ACKNOLEDGEMENTS

DECLARATION

This thesis entitled "Congenital heart defects and placental dysfunction" has been composed by me, Ilaria Fantasia, and the work on this thesis is my own. This research project was composed by me with advice from my supervisors Prof. Giuseppe Ricci and Dr. Tamara Stampalija. As part of my European PhD project, data collection and statistical analysis were held in two hospitals in UK, King's College Hospital, under the supervision of Professor Kypros Nicolaides and Medway Maritime Hospital, under the supervision of Professor Ranjt Akolekar.

I was responsible for part of patient recruitment and acquisition of biophysical and biochemical markers at both hospitals and I participated in part of the fetal echocardiography performed under the supervision of Dr. Vita Zidere.

I was directly responsible for the collection of data and creation of the database as well as for the process of obtaining pregnancy outcomes. I wrote and composed this thesis and where information has been derived from other sources, I confirm that this has been indicated in the thesis. I contributed to writing the published paper incorporated in this thesis. This work has not previously been submitted, in part or whole, for consideration in any other degree or professional qualification.

Ilaria Fantasia

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ABBREVIATIONS

AC	Abdominal circumference
aCGH	array Comparative genomic hybridization
APS	Antiphospholipid syndrome
APVR	Abnormal pulmonary vein return
ART	Assisted reproductive techniques
CAT	Common arterial trunk
CHDs	Congenital heart defects
CNV	Copy number variant
CoA	Coarctation of the aorta
CPR	Cerebroplacental ratio
CRL	Crown-rump length
DORV	Double outlet right ventricle
DV	Ductus venosus
FGR	Fetal growth restriction
FL	Femur length
FVT	Fetal thrombotic vasculopathy
HC	Head circumference
HELLP	Haemolytic anemia, eleveted liver enzymes, low platelet count
HLHS	Hypoplastic left heart syndrome
HRHS	Hypoplastic right sided lesions
ICSI	Intracytoplasmatic sperm injection
IQR	Interquartile range
IUGR	Intrauterine growth restriction
IVF	In-vitro fertilization
IVS	Intervilluous space
ISUOG	International Society of Ultrasound in Obstetric and Gynecology
LSOL	Left-sided obstructive lesions
MCA	Middle cerebral artery
MoM	Multiple of the median
MRI	Magnetic resonance imaging
NDD	Neurodevelopmental delay
NT	Nuchal translucency
O2	Oxygen

OR	Odds ratio
PAPP-a	Pregnancy associated plasma protein A
PE	Preeclampsia
PlGF	Placental growth factor
RSOL	Right-sided obstructive lesions
sFlt-1	Soluble Fms-like tyrosine kinase-1
SGA	Small for gestational age
SLE	Systemic lupus erythematosus
SNAs	Synticial nuclear aggregates
SVDs	Single ventricle defects
TBV	Total brain volume
TGA	Transposition of the great arteries
TOF	Tetralogy of Fallot
TOP	Termination of pregnancy
TR	Tricuspid regurgitation
UA-PI	Umbilical artery pulsatiliy index
UK	United Kingdom
US	Ultrasound
UtA-PI	uterine artery pulsatility index
VEGF	Vascular enfothelial growht factor
VSD	Ventricular septal defect
WES	Wide exome sequencing
β-HCG	Human chorionic gonadotropin

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Chapter 1 INTRODUCTION

1.1   CONGENITAL HEART DEFECTS: BACKGROUND

Under the name of congenital heart defects (CHD) goes a large set of structural and functional abnormalities whose origin take place in the period of embryogenesis, and that are summarized in Figure 1.1.
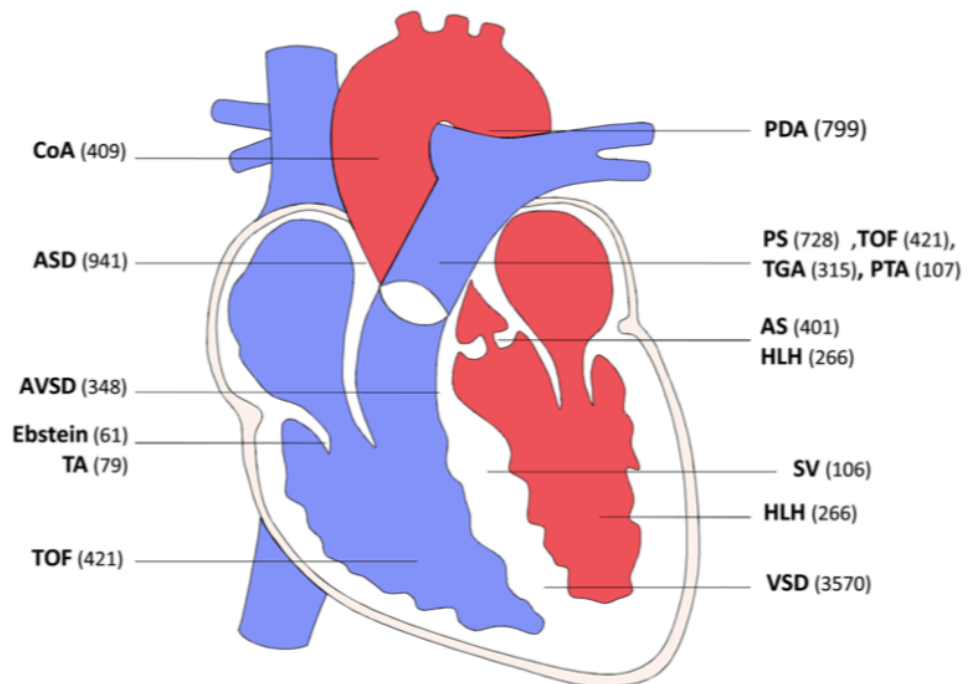
Figure 1.1 Locations of heart malformations that are usually identified in infancy. Numbers in brackets indicate the birth prevalence per million live births. Abbreviations: CoA, Coarctation of the aorta; AS, aortic stenosis; ASD, atrial septal defect; AVSD, atrioventricular septal defect; Ebstein, Ebstein anomaly; HLH, hypoplastic left heart; MA, mitral atresia; PDA, patent ductus arteriosus; PS, pulmonary stenosis; PTA, persistent truncus arterious; TA, tricuspid atresia; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defects; SV, single ventricle (From Fahed AC et al. Genetics of congenital heart disease: the glass half empty. Circ Res. 2013 15; 112:707-720).

CHDs are the most common type of congenital defects accounting for one third of all major congenital defects and occur in ~1% of live-born children (Matthiesen *et al,* 2016). CHDs represent an important medical challenge both in prenatal diagnosis, due to the required expertise in prenatal ultrasound for the correct diagnosis of the defect, and in the postnatal management, since part of these malformations requires a prompt intervention in the very first days of neonatal life.

The causes of CHD are still largely unknown. Chromosomal and single gene defects affect up to a quarter of all cases of CHD leaving the majority without an apparent explanation. Is it possible that more than half of CHDs is caused by casual "errors" during the embryogenesis or could it be that intervening external factors are able to disrupt the normal cardiac development causing the malformation?

Multi-factorial etiology, including environmental and epigenetic factors, could have a role in the pathogenesis of these "unexplained" cases, however it is still unclear how these factors interact to determine the disease. However, studying the early weeks of embryogenesis, when most of the fetal structures complete their morphogenesis, is difficult due to a lack of non-invasive techniques that allow studying the embryo in this time-frame.

In recent years, researchers shifted their attention to the first trimester of pregnancy as the time in which pregnancies at risk to develop subsequent complications, like preeclampsia, can be identified and an intervention can be started (Rolnik *et* al, 2017). Placenta can be thought as a bridge connecting the mother and the fetus and, therefore, by studying maternal blood we can gather several information on placenta and fetal health: regarding the matter of our discussion, the concentration of angiogenic and anti-angiogenic factors in maternal blood can give us information on placental function and some evidences show that an imbalance of these factors is present in fetuses with CHD (Llurba *et al,* 2013).

1.1.1 Genetic, epigenetic and environmental factors

Un underlying parental genetic cause is known to be one of the causes of CHD: a positive family history, defined as the presence of a CHD in a first-degree relative, increases the risk for the current offspring of being diagnosed with a cardiac defect and is one of the indication for a detailed fetal echocardiography. If one of the parents is affected the risk of having a child with a cardiac anomaly is 10.7% (Huhta *et al,* 2013). If a previous child had a heart abnormality the recurrence risk in the subsequent pregnancy is between 1 and 4% but it is 3 to 4 times higher if two previous children were affected (Huhta *et al,* 2013). It is, therefore, evident how much the familiar background is determinant for the onset of CHDs, however the exact genetic risks have been difficult to identify since 90-97% of subsequent pregnancies after an affected child proceed without recurrence (Huhta *et al,* 2013).

The genetic cause for each specific type of cardiac abnormalities is heterogeneous and seems that genetic factors, together with epigenetic and environmental factors, are responsible for the cause of CHD. However, the way in which environmental and epigenetic factors interact with genes remains poorly understood.
Chromosomal and genetic abnormalities are a well-known risk of CHD and the incidence of chromosomal abnormalities is around 18-22% (Jansen *et al*, 2015). The most frequent chromosomal abnormalities are trisomy 21, trisomy 18, trisomy 13 and monosomy X.

Microdeletion or microduplication genes syndromes are also involved in CHD. The most frequent is the 22q11 deletion, also known as DiGeorge syndrome, typically associated to conotruncal heart defect and linked to the haploinsufficiency of three genes (TBX1, CRKL, and ERK2) which causes dysfunction in the neural crest cells and anterior heart field, acting through gene inactivation, altered gene expression, or by encoding non-functional proteins (Momma 2010). Other genetic conditions frequently associated with CHD are the Williams-Beuren syndrome, also known just as Williams syndrome, due to a microdeletion the q11.23 region of chromosome 7 that encompass the elastin gene (ELN), and monogenetic defects, like Noonan syndrome (Jansen2015).

The importance of identifying a chromosomal or a genetic defect in fetuses with CHD is related to a higher risk of associated neurodevelopment delay (NDD). However, the majority of heart defects still remains without a clear genetic or chromosomal cause in the background.

The introduction of new technologies in the genetic analysis, like single nucleotide polymorphism array, next-generation sequencing and copy number variant (CNV) platforms are widening the range of known genetic causes of cardiac malformation. This is of crucial importance when counseling the parents. A recent meta-analysis on the clinical contribution of array comparative genomic hybridization (aCGH) reported that, for isolated CHD and after karyotyping and 22q11 FISH analysis, the incremented yield was 3.4% (95% CI; 0.3-6.6%), while it was 9% for non-isolated CHD (Jansen et al, 2015). Whole exome sequencing (WES) analysis in familial cases of CHD with Mendelian inheritance without a previous known genetic cause is able to identify a likely pathogenic and pathogenic mutation in 33% of cases (LaHaye *et al*, 2016).

However, some isolated CHD does not follow a familial inheritance and analysis of exome sequencing in children affected by CHD found that de novo point mutations are present in several hundreds of genes that collectively contribute to 10% of severe CHD (Fahed *et al,* 2013). New hypothesis to explain the occurrence of de novo mutations are based on epigenetic and environmental factors that can alter the genetic background. Maternal/placental microenvironment prior to and within 5-8 weeks of conception may influence the development of the fetal organs, such as heart and central nervous system. Environmental factors that can interfere with early heart development are different and include:

- environmental teratogens (i.e. dioxin, pesticides);
- maternal exposure (alcohol, isotretinoin, thalidomide, anti-epileptic drugs);
- infectious agents (i.e. rubella);
- folate deficiency;
- maternal pre-gestational diabetes.

Maternal diabetes is a well-recognized risk factor for cardiac abnormalities associated with a 4-fold increase in offspring of CHD (Oyen *et al*, 2016). It has been suggested that maternal hyperglycemia could alter the normal process of embryogenesis, but a population study on around 2 million births over a 34-year period, showed that improvement in perinatal care did not change significantly the rate of CHD. Therefore, other factors, like obesity, increased maternal age and pro-inflammatory state, can contribute to the strong association with heart defect in diabetic mothers. Gestational diabetes, on the opposite, is not associated to an increased risk of CHD, supporting the idea that an abnormal embryonic environment is responsible of the onset of CHD, maybe inducing epigenetic modification of CHD related genes (Oyen *et al*, 2016).

DNA methylation and histone modification are the most known epigenetic modifications that change chromatin regulation and thus genes expression (Chan *et al,* 2012). Epigenetic regulation of gene expression is one of the mechanisms involved in fetal programming. Specific gene can be activated, silenced of modulated by small non-coding RNAs (microRNA), DNA methylation status and histone modifications (Feinberg 2007). In the early embryo, DNA after fertilization undergoes progressive demethylation and becomes hypomethylated during the pluripotential stages. DNA methyltransferases are the enzymes responsible for DNA methylation. A principle source of methyl groups in the cell is S-adenosylmethionine synthetized by the folic acid metabolic cycle. Studies on mouse embryo showed that the observed cardiac defects are preventable if an adequate supplementation with folic acid is supplied early after conception and possibly at higher dose than the recommend multivitamins (Huhta *et* al, 2013). Therefore, epigenetic is an important area for analysis in relation to birth defects.

Given that a high percentage of pregnancies are unintended, the mechanism of action of external factors, such as folate intake, and intersecting pathways during early gestation together with prophylactic mechanism involving epigenetic effects are critical in understanding the placenta-heart axis.

1.1.2 Placental factors

Early in human gestation following conception, the fertilized embryo implants in the uterine wall. The successful implantation requires adequate maternal uterine perfusion and endogenous hormone preparation to allow initial embryonic survival and later organ development and growth for a term gestation.

Shortly after implantation cardiomyocytes specification and commitment take place between days 16 and 19 post conception. The circulation and a beating tubular heart are established by 21 days post conception in human pregnancy. Next, a beating, linear, tubular heart forms that then loops and septates to form a four-chambered heart (4-6 weeks of human gestation). Human cardiac morphological development is complete by 53 days post conception (8 weeks of human gestation) (Figure 1.2).
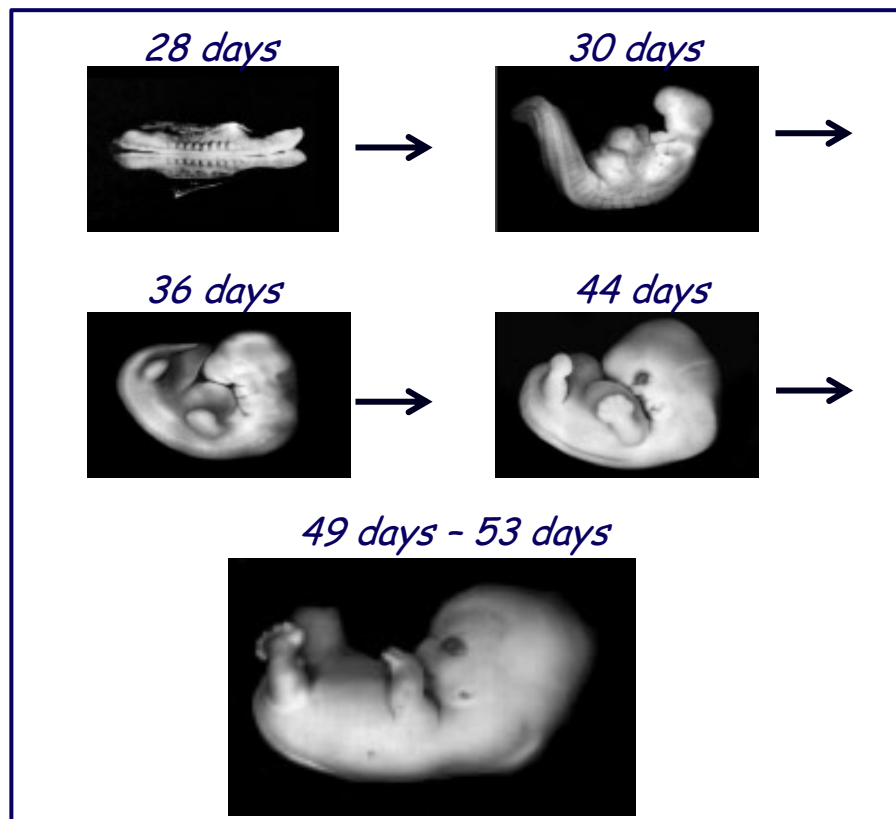
Figure 1.2 Embryonic development from day 2 to week 8, when development of the

heart   is   completed   (taken   from
http://www.embryology.ch/anglais/iperiodembry/carnegie02.html).

Initial steps in placentation occur in relatively low oxygen ambient, since in the first stages there is no maternal blood flow in the developing placenta. Histological studies on placentas of human early pregnancy (from 43 to 130 days of gestation) showed that before 8 weeks of pregnancy aggregates of cytotrophoblast cells virtually occlude the mouths of the maternal spiral arteries, ensuring that any flow into the intervillous space is a slow seepage of blood flow or even plasma filtrate. Only after this period direct channels can be observed, with increasing size and delineated shape after 11-12 weeks (Burton *et al*, 1999). These findings, published back in 1999, were recently confirmed by a study in which microvascular filling of the intervillous space (IVS) was demonstrated by contrast-enhanced ultrasound, giving an intravenous infusion of lipid-shelled octofluoropropane microbubbles, from 6 weeks onwards in 34 pregnant women: results showed that there is an increasing blood flow to the IVS starting from 6-7 weeks (Roberts VHJ *et al*, 2017).

But how the development of the embryo can happen in this under perfused environment? There are evidence that, at this stage, low levels of oxygen are essential to the normal development of the embryo for two reasons: the first is that the system is still immature to protect itself from oxidative agents; the second is that it represents a trigger for angiogenesis stimulating the production of vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and angiopoietin essential to the growth of the villous and harborization of the villous tree (Charnock-Jones *et al*, 2000).

At later stages, physiological remodeling of the spiral arteries provides adequate blood supply increasing O_2 concentration that is essential for the normal development of the fetus: chronic states of hypoxia or sudden increased concentration of O_2 can alter the normal development of embryonic tissue in these early stages. Studies on animals proved that exposure of mice embryos to reactive oxygen species increased the risk of congenital abnormalities (Dennery 2007). It, therefore, seems that a normal embryonic development depends on a correct balance of O_2 levels and either too low or too high

oxygen concentrations could damage the embryonic development. A normal fetal-placental homeostasis is guaranteed by O_2 concentration and by the consequent production of angiogenic factors that promote formation of the highly arborized vascular bed. There is evidence from animal studies that angiogenic factors may be implicated in cardiac morphogenesis (Llurba *et al,* 2013).

VEGF has many direct actions on endothelial cells, which are in some ways linked to the process of angiogenesis, and include vasodilatation, increase in micro-vascular permeability, protease release, migration and proliferation of endothelial cells and lumen foration. Studies on mice embryos have shown that it could also be involved in cardiac morphogenesis: VEGF expression is found in most endocardial cells located at point of cushion formation (Armstrong *et al*, 2004). In zebrafish embryos, blockage of VEGF receptors resulted in functional and structural defect of cardiac valve development, suggesting that these receptors are implicated in the formation of heart valves (Lee *et al*, 2006). On the basis of these findings, Lambrechts *et al.* performed selective genotyping on 148 families with isolated TOF and showed that the presence of specific haplotype, the AAG haplotype, which lowers VEGF expression increases the risk 1.8-fold of Tetralogy of Fallot (TOF) and is transmitted in 61% of the affected children (Lambrechts *et al*, 2005). On the other side, a 2 to 3-fold overexpression of VEGF in mutant mice embryos was also found to result in severe abnormalities of cardiac development, including an attenuated compact layer of myocardium, overproduction of trabeculae, defective ventricular septation and remodeling of the outflow tract (Miquerol *et al,* 2000).

VEGF production is increased as a response to hypoxia in order to recruit new blood vessels and improve cellular perfusion. Further studies on mice embryo showed that increased concentrations of VEGF secondary to hypoxia act as a teratogen factors on the heart, instead of being protective, thus increasing the rate of CHD (Dor *et al,* 2001). VEGF production seems to be regulated also by its most important antagonist, the soluble fms-like tyrosine kinase-1 (sFlt-1), which is highly expressed by trophoblast and could act as a protective agent when VEGF is overproduced (Charnock-Jones *et al*, 2000). Ablation of the sflt-1 genes leads to aberrant angioblast commitment and inappropriate vascular channel development in chimeric embryos (Fong *et al*, 1999).

Therefore, VEGF expression is not only related to specific genetic background but depends also on environmental factors, supporting the multi-factorial etiology of fetal heart defects.

PlGF, a glycoprotein belonging to the family of vascular endothelial growth factors (VEGF), is another important angiogenic factor produced by the placenta and induces proliferation, migration and activation of endothelial cells. PlGF is highly expressed by trophoblastic cells and is known to be involved in the regulation of placental vascular development. There is extensive evidence that in pregnancies with impaired placentation PlGF level, as well others angiogenic factors, is reduced and such a decrease is present from the first trimester (Tsiakkas *et al*, 2016).

There is just one study so far, from Llurba and collegues, assessing the possible relation between PlGF concentration and CHD (Llurba *et al,* 2013). They showed that in fetuses with major heart defects the concentrations of maternal serum PlGF were significantly decreased compared to controls (p<0.0001), suggesting an impaired placental angiogenesis that is present since the first trimester of pregnancy. Notably, values of uterine artery pulsatility index (UtA-PI) were within the normal ranges implying that a normal perfusion on the maternal side is present (p=0.396). The authors concluded that in fetuses with CHD there is a primary abnormal dysfunction of the placenta, reflected by lower levels of angiogenic factors, that seems to affect both placental and heart development and that is not related to abnormal placental perfusion. They, then, analysed PlGF according to three different subgroups of cardiac defect: valvar (32 cases), conotruncal (25 cases) and left-sided defects (11 cases). Significant reduction of PlGF maternal serum blood concentrations was observed in the first two groups (p<0.0001 and p=0.003) but not in the last, where the small number of cases could explain the lack of significance (p=0.863). PAPP-a and beta-HCG maternal serum levels did not show any significant correlation with CHD (p=0.292 and 0.616, respectively). The conclusions are that, though lower levels of angiogenic factors are present in pregnancies with CHDs, this is not true for LVOT defects that may be a consequence of different pathogenic causes.

The presence of an unbalanced angiogenic status in fetuses with a CHD is also supported by histological findings from placenta tissue in newborns with hypoplastic left heart syndrome (HLHS): they documented a reduction in the numbers of terminal villi and reduced villous vasculature (p=0.001), lower expression of PlGF RNA (p<0.05), increased in Syncityal Nuclear Aggregates (SNAs) (p<0.01) and overall reduced placental weight (p=0.02) compared to controls (Jones *et al,* 2015). Therefore, in fetuses with CHDs, placenta fails to expand its villous tree and to develop terminal villi. Stanek found similar findings on a group of fetuses affected by what he calls "postplacental hypoxia": in these cases, normal perfusion is provided on maternal side but the fetus does not have enough oxygen due to the presence of specific malformation like cardiac defects, umbilical knots, etc. He found thinner and longer villi due to poor branching on the placental side as a reflection of a compromised fetal circulation (Stanek 2015). Another larger study on 120 placental histology of 120 fetuses with CHD showed that the placental weight-to-birth weight ratio was significantly reduced in CHDs than in controls and histological findings of villous hypomaturity, thrombosis, chorioangiosis and placental infarction (Rychick *et al.,* 2018).

The same group of Llurba studied the expression of angiogenic and antiangiogenic factors in 65 fetuses with CHDs in fetal cord blood (39 cases), heart tissue of cases underwent to termination of pregnancy (TOP) (23 cases) and in maternal blood at the second and third trimester of pregnancy (65 cases) (Llurba *et al,* 2014). The results showed that, compared to controls, PlGF was significantly reduced (p<0.0001) and sFlt-1 significantly increased (p=0.0438) in maternal serum, while in heart tissue and fetal cord blood sFlt-1 and VEGF were significantly increased but not PlGF. Interestingly, analysis of heart tissue of fetuses with CHD underwent to TOP showed an increased expression of sFlt-1 and transcript levels of proteins related to hypoxia, such as hypoxia inducible factor (HIF)-2α. Therefore, also in the second and third trimester there is a dysregulation of the anti/angiogenic status in the maternal blood and a degree of hypoxia in the fetal heart tissue associated to a prevalent antiangiogenic environment.

Linask *et al*., have introduced the concept of the "heart-placenta axis" (Fig. 1.3) based on the hypothesis that cardiac and placental abnormalities may coexist through polymorphisms in genetic developmental pathways common to both organs, in particular those regulated by Wnt/ß- catenin signaling, or through a lack of key micronutrients, such as folate (Linask *et al.* 2014).
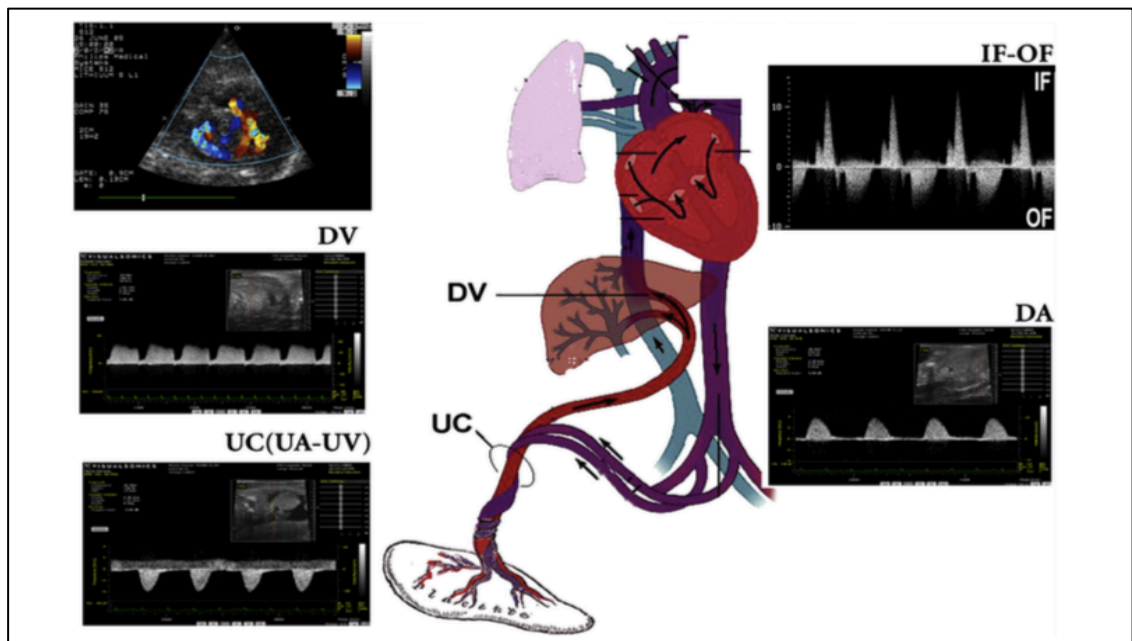
Figure 1.3 Figure of the heart-placenta axis*,* showing the vascular connections between the placenta and the fetus through multiple fetal vessel that constitute the fetal circulation: UC, umbilical cord; UA, umbilical artery; UV, umbilical vein; DV, ductus venosus; IF-OF: inflow-outflow; DA, ductus arteriosus (from Linask *et al*. Changes in vitelline and utero-placental hemodynamics: implications for cardiovascular development. *Front. Physiol* 2014*.* 5:390.

The heart-placental axis is associated with parallel development of the placenta and heart that utilizes many common molecules and genes and reflects intimate and synergistic growth of both organs.

However, as shown in Fig. 1.2, heart development is completed by around 8 weeks' gestation before placental circulation has established.

In an interesting review by Burton *et al.,* on the relationship between human placenta and heart development, the authors stress the concept that in the first weeks of embryonic life the circulation is guaranteed by the presence of the yolk sac, as shown in Figure 1.4.
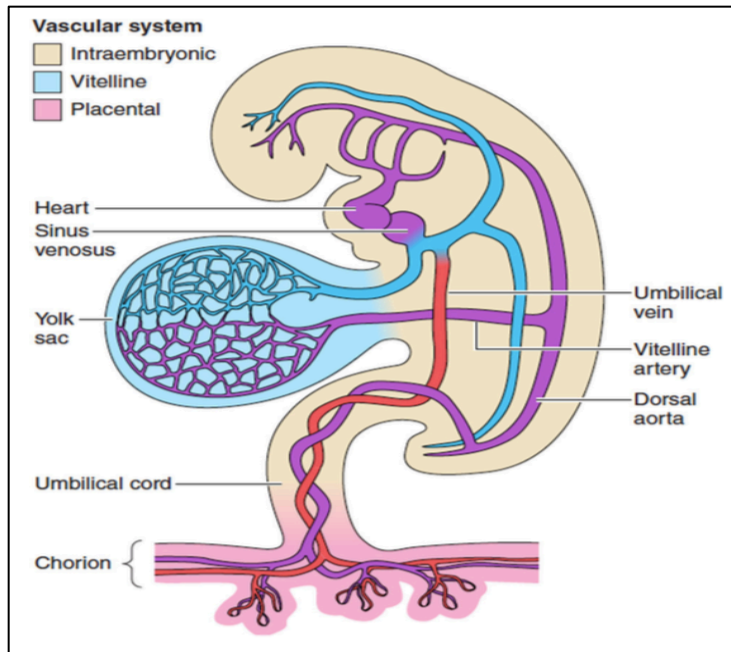
Figure 1.4 The extravillous circulations. The yolk sac is the first of the extraembryonic membranes to be vascularized, and likely plays a key role in maternal-fetal transport during the period of organogenesis before the chorionic circulation is fully established at ~12 weeks. Changes in the resistance offered by each circulation may affect gene expression and differentiation of the fetal cardiomyocytes (From Burton *et al.* 2018)

Extensive remodeling occurs toward the end of the first trimester when the definitive placenta is formed. Villi initially develop over the entire gestational sac but starting from around 8 weeks of gestation the villi over the superficial pole begin to regress, forming the smooth membranes or chorion laeve. Regression is associated with the progressive onset of the maternal arterial circulation to the placenta, first in the periphery and then in the rest of the placenta. This process is mediated by the migration of extravillous trophoblastic cells (EVT) into the placental bed and modulated by locally high levels of oxidative stress within the villi (Jauniaux et al., 2003).

Events at this stage of development play a key role in determining the final size and shape of the placenta, and so may impact development of the fetal heart.

1.2 Congenital heart defects and nuchal translucency

Nuchal translucency (NT) is a well-recognized marker for CHDs. In a study on almost 30000 pregnancies, including 50 cases with major CHDs, the presence of a NT above

the 95th centile achieved a detection rate for CHD of 56% (Hyett *et al,* 1999). The prevalence of CHDs increases with increasing value of NT (3% for NT between 3.5 and 4.5 mm and 20% for NT ≥5.5 mm). No differences were found according to the type of CHD, however strongest associations were seen for left-sided lesions such as HLHS and CoA. These findings are supported also by other studies that show how an increased NT thickness constitutes a risk factor for CHDs independently from the nature of the cardiac defect (Atzei *et al*, 2005; Syngelaki *et al,* 2011).

The relation between increased NT and CHDs is, however, not fully understood. Some authors advocate the presence of impaired diastolic function that leads to increased NT with a mechanism similar to that observed in severe cardiac dysfunction and fetal hydrops at later gestations, where a rise in systemic venous pressure and in hydrostatic pressure may lead to the accumulation of nuchal fluid in the first trimester (Hyett *et al,* 1996*)*. In support of this theory, it is known that tricuspid regurgitation (TR) and reversed flow in the ductus venosus (DV), both signs of impaired diastolic cardiac function are more frequent in fetuses with CHDs and increased NT. However, one study examined the cardiothoracic ratio and the left ventricular ejection fraction in fetuses with HLHS and isolated ventricular septal defects and increased NT in the first trimester: they failed to prove the presence of cardiac dysfunction, though the assessment was done in the second trimester and, therefore, transient period of cardiac dysfunction that caused increased NT cannot be excluded with certainty (Simpson *et al,* 2000). Furthermore, additional signs of heart failure, like pericardial and pleural effusion, edema, cardiomegaly and ascites are all usually absent and increased NT can be associated to several congenital abnormalities, other than just CHDs, suggesting that there are different pathogenic pathways to the presence of a CHD and increased NT.

The possible correlation between increased NT and CHD was evaluated in a review where 3309 relevant genes in cardiovascular development and heart morphology were manually cross-checked with 105 relevant genes in lymphatic development in mouse embryos (Burger *et al*, 2015). All these genes were identified through a genetic search on Mammalian Phenotype Browser. Following the cross-check, 3399 of 3414 were

excluded as they were not a mutual gene involved in both cardiac and lymphatic vascular development. Consequently, 15 genes were identified as potentially mutual genes in cardiac and lymphatic vascular development. Mutations in all but one gene (Pik3ca) resulted in a cardiac defects, abnormal lymphatic development and nuchal edema. All genes were involved in the regulation of endothelial differentiation strengthening the hypothesis that abnormal endothelial differentiation, rather than cardiac failure, is the common etiologic pathway underlying both defects. No specific CHD was identified, and no specific gene was responsible for a specific CHD and this is similar to findings in clinical practice and previous studies on human fetuses with increased NT, that showed no relation between a specific cardiac defects and increased NT (Haak *et al*, 2005; de Mooji *et al,* 2010*)*. The numerous potential interferences in this pathway explain the relative common phenotype of increased NT. However, the presence of a mutual genetic background could explain the strongest association between cardiac defects and nuchal edema, which is not marked for other fetal defects.

Fewer evidences are available on how maternal serum angiogenic and anti-angiogenic status may influence NT in fetuses with a CHD. The only study that investigated on this aspect is the one from Llurba et al. where angiogenic factors in fetuses with CHDs in the first trimester were analyzed: despite the fact that PlGF is overall reduced in all CHDs compared to controls, when sub-analysis was performed based on the presence or absence of increased NT, they found that in the heart defect group there was a significant association between log10PlGF-MoM and delta NT (r = -0.307; p<0.0001) but this observation was not found in the control group. They, then, classified the cardiac defect group according to NT thickness, and only patients with abnormal NT had statistically significant lower levels of PlGF than did the control group (Figure 1.5) (Llurba *et al,* 2013).
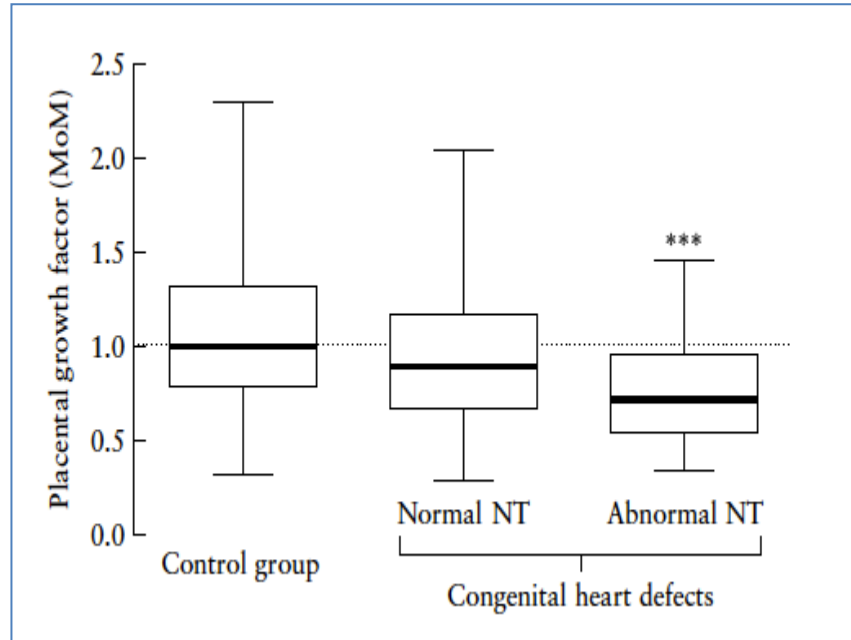
Figure 1.5. Box-and-whisker plot of PlGF-MoM values in the control group and in fetuses with CHD classified according to normal or abnormal NT (From Llurba *et al,* 2013).

These are the first data on a possible relationship between PlGF and increased NT in fetuses with CHD however the mechanism of interaction between these two entities has not been explained. VEGF genes play an essential role in the development of lymphatic endothelial cells from veins, and mutations in some VEGF allele cause dysfunction of lymph vessels and severe systemic edema (Shibuya *et al*, 2008). Since PlGF belongs to the family of VEGF the same action could be apply, however these conclusions remain speculative since no specific study was performed so far.

1.3 Congenital heart defects and obstetric adverse outcomes

1.3.1 Preeclampsia

Different studies have shown that in pregnancies with impaired placentation, PlGF production is reduced from the first trimester and is related to specific complications

of pregnancy such as preeclampsia (PE) and fetal growth restriction (FGR) (Poon *et al,* 2008; Akolekar *et al,* 2011). However, in these cases, there is an impaired perivascular and endovascular trophoblastic invasion of the spiral arteries. As a consequence, spiral arteries fail to become low-resistance vessels, and this is reflected in increased resistance to flow in the UtA (Meekins *et al*, 1994).

VEGF, PlGF and s-Flt-1 are highly expressed by cytotrophoblast cells and it has been shown that their expression is altered in placenta tissue of women with preeclampsia (Zhou *et al, 2002)*. Thus, apparently, pregnancies at risk for developing PE and pregnancies with fetus affected by a CHD share similar imbalances in the placental angiogenic environment.

Based on these findings, the relationship between fetal CHD and maternal PE has been recently investigated: in a retrospective study on 279 pregnancies with a fetal CHD, the onset of PE, subdivided as mild, severe and HELLP syndrome, was reported in 5.7% cases (Ruiz *et al,* 2016). In a cohort of almost 2 million Danish singleton pregnancies, Boyd et colleagues examined the possibility of a relationship between fetal cardiac defect and PE: CHD was present in 0.9% of this population; early preterm and late preterm PE was present in 0.2% and 0.3%, respectively; term PE was registered in 2-3% and gestational hypertension in 0.9% of pregnancies with CHD, respectively (Boyd *et al,* 2017). Results of this study showed that women carrying a fetus with a CHD have a 7-fold increased risk of developing early preterm PE and a 3-fold risk of late preterm PE, while the risk was lower for late PE and gestational hypertension. These data show that there is a strong correlation between impaired placental function and fetal CHD, which seems to be true for early-onset forms but not for the late one and is consistent regardless the type of CHD. Moreover, if the woman developed early preterm PE in one of the previous pregnancies the risk for the offspring of being affected by a CHD is eight times higher for preterm PE and three times for late preterm PE. Correlations with PE were proved also if a previous pregnancy was complicated by a fetal CHD: the risk of developing preterm PE was a two-fold higher and the risk for term PE was 25%.

Gestational hypertension didn't show significant impact in any of these cases. This study provides evidence that maternal PE and fetal CHD share a common pathway most likely linked to an endothelial dysfunction secondary to poor placental perfusion and placental insufficiency typical of earlier forms of PE, also known as "placental" forms. Unfortunately, due to the retrospective nature of the study, data on UtA-PI and maternal serum analysis of anti/angiogenic factors were not part of the analysis.

To date, there is just one retrospective study that analyzed UtA-PI in pregnancies affected by a fetal CHD in the second and third trimester: no significant differences in UtA-PI Z-score were observed in CHD cases compared to controls but UtA-PI Z-score showed a quadratic increase with gestational age in the whole population studied and 61% of cases had UtA-PI values > 95th centile at the end of the pregnancy. Any PE was reported in 5% of the total population studied but whether the occurrence was somehow higher in the group with increased UtA-PI was not specified (Ruiz *et al,* 2017).

1.3.2   Fetal growth and neurodevelopmental delay

The association between any type of fetal defect and fetal growth restriction is long time known. In 1987 Khoury *et al.,* reported that the presence of an isolated congenital defect doubles the risk for the fetus of being small for gestational age at birth (RR=2.2) and that this risk further increases if two or more defects are associated (from 20.5% among those with two defects to 58.6% among those with ten or more defects) (Khoury *et al.,* 1987). In the early era of prenatal diagnosis, the assessment of body proportionality was used to provide information on the underlying pathophysiologic changes of fetal growth restriction and, clinically, at least two types of intrauterine growth restriction (IUGR) were recognized according to the relationship between birth weight and length: IUGR with affected body length (symmetric intrauterine growth restriction) and intrauterine growth retardation with normal body length (asymmetric intrauterine growth retardation). The underlying pathogenetic mechanism was considered to be different for these two forms of

IUGR, with an early embryonic insult responsible for the early-onset symmetric form, while the presence of placental insufficiency would be responsible for the late-onset asymmetric one (Campbell *et al.,* 1977; Trudinger 1985; Wagner *et al.,* 2016; Dashe *et al.,* 2000). Studies on fetal growth and Doppler have shown that growth restriction secondary to placental insufficiency is characterized by increased impedance to flow in the uterine and umbilical arteries as a result of reduced placental function, while growth restriction in fetuses with fetal malformations or chromosomal abnormalities is more frequently characterized by normal Doppler values of UtA-PI and slightly higher values of UA-PI (Snijders *et al.,* 1993; Hiersch *et al.,* 2018).

In the Baltimore – Washington Infant Study in 1991 (Rosenthal *et al,* 1991) birth weight was studied according to different types of CHD and it was shown that weight at birth differs for each type of CHD and may depend on the fetal circulation determined by the defect itself. These findings were confirmed in a subsequent study by the same group (Rosenthal 1996). Fetal growth was analyzed in 4 types of CHD (TGA; TOF; HLHS; CoA) and they found that, overall, fetuses with CHD are smaller compared to controls, but the biometric parameters were different according to the type of CHD and consistent with the altered fetal circulation determined by the CHD. For example, fetuses with TGA, where deoxygenated blood is directed to the head and oxygenated blood to the body, had smaller head volume compared to the body, while fetuses with TOF, where there is a mixture of oxygenated and deoxygenated blood, were symmetrically smaller for all biometric parameters.
Around 20% of fetuses carrying a CHD are SGA and the presence of a CHD increases the risk of fetal growth impairment by two to three times (OR: 2.09) (Malik *et al,* 2015). Fetal growth seems to be affected already from the second trimester with a relative growth slope in the subsequent trimesters and HC values being the most affected (Williams *et al,* 2015). The relationship between CHD and fetal smallness is still unclear but it is possible that there is a shared etiologic pathway.

The importance of assessing fetal growth in fetuses with a CHD is related to the increased incidence of adverse outcomes in children that will undergo to either

corrective or palliative surgery: the degree of preoperative growth failure has been associated with longer time on the ventilator, difficulties in postoperative feeding, higher risk of infection, longer hospitalization time and poor postoperative growth catch-up. Furthermore, in a recent publication, from the EPICARD study group, there are evidence that being born SGA with a major CHD requiring surgery is significantly associated with lower cognitive score than in the non-SGA group (Calderon *et al,* 2017). Therefore, intrauterine fetal growth is critically important in these fetuses and the presence of fetal growth restriction constitutes a negative prognostic factor especially for those cases in need of postnatal surgery.

Improvements in mortality following surgery for complex CHD brought the attention on neonatal morbidities, such as neurodevelopmental outcome. Neurodevelopmental delay (NDD) is a well-established complication in newborns with CHDs. Different reports indicate that, in complex CHD, up to 50% of cases have NDD, which can be variable and involving different aspects such as mild impairments in cognition, fine and gross motor skills, executive functioning, visual construction and perception, social interaction and core communication skills (Marino *et al,* 2012) (Fig. 6).
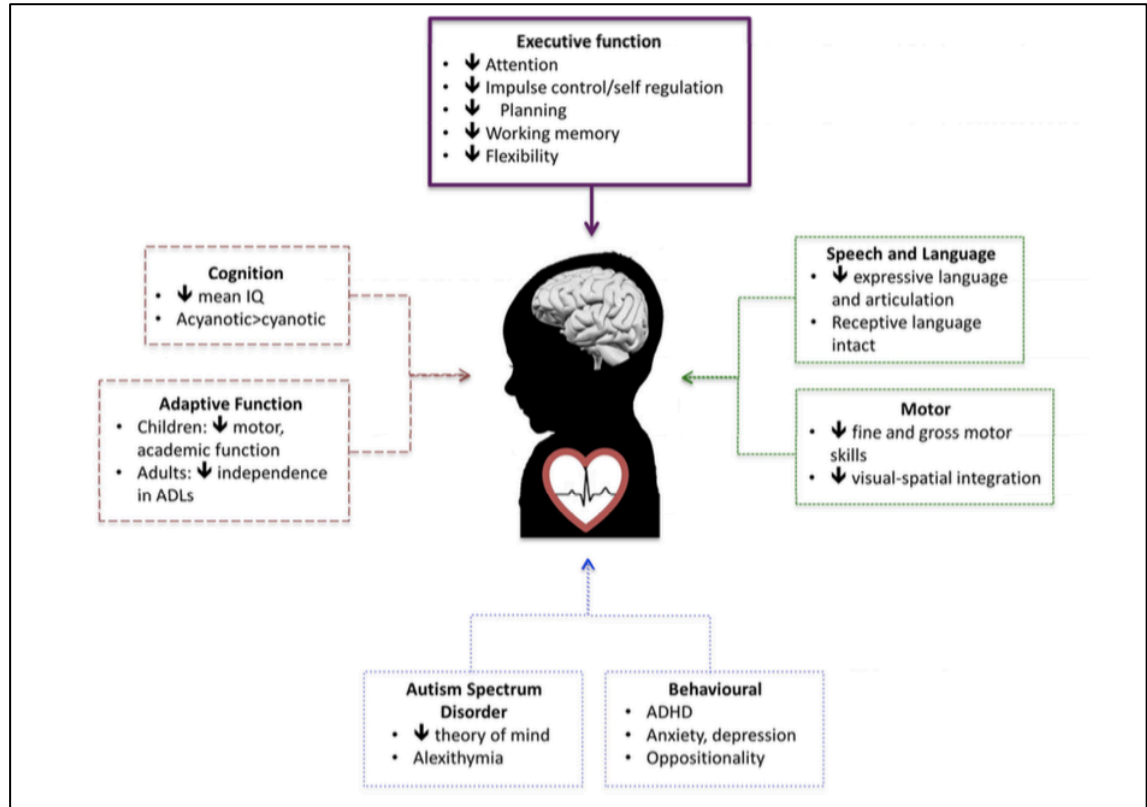
Figure 1.6 A summary of the neurodevelopmental deficits observed in children who underwent corrective surgery for CHD (From Nattel *et al.,* 2017).

NDD has been usually attributed to perioperative conditions occurred during surgery resulting in cerebral hypoxia and thrombo-embolic events. However, more recently, different studies reported the presence of brain lesions at neuroimaging already before cardiac surgery as well as in cases where surgery was not performed (Khalil *et al,* 2016). Magnetic resonance (MRI) studies found that the most commonly observed lesions were white matter injury, periventricular leukomalacia and stroke. Such brain lesions are reported in 19-52% of cases (Brossard-Racine *et al,* 2016; Mulkey *et al,* 2014). Despite different factors, like extent and duration of cerebral desaturation prior to surgery and intra-operative hypoxia, are important determinants of neurocognitive outcomes, the question whether brain insult is already present before surgery has arisen (Nattel *et al.,* 2017). The first studies on the assessment of NDD were carried out on TGA cases because of the relatively easy surgery

needed to correct these defects that shouldn't expose to serious degree of intra-operative hypoxia (Shillingford *et al,* 2008).

There are two main theories to explain the presence of NDD in fetuses with CHDs: the first is based on genetic and epigenetic factors that could affect the normal development of the brain in the presence of a CHD and whether any alterations of these pathways leads to abnormal development of both organs, heart and brain, with increased susceptibility of the brain tissue to hypoxic insults; the second is that the presence of a cardiac defect causes various degree of hypoxia due to the pathological fetal circulation established that secondarily affects the brain (Hinton *et al,* 2008). Quite interestingly, various degrees of NDD are observed also in minor CHDs, like ventricular septal defects (VSD) not requiring surgery (Khalil *et al,* 2016).

Advanced MRI techniques have allowed the measurement of brain metabolism, cortical development and cerebral oxygenation. Some studies showed a progressive decline starting in the 3rd trimester, with reduced cerebral oxygenation and metabolism, smaller brain volumes and delayed cortical gyrification (Limperopoulous *et al.,* 2010; Clouchoux *et al.,* 2012). In Figure 7 we can see how the Total Brain Volume (TBV) decreases with advancing of gestational age and the third trimester (~ 28 weeks, red arrow) seems to be the critical period when high energy dependent process happening in the fetal brain meet conditions that restrict cerebral oxygen or substrate supply (Limperopoulous *et al.,* 2010).
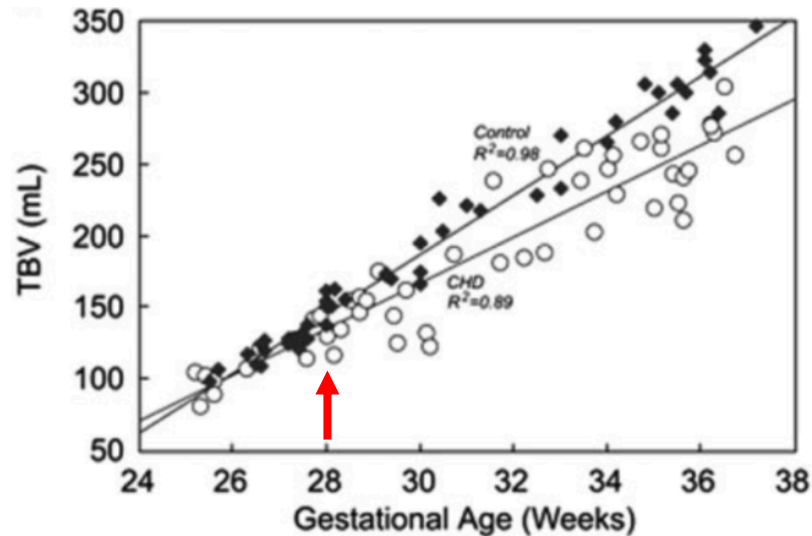
Figure 1.7 Relationship between gestational age and Total brain volume (TBV) in fetuses with CHD (open circles) and controls (solid diamonds) (From Limperopoulous *et al.* Circulation, 2010)

Therefore, the third trimester seems to be the most sensitive time of gestation for brain maturation including neuronal migration and arborization, synaptogenesis, programmed cell death, oligodendrocyte maturation, and extensive reorganization of synaptic connections. McQuillen et al. suggest that structural brain abnormalities in CHD are related to alterations in cerebral blood flow at key moments in development (McQuillen *et al.,* 2007). They suggest that damage occurs as a result of a unique vulnerability in late oligodendrocyte progenitor cells and subplate neurons, which play a critical role in myelination and white matter track development and are particularly vulnerable to hypoxia-ischemia. Myelination is energy-consuming, and oligodendrocyte precursors release self-inhibitory signals if energy demands aren't met. Fundamental cerebrovascular abnormalities in CHD brains may cause focal arrest of cells that a) inhibit appropriate myelination and b) are susceptible to vascular insult, contributing to white matter injury. In Figure 8 there is a schematic representation of the pathophysiology of neurodevelopmental deficits seen in CHD patients.
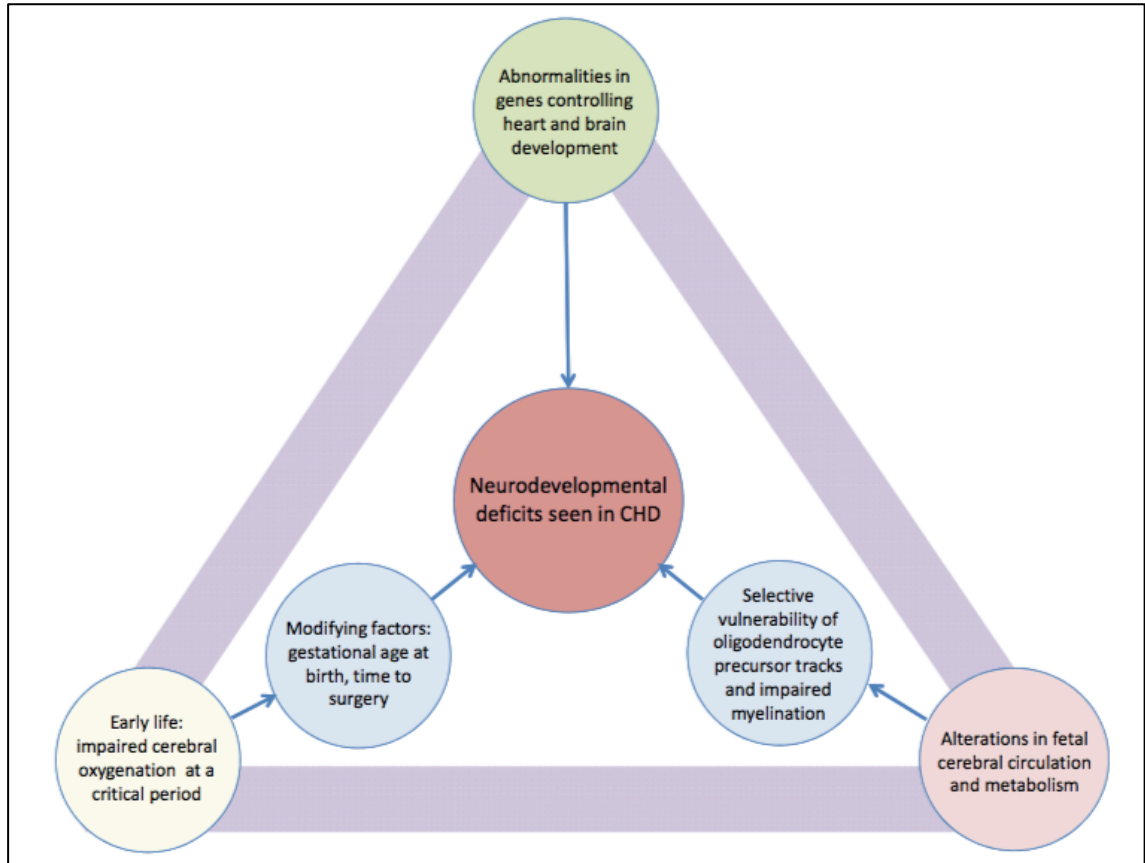
Figure 1.8 A schematic representation of the pathophysiology of neurodevelopmental deficits seen in CHD patients (From Nettel *et al.,* 2017).

Considering these emerging evidences that seems to collocate, in some fetuses with CHDs, the timing of the brain insult in the intrauterine life, different authors started investigating on US assessment of HC and blood flow to the middle cerebral artery (MCA) as prenatal parameters potentially able to identify fetuses at risk of NDD. The hypothesis on HC originated from the observation that neonates with a CHD are born with relatively smaller heads than normal babies and from the assumption that if in fetuses with CHDs there is chronic hypoxia because of the heart defect, brain volume would have been smaller and therefore the HC is smaller (Sun *et al*, 2015). The hypothesis on MCA-PI, and its ratio with the umbilical artery pulsatility index (UA-PI), is based on the findings in FGR fetuses.

FGR is most commonly caused by placental insufficiency, which exposes the fetus to a situation of chronic hypoxia. As a response to hypoxia the fetus redistributes its cardiac output to maximize oxygen and nutrient supply to the brain in a mechanism known as "brain sparing". This happens because the fetal circulation is a parallel circuit where the majority of the right ventricular output is shunted to the descending aorta through the ductus arteriosus while the left ventricle mainly supplies the upper body and the brain. In case of placental insufficiency there is vasoconstriction of peripheral vascular beds, due to placental damage, that increases the right ventricular afterload but, on the other side, the presence of vasodilation of the cerebral arteries, due to vasodilation of the MCA, causes a decrease in the left ventricular afterload. These changes result in a preferential shift of the cardiac output in favor of the left ventricle, enhancing blood supply to the brain in what is called cerebral redistribution (Coehn et al, 2015). Changes in cerebral blood flow can be detected by measuring MCA-PI and by its ratio with the UA-PI (cerebroplacental ratio; CPR) by Doppler ultrasound.

The "brain-sparing" effect was considered a reactive mechanism in IUGR fetuses to protect the brain from hypoxia. However, there are evidence that the presence of either low MCA-PI or low CPR is significantly associated to adverse perinatal outcome and to increased risk of delay in neonatal motor and state organization, lower communication and problem–solving score at 2 years of age (Miller *et a,* 2016).

In CHDs the altered circulation secondary to the anatomic defects can lead to various degrees of hypoxia. Simplifying, there are at least three circulatory mechanism that appear to be impacting the oxygen content of blood supplied to the brain in fetuses with CHD, shown in Figure 9.
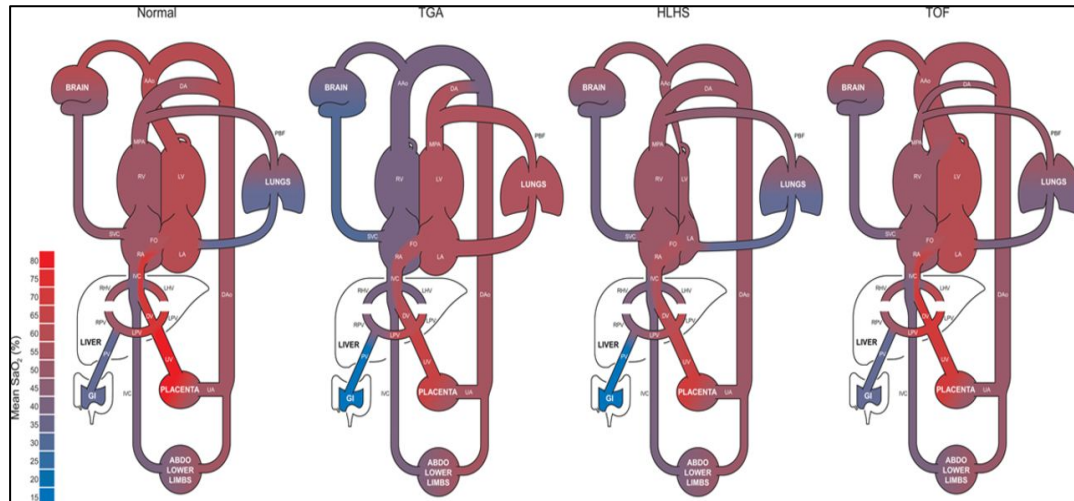
Figure 1.9 Oxygen saturations across the circulations of representative examples of a normal fetus and fetuses with hypoplastic left heart syndrome (HLHS), transposition of the great arteries (TGA) and tetralogy of Fallot (TOF) by MRI. In the normal fetal circulation, there is streaming of oxygenated blood from the placenta to the fetal cerebral circulation via the ductus venosus and foramen ovale. In each of the examples of CHD, this pathway is disrupted (from Sun *et al.,* 2016).

The first authors reporting on reduced HC values and MCA-PI in fetuses with a CHD in the third trimester were Donofrio et al (Donofrio *et al,* 2003). The study was conducted on 41 fetuses with CHD, divided in HLHS (12), TGA (4), TOF (11), left-sided obstructive lesions (LSOL) (4) and hypoplastic right heart syndrome (HRHS) (5), compared to 22 controls at 34 weeks. The mean HC to fetal weight ratio (HC/Wt) was overall significantly altered in the affected group (p=0.09). Sub-analysis by each type of CHD showed that significance was reached just in TOF cases (p<0.03) and not in the others, including HLHS, while CPR was significantly reduced in all type of CHD apart from LSOL and TOF; MCA-PI, however, was not significantly reduced in HRHS and LSOL suggesting that, though UA had normal values there could be a contribution in the calculation of the CPR explaining these differences. In a subsequent study by Kaltman at, CHDs were divided into left-sided (HLHS, 28 cases, and LSOL, 13 cases) and right-sided obstructive lesions (RSOL, 17 cases) and they found that MCA-PI was significantly reduced in HLHS (p<0.001) while MCA-PI was significantly increased (p<0.001) in RSOL compared to HLHS and also UA-PI showed significantly higher values compared to normal (p=0.045) (Kaltman

et al, 2005). They explain the higher MCA-PI values in RSOL to be due to auto-regulation of the MCA to the increased blood flow directed to the brain in such lesions. The discrepancy in results with the article from D'Onofrio may be explained by the different number of cases included and the authors recommend caution in using CPR in fetuses with CHD because reference values were established in IUGR fetuses with a normal heart and they could not be of value in assessing fetuses with CHDs.

Many other studies were published on this topic but there is a high degree of heterogeneity for type of study, inclusion criteria (like gestational age, biometric parameters analyzed), classification of the heart defect adopted.

A recent meta-analysis on HC values report that in fetuses with a CHD a smaller HC is present with a value of only 0,5 SD below the population mean (Jansen *et al,* 2016). Data on each type of CHD could not be evaluated because of the small numbers available for each category. Fewer data are available on AC and FL values.

LVOT lesions, HLHS, TGA and TOF are the defects that were examined more frequently. MCA-PI and CPR were found to be lower in left-sided lesions, HLHS and TOF with the lowest values found in the HLHS groups (Ruiz *et al*, 2017; Masoller *et al,* 2016; Szwast *et al,* 2012; Kaltman *et al,* 2005; Yamamoto *et al,* 2013; Williams *et al,* 2012) while contradictory results were found for TGA: some studies reported lower values while other found similar values to controls (Ruiz *et al,* 2017; Jouannic *et al,* 2002; Yamamoto *et al*, 2013; Berg *et al,* 2009). Fewer studies evaluated MCA-PI in right-sided lesions, but in two that specifically compared left and right-sided lesions with healthy controls, they found the presence of a reduced MCA-PI and CPR in left-sided and increased MCA-PI in right-sided lesions (Szwast *et al,* 2012; Kaltman *et al;* 2005). Contradictory results were reported also for the UA-PI because in some reports increased values of PI were reported in fetuses with CHD, while in others there were no significant differences (Kaltman *et al;* 2005; Meise *et al,* 2001; Szwast *et al*).

Table 1 report a summary of the studies evaluated in this thesis.

Tab. 1.1 Summery of the literature

Study (First Author, journal, Year of Publication)	Study design, No. Infants	CHD	Age	Methods	Findings
Ruiz et al, Ultrasound Obstet Gynecol, *2016*	Retrospective study, N= 119	Mixed	II and III trimester	Ultrasound (biometry, Doppler)	Normal MCA-PI and CPR during second trimester; 18% MCA-PI and CPR less than 5th percentile at 1st examination Lower MCA-PI in group with severe impairment of cerebral blood flow UA-PI increased with GA
Hahn et al, Ultrasound Obstet Gynecol*, 2016*	Retrospective study, *N* = 133	SVA	II and III trimester	Ultrasound (biometry, Doppler)	Lower MCA-PI and decreased more as GA progressed Smaller HC at 24–29 wk GA and >34 wk GA Fetal HC predictor of neonatal HC from 30 wk GA MCA-PI not associated with fetal and neonatal HC
Zeng et al, Ultrasound Obstet Gynecol*, 2015*	Case-control study, *N* = 73/168	Mixed	II and III trimester	Ultrasound (biometry, Doppler)	Lower MCA-PI Total intracranial volume, frontal lobe volume, cerebellar volume, and thalamus volume progressively decreased from 28 wk GA Largest decrease in frontal lobe volume, followed by total intracranial volume and cerebellar volume Smaller HC and BPD from 33 wk GA
Zeng et al, Ultrasound Obstet Gynecol*, 2015*	Case-control study, *N* = 112/112	Mixed	20-30 wks	Ultrasound (Doppler)	Lower MCA-PI in HLHS, MCA-PI tended to be lower in LSOL, normal MCA-PI in TGA and RSOL

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					Higher cerebral blood flow, vascularization index, flow index, and vascularization fow index of the total intracranial volume and 3 main arteries higher in HLHS and LSOL and of the anterior cerebral artery in TGA
Masoller et al, Ultrasound Obstet Gynecol, *2014*	Case-control study, *N* = 95/95	Mixed	20-24 wks	Ultrasound (biometry, Doppler)	Lower MCA-PI and CPR and higher fractional moving blood volume
					Fractional moving blood volume >95th percentile in 81% compared with 11% in controls
					No differences in MCA-PI and fractional moving blood volume between CHD diagnostic groups
					Smaller BPD and HC No differences in BPD and HC between CHD diagnostic groups
Williams et al, Am Heart J, *2013*	Cohort study, *N* = 134	SVA	18-38 wks	Ultrasound (Doppler)	MCA-PI at first fetal echocardiogram −0.95 ± 1.5
					22% MCA-PI < −2.0 at least once across gestation
Yamamoto et al, Ultrasound Obstet Gynecol, *2013*	Case-control study, *N* = 89/89	Mixed	32 wks	Ultrasound (biometry, Doppler)	Lower MCA-PI, higher UA-PI and lower CPR in HLHS and CoA
					CoA with retrograde aortic arch flow, lower MCA-PI and CPR, and higher UA-PI compared with CoA with antegrade flow
					Normal MCA-PI, UA-PI, and CPR in TGA and POTO
					Smaller HC at birth in TGA and CoA
Szwast et al, Ultrasound Obstet Gynecol, *2012*	Retrospective study, *N* = 131/92	SVA	18-40 wks	Ultrasound (Doppler)	Lower MCA-PI and lower CPR in aortic arch obstruction compared with controls and compared

					with pulmonary obstruction
					MCA-PI decreased during gestation for aortic obstruction
					MCA-PI increased during gestation for pulmonary obstruction
					Normal UA-PI
Williams et al, Ultrasound Obstet Gynecol*, 2012*	Pilot study, *N* = 13	Mixed	20-24 wks	Ultrasound (Doppler)	MCA-PI −1.7 ± 1.1 56% CPR < 1.0 (no *z* scores) HLHS and TOF lowest MCA-PI (−2.4 and −2.01, respectively), TGA −0.75
Arduini et al, J Matern Fetal Neonatal Med*, 2011*	Case-control study, *N* = 60/65	Mixed	30-38 wks	Ultrasound (biometry, Doppler)	Lower MCA-PI and CPR (no *z* scores) HLHS and CoA lowest and TOF and TGA highest CPR
					Smaller HC and HC/AC HLHS and CoA lowest and TOF and TGA highest HC/AC
Itsukaichi et al, Fetal Diagn Ther*, 2011*	Retrospective study, *N* = 44/140	Mixed	28-34 wks	Ultrasound (biometry, Doppler)	MCA-RI measurements more often less than 5th percentile and UA-RI >90th percentile
					Similar biometry measurements in fetuses <10th and >10th MCA-RI percentile
Berg et al, Ultrasound Obstet Gynecol*, 2009*	Case-control study, *N* = 113/137	Mixed	19-41 wks	Ultrasound (biometry, Doppler)	Smaller HC at birth, normal MCA-PI and CPR in TGA
					Smaller HC at birth, lower MCA-PI and CPR in HLHS
					Normal biometry and Doppler parameters in PA, AoS, and TOF
Guorong et al, Fetal Diagn Ther*, 2009*	Case-control study, *N* = 45/275	Mixed	20-40 wks	Ultrasound (Doppler)	Normal MCA-PI MCA-PI tended to be lower in LSOL and was lower in congestive heart failure
					Higher UA-PI and higher u/C PI ratios No traditional "brain

					sparing" as MCA-PI was normal, whereas U/C PI was higher
Kaltman et al, Ultrasound Obstet Gynecol*, 2005*	Case-control study, *N* = 58/114	Mixed	20-40 wks	Ultrasound (Doppler)	Lower MCA-PI in HLHS Higher MCA-PI in RSOL compared with HLHS Higher UA-PI in RSOL
Donofrio et al, Pediatr Cardiol*, 2003*	Case-control study, N= 36/21	Mixed	II and III trimester	Ultrasound (Doppler)	Lower MCA-RI and CPR Normal UA-RI HLHS and HRHS infants had highest incidence of abnormally low CPR (58% and 60%)
Jouannic et al, Ultrasound Obstet Gynecol*, 2002*	Case-control study, N= 23/40	TGA	36-38 wks	Ultrasound (Doppler)	Lower MCA-PI Normal UA-PI, DV-PI, and Ao-PI (no *z* scores)
Meise et al, Ultrasound Obstet Gynecol*, 2001*	Case-control study, N= 115/100	Mixed	19-41 wks	Ultrasound (Doppler)	Normal MCA-PI Higher UA-PI No difference in UA-PI >95th percentile
Masoller et al, Ultrasound Obstet Gynecol*, 2016*	Case-control study, *N* = 116/116	Mixed	30-38 wks	Ultrasound (biometry, Doppler)	Lower MCA-PI and CPR and higher fractional moving blood volume CHD diagnostic groups Smaller BPD and HC No differences in AC and FL between CHD and controls
Modena et al, Am J Obstet Gynecol*, 2006*	Case-control study, *N* = 71/71	Mixed	24-28 wks	Ultrasound (Doppler)	Normal MCA-PI, UA-PI, and CPR MCA-PI more often less than 5th percentile (5/71 vs 0/71) CPR more often less than 5th percentile (8/71 vs 2/71) No difference in UA-PI >95th percentile (6/71 vs 3/71)

CHD, congenital heart defects; MCA-PI, middle cerebral artery pulsatility index; CPR, cerebro-placental ratio; UA-PI, umbilical artery pulsatility index; GA, gestational age; AC, abdominal circumference; Ao, aorta; AoS, aortic stenosis; BPD, biparietal diameter; CoA, coarctation of the

aorta; DV-PI, pulsatily index of the ductus venosus; HC/AC, head circumference/abdominal circumference; HRHS, hypoplastic right heart syndrome; LSOL, left-sided obstructive lesion; POTO, pulmonary outflow tract obstruction; RSOL, right-sided obstructive lesion; SVA, single ventricle anomaly; TOF, tetralogy of Fallot; U/C PI, pulsatility index of the umbilical artery/pulsatility index of the middle cerebral artery.

Overall, as found in a recently published meta-analysis (Mebius *et al,* 2017), it can be concluded that the existing evidence suggest a tendency towards a brain vasodilation, as reflected by MCA evaluation, mainly in those cardiac defects with impaired blood flow to the brain. However, it is not clear whether the clinical meaning of these findings is the same as in SGA fetuses.

Despite the efforts put in identifying markers of impaired intrauterine brain development in the presence of isolated major congenital heart defects, the controversial data from the studies published so far do not allow a proper counseling for each type of cardiac defects in the prediction of the risk of post-natal NDD. A recent consensus statement from the International Society of Ultrasound on Obstetrics and Gynecology (ISUOG) recommend a cautious prenatal counseling of fetuses with CHDs since there is a lack of evidence in the relationship between CHDs, HC growth, prenatal Doppler evaluation and post-natal NDD (ISUOG, 2017).

Chapter 2 HYPOTHESIS

2.1   Main hypothesis

Isolated major CHDs are characterized by the presence of placental dysfunction.

2.2   Specific hypothesis

1. Placental dysfunction in fetuses with isolated major CHDs is reflected in an impaired angiogenic status since the first trimester of pregnancy.
2. Placental dysfunction affects fetal growth in the second and third trimester of pregnancy.
3. The combination of a fetal CHD and placental dysfunction is reflected in abnormalities of fetal-placental Doppler.

Chapter 3 OBJECTIVES

3.1   Main objective

To evaluate the correlation between isolated major CHDs and placental dysfunction and the impact on intrauterine growth and fetal Doppler throughout the pregnancy.

3.2   Specific objective

1. To evaluate the relationship between isolated major CHDs and markers of placental perfusion and function in the first trimester of the pregnancy.
2. To evaluate the relationship between isolated major CHDs, fetal growth pattern and markers of placental perfusion in the second and third trimester of the pregnancy.
3. To evaluate the relationship between isolated major CHDs, placental perfusion and fetal-placental Doppler (MCA-PI and UA-PI) in the third trimester of pregnancy.

Chapter 4 METHODS

4.1   Study population

The data for this study were derived from prospective screening for adverse obstetric outcomes in women attending for routine pregnancy care at King's College Hospital and Medway Maritime Hospital, United Kingdom. The women were screened between March 2006 and October 2015 and gave written informed consent to participate in the study, which was approved by the Ethics Committee.

At the first visit, a complete recording of maternal demographic characteristics and obstetric and medical history was taken with measurement of maternal weight and height.

The first trimester ultrasound examination was performed at 11-13+6 weeks' gestation and included: measurement of the fetal crown-rump length (CRL) to determine gestational age (Robinson *et al* 1975), measurement of the fetal nuchal translucency (NT) thickness (Nicolaides *et al* 1994), examination of the fetal anatomy for the diagnosis of major fetal defects (Syngelaki *et al* 2011), and transabdominal colour Doppler ultrasound for the measurement of UtA-PI (Plasencia *et al* 2007). The policy of both hospitals for the anatomical evaluation of the fetal heart in the first trimester is to get a color-Doppler view of the 4-chamber and the confluence of the aorta and pulmonary artery also known as "V-sign" (Syngelaki *et al.,* 2011). Maternal serum PAPP-A and free ß-human chorionic gonadotropin were sampled for combined screening for fetal aneuploidies (Nicolaides *et al* 2011), while maternal serum PlGF was sampled for research purposes.

The second trimester ultrasound examination was performed at 20+0-23+6 weeks and included: estimation of fetal size from transabdominal ultrasound measurements of fetal head circumference (HC), abdominal circumference (AC) and femur length (FL) plus transabdominal color Doppler ultrasound for the measurements of the UtA-PI (Albaiges *et al* 2000).

This scan involved systematic detailed examination of the fetus, including a sweep

through the heart in a transverse plane to include the four-chamber view, outflow tracts and three vessel view of the heart and great vessels with and without color Doppler.

If a fetal abnormality was suspected, the case was examined by a fetal medicine specialist. Likewise, all cases of suspected fetal cardiac defect were examined by a fetal cardiologist. In addition, the cardiologist carried out a fetal echocardiography at 11-14 weeks in those with NT values above the 99th centile and at 20 weeks in those with a NT between the 95th and 99th centiles.

All cases with a normal second trimester scan were offered a routine third trimester scan at 36 weeks' gestation while those with a diagnosis of CHD were offered serials growth scan at 24+0 - 28+6 weeks, at 30+0 - 34+6 weeks, and at 35+0 - 37+6 weeks.

The ultrasound examination included, for both groups, estimation of fetal size from transabdominal ultrasound measurements of HC, AC and FL. Transabdominal colour Doppler ultrasound was used to visualize the umbilical artery (UA), the middle cerebral artery (MCA) and the UtA. Doppler parameters were recorded automatically from consecutive waveforms and measured during periods of fetal quiescence. An angle of insonation below 30° was employed. The UA waveform was recorded by assessing a single free loop of umbilical cord using the colour Doppler. The UA-PI was calculated by applying a standard formula (Acharya *et al* 2005). The MCA Doppler was recorded according to a standard protocol by obtaining a transverse section of the fetal head and identifying the vessel close to the circle of Willis using colour Doppler and pulsed-wave Doppler to assess impedance to flow and PI was measured when three similar consecutive waveforms were observed for all three vessels (Bahlmann *et al.* 2002; Vyas *et al* 1990).

The left and the right uterine arteries were identified at the level of the crossover with the external iliac artery using colour Doppler (Albaiges *et al.,* 2000). After identification of each UtA, pulsed-wave Doppler was used with the sampling gate set at 2 mm to cover the whole vessel. Care was taken to ensure that the angle of insonation was less than 30◦ and the peak systolic velocity was greater than 60 cm/s to ensure that the UtA, rather than the arcuate artery, was being examined. When three similar waveforms had been obtained consecutively, the PI was measured, and the mean PI of the left and right arteries was calculated.

All neonates were examined by a pediatrician. Prenatal and neonatal findings were recorded in computerized databases. Data on pregnancy outcome from women who booked for obstetric care in our hospitals but delivered in other hospitals were obtained either from the maternity computerized records in these hospitals or the general medical practitioners of the women.

4.2   Inclusion and exclusion criteria

All cases with major cardiac defects diagnosed by pediatric cardiologists either antenatally and / or in the neonatal period were included if the subsequent criteria were met:

- measurements of maternal serum PAPP-a, βHCG, PlGF levels in the 11-13 weeks group and color Doppler UT-PI;
- measurements of fetal biometry and UtA-PI in the 20-24 weeks group;
- measurements of fetal biometry and UtA-PI, UA-PI and MCA-PI in the 30-38 weeks group.

Abnormalities suspected antenatally but not confirmed in the neonates were not included. In contrast, the prenatal diagnosis in cases of terminations and miscarriages at < 24 weeks or stillbirths at > 24 weeks were assumed to be correct because in these cases postmortem examination was not performed systematically.

The following fetal cardiac defects were not included: firstly, ventricular septal defects not requiring surgery because they are generally not considered to be major defects, secondly, right aortic arch, persistent left superior vena cava and aberrant right subclavian artery because they are not supposed to cause fetal hemodynamic changes and thirdly, cardiac tumors developing during the second and third trimesters of pregnancy because these defects would not be expected to have any manifestations during the 11-13 weeks scan.

All cases with aneuploidies and/or non-cardiac defects diagnosed prenatally or in the neonatal period were excluded as well as pregnancies with no abnormal fetal findings at the 11-13 weeks scan and / or the 20-24 weeks scan which resulted in termination, miscarriage or stillbirth and those lost to follow up.

4.3   Statistical analysis

Data from continuous variables were expressed as medians and interquartile ranges and from categorical data as n (%). Comparison of the maternal characteristics between the outcome groups was by the χ2-square test or Fisher's exact test for categorical variables and Mann-Whitney U-test for continuous variables, respectively. A p value of < 0.05 was considered significant. Post-hoc Bonferroni correction was used for multiple comparisons.

For the first trimester the measured values of PAPP-A, PIGF and UTPI were log10 transformed to make their distributions Gaussian and each value was expressed as a multiple of the normal median (MoM) after adjustment for those characteristics that provide a substantial contribution to the log10 transformed value. The measured fetal NT was expressed as a difference from the expected normal mean for fetal CRL (delta value). Median MoM values of biomarkers were compared between outcome groups. We divided congenital cardiac defects into those with fetal NT< 3.5 and those with measurements ≥ 3.5 and compared the significant of difference in the biomarkers in each group. Non-parametric bivariate correlation analysis was used to examine the association between biomarkers in pregnancies with congenital cardiac defects and those with normal cardiac anatomy.

For the second and third trimester the observed measurements of fetal HC, AC and FL were expressed as the respective *Z*-score corrected for gestational age. UtA-PI was analyzed as described for the first trimester.

For the third trimester, UA-PI and MCA-PI were log10 transformed to make their distributions Gaussian and each value was expressed as a multiple of the normal median (MoM) after adjustment for those characteristics that provide a substantial contribution to the log10 transformed value.

The statistical software package SPSS 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp, 2013) was used for the data analyses.

4.4   Classification of cardiac defects: first and second trimester

Major cardiac defects were subdivided into three groups based on the previous

publication from Llurba *et al.* (Llurba *et al.,* 2013):

1) **Conotruncal defects:** tetralogy of Fallot (TOF), transposition of great arteries (TGA), double outlet right ventricle (DORV), and common arterial trunk.

2) **Left ventricular outflow tract (LVOT) defects:** hypoplastic left heart syndrome (HLHS), aortic stenosis (AS), coarctation of aorta (CoA), and interrupted aortic arch.

3) **Valvular defects:** atrioventricular septal defects (AVSD), tricuspid stenosis or atresia (TS or TA), pulmonary stenosis or atresia (PS or PA), and Ebstein's anomaly.

CHAPTER 5   RESULTS

5.1   First trimester

5.1.1   Maternal and pregnancy characteristics

The 50,094 singleton pregnancies fulfilling the entry criteria included 49,898 pregnancies with a normal cardiac anatomy and 196 (0.4%) with major congenital cardiac defects. Of those, 73 (37.2%) were with conotruncal defects, 63 (32.1%) with LVOT defects and 60 (30.6%) with valvular abnormalities, respectively. The maternal and pregnancy characteristics are represented in Table 5.1.

Table. 5.1 Demographic and pregnancy characteristics in fetuses with congenital heart defects, stratified according to sub-groups, compared to those with normal cardiac anatomy.

Maternal characteristics	No cardiac defect (n=49,898)	All cardiac defects (n=196)
Age, median (IQR)	31.2 (26.7-34.8)	31.7 (26.1-36.1)
Weight, median (IQR)	66.9 (59.1-77.7)	67.0 (58.5-78.9)
Height, median (IQR)	1.65 (1.60-1.69)	1.65 (1.59-1.70)
Racial origin		
Caucasian, n (%)	36,327 (72.8)	146 (74.5)
Afro-Caribbean, n (%)	8,823 (17.7)	33 (16.8)
South Asian, n (%)	2,296 (4.6)	9 (4.6)
East Asian, n (%)	1,123 (2.3)	5 (2.6)
Mixed, n (%)	1,329 (2.7)	3 (1.5)
Method of conception		
Spontaneous, n (%)	48,317 (96.8)	189 (96.4)
Assisted conception, n (%)	1,581 (3.2)	7 (3.6)
Cigarette smoking, n (%)	4,595 (9.2)	16 (8.2)
Chronic hypertension, n (%)	735 (1.5)	2 (1.0)

SLE / APS, n (%)	114 (0.2)	0
Diabetes mellitus, n (%)	435 (0.9)	5 (2.6) *
Nulliparous, n (%)	25,003 (50.1)	101 (51.5)
Inter-pregnancy interval, median (IQR)	3.0 (2.0-4.9)	2.8 (1.8-3.9)

Post hoc Bonferroni correction for multiple comparisons; * = p< 0.0167; LVOT = left ventricular outflow tract; IQR = interquartile range; SLE = systemic lupus erythematosus; APS = antiphospholipid syndrome.

5.1.2   Biomarkers in outcome groups

In the cardiac defect group, compared to the normal cardiac anatomy group, the median PlGF MoM and PAPP-A MoM were lower, fetal delta NT was higher and UT-PI MoM was not significantly different. In all the sub-groups of congenital cardiac defects, this trend was maintained with lower PLGF and PAPP-A MoMs, higher delta fetal NT but no significant difference in UT-PI MoM (Table 5.2, Figure 5.1).

Table 5.2. First trimester median and interquartile range of biomarkers in fetuses with congenital cardiac defects compared to those with a normal cardiac anatomy.

Marker	No cardiac defect (n=49,898)	All cardiac defects (n=196)	Conotruncal defects (n=73)	LVOT defects (n=63)	Valvular defects (n=60)
Serum PAPP-A MoM	1.00 (0.69-1.42)	0.81 (0.52-1.27)**	0.73 (0.57-1.18)*	0.73 (0.44-1.29)*	0.90 (0.55-1.32)
Serum PlGF MoM	1.00 (0.77-1.29)	0.78 (0.56-1.07)**	0.75 (0.55-0.97)**	0.80 (0.56-1.19)*	0.74 (0.61-1.17)*
UTPI MoM	1.00 (0.81-1.22)	1.01 (0.83-1.26)	0.97 (0.81-1.28)	1.05 (0.84-1.29)	1.00 (0.89-1.25)
Delta fetal NT	0.00 (-0.20-0.22)	0.28 (-0.06-0.86)**	0.19 (-0.11-0.68)**	0.47 (-0.02-0.89)**	0.24 (-0.04-0.96)**

Significance value *p<0.01; **p<0.001; *post hoc* Bonferroni correction for multiple comparisons; LVOT = Left ventricular outflow tract; PAPP-A = pregnancy associated plasma protein-A; PLGF = placental growth factor; UTPI = uterine artery pulsatility index ; NT = nuchal translusency; MoM = Multiple of normal median.

Figure 5.1 First trimester maternal serum placental growth factor in pregnancies with major congenital cardiac defects compared to those without defects and for each group of cardiac defects. The cardiac defect group is subdivided according to high or normal NT and according to type of defect.
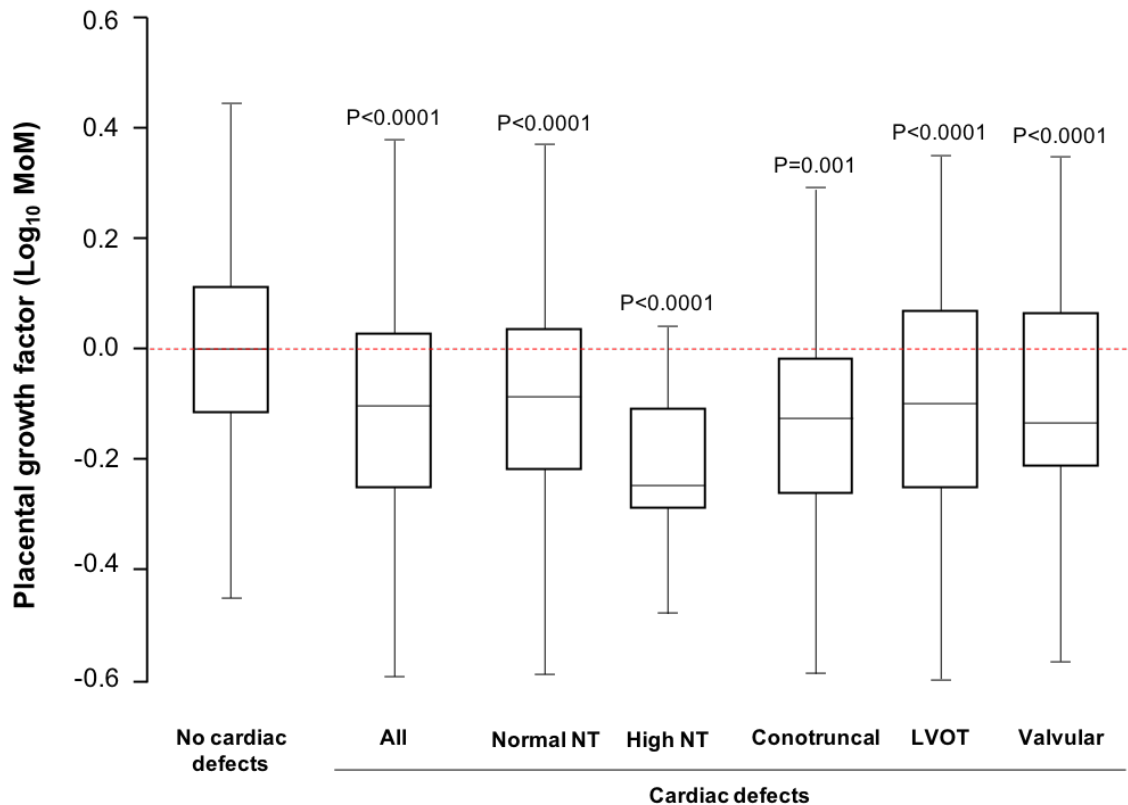
LVOT, left ventricular outflow tract

A significant association between PlGF MoM and delta fetal NT was found in the group with cardiac defects but not in those without defects (Table 5.3). In fetuses with cardiac defects and increased NT PlGF MoM was significantly lower in those with increased NT, compared to those with normal NT (0.56 vs 0.83 MoM; p=0.007) (Figure 5.2); there was no significant difference in PAPP-A MoM between the two groups (0.84 vs 0.79 MoM; p=0.586).

Table 5.3 First trimester correlations between biophysical and biochemical markers in fetuses with and without congenital cardiac defects.

Marker	Congenital cardiac defects			
	Serum PlGF MoM	Serum PAPP-A MoM	Uterine artery PI MoM	Delta fetal NT
Serum PlGF MoM	-	r = 0.34; p<0.0001	r = -0.23; p = 0.001	r = -0.15; p = 0.03
Serum PAPP-A MoM		-	r = -0.22; p = 0.002	r = -0.010; p = 0.9
Uterine artery PI MoM			-	r = -0.08; p = 0.3
Delta fetal NT				-
	Normal cardiac anatomy			
	Serum PlGF MoM	Serum PAPP-A MoM	Uterine artery PI MoM	Delta fetal NT
Serum PlGF MoM	-	r = 0.29; p<0.0001	r = -0.13; p<0.0001	r = 0.004; p = 0.4
Serum PAPP-A MoM		-	r = -0.15; p<0.0001	r = 0.02; p <0.0001
Uterine artery PI MoM			-	r = -0.006; p = 0.2
Delta fetal NT				-

PAPP-A = pregnancy associated plasma protein-A; PLGF = placental growth factor; UTPI = uterine artery pulsatility index ; NT = nuchal translusency; MoM = Multiple of normal median.

5.2   Second trimester

5.2.1   Maternal and pregnancy characteristic

The 93,408 singleton pregnancies fulfilling the entry criteria included 92,779 pregnancies with a normal cardiac anatomy and 629 (0.7%) with major congenital cardiac defects. The maternal and pregnancy characteristics in the outcome groups are represented in Table 5.4.

Table 5.4 Second trimester maternal and pregnancy characteristics in fetuses with congenital cardiac defect compared to those with normal cardiac anatomy

Maternal characteristics	No cardiac defect (n=92,779)	All cardiac defects (n=629)
Age, median (IQR)	30.9 (26.3-34.9)	31.0 (26.0-35.3)
Weight, median (IQR)	67.0 (56.9-77.6)	64.0 (59.1-72.2)
Height, median (IQR)	164.0 (160.0-168.5)	165.0 (160.0-167.74)
Racial origin		
Caucasian, n (%)	65837 (71.0)	489 (77.7)
Afro-Caribbean, n (%)	17059 (18.4)	74 (11.8) **
South Asian, n (%)	5093 (5.5)	47 (7.5) *
East Asian, n (%)	2583 (2.8)	8 (1.3)
Mixed, n (%)	2207 (2.4)	11 (1.7)
Method of conception		
Spontaneous, n (%)	90593 (97.6)	611 (97.1)
Assisted conception, n (%)	2186 (2.4)	18 (2.9)
Cigarette smoking, n (%)	9456 (10.2)	64 (10.2)
Chronic hypertension, n (%)	1207 (1.3)	1 (0.2) *
SLE / APS, n (%)	179 (0.2)	1 (0.2)
Diabetes mellitus, n (%)	859 (0.9)	6 (1.0)
Nulliparous, n (%)	46294 (49.9)	286 (45.5)
Inter-pregnancy interval, median (IQR)	2.8 (1.8-4.7)	3.0 (2.6-3.4)

Post hoc Bonferroni correction for multiple comparisons; * = $p < 0.05$; ** = $p < 0.001$; IQR = interquartile range; SLE = systemic lupus erythematosus; APS = antiphospholipid syndrome.

Overall, the incidence of CHDs was significantly higher in Afro-Caribbean and South Asian ethnic groups compared with controls (11.8% and 7.5%, p<0.001 and p<0.005,

respectively). In women with chronic hypertension there was a lower incidence of CHD compared to control (1.0% vs 0.9%, p<0.05).

5.2.2 Fetal biometry and UtA Doppler in all CHDs compared to control group

Results for fetal biometry are shown in Table 5.5 and Figure 5.2. The calculated Z-score for HC, AC and FL measurements in pregnancies with CHD is compared to controls: Z-scores of HC, AC and FL were significantly lower than controls (-0.26, -0.16 and -0.26, respectively, $p < 0.001$) while there was no difference in UtA-PI between the two groups (1.00 vs 1.03).

Table 5.5 Second trimester calculated z-score for head circumference, abdominal circumference and femur length, in fetuses with congenital heart defects compared to those with normal cardiac anatomy.

Marker	No cardiac defect (n=92,779)	All cardiac defects (n=629)
Head circumference z-score	0.00 (-0.46 – 0.47)	-0.26 (-0.92 – 0.30)**
Abdominal circumference z-score	0.00 (-0.43 – 0.42)	-0.16 (-0.66 – 0.42)**
Femur length z-score	0.00 (-0.45 – 0.46)	-0.26 (-0.87 – 0.25)**
Uterine artery PI MoM	1.00 (0.84 – 1.20)	1.03 (0.87 – 1.31)

Significance value * p<0.01; ** p<0.001; *post hoc* Bonferroni correction for multiple comparisons

Figure 5.2 Second trimester box-and-whisker plots of Z-scores for fetal head circumference (a), abdominal circumference (b), femur length (c) and uterine artery pulsatility index (d) in fetuses with congenital heart defects compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.
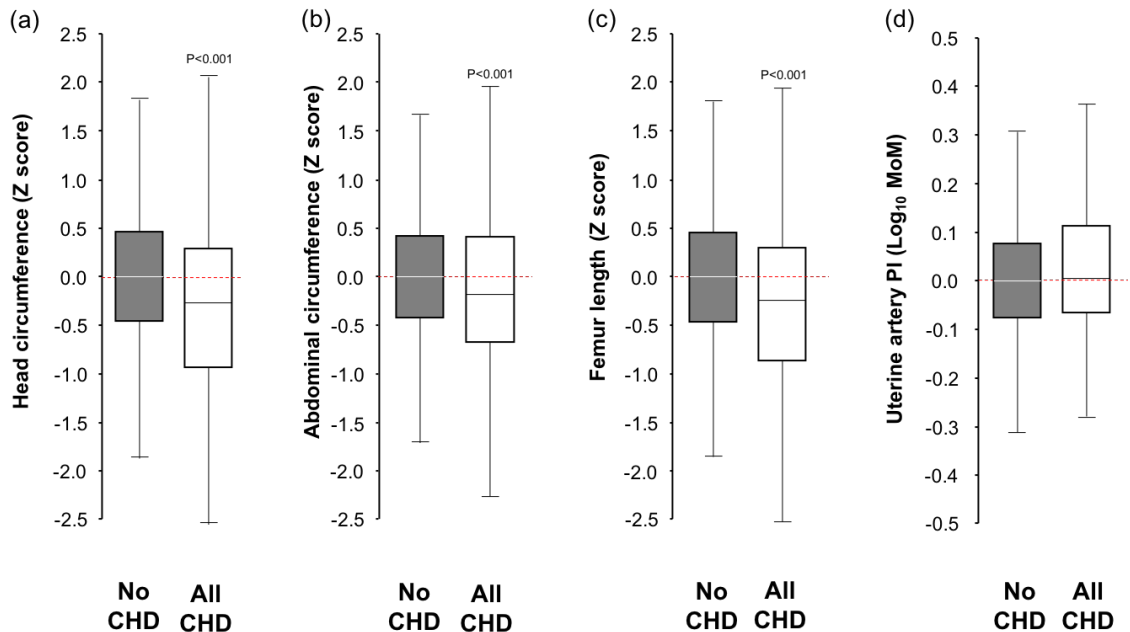
CHD, congenital heart defects

5.2.3 Fetal biometry and UtA Doppler in CHD, divided by sub-groups and compared to controls

Table 5.6 shows the Z-scores for HC, AC and FL measurements in pregnancies with CHD, divided in three sub-groups and compared to control group: HC and FL were significantly smaller in all subgroups of CHDs (p < 0.001); significantly lower values for AC Z-score were found in LVOT defects and, though a trend towards lower Z-score values was observed also for conotruncal and valvular defects, the difference did not reach a statistical significance. Same results are shown in Figure 5.4, Figure 5.5 and Figure 5.6 with the box-and-whiskers plot graphs for HC, AC and FL Z-score, respectively.

Table 5.6 Second trimester median and interquartilie range of fetal biometric parameter in fetuses with congenital cardiac defects, stratified according to sub-groups, compared to those with a normal cardiac anatomy.

Marker	No cardiac defect (n=92,779)	Conotruncal defects (n=269)	LVOT defects (n=225)	Valvular defects (n=135)
Head circumference z-score	0.00 (-0.46 – 0.47)	-0.29 (-0.98 – 0.20)**	-0.15 (-0.84 – 0.44)**	-0.39 (-0.87 – 0.30)**
Abdominal circumference z-score	0.00 (-0.43 – 0.42)	-0.14 (-0.69 – 0.45)	-0.22 (-0.68 – 0.29)**	-0.08 (-0.63 – 0.61)
Femur length z-score	0.00 (-0.45 – 0.46)	-0.24 (-0.83 – 0.33)**	-0.26 (-0.93 – 0.22)**	-0.32 (-0.83 – 0.33)**

Significance value * $p<0.01$; ** $p<0.001$; *post hoc* Bonferroni correction for multiple comparisons; LVOT = Left ventricular outflow tract

Fig. 5.4 Second trimester Box-and-whisker plots of Z-scores for head circumference (HC) in fetuses with congenital heart defects, stratified according to sub-groups, compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.
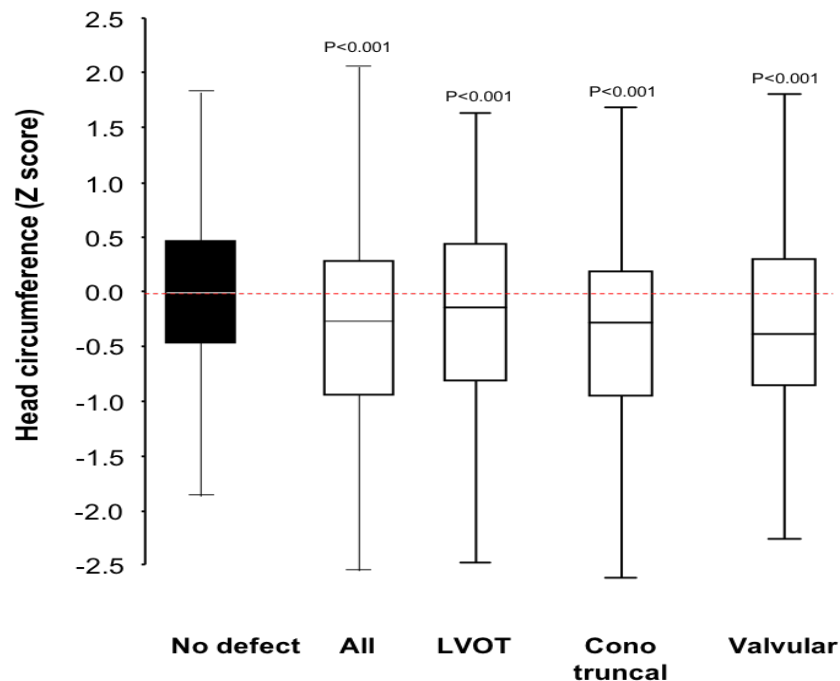
LVOT, left ventricular outflow

Fig. 5.5 Second trimester box-and-whisker plots of Z-scores for abdominal circumference (AC) in fetuses with congenital heart defects, stratified according to sub-groups, compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.

LVOT, left ventricular outflow

Fig. 5.6 Second trimester box-and-whisker plots of Z-scores for femur length (FL) in fetuses with congenital heart defects, stratified according to sub-groups, compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.
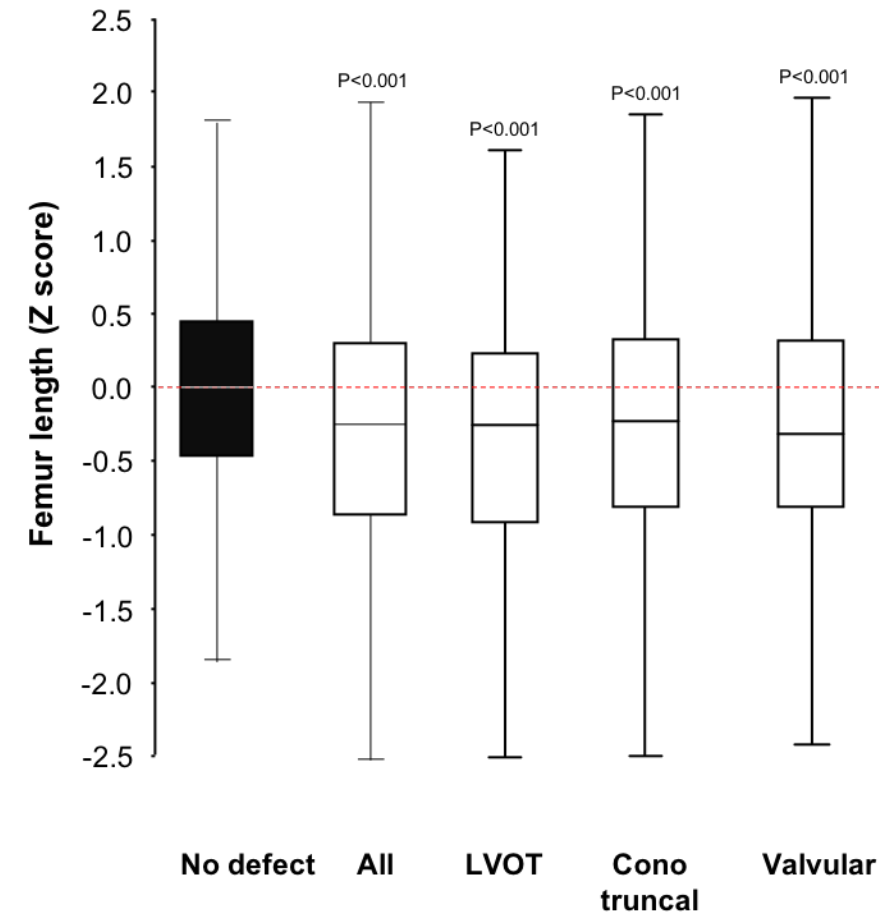
LVOT, left ventricular outflow

5.3   Third trimester

5.3.1   Maternal and pregnancy characteristics

The 47,884 singleton pregnancies fulfilling the entry criteria included 47,716 pregnancies with a normal cardiac anatomy and 168 (0.35%) with major congenital cardiac defects. The maternal and pregnancy characteristics in the outcome groups are represented in Table 5.7. An increased incidence of CHD was observed in women with a lower weight than in the control group (p < 0.001).

Table 5.7 Third trimester maternal and pregnancy characteristics in fetuses with congenital cardiac defect compared to those with normal cardiac anatomy

Maternal characteristics	No cardiac defect (n=47,716)	All cardiac defects (n=168)
Age, median (IQR)	31.3 (26.7 – 35.1)	30.5 (24.7 – 35.0)
Weight, median (IQR)	76.6 (68.5 – 87.0)	65.0 (60.1 – 77.8)**
Height, median (IQR)	164 (160 – 169)	165 (160 – 170)
Racial origin		
Caucasian, n (%)	33,725 (70.7)	127 (75.6)
Afro-Caribbean, n (%)	8,935 (18.7)	25 (14.9)
South Asian, n (%)	2,527 (5.3)	11 (6.5)
East Asian, n (%)	1,290 (2.7)	1 (0.6)
Mixed, n (%)	1,239 (2.6)	4 (2.4)
Method of conception		
Spontaneous, n (%)	46,124 (96.7)	161 (95.8)
Assisted conception, n (%)	1,592 (3.3)	7 (4.2)
Cigarette smoking, n (%)	4,432 (9.3)	16 (9.5)
Chronic hypertension, n (%)	666 (1.4)	0
SLE / APS, n (%)	119 (0.2)	1 (0.6)
Diabetes mellitus, n (%)	465 (1.0)	0
Nulliparous, n (%)	23,305 (48.8)	82 (48.8)
Inter-pregnancy interval, median (IQR)	2.9 (1.9 – 4.8)	3.0 (2.9 – 3.0)

Post hoc Bonferroni correction for multiple comparisons; ** = p < 0.001; IQR = interquartile range; SLE = systemic lupus erythematosus; APS = antiphospholipid syndrome.

5.3.2 Fetal biometry and fetal-maternal Doppler in CHD compared to control group

Results for fetal biometry and fetal-maternal Doppler parameters are shown in Table 5.8, Figure 5.7 and Figure 5.8. Overall, the calculated Z-scores for HC, AC and FL measurements in pregnancies with CHD were significantly lower than in control group ($p < 0.001$). UtA-PI and UA-PI were significantly higher in fetuses with CHD than in control group ($p < 0.01$ and $p < 0.001$, respectively) while there was no difference in MCA-PI between the two groups (1.01 vs 1.02).

Table 5.8 Third trimester median and interquartilie range of biomarkers in fetuses with congenital cardiac defects compared to those with a normal cardiac anatomy

Marker	No cardiac defect (n=47,716)	All cardiac defects (n=168)
Head circumference z-score	0.00 (-0.41 – 0.44)	-0.18 (-0.70 – 0.39)**
Abdominal circumference z-score	0.00 (-0.40 – 0.41)	-0.26 (-0.87 – 0.35)**
Femur length z-score	0.00 (-0.45 – 0.46)	-0.53 (-1.27 – 0.22)**
Uterine artery PI MoM	1.00 (0.85 – 1.19)	1.05 (0.89 – 1.37)*
Umbilical artery PI MoM	1.01 (0.91 – 1.13)	1.11 (0.96 – 1.27)**
Middle cerebral artery PI MoM	1.01 (0.90 – 1.12)	1.02 (0.84 – 1.14)

Significance value * $p<0.01$; ** $p<0.001$; *post hoc* Bonferroni correction for multiple comparisons; MoM = Multiple of the Median

Figure 5.7 Third trimester box-and-whisker plots of Z-scores for fetal head circumference (a), abdominal circumference (b), femur length (c) in fetuses with congenital heart defects compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.
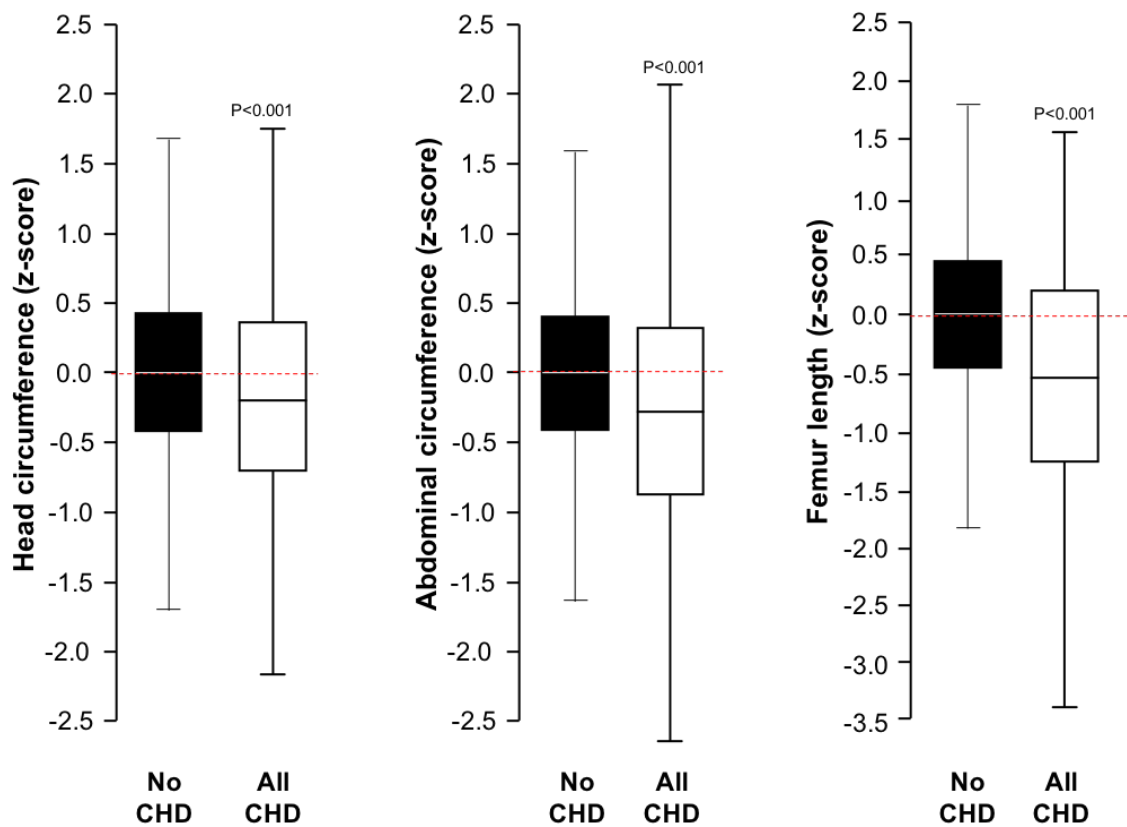
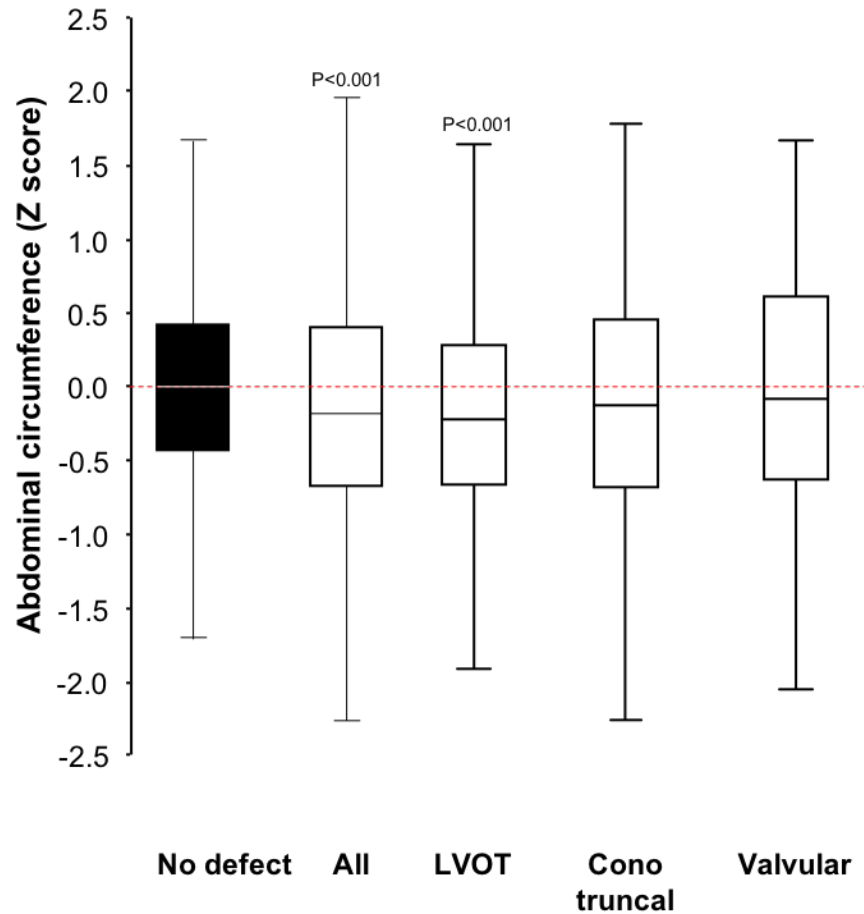CHD, congenital heart defects

Figure 5.8 Third trimester box-and-whisker plots of Z-scores for umbilical artery pulsatility index (a), middle cerebral artery pulsatility index (b) in fetuses with congenital heart defects compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.
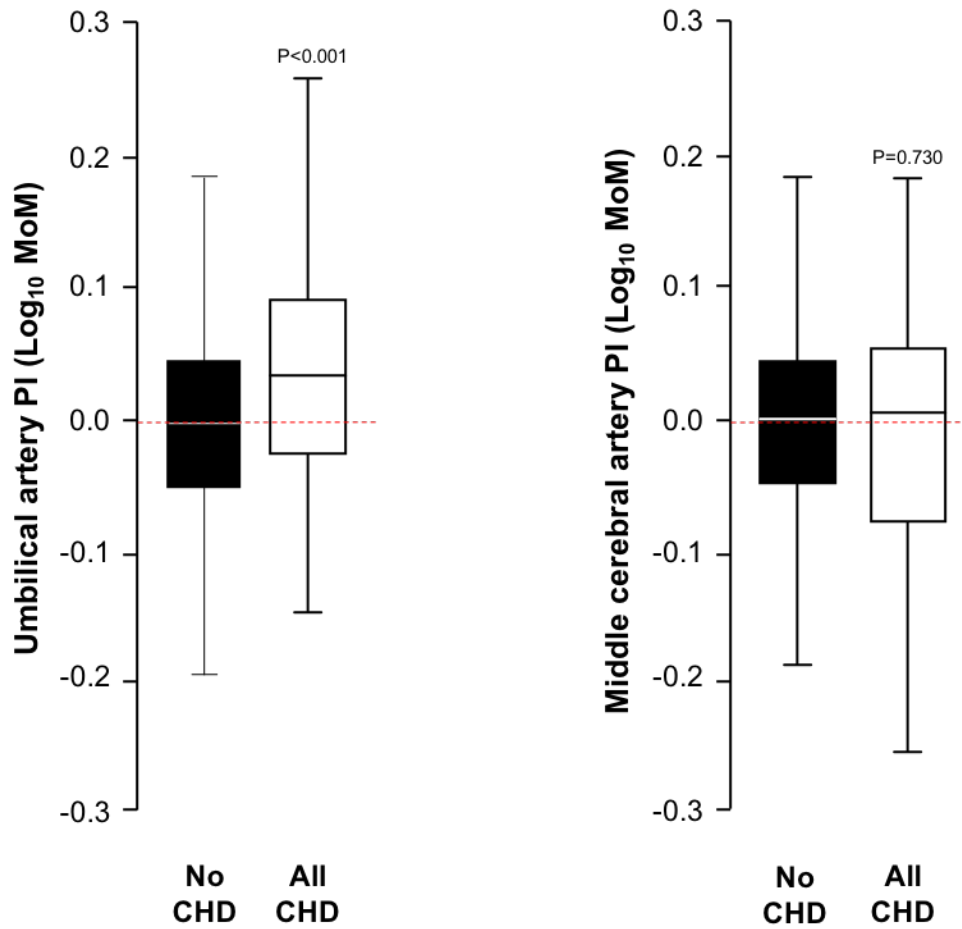
CHD, congenital heart defects

5.3.3 Fetal biometry and fetal-maternal Doppler in CHD, divided by sub-groups, compared to control group

Table 5.9 shows the Z-scores for HC, AC and FL measurements in pregnancies with CHD, divided in the three sub-groups, and compared to control group:

The HC was significantly smaller just in LVOT subgroup but not in valvular and conotruncal defects; AC was significantly smaller in LVOT and valvular defect but not in conotruncal, respectively; FL was significantly smaller in all CHD subgroups ($p < 0.0167$). For Doppler parameters, UA-PI was significantly higher in all CHD subgroups, while MCA-PI was significantly reduced just in LVOT defects subgroup. There were no differences in UtA-PI among the study groups.

Table 5.9 Third trimester median and interquartile range of biomarkers and Doppler parameters in fetuses with congenital cardiac defects, stratified according to sub-groups and compared to those with a normal cardiac anatomy.

Marker	No cardiac defect (n=47,716)	Conotruncal defects (n=62)	LVOT defects (n=48)	Valvular defects (n=58)
Head circumference z-score	0.00 (-0.41 – 0.44)	-0.22 (-0.76 – 0.52)	-0.32 (-0.66 – 0.28)*	0.03 (-0.61 – 0.31)
Abdominal circumference z-score	0.00 (-0.40 – 0.41)	-0.29 (-0.79 – 0.30)	-0.74 (-1.15 – 0.14)*	-0.38 (-1.06 – 0.14)*
Femur length z-score	0.00 (-0.45 – 0.46)	-0.49 (-1.01 – 0.15)*	-0.61 (-1.64 – 0.23)*	-0.56 (-1.30 – 0.52)*
Uterine artery PI MoM	1.00 (0.85 – 1.19)	1.12 (0.93 – 1.50)	0.99 (0.90 – 1.50)	1.01 (0.85 – 1.28)
Umbilical artery PI MoM	1.01 (0.91 – 1.13)	1.05 (0.94 – 1.27)*	1.14 (0.95 – 1.29)*	1.13 (0.99 – 1.24)*
Middle cerebral artery PI MoM	1.01 (0.90 – 1.12)	1.06 (0.84 – 1.15)	0.90 (0.80 – 1.04)*	1.07 (0.91 – 1.20)

Significance value * p<0.0167; *post hoc* Bonferroni correction for multiple comparisons;
LVOT = Left ventricular outflow tract; PI = pulsatility index; MoM = Multiple of normal median.

Same results are shown in Figure 5.9, Figure 5.10, Figure 5.11, Figure 5.12 and Figure 5.13 with the box-and-whiskers plot graphs for HC, AC, FL, UA-PI and MCA-PI Z-score, respectively.

Figure 5.9 Third trimester box-and-whisker plots of Z-scores for head circumference in fetuses with congenital heart defects, stratified according to sub-groups, compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.

Figure 5.10 Third trimester box-and-whisker plots of Z-scores for abdominal circumference in fetuses with congenital heart defects, stratified according to sub-groups, compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.

Figure 5.11 Third trimester box-and-whisker plots of Z-scores for femur length in fetuses with congenital heart defects, stratified according to sub-groups, compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.

Fig. 5.12 Third trimester Box-and-whisker plots of Z-scores for umbilical artery pulsatility index in fetuses with congenital heart defects, stratified according to sub-groups, compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.

Fig. 5.13 Third trimester box-and-whisker plots of Z-scores for middle cerebral artery pulsatility index in fetuses with congenital heart defects, stratified according to sub-groups, compared to those with normal cardiac anatomy. Boxes with interlines represent median and interquartile range and whiskers are range.

CHAPTER 6   DISCUSSION

Principal findings of the study: in fetuses with CHDs there is a placental dysfunction, supported by lower concentration of maternal serum PlGF and PAPP-a, in the presence of normal uterine perfusion. In the second trimester, all components of fetal growth are affected in the presence of normal placental perfusion. In the third trimester, again, fetal growth is overall affected with no difference in MCA-PI while the presence of placental dysfunction is reflected by increased resistance in the UA and UtA-PI Doppler velocimetry.

6.1 First trimester

Previous investigation from Llurba *et al.*, have raised the hypothesis that in pregnancies where the fetus carries an isolated major cardiac defect there are signs of placental dysfunction in the presence of a normal uterine perfusion in certain types of cardiac defects (Llurba *et al.,* 2013). We wanted to verify this hypothesis on a larger population sample keeping the same classification of cardiac defects described by Llurba *et al*.
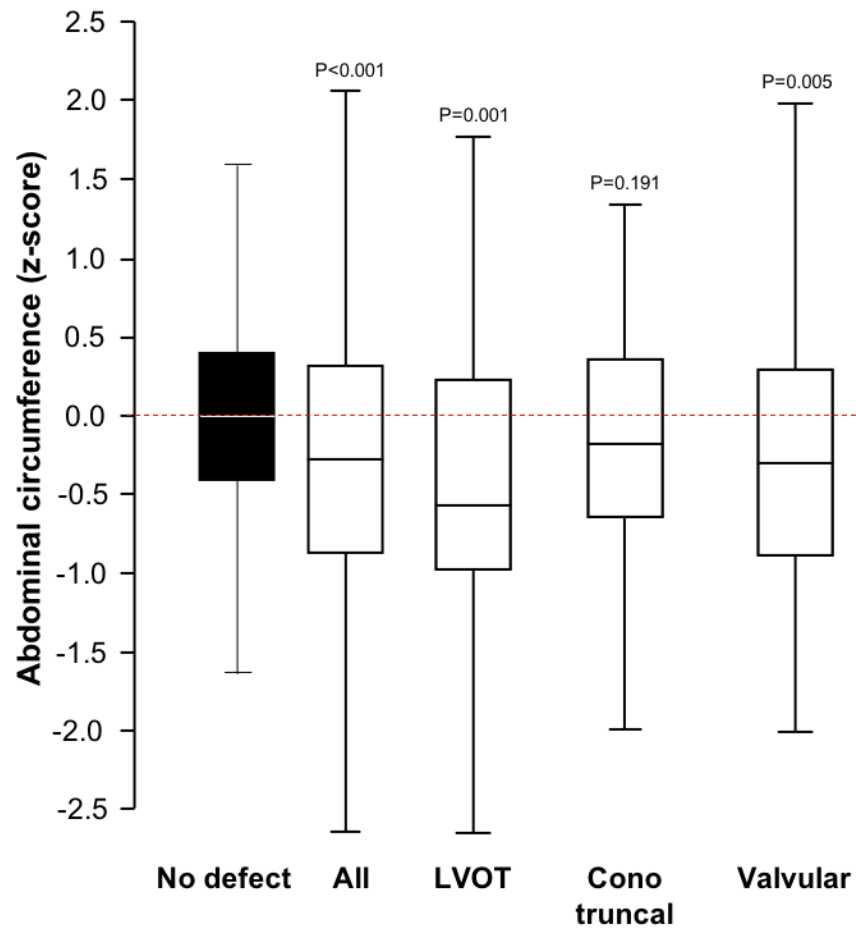
6.1.1 Placental factors

The main result in our population of fetuses with CHD, within the first trimester of pregnancy, is the evidence of a primary placental dysfunction.

The results obtained in our population are significantly different from the first study from Llurba *et al* in many aspects (Table 6.1). In the study from Llurba *et al*, while PlGF was significantly reduced in all pregnancies with CHD, PAPP-a was not (Llurba *et al*, 2013). Furthermore, subanalysis by heart defect subgroups showed that this was true for valvular and conotruncal abnormalities but not for LVOT. Different reasons were advocated to explain these findings, and one was the possible multifactorial pathogenesis of such defects. In this population, we substantially increased the number of cases analyzed for each subgroup and, particularly so, for LVOT defects: we had 63 cases compared to 11 included from Llurba *et al*. Therefore, it seems reasonable to conclude that pregnancies with CHD are characterized by lower concentrations of PlGF

in maternal serum, regardless of the type of cardiac defect. Similarly, PAPP-a showed a significant reduction in all CHD subgroups.

Table 6.1 Comparisons between the results reported by Llurba *et al* and results of our population

Llurba et al 2013		*Fantasia et al 2016*	
LVOT	11	LVOT	63
Conotruncal	25	Conotruncal	73
Valvar	35	Valvar	60
All CHDs		**All CHDs**	
reduced PlGF MoM	p < 0.001	reduced PlGF MoM	p < 0.001
increased dNT	p < 0.001	increased dNT	p < 0.001
PAPP-a	NS	PAPP-a	p < 0.001
UtA-PI	NS	UtA-PI	p < 0.001
PlGF in CHD subgroups:		**PlGF in CHD subgroups:**	
Valvar	p < 0.001	Valvar	p < 0.001
Conotruncal	p < 0.001	Conotruncal	p < 0.001
LVOT	ns	LVOT	p < 0.001
Log$_{10}$PlGF-MoM and dNT:		**Log$_{10}$PlGF-MoM and dNT:**	
all CHD	p < 0.0001	all CHD	p < 0.0001
controls	ns	controls	ns
dNT in all CHD and PlGF:		dNT in all CHD and PlGF:	
abnormal NT	p < 0.0001	abnormal NT	p < 0.0001
normal NT	ns	normal NT	p < 0.0001

LVOT, left ventricular heart defects; CHD, congenital heart defects, PlGF, placental growth factor; NT, nuchal translucency; dNT, delta nuchal translucency; NS, not significant.

Both PAPP-a and PlGF are markers of impaired placental function in pregnancies at risk of developing adverse obstetrical outcomes, like preeclampsia and IUGR (Poon and Nicolaides, 2014). However, in these cases, the physiological process of remodeling of the spiral arteries is inadequate resulting in high resistance in the UtA. Since blood perfusion on maternal side is defective, certain degree of placental insufficiency is established causing lower concentrations of placenta-related factors, such as PlGF and

PAPP-A, in maternal blood (Akolekar *et al,* 2008).

We showed, on a large population sample, that in fetuses with CHDs signs of placental dysfunction are present, as confirmed by lower concentrations of PLGF and PAPP-a, but the utero-placental perfusion on maternal side is normal, as confirmed by normal values of Ut-PI. This would suggest that these pregnancies are characterized by a primary placental dysfunction.

6.1.2 Environmental factors

The importance of environmental factor on the etiology of CHDs is supported by the analysis of maternal characteristics in our population: the presence of diabetes mellitus was significantly associated with the presence of CHD suggesting that hyperglycemia during early embryogenesis may alter gene expression in key cellular components of the developing heart. However, the mechanism producing this altered gene expression is still unclear (Kumar *et al,* 2007; Morgan *et al,* 2008).

Chronic hypertension and smoking were not significantly associated with any subgroup of CHDs and neither was the method of conception. While, indeed, the first two are not associated to CHDs, there is some evidence to support that pregnancies conceived through assisted reproductive techniques (ART) have an increased risk of fetal CHDs (REF). The American Heart Association (AHA) considers *in-vitro* fertilization (IVF), and mainly intracytoplasmatic sperm injection (ICSI), as an indication for a detailed cardiac scan with recommendation class/level of evidence of IIa/A and an absolute risk of 1.1-3.3% among live births (Donofrio *et al,* 2014). The risk for CHDs seems to be higher in pregnancies conceived with IVF/ICSI techniques rather than ovulation induction (Tararbit *et al,* 2013). In a recent meta-analysis on the incidence of CHDs in pregnancies conceived through IVF/ICSI, the odd ratio (OR) for CHDs was higher in the IVF/ICSI group than in naturally conceived infants (OR 1.55; 95% CI 1.21–1.99; p=0.18) (Giorgione *et al,* 2017). Quite interestingly, an association between pregnancies conceived by IVF techniques and low maternal serum concentrations of PAPP-a has been reported by different authors (Tul *et al.,* 2006; Amor *et al.,* 2009), suggesting a

causative relationship between IVF/ICSI technique, low PAPP-a and CHDs. However, no studies investigated this aspect on IVF pregnancies. In our cohort the total number of IVF pregnancies compared to the spontaneous (7 vs 189, respectively) was too little to perform a comparison between the two groups. Nonetheless, the findings that pregnancies carrying a fetus with CHD are characterized by lower levels of PAPP-a and that this factor could be someway responsible or linked to the increased incidence of heart defects in IVF pregnancies, open the possibility of new fields of investigation on this specific topic.

6.1.3 Nuchal translucency

In pregnancies with major cardiac defects fetal NT is increased, and in those with high NT serum PlGF is lower than in those with normal NT, while there were no significant differences in PAPP-a between the two groups. We also confirmed these results finding a significant inverse correlation between NT and PlGF for each subgroup of CHD and no association with PAPP-a. These results confirm, on a larger sample, what has been already found by Llurba *et al.*, who hypothesized a common pathophysiological mechanism for high NT and low PLGF but not for PAPP-a (Llurba *et al.,* 2013). PlGF, as part of the family of VEGF, may act on the same routes described for VEGF that are essential for the development of normal lymphatic vessels (Shibuya *et al*, 2008). Therefore, these data suggest that there might be a relation between PlGF and increased NT however, the etiology is still unknown.

6.2 Second trimester

Previous investigations have reported that fetuses with CHDs are at risk of being small for gestational age at birth (Marino *et al.,* 2012; Matthiesen *et al., 2016).* We wanted to verify if the presence of placental dysfunction, reported in the first trimester, have an impact on second trimester fetal growth.

6.2.1 Fetal growth restriction

Results from the analysis in the second trimester show that in fetuses with CHDs all components of fetal biometry are significantly affected when compared to controls.

Further analysis by subgroups show that, while HC and FL are significantly smaller in all three subgroups, AC is significantly affected just in LVOT defects. A negative trend was observed also for valvar and conotruncal defects, though the difference was not significant. Uterine arteries perfusion is normal as shown by normal values of UtA-PI in fetuses with CHD as in controls.

The association between the presence of various types of congenital malformations and fetal growth restriction (FGR) is well known (Sarkar *et al.,* 2013; Raynor *et al.,*1997). In fetuses with CHDs, the question if fetal systemic hemodynamic changes have an impact on fetal growth, secondarily to the cardiac abnormality, has arisen. Specifically, the link between low birthweight in CHDs and poor neurodevelopmental outcomes (Walden *et al.,* 2007), led different authors to investigate on the sole HC as a prenatal marker of NDD assuming that if brain perfusion is impaired, because of the cardiac abnormality, also brain growth would have been affected with smaller prenatal HC (Williams *et al.,* 2015; Masoller *et al.,* 2014; Turan *et al.,* 2016; Ruiz *et al.,* 2016; Haveman *et al.,* 2018; Hahn *et al., 2016;* Jansen *et al.,* 2016). Few studies considered AC and FL in their analysis and results are contradictory: if they all agreed that HC was smaller than controls, lower values of AC and FL were reported in some of them (Jansen *et al.* 2016; Turan *et al.* 2016; Williams *et al,* 2015*)* but not in others (Masoller *et al.* 2014*,* Ruiz *et al.* 2016).

The question, here, that need to be answered is: is it just the head that is smaller because of an impaired blood flow to the brain or the whole components of fetal growth are affected because of some elements of placental dysfunction?

In our study, done on a large population sample, we found that these fetuses are characterized by a symmetric fetal growth restriction, which is supported by the findings of Turan *et al.* and Williams *et al.,* where the calculation of the HC/AC ratio did not identify body asymmetry in any of the cardiac abnormality groups (Williams *et al.,* 2015, Turan *et al.,* 2016).

In Table 6.2, our results are compared with those studies that reported Z-score for, at least, HC and AC. It is clear that a negative trend is present for HC and FL, with small deviations from median values, as for our results. AC seems to be less affected in the study of Masoller *et al.* and Ruiz *et al.,* but significance is obtained when bigger numbers are included in the analysis as for Jansen *et al.* and our study. No studies reported the presence of true macrocephaly, defined as a HC < -3SD, or severe fetal growth restriction, defined as AC or EFW < 3rd centile, and the clinical relevance of the decrease in fetal size is overall small.

Table 6.2 Comparison between our results and other studies reporting Z-score for HC, AC and FL in the second trimester of pregnancy.

	Masoller et al, 2014	Ruiz et al, 2016	Jansen *et al*, 2016	Our results
Total N CHDs	95	119	343	629
HC Z-score	-0.89 ± 1.43	-0.79 ± 1.02	-0.06 (-0.18-0.06)	-0,26 (-0.92 – 0.30)
AC Z-score	0.27 ± 0.79	0.49 ± 0.88	-0.05 (-0.15-0.06)	-0,16 (-0.66 – 0.42)
FL Z-score	-0.09 ± 1.26	-0.04 ± 0.49	NR	-0,26 (-0.87 – 0.25)

HC, head circumference; AC, abdominal circumference; FL, femur length; NR, not reported

The finding of a small deviation from median biometric values in newborns with CHDs is also supported by the largest series on a Danish population of 924422 newborns, including 5519 babies with different types of CHDs, showing that the mean z-score difference in birthweight between all infants with CHD and the general population was –0.10 (95% confidence interval [CI], –0.13 to –0.08) and that, overall, birthweight z-score was modestly smaller than HC resulting in symmetrically small infants (Matthiensen *et al.,* 2016).

6.2.2 Placental factors

In our population, the presence of FGR happens in the presence of a normal uterine perfusion as supported by normal values of UtA-PI that imply a normal trophoblastic

arrangement in the first trimester. There is just one study, from Ruiz *et al.,* that included the assessment of the UtA-PI in the second trimester of pregnancy and they found that, despite the smaller biometry, UtA-PI was within normal ranges supporting the hypothesis that fetal smallness in the presence of a CHD is not related to abnormal uterine perfusion. Data on placental histology identified a specific pattern of lesion, called fetal thrombotic vasculopathy (FTV), characterized by regionally distributed avascular villi and often accompanied by upstream thrombosis in placental fetal vessels. Saleemuddin *et al.,* reported a 6-fold increased risk of fetal cardiac abnormalities, including ventricular or atrial septal defects, tetralogy of Fallot, aortic coarctation, double outflow tract, cardiomegaly, dextrocardia, and aortic stenosis, and a 2-fold increased risk of intrauterine growth restriction, when FVT was reported (Saleemuddin *et al.,* 2010). It is quite difficult to identify specific clusters of placental abnormalities related to defined fetal or maternal conditions because different maternal risk factors, like obesity and preeclampsia, frequently coexist in the same pregnancy (Stanek *et al.,* 2012). However, some histological findings are more specific than others for some conditions: for example, placenta of pregnancies affected by preeclampsia are usually smaller and characterized by the presence of chorionic villi defined "hypermature" for gestational age with aspects resembling those of a later gestational age, while placenta from women with diabetes can be larger or smaller with signs of hypervascularization and chorangiosis (Tyson *et al.,* 2008). Despite the few data exploring this aspect, there are some evidences to support the fact that the histological placental pattern of fetuses with CHDs is different from that of pregnancies with placental insufficiency secondary to uterine underperfusion that are, usually, characterized by more severe pattern of FGR. This might explain why fetal size is just moderately affected in fetuses with CHDs, but further studies are needed to investigate on the correlation between CHDs, FGR, placental histology and uterine perfusion.

6.2.3 Environmental factors

In the second trimester, there was a significantly higher number of CHDs in the group of women of Afro-Caribbean and South-Asian racial origin, while this was not the case in the first trimester. This might be due to the different numbers of CHDs included, which

was much higher in the second trimester than in the first (629 vs 196, respectively). Previous studies on the distribution of maternal biomarkers by ethnicity, showed that women of Afro-Caribbean and South-Asian ethnicity have higher maternal PlGF and PAPP-a serum concentrations (Tsiakkas *et al.,* 2015), and this seems to contradict our results in the first trimester population. However, in a recent analysis on the incidence of CHDs by ethnicity in a population of 5350 infants born in United Kingdom (UK), the overall incidence of CHDs was significantly higher in Asian and Black ethnic groups compared to the Caucasian population. The analysis of these subgroups by regional areas highlighted that British non-White ethnic populations were more likely to be living in more socioeconomically deprived areas, therefore highlighting the importance of maternal environmental factors on the etiology of fetal CHD.

Another finding emerging from the analysis, is that women with chronic hypertension have a lower incidence of CHDs compared to controls (0.2% vs 1.3%, p<0.05). A recent study on the incidence of preeclampsia in women with chronic hypertension found that maternal serum concentrations of PlGF and PAPP-a are higher in women with chronic hypertension (Panaitescu *et al.,* 2017). However, a definitive explanation for this finding cannot be given.

6.3 Third trimester

There is some evidence that fetuses with CHDs have a smaller HC and peculiar patterns of MCA-PI depending on the type of cardiac defects (Donofrio *et al.,* 2003; Berg *et al.,* 2009; Kaltman *et al,* 2005; Masoller *et al;* 2016*;* Ruiz *et al* 2016). We wanted to investigate fetal growth and fetal-maternal Dopplers in our population in all fetuses with CHDs and according by sub-groups.

6.3.1 Fetal biometry and fetal-maternal Dopplers

Our data show that in fetuses with CHDs, fetal biometry is overall significantly reduced with a moderate deviation from median values. Analysis by subgroups shows that HC is significantly smaller only in LVOT defects. AC is significantly smaller than controls in LVOT and valvar defects, while for FL significance is reached in all subgroups. A

tendency towards negative values was present also for the other components of fetal biometry, but the difference was not significant. Again, as for the second trimester, the degree of FGR is mild and follows a symmetrical pattern.

The presence of moderately increased resistance in the UtA flow, not reported in the first and second trimester group, is a new finding. This is in line with the only study evaluating UtA-PI in fetuses with a major CHD from Ruiz *et al.*: while they found normal values of UtA-PI in the second trimester, they observed a quadratic increase for both UA-PI and UtA-PI in the third trimester (Ruiz *et al.,* 2016). Based on these findings we may speculate that increased resistance in the uterine arteries are secondary to the presence of placental dysfunction and that such changes become evident only in the third trimester either because of worsen degrees of the primary defect or because of additional effects of placental aging. Placental histology and serial measurements of maternal biomarkers in the second and third trimester could be helpful in the understanding of such phenomenon.

Our data show that also UA-PI is moderately increased in fetuses with CHDs compared to controls and most severe patterns, like absent or reversed flow, where never observed. Previous studies reporting on the UA flow pattern (Kaltman et al; Masoller et al, 2016; Turan et al, 2016) give contradictory results. Kaltman *et al.* found significantly higher UA-PI in right-sided lesion and in HLHS and left-sided lesions but not to a significant degree; Yamamoto *et al.,* observed an increased UA-PI in all cardiac defects (Yamamoto *et al.,* 2013); others did not find any difference in UA-PI in any of the CHDs analyzed (Masoller *et al.* 2016*;* Ruiz *et al.,* 2016). In the article from Turan *et al.,* CHDs were divided in 4 groups of CHDs: 1) TGA; 2) left-sided lesions with retrograde aortic flow; 3) left-sided lesions with anterograde aortic flow; 4) right-sided lesions. They found that UA-PI z-score was higher in those CHDs with retrograde aortic arch flow and in those with pulmonary obstruction (p < 0.001 and p = 0.03, respectively), while it was normal in Group 1 and 3 (Turan *et al.,* 2016).

Different mechanisms have been advocated to explain the presence of increased resistance in fetuses with CHDs: some, like Kaltman *et al.,* have speculated that reverse

shunting through the ductus arterious to the obstructed arterial system was decreasing diastolic blood flow in the UA, thereby elevating UA-PI (Kaltman *et al.,* 2005). Others either did not provide any explanation for the finding (Turan *et al.,* 2016) or related it to the presence of placental abnormalities secondary to unknown genetic or epigenetic condition (Yamamoto *et al*., 2013). Our data strengthen the hypothesis that in fetuses with CHD there is a primary placental dysfunction responsible of increased resistance in the placental bed, supported by low levels of PIGF and PAPP-a in the first trimester and reduced intrauterine fetal growth in the second and third trimester.

The analysis of MCA-PI showed that, overall, there was no difference between CHDs and controls. However, subanalysis by different subgroups showed that MCA-PI is significantly lower in fetuses with LVOT defects than in valvar and conotruncal which, instead, were characterized by higher levels of MCA-PI though the difference with controls was not significant.

Stating first that, due to the heterogenicity between all studies in the clustering of cardiac defects included, a comparison between the findings have some limitations, we can see a tendency toward similar results: Masoller et al., in an interesting study on fetal biometry, Doppler ultrasound and functional MRI in fetuses with CHD identified two groups: group A with a low oxygen content and group B with a near-normal oxygen content and they found that MCA-PI Z-score was significantly lower in both groups (p<0.001) but Group A had a tendency towards lower values (Group A: –0.95 ± 0.95; Group B: –0.29 ± 1.14). Szwast *et al* compared single ventricle (SV) defects, divided in those with aortic obstruction and those with pulmonary obstruction: they found that MCA-PI Z-score was significantly lower in SV defects with aortic obstruction and significantly higher in SV defects in pulmonary obstruction (p<0.001) (Szwast *et al*., 2012). Autoregulation of the cerebral circulation to the blood flow directed to the brain was advocated as a possible explanation of these findings: a decrease in cerebrovascular resistance represents the brain's attempt to increase blood flow in the face of an insufficient source of flow. Conversely, when a pulmonary obstruction is present, the vast majority of blood volume exits into an unobstructed aorta. The cerebral circulation therefore receives a substantially increased fraction of blood relative to normal, and autoregulates by

increasing cerebrovascular resistance in order to limit blood flow and prevent hyper-perfusion. Again, similar findings were reported by Kaltman *et al.* who found significantly higher MCA-PI Z-score in fetuses with HLHS than in fetuses with right-sided lesions, though in these defects the difference was not significant (Kaltman *et al.*, 2005).

There is, therefore, some evidence that the interpretation of fetal brain Doppler in the presence of a CHD should take into account the systemic fetal hemodynamics determined by each CHD and the principles applied to FGR fetuses should not be simply transferred to CHDs defects. Because of the different hemodynamic circumstances in CHDs versus growth restriction, conclusions should not be interchangeable. The presence of a major cardiac defect causes a subverted systemic circulation, that is not present in FGR fetuses, and that varies for each type of defect. The consequences of a possible reduced oxygenation on the MCA-PI pattern need to take into account also the impact of an altered systemic circulation and caution should be used in the inclusion of such findings as prognostic factors in the prenatal counseling.

Moreover, studies that evaluated the correlation between fetal growth and post-natal neurodevelopmental outcome have found that there is a poor correlation between HC prenatal values and neurodevelopmental outcome. In their study, Williams *et al*. assessed the neurodevelopmental outcome of 18-months infants with CHDs (HLHS, TGA and TOF) by using the Bayleys Scales of Infant Development. They found that fetal biometry variables that correlated with Cognitive score included AC ($r = 0.32$, $P = 0.049$), HC/AC ($r = -0.39$, $P = 0.02$), and mean HC/AC ($r = -0.4$, $P = 0.007$) meaning that a larger abdominal relative to head circumference was associated with a higher score; language score correlated significantly with FL/BPD ($r = 0.32$, $P = 0.037$) and mean HC/AC ($r = -0.3$, $P = 0.045$). They did not see any association between head size at any time in pregnancy, or in the neonatal period, and neurologic function (Williams *et al.,* 2015). Similar findings were found by Hahn *et al.,* in the analysis of both left and right single-ventricle defects, where neither MCA-PI Z-score nor changes in MCA-PI Z-score, as gestation progressed, correlated with fetal HC Z-score or with post-natal neurodevelopmental outcome, while the only significant finding was that the mean of the individual fetal HC/AC ratios had a negative correlation with psychomotor developmental

outcome (r=−0.25, P=0.037; n=71) and as the HC/AC ratio decreased, PDI increased (Hahn *et al.,* 2016).

These findings highlight the importance of body symmetry for ultimate brain development, more so than the HC alone, and the lack of association may be because no true mechanistic relationship exists between fetal head growth and cerebral blood flow, with fetal head growth perhaps being influenced more by genetics, maternal factors such as nutrition and placental insufficiency, or other hemodynamic features not captured by repeated Doppler measures of the MCA-PI.

6.4 Strength and limitations

This study included a large population of pregnant women who attended for routine care in a gestational age range widely used for the assessment of the risk of chromosomal abnormalities. This allowed us to include a large number of patients whose fetus was affected by a major congenital heart defect, in order to systematically assess fetal biometry in relation to the uterine arteries perfusion in the second and third trimester of pregnancy. Furthermore, the study of the fetal anatomy was based on a specific protocol which included a detailed screening examination, review by fetal cardiologist in those with suspected abnormalities and neonatal examination by a pediatrician in all cases. Finally, a specific methodology was used by appropriately trained doctors to obtain measurements of fetal growth UtA-PI, UA-PI and MCA-PI.

A limitation of the study is the absence of placental histology examination in order to identify, on a large scale, a specific pattern of the placental structure in fetuses with a CHD and to correlate it with the UtA flow. Moreover, we did not sample maternal serum PlGF in the second and third trimester in order to see its pattern also at later gestational ages and its possible relation to fetal growth.

6.5 Future studies

More studies are needed to answer the question of the pathogenic relationship between placental dysfunction and CHD. However, in the very first weeks of human gestation, investigating on placental function is difficult because of both ethical problems and unknow condition of the state of pregnancy in many cases. IVF pregnancies could be a

population eligible for an early assessment of placental function since pregnancy is diagnosed at a very early stage; moreover, the latest evidence that report an increased risk for CHD, made this population of particular interest in the field.

Future research could try to add information to this aspect, for example, analyzing placental factors earlier in gestation or by studying not invasively placental volume by 3D acquisition images. Reus *et al.,* have shown that the assessment of the trophoblast volume by using the three-dimensional (3D) ultrasound technique with Virtual Organ Computer-aided AnaLysis (VOCAL) can accurately assess placental volume from the 6th weeks of pregnancy and that smaller first-trimester trophoblast volume as well as reduced trophoblast growth were related to higher risk of miscarriage (Reus *et al.,* 2013). Papastefanou *et al.* combined the assessment of placental volume by VOCAL acquisition with serum maternal levels of PAPP-a, beta-HCG and UtA-PI at 11-13 weeks' gestation and they found that there is a strong association between low PAPP-a levels and small fetal placental volume and this relation was independent from status of conception and maternal perfusion (Papastefanou *et al.,* 2014). It could be useful to study placental volume in combination with maternal angiogenic and antiangiogenic factors and maternal uterine arteries blood flow in pregnancies from IVF technique in the period from 6 to 10 weeks' gestation.

A better understanding of how aberrations in vasculogenesis and angiogenesis may impact the manifestation of CHD and associated placental abnormalities will improve our ability to predict those fetuses that will have significant growth abnormalities. Moreover, the prediction of an increased risk of CHD in the presence of family history or previous preeclampsia should bring the clinician to establish preventive measures such as high dose of folic acid or in case of low PlGF or PAPP-a detected at the time of the first trimester screening for aneuploidies or preeclampsia, the use of low-dose aspirin in the prevention of CHD should be assessed.

At later stage of pregnancy, alternative ways to MCA-PI analysis should be found to assess those fetuses who are potentially at risk of poor neurodevelopmental outcome. One could be the assessment of the umbilical vein (UV) flow. Sun *et al.,* designed a study using phase contrast MRI to evaluate blood flow quantification, fetal brain volumetry and

oximetry in fetuses with single ventricle defects, TGA and TOF and they found that a reduced UV flow and reduced O_2 saturation positively correlates with a reduced cerebral O_2 delivery and consumption (Sun *et al.,* 2016). Fetal umbilical vein blood flow (UVBF) has been assessed by US in pregnancies affected by FGR and has been shown to be a more direct surrogate of the amount of oxygen and nutrients reaching the fetus (Parra-Saavedra *et al.,* 2013; Ferrazzi *et al.* 2000). This open a new possibility in the field of prenatal evaluation of CHD by US: studies of the blood flow in the UV will possibly help in understanding the hemodynamic consequences of the cardiac defect on the systemic circulation, its relation to placental dysfunction and blood flow to the brain. Concomitant assessment of prenatal and postnatal fetal brain volume and function, by functional MRI study, and an adequate neonatal follow-up will hopefully help in the definition of those CHDs at increased risk of NDD.

CHAPTER 8   CONCLUSIONS

The findings of this thesis suggest that in pregnancy with major cardiac defects there is placental dysfunction from as early as 11-13 weeks' gestation in the absence of impaired placental perfusion. The presence of an angiogenic imbalance status does not correlate to the type of cardiac defect as does not the uterine perfusion.

In the second and third trimester of pregnancy we showed, on a large population, that HC is not the only parameter affected by the presence of the CHD but there is a symmetrical fetal growth restriction involving all components of the fetuses with moderate negative deviations from median values. Uterine arteries perfusion is still within the normal range in the second trimester but slightly higher in the third trimester reflecting a degree of placental dysfunction.

Analysis of fetal Doppler in the third trimester showed that the presence of placental dysfunction is reflected in increased resistance in the umbilical artery without the typical pattern of a severe placental insufficiency. The blood flow to the brain, assessed by the measurements of the MCA-PI, showed that there is a reactive vasodilation in those cardiac defects with a predicted low blood flow and oxygenation to the brain but there is a tendency towards higher levels of MCA-PI in those with a predicted normal or increased blood flow to the brain, suggesting the presence of a mechanic autoregulation of the MCA depending on the quantity of flow more than on quantity of oxygen.

In conclusion, we showed that fetuses with an isolated major congenital heart defect have a moderate degree of fetal growth restriction that affects all components of fetal body, in the presence of normal uterine perfusion. Such results suggest that the presence of fetal growth restriction in fetuses with CHDs is mainly related to a primary placental dysfunction supported by abnormal uterine and umbilical arteries Doppler in the third trimester. Significance of MCA-PI pattern still need to be clarified, however there is some evidence that different autoregulatory mechanism, other than low oxygenation, are responsible of MCA-PI in the different type of cardiac defect.

CHAPTER 9   REFERENCES

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Appendix 1 PUBLISHED STUDIES

Study Fantasia I, Kasapoglu D, Kasapoglu T, Syngelaki A, Akolekar R, Nicolaides KH. Major cardiac defects and placental dysfunction at 11-13 weeks' gestation. Ultrasound Obstet Gynecol. 2017 Aug 18. doi: 10.1002/uog.18839. [Epub ahead of print]

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