Selective fetal growth restriction in identical twins: from womb to adolescence

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Part IV

Summary and general discussion

Summary

This thesis consists of studies in monochorionic (MC) twins with selective fetal growth restriction (sFGR) investigating placental mechanisms (part I), short-term outcomes (part II) and long-term outcomes (part III). sFGR is a complication characterized by a large intertwin growth discrepancy during pregnancy, resulting in a large birth weight discordance (BWD) that is associated with an increased risk of perinatal morbidity and mortality as well as adverse long-term outcomes. Yet, a large gap in knowledge persists, impeding proper parent counseling and risk assessment. Simultaneously, this unique identical twin model can be used to investigate the early origins of disease after adverse intrauterine circumstances, by comparing a growth-restricted twin with a genetically identical, appropriately-grown co-twin with similar parental and obstetrical factors.

From unequal placental sharing to a discordant intrauterine environment (Part I)

In **Chapter 1** we investigated the placental characteristics after color-dye injection according to the classification for sFGR proposed by Gratacós based on the Doppler flow pattern in the umbilical artery (UA) of the smaller twin, delineating three types: type I with positive end-diastolic flow, type II with persistent absent/reversed end-diastolic flow and type III with intermittent absent/reversed end-diastolic flow. We found that type III placentas presented with the largest diameter of arterio-arterial (AA) anastomoses and the largest placental share discordance compared to type I and type II placentas. The larger AA anastomosis was thereby thought to primarily be responsible for the unpredictable clinical course of type III pregnancies, by allowing for acute feto-fetal transfusion in case of fetal demise in the smaller twin.

Chapter 2 further explored the relationship between BWD and placental share discordance, as well as the compensatory mechanism of large bidirectional anastomoses (AA and veno-venous anastomoses) in 449 MC placentas. The twins with a BWD ≥ 20% were classified according to the Gratacós classification. BWD appeared to be strongly associated with placental share discordance. Yet, the amount of BWD was relatively smaller than expected for the given amount of placental share discordance. A larger AA diameter was found to mitigate the effect of unequal placental sharing on BWD. In type II and type III, a distinct pathophysiology was identified with an increased importance of AA diameter as opposed to placental sharing. So, larger AA anastomoses are also beneficial for prenatal growth of the smaller twin, by allowing for a rescue transfusion from the larger to the smaller twin.

From fetus to newborn (Part II)

Chapter 3 comprises a systematic literature review of twelve articles on the optimal timing of delivery in MC twins with sFGR according to the Gratacós classification. We described that type I pregnancies are generally delivered at a later gestational age (33.0-35.0 weeks) and have lower rates of fetal demise, neonatal mortality and cerebral injury when compared to type II (27.8-32.4 weeks) and type III (28.3-33.8 weeks). Timing of delivery varied greatly in type II and type III, which was the result of heterogenous studies using different antenatal diagnostic criteria and definitions of outcome measures. This illustrated that uncertainty regarding optimal timing of delivery in MC twins with sFGR persists.

In **Chapter 4**, we showed that the larger twin in MC twins with sFGR has a doubled risk of developing respiratory failure at birth requiring mechanical ventilation and/or surfactant, while the smaller twin has a more than doubled risk of developing bronchopulmonary dysplasia characterized by respiratory insufficiency requiring treatment with >21% oxygen for at least 28 days, highlighting a pathophysiological effect of fetal growth restriction (FGR) on fetal lung development.

Chapter 5 presents the first results of the Twinlife study (Twin Longitudinal Investigation of FEtal discordance), in which MC twins are longitudinally followed up starting antenatally until eight years of age to uncover the early origins of disease using this unique discordant identical twin model. We analyzed the neonatal cardiac ultrasounds of 100 twin pairs, measuring the cardiac valve annuli diameters, left ventricular dimensions and aortic pulse-wave velocity as a surrogate marker for aortic stiffness. Z-scores were calculated based on gestational age at birth, describing the relationship between the measurement and the mean of a reference population. We found that the z-scores of the cardiac structures were all lower for the smaller twin when compared to the larger twin. Yet, the birth weight difference tended to be more pronounced than the difference in cardiac structure, indicative of heart sparing. These findings are suggestive of early structural cardiovascular remodeling after FGR.

In **Chapter 6** we retrospectively analyzed the first neonatal cerebral ultrasound after birth in 58 MC twins with sFGR, performing structural cerebral measurements to assess brain growth. These measurements were compared between the smaller and larger twin and with a sex- and gestational age-matched appropriately-grown singleton. The smaller twin presented with an overall restriction in brain growth, including smaller cerebral structures (corpus callosum, vermis, cerebellum),

white/deep gray matter and overall brain size, when compared to both the larger twin and the matched singleton. These differences remained after correction for intracranial volume, indicating a proportional decrease in brain growth.

From infant to adolescent (Part III)

Chapter 7 consists of a systematic literature review on the impact of sFGR on long-term neurodevelopmental outcomes. Five articles were included, all pointing in the same direction: substantial rates of neurodevelopmental impairment (NDI) for MC twins with sFGR with a trend towards a within-pair disadvantage for the smaller twin. These studies, however, had a high degree of heterogeneity resulting from substantially different methodologies, study populations and outcome measures. Therefore, this review primarily stresses the lack of knowledge of the long-term neurodevelopment after sFGR.

Chapter 8 presents the first results of the LEMON study (Long-term Effects of selective fetal growth restriction in MONochorionic twins), in which MC twins with sFGR born between 2002-2017 in our center and aged between 3-17 years were invited for follow-up. An age-appropriate neurodevelopmental test and a standardized neurological examination were performed as part of this follow-up in 47 included twin pairs. A within-pair comparison revealed that the smaller twin has a significantly lower intelligence quotient (IQ) across all domains of intelligence (6-point lower full scale IQ) as well as a substantially higher rate of mild NDI, with 36% vs. 11% (defined as a full scale IQ < 85, simple or complex minor neurological dysfunction or any mild visual or hearing impairments) when compared to the larger twin. The odds of developing mild NDI for the smaller twin was nearly five-fold higher than for the larger twin (OR 4.8). These findings indicated that FGR poses a substantial risk for long-term neurodevelopment, irrespective of genetic predisposition or obstetrical factors.

In **Chapter 9** we investigated the psychosocial development and school functioning of the LEMON study population using multiple parent-report questionnaires. We established that MC twins with sFGR (equally for the smaller and larger twin) presented with significantly more attachment insecurity (34%) than the general population (16%). Ambivalent/resistant attachment was most prevalent, in which the child constantly seeks attention from caregivers while also being resistant to contact. In addition, the smaller twin experienced more negative behaviors and emotions turned inwards (internalizing problems) and had a temperament that predisposes to

experiencing more negative emotions when compared to the larger twin. This information facilitates early detection, prevention and intervention strategies.

In **Chapter 10,** we analyzed the childhood growth patterns of the LEMON study population using a mixed-effects model to assess whether catch-up growth (defined as growth within target height range (target height +/- 0.8 standard deviation score)) occurs. Growth measurements as documented by a systematic primary care system in the Netherlands were collected and height, weight and head circumference were measured at follow-up. On average, smaller twins catch-up to a height within their target height range within 8-11 years after birth. A within-pair analysis showed that differences in height, body mass index and head circumference generally persisted, with smaller twins remaining smaller, lighter and lower in head circumference than their larger co-twins. These findings were suggestive of a persistent inhibitory effect of FGR on childhood growth.

General discussion

Selective fetal growth restriction (sFGR) in monochorionic (MC) twins is a prevalent complication in which the placenta is unequally shared, leading to a discordant antenatal growth pattern with a subsequently large birth weight discordance (BWD). These twins are at risk for adverse short- and long-term outcomes. Research on sFGR has been on the rise over the past decade (Figure 1). sFGR in the absence of twin-twin transfusion syndrome (TTTS) and twin anemia polycythemia sequence (TAPS) is increasingly recognized as a distinct entity in MC twin complications with its own course of disease. The uniqueness of these twins is also increasingly acknowledged: them being monozygotic (i.e., genetically identical) and in the same womb while experiencing a vastly discordant environment. The prenatal malnutrition that the smaller twin experiences is thought to predispose to disease in adulthood according to the early origins of disease hypothesis.

The aim of this thesis was to investigate the placental pathophysiology, short- and long-term outcomes of MC twins with sFGR as well as the early origins of disease after adverse intrauterine circumstances in this unique identical twin model.

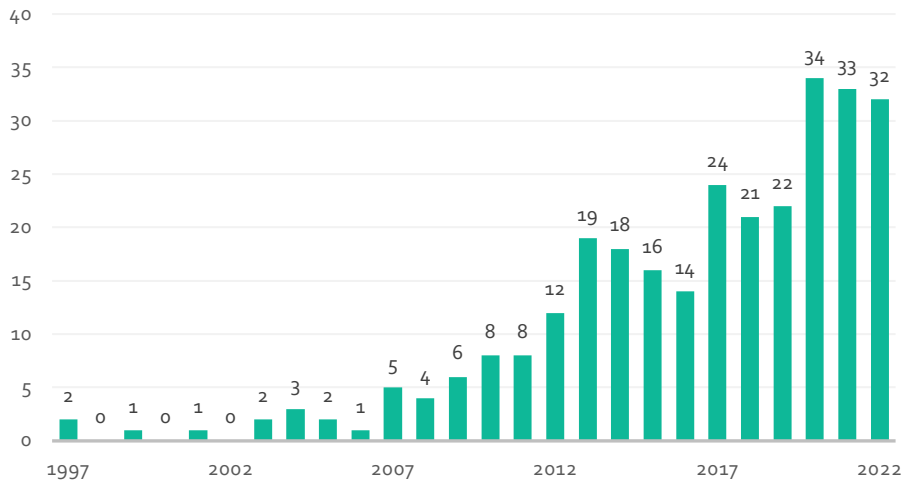

Figure 1. An overview of PubMed search results for the combination of the keywords 'selective fetal growth restriction', 'selective intrauterine growth restriction and 'monochorionic twins' (date 17-10-2022).

Placental pathophysiology

The importance of colored dye injection

Colored dye injection of MC twin placentas visualizing placental sharing and vascular anastomoses is paramount in understanding the pathophysiological processes behind the complications that can occur. Placenta injection with colored dye is part of standard care for all MC twins born in the Leiden University Medical Center (LUMC), including twins born after uncomplicated pregnancies to discern between what is normal and abnormal. This ensures that both clinicians involved in the day-to-day care for these twins and twin researchers gain familiarity with the complications that can occur in this vulnerable patient group. This thesis began with nearly twenty years of data gathered from the injection of placentas (n = 1168). The ensuing four years have led to an additional 445 placentas that have been added to this track record (Figure 2).

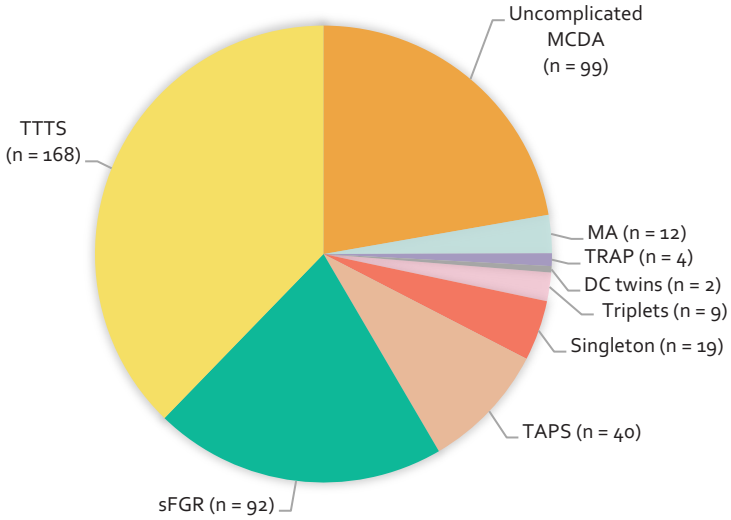

Figure 2. The injected placentas (n = 445) per category in the four years of this thesis (date 17-10-2022). Singleton placentas were from friends and colleagues or used as a demonstration in educational courses (n = 19).

But what lies beneath?

The placenta in MC twin pregnancies with sFGR is still a black box despite the colored dye injection: its exact internal mechanisms remain relatively unknown. We have now studied sharing by measuring the placental surface and have found that it strongly correlates to birth weight. Yet, much lies beneath the surface that has scarcely been

researched. Future studies should investigate the role of placental weight in determining birth weight in MC twins, to assess whether placental surface is indeed an adequate proxy for sharing. It is well-known that placental weight is correlated to birth weight[1-3]. Low placental weight is considered an expression of reduced functional mass in which exchange of nutrients and oxygen can occur, thereby leading to FGR. Yet, when separating the MC placentas along the vascular equator the fetal weight/placental weight ratio appeared to be higher for the smaller twin indicative of a more efficient perfusion despite the smaller share[4]. Studying the correlation between individual placental surface and placental weight in MC twins with sFGR can potentially lead to a more precise method of quantifying the actual placental sharing.

A crucial limitation in the generalizability of the results from our twin studies to singletons that was repeatedly considered in this thesis was the substantially different pathophysiological mechanisms of (s)FGR. Where sFGR in MC twins is thought to primarily be caused by unequal placental sharing, FGR in singletons generally finds its origin in placental insufficiency characterized by malperfusion of the placenta multifactorial in origin[5]. These differences may lead to incomparability of outcomes due to a distinct course of disease that has its own impact on fetal development. It should be investigated whether placental insufficiency is also at play in the smaller share of sFGR placentas. Current literature on the pathology of discordant MC placentas is conflicting: some report low placental weight (an expression of unequal placental sharing) combined with a velamentous cord insertion as the main culprit, while others also find more vascular thrombotic lesions (common in FGR placentas) in the smaller share[2,3,6]. Additionally, if pathophysiological mechanism do differ, it should be researched whether this does in fact lead to different health outcomes or if the consequences for health and development are the same. As of September 2020, we have included a pathological examination of the placenta in our standard care for complicated MC twins and/or participants in our prospective study (Twinlife).

Placental imaging: personalized medicine for monochorionic twins

Our new knowledge of placental mechanisms in sFGR can be used in clinical practice by improving antenatal visualization of the placenta and anastomoses. Current available imaging modalities include ultrasound and MRI. Three-dimensional color Doppler ultrasound can be used to assess the vascularization of the placenta as well as placental volumes[7-10]. Functional MRI can provide information on placental perfusion[11]. Ideally, imaging of the placenta would allow for quantification of exact placental sharing and the amount of transfusion that passes over anastomoses. With

this knowledge, the risk of fetal death in case of severe placental share discordance as well as the risk of acute feto-fetal transfusion can be estimated more accurately per pregnancy. When imaging modalities are further improved in the future, prognostication and management strategy can in turn become increasingly individualized.

Diagnosis

Fifteen years of the Gratacós classification – time for an update?

In 2007, Gratacós et al. introduced the classification system for sFGR in MC pregnancies based on the Doppler flow patterns in the umbilical artery (UA) of the smaller twin[12]. Fifteen years later, this classification system is still widely used, marking persistent (type II) or intermittent (type III) absent/reversed end-diastolic flow (A/REDF) as especially at risk for perinatal morbidity and mortality[13]. Management of these pregnancies is adapted accordingly, with daily fetal surveillance (in our center from 28 weeks onwards). If fetal distress of either twin is observed, the twins are delivered.

Even though this classification system has been widely used for fifteen years, the cause of the abnormal Doppler flow patterns is still speculated about. We described the distinct placental characteristics of each type in Chapter 2, primarily identifying type III placentas as 'the odd one out'. The intermittent A/REDF as observed in type III pregnancies is thought to be the consequence of the large AA anastomoses in which the two systolic waveforms collide. The cyclical nature of this flow pattern can be explained by the fluctuation in synchronicity of fetal heart rates[14]. Interestingly, we did not identify any specific placental characteristics for type II placentas. These cannot be discerned from a type I placenta with the naked eye. It remains unclear how the type II flow pattern arises, but it may be indicative of underlying placental insufficiency.

Importantly, chapter 2 and 3 of this thesis have touched upon a great obstacle in using the current Gratacós classification: the insurmountable international differences in its application in relation to the dynamic nature of the UA Doppler flow patterns. The changing patterns throughout pregnancy, sometimes fluctuating between type I, type II and type III, impede a proper determination of the 'definitive' Gratacós type[15]. As each center has its own methods of dealing with these changing flows in the classification (which are often not recorded in published studies), this ultimately leads to fundamental incomparability of reported outcomes for each type. Additionally,

intra- and interobserver variability in the interpretation of flow patterns (especially of type II and type III) have never been assessed. Hence, an update of the Gratacós classification is drastically necessary to further improve antenatal prognostication and management of sFGR pregnancies. A key element of a new system should be a guideline on how to interpret and classify changes in Doppler flow patterns throughout pregnancy. The first step in devising such a guideline is to research how placental characteristics relate to these changing patterns, to gain more understanding of underlying pathophysiology and consecutive risks. Moreover, it is desirable to incorporate brain sparing, measured by the cerebroplacental ratio, into a new classification as well, as it is considered a marker for a greater severity of FGR in singletons. Uniformity in the formation of a new classification for sFGR can only be achieved by international collaboration between fetal therapy centers. Multicenter research is necessary to 1) properly establish the variability in the application of the former classification, 2) identify relevant parameters that should be included in a new classification, 3) build a large retrospective cohort with available ultrasound imaging to re-classify cases and assess the differences in clinical outcomes with the former classification and 4) to start a large prospective cohort to apply the new classification.

Antenatal management

Overlapping complications

An important consideration in the antenatal management of MC twin complications, is that these are not mutually exclusive. Figure 3 shows the possibilities of overlap between the various complications. A previous study by our research group has confirmed that 60% of TTTS cases also present with an intertwin growth discordance before fetoscopic laser coagulation (Table 1)[16,17]. After birth, this drops to 25% that presents with a BWD ≥ 20% as intertwin transfusion stops when treatment is successful and the donor twin can largely recover. It is unknown how many TAPS cases present with a growth discordance at diagnosis, but this was estimated at 15-25%. In contrast with TTTS, the percentage of TAPS cases born with a BWD ≥ 20% increases to 35-45% due to the chronic nature of the intertwin transfusion. The donor becomes increasingly growth-restricted following an extended period of progressive anemia and hypoalbuminemia[18]. This is also illustrative of the different pathophysiological mechanisms behind growth discrepancies in TTTS or TAPS. Yet, the exact numbers for these combinations are still lacking. Future research must make out the true overlap between TTTS, TAPS and sFGR at diagnosis and at birth also specified according to treatment modality. Importantly, the overlapping complications can result in confusing terminology, e.g., the Gratacós classification

being applied to TTTS pregnancies. This may lead to substantial international differences in diagnosis and management. We need to speak the same language to stay on the same page. The distinct complications and classifications should be considered in the antenatal management of MC twin pregnancies: TTTS using the Quintero staging system, TAPS using the classification based on delta middle cerebral artery peak systolic velocity and isolated sFGR using the Gratacós classification[12,19,20]. A TTTS case that presents with simultaneous anemia-polycythemia or sFGR should always be primarily treated as TTTS.

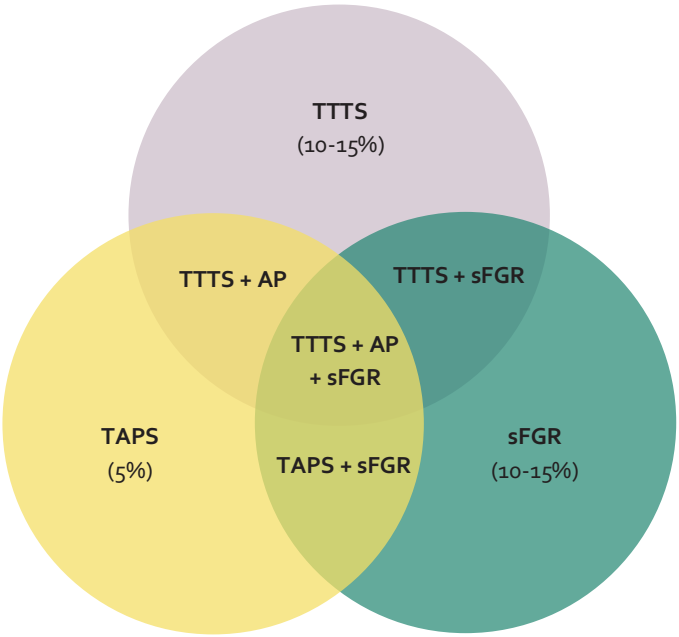

Figure 3. A Venn-diagram of the concurrent complications in MC twin pregnancies.

Management for sFGR

Two primary management strategies for sFGR can currently be identified: expectant management with fetal monitoring in case of abnormal UA Doppler flows or selective feticide of the smaller twin. Expectant management is the most widely used strategy to date, but frequency of ultrasounds and the threshold for admittance varies greatly internationally. A uniform management protocol is therefore still necessary, especially in light of the recent discussion on fetal viability in the Netherlands that may be adjusted to 23 weeks of gestation. In our center, fetal growth is monitored fortnightly including Doppler flow measurements. The frequency of these Doppler flow measurements can be intensified to weekly or even biweekly in case of type II or type

III sFGR. Ultrasounds are primarily performed at the outpatient clinic. In type II and type III, or in case of severe growth restriction (EFW < 3rd centile) and/or stagnating growth, it can be decided (in consultation with parents) to admit the mother for daily fetal surveillance using cardiotocography once or twice a day. The threshold for admittance in our center is 28 weeks of gestation, yet this may be earlier in other centers.

Table 1. An approximation of TTTS and TAPS with a concurrent intertwin growth discrepancy at diagnosis and at birth.

Intertwin growth discordance	**TTTS**	**TAPS**
At diagnosis *EFW < 10th centile + EFW discordance ≥ 20%*	60%	15-25% (?)
Pathophysiology	Combination of unequal sharing and amniotic fluid imbalance, disadvantageous for growth of donor.	Chronic intertwin transfusion resulting in anemia and hypoalbuminemia in donor.
At birth *BWD ≥ 20%*	20-30% after laser (?) 40-50% if no/unsuccessful laser (?)	35-45% (?)
Pathophysiology	Unequal placental sharing, intertwin transfusion no longer present if laser is successful. If no/unsuccessful laser, intertwin transfusion and amniotic fluid imbalance persists.	Progressive anemia and hypoalbuminemia in donor; donor generally has a larger placental share in TAPS.

TTTS: twin-twin transfusion syndrome, TAPS: twin anemia polycythemia sequence, sFGR: selective fetal growth restriction, EFW: estimated fetal weight, BWD: birth weight discordance.

Selective feticide is discussed with parents in cases in which the smaller twin is already severely growth-restricted in an early stage in pregnancies with abnormal UA Doppler flow patterns and/or presenting with additional signs of compromise (e.g., further stagnation in growth, cerebral abnormalities, cardiac compromise or echogenic bowels). As previously mentioned, there is a risk of acute feto-fetal transfusion through large bidirectional anastomoses after sudden fetal demise of the smaller twin

that may lead to neurological damage or death of the larger twin. Selective feticide aims to protect the larger twin, who is given the best chances of survival.

Another available treatment modality is fetoscopic laser coagulation. Yet, this is still a disputable option for sFGR to date. The idea behind performing fetoscopic laser coagulation in pregnancies with abnormal UA Doppler flow patterns (type II/type III) is that it eliminates the risk of acute feto-fetal transfusion in case of demise of the smaller twin, similar to selective feticide. As stressed by chapter 2, however, the compensatory function of the large AA anastomoses is also lost after coagulation, leading to high rates of fetal demise in the smaller twin (67-77%)[21-24]. In addition, the cause of sFGR is not an imbalance of blood flow as in TTTS or TAPS, so coagulation of anastomoses does not solve the underlying problem. Lastly, fetoscopic laser coagulation in sFGR comes with more technical challenges due to the absence of an amniotic fluid discordance. Overall, we do not deem it a feasible treatment option for sFGR in our center.

Optimal timing of delivery

Chapter 3 has illustrated that there is still no guideline for optimal timing of delivery in MC twins with sFGR. The risk of fetal demise of the smaller twin is thought to increase over the course of the pregnancy, as the placental share can become more insufficient in providing nutrients and oxygen. Simultaneously, the risk of neonatal morbidity and mortality increases with decreasing gestational age, thereby substantially impairing long-term neurodevelopment as well. Preferably, we must identify the moment at which the risk of fetal demise outweighs the risk of severe neonatal morbidity and mortality for each Gratacós type as has previously been done for monoamniotic twin pregnancies (Figure 4)[25]. Finding this balance proves difficult as many other factors play a role during sFGR pregnancies, such as a deterioration of fetal condition or stagnation of growth in the third trimester and, importantly, fetal distress. The first step in devising a guideline is to equalize reported outcome measures to uniformly assess clinical outcomes at birth and in childhood. As mentioned in Chapter 3, a wide variety of outcome measures is used in available literature leading to incomparability between studies. Reason for delivery is generally not recorded while this is crucial information. Moreover, as mentioned earlier, a new uniformly applicable classification for sFGR is a prerequisite for proper prognostication.

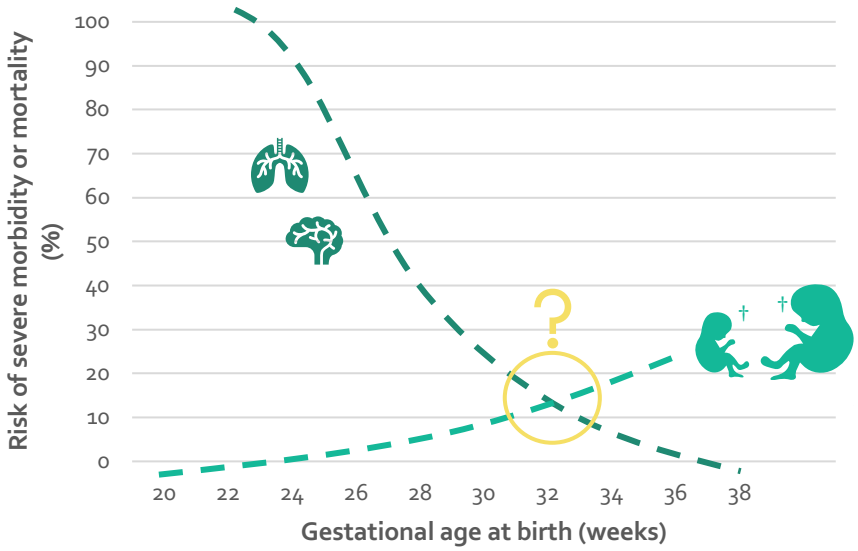

Figure 4. How to find the optimal timing of delivery in MC twins with sFGR.

Respiratory health

FGR has repeatedly been linked to adverse respiratory outcomes through abnormal fetal lung development, in which persistent structural (impaired vascularization and alveolarization) and functional (inefficient oxygenation) changes are induced following the chronic state of hypoxia fetus experiences[26,27]. Bronchopulmonary dysplasia (BPD), a chronic form of respiratory insufficiency, is thought to be an expression of this impaired lung development. As shown in Chapter 4, the smaller twin had a more than doubled odds of developing BPD. BPD as well as FGR in itself can in turn predispose to increased respiratory morbidity in childhood. Studies describing lung function after FGR at school age show conflicting results and primarily focus on very preterm infants[28-31]. Many other confounding factors can be at play, such as genetic susceptibility and parental smoking habits[32,33]. Identical twin models are ideally suited to control for these confounding factors. A large twin study including 752 twins (both mono- and dizygotic) found that lung function was reduced with decreasing birth weight. The smaller twin had five-fold higher odds of having a clinically relevant deficit. Yet, chorionicity and discordance were unreported[34]. It remains to be seen whether our observed within-pair differences in short-term respiratory morbidity also persist into childhood. We have included spirometry with add on of lung diffusion capacity and volumetric measurements for MC twins with sFGR aged between 4-17 years in the LEMON study (Netherlands Trial Register ID

NL9833), which are currently still being scheduled. These data will allow us to assess the prevalence of respiratory diseases, such as asthma, but also provides information on subtler changes in lung structure and functioning that are primarily associated with FGR[29].

Cardiovascular health

The early origins of disease hypothesis all began with cardiovascular disease (CVD). David Barker laid the foundation for this hypothesis in 1986 by proposing a link between prenatal nutrition and ischemic heart disease after he observed that the rate of ischemic heart disease was higher in areas with poor living conditions[35]. Research on the Dutch Hunger Winter, a severe famine at the end of World War II, has further substantiated this hypothesis by demonstrating that individuals who were exposed to prenatal famine in this period presented with an adverse risk profile of metabolic disease in adulthood[36-40]. MC twins now present the ideal model to further study the effect of early life adversity on lifelong health outcomes in the Twinlife study, limiting confounding by genetic, obstetrical or parental factors to an even greater extent than siblings [41]. By longitudinally following both concordant and discordant MC twins from fetus to adolescent, we can follow early cardiovascular differences after adverse intrauterine circumstances and consecutive development of risk factors for CVD[42,43].

The first step in cardiovascular tracking of these twins is presented in Chapter 5, reporting that early structural cardiovascular remodeling after FGR is already visible on neonatal cardiac ultrasound within one week after birth. These findings form the baseline for the Twinlife cohort. Previous research indicates that the adaptive process following prenatal adversity is more gradual throughout childhood[44,45]. Follow-up in the Twinlife study can explore whether the cardiovascular adaptations at birth are still present throughout childhood or whether new adaptations can be observed over time. Ideally, twins will be tracked well into adulthood to investigate whether CVD indeed occurs at a later age. Similarly, the older MC twins with sFGR included in the LEMON study are still being seen for a cardiac ultrasound. This will provide us with a look into the future to identify outcomes of interest for Twinlife. A measurement of the carotid intima-media thickness is performed in this population as well, a widely used surrogate marker for atherosclerosis and predictor of cerebral and cardiovascular events[46]. Ultimately, MRI studies including cardiovascular functioning and extensive metabolic imaging (e.g., body fat distribution) could provide novel insights into the effects of prenatal adversity on CVD risk.

Neurodevelopmental health

Structural brain development – Imaging

As mentioned, 'brain sparing' in FGR constitutes the redistribution of cardiac output towards major organs like the brain. It is considered as misnomer as the brain is not in fact spared. Brain sparing is indicative of a more severe form of FGR in which the fetus experiences chronic hypoxia that impairs the development of major organs. We have shown this in Chapter 7 in which the smaller twin of sFGR twins presented with an overall restriction in brain growth on neonatal cerebral ultrasound (cUS) when compared to the larger co-twin and a matched singleton. We were unable to find any differences in cerebral maturation on cUS, possibly because we used a relatively old scoring system that looks at a few rough markers of maturation in different planes. cUS is widely accepted as an important bedside tool to screen for brain injury and follow brain growth during the neonatal period. However, compared to cUS, MRI is able to detect injurious and maturational changes in the developing brain in far more detail as it allows the quantification of brain volume and cortical thickness, and can be used to assess functional brain development and connectivity[47-50]. All these parameters are important markers of brain development and maturation and all can be altered by early-life brain insults such as FGR.

Previous studies on structural cerebral adaptations after FGR in singletons have shown atypical brain development, including reduced total and cortical gray matter volumes, reduced cortical complexity, reduced and delayed myelination, altered hippocampal and cerebellar development, and reduced connectivity of specific brain networks[51-53]. However, the available literature is scarce. There are also many other early life adversities that can have an impact on brain development, such as socio-economic status, maternal stress, pregnancy complications and genetic factors[54]. Both fetal and neonatal term age equivalent MRI in MC twins with sFGR would be the next step to provide more robust mechanistic insight into brain development after FGR.

Functional brain development – Neurodevelopment

Chapter 7 has identified the poignant shortage of long-term neurodevelopmental outcomes of MC twins with sFGR in literature, with only five available studies reporting on a variety of outcome measures using different assessments and study populations. This review was the foundation for the LEMON study that ultimately provided the answer to a pressing question: are MC twins with sFGR at risk of long-term NDI? In Chapter 8 we have established that the smaller twin had a considerably

higher rate of mild neurodevelopmental impairment (NDI) as well as a lower intelligence quotient (IQ) across all domains of intelligence when compared to the larger twin. Additionally, the smaller twin was found to have a tendency towards negative emotions and internalizing behaviors, as well as a lower secondary school level than the larger twin, described in Chapter 9. These findings have validated that (s)FGR indeed has a long-lasting effect on functional brain development. It remains to be researched which structural cerebral changes lead to these functional consequences and whether these changes can already be identified in an early stage. Aside from the earlier proposed fetal and neonatal term age equivalent MRIs, an MRI at school age in the LEMON study population would allow us to directly link the observed functional changes we found to changes in brain growth, maturation and connectivity on MRI. This would in turn identify points of interest for longitudinal MRIs in a prospective setting.

The valuable viewpoint of parents

sFGR not only affects gross neurodevelopment, but also the more fine-grained aspects of childhood development as shown in Chapter 9. Importantly, (sub)clinical attachment insecurity rate was high (34%). We hypothesized that this resulted from the complicated pregnancy course and subsequent prematurity, impairing the formation of the parent-child relationship. Conversations with parents in the LEMON study have brought forward the impact of the option to perform selective reduction that was presented to them during pregnancy. This has, in their own words, resonated with them even years later. Therefore, we see added value of qualitative studies about the impact of a MC twin pregnancy and its sequelae on parents. This can establish major themes and problems that can then be addressed during pregnancy and in the first years after birth. Parents can be offered appropriate psychological support to ensure safe attachment and to identify any problems, such as post-traumatic stress or depression, in an early stage to facilitate early intervention.

Single survivors

An essential limitation of the LEMON study is the sole inclusion of double survivors. This may result in the selection of cases with relatively favorable outcome, as both twins have survived. Single fetal demise is thought to potentially lead to severe neurological damage of the surviving twin due to acute feto-fetal transfusion. Similarly, neonatal mortality is primarily caused by the complications of extreme prematurity. The neurodevelopment and psychosocial development of the surviving twin may therefore be more severely affected. This would mean that our reported

outcomes are only the tip of the iceberg. Research on long-term outcomes in this subgroup of single survivors after sFGR is necessary.

Childhood growth patterns

Once smaller, always smaller

Postnatal catch-up growth has been a prominent topic in research on FGR over the past years. It is well-known that the majority of infants born after FGR catches up to a normal height range within a few years after birth. Yet, comparisons with population growth curves do not tell the whole story. In Chapter 10, we have shown that the smaller twin at birth will remain smaller throughout childhood in the majority of cases, suggesting a persistent growth-perturbing effect of FGR on lifelong growth. Nonetheless, significant catch-up growth was observed in the smaller twin that continued up to ten years after birth. While catch-up growth after FGR is generally considered to be a positive phenomenon reflecting recovery, it is also thought to come at a cost. A previous study in twins (mono- and dizygotic) found that catch-up growth in weight SDS relative to birth weight SDS in the first two years of life was negatively correlated with IQ at age 12 and 18 years[55]. Chapter 8 supports this hypothesis, as the smaller twin had a lower IQ across all indexes[56]. Moreover, in Chapter 10 we have demonstrated that head circumference of the smaller twin remains significantly smaller throughout childhood, which is considered a predictor of adverse neurodevelopmental outcome in itself[57,58]. Similarly, Chapter 10 shows that within-pair difference in BMI decreases in the first year and then stabilizes with the smaller twin continuing to have a lower BMI throughout childhood. Multiple studies report high rates of obesity in adulthood after FGR[59]. It is thought that both fetal malnutrition and subsequent catch-up growth in early life alter insulin sensitivity and result in an adverse body composition with a more central body fat distribution, increasing susceptibility to metabolic syndrome and cardiovascular disease[60]. The relationship between neurodevelopmental, cardiovascular and metabolic outcomes and catch-up growth will be explored in the LEMON study in the near future.

The importance of long-term outcomes

Long-term outcomes are fundamental to reflect on the decisions clinicians make in daily practice. In available literature on MC twins with sFGR, follow-up generally extended up to two years of age (often only for research purposes) and largely encompassed questionnaires as opposed to an actual examination of the children. This thesis has illustrated that there is more to clinical outcome than survival and cerebral injury alone. Our research on neurodevelopmental outcome, psychosocial

and school functioning and growth patterns has substantiated that MC twins with sFGR experience more adversity later in life, with a disadvantage for the smaller twin. With the knowledge from this thesis we can now adequately counsel parents and remain watchful throughout the pregnancy and childhood to timely intervene and optimize the development of these children and address any psychological difficulties in parents in an early stage. Standardized follow-up in both the smaller and larger twin is essential to facilitate this, as the larger twin is not exempt from impairments in this vulnerable patient group.

Epigenetics: the link between prenatal adversity and lifelong health

What remains is to now explore the link between prenatal adversity and lifelong health in the epigenome. Epigenetics encompasses modifications to the DNA that can change the regulation of gene expression without altering the genetic code itself. The two primary molecular mechanisms underlying epigenetic programming are DNA methylation and modifications of histones[61]. Epigenetic changes after malnutrition in early fetal development are persistent into adulthood, predisposing individuals to health deficits at a later age as previously described in the Dutch Hunger Winter research[36,40]. Chapter 4-6 and Chapter 8-10 of this thesis have identified these short- and long-term health deficits in the smaller twins after sFGR. The epigenetic profiles of the umbilical cord derived MSCs, that can in turn be cultured into a spectrum of cell types that can be found in the human body, collected in Twinlife can now shine a light on how these adverse outcomes are indeed programmed in utero.

An identical twin model

The 'ideal' twin model?

There is a major pitfall in using MC twins as a model for the early origins of disease: the intertwin blood flow through the vascular anastomoses. When unbalanced, in case of TTTS, TAPS or other hematological imbalances at birth, intertwin blood transfusion can dilute the true effect of FGR we primarily aim to uncover[62]. In addition, it hampers extrapolation of our results to singletons, that do not experience these hematological shifts throughout pregnancy. Hence, another twin model may be even better suited: monozygotic dichorionic (DC) twins with sFGR. These twins are also genetically identical, but do not share a single placenta and therefore do not have vascular anastomoses. In this study design, the interference of intertwin transfusion would be eliminated. In addition, the pathophysiology of sFGR in DC twins is supposedly more similar to the pathophysiology in singletons, namely placental insufficiency instead of unequal sharing. Yet, using this twin model poses many challenges. Monozygotic DC

twins with sFGR are extremely rare. Approximately 175.000 children are born in the Netherlands each year, of which 3 to 4 in 1000 is monozygotic. This amounts to 700 monozygotic twins on a yearly basis, of which only a quarter (175) is DC[63]. sFGR occurs in 10-15% of all DC twins, regardless of zygosity[64]. Ultimately, this would lead to the birth of 18-27 monozygotic DC twin pairs with sFGR yearly, in the 'best case' scenario. Moreover, zygosity need to be determined after birth as same-sex DC twins can also be fraternal[65]. In the context of research this would mean additional costs (zygosity tests cost approximately €100 per twin pair) and either antenatal inclusion of cases that appear to be fraternal after birth or postnatal inclusion with limited antenatal measurement. So, despite the potential superiority of a monozygotic DC twin model, it is not as 'practical' as the MC twin model we have used.

The bare necessities for a longitudinal twin study

To facilitate a prospective study in a MC twin model that aims to investigate the early origins of disease, a few criteria must be met. Firstly, collaboration between research disciplines is essential. Studies should be translational to warrant the link between fundamental research (in this case epigenetics) and clinically relevant outcomes in all fields of health research. Secondly, extensive antenatal documentation of fetal condition (including Doppler flow measurements, fetal growth and imaging of the heart and brain) should be performed as this forms the basis of the assessment of what an 'adverse' intrauterine environment actually entails. Lastly, follow-up throughout childhood and into adulthood is crucial. Many surrogate markers can already be investigated in childhood, but whether these actually unearth into health problems at later age should ideally be examined as well.

Final conclusions

To conclude, this thesis has provided novel insights into the short- and long-term outcomes of MC twins with sFGR, including placental pathophysiology. We have thoroughly examined the clinical course of sFGR from womb to adolescence, thereby improving our knowledge regarding this vulnerable patient group. Additionally, we have explored the early origins of disease in this unique identical twin model discordant for intrauterine environment, eliciting the effects of prenatal adversity on lifelong health.

As mentioned, this thesis has also raised new questions that form the basis for future research in MC twins with sFGR. The following themes can be identified (Figure 5):

- **Placental mechanisms**, including:
    - The combination of placental surface and weight as an enhanced proxy for placental sharing.
    - Pathological examination of placental tissue to unearth any abnormalities that can play a role in the development of sFGR in MC twins, including (dis)similarities to FGR in singletons.
    - Antenatal imaging (ultrasound and MRI) of the shared placenta and its angioarchitecture to quantify the amount of placental share discordance and intertwin transfusion.
- **Antenatal management strategies**, including:
    - A much-needed update of the Gratacós classification that incorporates the changing UA Doppler flow patterns throughout pregnancy and brain sparing.
    - A guideline on optimal timing of delivery at which the risk of fetal demise outweighs the risk of neonatal morbidity and mortality.
- **Childhood outcomes**, including:
    - Childhood spirometry with added lung diffusion capacity and measurement of lung volumes, to uncover the persistent consequences of (s)FGR for lung development.
    - Cardiovascular follow-up, including more extensive metabolic imaging at school age, from fetus to adult to track surrogate markers for CVD throughout childhood and subsequent incidence of CVD in adulthood after (s)FGR.
    - MRI studies to provide the missing link between structural and functional brain development after (s)FGR.
    - Qualitative research on the impact of a complicated MC twin pregnancy and its sequelae on parents.
    - The possible deleterious effects of catch-up growth after (s)FGR for neurodevelopmental, metabolic and cardiovascular outcomes.
    - The role of the epigenome in the fetal programming of lifelong respiratory, cardiovascular and neurodevelopmental health, and growth patterns after (s)FGR.

In short, we are far from done. There is still so much to learn about sFGR, from womb to adolescence, to improve current management strategies and thereby perinatal and childhood outcomes for MC twins with sFGR. Simultaneously, we can use this unique identical twin model to take new steps in uncovering the early origins of disease. By

combining the expertise from different research disciplines within our center as well as from internationally renowned fetal therapy centers, we can join forces to do what is necessary to offer MC twins with sFGR the best possible care.

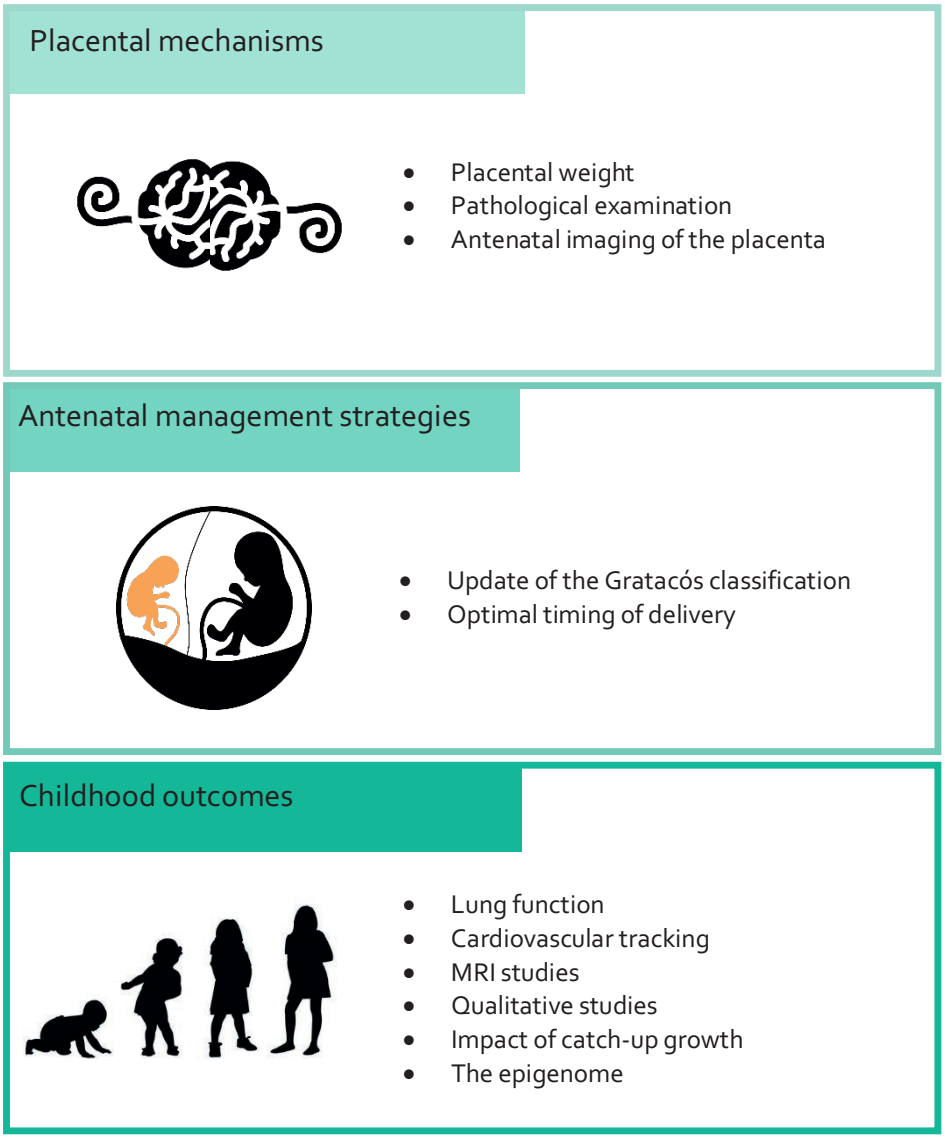

Figure 5. Future perspectives in research on MC twins with sFGR.

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