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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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ABSTRAKT

Cíle: Předkládaná studie se zabývala možností využití extracelulárních mikroRNA jako nových neinvazivních biomarkerů pro diagnostiku komplikací souvisejících s těhotenstvím, jako je gestační hypertenze (GH), preeklampsie (PE) a omezení růstu plodu (FGR). Nejprve jsme identifikovali s těhotenstvím asociované mikroRNA (tj. placentárně specifické mikroRNA) v mateřské cirkulaci u těhotenství s normálním průběhem. Poté jsme kvantifikovali vybrané extracelulární C19MC mikroRNA v průběhu těhotenství (od prvního do třetího trimestru) v mateřské cirkulaci u těhotenství s normálním průběhem. Následně jsme porovnali expresní profily C19MC mikroRNA v mateřském oběhu mezi těhotenstvím s klinicky manifestními závažnými těhotenskými komplikacemi (PE, FGR, GH) a zdravými kontrolami odpovídajícího gestačnímu stáří. Nakonec bylo provedeno monitorování vybraných placentárně specifických C19MC mikroRNA v mateřském cirkulaci v průběhu prvního trimestru těhotenství s cílem identifikovat extracelulární C19MC mikroRNA schopné rozlišovat mezi normálními těhotenstvím a těmi, které jsou vystavena zvýšenému riziku pozdějšího rozvoje závažných těhotenských komplikací.

Výsledky: Hladiny a expresní profily extracelulárních placentálně specifických mikroRNA v oběhu netěhotných jedinců a těhotných žen byly stanoveny metodou polymerázové řetězové reakce v reálném čase. Výběr vhodných mikroRNA s diagnostickým potenciálem pro uplatnění v neinvazivní prenatální diagnostice byl založen na následujících kritériích: (1) detekce 100% v placentární tkáni, (2) detekce ≥ 67% v mateřské plazmě během těhotenství (3) míra detekce 0% ve vzorcích periferní krve a plazmy získané od netěhotných kontrolních subjektů.

Na základě našich výsledků byly identifikovány nové extracelulární biomarkery, C19MC microRNA, které by mohly přispět k potvrzení diagnózy preeklampsie nebo předpovědět pozdější výskyt GH a preeklampsie. Brzké stanovení diagnózy závažných těhotenských komplikací by mohlo přispět k zahájení včasné léčby nebo dokonce zahájení včasné prevence pozdějšího vývoje závažných těhotenských komplikací.

ABSTRACT

Objectives: Our study 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.

Results: 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.

Conclusion: On the base of our results, new 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.

1. INTRODUCTION

1.1 MicroRNAs and their function in gene expression regulation

1.1.1 Discovery/History of microRNAs

Previous decades unveiled a number of important cellular roles for diverse subset of RNA molecules distinct from the information-encoding messenger RNAs (mRNAs). These non-coding RNA (ncRNA) molecules are not translated into a protein product, but function as critical structural molecules or regulators of various cellular processes. Besides historically well-characterized transfer RNAs (tRNAs), decoding the codons of the mRNA and carrying amino acid residues for protein synthesis, and ribosomal RNAs (rRNAs) function in both, a structural and catalytic part of ribosome, other classes of ncRNAs were described. Among them small nuclear RNAs (snRNAs) of the spliceosome, that recognize splice sites and catalyze alternate splicing of pre-mRNA transcripts (Padgett et al., 1986), small nucleolar RNAs (snoRNAs), which are involved in covalent modification of other RNAs (Smith and Steitz, 1997) and 7SL RNA, that served as a part of the signal recognition particle that channels proteins into the endoplasmatic reticulum during synthesis (Walter and Blobel, 1982), were characterized. Presence and function of the telomerase complex RNA, which is used as a template for extending the ends of chromosomes (Feng et al., 1995; Theimer and Feigon, 2006), and the noncoding XIST RNA, which is required for X-chromosome silencing and dosage compensation in human females (Brown et al., 1992; Clemson et al., 1996; Plath et al., 2002), in the cell was described.

Over the past two decades new classes of ncRNAs, such as long non-coding RNAs (lncRNAs) and especially short non-coding RNAs called microRNAs (miRNAs) have come into the spotlight.

The pioneer organism for study of short non-coding RNAs and their functions in cells become 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. Lin-14

protein is normally abundant in the nuclei of late-stage embryos and younger L1 larvae, but it is barely detectable in the L2 larvae (Ruvkun and Giusto, 1989). 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 two transcripts, which are called lin-4S (short; 22 nucleotides in length) and lin-4L (long; 61 nucleotides in length). Sequence analysis showed that lin-4S was part of lin-4L: it was predicted that lin-4L form a stem-loop structure, where lin-4S is located in its 5' arm. Lin-4S was identified to be complementary to seven repeated sequences in the 3´untranslated region (3´UTR) of the messenger RNA encoding the lin-14 protein. Since *lin-14* transcripts are constant throughout development, the results of the study of Wightman et al. (1993) indicate that *lin-14* is negatively regulated on posttranscriptional level. On the base of these results Lee and colleagues hypothesized that *lin-4* could regulate *lin-14* mRNA translation via antisense RNA:mRNA interaction between lin-4S and 3´NTR of *lin-14*.

In 2000 Gary Ruvkun's group discovered a second short regulatory RNA, *let-7*, that manages the later stages of C. elegans development. *Let-7* negatively regulates *lin-14, lin-28, lin-41, lin-42* and *daf-12* in a similar manner to *lin-4* (Reinhart et al., 2000). Because *lin-4* and *let-7* function as key regulators of developmental timing and their appearance is regulated during development, they were referred to as small temporal RNAs (stRNAs). It was soon discovered that both *lin-4* and *let-7* were evolutionarily conserved from flies to humans, implicating a more universal role for these genes in animals (Pasquinelli et al., 2000; Lagos-Quintana et al., 2001). After several months more than one hundred new tiny regulatory RNA molecules were described in both plant and animals (Lau et al., 2001; Lee and Ambros, 2001; Mourelatos et al., 2002; Reinhart et al., 2002; Houbaviy et al., 2003; Lim et al., 2003). These molecules are known from 2001 as microRNAs and nowadays more than 2588 mature microRNAs were

identified and submited to the online microRNA sequence repository database (miRBase)(www.mirbase.org; 6.6.2017, 19:56).

1.1.2 MicroRNAs – IN THE HUMAN GENOME

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. The microRNAs within a genomic cluster are often, though not always, related to each other; and related microRNAs are sometimes but not always clustered (Lagos-Quintana et al. 2001, Lau et al. 2001). In humans, more than one hundred microRNA clusters were successively discovered (Yuan et al., 2009). Up to 60% of known human microRNA genes are found in clusters carrying from two to as many as 46 microRNAs, as seen in the largest microRNA cluster, chromosome 19 microRNA cluster (C19MC), that is specific microRNA cluster found only in primates (Bentwich et al., 2005; Megraw et al., 2007; Wang et al., 2008; Zhang et al., 2008; Cano and Nieto, 2008; Bortolin-Cavaille et al. 2009). 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). It was believed that microRNAs encoded in cluster affected identical targets (Korpal et al., 2011, Dykxhroon et al., 2009), however recent evidence suggested that clustered microRNAs target different genes that are involved in a particular specific pathway or create various protein complexes (Kim et al., 2009; Yuan et al.,2009; Sass et al., 2011).

Depending on their genomic origin microRNAs can be categorized into four sub-types 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) between two consecutive protein coding genes, where are located DNA sequences that contain few or no protein-coding genes (Saini et al., 2008; Corcoran et al., 2009). Since primary transcripts of intergenic microRNAs are transcribed from individual, non-protein-coding genes, they have their own promoters like the coding genes and the same transcription factors that are used for mRNA transcription are utilized also for miRNAs (Lee et al., 2004; Aguda et al., 2008; Pichiorri et al., 2010; Wang et al., 2010).

Intragenic microRNAs can be located within introns of either protein-coding genes or non-coding transcription units (TUs) or within exons of long nonprotein-coding transcripts also known as mRNA-like non-coding RNAs (mlncRNAs) (Erdmann et al. 2000; Rodriguez et al., 2004; Saini et al., 2008; Corcoran et al., 2009). In some cases intragenic microRNA genes overlap with two or more TUs transcribed on opposite DNA strand or can be located in either an exon or an intron (so-called “mixed” microRNAs) depending on alternative splicing of the host transcript (Rodriguez et al., 2004). 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). Minor group of intragenic microRNAs are transcribed in the antisense orientation to host gene, suggesting that they form their own independent transcription units (Aguda et al., 2008; Pichiorri et al., 2010; Wang et al., 2010).

Various microRNAs are produced as per requirement of the cell but the regulation mechanism(s) of miRNA expression remains largely unknown and are yet to be elucidated. It was hypothesized that in response to variety of endogenous and exogenous factors microRNA gene expression can be regulated

by transcription factors and also by other microRNAs (O´Doneell et al., 2005; Kulshreshtha et al., 2007a,b; Sylvestre et al., 2007; Woods et al., 2007; Davis and Ross, 2008; Fidler et al., 2008). Proteins such as HnRNPA1, SMAD1 and SMAD5 were observed to interact with miRNA precursors and regulate their subsequent processing to mature microRNAs (Davis et al., 2010; Michlewski et al., 2008). Besides that some regulatory proteins can also bind mature microRNAs to direct their degradation. It was also estimated that 10% of microRNA expression is controlled through DNA methylation (Han et al., 2007).

1.1.3 MicroRNA BIOGENESIS

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 majority of microRNAs is transcribed by RNA polymerase II (pol II), which generates primary microRNAs molecules with 7-methyl guanylate (m7G) caps and polyadenylated (poly(A)) tails (Cullen 2004a, b; Saini et al., 2007; Altuvia et al., 2005; Yu et al., 2006; Lee et al., 2004; Cai et al., 2004; Bartel and Chen, 2004).

However, it was also speculated that microRNA can be transcribed by RNA polymerase III (pol III) which specifically synthesizes other small non-protein coding RNAs, like tRNA and 5S rRNA, involved in regulation of cell cycle and growth (Goodfellow and White, 2007; Felton-Edkins et al., 2003; Scott et al., 2001; White et al., 1995; Costanzo et al., 2001; Young et al., 1991; Besser et al., 1990; Kassavetis et al., 1989, Chen et al., 2004; Borchert et al., 2006).

In 2006, Borchert et al. (2006) published study investigating C19MC microRNA cluster located on the human chromosome 19 that encoded 46 microRNAs transcribed by pol III. These microRNAs were described to be scattered within Alu repeats containing promotor for RNA poly III. Later study by Gu *et al*. (2009) identified 68 new microRNAs that appear to be transcribed by an Alu-dependent pol III mechanism. Controversial, study of Bortolin-Cavaillé et al. (2009) re-examined microRNA gene organization and expression at C19MC and demonstrated that involvement of *Alu*-embedded Pol-III in transcribing microRNA genes at the C19MC locus is not supported by the miRNA- *Alu* gene organization.

Since the vast majority of these putative Pol-III promoters (82%) are inserted in the reverse orientation with respect to the direction of transcription of the pre-microRNA genes and even those *Alus* in the correct orientation are unlikely to drive expression of microRNAs because of several Pol-III transcriptional stop sinals (poly(T) stretches of T_4 or T_5) found upstream of the pre-microRNA sequences, Bortolin-Cavailee (2009) demonstrated that C19MC microRNA genes are unlikely to be significantly expressed as Pol-III-dependent transcription units. They privilege a model whereby C19MC microRNAs are processed from previously unidentified, Pol-II placenta-specific non-protein-coding transcripts.

● Nuclear processing and export of microRNAs

Localization of microRNA gene in genome, being either intergenic or protein-coding intronic, seems to determine nuclear pathway the microRNA will proceed through (Rodriguez et al., 2004; Lin et al., 2008).

Intergenic microRNAs, transcribed by pol II produce a large stem-loop pri-microRNA with single-stranded RNA extensions at both ends (Lee et al., 2004; Borchert et al., 2006; Han et al., 2006). Only pri-microRNA of appropriate stem length, with large flexible terminal loop and the capability of producing 5’ and 3’ single-stranded RNA overhangs will be efficiently processed and mature into

functional microRNA (Lee et al., 2003; Abbott et al., 2005; Zeng et al., 2005; Zeng and Cullen, 2003, 2005; Zhang and Zeng, 2010). The maturation cleavage process of pri-microRNA begins in nucleus and it is mediated by a protein complex known as “the microprocessor”. This complex comprises of the RNase III endonuclease Drosha with catalytic activity and the double-stranded RNA-binding protein DGCR8 (DiGeorge syndrome critical region gene 8) with RNA binding domains that enable and coordinate interaction of Drosha with pri-miRNA sequence (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Lee et al., 2002, 2003; Faller et al., 2010). DGCR8 recognizes the pri-microRNA at the single-stranded RNA (ssRNA)-double stranded RNA (dsRNA) junction and directs Drosha to a specific cleavage site ~11 base pairs (bp) from the junction on the stem where Drosha cuts pri-microRNA to liberate a 60-80 bp microRNA hairpin precursor (pre-microRNA) with phosphate on 5´end and approximately 2 nucleotides 3’ single-stranded RNA overhang (Han et al., 2004; Lee et al., 2002, 2003; Han et al., 2006; Zeng and Cullen, 2003; Gregory and Shiekhattar, 2005; Zeng et al., 2003).

Observations reported by Han et al. (2009) and Triboulet et al. (2009) describe unique crossregulation between DGCR8 and Drosha. While DGCR8 stabilizes Drosha *via* protein-protein interaction and assists in controlling Drosha protein levels, Drosha negatively regulates DGCR8 mRNA post-transcriptionally by cleaving an 88 nt hairpin located in the 5’ untranslated region (5´UTR) which destabilizes the transcript (Han et al., 2009; Triboulet et al., 2009). Processing of several pre-microRNAs requires association of additional proteins (such as hnRNPA1) with microprocessor complex (Michlewski et al., 2008; Guil and Caceres, 2007).

MicroRNAs located within an intron of a protein coding gene are transcribed by pol II as part of the precursor mRNA (pre-mRNA)(Rodriguez et al., 2004). The microRNA sequence is excised from pre-mRNA by microprocessor or by spliceosomal components that releases mirtrons (short hairpin introns) (Berezikov et al., 2007; Rainer et al., 2009; Ruby et al., 2007), pre-microRNAs, , and primary microRNAs (Ying and Lin, 2004; Lin et al., 2003)(Figure 1). Pre-

microRNAs are exported to the cytoplasm for other processing, and primary microRNAs undergo same microprocessor cleavage as intergenic pri-microRNA to generate pre-miRNA (Ying and Lin, 2004; Lin et al., 2003)(Figure 1).

Figure 1. Nuclear component of microRNA biogenesis

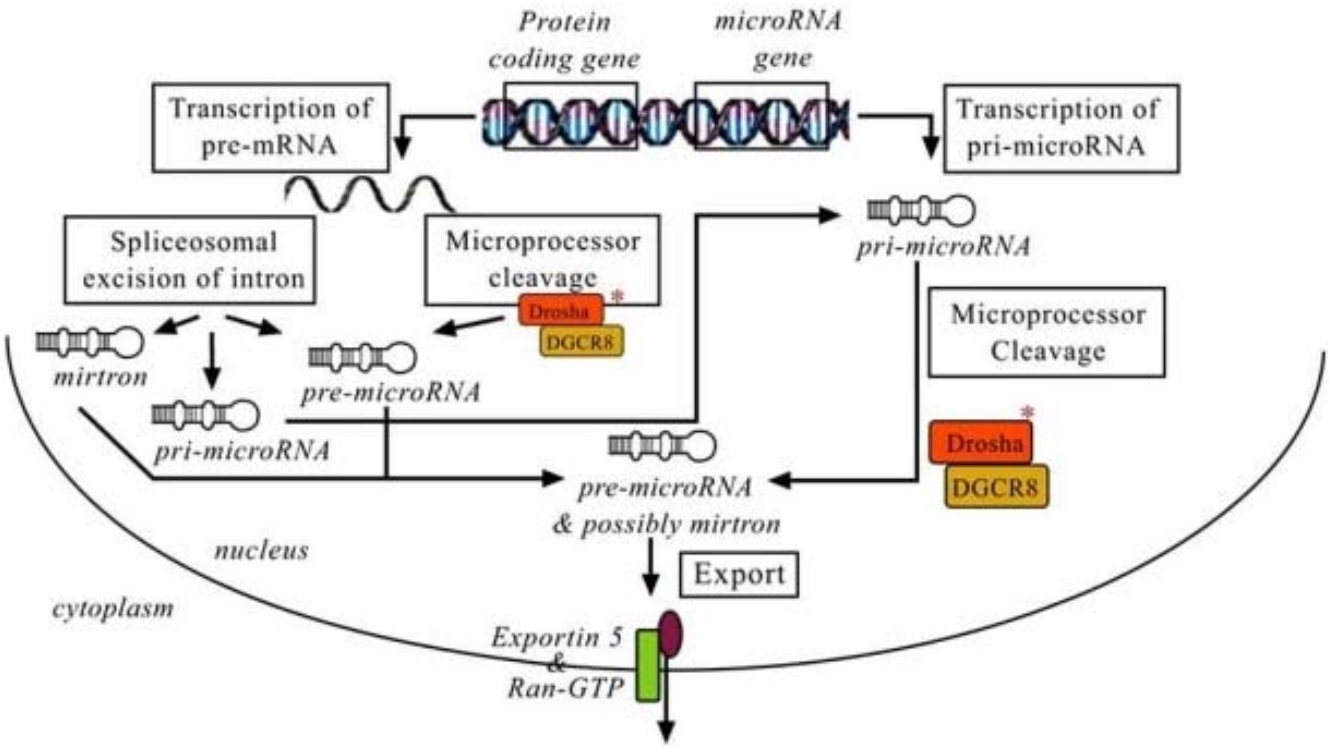

Fig. 1

Intergenic microRNAs are transcribed into primary microRNA (pri-microRNA) molecules, which are processed into a precursor microRNA (pre-microRNA) by the microprocessor complex comprised of DGCR8 and Drosha. Pre-microRNA are exported to the cytoplasm in a nukleocytoplasmic transporter containing Exportin 5 and Ran-GTP. MicroRNA within introns are transcribed as part of precursor mRNA (pre-mRNA) by RNA polymerase II. The microRNA sequence is excised from the pre-mRNA by spliceosomal components or the microprocessor to liberate a mirtron or a pre-microRNA that is exported. Alternatively, a primary miRNA (pre-microRNA) is released which undergoes microprocessor cleavage to generate pre-miRNA. (Adopted from MacFarlane and Murphy, Current Genomics 2010;11:537-561).

● Cytoplasmatic processing of microRNAs

Subsequent modifications of pre-microRNA take place in cytoplasm. Pre-microRNA (and possibly mirtron) forms a complex with the nucleocytoplasmic transporter factor Exportin-5 and GTPase RAN, which prevents nuclear degradation and facilitates translocation into the cytoplasm (Bohnsack et al.,

2004; Lund and Dahlberg, 2006; Okada et al., 2009; Yi et al., 2003; Zeng and Cullen, 2004). Exportin-5 interacts with dsRNA (pre-RNA) independently on its sequence (Bohnsack et al., 2004). During translocation of pre-miRNA through the nuclear pore, GTP in GTP-binding nuclear protein Ran is hydrolyzed to GDP, which leads to disassembly of the complex and the release of the pre-microRNA into the cytoplasm, when it undergoes further processing.

Three possible ways of pre-microRNA processing in cytoplasm were described. First (and most expanded) model is based on pre-microRNA incorporation into the performed pre-microRNA processing complex composed of the RNase III endonuclease Dicer, a multi-domain protein, and the associated RNA-binding proteins TRBP (transactivation-response RNA-binding protein) or PACT (protein kinase R-activating protein) (Perron and Provost, 2008; Haase et al., 2005; Chendrimada et al., 2005; Lee et al., 2006). Dicer protein comprises of a N-terminal ATPase/ Helicase domain, DUF283 domain of unknown function, PAZ (Piwi/Argonaute/Zwilli) domain and two tandem RNaseIII nuclease domains (RNase IIIa and RNase IIIb) located at the C-terminal followed by a dsRNA-binding domain (dsRBD) (Provost et al., 2002). Functionally most important domains are the PAZ domain, that recognizes last two nucleotides on 3'-overhang of the pre-microRNA and initiates binding (Zhang et al., 2002, 2004; Song et al. 2003), and two RNaseIII domains forming a single processing center with two catalytic sites (RIIIa and RIIIb) that cleaves the pre-microRNA. The resulting product is a double stranded miRNA duplex with 3' overhanging ends (Zhang et al., 2004).

Second model created by Cifuentes *et al*. (2010) describes Dicer-independent pre-miRNA processing pathway associated with the Argonaute 2 protein (Ago2), containing a Piwi domain and a PAZ domain with endonuclease activity (Cifuentes et al., 2010; Song et al., 2004; O´Carroll et al., 2007; Meister et al., 2004).

Third model proposed by Diedrichs and Haber (2007) suggests that Dicer, TRBP and Argonaute 2 protein (Ago2) form a protein complex that recognizes and binds the pre-microRNA through the PAZ domain of Dicer and/or Ago2.

Ago2 subsequently cleaves a single strand of the pre-microRNA and generates a nicked hairpin structure referred to as “Ago2-cleaved precursor microRNA” or “ac-pre-microRNA”, the Dicer substrate cleaves to give rise to the double stranded microRNA product of 19-22 nucleotides in length.

Cutting of the pre-microRNA leads to double stranded microRNA molecule consisting of a mature microRNA strand (also referred to as guide strand), a passenger strand commonly denoted microRNA:microRNA*, where the passenger strand is designated microRNA* (Bartel, 2004; Kim, 2005; Lau et al., 2001). The RNA duplex is unwound and the single strand mature microRNA is incorporated into the final effector protein complex RISC (RNA induced silencing complex) (Bartel, 2004). The core components of the RISC loading complex are Dicer, Ago2, PACT and TRBP (Lee et al., 2006; Maniataki and Mourelatos, 2005; Macrae et al., 2008). RISCs that load microRNA are designated microRNA containing ribonucleoprotein complex (miRISC or miRNP). Depending on composition of Ago proteins (Ago1-4) RISC complexes in humans could be divided into eight classes (Macrae et al., 2008).

The mechanism of duplex unwinding is not completely known, however several hypotheses were stated. Dicer could cleave the passenger strand leading to initiation of unwinding of the duplex and separated mature single strand microRNA could be captured by Ago2. Alternatively, microRNA duplex forms complex with Ago2 (the only argonaute protein with “slicer” activity) that catalyzes cleavage of the passenger strand and keeps the microRNA guide. Conformational changes in RISC during its assembly and/or RNA Helicase A (RHA) activity could also be responsible for RNA duplex unwinding (Maniataki and Mourelatos, 2005; Robb and Rana, 2007)(Figure 2).

Figure 2. Cytoplasmic component of microRNA biogenesis

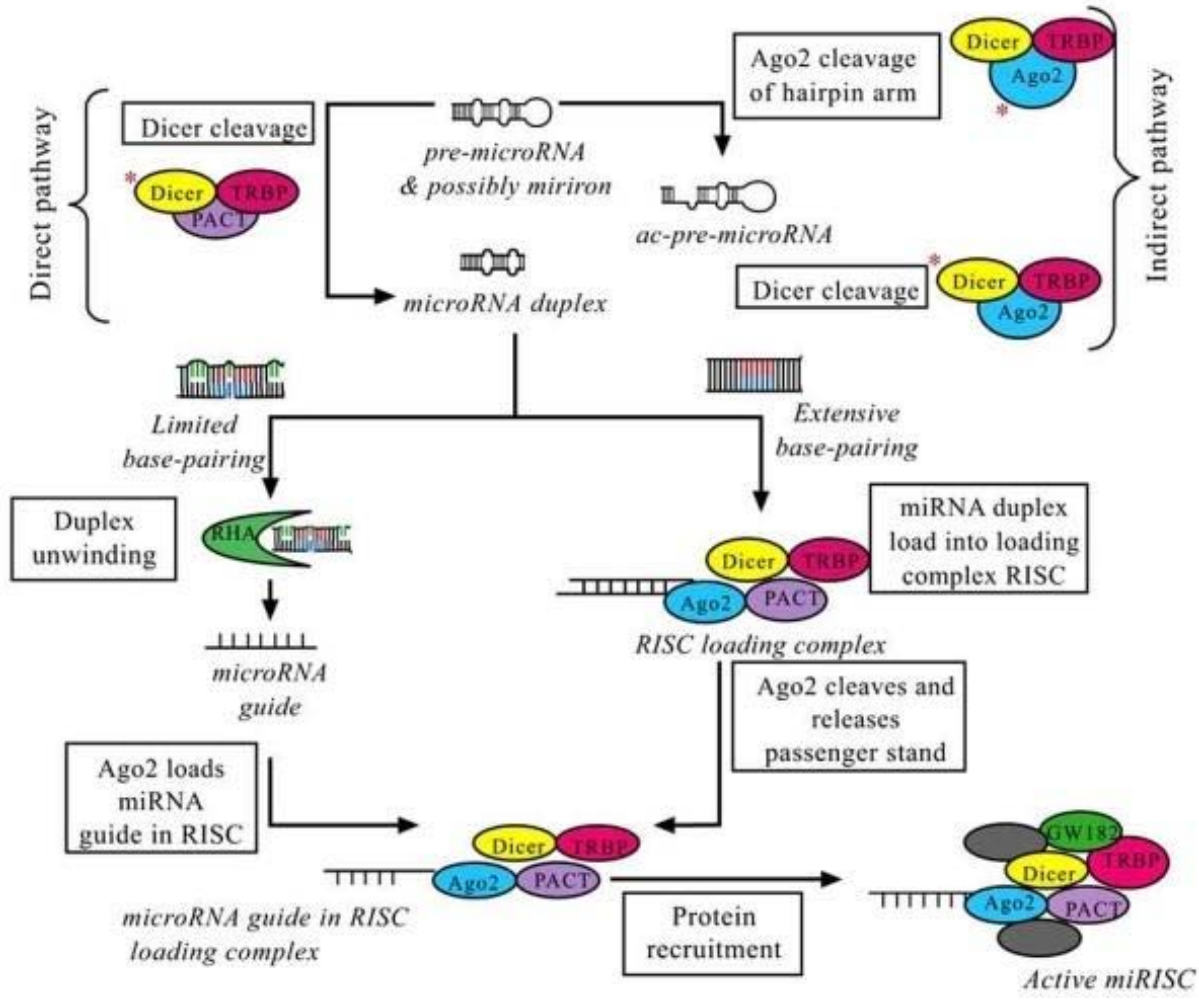

Fig 2.
Pre-microRNA is cleaved by Dicer to generate microRNA duplex or Ago2 to generate an Ago2-cleaved precursor microRNA (ac-pre-microRNA) that subsequently acts as a substrate for Dicer. The microRNA duplex liberates the mature microRNAto assemble into RISC loading complex comprised of Ago2, TRBP, PACT and Dicer.
(Adopted from MacFarlane and Murphy, Current Genomics 2010;11:537-561).

1.1.4 MicroRNA FUNCTION

It is widely accepted that microRNA guide strand in activated RISC binds through Watson-Crick base-pairing to 3'UTR of the target mRNA and negatively regulate its expression (Wightman and Ruvkun, 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).

Recognition of target mRNA relies heavily on base pairing between the seed (region from 2nd to 8th nucleotide at the 5'end) of the microRNA guide and target mRNA sequence (Brennecke et al., 2005; Doench and Sharp, 2004; Krek et al., 2005; Lewis et al., 2003). Moreover, the degree and nature of their complementarity appear to determine one of two distinct gene silencing mechanism; irreversible mRNA cleavage or reversible translation inhibition (Bartel, 2004; Yekta et al., 2004; Brengues et al., 2005; Valencia-Sanchez et al., 2006; Maroney et al., 2006a, b; Nottrott et al., 2006).

When the target mRNA and microRNA are extensively (near-perfect) base-paired over seed region of the guide, Ago2 catalyzes microRNA-directed mRNA cleavage (Meister et al., 2004a; Yekta et al., 2004; Liu et al., 2004; Mallory et al, 2004; Mallory and Vaucheret, 2004). Degradation of cleavage products begins with the deadenylation of the mRNA to remove the poly (A) tail (Valencia-Sanchez et al., 2006; Parker and Song 2004). Subsequent degradation could be set in exosome, a multi-protein complex with 3'- to-5' exonuclease activity, or the mRNA can undergo decapping by the enzymes Dcp1 and Dcp2 which facilitates 5'-to-3' degradation by the exoribonuclease Xrn1p (Valencia-Sanchez et al., 2006; Coller and Parker, 2004).

Complementary sites with moderate (limited, imperfect) base-pairing create bulges in the microRNA:mRNA duplex that inhibit the slicer activity of Ago2 (Wightman and Ruvkun, 1993; Lee and Ambrose, 1993; Baretl, 2004; Valencia-Sanchez et al., 2006; Pillai, 2005; Pillai et al., 2005), but the argonaute proteins ability to repress translation of the target mRNA remains unchanged (Bartel, 2004; Yekta et al., 2004; Liu et al., 2004; Meister et al., 2004a,b; Pillai et al., 2004). Competition between Ago2 from miRISC complex and initiation factor eIF4E for binding the mRNA methylguanosine 5´cap, as well as the anti-

association factor eIF6, which inhibits the assembly of the ribosomes on the mRNA (Wu and Belasco, 2008), leads to blocking of the translational machinery.

Premature termination of polypeptide synthesis and degradation of the newly formed chain also contribute to translational inhibition, but those processes are not fully understood (Araldi and Schipani, 2010). Translation can also be regulated indirectly by spatial separation of components, such that microRNA-targeted mRNA is sequestered away from translational machinery into cytoplasmic P-bodies (also known as processing bodies, GW bodies and Dcp bodies) (Brengues et al., 2005; Lui et al., 2005a, b; Kedersha et al., 2005; Andrei et al., 2005; Anderson and Kadesha, 2006; Teixeira et al., 2005). P-bodies are heterogenous cytoplasmic granula containing mRNAs along with Ago proteins and GW182 (glycine-tryptophane repeat containing protein, 182 kDa), the major protein component of P-bodies (Ding and Grosshans, 2009; Eulalio et al., 2007). Recent studies suggested that mRNA in P-bodies is not only inhibited, but also protected against degradation (Castilla-Llorente et al., 2012).

Moreover, cytoplasmic RISC complexes with microRNA can also accelerate target mRNA deadenylation and decapping effecting translation initiation efficiency and/or transcript stability (Wu et al., 2006; Bagga et al., 2005; Fischer and Weis, 2002; Coller et al., 2001) leading to target mRNA enzymatic degradation *via e*xosome or Xrn1p (Wu et al., 2006; Coller and Parker, 2004).

1.2 Extracellular microRNAs

For the first time, extracellular microRNAs were described in serum and plasma by several independent groups in 2008. Lawrie et al. detected microRNAs in serum of patients with lymphomas (Lawrie et al., 2008) and Mitchell et al. (2008) demonstrated the presence of stable microRNAs in the human plasma of healthy subjects and prostate cancer patients. Plasma microRNA levels correlated with serum levels indicating the possible clinical use of extracellular microRNAs from, both plasma and serum, in tumor detection (Mitchell et al., 2008). In the same year presence of placental microRNAs in maternal plasma was

also reported (Chim et al., 2008). MicroRNAs were subsequently detected in other body fluids and biological samples including saliva (Park et al., 2009), sperm (Li et al., 2012), urine (Hanke at al., 2010) and milk (Pigati et al., 2010).

Further research confirmed the high stability of microRNAs in bodily fluids. Study by Turchinovich et al. (2011) demonstrated that incubation of the sample at room temperature for 24 and 96 hours resulted in only 10% and 20% decrease in concentration of the endogenous microRNAs in serum and plasma, respectively, while addition of exogenous chemically synthesized microRNAs to the sample led to their rapid degradation (Turchinovich et al., 2011). Similarly, Park et al. (2009) compared the degradation rate of endogenous and exogenous microRNAs that were added to the saliva sample, and confirmed rapid degradation of exogenous microRNAs since less than 10% of the original amount was detected after three minutes of incubation. On the other hand, endogenous microRNAs were degraded more slowly, and after 30 minute incubation approximately 30% of the original microRNA amount was still detectable (Park et al., 2009). Considerable resistance of the microRNAs to extreme pH values was also described, with maximal 50% reduction of the microRNAs amount after a three-hour incubation of the sample with pH 1 or pH 12 medium. In biological samples microRNAs remain stable even after 10 cycles of freezing and thawing (Chen et al., 2008). The above mentioned observations indicate that microRNAs are protected in the extracellular environment against the effects of RNases and degradation.

1.2.1 Mechanisms stabilizing extracellular microRNAs

For the first time, presence of extracellular microRNAs was detected in exosomes that were released into the medium by cultured cells (Valadi et al., 2007). Later Hunter et al. detected microRNAs also in microvesicles and apoptotic bodies in peripheral blood samples (Hunter et al., 2008). These observations indicate that microRNAs could be protected against degradation in the extracellular environment by their encapsulation into membrane structures.

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)(Figure 2).

Association of microRNAs with Argonaute-2

Arroyo et al. (2011) systematically characterized the presence of microRNAs in human plasma and serum by differential centrifugation, size-exclusion chromatography, immunoprecipitation and western blot. After removing of the cellular fragments from the plasma sample and medium by ultracentrifugation (up to 120,000 x g), over 97% of microns remained in the supernatant, and only a very small portion was detected in a fraction corresponding to exosomes and microvesicles. Size-exclusion chromatography on the Sephadex column identified two fractions of extracellular microRNAs; most microRNAs were enriched in protein complexes and only a small amount of microRNAs was present in the fraction corresponding to lipid vesicles. In order to verify the hypothesis that the protein complexes are predominantly responsible for the stability of extracellular microRNAs, the individual fractions of the plasma sample were incubated with proteinase K that proteolytically cleaves ribonucleoprotein complexes, which resulted in rapid degradation of microRNAs in plasma fractions. These experiments suggest that the vast majority of extracellular microRNAs occurs in plasma in the form of complexes with proteins that stabilize microRNAs and protect them from RNases degradation (Arroyo et al, 2011). These findings were supported by Turchinovich and colleagues (2011) who performed ultracentrifugation of plasma and cell culture media through nanomembranes of diverse pore size and found that most of microRNAs were detected in the sample even after passage through 300kDa filter that eliminates the possible binding of microRNAs into microvesicles and other membrane structures. Since only a negligible amount of microRNAs passed 50 kDa filters, the magnitude of the protein complex carrying the microRNA in the plasma was

estimated to be between 300-50 kDa. Subsequent western blot immunoassay revealed that extracellular miRNA precipitated together with the 96kDa Ago2 protein (Turchinovich et al., 2011).

These studies indicate that most of the extracellular microRNAs in plasma (and possibly by other body fluids) and cell culture medium occurs in the complexes with Ago2, an effector protein of the cytoplasmic RISC complex that protects microRNAs against RNAase degradation (Arroyo et al.,2011; Turchinovich et al., 2011).

Association of microRNAs with Nucleophosmin-1

Nucleophosmin-1 (NPM1) is a multifunctional nuclear phosphoprotein that constantly passes between the nucleus and the cytoplasm and functions as a chaperon that prevents the aggregation of nuclear proteins (Borer et al., 1989). It may also have an important role in cell cycle regulation because it interacts with cyclin E / CDK2 complex and regulates p53 activity (Okuda et al. 2000; Colombo et al., 2002). Depending on its expression level, cellular localization and binding partners, NPM1 may have a tumor suppressor or oncogenic function. Mutation in the NPM1 gene is an important prediction factor for some types of leukemia (e.g. AML)(Verhaak et al., 2005). NPM1 binds with high affinity to single-stranded nucleic acids indicating its possible association with extracellular microRNAs. Wang and colleagues (2010) performed experiments with cell cultures A549 (derived from epithelial tumor) and HepG2 (human liver cancer cell line) and monitored the change in expression of extracellular microRNAs in response to stress caused by serum deprivation of cells cultivated in pure media. Within two hours after incubation in pure media without serum, significant increase in microRNA levels in medium accompanied with decrease in intracellular microRNA levels was observed. Simultaneously, the level of RNA-binding proteins including NMP1 increased in cell culture medium. The ability of NPM1 to bind microRNAs and protect them against RNase degradation was demonstrated

experimentally. These results support the hypothesis that extracellular microRNAs could by associated with NPM1.

MicroRNA association with membrane structures

The presence of microRNAs in microvesicles originating from the HMC-1 bone marrow cell culture medium was detected by Valadi et al. in 2007. Subsequent studies identified the presence of microRNAs in other membrane structures including exosomes and apoptotic bodies (Hunter et al., 2008; Wang et al., 2010; Zernwcke et al., 2009; Yang et al., 2011; Mathivanan et al., 2012). Extracellular vesicles (exosomes, ectosomes/shedding microvesicles, and apoptotic bodies) are small cytoplasmic compartments bounded by a phospholipid layer derived from the cell membrane that are released into the extracellular environment by almost all cell types, under normal as well as pathological conditions. These vesicles, in size ranging from 50nm up to 5µm in diameter, contain lipids, proteins and also nucleic acids including mRNA and microRNAs in complex with Ago2. Furthermore, all of the RISC complex components are present in these bodies, playing role in incorporation of microRNAs into the vesicles.

Exosomes are small vesicles of 30-100nm in diameter which are secreted into the extracellular environment by cells. They are formed within cells by inward budding of the peripheral membrane of the late endosomes called multivesicular bodies (MVBs). The molecular constituents in exosomes originate from various cellular compartments including: the Golgi apparatus, the endoplasmic reticulum, the plasma membrane, the nucleus and the cytosol. MVBs have two options how to be further processed in all cells: 1. they fuse with the lysosomal compartment where membranes and other content are degraded 2. or they fuse with the plasma membrane, which leads to the consecutive release of exosomes (Harding et al., 2013; Raiborg and Stenmark, 2009). MicroRNAs contained in the exosomes are released via the ceramide secretion

pathway by enzyme neutral sphingomyelinase-2, which hydrolyses sphingomyeline to ceramide (Trajkovic et al., 2008; Kosaka et al., 2010).

Ectosomes or shedding microvesicles are larger than exosomes, with size ranging from 100nm to 1000nm in diameter. They result from cell membrane bulging into the outer space and subsequent strangulation and shedding of the vesicle (Cocucci et al., 2009). MicroRNAs may be incorporated to microparticles derived from almost all cell types. These vesicles can also transfer chemokines, receptors and adhesive molecules (Prokopi et al., 2009).

Apoptotic bodies are the largest particles (1-5µm) that participate in vesicular transport of microRNAs. These are irregular membrane-bound vesicles containing a portion of cytoplasm with disintegrated cellular organelles, fragmented DNA and RNA including microRNA that are generated by cell undergoing programmed cell death – apoptosis (Zernecke et el., 2009; Hristov et al., 2004). Apoptotic bodies are engulfed by phagocytic cells and their components can be recycled.

MicroRNA association with lipoprotein particles

A small fraction of extracellular microRNAs precipitates with cellular high-density lipoprotein (HDL) and, low-density lipoprotein (LDL) to lesser extent (Wagner et al., 2013). MicroRNAs incorporated in lipid particles bounded by monolayer of phospholipids with apo-lipoproteins (ApoA-I) appear to be associated with Ago2 proteins as well. Interestingly, a ceramide pathway that positively affects microRNA secretion into exosomes suppresses the association of microRNAs with HDL particles. As expected, the presence of specific microRNAs in the complex with HDL lipoproteins differs between healthy subjects and patients with hypercholesterolemia (Vickers et al., 2011). Lipoprotein particles bind to the recipient cells by the surface receptor SR-BI responsible for the selective absorption of cholesterol esters and their transfer into the cytoplasm. Since this mechanism bypass the lysosomal transport

pathway, incorporation of microRNAs into lipoprotein particles could lead to their higher stability (Vickers et al., 2011).

1.2.2 Secretion of extracellular microRNAs

Extracellular microRNAs, which are bound only to protein complexes, are apparently released passively during cell death (for example during inflammation) and can be detected thanks to their stability in extracellular environment (Turchinovich et al., 2012). This is supported by observation that increasing cell counts or cell death rates in the tissue lead to increasing tissue-specific microRNA levels in peripheral blood (Laterza et al., 2009). However, work by Wang et al. (2010) suggests that the export of microRNA -protein complexes is probably an active process because blocking of the cellular respiration by rotenone (isoflavone, that inhibits mitochondrial complex I) led to suppression of microRNA levels in medium. In contrast, restriction of proteins secretion in the Golgi apparatus and exosomes formation does not influence secretion of microRNA-protein complexes indicating that microRNAs associated with protein complexes could be released from the cell without utilization of exosomal pathway.

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).

1.3 MicroRNAs in normal pregnancy

During pregnancy highly specialized vital fetal organ – placenta, arising temporarily in maternal body to support normal growth and development of the fetus (Gude et al., 2004). Growth and function of the placenta are precisely regulated and coordinated to ensure the exchange of nutrients and oxygen as well as removing of carbon dioxide and other waste products between the

maternal and fetal circulation operates with maximal efficiency. Other important function of the placenta is secretion of hormones and growth factors into both the maternal and fetal circulation. Placenta serves also as a barrier that can help to protect the fetus against certain xenobiotic molecules, infections and maternal diseases (Gude et al., 2004; Knipp et al., 1999).

1.3.1 Expression of microRNAs in placental tissue

Numerous studies have shown that a large number of microRNAs is expressed in trophoblast cells and human placental tissue (Lee et al., 2011; Mayor-Lynn et al., 2011; Gauster et al., 2012; Luo et al., 2012; Barad et al., 2004; Donker et al., 2007; Zhu et al., 2009; Mouillet et al., 2010; Landgraf et al., 2007; Luo et al., 2009; Pineles et al., 2007; Umemura et al., 2013; Ling et al., 2007). Further studies confirmed that the microRNA biogenesis pathway is active in human placenta, since the presence of key molecules involved in microRNA biogenesis, such as Drosha, Exportin 5, Dicer, Argonaute 2 (Ago2) and DP103, was proved in trophoblast cells (Donker et al., 2007; Forbes et al., 2012). Knockout studies of these proteins confirmed the requirement of functional microRNAs in reproduction. Loss of Dicer within ovarian granulosa cells, luteal tissue, oocyte, oviduct and, potentially, the uterus results in female infertility (Hong et al., 2008). Disruption of the gene for Ago2 leads to a phenotype similar to the disruption of Dicer1, but with embryonic lethality early after the implantation stage (Morita et al., 2007). It was revealed that microRNAs expressed in placenta exert regulatory effects on trophoblast cell differentiation, proliferation, apoptosis, migration and invasion, as well as on angiogenesis regulation. Some of these microRNAs also regulate trophoblast cell metabolism. It follows that microRNAs can regulate placental development and function.

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).

The C19MC, maps to chromosome 19q13.41, is one of the largest miRNA clusters in humans that spans a ~100 kb long region (Landgraf et al., 2007; Donker et al., 2012; Bentwich et al., 2005; Bortolin-Cavaillé et al., 2009). This cluster harbors 46 pri-microRNA genes, yielding 59 mature microRNAs that are found exclusively in primates (Ouyang et al., 2014). C19MC is encoded within imprinted domains and is exclusively expressed from the paternally inherited chromosome (Noguer-Dance et al., 2010). Even though the C19MC microRNA expression is mainly restricted to the reproductive system and placenta (Bentwich et al., 2005; Liang et al., 2007; Lin et al., 2010), some C19MC members are highly expressed in human embryonic stem cells (ESCs) (Bar et al. 2008; Ren et al., 2009), as well as in some tumors (Huang et al., 2008a; Li et al., 2009, Flor and Bullerdiek, 2012; Fornari et al., 2012). Moreover, expression one of C19MC microRNA, miR-498, was also reported in fetal brain in 20 weeks of gestation (Flor and Bullerdiek, 2012).

The C14MC cluster, also referred to as the Mirg cluster (Bortolin-Cavaille et al., 2009), the miR-379/miR-410 cluster (Noguer-Dance et al., 2010) or the miR-379/miR-656 cluster (Glazov et al., 2008) comprises 34 microRNA genes located in the imprinted DLK-DIO3 domain on the human 14q32 chromosome (Seitz et al., 2004; Gardiner et al., 2012; Morales-Prieto et al., 2012). C14MC microRNAs are encoded by alleles expressed solely from the maternally inherited chromosome (Seitz et al., 2004) and some of them are predominantly expressed in placenta (Liang et al., 2007).

The miR-371-3 cluster, located on chromosome 19 within a 1050 bp region adjacent to the C19MC cluster (Suh et al., 2004), contains mainly 3 microRNAs sharing the same seed sequence (Griffiths-Jones et al., 2006; Persson et al., 2011), that are predominantly expressed in placenta (Bentwich et al., 2005), however high expression levels decreasing during development were also detected in human ESCs (Suh et al., 2004; Laurent et al., 2008).

Expression level of C19MC microRNAs in trophoblast cells increases significantly from first to third trimester while C14MC microRNA level has the opposite pattern (Morales-Prieto et al., 2012). A small increase in microRNA levels within miR-371-3 cluster with advancing pregnancy has also been observed (Morales-Prieto et al., 2012). Changes in expression levels of additional microRNAs in trophoplast cells and placental tissue obtained in different gestational stages were demonstrated (Morales-Prieto et al., 2012; Luo et al., 2012; Fu et al., 2013), which supported the idea that such microRNAs have stage-specific function during pregnancy.

Regulation of MiRNA Expression in placenta

MicroRNA expression in the placenta could be regulated by environment factors, signaling pathways, and epigenetic modification (Donker et al., 2007; Ji et al., 2012; Seitz et al., 2004).

Since oxygen tension was shown to play critical role in placental development (Genbacev et al., 1996; Caniggia et al., 2000; Adelman et al., 2000), it was supposed that hypoxia may be the major regulator of microRNA expression in placenta. In vitro experiment performed on trophoblast cells cultured in 1% oxygen for 48 h confirmed up-regulation of miR-93, miR-205, miR-224, miR-335, miR-451, and miR-491 as well as down-regulation of miR-424 in response to hypoxia (Mouillet et al., 2010). Whereas low oxygen tension stimulates expression of hypoxia-inducible *AK123483* gene (Camps et al., 2008), it also results in increasing miR-210 expression, because the miR-210 gene is located within its intron. Therefore, miR-210 is well known as a sensor of hypoxia (Chan and Loscalzo, 2010; Devlin et al., 2011) in many different cell types including trobhoblast cells and its up-regulation is associated with diseases, such as cancer and preeclampsia (PE) (Chan and Loscalzo, 2010; Lee et al., 2011). It was suggested that the changes of microRNA levels in response to several environmental factors could be caused by transcriptional regulation of several microRNA genes (Chan and Loscalzo, 2010; Cummins and Taylor, 2005;

Kulshreshtha et al., 2007; Kelly et al., 2011; Zhang et al., 2012; Bamberger et al., 2004; Marzioni et al., 2010; Dai et al., 2011).

Other studies showed contribution of signaling molecules and environmental toxins in the control of microRNA expression in trophoblast cells. Leukemia inhibitory factor (LIF), a major regulator of trophoblast functions, up-regulates miR-21 and miR-93, but down-regulates miR-141 expression in JEG-3 cells (Morales-Prieto et al., 2011). Meanwhile, endocrine disruptors, such as Bisphenol A (BPA), significantly increased the expression of miR-146a in trophoblast cell lines, their exposition to nicotine and benzo(a)pyrene (Maccani et al., 2010) led to down-regulation of miR-146a (Avissar-Whiting et al., 2010; Maccani et al., 2010). In addition, miR-16, miR-21 and miR-146a levels are also significantly lower in placentas obtained from women who smoked during pregnancy (Maccani et al., 2010).

Epigenetic regulation, particularly DNA methylation status of a CpG-rich and Germ-Line-Derived differentially methylated regions (DMR), located upstream of the C19MC and C14MC clusters, respectively, controls tissue-specific expression of miRNAs in placenta. These regions are hypomethylated in placenta but hypermethylated in other cells (Seitz et al., 2004; Tsai et al., 2009; Noguer-Dance et al., 2010).

<u>The role of microRNAs in regulation of placental development and function</u>

• MicroRNAs and Trophoblast Cell Proliferation and Apoptosis

MicroRNAs regulate trophoblast cell proliferation and apoptosis during placental development. While several microRNAs were reported to enhance trophoblast cell proliferation and/or survival (miR-378a-5p, miR-376c, miR-141, and miR-182)(Fu et al., 2013; Luo et al., 2012; Pineles et al., 2007; Morales-Prieto et al., 2011; Segura et al., 2009), the other microRNAs inhibit trophoblast cell proliferation and/or induce apoptosis of trophoblast cells (miRNA-155, miR-675, and miR-29b)(Li et al., 2013; Dai et al., 2012; Gao et al., 2012; Keniry et al., 2012).

• MicroRNAs and Trophoblast Cell Migration and Invasion

Migration and invasion of extravillous trophoblasts (EVTs) to the decidua and myometrium are critical events during placentation (Chelbi et al., 2008). MicroRNAs can exert positive effect (miR-195, miR-376c, and miR-378a-5p)(Bai et al., 2012; 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 signaling pathways, enzymes, and adhesion molecules.

• MicroRNAs and Placental Angiogenesis

Direct evidence supporting the role of microRNAs in placental vascularization and spiral artery remodelling is limited. MiR-20b targets ephrin receptor B4 (EPHB4) and ephrin-B2 (Red-Horse et al., 2005), which play important role in placental angiogenesis (Chennakesava et al., 2006). It was speculated, but not yet determined, that miR-20b may play a role in cytotrophoblast remodelling of arterioles during human placentation. As reported by Burton at al. (2009) miR-29b reduces trophoblast cell invasion and tube-formation. Moreover, miR-29b and miR-16 suppress expression of vascular endothelial growth factor-A (VEGFA) (Li et al., 2013), known to be a key regulator of placental angiogenesis (Burton et al., 2009; Wang et al., 2012).

1.3.2 Expression of microRNAs in maternal circularion

Extracellular microRNAs are relatively stable and well protected in blood or extracellular compartment from digestion by RNases thanks to their package in microparticles (Valadi et al., 2007; Arroyo et al., 2011). Extracellular microRNAs may be divided into two groups: phospholipid membrane vesicle-encapsulated microRNAs, which are released into extracellular environment as the cargo of different types of microparticles, including microvesicles, exosomes, and apoptotic bodies (Valadi et al., 2007; Montecalvo et al., 2012; Chen et al.,

2012; Raposo and Stoorvogel, 2013; El Andaloussi et al., 2013), and protein-bound microRNAs, in which the naked microRNAs are complexed with proteins such as Ago2, nucleophosmin1, or high-density lipoproteins (Arroyo et al., 2011; Vickers et al., 2011; Wang et al., 2010).

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) suggesting a new mechanism for transmitting microRNA-based mRNA silencing to distant maternal cells. This is how placenta-derived microRNAs in the maternal circulation could influence gene expression in various maternal cell types that seem to play a pivotal role in adaptation of maternal organism to pregnancy, especially with regard to inducement of maternal immune tolerance against fetus.

Further studies confirmed that trophoblast-specific and nonspecific microRNAs are released to the extracellular fluid and are detectable in the maternal circulation during pregnancy. In 2008, Chim et al. compared expression profile of 157 microRNAs in normal placentas, whole maternal peripheral blood cells during pregnancy and maternal postdelivery plasma samples. They identified 34 microRNAs that were present in placenta at expression level >10-fold higher than in maternal blood cells during pregnancy, however only those 17 microRNAs that were not detected in the 24-h postdelivery maternal plasma were considered as candidate markers for monitoring pregnancy in maternal plasma. Subsequent analysis revealed that 4 microRNAs with the highest expression in placenta (miR-141, miR-149, miR-299-5p, and miR-135b) were also present in maternal plasma, with the highest expression rates in late pregnancy (near before the delivery), and showed reduced detection rates in postdelivery plasma (Chim et al., 2008).

In the same year, Gilad et al. (2008) reported that serum microRNAs might be used as promising new biomarkers for differentiation between pregnant and nonpregnant women. This study dealt with relative serum levels of 28 microRNAs, including microRNAs reported to be placenta-specific as well as

broadly expressed microRNAs in sera of non pregnant women and women in the first trimester and in the third trimester of pregnancy. All tested placental microRNAs were found at higher levels in sera from pregnant women and their levels rose with gestational age. Specifically, amounts of miR-526a and miR-527 were dramatically higher in sera of third trimester pregnant women (elevated by more than 600 fold). Results suggested that 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).

In another study, Miura et al. (2010) performed microarray-based screening of 723 human microRNAs and selected those microRNAs that exhibited signal intensities >100 times higher in placental tissues than in the corresponding whole peripheral blood samples. Of these 82 microRNAs, 24 showed significantly decreased concentrations in the maternal plasma after placenta delivery and were therefore identified as pregnancy-associated miRNAs. Only 6 microRNAs (miR-515-3p, miR-517a, miR-517c, miR-518b, miR-526b, and miR-323-3p) that showed the most significantly decreased concentrations in maternal plasma after pregnancy termination were selected for subsequent time-depending analysis in maternal plasma that revealed significant increase in plasma concentration of the following extracellular C19MC microRNAs (miR-515-3p, miR-517a, miR-517c, miR-518b, miR-526b) with advancing pregnancy. Contrary, as the pregnancy progressed into the third trimester, concentration of extracellular miR-323-3p encoded by gene on chromosome 14q32.31 showed no change in maternal plasma (Miura et al., 2010).

In 2012, maternal plasma miRNA expression profiles were detected by SOLiD sequencing in different pregnancy stages. 147 microRNAs were sequenced from maternal plasma, among them, 90 microRNAs were found in all samples, while 136, 108, and 99 miRNAs were found only during the first, the second, and the third trimesters of pregnancy, respectively. Expression levels of these pregnancy-related microRNAs were changing during pregnancy. Three microRNA clusters (miR-200c cluster, miR-200b cluster, and miR-222 cluster) were detected

in maternal plasma. In different gestational ages, the expression levels of cluster members changed with same trend. The function and functional relationship analysis of target genes showed that genetic disorders (Alzheimer´s disease), immunological diseases, cell signaling (insulin signaling pathway, ErbB signaling pathway, TGF-beta signaling pathway, Toll-like receptor signaling pathway, B/T cell receptor signaling pathway, and MAPK signaling pathway), cancer (non-small cell lung cancer, endometrial cancer, colorectal cancer, and prostate cancer), and cell cycle were involved in enriched pathways (Li et al., 2012).

Later, Williams et al. (2013) developed a nanogram-scale small RNA deep-sequencing method with sample multiplexing to profile circulating microRNAs in plasma of men and nonpregnant women as well as pregnant womens´ plasma, the umbilical cord blood of their offspring, and their placentas. Results revealed that relative levels of C19MC microRNA cluster members and, to a lesser extent, miR-127 and miR-134, were high in placental tissue and in umbilical cord blood sampled in mothers, but were very low in nonpregnant women and fathers. Tissue expression profiling demonstrated that, although miR-127 and miR-134 were expressed in liver and kidney; C19MC microRNA expression was unique to placenta. When compared with paternal plasma, C19MC microRNA members were 5.240-fold, 28-fold, and 180-fold more abundant in placenta, maternal plasma, and fetal plasma, respectively, which is in agreement with placental-specific expression of this cluster. This finding implies transmission of placental microRNAs into fetal and maternal circulation.

1.4 Pregnancy-related complications

1.4.1 Hypertension in pregnancy

Hypertension is defined as either a systolic blood pressure (BP) of 140 mm Hg or greater, a diastolic blood pressure of 90 mm Hg or greater, or both. 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 (Emuveyan, 1995; Myers and Baxer, 2002).

Hypertensive disorders of pregnancy are classified into 4 categories:

• Chronic hypertension

- defined as high BP known to predate conception or detected before 20 weeks of gestation or persisting longer than 12 weeks after delivery

• Preeclampsia/eclampsia

- preeclampsia is a multiorgan disease characterized by hypertension and proteinuria that occurs after 20 weeks of gestation; severe forms are accompanied with thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, or cerebral or visual symptoms

- eclampsia is the onset of seizures (convulsions) in a woman with pre-eclampsia; eclamptic seizures are usually generalized 60- to 90-second life-threatening seizures that may be followed by either a period of confusion or coma.

• Preeclampsia superimposed on chronic hypertension

- preeclampsia may complicate all other hypertensive disorders such as chronic hypertension and gestational hypertension

- the diagnostic criteria for superimposed preeclampsia include “new-onset proteinuria” in a woman with hypertension before 20 weeks of gestation, a sudden increase in proteinuria if already present in early gestation, a sudden increase in hypertension, or the development of HELLP syndrome (AJOG 2000). Women with chronic hypertension who develop headache, scotomata, or epigastric pain also may have superimposed preeclampsia (ACOG 2002)

• Gestational hypertension (transient hypertension of pregnancy or chronic hypertension identified in the latter half of pregnancy).

- is characterized as new-onset elevations of blood pressure after 20 weeks of gestation, without the presence of proteinurina 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.

1.4.2 Preeclampsia

Preeclampsia (PE) is pregnancy-related clinical syndrome characterized by new-onset hypertension in the second half of pregnancy often accompanied by new-onset proteinuria and many other signs and symptoms, including visual disturbances, headaches, epigastric pain, and the rapid development of edema (ACOG 2002, 2013). It 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).

Preeclampsia tends to occur more commonly (7.5fold higher risk) in healthy nulliparous women and young mothers where it is thought that novel exposure to paternal antigens is involved (Duckitt and Harrington, 2005; Tuffnell et al., 2005). Similarly, association between change in paternity from previous pregnancy (Tubbergen et al., 1999), increased interpregnancy interval (Skjaerven et al., 2002), use of barrier contraception (Klonoff-Cohen et al., 1989) as well as conception by donor sperm injection (Chiriva-Internati et al., 2002) implicate limited recent exposure to paternal antigen as a predisposing factor. In addition, it appears that various paternal factors can also increase a risk of preeclampsia such as an appearance of preeclampsia in the mother of the man who is currently involved in a conception. Furthermore, history of preeclampsia in anamnesis of another woman who was previously pregnant with this partner is considered as a potential risk (Esplin et al., 2001; Duckitt and Harrington, 2005;

Lie et al., 1998). Multiple gestation is an additional risk factor; triplet gestation is a greater risk than twin gestation. The risk of preeclampsia is 7-fold higher if previous pregnancy (pregnancies) were complicated with preeclampsia and increases up to 5-fold and 2-fold if a patient has a first- and second-degree relative with a medical history of the disorder, respectively (Graves, 1998; Salonen Ros et al., 2000; Duckitt and Harrington, 2005; Carr et al., 2005). Cardiovascular risk factors (age over 40, obesity, diabetes) and pre-existing vascular (hypertension, diabetes, and nephropathy) or thrombotic diseases such as the antiphospholipid syndrome are also associated with increased probability of preeclampsia (ACOG 2002, 2013).

Diagnosis of preeclampsia

Preeclampsia is characterized as new onset hypertension and proteinuria after 20weeks of gestation (ACOG 2002; Roberts and Hubel, 2009).

Diagnostic criteria include:

• blood pressure

- greater than or equal to 140 mm Hg systolic or greater than or equal to 90 mm Hg diastolic on two occasions at least 4 (rather 6) hours apart after 20 weeks of gestation in a woman with a previously normal blood pressure

• proteinuria

- the excretion of 300 mg or more of protein in a 24 hour urine collection (or this amount extrapolated from a timed collection)
- or protein/creatinine ratio greater than or equal to 0.3 (each measured as mg/dL)
- or dipstick reading of 1+ (used only if other quantitative methods are not available)

However the American College of Obstetricians and Gynecologists (ACOG) has recently stated in their revised guidelines that proteinuria is no longer

absolutely required for diagnosis of preeclampsia (ACOG 2013). The diagnosis may be established, in the absence of proteinuria, by the hypertension associated with other clinical signs:

- thrombocytopenia
- impaired liver function
- elevated serum creatinine
- pulmonary edema
- new-onset cerebral or visual disturbances

On the base of severity of clinical signs diagnosis of preeclampsia may be divided to severe PE and mild PE. Preeclampsia with the absence of severe manifestations meets basic criteria (blood pressure ≥140/90mmHg combined either with proteinuria or additional clinical signs) is characterized as "mild."
According to ACOG bulletin published in 2002, preeclampsia is considered severe if one or more of the following criteria is present:

• Blood pressure of 160 mm Hg systolic or higher or 110 mm Hg diastolic or higher on two occasions at least 6 hours apart while the patient is on bed rest
• Proteinuria of 5 g or higher in a 24-hour urine specimen or 3+ or greater on two random urine samples collected at least 4 hours apart
• Oliguria of less than 500 mL in 24 hours
• Cerebral or visual disturbances
• Pulmonary edema or cyanosis
• Epigastric or right upper-quadrant pain
• Impaired liver function
• Thrombocytopenia
• Fetal growth restriction (FGR)

Recent revision of ACOG guidelines (2013) maintains some above mentioned criteria for diagnosis of severe preeclampsia, such as:

• Systolic blood pressure of 160 mm Hg or higher, or diastolic blood pressure of 110 mm Hg or higher on two occasions at least 4 hours apart while the patient is on bed rest (unless antihypertensive therapy is initiated before this time)

• Thrombocytopenia (platelet count less than 100,000/microliter)

• Impaired liver function as indicated by abnormally elevated blood concentrations of liver enzymes – aspartate aminotransferase (AST), alanine aminotransferase (ALT) - to twice normal concentration, severe persistent right upper quadrant or epigastric pain unresponsive to medication and not accounted for by alternative diagnoses, or both

• Progressive renal insufficiency (serum creatinine concentration greater than 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal disease)

• Pulmonary edema

• New-onset cerebral or visual disturbances.

However, depending on recent studies that indicate a minimal relationship between the quantity of urinary protein and pregnancy outcome, massive proteinuria (greater than 5 g) was eliminated from the consideration of preeclampsia as severe. Additionally, since fetal growth restriction is presented similarly in pregnant women with and without preeclampsia, it has been removed as diagnostic criteria indicating severe preeclampsia (ACOG 2013).

In approximately 20% of pregnancies complicated by preeclampsia with severe features **HELLP syndrome** may occur (ACOG 2013, Sibai et al., 1993; Weinstei, 1982), nevertheless HELLP syndrome may not be considered a subtype of preeclampsia, since atypical HELLP syndrome can be diagnosed without meeting the blood pressure criteria for the diagnosis of preeclampsia in less than 1% of all pregnancies (ACOG 2013; Sibai, 2004). Name of the syndrome that was described for the first time by Weinstein in 1982, is the acronym for hemolysis (H), elevated liver enzymes (EL), and thrombocytopenia (low platelet count – LP) (Sibai, 2004).

Typical clinical symptoms of the HELLP syndrome are right upper abdominal quadrant or epigastric pain, nausea, and vomiting. The upper abdominal pain may be fluctuating, colic-like. Up to 30–60% women have headache and about 20% women have visual symptoms. In 70% of cases, the disorder is diagnosed antepartum: 10% before 27 weeks, 70% between 27-37 weeks, 20% after 37 weeks of gestation (Rahman and Wendon, 2002). In 30% of cases it is diagnosed postpartum. In the postpartum period, HELLP syndrome usually develops within the first 48 hours in women who had proteinuria and hypertension prior to delivery. The early diagnosis of HELLP syndrome is based on the detection of hemolysis, altered liver tests, and renal dysfunction (Sibai, 2004; Rahman and Wendon, 2002; O´Brien et al., 2007; Ch´ng et al., 2002).

Approximately 20% of all women with HELLP syndrome develop disseminated intravascular coagulation (DIC), which carries a poor prognosis for both mother and fetus, and 6% develop pulmonary edema. When HELLP is complicated by acute renal failure, incidence of DIC and pulmonary edema increases to 84% and 44%, respectively (Sibai et al., 1993; Sibai and Ramadan, 1993).

The most feared complication of preeclampsia is **eclampsia**, defined as the presence of new-onset grand mal seizures and/or unexplained coma during pregnancy or postpartum, with reported incidence ranges from 4 to 6 cases per 10,000 pregnancies in developed countries and higher in developing countries. Eclampsia occurs in about 0.5% of mild preeclampsia and 2-3% of severe preeclampsia. Ninety-one percent of cases of eclampsia develop at or beyond 28 weeks, 7.5% cases occur between 21 and 27 weeks of gestation and remaining 1.5% at or prior to 20 weeks of gestation. Eclamptic seizures are usually generalized 60- to 90-second seizures leading to hypoxia-related bradycardia in fetus.

Pathogenesis of preeclampsia

In view of the fact that preeclampsia occurs only in the presence of the placenta, even when there is no fetus (as in hydatidiform mole) and that the pre-

eclampsia symptoms disappeared, usually when the placenta is delivered, it is evident that abnormal placenta is the major organ involved in pathogenesis of PE (Matsuo et al., 2007; Soto-Wright et al., 1995; Redman, 1991; Sunanda and Johanson, 2001).

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).

During normal placentation, the embryo-derived cytotrophoblast cells form highly invasive extravillous trophoblasts (EVTs) that can migrate into the decidua and invade the first third of the myometrium. After invasion, cytotrophoblasts undergo pseudovasculogenesis by adopting an endothelial phenotype, they switch their adhesion molecules to mimic those of vascular cells (Zhou et al., 1993; Whitley and Cartiwrigh., 2010). In the smooth muscle and endothelial layers of the maternal spiral arteries cytotrophoblasts replace the endothelium of these arteries and induce their remodelling into high-capacitance and low-resistance vessels creating vascular system that allows a 10-fold increase in blood flow, that provide access to maternal oxygen and nutrients essential for fetal and placenta growth (Aplin, 1991; Brosens et al., 1972; Whitley and Cartwright, 2010; Zhou et al., 1997; Pijnenborg et al., 1981; Robertson et al., 1984).

This physiological placentation caused by EVT invasion and vascular remodelling of maternal spiral arteries is insufficient in preeclampsia (Brosens et al., 1972). The trophoblastic invasion of maternal spiral arteries is poor and limited only to spiral arteries present in superficial deciduas (Zhou et al., 1993),

since preeclamptic EVT cells do not adopt an endothelial adhesion phenotype (Zhou et al., 1997). Maternal spiral arteries fail to be invaded or remodeled, resulting in constricted, high-resistance blood vessels (Gerretsen et al, 1983), which are unable to transport adequate blood to the placenta, which then leads to placental ischemia (Gerretsen et al., 1983; Eastabrook et al., 2011; Genbacev et al., 1996; Zhou et al., 1998; Kingdom and Kaufman, 1999; Roberts and Gamill, 2005). In addition, signs of acute atherosis (such as disruption of the endometrium, fibrinoid necrosis, and leukocyte infiltration) of spiral arteries occur, which lead to their partial or complete blockage of the arteries (de Wolf et al., 1975). Hypoxia and arterial blockage cause placental infarcts, proliferation of cytotrophoblasts and outgrowth lesions of syncytiotrophoblasts resulting in the shedding of trophoblast cells and microparticles into maternal circulation (Kovo et al., 2010; Redman and Sargent, 2005).

The ischemic placenta is thought to release soluble circulating factors during the third trimester that cause diffuse maternal endothelial dysfunction manifested as glomerular capillary endotheliosis, increased levels of endothelial extracellular matrix components, raised plasma levels of fibronectine and von Willebrand factor, increased vascular permeability, enhance vascular resistance, and platelate aggregation (Sargent et al., 2003).

<u>Factors contributing to the development of preeclampsia</u>

• Proangiogenic vs. antiangiogenic factors

Clinical manifestation of preeclampsia results, in part, from an imbalance between circulating pro-angiogenic and anti-angiogenic factors in maternal circulation (Ahmad and Ahmed, 2004; Chaiworapongsa et al., 2004, 2005; Levine et al., 2004, 2006; Maynard et al., 2003; Noori et al., 2010; Venkatesha et al., 2006). Since the vascular endothelium relies on proangiogenic factors, the release of antiangiogenic factors by the placenta into maternal circulation causes endothelial dysfunction observed in preeclampsia. Soluble fms-like tyrosine kinase 1 (sFlt1, sVEGFR-1) was reported to be a potent inhibitor of vascular

endothelial growth factor (VEGF) and placental growth factor (PlGF) activity ***in vitro*** **(Kendall and Thomas, 1993)**. Upregulation of Flt-1 was identified in placentas delivered from women with preeclampsia (Maynard et al., 2003; Thomas et al., 2009; Sela et al., 2008; Haydarian et al., 2009).

Another anti-angiogenic protein, and mainly its soluble form, soluble endoglin (sEng), may combine with sFlt-1 to induce features of severe preeclampsia including liver dysfunction, fetal growth restriction, coagulation, and neurologic abnormalities (Venkatesha et al., 2006; Maharaj et al., 2008). Faupel-Badger et al. (2011) demonstrated 4 fold higher Eng levels in placentas derived from preeclamptic women when compared with normal pregnancies. Subsequent *in vitro* studies demonstrated that sEng reduces the binding of transforming growth factor β1 (TGFβ1) to its receptor and blocks TGFβ1 induced vasodilation of rat vessels, likely through down regulation of nitric oxide synthase (Venkatesha et al., 2006; Maharaj et al., 2008). Epidemiologic studies done in women with preeclampsia revealed altered blood levels of sFlt-1 and PlGF during and before clinical signs and symptoms of the disease. While increased levels of sFlt-1 were detected at least 5 weeks prior to the onset of clinical signs of preeclampsia and correlated with the severity of the disease (Chaiworapongsa et al., 2004, 2005; Levine et al., 2004; Hertig et al., 2004), levels of free PlGF were reported to be depressed in women with preeclampsia. Similarly, low urine PlGF level in the first trimester of gestation was determined as risk factor for subsequent development of preeclampsia that also correlates with the severity of the disease (Levine et al., 2004, 2005; Thadhani et al., 2004). Studies on sEng levels performed in women with and without preeclampsia (Levine et al., 2006) are consistent with the studies done on animal experimental models (rodents – pregnant rats injected with both sFlt-1 and sEng; mice injected with adenoviruses carrying both sFlt-1 and sEng genes) (Venkatesha et al., 2006; Maharaj et al., 2008). In eclampsia, sFlt-1, and sEng are altered in a similar manner as in patients with severe preeclampsia (Vaisbuch et al., 2010; Maharaj et al., 2008).

● Placental Hypoxia

The effect of hypoxia on Flt-1 expression was investigation *in vitro* (cytotrophoblast cell culture). Low oxygen tension was shown to induce the expression of Flt-1 and release of sFlt-1 (Nagamatsu et al., 2004; Gerber et al., 1997). In pregnant animal models (Sprague-Dawley rats and baboons *Papio hamadryas*) surgical uteroplacental ischemia evoked by reduction of blood flow (clipping, ligation) in one or both branches of ovarian arteries that supply the uterus induced elevated circulating sFlt-1 levels (Makris et al., 2007; Gilbert et al., 2007). Overexpression of the stabilized form of hypoxia inducible factor 1-α (HIF-1α) during mouse pregnancy (C57BL/6J mice) is associated with a preeclampsia-like phenotype including elevated levels of sFlt-1 and sEng (Tal et al., 2010).

● Immune Factors

Normal placentation requires an inducement of maternal immune tolerance against fetal (paternally-inherited) antigens, which may be altered in preeclampsia. Signs of chronic inflammation, increased number of dendritic cells and macrophage infiltration as well as dysregulation of complement system were observed in preeclamptic placentas (Salafia et al., 1995; Huang et al., 2008b; Lockwood et al., 2006; Hiby et al., 2004; Girardi et al., 2006). Associations between polymorphisms in killer immunoglobulin receptors (KIRs, present on NK cells), HLA-C (KIR ligands present on trophoblasts) and preeclampsia confirmed involvement of immune dysregulation in pathogenesis of the disease (Hiby et al., 2004).

● Renin-Angiotensin-Aldosterone Pathway

Decreased levels of renin, angiotensin, and aldosterone were detected in blood samples derived from preeclamptic pregnancies oppose to normotensive controls (Rinsler and Rigby, 1957; Weir et al., 1970, 1973; Gant et al., 1973, 1980). Despite that, women suffering from preeclampsia exhibit symptoms, such as hypertension and renal damage, which could be not attributed to an excess of ANG II but might probably be related to activation of angiotensin receptor 1 (AT_1). This puzzling situation was solved in 1999 by Wallukat *et al.* who reported, that women with preeclampsia harbor an agonistic autoantibody (AT_1-AA) that stimulates the AT_1 receptor (Wallukat et al., 1999). Extensive investigation into the contribution of AT_1-AA to the pathogenesis of preeclampsia show that AT_1-AA bind to AT_1 receptors on a variety of cells, including trophoblasts, and increase factors attributed to the pathogenesis of preeclampsia such as sFlt-1, PAI-1, and reactive oxygen species (ROS)(Zhou et al., 2008a, b; Bobst et al., 2005; Xia et al., 2002, 2003; Gutteridge and Halliwell, 2000; Burton et al., 2003; van Tuyl et al., 2005; Hubel, 1999). Consequently, while normotensive pregnant women demonstrate decreased vascular sensitivity to ANG II, preeclamptic women, exhibit increased sensitivity of the adrenal cortex and vascular system to ANG II (Gant et al., 1973, 1980; Abdalla et al., 2001; Quitterer et al., 2004), which resulted in vasoconstriction, increase of peripheral resistence and a subsequent increase in blood pressure (Signore et al., 2006; Wallukat et al., 1999).

● Alterations of Placental Enzymes

Experimental animal model (COMT knockdown and knockout mice) showed that COMT (catechol-O-methyltransferase) knockdown leads to a deficiency of 2-methoxyestradiol (2-ME), an inhibitor of the HIF 1α. It finally results in placental hypoxia, hypertension, proteinuria and modestly elevated levels of sFlt-1. Similarly, decrease of 2-ME was also demonstrated in serum of women with preeclampsia (Kanasaki et al., 2008). *In vitro* studies demonstrated that overexpression of heme oxygenase-1 (HO-1, placental enzyme producing carbon monoxide) and/or elevated CO production inhibits sFlt-1 release from

placental explants (Cudmore et al., 2007). Baum and colleagues (2000) observed lower end-tidal CO levels in women with preeclampsia (Baum et al., 2000). These findings (i.e., suppression of sFlt-1 by CO) may explain the lower risk for preeclampsia in smokers (Karumanchi et al., 2010).

● Oxidative Stress/Placental Debris

In preeclampsia, signs of oxidative stress have been demonstrated both in placenta and in maternal circulation (Raijmakers et al., 2004; Hubel, 1999). Preeclamptic placentas compared with normal ones produce greater quantities of superoxide and had less antioxidant capacity. Moreover, placental abnormalities and uteroplacental ischemia may induce the shedding of placental microparticles into maternal circulation. Maternal sera derived from preeclamptic pregnancies showed signs of oxidative modification of protein and lipoprotein particles, decreased levels of antioxidants, elevated circulating levels of placental debris, inflammation and vascular damage (Redman and Sargent, 2005; Knight et al., 1998; Chua et al., 1991; Lok et al., 2008).

● Genetics involvement

Many studies showed a strong familial predisposition to PE. Genomic imprinting controls expression of maternally or paternally derived genes in a parent-of-origin manner by epigenetic modifications that silence either the paternal or maternal allele. Whereas expression of maternal genes inhibit placental trophoblast invasion and placental growth and is responsible for the adaptive immune response to pregnancy (Dekker, 2011), paternally expressed genes are necessary for normal development of trophoblasts and extra-embryonic membranes and tend to enhance fetal growth (Dekker and Sibai, 2001).

A large genetic association study on PE by Goddard et al. (2007) reported six genes (IGF1, IL4R, IGF2R, GNB3, CSF1, and THBS4) with a significant maternal-fetal genotype interaction related to PE.

1.4.3 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).

FGR and SGA definition

FGR and SGA refer to the fetus who does not achieve the expected in utero growth potential due to genetic or environmental factors. It is defined as an estimated fetal weight below 10th percentil.

FGR is diagnosed by combining biometric measurement of fetal size with certain functional parameters. To distinguish FGR from small for gestational age (SGA), following criteria were estabilished:

• Biometric measurement:

- abdominal circumference below the 3rd percentile
- estimated fetal weight below 10th percentile

• Functional parameters

- either a solitary parameter - absent end-diastolic flow (AEDF) in the umbilical artery (only FGR)
- or contributory Doppler ultrasound parameters:
  - pulsatility index (PI) of the umbilical or uterine artery below 95th percentile
  - or cerebroplacental ratio (CPR) below 5th percentile

(Bamberg and Kalache, 2004; Nardozza et al., 2012; Seravalli and Baschat, 2015; Gordijn et al., 2016)

Etiology of fetal growth restriction can be broadly categorized into maternal, fetal, and placental factors:

Maternal factors:

• Maternal medical conditions

Maternal medical conditions include any chronic disorder that is associated with vascular disease (Ounsted et al., 1985; Cunningham et al., 1990;

Duvekott et al., 1995), such as pregnancy-related hypertensive diseases (Ounsted et al., 1985). Antiphospholipid syndrome (APS), an acquired immunomediated thrombophilic state, was also demonstrated to be associated with the occurrence of fetal growth restriction (ACOG 2012).

• Maternal Nutrition

Maternal malnutrition, especially extremely poor protein intake before 26 weeks of gestation as well as severe caloric restriction (i.e. intake of 600–900 kcal daily) was associated with modest reduction in birth weight (Antonov, 1942; Smith, 1947).

• Substance use and abuse

Tobacco use, as well as alcohol, cocaine and other narcotics intake during pregnancy were observed to be associated with low fetal birth weight (Ounsted et al., 1985; Bada et al., 2005; Shu et al., 1995; Mills et al., 1984; Virji, 1991; Naeye et al., 1973).

• Teratogene exposure

Exposure to certain maternal medications, use of antineoplastic medications (e.g. cyclophosphamide), antiepileptic drugs (e.g. valproic acid), and antithrombotic drugs (e.g. warfarin), were associated with an increased risk of fetal growth restriction (Battino et al., 1992; Mastroiacovo et al., 1988; Aviles et al., 1991).

<u>Fetal factors</u>:

• Multiple Gestation

Twin pregnancies account for 10–15% of adverse neonatal outcomes and are associated with an increased frequency of low fetal birth weight (Powers and Kiely, 1994; Houlton et al., 1981). The risk of small-for-gestational age (SGA) in multiple gestations is as high as 25% for twin pregnancies and 60% for triplet and quadruplet pregnancies (Mauldin and Newman, 1998). In addition, monochorionic twin pregnancies are at higher risk because of unequal placental sharing and twin to twin transfusion syndrome (Denbow et al., 2000).

• Infectious Diseases

It was estimated that 5-10% cases of fetal growth restriction may be primarily caused by intrauterine infection. Especially malaria, cytomegalovirus, rubella, toxoplasmosis, varicella, and syphilis infection was implicated in pathogenesis of fetal growth restriction (Desai et al., 2007; Iqbal et al., 2010).

• Genetic and Structural Disorders

At least 50% association of fetal growth restriction was observed in fetuses with trisomy 13 or trisomy 18 (Eydoux et al., 1989). Confined placental mosaicism was also observed to be associated with fetal growth restriction (Wolstenholme et al., 1994).

Fetuses with structural malformations (without chromosomal or genetic abnormalities), such as congenital heart disease or gastroschisis have increased incidence of fetal growth restriction as well (Khoury et al., 1988; Wallenstein et al., 2012; Malik et al., 2007; Raynor and Richards, 1997).

Placental factors:

• Placental Disorders and Umbilical Cord Abnormalities

Association between the presence of either abnormal placentation resulting in poor placental perfusion (e.g. placental insufficiency), placental disorders (e.g. abruption, infarction, circumvallate shape, hemangioma, and chorioangioma), or umbilical cord abnormalities (e.g. velamentous or marginal cord insertion) and the occurrence of fetal growth restriction has been confirmed (Salafia et al., 1997; Laurini et al., 1994; Shanklin, 1970; Ananth et al., 2001; Ananth and Wilcox, 2001). Single umbilical artery occurrence, complicating approximately 1% of all pregnancies, can also lead to the onset of fetal growth restriction (Pollack and Divon, 1992; Thummala et al., 1998; Heifetz, 1984).

Pathological factors associated with FGR

● Reduced placental angiogenesis and hypoxic stress in FGR

Angiogenesis is a complex biological mechanism involved in the process of placentation, wherein new blood vessels are formed from pre-existing ones in response to hypoxia (Biyashev and Qin, 2011). Normal pregnancy is associated with a balanced angiogenic state, while FGR pregnancies are characterized by an anti-angiogenic tendency with enhanced levels of anti-angiogenic factors, such as soluble fms-like tyrosine kinase-1 (sFlt1), and decreased levels of proangiogenic factors, such as neuropilin-1 and placental growth factor (PIGF) in maternal circulation and the placenta (Girardi et al., 2006; Herraiz et al., 2015).

Hypoxic stress is common in placental insufficiency. During the first trimester of pregnancy, hypoxic intervillous space (IVS) is pivotal for both placental and embryonic development, however abnormal maintenance of IVS hypoxia results in shallow EVT invasion and is a major factor leading to FGR (Genbacev et al., 1997; James et al., 2006).

In hypoxic placenta elevated levels of hypoxia-inducible factor 1-alpha (HIF-1α) promotes the increase expression of a Mcl-1 ubiquitin ligase E3 (MULE) which promotes trophoblast apoptosis by targeting the Bcl-2 family member, Mcl-1 (Rolfo et al., 2012).

Soluble fms-like tyrosine kinase-1 (sFlt-1), a soluble truncated variant of the type 1 VEGF receptor (Flt-1) is produced and secreted in higher amount from FGR placenta to the maternal circulation. sFlt-1 binds to pro-angiogenic factors VEGF and PIGF and reduces their bioavailability.

Interestingly, in FGR pregnancies during the third trimester, endocrine gland-derived vascular endothelial growth factor (EG-VEGF) secretion in placenta significantly increases, which leads also to higher concentration of EG-VEGF in maternal serum. EG-VEGF interacts with prokineticin receptor (PROKR1), stimulates proliferation of cytotrophoblasts and increases placental vascularization (Brouillet et al., 2013; Murthi et al., 2015), which may act as compensatory mechanism to ensure proper pregnancy progress.

Placenta serves as the fetal renal, respiratory, hepatic, gastrointestinal, endocrine and immune system organ (Guttmacher et al. 2014, Lanner, 2014). Placental dysfunction (an inefficient functioning of the placenta) is associated with the development of FGR.

Limited invasion of extravillous trophoblasts (EVTs) is associated with defective remodelling of maternal spiral arteries, which results in abnormal placental formation. This causes the hypoperfusion–reperfusion phenomenon that damages the villous architecture, eventually impairing the maternal–fetal exchange (Burton et al., 2009).

Invasion of EVT is a precisely controlled and regulated, spatially- and temporally-dependent process (Zhu et al., 2014) associated with interstitial trophoblast invasion, synthesis of nitric oxide (NO) and endothelial adhesion molecules in extravillous trophoblasts, and the expression of selectins in maternal uterine endothelial cells (Bischof et al., 2000; Lala and Chakraborty, 2003; Bischof and Irminger-Finger, 2005). EVT invasion involves proteolytic degradation of decidual and endothelial extracellular matrix (ECM) to enable appropriate adhesion to ECM elements and active cell migration (Mareel and Leroy 2003; Burrows et al. 1996). This process relies on the action of proteases, particularly metalloproteinases (MMPs; MMP2, MMP9, MMP8, MMP11)(Staun-Ram et al. 2004, Cohen et al. 2006), that are secreted as latent enzymes. Their activities are further regulated by the local concentration of their major natural tissue inhibitors (TIMP1 and TIMP2), which normally bind the MMPs with a ratio 1:1 (Huppertz et al. 1998). The local balance between MMPs and TIMPs at the invasive site is crucial for EVT invasion to decidua. Both, increased levels of matrix metalloproteinases (MMPs) and reduced levels of TIMP-1 and TIMP-3 were reported to be related to FGR pregnancies (Zhu et al., 2014).

● Modulation of fetal growth in FGR pregnancies by inflammation and interaction between decidual natural killer (dNK) cells and EVT

Normal pregnancy is usually defined as a controlled naturally-ongoing systemic inflammatory process, since pregnant women have to maintain immune tolerance against non-self-antigens (paternally-inherited allels) of the fetus. FGR is characterized by altered course of maternal inflammation mediated by specific cytokines. Comparison between the cytokine profiles, produced by maternal lymphocytes in normal pregnancies and FGR group with placental insufficiency showed a stronger pro-inflammatory profile characterized by enhanced levels of pro-inflammatory cytokines such as interleukin-8 (IL-8), interleukine-12 (IL-12), interferon gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α), and decreased levels of anti-inflammatory cytokines such as IL-13 and IL-10 in FGR group (Raghupathy et al., 2012).

In normal pregnancy, the walls of uteroplacental arteries are invaded by the trophoblasts with minimal participation of macrophages located around them. In FGR pregnancies, enhanced infiltration of activated macrophages, which produce high levels of TNF-α, around maternal spiral arteries was observed. Trophoblast cells, which express TNF receptor 1 (TNF-R1), undergo macrophage-induced apoptosis induced by TNF-α. Ongoing macrophage-induced apoptosis attracts and activates more macrophages, which leads to deficient trophoblast invasion (Hunt, 1990; Yui et al., 1994, 1996; Cotechini et al., 2014a, b).

FGR associated with antibody-dependent acquired immune response is frequently mediated by diverse prenatal infections caused by Toxoplasma gondii, Rubella, Cytomegalovirus (CMV), Herpes simplex virus (HSV), Varicella-zoster virus (VZV) and Treponema pallidum (Longo et al., 2014). The infection activates maternal immune and inflammatory responses, characterized by elevated levels of Th1 type cytokines (e.g. TNF-α, IFN-γ and IL-2) and reduced levels of Th2-type cytokine (IL-10) in maternal serum, which indicates a shift from placental anti-inflammatory Th2- to pro-inflammatory Th1-type of immune response (Lin et al., 2003 a, b).

Pregnancy-related complications are often associated with the innate immune system activation. Frequent deposition of one of complement components (C4d) in syncytiotrophoblasts was detected in preeclampsia and/or FGR patients, which leads to maternal vascular anderperfusion (MVU) (Kim et al., 2015). Complement activation causes dysregulation of angiogenic factors required for normal vascularization, which leads to vascular underperfusion.

The decidua basalis, part of the endometrium in pregnant women that participates with the chorion in the formation of the placenta, and the main tissue site, where cells from two individuals (mother and fetus) intermingle, is different from endometrium of non-pregnant women (decidua reveals changed profiles of signal messengers/intermediates, transcription factors, hormones/growth factors, cytokines, chemokines, adhesion molecules, ligands/receptors, cytoskeleton organization, composition of extracellular matrix, ion and water transport, cell cycle regulation, cell trafficking, migration and functions, and angiogenesis)(Brar et al., 2001; Evans et al., 2008; Takano et al., 2007; Garrido-Gomez et al., 2011). Decidua basalis is characterized by the presence of EVT and a diverse population of leucocytes, such as dNK cells, the other parts decidual macrophages (dMϕ) and decidual T cells (dT) (Vesce et al., 2014). Implantation and placental development is partly mediated by close interaction between natural killer cell receptors (NKR) on dNK cell surface and major histocompatibility complex (MHC) antigens on decidual EVT (Colucci et al., 2011). Significantly reduced proportion of dNK cells were found in human placentas obtained from pregnancies complicated by FGR compared to control cases (Eide et al., 2006; Williams et al. 2009).

dNK cells exhibit, compared to peripheral blood NK cells, distinct phenotype characterized by enhanced expression of cell surface receptors including leukocyte immunoglobulin-like receptor B1 (LILRB1), killer-cell immunoglobulin-like receptors (KIRs), NKp46, NKG2D and NKp30, and sphingosine-1-phosphate receptor-5 (S1PR5) (El Costa et al., 2009). Both, expression of these receptors and EVT invasion-promoting capacity of dNK cells

are affected by a steep decrease in the oxygen gradient from arteries to the intervillous space. This event signifies that interaction between dNK cells and EVT regulates EVT invasion and spiral artery remodelling (Ain et al., 2003, 2004; Wallace et al., 2013).

EVT cell express human leukocyte antigen class I ligands (HLA-E, HLA-G, and HLA-C) that are recognized by dNK cell surface receptors, such as CD94/NKG2, LILR and KIRs. Of these ligands and receptors, KIR and HLA-C genes encoding the KIR/HLA-C system are more polymorphic. Thus, particular combinations of maternal KIR and fetal HLA-C could lead to birth weight difference. For example,, binding of the strongly inhibitory KIR to HLA-C2 inherited from the father leads to reduced secretion of cytokines from dNK cells, such as VEGF-A, PIGF, angiopoietin-1 (ANGPT1), ANGPT2, MMPs, IL-8, IFN-γ, IL-1β and chemokine ligand 2 (CCL2/MCP-1), further causing low birth weight (Lima et al., 2014).

Alternatively, the interaction between the the activatory KIR2DSS1 receptor and fetal HLA-C2 is associated with increased birth weight. Thus, maintenance of normal birth weight is dependent on the frequency of combinations between activatory or inhibitory KIR and HLA-C2 (Lash et al. 2011; Robson et al. 2012; Xiong et al. 2013).

Pregnancy with high uterine artery Doppler resistance index (RI) in the first trimester is at a high risk of development of FGR. It is associated with reduced number of dNK cells expressing activatory KIR2DL/S1,3,5 and LILRB1 receptors, and reduced expression of HLA-C and HLA-G on EVT in decidual tissue, which results in higher expression of TNF-α and lower expression of CXCL10 (Moffett and Colucci 2015, Wallace et al. 2015).

● Imprinted genes and epigenetic changes involved in the control of birth weight and FGR pathogenesis.

Imprinted genes play a role in growth related genes in mammals. Genomic imprinting is the epigenetic phenomenon that results in gene

expression in a parent-of-origin-specific manner (Hutter et al., 2010). Imprinted genes that are exclusively expressed in the placenta play a role in the distribution of maternal resources to the fetus (Lambertini et al., 2011). According to the genetic conflict theory of imprinting, maternally imprinted genes that are paternally expressed, such as *IGF2*, *PEG1/MEST* and *PEG3*, promote fetal growth, while paternally imprinted genes that are maternally expressed, such as *H19*, *IGF2R*, *CDKN1C*, *PHLDA2* and *GRB10*, act as growth suppressors (Miguel Constância et al., 2002; Tunster et al., 2011).

To analyze the impact of imprinted genes on birth weight, a study group by Kappil et al. (2015) investigated the association between the expression of imprinted genes expressed in human placenta and birth weight.

Diplas and colleagues (2009) found four downregulated genes (*CDKAL1*, *DHCR24*, *PLAGL1* and *ZNF331*) and five upregulated genes (*CCDC86*, *ILK*, *NNAT*, *PEG10 and PHLDA2*) in placentas of FGR patients. Paternally expressed *PLAGL1* seems to mediate a gene network for growth. PLAGL1 binds to the shared enhancers of *H19*/*IGF2* in placenta. Significant correlations between *PLAGL1* levels and *H19* and *IGF2* expression levels were observed. In addition, PLAGL1 binding and expression also correlate with expression levels of metabolic regulator genes such as *SLC2A4*, *TCF4* and *PPARγ1*.

Interestingly, expression of PLAGL1 shows gender differences in FGR placentas. Total PLAGL1 expression was significantly lower in the placentas of growth-restricted girls than in placentas of growth-restricted boys. This difference was considered to be linked to hormonal differences between sexes (Iglesias-Platas et al., 2014).

IGF2 is one of the well-studied maternally imprinted genes expressed in maternal, fetal and placental tissues during early pregnancy contributing to fetal growth. Significantly decreased IGF2 mRNA levels were observed in human placentas from pregnancies complicated by FGR. This was attributed to altered CpG island DNA methylation and relaxation of imprinting (Koukoura et al., 2011a; Piyasena et al., 2015). Placenta from women diagnosed with FGR showed

significantly enhanced expression of H19 and decreased methylation in H19 promoter, which is consistent with a notion that *H19* produces an untranslated RNA that suppresses growth (Koukoura et al., 2011b; Piyasena et al., 2015).

These results indicate the important role of imprinted genes that are highly expressed in placenta and their epigenetic modifications in FGR pathogenesis.

1.5 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 (for detailed information see Supplement Table 1)

Inconsistent results on expression of microRNAs in placentas derived from PE and/or FGR pregnancies are caused by differences in methodological approaches. There are several methods of microRNA profiling. Most frequently utilized techniques - microarrays and microRNA microarrays (Barad et al., 2004; Zhu et al., 2009; Landgraf et al., 2007, Pineles et al., 2007; Enquobahrie et al., 2011; Wu et al., 2012) provide results that need to be optimally validated and confirmed with more sensitive techniques, such as quantitative real-time PCR. Other methods, such as Northern blot analyses (Donker et al., 2007; Mouillet et al., 2010), quantitative real-time PCR (Luo et al., 2009, 2012; Luque et al., 2014), in situ hybridization (ISH) (Umemura et al., 2013) and highthroughput technologies such as next-generation sequencing (Landgraf et al., 2007; Yang et al., 2011) were used by research groups for microRNA identification and expression profiling. Considerable variability between studies was also observed in statistical methods used for detecting differentially expressed genes as well as in the study sizes (which have been relatively small in majority of cases) and

different definitions of pregnancy-related complications inclusive of patient inclusion/exclusion criteria.

Role of representative microRNAs in pathogenesis of pregnancy-related complications

With regard to the essential role in gene regulation, alterations in microRNA expression may result in dysregulation of several key processes, including programmed-cell death (Pineles et al., 2007), innate/adaptive immunity (Pineles et al., 2007; Enquobahrie et al., 2011), cell cycle regulation, adhesion and migration (Enquobahrie et al., 2011). In addition, microRNAs may influence signal transduction events (Hu et al., 2009), vascular remodelling and angiogenesis (Wang et al., 2012), particularly cancer metastasis (Su et al., 2010), calcium and lipid metabolism (Pineles et al., 2007) and organ/system development (Hu et al., 2009).

Women with preeclampsia exhibit, for example increased placenta expression levels of **miR-210** typically induced by HIF-1α (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 by inhibiting trophoblast invasion (Anton et al., 2013), stimulating mitochondrial respiration targeting (Muralimanoharan et al., 2012; Colleoni et al., 2013; Myatt et al., 2014) or over-stimulating the immune system by inhibition of the STAT6/Interleukin-4 Pathway (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. Tili et al. (2007) reported that lipopolysaccharide (LPS) stimulation of macrophages resulted in the upregulation of miR-155 and TNF-α, which is one of the main cytokines involved in the response to LPS. Simultaneously, miR-155 was shown to function as an oncogene and as a common target of a broad range of inflammatory mediators, suggesting that

miR-155-inducing signals use the c-Jun N-terminal kinase (JNK) pathway (O'Connell et al., 2007).

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

The expression of **miRNA-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*).

2. AIMS

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

HYPOTHESIS

Extracellular pregnancy-associated microRNAs are reliably detected (with sufficient sensitivity and specificity) in maternal circulation in normally ongoing pregnancies.

Extracellular C19MC microRNAs are detectable in maternal circulation during the whole period of gestation in normally ongoing pregnancies.

Levels of extracellular C19MC microRNAs differ significantly between pregnancies with clinically established pregnancy-related complications (GH, PE, FGR) and gestational-age-matched controls.

Levels of extracellular C19MC microRNAs during the first trimester of gestation differentiate between patients at risk of latter development of pregnancy-related complications and normal pregnancies.

3. METHODS

The following methods were used to meet the objectives of this study:

Processing of blood and placenta samples

Peripheral blood (9 ml) samples were collected into EDTA-containing tubes. The plasma was prepared using double centrifugation at 1200 g for 10 minutes. Plasma samples were stored at -80 °C until subsequent processing.

Placenta samples were collected into RNAlater (Ambion) at the delivery room and processed immediately after the delivery. In order to remove erythrocytes, the placenta samples passed through a series of PBS washing steps and further were stored in RNAlater to stabilize RNAs at -80°C until the usage.

Total RNA isolation and RNA enrichment for small RNAs including microRNAs

Total RNA was extracted from 1 ml of maternal plasma samples using Trizol LS Reagent (Invitrogen) and chloroform. Then, RNA was highly enriched for small RNA fraction (< 200 nucleotides) containing microRNAs using mirVana miRNA Isolation kit (Ambion) and modified manufacturer's protocol.

Placenta samples (25mg) were homogenized and disrupted using a rotor-stator homogenizer (Tissue Ruptor, QIAgen) and RNA enriched for small RNA fraction was isolated using mirVana miRNA Isolation kit (Ambion) according to manufacturer's instruction.

To obtain DNA-free RNA samples, Deoxyribonuclease I (DNase I, Fermentas, Onatario, Canada) treatment was carried out to remove any contaminating DNA. RNA concentration and purity were determined by spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies).

Real-time RT-qPCR analysis

Detection of pregnancy-associated microRNAs in maternal plasma and placentas was performed using quantitative real-time RT-PCR. The reverse transcription (RT) step (microRNA reverse transcription into cDNA) was realized using a single miRNA-specific stem-loop RT primer to achieve better specificity to

discriminate similar microRNAs and enhance the stability of microRNAs to improve RT efficiency for relatively short RT primers. Further, PCR products were amplified from cDNA samples using a specific TaqMan MicroRNA Assay together with TaqMan Universal PCR Master Mix (Applied Biosystems) on a 7500 Real-Time PCR system (Applied Biosystems). A sample was considered positive if the amplification signal occurred before the 40th threshold cycle. All PCRs were performed in multiple replicates and multiple negative controls such as NTC (non-template control; water instead of cDNA sample) and NAC (non-amplicon control; non-transcribed RNA sample) were involved in each PCR analysis.

Quantification of microRNAs

• Absolute quantification

Concentrations of individual microRNAs were expressed as pg (picograms) of total RNA enriched for small RNAs per milliliter of plasma. Serially diluted RNA derived from the fetal part of the placenta (the part of the placenta derived from the chorionic sac that encloses the embryo, consisting of the chorionic plate and villi) was used to create the standard curves. The two-step RT-qPCR with TaqMan MicroRNA Assays showed reliable and reproducible results (slope and R2 coefficient of determination) for all tested microRNAs in placental tissues and was also applied to plasma samples.

• Relative quantification

The expression of particular microRNA in maternal plasma was determined using the comparative Ct method (Livak and Schmittgen, 2001) relative to the expression of the same microRNA in the reference sample, randomly selected placenta derived from gestation with normal course.

Due to a lack of generally accepted standards, former results were assessed relatively to normalization factor characterized as genomic mean of ubiquitous miR-16 and let-7d (their concentration in our study cohort was not influenced by clinical stage of pregnancy). However, since other researchers reported the dysregulation miR-16 and let-7d in samples derived from pregnant

women, synthetic C. elegans microRNA (cel-miR-39, Qiagen, Hilden, Germany) has been started to be used as an internal control. All experimental real-time RT-qPCR data were normalized to cel-miR-39, as it shows no sequence homology to any human microRNA. 1 μl of 0.1 nM cel-miR-39 was spiked in after incubation with Trizol LS reagent to all maternal plasma samples.

Statistical analysis

Different methods of statistical analysis were used depending on the normality of data distribution in the study cohorts and the needs of the study.

Statistical analysis was performed using Statistica software (version 9.0; StatSoft, Inc., USA).

Data distribution was assessed using the Shapiro-Wilk test; the results of the Shapiro-Wilk test determined what kind of statistical tests would be utilized for further analyses (non-parametric or parametric tests):

- Non-parametric tests
  - Mann-Whitney U test for the comparison between two groups
  - Kruskal-Wallis test for the comparison between three or more groups
- Parametric tests
  - Students *t*-test for the comparison between two groups

Spearman's rank correlation coefficient (ρ)

Correlation between variables including absolute and/or relative microRNA quantification and Doppler ultrasonography parameters (the pulsatility index in the umbilical artery, the pulsatility index in the middle cerebral artery and the cerebroplacental ratio) was calculated using the Spearman's rank correlation coefficient (ρ). If the correlation coefficient value is -1 or 1, there is a perfect negative or positive correlation. If it ranges within <-1; -0.5> or <0.5; 1>, there is a strong negative or positive correlation. If it varies from -0.5 to 0 and from 0 to 0.5, there is a weak negative or positive correlation. If the

ρ-value reaches 0, there is no correlation. Significant levels of correlation were set at a *P* value of < 0.05.

Bonferroni correction

To address the problem of multiple comparisons, the Bonferroni correction, which find the critical value (alpha) for an individual test by dividing the familywise error rate (usually 0.05) by the number of tests, was used.

Receivers operating characteristic (ROC) curves

Receivers operating characteristic (ROC) curves were constructed to calculate the area under the curve (AUC) and the best cut-off point for particular placental specific microRNA was used in order to calculate the respective sensitivity, specificity, predictive values and likelihood ratios in the prediction of pregnancy-related complications.

Comparison of ROC curves was done with the method of DeLong et al. (1988) using MedCalc statistical software (MedCalc Software bvba, Ostend, Belgium). The software gave the difference between the areas under the ROC curves, with standard errors, 95% confidence intervals and p-values.

Prediction of target genes

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. The data were collected from miRDB database (http://mirdb.org/miRDB/). All the targets were predicted by a bioinformatics tool MirTarget2, which was developed by analyzing thousands of genes impacted by microRNAs with an SVM learning machine.

The methodology is described in detail in individual publications in Section 4.

4. PUBLICATIONS

4.1 Placental-specific microRNAs in maternal circulation – identification of appropriate pregnancy-associated microRNAs with diagnostic potential

Kotlabova K, Doucha J, Hromadnikova I.
J Reprod Immunol. 2011 May;89(2):185-91.

4.1.1 Overview

Approximately 98% of human transcriptome is composed of non-coding RNAs (ncRNA) (Mattic et al., 2001) including well-characterized transfer (tRNA) and ribosomal (rRNA) RNAs, however recently expanded to include microRNAs (miRNAs), a class of short (~18-25 nucleotides), single-stranded RNAs that target specific messenger RNAs (mRNAs) and negatively modulate gene expression. Besides, ncRNAs include as well as small RNAs such as snoRNAs small nucleolar RNA), siRNAs (small interfering RNA), snRNAs (small nuclear RNA), exRNAs (extracellular RNA), piRNAs (Piwi-interacting RNA) and scaRNAs (small Cajal body-specific RNAs) and the long ncRNAs.

Recent data indicates that microRNAs regulate cell development, proliferation, communication (Bartel, 2004) and death (Baehrecke, 2003), as well as tissue differentiation and the maintenance of tissue integrity (Ason et al., 2006; Kloosterman and Plasterk, 2006). They are also implicated in pathogenesis of various human diseases.

Comprehensive studies revealed that some microRNAs are expressed abundantly in placenta (Barad et al., 2004). Functional impairment of placenta leads to severe abnormal pregnancies, such as preeclampsia and/or fetal growth restriction. Several studies have shown that preeclampsia is associated with microRNA expression alterations in the placenta (Hu et al., 2009; Pineles et al., 2007; Zhu et al., 2009). An increased microRNA expression gives rise to a down-regulation of potential target genes, which may contribute to pathophysiology of preeclampsia (Pineles et al., 2007). Zhu and colleagues (2009) demonstrated 11 microRNAs up-regulated and 23 microRNAs (including miR-195) down- regulated

in preeclamptic placentas vs. normal placental tissues. Similarly, a significant increase of seven microRNAs (miR-16, miR-29b, miR-195, miR-26b, miR-181a, miR-335 and miR-222) was found in placentas from Chinese women with severe preeclampsia compared to healthy controls, including miR-195 which was nevertheless reported by Zhu et al. (2009) to be down-regulated.

However, application to clinical practice requires non-invasive monitoring of pregnancy-related complications, related to a particular extracellular microRNA profile, with readily available biological samples, such as maternal plasma. For example, Chim et al. demonstrated that four microRNAs (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 (Chim et al., 2008).

4.1.2 Aims and methods

The main goal of this study was to identify pregnancy-associated microRNAs in maternal circulation and on the basis of their levels in maternal circulation differentiate between pregnant and non-pregnant individuals.

To achieve this goal, we tested microRNAs (miR-34c, miR-135b; miR-372) previously identified as pregnancy-associated microRNAs (Chim et al., 2008), miR-518b selected on the basis of the study describing up-regulation in preeclamptic placentas (Zhu et al., 2009) and other 16 microRNAs (miR-512-5p, miR-515-5p, miR-224, miR-516-5p, miR-517*, miR-136, miR-518f*, miR-519a, miR-519d, miR-519e, miR-520a*, miR-520h, miR-524-5p, miR-525, miR-526a and miR-526b) which have been reported, according to the miRNAMap database and the study presented by Liang and colleagues (2007), to be placenta specific. These selected 16 microRNAs have been reported to be highly expressed in placental tissues, while showing no or minimal expression in other tissues.

In our study, we tested the levels of these 20 selected microRNAs in whole peripheral blood and plasma samples derived from 10 healthy non-

pregnant individuals and 23 uncomplicated singleton pregnancies with normal course of gestation within the first-, second- and third-trimesters.

4.1.3 Results and discussion

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 $\geq$ 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 later 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.

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Placental-specific microRNA in maternal circulation – identification of appropriate pregnancy-associated microRNAs with diagnostic potential

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ABSTRACT

The goal of this study was to identify placental specific microRNAs present in maternal plasma that differentiate between women with normal pregnancies and nonpregnant individuals. The selection of appropriate pregnancy-associated microRNAs with diagnostic potential was based on the following criteria: (1) detection rate of 100% in full-term placentas, (2) detection rate of ≥67% in maternal plasma throughout gestation (at least four positive wells out of six tested wells) and (3) not detectable in whole peripheral blood and plasma samples of nonpregnant individuals. Initially, we tested microRNAs (miR-34c, miR-372, miR-135b and miR-518b), which had been previously identified as pregnancy-associated microRNAs. Additionally, we selected 16 other highly specific placental microRNAs (miR-512-5p, miR-515-5p, miR-224, miR-516-5p, miR-517*, miR-136, miR-518f*, miR-519a, miR-519d, miR-519e, miR-520a*, miR-520h, miR-524-5p, miR-525, miR-526a, and miR-526b) from the miRNAMap database. Seven microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525 and miR-526a) were identified as new pregnancy associated microRNAs with diagnostic potential. Their levels in maternal plasma during the 36th week of gestation corresponded to 45.0–427.0 pg of total RNA (enriched for small RNAs) per milliliter of maternal plasma.

1. Introduction

Approximately 98% of the human transcriptome is composed of nonprotein-coding RNA (ncRNA) (Mattick, 2001), including well-characterized transfer (tRNA) and ribosomal (rRNA) RNAs. However, the ncRNA fraction was recently expanded to include microRNAs, a class of short (approximately 18–25 nucleotides), single-stranded RNAs that target specific messenger RNAs (mRNAs), providing them with the ability to negatively modulate gene expression.

Recent data indicate that microRNAs play a fundamental role in a variety of physiological and pathological processes in animals. They are believed to be critical in cell development, proliferation, communication (Bartel, 2004), and death (Baehrecke, 2003), as well as in tissue differentiation and the maintenance of tissue identity (Ason et al., 2006; Kloosterman and Plasterk, 2006). In addition, microRNAs have been implicated in a variety of human diseases, such as cardiovascular disease (Thum et al., 2008), primary muscular disorders (Eisenberg et al., 2007), and cancer (Huang et al., 2010). MicroRNA analyses indicate that diverse affected tissues display microRNA expression profiles that are significantly different from normal tissues (Calin and Croce, 2006), which may be useful for a wide range of applications in clinical diagnostics (Rosenfeld et al., 2008).

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Studies of microRNA expression across several organs have revealed that microRNA expression is tissue-specific (Lagos-Quintana et al., 2002), and that some microRNAs are also expressed abundantly in placenta (Barad et al., 2004). Functional impairment of this organ occasionally leads to severely abnormal pregnancies, such as preeclampsia (caused by poor placentation with impaired remodeling of the spiral arteries) and intrauterine growth restriction (IUGR). Nevertheless, the fundamental mechanisms that underlie placental pathology and their contribution to the clinical manifestation are not completely clear. However, recent studies have shown that preeclampsia is associated with alterations in microRNA expression in the placenta (Hu et al., 2009; Pineles et al., 2007; Zhu et al., 2009). The increased microRNA expression suggests the down-regulation of potential target genes, which may contribute to the pathology of preeclampsia (Pineles et al., 2007). Zhu et al. (2009) carried out a study that offered a comprehensive analysis of microRNA expression profiles in preeclamptic pregnancies, which demonstrated that 11 microRNAs were up-regulated and 23 microRNAs were down-regulated in preeclamptic placentas vs. normal placentas. Similarly, a significant increase of another seven microRNAs (Hu et al., 2009) was found in placentas from Chinese women with severe preeclampsia compared with healthy controls, including miR-195, which was nevertheless reported by Zhu et al. to be down-regulated. However, application of these findings to clinical practice requires monitoring of pregnancy complications, related to a particular microRNA profile, with readily available biological samples, such as maternal plasma. Chim et al. demonstrated that four placental microRNAs (miR-141, miR-149, miR-299-5p, and miR-135b) were abundant in the plasma of pregnant women and rapidly cleared from the maternal circulation after delivery (Chim et al., 2008).

The main goal of our study was to identify placenta-specific microRNAs with a plasma expression profile that differed significantly between pregnant and nonpregnant individuals.

Initially, we tested in maternal plasma microRNAs (miR-34c, miR-135b, and miR-372) that had been previously identified as pregnancy-associated microRNAs (Chim et al., 2008) and miR-518b selected on the basis of the above-mentioned up-regulation in preeclamptic placentas (Zhu et al., 2009). Additionally, we selected another 16 microRNAs (miR-512-5p, miR-515-5p, miR-224, miR-516-5p, miR-517*, miR-136, miR-518f*, miR-519a, miR-519d, miR-519e, miR-520a*, miR-520h, miR-524-5p, miR-525, miR-526a, and miR-526b), which, according to the miRNAMap database and the study presented by Liang et al. (2007), have been reported to be placenta-specific (i.e., to be significantly expressed in the placenta while showing no or minimal expression in other tissues).

2. Materials and methods

2.1. Sample collection

Samples of peripheral blood (5 mL) were collected into tubes containing EDTA from 23 pregnant women and 10 healthy nonpregnant participants. The study was approved by the ethics committee board, and blood samples were taken after receiving written consent from the study participants. We recruited serial samples obtained from uncomplicated singleton pregnancies within the first, second, and third trimester attending the prenatal clinic at the Department of Obstetrics and Gynecology at the University Hospital Motol, Prague. Women with normal pregnancies were defined as those without medical, obstetrical, or surgical complications at the time of the study and who subsequently delivered at term (>37 weeks' gestation) an appropriate-for-gestational age newborn without neonatal complications. Term placentas used as positive controls for detection of selected microRNAs were obtained at the Institute for Care of Mother and Child, Prague.

2.2. Total RNA extraction and enrichment procedure for microRNAs

To harvest cell-free plasma, we centrifuged whole blood samples twice at $1200 \times g$ for 10 min at room temperature. A cube measuring 10 mm^3 of placental tissue was preserved in RNAlater (Ambion, Austin, TX, USA) immediately after delivery. All samples were stored at −80 °C until further processing.

Total RNA was isolated from 200 μL of whole peripheral blood from healthy nonpregnant participants using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and further used for the detection of selected microRNAs.

Total RNA was extracted from 1 mL plasma samples, derived from both pregnant and nonpregnant participants, and 25 mg of placental tissue. Extraction was then followed by an enrichment procedure for small RNAs according to the manufacturer's instructions using a mirVana microRNA Isolation kit (Ambion). With regard to plasma samples, Trizol LS reagent and the manufacturer's protocol for total RNA extraction from biological fluids (Invitrogen, Carlsbad, CA, USA) preceded the small RNA enrichment procedure.

To minimize DNA contamination, we treated the eluted RNA with 5 μL of DNase I (Fermentas International, Burlington, ON, Canada) for 30 min at 37 °C.

2.3. Reverse transcriptase reaction using a stem-loop primer

Each microRNA was reverse transcribed into complementary DNA (cDNA) using a TaqMan MicroRNA Assay (Applied Biosystems, Branchburg, NJ, USA) containing microRNA-specific stem-loop RT primers and a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Branchburg, NJ, USA) in a total reaction volume of 50 μL, and carried out according to the manufacturer's instructions.

Reverse transcriptase reactions were performed using a 7500 real-time PCR system (Applied Biosystems) and the following thermal cycling parameters: 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C, and then held at 4 °C.

Table 1
Characteristics of selected microRNAs.

microRNA	Tissue specificity	Sequence of microRNA	Ref. [n.]	Reason for the involvement in the study
miR-16	Ubiquitous	5′-UAGCAGCACGUAAAUAUUGGCG-3′	[47]	Endogenous control
miR-34c	Tissue nonspecific	5′-AGGCAGUGUAGUUAGCUGAUUGC-3′	[31]	High expression in placenta
miR-135b	Tissue nonspecific	5′-UAUGGCUUUUCAUUCCUAUGUG-3′	[31]	Positive in maternal plasma
Undetectable postpartum				
miR-136	Placenta, other tissues	5′-ACUCCAUUUGUUUUGAUGAUGGA-3′	[miRNAMap]	High expression in placenta
miR-224	Placenta, other tissues	5′-CAAGUCACUAGUGGUUCCGUUUA-3′	[miRNAMap]	High expression in placenta
miR-372	Placenta, testes	5′-AAAGUGCUGCGACAUUUGAGCGU-3′	[31]	High expression in placenta
miR-512-5p	Placenta	5′-CACUCAGCCUUGAGGGCACUUUC-3′	[miRNAMap]	High expression in placenta
miR-515-5p	Placenta	5′-UUCUCCAAAAGAAAGCACUUUCUG-3′	[miRNAMap]	High expression in placenta
miR-516-5p	Placenta	5′-CAUCUGGAGGUAAGAAGCACUUU-3′	[miRNAMap]	Placenta-specific microRNA
miR-517*	Placenta	5′-CCUCUAGAUGGAAGCACUGUCU-3′	[miRNAMap]	High expression in placenta
Up-regulation in preeclamptic placenta				
miR-518b	Placenta	5′-CAAAGCGCUCCCCUUUAGAGGU-3′	[29]	High expression in placenta
Up-regulation in preeclamptic placenta				
miR-518f*	Placenta	5′-CUCUAGAGGGAAGCACUUUCUCU-3′	[miRNAMap]	High expression in placenta
miR-519a	Placenta	5′-AAAGUGCAUCCUUUUAGAGUGUUAC-3′	[miRNAMap]	Placenta-specific microRNA
miR-519d	Placenta	5′-CAAAGUGCCUCCCUUUAGAGUGU-3′	[miRNAMap]	High expression in placenta
miR-519e	Placenta	5′-UUCUCCAAAAGGGAGCACUUUC-3′	[miRNAMap]	High expression in placenta
miR-520a*	Placenta	5′-CUCCAGAGGGAAGUACUUUCU-3'	[miRNAMap]	High expression in placenta
miR-520h	Placenta	5′-ACAAAGUGCUUCCCUUUAGAGU-3′	[miRNAMap]	High expression in placenta
miR-524-5p	Placenta	5′-CUACAAAGGGAAGCACUUUCUC-3′	[miRNAMap]	High expression in placenta
miR-525	Placenta	5′-CUCCAGAGGGAUGCACUUUCU-3′	[miRNAMap]	High expression in placenta
miR-526a	Placenta	5′-CUCUAGAGGGAAGCACUUUCU-3′	[miRNAMap]	High expression in placenta
miR-526b	Placenta	5′-CUCUUGAGGGAAGCACUUUCUGUU-3′	[miRNAMap]	High expression in placenta

miRNAMap – miRNAMap database (mirnamap.mbc.nctu.edu.tw).

2.4. Absolute quantification of individual microRNAs using real-time PCR

Fifteen microliters of cDNA, corresponding to each selected microRNA, and 4.4 μL of cDNA corresponding to ubiquitous miR-16, were mixed with specific primers and the TaqMan MGB probe (TaqMan MicroRNA Assay; Applied Biosystems), and the ingredients of the TaqMan Universal PCR Master Mix (Applied Biosystems) in a total reaction volume of 35 μL. TaqMan PCR conditions were set up as described in the TaqMan guidelines with the exception of an increase to 50 cycles. The analysis was performed using a 7500 real-time PCR system (Applied Biosystems). All PCRs were performed in multiple replicates ranging from 3 to 10 wells depending on the type of biological material. Multiple negative controls such as NTC (water instead of cDNA sample), NAC (nontranscribed RNA samples) and genomic DNA isolated from the same biological samples, did not generate any signals during the PCR reactions. A participant's specimen was considered positive if the amplification signal occurred before the 40th threshold cycle ($C_t < 40$). In the case of placenta, we assessed the requirements for the positivity (three positive wells out of three tested wells). In the case of maternal plasma samples, we assessed the

Table 2
Selection of appropriate pregnancy-associated microRNAs with diagnostic potential.

MicroRNA	Placenta	Nonpregnant controls		Pregnant women – plasma			Potential for diagnosis
		Whole blood	Plasma	12th week of gestation	16th week of gestation	36th week of gestation	
miR-16	3/3+	3/3+	3/3+	6/6+	6/6+	6/6+	Endogenous control
miR-34c	3/3+	2/6+	ND	2/6+	1/6+	1/6+	No
miR-135b	3/3+	0/3+	ND	1/6+	2/6+	6/6+	Third trimester only
miR-136	3/3+	0/3+	ND	0/6+	0/6+	0/6+	No
miR-224	3/3+	3/3+	ND	4/6+	5/6+	6/6+	No
miR-372	3/3+	0/3+	ND	0/6+	3/6+	0/6+	No
miR-512-5p	3/3+	3/6+	ND	3/6+	3/6+	5/6+	No
miR-515-5p	3/3+	2/6+	ND	3/6+	3/6+	6/6+	No
miR-516-5p	3/3+	0/3+	0/10+	6/6+	4/6+	6/6+	Yes
miR-517*	3/3+	0/3+	0/10+	4/6+	5/6+	5/6+	Yes
miR-518b	3/3+	0/3+	0/10+	6/6+	6/6+	6/6+	Yes
miR-518f*	3/3+	2/6+	ND	1/6+	0/6+	1/6+	No
miR-519a	3/3+	0/3+	ND	0/6+	0/6+	0/6+	No
miR-519d	3/3+	3/3+	ND	6/6+	6/6+	6/6+	No
miR-519e	3/3+	2/6+	ND	1/6+	1/6+	1/6+	No
miR-520a*	3/3+	0/3+	0/10+	6/6+	6/6+	6/6+	Yes
miR-520h	3/3+	0/3+	0/10+	4/6+	6/6+	6/6+	Yes
miR-524-5p	3/3+	0/3+	ND	2/6+	2/6+	2/6+	No
miR-525	3/3+	0/3+	0/10+	6/6+	6/6+	6/6+	Yes
miR-526a	3/3+	0/3+	0/10+	5/6+	5/6+	5/6+	Yes
miR-526b	3/3+	0/3+	ND	0/6+	1/6+	1/6+	No

Detection rate is expressed as the number of positive PCR wells among the number of tested wells per particular case.
ND, not done.
The newly identified microRNAs with diagnostic potential are shown in bold.
The identification of appropriate pregnancy-associated microRNAs with diagnostic potential was based on the following criteria:
1. Detection rate of 100% in term placenta samples.
In the case of placenta we assessed the requirements for the positivity (three positive wells out of three tested wells).
2. Detection rate of ≥67% in maternal plasma throughout gestation.
In the case of maternal plasma samples, we assessed the requirements for the positivity (four to six positive wells out of six tested wells).
3. No detection in nonpregnant individuals (whole peripheral blood and plasma samples).
In the case of nonpregnant controls (whole blood) we assessed the requirements for the negativity (three negative wells out of three tested wells).
Finally, those microRNAs, which were clearly positive in placenta and maternal plasma samples and concurrently negative in whole blood of nonpregnant controls, were further tested in plasma samples derived from nonpregnant controls. If they fulfilled the requirements of negativity (no positive replicate out of ten tested wells), the microRNAs were finally considered to be placenta-specific microRNAs and selected for further research.

requirements for the positivity (at least four positive wells out of six tested wells).

Four-fold diluted RNA derived from the fetal side of the placenta (the part of the placenta derived from the chorionic sac that encloses the fetus, consisting of the chorionic plate and villi) was used to create the standard curves. The two-step RT-PCR with TaqMan MicroRNA assays showed reliable and reproducible results (slope and R2 coefficient of determination) for all tested microRNAs in placental tissues and was also applied to plasma samples. Concentrations of individual microRNAs were expressed as picograms of total RNA enriched for small RNAs per milliliter of plasma.

3. Results

Initially, we verified the specificity of selected microRNAs (Table 1) by testing whole peripheral blood derived from healthy nonpregnant women. Next, we tested the sensitivity of individual microRNA assays on plasma samples of women with normal pregnancies throughout the entire gestation period.

The selection of appropriate pregnancy-associated microRNAs with diagnostic potential was based on the following criteria: (1) detection rate of 100% in full-term placenta tissue samples, (2) detection rate of ≥67% in maternal plasma throughout gestation (at least four positive wells out of six tested wells) and (3) not detectable in whole peripheral blood and plasma samples taken from nonpregnant women.

All 20 selected microRNAs were reliably detectable in the fetal side of the placenta, when a fixed concentration of RNA (5 ng/μL) was used in the analysis; however, their expression differed significantly with respect to various C_t values, ranging from 17.4 to 35.2.

MicroRNAs with later amplification in the placenta, miR-136 and miR-519a, with a mean C_t value of 31.0 and 35.2, respectively, were undetectable in maternal plasma. Six microRNAs (miR-34c, miR-372, miR-518f*, miR-519e, miR-524-5p, and miR-526b) displayed very poor amplification results throughout the entire gestation period. Seven other microRNAs (miR-224, miR-512-5p, miR-515-5p, miR-516-5p, miR-517*, miR-520h, miR-526a, and miR-135b) were reliably detectable only during the third trimester of gestation, while only four microRNAs (miR-518b, miR-519d, miR-520a*, and miR-525) were detectable throughout the gestation period.

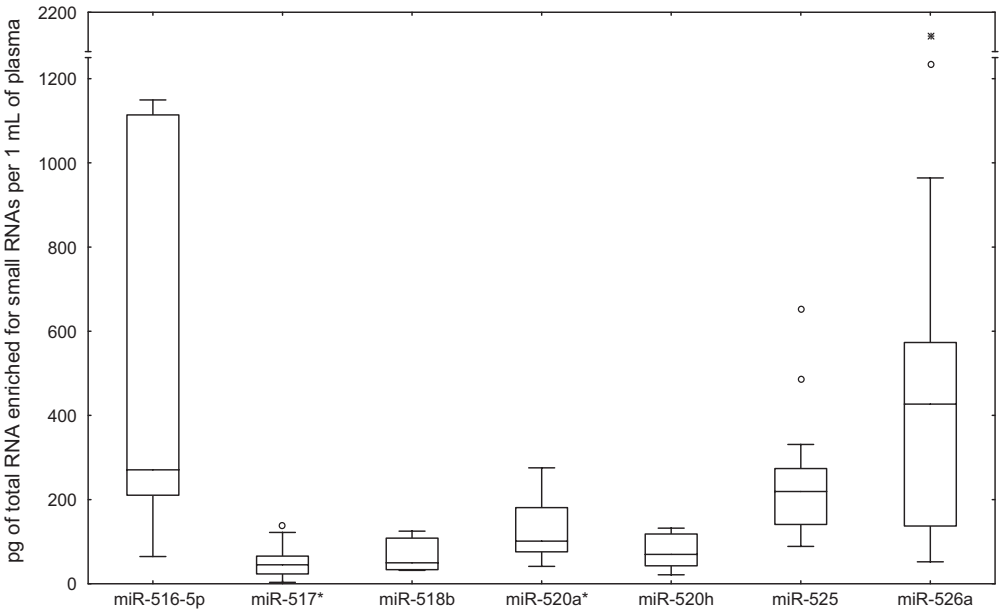

Fig. 1. Quantification of newly identified pregnancy-associated microRNAs in maternal plasma during the 36th week of gestation. Box plots of miRNA concentrations in plasma samples of normal pregnancies during the 36th week of gestation. The upper and lower limits of the boxes represent the 75th and 25th percentiles. The upper and lower whiskers represent maximum and minimum values. The median is indicated by the line in each box. Outliers are indicated by circles and extremes by asterisks.

From the group of 11 microRNAs present in maternal plasma samples, seven microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) were simultaneously negative in the whole blood derived from the healthy, nonpregnant women (three negative wells out of three tested wells). The negative result of that amplification was further confirmed by testing autologous plasma samples (10 negative wells out of 10 tested wells).

On the basis of these results, 7 microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) were finally identified to be pregnancy-associated with diagnostic potential. All results are summarized in Table 2.

The levels of newly identified pregnancy-associated microRNAs in maternal plasma during the 36th week of gestation corresponded to 45.0–427.0 pg of total RNA enriched for small RNAs per mL of maternal plasma (Fig. 1).

4. Discussion

Nucleic acids (DNA and RNA) of placental origin released into the maternal circulation (Ng et al., 2003; Chim et al., 2005), in the form of apoptotic bodies (Ishihara et al., 2002) during gestation, have been used as a non-invasive source for prenatal diagnosis (Lo et al., 1997, 1998; Costa et al., 2001; Faas et al., 1998; Honda et al., 2002; Hromadnikova et al., 2005; Rijnders et al., 2001; Sekizawa et al., 2003; Zhong et al., 2000) and assessment of placental insufficiency-related complications associated with excessive apoptosis of placental trophoblasts (Caramelli et al., 2003; Hromadnikova et al., 2009, 2010; Lo et al., 1999; Sekizawa et al., 2003; Smid et al., 2001; Tsui et al., 2007). It is now crucial to discover whether placenta-specific microRNAs are also released into the maternal circulation and, if so, whether they can be exploited for non-invasive prenatal diagnosis or as predictors of pregnancy-related complications. Luo et al. recently demonstrated that placenta-specific microRNAs are likely constituents of chorionic villous trophoblasts and are released extracellularly into the maternal circulation during pregnancy via exosomes (Luo et al., 2009).

As described previously, miR-16 is ubiquitous in almost all somatic tissues (Lagos-Quintana et al., 2003) and hence we used this microRNA to introduce and optimize microRNA detection in various biological samples. Initially, we tested microRNAs (miR-518b, miR-34c, miR-372, miR-135b) selected from the above-mentioned publications (Zhu et al., 2009; Chim et al., 2008). In particular, miR-518b was observed to be elevated in preeclamptic placenta, compared with normal (nonpreeclamptic) placenta (Zhu et al., 2009). miR-34c, miR-372, and miR-135b were observed to be expressed at a concentration 10 times or higher in the placenta, compared with blood cells of pregnant women (Chim et al., 2008).

Only miR-518b fulfilled our established criteria for the selection of an optimal microRNA marker for potential diagnostic and/or prognostic purposes. Unfortunately, miR-372 was not present in maternal circulation throughout gestation. However, in our setting, miR-34c showed positive amplification in whole blood derived from nonpregnant healthy women together with inconsistent detection in maternal plasma samples (the amplification occurred in less than four wells out of six tested wells throughout gestation). Therefore, it was excluded from further analysis. Like Chim et al. (2008), we demonstrated the absence of miR-135b in nonpregnant women and its presence in the maternal circulation, particularly during late gestation, when it can be reliably detected. The detection rates of miR-135b in the 12th, 16th, and 36th week of gestation in maternal plasma were 17%, 33%, and 100%, respectively.

The other 16 microRNAs (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) chosen for this study were shown to be expressed only in the placenta and/or at a significantly higher levels in the placenta, compared with other human tissues (Liang et al., 2007). Unfortunately, miR-512-5p, miR-515-5p, miR-518f*, miR-519d, and miR-519e were detected in blood cells of healthy nonpregnant women and therefore, were excluded from further analysis.

Finally, those microRNAs with no positive signals in whole blood samples as well as those with negative results in the plasma of healthy, nonpregnant women were also considered to be placenta-specific. However, three microRNAs (miR-519a, miR-524-5p, and miR-526b) were excluded from follow-up testing since they displayed negative and/or inconsistent amplification (less than four positive wells out of six tested wells) in the maternal circulation throughout gestation.

In conclusion, we identified seven placenta-specific microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525 and miR-526a) that were reliably detectable in the maternal circulation throughout the whole period of gestation. Similarly, another concurrently running study identified 24 pregnancy-associated microRNAs (Miura et al., 2010). However, the investigators finally selected for further analysis only five microRNAs (miR-515-3p, miR-517a, miR-517c, miR-518b, and miR-526b) that showed the significant increase in maternal plasma concentrations throughout gestation and the significant decrease after pregnancy termination. A comparison of these two studies revealed that we identified at least two pregnancy-associated microRNAs that have not been reported previously (miR-516-5p and miR-520h). Because microRNAs are exceptionally stable in plasma (Mitchell et al., 2008) they hold promise as markers that can be used in the clinical setting. The measurement of placenta-specific microRNAs in maternal plasma for prenatal monitoring and diagnosis shows promise and warrants further research.

The association between pathological pregnancy and altered microRNA expression suggests the possibility of a functional role for microRNA in pregnancy complications; therefore, it will be interesting to determine whether possible changes in the expression profiles of these microRNAs in maternal plasma can serve as diagnostic markers of pregnancy complications associated with placental insufficiency, such as preeclampsia or fetal growth restriction.

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4.2 Absolute and relative quantification of placenta-specific microRNAs in maternal circulation with placental insufficiency-related complications

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4.2.1 Overview

Growth and differentiation of the placenta, a vital fetal organ, is fundamental to mammalian reproduction, hence its functional impairment leads to development of placental insufficiency related complications (PIRCs), the major cause of maternal and perinatal morbidity and mortality worldwide. In order to reduce the most severe consequences of PIRCs, new effective biomarkers for early diagnosis and prediction (prior to the onset of clinical signs of the disease) have been intensively investigated.

During the last decades new approaches to monitor an excessive placental trophoblast apoptosis in pregnancies with placental insufficiency disorders using cell-free fetal nucleic acids quantification approach based on the detection of paternally inherited sequences (*SRY*, *DYS-14* or hypermethylated *RASSF1A*) in maternal circulation were developed.

Most quantification studies revealed higher concentrations of fetal DNA in maternal plasma in preeclampsia (Lo et al., 1999, Huang et al., 2010; Vandesomple et al., 2002; DiFederico et al., 1999; Allaire et al., 2000). It was suggested that a rise in fetal DNA represents a valuable marker for monitoring of placental insufficiency-related pregnancy complications. Using this approach, preeclampsia could be predicted several weeks before its clinical manifestation (Farina et al., 2004; Zhong et al., 2007; Tsui et al., 2007; Hromadnikova et al., 2010). With regard to intrauterine growth restriction, a rise in fetal DNA was less well correlated (Carameli et al., 2003; Sekizawa et al., 2003; Tsui et al., 2007).

Nevertheless, recent findings have offered the possibility of exploitation of new molecular biomarkers, microRNAs (miRNAs), and verification of their

potential for diagnosis and prediction of pregnancy-related complications, such as preeclampsia and IUGR.

4.2.2 Aims and methods

The aims of the current pilot study was (i) quantify placental specific microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, miR-526a) in maternal circulation in normal pregnancies, (ii) determine if they could differentiate between pregnancies with the onset of PIRCs and normally progressing pregnancies and (iii) determine if they are able to differentiate during early stages of gestation between normal pregnancies and patients at risk of later development of preeclampsia and/or IUGR. We performed retrospective study on a cohort consisted of Caucasian women involving 10 healthy non-pregnant individuals, 50 normal pregnancies, 32 pregnancies with clinically established PIRCs (16 preeclampsia, 5 preeclampsia with IUGR and 11 IUGR) and 7 pregnancies at various gestational stages, which later developed preeclampsia and/or IUGR (3 before the 34^{th} week of gestation and 4 after the 34^{th} week of gestation).

4.2.3 Results and discussion

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 PIRCs 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 12^{th} and 16^{th} weeks of gestation). During late gestation (from the 20^{th} 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.

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Absolute and Relative Quantification of Placenta-Specific MicroRNAs in Maternal Circulation with Placental Insufficiency–Related Complications

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Placental insufficiency–related complications are one of the leading causes of maternal and perinatal morbidity and mortality. This study investigated the quantification of placenta-specific microRNAs (miRNAs) in the maternal circulation during gestation in a cohort of women with normally progressing pregnancies, the differentiation between placental insufficiency–related complications and normally progressing pregnancies, and the differentiation between placental insufficiency and normally progressing pregnancies during the early stages of gestation. Both absolute and relative quantification of placenta-specific miRNAs (ie, miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) was determined in 50 women with normally progressing pregnancies, 32 with complicated pregnancies [21 with preeclampsia with or without intrauterine growth retardation (IUGR) and 11 with IUGR], and 7 women with pregnancies at various gestational stages who later developed preeclampsia and/or IUGR using real-time PCR and a comparative C_T method relative to normalization factor (ie, geometric mean of ubiquitous miR-16 and let-7d). Both quantification approaches revealed significant increases in extracellular placenta–specific miRNA levels over time in women with normally progressing pregnancies; however, they were not able to differentiate between normally progressing and complicated pregnancies at the time of preeclampsia and/or IUGR onset. Nevertheless, significant elevation of extracellular miRNA levels was observed during early gestation (ie, within the 12th to 16th weeks) in pregnancies with later onset of preeclampsia and/or IUGR. Early gestation extracellular miRNA screening can differentiate between women with normally progressing pregnancies and those who may later develop placental insufficiency–related complications. ***(J Mol Diagn 2012, 14:160-167; DOI: 10.1016/j.jmoldx.2011.11.003)***

Because placental insufficiency–related complications (PIRCs) are one of the leading causes of maternal and perinatal morbidity and mortality, it is imperative to develop effective methods for their prediction before clinical signs (optimally during the first trimester of gestation). There has been a trend in prenatal medicine during the last 10 years to develop noninvasive methods to monitor an excessive placental trophoblast apoptosis associated with placental insufficiency using cell-free fetal nucleic acid quantification in maternal plasma. Initially, researchers focused on the detection of male fetal-derived DNA in maternal circulation; most often this was done using the single-copy sex-determining region Y (*SRY*) sequence on the Y chromosome, which is absent in the maternal genome.[1–3] Subsequently, Chan et al[4] introduced the *RASSF1A* sequence as a promising universal fetal DNA marker because the promotor of the *RASSF1A* gene turned out to be hypermethylated in the fetal part of the placenta and therefore resistant to methylation-sensitive restriction enzyme digestion, whereas maternally derived hypomethylated *RASSF1A* sequences were completely digested.

Most quantification studies revealed higher concentrations of fetal DNA in PIRCs. Lo et al[5] showed a fivefold increase in the median concentration of extracellular fetal DNA, using real-time quantitative PCR assays for the *SRY* gene on the Y chromosome, in women with preeclamptic pregnancies compared with gestation age–matched controls. These data were later confirmed by other investigators[2,6–9] using real-time quantitative PCR and either the *SRY* gene or the *DYS-14* sequence as markers to differentiate between normally progressing and complicated pregnancies. It was suggested that an increase in

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fetal DNA represented a valuable marker of placenta-related pregnancy complications, which could predict preeclampsia several weeks before clinical manifestation[10–13]; however, with regard to intrauterine growth restriction (IUGR), an increase in fetal DNA was less well correlated.[14]

Caramelli et al[3] demonstrated only a small increase in fetal DNA concentrations, determined using *SRY* TaqMan PCR assays, in pregnant women with IUGR, and Sekizawa et al[14] demonstrated that fetal DNA concentrations were similar in both pregnant women with IUGR and women with normally progressing pregnancies determined using *DYS-14* sequence quantification in maternal plasma.

Subsequently, Tsui et al[12] reported a 4.3-fold higher concentration of hypermethylated *RASSF1A* sequences in plasma of women with preeclamptic pregnancies compared with controls. Similarly, our previous studies revealed significantly increased levels of extracellular fetal and total DNA in pregnant women with preeclampsia with or without IUGR, relative to controls (using *SRY*, hypermethylated *RASSF1A* sequence, and *GLO* as markers and real-time PCR).[13,15] However, we observed that the *DYS-14* sequence was not an optimal marker for extracellular fetal DNA quantification, for differentiation between normally progressing and complicated pregnancies, because of considerable variations in *DYS-14* copy numbers in males and discrepancies in *DYS-14* copy numbers between extracellular fetal DNA and the original fetal genome.[15]

Although our data indicated elevation of extracellular DNA in patients at risk of PIRCs before onset, this phenomenon was strongly individualized, could not be generalized to all cases, and was probably dependent on excessive placental trophoblast apoptosis. Interestingly, *SRY* and *GLO* quantification provided superior results compared with the hypermethylated *RASSF1A* sequence.[13]

Nevertheless, recent studies have offered the possibility of exploiting a new class of molecular markers, microRNAs (miRNAs), for diagnosis and prediction of pregnancy-related complications, such as preeclampsia and IUGR. The miRNAs belong to a family of small, noncoding RNAs (18 to 25 nucleotides) that regulate gene expression at the posttranscriptional level by degrading or blocking translation of mRNA targets.[16,17] Moreover, recent research shows that many miRNAs are abundantly expressed in the human placenta. Pineles et al[18] were the first to report differential expression of miR-210 and miR-182 between preeclampsia placentas and controls. Zhu et al[19] performed a comprehensive analysis of 455 miRNA expression profiles in preeclampsia versus healthy placentas and revealed 11 miRNAs as overexpressed and another 23 as underexpressed in patients with preeclampsia. Hu et al[20] performed a high-throughput miRNA microarray study and revealed significantly increased levels of seven miRNAs (miR-16, miR-29b, miR-195, miR-26b, miR-181a, miR-335, and miR-222) in preeclampsia placentas compared with healthy controls.

However, application of these findings into routine practice requires monitoring of placenta-specific miRNAs in the maternal circulation. Chim et al[21] identified 17 of 157 miRNAs in significantly higher concentrations in healthy placentas than in maternal blood cells and the four most abundant placental miRNAs (miR-141, miR-149, miR-299-5p, and miR-135b) in maternal plasma samples.

The main goal of our study was to identify placenta-specific miRNAs with a plasma expression profile that differs significantly between normally progressing pregnancies and pregnancies with PIRCs. Initially, we tested 20 miRNAs, which had been selected on the basis of previous findings,[19,21] placenta specificity according to the miRNAMap database (*mirnamap.mbc.nctu.edu.tw*; last accessed December 12, 2011), and a study presented by Liang and colleagues.[22] The selection of appropriate miRNAs with diagnostic potential was based on the following criteria: detection rate of 100% in full-term placentas and maternal plasma throughout gestation and detection rate of 0% in whole peripheral blood and plasma samples of nonpregnant women. Seven miRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) have been recently identified as pregnancy associated with diagnostic potential.[23]

The aims of the current pilot study were to i) quantify placenta-specific miRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) in maternal circulation in women with normally progressing pregnancies, ii) determine whether they could differentiate between pregnancies with the onset of PIRCs and normally progressing pregnancies, and iii) determine whether they are able to differentiate during early stages of gestation between women with normally progressing pregnancies and patients at risk of developing preeclampsia and/or IUGR.

Materials and Methods

Patients

The study was designed in a retrospective manner. The studied cohort consisted of white women, including 10 healthy nonpregnant women, 50 women with normally progressing pregnancies, 32 women with pregnancies with clinically established PIRCs (16 with preeclampsia, 5 with preeclampsia and IUGR, and 11 with IUGR), and 7 women with pregnancies at various gestational stages who later developed preeclampsia and/or IUGR (3 before the 34th week of gestation and 4 after the 34th week of gestation). Plasma samples were collected at the Institute for the Care of Mother and Child and stored at −80°C until further processing. The samples for study were chosen on the basis of equal times in storage and gestation age. Gestational age was assessed using ultrasonography.

Women with normally progressing pregnancies were defined as those without medical, obstetric, or surgical complications at the time of the study and who subsequently delivered full-term, singleton, healthy infants weighting >2500 g after 37 completed weeks of gestation.

Preeclampsia was defined as blood pressure >140/90 mm Hg in two determinations 4 hours apart or by a diastolic blood pressure >110 mm Hg that was associ-

ated with a protein level >300 mg/24 h after 20 weeks of gestation (American Congress of Obstetricians and Gynecologists Committee on Practice Bulletins–Obstetrics, unpublished data).

IUGR is the failure to achieve genetically predetermined growth potential and can be caused by maternal, fetal, placental, and external factors. IUGR was diagnosed when the estimated fetal weight, calculated using the Hadlock formula (Austraia Software, Munich, Germany), was below the 10th percentile for the evaluated gestation age; adjustments were made for the appropriate population standards in the Czech Republic.

All patients who participated in this study provided written informed consent. The study was approved by the local ethics committee.

Processing of Samples and Preparation of miRNAs

Nine milliliters of peripheral blood were collected into EDTA tubes and centrifuged twice at 1200 × *g* for 10 minutes at room temperature. Plasma samples were stored at −80°C until subsequent processing.

Total RNA was extracted from 1 mL of plasma derived from nonpregnant individuals, those with normally progressing pregnancies, and those with pathologic pregnancies and 25 mg of healthy placental tissue preserved in an RNAlater (Ambion, Austin, TX) followed by an enrichment procedure for small RNAs (small interfering RNAs and miRNAs), according to the manufacturer's instructions using a mirVana miRNA isolation kit (Ambion). Trizol LS reagent was used in plasma samples according to manufacturer's protocol for total RNA extraction from biological fluids (Invitrogen, Carlsbad, CA) and preceded the small RNA enrichment procedure. An RNA fraction highly enriched in RNA species <200nt was obtained using this novel approach, and the concentration was determined measuring the absorbance at 260 nm on a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE) and expressed as nanograms per milliliter of plasma.

To minimize DNA contamination, we treated the eluted RNA with 5 μL of DNase I (Fermentas International, Ontario, Canada) for 30 minutes at 37°C.

Reverse Transcription Reaction Using a Stem-Loop Primer

Each miRNA (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, miR-526a, miR-16, and let-7d) was reverse transcribed into complementary DNA (cDNA) using TaqMan MicroRNA Assay (Applied Biosystems, Branchburg, NJ), containing miRNA-specific stem-loop RT primers and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) in a total reaction volume of 50 μL according to the manufacturer's instructions on a 7500 Real-Time PCR system (Applied Biosystems) with the following thermal cycling parameters: 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and then held at 4°C.

Absolute and Relative Quantification of Individual miRNAs by Real-Time PCR

A total of 15 μL of cDNA, corresponding to each selected miRNA, and 4.4 μL of cDNA, corresponding to ubiquitous miR-16 and let-7d, were mixed with specific primers, the TaqMan MGB probe (TaqMan MicroRNA Assay; Applied Biosystems), and the ingredients of the TaqMan Universal PCR Master Mix (Applied Biosystems) in a total reaction volume of 35 μL. TaqMan PCR conditions were set as described in the TaqMan guidelines, with the exception of an increase to 50 cycles. The analysis was performed using a 7500 Real-Time PCR System. All PCRs were performed in three replicates. Multiple negative controls, such as non-template control (water instead of cDNA sample), non-amplicon control (nontranscribed RNA samples), and genomic DNA (isolated from equal biological samples), did not generate any signal during PCR reactions. A sample was considered positive if the amplification signal occurred before the 40th C_T. The calibration curve for individual miRNAs plotted the C_T against known concentrations of serially diluted samples whose concentrations ranged from 50 ng to 50 pg per well. RNA fraction highly enriched for small RNA isolated from the fetal part of the placenta (the part of the placenta derived from the chorionic sac that encloses the embryo, consisting of the chorionic plate and villi) was used to build up the standard curves. Concentrations of individual miRNAs were expressed as picograms of total RNA enriched for small RNAs per milliliter of plasma.

The expression of particular miRNA present in plasma samples was determined using the comparative C_T method[24] relative to normalization factor (geometric mean of ubiquitous miR-16 and let-7d),[25–27] which also served as positive controls for successful extraction of RNA from all samples.

Statistical Analysis

The miRNA expression levels were compared between groups using the Mann-Whitney *U*-test using Statistica software (StatSoft, Inc., Tulsa, OK). Because the Bonferroni correction was used to address the problem of multiple comparisons, the significance level was established at $P < 0.025$.

Absolute and relative quantification data were expressed as box plots of individual miRNAs in cohorts of nonpregnant individuals, those with normally progressing pregnancies, and those with complicated pregnancies using Statistica software. The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers represent the maximum and minimum values that are no more than 1.5 times the span of the interquartile range (range of the values between the 25th and the 75th percentiles). The median is indicated by the line in each box. Outliers are indicated by circles and extremes by asterisks.

Correlation among variables, including absolute quantification of individual miRNAs in maternal plasma and the storage life, was calculated using Spearman's rank cor-

Table 1. Stability of miRNAs in Maternal Plasma with Long-Term Storage Life

	Normal		PIRCs		Normal and PIRCs	
miRNA	ρ	*P*	ρ	*P*	ρ	*P*
miR-16	−0.421	0.013	−0.787	<0.001	−0.59	<0.001
let-7d	−0.449	0.072	−0.267	0.025	−0.388	0.053
miR-520a*	−0.44	0.009	−0.577	0.001	−0.521	<0.001
miR-520h	−0.387	0.048	−0.306	0.03	−0.398	0.009
miR-525	−0.542	0.001	−0.555	<0.001	−0.55	<0.001
miR-526a	−0.107	0.525	−0.125	0.474	−0.131	0.276
miR-516-5p	−0.311	0.062	−0.620	0.006	−0.432	0.013
miR-517*	−0.399	0.013	−0.81	<0.001	−0.675	0.008
miR-518b	−0.414	0.011	−0.666	0.003	−0.541	0.001

miR-16, let-7d, miR-520a*, miR-520h, miR-525, miR-516-5p, miR-517*, and miR-518b levels showed a significant decrease in maternal plasma samples derived either from those with normally progressing pregnancies or those with PIRCs with long-term storage life (range, 12.2 to 58.5 months; mean, 43.4 months; median, 45.1 months).

relation coefficient (ρ). If the correlation coefficient value is −1 or 1, there is a perfect negative or positive correlation. If it ranges within <−1;0.5> or <0.5;1>, there is a strong negative or positive correlation. If it varies from −0.5 to 0 and from 0 to 0.5, there is a weak negative or positive correlation. If the ρ value reaches 0, there is no correlation. Significant levels of correlation were set at $P < 0.05$.

Results

Stability of Extracellular miRNAs in Maternal Plasma

Initially, we investigated the stability of miRNAs in maternal plasma. Short-term storage (range, 0 to 2.57 months; mean, 0.82 months; median, 0.53 months) of plasma samples at −80°C had minimal effects on levels of individual miRNAs in maternal plasma.

However, we observed a significant decrease of certain miRNA (miR-16, let-7d, miR-520a*, miR-520h, miR-525, miR-516-5p, miR-517*, and miR-518b) levels in maternal plasma associated with long-term storage (range, 12.2 to 58.5 months; mean, 43.4 months; median, 45.1 months) (Table 1). Therefore, we decided that the next study should only include those samples that had been stored for <2 months.

Absolute and Relative Quantification of Extracellular miRNAs in Maternal Circulation During Normal Gestation

Absolute quantification analysis revealed no differences between the levels of ubiquitous miR-16 and let-7d during gestation in a cohort of women with normally progressing pregnancies (Figure 1). However, the levels of all examined extracellular placenta-specific miRNAs determined using both absolute and rel-

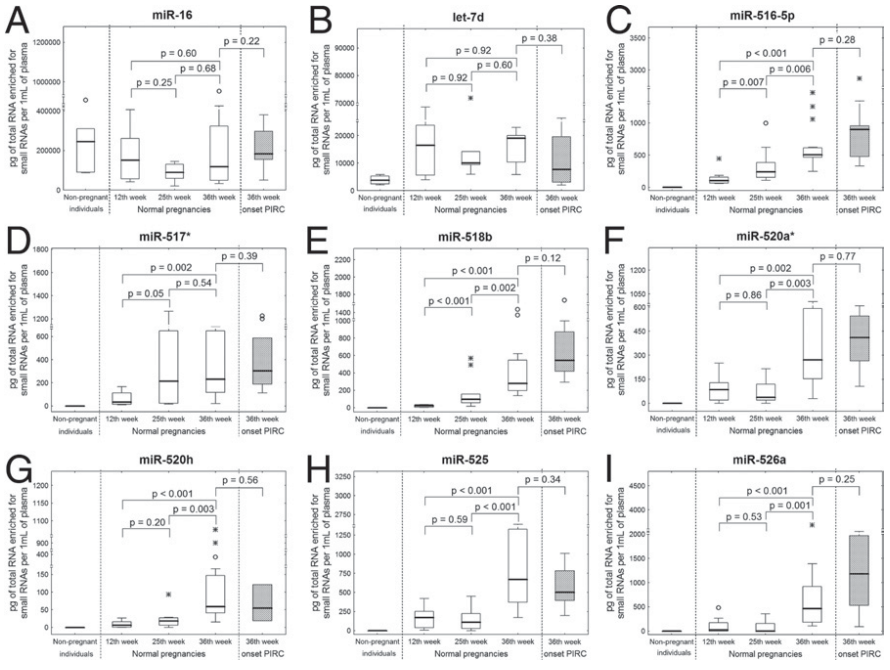

Figure 1. Absolute quantification of extracellular miRNAs in maternal circulation in women with normally progressing pregnancies and those with PIRCs. Although no differences between the levels of ubiquitous miR-16 and let-7d were found over time in those with normally progressing pregnancies, a statistically significant difference was observed between the first and the third trimesters of gestation in the cohort of women with normally progressing pregnancies for all placenta-specific miRNAs in maternal circulation. No difference in the levels of extracellular miRNAs was observed between those with normally progressing pregnancies and those with PIRCs.

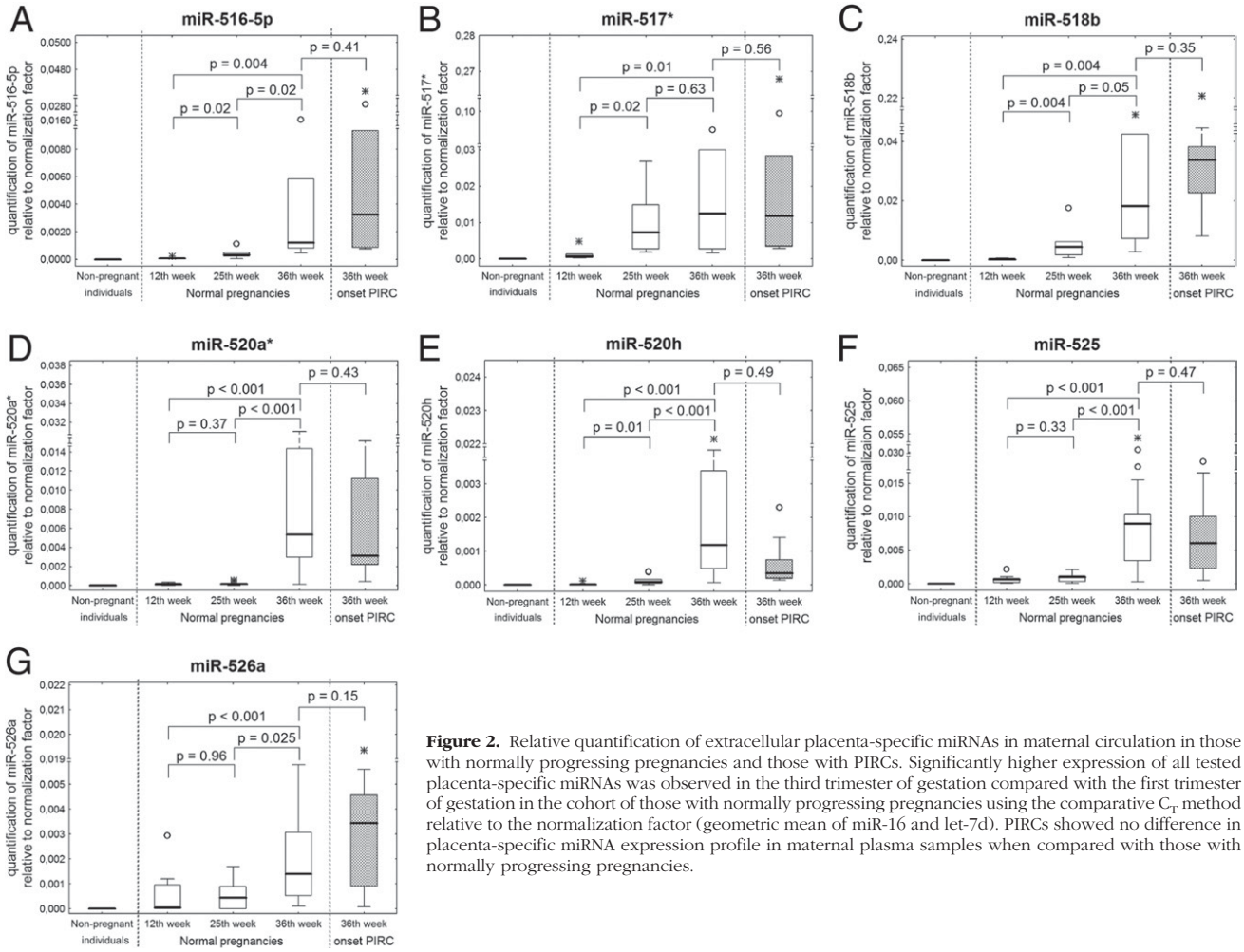

Figure 2. Relative quantification of extracellular placenta-specific miRNAs in maternal circulation in those with normally progressing pregnancies and those with PIRCs. Significantly higher expression of all tested placenta-specific miRNAs was observed in the third trimester of gestation compared with the first trimester of gestation in the cohort of those with normally progressing pregnancies using the comparative C_T method relative to the normalization factor (geometric mean of miR-16 and let-7d). PIRCs showed no difference in placenta-specific miRNA expression profile in maternal plasma samples when compared with those with normally progressing pregnancies.

ative quantification approaches significantly increased with advancing gestation (Figures 1 and 2). Interestingly, the highest concentrations and expression of circulating placenta-specific miRNAs were observed during the third trimester (36th week of gestation), whereas no differences were found between the first (12th week of gestation) and the second (25th week of gestation) trimesters for some placenta-specific miRNAs (miR-520a*, miR-525, and miR-526a). Similarly, no difference was observed between the second and the third trimesters of gestation in a case of placenta-specific miR-517*.

Absolute and Relative Quantification of Extracellular miRNAs in Maternal Circulation in Those with Normally Progressing Pregnancies and Those with the Onset of PIRCs

In addition, we determined the concentrations of ubiquitous miR-16 and let-7d and placenta-specific miRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) in plasma samples derived from those with normally progressing pregnancies and those with onset of PIRCs. Comparable levels of all tested extracellular miRNAs were found in all maternal plasma samples, regardless of the course of gestation (Figure 1).

As with absolute quantification, no significant difference in placenta-specific miRNA expression profiles were observed between cohorts of those with PIRCs and gestational age–matched women with normally progressing pregnancies (Figure 2).

Quantitative Aberrations of Extracellular miRNAs in Maternal Circulation Occurring During Early Gestation May Predict Late Onset of PIRCs

Subsequently, we tested plasma samples derived at various gestational stages from pregnant women who later developed PIRCs (three severe cases with the onset before the 34th week of gestation and four with preeclampsia with the onset after the 34th week of gestation). Both quantification approaches revealed significant elevation of extracellular miRNA levels and expression in maternal circulation during early gestation in all cases (between the 12th and 16th weeks of gestation). During late gestation (from the 20th week until term), the levels of extracellular miRNAs decreased significantly until they finally reached the corre-

Table 2. Quantitative Aberrations of Placenta-Specific miRNAs in the Maternal Circulation Occurring During Early Gestation in a Cohort of Patients Who Developed PIRCs

			miR-516-5p		miR-517*		miR-518b		miR-520a*		miR-520h		miR-525		miR-526a		Total	
UPN	Diagnosis	Week	AQ	RQ	AQ	RQ	AQ	RQ	AQ	RQ	AQ	RQ	AQ	RQ	AQ	RQ	AQ	RQ
2327	IUGR onset	15	+	+	+	+	+	+	+	+	+	+	+	+/−	+	−	7/7	5/7
	at 34th	30	−	−	−	−	−	−	−	−	−	−	−	−	−	+	0/7	1/7
	week	32	−	−	−	−	−	−	−	−	−	−	−	−	−	+	0/7	1/7
		34	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/7	0/7
2397	PEP onset	13	+	+	+	+/−	+	+	−	−	+	+	−	−	+	+	5/7	4/7
	at 29th	25	−	−	−	−	−	−	−	−	−	−	−	−	+	−	1/7	0/7
	week																	
2335	PEP and	20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/7	0/7
	IUGR	25	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/7	0/7
	onset at	31	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/7	0/7
	33rd	33	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/7	0/7
	week																	
1875	PEP onset	12	+	+	+	+	+	+/−	+	+	−	−	+	+	+	+	6/7	5/7
	at 39th	16	+	+	+	+	−	−	−	−	−	−	−	+	+	+	3/7	4/7
	week	36	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/7	0/7
1857	PEP onset	12	+	+	+	+	+	+	−	−	−	−	−	+/−	−	+/−	3/7	3/7
	at 39th	16	+	+	+	+	−	+/−	+	+	−	−	−	+/−	−	−	3/7	3/7
	week																	
2000	PEP onset	12	+	+	+	+	−	−	−	−	−	+/−	−	+/−	−	+/−	2/7	2/7
	at 39th	16	+	+	+	+	−	−	−	+/−	+	+	−	+/−	+	+	4/7	4/7
	week	36	+	−	−	−	−	−	−	−	−	−	−	−	−	−	1/7	0/7
2345	PEP onset	12	+	+	+	+	+	+	+	+	+	+	+	+/−	+	+	7/7	6/7
	at 38th	16	+	+	−	−	−	−	+/−	+	−	+	+	+/−	+/−	+	2/7	4/7
	week	21	−	−	−	−	−	−	−	+	−	−	−	−	−	−	0/7	0/7
		35	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0/7	0/7

+, extracellular miRNAs differentiating between those with normally progressing pregnancies and those who later developed PIRCs (the values above the cutoffs); −, extracellular miRNAs not differentiating between those with normally progressing pregnancies and those who later developed PIRCs (the values under the cutoffs); +/−, threshold values of extracellular miRNAs in maternal circulation (the values close to the cutoffs).

AQ, absolute quantification; IUGR, intrauterine growth retardation; PEP, preeclampsia; RQ, relative quantification.

sponding levels of gestational age–matched women with normally progressing pregnancies (Table 2, Figure 3).

Our defined experimental cutoffs (the mean of the levels of miRNAs in maternal plasma in those with normally progressing pregnancies during the 12th, 25th, and 36th weeks of gestation plus 2 SDs) showed the maximum feasible specificity (>95%) for all placenta-specific miRNAs to differentiate between those with normally pro-

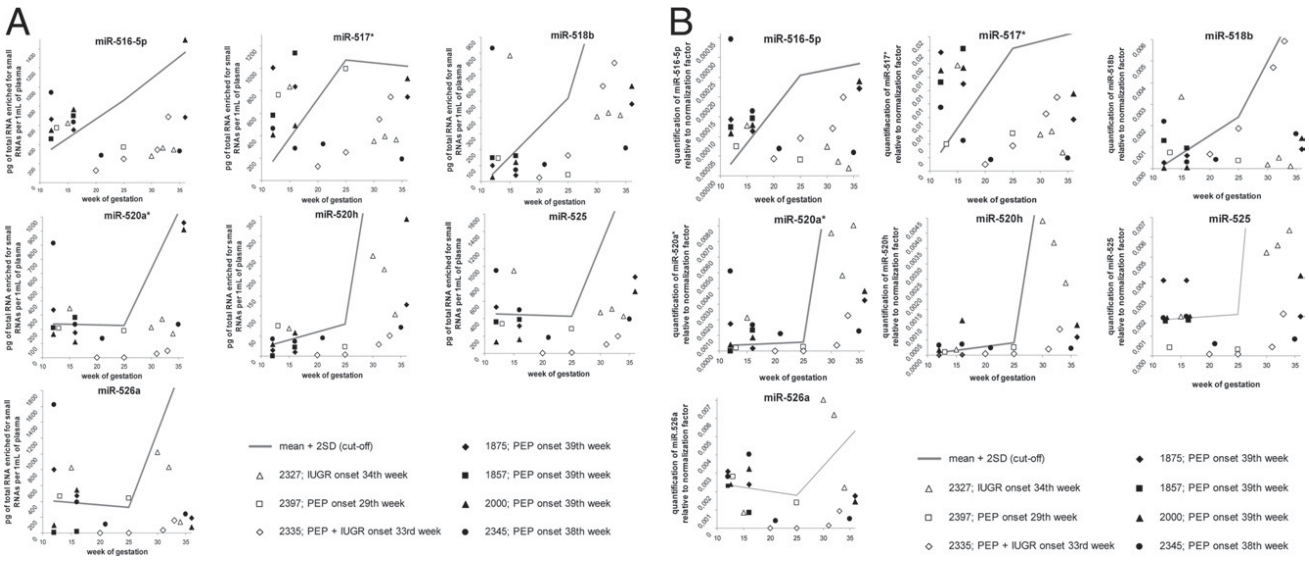

Figure 3. Demonstration of quantitative aberrations of extracellular placenta-specific miRNAs in maternal circulation throughout gestation in patients who later developed preeclampsia and/or IUGR. Significant elevation of the concentrations (**A**) and expression (**B**) of extracellular placenta-specific miRNAs was observed during early gestation (within the 12th to 16th weeks of gestation) in pregnant women who later developed preeclampsia and/or IUGR. The mean ± 2 SDs for extracellular miRNAs was used as the cutoff to differentiate between those with PIRCs and those with normally progressing pregnancies. This cutoff showed the maximum feasible specificity (the percentage of those with normally progressing pregnancies identified as not having the condition) for each miRNA assay.

gressing pregnancies and those who later developed pregnancies with PIRCs.

Discussion

The absolute quantification study revealed increasing levels of all examined placenta-specific miRNAs in maternal circulation during progression of normally progressing pregnancies, which may be linked to the accruing mass of the placenta.

Unfortunately, absolute and relative quantification of placenta-specific miRNAs in maternal circulation did not meet our expectations, that is, extracellular miRNA concentrations would be significantly elevated in maternal circulation at the onset of PIRCs due to excessive placental trophoblast apoptosis.[28–31]

Interestingly, the levels of selected placenta-specific miRNAs in maternal plasma showed no difference (miR-520a*, miR-520h, miR-525, miR-526a, miR-516-5p, miR-517*, and miR-518b) between those with normally progressing pregnancies and those with clinically established preeclampsia and/or IUGR.

Because miR-16 and let-7d are ubiquitously expressed in most tissues, it argues for their use as endogenous controls to study expression profiles of various miRNAs.[25,26] We found that normally progressing pregnancies and the manifestation of PIRCs do not influence the levels of miR-16 and let-7d in maternal plasma.

To verify the results of absolute quantification, we chose a relative quantification approach, where we compared the expression of placenta-specific miRNAs to invariable miR-16 and let-7d (normalization factor) using a comparative C_T method. With this approach, the inability of placenta-specific miRNAs to differentiate between normally progressing and complicated pregnancies during the onset of preeclampsia and/or IUGR was confirmed.

Surprisingly, we found that the analysis of maternal plasma samples enabled differentiation between patients at risk of later PIRC development and normally progressing pregnancies during early stages of gestation. The levels of examined placenta-specific miRNAs exceeded, within the 12th to 16th weeks of gestation, the explicitly defined cutoffs (mean plus 2 SDs) of the control cohort at the appropriate gestational age. Thereafter, the levels of extracellular miRNAs did not differ from the controls, even at the time of clinical manifestation of preeclampsia and/or IUGR, respectively.

This phenomenon might reflect the temporarily increased expression of particular miRNAs in placentas with incipient placental insufficiency. Although the original causes of preeclampsia and IUGR are still unknown, both entities are characterized by defective placentation eliciting inadequate uteroplacental blood perfusion and ischemia.[32] Normal placentation comprises trophoblast cell invasion of the spiral arteries, which results in reversible changes of the normal arterial wall architecture.[33] Physiologic trophoblastic invasion of the spiral arteries develops from 8 weeks of gestation and is believed to be mostly completed by 16 to 20 weeks of gestation.[32,34–36] As we demonstrated in our cohort of patients, placental injury had attenuated miRNA biogenesis and increased exosome-dependent or exosome-independent release of miRNAs to the plasma within 11 and 16 weeks of gestation. After the 20th week of gestation, transiently enhanced, extracellular, placenta-specific miRNA levels had been subsequently normalized. Unfortunately, limited data comparing extracellular placenta-specific miRNA levels between the groups of those with normally progressing and complicated pregnancies are available.[37]

Our findings may be partially supported by the studies of Illanes et al,[38] Sifakis et al,[39] and Cotter et al,[40] which demonstrated significant increases of cell-free fetal DNA levels in early pregnancies in women who subsequently developed preeclampsia and/or IUGR. However, our finding is inconsistent with 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.[10–15] We suggest that the normalized expression of placenta-specific miRNAs in preeclamptic and/or IUGR placentas together with the increased placental trophoblast apoptosis trended to a higher level of some extracellular placenta-specific miRNAs (absolute quantification: miR-516-5p, miR-520a*, miR-518b, and miR-526a; relative quantification: miR-518b and miR-526a) in PIRCs; however, it did not reach statistical significance when compared with gestational age–matched controls. Our findings may be supported by the study of Mouillet et al, who have recently also observed no significant difference in relative placenta-specific miRNA levels (miR-517a and miR-518b) in placenta and plasma samples from those with normally progressing and fetal growth restriction pregnancies.[37] Another study of Zhu et al[19] reported an increased expression of placenta-specific miRNAs (miR-517a and miR-518b) only in severe preeclamptic placentas.

We are aware that this pilot study involves a small cohort of patients and it is difficult to come to definitive conclusions. However, the data resulting from this study suggest the potential of extracellular miRNAs to differentiate, at the beginning of gestation between patients at risk of later development of PIRCs and those with normally progressing pregnancies; these interesting findings should be further confirmed using larger cohorts. These data strongly support the need for a more detailed exploration of extracellular miRNAs in maternal circulation with the view toward routine assessment into everyday practice and recognition as potential biomarkers for PIRCs.

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4.3 Circulating C19MC microRNAs in preeclampsia, gestational hypertension, and fetal growth restriction

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4.3.1 Overview

Physiologic changes in normal pregnancy are part of an acute-phase reaction, which is generated by an inflammatory response. Since the placenta is being continuously remodelled during normal development, extracellular nucleic acids of both fetal and placental origin, packed into either trophoblast-derived apoptotic bodies or shedding syncytiotrophoblast microparticles, may be detected in maternal circulation during the course of gestation (Nelson et al., 1996; Oudejans et al., 2003; Huppertz and Kingdom, 2004; Orozco et al., 2006; Hromadnikova, 2012). It was hypothesized that circulating syncytiotrophoblast debris and the microparticles shed during pregnancy are proinflammatory particules that contribute to maternal inflammation and some of the features of the maternal syndrome (Redman and Sargent, 2005). Signs of maternal inflammation which appear to be present in normal pregnancies at term are exaggerated in preeclampsia (PE) and fetal growth restriction (FGR) and can account for their clinical features (Redman and Sargent, 2004; Khong et al., 1986).

Since hypoxic environment induces excessive trophoblast cell death and increased shedding of placenta debris into the maternal circulation, placental insufficiency related pregnancy complications are associated with abnormal levels of extracellular fetal DNA and mRNA transcripts (Orozco et al., 2006; Reddy et al., 2008).

There has been a trend over the last 10 years to develop non-invasive methods utilizing quantification of cell-free nucleic acids inclusive of microRNAs in maternal circulation. The diagnostic potential of particular molecular

biomarkers and their implementation in the current predictive and diagnostic algorithms for pregnancy related complications are subject of interest.

Recent studies have shown that preeclampsia is associated with alterations in extracellular microRNA expression. Gunel et al. (2011), Yang and colleagues (2011), and Wu et al. (2012) demonstrated specific profiles of up- and down-regulated microRNAs in patients with preeclampsia. On the other hand, Mouillet et al. (2010) did not observed any difference between pregnancies with normal and fetal growth-restricted fetuses when compared circulating microRNA expression levels.

Anyway, most investigators focused on those microRNAs, whose genes are located outside chromosome 19 miRNA clusters (C19MC and miR-371-3 cluster) or the chromosome 14 miRNA cluster (C14MC) that encode pregnancy-associated microRNAs (Seitz et al., 2004; Liang et al., 2007; Bentwich et al., 2005; Lin et al., 2010; Morales-Prieto et al., 2013).

4.3.2 Aims and methods

This study is a follow-up of our previous studies and describes comprehensively the expression profile of circulating C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525 and miR-526a) in the entirely new sample set of patients with clinically established pregnancy-related complications. The studied cohort consisted of 63 preeclampsia (PE) w or w/o fetal growth restriction (FGR), 27 FGR, 23 gestational hypertension (GH) and 55 control plasma samples. Plasmatic concentrations and/or expression profile of C19MC microRNAs were analyzed in relation to the severity of the disease with respect to the degree of clinical signs (mild and severe preeclampsia) and requirements for the delivery (before and after 34 week of gestation). Function and functional relationship analysis of microRNAs and their target genes were performed by bioinformatics tool MirTarget2.

4.3.3 Results and discussion

The results of our pilot study indicated no difference in C19MC microRNA profiles between normal and complicated pregnancies during the onset of the disease, but could not come to definitive conclusions due to the low number of studied subjects involved. As expected, this 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 showed 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.

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Research Article

Circulating C19MC MicroRNAs in Preeclampsia, Gestational Hypertension, and Fetal Growth Restriction

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The objective of the study was to identify the profile of circulating C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) in patients with established preeclampsia ($n = 63$), fetal growth restriction ($n = 27$), and gestational hypertension ($n = 23$). We examined the correlation between plasmatic concentrations and expression levels of microRNAs and the severity of the disease with respect to clinical signs, requirements for the delivery, and Doppler ultrasound parameters. Using absolute and relative quantification approaches, increased extracellular C19MC microRNA levels (miR-516-5p, $P = 0.037$, $P = 0.009$; miR-517*, $P = 0.033$, $P = 0.043$; miR-520a*, $P = 0.001$, $P = 0.009$; miR-525, $P = 0.026$, $P = 0.01$; miR-526a, $P = 0.03$, $P = 0.035$) were detected in patients with preeclampsia. The association analysis pointed to no relationship between C19MC microRNA plasmatic concentrations and expression profile and identified risk factors for a poorer perinatal outcome. However, the dependence between the levels of plasmatic C19MC microRNAs and the pulsatility index in the middle cerebral artery and the values of cerebroplacental ratio was demonstrated. The study brought the interesting finding that the upregulation of miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a is a characteristic phenomenon of established preeclampsia.

1. Introduction

Normal pregnancy is associated with a systemic inflammatory response. Many of the physiologic changes of normal pregnancy are part of an acute-phase reaction, which is generated by an inflammatory response. The placenta is the proximal cause of these problems [1]. Since the placenta is being continuously remodelled during normal placental development, extracellular nucleic acids of both fetal and placental origin, packed into either trophoblast-derived apoptotic bodies or shedding syncytiotrophoblast microparticles, may be detected in maternal circulation during the course of normal gestation [2–6].

Circulating syncytiotrophoblast debris has been hypothesized to contribute to maternal inflammation and some of the features of the maternal syndrome [7]. Signs of maternal inflammation which appear to be present in normal pregnancies at term are exaggerated in preeclampsia (PE) and fetal growth restriction (FGR) and can account for their clinical features [1, 8].

Preeclampsia and fetal growth restriction (FGR) are major complications affecting about 2–10% of pregnancies responsible for maternal and perinatal morbidity and mortality [9, 10]. Preeclampsia usually develops after 20 weeks of gestation and is characterized by chronic or gestational hypertension combined with proteinuria, which results from defective placentation eliciting inadequate uteroplacental blood perfusion and ischemia [8, 11, 12]. The causes of preeclampsia and FGR remain unknown. Trophoblastic debris and the microparticles shed during normal pregnancy are proinflammatory and this process is amplified in preeclampsia [13]. A hypoxic environment induces excessive

trophoblast cell death and increased shedding of placenta debris into the maternal circulation; as a result, placental insufficiency related pregnancy complications are associated with abnormal levels of extracellular fetal DNA and mRNA transcripts [5, 14].

Recent evidence suggests that preeclampsia can be further subdivided into early PE (before 34 weeks of gestation), intermediate PE (between 34 and 37 weeks of gestation), and late PE (after 37 weeks of gestation) [15, 16]. The concept of early and late PE is modern, and it is widely accepted that these two entities have different etiologies and should be regarded as different forms of the disease, where early onsets of PE and IUGR are considered to be placenta-mediated diseases [17–19].

There has been a trend over the last 10 years to develop noninvasive methods utilizing quantification of cell-free nucleic acids inclusive of microRNAs in maternal circulation [6, 20–39]. The diagnostic potential of particular molecular biomarkers and their implementation in the current predictive and diagnostic algorithms for pregnancy related complications is subject of interest [6].

MicroRNAs belong to a family of small noncoding RNAs that regulate gene expression at the posttranscriptional level by degrading or blocking translation of messenger RNA (mRNA) targets [40, 41].

Recent studies have shown that preeclampsia is associated with alterations in extracellular microRNA expression. Using real-time PCR analysis, Gunel et al. [42] demonstrated the upregulation of miR-210 and downregulation of miR-152 in patients with preeclampsia. The application of next generation sequencing technology revealed a broader profile of dysregulated circulating microRNAs in preeclampsia. Compared to controls, 15 microRNAs were found to be upregulated (miR-521, miR-520h, miR-517c, miR-519d, miR-520g, miR-517b, miR-542-3p, miR-136, let-7f-1*, miR-518e, let-7a*, miR-125b, miR-125 a-5p, miR-519a, and miR-29a) and 7 microRNAs were found to be downregulated (let-7f, miR-223, miR-1260, let-7d, miR-320c, miR-185, and miR-1272) in four examined preeclamptic serum samples [43].

Later, using microarray analysis Wu et al. [44] reported the upregulation of 13 microRNAs (miR-574-5p, miR-26a, miR-151-3p, miR-130a, miR-181a, miR-130b, miR-30d, miR-145, miR-103, miR-425, miR-221, miR-342-3p, and miR-24) and down-regulation of 2 microRNAs (miR-144, miR-16) in patients with severe preeclampsia. Seven of these 13 microRNAs (miR-574-5p, miR-26a, miR-130b, miR-181a, miR-342-3p, miR-103, and miR-24) were validated by real-time PCR analysis to be elevated in plasma from severe preeclamptic pregnancies.

In a small-scale analysis, Mouillet et al. [45] did not observe any differentiation between pregnancies with normal and fetal growth restricted fetuses when compared circulating microRNA expression levels (miR-27a, miR-30d, miR-141, miR-200c, miR-205, miR-424, miR-451, miR-491, miR-517a, miR-518b, miR-518e, and miR-524).

However, most of investigators focused on the study of those microRNAs, whose genes are located outside chromosome 19 miRNA clusters (C19MC and miR-371-3 cluster) or the chromosome 14 miRNA cluster (C14MC) that encode pregnancy-associated microRNAs [46–50].

We have previously identified C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) present in maternal plasma differentiating between normal pregnancies and nonpregnant individuals [51]. We selected from the chromosome 19 microRNA cluster, which involves 46 microRNA genes altogether, [48–50, 52] preferentially those microRNAs that were previously demonstrated to be exclusively expressed in placental tissues (miR-520a*, miR-516-5p, miR-517*, miR-518b, miR-519a, miR-524-5p, miR-525, miR-526a, miR-526b, and miR-520h) and those microRNAs that were reported to be highly expressed in placental tissues (miR-512-5p, miR-515-5p, miR-518f*, miR-519d, and miR-519e*) [51, 53, 54].

Later, we demonstrated significant increases in extracellular C19MC microRNAs levels (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) over time in normally progressing pregnancies [51, 54].

The results of our pilot study indicated no differentiation between normal and complicated pregnancies, but could not come to definitive conclusions due to the low number of studied subjects involved [51, 54]. The current study is a followup of our previous studies [51, 54] and describes comprehensively for the first time the expression profile of circulating C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) in the entirely new sample set of patients with clinically established preeclampsia and/or fetal growth restriction. To our knowledge, no study describing the profile of circulating C19MC microRNAs in gestational hypertension has been carried out.

2. Materials and Methods

2.1. Patients. The studied cohort consisted of Caucasian women involving 63 preeclampsia (PE) w or w/o fetal growth restriction (FGR), 27 FGR, 23 gestational hypertension (GH), and 55 controls. Twenty-four women had signs of mild preeclampsia, 39 women were diagnosed with severe preeclampsia, 24 preeclamptic patients required the delivery before 34 weeks of gestation and 39 patients delivered after 34 weeks of gestation. In 18 cases, preeclampsia superposed on previous hypertension; otherwise, it occurred in normotensive patients (45 cases). Eight growth-retarded foetuses were delivered before 34 weeks of gestation and 19 those after 34 weeks of gestation. Oligohydramnios or anhydramnios were present in 7 growth restricted foetuses.

Doppler studies showed an abnormal pulsatility index (PI) in the umbilical artery (14 preeclampsia ± FGR and 14 FGR) and/or in the middle cerebral artery (10 preeclampsia ± FGR and 11 FGR). Cerebroplacental ratio (CPR), expressed as a ratio between umbilical artery and middle cerebral artery pulsatility index, was below the fifth percentile in 21 cases (9 preeclampsia ± FGR and 12 FGR). Absent or reversed enddiastolic velocity waveforms in the umbilical artery occurred in 8 cases (2 preeclampsia + FGR and 6 FGR).

Normal pregnancies were defined as those without complications who delivered full term, singleton, healthy infants

Table 1: Correlation between Doppler ultrasonography parameters and C19MC microRNAs expression levels and plasmatic concentrations.

	Spearman's rank correlation											
	Absolute quantification						Relative quantification					
	A. umbilicalis PI		Middle cerebral artery PI		Cerebroplacental ratio		A. umbilicalis PI		Middle cerebral artery PI		Cerebroplacental ratio	
	ρ	P	ρ	P	ρ	P	ρ	P	ρ	P	ρ	P
miR-516-5p	−0.023	0.845	**−0.393**	**0.005**	−0.219	0.125	−0.040	0.736	**−0.307**	**0.030**	−0.190	0.183
miR-517*	0.032	0.791	**−0.328**	**0.020**	−0.215	0.131	0.073	0.542	**−0.288**	**0.041**	−0.269	0.060
miR-518b	−0.025	0.834	−0.152	0.269	−0.074	0.606	−0.019	0.872	−0.220	0.120	−0.167	0.242
miR-520a*	0.092	0.441	**−0.314**	**0.027**	**−0.261**	**0.050**	0.085	0.477	**−0.339**	**0.017**	**−0.339**	**0.018**
miR-520h	0.016	0.893	−0.238	0.093	−0.178	0.214	0.038	0.748	−0.256	0.071	−0.219	0.126
miR-525	0.009	0.941	**−0.358**	**0.011**	−0.190	0.183	−0.028	0.812	**−0.357**	**0.012**	−0.185	0.194
miR-526a	0.156	0.196	**−0.304**	**0.032**	**−0.340**	**0.017**	0.131	0.277	**−0.286**	**0.043**	**−0.329**	**0.021**

The association between the plasmatic concentration (absolute quantification) and gene expression levels (relative quantification) of C19MC microRNAs and Doppler ultrasonography parameters such as arteria umbilicalis pulsatility index, middle cerebral artery pulsatility index, and cerebroplacental ratio.
PI: pulsatility index; ρ: Spearman's correlation coefficient; P: level of significance; bold font: statistically significant results.

weighting >2500 g after 37 completed weeks of gestation. Preeclampsia was defined as blood pressure >140/90 mmHg in two determinations 4 hours apart that was associated with proteinuria >300 mg/24 h after 20 weeks of gestation. Severe preeclampsia was diagnosed by the presence of one or more of the findings according to the guidelines of ACOG Committee [11].

Fetal growth restriction was diagnosed when the estimated fetal weight (EFW), calculated using the Hadlock formula (Astraia Software GmbH), was below the tenth percentile for the evaluated gestational age.

Gestational hypertension was defined as high blood pressure that developed after the twentieth week of pregnancy.

All patients provided written informed consent. The study was approved by the Ethics Committee of the Third Faculty of Medicine, Charles University in Prague. The samples for the study were chosen on the basis of equal times in storage and gestation age. Gestational age was assessed using ultrasonography.

2.2. Processing of Samples. Nine millilitres of peripheral blood were collected into EDTA tubes and centrifuged twice at 1200 g for 10 min at room temperature. Plasma samples were stored at −80°C until subsequent processing.

Total RNA was extracted from 1 mL of plasma and 25 mg of normal placental tissue preserved in RNAlater (Ambion, Austin, USA) followed by an enrichment procedure for small RNAs using a mirVana microRNA Isolation kit (Ambion, Austin, USA). Trizol LS reagent was used in plasma samples for total RNA extraction from biological fluids (Invitrogen, Carlsbad, USA) and preceded the small RNAs enrichment procedure. To minimize DNA contamination, we treated the eluted RNA with 5 μL of DNase I (Fermentas International, ON, Canada) for 30 min at 37°C.

2.3. Reverse Transcriptase Reaction. Each microRNA was reverse-transcribed into complementary DNA using TaqMan MicroRNA Assay, containing microRNA-specific stem-loop RT primers (Table 1), and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Branchburg, USA) in a total reaction volume of 50 μL on a 7500 real-time PCR system (Applied Biosystems, Branchburg, USA) with the following thermal cycling parameters: 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and then held at 4°C.

2.4. Quantification of MicroRNAs. 15 μL of cDNA corresponding to each microRNA was mixed with components of TaqMan MicroRNA Assay and the ingredients of the TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, USA) in a total reaction volume of 35 μL. TaqMan PCR conditions were set as described in the TaqMan guidelines. The analysis was performed using a 7500 real-time PCR system. All PCRs were performed in duplicates. A sample was considered positive if the amplification signal occurred before the 40th threshold cycle. Concentrations of individual microRNAs were expressed as pg of total RNA enriched for small RNAs per millilitre of plasma.

The expression of particular microRNA in maternal plasma was determined using the comparative Ct method [55] relative to the expression of the same microRNA in the reference sample, randomly selected placenta derived from gestation with normal course.

RNA fraction highly enriched for small RNA isolated from the fetal part of the placenta (the part of the placenta derived from the chorionic sac that encloses the embryo, consisting of the chorionic plate and villi) was used to build-up the standard curves and as a reference sample for relative quantification throughout the study.

Synthetic C. elegans microRNA (cel-miR-39, Qiagen, Hilden, Germany) was used as an internal control for variations during the preparation of RNA, cDNA synthesis, and real-time PCR. Due to a lack of generally accepted standards, all experimental real-time qRT-PCR data were normalized to cel-miR-39, as it shows no sequence homology to any human microRNA. 1 μl of 0.1 nM cel-miR-39 was spiked in after incubation with Trizol LS reagent to the human plasma samples.

2.5. Statistical Analysis. MicroRNA levels were compared between groups by nonparametric tests (the Mann-Whitney U test for the comparison between two groups and the Kruskal-Wallis test for the comparison between three or more groups) using Statistica software (StatSoft Inc., USA). Correlation between variables including absolute and/or relative microRNA quantification and Doppler ultrasonography parameters (the pulsatility index in the umbilical artery, the pulsatility index in the middle cerebral artery, and the cerebroplacental ratio) was calculated using the Spearman's rank correlation coefficient (ρ). If it varies from −0.5 to 0, there is a weak negative correlation. The significance level was established at a P value of $P < 0.05$.

3. Results

3.1. Circulating C19MC MicroRNAs Differentiate between Complicated and Normal Pregnancies. Overall, increased plasmatic levels of miR-516-5p ($P = 0.008$), miR-517* ($P = 0.003$), miR-520a*. ($P < 0.001$), miR-525 ($P = 0.003$), and miR-526a ($P = 0.004$) were observed in women with pregnancy-related complications (gestational hypertension, preeclampsia and fetal growth restriction) compared to normal pregnancies.

Similarly, the difference in gene expression of circulating microRNAs between pregnancy-related complications and the control cohort (normal pregnancies) achieves statistical significance for miR-516-5p ($P < 0.001$), miR-517* ($P = 0.005$), miR-520a* ($P = 0.001$), miR-525 ($P = 0.001$), and miR-526a ($P = 0.004$).

3.2. Upregulation of Circulating C19MC MicroRNAs in Pregnancies with Established Preeclampsia. Consecutive detailed group analysis confirmed a difference in the levels of extracellular microRNAs in 5/5 C19MC microRNAs (miR-516-5p, $P = 0.037$; miR-517*, $P = 0.015$; miR-520a*, $P = 0.003$; miR-525, $P = 0.026$; and miR-526a, $P = 0.032$).

While plasmatic levels of microRNAs between the control cohort and the cohorts of patients with FGR and GH did not differ, increased levels were detected in the group of patients with established preeclampsia (miR-516-5p, $P = 0.037$; miR-517*, $P = 0.033$; miR-520a*, $P = 0.001$; miR-525, $P = 0.026$; and miR-526a, $P = 0.030$) (Figures 1(a)–1(e)).

Parallel, significant difference in microRNA gene expression was found between groups of preeclampsia, gestational hypertension, fetal growth restriction, and controls (miR-516-5p, $P = 0.005$; miR-517*, $P = 0.028$; miR-520a*, $P = 0.011$; miR-525, $P = 0.01$; miR-526a, $P = 0.034$). Again, while the expression of microRNAs between the control cohort, gestational hypertension, and fetal growth restriction did not differ, the highest expression was detected in the group of patients with preeclampsia (miR-516-5p, $P = 0.009$; miR-517*, $P = 0.043$; miR-520a*, $P = 0.009$; miR-525, $P = 0.01$; miR-526a, $P = 0.035$) (Figures 1(f)–1(j)).

3.3. The Association Study of Circulating C19MC MicroRNAs and the Severity of the Disease with respect to Clinical Signs and Requirements for the Delivery. Plasmatic concentrations and/or expression profile of C19MC microRNAs were analysed in relation to the severity of the disease with respect to the degree of clinical signs (mild and severe preeclampsia) and requirements for the delivery (before and after 34 weeks of gestation). No effect of the severity of the disease either on plasmatic C19MC microRNA concentrations (miR-516-5p, $P = 0.396$; miR-517*, $P = 0.226$; miR-520a*, $P = 0.08$; miR-525, $P = 0.237$; and miR-526a, $P = 0.201$) or C19MC microRNA expression levels (miR-516-5p, $P = 0.476$; miR-517*, $P = 0.58$; miR-520a*, $P = 0.239$; miR-525, $P = 0.397$; miR-526a, $P = 0.646$) was observed.

Further, the association between C19MC microRNA plasmatic levels and/or gene expression and the occurrence of previous hypertension in the cohort of patients with preeclampsia was determined. No difference between the group of preeclampsia superposed on chronic hypertension and/or gestational hypertension and the group of patients with unexpected onset of preeclampsia was revealed (absolute quantification: miR-516-5p, $P = 0.885$; miR-517*, $P = 0.538$; miR-520a*, $P = 0.342$; miR-525, $P = 0.909$; miR-526a, $P = 0.273$; relative quantification: miR-516-5p, $P = 0.721$; miR-517*, $P = 0.621$; miR-520a*, $P = 0.885$; miR-525, $P = 0.568$; miR-526a, $P = 0.201$).

3.4. The Association Study of Circulating C19MC Micrornas and the Severity of the Disease with respect to Doppler Ultrasonography Monitoring. The association between the plasmatic concentration and gene expression levels of C19MC microRNAs and Doppler ultrasonography parameters (the pulsatility index in the umbilical artery the pulsatility index in the middle cerebral artery, and the cerebroplacental ratio) was studied in the cohort of pregnancies complicated with preeclampsia and/or fetal growth restriction.

No difference within the group of complicated pregnancies with normal and abnormal values of flow rate in the umbilical artery was found out with the exception of miR-526a, which was upregulated in the group of patients with abnormal blood flow velocity waveforms (absolute quantification: $P = 0.038$; relative quantification: $P = 0.05$).

Further, the statistical analysis showed no effect of the pulsatility index in the middle cerebral artery and the cerebroplacental ratio on the plasmatic concentrations (A. cerebri media: $P = 0.479$, $P = 0.826$, $P = 0.528$, $P = 0.625$, $P = 0.154$; CPR: $P = 0.426$, $P = 0.479$, $P = 0.443$, $P = 0.867$, and $P = 0.181$) and expression levels (A. cerebri media: $P = 0.826$, $P = 0.931$, $P = 0.427$, $P = 0.639$, and $P = 0.297$; CPR: $P = 0.517$, $P = 0.288$, $P = 0.198$, $P = 0.984$, and $P = 0.195$) of all microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) that were identified to be upregulated in plasma samples derived from preeclampsia with or without fetal growth restriction.

The correlation between variables including absolute and/or relative quantification of particular microRNA in maternal plasma and the values of flow rate in the umbilical artery and the fetal blood vessel was calculated using Spearman's rank correlation coefficient. The pulsatility index in the umbilical artery did not show any correlation with microRNA plasmatic concentrations and/or microRNA gene expression. However, a weak negative correlation between the

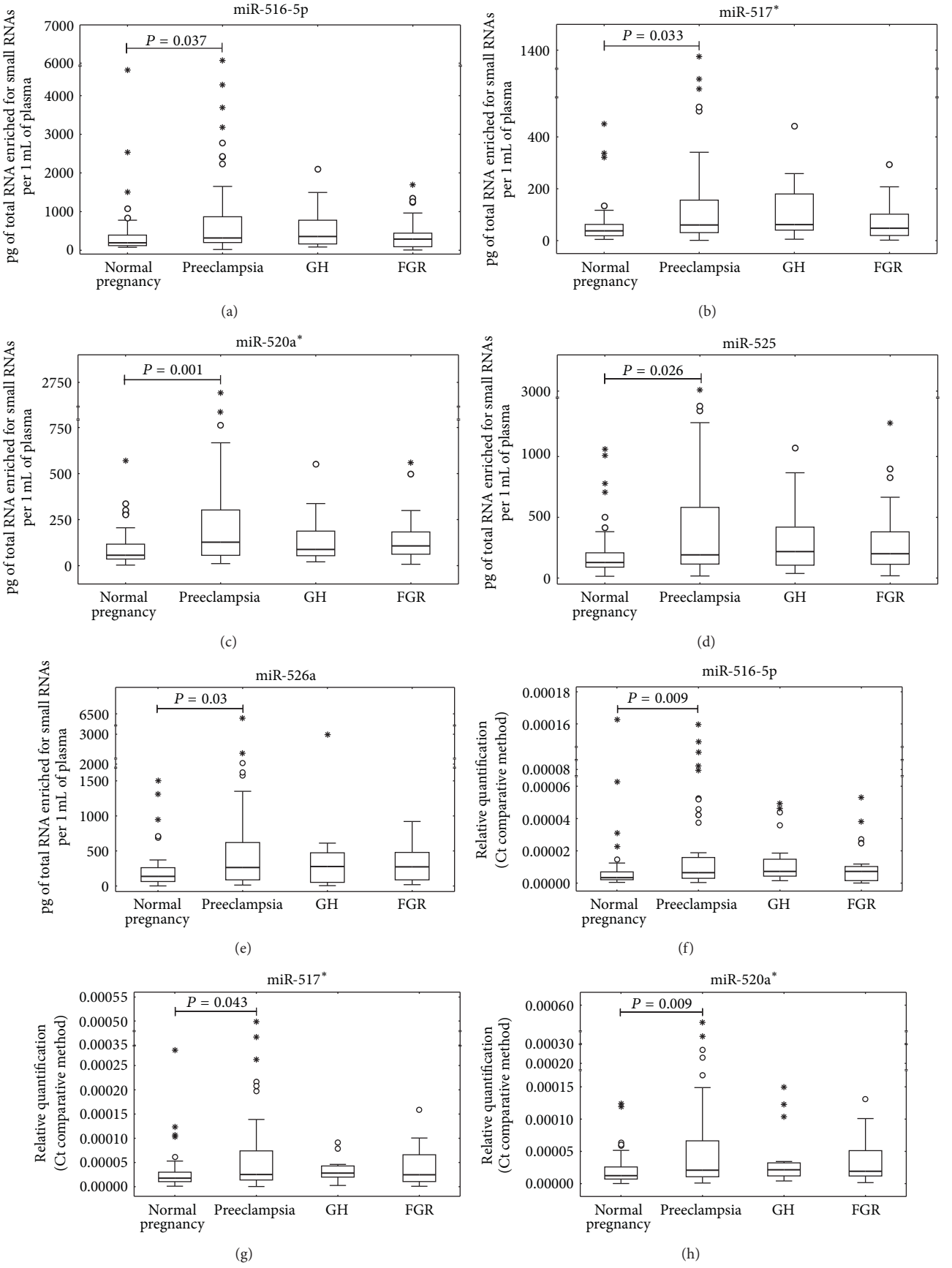

Figure 1: Continued.

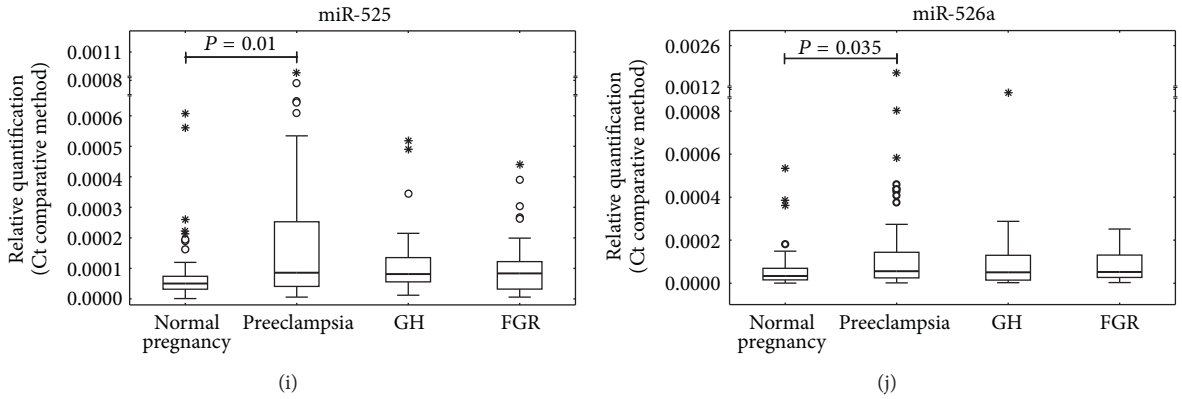

Figure 1: Upregulation of circulating C19MC microRNAs in pregnancies with preeclampsia. Absolute ((a), (b), (c), (d), and (e)) and relative ((f), (g), (h), (i), and (j)) quantification data were expressed as box plots of individual microRNAs in cohorts of normal and complicated pregnancies using Statistica software. The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers represent the maximum and minimum values that are no more than 1.5 times the span of the interquartile range (range of the values between the 25th and the 75th percentiles). The median is indicated by the line in each box. Outliers are indicated by circles and extremes by asterisks.

pulsatility index in the middle cerebral artery and microRNA plasmatic concentrations (miR-516-5p: $\rho = -0.393$, $P = 0.005$; miR-517*: $\rho = -0.328$, $P = 0.020$; miR-520a*: $\rho = -0.314$, $P = 0.026$; miR-525: $\rho = -0.358$, $P = 0.011$; miR-526a: $\rho = -0.304$, $P = 0.031$) or microRNA gene expression (miR-516-5p: $\rho = -0.307$, $P = 0.030$; miR-517*: $\rho = -0.288$, $P = 0.041$; miR-520a*: $\rho = -0.339$, $P = 0.017$; miR-525: $\rho = -0.357$, $P = 0.012$; miR-526a: $\rho = -0.286$, $P = 0.043$) was observed. Furthermore, a weak negative correlation between cerebroplacental ratio and microRNA plasmatic concentrations (miR-520a*: $\rho = -0.261$, $P = 0.050$; miR-526a: $\rho = -0.340$, $P = 0.017$; Figure 2(a)) or microRNA gene expression (miR-520a*: $\rho = -0.339$, $P = 0.018$; miR-526a: $\rho = -0.329$, $P = 0.021$; Figure 2(b)) was found (Table 1).

3.5. Function and Functional Relationship Analysis of Target Genes of Differentially Expressed Extracellular C19MC MicroRNAs in 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 (Table 2). The data were collected from miRDB database (http://mirdb.org/miRDB/). All the targets were predicted by a bioinformatics tool MirTarget2, which was developed by analyzing thousands of genes impacted by miRNAs with an SVM learning machine.

4. Discussion

The results of our previous pilot study strongly supported the need for a more detailed exploration of extracellular microRNAs in maternal circulation with the view toward their recognition as potential biomarkers for placental insufficiency related complications [51, 54].

Initially, some extracellular placental specific microRNAs (miR-516-5p, miR-520*, miR-518b, and miR-526a) trended just to a higher level in the small cohort of patients with placental insufficiency related complications (16 preeclampsia, 5 preeclampsia with IUGR and 11 IUGR), however, did not reach statistical significance when compared to gestational-age-matched controls. Although we have previously demonstrated that normal pregnancies and the manifestation of placental-insufficiency-related complications did not influence the levels of miR-16 and let-7d in maternal plasma [51, 54], it is now no doubt that ubiquitously expressed miR-16 and let-7d should not be further used to normalize expression profiles of various extracellular microRNAs as was done before in our pilot study. Our latest research revealed that the expression levels of miR-16 were significantly decreased in placental tissues derived from patients with preeclampsia (data submitted for publication). Similarly, Maccani et al. [56] also reported that reduced expression of miR-16 in placental tissue may be relevant to the low birth weight in term infants born small for gestational age. In contrast, miR-16 was previously observed to be overexpressed in placental tissues affected with severe preeclampsia, respectively [38]. The latest study of Wu et al. [44] brought the evidence of circulating mir-16 down-regulation in patients with severe preeclampsia. Furthermore, Yang et al. [43] showed decreased expression of circulating let-7d in preeclamptic patients applying more sophisticated approach such as next generation sequencing technology.

For that reason, alternative endogenous control candidates to normalize extracellular microRNA gene expression data should be used.

In the current study, the cohort of pregnancy related complications was expanded to achieve adequate power of the study. This time, the normalization of circulating microRNA expression was done against synthetic C. elegans microRNA (cel-miR-39). Using both absolute and relative quantification approaches, the ability of extracellular C19MC microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) to differentiate between normal and complicated pregnancies

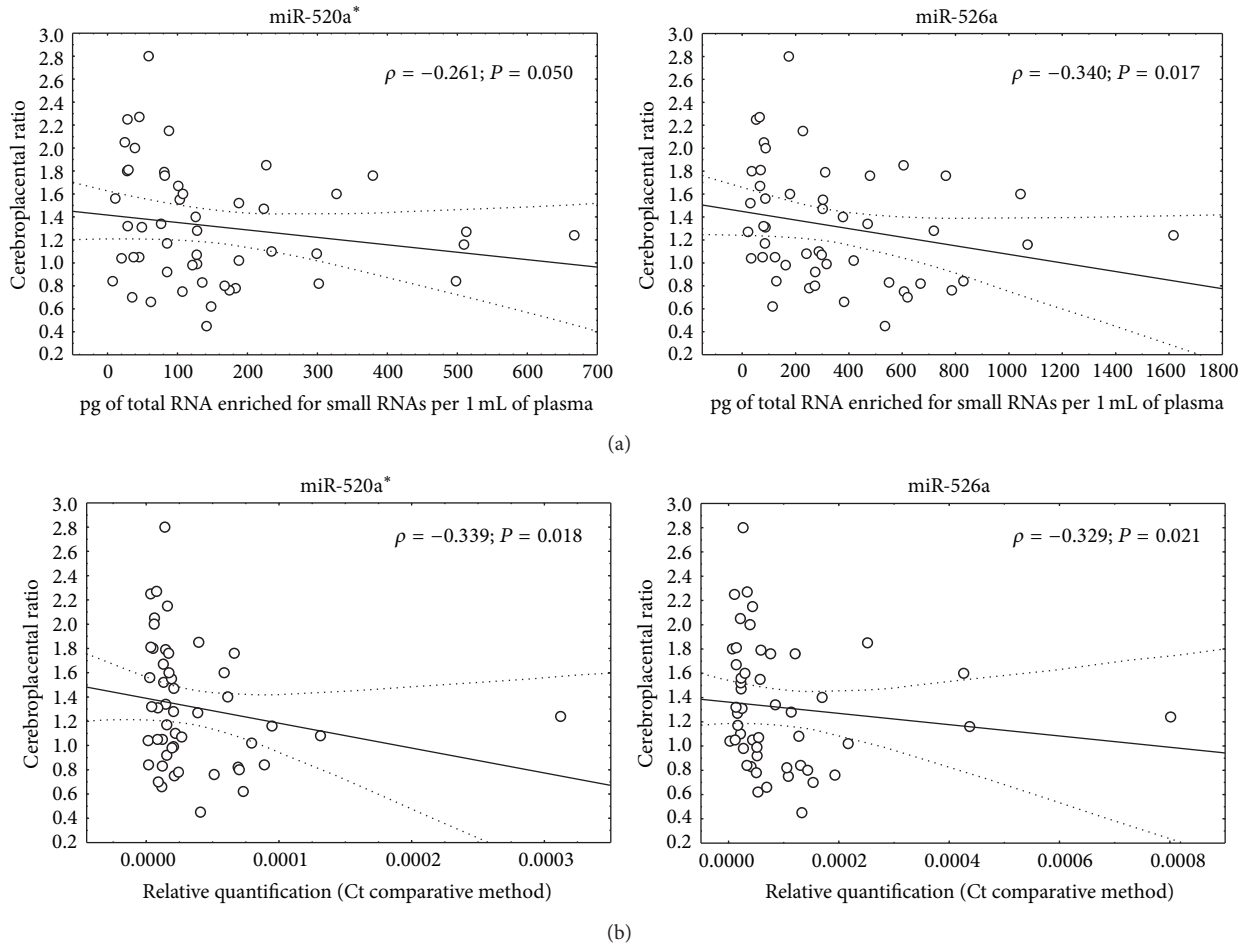

Figure 2: The association between the plasmatic concentration (a) and expression levels (b) of C19MC microRNAs and Doppler ultrasonography parameters. ρ: Spearman's correlation coefficient; P: level of significance.

during the onset of preeclampsia w or w/o fetal growth restriction was confirmed.

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. [43], who observed the upregulation of extracellular miR-520h in four patients with preeclampsia.

Our findings may be supported by Mouillet et al. [45], who have recently also observed no significant difference in relative placental specific microRNA levels (miR-518b) in plasma samples from those with normally progressing and fetal growth restriction pregnancies.

On the basis of the results of our study, we further studied the association between circulating C19MC microRNAs and the severity of the disease with respect to the degree of clinical signs, requirements for the delivery (before and after 34 weeks of gestation), and Doppler ultrasound examination.

The association analysis pointed to no relationship between C19MC microRNA plasmatic concentrations and/or gene expression and identified risk factors for a poorer perinatal outcome. There was no difference in microRNA plasmatic levels and/or gene expression between pregnancies with mild and severe preeclampsia, pregnancy-related complication with the need for the delivery before 34 weeks of gestation, and those who delivered after this critical period and pregnancies with abnormal and normal blood flow velocity waveforms. Nevertheless, the levels of miR-526a were significantly increased in the group of patients with abnormal values of flow rate in the umbilical artery.

On the other hand, the dependence between the levels of plasmatic C19MC microRNAs and the pulsatility index in the middle cerebral artery and the values of cerebroplacental ratio was demonstrated. The relation between the increased levels of plasmatic C19MC microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) and decreased values of flow rate in the middle cerebral artery reached a statistical significance in complicated pregnancies.

Similarly, the relationship between increased levels of plasmatic C19MC microRNAs (miR-520a* and miR-526a) and decreased values of cerebroplacental ratio was revealed.

In conclusion, microRNAs play a fundamental role in a variety of physiological and pathological processes involving

Table 2: (a) Function and functional relationship analysis of target genes of differentially expressed extracellular C19MC microRNAs in preeclampsia in relation to immune system response. (b) Function of target genes of differentially expressed extracellular C19MC microRNAs in preeclampsia in immune system response.

(a)

microRNA	miR-516-5p	miR-517*	miR-526a	miR-525	miR-520a*
Number of predicted target genes	349	179	212	340	352
		Unique target genes			Shared with miR-525
	CCR2	FAS	BCAP29	TOX	ACVR2B
	CD109	IL6ST	CD24		AHSA2
	CD1A	IL9R	CD302		ATRN
	DNAJC25	IRAK3	CFLAR		CD2
	FLT1	LILRA2	DNAJC21		CD300LB
	IL17RE	MTDH	HSP90AA1		CD46
	IRAK1	PAPPA	IGFBP1		CD93
	LILRB5		TLR2		HSF5
	PDCD6IP		TNFRSF19		IGF1R
	SOCS2		TNFSF15		IL10RA
			TRAF6		MMD2
					PPARA
					TLR7
					VSIG4

All the targets were predicted by a bioinformatics tool MirTarget2 using miRDB online database.

(b)

Gene official symbol	Gene full name	The role in immune system response
ACVR2B	Activin A receptor, type IIB	Activins belong to the TGF-β superfamily
AHSA2	AHA1, activator of heat shock 90 kDa protein ATPase homolog 2 (yeast)	Hsp90 is an inducible molecular chaperone protecting stressed cells
ATRN	Attractin	Involvement in initial immune cell clustering during inflammatory responses that may regulate the chemotactic activity of chemokines
BCAP29	B-cell receptor-associated protein 29	Involvement in CASP8-mediated apoptosis
CCR2	Chemokine (C-C motif) receptor 2	Binds monocyte chemoattractant protein-1 involved in monocyte infiltration during inflammation
CD109	CD109 molecule	Encodes GPI-linked glycoprotein that negatively regulates signaling of TGF-β
CD1A	CD1a molecule	Encodes glycoproteins structurally related to MHC proteins mediating the presentation of lipid and glycolipid antigens
CD2	CD2 molecule	A surface antigen of thymocytes, T, and NK cells
CD24	CD24 molecule	Encodes a sialoglycoprotein expressed on mature granulocytes and B cells
CD300LB	CD300 molecule-like family member b	A nonclassical activating receptor of the Ig superfamily expressed on myeloid cells
CD302	CD302 molecule	A C-type lectin receptor involved in cell adhesion, migration, endocytosis, and phagocytosis
CD46	CD46 molecule, complement regulatory protein	Has cofactor activity for inactivation of complement components C3b and C4b by serum factor I
CD93	CD93 molecule	Involvement in intercellular adhesion and in the clearance of apoptotic cells
CFLAR	CASP8 and FADD-like apoptosis regulator	Regulator of apoptosis structurally similar to caspase-8

(b) Continued.

Gene official symbol	Gene full name	The role in immune system response
DNAJC21	DnaJ (Hsp40) homolog, subfamily C, member 21	A molecular chaperone protein protecting against cellular stress
DNAJC25	DnaJ (Hsp40) homolog, subfamily C, member 25	A molecular chaperone protein protecting against cellular stress
FAS	FAS cell surface death receptor (FAS)	Plays a central role in regulation of programmed cell death
FLT1	fms-related tyrosine kinase 1	A member of vascular endothelial growth factor receptor (VEGFR) playing an important role in angiogenesis and vasculogenesis
HSF5	Heat shock transcription factor family member 5	A transcriptional activator of heat shock genes
HSP90AA1	Heat shock protein 90 kDa alpha (cytosolic), class A member 1	An inducible molecular chaperone protecting stressed cells
IGF1R	Insulin-like growth factor 1 receptor	Antiapoptotic agent enhancing cell survival
IGFBP1	Insulin-like growth factor binding protein 1	Prolongs the half-time of IGFs in plasma that regulate cell growth and development
IL10RA	Interleukin 10 receptor, alpha	Involvement in inhibition of the synthesis of proinflammatory cytokines
IL17RE	Interleukin 17 receptor E	Participation in MAPK pathway
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	A signal transducer shared by IL-6, LIF, and oncostatin M
IL9R	Interleukin 9 receptor	Mediates IL-9 effects like stimulation of cell proliferation and prevention of apoptosis
IRAK1	Interleukin-1 receptor-associated kinase 1	Responsible for IL-1 induced upregulation of the transcription factor NF-kappa B
IRAK3	Interleukin-1 receptor-associated kinase 3	Functions as a negative regulator of Toll-like receptor signaling
LILRA2	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 2	An activatory cell-surface receptor expressed on monocytes, B cells, dendritic, and NK cells
LILRB5	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 5	An inhibitory cell-surface receptor expressed on immune cells
MMD2	Monocyte to macrophage differentiation-associated 2	Modulates Ras signaling
MTDH	Metadherin	Involvement in HIF-1 alpha mediated angiogenesis and RNA-induced silencing complex and miRNA functions
PAPPA	Pregnancy-associated plasma protein A, pappalysin-1	Involvement in local proliferative processes such as wound healing
PDCD6IP	Programmed cell death 6 interacting protein	Protects against cell death
PPARA	Peroxisome proliferator-activated receptor alpha	Affects the expression of genes involved in cell proliferation, cell differentiation, and in immune and inflammation responses
SOCS2	Suppressor of cytokine signaling 2	A negative regulator of JAK/STAT cytokine signaling pathway
TLR2	Toll-like receptor 2	Plays a fundamental role in activation of innate immunity, stimulates NF-kappa B
TLR7	Toll-like receptor 7	Plays a fundamental role in activation of innate immunity
TNFRSF19	Tumor necrosis factor receptor superfamily, member 19	Interacts with TRAF family members, induces apoptosis by a caspase-independent mechanism

(b) Continued.

Gene official symbol	Gene full name	The role in immune system response
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	A cytokine induced by TNF and IL-1 alpha activating NF-kappa B and MAP kinases inducing apoptosis in endothelial cells
TOX	Thymocyte selection-associated high mobility group box	Highly expressed in thymus, the site of development of T cells
TRAF6	TNF receptor-associated factor 6, E3 ubiquitin protein ligase	Functions as a signal transducer in the NF-kappa B pathway, activates Ikappa B kinase in response to proinflammatory cytokines
VSIG4	V-set and immunoglobulin domain containing 4	A negative regulator of T-cell responses structurally related to the B7 family of immune regulatory proteins

pregnancy-related complications. Current study demonstrated for the first time that circulating C19MC microRNAs might play a role in the pathogenesis of preeclampsia, but not in the pathogenesis of gestational hypertension and fetal growth restriction. The study brought interesting finding that the upregulation of circulating C19MC microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) is a characteristic phenomenon of established preeclampsia.

5. Conclusion

The study brought the interesting finding that the upregulation of circulating C19MC microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) is a characteristic phenomenon of established preeclampsia.

Conflict of Interests

The authors report no conflict of interests.

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4.4 First trimester screening of circulating C19MC microRNAs can predict subsequent onset of gestational hypertension

Hromadnikova I, Kotlabova K, Hympanova L, Doucha J, Krofta L.
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4.4.1 Overview

Latest findings revealed that microRNAs are packed within exosomal nanoparticles released from placenta (that undergoes continuous remodelling during development) into the blood and extracellular compartments, where they mediate the communication with diverse types of cells including either neighbouring or distant cells (Montecalvo et al., 2012; Chen et al., 2012; Ouyang et al., 2014).

Recent studies have shown that clinically established preeclampsia is associated with alterations in extracellular microRNA levels (Gunel et al., 2011; Yang et al., 2011; Wu et al., 2012; Li et al., 2013). Studies dealt with fetal growth-restriction reported no differentiation between pregnancies with normal and abnormal course, when circulating microRNA levels were compared between normal and fetal growth-restricted foetuses (Mouillet et al., 2010; Higashijima et al., 2013; Xu et al., 2014). Nevertheless, Whitehead et al. (2013) revealed up-regulation of several hypoxia-regulated circulating microRNAs in pregnancies complicated by severe preterm fetal growth restriction compared to gestational-age-matched controls.

The data resulting from our pilot study suggested the potential of extracellular C19MC microRNAs to differentiate, at the beginning of gestation (within 12^{th} to 16^{th} weeks), between patients at risk of later development of placental insufficiency related complications and normal pregnancies (Hromadnikova et al., 2012). These data strongly supported the need for a more detailed exploration of extracellular C19MC microRNAs in maternal circulation with the view toward routine assessment into everyday practice, and recognition as potential biomarkers for placental insufficiency related pregnancy complications.

4.4.2 Aims and methods

In this prospective study, we discuss the effectiveness of circulating C19MC microRNAs to predict at the first trimester of gestation later occurrence of gestational hypertension. Plasma samples were obtained from singleton pregnant women enrolled at first trimester screening at 10 to 13 weeks. The case cohort included 18 pregnancies which developed gestational hypertension and 28 normal, gestational age-matched pregnancies.

Receivers operating characteristic (ROC) curves were constructed to calculate the area under the curve (AUC) and the best cut-off point for particular placental specific microRNA was used in order to calculate the respective sensitivity, specificity, predictive values and likelihood ratios in the prediction of gestational hypertension.

The function and functional relationship analysis of predicted targets of studied C19MC microRNAs were performed; targets were predicted by a bioinformatics tool MirTarget2.

4.4.3 Results and discussion

In our study performed on the samples collected during the first trimester screening, we found 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.

The predictive accuracy of single first trimester plasmatic microRNA biomarkers was assessed. 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%.

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%. 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.

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.

PLOS ONE

RESEARCH ARTICLE

First Trimester Screening of Circulating C19MC microRNAs Can Predict Subsequent Onset of Gestational Hypertension

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Abstract

Objective: The objective of the study was to evaluate risk assessment for gestational hypertension based on the profile of circulating placental specific C19MC microRNAs in early pregnancy.

Study design: The prospective longitudinal cohort study of women enrolled at first trimester screening at 10 to 13 weeks was carried out (n=267). Relative quantification of placental specific C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525 and miR-526a) was determined in 28 normal pregnancies and 18 pregnancies which developed gestational hypertension using real-time PCR and a comparative Ct method relative to synthetic C. elegans microRNA (cel-miR-39).

Results: Increased extracellular C19MC microRNA plasmatic levels (miR-516-5p, $p<0.001$; miR-517*, $p=0.007$; miR-520h, $p<0.001$; miR-518b, $p=0.002$) were detected in patients destined to develop gestational hypertension. MiR-520h had the best predictive performance with a PPV of 84.6% at a 7.1% false positive rate. The combination of miR-520h and miR-518b was able to predict 82.6% of women at the same false positive rate. The overall predictive capacity of single miR-518b (73.3% at 14.3% FPR), miR-516-5p (70.6% at 17.9% FPR) and miR-517* (57.9% at 28.6% FPR) biomarkers was lower.

Conclusion: The study brought interesting finding that the up-regulation of miR-516-5p, miR-517*, miR-520h and miR-518b is associated with a risk of later development of gestational hypertension. First trimester screening of extracellular

miR-520h alone or in combination with miR-518b identified a significant proportion of women with subsequent gestational hypertension.

Introduction

Since the placenta is being continuously remodelled during normal placental development, extracellular nucleic acids of both fetal and placental origin, packed into either trophoblast-derived apoptotic bodies or shedding syncytiotrophoblast microparticles, may be detected in maternal circulation during the course of normal gestation [1–5]. Latest findings revealed that microRNAs are also packed within exosomal nanoparticles released into the blood and extracellular compartment mediating the communication between diverse types of neighbouring or distant cells [6–9].

There has been a trend over the last 10 years to develop non-invasive methods utilizing quantification of cell-free nucleic acids inclusive of microRNAs in maternal circulation [5, 10–29]. The diagnostic potential of particular molecular biomarkers and their implementation in the current predictive and diagnostic algorithms for pregnancy related complications are subject of interest [5].

MicroRNAs belong to a family of small noncoding RNAs that regulate gene expression at the posttranscriptional level by degrading or blocking translation of messenger RNA (mRNA) targets [30, 31].

Recent studies have shown that clinically established preeclampsia is associated with alterations in extracellular microRNA expression [32–35]. Nevertheless, no differentiation between pregnancies with normal and fetal growth-restricted foetuses was observed when circulating microRNA expression levels were compared [36–38]. Nevertheless, the recent study of Whitehead et al. revealed up-regulation of several hypoxia-regulated microRNAs in pregnancies complicated by severe preterm fetal growth restriction compared to gestation-matched controls [39].

However, most of investigators focused on the study of those microRNAs, whose genes are located outside chromosome 19 miRNA clusters (C19MC and miR-371-3 cluster) or the chromosome 14 miRNA cluster (C14MC) that encode pregnancy-associated microRNAs [40–44].

We have previously identified C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525 and miR-526a) present in maternal plasma differentiating between normal pregnancies and non-pregnant individuals [45]. Significant increases in extracellular C19MC microRNAs levels (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525 and miR-526a) over time in normally progressing pregnancies were observed [45, 46].

The results of our follow-up 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 [47].

The data resulting from our pilot study suggested the potential of extracellular C19MC microRNAs to differentiate, at the beginning of gestation (within 12th to 16th weeks), between patients at risk of later development of placental insufficiency related complications and normal pregnancies [46]. These data strongly supported the need for a more detailed exploration of extracellular microRNAs in maternal circulation with the view toward routine assessment into everyday practice, and recognition as potential biomarkers for placental insufficiency related pregnancy complications.

To our knowledge, no prospective study of women enrolled at first trimester screening to describe the profile of circulating C19MC microRNAs in the women at risk of subsequently developing gestational hypertension has been carried out. Here, we discuss for the first time the effectiveness of circulating C19MC microRNAs to predict the later occurrence of gestational hypertension.

Consequently, the function and functional relationship analysis of predicted targets of aberrantly expressed C19MC microRNAs in patients destined to develop gestational hypertension, was performed.

Materials and Methods

Patients

The study was designed in a prospective manner. The study cohort consisted of 267 consecutive Caucasian singleton pregnant women enrolled at first trimester screening at 10 to 13 weeks. The case cohort included 18 pregnancies which developed gestational hypertension and the control cohort that was chosen on the basis of equal times in storage and gestational age, included 28 normal pregnancies.

Gestational hypertension was defined as high blood pressure that developed after the twentieth week of pregnancy. Normal pregnancies were defined as those without complications who delivered full term, healthy infants weighting $>$2500 g after 37 completed weeks of gestation.

All patients provided written informed consent. The study was approved by the Ethics Committee of the Third Faculty of Medicine, Charles University in Prague. Gestational age was assessed using ultrasonography at 11 to 13 weeks and 6 days.

Processing of samples

Nine millilitres of peripheral blood were collected into EDTA tubes and centrifuged twice at 1200 g for 10 min at room temperature. Plasma samples were stored at -80℃ until subsequent processing.

Total RNA was extracted from 1 mL of plasma and 25 mg of normal placental tissue preserved in RNAlater (Ambion, Austin, USA) followed by an enrichment procedure for small RNAs using a mirVana microRNA Isolation kit (Ambion, Austin, USA). Trizol LS reagent was used in plasma samples for total RNA extraction from biological fluids (Invitrogen, Carlsbad, USA) and preceded the

small RNAs enrichment procedure. To minimize DNA contamination, we treated the eluted RNA with 5 μL of DNase I (Fermentas International, Ontario, Canada) for 30 min at 37℃.

Reverse transcriptase reaction

Each microRNA was reverse transcribed into complementary DNA using TaqMan MicroRNA Assay, containing microRNA-specific stem-loop RT primers, and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Branchburg, USA) in a total reaction volume of 50 μL on a 7500 Real-Time PCR system (Applied Biosystems, Branchburg, USA) with following thermal cycling parameters: 30 minutes at 16℃, 30 minutes at 42℃, 5 minutes at 85℃, and then held at 4℃.

Quantification of microRNAs

The characteristics of studied C19MC microRNAs are outlined in table 1.

15 μL of cDNA corresponding to each microRNA was mixed with components of TaqMan MicroRNA Assay, and the ingredients of the TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, USA) in a total reaction volume of 35 μL. TaqMan PCR conditions were set as described in the TaqMan guidelines. The analysis was performed using a 7500 Real-Time PCR System. All PCRs were

Table 1. Characteristics of selected C19MC microRNAs.

Assay name	miRBase ID	NCBI Location Chromosome	microRNA sequence	Expression in placenta
hsa-miR-516-5p	hsa-miR-516b-5p	Chr.19: 58920508 - 58920592 [+]	5′-CAUCUGGAGGUAAGAAGCACUUU-3′	exclusively expressed
hsa-miR-517*	hsa-miR-517-5p	Chr.19: 54215522 - 54215608 [+]	5′-CCUCUAGAUGGAAGCACUGUCU-3′	high expression
hsa-miR-518b	hsa-miR-518b	Chr.19: 54205991 - 54206073 [+]	5′-CAAAGCGCUCCCCUUUAGAGGU-3′	exclusively expressed
hsa-miR-520a*	hsa-miR-520a-5p	Chr.19: 54194135 - 54194219 [+]	5′-CUCCAGAGGGAAGUACUUUCU-3′	high expression
hsa-miR-520h	hsa-miR-520h	Chr.19: 54245766 - 54245853 [+]	5′-ACAAAGUGCUUCCCUUUAGAGU-3′	exclusively expressed
hsa-miR-525	hsa-miR-525-5p	Chr.19: 54200787 - 54200871 [+]	5′-CUCCAGAGGGAUGCACUUUCU-3′	exclusively expressed
hsa-miR-526a	hsa-miR-526a	Chr.19: 54209506 - 54209590 [+]	5′-CUCUAGAGGGAAGCACUUUCU-3′	high expression

C19MC microRNAs were divided into two categories (microRNAs exclusively expressed in the placental tissue and those with high expression in the placental tissue) based on information in miRNAMap 2.0 database (http://mirnamap.mbc.nctu.edu.tw/index.php), where the Q-PCR experiments for monitoring the expression profiles of 224 human miRNAs in eighteen major normal tissues in humans are provided. For example, we indicated miR-516-5p and miR-518b as those to be exclusively expressed in the placental tissue, since according to the miRNAMap 2.0 database miR-516-5p was shown to be expressed only in the placental tissue and miR-518b to be highly expressed in the placental tissue and rarely expressed in testes. On the other hand, for instance miR-520a* showed besides high expression in the placental tissue also low expression in other human tissues involving adipose, bladder, brain, cervix, heart, kidney, liver, lung, muscle, ovary, prostate, small intestine, spleen, testes, thymus, thyroid and trachea.

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performed in duplicates. A sample was considered positive if the amplification signal occurred before the 40th threshold cycle.

The expression of particular microRNA in maternal plasma was determined using the comparative Ct method [48] relative to the expression of the same microRNA in the reference sample. RNA fraction highly enriched for small RNA isolated from the fetal part of one randomly selected placenta derived from gestation with normal course (the part of the placenta derived from the chorionic sac that encloses the embryo, consisting of the chorionic plate and villi) was used as a reference sample for relative quantification throughout the study.

Synthetic C. elegans microRNA (cel-miR-39, Qiagen, Hilden, Germany) was used as an internal control for variations during the preparation of RNA, cDNA synthesis, and real-time PCR. Due to a lack of generally accepted standards, all experimental real-time qRT-PCR data were normalized to cel-miR-39, as it shows no sequence homology to any human microRNA. 1 μl of 0.1 nM cel-miR-39 was spiked in after incubation with Trizol LS reagent to human plasma and reference samples. The following form of equation was used to compare the gene expression between various samples:

$$2^{-\Delta\Delta Ct} = [(\text{Ct particular C19MC microRNA} - \text{Ct cel-miR-39})\ \text{tested sample} - (\text{Ct particular C19MC microRNA} - \text{Ct cel-miR-39})\ \text{reference sample}]$$

Statistical analysis

Imprecision of the assays is indicated as percentage coefficients of variations (%CV). Normality of the data was assessed using Shapiro-Wilk test, which indicated that our data did not follow a normal distribution. Therefore, microRNA levels were compared between groups by non-parametric test (the Mann-Whitney U test) using Statistica software (version 9.0; StatSoft, Inc., USA). Since the Bonferroni correction was used to address the problem of multiple comparisons (altogether 6 placental specific microRNAs were analysed), the significance level was established at a *p*-value of $p<0.0083$ ($\alpha=0.05/6$).

Receivers operating characteristic (ROC) curves were constructed to calculate the area under the curve (AUC) and the best cut-off point for particular placental specific microRNA was used in order to calculate the respective sensitivity, specificity, predictive values and likelihood ratios in the prediction of gestational hypertension.

Comparison of ROC curves was done with the method of DeLong et al. [49] using MedCalc statistical software (MedCalc Software bvba, Ostend, Belgium). The software gave the difference between the areas under the ROC curves, with standard errors, 95% confidence intervals and p-values.

Function and functional relationship analysis of target genes of studied C19MC microRNAs

The function and functional relationship analysis of predicted targets of studied C19MC microRNAs were performed. Mainly the predicted targets with close relation to gestation were subject of interest. The data were collected from miRDB database (http://mirdb.org). All the targets were predicted by a bioinformatics tool MirTarget2, which was developed by analysing thousands of genes impacted by miRNAs with an SVM learning machine.

The miRDB database is interconnected to the NCBI database (http://www.ncbi.nlm.nih.gov/gene/), where the description of proteins encoded by predicted genes is provided. Comprehensive and systematic search for each predicted target of particular mature C19MC microRNAs (miR-516-5p, miR-517*, miR-520h and miR-518b), that have been shown to be upregulated in patients with later onset of gestational hypertension, in relation to gestation was made using the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/).

Results

Of 267 pregnant women enrolled at first trimester screening, 25 were lost for follow-up, 18 developed gestational hypertension (6.7%), 15 were diagnosed with other pregnancy-related complications (10 preeclampsia, 3 intrauterine growth restriction, 2 small for gestational age foetuses) and 209 had normal course of gestation (78.3%).

Unfortunately, miR-526a displayed late amplification curves (median Ct 39.61) in first trimester plasma samples, and therefore it was excluded from further analysis.

Intra- and inter- assay variability for particular microRNA assays

Intra- and interassay reproducibility testing for particular microRNA assays using plasma samples derived from both pregnancies with normal and complicated course of gestation showed an imprecision of 3.01%–5.68% in within-assay comparisons (miR-516-5p: 3.67%, miR-517*: 3.01%, miR-518b: 3.55%, miR-520a*: 5.16%, miR-520h: 3.12%, and miR-525: 5.68%) and 12.61%–14.92% in between-assay comparisons (miR-516-5p: 14.92%, miR-517*: 13.53%, miR-518b: 14.23%, miR-520a*: 13.01%, miR-520h: 13.98%, and miR-525: 12.61%), respectively.

Up-regulation of circulating C19MC microRNAs in pregnancies which subsequently developed gestational hypertension

Overall, increased plasmatic levels of **miR-516-5p** ($p<0.001$), **miR-517***($p=0.007$), **miR-520h** ($p<0.001$) and **miR-518b** ($p=0.002$) were observed in maternal plasma samples derived from first trimester screening of those women who subsequently developed gestational hypertension compared to normal

pregnancies. No difference in plasmatic levels of **miR-520a*** (p=0.044) and **miR-525** (p=0.422) between the control cohort and the cohort of patients destined to develop gestational hypertension was identified (Fig. 1).

First trimester screening of circulating C19MC microRNAs in the identification of pregnancies with later onset of gestational hypertension

Table 2 and 3 display the predictive accuracy of maternal plasma concentrations of placental specific microRNAs in early pregnancy in the identification of gestational hypertension using cut-offs derived from the ROC curves. Firstly, the predictive accuracy of single first trimester plasmatic microRNA markers was assessed. The largest area under the curve was observed for miR-516-5p (0.850), miR-520h (0.817) and miR-518b (0.786). Using miR-517* and miR-520a* prediction rules for gestational hypertension had lower area under the curve of 0.752/0.688, respectively (Fig. 2, Table 2). The predictive performance of miR-525 was not reported since the AUC was not significant (0.576, p=0.4685). Pairwise comparison between ROC curves revealed that the difference between AUCs of miR-516-5p and miR-518b was 0.064 and this difference was significant (95% CI, 0.012–0.116, p=0.014), while the difference between AUCs of miR-516-5p and miR-520h (0.033), was not significant (95% CI, −0.021–0.088, p=0.234). Similarly, no difference was observed between AUCs of miR-520h and miR-518b (0.031), (95% CI, −0.022–0.084, p=0.253).

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%. Overall, the likelihood ratios for a positive test for these three best placental specific microRNA markers were large to small, ranging between 10.27 and 4.48.

However, whilst raised plasmatic levels of miR-517* have been observed in the first trimester, the overall predictive capacity for gestational hypertension was lower (sensitivity 73.3%, specificity 71.4%, and PPV 57.9%).

The areas under the curves were comparable between various combinations of three selected C19MC microRNA biomarkers (miR-516-5p, miR-520h and miR-518b). Pairwise comparison between ROC curves revealed no difference between AUCs of the following combinations of C19MC microRNAs (miR-516-5p and miR-518b vs. miR-516-5p and miR-520h: 0.788 vs. 0.828, 95% CI, −0.007–0.089, p=0.097; miR-516-5p and miR-518b vs. miR-518b and miR-520h: 0.788 vs. 0.786, 95% CI, −0.090–0.093, p=0.969; miR-516-5p and miR-520h vs. miR-518b and miR-520h: 0.829 vs. 0.786, 95% CI, −0.014–0.099, p=0.137).

First trimester screening based on the combination of two placental specific microRNAs (miR-520h and miR-518b) showed the highest accuracy for the prediction of gestational hypertension; it was able to identify women at risk of

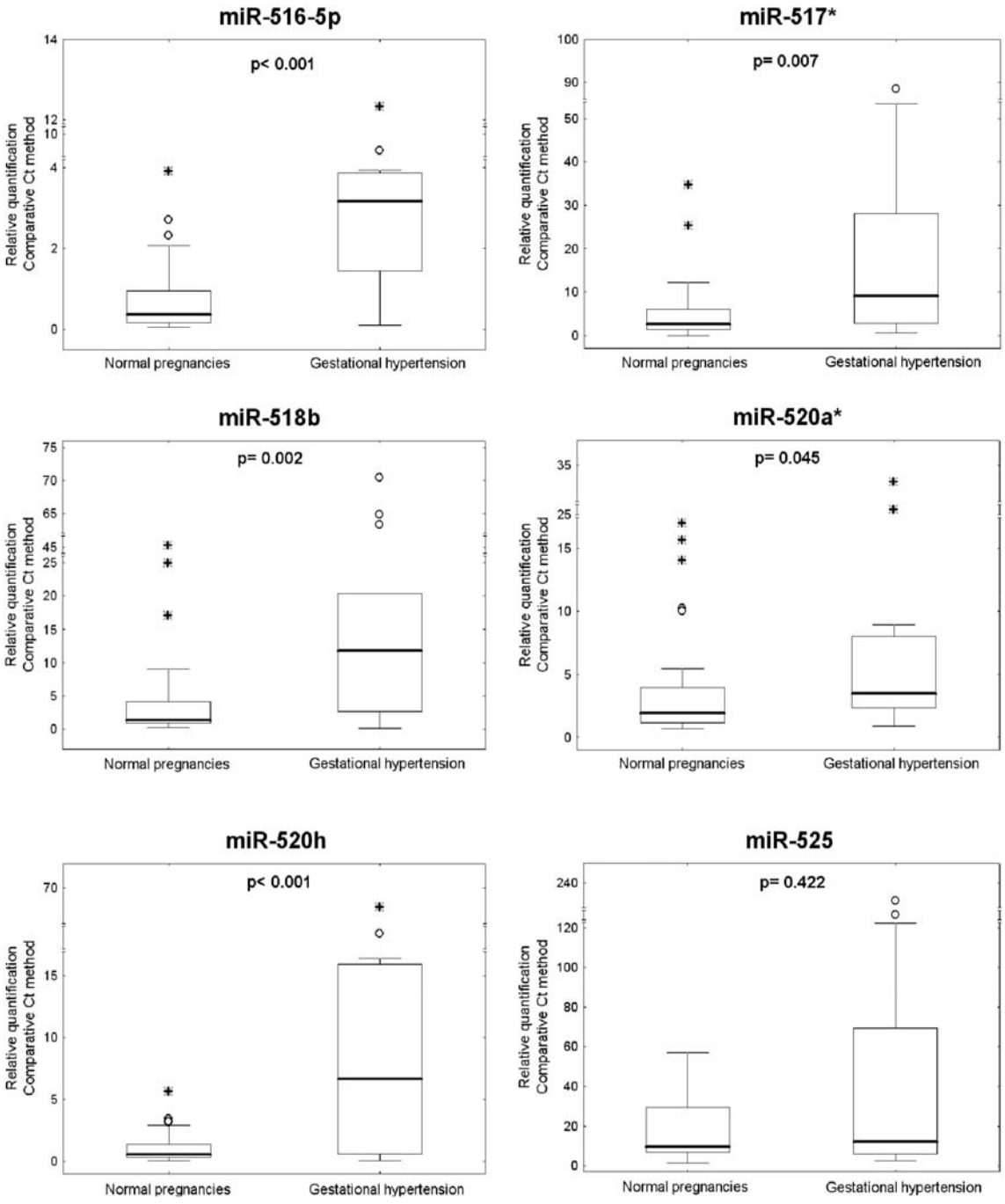

Fig. 1. Up-regulation of circulating C19MC microRNAs in pregnancies which developed gestational hypertension. Relative quantification data were expressed as box plots of individual microRNAs in cohorts of normal and complicated pregnancies using Statistica software. The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers represent the maximum and minimum values that are no more than 1.5 times the span of the interquartile range (range of the values between the 25th and the 75th percentiles). The median is indicated by the line in each box. Outliers are indicated by circles and extremes by asterisks.

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Table 2. Predictive accuracy of first trimester maternal plasma C19MC microRNA markers for the diagnosis of gestational hypertension.

miRNA	*AUC*	*ROC curve*	*Cutoff*	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*Positive likelihood ratio*	*Negative likelihood ratio*
	(95% CI)	*p-value*						*(95% CI)*	*(95% CI)*
miR-520h	0.817 (0.669−0.918)	0.0002	>3.1986	73.30%	92.90%	84.60%	86.70%	10.27 (2.6−40.4)	0.29 (0.1−0.7)
miR-518b	0.786 (0.634−0.896)	0.0004	>5.5955	73.30%	85.70%	73.30%	85.70%	5.13 (2.0−13.4)	0.31 (0.1−0.7)
miR-516-5p	0.85 (0.708−0.940)	<0.0001	>1.2013	80.00%	82.10%	70.60%	88.50%	4.48 (1.9−10.3)	0.24 (0.09−0.7)
miR-517*	0.752 (0.597−0.871)	0.0021	>3.761	73.30%	71.40%	57.90%	83.30%	2.57 (1.3−5.0)	0.37 (0.2−0.9)
miR-520a*	0.688 (0.529−0.820)	0.031	>2.2471	80.00%	67.90%	57.10%	86.40%	2.49 (1.4−4.5)	0.29 (0.1−0.8)

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subsequently developing complication with a PPV of 82.6% at a specificity of 92.9% (Fig. 2, Table 3). The combination of these two placental specific microRNAs showed the same specificity as the miR-520h biomarker, and higher a PPV (82.6%) compared to the single miR-518b biomarker. 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 (Fig. 2, Table 3). Thats why there was no additive effect of using of miR-516-5p in the combination with miR-520h and/or miR-518b, and the use of all three C19MC microRNA biomarkers had no advantage over using single miR-520h biomarker and in combination with miR-518b.

Table 3. Predictive accuracy of first trimester maternal plasma C19MC microRNA markers combination for the diagnosis of gestational hypertension.

miRNA	*AUC*	*ROC curve*	*Cutoff*	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*Positive likelihood ratio*	*Negative likelihood ratio*
	(95% CI)	*p-value*						*(95% CI)*	*(95% CI)*
miR-520h and miR-518b	0.786 (0.685−0.867)	<0.0001	>5.6161	63.30%	92.90%	82.60%	82.50%	8.87 (3.3−23.7)	0.39 (0.2−0.6)
miR-516-5p and miR-520h	0.829 (0.733−0.902)	<0.0001	>0.0002	70.00%	87.50%	75.00%	84.50%	5.6 (2.7−11.6)	0.34 (0.2−0.6)
miR-516-5p and miR-518b	0.788 (0.687−0.869)	<0.0001	>0.0002	80.00%	73.20%	61.50%	87.20%	2.99 (1.9−4.8)	0.27 (0.1−0.6)
miR-516-5p, miR-520h and miR-518b	0.796 (0.716−0.862)	<0.0001	>0.0002	73.30%	79.80%	66.00%	84.80%	3.62 (2.3−5.7)	0.33 (0.2−0.5)

AUC: area under the curve, ROC: receiver operating characteristic; PPV: positive predictive value; NPV: negative predictive value.

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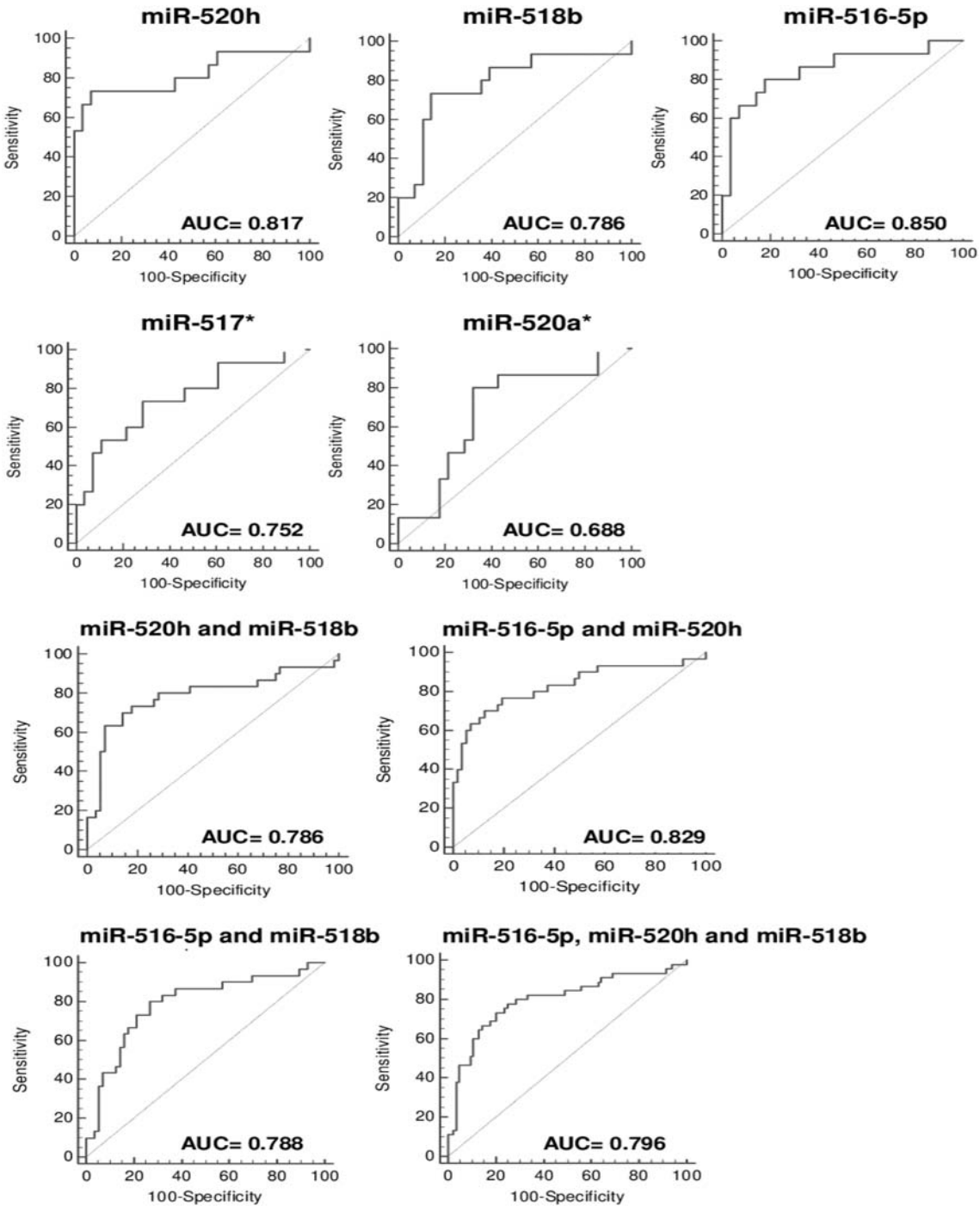

Fig. 2. Receiver operating characteristic curves – evaluation of the effectiveness of circulating C19MC microRNAs to predict the development of gestational hypertension.

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Function and functional relationship analysis of target genes of differentially expressed extracellular C19MC microRNAs in early pregnancy in patients destined to develop gestational hypertension

The function and functional relationship analysis of predicted targets of up-regulated extracellular C19MC microRNAs in patients who subsequently developed gestational hypertension 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) (Table 4; S1–S4 Tables).

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 (miR-516-5p: GYS1, PDGFRA, SP1, DDAH1, KNG1, NOS1, FLT1, KCNQ3, STC1, SOCS2, SLC9A1, MTHFR, GRB2 and SLC6A2; miR-517*:-CUL4B, SFRP4, SDC4, FAS, SH3BGRL2, CASP10 and PAPPA; miR-518b: TOLLIP and PRDX6; miR-520h: NOX4, VAV3, FBN1, LAMP2, HIF1A, GNA14, TLR5, PRCP, CALU, OCLN, EDNRA, SLC41A1, F3, CB1, ITGAV, SDC2, E2F1, GLRX, CDKN1A, TGFB2, LIN28B, ABCA1, OLR1, ACP1, PAFAH1B2, NTRK2 and ANGPT1) (S1–S4 Tables).

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 (S1, S2, S4 Tables).

Discussion

Although individual maternal plasma/serum markers have not usually performed well as a screening test for preeclampsia [50, 51, 53–69] and fetal growth restriction [52, 57, 58], combined screening tests to assess the risk of preeclampsia are currently used in practice [66]. In a proposed new approach to prenatal care, screening by 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 about 95% of cases with early onset of preeclampsia for a false-positive rate of 10% [66].

Further research is needed to discover other biomarkers with better diagnostic performance in order to improve the prediction of placental-insufficiency related complications. Recent study of Luque et al. demonstrated no predictive value of first trimester maternal serum miRNA assessment for early preeclampsia analysing a total of 754 miRNAs [70]. Initially, miRNA profiling on high-throughput OpenArray™ system revealed differential abundance profile of 7 microRNAs in

Table 4. Function and functional relationship analysis of target genes of differentially expressed extracellular C19MC microRNAs in patients developing gestational hypertension in relation to pregnancy.

microRNA	Total no. of predicted target genes/ no. of target genes with relation to pregnancy	Target genes with relation to pregnancy
miR-516-5p	349/53	GYS1, MAPK10, MSRB3, EGLN3, ITGA9, PMP22, CD177, PDGFRA, PSG5, LIN28B, TFCP2L1, PSG9, CHAF1B, IL17RE, PSG6, PSG2, DDAH1, KNG1, NOS1, FUT1, FLT1, UMPS, KCNQ3, SPRY2, PSG11, DAZ1, CHM, OBSL1, WARS, SPAST, SH3BGR, MEOX2, CD1A, PSG3, CCNG1, STC1, CCNA2, CCR2, EGR1, SOCS2, ICOSLG, KCNB1, SLC1A1, SLC9A1, MTHFR , IRAK1, TYRO3, CELF4, ANXA4, MS4A1, ADAMTS14, GRB2, SLC6A2
miR-517*	179/21	RND3, CUL4B, SFRP4, SDHC, MTDH, FUT1, LHCGR, SDC4, FUT9, MBD2, ZCCHC10, CCNG1, DGKE, LMNB2, FAS, SH3BGRL2, CASP10, NFIB, TWIST1, GCLC, PAPPA
miR-518b	42/4	TOLLIP, SLCO4C1, PRDX6, CXADR
miR-520h	509/65	NOX4, VAV3, KLF12, FBN1, JAZF1, CAPN2, TRPC5, ANXA4, LAMP2, HIF1A, RND3, EIF4E, GNA14, PAK2, NR4A2, GBP6, TLR5, GCH1, PHF14, PRCP, SENP1, MYCN, TFAM, CALU, OCLN, CCNB1, EDNRA, SLC41A1, F3, CNR1, ITGAV, SDC2, E2F1, GLRX, CGA, RGS2, ADAMTS5, PRIM2, CDKN1A, PCK1, TGFB2, LIN28B, ABCA1, UNG, AHRR, CDKN2B, SLK, OLR1, NOD2, PKHD1, LRP8, S1PR3, ACP1, BCOR, PAFAH1B2, IRS1, PAH, GRIP1, DMD, GPLD1, NTRK2, FURIN, ANGPT1, TBX1, PTPN1

All the targets were predicted by a bioinformatics tool MirTarget2 using miRDB online database.

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early preeclampsia (miR-192, miR-143 and miR-125b were overrepresented and miR-127, miR-942, miR-126 and miR-221 were underrepresented in preeclampsia). Consequently, validation by real-time quantitative stem-loop RT-PCR analysis revealed no significant differences between preeclampsia and controls.

On the other hand, Winger et al. were able to predict with great accuracy miscarriage and late preeclampsia during the first trimester of pregnancy via screening of 30 non-placental microRNAs (miR-340-5p, miR-424-5p, miR-33a-5p, miR-7-5p, miR-1229, miR-1267, miR-671-3p, miR-1, miR-133b, miR-144-3p, miR-582-5p, miR-30e-3p, miR-199a-5p, miR-199b-5p, miR-210, miR-221-5p, miR-575, miR-301a-3p, miR-148a-3p, miR-193a-3p, miR-219-5p, miR-132, miR-513a-5p, miR-1244, miR-16, miR-146a, miR-155, miR-181a, miR-196a and miR-223) in maternal peripheral blood mononuclear cells using quantitative RT-PCR [71]. Results for each microRNA were arranged from highest to lowest Ct value, scored using a devised scoring system giving points to each patient sample where the result of microRNA quantification fell within the topmost eight results. Finally, the results of all microRNAs were summed for each patient and individual pregnancy risk score was assessed. Four microRNAs (miR-33a-5p, miR-219-5p, miR-424-5p and miR-513-5p) demonstrated very low readings so were considered technically unsuitable for analysis and excluded from the scoring system.

Similarly, Ura et al. identified 19 differentially expressed mature miRNAs including 12 upregulated (miR-1233, miR-650, miR-520a, miR-215, miR-210, miR-25, miR-518b, miR-193a-3p, miR-32, miR-204, miR-296-5p and miR-152) and 7 dowregulated (miR-126, miR-335, miR-144, miR-204, miR-668, miR-376a and miR-15b) at early stages of gestation in the serum of pregnant women, who later developed severe preeclampsia using microarray analysis and subsequently validated the expression of 4 miRNAs (miR-1233, miR-520a, miR-210 and miR-

144) using quantitative real-time PCR (72). Mir-1233 was the most overexpressed (5.6 fold change), mir-520a showed a 3.5 fold-increase, miR-210 a 3.3 fold-increase and miR-155 a 0.4 fold-decrease in the serum of women who later developed severe preeclampsia.

The results of our previous pilot study strongly supported the need for a more detailed exploration of extracellular placental specific C19MC microRNAs in maternal circulation with the view toward their recognition as potential biomarkers for placental insufficiency related complications (46, 47). Using both the absolute and relative quantification approaches, the ability of extracellular C19MC microRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525 and miR-526a) to differentiate between normal pregnancies and all women destined to develop preeclampsia and/or intrauterine growth restriction in early pregnancy (between the 12th and 16th weeks of gestation) was outlined. This pilot study included 6 pregnant women altogether (1 early preeclampsia, 4 late preeclampsia and 1 early IUGR) [46]. Ura et al. significantly contributed to the confirmation of the results of our pilot study when reported up-regulation of placental specific miR-520a in sera from 12–14 week-gestation in the group of women who later developed severe preeclampsia [72]. Mir-520a (miR-520a-3p) is derived from miR-520a stem-loop together with miR-520a* (miR-520a-5p), which was a subject of interest in our study (http://mirdb.org). Similarly, as Ura et al. we observed in our pilot study upregulation of circulating miR-517* in early pregnancy destined to develop preeclampsia, but this finding was not confirmed in validation experiments performed by Ura et al. [72]. Concerning the selection of microRNAs that were evaluated as early biomarkers of pregnancy-related complications, there is no overlap between our pilot study and studies performed by Luque et al. and Winger et al. [46, 47, 70, 71].

Consecutive large scale studies are needed to assess sensitivity, specificity and positive predictive value of C19MC microRNAs for preeclampsia and/or intrauterine growth restriction. In addition, the diagnostic performance of placental specific microRNAs in relation to the severity of the disease with respect to clinical signs, requirements for the delivery and Doppler ultrasound parameters should be evaluated.

To our knowledge, this is the first longitudinal cohort study in an unselected population reported to date evaluated risk assessment for gestational hypertension, based on maternal plasma concentrations of placental specific C19MC microRNAs in early pregnancy. The study brought interesting finding that the up-regulation of circulating C19MC microRNAs (miR-520h, miR-518b, miR-516-5p, and miR-517*) is a characteristic phenomenon of early pregnancy destined to develop not only placenta-insufficiency related complications, but gestational hypertension as well. In addition, the presence of first trimester higher plasmatic levels of miR-520h, miR-518b and miR-516-5p alone certainly appears to be predictive of subsequent gestational hypertension. Effective screening for onset of gestational hypertension can be achieved in the first-trimester of pregnancy using a single C19MC placental specific microRNA biomarker (miR-520h). 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. Consecutive multi-centre large scale studies involving the patients from all populations are needed to verify that a single plasmatic miR-520h biomarker or a combination of miR-520h and miR-518b biomarkers represent promising tools in the risk assessment for gestational hypertension. The increased levels of extracellular C19MC microRNAs during the first trimester of gestation may be related to down-regulation of some proteins and hormones that have been studied as potential early markers for gestational hypertension, preeclampsia, SGA, preterm delivery, miscarriages, stillbirth or Downs syndrome (e.g. pregnancy-specific glycoproteins (SP1), PAPP-A, LHCGR (Table 4, S1, S2 Tables).

However, none has achieved sufficiently good discrimination to be used alone in a clinical context, although combinations of second trimester biochemical markers and biochemical and ultrasound markers have been proposed. On the other hand, some predicted targets of C19MC microRNAs, that were subject of interest in the current study, were shown previously to be upregulated in first trimester maternal plasma/serum samples derived from patients with pre-eclampsia, SGA, preterm delivery, miscarriage or stillbirth (e.g. soluble Flt-1, Ang-1/Ang-2 ratio) (Table 4, S1, S4 Tables).

Nevertheless, multiple miRNAs can regulate a single gene. Although methods to comprehensively identify miRNAs that regulate individual genes of interest are currently available, pathways involving miRNAs are often complex regulatory networks, whose regulation is difficult to understand and make the direct interpretation of experimental data elaborate. Many genes are targeted for repression by a high number of miRNAs, which seem to regulate those genes cooperatively [73].

In conclusion, microRNAs play a fundamental role in a variety of physiological and pathological processes involving pregnancy-related complications. Current study demonstrated for the first time that circulating C19MC microRNAs might play a role in early pregnancy in the inducement of not only preeclampsia and fetal growth restriction, but gestational hypertension as well.

Supporting Information

S1 Table. Function of target genes of miR-516-5p (miR-516b-5p) in relation to pregnancy.
doi:10.1371/journal.pone.0113735.s001 (DOCX)

S2 Table. Function of target genes of miR-517* (miR-517-5p) in relation to pregnancy.
doi:10.1371/journal.pone.0113735.s002 (DOC)

S3 Table. Function of target genes of miR-518b in relation to pregnancy.
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S4 Table. Function of target genes of miR-520h in relation to pregnancy.
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Author Contributions

Conceived and designed the experiments: IH KK LH JD LK. Performed the experiments: KK LH. Analyzed the data: IH KK JD LK. Contributed reagents/materials/analysis tools: LH JD LK. Contributed to the writing of the manuscript: IH KK LH JD LK.

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4.5 First trimester screening of circulating C19MC microRNAs and the evaluation of their potential to predict the onset of preeclampsia and IUGR

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4.5.1 Overview

The diagnostic potential of specific molecular biomarkers and their implementation in predictive and diagnostic algorithms for pregnancy-related complications are subjects of considerable interest (Hromadnikova, 2012).

Although the study by Luque et al. (2014) demonstrated that maternal serum microRNA assessment at the end of the first trimester of pregnancy did not appear to have any predictive value for early preeclampsia (requiring delivery before 34 weeks of gestation), other studies reported opposite results. Ura et al. (2014) have shown that severe preeclampsia is associated with alterations in extracellular microRNA expression (miR-1233, miR-520, miR-210 and miR-144) during early stages of gestation (12-14 weeks). Winger et al. (2014, 2015) demonstrated that profiling of selected microRNA biomarkers in maternal peripheral blood mononuclear cells, prior the end of the first trimester, may successfully predict adverse outcomes such as preeclampsia and miscarriage.

Our pilot study suggested the potential of using circulating C19MC microRNAs (miR-520h, miR-518b, miR-516b-5p, and miR-517-5p) to differentiate, at the beginning of gestation (10–13 weeks), between patients that will develop gestational hypertension and those that will have normal pregnancies (Hromadnikova et al., 2013). First trimester screening of extracellular miR-520h alone or in combination with miR-518b was able to identify a significant proportion of women that went on to develop gestational hypertension (2014).

4.5.2 Aims and methods

This study is a part of a long-term research program focused on the description of the complex pathogenesis mechanisms involved in pregnancy-

related complications, with the goal of identifying of novel biomarkers that can diagnose and/or predict pregnancy-related complications.

The case cohort in this study included 21 preeclamptic pregnancies, 18 IUGR pregnancies, and the control cohort (58 normal pregnancies), which were chosen based on equal blood sample storage times and gestational age.

Receivers operating characteristic (ROC) curves were constructed to calculate the area under the curve (AUC) and the best cut-off point for particular placental specific microRNA was used in order to calculate the respective sensitivity, specificity, predictive values, and likelihood ratios for prediction of preeclampsia and IUGR.

4.5.3 Results and discussion

The current work is oriented towards novel insights into pathogenesis of preeclampsia and IUGR and potential improvement of diagnostical modalities. In order to unravel the causes of the failures in the maternal-fetal dialogue we focused on early pregnancy.

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 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.

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).

In this study we tested predictive accuracy of C19MC microRNA biomarkers for prediction of later onset of preeclampsia and IUGR on first trimester maternal plasma samples. 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%. 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.

Certainly, consecutive large scale studies are needed to assess sensitivity, specificity, and predictive value of the circulating miR-517-5p as a single biomarker for preeclampsia. In addition, the diagnostic performance of the placental specific miR-517-5p biomarker, in relation to the severity of the disease with respect to clinical signs, requirements for the delivery, and Doppler ultrasound parameters, should be evaluated.

PLOS ONE

RESEARCH ARTICLE

First trimester screening of circulating C19MC microRNAs and the evaluation of their potential to predict the onset of preeclampsia and IUGR

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Abstract

Objectives

A nested case control study of a longitudinal cohort comparing pregnant women enrolled at 10 to 13 gestational weeks was carried out to evaluate risk assessment for preeclampsia and IUGR based on circulating placental specific C19MC microRNAs in early pregnancy.

Methods

The expression of placental specific C19MC microRNAs (miR-516b-5p, miR-517-5p, miR-518b, miR-520a-5p, miR-520h, and miR-525-5p) was determined in plasma samples from pregnancies that subsequently developed preeclampsia (n = 21), IUGR (n = 18), and 58 normal pregnancies using real-time PCR and comparative Ct method relative to synthetic *Caenorhabditis elegans* microRNA (cel-miR-39).

Results

Circulating C19MC microRNAs were up-regulated (miR-517-5p, p = 0.005; miR-518b, p = 0.013; miR-520h, p = 0.021) or showed a trend toward up-regulation in patients destined to develop preeclampsia (miR-520a-5p, p = 0.067; miR-525-5p, p = 0.073). MiR-517-5p had the best 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%. The combination of all examined circulating C19MC microRNAs had no advantage over using only the miR-517-5p biomarker to predict the occurrence of preeclampsia (a sensitivity of 20.6%, a specificity of 90.8%, a PPV of 44.8%, and a NPV of 76.0%).

Conclusions

Up-regulation of miR-517-5p, miR-518b and miR-520h was associated with a risk of later development of preeclampsia. First trimester screening of extracellular miR-517-5p

identified a proportion of women with subsequent preeclampsia. No circulating C19MC microRNA biomarkers were identified that could predict later occurrence of IUGR.

Introduction

MicroRNAs belong to a family of small noncoding RNAs that regulate gene expression at the post-transcriptional level by degrading or blocking translation of messenger RNA (mRNA) targets [1, 2]. The diagnostic potential of specific molecular biomarkers and their implementation in predictive and diagnostic algorithms for pregnancy related complications are subjects of considerable interest [3].

Although the study by Luque et al. [4] demonstrated that maternal serum microRNA assessment at the end of the first trimester of pregnancy did not appear to have any predictive value for early preeclampsia (requiring delivery before 34 weeks), the data from other studies strongly supported the need for a more detailed exploration of microRNAs in maternal circulation with the view toward routine assessment in everyday practice, and recognition that they represent potential biomarkers for pregnancy related complications [5–8].

Our pilot study suggested the potential of using circulating C19MC microRNAs (miR-520h, miR-518b, miR-516b-5p, and miR-517-5p) to differentiate, at the beginning of gestation (weeks 10–13), between patients that will develop gestational hypertension and those that will have normal pregnancies [9]. First trimester screening of extracellular miR-520h alone or in combination with miR-518b was able to identify a significant proportion of women that went on to develop gestational hypertension [9].

Recent studies of Ura et al. [5] have shown that severe preeclampsia is associated with alterations in extracellular microRNA expression (miR-1233, miR-520, miR-210 and miR-144) during the early stages of gestation (12–14 weeks).

Latest preliminary studies by Winger et al. [6, 10] demonstrated that profiling of selected microRNA biomarkers in maternal peripheral blood mononuclear cells, prior the end of the first trimester, may successfully predict adverse outcomes such as preeclampsia and miscarriage. In addition, microRNA quantification of maternal blood cells was also able to predict the occurrence of late preeclampsia.

As far as we know, this is the first study using first trimester screening of women at risk of developing preeclampsia or IUGR relative to circulating C19MC microRNAs (miR-516b-5p, miR-517-5p, miR-518b, miR-520a-5p, miR-520h, and miR-525-5p). Here, we discuss for the first time the effectiveness of circulating C19MC microRNAs as predictors of preeclampsia and IUGR. This study is a part of a long-term research program focused on the description of the complex pathogenesis mechanisms involved in pregnancy-related complications, with the goal of identifying of novel biomarkers that can diagnose and/or predict pregnancy-related complications [8, 9, 11].

Materials and methods

Patients

The study was retrospective, designed to run from 2012–2016. The study cohort consisted of 1464 consecutive Caucasian singleton pregnant women undergoing first trimester screening at 10–13 gestational weeks. Of 1464 pregnant women participating in first trimester screening, 359 were lost to follow-up (they underwent first trimester screening at the Center of Prenatal Diagnosis in our health care facility, but delivered at another health care facility), 21 developed

preeclampsia (1.9%) and 18 pregnancies (1.63%) were complicated by intrauterine growth restriction (IUGR). The clinical characteristics of normal and complicated pregnancies are presented in Table 1 and also provided as a supplementary table in Excel format (S1 Table).

The case cohort included all 21 preeclamptic pregnancies, 18 IUGR pregnancies, and the control cohort (58 normal pregnancies), which were chosen based on equal blood sample storage times and gestational age. Of the 21 patients with preeclampsia, 7 had symptoms of mild preeclampsia and 14 were diagnosed with severe preeclampsia. Six preeclamptic patients required delivery before 34 weeks of gestation and 15 patients delivered after 34 weeks of gestation. Preeclampsia occurred both in previously normotensive patients (18 cases), or was superimposed on pre-existing hypertension (3 cases).

Three growth-retarded fetuses were delivered before 34 weeks of gestation and 15 after 34 weeks of gestation. Oligohydramnios or anhydramnios were present in 2 growth-restricted fetuses. The cerebro-placental ratio (CPR), expressed as a ratio between the middle cerebral artery and the umbilical artery pulsatility indexes was below the fifth percentile in 11 IUGR cases. Absent or reversed end-diastolic velocity waveforms in the umbilical artery occurred in 1 IUGR case.

Preeclampsia was defined as blood pressure $>$ 140/90 mmHg on two determinations 4 hours apart that was associated with proteinuria $>$ 300 mg/24 h after 20 weeks of gestation [12]. Severe preeclampsia was diagnosed by the presence of one or more of the following findings: 1) a systolic blood pressure $>$ 160 mmHg or a diastolic blood pressure $>$ 110 mmHg, 2) proteinuria greater than 5 g of protein in a 24-hour sample, 3) very low urine output (less than 500 ml in 24 h), 4) signs of respiratory problems (pulmonary edema or cyanosis), 5) impaired liver functions, 6) signs of central nervous system problems (severe headache, visual disturbances), 7) pain in the epigastric area or right upper quadrant, 8) thrombocytopenia, and 9) the presence of severe fetal growth restriction [12].

Fetal growth restriction was diagnosed when the estimated fetal weight (EFW), calculated using the Hadlock formula (Astraia Software GmbH), was below the tenth percentile for the evaluated gestational age, adjustments were made for the population standards of the Czech Republic. In addition to fetal weight below the threshold of the 10th percentile, IUGR fetuses had at least one of the following pathological finding: an abnormal pulsatility index in the umbilical artery, absent or reversed end-diastolic velocity waveforms in the umbilical artery, an abnormal pulsatility index in the middle cerebral artery, a sign of a blood flow centralization, and a deficiency of amniotic fluid (anhydramnios and oligohydramnios).

Centralization of the fetal circulation represents a protective reaction of the fetus against hypoxia that manifests itself in redistribution of the circulation to the brain, liver, and heart at the expense of the flow reduction in the periphery [13, 14]. The cerebroplacental ratio (CPR) quantifies redistribution of cardiac output by dividing Doppler indices from representative cerebral and fetoplacental vessels.

Normal pregnancies were defined as those without complications that delivered full term, healthy infants weighting $>$ 2500 g after 37 completed weeks of gestation.

All patients provided written informed consent. The study was approved by the Ethics Committee of the Third Faculty of Medicine, Charles University. Gestational age was assessed using ultrasonography between weeks 10–13 weeks plus 6 days.

Processing of samples

Nine milliliters of peripheral blood were collected into EDTA tubes and centrifuged twice at 1200 g for 10 min at room temperature. Plasma samples were stored at −80˚C until subsequent processing.

Table 1. Maternal and neonatal characteristics of normal and complicated pregnancies.

	Normal pregnancies (n = 58)	Preeclampsia (n = 21)	IUGR (n = 18)	p-value[1]	p-value[2]	p-value[3]
Maternal age (years)	32.71±0.49 (27–42)	34.33±1.13 (27–42)	34.56±1.08 (27–43)	0.145	0.127	0.085
GA at sampling (weeks)	10.86±0.12 (10–13)	11.18±0.31 (10–13.56)	10.58±0.17 (10–13.28)	0.191	0.244	0.250
GA at delivery (weeks)	39.91±0.16 (38–41.56)	35.0±1.05 (21.42–40.7)	36.21±0.62 (28.28–39.0)	**<0.001**	**<0.001**	**<0.001**
Mode of delivery						
● Vaginal	54 (93.1%)	2 (9.5%)	3 (16.7%)	**<0.001**	**<0.001**	**<0.001**
● Cesarean section	4 (6.9%)	19 (90.5%)	15 (83.3%)			
Fetal birth weight (g)	3450.7±66.62 (2930–4340)	2627.5±205.93 (930–3860)	2079.6±127.48 (746–2840)	**<0.001**	**<0.001**	**<0.001**
Fetal sex						
● Boy	30 (51.7%)	12 (57.2%)	7 (38.9%)	0.517	0.669	0.341
● Girl	28 (48.3%)	9 (42.8%)	11 (61.1%)			
Primiparity						
● Yes	28 (48.3%)	14 (66.7%)	12 (66.7%)	0.209	0.147	0.172
● No	30 (51.7%)	7 (33.3%)	6 (33.3%)			

Data are presented as mean±SE (range) for continuous variables and as number (percent) for categorical variables.

Statistically significant results are marked in bold.

p-value[1]: the comparison among three groups.

Continuous variables were compared using ANOVA. Categorical variables were compared using Fisher´s exact test.

p-value[2]: the comparison among preeclampsia and normal pregnancies.

p-value[3]: the comparison among IUGR and normal pregnancies.

Continuous variables were compared using the t-test. Categorical variables were compared using a chi-square test.; GA, gestational age.

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Total RNA was extracted from 1 mL of plasma and 25 mg of normal placental tissue preserved in RNAlater (Ambion, Austin, USA), followed by an enrichment procedure for small RNAs using a mirVana microRNA Isolation kit (Ambion, Austin, USA). Trizol LS reagent was used in plasma samples for total RNA extraction from biological fluids (Invitrogen, Carlsbad, USA) and preceded the small RNAs enrichment procedure. To minimize DNA contamination, we treated the eluted RNA with 5 μL of DNase I (Fermentas International, Ontario, Canada) for 30 min at 37˚C.

Reverse transcriptase reaction

Each microRNA was reverse transcribed into complementary DNA using TaqMan MicroRNA Assay, containing microRNA-specific stem-loop RT primers, and a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Branchburg, USA) in a total reaction volume of 50 μL on a 7500 Real-Time PCR system (Applied Biosystems, Branchburg, USA) with the following thermal cycling parameters: 30 minutes at 16˚C, 30 minutes at 42˚C, 5 minutes at 85˚C, and then held at 4˚C.

Quantification of microRNAs

15 μL of cDNA corresponding to each microRNA was mixed with components of TaqMan MicroRNA Assay, and the ingredients of a TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, USA) in a total reaction volume of 35 μL. TaqMan PCR conditions were set as described in the TaqMan guidelines. The analysis was performed using a 7500 Real-Time PCR System. All PCRs were performed in duplicates. A sample was considered positive

Table 2. Characteristics of selected C19MC microRNAs.

Assay name	miRBase ID	NCBI Location Chromosome	microRNA sequence	Expression in placenta
hsa-miR-516-5p	hsa-miR-516b-5p	Chr.19: 58920508–58920592 [+]	5´-CAUCUGGAGGUAAGAAGCACUUU-3´	exclusively expressed
hsa-miR-517*	hsa-miR-517-5p	Chr.19: 54215522–54215608 [+]	5´-CCUCUAGAUGGAAGCACUGUCU-3´	high expression
hsa-miR-518b	hsa-miR-518b	Chr.19: 54205991–54206073 [+]	5´-CAAAGCGCUCCCCUUUAGAGGU-3´	exclusively expressed
hsa-miR-520a*	hsa-miR-520a-5p	Chr.19: 54194135–54194219 [+]	5´-CUCCAGAGGGAAGUACUUUCU-3´	high expression
hsa-miR-520h	hsa-miR-520h	Chr.19: 54245766–54245853 [+]	5´-ACAAAGUGCUUCCCUUUAGAGU-3´	exclusively expressed
hsa-miR-525	hsa-miR-525-5p	Chr.19: 54200787–54200871 [+]	5´-CUCCAGAGGGAUGCACUUUCU-3´	exclusively expressed

C19MC microRNAs were divided into two categories (microRNAs exclusively expressed in the placental tissue and those with high expression in the placental tissue) based on information in miRNAMap 2.0 database (http://mirnamap.mbc.nctu.edu.tw/index.php), where the Q-PCR experiments for monitoring the expression profiles of 224 human miRNAs in eighteen major normal tissues in humans are provided. For example, we indicated miR-516b-5p and miR-518b as those to be exclusively expressed in the placental tissue, since according to the miRNAMap 2.0 database miR-516b-5p was shown to be expressed only in the placental tissue and miR-518b to be highly expressed in the placental tissue and rarely expressed in testes. On the other hand, for instance miR-520a-5p showed besides high expression in the placental tissue also low expression in other human tissues involving adipose, bladder, brain, cervix, heart, kidney, liver, lung, muscle, ovary, prostate, small intestine, spleen, testes, thymus, thyroid and trachea.

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if the amplification signal occurred before the 40th threshold cycle. The characteristics of studied C19MC microRNAs are outlined in Table 2.

The expression of particular microRNA in maternal plasma was determined using the comparative Ct method [15] relative to the expression of the same microRNA in a reference sample. A RNA fraction, highly enriched for small RNA, isolated from the fetal part of one randomly selected placenta derived from gestation with normal course (the part of the placenta derived from the chorionic sac that encloses the embryo, consisting of the chorionic plate and villi) was used as a reference sample for relative quantification throughout the study.

Synthetic *C. elegans* microRNA (cel-miR-39, Qiagen, Hilden, Germany) was used as an internal control for variations during the preparation of RNA, cDNA synthesis, and real-time PCR. Due to a lack of generally accepted standards, all experimental real-time qRT-PCR data were normalized to cel-miR-39, since it shows no sequence homology to any human microRNA. 1 μl of 0.1 nM cel-miR-39 was spiked in after incubation with Trizol LS reagent to human plasma and reference samples. The following equation was used to compare gene expression between various samples:

$$2^{-\triangle\triangle Ct} = [(Ct\ particular\ C19MC\ microRNA - Ct\ cel-miR-39)tested\ sample - (Ct\ particular\ \ C19MC\ microRNA - Ct\ cel-miR-39)reference\ sample]$$

Statistical analysis

Data normality was assessed using the Shapiro-Wilk test, which showed that our clinical data (maternal age) followed a normal distribution. Therefore, microRNA levels were compared between groups using the parametric test (*t*-test) with Statistica software (version 9.0; StatSoft, Inc., USA). Since the Bonferroni correction was used to address the problem of multiple comparisons, the significance level was established at $p < 0.025$.

Receivers operating characteristic (ROC) curves were constructed to calculate the area under the curve (AUC) and the best cut-off point for particular placental specific microRNA was used in order to calculate the respective sensitivity, specificity, predictive values, and likelihood ratios for prediction of preeclampsia.

Data analysis was performed, and box plots were generated using Statistica software (version 9.0; StatSoft, Inc., USA). Each box encompasses the mean (dark horizontal line) of

normalized gene expression values for microRNAs of interest in cohorts, one standard error above and below the mean in the box, and the 95% confidence interval are shown as bars (standard deviation). Outliers are indicated by circles, and extremes are indicated by asterisk.

Results

Up-regulation of circulating C19MC microRNAs in pregnancies that developed preeclampsia

Overall, increased levels of **miR-517-5p** (mean 17.770 ± 6.107 vs. 5.713 ± 1.271, p = 0.005), **miR-518b** (mean 5.872 ± 3.024 vs. 1.204 ± 0.217, p = 0.013), and **miR-520h** (mean 4.826 ± 3.050 vs. 0.542 ± 0.088, p = 0.021) were observed during the first trimester of gestation in maternal plasma samples derived from the women who developed preeclampsia compared to women with normal pregnancies. Simultaneously, a trend towards increased plasma levels of **miR-520a-5p** (mean 5.227 ± 1.984 vs. 2.628 ± 0.452, p = 0.067), **miR-525-5p** (mean 27.365 ± 13.186 vs. 12.136 ± 1.786, p = 0.073), and **miR-516b-5p** (mean 1.431 ± 0.580 vs. 0.678 ± 0.207, p = 0.127) in patients destined to develop preeclampsia was identified (Fig 1).

First trimester screening of circulating C19MC microRNAs does not differentiate between pregnancies with later onset of IUGR and pregnancies with normal course of gestation

No difference in plasma levels of **miR-516b-5p** (mean 0.678 ± 0.207 vs. 0.515 ± 0.205, p = 0.694), **miR-517-5p** (mean 5.713 ± 1.271 vs. 5.674 ± 0.948, p = 0.932), **miR-518b** (mean 1.204 ± 0.217 vs. 0.975 ± 0.393, p = 0.606), **miR-520a-5p** (mean 2.628 ± 0.452 vs. 2.262 ± 1.116, p = 0.809), **miR-520h** (mean 0.542 ± 0.088 vs. 0.317 ± 0.125, p = 0.242), and **miR-525-5p** (mean 12.136 ± 1.786 vs. 7.746 ± 2.872, p = 0.210) between the control group and the group of patients destined to develop IUGR was found.

First trimester screening of circulating C19MC microRNAs in the identification of preeclampsia pregnancies

First, the predictive accuracy of single first trimester plasma microRNA biomarkers for preeclampsia was assessed. The largest area under the curve (AUC) was observed for **miR-517-5p** (0.700, p = 0.045). Using **miR-516-5p** (0.608, p = 0.146), **miR-518b** (0.550, p = 0.507), **miR-520a-5p** (0.495, p = 0.951), **miR-520h** (0.451, p = 0.538), and **miR-525-5p** (0.475, p = 0.755) prediction rules for preeclampsia had smaller areas under the curve and the predictive performance was not significant (Fig 2, Table 3). MiR-517-5p predicted preeclampsia with a sensitivity of 42.9%, a specificity of 86.2%, a PPV of 52.9%, and a NPV of 80.6%. First trimester screening based on the combination of all 6 tested circulating placental specific microRNAs (miR-516b-5p, miR-517-5p, miR-518b, miR-520a-5p, miR-520h, and miR-525-5p) was able to identify women at risk of developing preeclampsia with a sensitivity of 20.6%, a specificity of 90.8%, a PPV of 44.8%, and a NPV of 76.0%. Table 3 displays the predictive accuracy of maternal plasma concentrations of placental specific microRNAs in early pregnancy in the identification of preeclampsia using cut-offs derived from the ROC curves.

Discussion

The current work is oriented towards novel insights into pathogenesis of preeclampsia and IUGR and potential improvement of diagnostical modalities. In order to unravel the causes of the failures in the maternal-fetal dialogue we focused on early pregnancy. To our knowledge, this is the first nested case control study from a longitudinal cohort reported to evaluate 1st

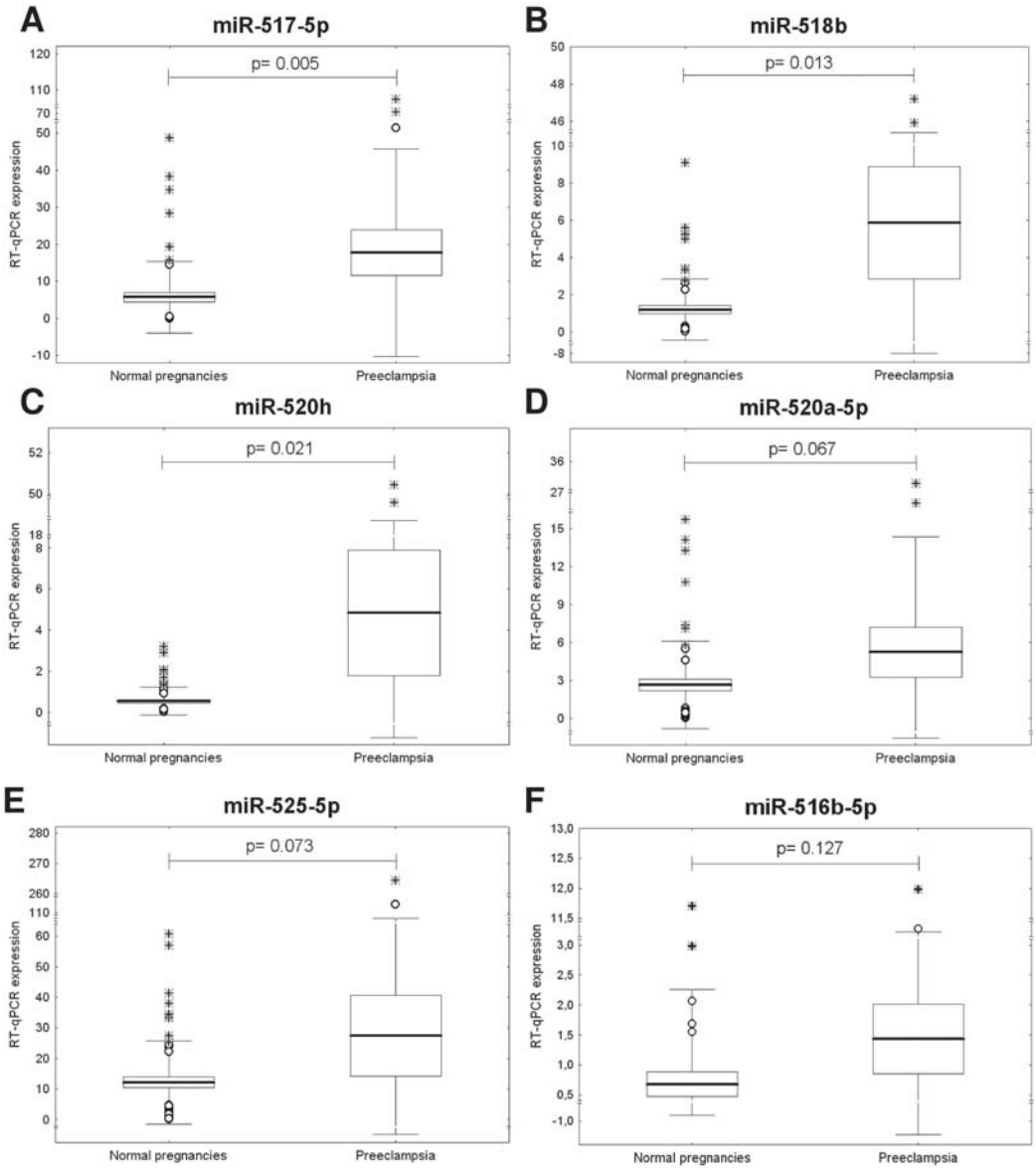

Fig 1. Up-regulation of circulating C19MC microRNAs in pregnancies destined to developed preeclampsia.

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trimester maternal plasma concentrations of placental specific C19MC microRNAs present in pregnancies that went on to develop preeclampsia and IUGR. We were specifically interested in how the evolutionary conflict in parent-offspring relations in the placental bed is manifested in maternal circulation during early gestation. Genetic factors account for more than half of the incidence of preeclampsia, with maternal genes contributing more than fetal genes; couple effects can also occur because of the interaction between genes of the mother and the father [16, 17]. The C19MC cluster is imprinted, and exclusively expressed in the placenta from the paternally inherited allele [18]. C19MC microRNAs are expressed predominantly in placental trophoblasts during pregnancy, although they have also been detected in the testis, embryonic stem cells, and specific tumors [19–27]. The expression level of the C19MC cluster markedly

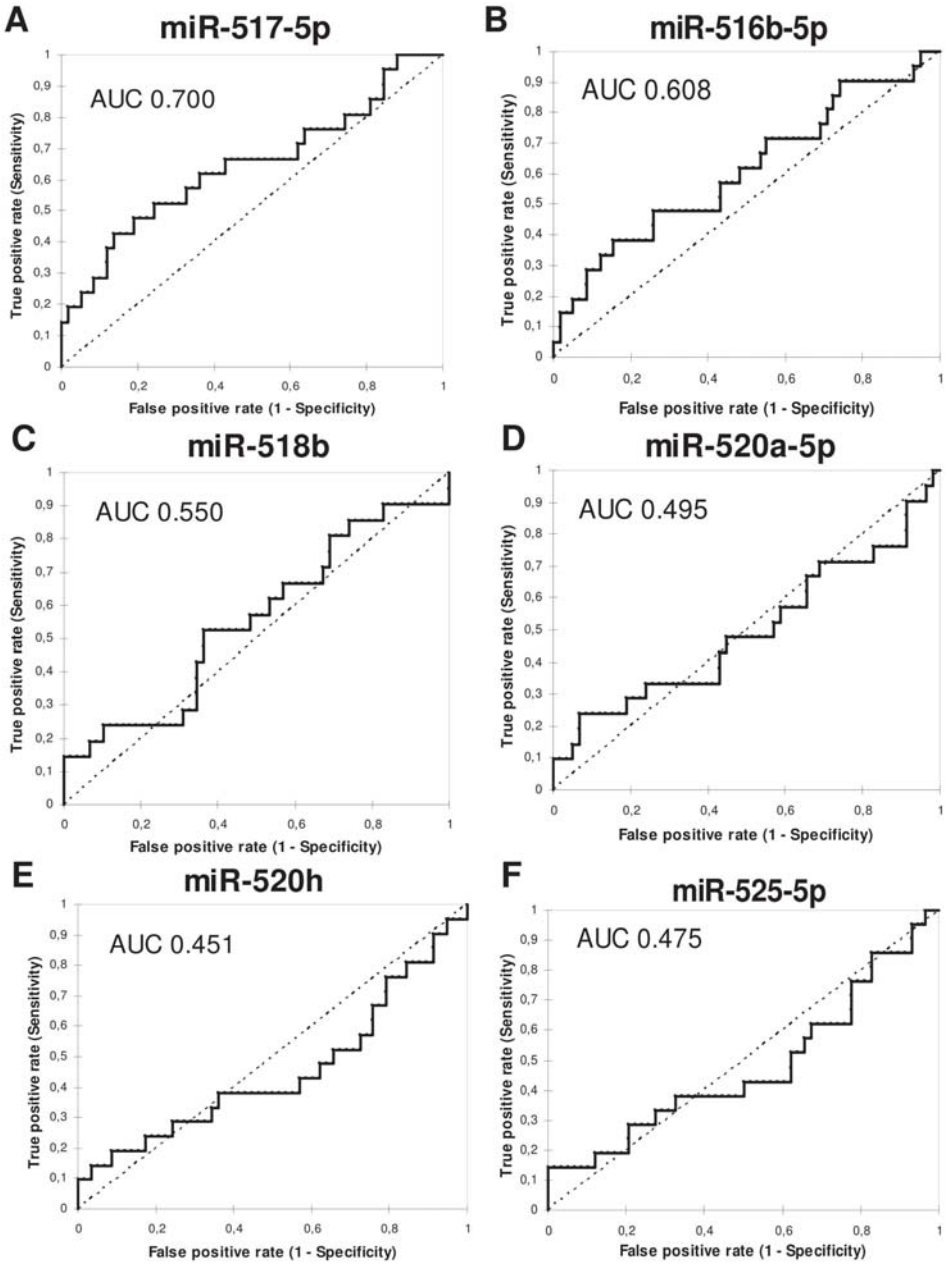

Fig 2. Receiver operating characteristic curves for prediction of development of preeclampsia.

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increases in placental trophoblasts [28, 29] and maternal plasma from the first to the third trimester [30–34]. Our previous study demonstrated that upregulation of circulating C19MC microRNAs (miR-516b-5p, miR-517-5p, miR-520a-5p, miR-525-5p, and miR-526a) was associated with clinically established preeclampsia [8]. Furthermore, the dependence between the levels of plasma C19MC microRNAs and the pulsatility index in the middle cerebral artery (miR-516b-5p, miR-517-5p, miR-520a-5p, miR-525-5p, and miR-526a) and the values of the cerebroplacental ratio (miR-520a-5p, and miR-526a) was demonstrated in a cohort of pregnancies complicated with preeclampsia and/or fetal growth restriction [8]. The current study

Table 3. Predictive accuracy of circulating C19MC microRNA biomarkers for preeclampsia.

miRNA	AUC (95% CI)	ROC curve p-value	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	PLR	NLR	TP	TN	FP	FN
miR-517-5p	0.700 (0.497–0.792)	0.045	42.9% (24.5–63.5)	86.2% (74.7–93.0)	52.9%	80.6%	3.107	0.663	9	50	8	12
miR-516b-5p	0.608 (0.462–0.775)	0.146	38.1% (20.8–59.2)	84.5% (72.7–91.8)	47.1%	79.0%	2.455	0.733	8	49	9	13
miR-518b	0.550 (0.402–0.698)	0.507	52.4% (32.4–71.6)	63.8% (50.9–74.9)	34.4%	78.7%	1.447	0.746	11	37	21	10
miR-520a-5p	0.495 (0.337–0.653)	0.951	23.8% (10.4–45.6)	93.1% (83.0–97.7)	55.6%	77.1%	3.452	0.818	5	54	4	16
miR-520h	0.451 (0.294–0.607)	0.538	14.3% (4.3–35.7)	96.6% (87.4–99.7)	60%	75.7%	4.143	0.888	3	56	2	18
miR-525-5p	0.475 (0.321–0.630)	0.755	14.3% (4.3–35.7)	100% (92.4–100.0)	100%	76.3%	ND	0.857	3	58	0	18
6 C19MC microRNAs	0.545 (0.484–0.606)	0.144	20.6% (14.5–28.6)	90.8% (87.3–93.4)	44.8%	76.0%	2.244	0.874	26	316	32	100

PPV; positive predictive value, NPV; negative predictive value, PLR; positive likelihood ratio, NLR; negative likelihood ratio, TP; true positive, TN; true negative, FP; false positive, FN; false negative

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produced an interesting finding, i.e., up-regulation of circulating C19MC microRNAs (miR-517-5p, miR-518b, and miR-520h) is present in early pregnancy in those women destined to develop preeclampsia; while the other examined circulating C19MC microRNAs (miR-516b-5p, miR-520a-5p, and miR-525-5p) showed a trend toward up-regulation at 10 to 13 weeks of gestation in patients at risk of preeclampsia. Interestingly, up-regulation of miR-516b-5p, miR-517-5p, miR-520h, and miR-518b was also recently reported to be associated with a later occurrence of gestational hypertension [9].

In addition, the presence of higher first trimester plasma levels of miR-517-5p appears to be predictive of preeclampsia. 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%. 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, first trimester screening of women for C19MC microRNA biomarkers had no clinical utility relative to development of IUGR.

Individual maternal plasma/serum markers have not usually performed well as screening tests for preeclampsia and fetal growth restriction [35–45]. 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 [46–48]. Usually biochemical and biophysical tests are combined to assess placentation and maternal disease susceptibility [49]. 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% [46, 47]. 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 [48].

The addition of more efficient biomarkers for first trimester screening would certainly increase the predictive value of the diagnostic panel for preeclampsia.

Early prediction of severe pre-eclampsia would allow closer surveillance and earlier intervention to improve outcomes. It is likely that prospective treatments would need to start as early as possible, ideally before the 16th week of gestation, in order to alter the pathogenesis [49].

Nevertheless, consecutive large scale studies are needed to assess sensitivity, specificity, and predictive value of the circulating miR-517-5p biomarker for preeclampsia. In addition, the diagnostic performance of the placental specific miR-517-5p biomarker, in relation to the severity of the disease with respect to clinical signs, requirements for the delivery, and Doppler ultrasound parameters, should be evaluated.

While the full repertoire of the biological action of C19MC microRNAs remains to be established, data from various expression studies of C19MC microRNAs imply a role for them in cell proliferation, self-renewal, angiogenesis, and particularly in pro-/anti-cancer activity [26, 28, 50]. In fact, there is not much research data about the function of miR-517-5p, miR-518b, and miR-520h in the literature. However, it is likely that similar mechanisms as those present in cancer development and tumor progression may also be in place during human placentation, which starts just after the implantation of the blastocyst into the epithelium of the uterus, and during vasculogenesis of the placental villi, which begins about the 5th week of gestation. Since placental blood vessel formation happens in a relatively hypoxic environment until 10–12 weeks of gestation, there is a certain parallel with tumor biology. Hypoxia, which is a pivotal factor in tumor pathophysiology and a characteristic feature of locally advanced solid tumors, can promote tumor progression, since it is associated with restrained proliferation, differentiation, necrosis, and/or apoptosis [51].

It has been proposed that miR-518b may function as a tumor suppressor by targeting Rap1b [52], since Rap1b expression is negatively regulated by miR-518b. Rap1b is an isoform of Rap1, a small GTPase regulating adhesion, migration, polarity, differentiation, growth, and angiogenesis [53, 54]. Mir-518b has been shown to suppress cell proliferation by inducing apoptosis in tumor cells and invasion by targeting Rap1b [52]. Similarly, mir-520h targets ABCG2, which is highly expressed in several tumors, and alters cellular epigenetic programming to promote cell survival. Functional studies have indicated that loss of miR-520h expression is accompanied by subsequent activation of ABCG2 expression, which represent critical events in the invasion and migration of human pancreatic cancer cells [55]. Moreover, it has been shown that miR-520h functions as a potent suppressor of migration and invasion of human pancreatic cancer cells through down-regulation of ABCG2 expression [55]. MiR-520h is also crucial for DAPK2 (Death-associated protein kinase 2) regulation in breast cancer progression [56]. Mir-520h induced suppression of DAPK2 is associated with a poorer prognosis and lymph node metastasis in breast cancer patients [56].

Furthermore, Rg-3-induced overexpression of miR-520h results in the reduction of EphB2 and EphB4 and in subsequent angiosuppression [50]. Ephrins (Eph) mediate the critical steps of angiogenesis and vascular-network formation, including endothelial cell-to-endothelial/mesenchymal-cell interactions, cell adhesion to the extracellular matrix, cell proliferation, and migration [57]. Eph/ephrin signaling mechanisms may also correlate with VEGF-induced angiogenesis and VEGFR function in developmental and tumor angiogenesis [50, 58, 59]. Mir-520h induced down-regulation of EphB2 and EphB4 in endothelial cells could lead to inhibition of VEGFR-2 expression and angiosuppression [50]. In addition, silencing of CXCR4, a rhodopsin-like G-protein-coupled receptor that selectively binds CXCL12 chemokine, by miR-520h has been shown to successfully block invasion and metastasis of cancer cells [60]. The binding of CXCL12 to CXCR4 activates various signaling pathways such as calcium

influx, phosphoinositide 3 (PI3) kinase, mitogen-activated protein (MAP) kinase, Src kinase and Rho [61]. Additionally, altered CXCR4 expression results in tumor growth, angiogenesis, invasion, and metastasis [62, 63].

Unfortunately, functional roles for miR-517-5p have not yet been demonstrated. Available prediction algorithms usually predict hundreds of potential target genes for a single microRNA, but often generate false-positive candidates [64]. We reported a list of predicted target genes of differently expressed C19MC microRNAs in pregnancy-related complications, in relation to immune system and the inflammatory response, in our previous study dedicated to the expression profile of C19MC microRNAs in placental tissues [3].

Different C19MC microRNA expression profiles in different cell types within villous tissue and in different areas of placental tissues were documented. The expression of C19MC microRNAs has been observed at least in first-trimester and full-term placental tissues [29, 65], human first and third trimester trophoblast cell lines, ACH-3P and AC1-M59 [66], and placenta-derived stromal cells [67]. In our initial study, we have observed the presence of all 16 tested C19MC microRNAs (miR-512-5p, miR-515-5p, miR-224, miR-516-5p, miR-517*, miR-136, miR-518f*, miR-519a, miR-519d, miR-519e, miR-520a*, miR-520h, miR-524-5p, miR-525, miR-526a and miR-526b) on the fetal side of the placenta [31]. In addition, the set of microRNAs (miR-517c, miR-518a, miR-519d, and miR-520h) forming a cluster on chromosome 19q13 was observed to be expressed in umbilical cord blood CD34+ cells [68]. Gu et al. [69] previously showed that the optimal solution was to test the microRNA expression profile in whole villous tissue containing cytotrophoblasts, syncytiotrophoblasts, mesenchymal/stromal cells, villous core fetal vessel endothelium, etc. The microRNA expression profile in whole villous tissue closely resembles microRNA expression without disruption of tissue integrity in an in vivo situation [69]. Therefore, we previously analyzed C19MC microRNA gene expression in whole villous tissue, but in the specific area of the central cotyledon zone, where the umbilical cord inserts into the chorionic plate. Although, we did not specifically examine the localization of C19MC microRNAs within villous tissue, our data suggested that pregnancy-related complications were associated with alterations in placental microRNA expression [3]. The retrospective study design enabled us to test diverse biological material of equal patients (i.e., placental tissues, maternal plasma samples collected at 10 to 13 weeks of gestation, and during the onset of pregnancy-related complications). Nevertheless, in contrast to maternal circulation, down-regulation of C19MC microRNAs was found in placental tissues derived from patients with (1) gestational hypertension (miR-517-5p, miR-519d, miR-520a-5p and miR-525), (2) fetal growth restriction (miR-517-5p, miR-518f-5p, miR-519a, miR-519d, miR-520a-5p and miR-525), and (3) clinically established preeclampsia (miR-515-5p, miR-517-5p, miR-518b, miR-518f-5p, miR-519a, miR-519d, miR-520a-5p, miR-520h, miR-524-5p, miR-525 and miR-526a) [3]. Other independent studies have also observed decreased expression at least of some C19MC microRNAs in preeclamptic placentas (miR-518b and miR-525, [70]) or FGR (miR-515-5p, miR-518b, miR-519d, miR-520h, and miR-526b, [71]). Although, C19MC microRNAs were found to be down-regulated around the central cotyledon in patients with clinically established preeclampsia [3], they can be up-regulated in other areas of placenta tissues, as has been shown in several independent studies. For example, Xu et al. [72] observed up-regulated expression of miR-518b in basal plates of severe preeclamptic placentas and Ishibashi et al. [73] revealed up-regulation of miR-525, miR-518f-5p, miR-526b and miR-519e-5p in preeclamptic placentas, but no details regarding the sampling location of placental tissue was provided in the study. We believe that variable levels of circulating C19MC microRNAs in patients affected with pregnancy-related complications such as gestational hypertension, preeclampsia, and FGR can be influenced by compilations stemming from several factors. At the very least, an expression of particular circulating C19MC microRNA is represented by the

total sum of expression of this particular C19MC microRNA in individual cells located in different areas of placenta, which actively secrete exosomes mediating intercellular communication, currently undergo apoptosis, or release placental debris into the maternal circulation. It has been clearly demonstrated that the establishment of a balance between trophoblast proliferation and apoptosis is crucial during normal placental development [74]. Both aging syncytiotrophoblasts and extravillous cytotrophoblasts undergo apoptosis [75–77]. Therefore, extracellular nucleic acids (DNA, mRNA, and microRNAs) of both fetal and placental origin, packed into trophoblast-derived apoptotic bodies, can be detected in the maternal circulation during the normal course of gestation [78]. In addition, human chorionic villi can secrete microRNAs extracellularly via exosomes, which can enter the maternal circulation [30]. Several recent studies have showed increasing levels of circulating nucleic acids (fetal DNA, placental specific mRNA transcripts, and C19MC microRNAs) with advancing gestation, which reflects the growth of the placenta [7, 31, 79–82]. Both absolute and relative quantification approaches have revealed significant increases, over time, in extracellular placental specific C19MC microRNA levels (miR-516-5p, miR-517-5p, miR-518b, miR-520a-5p, miR-520h, miR-525 and miR-526a) in women with normally progressing pregnancies [7]. The highest concentrations and expression of circulating C19MC microRNAs have been observed during the third trimester (36^{th} week of gestation), whereas differences have been found between the first (12^{th} week of gestation) and the second (25^{th} week of gestation) trimesters only in 4 out of 7 tested placenta-specific microRNAs (miR-516-5p, miR-517-5p, miR-518b, and miR-520h) [7].

Chronic placental hypoxia is one of the root causes of placental insufficiencies that result in preeclampsia and maternal hypertension [83]. One of the most probable hypothesis to describe the etiology of preeclampsia is based on a failure of extravillous trophoblasts to invade the uterine spiral arteries in the placental bed. This results in placental hypoxia and subsequent damage to villous trophoblasts [83]. Hypoxic environment induces excessive trophoblast cell death and increased shedding of placenta debris into the maternal circulation. Increased apoptosis has been observed in extravillous trophoblasts of placentas; although, mainly in pregnancies complicated by preeclampsia [84]. As a result, placental insufficiency related pregnancy complications (preeclampsia) have also been associated with abnormal levels of extracellular fetal DNA, mRNA transcripts, and circulating C19MC microRNAs (miR-516b-5p, miR-517-5p, miR-520a-5p, miR-525-5p, and miR-526a) [8, 77, 85].

Similarly, increased extravillous trophoblast sensitivity to apoptotic signals in the first trimester of gestation, due to reduced NO synthesis in pregnancies at higher risk of developing preeclampsia, can cause higher levels of circulating C19MC microRNAs. Whitley et al. [86] showed that first trimester extravillous trophoblasts from pregnancies with high uterine artery resistance were inherently more sensitive to apoptotic stimuli, which can be associated with reduced remodeling of the maternal spiral arteries.

Fetal growth restriction is a complex disease, resulting from an array of diverse etiologies, which is characterized by a failure of the fetus to reach its growth potential. Recent studies have demonstrated that plasma levels of the majority studied microRNAs were not significantly different in women with FGR [87], despite the fact that their expression has been shown to be altered by hypoxia in trophoblasts under *in vitro* conditions [88]. Analogous to our previous and current study [8, 71] also observed reduced expression of certain C19MC microRNAs in placentas of FGR patients, but circulating levels of these substances in maternal plasma showed no significant differences between FGR and uncomplicated pregnancies. The most likely explanation is a hypothesis presented by Huppertz et al. [89] suggesting that during intra-uterine growth restriction, placental oxygenation may be increased rather than decreased, and therefore at least some IUGR patients have no signs of trophoblast injury and

consequential placental dysfunction, which can lead to increased data variability for IUGR pregnancies [88].

In conclusion, C19MC microRNAs play a role in the pathogenesis of pregnancy-related complications. Our current and previous studies demonstrated for the first time that circulating C19MC microRNAs are dysregulated in maternal circulation early in the pregnancy and might play a role in the inducement of gestational hypertension and preeclampsia.

Supporting information

S1 Table. S1 Table.
(XLS)

Author Contributions

Conceptualization: IH LK.

Data curation: KK.

Formal analysis: IH KK.

Funding acquisition: IH.

Investigation: KK KI.

Methodology: KK IH.

Project administration: IH.

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Supervision: IH LK.

Visualization: KK.

Writing – original draft: IH KK.

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5. DISCUSSION

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.

The research started with the selection of potential candidate microRNAs through the search of accessible databases and literature and continued by subsequent identification of extracellular placental specific microRNAs in maternal circulation, whose plasma levels and expression profile significantly differed between pregnant women and non-pregnant healthy individuals.

Initially, we selected 20 microRNAs to be tested (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) that have been previously reported to be highly or exclusively expressed in placenta with no or minimal expression in other human tissues (Liang et al., 2007).

The study was based on the original idea that extracellular nucleic acids (DNA and mRNA) of both, placental and fetal origin are present in maternal circulation in the form of trophoblast-derived apoptotic bodies (Ishihara et al., 2002) (Ng et al., 2003; Chim et al., 2005) during the normal course of gestation and could be used as a non-invasive source of genetic information for prenatal diagnosis (Lo et al., 1997; Costa et al., 2002; Faas et al., 1998; Honda et al., 2002; Hromadnikova et al., 2005a, 2005b; Lo et al., 1998; Rijnders et al., 2001). After discovery of short non-coding RNAs, and mainly microRNAs that significantly affect gene expression, several questions have arised 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 selected microRNAs, 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 of microRNAs with potential use in noninvasive prenatal diagnostics, 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. Our findings were supported by concurrently running study by Miura et al. (2010) which identified 24 pregnancy associated microRNAs that showed significantly increased levels in maternal plasma through gestation and significant decrease after pregnancy termination. Within these 24 extracellular microRNAs, five extracellular C19MC microRNAs (miR-515-3p, miR-517a, miR-517c, miR-518b and miR-526b) have also been identified. A comparison between these two studies revealed that we identified at least other five extracellular pregnancy associated C19MC microRNAs previously unreported (miR-516-5p, miR-517*, miR-520a*, miR-520h, and miR-525).

Since 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), we examined if placenta specific C19MC microRNAs would have been conducting similarly. 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, whereas differences were found only in 3 out of 7 tested placenta specific C19MC microRNAs (miR-516-5p, miR-517*, and miR-518b) between the first and the second trimesters 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 initially 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 have also retrospectively tested plasma samples from 7 pregnant women, who later developed various placental insufficiency-related complications. We surprisingly revealed that levels of examined placental specific C19MC microRNAs exceeded, within 12^{th} to 16^{th} weeks of gestation, in patients who later develop PIRCs, the explicitly defined cut-offs (mean plus two standard deviations) of the control cohort at the appropriate gestational age. Thereafter, the levels of extracellular microRNAs did not differ from the controls. 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., 1986).

Normal placentation comprises trophoblast cell invasion of the spiral arteries, beginning in 8 week and completed by 20 week of gestation, resulting in reversible changes of arterial wall architecture (Khong et al., 1986; Brosens et al., 1967; Pijnenborg et al., 1980; De Wolf et al., 1980; Bujold et al., 2010). Our results suggested that placental injury and/or hypoxia attenuated microRNA biogenesis and increased both exosome-dependent or independent release of microRNAs to maternal circulation within 11 and 16 weeks of gestation. Unfortunately, no data evaluating extracellular placental specific C19MC microRNA levels in the first trimester plasma in women at risk of subsequently developing preeclampsia and/or IUGR are available till now.

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 develop 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. Although an extensive study performed by Luque et al. (2014) using high-throughput OpenArray system for 754 microRNAs revealed differential profile of 7 microRNAs in early preeclampsia (up-regulated: miR-192, miR-143, and miR-125b; down-regulated: miR-127, miR-942, miR-126, and miR-221), consequent validation of results by real-time quantitative stem-loop RT-PCR analysis revealed no significant differences between preeclampsia and controls. On the other hand, Winger et al. (2014) were able to predict miscarriage and late preeclampsia during the first trimester of pregnancy via screening of 30 non-placental microRNAs in maternal peripheral blood mononuclear cells with great accuracy. Similarly, Ura et al. identified 19 differentially expressed mature miRNAs at early stages of gestation in sera of pregnant women, who later developed severe preeclampsia using microarray analysis. Subsequent validation analysis confirmed up-regulation of miR-1233, miR-520a, and miR-210 in sera of women who later developed severe preeclampsia. This partially correlated with our results since placental specific C19MC microRNA, miR-520a, reported to be up-regulated in sera derived from 12 - 14 weeks of gestation in the group of women who later developed severe preeclampsia, is derived from miR-520a stem-loop together with miR-520a* (miR-520a-5p), which was a subject of

interest in our study. Similarly, up-regulation of circulating miR-517* in early pregnancy destined to develop preeclampsia initially observed by Ura et al. supported our previous finding, but miR-517* was not finally confirmed to be upregulated, when validation experiments had been performed (Ura et al., 2014). Akehurst et al. (2015) assessed microRNA expression in maternal plasma samples obtained at 16th and 28th weeks of gestation, however only serum miR-23a* levels differentiated in early gestation (16th week) between women who developed preeclampsia and women with normotensive pregnancies. Recently, Zhang et al. (2017) investigated circulating miR-942 levels in early phase of mid-pregnancy (12-20 weeks of gestation) and revealed significantly lower levels of miR-942 in women who later developed preeclampsia.

Although a large number of different biochemical maternal plasma/serum biomarkers have been found to date for screening of preeclampsia and IUGR, the predictive ability of individual biomarkers has been low and therefore different combinations of several markers are currently used in clinical practice (Kusanovic et al., 2009; Zhao et al., 2012; Pihl et al., 2009; Asvold et al., 2014; Karahasanovic et al., 2014; Chambers et al., 2012; Spencer et al., 2008; Neale and Mor, 2005; Whitley et al., 2007; Sziller et al., 2005; Robinson et al., 2009; Ciarmela et al., 2010; Tal, 2012; Sezer et al., 2013; Taylor et al., 1990; Nishikawa et al., 2000; Poon et al., 2014). Screening approach combining maternal risk factors, mean arterial pressure, Doppler uterine artery, 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; Scazzocchio et al., 2016). Screening model for fetal growth restriction investigating maternal characteristics, mean arterial pressure, Doppler uterine artery, placental growth factor, and soluble Fms-like tyrosine kinase-1 achieved an overall detection rate of 71.4% with a 10% false positive rate (Croveto et al., 2016).

6. CONCLUSION

Early diagnosis of severe pre-eclampsia would allow early interventions. Increased plasmatic levels and gene expression of 5 extracellular C19MC microRNAs (miR-516-5p, miR-517*, miR-520a*, miR-525, and miR-526a) were observed in circulation of women with clinically established preeclampsia, and could be used as non-invasive biomarkers to monitor the development of preeclampsia after 20 weeks of gestation.

Early identification of patients at risk of later development of preeclampsia and FGR would allow early interventions. Usually the usage of low-dose aspirin (dose 80-100 mg/day) is indicated, ideally before the 16th week of gestation. It is believed that acetylsalicylic acid improves the course of endangered pregnancies and prevents the development of the most severe symptoms of the disease (Leslie et al., 2011). 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%. In addition, 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%.

It is evident that the addition of other specific biomarkers into the first trimester screening portfolio could improve the predictive value for the later occurrence of severe pregnancy-related complications. Our study significantly contributed to this area, since the involvement of extracellular C19MC microRNAs into current screening approaches has not yet been described. 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.

Mainly, it is necessary to assess sensitivity, specificity, and predictive value of circulating C19MC microRNA biomarkers to predict gestational hypertension and preeclampsia. The relation between circulating C19MC microRNAs and the

severity of the disease (preeclampsia) with respect to clinical signs, requirements for the delivery, and Doppler ultrasound parameters, should be also evaluated.

7. SUPPLEMENTARY MATERIAL

Supplement Table 1. Aberrant expression of microRNAs in pregnancies complicated with PE and/or FGR (IUGR/SGA)

Pregnancy-related complication	Number of cases in studied cohorts	Detection method	microRNA expression	References
PE	35 together: 30 PE, 15 NP	RT-qPCR	↑ miR-210	Zhang Y et al., 2012
PE/PE+SGA	36 together: 9 PE, 9 SGA, 9 PE+SGA, 9 NP	RT-qPCR	↑ miR-210, miR-182 (PE) ↑ miR-210, miR-182, miR-154*, miR-155, miR-181b, miR-182, miR-182*, miR-183, miR-200b (PE+SGA)	Pineles et al., 2007
PE	12 together: 6 PE, 6NP	RT-qPCR	↑ miR-210	Muralimanoharan et al., 2012
PE	18 together: 8 PE, 10 NP	Large-scale profiling, RT-qPCR	↑ miR-10b, miR-18a, miR-19a, miR-20a, miR-22, miR-126, miR-142-3p, miR-144, miR-146b-3p, miR-185, miR-193b, miR-193b*, miR-210, miR-451, miR-517c, miR-518c, miR-518f, miR-519e, miR-520a-3p, miR-525-5p, miR-526b, miR-590-5p	Ishibashi et al., 2012
PE	40 together: 20 PE, 20 NP	Microarray, RT-qPCR	↑ miR-210 ↓ miR-328, miR-584, miR-139-5p, miR-500, miR-1247, miR-34c-5p, miR-1	Enquobahrie et al., 2011
PE	53 together: 20 PE, 33 NP	MiRNA microarray, RT-qPCR	↑ miR-210, miR-30a-3p, miR-518b, miR-524, miR-17-3p, miR-151, and miR-193b ↓miR-195, miR-223, miR-218, miR-17, miR-18a, miR-19b1, miR-92a1, miR-379, miR-411	Xu et al., 2014
PE	34 together: 23 PE, 11 NP	MiRNA microarray, RT-qPCR	↑ miR-181a, miR-584, miR-30a-3p, miR-210, miR-517*, miR-518b, miR-519e*, miR-638, miR-296, miR-362	Zhu et al., 2009

			↓ miR-101, miR-10b, miR-218, miR-590, miR-204, miR-32, miR-126*, miR-18a, miR-19a, miR-411, miR-377, miR-154*, miR-625, miR-144, miR-195, miR-150, miR-1, miR-18b, miR-363, miR-542-3p, miR-450, miR-223, miR-374	
PE	14 together: 7 PE, 7 NP	Microarray, RT-qPCR	30 upregulated and 48 downregulated (data not available)	Mayor-Lynn et al., 2011
PE	20 together: 10 PE, 10 NP	MiRNA microarray, RT-qPCR	↑ miR-17, miR-20a, miR-20b	Wang et al., 2012
PE	40 together: 20 PE, 20 NP	RT-qPCR	↑ miR-155	Zhang et al., 2010
PE	36 together: 18 PE, 18 NP	MicroRNA microarray, RT-qPCR	↓ let-7b*, let-7f-1*, miR-10b*, miR-1225-3p, miR-125a-3p, miR-1273c, miR-1275, miR-1539, miR-18b*, miR-191*, miR-2116*, miR-23c, miR-30c-1*, miR-3162, miR-3163, miR-3180-5p, miR-33b*, miR-345, miR-3663-3p, miR-370, miR-422a, miR-425*, miR-509-3-5p, miR-513b, miR-550a, miR-614, miR-650, miR-662, miR-718, miR-874, miR-933	Gunel et al., 2017
PE	50 together: 24 PE, 24 NP	Microarray, RT-qPCR	↑ miR-16, miR-29b, miR-195, miR-26b, miR-181a, miR-335 and miR-222	Hu et al., 2009
PE	10 together: 5 PE, PNP	RT-qPCR	↑ let-7b, miRNA-302*, miRNA-104, miRNA-128a, miRNA-182*, miRNA-133b	Noacket al., 2011
PE	70 together: 35 PE, 35 NP	RT-qPCR	↑ miR-210	Adel et al., 2017
PE	40 together: 20 PE, 20 NP	Microarray, RT-qPCR	↑ miR-335, miR-584, miR-21, miR-181a, miR-210, miR-451a, miR-17, miR-152, miR-516, miR-135b, miR-182, miR-96 ↓ miR-32, miR-377, miR-196, miR-126, miR-182, miR-362-3p	Jiang et al., 2015
PE	37 together: 15 PE, 22 NP	RT-qPCR	↓miR-376c	Fu et al., 2013
PE	37 together: 15 PE, 22 NP	RT-qPCR	↓miR-378a-5p	Luo et al., 2012
PE	22 together: 15 PE, 17 NP	RT-qPCR	↓miR-195	Bai et al., 2012
PE	48 together: 24 PE, 24 NP	RT-qPCR	↓miR-675	Gao et al., 2012
SGA	107 together: 32 SGA, 75 NP	RT-qPCR	↑ miR-16, miR-21, miR-93, miR-135b, miR-146a, miR-182	Maccani et al., 2011
FGR	95 together: 45 FGR, 50 NP	RT-qPCR	↓miR-518b, miR-1323, miR-516b, miR-515-5p, miR-520h, miR-519d, miR-526b	Higashijima et al., 2013
FGR	31 together: 10 FGR, 21 NP	MicroRNA microarray, RT-qPCR	↑ miR-10b, miR-363	Thamotharan et al., 2017

PE/IUGR	71 together: 16 PE, 26 IUGR	RT-qPCR	↓miR-194	Guo et al., 2013
PE/IUGR	27 together: 14 PE, 6 IUGR, 6 NP	RT-qPCR	↑ miR-21 (IUGR, 7 PE cases with abnormal Doppler)	Cindrova-Davies et al., 2013
PE/GH/FGR	162 together: 63 PE, 21 GH, 36 FGR, 42 NP	RT-qPCR	↓ miR-515-5p, miR-517-5p, miR-518b, miR-518f-5p, miR-519a, miR-519d, miR-520a-5p, miR-520h, miR-524-5p, miR-525, miR-526a (PE) ↓ miR-517-5p, miR-519d, miR-520a-5p, miR-525 (GH) ↓ miR-517-5p, miR-518f-5p, miR-519a, miR-519d, miR-520a-5p, miR-525 (FGR)	Hromadnikova et al., 2015a
PE/GH/FGR	160 together: 80 PE, 35 GH, 35 FGR, 20 NP	RT-qPCR	↑ miR-499a-5p (PE, GH, FGR), miR-1-3p (FGR) ↓ miR-16-5p, miR-26a-5p, miR-100-5p, miR-103a-3p, miR-122-5p, miR-125b-5p, miR-126-3p, miR-143-3p, miR-145-5p, miR-195-5p, miR-199a-5p, miR-221-3p, miR-342-3p, miR-574-3p (FGR)	Hromadnikova et al., 2015b
PE/SGA/PE+SGA	221 together: 52 PE, 66 SGA, 31 PE+SGA, 72 NP	RT-qPCR	↑ miR-210	Le et al., 2011

PE, preeclampsia; GH, gestational hypertension; FGR, fetal growth restriction; IUGR, intrauterine growth restriction; SGA, small-for-gestational age; NP, normal pregnany; RT-qPCR, real-time quantitative polymerase chain reaction

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