“FEDERICO II” UNIVERSITY OF NAPLES

SCHOOL OF MEDICINE

PhD PROGRAM IN NEUROSCIENCE

XXXIII CYCLE

IDENTIFICATION OF HYPOXIA-REGULATED MICRORNAs IN MATERNAL BLOOD AS EARLY PERIPHERAL BIOMARKERS FOR FETAL GROWTH RESTRICTION

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ACADEMIC YEAR 2019-2020

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1. INTRODUCTION

1.1 Placenta biology

The mature placenta is a highly vascularized tree-like structure of chorionic villi that are the functional units of maternal-fetal oxygen exchange and nutrient transport. The villi are composed of three component layers: 1) a syncytio-cytotrophoblast bilayer that covers the entire surface of the villous tree; 2) a mesenchyme-derived core that includes fibroblast cells, macrophages and pockets of blood islands; 3) fetal vessels that include vascular smooth muscle cells, perivascular cells, and endothelial cells.

It is the outer layer of multinucleated syncytium that is directly bathed in maternal blood. This syncytium provides a barrier to the migration of maternal cells and bacteria, while allowing substances to be transported from maternal blood to fetal blood (and vice versa) across this interface through both active and passive processes. Syncytial trophoblast is also the main source of many placenta-specific growth factors and hormones that are key indicators of placental growth and health (Wang et al., 2010b). These include, for example, human chorionic gonadotropin (hCG), progesterone, human placental lactogen, vascular endothelial growth factor (VEGF), VEGF-receptor (FLT1), placental growth factor (PlGF). Syncytiotrophoblast-derived particles can also enter maternal circulation through apoptosis/necrosis at the placental surface or through the production of micro-vesicles. Deported trophoblast structures are found in the uterine vein blood of normal pregnancies and may be transcriptionally active and synthesize a significant proportion of placental mRNA and proteins (Rajakumar et al., 2012).

From the tips of the chorionic villi, invasive extra-villous trophoblasts (EVTs) migrate into and remodel the uterine spiral arteries leading to loss of the endothelial lining and musculo-elastic tissue in these vessels. Because invasion of the maternal uterine vasculature is a normal property of EVTs, they can be found at low rates in maternal circulation (~ 1 fetal/placental cell to 100,000 maternal cells) (Gammill et al., 2013). This invasion process is dependent on the health of the maternal uterine natural killer cells and macrophages at the placental interface, and is often incomplete in early-onset/severe preeclampsia (PE), causing reduced blood flow to the maternal-fetal interface. While the mesenchymal cells from the inner core of the villi are less studied, they can produce substances that may be transported through the trophoblast layer into maternal blood. Early in development, macrophages modulate trophoblast migration and villous tree branching by producing angiogenic factors including VEGF, fetal growth factor (FGF), vasculotropin, and vascular endothelial cell proliferation factor, while later they provide

immune protection and may be altered in number in placentae exhibiting inflammation (Tang et al., 2011). Hence, early indicators of susceptibility may be non-specific, e.g. maternal pro-inflammatory signals, but more accurate detection may only be possible later when inflammation is more pronounced.

Several studies showed that distinct molecular changes in the placenta may be associated with specific pathologies; these changes may not directly correspond to biomarkers present in maternal blood (Wilson et al., 2015). Further, the ability to predict certain conditions before their clinical presentation depends upon the time in pregnancy when the underlying pathology originates. Pregnancy complications can result from failure of key processes, such as 1) trophoblast proliferation, migration, and invasion; 2) maternal-fetal tolerance; and 3) endocrine signaling important for nutrient transfer.

The term “placental insufficiency” refers to the most important pregnancy complication in which the placenta does not function adequately, because of insufficient invasion and remodeling of maternal spiral arteries that then leads to downstream effects resulting from reduced perfusion and oxidative stress, causing both fetal growth restriction (FGR) and PE (Roberts and Escudero, 2012). PE is defined by maternal hypertension and proteinuria and is itself a major cause of FGR and preterm birth (Figure 1.1).

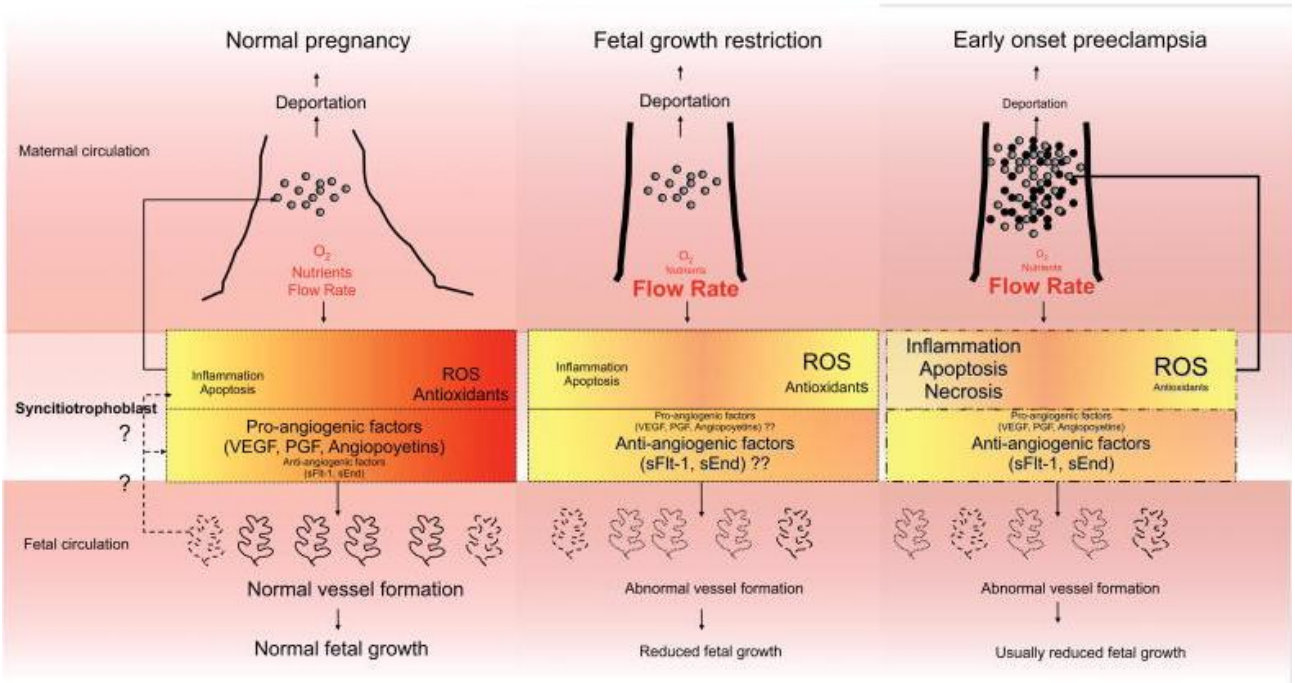


Figure 1.1 Schematic Representation of Human Placentation (by Roberts and Escudero, 2012).

1.2 Placenta associated disorders: Fetal Growth Restriction

Fetal growth restriction (FGR) occurs in 5–10% of all pregnancies. Placental insufficiency is the most common etiology. FGR is diagnosed by combining biometric measurement of fetal size with certain functional parameters to distinguish it from small for gestational age (SGA). Biometric measurement of fetal size signifying abdominal circumference or estimated fetal weight below the 3rd or 10th percentile indicates FGR. Functional parameters include either a solitary parameter, namely, absent end-diastolic flow (AEDF) in the umbilical artery (UA) or contributory parameters such as pulsatility index (PI) of the umbilical or uterine artery (UtA) >95 centile, or cerebro-placental ratio (CPR) <5 centile (Gordijn et al., 2016).

A slowing of growth that leads to preterm fetal growth restriction occurs because the under-functioning placenta cannot meet the nutrient and oxygen demands of the fetus for normal growth velocity. If severe, it can progress to fetal hypoxia and acidemia. The acidemia arises from an accumulation of lactic acid, produced because the fetus switches to anaerobic metabolism in the face of hypoxia. The lactic acid can cause asphyxia neuronal and myocardial injury, culminating in stillbirth. Low birth weight is linked with morbidity and mortality in early infancy as well as with an increased risk for certain diseases later in life, particularly neurodevelopmental (Figure 1.2) (Miller et al., 2016), cardiovascular (Zohdi et al., 2015) and metabolic diseases (Bale, 2015). FGR also affects hormonal regulation and is associated with permanent changes in hormone levels. Additionally, individuals with FGR have a higher tendency to develop obesity (Ornoy, 2011).

FGR evolves from a preclinical phase to clinically apparent growth delay and may eventually progress to fetal deterioration. Increased blood flow resistance in the maternal uterine arteries or the fetal umbilical arteries indicates that the vascular mechanisms that are important for maternal nutrient delivery or fetal nutrient uptake and waste exchange are deficient. When transplacental gas transfer becomes abnormal, leading first to hypoxemia and then hypercarbia, additional fetal responses such as decreased activity, decreased blood flow resistance, or increased peak systolic velocity in the middle cerebral artery (MCA) may be observed. The aspects of placental function, which are predominantly affected, determine the clinical phenotype of FGR at the time of diagnosis and progression as placental dysfunction worsens. Placental lesions that are associated with underperfusion in the fetal and maternal compartments are more common at earlier gestational age. Although placental pathology probably changes over the continuum of

gestational age, expert opinion considers FGR presenting before 32 weeks as “early onset” and thereafter as “late onset” (Baschat, 2018).

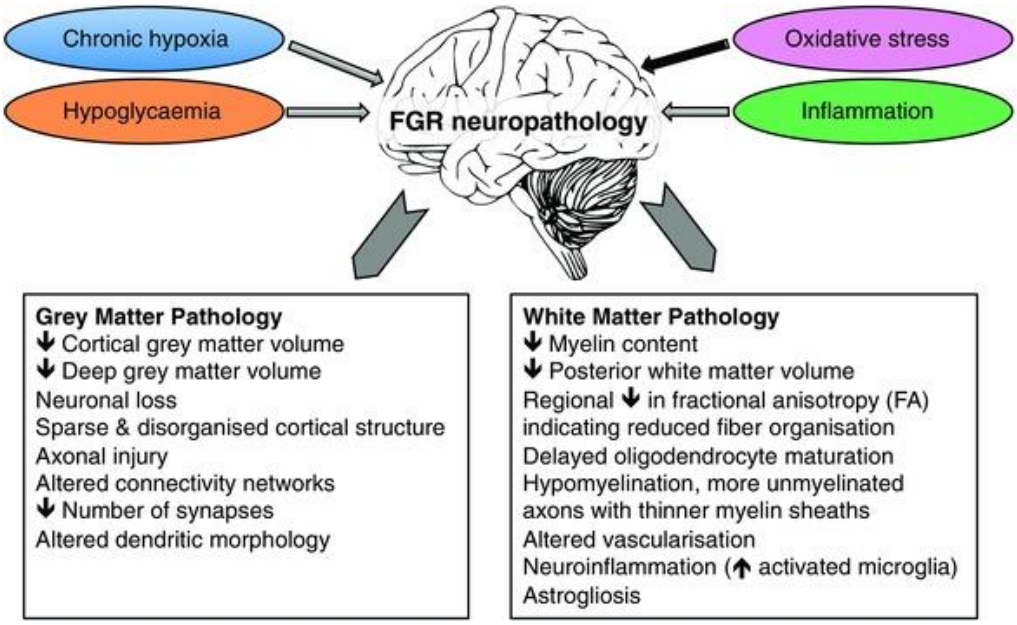


Figure 1.2: Principal adverse mechanisms contributing to grey matter and white matter pathology in FGR (by Miller et al., 2016).

1.2.1 Early-onset FGR

The early-onset (<32 weeks) represents 20–30% of all FGRs and it is associated with severe placental insufficiency, Doppler abnormalities and preeclampsia (50% of cases). According to some studies, the pathophysiology of early-onset is a higher prevalence of villous perfusion abnormalities, with a reduction of more than 30% in the vascular area of the chorionic villi, and failure of conversion of utero-placental arteries, resulting in severe placental insufficiency and chronic fetal hypoxia (Figueras and Gratacós, 2014; Baschat, 2010).

It is the rate of increase in umbilical artery blood flow resistance and specifically how rapidly end-diastolic velocity (EDV) is lost that determines the rate and degree of fetal deterioration. In the early-onset FRG fetus with increased UA blood flow resistance, MCA brain sparing may be present or develop as a sign of progressive cardiovascular responses to placental dysfunction. Further increasing UA blood flow resistance, worsening acidemia, and superimposing cardiac dysfunction eventually can lead to abnormal precordial venous Doppler parameters. This degree of cardiovascular deterioration typically precedes an abnormal biophysical profile score or stillbirth. The

latency from diagnosis to late cardiovascular changes may range between 4 and 6 weeks and is determined by the rate at which UA-EDV is lost and by the gestational age.

According to Baschat, the longitudinal progression of abnormal Doppler waveforms in the early FGR deterioration of utero-placental function is the following: elevated UA blood flow resistance and reduced umbilical vein flow volume precede the onset of a growth delay, followed by decreased MCA impedance and increased brain venous blood flow velocities which characterize the “brain sparing effect” (Baschat, 2018). These early responses are physiologically followed by late-onset Doppler abnormalities such as absent/reversed UA-EDV, absent/reversed ductus venosus (DV) waves, and cCTG abnormalities (Figure 1.3).

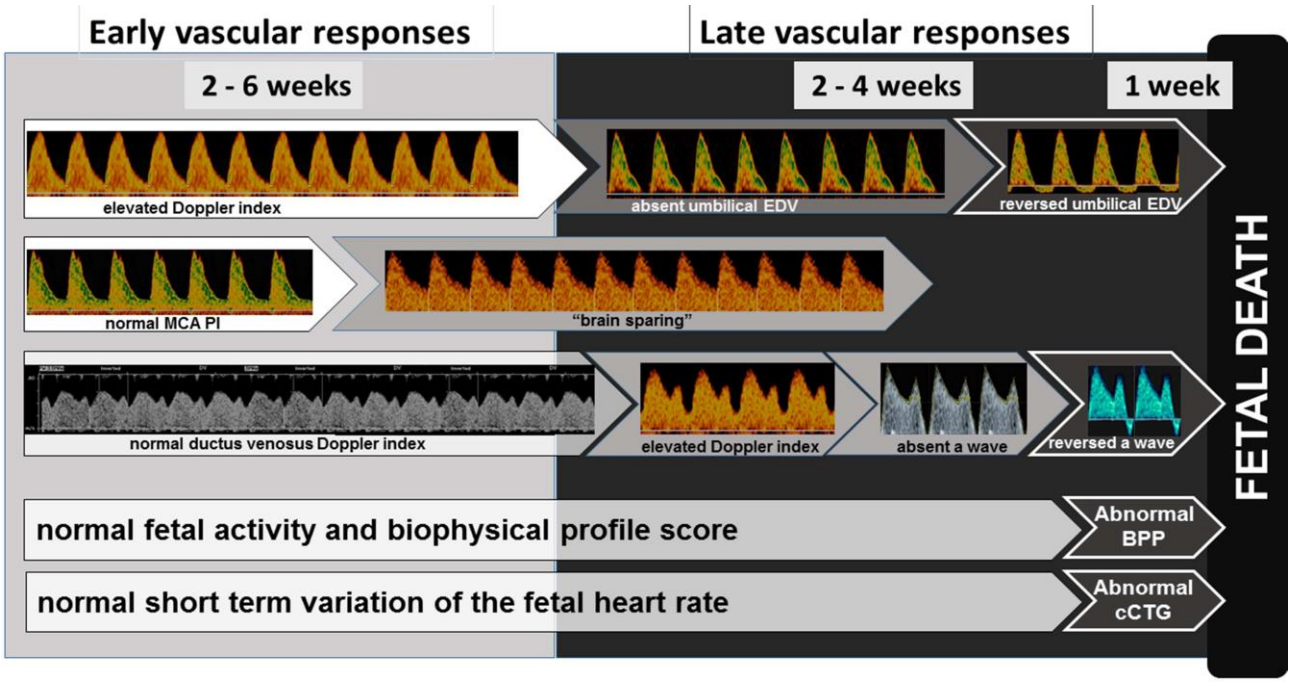


Figure 1.3: Clinical evolution of early-onset FGR (by Baschat, 2018).

1.2.2 Late-onset FGR

The late-onset (>32 weeks) represents 70–80% of all FGRs, so the incidence is much higher than early-onset FGR. The pathophysiology of the late-onset is the insufficient maturation of the chorionic villi or a moderate reduction of their area in the placenta, resulting in mild placental insufficiency and normal Doppler velocimetry (Esposito et al., 2019). Owing to the higher prevalence of villous diffusion abnormalities and a lesser degree of perfusion abnormalities, late-onset FGR may present with little or no UA index elevation but rather uterine artery Doppler index increase or a decrease in umbilical venous volume flow. Despite the seemingly “normal” placental function in the presence of

a normal UA Doppler index, MCA brain sparing or a decrease in the cerebro-placental Doppler ratio (CPR) may be observed documenting decreased placental O_2 transfer.
In the clinical progression, the UA Doppler and the DV are almost always regular, while a decreased CPR progressing to brain sparing can be observed. Additional signs of deterioration preceding stillbirth include a decline in amniotic fluid volume or abnormal fetal heart rate (FHR) parameters. Because UA Doppler is mostly normal, the latency from diagnosis to delivery may be up to 9 weeks and is determined by the rate of progression in cerebral blood flow abnormalities (Baschat, 2018) (Figure 1.4).

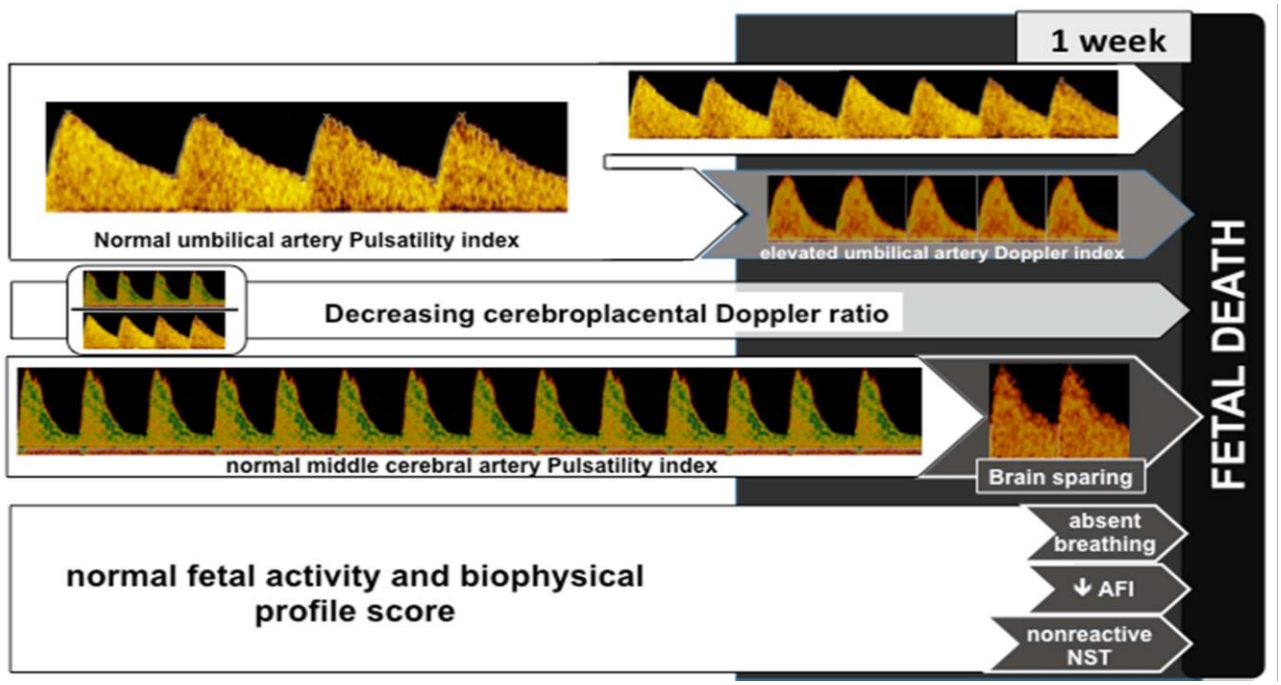


Figure 1.4: Clinical evolution of late-onset FGR (by Baschat, 2018).

1.2.3 Identification of fetuses at greatest risk for compromise

Fetal growth delay that is due to restricted nutrient transfer across the placenta typically leads to sequential lagging of abdominal circumference (AC) and head circumference (HC) growth, finally leading to a decrease in the sonographically estimated fetal weight (SEFW). Given these relationships between placental dysfunction and size, the tenth, fifth, or third percentiles for the AC or SEFW may be chosen for the diagnosis of FGR. Utilizing the AC alone as a diagnostic cut-off is the most sensitive approach, but it is less specific and may include constitutionally small but normally grown fetuses. Choosing an SEFW below the tenth, fifth, and third percentiles successively increases the specificity of identifying FGR.
Most national societies agree on the tenth percentile for the SEFW as a diagnostic cut-off for small for gestational age. The disadvantage of this cut-off is the inclusion of a variable

number of normal constitutionally small fetuses that do not require surveillance. Using an SEFW less than the third percentile or a decreased AC growth rate is more likely to identify “true FGR”, but it has the disadvantage that less severe forms of FGR at risk for deterioration or stillbirth are missed.

Including other parameters of placental dysfunction, such as oligohydramnios and abnormal placental and cerebral Doppler, carries the benefit of identifying the small fetus at risk for prenatal deterioration. Accordingly, such an approach is not only designed to improve diagnosis of true FGR but also to select patients who require fetal surveillance. To capture placental dysfunction across the phenotype of early- and late-onset FGR, inclusion of the uterine and especially MCA Doppler is required (Baschat, 2018).

1.2.4 Surveillance of fetal well-being in FGR

To date, timely and accurate prenatal detection of FGR is still a challenge. The goal of fetal surveillance tests is the accurate estimation of the risk for hypoxemia, prelabor acidemia, or stillbirth, as well as the rate of clinical deterioration. These estimates are required to make decisions on delivery timing and to choose the appropriate interval to the next surveillance visit.

Fetal deterioration in FGR can manifest itself in abnormal Doppler, amniotic fluid, FHR, and biophysical parameters; yet the correlation with acide-base balance has predominantly been studied considering each testing modality in isolation (Baschat, 2003).

In principle, the fetal pH drops proportional to the reduction of EDV, and acidemia is therefore most prevalent when EDV is absent or reversed. However, because UA Doppler may be associated with a wide range of additional Doppler and biophysical abnormalities, its consideration in isolation has an inconsistent relationship with pH. Decrease in the cerebro-placental Doppler ratio or MCA Pulsatility index is associated with a decrease in fetal pH by two standard deviations (Hecher et al., 1995). Studies that evaluated the relationship between MCA Doppler and pH in late-onset FGR have done this for intrapartum fetal distress or neonatal acidosis rather than prelabor acidebase status. However, even near term, addition of cerebral Doppler adds little accuracy to the pH prediction because a range of additional surveillance parameters may be abnormal (Akolekar et al., 2015). Because abnormal venous Doppler occurs in the setting of advanced placental dysfunction in early-onset FGR, it has to date provided the most consistent relationship with declining pH in this subset of patients. An increase in

precordial venous indices is associated with a decrease in umbilical venous pH by approximately 4 standard deviations (Baschat et al., 2004).

When FHR analysis is used in FGR monitoring, a reactive cardiotocographic trace (CTG) virtually excludes hypoxemia, while a nonreactive CTG is associated with a wide range of pH values. Acidemia and in particular a cord artery pH < 7.20 at birth is most accurately predicted by a short-term variation (STV) below 3.5 ms in the computerized CTG (cCTG).

The relationship between biophysical variables and fetal pH resembles the observations made for Doppler, as each individual component (tone, gross body movement, breathing movement, amniotic fluid volume, and heart rate reactivity) is independently altered by hypoxemia but their combined consideration in a composite score best predicts pH and outcome. An abnormal biophysical profile score (BPS) of 4 or less is associated with a mean pH of less than 7.20 and a score of less than 2 has a 100% sensitivity for acidemia (Turan et al., 2007). It is noteworthy that the biophysical parameters, especially loss of tone and movement, have a closer association with pH than the Doppler parameters. Furthermore, this relationship is consistent across gestational age until term and independent of the underlying fetal disease.

Integrated fetal testing is based on the recognition that FGR may present with significant variations in the clinical evolution, that are inadequately captured by utilizing one surveillance modality alone. The trial of umbilical and fetal flow in Europe (TRUFFLE) compared outcomes of early-onset FGR, which were managed by cCTG alone, with those that had combined monitoring with early DV ductus venosus (DV) Doppler and cCTG, and a third arm with late DV changes and cCTG. In the two arms managed with DV Doppler, between 23% and 33% of participants were delivered for “rescue” cCTG criteria. The trial confirmed that combined surveillance led to better composite short-term and 2-year developmental outcome (Lees et al., 2015).

The translation of the TRUFFLE findings into uniform acceptance of combined surveillance will pose challenges, as the cCTG is not as widely available as Doppler and biophysical profile scoring. In early-onset FGR, it has been demonstrated that combined surveillance where the cCTG is substituted with biophysical variables provides equivalent prediction of the delivery pH. When a combined monitoring approach is utilized, loss of fetal gross body movement in the fetus with minimal UA-EDV and elevated DV Doppler index is an accurate predictor of a cord artery pH below 7.20. The additional loss in fetal tone is strongly associated with a pH <7.00 or a base excess <-12 (Ganzevoort et al., 2017). It is important to recognize that the TRUFFLE study provides level 1 evidence on the

surveillance approach and delivery criteria for early-onset FGR. On the contrary, late-onset FGR is usually undiagnosed and there are no comparable evidences for the management approach, especially with respect to Doppler-based surveillance.

1.2.4.1 Electronic Fetal Heart Rate monitoring

The electronic Fetal Heart Rate (FHR) (or cardiotocographic) monitoring is one of the most widespread not invasive method to evaluate the fetal well-being during the antenatal period, especially in pregnancies complicated by FGR.

The monitoring is performed starting from the 37th week of gestation once a week in healthy pregnancies, but it can be started before 37th week and repeated more frequently in case of high-risk pregnancies. The cardiotocograph consists of an ultrasound transducer, which allows detecting the FHR, and a pressure transducer (tocodynamometer), which allows detecting uterine contractile activity. The pressure transducer is placed on the uterine fundus to detect the tone of the myometrium. The ultrasound transducer is placed on the maternal abdomen as close as possible to the focus of maximum auscultation of the fetal heart, in order to detect the movement of the heart valves and defines the duration of the cardiac cycle, using the opening and closing echoes of the valves as triggers. During FHR monitoring the pregnant woman is lying on an armchair or a bed, preferably in left lateral decubitus to avoid possible supine hypotension (Figure 1.5). The cardiotocograph therefore allows obtaining two tracings that can be recorded simultaneously on the same strip of paper. A centimeter scale is shown on the horizontal axis of the trace. The FHR values are highlighted on the upper portion of the trace (range of 50-210 beats per minute). On the lower portion, uterine activity is recorded, measured in Relative Units (0-100) (Figure 1.6a).

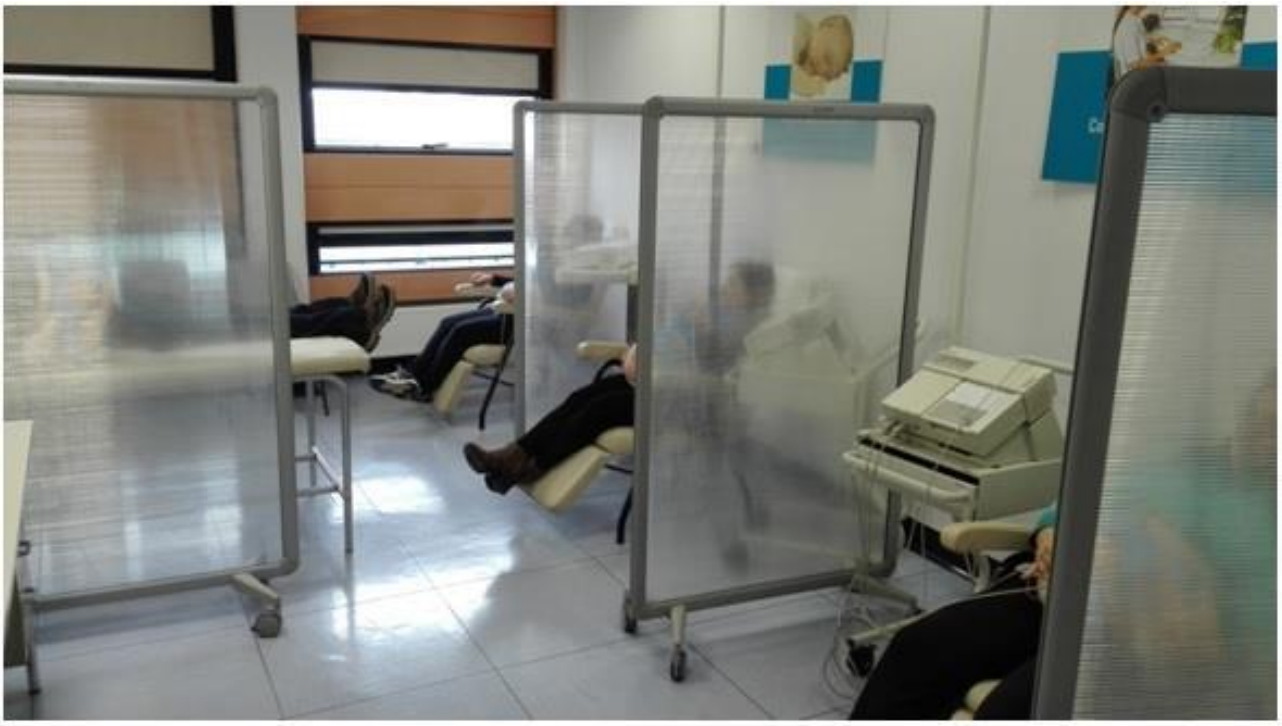
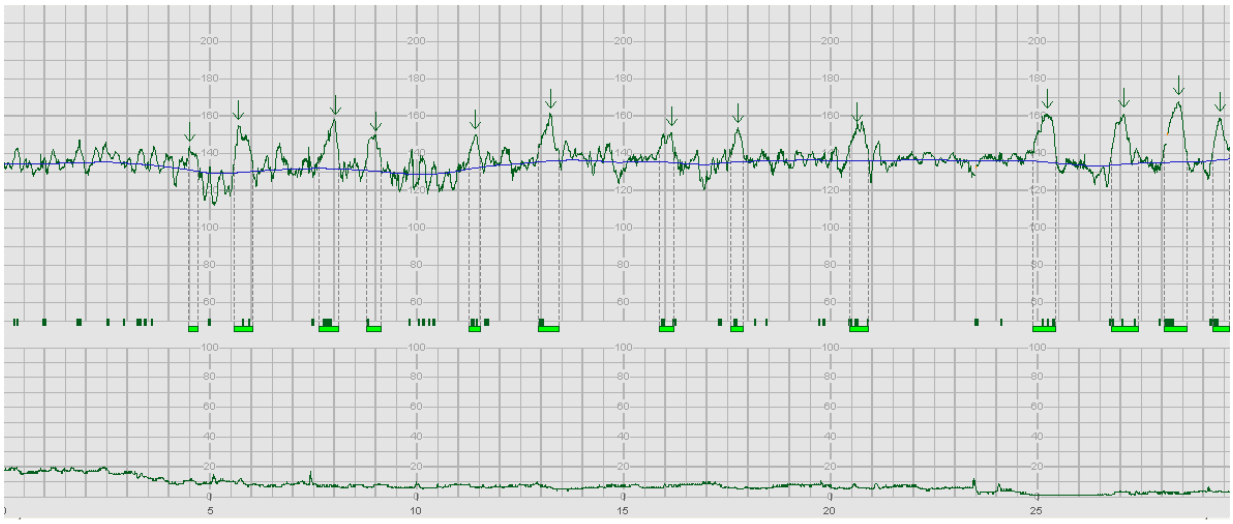


Figure 1.5: Monitoring room. Conventional and Computerized Cardiotocography Service, AOU Federico II.

Many efforts have been made to understand the mechanisms of regulation of FHR variability in Healthy and FGR fetuses. Computerized Cardiotocography provides a standardized method to evaluate conventional CTG parameters and introduces quantitative measures of linear and nonlinear indices related to FHR variability as a multiparametric analysis of fetal cardiovascular and nervous activity. The presence of significant beat to beat variation suggests intact baroreflex, sympathetic/parasympathetic tone and central control indicating normal central nervous system (CNS) responsiveness and normal local CNS metabolic environment reflecting fetal health (Esposito et al., 2019).
The Short Term Variability is the most significant indicator of fetal homeostasis, especially when it is compared to long and medium term variability. In fact, STV is the most extensively studied parameter of cCTG, because it is able to assess the baroreflex and the integrity of the autonomic nervous system (ANS) and its connections with the CNS: normal STV values reflect a healthy ANS, normal activity of chemoreceptors, baroreceptors and cardiac responsiveness, while low STV values are associated with impending deterioration of fetal oxygen supply and therefore fetal distress (Serra et al., 2008).
Figure 1.6b shows a report of computerized analysis processed by the 2CTG2 system used in our cardiotocography service (Arduini et al., 1993).



TRACCIATO CARDIOTOCOGRAFICO

Num. registrazione:
Cognome coniugata:
Indirizzo:
Ultima mestruazione:
Numero gravidanza:

Eseguito il:
Data di nascita:
Tel:
Gestazione:
Parità:

Durata: 27 minuti
Codice paziente:
Correzione ecografica:

Analisi automatica del CTG

	Totali	/Ora
FCF basale Media (FCFB bpm)	136.50	
Rapporto grandi / piccole accelerazioni	---	
Numero decelerazioni > 20 bpm per 30 sec o > 10 bpm per 60 sec	0.00	---
Numero movimenti (marker)	0.00	---
Numero grandi accelerazioni > 15 bpm per 15 sec	13.00	---
Numero piccole accelerazioni > 10 bpm e < 15 bpm per 15 sec	0.00	---
Numero contrazioni	2.00	---

Analisi automatica parametri Frequenza Cardiaca Fetale (FCF)

	Media	Mediana	Interquantile 25%	Interquantile 75%
FCF basale (FCFB bpm)	136.50	136.51	133.77	139.51
Variabilità breve periodo (STV msec)	7.26	6.13	5.67	9.04
Variabilità lungo periodo (LTI msec)	23.82	25.73	22.78	26.63
Ampiezza della variabilità (Delta msec)	45.22	41.00	35.70	55.87
Interval index (II)	0.79	0.77	0.66	0.87
Entropia approssimata (ApEn)	1.45	1.33	1.29	1.64
Contributo spettrale bassa frequenza (LF)	76.74	78.92	68.05	83.61
Contributo spettrale media frequenza (MF)	13.21	11.60	9.59	18.31
Contributo spettrale alta frequenza (HF)	10.05	9.48	6.80	13.64
LF/(HF+MF)	2.26	1.85	0.00	3.94

Qualità del segnale CTG: 5 **Percentuale tracciato analizzato: 54%** **Operatore:** **ID cardiotocografo:**
Note:
Campo utente 1: Campo utente 2:

Figure 1.6: a. Antenatal cardiotocographic trace. b. Report of computerized cardiotocographic analysis (2CTG2 system).

1.2.5 Selecting the appropriate surveillance frequency

Given there are no therapies to rescue in utero placental insufficiency, the current clinical management for preterm fetal growth restriction is to leave fetuses in utero to gain gestation, but to deliver promptly if there is evidence that the fetus is significantly acidemic (Hannan et al., 2020).

Ultrasound-based tests and the cardiotocography are commonly used to detect the presence of fetal hypoxia/acidemia. While these tests have improved outcomes including perinatal survival, none of these existing tests can reliably determine which pregnancies

are affected by significant fetal acidemia. Therefore, a part of preterm FGR are still lost to stillbirth or suffer significant perinatal asphyxia injury (Baschat et al., 2007).

In the fetus that does not yet meet delivery criteria, ongoing surveillance is required for re-evaluating fetal deterioration, and to determine if there is a need for intervention. The growth restriction intervention trial (GRIT) demonstrated that delayed delivery was associated with less prematurity-related immediate mortality and less developmental delay (GRIT Study Group, 2003). However, delay in delivery was associated with significantly higher stillbirth rate highlighting the importance to choose surveillance intervals that are appropriate for the degree of fetal deterioration. Unfortunately, the optimal surveillance pattern remains the object of much debate and research has primarily focused on intervention thresholds. There is no general consensus between national guidelines on the appropriate frequency of testing, and they are based on expert opinion of key authors because there is no high quality evidence to guide practice. In the authors' opinion, it is best to institute longitudinal surveillance starting at 24-26 weeks with integrated fetal testing, including multi-vessel Doppler examination, cCTG if available, and assessment of fetal activity through biophysical profile scoring (Wolf et al., 2017). The monitoring frequency needs to be increased when there are additional signs of deterioration until the delivery threshold is reached. It is critical to recognize that signs of clinical acceleration differ according to the gestational age when FGR is diagnosed. In early-onset FGR, it is the loss of UA-EDV that predisposes to venous Doppler abnormalities and the latter in turn to an abnormal biophysical profile score. UA absent EDV (AEDV) typically evolves over 4 e 6 weeks after diagnosis, and when this occurs, the average time to develop late decelerations is 2 weeks (Baschat et al., 2001). The daily rate of new cCTG abnormalities meeting delivery thresholds is 5% and unpredictable by the Doppler abnormalities and therefore requiring a more frequent surveillance frequency. Once forward velocities in the DV become absent or reversed, fetal survival of longer than 1 week is unlikely (Turan et al., 2011).

In late-onset FGR, an increase in the UA PI and a decrease in the CPR or in the MCA PI may present themselves as signs of clinical acceleration. The latency to delivery is longer and may be up to 9 weeks. CPR or MCA Doppler abnormalities may occur even if the UA Doppler is normal (Oros et al., 2011). MCA Doppler deterioration can occur unanticipated at a median of 4 days before stillbirth (Harrington et al., 1999) and therefore is the most important Doppler parameter to guide monitoring intervals.

In order to improve the late-onset FGRs management and consequently plan their delivery before adverse events occur international guidelines suggest performing the last ultrasound for checking fetal growth at 28-32 weeks of gestation. The low diagnostic rate of late-onset FGR contributes significantly to pregnancy stillbirths. Because late FGR may undergo rapid deterioration leading to severe injury or perinatal death without observable late-stage signs as in early FGR. This might be explained by a combination of causes, which could include the low tolerance of term fetuses to hypoxia in comparison with early FGR and episodes of rapid placental function failure. Contrary to early FGR, where the management is the real problem, in late FGR the most challenging issue is the timely and adequate diagnosis (Baschat, 2003).

This condition would greatly benefit from the availability of early diagnostic tests before clinical signs occurs, in order to give an opportunity for early prevention or intervention. The early identification of severe placental insufficiency could improve maternal-fetal outcomes, in order to flag fetuses that are extremely compromised at imminent risk of stillbirth or severe injury in utero, leading to permanent disability after birth and finally to substantially contain the public health cost.

1.3 Biomarkers to predict pregnancy outcomes

The process of identifying biomarkers to predict pregnancy outcomes requires: 1) understanding the factors that affect biomarker measurement, including technical variables, confounding maternal influences, and the underlying placental processes; 2) defining the distinct placental phenotypes that we are trying to predict; and 3) relating the placental phenotypes to the diverse and overlapping clinical outcomes (FGR, PE, preterm birth, etc) of interest.

Although the placenta is developmentally distinct from somatic tissues, the molecular profile of the placenta may offer a glimpse into the exposures that lead to developmental defects of the baby. Cord blood may also provide a source of biomarkers for fetal outcomes, but can be affected by transient variability in blood cell composition associated with perinatal factors, that must be accounted for. Beyond what the placenta can tell us about fetal health, there is a keen interest in using placental biomarkers at birth to predict postnatal neurodevelopment, metabolic syndrome, and other health outcomes.

1.3.1 Cell-free "fetal" DNA and RNA

Cell-free "fetal" DNA in maternal plasma has attracted much attention since its discovery in 1997 as a novel approach for prenatal screening. This DNA is mainly syncytial trophoblast in origin arising mainly from either apoptosis/necrosis or through formation of microvesicles. While its low level (~10% of the total cell-free DNA) relative to maternal cell-free DNA is the main technical limitation, it has been successfully used to determine fetal sex, RHD blood group, and carrier status for common monogenic diseases (Drury et al., 2016). This 'fetal' DNA, more accurately referred to as cell-free placental (cfp)-DNA, is increasingly used in clinical settings for fetal aneuploidy screening based on genomic sequencing data, yielding high detection rates for T21 (>99%), T18 (96%), T13 (92%), and sex chromosome aneuploidies (90%) (Gil et al., 2015). The placental origin of this DNA has some disadvantages for prenatal diagnosis, as false-positive results can result from confined placental mosaicism, which occurs in up to 2% of all pregnancies.

In addition to DNA, mRNA from the placenta also circulates in the maternal blood and offers a greater range of possibilities to monitor placental function (Tsui et al., 2014). Similar to cfp-DNA, placenta-specific mRNA transcripts are detectable in maternal circulation at the first weeks of pregnancy, and are rapidly cleared from plasma in a few hours after delivery. This placental mRNA can both act as a messenger or be packed in debris (apoptotic bodies or microvesicles) as a result of cell death (Ng et al., 2003). Trophoblast miRNAs are extremely attractive as biomarkers due to their stability, specificity, and relative abundance in maternal plasma. They are associated with all types of placental debris, but are thought to have regulatory function when allied with exosomes and protein complexes (Arroyo et al., 2011).

The regulation of gene expression by miRNAs by targeting mRNAs to transiently block translation or degrade the mRNA without altering the DNA sequence, classifies miRNAs as epigenetic regulators (Krol et al., 2010). These epigenetic mechanisms are influenced by multiple factors during pregnancy: intrauterine nutrient availability (determined by maternal nutrition and placental function), maternal age, use of drugs, endocrine disruptors, toxins, and infectious agents.

The human placenta has a unique miRNA profile, originating in part from 2 large miRNA clusters on chromosomes 14 and 19 (Bentwich et al., 2005).

Many placenta-specific mRNA circulating in maternal plasma may be differentially expressed in pregnancies complicated by early-onset preeclampsia and fetal growth restriction (Whitehead et al., 2016).

However, the main limitation to the use of placental mRNA as a biomarker of pregnancy is that mRNA can degrade rapidly and requires strict handling guidelines. Furthermore, the mRNAs evaluated as predictive biomarkers in maternal blood often do not show improved sensitivity as compared to the corresponding protein product. The low rate of reproducibility may be related to our incomplete knowledge of their origin and biological function, as well as insufficient study power. Furthermore, the alterations in miRNAs may depend on gestational age, population studied, and other clinical measures (e.g. maternal blood pressure and/or the uterine artery Doppler pulsatility index). Larger studies considering potential confounding variables are needed to refine the utility of placental miRNAs.

1.4 Non-Coding RNA: specificity and function of MicroRNA

The “central dogma” dominating molecular biology for many years asserted that the protein production was mediated by the DNA-RNA-protein axis, which only involves the mechanisms of transcription and translation to allow the human genome to be decoded for the production of specific proteins. However, over recent years, the discovery of the world of non-coding RNA has radically revolutionized this theory, defining new mechanisms involved in regulating protein synthesis. The human genome project revealed that protein-coding sequences only constitute the 1.5% of the entire genome, whereas the remaining 98.5% of the genome is composed of introns, regulatory DNA sequences, interspersed elements and non-coding RNA (ncRNA) molecules (International Human Genome Sequencing Consortium, 2001). Indeed, the majority of mammalian genomes are transcribed into ncRNAs, many of which are alternatively spliced or processed into smaller products. To date, two types of non-coding RNAs have been identified and academically classified: short non-coding RNA and long non-coding RNA molecules. The short non-coding RNA molecules can further be subdivided into microRNA (miRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), piwi-interacting RNA (pi-RNA), transfer RNA (tRNA), ribosomal RNA (rRNA) and other uncharacterized small molecules. Among all the regulatory molecules, miRNAs are the most studied and better characterized, particularly as regulators in human diseases, and one of the main proofs is that miRNA expression profiles very often show significant modifications in response to a disease state, suggesting that miRNAs represent key regulators of disease-associated pathways. The first miRNA was discovered over 30 years ago in the nematode Caenorhabditis elegans with the identification of the developmental

regulator lin-4 (Lee et al., 1993). Currently, miRNAs are defined as RNA molecules of 18-24 nucleotides in length, transcribed from specific genes or from intronic regions of other genes, and able to regulate gene expression either by promoting mRNA degradation or by inhibiting protein translation (Watson, 2015). Based on computational prediction, it has been estimated that more than 60% of mammalian mRNAs are targeted by at least one miRNA (Friedman et al., 2009).

Recent developments in the field of miRNA, in relation to human diseases, have revealed that miRNAs represent valuable tools as biomarkers and as potential disease-modifying agents (Basak et al., 2016).

1.4.1 MicroRNA Biogenesis pathway

MiRNAs are evolutionarily conserved regulatory molecules that are synthesized after processing by both nuclear and cytosolic proteins (Hammond, 2015). In the nucleus, non-coding RNA molecules, called primary miRNAs (pri-miRNA), are transcribed from miRNA-encoding genomic sequences by RNA polymerase II. These primary miRNA transcripts are often several hundred nucleotides long and are modified similarly to protein-coding transcripts by the addition of a 5' cap and a 3' poly-A tail. The defining feature of all miRNA genes is the stem-loop precursor RNA structure, with one (but sometimes both) of strands of the stem that represents the source of the mature miRNA (Figure 1.7).

Following this initial transcription event a protein complex comprised of the RNase III enzyme, Drosha and DGCR8 (in addition to several cofactors), recognizes the pri-miRNA and cleaves the 50 and 30 arms of the pri-miRNA hairpin to form the premature miRNA (pre-miRNA) of 70–110 nucleotides. Then, Exportin-5 recognizes and transports the pre-miRNA to the cytosol for further processing via a RAN-GTP-dependent mechanism. In the cytosol, a protein complex consisting of Dicer (RNase) and double-stranded RNA binding domain proteins TRBP, PACT and Ago2 further processes the pre-miRNAs into 22 nucleotide mature miRNA duplexes. Typically, one strand of this mature miRNA duplex, termed the guide strand, associates with the RNA-induced silencing complex (RISC), in particular with Ago2. While it is generally believed that upon incorporation into the RISC complex, the other strand (passenger strand) is separated from the guide strand and degraded, there is evidence that in some cases, both strands of the miRNA duplex can be functional. Ago2 directly binds the mature miRNA and seeks target mRNAs that have complementarity to the miRNA (Watson, Zanichelli, 2015).

Alternatively, miRNAs can be generated without the RISC involvement, but from short hairpin introns, snoRNAs, tRNAs and endogenous short hairpin RNAs as a result of splicing, debranching and complex processing mechanisms (Miyoshi et al., 2010).

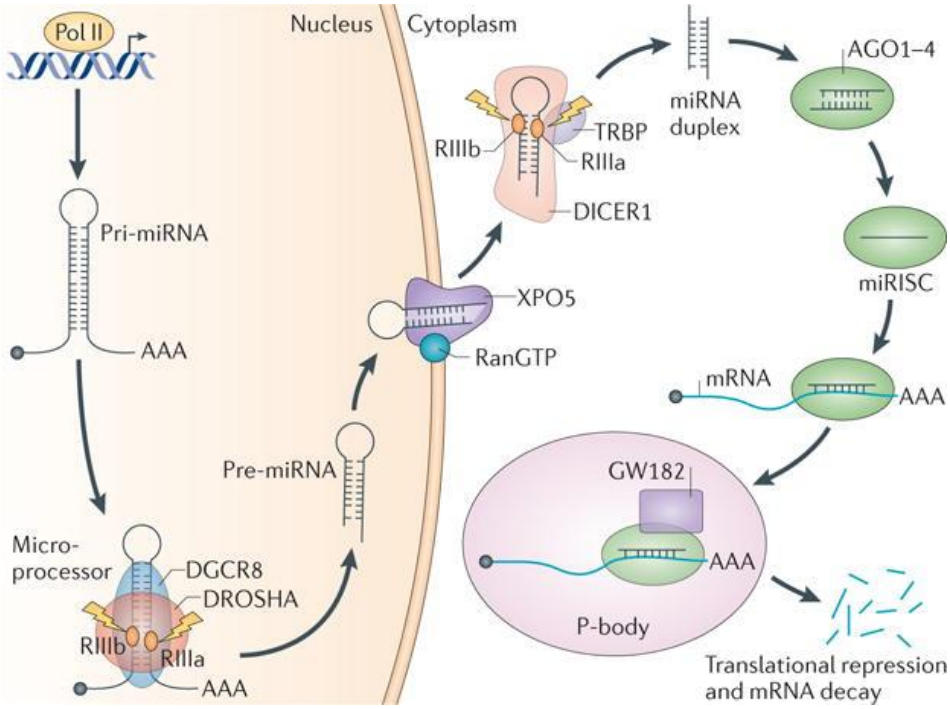


Figure 1.7: Overview of miRNA biogenesis pathway (by Lin and Gregory, 2015).

1.4.2 Nomenclature of MicroRNA

The discovery of an increasing number of microRNA has led researchers to adopt a uniform system of annotation to ensure uniformity and ease the cataloguing of miRNAs (Bhaskaran and Mohan, 2014; Ambros et al., 2003; Griffiths-Jones, 2004). First of all, miRNAs are numbered sequentially in the time order that they are discovered. In particular, when a new miRNA molecule is confirmed, it is assigned a number that is attached to the prefix “miR” followed by a dash (eg, miR-1). To give an indication of the organism in which the novel miRNA has been identified, three letters are added before the word “miR” (eg, “hsa” for Homo sapiens, “mmu” for mouse and “rno” for rat). Moreover, the mature miRNA is denoted with a capitalized “R” (eg miR-1); on the contrary, the uncapitalized “r” refers to both the miRNA gene and the miRNA precursor (eg mir-1). It can often occur that identical mature miRNA sequences originate from different precursor sequences and genomic loci: in this case a numeric suffix will be used to distinguish them, such as miR-103-1 and miR-103-2. This situation is different from that in which mature sequences differ by few nucleotides (1 or 2), and are named with a lettered suffix. This would mean that, for example, hsa-miR-130a and has-miR-130b are derived from specific

and discrete precursors. In addition, deep sequencing studies have revealed that individual pre-miRNAs can give rise to multiple mature miRNA species, “termed isomirs”, with little differences in length or sequence because subjected to a variety of modifications. Finally, in some cases, miRNA precursors could give rise to two mature miRNAs: if the predominantly expressed miRNA specie can be definitively established, it is named as mentioned above (eg miR-136), while the one originating from the opposite strand of the precursor is designated the same name but with an asterisk next to the number (miR-136*). However, determination of the predominantly expressed species often is not possible and so identifiers such as miR-223-5p (from the 5′ strand) and miR-223-3p (from the 3′ strand) are assigned.

1.4.3 Biological Functions of MicroRNA: Target Prediction

The development of precise and fast assays for miRNA target identification has played a significant role in the study of miRNA functions and the biological processes in which they are involved. Several effective algorithms have been developed for the prediction of miRNAs targets in animals (Huang et al., 2010). In particular, there are different prediction criteria that are exploited from these software. First of all, the complementarity of miRNA sequence to the 3′-UTR sequence of potential target mRNAs. Indeed, the strong binding of the 5′ end of the mature miRNA to the 3′-UTR sequence of mRNA is very important for targeting. In particular, there are three structural types of target sites (Figure 1.8):

- The condition in which canonical sites have good or perfect complementarity at both the 5′ and 3′ ends of the miRNA, with a characteristic bulge just in the middle (Figure 1.8A);
- A situation in which there is perfect seed complementarity in the 5′ end of the miRNA, but poor 3′ complementarity (Figure 1.8B);
- The possibility that compensatory sites have a mismatch or wobble in the 5′ seed region of miRNA, that are compensated through excellent complementarity at the 3′ end (Figure 1.8C).

Another very important aspect to be considered concerns the thermodynamic properties of miRNA-mRNA duplexes, that are assessed by calculating free-energy (ΔG) of the putative binding. The energetically more favorable state is when the complementary miRNA and mRNA are hybridized. Lower values of free energy of two paired RNA strands correspond to more energy that is needed to disrupt this duplex formation. Thus, when the free energy is low, the binding of the miRNA to the mRNA is stronger (Lewis et al., 2005). Moreover,

the conservation of miRNA target sites among different species is another important element to be considered for an optimum prediction, because using predicted binding sites that are conserved in multiple species allows to reduce the number of false positives.

TargetScan is a computational method to predict the targets of conserved vertebrate miRNAs, integrating the model of interaction miRNA-mRNA on the basis of thermodynamics and sequence alignment analysis between miRNA binding sites among different species (Lewis et al., 2003). In this way many false positives are filtered from the beginning of the prediction process, so resulting to be estimated in a range between 22% and 31%. In particular, **TargetScan 7.2** uses an improved method to predict targeting efficacy (the context++ model, Agarwal et al., 2015), uses 3' UTR profiles that indicate the fraction of mRNA containing each site.

The algorithm of **DIANA-microT-CDS** integrates computational and experimental approaches (Kiriakidou et al., 2004). The search is performed in the UTRs for stringent seed to the miRNA, but also putative target sites with 6 consecutive base pairs or with seed matches containing one G:U wobble are accepted if they are compensated by a pairing to the 3′ end of the miRNA. Currently, the software considers also the conservation of the sites and the binding type. The total score of a target is the sum of the individual scores of each target site on the 3′-UTR.

RNAhybrid 2.2.1 is a tool characterized by the application on a single genome, basing on analyzing the secondary structure of the miRNA/mRNA duplex (Krüger and Rehmsmeier, 2006). This algorithm calculates the most favorable hybridization miRNA-mRNA in terms of energy, but user must specify the portion of the miRNA that should form a perfect helix corresponding to the seed site.

RNA22v2 is a method for identifying miRNA binding sites (Miranda et al., 2006), but it doesn’t exploit a cross-species sequence conservation filter. So the user, to restrict the number of the results, that in this way is increased, has to choice putative target sites considering the maximal free energy, the minimum number of base-pairs between miRNA and target, and the maximum number of unpaired bases of the miRNA seed region.

PicTar2 uses the criteria of co-expression in space and time of miRNAs and their targets, through combinations of different microRNAs (Krek et al., 2005). This algorithm requires that the binding stability of the putative miRNA-target interaction, measured by thermodynamic binding energy, is higher than a specified threshold.

miRDB is an online database for miRNA target prediction and functional annotations. All the targets in miRDB were predicted by a bioinformatics tool, MirTarget, which was

developed by analyzing thousands of miRNA-target interactions from high-throughput sequencing experiments. Common features associated with miRNA binding and target downregulation have been identified and used to predict miRNA targets with machine learning methods. MiRDB hosts predicted miRNA targets in five species: human, mouse, rat, dog and chicken (Chen and Wang, 2020).

miRWalk2.0 not only documents miRNA binding sites within the complete sequence of a gene, but also combines this information with a comparison of binding sites resulting from 12 existing miRNA-target prediction programs (DIANA-microTv4.0, DIANA-microT-CDS, miRanda-rel2010, mirBridge, miRDB4.0, miRmap, miRNAMap, doRiNA i.e., PicTar2, PITA, RNA22v2, RNAhybrid2.1 and Targetscan6.2) to build novel comparative platforms of binding sites for the promoter (4 prediction datasets), cds (5 prediction datasets), 5'- (5 prediction datasets) and 3'-UTR (13 prediction datasets) regions. It also documents experimentally verified miRNA-target interaction information collected via an automated text-mining search and data from existing resources (miRTarBase, PhenomiR, miR2Disease and HMDD) offer such information (Dweep and Gretz, 2015).

Finally, **tools4mirs.org** is a first, manually curated platform gathering at the present over 170 methods for the broadly-defined miRNA analysis. All tools in Tools4miRs are classified in the four general and seven more detailed categories. In each of the aforementioned sections user can additionally filter available methods according to his research needs, capabilities and preferences. Tools4miRs is also a web-based target prediction meta-server incorporating user-designated target prediction methods in the analysis of user-provided data (Lukasik et al., 2016).

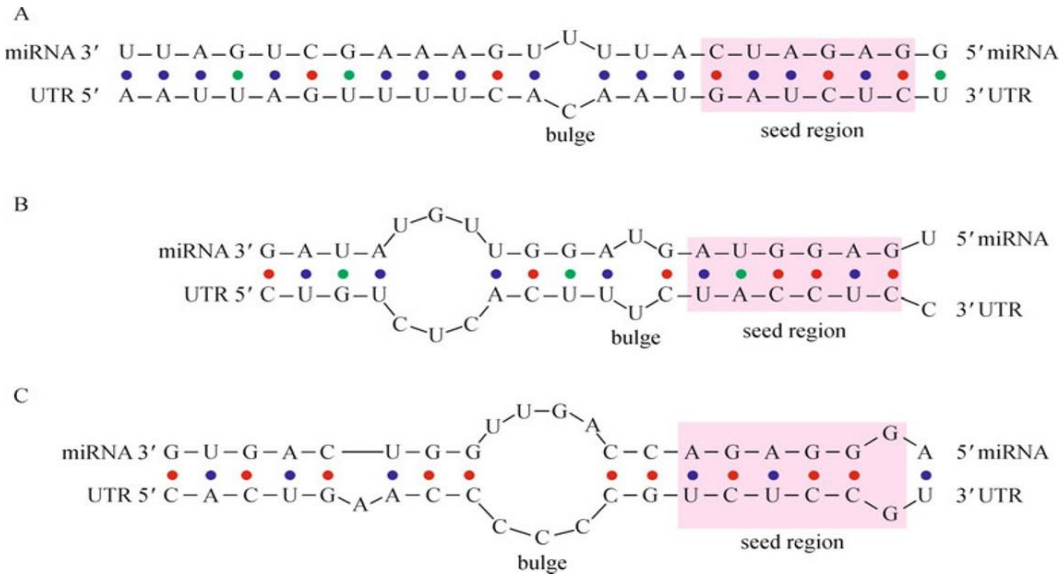


Figure 1.8: Approximate secondary structures of the three main types of target site duplex (by Huang et al., 2010.).

1.4.4 MicroRNA as Therapeutic Target

The miRNAs possess a unique characteristic which is very attractive in terms of drug development; they are small, with known sequences and are often conserved among species.

There are several expanding efforts to develop therapies that directly can target microRNA, and according to the type of miRNA, its expression and its function. Different approaches are used to overexpress or inhibit miRNA, therefore these drug candidates can be either miRNA mimics or antisense inhibitors (Hammond, 2015).

If the therapeutic goal is to replace the miRNA concentration, that was previously reduced, for example in pathologic conditions, a miRNA mimic is applied. This approach was developed in 2007 as gain-of-function tool for specific miRNAs and consists of synthetic double-stranded RNAs that are directly recognized by RNA-induced silencing complex (RISC) to form the miRNA: RISC complex (miRISC) (Wang, 2009). These miRNA-like RNA fragments have a 5' end that gets a motif with partial complementarity to the 3' UTR of the target genes, thus miming microRNA operate mode. This technology has been further developed by pharmaceutic companies, and a library of miRNA mimics is available for human miRNAs discovered until now. Moreover, these molecules undergo modifications in backbone and ribose to promote stability in vivo and to reduce problems related to pharmacodynamics. Nevertheless, important advances are being made using adeno-associated virus (AAV) vectors and tissue-specific promoters to enhance tissue specificity (van Rooij et al., 2012).

On the other hand, blocking a miRNA also can be useful in terms of disease intervention. Anti-miRNAs are antisense oligonucleotides that would bind to the mature guide strand of the miRNA, causing its inhibition (Basak et al., 2016). In order to create stable and deliverable miRNA inhibitors, several methods are used to add modifications, such as cholesterol conjugation and the use of Locked Nucleic Acid (LNA) (Elmen et al., 2008), generating a class of antagonists known as antagomirs. LNA modification increases the stability and nuclease resistance of antisense oligonucleotides, but also increases the efficiency of hybridization to single stranded RNA.

In case of tumors, the miRNAs which are overexpressed may be considered as oncogenes and are called "oncomirs". They are considered to be involved in tumor development by reciprocally inhibiting tumor suppressor genes. On the other hand, expression of some miRNAs (tumor suppressor) is lower in cancerous cells and usually

prevents tumor development by negatively inhibiting oncogenes, and aberrant expression results in oncogenic loss of differentiation.

There is a great excitement regarding miRNA use as therapeutic entities. In terms of scientific perspective, miRNA represents a novel and an attractive target which could manipulate the body functions. Consequently, there has been considerable increase in the number of patent applications filed over the decade.

However, various critical prerequisite data must be available; for example, identification of signature miRNAs, their mechanism of action, applicability by RNAi (miRNA injection), delivery of miRNAs, and their active form in vivo. Once this information is available, miRNA will have a bright future and become a novel therapeutic tool. The process of building miRNA therapeutics is similar to drug discovery and development. Following steps are involved in the discovery and development of miRNA therapeutics (Figure 1.9) (Christopher et al., 2016).

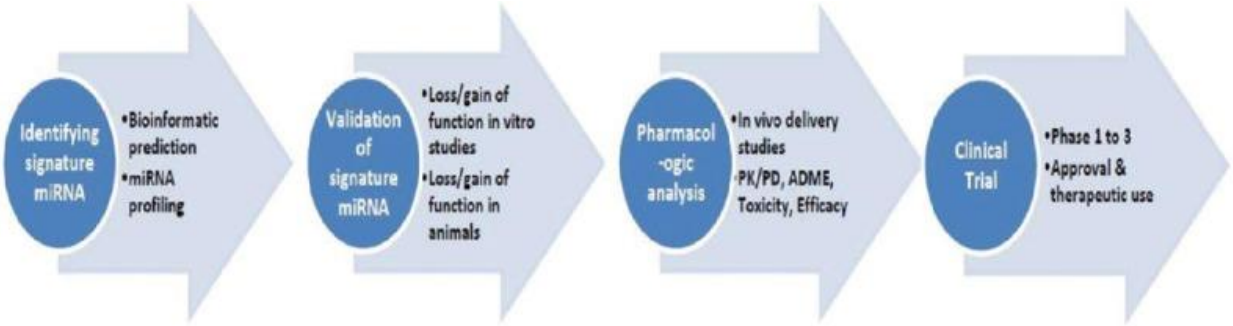


Figure 1.9: Process of microRNA discovery and development (by Christopher et al., 2016).

1.5 Role of MicroRNA in Human disease

In the past decade, the role of miRNAs in several human diseases has been gradually emerging. Gene expression profile studies have shown alterations in miRNA expression in several diseases. Indeed, many cancer types are related to alterations in specific miRNA expression, and in-depth studies have evidenced the role of some of these non-coding RNA, that can function as tumor suppressors or can promote tumor development (oncomiRs) depending on the functions of the target proteins they regulate. Luckily, each day a new miRNA is presented as a novel candidate for cancer diagnosis and prognosis, and an increasing number of studies are achieving good results for miRNA applications (by overexpression o by inhibition) in cancer treatment (Farazi et al., 2011). In cardiovascular diseases, the role of miRNA was investigated evidencing different expression profiles in hypertrophy and heart failure (van Rooij et al., 2006). MiRNA

dysregulation was also observed in autoimmune diseases (Qu et al., 2014) and in other diseases involving immune system (Contreras et al., 2012), such as in infectious diseases. In this context, miRNAs can influence the manifestation and pathogenesis of infectious diseases modulating the pathogenicity of individual pathogens and regulating the expression of genes that have a pivotal role in innate and adaptive immune responses. Neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's disease and amyotrophic lateral sclerosis were also found to have characteristic miRNA expression profiles (Karnati et al., 2015). There are many examples of miRNAs that are specifically expressed in brain tissue and play a critical role in regulating neuronal activity (Kuss and Chen, 2008). The tissue specificity of miRNAs is especially evident in neurons, as they regulate and influence key neuronal features such as neurogenesis, synaptic plasticity, neuronal differentiation and neuronal proliferation. In this scenario, significant number of miRNAs were identified to be enriched in a cell compartment-specific manner. For example, miR-132, miR-134 and miR-138 are abundant in nerve endings and have important regulatory roles in the synapses, such as the development of dendritic spines (Bicker et al., 2014; Schratt et al., 2006). Furthermore, miR-9, miR-124 and miR-128 represent brain-specific miRNAs, and their expression patterns change in disease states. Interestingly, miR-124a regulates neuronal differentiation and maintenance affecting the levels of many hundred non-neuronal gene transcripts (Conaco et al., 2006).

There are increasing evidences on the role of miRNAs in a variety of pregnancy-related complications such as FGR, for their potential use as diagnostic biomarkers due to their ability to enter maternal circulation and to be detectable in the maternal plasma (Luo et al., 2009).

Circulating RNA concentrations of some genes vary with such predictability across pregnancy that their maternal signature might be able to determine gestational age with an accuracy comparable to ultrasound fetal measurements (Ngo et al., 2018).

It is also possible that circulating RNAs could be used as novel clinical biomarkers of obstetric disease, in order to predict, diagnose and monitor fetal wellbeing by identifying fetuses with severe placental insufficiency and fetal acidemia that are at high risk of stillbirth or neonatal morbidity.

1.6 MicroRNAs Regulating Placental Function

Placenta is of great importance during pregnancy because it is located at the interface between the fetus and the mother, and plays a role in the exchange of oxygen and nutrients, buffering from deleterious maternal factors. In general, FGR is associated with abnormal placentation and an anti-angiogenic and pro-inflammatory bias. Furthermore, multiple layers of regulation are involved in the pathogenesis of FGR, including interaction between extravillous trophoblast and decidual leukocytes, altered expression of miRNA in placenta and maternal circulation, imprinted genes and their epigenetic modifications, among others. Although it is still unclear how these factors interact with each other to play a causal role in FGR, identifying these factors has helped us have a better knowledge of the physiology of fetal growth and the pathology of FGR. Furthermore, it is imperative to analyze valuable biomarkers and promising agents in a large cohort to examine whether they have a diagnostic/predictive or curative value, making it possible to recognize and treat FGR as early as possible, and to save the baby from an adverse pregnancy outcome.

During pregnancy, due to a normal extravillous trophoblast invasion, nucleic acids of the placental compartment are released into the maternal circulation in a stable form: this release occurs through the migration of microvesicles from apoptotic/necrotic cells and active cellular communication system, involving also nanovesicles/exosomes and subcellular fragments. Due to placental continuous remodelling, these extracellular nucleic acids may be detected in maternal blood during the course of gestation and can be measured to monitor placental function and allow early diagnosis of pregnancy complications (Chiofalo et al., 2017).

MiRNAs predominantly expressed in the placenta are probably involved in placental differentiation and in the maintenance of pregnancy (Mouillet et al. 2011), allowing communication between the fetus and mother (Chiu et al., 2010).

Previous studies (Miura et al., 2010; Kotlabova et al., 2011), revealed increased levels of placenta-specific miRNAs in maternal circulation during progression of normal pregnancies, which may be linked to the accruing mass, the aging, and/or the development of the placenta. In turn, the placenta is of critical importance to ensure the proper growth and development of the fetus. In fact, placenta weight in FGR pregnancies was significantly lower compared with cases of uncomplicated pregnancies.

Alterations of placental miRNAs expression may serve as a record of in utero exposure to extrauterine and intrauterine environmental during pregnancy, as pollution levels, ethnicity, maternal age and lifestyle (Addo et al., 2020) (Figure 1.10).

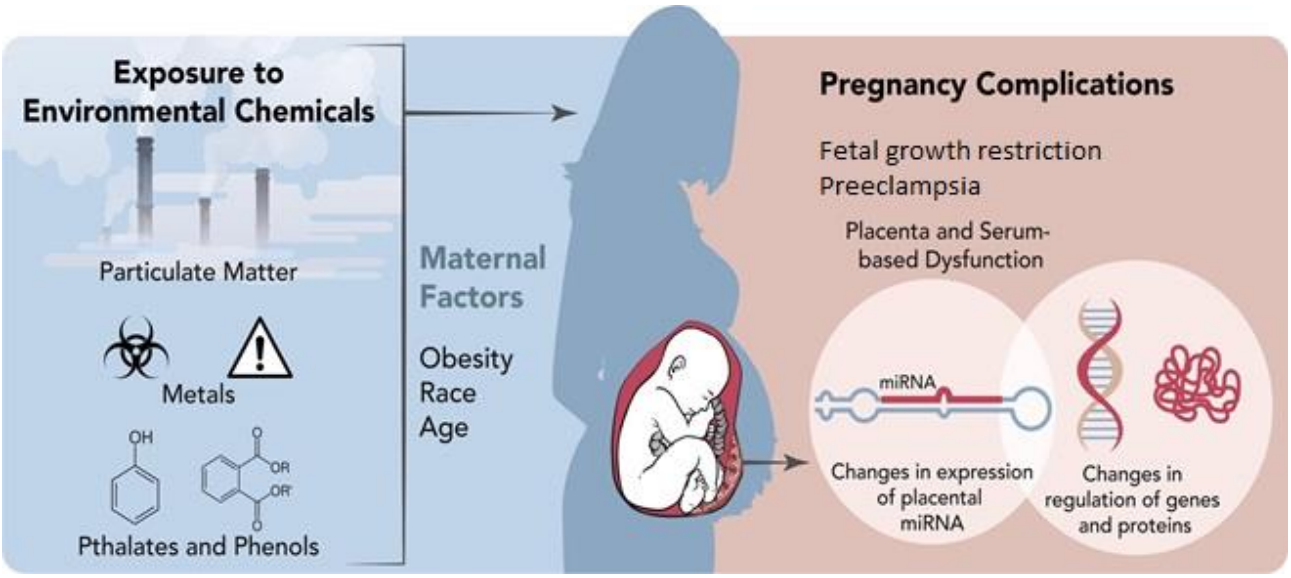


Figure 1.10 Various environmental factors are associated with pregnancy complications including FGR and preeclampsia (by Addo et al., 2020).

Most miRNAs expressed in human placenta are derived from two miRNA clusters, the chromosome 14-miRNA cluster (C14MC) and the chromosome 19-miRNA cluster (C19MC). Although C14MC produces many groups of miRNAs in the placenta, most of them are considered non-functional and the function of others remain unknown (Mouillet et al. 2015). To date, many studies focused their attention on C19MC, because comprises 46 miRNAs and it is the largest gene cluster of miRNAs in humans, exclusively expressed in undifferentiated cells and in placenta (Donker et al., 2012; Higashijima et al., 2013). Donker et al. compared 14 placentas from FGR pregnancies with 14 from normal pregnancies, they found that hypoxic stress affect the downregulation of miR-500c-3p in C19MC (Donker et al., 2012). The expression of miR-16 and miR-21 was markedly reduced in fetuses with severe growth restriction. MiR-16 is involved in regulating cell cycle progression and miR-21 in regulating cell cycling and cell proliferation (Maccani et al., 2011). Higashijima et al., demonstrated that the levels of seven placental-specific miRNAs (hsa-miR-518b, hsa-miR-1323, hsa-miR-516b, hsamiR-515-5p, hsa-miR-520h, hsa-miR-519d, and hsa-miR526b) located on C19MC were significantly lower in placentas from pregnancies complicated by FGR than in placentas from uncomplicated pregnancies, indicating that the aberrant expression of placental C19MC may cause placental dysfunction resulting in FGR. Moreover, four of the seven FGR placenta-specific miRNAs (hsa-miR-518b, hsamiR-1323, hsa-miR-520h and hsa-miR-519d), that showed the most significantly decreased expression level in placentas of FGR pregnancies, were at significantly decreased concentrations in maternal plasma after delivery and were

identified as FGR pregnancy associated, placenta-specific miRNAs (Higashijima et al., 2013). On the other hand, Hromadnikova et al. showed a significant elevation of seven extracellular placenta-specific miRNA levels (miR-516-5p, miR-517, miR-518b, miR-520a, miR-520h, miR-525, and miR-526a) during early gestation in seven pregnancies with later onset of preeclampsia and/or FGR. According to these data, an early screening (i.e., within the 12th to 16th weeks) of miRNA circulating levels may differentiate between women with normally progressing pregnancies and those who could later develop placental insufficiency–related complications (Hromadnikova et al., 2012). The same authors, detected a downregulation of six miRNAs (miR-517-5p, miR-518f-5p, miR-519a, miR-519d, miR-520a-5p, and miR-525) in placental tissues of 36 FGR pregnancies; these results seem more robust, compared to the previous studies, since that authors investigated more types of miRNAs and used those that were previously demonstrated to be exclusively expressed or highly expressed in placental tissues (Hromadnikova et al., 2015b). Last years, Hromadnikova's group found a downregulation of miR-100-5p, miR-125b-5p, and miR-199a-5p in 39 patients with gestational hypertension, in 68 with preeclampsia, and in 33 with fetal growth restriction compared with 55 healthy controls; in addition, they showed downregulation of miR-17-5p, miR-146a-5p, miR-221-3p, and miR-574-3p only in FGR pregnancies (Hromadnikova et al., 2016).

In a small-scale analysis, others found that a group of miRNAs that are altered by hypoxia in trophoblasts (miR-27a, miR-30d, miR-141, miR-200c, miR-424, miR-205 and miR-451, miR-491, miR-517a, miR-518b, miR-518e, and miR-524) were elevated in FGR pregnancies (14 FGR versus 14 controls) (Mouillet et al., 2010). Some of these miRNAs, such as miR-141, miR-200c, and miR-205 were studied also in animal models. In particular, it was found that they play important roles in the maintenance of the integrity of the folded trophoblast-endometrial epithelial bilayer in porcine placentas (Liu et al., 2015). Other experiments found that the expression of miR-518b was decreased, whereas miR-519a was significantly increased, in 30 FGR placentas (Wang et al., 2014).

Detailed investigation is required to elucidate the biological significance of these differentially expressed miRNAs by validating their specific target genes and determining the relevance of these target genes to placental development and functional activities.

1.6.1 Placenta-specific miRNAs and their target genes

There seems to be a confusion in the literature on the terminology used. Typically, placental miRNAs include placenta-specific, placenta-associated, and placenta-derived circulating miRNAs. While placenta-specific miRNAs are expressed largely or uniquely in the placental tissue, placenta-associated miRNAs are expressed ubiquitously in the placenta and other tissues. Placenta derived circulating miRNAs refer to the placenta-released circulating miRNA. Considering that placenta is a central organ for healthy pregnancy, studies regarding the placental miRNAs are important to understand the regulatory mechanisms of normal and complicated pregnancies. Until now, studies of placenta-specific miRNAs in the regulation of pregnancy are very limited focusing only on the basic biological characteristics.

The expression of miRNA-141 is elevated in the placenta of FGR pregnancies and is of high diagnostic value. MiRNA-141 decrease the mRNA level of E2F3 and the mRNA and protein levels of PLAG1 (Tang et al. 2013). The levels of miRNA-424, a hypoxia-regulated miRNA, were significantly increased in the placenta of women with FGR. Compared to controls, both mRNA levels and protein levels of mitogen-activated protein kinase 1 (MEK1) and fibroblast growth factor receptor 1 (FGFR1) were significantly reduced in placentae complicated with FGR. Furthermore, the increased levels of miRNA-424 were negatively correlated with the mRNA levels of MEK1 in placentae complicated with FGR.

Since FGFR1 mediates the functions of VEGF, it is possible that increased miRNA-424 contributes to FGR by affecting the normal vascularity in the placenta (Huang et al. 2013).

Using an in vitro model of nutrient deficiency to mimic that seen in FGRs, was provided evidence that specifically selected miRNAs (miR-10b, miR-363, miR-149) are up-regulated in the human term FGR placenta in vivo. Furthermore, inhibition of these miRNAs in vitro results in increased expression of their target genes, which include those important for angiogenesis and nutrient transfer. In particular, increased miR-10 in FGR placentas may reflect increased E-cadherin ensuring maturation of cell adhesions towards premature trophoblastic terminal differentiation rather than proliferation, underlying the small sized placentas. MiR-363 increased in FGR placenta causing reduced SNAT1 and SNAT2 concentrations and System A amino acid transport activity. SNAT1 and SNAT2 are sodium coupled neutral amino acid transporters, whose perturbed expression may affect overall trophoblastic uptake of small non-essential neutral amino acids such as alanine, glycine and serine (Thamotharan et al., 2017).

Several miRNAs have been demonstrated to regulate trophoblast invasion and migration through diverse downstream signaling pathways. MiRNA-135b mediates low oxygen-induced reduction of extravillous trophoblast-derived HTR-8/SVneo cell invasion by inhibiting its target gene CXCL12, an invasion-promoting factor (Tamaru et al. 2015). Overexpression of miRNA-155 in HTR-8/SVneo trophoblast- and stromal/mesenchymal-like cells reduced the level of cyclin D1 protein, decreasing cell proliferation and migration (Dai et al. 2012). MiRNA-204 suppressed the invasion of BeWo trophoblast-like cells by reducing the expression of its target gene matrix metalloproteinase-9 (MMP9) (Yu et al. 2015). Overexpression of miR-519d attenuated the migration of human EVT through their target genes associated with invasion, such as CXCL6, NR4A2 and FOXL2 (Xie et al. 2014). Overexpression of miR-125b-1-3p inhibits trophoblast cell invasion by targeting sphingosine-1-phosphate receptor 1 (S1PR1) in HTR8/SVneo cells (Li et al. 2014a). Remodelling of the uterine spiral arteries by the invading extravillous trophoblast (EVT) cells increases vessel diameter and results in uteroplacental perfusion. Abnormalities in this process can cause placental hypoxia, resulting in trophoblast dysfunction. Overexpression of miR-210 inhibits trophoblast invasion in primary EVT cells partly through extracellular signal regulated kinase (ERK) signaling (Anton et al. 2013). Studies on placental tissues in FGR and preeclampsia revealed that miR-210 is related to several target genes, which include EFNA3, HOXA9, APLN, CSF1, ITGAM, C3AR1, and SELE (Awamleh et al., 2019). MiRNA-144 inhibits human trophoblast cell invasion by inhibiting titin and its downstream signaling molecule such as the expression of ERK1/2 and the activity of MMP2/9 (Liang et al. 2014). MiR-29b inhibits the invasion and angiogenesis of trophoblast cells by inhibiting its target genes, myeloid cell leukemia sequence 1 (MCL1), MMP2, VEGF-A and integrin β1 (ITGB1) genes (Li et al. 2013).

The interaction between trophoblast and endothelial cell play an important role in normal placentation process. MiR-15b inhibits the invasion of HTR8/SVneo trophoblast-like cells and HUVECs endothelial-like cells tube formation by targeting the protein-encoding sequence of Argonaute 2 (AGO2) mRNA. In an aberrant inflammatory condition mimicked by adding a Lipopolysaccharides (LPS) to HTR8 cells, an increase of miR-15b expression mediated by LPS receptor TLR4 was seen which was consistent with the measured increased secretion of sFlt. sFlt is a soluble VEGFR1 isoform that is specifically expressed in the placenta and represents a well-established biomarker in the prediction of preeclampsia (Yang et al. 2016). In contrast to the above-mentioned functions of miRNAs, miR-376c promotes migration and invasion of human HTR-8/SVneo cells by inhibiting

Nodal and transforming growth factor-β (TGF-β) signalling. MiR-378a-5p promotes trophoblast cell migration and invasion partly by inhibiting Nodal. Nodal is a member of the TGF-β superfamily which inhibits trophoblast cell proliferation, migration and invasion in the human placenta (Luo et al. 2012) (Figure 1.11).

Downregulation of miR-519 and miR-379 and upregulation of miR-193b seems to be important in placentas complicated by either PE or FGR. MiR-193b-3p decreased the migration and invasion of trophoblast cells. MiR-379 has been found to target insulin-like growth factor I (IGF-I), which is crucial to and regulates placental and fetal growth and development. The downregulation of miR-379-3p is supposed to increase the expression of IGF-I, would constitute a compensatory mechanism for poor fetal growth. MiR-379 has also been found to inhibit cell proliferation and induce cell cycle arrest, indicating that low miR-379 expression would promote growth. Decreased expression of miR-379-3p, miR-369-5p, and miR-380-3p were found in SGA infants. The possibly more relevant predicted targeted genes were grouped according to their functions: the ubiquitin (or ubiquitin-like) system (UFL, UBE3D, COPS3, COMMD3); inflammation (IFNW1, NLRC4, MPO); angiogenesis (CLEC14A, EFNA4); metabolism of toxins (CYP2E1, KLHL1); and transcription factors (TFAP2A). Exploring these genes in placental tissue and their relationship with fetal growth in future studies may reveal new important mechanisms for FGR (Östlinga et al., 2019).

Low plasma serine peptidase inhibitor Kunitz type-1 (SPINT1) concentrations in placenta and plasma from pregnancies complicated by FGR at 36 weeks' gestation were found, showing that (1) has a step-wise association with SGA birthweight centiles <10th, 5th, 3rd centiles; (2) it is associated with reduced neonatal lean mass at birth; (3) it has a negative correlation with uterine artery blood flow resistance measured at 36 weeks' gestation, and (4) is correlated with placental weight (Kaitu'u-Lino et al., 2020).

Others colleagues identified two clusters of selected genes in placental tissue of FGR: the first cluster included the mRNAs STAR, CHST2, FOXL2, FGG, IGFBP1, TMEM132, PRUNE2, TNFRSF1 CPEB1, and the lnc-RNAs MED4-AS1 and EGFR-AS1, while the second cluster was mostly composed of mRNAs FBXO2, KCNK12, IL1R2, and CATSPER1 mRNAs and lnc-VAPA-1. Most of them were upregulated in FGR and they were mainly enriched in immunological processes including cytokine-cytokine receptor interaction, chemokine signaling pathway, and primary immunodeficiency, as well as allograft rejection and graft-versus-host disease (Medina-Bastidas et al., 2020).

Majewska et al. identified 28 differentially-expressed genes, 10 were upregulated and 18 were downregulated in FGR-affected placentas. Five of the most upregulated protein-coding genes were: ARMS2, ASTE1, ADAM2, TCHHL1, BTNL9. Five of the most downregulated gene were: THEMIS, PTPRN, FNDC4, SIRPG, SLC38A5. Functional enrichment annotation indicated, that most of the differentially expressed genes were implicated in the processes of inflammation and immune disorders (IL7R, PINLYP, FNDC4, ARMS2, LCK, ZAP70, BCL11B, SIRPG, ITK, BTNL9) related with FGR. Enrichment Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed differentially expressed genes engaged into three metabolic pathways: NF-kappa B signaling pathway (ZAP70, LCK, LTB), T cell receptor signaling pathway (ITK, ZAP70, LCK), and primary immunodeficiency (ZAP70, IL7R, LCK) (Majewska et al., 2019).

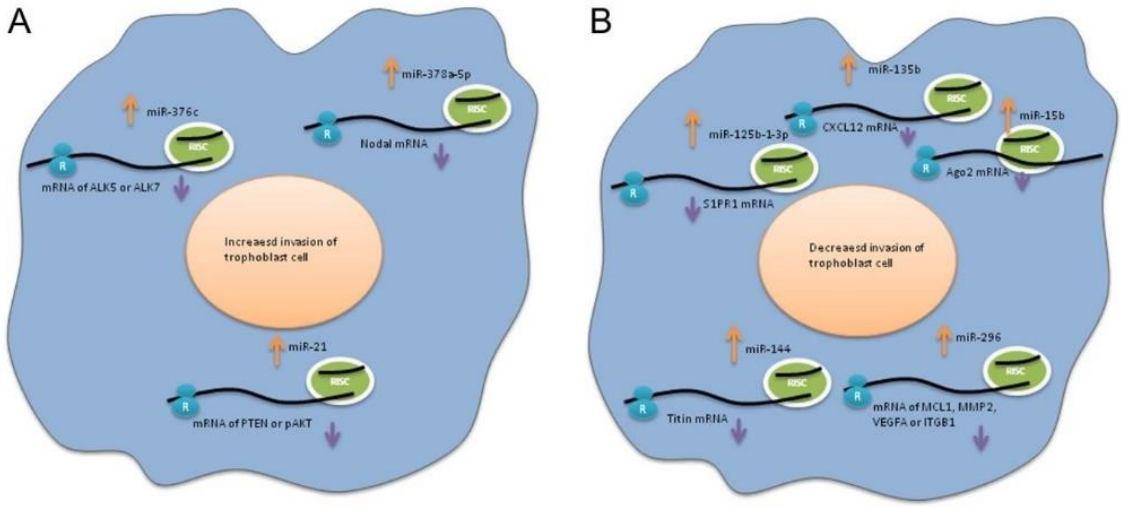


Figure 1.11: miRNAs linked to invasion of trophoblast cells. (A) Showing that three miRNAs are found to be able to promote invasion of trophoblast cells by degrading respective mRNAs; (B) showing that five miRNAs are found to decrease invasion of trophoblast cells by degrading respective mRNAs (by Yang et al. 2016).

Although the association of the above-mentioned miRNAs with FGR has not been carefully investigated, there is a great possibility that these miRNAs are dysregulated in the placenta or plasma of pregnancies complicated by FGR, as impaired trophoblast invasion and/or migration is a primary cause of FGR. The validity of these altered miRNAs in maternal blood from pregnancies complicated by FGR, as a predictor of FGR, warrants further investigation.

1.6.2 Circulating miRNAs in FGR pregnancies

As described above, there are no therapies to rescue in utero placental insufficiency. Therefore, it is of great importance to develop an early predictor for FGR with high specificity and sensitivity. Different biomarkers are required according to the time of onset and different pathophysiological evolution of growth restriction.

In the first trimester of pregnancy, increased maternal circulating IGFBP-4 is considered as a promising biomarker of FGR. It reflects the presence of abundant extravillous trophoblasts and decidual cells that highly expressed IGFBP-4. Importantly, it has a higher predictive value than many other biomarkers alone, including circulating human chorionic gonadotropin (b-hCG), α-fetoprotein (AFP), ADAM-12 (a disintegrin and metalloprotease-12), placental protein 13 (PP-13), soluble endoglin and soluble fms-like tyrosine kinase-1 (s-Flt1). Moreover, It is believed that insufficient insuline-like growth factor 2 (IGF-2) results in limited placenta growth, thereby restricting nutrient transfer to the developing fetus and leading to FGR (Qiu et al. 2012). Pregnancy-associated plasma protein-A (PAPP-A) is a protease that cleaves a subset of insulin-like growth factor binding proteins 4 (IGFBP4). The molecular function suggests its involvement in the IGF system that is vital for fetal growth and development. Lower PAPP-A levels measured in maternal serum in the first trimester of pregnant women delivering small neonates is reported to have a predictive value for FGR (Hansen et al. 2016).

In the second trimester, an imbalance between circulating antiangiogenic and proangiogenic proteins is associated with FGR. The combination of high circulating levels of soluble fms-like tyrosine kinase 1 (sFlt1) and low placental growth factor (PGF) is the best predictive biomarker of adverse pregnancy outcome during 16–19 weeks of gestation (Kim et al. 2016).

In the third trimester, pentraxin 3 (PTX3), a C-reactive protein family member expressed in response to inflammatory stimuli is helpful in differentiating growth restricted babies into physiological SGA and pathological FGR. Pregnancies complicated by FGR have higher PTX3 concentrations in the maternal blood than controls. This increase was related to the severity of FGR, since increased PTX3 reflects endothelial dysfunction in the placenta (Cozzi et al., 2012).

Currently, there are no predictors of late-onset FGR sufficiently accurate to be recommended for clinical use. Previous studies evaluating the prediction of late-onset FGR have relied on either first trimester biomarkers, ultrasound markers of abnormal placentation or routine third trimester biometry. Pregnancy associated plasma protein A

(PAPP-A) is an integral part of aneuploidy screening in the first trimester, and due to its association with FGR has been introduced into many clinical guidelines for prediction of late-onset FGR. However, PAPP-A <5th centile has a sensitivity of 10-15% for detecting any FGR and even lower for late-onset FGR (Zhong et al., 2015).

First and second trimester uterine artery Dopplers can be used to detect abnormal placentation but the sensitivity of first trimester uterine artery Dopplers for predicting FGR is only 15% (Velauthar et al., 2014). Second trimester uterine artery Dopplers perform better at predicting early-onset FGR, but not late-onset FGR (Savasan et., 2014). Recently, the efficacy of third trimester biometry at predicting late-onset FGR has gained increased attention but again the sensitivity is as low as 29 - 58 % (Sovio et al., 2015).

Successful establishment of pregnancy requires coordination and interactions of maternal and fetal genes, proteins, and essential nutrients. Growing evidence showed that inherited maternal miRNAs in the fetus are essential for fetal development. Maternal environmental and physiological factors can directly affect fetal miRNA expression, meaning that dysregulation of miRNA expression may lead to the fetal defects or even lifelong consequences. Moreover, fetal DNA and RNA, including miRNA, are presented in the maternal circulation and serve as important diagnostic tool for pregnancy and fetal disease (Cai et al., 2017).

Placenta-derived circulating miRNAs were initially introduced as biomarkers for pregnancy monitoring. The miRNA expression profiles in maternal plasma before and after delivery were analysed. It seems that a miRNA pool rather than individual miRNAs has a defined role as potential biomarker in pregnancy and fetal related disorders (Figure 1.12).

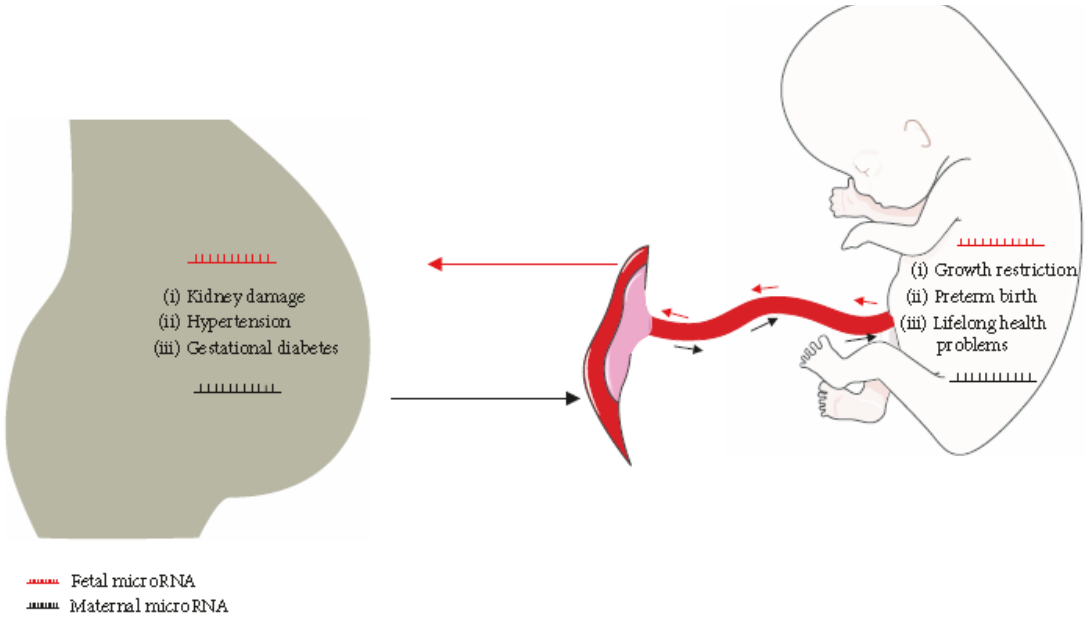


Figure 1.12: The mutual communication of miRNAs between fetus and mother (by Cai et al., 2017).

Many studies investigated the association between circulating maternal plasma levels of miRNA and development of FGR in several stage of pregnancy. Hromadnikova et al. observed high level of seven pregnancy-related extracellular miRNAs (miR-516-5p, miR-517, miR-518b, miR-520a, miR-520h, miR-525, and miR-526a) at the early stages of the pregnancy (between the 12th and the 16th week of gestation) in late-onset IUGR as opposed to normal pregnancies (Hromadnikova et al., 2012). Same authors found that upregulation of miR-499a-5p is a common feature of all placental insufficiencies such as preeclampsia, gestational hypertension, and FGR; they also demonstrated an upregulation of miR-1-3p in FGR pregnancies with abnormal Doppler of umbilical artery; finally, they found downregulation of a series of miRNAs (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, and miR-574-3p) in FGR requiring the delivery before 34 weeks of gestation (Hromadnikova et al., 2015a). Last studies of the same working group observed a down-regulation of miR-520a-5p in circulating plasma exosomes during the first trimester of gestation in women which developed growth-restricted fetuses (Hromadnikova et al., 2019). On the other hand, Guo et al. observed an up-regulation of miR-520a-5p and a down-regulation of miR-194 in FGR placentas (Guo et al., 2013). Higashijima et al., found four FGR placenta-specific microRNAs (hsa-miR-518b, hsa-miR-1323, hsa-miR-520h, and hsa-miR-519d) were significantly lower in placentas from FGR pregnancies than in placentas from uncomplicated pregnancies but their circulating levels in maternal plasma showed no significant differences between FGR and uncomplicated pregnancies (Higashijima et al., 2013). Recently, also Addo et al. observed that altered expression of miR-518 in plasma and placenta has been associated with FGR, low birth weight and preeclampsia (Addo et al., 2020). The maternal plasma levels of miR-27a-1, miR-30d, miR-93, miR-141, miR-200c, miR-205, miR-224, miR-335, mir-424, miR-451, miR-491 were found lower by 24% in FGRs than in controls (Mouillet et al., 2010).
Significantly, enhanced levels of a group of six hypoxia-induced miRNAs (miR 210, miR 21, miR 424, miR 199a, miR 20b, and miR 373) are seen in the maternal blood of women diagnosed with FGR, suggesting increased expression and secretion of these miRNAs under hypoxic conditions in the placenta of FGRs. Furthermore, the expression increased in keeping with the degree of in-utero hypoxia estimated by fetal Doppler velocimetry (Whitehead et al. 2013).

Moreover, some studies on SGA fetuses reveled that maternal plasma levels of hsa-miR-374a-5p, hsa-miR-191-5p, and hsa-let-7d-5p, were higher in SGA cases whereas hsa-miR-107, hsa-miR-30e-5p and hsamiR-4454+miR-7975 were lower in SGA cases compared to controls between 12+0 to 21+6 weeks of gestation (Kim et al., 2020). In an other study, miR-20b-5p, miR-942-5p, miR-324-3p, miR-223-5p and miR-127-3p maternal plasma levels were significantly lower in SGA cases compared to controls (Rodosthenous et al., 2017).

The identification of common miRNAs that are dysregulated in fetal growth restriction could revealed candidate gene targets primarily involved in FGR pathogenesis.

1.6.3 Circulating FGR-related miRNAs and their potential target genes

The data regarding miRNAs in placenta and plasma of FGR pregnancies are limited; therefore, investigation of FGR pregnancy-associated, placenta-specific miRNAs is likely to shed light on the molecular mechanisms of human FGR etiology.

Wang and coworkers evaluated the expression levels of six imprinted genes (Col9a3, Dlk1, Fuca1, Lilrb4, Sfrp2, and Ventx), which were differentially expressed in umbilical cord blood of FGRs versus controls. Three protein-coding genes in the umbilical cord blood (lncRNAs RP11_552M6.1, LINC01291, and Asgr1), and three in the maternal peripheral blood (Sfrp2, miR-432-5p, and miR-1306-3p) had significantly predictive power for FGR. These findings suggest a cluster of molecular signatures that are potentially diagnostic and predictive markers of FGR (Wang et al., 2020).

In women destined to develop late-onset FGR there was increased expression of Adrenomedullin (ADM), Activating Transcription Factor 3 (ATF3), Tachykinin 3 (TAC3), Insulin like growth factor 1 (IGF1), Kisspeptin 1 (KISS1), and Syncytin (ERWVE1) in maternal blood at 26-30 weeks gestation. To improve the discriminative performance an optimal combination of four biomarkers (ADM, ATF3, TAC3 and IGF1) was used. The sensitivity was 79% and specificity 88% for late-onset FGR < 3rd centile, with a likelihood ratio of 6.2 (Whitehead et al., 2016).

Among 339 differentially expressed protein-coding genes, 224 genes were down-regulated and 115 genes were up-regulated in the umbilical cord blood and maternal peripheral blood obtained from 12 FGRs respect to 12 controls. The gene ontology (GO) and signaling pathway enrichment analyses showed that these down-regulated genes were mainly enriched in plasma membrane, arachidonic acid binding, and neutrophil degranulation. Further signaling pathway analysis indicated that these genes were mainly

involved in osteoclast differentiation, phagosome, and lysosome. Additionally, the up-regulated genes were mainly enriched in MHC class I protein complex binding, the cellular defense response, natural killer cell-mediated cytotoxicity and Graft-versus-host disease. Further gene set enrichment analysis (GSEA) results showed that these genes were not only enriched in known FGR-related processes, but also significantly associated with metabolism, neural and cardiac systems, and immune system (Wang et al., 2020).

Knowledge of the molecular mechanisms underlying placental gene regulation may be enhanced by identifying miRNA biomarkers for FGR risk. In addition, these aberrant patterns of miRNA expression may lead to the identification of previously unknown pathways that are perturbed and that are targets for novel drug treatment or prevention strategies. Advances in characterizing placental mRNA expression associated with FGR placenta-specific miRNAs could provide a better understanding of the epigenetic regulatory mechanisms in the placenta. Future work characterizing associations between dysregulated FGR placenta-specific miRNA expression and assessments of fetal growth will be the key in understanding how dysregulated FGR placenta-specific miRNA expression may lead to FGR. Moreover, understanding how dysregulation of these miRNAs leads to dysregulation of their target gene protein levels will also be essential in further understanding the effects of environmental exposures on placental function and potentially downstream fetal programming. In addition, investigations about miRNA levels in both placenta and plasma should evaluate the possible overlapping among FGR, preeclampsia, and gestational diabetes, since they all have in common placental vascular alterations due to angiogenic disbalance (Awamleh et al., 2019).

1.6.3.1 MicroRNA-15/107 Family

As described above, the 5′ end-portion of miRNAs is particularly important to define the function of microRNA. Comparing the sequence of this portion, from human genes in the miRBase miRNA Registry (Griffiths-Jones et al., 2004), it was evidenced that different miRNAs share the sequence that is crucial for the interaction with 3'UTRs of target genes. An example is represented by miR-15/107 family, whose members are a group of 12 paralogous evolutionarly-conserved miRNAs, with the sequence AGCAGC in common, starting from the first of the second nucleotide of the 5' end of the mature miRNA (Finnerty et al., 2010; Wang et al., 2014b). In the Table 1.1 it's illustrated the list of miRNAs belonging to this family and their chromosome position. MiR-103 and miR-107 paralog genes reside in the pantothenate kinase (PANK) genes introns; these genes get important

metabolism-related cell functions, indeed they are central enzymes in the regulation of cellular Co-enzyme A levels. The location of miR-103 and -107 genes within introns of the PANK genes may be physiologically relevant, and some studies have showed that both miRNAs act symbiotically with the PANK proteins (Wilfred et al., 2007). Moreover, expression levels of this pair miRNAs and their host genes often are highly correlated, presumably because they are co-transcribed. On the other hand, miR-16 is located in genes related to cell proliferation, SMC4 and DLE2. The function of the genes related to the other members of miR-15/107 family is still unknown. Nevertheless, studies collected in the scientific literature provide clues that these miRNAs play key roles in gene regulation involved in physiological mechanisms, such as cell division, metabolism, stress response and angiogenesis, and implicated in pathological processes including cancers, cardiovascular disease and neurodegenerative diseases. All these function are due to the capacity of miR-15/107 group to target various mRNAs, including GRN, DICER1, BCL2, CDK5R1, BDNF, CDK6 and BACE1 (Finnerty et al., 2010). MiRNA profiling experiments have shown that miR-15/107 group miRNAs are expressed at moderate-to-high levels in many mammalian tissues. The most recent expression study has evidenced that similar anatomical tissues were generally clustered together; for example, heart and skeletal muscle samples demonstrated similar expression patterns (Wang et al., 2014b). However, generally, the highest-expressing miRNAs in humans tend to be miR-15a, miR-15b, miR-16, miR-103 and miR-107. Members of miR-15/107 are involved in different physiological functions, for example regulation of pathways related to mitosis. Specifically, it was shown that miR-16 and miR-103 target a highly disproportionate number of cell cycle genes, and transfection with these miRNAs led to G0/G1 arrest (Linsley et al., 2007). However, the main studies about the role of these miRNAs were performed on their involvement in the regulation of cellular metabolism. First of all, miR-107 expression was demonstrated to be altered following increased extracellular glucose (Tang et al., 2009) and after dietary intake of various lipids (Davidson et al., 2009). Furthermore, miR-103 and -107 are shown to be upregulated in obese mice, and their silencing led to improved glucose homeostasis and insulin sensitivity (Trajkovski et al., 2011). Indeed, caveolin-1, a critical regulator of the insulin receptor, was identified as a direct target gene of miR-103/107, suggesting that this protein is upregulated upon inactivation of miR-103 and miR-107 in adipocytes, contributing to the stabilization of the insulin receptor and enhance of insulin signalling. Multiple stress conditions have been associated with miR-15/107 function. MiR-16 expression increased in cultured cells exposed to ultraviolet light and then appeared to

participate in regulating the DNA-damage response (Pothof et al., 2009). On the other hand, miR-16 expression decreased in mice subjected to exposure with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a common environmental toxicological contaminant (Zhang et al., 2009). In addition, after induction of rodent traumatic brain injury miR-107 expression is down-regulated, and this reduction could mediate the activation of adaptive responses (Redell et al., 2009). Finally, miR-103 and miR-107 are strongly induced by hypoxia in vascular endothelial cells and by targeting AGO1 they are shown to promote angiogenesis (Chen et al., 2013), and more specifically microRNA-107 contributes to post-stroke angiogenesis by targeting Dicer-1 (Li et al., 2015). On the other hand, miR-16 appears to regulate VEGF, thus affecting angiogenesis (Karaa et al., 2009).

The involvement of miR-15/107 cluster in various human cell pathways suggests that the dysregulation of its gene expression may contribute to, or even cause, human diseases. For example, miR-16 constitute key tumor suppressors whose deletion contributes to cancer: in particular they are located in regions that when subjected to deletion might contribute to susceptibility to chronic lymphocytic leukemia (Calin et al., 2005). Moreover, granulin (GRN), which is regulated by miR-15/107 gene group members, is an active mitogen and growth factor relevant to many cancers.

MiRNAs get a direct relevance to human neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD) and frontotemporal dementia (FTD). Members of the miR-15/107 group have been shown to be downregulated in AD. Specifically, miR-107 is associated to the early stage of AD pathogenesis (Nelson and Wang, 2010; Wang et al., 2008), and in addition it targets the beta-amyloid cleavage enzyme 1 (BACE1) gene, so its downregulation may mediate the pathogenetic consequences. Moreover, miR-107, but also other group members, strongly regulate the expression of GRN, which is a gene that increases predisposition to FTD (Wang et al., 2010a). Members of the miR-15/107 group are also predicted to target neurodegeneration-related genes such as the β-amyloid precursor protein (involved in AD) and α-synuclein (involved in PD).

Table 1.1: The “AGCAGC” sequence for evolutionarily conservative miR-15/107 family (modified by Wang et al., 2019)

miRNAs	Sequence (from 5′ to 3′)	Transcript location	Chromosome position
hsa-miR-15a-5p	UAGCAGCACAUAAUGGUUUGUG	Intron	chr13: 50049119–50,049,201 [−]
hsa-miR-15b-5p	UAGCAGCACAUCAUGGUUUACA	Intron	chr3: 160404588–160,404,685 [+]
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	Intron	chr3: 160404745–160,404,825 [+]
		Intron	chr13: 50048973–50,049,061 [−]
hsa-miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA	Intron	chr5: 168560896–168,560,973 [−]
		Intron	chr20: 3917494–3,917,571 [+]
hsa-miR-107	AGCAGCAUUGUACAGGGCUAUCA	Intron	chr10: 89592747–89,592,827 [−]
hsa-miR-195-5p	UAGCAGCACAGAAAUAUUGGC	N/A	chr17: 7017615–7,017,701 [−]
hsa-miR-424-5p	CAGCAGCAAUUCAUGUUUUGAA	N/A	chrX: 134546614–134,546,711 [−]
hsa-miR-497-5p	CAGCAGCACACUGUGGUUUGU	N/A	chr17: 7017911–7,018,022 [−]
hsa-miR-503-5p	UAGCAGCGGGAACAGUUCUGCAG	N/A	chrX: 134546328–134,546,398 [−]
hsa-miR-646	AAGCAGCUGCCUCUGAGGC	N/A	chr20: 60308474–60,308,567 [+]
hsa-miR-6838-5p	AAGCAGCAGUGGCAAGACUCCU	Exon/intron	chr7: 44073378–44,073,433 [−]

1.6.3.2 MicroRNA-27b

MiR-27b is an intragenic microRNA located on chromosome 9q22.1 within the C9orf3 gene. More and more studies indicate that microRNA-27b is involved in affecting various biological processes, such as angiogenesis, proliferation, metastasis, and drug resistance, and thus may act as a promising therapeutic target in human cancers (Ding et al., 2017). MiR-27b was targeted and down-regulated by NR2F2 in human gastric cancer tissues and cells. The ectopic expression of miR-27b inhibited gastric cancer cell proliferation and tumor growth in vitro and in vivo (Feng et al., 2017). MiR-27b-3p significantly promotes the migration and invasion of colorectal cancer cells by targeting the HOXA10/integrin β1 cell signal axis (Yang et al., 2019). MiR-27b and miR-34a were significantly downregulated in docetaxel-resistant prostate cancer cells. Gain-of-function experiments showed that overexpression of miR-27b or miR-34a enhanced docetaxel sensitivity and inhibited

epithelial-to-mesenchymal transition in docetaxel-resistant prostate cancer cells. Moreover, miR-27b and miR-34a was demonstrated to directly suppress ZEB1 expression, one factor of the zinc finger E-box-binding (ZEB) family. Loss-of-function analysis disclosed that ZEB1 knockdown enhanced docetaxel sensitivity and suppressed epithelial-to-mesenchymal transition in docetaxel-resistant prostate cancer cells (Zhang et al., 2018). The expression of miR-27-3p was significantly increased in examined osteosarcoma cell lines compared with that in normal osteocyte cell lines. Up-regulation of miR-27-3p significantly accelerated osteosarcoma cell growth via promoting G1-S transition. In addition, the opposite result was observed in miR-27-3p-down-regulated cells (Ye et al., 2018). MiR-27 overexpression promoted multiple myeloma cell proliferation, facilitated cell cycle progression, and expedited cell migration and invasion; whereas miR-27 knockdown inhibited cell proliferation, induced cell cycle arrest, and slowed down cell motility. Besides, miR-27 ablation suppressed tumorigenicity of multiple myeloma cells in mouse xenograft models (Che et al., 2019).

In a context of myocardial cell damage induced by hypoxia/reoxygenation miR-27 prevents atherosclerosis by inhibiting inflammatory responses induced by lipoprotein lipase. Overexpression of miR-27b attenuates angiotensin-induced atrial fibrosis. MiR-27 repressed cardiomyocyte injury induced by hypoxia/reoxygenation via mediating TGFBR1 and inhibiting NF-κB signaling pathway (Zhang et al., 2019). Up-regulation of miR-27 increased cell proliferation and reduced cell apoptosis, while down-regulation of miR-27 suppressed cell growth and promoted cell apoptosis. Intercellular adhesion molecule 1 (ICAM1) was a predicted target gene and it is negatively regulated by miR-27 (Xiang et al., 2019). Moreover, miR-27b-3p induces acyl-CoA thioesterase 2 (ACOT2) expression in pre-adipose cells, enhancing the pre-adipocyte to adipocyte differentiation associated with lipid accumulation and intracellular triglyceride contents. Also that the knockdown of ACOT2 expression in pre-adipose cells suppresses lipid accumulation and adipocyte differentiation in both the presence and absence of miR-27b-3p treatment (Murata et al., 2019). MiR-27b plays an important role in preventing hypertrophic chondrogenesis of human bone marrow-derived mesenchymal stem cells (hBMSCs) by targeting core-binding factor β-subunit (CBFB) and is essential for maintaining a hyaline cartilage state. In fact, miR-27b-overexpressing hBMSC chondrogenic pellets had better hyaline cartilage morphology and reduced expression of hypertrophic markers and tend to increase repair efficacy in vivo (Lv et al., 2020). Finally, miR-27b expression was decreased in fibroblast-like synoviocytes (FLSs) cells stimulated by TNF-α. Consistently, enhanced miR-27b

expression results in the suppression of proliferation and the promotion of apoptosis in FLSs cells stimulated by TNF-α, partially by regulating IL-1β expression and NF-κB signaling (Lei et al., 2019).

2. AIM

Hypoxia may occur acutely during labour and birth, or develop gradually across pregnancy in cases of FGR due to placental dysfunction (ACOG, 2013; Baschat, 2011).

Ultrasound-based tests and the cardiotocography (CTG) are commonly used to detect the presence of fetal hypoxia/acidemia. While these tests have improved outcomes including perinatal survival, none of them can reliably determine which pregnancies are affected by significant fetal acidemia. Therefore, a part of preterm FGR are still lost to stillbirth or suffer significant perinatal asphyxia injury (Baschat et al., 2007), highlighting the importance to choose surveillance intervals that are appropriate for the degree of fetal deterioration. Unfortunately, the optimal surveillance pattern remains the object of much debate and research has primarily focused on intervention thresholds. There is no general consensus between national guidelines on the appropriate frequency of testing, and they are based on expert opinion of key authors because there is no high quality evidence to guide practice. Integrated fetal testing, including multi-vessel Doppler examination and computerized cardiotocography (cCTG) is appropriate (Wolf et al., 2017). The monitoring frequency needs to be increased when there are additional signs of deterioration until the delivery threshold is reached. It is critical to recognize that signs of clinical acceleration differ according to the gestational age when FGR is diagnosed.

In recent years, miRNAs have been identified in the maternal blood during pregnancy, which may reflect both physiological and pathological placental conditions. This offers a potential new avenue to develop a screening test for pregnancy complications. Hypoxia has a profound effect on gene regulation and miRNAs play a vital role in the cellular response to hypoxia. Therefore, we hypothesized that measuring hypoxia regulated circulating miRNAs in the maternal blood may be a promising strategy to identify biomarkers for fetal hypoxia in-utero.

1) The first aim was to investigate the role of ultrasound biometry, maternal and fetal Doppler to discriminate between early- and late-onset FGRs and assess the severity of FGRs (see “Placenta associated disorders: Fetal Growth Restriction” section);

2) The second aim was to introduce new computerized Cardiotocographic (cCTG) parameters to improve the surveillance and management of FGRs in relation to the timing of delivery (see “Electronic Fetal Heart Rate monitoring” sub-section);
3) The third aim was to examine the expression levels of several maternal blood microRNAs known to be regulated by hypoxia with the intent to identify putative markers of fetal deterioration and their potential target genes (see “MicroRNAs Regulating Placental Function” section).

3. MATERIALS AND METHODS

3.1 Experimental groups

All subjects were pregnant women between 18 and 46 years old hospitalized at the Department of Obstetrical-Gynaecological and Urological Science and Reproductive Medicine of the Federico II University (Italy) in a period of two years (2018-2020). All pregnant women gave their written informed consent prior to inclusion in the study protocol.
The diagnosis of FGR was based on the evaluation of estimated weight below the 10^{th} centile and/or estimated abdominal circumference below the 10th centile, according to the gestational age. The diagnosis was also based on Pulsatility Index (PI) of umbilical artery (UA) >95^{th} centile for the gestational age in early-onset FGR, irrespective of the presence or absent of reversed end-diastolic flow; while in late-onset FGR a cerebro-placental ratio (CPR) <5^{th} centile or UA-PI >95^{th} centile was used.
To discriminate between early- and late-onset FGR, the study population was divided into two subgroups according to the gestational age at delivery: (group I) <32^{nd} weeks of gestation; (group II) from 32^{nd} to 37^{th} weeks of gestation (Gordijn et al., 2016).
Healthy group included fetuses whose growth were appropriate for gestational age without any chromosomal or major congenital anomalies. Healthy pregnancies were admitted in hospital for preterm contractions, without premature rupture of membranes (PPROM) or vaginal swab positive for infections, resolved spontaneously without premature delivery. All healthy pregnant women subsequently delivered appropriately grown neonates at term without obstetric complications. Healthy group was divided into two subgroups to match them to FGR fetuses: (group I) <32^{nd} weeks of gestation; (group II) from 32^{nd} to 37^{th} weeks of gestation.
Starting from a population of 1400 pregnant women, 77 pregnant women composed of 34 complicated by FGR and 43 with healthy fetuses fulfilled the criteria of the study.

3.2 Inclusion criteria

Inclusion criteria were Caucasian ethnicity; singleton pregnancy; certain pregnancy dating (calculated from the first day of the last menstrual period and confirmed by ultrasound measurements, according to the population nomograms) (Butt and Lim, 2014); gestational age from 26th to 37th week. All FGRs and Healthy fetuses were evaluated by ultrasound biometry, Doppler measurements and cCTG monitoring. Only the last cCTG record within 24h before delivery was considered for FGRs. cCTG records with a signal loss more than 15% over the whole record were excluded.

Maternal whole blood samples were collected after two weeks of hospitalization for both FGRs and gestation matched controls, and they were sent immediately to the Pharmacology Unit in the Neuroscience Department for analysis. Blood samples collected in the last 48 hours before labour or elective/urgent cesarean section were excluded in FGRs. Pre-existing maternal disease, such as hypertension, diabetes, epilepsy, heart and renal disease, or autoimmune disorders were excluded. Maternal diseases arising or related to pregnancy were also excluded. Smoking, coffee, alcohol and drug abuse were excluded. Fetuses with chromosomal, genetic and congenital anomalies diagnosed during pregnancy or after birth were excluded. As regards delivery, only pregnant women whose elective or urgent delivery occurred for fetal indication were considered in the FGR group, excluding maternal or gestational indications. In this way, they were selected only pregnancies complicated by fetal growth retardation, avoiding the overlap with preeclampsia and gestational diabetes that share common placental vascular alterations due to angiogenic disbalance.

Newborn baby data (sex, weight, Apgar score, malformations at birth, umbilical artery gas and pH values, access to neonatal intensive care) were recorded. Newborns with missing data or inadequate umbilical cord samples at birth (insufficient blood sampling and/or errors in pH and gas analysis by the pH meter) were excluded.

Neonatal and infant data (included survival without cerebral palsy, severe neurosensory impairment, or low Bayley Scales of Infant Developmental score) at 1 years of age were collected.

3.3 Assessment of growth restriction severity

The severity of the growth restriction was assessed by ultrasound biometry once a week, fetal Doppler velocimetry of UA, middle cerebral artery (MCA), ductus venosus (DV) twice a week and daily cCTG monitoring. Doppler velocimetry was considered abnormal when

PI of UA and DV was >95th centile according to the gestational age (Figueras and Gratacos, 2014); when absent or reverse end-diastolic flow in UA and in DV were detected (Baschat, 2011; Kessous et al., 2014) or MCA-PI was <5th centile according to the gestational age (Ebbing et al., 2007; Bhide et al., 2013).

The conventional CTG monitoring was evaluated as normal, suspicious or pathological according to the classification criteria of FIGO guidelines, which includes FHR baseline, variability, decelerations, large/small accelerations, and contractions estimation (Ayres-de-Campos et al., 2015). Additional computerized analysis was evaluated according to the standard of computerized system analysis (Arduini et al., 1993). In particular, abnormal cCTG was defined as FHR STV < 2.6 ms between 26 + 0 and 28 + 6 weeks of gestation, STV < 3 ms between 29 + 0 and 31 + 6 weeks', STV < 3.5 ms between 32 + 0 and 33 + 6 weeks', STV < 4.5 ms between 34 + 0 and 36 + 6 weeks' gestation. Abnormal cCTG analysis was absolute indications for delivery. In many cases, the delivery occurred after at least 24h the administration of maternal steroids before 34 weeks.

Healthy fetuses were subjected to ultrasound biometry, Doppler and cCTG monitoring with the same frequency and at the same gestational age of FGR ones.

3.3.1 Signal Acquisition

The antepartum Doppler velocimetry was performed using a Voluson E8 (General Electric Healthcare Technologies, Milwaukee, WI, USA) ultrasound machine equipped with a 2–8 MHz transabdominal transducer. Color Doppler imaging was used to identify the UA and MCA vessels. Doppler flow spectra were obtained from the UA at the midsection of the umbilical cord and the distal portion of the MCA by methods that have been previously described (Oros et al., 2011).

The cCTG records were obtained using Philips Avalon FM30, equipped with an ultrasound transducer and a transabdominal tocodynamometer and lasted at least 60 minutes. These fetal monitors use an autocorrelation technique to compare the demodulated Doppler signal of a heartbeat with the next one, in order to obtain the heart period (the equivalent of RR period). The resulting heart period is then converted into a heart frequency in beats per minute as soon as a new heart event is detected and accepted. Philips monitors produce a FHR value in bpm every 250 ms (4 values/second).

The cardiotocograph was connected to the 2CTG2 system (Arduini et al., 1993), which reads both FHR and toco signals two times per second. This frequency represents a

reasonable compromise to achieve enough bandwidth and acceptable accuracy for an advanced analysis including non-linear parameters (Signorini et al., 2020). Each CTG recording was divided in subintervals of either 120 (60 s) and 360 points (180 s), after noise and artifacts removal. The use of 1-minute or 3-minutes subintervals is related to differences in the extracted parameters as explained in the next section.

3.3.2 FHR analysis

A set of parameters according to the standard CTG analysis were computed. This set includes time domain measures: FHR mean over 1 min, Delta FHR, Short Term Variability (STV), Long Term Irregularity (LTI), Interval Index (II) as reported in Arduini et al. (Arduini et al., 1993).

Moreover, the 2CTG2 software allows computing frequency domain and nonlinear parameters, which aim at connecting FHRV behavior to physiological heart controlling systems. Power Spectral Density provide frequency components in specific bands with their associated power: Low Frequency, LF; Movement Frequency, MF; High Frequency, HF; LF/ (HF+MF) ratio, (LF/ (HF+MF) (Signorini et al., 2003). Approximate Entropy (ApEn) quantifies regularity and complexity of the FHR time series (Richman et al., 2000). In order to obtain a reliable estimation for these parameters, they were calculated on 3 min subintervals (360 points). For a more detailed description of cCTG parameters please refer to (Signorini et al., 2014).

3.4 Plasma samples collection

Human blood samples were withdrawn from pregnant women and collected in BD Vacutainer tubes (K3 EDTA 5.4mg). For plasma separation, blood samples were centrifuged in ALC PK 120 centrifuge at 1500 x g (2900rpm) for 8 minutes at room temperature. The supernatant plasma was transferred to sterile 2.0ml tubes and centrifuged in Eppendorf centrifuge at 11,000rpm to clean the sample from any cellular residues. The absorbance at 415nm of a 50µl aliquot for each sample was measured in a Bio-rad Microplate Reader to evaluate the possibility of a previous haemolytic process during blood withdrawal. Only samples with absorbance values less than 0.5OD were used for next analyses.

Maternal plasma samples were collected after two weeks of hospitalization in all cases and at least 48 hours before delivery in FGRs. In this way, pregnant women shared the

same hospital setting and they had well understood and accepted intensive fetal surveillance until delivery. It was collected a single maternal plasma sample per patient.

3.5 MicroRNA isolation and assessment by Real-Time Polymerase Chain Reaction

MiRNA isolation from plasma samples was performed by using miRNeasy Serum/Plasma Kit (Qiagen) according to the manufacturer's protocol. Precise volumes (5µl) of RNA were retrotranscribed by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and Taqman probes (Taqman MicroRNA Assays, Thermo Fisher Scientific), following TaqMan Small RNA Assays Protocol (16˚C for 30 minutes, 42˚C for 30 minutes and 85˚C for 5 minutes). Quantitative real-time polymerase chain reaction was performed with TaqMan Universal PCR Master Mix II (Applied Biosystems) in a 7500 Fast Real-Time PCR System (AB Applied Biosystems). cDNA samples were amplified simultaneously in triplicate in 1 assay run, following the protocol for Taqman assays: 50°C for 2 minutes, 95°C for 10, 40 cycles of amplification of 95°C for 15 seconds and 60°C for 1 minute. Results were analysed and exported with 7500 Fast System SDS Software.

TaqMan probes used are the following: hsa-miR-16 (ID: 000391); hsa-miR-103 (ID: 000439); hsa-miR-107 (ID: 000443); hsa-miR-27b (ID: 000409); miRNA Control Assay U6 snRNA (ID: 001973).

3.6 Information on microRNA-gene-Disease ontology interactions

miRWalk (available: http://mirwalk.umm.uni-heidelberg.de), MiRDB (available: http://mirdb.org/miRDB/) and Targetscan databases (available: http://www.targetscan.org/vert_72/) were used to predict targets of four microRNAs whose expression in blood samples of pregnant women was affected in case of FGR. These databases includes predicted and validated biological targets of miRNAs, by searching for the presence of conserved sites that match the seed region of each miRNA, to provide information on interaction.

3.7 Statistical analysis

Values were expressed as means ± SEM. In particular, Real-Time PCR results are expressed as fold change ($2^{-\Delta\Delta Ct}$) compared to the control group, following the instructions provided by the literature (Livak and Schmittgen, 2001). Briefly, difference between Ct values of gene of interest and internal control (ΔCt) is calculated for both control sample and target sample. Then, difference between ΔCt of target sample and control sample

(ΔΔCt) is calculated. Fold change of gene expression of target samples compared to control sample is calculated as $2^{-\Delta\Delta Ct}$.

Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA), using t-test for continuous variables and chi-square test for categorical variables. Statistical significance was accepted at the 95% confidence level ($p < 0.05$).

4. RESULTS

All pregnant women were hospitalized at the Division of Obstetric and Gynaecology, School of Medicine, University of Naples "Federico II", so that each group and subgroup included patients with the same lifestyle, that is the same low-calorie diet with similar energy consumption, no alcohol or smoking consumption. Moreover, the same clinical environment shared for long time helped pregnant women to better understand and accept the intensive pregnancy monitoring, to trust medical choices and to reduce anxiety levels.

Patients were matched according to the maternal age, BMI, number of previous pregnancies and parity, in order to reduce confounding factors.

Most of Healthy fetuses were delivered spontaneously between the 37th and 41th week of gestation, while all FGRs were delivered with cesarean section between the 30th and 36th week of gestation. The time interval between maternal blood sampling and delivery was few days for FGRs and few weeks for healthy fetuses, because all healthy fetuses were delivered at term.

As regards neonatal data, no difference in pH or blood gas analysis umbilical artery values at birth were found. The 1' and 5' minute Apgar score at birth were also similar between the two groups. Female newborns were more numerous in FGR than Healthy group. As expected, the difference in newborns weight was due to both growth retardation and the need for preterm delivery in FGRs respect to Healthy fetuses. The percentage of NICU admission in FGRs was significantly higher than Healthy group, mainly related to the different newborns weight between the two groups; under 2000 gr. almost all newborns were admitted in NICU (Table 4.1a). The comparison between the two FGRs subgroups did not show differences in maternal data, as age, BMI, gravidity, parity and timing of maternal blood sampling. Early-onset FGRs were delivered before and the newborns had lower birth weight than late-onset FGRs, according to the different severity of growth retardation. None of FGRs was delivered in conditions of severe late deterioration, as shown by umbilical artery pH and blood gas analysis data at birth, and no stillbirth cases occurred. Finally, female newborns were prevalent in late-ones FGRs than early-ones

(Table 4.1b). A stratified analysis according to the newborns sex showed no differences (data not shown).

Table 4.1a. Differences in maternal and neonatal data between FGR and Healthy fetuses

	FGR (n=34) (mean ± std†)	**Healthy (n=43) (mean ± std)**	**p-value***
Maternal data			
Age (yrs)	33,1 ± 6,3	31,8 ± 6,7	0,423
BMI	29,29 ± 4,58	30,25 ± 6,07	0,15
Gravidity (n)	1,8 ± 1,0	1,6 ± 0,8	0,595
Parity (n)	0,1 ± 0,4	0,1 ± 0,4	0,851
Week of delivery (wks)	33,1 ± 2,9	39,2 ± 2,6	**<0,001****
Cesarean section (%)	100	35	**<0,001**
Timinig of sampling before delivery (dys)	7 ± 4	38 ± 18	**<0,001**
Neonatal data			
Fetal pH at birth	7,27 ± 0,10	7,32 ± 0,07	0,317
pCO_2 (mmHg)	50,35 ± 14,67	37,44 ± 3,11	0,062
pO_2 (mmHg)	22,79 ± 11,82	25,44 ± 6,99	0,671
Base Excess (mmol/l)	-4,08 ± 3,40	-4,44 ± 7,24	0,856
Lactate (mEq/l)	4,53 ± 1,26	4,63 ± 2,58	0,935
1 min Apgar score	7,71 ± 0,91	7,37 ± 1,27	0,487
5 min Apgar score	8,47 ± 0,68	8,60 ± 0,89	0,700
Female (%)	68,9	56,4	**0,04**
Birth weight (gr)	1592 ± 465	2544 ± 711	**<0,001**
NICU (%)	58,8	5,1	**<0,001**

†STD, standard deviation.

*p-value for comparison between FGR and Healthy using T student two sides for continuous variables and chi-square test for categorical variables (values are expressed as a percentage).

**values in bold are statistically significant.

BMI: Body Mass Index; NICU: Neonatal Intensive Care Unit.

Table 4.1b. Differences in maternal and neonatal data between early- and late-onset FGRs

	FGR<32 wks (n=18) (mean ± std†)	FGR 32-37 wks (n=16) (mean ± std)	p-value*
Maternal data			
Age (yrs)	32,9 ± 4,2	33,3 ± 8,3	0,862
BMI	29,29 ± 4,58	31,25 ± 6,07	0,22
Gravidity (n)	1,7 ± 0,9	1,8 ± 1,2	0,804
Parity (n)	0,1 ± 0,2	0,2 ± 0,6	0,352
Week of delivery (wks)	31,8 ± 1,1	34,7 ± 2,1	**0,004****
Timinig of sampling before delivery (dys)	7 ± 4	8 ± 5	0,31
Neonatal data			
Fetal pH at birth	7,27 ± 0,13	7,27 ± 0,07	0,859
pCO_2 (mmHg)	49,26 ± 17,4	51,53 ± 11,56	0,685
pO_2 (mmHg)	24,76 ± 17,89	20,81 ± 4,18	0,728
Base Excess (mmol/l)	-4,64 ± 3,55	-3,44 ± 3,23	0,343
Lactate (mEq/l)	4,29 ± 2,63	4,65 ± 0,55	0,784
1 min Apgar score	7,06 ± 1,48	7,71 ± 0,91	0,165
5 min Apgar score	8,25 ± 0,68	8,71 ± 0,61	0,061
Female (%)	55,5	62,5	**0,04**
Birth weight (gr)	1303 ± 422	1922 ± 337	**<0,001**
NICU (%)	83,8	35,4	**<0,001**

†STD, standard deviation.

*p-value for comparison between early- and late-onset FGR using T student two sides for continuous variables and chi-square test for categorical variables (values are expressed as a percentage).

**values in bold are statistically significant.

BMI: Body Mass Index; NICU: Neonatal Intensive Care Unit.

Comparing Doppler measurements, UA_PI values had a high discriminatory power between FGR and Healthy before the 32nd week, while no difference was found between the 32nd and 37th week. On the other hand, lower MCA_PI values were found both in early- and late-onset FGRs than matched controls, consistently with results published by Baschat (Baschat, 2018). No early-onset cases with ductus venosus (DV)-PI >95th centile

or absent/reversed waves A flow were observed, because delivery was undertaken before it occured.

Comparison analysis of cCTG parameters, in order to identify which parameter or parameter set was most efficient in the discrimination task between hypoxic and non-hypoxic fetuses, reveled statistical significant difference for STV values investigated between FGR and matched Healthy fetuses <32nd week. No statistically significant difference was found for others cCTG parameters, except for HF between 32nd and 37th week (Table 4.2).

Table 4.2. Differences in Doppler and cCTG data between FGR and Healthy fetuses matched for gestational age.

Doppler and cCTG data	**FGR (n=34) (mean ± std†)**	**Healthy (n=43) (mean ± std)**	**p-value***
UA_PI			
< 32nd	1,62 ± 0,52	1,10 ± 0,19	**<0,01****
32nd - 37th	1,14 ± 0,31	0,99 ± 0,26	0,06
MCA_PI			
< 32nd	1,16 ± 0,27	1,44 ± 0,18	**0,02**
32nd - 37th	1,18 ± 0,35	1,41 ± 0,22	**0,01**
Time parameter			
FCF (bpm)			
< 32nd	141,97 ± 9,04	140,93 ± 11,88	0,751
32nd - 37th	136,64 ± 8,71	143,80 ± 9,08	0,070
STV (ms)			
< 32nd	3,46 ± 2,13	5,09 ± 1,94	**0,032**
32nd - 37th	5,99 ± 2,31	7,10 ± 2,05	0,277
LTI (ms)			
< 32nd	20,16 ± 6,20	18,06 ± 5,36	0,320
32nd - 37th	20,05 ± 5,80	24,27 ± 5,85	0,106
Delta (ms)			
< 32nd	35,99 ± 10,95	34,89 ± 11,72	0,774
32nd - 37th	38,62 ± 12,00	44,87 ± 7,31	0,225
Interval Index			
< 32nd	1,01 ± 1,09	0,87 ± 0,07	0,693

32nd - 37th	0,84 ± 0,04	0,84 ± 0,04	0,863
Non Linear Parameters			
ApEn			
< 32nd	1,23 ± 0,14	1,27 ± 0,17	0,475
32nd - 37th	1,27 ± 0,13	1,21 ± 0,17	0,329
Spectral analysis			
LF (ms^2)			
< 32nd	81,00 ± 5,76	82,33 ± 4,54	0,493
32nd - 37th	80,32 ± 5,88	81,52 ± 3,46	0,631
MF (ms^2)			
< 32th	14,84 ± 4,38	13,94 ± 2,99	0,535
32th - 37th	15,26 ± 4,13	14,38 ± 2,19	0,614
HF (ms^2)			
< 32nd	4,15 ± 1,75	3,72 ± 1,79	0,476
32nd - 37th	4,42 ± 2,02	10,11 ± 15,19	**0,027**
LF/(HF+MF)			
< 32nd	3,52 ± 2,12	3,79 ± 2,07	0,718
32nd - 37th	4,03 ± 1,99	3,51 ± 1,83	0,553

†STD, standard deviation.

*p-value for comparison between Healthy and FGR using T student two sides, according to the gestational age.

**values in bold are statistically significant.

UA_PI: Umbilical Artery Pulsatility Index; MCA_PI: Middle Cerebral Artery Pulsatility Index; FHR: Fetal Heart Rate; STV: Short Term Variability; LTI: Long Term Irregularity; Delta: Delta Index; II: Interval Index; ApEn: Approximate Entropy; LF: Low Frequency; MF: Movement Frequency; HF: High Frequency; LF/(HF+MF), the LF/(HF+MF) ratio.

miR-16-5p, miR-103-3p, and miR-27b-3p increase in blood of FGR pregnant women <32nd week of gestation.

The expression levels of four microRNAs were evaluated by real-time PCR in plasma samples of pregnant women complicated by FGR before the 32nd week and between the 32nd and the 37th week of gestation, compared to control samples withdrawn at the same gestational age (Figure 4.1).

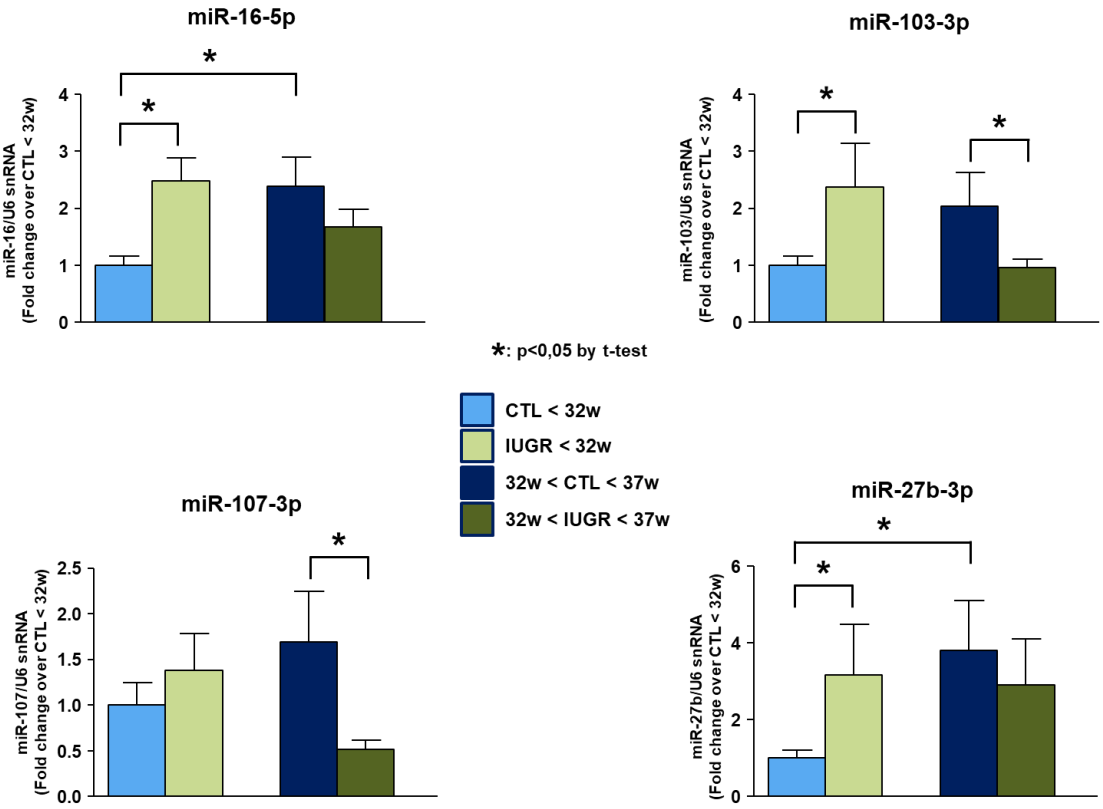


Figure 4.1. Expression analysis by Real-Time PCR of hypoxia induced miRNA in maternal blood from women with preterm FGR compared to gestation matched controls.

MicroRNA levels analyzed by Real-Time PCR in plasma samples are expressed as fold change of relative expression levels over the healthy group <32nd week of gestation setted at 1. Each column represents the mean ± S.E.M. Results of microRNAs expression were normalized with respect to U6 snRNA as internal control. *: p<0.05

In early-onset FGR, miR-16-5p, miR-103-3p and miR-27b-3p expression levels were upregulated than matched controls. In late-onset FGR, miR-103-3p and miR-107-3p were downregulated than matched controls. In Healthy group, miR-16-5p and miR-27b-3p were upregulated as pregnancy progressed. Interestingly, all four miRNA investigated showed a progressive trend to downregulation in FGRs and upregulation in Heathy fetuses, as pregnancy progressed. The high levels of miR-16-5p, miR103-3p and miR-27b-3p in FGR before the 32nd week of gestation could be an early indicator of gene regulation pathways underlying the FGR development.

Information on microRNA-gene-Disease ontology interactions

The association between microRNA expression in plasma blood samples of pregnant women with FGR and predicted or validated targets genes could indicate that a large group of genes may be potentially dysregulated in response to chronic hypoxia in utero (Table 4.3).

Table 4.3. A list of predicted or validated targets of miRNA dysregulated in plasma samples of pregnant women with FGR using miRWalk, miRDB and Targetscan databases.

Candidate miRNA	Molecular Targets	Predictive Softwares used	Pathway involved in FGR/IUGR
miR-16-5p	APP	miRWalk, Targetscan	Metabolic
	APLN	miRDB, Targetscan	
	COMT	miRWalk	Abnormal umbilical artery Doppler waveform
	EGFR	miRWalk	Smaller placental size
	FGFR1	miRDB, Targetscan	Fibroblast growth
miR-103-3p	APLN	miRWalk	VEGF signaling and angiogenesis
	FGF2	miRWalk, Targetscan	Placental development
	MAPK8	miRWalk, Targetscan	Differentiation and proliferation regulation
	PDE4D2	miRWalk, Targetscan	Retarded growth
	PIK3R1	miRWalk, Targetscan	Insulin secretion
miR-27b-3p	FGF	miRWalk, Targetscan	Signaling pathway
	EGFR	miRDB	Signaling pathway
	VEGF	miRDB, Targetscan	Signaling pathway
miR-107-3p	CLOCK	miRWalk, Targetscan	Metabolic following circadian rhythm
	FASN	miRWalk	Metabolic

Correlation analysis revealed candidate gene targets primarily involved in angiogenesis and placental development, secondly in metabolic pathways such as cell proliferation and growth. In particular, APLN, EGFR and FGF are targets shared by several miRNA, providing greater value to this correlation.

Infant neurological outcome at 1 years of age

The secondary outcome was to evaluate the one-year survival without neurological impairment of FGR fetuses. Normal neurological outcome occurred more often in late-onset (87.5%) than early-onset group (73.3%) (p=0.04). Women with a normal infant outcome had been randomized at a higher gestational age and the estimated fetal weight and birthweight were larger than in women with an infant who died or had abnormal development. Three neonatal deaths (16.7%) occurred in the early-onset group. No neonatal death was observed in late-onset group. Two of three death had a birth weight less than 1000gr., while the other one had a birth weight just over 1000gr. Causes for neonatal death were acute respiratory distress (despite corticosteroid prophylaxis), multi-organ failure or clinical sepsis.

Six infants (40%) in early-onset group had impaired neurological development at birth, but only four infants showed abnormal development at one year (26.7%). On the other hand, only one infant (6.2%) in late-onset group showed abnormal neurological outcome at birth and at one year, while another one infant developed severe morbidity during the first year of life. Cerebral hemorrhage and periventricular leucomalacia (PVL) have been found at birth in four of six infants with impaired neurological delivered before 32nd week, while the other two infants developed a clinical sepsi. Cerebral hemorrhage has been found also in one infant of late-onset group with neurological impairment at birth, while the other one developed severe encephalopathy at six months of life (Figure 4.2).

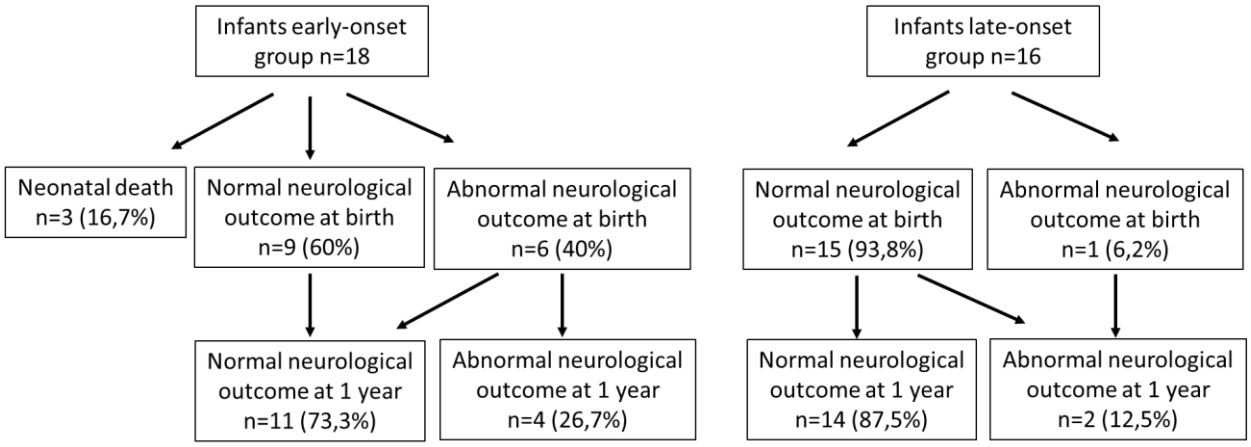


Figure 4.2. Infant neurodevelopment outcome at birth and 1 year after delivery.

5. DISCUSSION

The target of the present study was FGR with placental insufficiency excluding fetal growth restriction associated with preeclampsia, chromosomal and/or genetic abnormalities and maternal pathologies. The goal was to use new methods for early identification of fetuses with chronic hypoxia that require delivery before major morbidity or stillbirth occurs.

Inclusion criteria were based on UA-PI >95th centile for the gestational age in early-onset FGR, irrespective of the presence or absent of reversed end-diastolic flow; and a cerebro-placental ratio (CPR) <5th centile or UA-PI >95th centile in late-onset FGR. In the context of longitudinal Doppler monitoring of early-onset FGR, a progressive deterioration of UA-PI was observed with development of absent end-diastolic flow in many cases. No early-onset cases with reverse umbilical artery end-diastolic flow were observed. A brain-sparing effect with progressive deterioration of MCA_PI indices appeared in all early-onset cases. On the other hand, higher UA-PI indices were found only in few late-onset FGRs than matched controls, while lower MCA-PI indices were showed in all late-onset cases, resulting in a CPR<5th centile, according to the inclusion criteria of the study. No late-onset cases with absent or reverse umbilical artery end-diastolic flow were observed.

Clinical evolution of early-onset FGR is associated with severe placental insufficiency which has two direct effects on fetal cardiovascular development. First, reduced oxygen and nutrients supply and second, leads to increasing of placental resistance and chronic cardiac afterload. Therefore, the approach used to identify early-onset FGR is through serial ultrasound-based measures of fetal growth and Doppler sampling of the UA, MCA and DV. In late-onset cases, villous hypoplasia/thrombosis do not have a significant extent in order to increase the UA_PI. The first hemodynamic alteration in the presence of hypoxia is the cerebral vasodilatation. Vasodilatation itself is a method of neuro-protection, but it cannot completely compensate the effects of hypoxia, which rapidly causes severe brain injury without the classical sequence of Doppler modifications and cardiac insufficiency, leading to adverse perinatal outcomes and potential long-term neurological sequels (Baschat, 2018). Therefore, the diagnostic approach used for early-onset FGR is not accurate for late-onset FGR, which frequently results in moderate decrease in MCA-PI and normal UA-PI with signs of late deterioration, as a non-reactive cCTG.

In the present study, abnormal low STV values were observed in early-onset FGR than matched controls before 32nd week. Computerized CTG is the only objective measure of fetal heart rate that has been validated against invasive testing in fetal hypoxemia and acidemia. It is also able to identify late repetitive decelerations. Simple visual interpretation

of a regular CTG may not be sufficiently informative or sufficiently objective to provide reassurance about the fetal condition. When a conventional CTG trace is reassuring could exclude hypoxemia, while a non-reassuring CTG is associated with a wide range of pH values at birth (Vintzileos et al., 1991), showing a low specificity and low positive predictive value for fetal hypoxia (ACOG, 2013; Visser and Ayres-de-Campos, 2015). In the clinical practice, abnormal low STV values reflect acute changes in fetal condition and they are associated with an increased risk of motor and neurological delay and damage in specific brain areas with cognitive effects as gestation advances. This neurological delay mainly affects early-onset FGRs, because they are more exposed to chronic hypoxia involving all stages of biophysical development and the autonomic nervous system (ANS) maturation (Serra et al., 2008). On the contrary, no abnormal cCTGs were observed in late-onset FGR, probably because mild-moderate placental insufficiency determines a minor impact on fetal neurodevelopment. According to Baschat, major abnormalities in cCTG parameters occur when fetuses are affected by severe chronic hypoxia or metabolic acidosis, while all FGRs analyzed in the study were delivered before signs of late deterioration occurred (Baschat, 2018). Moreover, other experimental cCTG parameters were used to assess fetal condition in FGR; none of them showed a significant discrimination between “hypoxic” and “non hypoxic” fetuses nor have yet been validated for clinical management.

On the contrary, late-onset FGR is usually undiagnosed and there are no comparable evidences on the appropriate management and frequency of monitoring, especially with respect to Doppler-based surveillance. Regarding the delivery time, the DIGITAT study affirmed the best time to deliver a late-onset FGR is at 38 weeks, because it is associated with the lowest stillbirth and neonatal morbidity rates, without affects operative vaginal delivery or cesarean section rate (Boers et al., 2010). Moreover, RCOG guidelines recommend delivery of late-onset FGR when brain sparring effect manifest, because it is predictive of acidosis at birth and long-term sequelae (RCOG, 2014). In fact, a reduction in MCA-PI indices are more specific to detect late-onset FGR at risk for adverse outcome, because they could indicate an advanced stage of increased brain blood flow, which it is not an entirely protective mechanism in late-onset FGR. In fact, also mild degrees of hypoxia can induce permanent adaptative changes in the developing brain of late preterm fetuses, which in turn could have effects on neurocognitive development at two years of age (Cruz-Martinez et al., 2009).

In the present study, FGR infants were followed with frequent neurological checks for up to one year of life, in order to assess whether they had a normal neurological development. A normal outcome was less frequent in infants of early-onset group (73.3%) than late-onset group (87.5%). These results were in agreement with those provided by the TRUFFLE study for early-onset FGR (Ganzevoort et al., 2017), which showed that timing of delivery based on Doppler measurement and cCTG criteria improves long-term (2-year) neurodevelopmental outcome in surviving infants.

In the current study, neurological abnormalities are principally linked to the severe prematurity of newborns; in fact, six of eight with neurological sequelae at birth were delivered severely premature, while other two ones were delivered between 32nd and 34th week. On the other hand, the gap existent between fetal well-being monitoring and neonatal neurological status questions the validity of prenatal screening tests. In fact, cCTG monitoring, umbilical artery pH and blood gas analysis at birth give us information only on the last hours of the fetus's life. Whether an acute fetal hypoxic event occurred in the last few weeks of pregnancy cannot be accurately estimated.

Therefore, integrated fetal surveillance is fundamental for both the diagnosis and management of clinical evolution of early- and late-onset FGR, that are inadequately identified with a single-modality surveillance method (Baschat, 2003). In particular, combined Doppler and cCTG monitoring seems to be the best way forward in managing early-onset cases and to choose the best delivery time. However, the current clinical methods available are unable to accurately diagnose fetal hypoxia before it occurs or even in the early stages, nor quantify its severity.

5.1 Circulating miRNAs as diagnostic marker of FGR

In order to improve the detection rate for hypoxia, a panel of hypoxia-induced miRNA were identified for the first time. The identification and the choice of these microRNAs was made by a study group of Pharmacology Unit in the Neuroscience Department, combining literature studies which have verified the role of these miRNA in ischemic injury pathogenesis or in stroke diagnosis and previous experiments of the same study group (some of these data not yet published). About the correlation of these miRNAs and hypoxic insult new details have been added. Moreover, in other two studies not yet published by the study group, it was performed a microarray analysis to obtain a general pattern of differentially modulated microRNAs in ischemic brain. Interestingly, the expression of these miRNAs was also validated by real-time PCR in brain tissue of rats

subjected to cerebral ischemia; their levels were significantly increased compared to the control group (Cuomo et al., 2019; Vinciguerra et al., 2020). Thus, at the beginning of this current study we assessed the expression of also other microRNAs, such as miR-143-3p, let-7a-5p, miR-223-5p, miR-101a-3p and miR-218a-5p, whose modulation has been strongly correlated with stroke, but we did not observe any variation in plasma levels of FGR samples (data not shown).

Instead, miR-16-5p, miR-103-3p, miR-107-3p and miR-27b-3p were differentially expressed in the maternal blood of pregnancies complicated by FGR compared controls with healthy fetuses. In particular, miR-16-5p, miR-103-3p and miR-27b-3p were up-regulated in FGR before the 32nd week of gestation, while miR-103-3p and miR-107-3p were down-regulated in FGR between the 32nd and 37th week of gestation. Moreover, all four miRNA investigated showed a progressive trend to downregulation in FGRs and upregulation in Heathy fetuses, as pregnancy progressed.

On the contrary, Hromadnikova et al. analyzed a set of miRNA from placental tissues of pregnancies complicated by FGR. They found several miRNA, including miR-16-5p and miR-103a-3p, were down-regulated or exhibited a trend to down-regulation in FGRs requiring the delivery before 34 weeks of gestation (Hromadnikova et al., 2015a). Also Maccani et al. showed that the expression of miR-16 was markedly reduced in fetuses with severe growth restriction, and it was involved in regulating cell cycle progression (Maccani et al., 2011). Among molecular targets, a study evidenced that miR-16 appears to regulate VEGF, thus affecting angiogenesis (Karaa et al., 2009).

More and more studies indicate that miR-27b is involved in affecting various biological processes, such as angiogenesis, proliferation, metastasis, and drug resistance, and thus may act as a promising therapeutic target in human cancers (Ding et al., 2017). An animal study showed that high expression of miR-27b-3p in mice epididymal white adipose tissue (eWAT) inhibits browning ability and leads to visceral fat accumulation. It is suggested miR-27b-3p may become a potential therapeutic option for visceral obesity and its associated diseases (Yu et al., 2019). Another study demonstrated that elevated levels of a transcription factor c-Myc plays an important role in downregulation of miR-27b in embryonic brain cortices during hypoxia-induced neuronal apoptosis, resulting in additional neuronal apoptosis (Chen et al., 2015). Finally, it was also showed that, miR-27b is an endogenous inhibitory factors of Apaf-1 expression and regulate the sensitivity of neurons to apoptosis in central neural system after hypoxic injuries caused by fetal distress, having

implications for the potential target role of miR-27b in the treatment of neuronal apoptosis-related diseases (Chen et al., 2014).

About miR-107, a study on SGA fetuses reveled that maternal plasma levels of miR-107 were lower in SGA compared to controls between 12+0 to 21+6 weeks of gestation (Kim et al., 2020). Several studies showed that miR-103 and miR-107 are strongly induced by hypoxia in vascular endothelial cells and by targeting AGO1 they are shown to promote angiogenesis (Chen et al., 2013), and more specifically miR-107 contributes to post-stroke angiogenesis by targeting Dicer-1 (Li et al., 2015). In addition, after induction of rodent traumatic brain injury, miR-107 expression is down-regulated, and this reduction could mediate the activation of adaptive responses (Redell et al., 2009). In other animal studies, miR-103 and -107 are shown to be upregulated in obese mice, and their silencing led to improved glucose homeostasis and insulin sensitivity. Indeed, caveolin-1, a critical regulator of the insulin receptor, was identified as a direct target gene of miR-103/107, suggesting that this protein is upregulated upon inactivation of miR-103 and miR-107 in adipocytes, contributing to the stabilization of the insulin receptor and enhance of insulin signalling (Trajkovski et al., 2011). Also in-vitro studies confirmed the involvement of miR-103 and -107 in metabolic pathways. In fact, the location of miR-103 and -107 genes within introns of the PANK genes may be physiologically relevant, and some studies have showed that both miRNAs act symbiotically with the PANK proteins, which regulates human metabolic pathways (Wilfred et al., 2007). Other authors confirmed that miR-107 expression was altered following increased extracellular glucose (Tang et al., 2009) and after dietary intake of various lipids (Davidson et al., 2009).

5.2 Expression profile of miRNAs in chronic hypoxia and placental development mechanisms

In the context of the available data, we hypothesized that chronic fetal hypoxia, due to impaired uteroplacental blood flow, activates oxygen-sensitive miRNAs. By regulating target genes, these miRNAs act as triggers for the signaling cascades associated not only with responses to chronic hypoxia but also with placental development mechanisms. In placenta there are important vascular and trophoblast cell functions, impairment of these functions can result in abnormal maternal spiral arteries remodeling, placental maldevelopment and insufficiency, which in turn contributes to the etiology of FGR.

This would explain how FGR, especially early-onset, represents the final phase of an etiopathogenetic process that originates in a very early stage of pregnancy.

In fact, the only effective treatment for chronic fetal hypoxia is the choice of the best time to delivery before severe hypoxia occurs, in order to avoid the commonest causes of stillbirth, neonatal morbidity and long-term neurological sequelae (Baschat, 2011).

A non-invasive measure of fetal hypoxia would be ideal, yet we are not aware of any known maternal biomarker of fetal hypoxia. Several studies have investigated on the association between circulating levels of miRNAs and gestational disorders, to better understand molecular mechanisms involved in the physiopathology of these diseases.

The aforementioned scientific literature well describes different miRNA expression in mother-child pairs with adverse pregnancy outcomes respect to controls, suggesting that the evaluation of miRNA levels may serve as useful prevention and clinical tools.

However, results from several works are inconsistent and the spectrums of miRNAs observed by different studies are rather controversial. Such inconsistency or discrepancies can be attributed to differences in sample type, sample handling, gestational age at sampling, techniques used for miRNA profiling, and population characteristics, such as age, ethnicity, and lifestyle (Chiofalo et al., 2017).

In this study, we have identified a novel approach to noninvasively diagnose of chronic fetal hypoxia by measuring circulating levels of miRNAs in the maternal blood during pregnancy.

6. CONCLUSIONS

Overall, from our results it is conceivable that measurement of miRNAs in maternal blood may form the basis for a future diagnostic test to determine the degree of fetal hypoxia in FGR, thus allowing the initiation of appropriate therapeutic strategies in order to alleviate the burden of this disease. Probably, the development of new screenings to assess miRNA levels in maternal blood during the first trimester of pregnancy could identify fetal hypoxia before FGR occurs. In addition, the identification of potential targets for the selected miRNAs will pave the way for the development of innovative pharmacological strategies exploiting these druggable targets.

7. AKNOWLEDGEMENTS

I would like to deeply thank all the people I worked with, during the three years of my PhD experience: first of all, Dr. Giuseppe Maria Maruotti, Dr. Marta Campanile and Prof. Fulvio Zullo. Special thanks go to Pharmacology Unit in the Neuroscience Department: Prof. Giuseppe Pignataro, Dr. Pasquale Cepparulo and Dr. Antonio Vinciguerra, because this work would not have been possible without them.

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