PLACENTAL FUNCTION AND OBSTETRIC OUTCOMES IN TWIN PREGNANCIES: A COHORT STUDY

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To Ana, who never lets me give up;

To Sofia, my self-awareness;

To Francisca, my force of nature;

To all parents of twins, heroes in this adventure;

And especially to Teresinha, my forever "Queen of Twins"!

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To my parents, who instilled in me the belief that conducting research alongside clinical practice in medicine was an achievable dream.

Finally, I would like to acknowledge my dear colleagues who contributed to the collection of clinical data and supported me throughout this project in countless ways.

EPIGRAPH

“Twinning and increased litter size come with high maternal and offspring mortality risks, reducing offspring quality and often negating any fitness advantages of bearing multiples. These risks, along with low twinning rates in humans and other catarrhine primates, indicate that these species are adapted to bear only one offspring per gestation. Twinning can be not a adaptive but may be a by-product of 'insurance ovulation' to mitigate early embryo loss and ensure the survival of at least one viable zygote. Consequently, bearing twins may be a costly error in an adaptive brood reduction system.”

Robson SL, Smith KR. Twinning in humans: maternal heterogeneity in reproduction and survival. Proc Biol Sci. 2011 Dec 22;278(1725):3755-61.

PREFACE

In 2011, when I finished my Obstetric specialization and started working in the prenatal diagnosis center, the assessment of uterine perfusion through arterial Doppler measurement had become common practice in singleton pregnancies. Women with elevated placental perfusion resistance were identified as high risk for developing fetal growth restriction or hypertensive disorders in pregnancy. The association of fetal growth restriction with placental insufficiency was increasingly recognized, and the first steps in screening for pre-eclampsia in the first trimester were taken. Then, in 2017, unequivocal clinical evidence came through the ASPRE trial! With early identification of women at risk and the institution of prophylactic aspirin in the first trimester, a significant reduction in the risk of these complications was possible!

And in twins? Twin pregnancy is recognized as a high-risk pregnancy for maternal and fetal complications, but unfortunately, it is a group often excluded from clinical trials. In 2013, I began to focus on the surveillance of twin pregnancy, both in maternal follow-up and in ultrasound evaluation. Doppler assessment of uterine arteries was not routine in twin pregnancy at my center. "Twins all have low uterine resistances," argued one colleague, "There is no evidence in twin pregnancy for prophylaxis, so it is useless to evaluate the uterine arteries in Twins" asserted another colleague. These statements made no sense to me, first because they were not biologically plausible, and second because I hypothesized that women destined to develop placental insufficiency would likely have worse outcomes if they carried twins instead of a singleton pregnancy. Adding to those common practice beliefs, the reference values for uterine arterial Doppler in our ultrasound software were not, and still are not, adjusted for twins; therefore, many evaluations were considered to have “normal” parameters without actually being so.

I began to observe that in clinical practice, pregnant women with values considered "normal" for a singleton pregnancy but probably “higher” for twins often experienced complications such as preterm birth associated with fetal growth restriction or hypertensive disorders. I began to systematically evaluate uterine artery Doppler in twins and included this hypothesis in my doctoral research proposal. I also sought the collaboration of my colleagues from the prenatal diagnosis center to assist me in this challenge. The results of my last decade of clinical practice and research are reflected in this thesis. I thank the readers of this work, hoping that they will appreciate it and

that it will contribute to insights in their clinical practice. For me, it was a pleasant journey with some surprising findings!

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ABSTRACT

Introduction: Twin pregnancies (TwPs) carry a higher risk of poor obstetric outcomes, such as preterm birth (PTB), fetal growth restriction (FGR), and hypertensive disorders of pregnancy (HDP). These conditions can often be manifestations of placental dysfunction, particularly when early PTB is associated with FGR and/or HDP. However, well-fitted predictive models for these outcomes are lacking in TwPs, and consensus on the management varies between different medical societies and institutions.

Objective: This study aims to investigate the associations between placental function and obstetric outcomes in TwPs, focusing on the role of first- and second-trimester uterine artery Doppler (UtA-PI) measurements and first trimester maternal serum biomarkers in relation to PTB, SGA, FGR, and HDP.

Methods: This is a retrospective cohort study of TwPs managed at the Dr. Alfredo da Costa Maternity, Unidade Local de Saúde de São José (ULSSJOSE), Lisbon, Portugal, between January 2010 and December 2022. The study analyzed data from first-trimester screening, including UtA-PI and maternal serum biomarkers, pregnancy-associated plasma protein-A (PAPP-A) and β-human chorionic gonadotropin (β-hCG), as well as second-trimester UtA-PI. These data were combined with maternal and pregnancy characteristics.

The primary outcomes evaluated were PTB occurring < 32 weeks (w), < 34w, and < 36w, small for gestational age (SGA) defined as birthweight below the 3rd, 5th, and 10th percentiles, and HDP which included gestational hypertension (GH), early-onset preeclampsia (PE), late-onset PE, and HELLP syndrome. Secondary outcomes included fetal demise, FGR, spontaneous and medically indicated PTB, birth weight discordance (BWD) ≥ 25% or more, mean neonatal birth weight, composite outcomes of PTB associated with FGR and/or HDP, and neonatal and perinatal morbidity and mortality.

Univariable analysis of the primary outcomes was performed using Mann-Whitney, Chi-square, or Fisher's exact tests, as appropriate. Multivariable logistic regression (LR) models were used to analyze the composite outcomes of PTB related to FGR, SGA, and/or HDP. Discriminative ability and calibration of these models were assessed by the area under the receiver-operating characteristic curve (AUC) and the Hosmer-Lemeshow (HL) goodness-of-fit test, respectively.

Results: The median first-trimester UtA-PI was significantly lower in TwPs compared to singleton pregnancies (SP), with the lowest values observed in DC pregnancies. In

univariable analysis, UtA-PI ≥ 95th percentile was associated with increased odds of PTB < 32 w and < 34 w, all categories of SGA, and higher neonatal morbidity. A 100% detection rate for early-onset PE was achieved when using the UtA-PI 90th percentile cut-off for twins. However, when singleton references were used, the detection rate dropped to 50%. Late-onset PE, GH, and HELLP syndrome were not associated with high UtA-PI.

In the multivariable analyses for PTB, SGA, and the composite outcome of PTB concurrent with FGR, SGA, and/or HDP, UtA-PI ≥ 95th percentile proved to be an independent risk factor for the outcomes studied. The highest odds were observed for PTB < 32 w concurrent with FGR, SGA, and/or HDP.

Low PAPP-A was also found to be an independent risk factor for SGA < 3rd percentile, PTB < 34 w, and the composite outcome of PTB < 32 w and < 34 w concurrent with FGR, SGA, and/or HDP. Importantly, none of the women with PAPP-A MoM > 90th percentile developed early-onset PE or PTB < 35 weeks.

The best multivariable model for predicting PTB concurrent with FGR, SGA, and/or HDP integrated both first and second trimester data, achieving an 85% detection rate with a false positive rate of 16%.

Conclusions: We emphasize the need for specific adjustments in clinical research for TwPs, including the establishment of UtA Doppler reference values based on larger datasets, the use of twin-specific birth weight charts, and the consideration of maternal and pregnancy characteristics, which may have a greater impact in TwPs. While clinicians await more robust evidence regarding the use of prophylactic aspirin in TwPs to prevent PE and possible FGR, we recommend that first-trimester UtA Dopplers (adjusted for twins) be included in clinical guidelines as a key risk factor for placental dysfunction-related conditions. Unless other significant risk factors for HDP are present, women with low UtA-PI and high PAPP-A levels are unlikely to benefit from aspirin prophylaxis. Nevertheless, close monitoring of all TwPs for HDP and FGR remains essential.

RESUMO

Introdução: As gravidezes gemelares apresentam um maior risco de resultados obstétricos adversos, como parto pré-termo (PPT), restrição de crescimento fetal (RCF) e distúrbios hipertensivos da gravidez (DHG). Estas condições podem frequentemente ser manifestações de disfunção placentar, especialmente quando o PTB precoce está associado a RCF ou DHG. No entanto, faltam modelos preditivos bem ajustados para estes desfechos em gravidezes gemelares, e o consenso sobre as condutas clínicas destas gravidezes varia entre diferentes sociedades médicas e instituições.

Objetivo: Este estudo tem como objetivo investigar as associações entre a função placentária e os desfechos obstétricos em gestações gemelares, com foco no Doppler das artérias uterinas no primeiro e segundo trimestres e dos biomarcadores séricos maternos do primeiro trimestre em relação ao PTB, recém-nascido leve para a idade gestacional (LIG), RCF e DHG.

Métodos: Este é um estudo de coorte retrospetivo de gravidezes gemelares acompanhadas na Maternidade Dr. Alfredo da Costa, Unidade Local de Saúde de São José (ULSSJOSE), Lisboa, Portugal, entre Janeiro de 2010 e Dezembro de 2022. Foram analisados dados do rastreio do primeiro trimestre, incluindo o UtA-PI e os biomarcadores séricos maternos proteína plasmática associada à gravidez - A (PAPP-A) e β-gonadotrofina coriónica humana (β-hCG), bem como o UtA-PI no segundo trimestre. Estes dados foram combinados com características maternas e da gravidez.

Os desfechos primários avaliados foram: PPT < 32 semanas (s), < 34 s e < 36 s, leve para a idade gestacional (LIG), definido como peso ao nascimento abaixo do percentil 3, 5 e 10, e DHG incluindo hipertensão gestacional (HG), pré-eclâmpsia (PE) precoce, PE tardia e síndrome HELLP. Os desfechos secundários avaliados incluíram: morte fetal, RCF, PPT espontâneo e induzido, discordância de peso ao nascer ≥ 25%, peso médio dos recém-nascidos, desfechos compostos de PPT associado a RCF e/ou DHG, morbilidade e mortalidade neonatal e perinatal.

A análise univariável dos desfechos primários foi realizada utilizando os testes de Mann-Whitney, qui-quadrado ou exato de Fisher, conforme apropriado. Modelos de regressão logística multivariável foram utilizados para analisar os desfechos compostos de PPT relacionados com RCF, LIG e/ou DHP. A capacidade discriminativa e a calibração desses modelos foram avaliadas pela área sob a curva ROC (AUC) e pelo teste de ajustamento de Hosmer-Lemeshow (HL), respetivamente.

Resultados: A mediana da UtA-PI do primeiro trimestre foi significativamente mais baixa em gestações gemelares em comparação com gestações únicas, sendo os valores mais baixos observados em gestações dicoriónicas. Na análise univariável, o UtA-PI ≥ percentil 95 foi associado a maiores probabilidades de PPT < 32 s e < 34 s, a todas as categorias de LIG e a uma maior morbilidade neonatal. Foi alcançada uma taxa de deteção de 100% para PE precoce ao utilizar o ponto de corte do percentil 90 do UtA-PI para gravidez gemelar. No entanto, quando utilizadas referências dos Dopplers das artérias uterinas nas gravidezes simples, a taxa de deteção baixou para 50%. Não se encontrou associação entre um UtA-PI elevado e a PE tardia, HG e a síndrome HELLP.

Nas análises multivariáveis para PPT, LIG e o desfecho composto de PPT associado com PPT, RCF e/ou DHG, o UtA-PI ≥ percentil 95 provou ser um fator de risco independente para os desfechos estudados, com a maior razão de possibilidades observada para o PPT < 32 s associado a RCF, LIG e/ou DHG.

Um valor baixo de PAPP-A também foi identificado como um fator de risco independente para LIG < percentil 3, PPT < 34s e o desfecho composto de PPT < 32 s e < 34 s com RCF, LIG e/ou DHG. Importa salientar que nenhuma das mulheres com PAPP-A MoM > percentil 90 desenvolveu PE precoce ou PPT antes das 35 semanas.

O melhor modelo de associação multivariável para o desfecho de PPT associado a RCF, LIG e/ou DHG integrou dados do primeiro e segundo trimestre, atingindo uma taxa de deteção de 85% dos casos, com uma taxa de falsos positivos de 16%.

Conclusões: Os autores defendem a necessidade de ajustes específicos na investigação clínica para gravidezes gemelares, incluindo o estabelecimento de valores de referência para os Dopplers das artérias uterinas com base em maiores bases de dados, a utilização de curvas de peso ao nascer específicas para gémeos e a consideração das características maternas e factores da gravidez, que podem ter um maior impacto nas gravidezes gemelares.

Enquanto os clínicos aguardam evidências mais robustas sobre o uso de aspirina profilática para a prevenção de PE precoce, e eventualmente de RCF na gravidez gemelar, recomendamos que o Doppler das artérias uterinas do primeiro trimestre seja incluído nas recomendações clínicas para identificar as grávidas com maior risco de desfechos relacionados com a disfunção placentar. Excetuando os casos em que estejam presentes fatores de risco significativos para DHG, as grávidas com UtA-PI baixos e níveis elevados de PAPP-A provavelmente não beneficiarão da profilaxia com aspirina. Assim sendo, sugerimos que as diretrizes atuais para a instituição de aspirina

profilática na gravidez sejam reconsideradas tendo em conta a informação do Doppler das artérias uterinas no primeiro trimestre. No entanto, a vigilância frequente de todas as gravidezes gemelares para os DHG e RCF permanece recomendada.

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LIST OF ABBREVIATIONS

AM – accelerated maturation
ART – assisted reproduction techniques
AUC – area under the receiver-operating characteristic curve
BP – blood pressure
BWD – birth weight discordance
CL – cervical length
CRL – crown-rump length
DA – diamniotic
DC – dichorionic
EFW – estimated fetal weight
EVT – extravillous trophoblast
(s)FGR – (selective) fetal growth restriction
FM – female/male
FVM – fetal vascular malperfusion
GD – gestational diabetes
GH – gestational hypertension
HDP – hypertensive disorders of pregnancy
HELLP – elevated liver enzymes and low platelets
HL – Hosmer–Lemeshow test
ISUOG – International Society of Ultrasound in Obstetrics and Gynecology
ldASA – low dose aspirin
LMWH – low molecular weight heparin
LR – logistic regression
MAP – mean arterial pressure
MC – monochorionic
MoM – multiple of the median
MVM – maternal vascular malperfusion
NICU – Neonatal Intensive Care Unit
OR – Odds Ratio
PAPP-A – pregnancy-associated plasma protein-A
PE – preeclampsia
SP – singleton pregnancy
PTB – preterm birth
sFlt1/PlGf – soluble fms-like tyrosine kinase-1/placental growth factor ratio
sEng – soluble endoglin
SGA – small for gestational age
TORCH – toxoplasmosis, others (syphilis, hepatitis B), rubella, cytomegalovirus, herpes simplex infections
TwPs(s) – twin pregnancy(ies)
US – ultrasound
UtA-PI – uterine artery pulsatility index
VML – vascular malperfusion lesions
VUE – villitis of unknown etiology
W – week(s)
β-hCG – β-human chorionic gonadotropin

CHAPTER 1 – INTRODUCTION

Twin pregnancy is a rare phenomenon in humans, occurring in only a small fraction of pregnancies, but it has always fascinated societies throughout history. In ancient civilizations, the birth of twins was often seen as a mystical event, laden with spiritual significance. In many cultures, mothers of twins were revered and celebrated as symbols of fertility and divine blessings. It was believed that the arrival of twins was an intervention of the gods or a manifestation of supernatural forces, and thus, these mothers were accorded a special status within the community.

One of the first to attempt a scientific understanding and description of twin pregnancy was the Greek philosopher Aristotle. In the 4th century BC, he made pioneering observations on fetal development and speculated on the origins of twins. Although his theories were limited by the knowledge of his time, Aristotle paved the way for the study of twinning, laying the groundwork for a deeper understanding of this phenomenon.

Over the centuries, the perception of twin pregnancy evolved, shifting from a phenomenon shrouded in myths and symbolism to a field of scientific inquiry. However, even with the advances of modern medicine, twin pregnancies continue to present unique challenges. Women carrying twins face higher risks for both maternal and fetal health, including an increased incidence of complications such as preterm birth, fetal growth restriction, and gestational hypertension.

The complexity of twin pregnancy, combined with its rarity and medical implications, keeps this type of gestation a field of great interest, both in medicine and reproductive biology. The combination of medical challenges and the rich historical and cultural context makes twin pregnancy a fascinating and multifaceted topic that continues to capture the imagination and attention of professionals and the general public alike.

This thesis seeks to enhance medical understanding of the challenges and adverse outcomes associated with twin pregnancy. Recognizing placental function as a key factor in these outcomes, the research delves into the relationship between placental function parameters and obstetric results.

CHAPTER 2 – LITERATURE REVIEW

2.1 PATHOPHYSIOLOGY AND EVALUATION OF THE PLACENTA FUNCTION

The placenta is a vital organ that develops during pregnancy, playing several crucial roles in supporting fetal growth and development. It establishes the interface for nutrient and gas exchange between maternal and fetal circulations and induces maternal immune, cardiovascular, and metabolic adaptations to promote fetal growth and ensure successful delivery. With such an important role, it is not surprising that defective early placental development is the primary cause of common pregnancy disorders [1]. Additionally, adverse pregnancy conditions may also affect the life-long health of the fetus via developmental programming [2].

Human placentation is highly invasive, and by 11 days post-fertilization, the conceptus becomes completely embedded within the wall of the uterus. One of the crucial roles of the extravillous trophoblast (EVT) cells is to mediate vascular remodeling, invading the maternal decidua towards the spiral arteries, where they destroy the smooth muscle media and transform the arteries into high conductance vessels. The spiral artery transformation, facilitated by EVT invasion, is critical to pregnancy success. Lack of balanced regulation of EVT invasion leads to deficient spiral artery remodeling and inadequate utero-placental vascularization associated with placental dysfunction related conditions including recurrent miscarriage, fetal growth restriction (FGR), preeclampsia (PE), preterm birth (PTB) and stillbirth [3].

Different studies have attempted to integrate various clinical and laboratory findings to support the theory of placental dysfunction due to defective implantation and subsequent poor placental perfusion occurring in the early stages of pregnancy. The most common methods used to evaluate placental function include:

- Ultrasound and MRI that analyze placental volume and structure [4];
- Detailed Doppler studies of the uterine artery (UtA), umbilical artery, and fetal middle cerebral artery [5,6];
- Placental histologic examinations [7,8];
- Maternal serum biomarkers [9];
- Genetic and epigenetic studies [10,11].

There is consistent evidence that abnormally elevated UtA resistance is associated with defective trophoblast migration, as observed in placental bed biopsies. The presence of placental vascular malperfusion lesions (VML) in cases of FGR, coupled with abnormal UtA Doppler velocimetry, closely resembles the pathology seen in preterm PE.

Conversely, placentas from pregnancies complicated by PE or FGR that present at or near term exhibit a significantly lower frequency of histological abnormalities compared to those with early-onset disease [12].

Angiogenic molecules resultant from ischemia-reperfusion injury in placental villi can be detected in maternal serum. Impaired uteroplacental perfusion reduces the placental secretion of the pro-angiogenic Placental Growth Factor (PlGF) and enhances secretion of the anti-angiogenic factors soluble FMS-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng). The altered circulating ratio of these placenta-derived proteins induces endothelial dysfunction that is responsible for the clinical manifestations of HDP, and sFlt1/PlGf ratio was found to be positively correlated with the severity of maternal vascular disease in singleton pregnancies (SP) [13,14].

Finally, genetic and epigenetic studies of the placenta are important for advancing our understanding of placental biology, its role in pregnancy outcomes, and its impact on long-term health outcomes for the offspring. In the future, these insights can lead to the development of better diagnostic, preventive, and therapeutic strategies for pregnancy-related disorders.

2.2 PARTICULARITIES OF TWIN PREGNANCIES

2.2.1 Definition and Epidemiology

A pregnancy with more than one fetus is called multiple pregnancy and twins are two offspring produced by the same pregnancy. Historically, the frequency of twins has been one in 80-90 deliveries but, in the past years, the rate of twin pregnancies (TwPs) is growing worldwide, mostly due to ovulation inductions, assisted reproduction techniques (ART) and advance maternal age [15].

Twins can be classified according to zygosity and chorionicity:

- Dizygotic: 70-75% of TwPs result from the fertilization of two eggs by two sperm cells (distinct genetics). They are dichorionic (DC), two separate placentas, and diamniotic (DA), two amniotic sacs. In rare cases, more frequently after ART, placental fusion can occur, and the pregnancy may clinically behave as a Monochorionic (MC) pregnancy.
- Monozygotic: 25-30% of TwPs result from the division of a single zygote formed by the fertilization of one egg by one sperm cell (identical genetics). These

pregnancies can be:

- Monochorionic-diamniotic (MC/DA), one single placenta with two amniotic sacs in 70-75% of cases
- Dichorionic-diamniotic (DC/DA) in 20-25% of cases
- Monochorionic-monoamniotic (MC/MA), one single placenta and one amniotic sac in 1-2% of cases
- Conjoined twins in <1% of cases, when embryos do not fully separate, resulting in physically connected bodies.

In clinical practice, classification based on chorionicity is often used, as MC twins are a group with a higher risk for complications and need careful monitoring and management. By sharing the same placenta, imbalanced vascular anastomoses between fetal circulations can result in conditions such as twin-to-twin transfusion syndrome (TTTS), twin anemia polycythemia sequence (TAPS), and selective fetal growth restriction (sFGR). These conditions lead to higher mortality and morbidity in MC TwPs.

In recent years, multiple birth rates in Europe have ranged from approximately 2% to 4% of all births, with PTB rates for multiples varying between 39.6% and 66.0%, compared to 4.1% to 7.6% for singletons. In 2022, twin deliveries accounted for 1.6% of all births in Portugal [16].

2.2.2 Placental studies in Twins

Studies on the placenta in TwPs are scarce compared to SP. TwPs are less common and are often excluded from clinical trials due to potential confounders related to complications exclusive to MC twins. This exclusion limits the understanding of placental dynamics and associated risks specific to TwPs, thereby creating a gap in the research and knowledge necessary for improving clinical outcomes in twins. Also, some authors compare placental histological findings of SP with those of TwPs, although the baseline characteristics of these populations differ, such as the rate of PTB, FGR or HDP. Consequently, "different possible causes" have been suggested to explain the complications observed in TwPs, though their underlying pathophysiological mechanisms remain unclear.

A study from Chan et al. evaluated histologic slides of 80 pairs of MC and 80 pairs of DC twin placentas and found that fetal vessel thrombosis occurred in 7.5% of MC and 3.1% of DC twins. These lesions were associated with FGR among the MC and with HDP

among the DC twins. On the other side, vascular anastomoses, TTTS, chorangiosis, and chorioamnionitis were not associated with fetal vessel thrombosis [17].

Another study from Barber et al. compared placental histopathology and neonatal outcomes between 66 DC twins and 500 SP complicated by small for gestational age (SGA). The groups differed in maternal age, parity, smoking habits, gestational age, and PE rates. Additionally, the SGA definition was not adjusted for twins' references. Placentas from SGA in SP exhibited more maternal VML compared to those from SGA in TwPs. The authors suggested that "different mechanisms" might be involved in abnormal fetal growth in TwPs [18].

Similar conclusions were made by Aviran et al. in a study comparing 144 DC twins to 768 singletons complicated by HDP. In this cohort, early-onset PE was significantly lower in twins than in singletons (26% vs. 60%, respectively). Twins had higher mean birth weights, less PTB, and were less likely to be SGA (non-specific references for TwPs). Unsurprisingly, twins were found to have fewer placental VML, and the authors state that these findings support the hypothesis that placental malperfusion is less relevant to the pathogenesis of HDP in TwPs, suggesting that other placental or non-placental factors are responsible for the increased risk of this outcome [19].

More recently, Weiner et al. study found a "dose-dependent" association between the presences of placental VML in 125 DC TwPs with the development of early and severe PE, as well as with SGA and adverse neonatal outcome. These findings emphasize the placental role in the development of early onset PE also in TwPs [20]. Dekalo et al. analyzed 126 FGR DC twins, 59 of them were also diagnosed as FGR according to twin specific charts. This group presented worse neonatal outcomes associated with underlying placental insufficiency, evidenced by a higher rate of placental VML lesions. These findings support using a twin-specific chart to diagnose FGR in twins and underscore the impact of poor placentation [21].

In summary, early studies have compared incomparable cohorts of TwPs and SP. Later studies have adopted more rigorous classification of SGA using twin-specific references and focused on early-onset complications related to placental insufficiency, which have a higher likelihood of VML.

2.3 MAJOR OBSTETRIC COMPLICATIONS IN TWIN PREGNANCIES

Despite being fascinating for couples and obstetricians, TwPs are considered high-risk gestations. Major obstetrical complications include PTB, FGR, HDP and gestational diabetes (GD)[22].

2.3.1 Preterm Birth

PTB is the most frequent complication in TwPs. The chances of having a newborn that weighs < 1500 g is 10 times greater in TwPs than in SP, and at least 60% of all twins are born < 37 weeks [23].

In Europe, about 75% of all neonatal deaths and 60% of all infant deaths occur to infants born preterm. Although survival of preterm infants has increased significantly in the past decade, these infants remain at higher risks of long-term motor and cognitive impairments [24].

PTB in TwPs is multifactorial and can be categorized into 3 groups: medically indicated because of maternal-fetal outcomes (58-62%), after spontaneous onset of preterm labor (30-32%) and after premature rupture of membranes (8-10%) [23].

Much of the research on PTB has focused on spontaneous PTB and second-trimester cervical length (CL) (see Chapter 2.5). However, in recent years, obstetricians have increasingly turned their attention to the prediction, prevention, and treatment of HDP and FGR, as these are the most common conditions that can lead to iatrogenic PTB as well as maternal and neonatal morbidity.

2.3.2 Fetal Growth Restriction

FGR refers to a condition in which a fetus is unable to achieve its genetically determined potential size and affects up to 5-10% of SP. FGR is associated with high incidence of intrauterine fetal demise, intrapartum fetal morbidity, and operative deliveries. The origins of FGR can be fetal, maternal, placental or environmental. Fetal causes are less common and include aneuploidy, genetic syndromes, fetal malformations and congenital infections [25].

It is indisputable that the majority of twins are born with lower birth weights compared to singletons. Additionally, about 60% of them are born before 37 weeks.

From 28-30 weeks onward, the rate of fetal growth in twins decreases, and with this adaptation, it is possible to prolong the gestational age at birth [26].

In a study that used the United States National dataset of all twins delivered from 1995 through 1997, weight-discordant twins were found to be delivered at a more advanced gestational age than concordant twins. Discordant growth may serve as an adaptive measure to promote maturity by reducing the inevitable "uterine overdistension" [27].

We could consider that, by definition, the vast majority of twins experience growth restriction since they are genetically programmed for a higher weight and, in most cases, would achieve a higher birth weight if they were singletons. Indeed, the combined mean estimated fetal weight of twins around 30 weeks corresponds to the mean estimated weight of a singleton fetus around 37 weeks [28].

However, in a significant proportion of cases, this adaptive phenomenon does not result in increased fetal demise or neonatal morbidity [29]. A fetus can be considered small but healthy, and in both SP and TwPs, the addition of Doppler parameters is used to distinguish the growth-restricted fetus at higher risk for adverse perinatal outcomes [30].

Also, we can postulate, for example, that maternal factors such as height can act as a proxy for uterine capacity. An argument could be made that maternal height is also driven by genetic factors, and this is the main reason taller mothers have bigger newborns. However, studies investigating pregnancies conceived using oocyte donation have shown that fetal weight is more closely associated with the height and constitution of the pregnancy carrier rather than the donor [31]. This finding implies that maternal factors can exert their effect via "uterine capacity" rather than genetic predisposition.

Albeit the previous considerations, FGR remains one of the most significant causes of perinatal morbidity and mortality, significantly impacting TwPs. In preterm FGR (early-onset represents 20-30%), particularly < 34 w, iatrogenic prematurity is frequent. The incidence of FGR and SGA in twins depends on the definition of FGR and birthweight references adopted from different societies [32,33]. Within one study, three separate criteria were applied for FGR - at least one twin with birth weight <10th percentile, or at least one twin with birth weight <5th percentile or a birth weight discordance (BWD) ≥ 20%, giving an incidence of 48%, 27% and 16%, respectively [34].

MC pregnancies present a higher rate of fetal morbimortality than DC. Apart from TTTS, which significantly impacts around 15% of these pregnancies, MC twins are more likely to be complicated by FGR than DC twins, with nearly twice the rate of FGR: 19.7% vs 10.5%, and perinatal mortality rate: 75.1/1000 vs 33.0/1000, in MC and DC, respectively [35,36]. The higher incidence of FGR in MC can be justified by a greater incidence of marginal or velamentous umbilical cord insertion, unequal sharing of the placental bed, unbalanced arterio-venous anastomoses, or higher rate of fetal malformations encountered in this type of placentation [37]. Moreover, sFGR in DC can also be a result of placental insufficiency affecting only one fetus.

In DC, birthweight can also be influenced by the co-twin sex. A study found an intrauterine effect of the male twin on birth weight of its female co-twin. The comparison of females in same-sex pairs with females in unlike-sex (FM) pairs, showed significantly higher birth weights of females in FM pairs. No significant difference was found when males in same-sex pairs were compared with males in FM pairs [38].

The management of sFGR in twins depends on chorionicity. In MC twins, the death of one twin can affect the co-twin with an increased risk of mortality and neurological sequelae. Therefore, the medical approach is more interventional once fetal viability is reached and if there is an imminent risk of one fetal death [39]. In cases of sFGR in DC pregnancies, the timing of delivery should be discussed with parents and neonatologists. However, there is clinical consensus to delay delivery until 30-32 weeks to protect the healthy fetus [40].

In certain severe early restricted cases, complicated by severe PE, selective reduction is possible in DC twin pregnancies. The FGR twin's selective feticide, or the single spontaneous fetal demise, may resolve PE and extend pregnancy duration to obtain better obstetric outcomes in a properly growing fetus [41].

2.3.3 Hypertensive Disorders of Pregnancy

Maternal conditions such as HDP are leading causes of maternal mortality and morbidity, especially PE. PE complicates approximately 2-3% of SP and 10% of TwPs. Compared to SP, the estimated relative risk for preterm PE is 8.7 for DC and 9.1 for MC TwPs [42].

Several risk factors for PE were identified, including primiparity, multifetal gestation, extreme maternal age, a previous personal or family history of PE, and medical comorbidities such as obesity, hypercoagulable states, chronic hypertension, renal disease, lupus, and diabetes mellitus [43].

PE is a complex medical disorder and a pregnancy-specific syndrome, defined as the presence of new-onset hypertension (> 140 mmHg systolic or > 90 mmHg diastolic occurring on two separate occasions, more than 6 hours apart) after 20 weeks' gestation accompanied by proteinuria (300 mg/24 h or a spot urine protein/creatinine ratio 30 mg/mmol) and/or evidence of maternal acute kidney injury, liver dysfunction, neurological features, hemolysis/thrombocytopenia, or FGR [44]. The hallmarks of PE include increased vasoconstriction (resulting in maternal hypertension and reduced uteroplacental blood flow), disturbed vascular endothelial integrity with increased vascular permeability and activation of the coagulation cascade [45].

Recently, a growing body of evidence has supported the theory of the adaptations of the maternal cardiovascular system during normal pregnancy and its maladaptation in PE. Taking into account the different roles of the placenta and the maternal cardiovascular system in the pathophysiology of PE, two phenotypes of PE have been described [43]. Type I PE may present earlier, characterized by placental dysfunction (shallow trophoblast invasion, inadequate spiral artery conversion, profound syncytiotrophoblast stress) elevated sFlt1, reduced PlGF levels, high peripheral vascular resistance, and low cardiac output. Type I is more often accompanied by FGR, and low PlGF levels have a measurable impact on maternal cardiac remodeling and function. Type II PE typically occurs in the later stages of pregnancy and entails an evolving maternal cardiovascular intolerance to the demands of pregnancy, with a moderately dysfunctional placenta and inadequate blood supply. The sFlt1/PlGF ratio may be normal or slightly disturbed, peripheral vascular resistance is low, and cardiac output is high, but these adaptations still fail to meet demand. Emergent placental dysfunction, coupled with an increasing inability to meet demand, more often appears with fetal macrosomia, multiple pregnancies, or prolonged pregnancy [43]. In both scenarios, we can conclude that the placenta cannot meet the demands of fetal necessities. This imbalance triggers a cascade of biological processes that, in extreme cases, can result in maternal or fetal death if medical intervention does not occur.

In SP, HDP are complicated by FGR in 30 to 40% of cases. If early FGR is present, the association with early PE is up to 50%. The association between HDP and FGR in DC

TwPs is similar in magnitude to that observed in singletons, so long as appropriate birth weight references for twins are applied [46].

2.4 SCREENING FOR PLACENTAL DYSFUNCTION RELATED CONDITIONS

Overall, the advantages of screening for placental dysfunction related conditions lie in the potential for early intervention, improved outcomes, and more efficient use of healthcare resources. The first trimester is considered the ideal time for early stratification of high-risk pregnancies because it allows for the establishment of prophylactic treatments that can reduce morbidity.

In SP, first-trimester screening using maternal factors, mean arterial pressure (MAP), UtA-PI, PAPP-A and/or PlGf to assess the risk for early-onset PE and FGR are well established [47-49]. The same model of first-trimester screening for PE was extended to TwPs, albeit with less robust conclusions about its effectiveness [50].

Additionally, the first-trimester screening for SGA utilizing a predictive competing-risks model (including maternal factors, MAP UtA-PI, PAPP-A, PlGf), in a large dataset of SP (57,131 women) achieved moderate performance [51]. In the case of TwPs, there are no published studies of first-trimester predictive models for FGR or SGA using similar methods as those used in SPs. The data on TwPs is scarce, and validating predictive models can be challenging. Some studies have analyzed the ability of discordance in the fetal crown-rump length (CRL) to identify sFGR. A CRL discrepancy of 7% or more in MC twins has a sensitivity of 92% for sFGR with a specificity of 76% [52]. In addition, in both MC and DC twins, CRL discordance $\geq$ 10% in the first trimester is associated with several adverse pregnancy outcomes, including preterm delivery < 34 w, BWD and perinatal loss. However, the accuracy of CRL discordance in predicting adverse outcome is poor and thus limits its routine use in clinical practice [53].

The second trimester screening for PE and FGR can also be attempted, but it does not offer the potential benefit of implementing early prophylactic treatment. The analysis of UtA Dopplers alone (adjusted for twins), showed a sensitivity of 36.4% for PE, 26.7% for FGR and 29.9% for birth weight discordance > 20%, with a SPR of 14.0% [54]. Moreover, evaluation of inter-twin estimated fetal weight (EFW) differences at 21-24 weeks' scans has poor positive predictive value for BWD, though it performs better in MC than in DC twins. On the other hand, the negative predictive value and sensitivity for MC twins were found to be 100%. Concordant EFWs at 21-24 weeks effectively exclude

BWD in MC twins, whereas discordant EFWs are poor predictors of BWD, especially in DC twins [55].

2.5 PREVENTION OF PRETERM BIRTH, PREECLAMPSIA AND FETAL GROWTH RESTRICTION

Different interventions are intended to lower the morbidity and mortality associated with PTB. Primary interventions target all women (e.g., preconception care, lifestyle, nutrition), secondary interventions attempt to eliminate or minimize existing risk (e.g., smoking cessation, treating urinary tract infections, CL measurement), and tertiary interventions improve outcomes for preterm infants (e.g., antibiotics, tocolytic drugs, and prenatal corticosteroids). To date, most efforts have focused on tertiary therapies proven to decrease perinatal morbidity and mortality, but these have had limited success [56].

In TwPs, most clinical efforts have been focused on predicting the spontaneous onset of preterm labor. This involves measuring CL or fetal fibronectin (fFN) levels to identify pregnancies at higher risk. Prophylactic treatments have been established for short CL, such as the administration of local progesterone, vaginal pessary insertion, cerclage procedures, or bed rest [57-59].

As mentioned before, these interventions address only about one-third of the etiology of PTB in TwPs and do not always have a substantial impact on improving perinatal outcomes. Instead, some strategies can increase the duration of antepartum admission without concomitant benefits, and therefore improvements in primary and secondary care are still necessary to prevent disabilities associated with spontaneous PTB [57].

On the other hand, iatrogenic PTB is often indicated due to maternal and fetal conditions related to placental dysfunction, and various prophylactic treatments have been explored to reduce PTB associated with poor placentation [60,61]. In clinical practice, ldASA and low molecular weight heparin (LMWH) are the most commonly used, with higher evidence of effectiveness and safety regarding adverse effects. In the case of SP, the use of ldASA for the prevention of preterm PE in high-risk patients is well established and is accompanied by a decrease in the risk of PTB and FGR [62]. It seems that ldASA can improve placentation if started before the 16th week of pregnancy and reduces the incidence of premature PE and severe form of FGR, thus reducing

prematurity (spontaneous or iatrogenic) caused by this process [63,64]. In the ASPRE clinical trial, the use of ldASA reduced the overall incidence of SGA < 10th percentile by about 40% in newborns at < 37 w' gestation and by about 70% in newborns at < 32 weeks [65].

A meta-analysis comparing the effectiveness of combined ldASA plus LMWH to ldASA alone for the prevention of PE in SP with previous PE, concluded that the combination of both drug is more effective than ldASA alone, with a significant reduction in the incidence of PE (RR: 0.54) and in delivery of SGA age neonates (RR: 0.54)[66]. Administration of both drugs (LMWH plus ldASA) is used for a long time and, although their effectiveness is only proven for cases with obstetric antiphospholipid syndrome and possibly second trimester pregnancy loss, their use is currently widespread in high risk pregnancies for poor perinatal outcome [67,68]. These drugs demonstrated *in vitro* studies direct actions on the developing trophoblast, attenuating placental apoptosis [69,70].

In TwPs there is a lack of evidence of the effectiveness of such interventions [71,72]. Clinical guidelines are not well-established and vary among different institutions. Previous studies addressed the potential benefits of aspirin intake in TwPs. A systematic review and meta-analysis showed that the administration of aspirin in women with TwPs reduced the risk of PE but not FGR. The overall quality of evidence is low and this highlighted the need for randomized controlled trials elucidating the actual role of aspirin in affecting maternal and perinatal outcomes in TwPs [73]. More recently, a multicentric clinical trial (ASPRE-T) funded by Fetal Medicine Foundation (UK) is being conducted to clarify the use of aspirin for the prevention of preeclampsia in TwPs [74].

2.6 SUMMARY

PTB is a major concern in TwPs and, in more than half of the cases, placental dysfunction and its consequences may be involved. In TwPs, there is scarce evidence of predictive models' performance for early-onset PE, FGR and/or SGA. Prediction and prevention should be evidence-based, and more research is needed to identify those TwPs at higher risk for poor obstetric outcomes. Clinical guidelines should consider the cost-benefit of implementing prophylactic drugs in all TwPs or alternatively in an individualized strategy, and also provide clarity for couples' counseling.

Therefore, if we aim to reduce the morbidity associated with twin pregnancies, this

can only be achieved by first correctly and early identifying those at risk, followed by the institution of pharmacological prophylaxis that potentially optimizes placental function and consequently reduces the incidence of PTB associated with FGR and early-onset PE.

CHAPTER 3 – OBJECTIVES AND METHODOLOGY

3.1 OBJECTIVES

General objective

To study the influence of placental function in obstetric outcomes in TwPs.

Specific objectives

1) To study the association of UtA Dopplers parameters (evaluated in first and second trimester) and first-trimester maternal serum placental biomarkers (free β-hCG, PAPP-A) in TwPs in:
   - the incidence of PTB
   - the incidence of SGA and FGR
   - the incidence of HDP
   - the incidence of neonatal morbidity and mortality

2) To obtain multivariable models integrating maternal and pregnancy factors with placental function parameters (UtA Doppler and serum biomarkers) for poor obstetric outcomes (PTB, FGR, SGA, HDP) in TwPs.

3) To propose a clinical strategy for identifying and implementing individualized prophylactic measures in TwPs for placenta-related pregnancy complications.

3.2 STUDY DESIGN

Study type: observational

Study design: single center cohort study.

3.3 SETTING, PARTICIPANTS AND FOLLOW -UP

This is a study of TwPs followed at the Dr. Alfredo da Costa Maternity, Unidade Local de Saúde de São José (ULSSJOSE), Lisbon, Portugal, between January 2010 and December 2022. This is a tertiary perinatal center that cares for the Lisbon area, serves as a referral center for the South of Portugal and follows around 100 women per year.

In 1994, Teresinha Simões, PhD, MD, founded the Multiple Pregnancy Outpatient Consultation at our center and is the principal investigator for the Twins Study Group. All clinical information about pregnancies and deliveries has been collected

prospectively in an informatic database since its inception by two dedicated and experienced obstetricians. To date, this database accounts for more than 3,500 patients followed.

Taking into account the long period of the Twins Study Group database and the temporal changes in clinical practice that occurred, this cohort can be divided into three groups: 1) a historical cohort from 1994 to 2006, which includes maternal data and obstetric outcomes but lacks reliable data on fetal biometry (without informatic record of ultrasound data); 2) a cohort from 2006 to 2010, which adds ultrasound data on fetal biometry retrieved from Astraia® software; 3) the most recent cohort, from 2010 onward, includes available data from first trimester screening, with additional variables of interest reflecting placental function (maternal serum biomarkers and uterine artery Doppler) that have been integrated into clinical practice and were considered for this study.

At our center, around half of the patients start pregnancy surveillance in the first trimester and complete the first trimester screening at our Fetal Medicine and Surgery Center. The screening for aneuploidies is performed by Fetal Medicine Foundation certified obstetricians, who utilize the Astraia software® for risk calculation. The maternal parameters (weight, height, conception method, and ethnicity) and medical history (parity, cigarette smoking, chronic hypertension, diabetes mellitus, systemic lupus erythematosus or antiphospholipid syndrome, previous PTB, FGR or PE, family history of PE) are included in the software. After 16 weeks, routine scans include standard fetal biometrics (biparietal diameter, head circumference, abdominal circumference and femur length) to calculate an EFW using a Hadlock formula (I), biophysical profile and fetal doppler assessment as indicated. For MC, the standard surveillance is an US every 2 weeks, regarding early detection of TTTS. For DC, a 4 to 6 weeks interval is appropriate. The EFW percentiles presented in the reports are adjusted for MC and DC TwPs (*Stirrup OT et al charts, 2015*) [75].

Obstetric interventions were done according to the institutional clinical guidelines in an individualized practice. After 32 weeks of gestation, the follow-up includes weekly cardiotocography to monitor fetal well-being. In normally progressing gestations, we offered elective termination of pregnancy at 36–38 completed weeks of gestation and iatrogenic preterm deliveries were carried out based on maternal and/or fetal conditions. Neonatal outcomes, in the event of Neonatal Intensive Care Unit (NICU) admission, were derived from the newborn files records of the same center and added

to the Twins Study Group database. Placental histopathologic examinations at ULSSJOSE were routinely conducted by various examiners until 2021, resulting in varied classification methods. In 2022, however, all histopathologic assessments were standardized under the expertise of Dr. Rosete Nogueira, PhD, MD, ensuring consistency in classification criteria. For this study, only the histopathologic examinations conducted by Dr. Nogueira in 2022 were included in the analysis. The study protocol is summarized in Figure 1.

Furthermore, to enable more precise statistical analysis in this study regarding the classification of SGA, we established our own "gold standard" birth weight population empirical percentiles. For this purpose, we analyzed all twins followed in our institution who had uncomplicated pregnancies (excluding maternal, obstetric and fetal diseases) and delivered two live newborns after 36 weeks between the years 1994 and 2002. Our population empirical percentiles for each gestational age were adjusted for chorionicity and based on ultrasound fetal weight estimations inserted in Astraia® software between 20 and 35 w, as well as birth weight data > 36 w, excluding birthweights of twins born prematurely < 36 w (unpublished data).

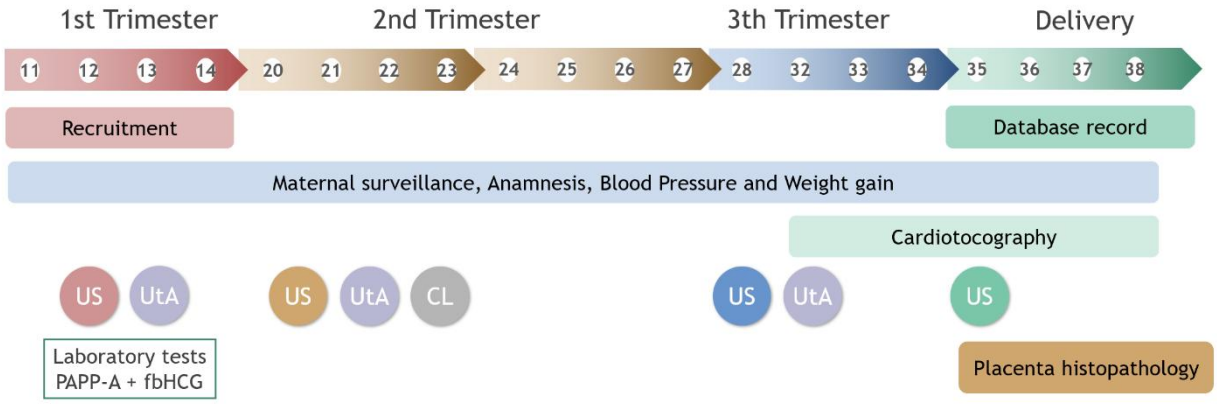


Figure 1 - Resume of the study protocol schedule

3.4 INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria:

- MC and DC viable TwPs followed at Dr. Alfredo da Costa Maternity
- Maternal age ≥ 18 years
- First trimester screening performed in our center
- Two live fetuses at the first-trimester scan
- Delivery ≥ 24 w gestation

Exclusion criteria:

- Multiple pregnancies ≥ 3 fetus
- Major fetal congenital structural anomalies, increased first trimester nuchal translucency ≥ 3.5 mm or genetic disorder
- abnormal umbilical cord (two vessels or velamentous insertions)
- TTTS or TAPS
- Medically induced fetal reduction, vanishing twin ≤ 14 w or single fetal demise < 24w
- MC monoamniotic twins
- TORCH infections or preterm deliveries related to COVID-19
- Less than two ultrasound data available
- Lost to follow-up

3.5 PRIMARY AND SECONDARY OUTCOMES

Primary Outcomes

- PTB < 32 w, < 34 w and < 36w
- SGA < 3rd, 5th and 10th percentile
- HDP: gestational hypertension (GH), early (< 34w) and late-onset (≥ 34 w) PE, HELLP syndrome

Secondary Outcomes

- Fetal demise ≥ 24w
- FGR
- Spontaneous PTB (< 28, < 32, < 34 and < 36 w)
- Medically indicated PTB (< 32, < 34 and < 36 w)
- BWD ≥ 25%
- Mean neonate birthweight
- Composite outcome of PTB (<32, <34 and <36w) associated with FGR, SGA (considered the < 5th percentile), and/or HDP
- Neonatal and perinatal mortality
- Neonatal morbidity: NICU admission, Respiratory Distress Syndrome (RDS), Hyaline Membrane disease (HMD), Bronchopulmonary dysplasia (BPD), peri-

intraventricular hemorrhage (IVH) Grade III/IV, retinopathy of prematurity (ROP), sepsis, Leukomalacia

3.6 PREDICTORS, POTENTIAL CONFOUNDERS AND EFFECT MODIFIERS

For multivariable analyses we will include maternal characteristics and pathologies (age, body mass index (BMI), parity, smoking habits, previous obstetric history, chronic hypertension, diabetes and thrombophilia), placental chorionicity, maternal biophysical parameters (UtA doppler, systolic and diastolic arterial pressure) and maternal serum placental origin biomarkers (fbHCG, PAPP-A).

Cervical insufficiency or other non-placental risk factor for PTB (for e.g. Infection) are potential confounders for poor obstetric outcome.

Patients with previous placenta-related pregnancy complications may be placed under preventive measures typically instituted in clinical practice. These patients may experience a reduction in the incidence of adverse outcomes due to the prescription of ldASA and/or LMWH, which can act as effect modifiers, reducing the clinical impact of placental dysfunction.

ART's resulting pregnancies are usually considered a high-risk group for obstetrics complications, but controlling for confounders like maternal age or chronic hypertension in a multivariable model, can lead to different conclusions.

3.7 DIAGNOSTIC CRITERIA AND MEASUREMENTS

- **Cervical length** was obtained according to the International Society of Ultrasound in Obstetrics and Gynecology (ISUOG) guidelines, in first and second trimester. In our center, measurements can be transabdominal if endocervical channel is clearly identified or transvaginal in all cases when short cervix (≤ 25 mm) is suspected or there is poor visualization.
- **Chorionicity** was established by ultrasonographic criteria: lambda or T-sign in DC or MC, respectively, confirmed by careful examination of the delivered placenta by experienced obstetricians and by histopathologic examination.
- **Estimated Fetal Weight** was calculated using a Hadlock formula (BPD, HC, AC, FL).
- **Fetal and Newborn Weight Discrepancy** was defined using the formula: (larger Twin – smaller Twin / larger) x 100; ≥25% is considered significant.

- **FGR** was defined according to the "C*onsensus definition and essential reporting parameters of selective FGR in twin pregnancy*" described by Khalil A *et al*. [76].
- **Genetic disorder**s were confirmed by fetal or newborn genetic testing.
- **Gestational age** was derived from the last menstrual period that was confirmed or corrected by the measurement of fetal crown–rump length of the larger twin in the first trimester scan or from the day of oocyte retrieval in pregnancies after assisted reproductive techniques (ART).
- **Gestation Diabetes** was defined according to the *International Association of Diabetes and Pregnancy Study Groups Recommendations on the Diagnosis and Classification of Hyperglycemia in Pregnancy* [77].
- **HDP** were defined according to the International Society for the Study of Hypertension in Pregnancy classification, diagnosis, and management recommendations for international practice [78].
- **Major Fetal congenital structural anomalies** were diagnosed in scans by experienced obstetricians and confirmed postnatally by newborn examination or autopsy exams.
- **Maternal serum PAPP-A** and **free β-hCG:** maternal blood samples were collected at 10+0 to 13+6 weeks and processed in our laboratory. Serum PAPP-A and β-hCG levels were measured using two immunoassay systems: Kryptor (Thermo Fisher Scientific, Clinical Diagnostics, Brahms GmbH, Henningsdorf, Germany) and Cobas (Roche Diagnostics, Basel, Switzerland). The results were then converted to multiples of the median (MoM) using Astraia® software.
- **Mean, Systolic and Diastolic Blood pressure (BP)** are measured by validated automated devices according to a standardized protocol recommended by *Fetal Medicine Foundation*.
- **Placental histopathology exams** were classified according to the criteria suggested by Redline [79] and the 2014 Amsterdam Placental Workshop Group [80]
- **SGA** was defined as birth weight < 3^{rd}, 5^{th} and 10^{th} based on empirical percentiles of our TwPs population (unshown data). The 5^{th} percentile was used in the definition of the composited outcome: PTB concurrent with FGR, SGA and/or HDP.
- **Thrombophilia** was defined as any hypercoagulability or a prothrombotic state, inherited or acquired, that necessitated thromboprophylaxis in pregnancy

according to the practice bulletins of the *American College of Obstetricians and Gynecologists* [81].

- **TTTS** is a complication of disproportionate blood perfusion in monochorionic multiples classified in five stages according to *Quintero* [82].
- **UtA Doppler** was obtained according to ISUOG guidelines: in the first trimester the uterine artery was visualized transabdominally along the side of the cervix at the level of the internal *os* [83]. The standard measurement is Pulsatility index (PI) = (systolic velocity - diastolic velocity / mean velocity), that is automatically calculated by ultrasound devices. The mean PIs of the left and right arteries were calculated and considered according to chorionicity and gestational age.

  Considering that the UtA-PI percentiles and their MoM conversions from Astraia® software were not adjusted for TwPs, a comparative study of UtA Doppler values between TwPs and SPs was conducted to establish the appropriate reference ranges and cut-off values for detecting HDP. To obtain empirical percentiles for first-trimester UtA-PI, TwPs that had undergone first-trimester screening in our unit between 11^{+0} and 13^{+6} weeks were considered. TwPs' UtA-PI values were compared with those from a group of SP that had undergone first-trimester screening in the unit during the same period. The comparison was done in a 1:2 proportion, matched for the same pregnancy and maternal conditions. First trimester empirical percentiles obtained from TwPs were adjusted for chorionicity and gestational age [84]. Second-trimester (measurements between 20 and 24 weeks) empirical percentiles were retrieved from the Twins Study Group dataset and adjusted for chorionicity.

3.8 BIAS

Prevention of bias from misclassification included:

- detailed patients' anamnesis and data verification by experienced obstetricians
- standard and validated methods for measurements
- classification of chronicity and FGR by experience US operators
- using international guidelines criteria for clinical conditions (e.g. HDP and Gestation Diabetes)

- neonatal morbidity classified by experienced neonatologist (independent of the study protocol)

3.9 STUDY SAMPLE SIZE

To calculate the required sample size and determine the appropriate time frame in this study, various factors needed to be considered, including the incidence rates of the outcomes of interest, the expected effect sizes (odds ratios), the desired level of statistical power (80% power and a significance level of 0.05), availability of data, and feasibility of recruitment. We based the odds ratio estimates on previous reports that analyzed first-trimester serum biomarkers and UtA dopplers in relation to obstetric outcomes [85-88].

Three major outcomes were considered in this study: PTB, SGA and HDP. To estimate the expected incidence of the primary outcomes in the cohort, the observed rates from historical cohorts in the database were calculated. The expected OR in high-risk pregnancies was estimated based on previous reports [50,51,54].

To analyse the risk of PTB, considering an incidence of 22% < 34 w in this cohort, with 10% of cases in the risk group (UtA Doppler PI $\geq 90^{th}$ percentile) and an expected OR of around 3.0, the sample size needed is 320 cases.

To analyse the outcome of SGA $< 10^{th}$ percentile, we considered an incidence of 32% affected pregnancies in this cohort, with 10% of cases in the risk group (PAPP-A $\leq 10^{th}$ percentile) and an expected OR of around 4.0, the sample size needed is 334 cases.

As early-onset PE has a low incidence (under 1%), typically a large sample size is required to detect significant differences and develop predictive models. Therefore, we did not calculate the sample size for this outcome.

In our preliminary analysis of the twins dataset, and considering the first-trimester data needed as well as the exclusion criteria and the potential lost to follow-up, we estimated that the sample size should be achieved by considering the temporal period from 2010 to 2022 in our institution.

3.10 STATISTICAL METHODS

An exploratory analysis of the variables under study was carried out with categorical variables being described as frequencies (percentages), and the remaining variables as

mean (standard deviation). Normal distribution of the quantitative variables was verified using the Shapiro-Wilk test. Outcome variables were associated with all clinical and demographic variables using Mann-Whitney, Kruskal-Wallis, Chi-square, or Fisher's exact tests, as appropriate. To study the association between the outcomes with maternal and pregnancy characteristics and placental function surrogate variables, linear regression and logistic regression models were used, as appropriate. For the multivariable models, all the variables that in the univariable analysis attained a p-value ≤ 0.25 were selected. Adjusted ORs were estimated with corresponding 95% confidence intervals (95% CI). Discriminative ability and calibration of these models were assessed by the area under the receiver-operating characteristic curve (AUC) and the Hosmer-Lemeshow (HL) goodness-of-fit test, respectively.

Although a significance level of α=0.05 was used, certain variables with p-values greater than 0.05 were retained in the final multivariable models due to their clinical relevance and contribution to improved discriminative ability. Data analysis was performed using the Statistical Package for the Social Sciences for Windows (SPSS, IBM Corp. Released 2021.Version 28.0), STATA 13.0 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP.) and R software (R: A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria, year = 2014, (http://www.R-project.org).

3.11 ETHICAL APPROVAL

This study utilizes data from a historical cohort spanning a lengthy period. Verbal informed consent for anonymous data collection and publication purposes was obtained from subjects before their inclusion in the Twins Study Group within the historical database. This database was approved by the Portuguese National Commission for Data Protection. Therefore, informed written consent was not sought before 2020 for the present study due to its retrospective nature and anonymous data collection.

Additional approval for the present study was granted by the Local Ethical Committees of Nova Medical School (n° 81/2020/CEFCM) and ULSSJOSE (n° 950/2020) (Appendices). After 2020, written consent was obtained from all subjects for participation and publication in the prospective cohort, in accordance with ethical committee guidelines.

CHAPTER 4 – RESULTS

4.1 PARTICIPANTS AND DESCRIPTION OF THE MATERNAL AND PREGNANCY CHARACTERISTICS

Of the 1175 TwPs followed between January 2010 and December 2022 in our center, the dataset included 572 TwPs that met the inclusion criteria (Figure 1). Of these, 466 completed first-trimester serum biochemical screening, 390 underwent first trimester UtA doppler evaluation, 391 second trimester UtA doppler evaluation, and 371 had complete data with all first trimester biomarkers. Maternal and pregnancy characteristics are summarized in Table 1. The dataset included 450 (78.7%) DC and 122 (21.3%) MC pregnancies, the majority of whom are Caucasian nulliparous women with spontaneous conceptions. Two single fetal demises occurred in two DC pregnancies, one at 25 weeks and another at 36 weeks. Neither fetus was suspected to be growth-restricted, although in the latter case, the surviving co-twin was SGA < 3rd percentile.

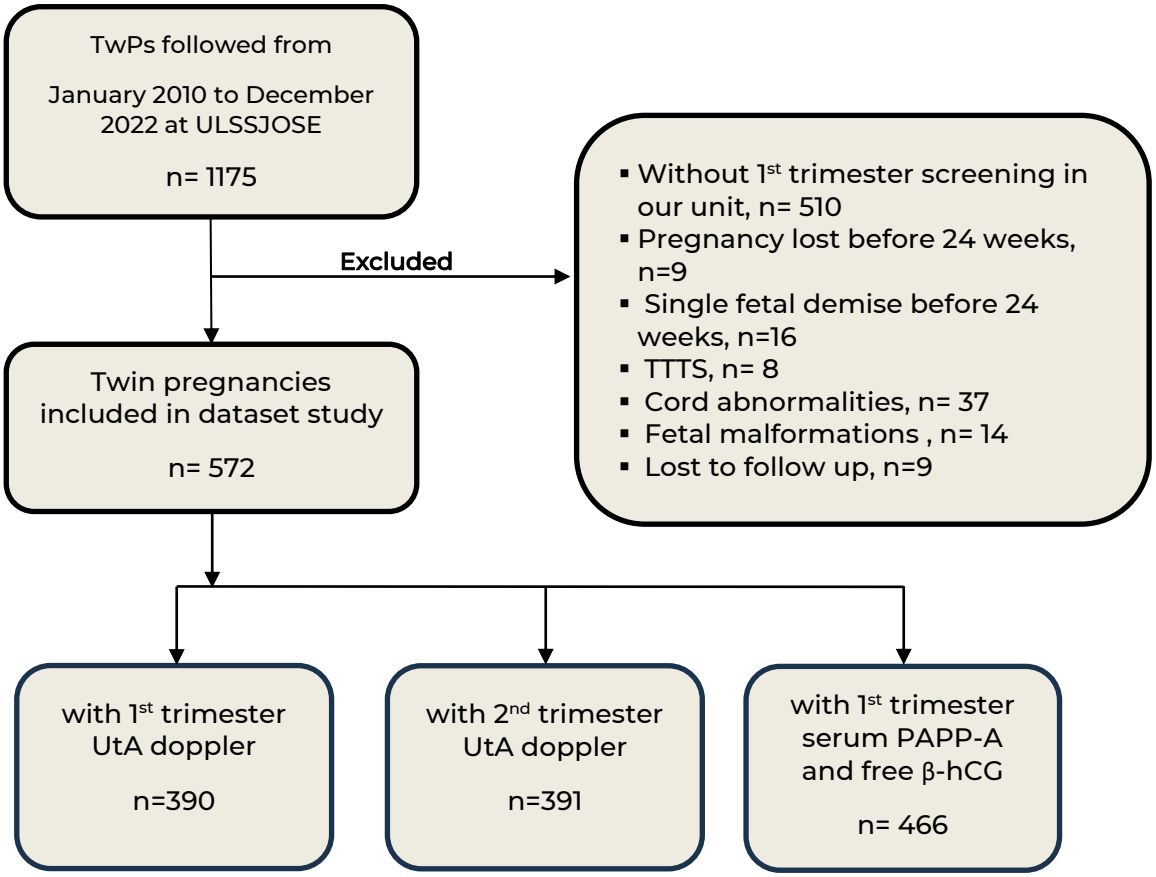


Figure 2. Flowchart showing the inclusion of pregnancies that underwent first trimester screening at ULSSJOSE.

ULSSJOSE – Unidade Local de Saúde de São José; HDP – Hypertensive Disorders of Pregnancy; SGA – Small to Gestational Age; TTTS – twin to twin transfusion syndrome; TwPs – twin pregnancies; UtA – uterine artery

TABLE 1 Demographic and pregnancy characteristics in 572 twin pregnancies who underwent 1st trimester screening.

Variables	Mean (SD) or n (%)
Maternal age (years)	32.7 (5.1)
Age ≥40 years	38 (6.6%)
BMI (Kg/m2)	24.4 (4.3)
BMI <20	74 (12.9%)
BMI ≥20<35	296 (51.7%)
BMI ≥25<30	140 (24.5%)
BMI ≥30	62 (10.8%)
Ethnicity	
Caucasian	493 (86.2%)
African	63 (11.0%)
East Asian	15 (2.6%)
mixed	1 (0.2%)
Parity	
Nulliparous	361 (63.1%)
Parous with prior PTB	22 (3.8%)
Parous with prior PE	8 (1.4%)
Parous with prior SGA	13 (2.3%)
Method of conception	
Spontaneous	345 (60.3%)
Ovulation inductions	21 (3.7%)
ART	206 (36.0%)
Chorionicity	
Monochorionic diamniotic	122 (21.3%)
Dichorionic	450 (78.7%)
Smoker	54 (9.4%)
Chronic hypertension	29 (5.1%)
Family obstetric history of PE	14 (2.4%)
SLE/APS/Thrombophilia	15 (2.6%)
Diabetes Mellitus	3 (0.5%)
Aspirin prophylactic intake (started <20 weeks)	73 (12.8%)
1st trimester Mean Arterial Pressure	85.2 (8.9)

Abbreviations: APS – Antiphospholipid syndrome; ART – artificial reproductive techniques; BMI – body mass index; PTB – preterm birth, PE – preeclampsia; SD – standard deviation; SGA – small for gestational age; SLE – Systemic lupus erythematosus.

4.2 DESCRIPTION OF CLINICAL MARKERS OF PLACENTAL FUNCTION IN TWINS

4.2.1 Uterine artery dopplers

The comparative study of UtA-PI between TwPs and SPs included 454 TwPs, 312 (68.7%) DC and 142 (31.3%) MC, and 908 SP, as summarized in Table 2. Second-trimester empirical percentiles were retrieved from the 391 measurements in the dataset.

The median first-trimester UtA-PI was significantly lower in TwPs compared to SP, with the lowest values observed in DC pregnancies (p<0.001; Figure 3 and Table 3). A weak evidence of a difference between the distributions of UtA-PI in DC and MC pregnancies was found (p=0.076), with the 95th percentile of UtA-PI obtained in MC pregnancies being similar to that derived from SP (1.74 vs 1.73, respectively). The 95th percentile of second trimester uterine UtA-PI was lower in DC compared to MC pregnancies, however, the difference was not statistically significant (p = 0.170; Table 4 and Figure 4).

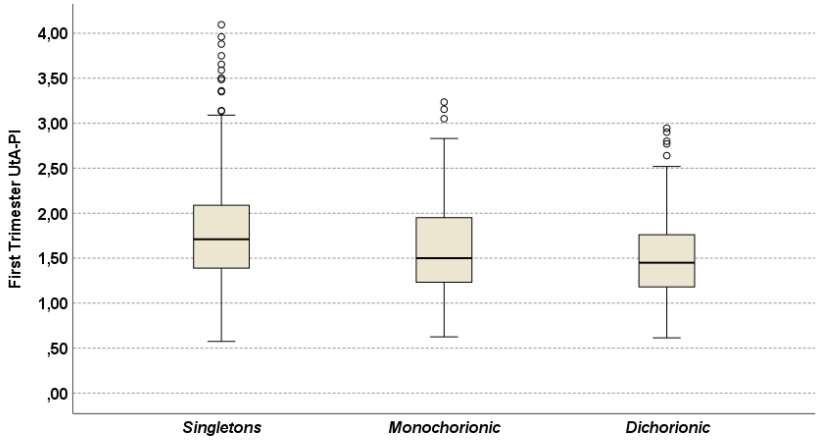


Figure 3. First Trimester uterine artery pulsatility index (UtA-PI) in singletons and twin pregnancies (circles represent moderate outliers).

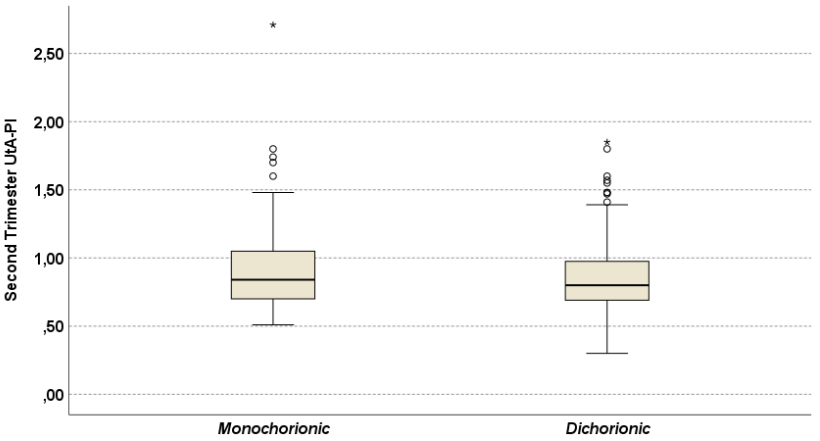


Figure 4. Second trimester uterine artery pulsatility index (UtA-PI) in twin pregnancies
(circles represent moderate outliers and asterisks extreme outliers).

TABLE 2 Demographic and pregnancy characteristics in twin pregnancies compared to a group of singleton pregnancies with first trimester uterine artery doppler evaluation.

	Twins, n=454	Singletons, n=908	
Variables	Mean (SD) or n (%)	Mean (SD) or n (%)	p-value
Maternal age (years)	32.9 (5.1)	33.3 (5.5)	0.145
Age ≥40 years	35 (7.7%)	96 (10.6%)	0.091
BMI (Kg/m2)	24.5 (4.5)	24.6 (4.7)	0.145
BMI <20	60 (13.2%)	108 (11.9%)	0.167
BMI ≥20<25	239 (52.6%)	433 (47.7%)	
BMI ≥25<30	102 (22.5%)	239 (26.3%)	
BMI ≥30	53 (11.7%)	128 (14.1%)	
Ethnicity			
Caucasian	393 (86.6%)	781 (86.0%)	0.803
African	45 (9.9%)	90 (9.9%)	1.000
East Asian	0 (0.0%)	4 (0.4%)	1.000
South Asian	15 (3.3%)	30 (3.3%)	1.000
Mixed	1 (0.2%)	3 (0.3%)	0.308
Parity			
Nulliparous	284 (62.6%)	568 (62.6%)	1.000
Primiparous/Multiparous	170 (37.4%)	340 (37.4%)	
Method of conception			
Spontaneous	280 (61.7%)	560 (61.7%)	1.000
Ovulation inductions	15 (3.3%)	30 (3.3%)	1.000
ART	159 (35.0%)	318 (35.0%)	1.000
Chorionicity			
Monochorionic diamniotic	142 (31.3%)	-	-
Dichorionic	312 (68.7%)	-	-
Smoker	48 (10.6%)	96 (10.6%)	1.000
Chronic hypertension	19 (4.2%)	38 (4.2%)	1.000
SLE/APS/Thrombophilia	14 (3.1%)	28 (3.1%)	1.000
Diabetes Mellitus	3 (0.7%)	6 (0.7%)	1.000
1st trimester Mean Arterial Pressure	85.6 (8.4)	86.2 (8.1)	0.532

Abbreviations: APS- Antiphospholipid syndrome; ART- artificial reproductive techniques; BMI- body mass index; PTB- preterm birth, PE- preeclampsia; SD- standard deviation; SLE- Systemic lupus erythematosus.

TABLE 3 First trimester (11^{+0} to 13^{+6} weeks) uterine artery pulsatility indices in twins according to chorionicity compared to singletons (matched for maternal characteristics).

	Singletons	MC	DC	Mean difference; p-value
n	908	142	312	MC vs SP 0.136; **<0.001** DC vs SP 0.269; **<0.001** MC vs DC 0.133; **0**.076
Mean GA (weeks) (SD)	12.2 (0.6)	12.4 (0.6)	12.5 (0.7)	
Mean (SD)	1.76 (0.53)	1.60 (0.54)	1.50 (0.46)	
Minimum	0.57	0.62	0.61	
Maximum	4.09	3.23	2.94	
Empirical UtA - PI percentiles				
P5	1.01	0.89	0.84	
P10	1.12	1.03	0.95	
P25	1.39	1.21	1.18	
P50	1.71	1.50	1.45	
P75	2.09	1.95	1.79	
P90	2.46	2.35	2.19	
P95	2.73	2.74	2.38	

TABLE 4 Second trimester (20 to 24 weeks) uterine artery pulsatility indices in twins according to chorionicity.

	MC	DC	Mean difference; p-value
n	84	307	MC vs DC 0.206; 0.170
Mean (SD)	0.925 (0.37)	0.851 (0.21)	
Minimum	0.50	0.30	
Maximum	2.70	1.90	
Empirical UtA - PI percentiles			
P5	0.55	0.57	
P10	0.59	0.60	
P25	0.70	0.84	
P50	0.84	0.80	
P75	1.05	0.97	
P90	1.25	1.16	
P95	1.67	1.24	

Abbreviations in Tables 3 and 4 :GA- gestational age; DC- Dichorionic Twins; MC – Monochorionic Twins; SP- Singleton Pregnancies; SD- standard deviation; UtA - PI- Uterine artery pulsatility index.

4.2.2 First trimester placental serum biomarkers

Serum biomarkers PAPP-A and β-hCG were available in 466 cases. Descriptive analysis of these biomarkers is summarized in Table 5 and Table 6.

No significant differences were observed between different chorionicities. The median was found to be around 1.0 for both biomarkers, which was expected if the conversion to MoM is well adjusted to our population.

TABLE 5 First trimester serum PAPP-A converted to MoM according to chorionicity.

	MC	DC	Mean difference; p-value
n	82	384	MC vs DC -0.066; 0.522
Mean (SD)	1.07 (0.56)	1.14 (0.60)	
Minimum	0.10	0.17	
Maximum	2.58	5.52	
Empirical PAPP-MoM percentiles			
P5	0.29	0.41	
P10	0.42	0.54	
P25	0.61	0.72	
P50	0.99	1.00	
P75	1.41	1.42	
P90	1.84	1.83	
P95	2.19	2.20	

TABLE 6 First trimester serum β-hCG converted to MoM according to chorionicity.

	MC	DC	Mean difference; p-value
n	82	384	MC vs DC 0.007; 0.883
Mean (SD)	1.11 (0.67)	1.11 (0.71)	
Minimum	0.32	0.26	
Maximum	3.00	5.33	
Empirical β-hCG MoM percentiles			
P5	0.34	0.41	
P10	0.39	0.49	
P25	0.60	0.65	
P50	0.99	0.93	
P75	1.41	1.36	
P90	2.32	1.94	
P95	2.60	2.44	

Abbreviations in Tables 5 and 6: GA- gestational age; DC- Dichorionic Twins; MC – Monochorionic Twins; SP- Singleton Pregnancies; SD- standard deviation; UtA - PI- Uterine artery pulsatility index.

4.3 ASSOCIATION OF MATERNAL FACTORS, PREGNANCY CHARACTERISTICS, FIRST TRIMESTER SERUM BIOMARKERS, AND UTERINE ARTERY DOPPLERS WITH OBSTETRIC OUTCOMES IN TWIN PREGNANCIES

4.3.1 Preterm birth

Overall, the observed PTB rates < 28 w, < 32 w, < 34 w, < 36 w were: 8/572 (1.4%), 39/572 (6.8%), 82/572 (14.3%) and 195/572 (34.1%), respectively (Figure 5 and Table 7). Before 36 weeks, 93 cases (16.3%) were medically indicated PTBs. Within the different gestational age categories for PTB, the iatrogenic rates for <28 w, < 32w, < 34 w, and < 36 w were 12.5%, 46.2%, 40.2%, and 47.7%, respectively.

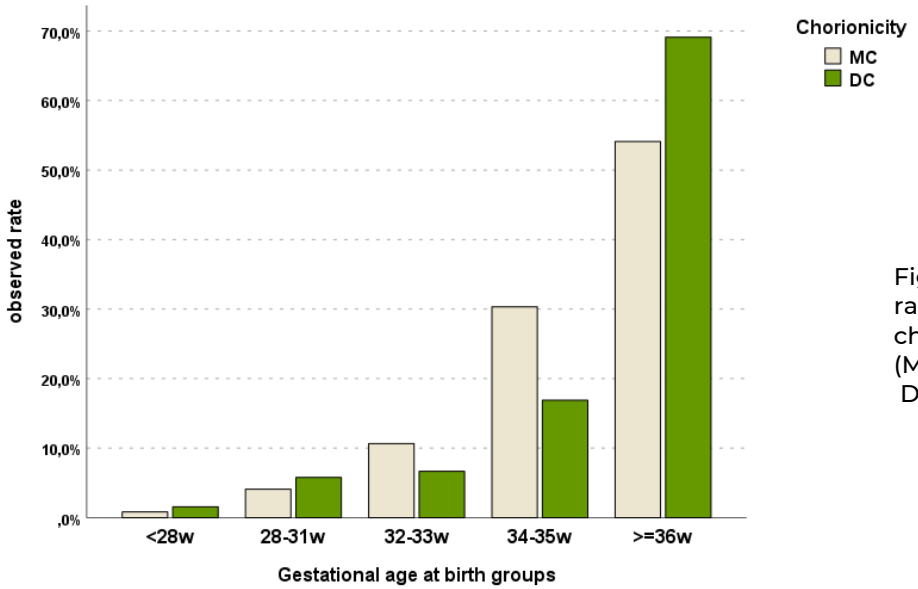


Figure 5. Preterm birth rates according to chorionicity (MC – monochorionic, DC – Dichorionic).

- **Maternal and pregnancy factors**

In the univariable analysis, MC twins presented higher rate of PTB than DC twins < 36 w, 56/122 (45.6%) vs 139/450 (30.9%), OR 1.8 (95%CI: 1.3-2.8, p=0.002), respectively (Figure 5 and Table 7).

Maternal height below the 3rd quartile (< 167 cm in our cohort) was associated with a higher rate of PTB < 32 w, < 34 w, and < 36 w, with OR of 7.8 (95% CI: 2.2-48.9, p<0.001), 4.2 (95%CI: 2.0-9.5, p<0.001), and 1.8 (95%CI: 1.2-2.7, p=0.004), respectively (Figure 6). Additionally, women with increased BMI ≥ 30 kg/m^2 showed an increased odds for PTB <34 w, 66/510 (12.9%) vs 16/62 (25.8%) OR 2.3 (95%CI: 1.3-4.3, p=0.006)(Table 7).

Nulliparous and parous women with obstetric past of PTB also presented increased odds for PTB <36 w, 57/211 (27.0%) vs 138/361 (38.2%) OR 1.6 (95%CI: 1.1-2.4, p=0.006), and 47/189 (24.5%) vs 10/22 (45.5%) OR 2.5 (95%CI: 1.0-6.2, p=0.040), respectively (Table 7).

Pregnancies resulting from ART presented a higher rate of PTB <32, <34 and <36 weeks, 20/366 (5.5%) vs 19/206 (9.2%) %, 47/366 (12.8%) vs 35/206 (17.0%) and 115/366 (31.4%) vs 80/206 (38.8%), respectively, although these differences did not reach statistical significance (p>0.05<0.174)(Table 7).

Women with higher mean first-trimester arterial pressure had increased odds of PTB at < 32 w and < 34 w, with OR 1.05 (95% CI: 1.00-1.09, p=0.027) and OR 1.03 (95% CI: 1.00-1.06, p=0.038), respectively.

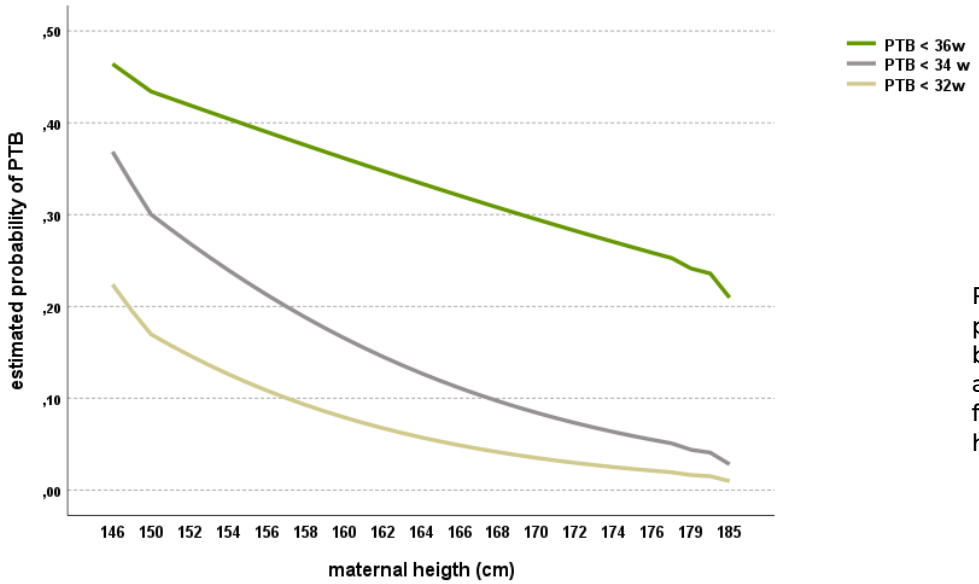


Figure 6. Estimated probability of preterm birth (PTB) < 32 w, < 34 w and < 36 weeks as a function of maternal height. (w-weeks)

In the second trimester, short CL ≤ 25 mm (corresponding to <5th percentile in this cohort) and ≤ 28 mm (corresponding to <10th percentile in this cohort) were observed in 4.3% and 9.7% of the cases, respectively. These women had an increased risk for all categories of PTB (< 28 w, <32 w, <34 w, and < 36 w), with the highest odds found in those with a CL ≤ 25 mm for PTB < 28 w, OR 18.8 (95% CI: 3.9–89.8, p=0.002).

In this cohort, no significant differences in PTB incidence were observed with respect to maternal age, ethnicity, smoking habits, chronic hypertension, thrombophilia, or aspirin prophylactic use.

TABLE 7 Univariable analysis of maternal and pregnancy characteristics and preterm birth in twin pregnancies.

Total n =572	**Unaffected n=377 (65.9%)**	**PTB < 32 weeks n= 39 (6.8%)**		**PTB < 34 weeks n= 82 (14.3%)**		**PTB < 36 weeks n= 195 (34.1%)**	
	Mean (SD) or n (%)	Mean (SD) or n (%)	OR (95%CI) p-value	Mean (SD) or n (%)	OR (95%CI) p-value	Mean (SD) or n (%)	OR (95%CI) p-value
Maternal age (years)	33.0 (4.9)	33.3 (4.5)	1.0 (0.9-1.1) 0.304	33.9 (4.4)	1.0 (0.9-1.1) 0.287	33.4 (9.0)	1.0 (0.9-1.0) 0.908
Age ≥40 years	25 (6.6%)	4 (10.3%)	1.6 (0.6-4..9) 0.317	6 (7.3%)	1.1 (0.4-2.7) 0.791	13 (6.7%)	1.0 (0.5-2.0) 0.987
Maternal heigh (cm)	163.3 (5.9)	160.4 (4.2)	0.9 (0.8-0.9) **0.003**	163.5 (5.9)	0.9 (0.8-0.9) **<0.001**	163.5 (5.9)	0.9 (0.8-0.9) **0.045**
BMI (Kg/m2)	25.0 (4.8)	24.7 (5.0)	1.0 (0.9-1.1) 0.448	24.6 (4.4)	1.0 (0.9-1.1) 0.243	23.9 (4.2)	0.9 (0.9-1.0) 0.700
BMI <20	46 (12.2%)	6 (15.2%)	1.2 (0.5-3.0) 0.637	12 (14.6%)	1.1 (0.6-2.3) 0.621	28 (14.4%)	1.2 (0.7-2.0) 0.466
BMI ≥20<25	198 (52.5%)	18 (46.2%)	0.7 (0.4-1.5) 0.469	37 (45.1%)	0.7 (0.5-1.1) 0.192	98 (50.3%)	0.9 (0.6-1.2) 0.608
BMI ≥25<30	98 (26.0%)	8 (20.5%)	0.8 (0.3-1.7) 0.551	17 (20.7%)	0.8 (0.4-1.4) 0.394	42 (21.5%)	0.8 (0.5-1.1) 0.240
BMI ≥30	35 (9.3%)	7 (17.9%)	1.9 (0.8-4.5) 0.139	16 (19.5%)	2.3 (1.3-4.3) **0.006**	27 (13.8%)	1.5 (0.9-2.6) 0.096
Ethnicity							
Caucasian	320 (84.9%)	34 (87.2%)	1.0 (0.4-2.8) 0.853	72 (87.8%)	1.1 (0.5-2.3) 0.647	173 (88.7%)	1.4 (0.8-2.4) 0.207
African	46 (12.2%)	5 (12.8%)	1.2 (0.4-3.2) 0.605	9 (11.0%)	0.9 (0.5-2.1) 0.990	17 (8.7%)	0.7 (0.4-1.2) 0.207
South Asian	10 (2.7%)	0 (0.0%)	0.615[a]	1 (1.2%)	0.4 (0.5-3.2) 0.708	5 (2.6%)	0.9 (0.3-2.9) 0.950
Mixed	1 (0.3%)	0 (0.0%)	- 1.000[a]	0 (0.0%)	- 1.000[a]	0 (0.0%)	- 1.000[a]
Parity							
Nulliparous	223 (59.2%)	30 (76.9%)	2.0 (0.9-4.6) 0.064	58 (70.7%)	1.5 (0.9-2.5) 0.122	138 (70.8%)	1.6 (1.1-2.4) **0.006**
Parous with prior PTB	12/154 (7.8%)	1/9 (11.1%)	1.0 (0.1-9.0) 1.000	6/24 (25.0%)	3.5 (1.2-10.2) **0.025**	10/57 (17.5%)	2.5 (1.0-6.2) **0.040**
Parous with prior SGA	8/154 (5.2%)	2/9 (22.2%)	4.9 (0.9-26.7) 0.099	3/24 (12.5%)	2.5 (0.6-9.9) 0.172	5/57 (8.8%)	1.7 (0.5-5.6) 0.344
Parous with prior PE	5/154 (3.2%)	0 (0.0%)	- 1.000[a]	3 (12.5%)	5.2 (1.1-23.3) 0.050	3 (5.3%)	1.6 (0.4-7.1) 0.448
Method of conception							
Spontaneous	236 (63.4%)	18 (46.2%)	0.5 (0.3-1.0) 0.061	43 (52.4%)	0.6 (0.4-1.0) 0.115	106 (54.4%)	0.7 (0.5-0.9) 0.038
Ovulation inductions	12 (3.2%)	2 (5.1%)	1.4 (0.3-6.5) 0.648	4 (4.9%)	1.4 (0.5-4.3) 0.524	9 (4.6%)	1.4 (0.6-3.5) 0.388
ART	126 (33.4%)	19 (48.7%)	1.7 (0.9-3.4) 0.087	35 (42.7%)	1.4 (0.8-2.2) 0.174	80 (41.0%)	1.4 (0.8-1.9) 0.073
Chorionicity							
Monochorionic diamniotic	56 (28.7%)	6 (15.4%)	0.6 (0.2-1.5) 0.422	19 (23.2%)	1.1 (0.6-1.9) 0.660	56 (28.7%)	1.8 (1.3-2.8) **0.002**
Dichorionic	139 (71.3%)	33 (84.6%)		63 (76.8%)		139 (71.3%)	
Smoker	36 (9.5%)	5 (12.8%)	1.4 (0.5-3.8) 0.401	8 (9.8%)	1.0 (0.4-2.2) 0.916	18 (9.2%)	0.9 (0.5-1.7) 1.000
Chronic hypertension	18 (4.8%)	3 (7.7%)	1.6 (0.4-5.6) 0.438	4 (4.9%)	0.9 (0.3-2.8) 1.000	11 (5.6%)	1.1 (0.5-2.5) 0.689
SLE/APS/Thrombophilia	11 (2.9%)	1 (2.6%)	0.9 (0.1-7.6) 1.000	1 (1.2%)	0.4 (0.1-3.2) 0.708	4 (2.1%)	0.7 (0.2-2.2) 0.539
Aspirin prophylactic intake (started <20 weeks)	45 (11.9%)	5 (12.8%)	1.0 (0.4-2.6) 1.000	13 (15.9%)	1.3 (0.7-2.6) 0.365	28 (14.4%)	1.2 (0.7-2.0) 0.410
1st trimester Mean Arterial Pressure	83.7 (9.0)	83.5 (4.9)	1.05 (1.00-1.09) **0.027**	85.1 (5.8)	1.03 (1.00-1.06) **0.038**	84.3 (7.0)	1.0 (0.9-1.0) 0.102

a) The odds ratio could not be estimated due to zero counts in one of the groups

Abbreviations: ART- artificial reproductive techniques; APS- Antiphospholipid syndrome; β-HCG- β-human chorionic gonadotropin; BMI- body mass index; CI – confidence interval; OR -odds ratio estimate; PE- preeclampsia; PTB- preterm birth; SGA- small for gestational age; SD- standard deviation; SLE- Systemic lupus erythematosus.

- **Uterine artery Dopplers**

In the univariable analysis, a higher first trimester UtA-PI (TwPs' references ≥ 90th and ≥ 95th empirical percentiles) was associated with increased odds of PTB < 32 w, < 34 w and < 36 w, with higher odds for PTB < 32 w when UtA-PI was ≥ 95th percentile, OR 4.3, (95% CI: 1.5–12.7, p=0.016) (Figure 6 and Table 10).

Women with higher second trimester UtA-PI also presented higher rates of PTB < 32 w, < 34 w, and < 36 weeks. More than half (68.4%) of the women with UtA-PI ≥ 95th percentile experienced preterm birth < 36 w, compared to 32.0% in the group with UtA-PI < 95th percentile, OR 4.6 (95%CI: 1.7–12.4, p=0.001) (Figure 7 and Table 8).

No differences were found in PTB < 28 w or < 30 w among women with higher UtA-PI in the first or second trimester.

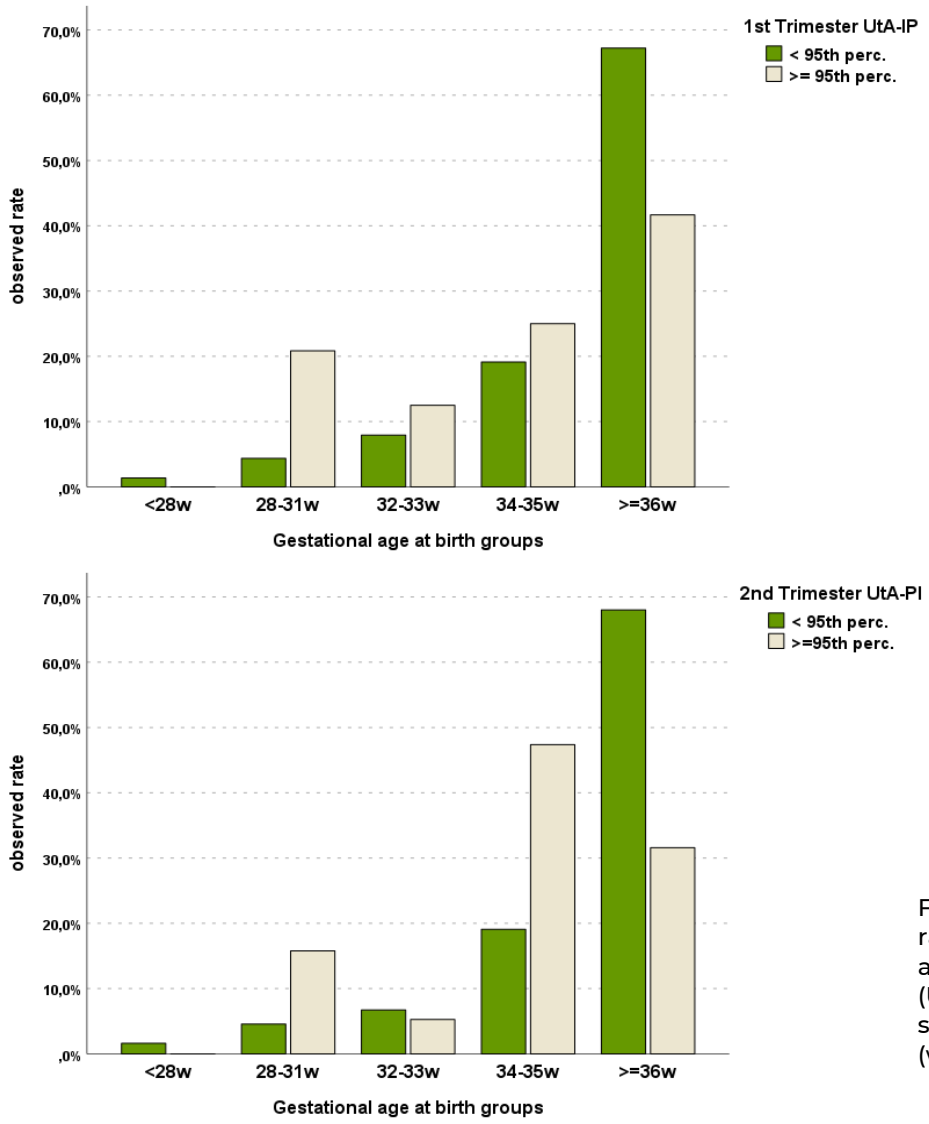


Figure 7. Preterm birth rates according to uterine artery pulsatility index (UtA-PI) in the first and second trimesters. (w-weeks)

- **First trimester serum biomarkers**

PAPP-A demonstrated a linear association with gestational age at birth ($\hat{\beta}$=0.26, 95%CI: 0.1-0.4, p=0.003)(Table 8 and Figure 8). Women with low PAPP-A <10th percentile exhibit higher odds for PTB < 32 w (OR 3.0, 95%CI: 1.3 – 7.2, p=0.015) and <36 w (OR 1.9, 95%CI: 1.0-3.6, p=0.030)(Figure 9). Despite these findings in univariable analyses, the use of PAPP-A < 10th percentile alone for predicting PTB is poor (Table 9). On the other hand, none of the women with high level of PAPP-A, over the 90th percentile corresponding to 1.82 MoM in our cohort, developed PTB < 35 w, whether spontaneous or iatrogenic (Figure 8).

In our cohort, there was no association between β-hCG levels and PTB (Table 8).

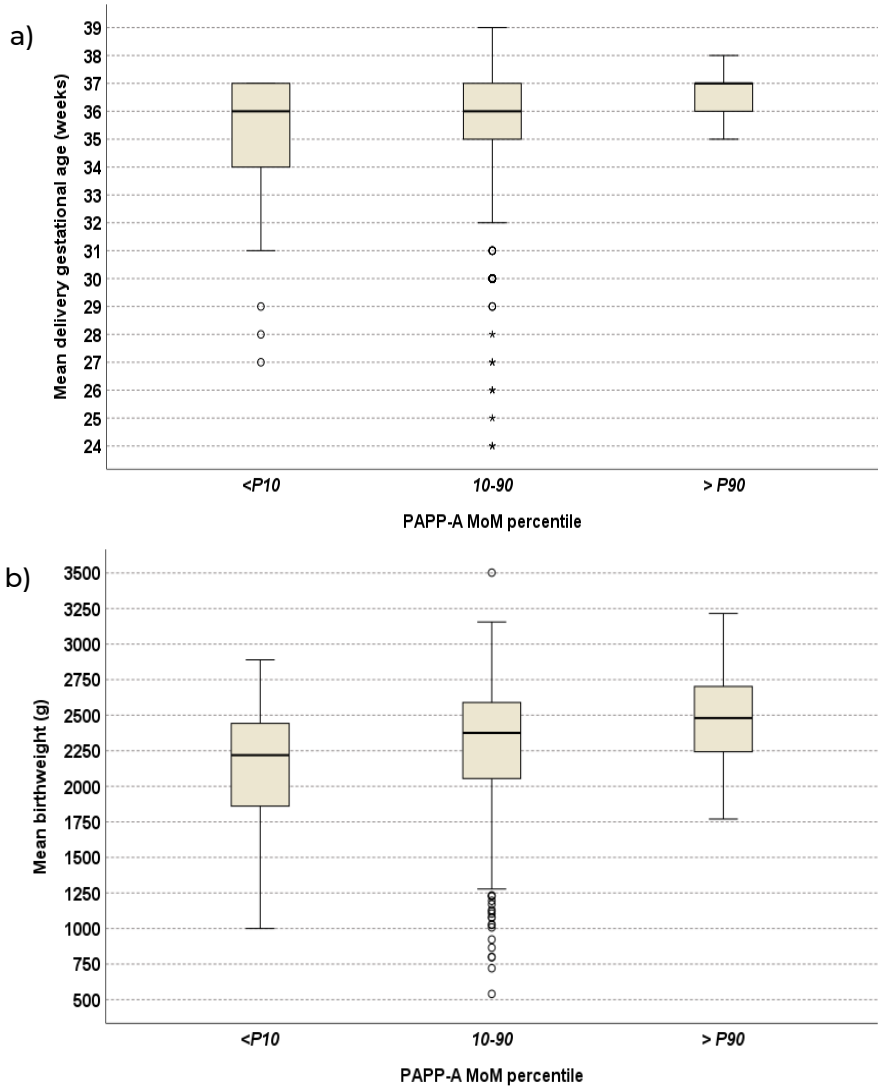


Figure 8. First trimester serum pregnancy-associated plasma protein-A (PAPP-A) in twin pregnancies and: a) mean gestational age; b) mean birth weight. (circles represent moderate outliers and asterisks extreme outliers).

TABLE 8 Univariable analysis of uterine artery Dopplers, first trimester serum biomarkers, 2nd trimester cervical length and PTB in twin pregnancies.

	Unaffected	**PTB < 32 weeks**		**PTB < 34 weeks**		**PTB < 36 weeks**	
	n (%)	n (%)	OR (95%CI) p-value	n (%)	OR (95%CI) p-value	n (%)	OR (95%CI) p-value
1st Trimester UtA-PI available data, n=390							
UtA-PI (continuous variable)	256 (65.6%)	26 (6.7%)	1.4 (0.6-3.1) 0.357	58 (14.9%)	1.4 (0.8-2.5) 0.199	134 (34.4%)	2.1 (1.4-3.3) **<0.001**
UtA-PI ≥ 90th percentile	19 (7.3%)	7 (16.7%)	3.5 (1.4-8.8) **0.014**	14 (32.6%)	3.4 (1.7-7.1) **<0.001**	25 (58.1%)	3.2 (1.7-6.2) **<0.001**
UtA-PI ≥ 95th percentile	6 (2.8%)	5 (21.7%)	4.3 (1.5-12.7) **0.016**	8 (34.8%)	3.2 (1.3-7.8) **<0.016**	14 (56.5%)	2.9 (1.2 - 6.7) **0.011**
2nd Trimester UtA-PI available data, n=391							
UtA-PI (continuous variable)	259 (66.2%)	26 (6.6%)	4.3 (1.3-14.4) **0.015**	52 (13.3%)	3.6 (1.6-9.7) **0.010**	132 (33.8%)	5.6 (2.3-13.3) **<0.001**
UtA-PI ≥ 90th percentile	19 (7.3%)	5 (19.2%)	2.1 (0.7-5.9) 0.179	10 (18.9%)	2.2 (1.0-4.8) **0.039**	21 (15.9%)	2.6 (1.3-5.0) **0.003**
UtA-PI ≥ 95th percentile	6 (2.3%)	3 (11.5%)	2.8 (0.7-10.4) 0.124	4 (7.7%)	1.8 (0.6-5.6) 0.299	13 (9.8%)	4.6 (1.7-12.4) **0.001**
1st trimester PAPP-A MoM available data, n=466							
PAPP-A MoM (continuous variable)	310 (66.5%)	35 (7.5%)	0.3 (0.1-0.8) **0.010**	70 (15.0%)	0.4 (0.2-0.6) **<0.001**	156 (33.5%)	0.7 (0.5-0.9) **0.026**
PAPP-A MoM < 10th percentile	24 (7.7%)	8 (22.9%)	3.0 (1.3-7.2) **0.015**	10 (14.3%)	1.6 (0.7-3.5) 0.179	22 (14.1%)	1.9 (1.0-3.6) **0.030**
PAPP-A MoM > 90th percentile	37 (11.9%)	0 (0%)	- **0.037**[a]	0(0%)	- **<0.001**[a]	9 (5.8%)	0.4 (0.2-0.9) **0.035**
1st trimester β-hCG MoM available data, n=466							
β-hCG MoM (continuous variable)	310 (66.5%)	35 (7.5%)	1.0 (0.6-1.7) 0.842	70 (15.0%)	1.0 (0.7-1.5) 0.727	156 (33.5%)	1.0 (0.8-1.3) 0.856
β-hCG MoM < 10th percentile	32 (10.3%)	4 (11.4%)	1.1 (0.4-3.5) 0.766	7 (10.0%)	1.0 (0.4-2.3) 1.000	14 (9.0%)	0.8 (0.4-1.6) 0.645
β-hCG MoM > 90th percentile	30 (9.7%)	4 (11.4%)	1.1 (0.4-3.5) 0.766	7 (10.0%)	1.0 (0.4-2.3) 1.000	16 (10.3%)	1.0 (0.6-2.0) 0.843
2nd Trimester cervical length (mm) available data, n =552							
CL mm (continuous variable)	368 (66.1%)	38 (6.8%)	0.9 (0.8-0.9) **0.002**	80 (14.4%)	0.9 (0.9-0.9) **<0.001**	189 (33.9%)	0.9 (0.9-0.9) **<0.001**
CL ≤ 25 mm (< 5th percentile)	7 (1.9%)	6 (15.8%)	5.2 (1.9-14.0) **0.004**	10 (12.5%)	4.7 (2.0-11.0) **<0.001**	17 (9.0%)	5.0 (2.0-12.5) **<0.001**
CL ≤ 28 mm (< 10th percentile)	17 (4.6%)	7 (18.4%)	3.2 (1.3-7.8) **0.016**	13 (16.3%)	3.1 (1.5-6.3) **0.001**	24 (12.7%)	3.0 (1.5-5.7) **<0.001**

Abbreviations: B – linear regression coefficient; CI – confidence interval; CL – cervical length; MoM – multiple of the median; OR – odds ratio estimate; PAPP-A – pregnancy-associated plasma protein-A; PTB preterm birth; SD – standard deviation; UtA-PI – Uterine artery pulsatility index; β-hCG – β-human chorionic gonadotropin.

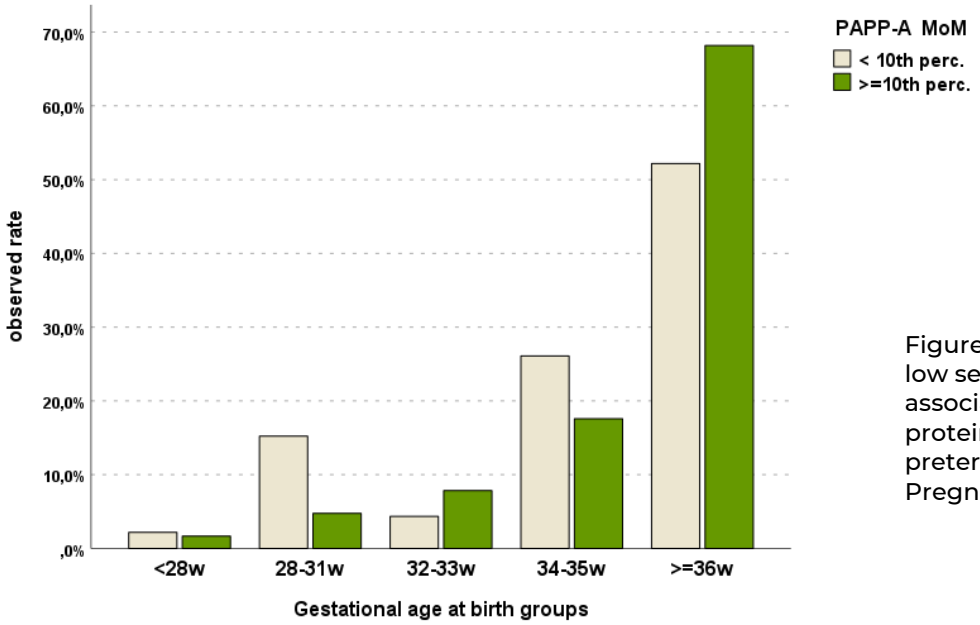


Figure 9. First Trimester low serum pregnancy-associated plasma protein-A (PAPP-A) and preterm birth in Twin Pregnancies.

- **Combined maternal and pregnancy factors with UtA-PI and serum biomarkers**

In the multivariable LR analysis, which incorporated first trimester maternal and pregnancy factors, serum biomarkers, and UtA-PI, the independent risk factors for PTB identified were: maternal height, BMI ≥ 30, MAP, UtA-PI ≥ 95th percentile, and PAPP-A MoM for PTB < 32 w and < 34 w; nulliparity, monochorionicity, PAPP-A MoM, and UtA-PI ≥95th percentile for PTB < 36 weeks. The highest odds were found for UtA-PI ≥95th percentile (OR 3.4, 95% CI: 1.05–11.3, p=0.049) for PTB < 32 weeks. The obtained AUCs of first-trimester association models for PTB ranged from 0.688 to 0.734, which corresponds to a detection rate of 44% to 49% for a fixed false positive rate of 20% (Table 10).

The multivariable LR analysis, incorporating maternal and pregnancy factors, first-trimester serum biomarkers, and second-trimester CL along with UtA-PI, demonstrated enhanced discriminative performance, achieving an AUC of 0.783 for detecting PTB before 32 weeks, with a sensitivity of 65% at a 20% false positive rate. The highest odds of PTB < 32 w were found for UtA-PI ≥ 95th percentile, OR 6.8 (95% CI: 1.1–43.1, p=0.039) and for short CL (≤ 25 mm), OR 6.2 (95% CI: 1.3–29.0, p=0.020). Additionally, conception by ART was identified as an independent risk factor for PTB < 36 w in the second-trimester multivariable LR analysis. Both models demonstrated good calibration, as assessed by the HL test (Tables 10 and 11).

TABLE 9 Performance of different cut-off of PAPPA-A levels in predicting adverse outcomes: SGA and FGR, SGA and/or HDP concurrent with PTB in twin pregnancies.

PAPPA-A < 0.5 MoM (10th percentile)					
	Sensitivity	False positive rate	Specificity	Positive Likelihood	Negative Likelihood
One or both SGA < 3rd percentile	16.5%	65.2%	91.8%	2.00	0.91
One or both SGA < 5th percentile	14.3%	60.9%	91.6%	1.71	0.94
One or both SGA < 10th percentile	15.0%	56.5%	91.6%	1.78	0.93
PTB < 32w with FGR, SGA and/or HDP	25.0%	87.0%	91.0%	2.76	0.82
PTB < 34w with FGR, SGA and/or HDP	13.0%	87.0%	90.5%	1.37	0.96
PTB < 36w with FGR, SGA and/or HDP	15.8%	67.4%	91.6%	1.89	0.92
PAPPA-A < 1.0 MoM					
	Sensitivity	False positive rate	Specificity	Positive Likelihood	Negative Likelihood
One or both SGA < 3rd percentile	60.8%	74.2%	53.3%	1.30	0.74
One or both SGA < 5th percentile	57.1%	68.6%	53.1%	1.22	0.81
One or both SGA < 10th percentile	54.2%	63.8%	52.6%	1.14	0.87
PTB < 32w with FGR, SGA and/or HDP	75.0%	92.2%	52.0%	1.56	0.48
PTB < 34w with FGR, SGA and/or HDP	58.7%	88.3%	51.7%	1.21	0.80
PTB < 36w with FGR, SGA and/or HDP	56.8%	76.5%	52.6%	1.20	0.82
PAPPA-A < 1.82 MoM (90th percentile)					
	Sensitivity	False positive rate	Specificity	Positive Likelihood	Negative Likelihood
One or both SGA < 3rd percentile	92.8%	78.3%	10.7%	1.04	0.67
One or both SGA < 5th percentile	94.4%	71.3%	11.6%	1.07	0.48
One or both SGA < 10th percentile	94.1%	65.3%	12.0%	1.07	0.49
PTB < 32w with FGR, SGA and/or HDP	100.0%	91.7%	10.7%	1.12	0.00
PTB < 34w with FGR, SGA and/or HDP	100.0%	83.3%	11.6%	1.13	0.00
PTB < 36w with FGR, SGA and/or HDP	94.2%	65.0%	11.9%	1.07	0.48

Abbreviations: FGR – fetal growth restriction; GH – gestational hypertension; HDP – hypertensive disorders of pregnancy; MoM – multiple of the median; PAPP-A pregnancy-associated plasma protein-A; PE – preeclampsia; PTB – preterm birth; SD – standard deviation; SGA – small to gestational age.

TABLE 10 Multivariable logistic regression analyses for PTB in twin pregnancies integrating first trimester clinical data.

Independent variables	Adjusted OR (95%CI)	p-value	AUC (95%CI) Sensitivity to a False Positive rate of 20%	Hosmer-Lemeshow test p-value
All PTB < 32 weeks				
Maternal height (cm)	0.9 (0.8-0.9)	**0.023**	0.722 (0.612-0.831) S 48%	0.303
MAP (continuous variable)	1.05 (1.0-1.09)	**0.040**		
PAPP-A MoM (continuous variable)	0.4 (0.1-1.0)	0.069		
UtA-PI ≥ 95th percentile	3.4 (1.0-11.3)	**0.049**		
All PTB < 34 weeks				
Maternal height (cm)	0.9 (0.8-0.9)	**<0.001**	0.734 (0.666-0.801) S 49%	0.175
BMI ≥ 30 (Kg/m2)	2.5 (1.1-5.7)	0.022		
MAP (continuous variable)	1.0 (0.9-1.1)	0.272		
PAPP-A MoM (continuous variable)	0.4 (0.2-0.8)	**0.015**		
UtA-PI ≥ 95th percentile	2.8 (1.1-7.8)	**0.036**		
All PTB < 36 weeks				
Nulliparas	2.2 (1.3-3.6)	**0.002**	0.668 (0.6.9-0.727) S 44%	0.747
BMI ≥ 30 (Kg/m2)	1.8 (0.9-3.4)	0.089		
Monochorionicity	2.0 (1.2-3.6)	**0.010**		
PAPP-A MoM (continuous variable)	0.7 (0.4-1.0)	0.078		
UtA-PI ≥ 95th percentile	3.0 (1.2-7.7)	**0.021**		

TABLE 11 Multivariable logistic regression analyses for PTB in twin pregnancies integrating 2nd trimester UtA Dopplers and cervical length.

Independent variables	Adjusted OR (95%CI)	p-value	AUC (95%CI) Sensitivity to a False Positive rate of 20%	Hosmer-Lemeshow test p-value
All PTB < 32 weeks				
Maternal height (cm)	0.9 (0.8-0.9)	**0.030**	0.783 (0.654-0.911) S 65%	0.446
1st trimester MAP (continuous variable)	1.1 (1.0-1.1)	**0.025**		
Cervical length ≤ 25 mm	6.2 (1.3-29.0)	**0.020**		
PAPP-A MoM (continuous variable)	0.4 (0.1-1.3)	0.142		
UtA-PI ≥ 95th percentile	6.8 (1.1-43.1)	**0.039**		
All PTB < 34 weeks				
Maternal height (cm)	0.9 (0.8-0.9)	**0.007**	0.796 (0.711-0.882) S 63%	0.416
BMI ≥ 30 (Kg/m2)	5.3 (1.8-15.1)	**0.002**		
1st trimester MAP (continuous variable)	1.0 (0.9-1.1)	0.192		
PAPP-A MoM (continuous variable)	0.4 (0.2-27.5)	**0.031**		
Cervical length < 28mm	4.9 (1.6-15.2)	**0.006**		
UtA-PI ≥ 95th percentile	5.4 (1.1-27.5)	**0.039**		
All PTB < 36 weeks				
Monochorionicity	2.7 (1.4-5.3)	0.004	0.664 (0.604-0.723) S 50%	0.172
BMI ≥ 30 (Kg/m2)	2.2 (1.0-5.0)	0.050		
Conception by ART	1.8 (1.0-3.2)	0.031		
Cervical length ≤28mm	6.3 (2.1-18.9)	<0.001		
UtA-PI ≥ 95th percentile	4.3 (1.2-15.3)	0.002		

Abbreviations: ART – assisted reproduction techniques; AUC – area under the receiver-operating characteristic curve; BMI – body mass index; MAP – mean arterial pressure; MoM – Multiple of the Median; OR – odds ratio estimate; PAPP-A – Pregnancy-Associated Plasma Protein-A; PTB – Preterm Birth; UtA-PI – uterine artery pulsatility index.

4.3.2 Fetal Growth Restriction and Small for Gestational Age

Overall, suspicion of FGR in one or both fetuses per pregnancy occurred in 152/572 (26.6%) of TwPs, including 94/572 (16.4%) with abnormal fetal Doppler findings. Pregnancies with one or both infants classified as SGA <3rd, <5th or <10th percentiles were 120/572 (20.9%), 157/572 (27.4%) and 190/572 (33.2%), respectively (Table 12). Newborns classified as SGA <3rd, <5th and <10th percentiles accounted for 145/1142 (12.7%), 191/1142 (16.7%) and 247/1142 (21.6%), respectively.

- **Maternal and pregnancy factors**

In the univariable analysis, suspicion of FGR in one or both fetuses per pregnancy occurred more frequently in MC, 36.1% compared to 24.0% in DC, OR 1.7 (95% CI: 1.2-2.5, p=0.007) (Figure 10). Nevertheless, no statistically significant differences were found in the incidence of SGA < 3rd , < 5th , and < 10th percentiles between MC and DC: 22.1% vs. 20.7%, OR 1.1 (95% CI: 0.7-1.7, p=0.725); 31.1% vs. 26.4%, OR 1.3 (95% CI: 0.8-1.9, p=0.302); and 39.3% vs. 31.6%, OR 1.4 (95% CI: 0.9-2.1, p=0.105), respectively (Table 12).

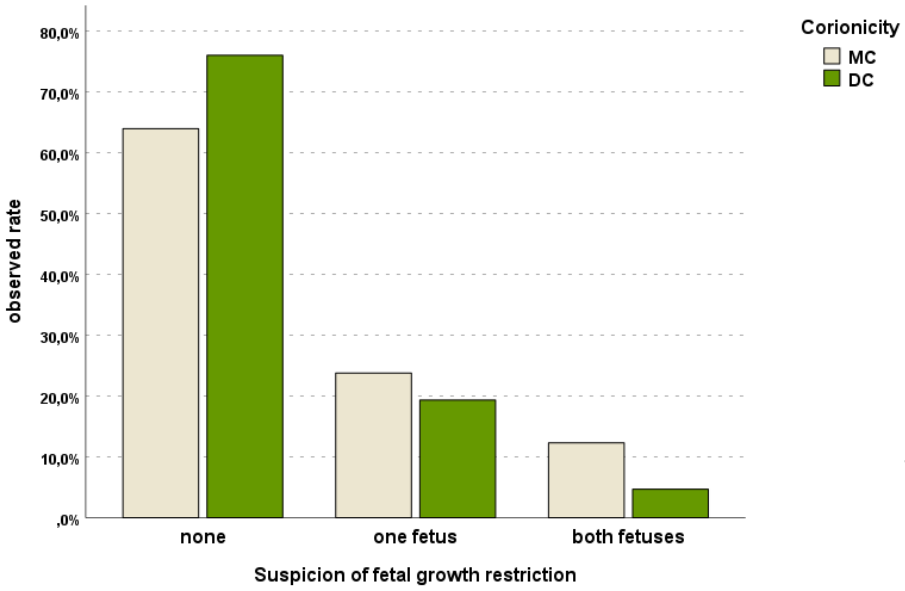


Figure 10. Suspicion of fetal growth restriction rates according to chorionicity.

Lower maternal height (< 167 cm) was associated with a higher rate of SGA < 3rd, < 5th and < 10th percentile, OR 2.1 (95% CI: 1.3-3.5, p=0.004), OR 1.9 (95% CI: 1.2-3.1, p=0.003) and OR 1.7 (95% CI: 1.1-12.6, p=0.008), respectively (Figure 11 and Table 12).

Women with low BMI (< 20Kg/m^2) showed increased odds of SGA < 3rd, < 5th and <10th percentiles, 31.1% vs 19.5%, OR 1.8 (95%CI: 1.0-3.1, p=0.022), 41.9% vs 25.3%, OR 2.1 (95%CI: 1.3-3.5, p=0.003) and 51.4% vs 30.5%, OR 2.4 (95%CI: 1.4-3.9, p<0.001), respectively (Table12).

Nulliparous women showed increased odds of SGA < 10th percentile, 36.3% vs 28.0%, OR 1.5 (95%CI: 1.0-2.1, p=0.041). Within parous women, those with a previous history of SGA had increased odds of SGA <3rd and <10th percentile, 53.8% vs 16.7%, OR 5.8 (95%CI: 1.8-18.4, p=0.004) and 61.5% vs 25.8%, OR 4.6 (95%CI: 1.4-14.7, p=0.009). Additionally, women under aspirin prophylaxis revealed increased odds for SGA < 5th percentile, 39.7% vs 25.7%, OR 1.9 (95%CI: 1.1-3.1, p=0.012)(Table 12).

Furthermore, women who reported smoking habits in the first trimester had increased odds of SGA in the subgroup >3rd <10th percentile, 24.1% vs 11.0%, OR 2.5 (95%CI: 1.3-5.0, p=0.005), but not to SGA < 3rd percentile: 20.4% vs 21.0%, OR 0.9 (95%CI: 0.5-1.9, p=0.908) (Table 12).

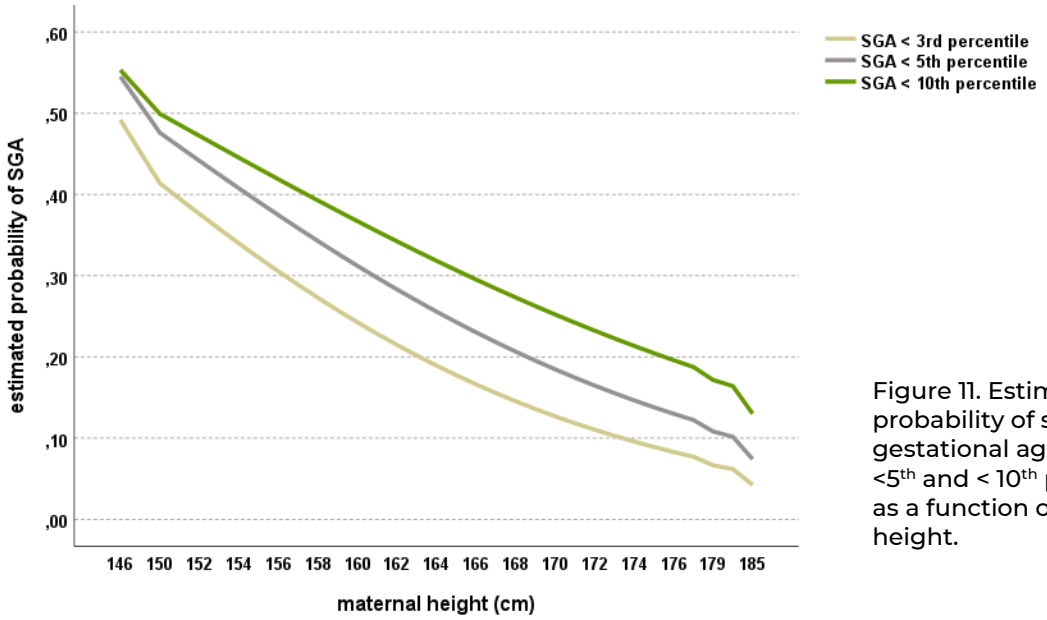


Figure 11. Estimated probability of small for gestational age (SGA) < 3rd, <5th and < 10th percentiles as a function of maternal height.

- **Uterine artery Dopplers**

In univariable analysis, women with higher first trimester UtA-PI (TwPs' references ≥ 90th or ≥ 95th empirical percentiles) presented an increased risk of SGA (Figure 12.a and Table 13). For example, women with first trimester Uta-PI ≥ 95th percentile showed and increased odds of one or both neonates SGA < 3rd, < 5th and < 10th percentiles, 54.2% vs 21.0 %, OR 4.4 (95%CI: 1.9-10.2, p<0.001), 58.3% vs 26.8%, OR 3.8 (95%CI: 1.6-8.9, p<0.001), and 58.3% vs 33.3%, OR 2.8 (95%CI: 1.2-6.4, p=0.013), respectively. In the second trimester UtA Doppler evaluation, statistically significant differences were found in the group with UtA-PI ≥ 90th percentile for SGA <3rd, <5th , and <10th percentiles, with the highest odds observed for one or both neonates SGA <3rd percentile, OR 2.4 (95% CI: 1.2-4.9, p=0.007).

TABLE 12 Univariable analysis of maternal and pregnancy characteristics and small for gestational age (one or both per pregnancy) < 10th, < 5th and < 3rd birth weight percentiles in twin pregnancies.

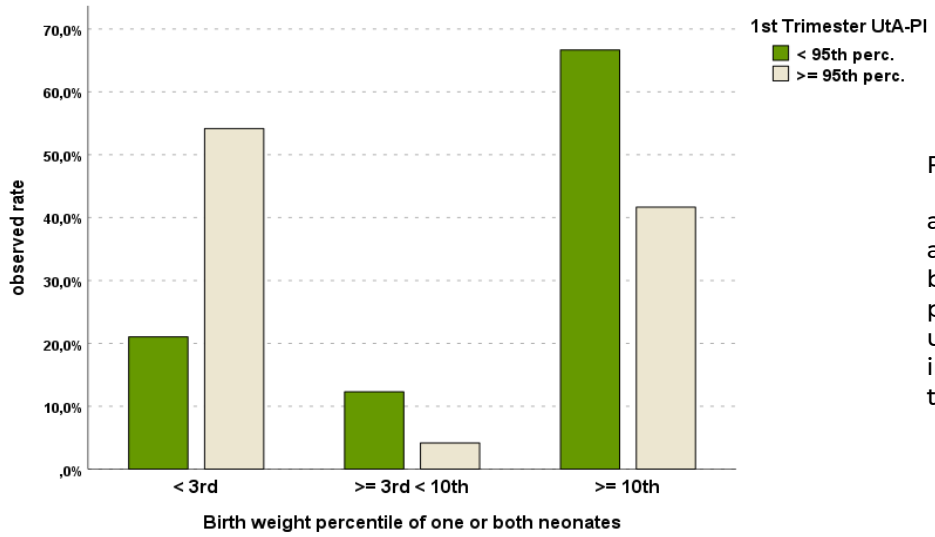
Total n =572	**Unaffected n=382 (66.8%)**	**SGA <10th n= 190 (33.2%)**		**SGA <5th n= 157 (27.4%)**		**SGA < 3rd n= 120 (20.9%)**	
	Mean (SD) or n (%)	Mean (SD) or n (%)	OR (95%CI) p-value	Mean (SD) or n (%)	OR (95%CI) p-value	Mean (SD) or n (%)	OR (95%CI) p-value
Maternal age (years)	32.6 (5.2)	33.0 (5.3)	1.0 (0.9-1.0) 0.688	32.9 (5.2)	1.0 (0.9-1.0) 0.517	33.0 (5.3)	1.0 (0.9-1.0) 0.486
Age ≥40 years	25 (6.5%)	13 (6.8%)	1.0 (0.5-2.0) 0.893	12 (7.6%)	1.2 (0.6-2.5) 0.555	10 (8.3%)	1.3 (0.6-2.9) 0.403
Maternal heigh (cm)	163.7 (5.8)	161.9 (5.7)	1.05 (1.02-1.09) **<0.001**	161.4 (5.6)	1.07 (1.03-1.11) **<0.001**	161.7 (5.9)	1.08 (1.04-1.12) **<0.001**
BMI (Kg/m2)	24.7 (4.4)	23.7 (4.1)	0.9 (0.8-0.9) **0.003**	23.5 (4.0)	0.9 (0.8-0.9) **0.006**	23.7 (4.1)	0.9 (0.9-1.0) 0.078
BMI <20	36 (9.4%)	38 (20.0%)	2.4 (1.4-3.9) **<0.001**	31 (19.7%)	2.1 (1.3-3.5) **0.003**	23 (19.2%)	1.8 (1.1-3.1) **0.022**
BMI ≥20<25	201 (52.6%)	95 (50.0%)	0.9 (0.6-1.2) 0.555	79 (50.3%)	0.9 (0.6-1.3) 0.674	59 (49.2%)	0.8 (0.6-1.3) 0.524
BMI ≥25<30	102 (26.7%)	38 (20.0%)	0.6 (0.4-1.0) 0.079	30 (19.1%)	0.6 (0.4-1.0) 0.066	24 (20.0%)	0.7 (0.4-1.2) 0.200
BMI ≥30	43 (11.3%)	19 (10.0%)	0.8 (0.4-1.5) 0.649	17 (10.8%)	0.9 (0.5-1.8) 0.996	14 (11.7%)	1.1 (0.6-2.0) 0.743
Ethnicity							
Caucasian	327 (85.6%)	166 (87.4%)	1.1 (0.7-1.9) 0.564	136 (86.6%)	1.0 (0.6-1.8) 0.853	101 (84.2%)	0.8 (0.5-1.4) 0.470
African	46 (12.0%)	17 (8.9%)	0.7 (0.4-1.2) 0.266	14 (8.9%)	0.7 (0.4-1.3) 0.325	12 (10.0%)	0.8 (0.4-1.7) 0.690
South Asian	8 (2.1%)	7 (3.7%)	1.7 (0.6-5.0) 0.275	7 (4.5%)	2.3 (0.8-6.6) 0.137	7 (5.8%)	3.4 (1.2-9.6) 0.013
Mixed	1 (0.3%)	0 (0.0%)	- 0.480[a]	0 (0.0%)	- 1.000[a]	0 (0.0%)	- 1.000[a]
Parity							
Nulliparous	230 (60.2%)	131 (68.9%)	1.4 (1.0-2.1) **0.041**	107 (68.2%)	0.7 (0.5-1.0) 0.124	80 (66.7%)	1.5 (1.0-2.3) 0.364
Parous with prior PTB	13/152 (8.5%)	9/59 (15.3%)	1.9 (0.7-4.7) 0.153	9/50 (18.0%)	2.5 (1.0-6.2) **0.045**	7/40 (17.5%)	2.2 (0.8-5.8) 0.146
Parous with prior SGA	5/152 (3.3%)	8/59 (13.6%)	4.6 (1.4-14.7) **0.009**	7/50 (14.0%)	4.2 (1.3-13.1) **0.015**	7/40 (17.5%)	5.8 (1.8-18.4) **0.004**
Parous with prior PE	5/152 (3.3%)	3/59 (5.1%)	1.5 (0.3-6.8) 0.689	3/50 (6.0%)	1.9 (0.5-8.6) 0.397	3/40 (7.5%)	2.6 (0.6-11.7) 0.177
Method of conception							
Spontaneous	236 (61.8%)	109 (57.4%)	0.8 (0.5-1.1) 0.310	90 (57.3%)	0.8 (0.5-1.2) 0.369	70 (58.3%)	0.9 (0.6-1.3) 0.618
Ovulation inductions	15 (3.9%)	6 (3.2%)	0.7 (0.3-2.0) 0.645	5 (3.2%)	0.8 (0.3-2.2) 0.703	3 (2.5%)	0.6 (0.2-2.1) 0.443
ART	131 (34.3%)	75 (39.5%)	1.2 (0.8-1.7) 0.224	62 (39.5%)	1.2 (0.8-1.8) 0.287	47 (39.2%)	1.2 (0.8-1.8) 0.418
Chorionicity							
Monochorionic diamniotic	74 (19.4%)	48 (25.3%)	0.7 (0.5-1.0) 0.105	38 (24.2%)	0.7 (0.5-1.2) 0.302	27 (22.5%)	0.9 (0.6-1.5) 0.725
Dichorionic	308 (80.6%)	142 (74.7%)		119 (75.8%)		93 (77.5%)	
Smoker	30 (7.9%)	24 (12.6%)	1.6 (0.9-2.9) 0.066	17 (10.8%)	1.2 (0.6-2.2) 0.485	11 (9.2%)	0.9 (0.5-1.9) 0.908
Chronic hypertension	17 (4.5%)	12 (6.3%)	1.4 (0.7-3.0) 0.338	12 (7.6%)	1.9 (0.9-4.1) 0.084	8 (6.7%)	1.4 (0.6-3.3) 0.370
SLE/APS/Thrombophilia	11 (2.9%)	4 (2.1%)	0.7 (0.2-2.3) 0.783	4 (2.5%)	0.9 (0.3-3.0) 1.000	3 (2.5%)	0.9 (0.3-3.4) 1.000
Aspirin prophylactic intake (started <20 weeks)	44 (11.5%)	29 (15.3%)	1.3 (0.8-2.2) 0.231	29 (18.5%)	1.9 (1.1-3.1) **0.012**	19 (15.8%)	1.3 (0.8-2.4) 0.257
1st trimester Mean Arterial Pressure	85.4 (8.5)	86.0 (8.7)	1.0 (0.9-1.0) 0.755	85.6 (8.9)	1.0 (0.9-1.0) 0.493	86.4 (8.5)	1.0 (0.9-1.0) 0.263

a) The odds ratio could not be estimated due to zero counts in one of the groups

Abbreviations: ART– artificial reproductive techniques; APS – Antiphospholipid syndrome; β-hCG – β-human chorionic gonadotropin; BMI – body mass index; CI – confidence interval; OR – odds ratio estimate; PE – preeclampsia; PTB – preterm birth; SGA – small for gestational age; SD – standard deviation; SLE – Systemic lupus erythematosus.

When analysing the first trimester UtA-PI values according to chorionicity and the number of neonates with SGA < 10th percentile, significant differences were found only in MC pregnancies affected with two SGA neonates (p=0.014). No significant differences in the UtA-PI were observed in DC or when only one neonate was SGA (p=1.000) (Figure 12.b). Although this finding was observed, the rates of SGA <3rd, 5th, and 10th percentiles were higher when the first trimester UtA-PI was ≥ 95th percentile for one or both neonates in MC pregnancies and for one neonate in DC pregnancies. Statistically significant differences were found only in DC TwPs for one neonate being SGA < 3rd and < 5th percentiles, with rates of 16.2% versus 50.0% (p=0.001) and 20.3% versus 50.0% (p=0.012), respectively. In MC pregnancies, differences were noted in the rates of SGA for both one and two neonates across all categories: < 3rd percentile (one SGA: 16.7% vs 33.3%, both SGA: 4.2% vs 16.7%, p=0.206); < 5th percentile (one SGA: 20.8% vs 50.0%, both SGA: 8.3% vs 16.7%, p=0.164); and < 10th percentile (one SGA: 26.4% vs 33.3%, both SGA: 9.7% vs 33.3%, p=0.164).

a)



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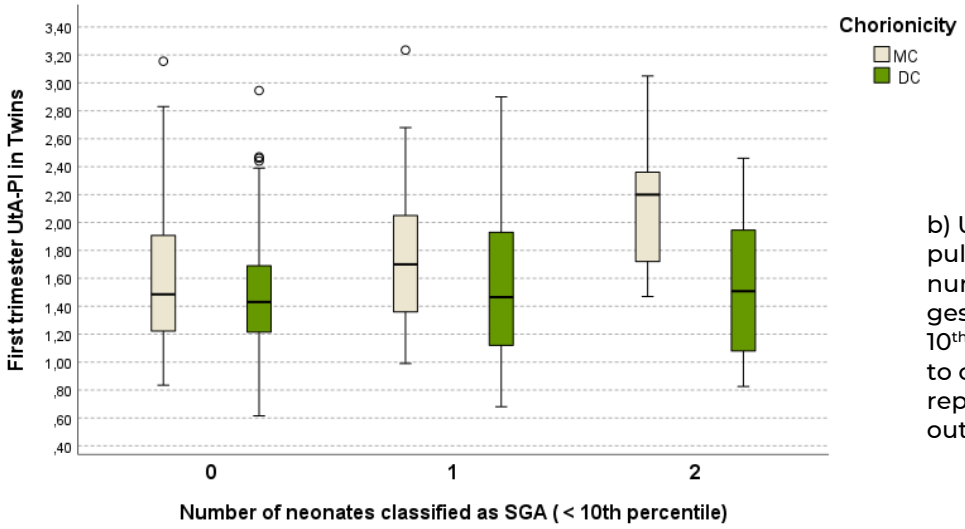

Figure 12.

a) Small for gestational age (SGA) rates of one or both neonates per pregnancy according to uterine artery pulsatility index (utA-PI) in the first trimester.

b) Uterine artery pulsatility index and number of small to gestational age under the 10th percentile according to chorionicity (circles represent moderate outliers).

TABLE 13 Univariable analysis of uterine artery Dopplers, first trimester serum biomarkers, and small for gestational age <10th, <5th and <3rd birthweight percentiles (one or both neonates per pregnancy).

	Unaffected	SGA <10th		SGA <5th		SGA < 3rd	
	n (%)	n (%)	OR (95%CI) p-value	n (%)	OR (95%CI) p-value	n (%)	OR (95%CI) p-value
1st Trimester UtA-PI available data, n = 390							
UtA-PI (continuous variable)	254 (65.1%)	136 (34.8%)	1.6 (1.0-2.4) **0.032**	112 (28.7%)	2.0 (1.3-3.2) **0.002**	90 (23.1%)	1.6 (1.0-2.4) **0.032**
UtA-PI ≥ 90th percentile	21 (8.3%)	21 (15.4%)	2.0 (1.1-3.8) **0.029**	21 (18.8%)	2.8 (1.4-5.4) **0.001**	18 (20.0%)	2.8 (1.4-5.5) **0.001**
UtA-PI ≥ 95th percentile	10 (3.9%)	14 (10.3%)	2.8 (1.2-6.5) **0.013**	14 (12.5%)	3.8 (1.6-8.9) **<0.001**	13 (14.4%)	4.4 (1.9-10.2) **<0.001**
2nd Trimester UtA-PI available data, n=391							
UtA-PI (continuous variable)	264 (67.5%)	127 (32.5%)	4.4 (1.8-10.2) <0.001	106 (27.1%)	4.1 (1.7-9.8) 0.001	88 (22.5%)	4.0 (1.6-9.8) 0.002
UtA-PI ≥ 90th percentile	21 (8.0%)	20 (15.7%)	2.2(1.1-4.1) **0.018**	18 (17.0%)	2.3 (1.2-4.5) **0.011**	16 (18.2%)	2.4 (1.2-4.9) **0.007**
UtA-PI ≥ 95th percentile	10 (3.8%)	9 (7.1%)	1.9 (0.7-4.8) 0.155	8 (7.5%)	2.0 (0.8-5.2) 0.132	7 (8.0%)	2.0 (0.8-5.4) 0.156
1st trimester PAPP-A MoM available data, n=466							
PAPP-A MoM (continuous variable)	313 (67.2%)	153 (32.8%)	0.6 (0.4-0.8) **0.014**	126 (27.0%)	0.5 (0.4-0.8) **0.006**	97 (20.8%)	0.5 (0.3-0.8) **0.006**
PAPP-A MoM < 10th perc.	26 (8.3%)	20 (13.1%)	1.6 (0.9-3.0) 0.105	18 (14.3%)	1.8 (0.9-3.4) **0.052**	16 (16.5%)	2.2 (1.1-4.2) **0.014**
PAPP-A MoM > 90th perc.	37 (11.8%)	9 (5.9%)	0.4 (0.2-0.9) **0.044**	7 (5.6%)	0.4 (0.2-1.0) **0.057**	7 (7.2%)	0.6 (0.3-1.5) 0.325
1st trimester β-hCG MoM available data, n=466							
β-hCG MoM (continuous variable)	313 (67.2%)	153 (32.8%)	1.0 (0.8-1.4) 0.827	126 (27.0%)	0.9 (0.7-1.3) 0.977	97 (20.8%)	1.0 (0.7-1.4) 0.713
β-hCG MoM < 10th perc.	28 (8.9%)	18 (11.8%)	1.3 (0.7-2.5) 0.338	17 (13.5%)	1.6 (0.9-3.1) 0.111	13 (13.4%)	1.5 (0.8-3.1) 0.190
β-hCG MoM > 90th perc.	32 (10.2%)	14 (9.2%)	0.8 (0.4-1.7) 0.715	12 (9.5%)	0.9 (0.5-1.8) 0.878	10 (10.3%)	1.0 (0.5-2.2) 0.871

Abbreviations: CI – confidence interval; MoM – multiple of the median; OR – odds ratio estimate; PAPP-A – pregnancy-associated plasma protein-A; SGA – small for gestational age; SD – standard deviation; UtA-PI- Uterine artery pulsatility index; β-hCG – β-human chorionic gonadotropin.

- **First trimester serum biomarkers**

PAPP-A demonstrated a linear association with mean neonates birth weight ($\hat{\beta}$=73, 95% CI: 38.9-104.1, p<0.001) (Figure 8). Additionally, women with low PAPP-A MoM (< 10th percentile, corresponding to 0.50 MoM in our cohort) had increased odds of SGA < 3rd percentile, 34.8% vs 19.2% OR 2.2 (95%CI: 1.1-4.3, p<0.014)(Table 13). However, the performance of using this cut-off for predicting SGA is poor, detecting only 16.5% of SGA < 3rd percentile with a false positive rate of 65.2% (Table 9).

In our cohort, there was no association between β-hCG levels and SGA (Table 13).

- **Combined maternal and pregnancy factors with UtA-PI and serum biomarkers**

In the multivariable LR analysis, which incorporated first trimester maternal and pregnancy factors, serum biomarkers, and UtA-PI, the independent risk factors for SGA identified were: advanced maternal age, nulliparity, low BMI (< 20 kg/m^2), PAPP-A MoM < 10th percentile and UtA-PI ≥ 95th percentile. The highest odds were found for UtA-PI ≥ 95th percentile, OR 5.1, 95% (CI: 2.1–12.9, p<0.001) for SGA < 3rd percentile. The obtained AUCs of first-trimester association models for SGA ranged from 0.648 to 0.666, which corresponds to a detection rate varying from 37% to 44% for a fixed false positive rate of 20% (Table 14).

TABLE 14 Multivariable logistic regression analyses for SGA and concurrent PTB in twin pregnancies integrating first trimester data.

Outcomes and independent variables	Adjusted OR (95%CI)	p-value	AUC (95%CI) Sensitivity to a False Positive rate of 20%	Hosmer-Lemeshow test p-value
One or both SGA < 3rd percentile				
Asian ethnicity	3.1 (0.9-10.1)	**0.051**	0.668 (0.601-0.735) S 48%	0.397
Maternal height < 167 cm	2.2 (1.1-4.15)	**0.015**		
MAP (continuous variable)	1.0 (0.9-1.0)	**0.066**		
PAPP-A MoM < 10th percentile	2.3 (1.0-5.1)	**0.035**		
UtA-PI ≥ 95th percentile	5.2 (2.0-13.1)	**<0.001**		
One or both SGA < 5th percentile				
Maternal height < 167 cm	1.8 (1.0-3.1)	**0.038**	0.666 (0.603-0.728) S 44%	0.869
Nulliparas	1.7 (1.0-2.8)	**0.044**		
BMI < 20 (Kg/m2)	2.1 (1.1-4.1)	**0.028**		
PAPP-A MoM (continuous variable)	0.7 (0.4-1.0)	0.120		
UtA-PI ≥ 95th percentile	4.8 (1.9-12.2)	**<0.001**		
One or both SGA < 10th percentile				
Maternal height < 167 cm	1.7 (1.0-2.9)	**0.036**	0.655 (0.697-0.713) S 37%	0.651
Nulliparas	2.0 (1.2-3.3)	**0.005**		
BMI < 20 (Kg/m2)	1.9 (1.0-3.6)	**0.048**		
PAPP-A MoM (continuous variable)	0.7 (0.5-1.1)	0.161		
UtA-PI ≥ 95th percentile	3.4 (1.4-8.8)	**0.009**		
One or both SGA < 3rd percentile and PTB < 32 weeks				
Maternal height < 167 cm	10.1 (1.2-83.6)	**0.031**	0.834 (0.750-0.918) **S 77%** (False positive rate = 17%)	0.388
Smoking habits	3.5 (0.9-13.2)	0.063		
MAP (continuous variable)	1.1 (1.0-1.1)	**0.012**		
PAPP-A MoM < 10th percentile	4.3 (1.1-16.0)	0.034		
UtA-PI ≥ 95th percentile	7.2 (1.8-28.5)	**0.005**		
One or both SGA < 3rd percentile and PTB < 34 weeks				
Maternal height (cont. variable)	0.9 (0.8-0.9)	**0.002**	0.768 (0.681-0.855) S 63%	0.956
BMI < 20 (Kg/m2)	2.9 (1.0-8.6)	**0.043**		
MAP (continuous variable)	1.0 (1.0-1.1)	0.052		
PAPP-A MoM (continuous variable)	0.5 (0.2-1.1)	0.088		
UtA-PI ≥ 95th percentile	6.1 (2.0-18.5)	**0.001**		

TABLE 14 (continuation) Multivariable logistic regression analyses for SGA and concurrent PTB in twin pregnancies integrating first trimester data.

One or both SGA < 3rd percentile and PTB < 36 weeks				
Maternal height (cont. variable)	0.9 (0.8-0.9)	**0.039**	0.716 (0.639-0.794) S 50 %	0.300
BMI < 20 (Kg/m2)	2.3 (0.9-5.4)	0.060		
MAP (continuous variable)	1.0 (0.9-1.0)	0.106		
PAPP-A MoM (cont. variable)	0.5 (0.3-1.0)	0.078		
UtA-PI ≥ 95th percentile	7.2 (2.7-18.6)	**<0.001**		
One or both SGA < 10th percentile and PTB < 32 weeks				
Maternal height < 167 cm	4.9 (1.1-22.9)	**0.039**	0.779 (0.672-0.886) **S 65%**	0.679
Smoking habits	2.6 (0.7-9.2)	0.128		
MAP (continuous variable)	1.1 (1.0-1.1)	**0.025**		
PAPP-A MoM < 10th percentile	3.0 (0.9-10.7)	0.079		
UtA-PI ≥ 95th percentile	5.1 (1.4-18.7)	**0.012**		
One or both SGA < 10th percentile and PTB < 34 weeks				
Maternal height < 167 cm	3.5 (1.3-9.6)	**0.014**	0.716 (0.629-0.802) S 49%	0.258
BMI ≥ 30 (Kg/m2)	2.3 (0.9-5.7)	0.063		
MAP (continuous variable)	1.0 (0.9-1.0)	0.210		
PAPP-A MoM (continuous variable)	0.5 (0.2-1.1)	0.102		
UtA-PI ≥ 95th percentile	3.6 (1.3-10.1)	**0.017**		
One or both SGA < 10th percentile and PTB < 36 weeks				
Maternal height < 167 cm	1.4 (0.8-2.6)	0.243	0.675 (0.608-0.741) S 42%	0.613
Nulliparas	2.4 (1.3-4.4)	**0.005**		
Monochorionic	1.9 (1.0-3.5)	**0.046**		
PAPP-A MoM (continuous variable)	0.6 (0.4-1.2)	0.148		
UtA-PI ≥ 95th percentile	3.8 (1.5-9.6)	**0.005**		

Abbreviations: AUC – area under the receiver-operating characteristic curve; BMI – body mass index; MAP – mean arterial pressure; MoM – Multiple of the Median; OR – odds ratio estimate; PAPP-A – Pregnancy-Associated Plasma Protein-A; PTB – Preterm Birth; SGA – Small for Gestational Age; UtA-PI – uterine artery pulsatility index.

4.3.3 Hypertensive Disorders of Pregnancy

Overall, observed rates of early- and late-onset PE, GH, and HELLP syndrome were 4/572 (0.7%), 19/572 (3.3%), 49/572 (8.6%), and 8/572 (1.4%), respectively (Table 15). Pre-gestational chronic hypertension was present in 29 (5.1%) women (Table 1). Among those women with pre-existing chronic hypertension, 8 (27.6%) experienced worsening of the disease, one developed PE (3.4%) and another (3.4%) developed HELLP syndrome.

- **Maternal and pregnancy factors**

In the univariable analysis, maternal and pregnancy factors associated with HDP were identified as follows: monochorionicity and a previous history of PE for early-onset PE; high maternal BMI, higher first-trimester MAP, and a previous history of PE for late-onset PE; and advanced maternal age, nulliparity, and conception by ART for GH (Figures 13, 14 and Table 15). No associations were found for HELLP syndrome in this cohort.

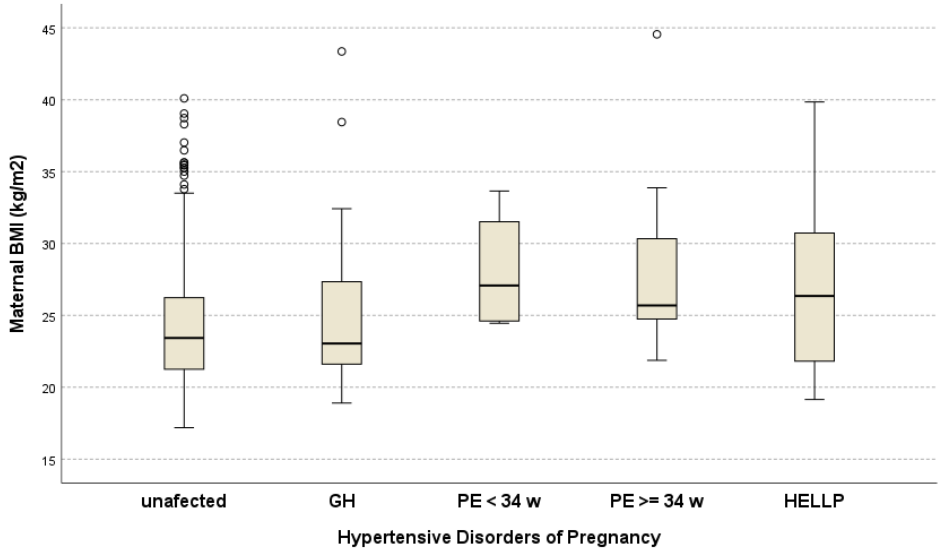

Figure 13. Pre-gestational maternal body mass index (BMI) and new onset of hypertensive disorders of pregnancy (circles represent moderate outliers).

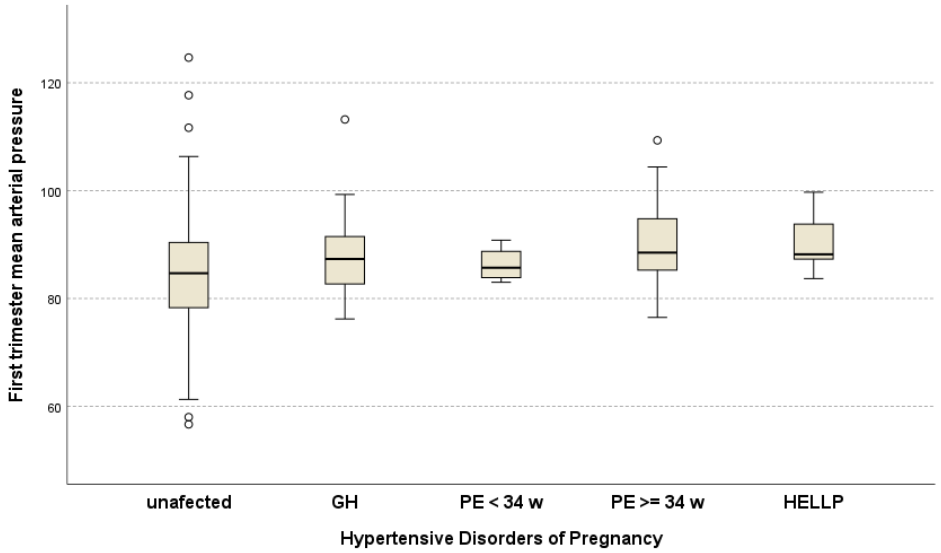

Figure 14. First trimester maternal mean arterial pressure and development of hypertensive disorders of pregnancy.

Abbreviations in Figure 13 and 14: BMI – body mass index, GH – Gestational Hypertension; PE – Preeclampsia; HELLP – Hemolysis, Elevated Liver enzymes, and Low Platelets syndrome, TwPs – Twin pregnancies.

TABLE 15 Univariable analysis of maternal and pregnancy characteristics and hypertensive disorders of pregnancy in twins pregnancies (HELLP syndrome not included)[c].

Total n =572	**Unaffected n= 493 (86.2%)**	**Early-onset PE<34weeks n= 4 (0.7%)**		**Late-onset PE≥34weeks n= 19 (3.3%)**		**Gestational Hypertension[b] n= 49 (8.6%)**	
	Mean (SD) or n(%)	Mean (SD) or n(%)	OR (95%CI) p-value	Mean (SD) or n(%)	OR (95%CI) p-value	Mean (SD) or n(%)	OR (95%CI) p-value
Maternal age (years)	32.5 (5.2)	34.0 (3.5)	1.0 (8.6-1.3) 0.626	33.2 (5.0)	1.0 (0.9-1.1) 0.721	34.3 (4.6)	1.07 (1.00-1.13) **0.032**
Age ≥40 years	29 (5.9%)	0 (0.0%)	- 1.000[a]	3 (15.8%)	2.7 (0.7-9.9) 0.125	5 (10.2%)	1.6 (0.6-4.5) 0.360
BMI (Kg/m2)	24.2 (4.1)	28.0 (4.3)	1.1 (0.9-1.3) 0.100	27.8 (5.4)	1.1 (1.0-1.2) **<0.001**	24.5 (4.7)	1.0 (0.9-1.1) 0.756
BMI <20	68 (13.8%)	0 (0.0%)	- 1.000[a]	0 (0.0%)	0.154[a]	5 (10.2%)	0.7 (0.3-1.9) 0.551
BMI ≥20<25	260 (52.7%)	2 (50.0%)	0.9 (0.1-6.6) 1.000	5 (26.3%)	0.3 (0.1-0.9) **0.024**	27 (55.1%)	1.1 (0.6-2.0) 0.623
BMI ≥25<30	116 (23.5%)	1 (25.0%)	1.0 (0.1-9.9) 1.000	9 (47.5%)	2.8 (1.1-7.2) **0.028**	12 (24.5%)	1.0 (0.5-1.9) 0.998
BMI ≥30	49 (9.9%)	1 (25.0%)	2.7 (0.3-27.0) 0.369	5 (26.3%)	3.1 (1.1-8.9) **0.045**	5 (10.2%)	0.9 (0.3-2.4) 0.881
Ethnicity							
Caucasian	425 (86.2%)	3 (75.0%)	0.4 (0.1-4.6) 0.449	14 (73.7%)	0.4 (0.2-1.2) 0.164	45 (91.8%)	1.8 (0.7-5.3) 0.231
African	55 (11.2%)	1 (25.0%)	2.7 (0.3-26.5) 0.374	4 (21.1%)	2.2 (0.7-6.9) 0.146	2 (4.1%)	0.3 (0.1-1.3) 0.105
South Asian	12 (2.4%)	0 (0.0%)	- 1.000[a]	1 (5.3%)	2.1 (0.3-17.1) 0.401	2 (4.1%)	1.7 (0.4-7.6) 0.373
Mixed	1 (0.2%)	0 (0.0%)	- 1.000[a]	0 (0.0%)	- 1.000[a]	0 (0.0%)	- 1.000[a]
Parity							
Nulliparous	299 (60.6%)	1 (25.0%)	5.1 (0.5-50.2) 0.144	14 (73.7%)	0.6 (0.2-1.7) 0.469	42 (85.7%)	3.8 (1.6-10.7) **< 0.001**
Parous with prior PTB	19/194 (9.8%)	2 (66.7%)	18.8 (1.6-216.6) **0.029**	1 (20.0%)	2.2 (0.2-20.6) 0.427	0 (0.0%)	- 1.000[a]
Parous with prior SGA	12/194 (6.2%)	1 (33.3%)	8.1 (0.6-96.5) 0.174	0 (0.0%)	1.000a	0 (0.0%)	- 1.000[a]
Parous with prior PE	4/194 (2.1%)	2 (66.7%)	67.3 (5.3-848.7) **0.004**	2 (40.0%)	22.2 (3.1-158.5) **0.012**	0 (0.0%)	- 1.000[a]
Method of conception							
Spontaneous	307 (62.3%)	4 (100.0%)	1.0 (1.0-1.0) 0.156	9 (47.4%)	0.5 (0.2-1.4) 0.241	22 (44.9%)	0.5 (0.3-0.9) **0.021**
Ovulation inductions	19 (3.9%)	0 (0.0%)	- 1.000[a]	1 (5.3%)	1.5 (0.2-11.6) 0.514	1 (2.0%)	0.5 (0.1-3.9) 1.000
ART	167 (33.9%)	0 (0.0%)	- 0.302[a]	9 (47.4%)	1.6 (0.6-4.0) 0.294	26 (53.1%)	2.1 (1.1-3.9) **0.009**
Chorionicity							
Monochorionic	104 (21.1%)	3 (75.0%)	11.2 (1.2-298.6) **0.032**	5 (26.3%)	0.7 (0.3-2.1) 0.573	7 (14.3%)	1.6 (0.7-3.8) 0.208
Dichorionic	389 (78.9%)	1 (25.0%)		14 (73.7%)		42 (85.7%)	
Smoker	49 (9.9%)	0 (0.0%)	- 1.000[a]	0 (0.0%)	0.242	5 (10.2%)	1.0 (0.4-2.9) 0.799
Chronic hypertension	27 (5.5%)	0 (0.0%)	- 1.000[a]	1 (5.3%)	1.0 (0.1-8.0) 1.000	---	---
SLE/APS/Thrombophilia	12 (2.4%)	0 (0.0%)	- 1.000[a]	2 (10.5%)	4.8 (1.0-23.3) 0.085	1 (2.0%)	0.7 (0.1-5.9) 1.000
Aspirin prophylactic intake (started <20 weeks)	60 (12.2%)	2 (50.0%)	7.0 (0.8-50.4) 0.081	7 (36.8%)	4.3 (1.6-11.3) **0.006**	3 (6.1%)	0.4 (0.1-1.3) 0.145
1st trimester Mean Arterial Pressure	84.5 (8.9)	86.3 (3.3)	1.0 (0.9-1.1) 0.796	90.5 (8.8)	1.06 (1.01-1.12) **0.011**	87.9 (7.5)	1.0 (0.9-1.1) 0.075

a) The odds ratio could not be estimated due to zero counts in one of the groups or to a small number of events; b) new onset, chronic hypertension excluded; c) HELLP syndrome accounts for 8 cases (1.4%)

Abbreviations: ART– artificial reproductive techniques; APS – Antiphospholipid syndrome; BMI – body mass index; CI – confidence interval; HELLP – Hemolysis, Elevated Liver enzymes, and Low Platelets syndrome; OR – odds ratio estimate; PE – preeclampsia; PTB – preterm birth; SGA – small for gestational age; SD – standard deviation; SLE – Systemic lupus erythematosus.

- **Uterine artery Dopplers**

Women who develop early-onset PE showed higher UtA-PI in the first and second trimester Dopplers, p=0.001 and p=0.006, respectively (Table 16). Between women with first trimester UtA Doppler evaluation, all observed cases (n=4) of early-onset PE presented first trimester for UtA-PI ≥ 90th percentile, considering TwPs' references for chorionicity and gestational age. This corresponds to a value of 2.19 for the first trimester of UtA-PI in DC TwPs and 2.35 for MC TwPs, as observed in the empirical percentiles (Table 3). On the other hand, if we were to consider Dopplers' references of SP, only two (50%) of the total cases would be categorized in the high-resistance UtA group (Figure 15.a). The remaining HDP groups, including late-onset PE, GH, and HELLP syndrome, were not associated with high resistance in the first trimester of UtA-PI. Notably, all women with HELLP syndrome exhibited low resistance in UtA Dopplers.

In the second trimester evaluation, all women who developed early-onset PE also maintained higher UtA-PI values ≥ 90th percentile, based on empirical references for TwPs, adjusted for chorionicity. This corresponded to a UtA-PI value of ≥ 1.5 in three MC pregnancies (Figure 15.b). Additionally, women with second trimester UtA-PI ≥95th percentile had increased odds of developing GH, OR 3.8 (95%CI: 0.039, p= 0.039)(Table 16).

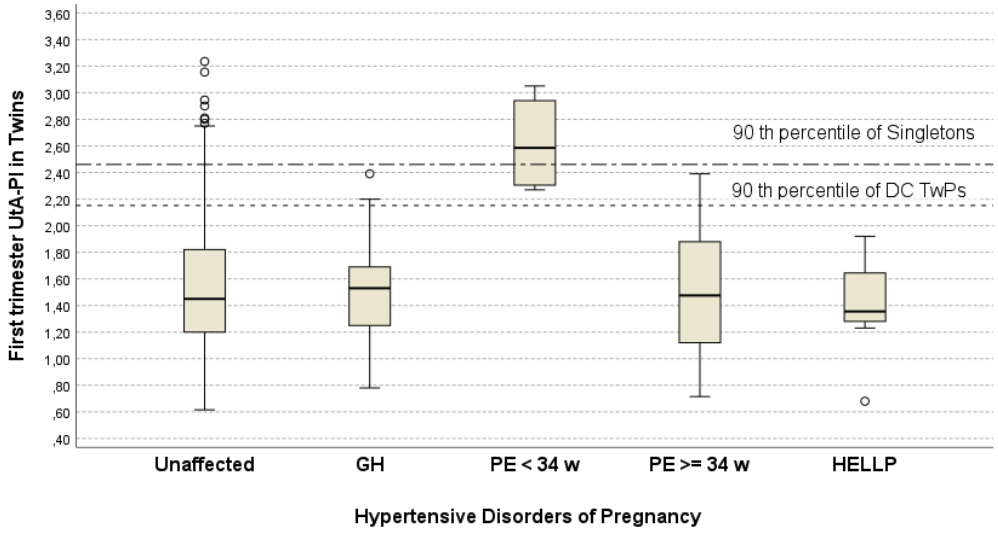

Figure 15.a - First trimester uterine artery pulsatility index (UtA-PI) in Twin Pregnancies and Hypertensive Disorders of Pregnancy (dotted line and dash-dotted line represent the 90th percentile of UtA-PI of DC twins and singletons, respectively, circles represent moderate outliers).

Abbreviations: GH – Gestational Hypertension; PE – Preeclampsia; HELLP – Hemolysis, Elevated Liver enzymes, and Low Platelets syndrome, TwPs – Twin pregnancies.

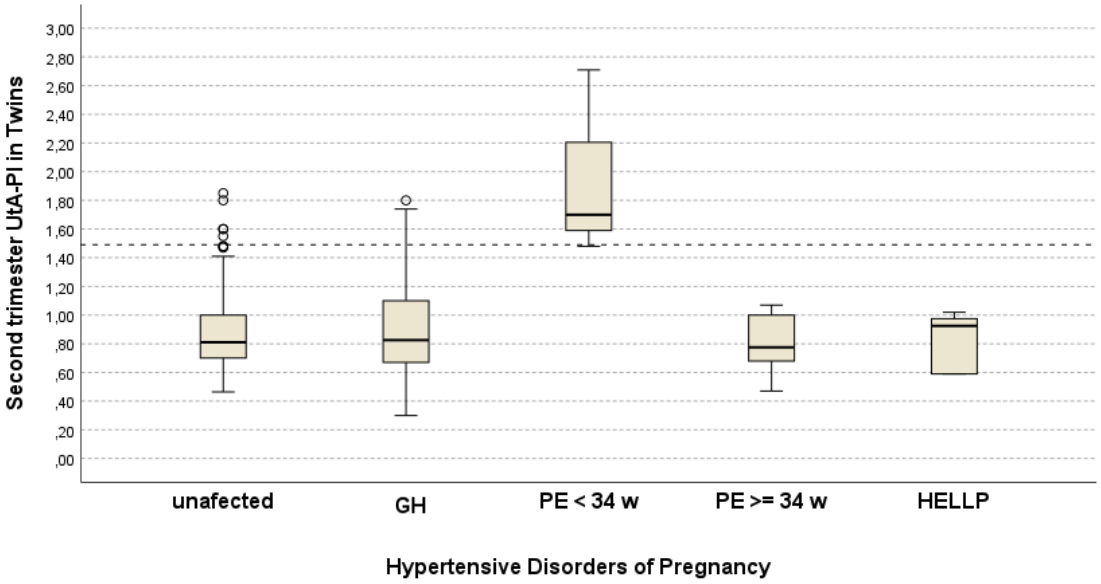

Figure 15.b - Second trimester uterine artery pulsatility index (UtA-PI) in Twin Pregnancies and Hypertensive Disorders of Pregnancy (dotted line represents the value 1.5 of the UtA-PI, circles represent moderate outliers).

Abbreviations: GH – Gestational Hypertension; PE – Preeclampsia; HELLP – Hemolysis, Elevated Liver enzymes, and Low Platelets syndrome, TwPs – Twin pregnancies.

TABLE 16 Univariable analysis of uterine artery Dopplers, first trimester serum biomarkers, and hypertensive disorders in twin pregnancies

	Unaffected	Early-onset PE<34weeks		Late-onset PE≥34weeks		Gestational Hypertension[b]	
	n (%)	n (%)	OR (95%CI) p-value	n (%)	OR (95%CI) p-value	n (%)	OR (95%CI) p-value
1st Trimester UtA-PI available data, n=390							
UtA-PI (continuous variable)	333 (85.4%)	4 (1.0%)	40.4 (4.6-354.2) **<0.001[a]**	17 (4.4%)	0.6 (0.2-1.8) 0.384	29 (7.4%)	0.9 (0.4-2.0) 0.854
UtA-PI ≥ 90th percentile	35 (10.5%)	4 (100.0%)	- **<0.001[a]**	1 (5.9%)	0.5 (0.1-3.9) 1.000	2 (6.9%)	0.6 (0.1-2.6) 0.755
UtA-PI ≥ 95th percentile	19 (5.7%)	3 (75.0%)	52.1 (5.1-522.9) **<0.001**	1 (5.9%)	0.9 (0.1-7.4) 1.000	1 (3.4%)	0.5 (0.1-4.0) 1.000
2nd trimester UtA-PI available data, n=391							
UtA-PI (continuous variable)	336 (85.9%)	3 (0.8%)	- **0.006[a]**	18 (4.6%)	0.3 (0.0-2.9) 0.309	28 (7.2%)	1.7 (0.5-6.6) 0.390
UtA-PI ≥ 90th percentile	33 (9.8%)	3 (100.0%)	- **<0.001[a]**	0 (0.0%)	- 0.237[a]	5 (17.9%)	1.9 (0.7-5.5) 0.197
UtA-PI ≥ 95th percentile	13 (3.9%)	2 (66.7%)	- **0.007[a]**	0 (0.0%)	- 1.000[a]	4 (14.3%)	3.8 (1.2-12.5) **0.039**
1st trimester PAPP-A MoM available data, n=466							
PAPP-A MoM (continuous variable)	395 (84.8%)	4 (0.9%)	0.2 (0.0-3.1) 0.297	19 (4.1%)	1.0 (0.5-2.1) 0.788	42 (9.0%)	0.6 (0.4-1.2) 0.221
PAPP-A MoM < 10th perc.	38 (9.6%)	0 (0.0%)	- 1.000[a]	3 (15.8%)	1.7 (0.5-6.2) 0.419	5 (11.9%)	1.2 (0.4-3.9) 0.591
PAPP-A MoM > 90th perc.	41 (10.4%)	0 (0.0%)	- 1.000[a]	2 (10.5%)	1.0 (0.2-4.8) 1.000	3 (7.1%)	0.6 (0.2-2.2) 0.789
1st trimester β-hCG MoM available data, n=466							
β-hCG MoM (continuous variable)	395 (84.8%)	4 (0.9%)	0.8 (0.1-4.0) 0.788	19 (4.1%)	1.3 (0.8-2.3) 0.258	42 (9.0%)	0.3 (0.1-0.7) **0.005**
β-hCG MoM < 10th perc.	35 (8.9%)	1 (25.0%)	3.0 (0.3-30.3) 0.341	1 (5.3%)	0.5 (0.1-3.8) 0.709	8 (19.0%)	2.3 (1.0-5.5) **0.053**
β-hCG MoM > 90th perc.	42 (10.6%)	0 (0.0%)	- 1.000[a]	3 (15.8%)	1.7 (0.5-6.2) 0.419	0 (0.0%)	- **0.025[a]**

Abbreviations: CI – confidence interval; MoM – multiple of the median; OR – odds ratio estimate; PAPP-A – pregnancy-associated plasma protein-A; PE – preeclampsia; SD – standard deviation; UtA-PI – Uterine artery pulsatility index; β-hCG – β-human chorionic gonadotropin.

- **First trimester serum biomarkers**

In our cohort, there was no association between PAPP-A levels and the development of HDP. However, an increased odds of developing GH was found in women with lower β-hCG levels (< 10th percentile), OR 2.3 (95% CI: 1.0-5.5, p=0.053), while no women with higher β-hCG levels (> 90th percentile) developed GH (Tabel 16).

- **Combined maternal and pregnancy factors with first trimester UtA-PI and serum biomarkers**

Due to the small number of cases and the fact that applying the TwPs' UtA-PI ≥ 90th percentile resulted in 100% detection, no multivariable model was fitted for this outcome. For late-onset PE and/or GH, maternal factors such as advanced age, nulliparity, obesity (BMI ≥ 30 kg/m²), and elevated mean arterial pressure (MAP) in the first trimester were associated with increased odds of HDP (Table 21). Low β-hCG levels were maintained as an independent variable for the development of GH. No significant association was found between HDP and chorionicity or conception by ART treatments. The AUCs indicated that the first trimester multivariable models had limited discriminative performance for late HDP, with values ranging from 0.568 to 0.727, which corresponds to a detection rate varying from 27% to 48% for a fixed false positive rate of 20% (Table 17).

TABLE 17 Multivariable logistic regression analyses for HDP in twin pregnancies integrating first trimester clinical data.

Independent variables	Adjusted OR (95%CI)	p-value	AUC (95%CI) Sensitivity to a False Positive rate of 20%	Hosmer-Lemeshow test p-value
Late onset PE (≥34 weeks)				
BMI ≥ 25 (Kg/m2)	5.2 (1.6-17.3)	0.005	0.568 (0.476-0.660) S 27%	0.928
MAP (continuous variable)	1.04 (109-1.1)	**0.095**		
Gestational Hypertension				
Maternal age	1.1 (1.1-1.2)	**0.029**	0.709 (0.636-0.783) S 48%	0.755
Nulliparas	2.8 (1.2-6.6)	**0.014**		
β-hCG MoM	0.3 (0.2-0.7)	**0.005**		
All Gestational Hypertension (new onset, chronic hypertension excluded)				
Maternal age (continuous variable)	1.1 (1.0-1.2)	**0.008**	0.734 (0.668-0.801) S 46%	0.598
Nulliparas	1.8 (0.9-3.6)	0.068		
BMI (Kg/m2) (continuous variable)	1.1 (1.0-1.2)	**0.006**		
MAP (continuous variable)	1.1 (1.0-1.1)	**0.001**		

Abbreviations: AUC – area under the receiver-operating characteristic curve; BMI – body mass index; MAP – mean arterial pressure; MoM – Multiple of the Median; OR – odds ratio estimate; PAPP-A – Pregnancy-Associated Plasma Protein-A; UtA-PI – uterine artery pulsatility index; β-hCG – β-human chorionic gonadotropin;

4.3.4 Composite outcome of PTB concurrent with FGR, SGA and/or HDP

Overall, the incidence of PTB < 32 w, < 34 w and < 36 w associated to FGR, SGA (< 5th percentile) and/or HDP was found to be 4.5%, 9.4% and 21.7%, respectively, with more than half (71.8%) of premature births < 32 w being associated with the presence of SGA < 10th percentile in one or both twins.

Considering the outcome SGA < 3rd percentile in one or both neonates per pregnancy, it was observed in 23 (59.0%) vs 97 (18.2%) cases in PTB < 32 w, OR 6.4 (95%CI: 3.2-12.7, p<0.001); in 40 (48.8%) vs 80 (16.3%) cases in PTB < 34 w, OR 4.8 (95%CI: 2.9-8.0, p<0.001); and in 73 (37.4%) cases vs 47 (12.5%) cases in PTB < 36 w, OR 4.2 (95%CI: 2.7-6.4, p<0.001).

Among women with iatrogenic PTB < 34 w (33 cases, corresponding to 40.2% of all PTB < 34 w), 30 (90.9%) had prenatal suspicion of FGR, and 31 (93.9%) had one or both neonates classified as SGA < 10th percentile.

Among women with PTB < 32 w (39 cases), HDP were observed in two cases (5.1%) of GH, one case (2.6%) of PE, and one case (2.6%) of HELLP syndrome; among those with PTB < 34 w, three cases (3.7%) of GH, four cases (4.9%) of PE, and three cases (3.7%) of HELLP syndrome were observed.

- **Maternal and pregnancy factors**

In the multivariable LR analysis models developed for the different outcomes studied, the maternal and pregnancy factors that demonstrated significant statistical evidence were as follows: maternal height, first trimester MAP, and second trimester short CL (≤ 28 mm) for PTB < 32 and <34 weeks concurrent with FGR, SGA, and/or HDP; monochorionicity, short CL, and conception via ART of PTB < 36 w concurrent with FGR, SGA, and/or HDP. Obesity was associated with increased odds of PTB < 34 w concurrent with FGR, SGA, and/or HDP. Nulliparity and monochorionicity were identified as independent risk factors for SGA <10th percentile in one or both fetuses born before 36 weeks. Additionally, smoking habits showed a weak level of evidence, with p-values >0.05 but <0.100, for PTB <32 w concurrent with FGR, SGA, and/or HDP (Tables 10, 11, 14, 17 to 19).

TABLE 18 Multivariable logistic regression analyses for PTB concurrent with FGR, SGA and/or HDP in twin pregnancies integrating first trimester clinical data.

Independent variables	Adjusted OR (95%CI)	p-value	AUC (95%CI) Sensitivity to a False Positive rate of 20%	Hosmer-Lemeshow test p-value
PTB < 32 weeks concurrent with FGR, SGA and/or HDP				
Maternal height < 167 cm	10.6 (1.3-86.6)	**0.027**	0.830 (0.751-0.910) **S 72%**	0.184
Smoking habits	3.2 (0.9-11.9)	0.079		
MAP (continuous variable)	1.1 (1.0-1.1)	**0.009**		
PAPP-A MoM < 10th percentile	3.8 (1.0-14.5)	**0.042**		
UtA-PI ≥ 95th percentile	6.5 (1.7-25.3)	**0.006**		
PTB < 34 weeks concurrent with FGR, SGA and/or HDP				
Maternal height < 167 cm	6.7 (1.9-23.8)	**0.003**	0.759 (0.680-0.839) S 55%	0.760
BMI < 30 (Kg/m2)	2.6 (1.0-6.5)	**0.043**		
MAP (continuous variable)	1.0 (0.9-1.1)	0.185		
PAPP-A MoM (continuous variable)	0.4 (0.2-0.9)	**0.040**		
UtA-PI ≥ 95th percentile	5.2 (1.8-14.7)	**0.002**		
PTB < 36 weeks concurrent with FGR, SGA and/or HDP				
Maternal height cm (continuous variable)	0.9 (0.9-1.0)	0.066	0.676 (0.608-0.744) S 48%	0.127
BMI (Kg/m2) (continuous variable)	0.9 (0.9-1.0)	0.084		
Nulliparas	1.6 (0.9-3.0)	0.082		
Monochorionic	1.6 (0.8-3.0)	0.139		
PAPP-A MoM (continuous variable)	0.7 (0.5-1.2)	0.234		
UtA-PI ≥ 90th percentile	3.7 (1.8-7.6)	**<0.001**		

Abbreviations: AUC – area under the receiver-operating characteristic curve; BMI – body mass index; FGR – fetal growth restriction; HDP – hypertensive disorders of pregnancy; MAP – mean arterial pressure; MoM – Multiple of the Median; OR – odds ratio estimate; PAPP-A – Pregnancy-Associated Plasma Protein-A; PTB – Preterm Birth; SGA – Small for Gestational Age; UtA-PI – uterine artery pulsatility index.

- **Uterine artery Dopplers**

In the multivariable LR analysis, a first trimester UtA-PI ≥ 95th percentile was identified as an independent risk factor for all PTB concurrent with all the categories of SGA, with the highest odds found for SGA < 3rd percentile born < 32 and < 36 w, OR of 7.2 (95% CI: 1.8–28.5, p=0.005), and 7.2 (95% CI: 2.5–16.4, p<0.001), respectively (Table 17). Moreover, the highest odds for PTB concurrent with FGR, SGA, and/or HDP were observed when integrating second-trimester data, including CL and UtA-PI, with a UtA-PI ≥ 95th percentile, yielding an OR of 27.9 (95% CI: 3.5–218.7, p=0.002)(Table 22). Despite the higher risk for this outcome, a first-trimester UtA-PI ≥ 95th percentile alone detected 5/19 (26.3%) affected cases, whereas in the second trimester, it detected 3/18 (16.7%) affected cases.

- **First trimester serum biomarkers**

In the multivariable LR analysis, women with low PAPP-A < 10th percentile exhibit higher odds for SGA < 3rd percentile and PTB < 32 weeks, OR 4.3 (95% CI 1.1-16.0, p=0.032),

as well as PTB < 32 w concurrent with FGR, SGA and/or HDP, OR 3.8 (95% CI 1.0-14.5, p=0.042) (Tables 14 and 18). Despite these findings, in the univariable analyses, the use the cut-off of PAPP-A <10th percentile alone for predicting adverse outcomes is poor. Although the best detection rate (25%) was observed for PTB < 32 w concurrent with FGR, SGA and/or HDP, it came with the highest false positive rate (87.5%). Even when considering a higher cut-off of 1.0 MoM, the sensitivity to detect PTB < 32 w concurrent with FGR, SGA and/or HDP improved to 75%, albeit with a high false positive rate of 92.2% (Table 9).

β-hCG levels did not reach statistical significance and were not included in the multivariable models analyzed for composite outcomes.

TABLE 19 Multivariable logistic regression analyses for PTB concurrent with FGR, SGA and/or HDP in twin pregnancies integrating first and second trimester clinical data.

Independent variables	Adjusted OR (95%CI)	p-value	AUC (95%CI) Sensitivity to a False Positive rate of 20%	Hosmer-Lemeshow test p-value
PTB < 32 weeks concurrent with FGR, SGA and/or HDP				
Maternal height < 167 cm	14.5 (1.2-166.3)	**0.031**	0.875 (0.780-0.971) **S 85%** (False positive rate = 16%)	0.721
BMI ≥ 30 (Kg/m2)	4.2 (0.9-18.8)	0.059		
Smoking habits	6.0 (0.9-39.1)	0.058		
1st trimester MAP (continuous variable)	1.1 (1.0-1.1)	**0.047**		
1st trimester PAPP-A MoM < 10th perc.	7.7 (1.4-44.3)	**0.021**		
2nd trimester Cervical length ≤28 mm	4.9 (0.9-26.1)	**0.060**		
2nd trimester UtA-PI ≥ 95th percentile	27.9 (3.5-218.7)	**0.002**		
PTB < 34 weeks concurrent with FGR, SGA and/or HDP				
Maternal height cm (continuous variable)	0.8 (0.8-0.9)	**<0.001**	0.827 (0.737-0.916) **S 80%**	0.637
1st trimester MAP (continuous variable)	1.1 (1.01-1.1)	**0.005**		
1st trimester PAPP-A MoM (cont. variable)	0.3 (0.1-0.9)	**0.032**		
2nd trimester Cervical length ≤28 mm	4.1 (1.2-14.3)	**0.029**		
2nd trimester UtA-PI ≥ 95th percentile	9.5 (1.6 -53.8)	**0.013**		
PTB < 36 weeks concurrent with FGR, SGA and/or HDP				
Monochorionicity	2.5 (1.2-5.4)	0.015	0.696 (0.615-0.777) S 55%	0.532
BMI ≥ 30 (Kg/m2)	2.3 (0.9-5.5)	0.056		
Conception by ART	1.9 (1.0-3.6)	0.049		
1st trimester PAPP-A MoM (cont. variable)	0.7 (0.4-1.2)	0.256		
2nd trimester Cervical length ≤28 mm	4.2 (1.5-11.9)	0.006		
2nd trimester UtA-PI ≥ 90th percentile	3.8 (1.6-9.2)	**0.002**		

Abbreviations: AUC – area under the receiver-operating characteristic curve; BMI – body mass index; FGR – fetal growth restriction; HDP – hypertensive disorders of pregnancy; MAP – mean arterial pressure; MoM – Multiple of the Median; OR – odds ratio estimate; PAPP-A – Pregnancy-Associated Plasma Protein-A; PTB – Preterm Birth; SGA – Small for Gestational Age; UtA-PI – uterine artery pulsatility index.

- **Performance of multivariable models combining maternal and pregnancy factors with first and second trimester UtA-PI and first trimester serum biomarkers**

The AUCs achieved in the multivariable LR models for the composite outcomes ranged from 0.675 to 0.875, corresponding to a detection rate varying from 42% to 85% for a fixed false positive rate of 20%. The best-performing association model was obtained for the outcome of PTB < 32 w concurrent with FGR, SGA and/or HDP (85% of sensitivity for a false positive rate of 16%) witch integrated second trimester UtA-PI (Table 19 and Figure 16). The different models demonstrated good calibration, as assessed by the HL test (Tables 14, 18 and 19).

Of the 13 patients affected by the outcome PTB < 32 w concurrent with FGR, SGA and/or HDP, two were not detected by the LR model. One case involved a nulliparous woman with a DC TwP following ART conception. One of the fetuses developed early and severe FGR. The parents declined genetic testing but agreed to terminate the pregnancy at 28 weeks to avoid fetal demise of the growth-restricted fetus. This neonate died two days after delivery due to complications related to severe prematurity and very low birth weight (715g), with no malformations found during the autopsy, while the co-twin experienced neonatal morbidity related to prematurity, resulting in long-term sequelae. Another case involved a nulliparous woman with Celiac disease and a DC pregnancy, who had low resistance first- and second-trimester UtA Dopplers. She experienced premature rupture of membranes at 31 w and delivered two neonates, both SGA below the 3rd percentile. Detailed placental histopathologic examinations were not performed in either case.

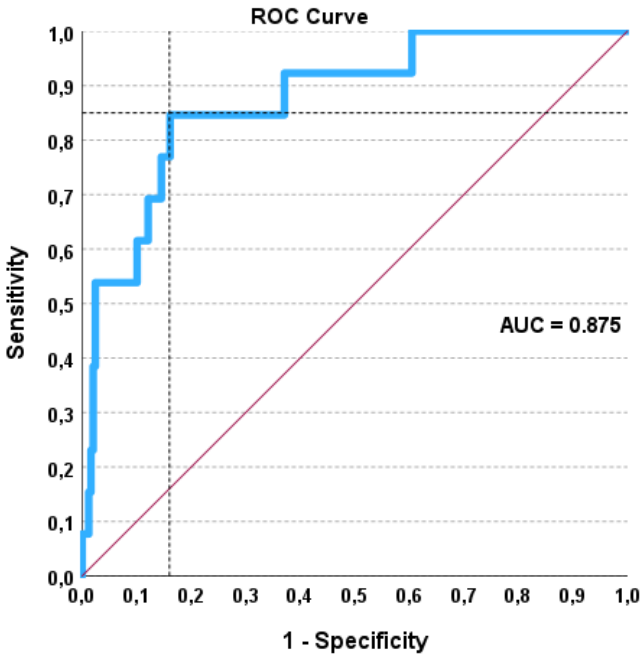

Figure 16 – Receiver Operating Characteristic (ROC) curve for preterm birth < 32 weeks concurrent with fetal growth restriction, small for gestational age and/or hypertensive disorders of pregnancy integrating first and second trimester data.

The dashed black lines are placed at values of 85% sensitivity and a 16% false-positive rate (1-specificity).

Abbreviations: AUC – area under the curve.

4.3.5 Neonatal outcomes

Two single fetal demises occurred in two DC pregnancies, one at 25 weeks and the other at 36 weeks. Neither fetus was suspected to be growth-restricted; however, in the latter case, the surviving co-twin was classified as SGA < 3rd percentile. No cases of fetal demise were observed in MC pregnancies.

Overall, the mean neonate birth weight observed was 2185g (409) in MC and 2312g (484) in DC, with a mean difference 154g (95%CI: 59-248, p=0.001). As MC pregnancies presented a higher rate of PTB, when considering only deliveries after 36 weeks, the mean neonatal birth weight was 2420g (235) in MC and 2526g (287) in DC, with a mean difference of 105g (95% CI: 39-171, p=0.002) (Figure 17). Birth weight discrepancy ≥ 25% was observed in 1.7% of MC and 2.5% of DC pregnancies.

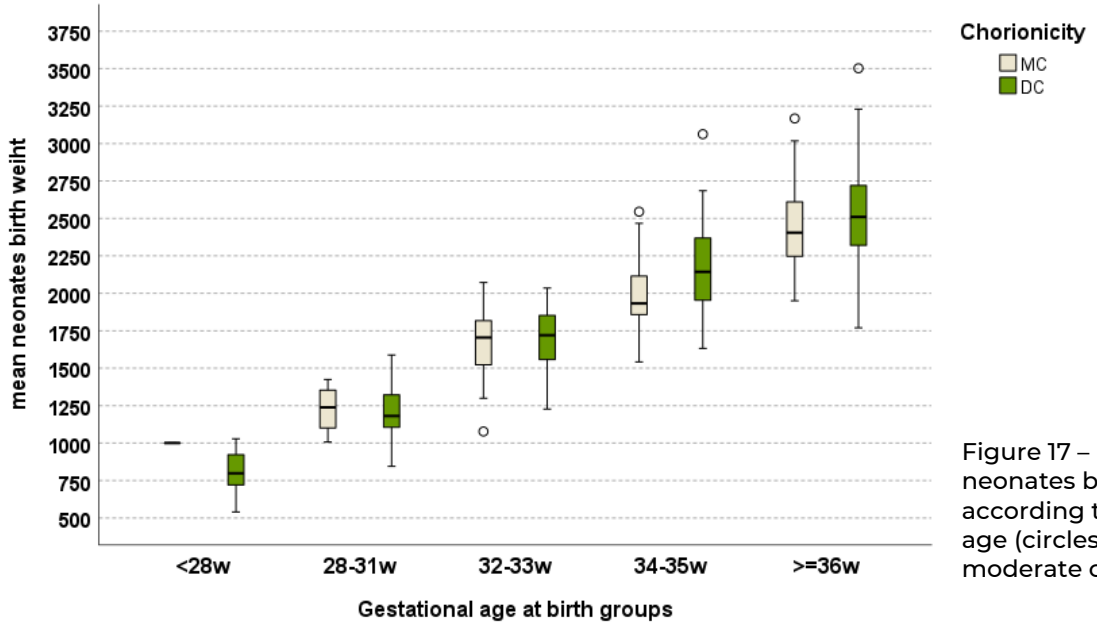

Figure 17 – Mean neonates birth weight according to gestational age (circles represent moderate outliers).

Neonatal deaths occurred in eight cases (0.9%) in DC, with no neonatal deaths reported in MC twins. Perinatal death in DC accounted for 10 cases, corresponding to an incidence of 1.1% per fetus and 1.7% per pregnancy.

The neonatal morbidity rates observed in the surviving neonates was as follows:

- Neonatal Care Unit admission ≥ 8 days: 32.7% in MC and 19.6% in DC
- Respiratory Distress Syndrome: 33.1% in MC and 21.9% in DC
- Hyaline Membrane Disease: 11.0% in MC and 10.6% in DC
- Bronchopulmonary Dysplasia: 0.8% in MC and 0.6% in DC
- Sepsis (early and/or late): 6.5% in MC and 2.6% in DC

- Retinopathy of prematurity (grade not specified): 1.6% in MC and 0.6% in DC
- Intraventricular hemorrhage grade III/IV: 0.4% in MC and 1.2% in DC
- Leukomalacia: 0.4% in MC and 0.8% in DC

- **Uterine artery Dopplers**

In univariable analysis, a higher first trimester UtA-PI ≥ 90th or ≥ 95th was associated with an increased risk of lower birth weight, $\hat{\beta}$= -306 (95%CI: -459 − -112, p<0.001) and $\hat{\beta}$= -325 (95%CI: -527 - -124, p=0.002), respectively, in both MC and DC pregnancies (Table 14). Additionally, neonates from women with higher UtA-PI values had a greater need of prolonged neonatal care admission (≥8 days) and higher rates of neonatal morbidity related to prematurity, specifically RDS and HMD. The highest odds of HMD were observed in the UtA-PI ≥ 90th percentile group, with an OR of 3.4 (95% CI: 1.9–5.9, p<0.001). For other less frequently observed neonatal outcomes, such as sepsis and ROP, no statistically significant differences were found, despite a higher observed rate in the neonates of the higher-risk women. Also, no cases of IVH grade III/IV were observed in this group (Table 20).

In the univariable analysis for second trimester UtA-PI, similar findings were observed, with statistical significance reached for the outcome of sepsis. The highest odds for this outcome was observed in the UtA-PI ≥ 90th percentile group, with an OR of 4.6. (95% CI: 1.3–13.7, p=0.037)(Table 21).

- **First trimester serum biomarkers**

In univariable analysis, PAPP-A demonstrated a linear association with neonates mean birth weight, p<0.001. A lower first trimester PAPP-A <10th percentile was associated with an increased risk of lower birth weight, $\hat{\beta}$ = -266 (95%CI: -372 - -84, p=0.002) (Table 16). For example, when comparing pregnancies with PAPP-A < 10th percentile to those with PAPP-A > 90th percentile, the mean birth weight was 2074g vs 2487g, respectively (Figure 8 and Table 22).

Additionally, neonates from women with PAPP-A < 10th percentile had a greater need for prolonged neonatal care admission, which was statistically significant in the subgroup of DC TwPs, and exhibited higher rates of neonatal morbidity related to prematurity, specifically RDS and HMD (Table 22).

For other less frequently observed neonatal outcomes, such as sepsis and ROP and IVH (grade III/IV), no statistically significant differences were found. Notably, no cases of severe neonatal morbidity were observed in the neonates from women with PAPP-A >90th percentile (Table 22).

No associations with neonatal outcomes were identified in relation to first trimester serum β-hCG levels (Table 23).

TABLE 20 Univariable analysis of first trimester uterine artery Dopplers and perinatal outcomes in twin pregnancies.

Total 1st trimester UtA-PI data n=390, MC=79, BC=311	1st trimester UtA-PI <90th percentile, n=348 MC= 70, DC=278	1st trimester UtA-PI ≥ 90th percentile, n=42 MC=9, DC=33		1st trimester UtA-PI ≥ 95th percentile, n=24 MC=6, DC=18	
	Mean (SD) or n (%)	Mean (SD) or n (%)	OR or $\hat{\beta}$= (95%CI) p-value	Mean (SD) or n (%)	OR or $\hat{\beta}$= (95%CI) p-value
Prenatal suspicion of FGR (one or both fetus per pregnancy)					
All twins	93 (26.7%)	19 (45.2%)	2.2 (1.2-4.3) **0.012**	13 (54.2%)	3.1 (1.4-7.3) **0.004**
Monochorionic	22 (31.4%)	6 (66.7%)	4.3 (0.9-19.0) 0.061	5 (83.3%)	10.8 (1.2-98.4) **0.019**
Dichorionic	71 (25.5%)	13 (39.4%)	1.8 (0.9-4.0) 0.090	8 (44.4%)	2.3 (0.8-6.0) **0.102**
Single fetal demise ≥ 24w (number of fetuses per pregnancy)					
Dichorionic	0 (0.0%)	1 (3.0%)	- 0.106[a]	1 (5.5%)	1.1 (0.9-1.2) 0.058
Gestational age at delivery					
All twins	35.6 (2.1)	34.4 (2.8)	-1.2 (-1.9 - -0.5) **<0.001**	34.4 (2.7)	-1.1 (-2.03 - -0.20) **0.016**
Monochorionic	35.2 (1.6)	34.0 (2.0)	-1.2 (-2.3 - -0.1) **0.036**	34.0 (2.2)	-1.2 (-2.6 – 0.2) 0.096
Dichorionic	35.7 (2.2)	34.5 (3.0)	-1.2 (-2.0 - -0.3) **0.006**	34.6 (2.8)	-1.1 (-2.2 - -0.04) 0.058
Mean neonates birthweight					
All twins	2307 (453)	1998 (594)	-306 (-459 - -112) **<0.001**	1965 (554)	-325 (-527 - -124) **0.002**
Monochorionic	2217 (377)	1851 (402)	-365 (-634 - -97) **0.008**	1882 (407)	-317 (-646 - -11) **0.058**
Dichorionic	2330 (468)	2039 (637)	-290 (-469 - -111) **0.002**	1995 (606)	-322 (-563 - -81) **0.009**
Birth weight discrepancy ≥ 25%					
All twins	24 (6.9%)	5 (11.9%)	1.8 (0.6-5.0) 0.222	3 (12.5%)	1.9 (0.5-6.6) 0.408
Monochorionic	4 (5.7%)	0 (0.0%)	- 1.000[a]	0 (0.0%)	- 1.000[a]
Dichorionic	20 (7.2%)	5 (15.2%)	2.3 (0.8-6.6) 0.164	0 (16.7%)	2.5 (0.6-9.1) 0.167
Neonatal Death (number of cases per neonates)					
Dichorionic	5/556 (0.9%)	2/65 (3.0%)	3.4 (0.4-17.9) 0.323	0/35 (0.0%)	- 1.000[a]
Perinatal Death (number of cases per fetuses)					
Dichorionic	5/556 (0.9%)	3/66 (4.5%)	3.5 (0.7-13.8) 0.176	1/36 (2.7%)	2.3 (0.1-15.8) 0.454
Neonatal Care Unit admission ≥ 8 days (number of cases per surviving newborns)					
All twins	143/691 (20.7%)	35/81 (43.2%)	2.9 (1.7-4.7) **<0.001**	21/47 (44.7%)	3.1 (1.7-5.7) **<0.001**
Monochorionic	44/140 (31.4%)	12/18 (66.6%)	4.3 (1.5-13.2) **0.005**	8/12 (66.6%)	4.0 (1.1-16.1) **0.045**
Dichorionic	99/551 (17.9%)	23/63 (36.5%)	2.6 (1.5-4.5) **0.001**	13/35 (37.1%)	2.5 (1.2-5.2) **0.008**
Neonatal morbidity (number of cases per surviving newborns)					
RDS	163/691 (23.6%)	36/81 (44.4%)	2.6 (1.6-4.1) **<0.001**	18/47 (38.3%)	1.8 (1.0-3.4) **0.043**
HMD	71/691 (10.3%)	23/81 (28.4%)	3.4 (1.9-5.9) **<0.001**	13/47 (27.6%)	3.0 (1.5-5.9) **0.003**
IVH Grade III/IV	7/691 (1.0%)	0/81 (0.0%)	- 1.000[a]	0/47 (0.0%)	- 1.000[a]
Sepsis	22/691 (3.1%)	3/81 (3.7%)	1.2 (0.3-3.6) 0.760	3/47 (6.4%)	2.2 (0.5-7.1) 0.361
ROP	5/691 (0.7%)	2/81 (2.4%)	3.4 (0.5-17.8) 0.322	2/47 (4.2%)	6.3 (0.8-33.2) 0.125

Abbreviations: $\hat{\beta}$ – linear regression coefficient estimate; BPD – Bronchopulmonary dysplasia; CI – confidence interval; DC – dichorionic; HMD – Hyaline membrane disease; IVH – Intraventricular hemorrhage; MC – Monochorionic; OR – odds ratio estimate; RDS – respiratory distress syndrome in newborn; ROP – Retinopathy of prematurity; SD – standard deviation; UtA-PI – Uterine artery pulsatility index.

TABLE 21 Univariable analysis of second trimester uterine artery Dopplers and perinatal outcomes in twin pregnancies.

Total 2nd trimester UtA-PI data n=391, MC=84, DC=307	2nd trimester UtA-PI < 90th percentile, n=350 MC=74, DC=276	2nd trimester UtA-PI≥ 90th percentile, n=41 MC=10, DC=31		2nd trimester UtA-PI≥ 95th percentile, n=19 MC=4, DC=15	
	Mean (SD) or n (%)	Mean (SD) or n (%)	OR or $\hat{\beta}$= (95%CI) p-value	Mean (SD) or n (%)	OR or $\hat{\beta}$= (95%CI) p-value
Prenatal suspicion of FGR (one or both fetus per pregnancy)					
All twins	81 (23.1%)	22 (53.7%)	3.8 (1.9-7.4) **<0.001**	10 (52.6)	3.3 (1.3-8.4) **0.008**
Monochorionic	24 (32.4%)	9 (90.0%)	18.7 (2.2-156.6) **<0.001**	4 (100.0%)	- **0.021**[a]
Dichorionic	57 (20.7%)	13 (41.9%)	2.7 (1.2-5.9) **0.007**	6 (40.0)%	2.3 (0.8-6.9) **0.104**
Single fetal demise ≥ 24weeks (number of fetuses per pregnancy)					
Dichorionic	2 (0.7%)	0 (0.0%)	- 1.000[a]	0 (0.0%)	- 1.000[a]
Gestational age at delivery					
All twins	35.6 (2.1)	35.0 (2.4)	-0.6 (-1.3 - -0.1) 0.109	34.5 (2.3)	-1.1 (-2.1 - -0.9) **0.034**
Monochorionic	35.2 (1.7)	33.9 (2.1)	- 1.2 (-2.4 - -0.08) **0.036**	33.0 (2.1)	-2.1 (-3.9 - -0.3) **0.021**
Dichorionic	35.7 (2.2)	35.3 (2.4)	-0.3 (-1.2 – 0.5) 0.434	34.9 (2.3)	-0.8 (-2.0 – 0.3) 0.169
Mean neonates birthweight					
All twins	2318 (472)	2074 (532)	-243 (-399 - -88) **0.002**	2002 (490)	-305 (-572 - -83) **0.007**
Monochorionic	2172 (398)	1801 (442)	-371 (-641 - -100) **0.008**	1694 (356)	-455 (-872 - -37) **0.033**
Dichorionic	2358 (483)	2162 (534)	-195 (-377 - -13) **0.036**	2084 (497)	-267 (-521 - -12) **0.040**
Birthweight discrepancy ≥ 25%					
All twins	22 (6.3%)	4 (9.8%)	1.6 (0.5-4.9) 0.336	1 (5.3%)	6.7 (0.6-68.4) 0.183
Monochorionic	4 (5.4%)	0 (0.0%)	- 1.000[a]	0 (0.0%)	-
Dichorionic	18 (6.5%)	4 (12.9%)	2.1 (0.6-6.7) 0.257	1 (6.7%)	6.8 (0.6-69.7) 0.184
Neonatal Death (number of cases per neonates)					
Dichorionic	5/550 (0.9%)	0/62 (0.0%)	- 1.000[a]	0 (0.0%)	- 1.000[a]
Perinatal Death (number of cases per fetus)					
Dichorionic	7/552 (1.2%)	0/62 (0.0%)	- 1.000[a]	0 (0.0%)	- 1.000[a]
Neonatal Care Unit admission ≥ 8 days (number of cases per surviving newborns)					
All twins	135/693 (19.5%)	28/82 (34.1%)	2.1 (1.2-3.4) **0.003**	16/38 (42.1%)	2.7 (1.4-5.3) **0.002**
Monochorionic	44/148 (29.7%)	12/20 (60.0%)	3.5 (1.3-9.6) **0.010**	6/8 (75.0%)	5.9 (1.2-43.9) **0.046**
Dichorionic	91/545 (16.7%)	16/62 (25.8%)	1.7 (0.9-3.1) 0.087	10/30 (33.3%)	2.6 (1.1-5.7) **0.023**
Neonatal morbidity (number of cases per surviving newborns)					
RDS	151/693 (21.8%)	30/82 (36.6%)	2.0 (1.2-3.3) **0.004**	14/38 (36.8%)	2.0 (1.0-3.9) **0.040**
HMD	71/693 (10.2%)	15/82 (18.3%)	1.9 (1.0-3.5) **0.039**	7/38 (18.4%)	1.8 (0.7-4.1) 0.264
IVH Grade III/IV	3/693 (0.4%)	0 (0.0%)	- 1.000[a]	0/38 (0.0%)	- 1.000[a]
Sepsis	14/693 (2.0%)	5/82 (6.1%)	3.1 (0.9-8.7) 0.082	4/38 (10.5%)	4.6 (1.3-13.76) **0.037**
ROP	7/693 (1.0%)	2/82 (2.4%)	2.4 (0.3-11.2) 0.489	2/38 (5.2%)	4.7 (0.6-20.6) 0.179

Abbreviations: $\hat{\beta}$ – linear regression coefficient estimate; BPD – Bronchopulmonary dysplasia; CI – confidence interval; DC – dichorionic; HMD – Hyaline membrane disease; IVH – Intraventricular hemorrhage; MC – Monochorionic; OR – odds ratio estimate; RDS – respiratory distress syndrome in newborn; ROP – Retinopathy of prematurity; SD – standard deviation; UtA-PI – Uterine artery pulsatility index.

TABLE 22 Univariable analysis of serum PAPP-A and perinatal outcomes in twin pregnancies.

Total n=466 MC=82, DC=384	**1st trimester PAPP-A MoM ≥10 ≤ 90th percentile, n=374 MC=60, DC=314**	**1st trimester PAPP-A MoM <10th percentile, n=46 MC= 14, DC=32**		**1st trimester PAPP-A MoM >90th percentile, n=46 MC=8, DC=38**	
	Mean (SD) or n (%)	Mean (SD) or n (%)	OR or $\hat{\beta}$= (95%CI) p-value	Mean (SD) or n (%)	OR or $\hat{\beta}$= (95%CI) p-value
Prenatal suspicion of FGR (one or both fetus per pregnancy)					
All twins	98 (26.2%)	15 (32.6%)	1.4 (0.7-2.8) 0.258	7 (15.2%)	0.4 (0.2-1.1) 0.085
Monochorionic	23 (38.3%)	4 (28.6%)	0.7 (0.2-2.6) 0.764	1 (12.5%)	0.2 (0.03-2.1) 0.254
Dichorionic	75 (23.8%)	11 (34.4%)	1.7 (0.8-3.7) 0.192	6 (15.8%)	0.6 (0.2-1.4) 0.214
Single fetal demise ≥ 24weeks (number of fetuses per pregnancy)					
Dichorionic	2 (0.6%)	0 (0.0%)	- 1.000[a]	0 (0.0%)	- 1.000[a]
Gestational age at delivery					
All twins	35.5 (2.3)	34.7 (2.6)	-0.8 (-1.5 - -0.1) **0.024**	36.4 (0.8)	1.0 (0.3-1.7) **0.004**
Monochorionic	34.8 (1.6)	35.0 (1.8)	0.01 (-1.1 - 1.2) 0.908	36.5 (0.5)	1.7 (0.2-3.1) **0.020**
Dichorionic	35.6 (2.3)	34.6 (2.5)	**-1.0 (-1.9 - -0.2) 0.018**	36.4 (0.8)	0.9 (0.1-1.7) **0.026**
Mean neonates birth weight					
All twins	2280 (481)	2074 (508)	-266 (-372 - -84) 0.002	2487 (343)	232 (86-378) **< 0.001**
Monochorionic	2115 (418)	2101 (468)	-70 (-324 - -184) 0.586	2529 (334)	416 (107-2210) **0.009**
Dichorionic	2312 (486)	2062 (531)	-268 (-443 - -93) **0.003**	2478 (348)	189 (27-352) **0.022**
Birth weight discrepancy ≥ 25%					
All twins	26 (6.9%)	2 (4.3%)	1.6 (0.4-7.7) 0.379	0 (0.0%)	- 0.629[a]
Monochorionic	5 (8.3%)	1 (7.1%)	5.1 (0.3-87.7) 0.314	0 (0.0%)	- 1.000[a]
Dichorionic	21 (6.7%)	1 (3.1%)	1.0 (0.1-8.7) 1.000	0 (0.0%)	- 0.611[a]
Neonatal Death (number of cases per neonates)					
Dichorionic	6/626 (0.9%)	1/64 (1.5%)	1.5 (0.0-12.5) 1.000[a]	1/76 (1.3%)	1.3 (0.0-10.3) 1.000
Perinatal Death (number of cases per fetus)					
Dichorionic	8/628 (1.3%)	1/64 (1.5%)	1.9 (0.04-15.5) 0.872	1/76 (1.3%)	1.0 (0.04-6.2) 1.000
Neonatal Care Unit admission ≥ 8 days (number of cases per surviving newborns)					
All twins	172/740 (23.2%)	30/91 (32.9%)	1.8 (1.1-2.9) **0.013**	3/91 (3.3%)	0.1 (0.02-0.3) **<0.001**
Monochorionic	27/120 (22.5%)	6/28 (21.4%)	1.1 (0.4-2.9) 0.850	0/16 (0.0%)	**- 0.045[a]**
Dichorionic	120/620 (19.3%)	24/63 (38.0%)	2.8 (1.6-4.9) **<0.001**	3/75 (4.0%)	0.1 (0.04-0.4) **<0.001**
Neonatal morbidity (number of cases per surviving newborns)					
RDS	192/740 (25.9%)	32/91 (35.1%)	1.6 (1.0-2.6) **0.003**	13/91 (14.3%)	0.5 (0.2-0.8) **0.011**
HMD	91/740 (12.3%)	18/91 (19.8%)	2.0 (1.1-3.5) **0.020**	0/91 (0.0%)	- **<0.001[a]**
IVH Grade III/IV	7/740 (0.9%)	0/91 (0.0%)	- 1.000[a]	0/91 (0.0%)	- 1.000[a]
Sepsis	29/740 (3.9%)	6/91 (6.6%)	1.9 (0.7-4.6) 0.170	0/91 (0.0%)	- **0.048[a]**
ROP	7/740 (0.9%)	2/91 (2.2%)	2.6 (0.3-14.1) 0.440	0/91 (0.0%)	- 0.781[a]

Abbreviations: $\hat{\beta}$ – linear regression coefficient estimate; BPD – Bronchopulmonary dysplasia; CI – confidence interval; DC – dichorionic; HMD – Hyaline membrane disease; IVH – Intraventricular haemorrhage; MC – Monochorionic; OR – odds ratio estimate; PAPP-A ¬ pregnancy-associated plasma protein-A; RDS – respiratory distress syndrome in newborn; ROP – Retinopathy of prematurity; SD – standard deviation;

TABLE 23 Univariable analysis of serum β-hCG MoM and perinatal outcomes in twin pregnancies.

Total n=466 MC=82, DC=384	1st trimester β-hCG MoM ≥10 ≤ 90th percentile, n=374 MC=58, DC=316	1st trimester β-hCG MoM <10th percentile, n=46 MC=13, DC=33		1st trimester β-hCG MoM >90th percentile, n=46 MC=11, DC=35	
	Mean (SD) or n (%)	Mean (SD) or n (%)	OR or $\hat{\beta}$= (95%CI) p-value	Mean (SD) or n (%)	OR or $\hat{\beta}$= (95%CI) p-value
Prenatal suspicion of FGR (one or both fetus per pregnancy)					
All twins	94 (25.1%)	14 (30.4%)	1.2 (0.6-2.5) 0.444	12 (26.1%)	1.0 (0.5-2.0) 0.956
Monochorionic	30 (34.5%)	4 (30.8%)	0.8 (0.2-2.9) 1.000	4 (36.4%)	1.1 (0.3-4.2) 1.000
Dichorionic	242 (23.4%)	10 (30.3%)	1.4 (0.6-3.1) 0.372	8 (22.9%)	0.9 (0.4-2.1) 0.873
Single fetal demise ≥ 24weeks (number of fetuses per pregnancy)					
Dichorionic	1 (0.3%)	1 (3.0%)	10.9 (0.7-179.0) 0.165	0 (0.0%)	0.9 (0.8-0.9) 1.000
Gestational age at delivery					
All twins	35.5 (2.3)	35.4 (2.6)	-0.03 (-0.7 – 0.6) 0.914	35.3 (2.2)	-0.2 (-0.8 – 0.5) 0.660
Monochorionic	34.8 (2.2)	35.3 (1.4)	0.4 (-0.7 – 1.6) 0.499	35.4 (0.8)	0.5 (-0.8 – 1.7) 0.476
Dichorionic	35.6 (2.3)	35.4 (3.0)	-0.1 (-0.9 – 0.7) 0.807	35.3 (2.5)	-0.3 (-1.1 – 0.5) 0.490
Mean neonates birth weight					
All twins	2286 (474)	2268 (540)	-13 (-161 – 134) 0.857	2242 (471)	-42 (-189 – 104) 0.568
Monochorionic	2152 (474)	2168 (394)	17 (-245 – 279) 0.897	2145 (241)	-10 (-292 – 271) 0.942
Dichorionic	2311 (471)	2308 (590)	1 (-175 – 178) 0.989	2272 (522)	-38 (-208 – 131) 0.654
Birthweight discrepancy ≥ 25%					
All twins	29 (7.8%)	3 (6.5%)	0.9 (0.3-3.0) 1.000	1 (2.2%)	0.2 (0.0-2.0) 0.233
Monochorionic	4 (6.9%)	2 (15.4%)	2.9 (0.5-18.1) 0.240	0 (0.0%)	- 1.000[a]
Dichorionic	25 (7.9%)	1 (3.0%)	0.4 (0.0-2.9) 0.494	1 (2.9%)	0.3 (0.0-2.7) 0.493
Neonatal Death (number of cases per neonates)					
Dichorionic	6/631 (0.9%)	2/65 (3.0%)	3.6 (0.3-21.0) 0.284	0/70 (0.0%)	- 0.925[a]
Perinatal Death (number of cases per fetus)					
Dichorionic	7/632 (1.1%)	3/66 (4.5%)	4.7 (0.8-21.4) 0.094	0/70 (0.0%)	- 0.461[a]
Neonatal Care Unit admission ≥ 8 days (number of cases per surviving newborns)					
All twins	160/741 (21.6%)	19/89 (21.3%)	0.9 (0.5-1.6) 0.832	26/92 (28.2%)	1.4 (0.8-2.3) 0.143
Monochorionic	39/116 (33.6%)	10/26 (38.4%)	1.1 (0.5-2.7) 0.718	9/22 (40.9%)	1.3 (0.5-3.3) 0.558
Dichorionic	121/625 (19.3%)	9/63 (14.3%)	0.6 (0.3-1.3) 0.285	17/70 (24.3%)	1.3 (0.7-2.4) 0.278
Neonatal morbidity (number of cases per surviving newborns)					
RDS	187/741 (25.2%)	20/89 (22.5%)	0.8 (0.5-1.4) 0.522	26/92 (28.2%)	1.1 (0.7-1.9) 0.486
HMD	84/741 (11.3%)	11/89 (12.3%)	1.0 (0.5-2.0) 0.868	14/92 (15.2%)	1.4 (0.7-2.5) 0.289
IVH Grade III/IV	3/741 (0.4%)	0/89 (0.0%)	- 0.980[a]	4/92 (4.3%)	12.4 (2.0-86.4) **0.005**
Sepsis	28/741 (3.7%)	3/89 (3.3%)	0.8 (0.2-2.6) 1.000	4/92 (4.3%)	1.1 (0.3-3.1) 0.938
ROP	9/741 (1.2%)	0/89 (0.0%)	- 0.798[a]	0/92 (0.0%)	- 0.773[a]

Abbreviations: $\hat{\beta}$ – linear regression coefficient estimate; BPD – Bronchopulmonary dysplasia; CI – confidence interval; DC – dichorionic; HMD – Hyaline membrane disease; IVH – Intraventricular haemorrhage; MC – Monochorionic; OR – odds ratio estimate; PAPP-A ¬ pregnancy-associated plasma protein-A; RDS – respiratory distress syndrome in newborn; ROP – Retinopathy of prematurity; SD – standard deviation; β-hCG – β-human chorionic gonadotropin;

4.4 ASSOCIATION BETWEEN HISTOPATHOLOGIC FINDINGS AND OBSTETRIC OUTCOMES IN TWINS

Placental histopathologic exams were performed in 49 TwPs, including 8 MC and 41 DC (Figure 18). Of these, 2 (4.1%) were PTB < 34 w, and 11 (22.4%) were PTB < 36 w. The pathologic placental findings per pregnancy (i.e., in one or both placentas per case) were as follows: accelerated maturation (AM) in 15 (30.6%), MVM lesions in 18 (36.7%), fetal vascular malperfusion (FVM) lesions in 9 (18.4%), chorioangiosis in 30 (61.2%), and inflammatory-immune processes most classified as villitis of unknow etiology (VUE) in 10 (20.4%)(Table 24).

Accelerated maturation was associated with PTB < 36 w, 15.8% vs 81.8%, OR 24.0 (95% CI: 4.1–139.9, p<0.001). Additionally, higher rates of FGR and SGA < 10th percentile were observed with this finding, although without reaching statistical significance: 25.0% vs 46.2%, OR 2.5 (95% CI: 0.7–9.6, p=0.178), and 25.7% vs 42.9%, OR 2.1 (95% CI: 0.6–7.9, p=0.309), respectively. MVM lesions were also more commonly found in cases of FGR and SGA, though without reaching statistical significance (Table 24).

No associations were found between HDP (including five cases of chronic hypertension, four GH, two PE, one HELLP syndrome) and the histopathologic findings (not presented in Table 24 due to small number of events).

TABLE 24 Placental histopathologic findings and obstetric outcomes in twin pregnancies.

n=49	**PTB < 36 weeks**			**FGR (one or both fetus)**			**SGA < 10th percentile (one or both fetus)**		
Histologic findings	*no* n=38 (77.6%)	*yes* n= 11 (22.4%%)	OR (95%CI) p-value	*no* n=36 (73.5%)	*yes* n=13 (26.5%)	OR (95%CI) p-value	*no* n=35 (71.4%)	*yes* n=14 (28.6%)	OR(95%CI) p-value
Accelerated maturation	6 (15.8%)	9 (81.8%)	24.0 (4.1-139.9) **<0.001**	9 (25.0%)	6 46.2%)	2.5 (0.7-9.6) 0.178	9 (25.7%)	6 (42.9%)	2.1 (0.6-7.9) 0.309
Maternal vascular malperfusion	15 (39.5%)	3 (27.3%)	0.5 (0.1-2.5) 0.724	11 (30.6%)	7 (53.8%)	2.6 (0.7-9.7) 0.184	12 (34.3%)	6 (42.9%)	1.4 (0.4-5.1) 0.572
Fetal vascular malperfusion	6 (15.8%)	3 (27.3%)	2.0 (0.4-9.7) 0.400	8 (22.2%)	1 (7.7%)	0.3 (0.0-2.6) 0.412	6 (17.1%)	3 (21.4%)	1.3 (0.3-6.2) 0.702
Chorioangiosis	24 (63.2%)	6 (54.5%)	0.7 (0.2-2.7) 0.729	22 (61.1%)	8 (61.5%)	1.0 (2.3-3.7) 0.978	21 (60.0%)	9 (64.3%)	1.2 (0.3-4.3) 0.781
Inflammatory-immune processes	9 (23.7%)	1 (9.1%)	0.3 (0.0-2.8) 0.419	7 (19.4%)	3 (23.1%)	1.2 (0.3-5.8) 1.000	7 (20.0%)	3 (21.4%)	1.0 (0.3-4.9) 1.000

Abbreviations: FGR – fetal growth restriction; OR – odds ratio estimate; PTB – Preterm Birth; SGA – Small for Gestational Age;

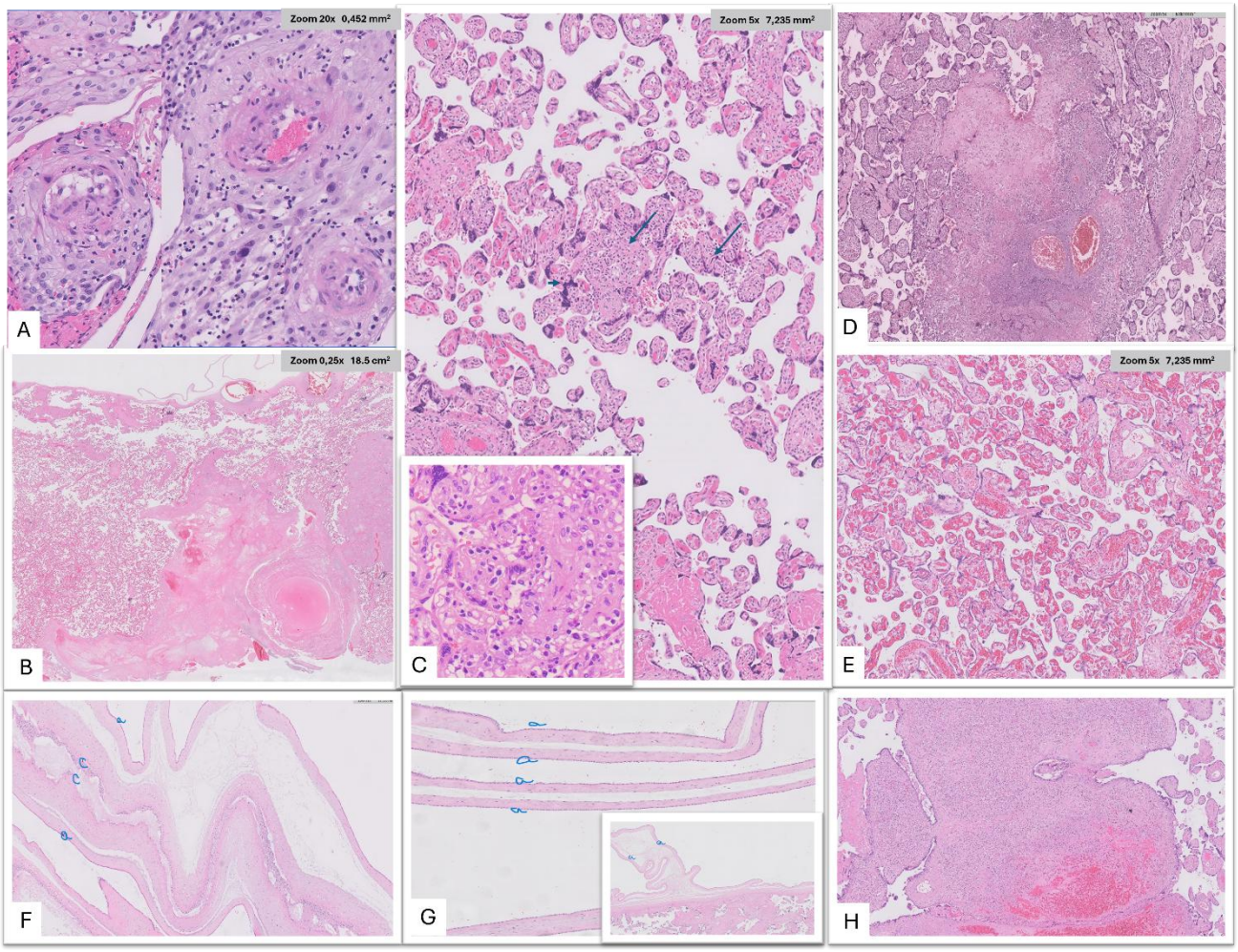

Figure 18 - Examples of placental microscopic findings in:

A and B, Case 52, delivery at 37 weeks, no FGR and no HDP, Dichorionic placenta, maternal vascular malperfusion combining decidual vasculopathy (A) and early infarction of villous tissue (B), the clot (bottom) is elevating the decidua basalis as an acute hematoma.;

C, Case 45, delivery at 36 weeks, one FGR with normal Dopplers, Monochorionic placenta, obvious acceleration of villous maturation and increased syncytial knots which appear enlarged and bulbous in shape (small arrow), and chronic villitis (long arrow, inset bottom);

D, E and H, Case 40, delivery at 37 weeks, no FGR and no HDP, Dichorionic placenta with fetal vascular malperfusion (stem vessels thrombosis and obliteration) and contiguous parenchymal early infarct (D), chorangiosis (E), and chorangioma (H);

F, Dichorionic dividing membranes with trophoblast remnants (*c*) observed between the fused membranes;

G, Monochorionic twin pregnancy with a 'T-shaped' section of dividing membranes (inset bottom) reveals only two single layers of cuboidal epithelial cells (amnio, *a*) and scant connective tissue, lacking blood vessels and trophoblast remnants.

CHAPTER 5 – DISCUSSION

5.1 INTERPRETATION OF MAIN FINDINGS

5.1.1 Principal findings

- **Differences between uterine artery dopplers in Twins vs Singleton Pregnancies**

This study demonstrated that first-trimester UtA-PI in TwPs significantly differs from that in singletons, showing lower resistance when compared to a control group matched for baseline maternal and pregnancy characteristics. Additionally, the 95th empirical percentile obtained for MC was similar to that of singletons, although larger data is needed for more robust conclusions.

- **Obstetric outcomes in Twin Pregnancies**

In this cohort of TwPs, a high rate of PTB (< 36 w) and SGA (in one or both neonates <10th percentile) was observed, affecting about one-third of the cases. HDP was a less frequent complication, occurring in approximately 14% of cases, with a low incidence of more severe forms, such as early-onset PE or HELLP syndrome, occurring in less than 1% of cases. Notably, PTB had a strong association with FGR, and/or SGA, particularly before 32 weeks. Almost all cases (around 90%) of iatrogenic PTB < 34 w were indicated due to FGR, which accounted for nearly half of the preterm deliveries under this gestational age. Overall, one fifth of TwPs were affected by PTB <36 weeks associated to FGR, SGA and/or HDP and iatrogenic PTB (< 36 w) was indicated in 16.3% of the cases.

In contrast, extreme prematurity (< 28 w) was uncommon in this cohort, around 1%, and second-trimester short CL (≤ 25 mm) was found to be associated with this outcome.

- **Association of maternal and pregnancy characteristics, uterine artery Dopplers, serum biomarkers and obstetric and neonatal outcomes**

In this study, both higher first- and second-trimester UtA Doppler measurements and low first-trimester serum PAPP-A levels were associated with obstetric adverse outcomes related to “poor placental function.” Additionally, the most common neonatal morbidities were significantly more frequent in these cases. However, the performance of an isolated marker is inadequate for detecting PTB concurrent with FGR, SGA, and/or HDP. For instance, both first-trimester UtA-PI ≥ 95th percentile and PAPP-A < 10th percentile identified only one-quarter of cases with this outcome before 32 weeks. Nevertheless, almost half of the women with higher first-trimester UtA-PI experienced PTB < 36 w concurrent with SGA < 3rd percentile. Differences were observed based on chorionicity concerning the association between high UtA-PI and SGA. In MC

pregnancies, there is a higher likelihood that one or both neonates will be SGA, whereas, in DC, it is more likely that only one fetus will be affected.

Notably, using a first trimester UtA-PI cut-off of ≥ 90th percentile (adjusted for TwPs) detected 100% of early-onset PE cases, an infrequent but highly morbid event for both mothers and infants. Other forms of HDP were not associated with higher resistance UtA Dopplers.

Multivariable analysis models for the different outcomes incorporated both UtA-PI and PAPP-A as risk factors, with UtA-PI ≥95th percentile having a stronger impact, improving the performance of almost all models except those related to HDP (excluding early-onset PE). Some baseline maternal and pregnancy characteristics, long recognized as risk factors for adverse obstetric outcomes, were also identified and contributed to improving the multivariable association models. Notably, maternal height significantly impacted the outcomes of PTB and SGA.

The best-performing LR models associated with poor obstetric outcomes related to placental function were observed when PTB occurred before 32 weeks. The model incorporating second-trimester UtA-PI detected nearly all affected cases with an acceptable false-positive rate. However, statistical internal validation of the prediction was not possible due to the small sample size. The model using only first-trimester data achieved slightly lower but still good performance, with the potential benefit of early detection of cases.

- **Histopathologic findings**

In this small sample, accelerated maturation was associated with PTB, and higher rates of FGR and SGA were observed with this finding and with MVM lesions. Additionally, a high rate of chorioangiosis was found in more than half of the cases, though no association with adverse outcomes was identified.

5.1.2 Explanations and comparison with existing literature

Twin pregnancies are considered high-risk due to an increased risk of obstetric complications, including FGR and HDP. These conditions are often associated with poor placental perfusion, especially when diagnosed before term. While this association has long been recognized in SP, it was only highlighted in twin pregnancies when TwPs' specific birth weight charts were used in data analysis [46].

In singletons, high resistance of UtA Doppler has long been considered as an important risk factor for adverse outcomes related to “poor placental perfusion” [48,49]. The resistance of the uterine arteries depends on placental mass and gradually decreases throughout pregnancy [89]. In TwPs, the placental mass is increased, so the Doppler measurements at each gestational age typically show lower values [54,87]. Similar to previous studies, we also demonstrated that TwPs have significantly lower UtA resistance compared to SP. However, we additionally found that UtA resistance is even lower in DC compared to MC pregnancies. This finding challenges previous beliefs from small sample studies in the first and second trimesters, which suggested that UtA Doppler values were similar in MC and DC pregnancies [87,90,91]. Some earlier studies on second-trimester uterine artery Dopplers in TwPs have shown conflicting results, with one study aligning more closely with our findings [92]. Consequently, we recommend that the reference ranges for UtA-PI be adjusted for TwPs and also for chorionicity.

The association of high-resistance UtA Doppler with early-onset PE and FGR in TwPs has been previously demonstrated, although the predictive value of this finding alone is poor. However, it improves when using TwPs-specific nomograms instead of SP references [54, 87]. Rizzo et al. described higher UtA-PI in pregnancies that developed SGA in both twins (without subgroup analysis between MC and DC) [87]. Conversely, we found higher rates for one or both neonates in MC pregnancies (though without statistical significance) and only for one SGA in DC. Differences in placental mass and fetal genetic factors may explain these discrepancies according to chorionicity.

Additionally, our study achieved a 100% detection rate for early-onset PE after adjusting first-trimester UtA-PI for chorionicity and gestational age, and using the 90th percentile as a cut-off. However, our findings may differ in larger datasets. Notably, the incidence of early-onset PE in our cohort was as low as 0.87%, which is similar to another larger dataset that observed 0.94% [50]. Additionally, late-onset PE, GH, and HELLP syndrome were not associated with high UtA-PI, which is consistent with findings from other studies [87]. In the case of HELLP syndrome, other underlying physiological mechanisms, such as immunologic and genetic factors, may contribute to the syndrome, and some cases present it clinically without hypertension [93]. However, the low incidence of these conditions makes it challenging to draw more definitive conclusions [94].

Regarding first trimester serum biomarkers, although this study found that women with low PAPP-A levels had an increased risk for adverse outcomes, this marker alone has limited predictive value for identifying most at-risk cases. Our findings are consistent with the conclusions of a systematic review and meta-analysis conducted in SP [85]. Conversely, a high PAPP-A measurement (> 90th percentile) excluded all cases of PTB < 35 w, whether spontaneous or iatrogenic, a finding not previously described in the literature. This may reflect a larger placental mass and improved trophoblastic function in those pregnancies, facilitating adequate fetal growth and prolonging gestation. PAPP-A plays a crucial role in pregnancy by regulating placental function, fetal growth, and placental development. Its biological mechanism involves the modulation of insulin-like growth factor activity, which influences various aspects of pregnancy and fetal development. Maternal concentrations reflect the placental volume and likely the amount of trophoblastic tissue [95].

Additionally, low levels of β-hCG were associated with GH, a finding that is not typically emphasized in clinical practice. Contradictory results were found by Euser AG et al., who reported a reduction in PE in a subgroup of TwPs with elevated hCG levels [96]. In our cohort, no women with high β-hCG developed early-onset PE or GH, suggesting that these women may not benefit from ldASA prophylaxis. In SP resulting from ART, low hCG concentrations in very early pregnancy have been associated with an increased risk of severe PE, but findings have been inconsistent across different studies [97, 98]. Low maternal serum concentrations of hCG early in pregnancy may indicate impaired trophoblast cell proliferation or invasion, potentially serving as a marker for poor placental development. However, there is no clear evidence to support the use of this biomarker to predict HDP in clinical practice.

Besides β-hCG and PAPP-A, there are many other candidate placental products to be employed as surrogates of placental function. A systematic review of early pregnancy biomarkers in PE found low predictive values using individual biomarkers which included a disintegrin and metalloprotease 12 (ADAM-12), inhibin-A, PAPP-A, PlGF and placental protein 13 (PP-13) [99]. PlGF and PAPP-A are the two most commonly used markers for the Fetal Medicine Foundation's first-trimester screening, with PlGF being more effective at improving predictive models. Unfortunately, in our center, PlGF is not routinely measured during first-trimester screening, so it could not be included in this study.

In SP, first trimester screening for early-onset PE and FGR is recommended in clinical practice, although the latter exhibits poorer performance [48,49]. Similar first-trimester screening for PE have been extended to TwPs, though conclusions about their effectiveness remain less robust [50, 100]. In those studies, UtA-PI > 95th percentile was associated with a 10 to 14-fold increase in risk of PE, and the combination of UtA-PI MoM, MAP and maternal factors performed well in predicting PE < 32 w' gestation. The authors recognized the need for better-fitted models using competing risks, and concluded that if the desired detection rate of preterm PE is 75%, then the risk cut-off and consequent false positive rate in TwPs would be about 1 in 15 and 40%, respectively [50]. Since early-onset PE had a low incidence compared to FGR, and we achieved a 100% detection rate for early-onset PE using the 90th percentile of UtA-PI for TwPs, we chose to develop a multivariable model for placental dysfunction-related conditions (FGR, SGA, and HDP) associated with PTB. Comprehensively, most events in these groups involved FGR and/or SGA. We also chose to analyze the outcome of one or both SGA per pregnancy together to simplify the statistical analysis. The AUCs obtained for detection of PTB < 32 w demonstrated good performance, with an acceptable false positive rate up to 20%. However, due to the small sample size, we cannot fully validate the predictive model.

The authors recognize that predictive models for low-incidence events like early-onset PE are particularly challenging in TwPs. PE is a complex condition caused by various factors, processes, and pathways [101]. Some maternal and pregnancy conditions are usually recognized as risk factors for poor obstetric outcomes. For example, overweight and obesity are associated with higher odds of Gestational Diabetes and HDP and consequent adverse maternal, fetal and neonatal outcomes [102,103]. Additionally, ART was found to be an independent risk factor for PTB and HDP in TwPs [22,104]. Also, MC pregnancies are at higher risk for PTB, FGR, HDP, and fetal demise. However, the exclusion of TTTS and fetal malformations in this study might explain why no perinatal deaths occurred in this subgroup [42,29]. Finaly, fetal sex can also play a role in modifying the odds of PTB or HDP in TwPs, as previous demonstrated but not explored in the present study [105,106]. However, in our small cohort with a limited number of events, not all maternal and pregnancy risk factors reached statistical significance and could be included in the multivariable analysis.

In TwPs, predictive models for FGR are lacking, despite the greater impact of this condition on these pregnancies. A primary obstacle is the lack of consensus among experts on the definition of growth restriction, the birth weight references used (twins vs. singletons), and the exclusion criteria applied in different studies [32]. In our study,

we excluded velamentous cord insertions due to their frequent association with FGR and adverse perinatal outcomes, particularly in MC twins [107, 108]. Velamentous cord insertions are anatomical abnormalities that lead to unequal placental sharing and underperfusion of the smaller twin, which is etiologically unrelated to impaired UtA perfusion. This exclusion might explain the similar rate of SGA <3rd percentile in both MC and DC twins in our dataset. Ignoring umbilical cord abnormalities can reduce the performance of predictive models for outcomes related to placental dysfunction.

In singletons, first trimester screening for SGA < 3rd percentile and PTB < 32 w, using a predictive competing-risks model with maternal factors, MAP, PAPP-A and UtA-PI, achieved moderate performance. In this large dataset (57,131 SP), the estimated AUC was 0.819, with a detection rate of 55% and 72% at false positive rates of 10% and 20%, respectively [51]. In the present study, the multivariable LR association model for SGA < 3rd percentile born prematurely < 32 w achieved a 77% detection rate with a 17% false positive rate. Since the etiology of FGR can vary, including factors such as maternal immune diseases and infections, a model that focuses solely on placental origin, like the one used in this study, may have limited performance. Indeed, of the four cases not detected by the first-trimester model, two had documented maternal autoimmune conditions, while another presented with a short cervix and experienced spontaneous PTB associated with histologic findings of chorioamnionitis and neonatal early-onset sepsis. Maternal height was also found to be an important risk factor for all PTB and for SGA < 3rd percentile concurrent with PTB [31,109].

Morover, it is important to remember that clinical interventions can introduce bias in observational studies, such as the administration of ldASA for the prevention of preterm PE. In SP, ldASA prophylaxis for high-risk patients is well-established [48]. The reduction in the risk of early-onset PE is accompanied by a decrease in the risk of PTB and FGR. In the ASPRE trial, use of ldASA reduced the overall incidence of SGA < 10th percentile by about 40% in newborns at < 37 w' gestation and by about 70% in newborns at < 32 weeks [49].

Some societies recommend, by consensus, the use of aspirin to reduce PE in TwPs. For example, the American College of Obstetricians and Gynecologists (ACOG) recommends the use of ldASA in all TwP due to the higher risk for hypertensive disorders [71]. In contrast, the National Institute for Health and Care Excellence (NICE) guidelines suggest ldASA if two moderate risks are present; for example, all nulliparous women with a twin gestation should take aspirin [72]. In the latter scenario, about 73% (all

nulliparas and multiparas with risk factors) of our patients in this cohort would be prescribed medication. At our center, we opted to prescribe ldASA exclusively to women with significant risk conditions, often regardless of UtA-PI values. This approach led to a lower rate of prophylaxis selection (12.8%) among women, and we cannot precisely estimate the potential impact on reducing adverse outcomes with this strategy. However, in our cohort, the incidence of SGA (< 5th percentile) was doubled among women who received ldASA prophylaxis. Conversely, the majority of women who experienced PTB < 34 w associated with FGR and/or HDP were not on aspirin prophylaxis. Moreover, most higher-risk women (70.6%), as identified by the better fitted LR model for placental-related adverse outcomes (PTB < 32 w concurrent with FGR, SGA, and/or HDP), were not receiving aspirin prophylaxis.

Regarding histopathologic findings, accelerated villous maturation is interpreted as a compensatory change due to MVM and is often diagnosed in placentas prior to term and may be found in mild, moderate, or severe forms of placental insufficiency, which includes FGR, PE, and PTB [110]. It´s recognized that TwPs experience accelerated placental maturation compared to SP. This accelerated maturation can be interpreted as an adaptive response to the increased demands of twin gestations. MVM lesions develop because of abnormal spiral artery flow and can result in accelerated villous maturation or villous infarcts, which are areas of ischemic necrosis caused by occluded spiral arteries [79].

Additionally, higher rates of chorioangiosis, characterized by capilar proliferation in villous stroma, were more frequently found in TwPs [111]. The pathogenesis of diffuse chorioangiosis is thought to result not from poor uteroplacental perfusion, but rather from preuterine factors that precondition the placenta by increasing resistance to ischemia-reperfusion injury during labor [111]. Villous hypervascularity in TwPs may thus represent an adaptive change, similar to that seen in pregnancies at high altitudes and in cases of FGR.

Our histopathologic findings are consistent with these explanations, but the small sample size does not allow us to draw more definitive conclusions.

5.2 CLINICAL AND RESEARCH IMPLICATIONS

5.2.1 Recommendations for clinical practice

In the era of non-invasive prenatal testing (NIPT), the importance of first-trimester screening, which combines ultrasound with biochemical and biophysical markers, remains paramount in prenatal care. Along with screening for aneuploidies, first-trimester combined screening offers the benefits of identifying pregnancies at increased risk of PE, spontaneous PTB, and FGR, this approach can be extended to TwPs, although with less conclusive evidence regarding its effectiveness. TwPs have higher odds of poor obstetric outcomes, therefore, frequent surveillance is recommended to ensure early detection of maternal and fetal complications.

Uterine artery Dopplers should be evaluated in TwPs, but clinicians must be aware that Doppler reference values need to be adjusted for this type of pregnancy. Failure to do so may lead to misinterpretation of normal findings, potentially neglecting patients at higher risk.

We recommend administering ldASA prophylaxis to patients with TwPs who have clinical conditions predisposing them to HDP, regardless of their first-trimester UtA-PI values. Conversely, those without identified risk factors — including multiparous women with no previous history of HDP or early-onset FGR, and those with low-resistance UtA Dopplers (adjusted for TwPs) — should generally not be candidates for ldASA prophylaxis. For women with a higher UtA-PI (≥ 90th percentile), there is an increased risk of preterm PE, and ldASA prophylaxis should be considered and discussed with the patient.

It remains unclear whether women with TwPs and low PAPP-A or low PlGF will benefit from ldASA prophylaxis for the prevention of FGR, as there are no established guidelines for this outcome in this population. In such cases, laboratory results should be discussed with the patient, and ldASA may be prescribed if there are no contraindications and both the clinician and patient agree on this approach. Higher levels of PAPP-A can be very reassuring for women and clinicians, as the likelihood of PTB < 35 w was found to be zero in those with elevated levels. However, this applies to only 10% of our population, and these findings should be validated in larger datasets.

Women of shorter stature undergoing fertility treatments should be informed about the increased risks of PTB and SGA associated with transferring two embryos, given the higher likelihood of a TwP. Although elective single embryo transfer is widely recognized

as the optimal approach, double embryo transfer remains common in countries such as Portugal. Our study aims to provide insights to better guide counselling for couples and ART professionals in current practice. However, no clinical cut-off for maternal height has been validated, and individualized, population-based evidence should be taken into consideration [109, 112].

Of equal importance, maternal conditions and modifiable lifestyle factors, such as smoking habits, nutritional support, and obesity prevention and treatment, should be thoroughly discussed with patients. Providing specialized support in these areas is crucial for improving both maternal and fetal outcomes.

Finally, placental pathology can be useful in identifying unsuspected conditions, such as chronic villitis, that are likely to recur in subsequent pregnancies and in uncovering the underlying causes of unexpected adverse outcomes. TwPs have a higher rate of morbidity related to this specific condition; therefore, recurrence in a subsequent SP is less likely to occur.

5.2.2 Recommendations for clinical research

Although twin pregnancies carry increased risks for adverse obstetric and neonatal outcomes, they are often underrepresented in clinical evidence due to their lower occurrence and frequent exclusion from clinical trials. Therefore, a collective effort through multicenter studies is essential to provide timely answers to practical questions regarding optimal clinical management, including monitoring, prevention, and treatment strategies.

In 2022, there were 1,272 twin deliveries in Portugal [16]. With strong collaboration, effective organization between institutions, and appropriate funding, it should be possible to conduct clinical trials in our country with an adequate sample size to address some of the controversies surrounding the management of TwPs within three to four years. To achieve this, it is essential to provide clinicians in Portugal with the necessary conditions, including training and dedicated time to meet the demands of conducting research.

In this study, we highlighted the significance of maternal height in the risk of PTB and SGA, underscoring its importance as a factor to be examined in future research. Additionally, some of our findings are novel and should be validated with larger datasets if deemed relevant.

We would like to emphasize and recommend that UtA Doppler measurements in TwPs be adjusted for chorionicity and other maternal conditions. These adjusted references should be used in predictive models with larger datasets and made available to the medical community to ensure more accurate interpretation of the findings. We also recommend using specific birth weight charts for classifying SGA, as the association with poor outcomes appears to be stronger when this adjustment is applied, as outlined in our methodology.

To clarify the use of aspirin for the prevention of PE in TwPs, a multicentric clinical trial (ASPRE-T) funded by the Fetal Medicine Foundation (UK) is currently underway [74]. While the primary outcome of this trial is the incidence of PE requiring delivery before 37 w in TwPs, secondary outcomes include iatrogenic PTB due to PE, GH, or FGR. Given that FGR leading to PTB is more common than early-onset PE, its potential impact on the trial's outcomes could be clinically more significant.

It would also be valuable to explore the use of other medications for the prevention or early treatment of FGR, such as LMWH. In SP, combining LMWH with ldASA has proven to be more effective than ldASA alone, reducing not only the incidence of early-onset PE but also FGR. Considering that FGR is more common in TwPs, this combined approach could potentially offer greater benefits.

5.3 STRENGTHS AND LIMITATIONS

This study was conducted at one of the Portuguese institutions with the most experience in monitoring TwPs. The professionals involved are well-versed in diagnosing and managing major obstetric complications in TwPs, as well as in providing care for extremely premature neonates. The pregnant women were monitored by two obstetricians specialized in TwPs, following uniform clinical practices. The clinical information was entered prospectively into the database by the obstetricians who managed the pregnancies, ensuring the reliability of the data.

Regarding the first trimester Doppler and serum biomarker data, this study is primarily retrospective. Unfortunately, we do not have a biobank, which would have allowed for the measurement of PlGF in the first trimester, a marker that has a slightly better performance than PAPP-A in predicting early-onset PE. Another limitation of our study is the fact that many pregnancies are only followed at our institution after 14 weeks, which makes it impossible to carry out first-trimester screening. Additionally, in the later participants, UtA Dopplers were not systematically measured, as it was not

routine practice in TwPs for all ultrasound operators. As a result, the sample size was reduced, limiting the statistical analysis.

Due to the small sample size, we chose not to convert Doppler values to MoM, which is commonly applied in first-trimester screening for HDP. Furthermore, as of now, the software ASTRAIA® used in our center does not specifically adjust UtA Doppler for TwPs. Although we could have calculated MoM based on chorionicity, the small dataset did not support adjustments for other maternal characteristics, which is a complex analysis. We conducted an exploratory analysis using first trimester unadjusted UtA MoM from our cohort but, the multivariable analysis did not show improved performance compared to the UtA-PI ≥ 95th percentile (unshown data). Despite these limitations, significant differences were observed in women with high-resistance UtA Dopplers regarding common adverse pregnancy and neonatal outcomes in TwPs.

Finally, placental histopathologic reports from the oldest participants were excluded due to insufficient and different classification criteria, and in many cases, placental specimens were not available. Funding for histopathologic exams was only secured in the last year of recruitment, which limited the sample size. As a result, only a very limited analysis was included in the present study, limiting the ability to fully document the underlying causes of PTB, FGR and HDP, with diagnostic hypotheses based mainly on clinical data.

CHAPTER 6 – CONCLUSIONS

This study aimed to demonstrate the impact of placental function on TwPs. The methodology employed sought to eliminate confounding factors unrelated to placental function that could contribute to adverse outcomes. However, the assessment of placental function was indirectly inferred through UtA Dopplers and first-trimester biomarkers, which serve as surrogate markers during early pregnancy. Additionally, the absence of histopathological exams in the majority of cases limits the ability to draw more robust conclusions.

In SP, it is well-established that deficient trophoblast implantation and consequent poor placental perfusion is the most common etiology of FGR and early-onset PE. In TwPs, the same mechanism is likely at play, but with the added challenge of supporting two fetuses, which significantly increases the demand on the uterus and placentas. This hypothesis supports the higher rates of these complications observed in TwPs, as well as the findings in this study. Similar results have been reported in other studies, although methodologies may differ.

Early detection of cases at risk for placental dysfunction and the institution of ldASA prophylaxis is now a widely accepted practice in SP within the medical community. However, in TwPs, guidelines and clinical practices vary, with no consensus or uniformity in the approach.

We highlight the need for specific adjustments in clinical research for TwPs, including the establishment of UtA Doppler reference values based on larger datasets, the use of twin-specific birth weight charts, and consideration of maternal and pregnancy characteristics, which may have a greater impact in TwPs. Without these adjustments, clinical interpretation and prediction models that are not tailored for twins could be misleading and ineffective, potentially leading to inadequate clinical practices.

As clinicians await more robust evidence and updated guidelines on the use of ldASA in twins, decisions in this area must carefully weigh the risks and benefits of initiating drug prophylaxis. We believe that first-trimester UtA Dopplers should be included in clinical guidelines alongside maternal risk factors. Unless other significant risk factors for HDP are present, women with low UtA-PI and high PAPP-A levels are unlikely to benefit from ldASA prophylaxis, suggesting that current guidelines should be revisited. Nevertheless, close monitoring of all TwPs for HDP and FGR remains essential.

Finally, there is hope for reducing the morbidity associated with TwPs through the introduction of additional pharmacological prophylaxis that could optimize fetal growth

and decrease the incidence of PTB linked to this condition. Also, collaborative efforts in clinical research on TwPs should be encouraged to achieve a better level of evidence for this high-risk pregnancy.

CHAPTER 7 – REFERENCES

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CHAPTER 8 – APPENDICES

8.1 Abstract of the author's studies in the context of this thesis

Simões T, Pereira I, Gomes L, Brás S, Nogueira I, Queirós A. Higher risk of preterm twin delivery among shorter nulliparous women. J Gynecol Obstet Hum Reprod. 2024 Jan;53(1):102694. doi: 10.1016/j.jogoh.2023.102694. Epub 2023 Nov 21. PMID: 37992965.

Objective: To determine if maternal height in nulliparous women influences pregnancy results in twin pregnancies.

Material and methods: Retrospective cohort analysis evaluating twin pregnancies followed at Centro Hospitalar Universitário Lisboa Central, between 1995 and 2020. Of the 2900 pregnancies followed in that period, 886 nulliparous women with dichorionic twin pregnancies were selected. Two groups were considered: A - maternal height <163 cm (<Q2) (n = 436) and B - maternal height ≥167 cm (≥Q3) (n = 234), The following results were compared: Age, body mass index (BMI), premature contractions, premature rupture of membranes (PROM), hypertensive disorders, gestational diabetes, gestational age at birth, delivery <28, <32, <34, < 36, ≥37 weeks (wks), average weight of newborns, very low birth weight, low birth weight, cesarean section rate, stillbirths, five minute Apgar score, neonatal death and perinatal death.

Result(s): PTB rates decreased along increasing maternal height. The comparison between group A and group B revealed no statistically significant differences in maternal characteristics (age, mode of conception - spontaneous or ART pregnancies, or BMI). Statistically significant differences were found in mean gestational age at birth (35.1 ± 1.8 vs. 36.0 ± 2.6 wks), PTB rates < 32, 34 and 36 wks, OR: 3.2, 2.3 and 2.4 respectively, p < 0.01. Shorter women had a 1.7× and 2.6× increased risk for significantly low (<2500 g) and very low (<1500 g) newborn birth weight (BW), respectively, and a 40 % increased risk of Cesarian delivery. No significant differences were shown with respect to stillbirths, neonatal and perinatal deaths, which had a low incidence in this study. In ART pregnancies we found the same results regarding PTB rates and newborn birthweight in shorter women. In Logistic Regression analysis, maternal height <Q2 is an independent risk factor for PTB under 32, 34 and 36 wks, adjusted OR: 2.0. 2.2 and 2.4, respectively, 95 % CI 1.1-3.7, p = 0.021.

Conclusion: Increased pregnancy risk in nulliparous shorter women should be taken into consideration in double embryo transfers.

Keywords: Art; Dichorionic twins; Maternal height; Nulliparous women; Perinatal outcomes.

Queirós A, Gomes L, Pereira I, Charepe N, Plancha M, Rodrigues S, Cohen Á, Alves M, Papoila AL, Simões T. First-trimester serum biomarkers in twin pregnancies and adverse obstetric outcomes-a single center cohort study. Arch Gynecol Obstet. 2024 Jul;310(1):315-325. doi: 10.1007/s00404-024-07547-6. Epub 2024 May 12. PMID: 38734998; PMCID: PMC11169060.

Purpose: This study aimed to determine the association of first-trimester maternal serum biomarkers with preterm birth (PTB), fetal growth restriction (FGR) and hypertensive disorders of pregnancy (HDP) in twin pregnancies.

Methods: This is a retrospective cohort study of twin pregnancies followed at Maternidade Dr. Alfredo da Costa, Lisbon, Portugal, between January 2010 and December 2022. We included women who completed first-trimester screening in our unit and had ongoing pregnancies with two live fetuses, and delivered after 24 weeks. Maternal characteristics, pregnancy-associated plasma protein-A (PAPP-A) and β-human chorionic gonadotropin (β-hCG) levels were analyzed for different outcomes: small for gestational age (SGA), gestational hypertension (GH), early and late-onset pre-eclampsia (PE), as well as the composite outcome of PTB associated with FGR

and/or HDP. Univariable, multivariable logistic regression analyses and receiver-operating characteristic curve were used.

Results: 466 twin pregnancies met the inclusion criteria. Overall, 185 (39.7%) pregnancies were affected by SGA < 5th percentile and/or HDP. PAPP-A demonstrated a linear association with gestational age at birth and mean birth weight. PAPP-A proved to be an independent risk factor for SGA and PTB (< 34 and < 36 weeks) related to FGR and/or HDP. None of the women with PAPP-A MoM > 90th percentile developed early-onset PE or PTB < 34 weeks.

Conclusion: A high serum PAPP-A (> 90th percentile) ruled out early-onset PE and PTB < 34 weeks. Unless other major risk factors for hypertensive disorders are present, these women should not be considered candidates for aspirin prophylaxis. Nevertheless, close monitoring of all TwP for adverse obstetric outcomes is still recommended.

Keywords: Fetal growth restriction; First trimester screening; Hypertensive disorders; Preterm birth; Serum biomarkers; Twin pregnancies.

Queirós A, Domingues S, Gomes L, Pereira I, Brito M, Cohen Á, Alves M, Papoila AL, Simões T. First-trimester uterine artery Doppler and hypertensive disorders in twin pregnancies: Use of twin versus singleton references. Int J Gynaecol Obstet. 2024 May 27. doi: 10.1002/ijgo.15706. Epub ahead of print. PMID: 38800867.

Objective: To determine the association of first-trimester uterine artery Doppler with hypertensive disorders of pregnancy in twin pregnancies.

Methods: This was a retrospective cohort study of twin pregnancies followed at the University Hospital Center of Central Lisbon, Portugal, between January 2010 and December 2022. First-trimester uterine artery pulsatility index (UtA-PI) was determined and compared between twin pregnancies (n = 454) and singleton pregnancies (n = 908), matched to maternal and pregnancy characteristics. Maternal characteristics and mean UtA-PI were analyzed for gestational age, birth weight, gestational hypertension, early- and late-onset pre-eclampsia, HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome, and preterm birth. Univariable and multivariable logistic regression models were used.

Results: The mean first-trimester UtA-PI was significantly lower in dichorionic twins than in singletons (P < 0.001). To study hypertensive disorders of pregnancy in twins, 390 pregnancies were included: 311 (79.7%) dichorionic and 79 (20.3%) monochorionic twins. The observed rates of early- and late-onset pre-eclampsia, gestational hypertension, and HELLP syndrome were 1.0%, 4.4%, 7.4%, and 1.5%, respectively. We achieved a 100% detection rate for early-onset pre-eclampsia using the UtA-PI 90th centile for twins. However, when singleton references were considered, the detection rate decreased to 50%. UtA-PI at or above the 95th centile was associated with increased odds for preterm birth before 32 weeks (adjusted odds ratio 4.1, 95% confidence interval 1.0-16.7, P = 0.043).

Conclusions: Unless other major risk factors for hypertensive disorders are present, women with low UtA-PI will probably not benefit from aspirin prophylaxis. Close monitoring of all twin pregnancies for hypertensive disorders is still recommended.

Keywords: HELLP syndrome; aspirin prophylaxis; first-trimester screening; hypertensive disorders; preterm birth; pre-eclampsia; twin pregnancies; uterine artery Doppler.

Queirós A, Bernardo A, Rijo C, Carocha A, Ferreira L, Martins AT, Cohen Á, Alves M, Papoila AL, Simões T. First-trimester screening and small for gestational age in twin pregnancies: a single center cohort study. Arch Gynecol Obstet. 2024 Dec 26. doi: 10.1007/s00404-024-07884-6.

Objective: This study aimed to investigate the association between maternal factors and first-trimester biophysical and biochemical markers with small for gestational age (SGA) neonates in twin pregnancies (TwPs).

Methods: Single-center retrospective cohort study of TwPs followed from January 2010 to December 2022 at a tertiary perinatal center, Portugal. Maternal and pregnancy characteristics, mean arterial pressure, pregnancy-associated plasma protein-A (PAPP-A), β-human chorionic gonadotropin (β-HCG), and uterine artery pulsatility index (UtA-PI) were analyzed. Univariable, multivariable logistic regression (LR) and receiver-operating characteristic curve analyses were performed. The main outcome measures considered were: SGA < 3rd, < 5th and < 10th percentile, the composite outcome of SGA combined with preterm birth (PTB) (< 32, < 34, and < 36 weeks).

Results: 572 TwPs were included, 450 (78.7%) DC and 122 (21.3%) MC. TwPs affected with SGA < 3rd, < 5th or < 10th percentiles were 120/572 (20.9%), 157/572 (27.4%) and 190/572 (33.2%), respectively. SGA < 3rd percentile was associated with a higher rate of PTB, 59.0% of cases < 32 weeks, OR 6.4 (95% CI: 3.2-12.7, p < 0.001). Shorter maternal height, UtA-PI ≥ 95th percentile, and low PAPP-A were identified as significant independent risk factors associated with SGA and SGA combined with PTB. The best LR model was obtained for the composite outcome SGA < 3rd percentile and PTB < 32 weeks, with an AUC of 0.834, a sensitivity rate of 77%, and a false positive rate of 17%.

Conclusion: The majority of pregnancies at risk for SGA combined with prematurity can be detected in the first trimester. However, larger datasets are necessary to develop robust predictive models.

Keywords: Aspirin prophylaxis; Fetal growth restriction; First-trimester screening; Preterm birth; Small for gestational age; Twin pregnancies.

8.2 Ethical and institutional approvals

MEDICAL SCHOOL FACULDADE DE CIÊNCIAS MÉDICAS

UNIVERSIDADE NOVA DE LISBOA

Decisão final sobre o projeto

"Placental function and obstetric outcome in twin pregnancies: a prospective cohort study"

A Comissão de Ética da NMS|FCM-UNL (CEFCM) decidiu, na sua reunião de 4 de novembro de 2020, aprovar por unanimidade, do ponto de vista ético, o projeto de investigação intitulado "***Placental function and obstetric outcome in twin pregnancies: a prospective cohort study***" (nº 81/2020/CEFCM), submetido pela Doutoranda Alexandra Sofia Queirós, no âmbito do Doutoramento em Medicina.

Lisboa, 9 de novembro de 2020

O Presidente da Comissão de Ética,

(Professor Doutor Diogo Pais)

TO WHOM IT MAY CONCERN

The Ethics Research Committee of NMS|FCM-UNL (CEFCM) has unanimously approved, in November 4, 2020 meeting, the Project entitled "***Placental function and obstetric outcome in twin pregnancies: a prospective cohort study***" (No. 81/2020/CEFCM) submitted by Alexandra Sofia Queirós, PhD student in Medicine.

Lisbon, November 9th, 2020

The Chairman of the Ethics Research Committee,

(Diogo Pais, MD, PhD)

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CENTRO HOSPITALAR
UNIVERSITÁRIO DE LISBOA
CENTRAL
CES

COMISSÃO DE ÉTICA PARA A SAÚDE

Parecer

Data: 28-01-2021

Processo nº 950/2020

Título: "Placental function and obstetric outcome in twin pregnancies: a prospective cohort study"

Relator: Dr. Gonçalo Cordeiro Ferreira

Investigador Principal: Dra. Alexandra Queirós - Grupo de Estudo da Gravidez Múltipla, MAC-CHULC

Projeto no âmbito de doutoramento na NOVA Medical School/Faculdade de Ciências Médicas da Universidade Nova de Lisboa.

Este projeto pretende estudar o impacto da função placentária no desfecho obstétrico na gravidez múltipla. Para tal são avaliados dados clínicos e laboratoriais maternos, dados ecográficos relativos ao crescimento fetal e de fluxometria doppler das artérias uterinas, e dados do parto e neonatais.

Os dados serão obtidos a partir da Base de dados de gémeos que o serviço tem vindo a construir desde 1994. (cuja criação teve autorização da CNPD)

Serão consideradas as gravidezes múltiplas seguidas na MAC entre janeiro 2010 e dezembro 2022.

É, pois, um estudo não intervencional com um componente retrospetivo e prospetivo.

Há uma semi-anonimização de dados, havendo lugar a um procedimento de consentimento informado para utilização dos dados recolhidos (componente prospetivo).

É garantida a anonimização total dos dados recolhidos na coorte histórica (retrospetiva).

Tem autorização hierárquica.

Conclusão:

Assim sendo, o estudo em análise não levanta questões do ponto de vista ético, pelo que esta Comissão entende emitir parecer favorável à sua realização.

O Presidente da Comissão de Ética

(Gonçalo Cordeiro Ferreira)

Secretariado – Manuela Ricoca Nunes - Antonio Couto
e-mail – comissao.etica@chlc.min-saude.pt - Tel. 213514410

Secretariado CA - Presidência

De: Salomé Almeida
Enviado: 15 de fevereiro de 2021 17:46
Para: Secretariado CA - Presidência; Secretariado CA - CHULC, EPE
Cc: projetos.inv@chlc.min-saude.pt
Assunto: projeto INV_134 - submissão do dossier final do projeto de investigação no âmbito de Doutoramento, para autorização do Conselho de Administração
Anexos: INV 134 - AGFC_Informação - 144_2020.pdf; INV 134 - CES 950_2020.pdf; INV_134 _ Informação para Consentimento Twins.pdf; INV_134 carta de submissao CA_ twins.pdf; INV_134_Anexo 7A_conformidade_assinado.pdf; INV_134_Anexo 9 _Compromisso_publicacao projecto twins AQ.pdf; INV_134_Anexo 10 _ConclusaoProjeto_Eliminacao de Dados Pessoais projecto Twins AQ.pdf; INV_134 _Anexo8_ConsentimentoTratamentoDadosPessoais Twins.pdf; INV_134_parecer hierarquico da instituição assinado_intenção AQ.pdf; INV_134_projecto twins AQ.pdf; INV_134_FICHA DE IDENTIFICACAO DE PROJETO.docx

Exma Dra Rosa Valente de Matos
Presidente do Conselho de Administração
Do Centro Hospitalar Universitário de Lisboa Central

Recebemos, no Centro de Investigação, o pedido de autorização para realização de projeto de investigação, com o título **Placental function and obstetric outcome in twin pregnancies: a prospective cohort study,** tendo sido atribuída a referência interna INV_134.

Projeto desenvolvido no âmbito do programa de Doutoramento em Medicina, na NOVA Medical School/Faculdade de Ciências Médicas da Universidade Nova de Lisboa (NMS/UNL)

Instituição/Unidade I&D: Consulta de Gravidez Múltipla, MAC-CHULC

Investigador Principal: Alexandra Queirós

Investigadores associados: Teresinha Simões, Ana Luisa Papoila (FCM-UNL)

Objetivo do estudo: Este projeto pretende estudar o impacto da função placentária no desfecho obstétrico na gravidez múltipla. Nesse sentido será estudada a influência de alguns marcadores placentários, como o doppler da artéria uterina (UtA) e biomarcadores no soro materno, de origem placentária, assim como a histopatologia da placenta. No final espera-se desenvolver uma proposta de estratégia clínica que permita um melhor acompanhamento da gravidez múltipla, com possibilidade de intervenção precoce, baseada na vigilância dos marcadores da função placentária.

O estudo tem uma componente retrospetiva, para identificação de correlações entre os marcadores propostos e o desfecho obstétrico. Os dados serão recolhidos a partir da base de dados de gravidez múltipla, existente no serviço. Este projeto tem uma componente prospetiva não intervencional, com a avaliação de dados clínicos e laboratoriais maternos, dados ecográficos relativos ao crescimento fetal e de fluxometria doppler das artérias uterinas, e dados do parto e neonatais.

Serão consideradas as gravidezes múltiplas entre 2010 e 2022.

As investigadoras preveem a publicação dos resultados obtidos, em revistas com revisão por pares, internacionais e indexadas

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Avaliação sumária do dossier do projeto, pelo GRAP:

A informação facultada inclui:

Protocolo do estudo, enquadrando o problema e a importância de validar cientificamente o impacto da função placentária no desfecho obstétrico nas gravidezes múltiplas

Metodologia de recolha de dados: Os dados serão obtidos a partir da Base de dados de gémeos que o serviço tem vindo a construir desde 1994. O protocolo descreve o tipo de dados a considerar e a forma como serão analisados para responder aos objetivos do estudo.

Informação e Consentimento informado: Foi apresentada a informação ao participante e a autorização de tratamento de dados pessoais (anexo 8), a aplicar na coorte prospetiva. É garantida a anonimização total dos dados recolhidos na coorte histórica (retrospetiva).

Finalidade da recolha de dados pessoais: no âmbito dos objetivos de investigação; na perspetiva de validação científica e melhoria das boas práticas clínicas.

Licitude da recolha de dados pessoais: para fins de investigação, no âmbito dos objetivos propostos

Proteção de dados: os dados de cada participante são pseudonimizados no momento da seleção, a partir da base de dados assistencial de gravidez múltipla. A base de dados de investigação é anónima; o acesso aos dados recolhidos é restrito. As investigadoras responsabilizam-se pela aplicação das medidas de proteção de dados e garantia da confidencialidade e privacidade dos titulares dos dados, em todos os momentos, incluindo para efeitos de divulgação e publicação. Este projeto está em conformidade com o RGPD e as práticas de proteção de dados em curso no CHULC.

Custos: Este projeto não tem custos para o CHULC

Parecer da Área de Gestão financeira e Contabilidade (AGFC 144/2020) – "É de autorizar, dado que não se perspetivam encargos adicionais para a Instituição".

Parecer da Comissão de Ética para a Saúde (CES 950/2020) - "O estudo em análise não levanta questões do ponto de vista ético, pelo que esta Comissão entende emitir parecer favorável à sua realização."

Documentos anexados:

Para efeitos de autorização deste projeto, remete-se o dossier contendo os seguintes documentos:

Ficha de Identificação do Projeto
Carta de Submissão
Parecer hierárquico
Protocolo de estudo
Informação para consentimento
Consentimento para tratamento de dados pessoais (modelo do anexo 8)
Compromisso de publicação (modelo do anexo 9)
Compromisso de eliminação (modelo do anexo 10)
Anexo 7A – conformidade com o RGPD
Parecer da Área de Gestão Financeira e Contabilidade, AGFC 144/2020
Parecer da Comissão de Ética para a Saúde, CES 950/2020

Uma vez avaliado, solicita-se o envio de documento assinado e digitalizado para o Gabinete de Registo e Apoio aos Projetos, para o e-mail projetos.inv@chlc.min-saude.pt.

Com os melhores cumprimentos

CENTRO HOSPITALAR DE LISBOA CENTRAL, EPE
Secretariado CA
Nº: CA1006
Entrada 19/02/2021
Saída 19/02/2021

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Salomé de Almeida
PhD, Genética Molecular
Assistente de Investigação

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PRESENTE À SESSÃO DO C.A. DE 18 de FEV de 2021
A Presidente Rosa Valente de Matos
O Vogal Jaime Alves
O Vogal Paulo Espiga
O Diretor Clínico Pedro Soares Branco
A Enfª Diretora Maria José Costa Dias
ATA Nº 8 / 2021

O CA autorize, em face do parecer da AGFC e dado que não implica encargos para o CHLC e

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