Published in final edited form as:

Placenta. 2015 October ; 36(10): 1078–1086. doi:10.1016/j.placenta.2015.08.003.

Hypoplastic left heart syndrome is associated with structural and vascular placental abnormalities and leptin dysregulation

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Abstract

Introduction—Hypoplastic left heart syndrome (HLHS) is a severe cardiovascular malformation (CVM) associated with fetal growth abnormalities. Genetic and environmental factors have been identified that contribute to pathogenesis, but the role of the placenta is unknown. The purpose of this study was to systematically examine the placenta in HLHS with and without growth abnormalities.

Methods—HLHS term singleton births were identified from a larger cohort when placenta tissue was available. Clinical data were collected from maternal and neonatal medical records, including anthropometrics and placental pathology reports. Placental tissues from cases and controls were analyzed to assess parenchymal morphology, vascular architecture and leptin signaling.

Results—HLHS cases ($n = 16$) and gestational age-matched controls ($n = 18$) were analyzed. Among cases, the average birth weight was 2993 grams, including 31% that were small for gestational age. When compared with controls, gross pathology of HLHS cases demonstrated significantly reduced placental weight and increased fibrin deposition, while micropathology showed increased syncytial nuclear aggregates, decreased terminal villi, reduced vasculature and increased leptin expression in syncytiotrophoblast and endothelial cells.

Discussion—Placentas from pregnancies complicated by fetal HLHS are characterized by abnormal parenchymal morphology, suggesting immature structure may be due to vascular

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abnormalities. Increased leptin expression may indicate an attempt to compensate for these vascular abnormalities. Further investigation into the regulation of angiogenesis in the fetus and placenta may elucidate the causes of HLHS and associated growth abnormalities in some cases.

Introduction

Hypoplastic left heart syndrome (HLHS) continues to be one of the most challenging cardiovascular malformations (CVM) to manage. Rapidly evolving treatment paradigms have resulted in both increased survival and improved long-term outcomes, but the underlying cause(s) remain poorly understood. The etiology of HLHS is thought to be multifactorial, attributed to a combination of complex inheritance and environmental factors [1]. Research has identified different influences that contribute to the manifestation of HLHS, including genetic predisposing factors, environmental risk factors and physiologic perturbations in the developing and growing heart [2, 3], but it remains unclear how these factors interact to impact pathogenesis.

HLHS infants are at increased risk of being small for gestational age (SGA), and both SGA and low birth weight (<2500 grams) are associated with adverse clinical outcomes [4, 5]. Growth patterns have been characterized in HLHS, and about 50% of fetuses are affected by growth abnormalities [6]. Recently, we described fetal growth in HLHS, and in a significant proportion of cases, weight and/or head circumference demonstrate diminished growth trajectories late in gestation [7–9]. Whether growth abnormalities in HLHS represent primary and/or secondary insults to the fetus is unknown, and the potential role of impaired placental development and/or function in the manifestation of these growth abnormalities remains unclear.

The placenta plays a central role in fetal growth, and abnormal placental implantation, growth, development and function can all negatively impact fetal growth and development [10, 11]. Gross placental pathology has not been identified in large studies examining all CVM collectively [12]. Several studies have shown pregnancies complicated by fetuses with HLHS have normal umbilical artery Doppler by ultrasound assessment [7, 13, 14], suggesting appropriate fetoplacental blood flow, but these studies have not assessed placental structure, vascularization or function. The significantly increased incidence of any growth abnormality in newborns with HLHS, approximately 40%, implicates placental dysfunction. As recently highlighted, placental insufficiency can underlie a significant proportion of late-onset fetal growth restriction cases without the presence of abnormal umbilical artery Doppler [15]. The role of the placenta in the manifestation of HLHS and associated growth abnormalities is unknown. One emerging concept proposes that primary placental abnormalities may be associated etiologically with primary CVMs [16, 17], suggesting the placenta may contribute to or be impacted by primary cause. This so-called heart-placental axis proposes that the heart, placental vasculature and villous tree develop concurrently in early pregnancy [17]. Recent studies in mice have shown that common factors exist, such as PPAR γ , that lead to CVM, reduced placental vasculature, impaired placental function and reduced somatic growth [18, 19]. The causal factors for HLHS may impact placental vasculature and further investigation into the regulation of

vasculogenesis, as well as angiogenesis, as it applies to the cardiovascular and placenta systems may help elucidate these factors.

Altered placental development or function and resulting aberrant fetal growth has previously been associated with dysregulation of several growth factors and adipokines such as IGF-1 and leptin [20, 21]. Leptin is an angiogenic and mitogenic hormone produced by the placenta that has both paracrine and autocrine effects [22, 23]. In a healthy pregnancy, leptin levels are positively correlated with placental weight and a number of specific indices of fetal growth, including body weight and length, head circumference, ponderal index, adiposity and bone mineral density [24]. Furthermore, it has been demonstrated that leptin has a pro-angiogenic effect in placental tissue [25] and an anti-apoptotic effect on trophoblast cells [22]. Several placental pathologies display altered leptin levels, including lower fetal serum concentrations in growth restricted fetuses and higher maternal serum levels in fetal macrosomia, diabetes mellitus, and recurrent fetal demise [27–30]. The adipokine, metabolic and angiogenic functions of placental leptin have not been examined in the context of CVM.

The objective of this study was to characterize placental micropathology in HLHS, correlate changes with associated fetal growth abnormalities, and identify mechanisms that contribute to pathogenesis. We hypothesized that placental tissue in HLHS is immature and characterized by morphological and vascular abnormalities in some cases, and these findings are more severe in those cases with growth abnormalities.

Methods

Study population

This cohort was assembled retrospectively as a single center case series. Maternal, fetal and neonatal clinical data were collected for term (> 37 weeks gestation) HLHS cases from Cincinnati Children's Hospital Medical Center (CCHMC) and Good Samaritan Hospital (GSH, Cincinnati, Ohio) from 2003–2010. Prematurity, multiple gestation pregnancies, and fetuses with genetic abnormalities or additional CVMs were excluded. In addition, cases with a history of maternal diabetes, preeclampsia or hypertension were excluded. HLHS was strictly defined as atresia or stenosis of the aortic and mitral valves, and hypoplasia of the left ventricle and ascending aorta [31], excluding other single ventricle lesions. Placental tissue was available in a subset of cases. Control placental tissue was obtained from uncomplicated term pregnancies with documented normal fetal cardiac structure and function. This study was approved by the Institutional Review Boards of CCHMC and GSH.

Maternal health

Clinical records were reviewed and the following data was collected: maternal age, pre-pregnancy body mass index (BMI, kg/m²), smoking habits and weight gain during pregnancy. Pre-pregnancy BMI and smoking habits were ascertained by self-reporting. Smoking was defined as any history of smoking during pregnancy, excluding cessation prior to pregnancy. In addition, mode of delivery and the presence of chorioamnionitis were noted.

Fetal and neonatal health

The diagnosis of HLHS was determined by neonatal echocardiogram. Estimated fetal weight and gestational age data were collected at the in utero stage. Anthropometrics, including birth weight, gender and gestational age at the time of delivery were collected at birth. Fetal and newborn percentiles were derived from the Olsen standard, based on growth curves derived from a large contemporary US sample adjusted for gestational age and gender [32]. Fetal Growth Restriction (FGR) was defined as an estimated fetal weight less than or equal to the tenth percentile. Small for gestational age (SGA) was defined as a birth weight less than or equal to the tenth percentile. Low birth weight (LBW) was considered a birth weight less than 2500 grams.

Placenta

Gross placental evaluation was based on routine clinical pathology reports generated at the time of delivery for all cases and controls. This evaluation included measures of stripped weight and dimensions, site of cord insertion, the presence of chorioamnionitis, inflammation, thrombi, infarction or fibrin deposition. Placentas from both Control and HLHS cohorts were formalin fixed immediately following delivery, and sent for gross pathological assessment. Placental tissue blocks were obtained subsequently for additional research analyses as described below.

Histochemistry and Immunohistochemistry

Serial sections were stained with Hematoxylin and Eosin (H&E) and assessed for evidence of inflammation and alterations in villous architecture as described below. For antibody staining, sections were incubated in target retrieval solution (sodium citrate (pH 6.0) for Leptin, high pH EDTA for Leptin-R, and proteinase K without boiling for VEGF). Sections were incubated with 3% hydrogen peroxide in methanol for 20 minutes. Following washes, slides were incubated with 5% donkey serum for 60 minutes. All primary antibodies were derived from rabbit serum: Leptin (LifeSpan Bioscience, B1459, 1:500), Leptin-R (AbCam, ab60042, 1:50), VEGF-A (Santa Cruz, SC53463 1:50), Flk1 (Novus Biologicals, NBP174001, 1:100), Cleaved-Caspase-3 (Cell Signaling, 1:200), CD31 (Abcam 1:50), and Ki67 (Abcam 1:50) RNAPolymerase II (Abcam 1:100). Sections were incubated overnight at 4°C and washed in PBS with triton 1%. Sections were incubated with secondary antibody (donkey, anti-rabbit) for 30 minutes, antibody binding was detected using DAB, and slides were dehydrated, mounted and counterstained with hematoxylin. Slides were examined under a Nikon Eclipse 80i microscope by a blinded observer and a scale of staining was developed where 0 was no staining and 5 was maximal staining across all slides, a score was then assigned to 10 high power fields across sections from each sample.

Syncytial Nuclear Aggregate Assessment

A Syncytial Nuclear Aggregate (SNA) was defined as a multi-layered aggregation of at least 10 syncytiotrophoblast nuclei protruding from the villous surface that was not in direct contact with adjacent villi [33]. The number of SNAs was counted manually, and villous area measured by sequential color thresholding, as previously described [34]. SNAs in 10 high-powered fields per slide were counted. Data were expressed as SNAs per high-powered

field and normalized to villous area to give a measure of the number of SNAs per mm² of villous tissue.

Villous Assessment

To assess the terminal branching of placental villi that normally occurs exponentially in the third trimester, terminal villi (40–80µm in diameter in normal placentas [35]) density in each high-powered field was assessed both qualitatively and the number of villi less than 80µm in diameter counted. To investigate the villous vasculature, the number of vessels per high-powered field was assessed initially by manual counting in 10 random high-powered fields across 3 sections following staining for CD31 for vessel visualization. Vessel area was calculated by manually identifying villous vessels, following CD31 staining, then using the area measurement tool in Nikon Elements software. Further, villous maturity was also assessed by counting vasculo-syncytial membranes [36] in 10 hpf across 3 sections for each sample.

Isolation and analysis of RNA from FFPE samples

RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) human placenta tissue blocks using the RecoverAll Total Nucleic Acid Isolation kit (Ambion). Three-20µm sections were cut from the center of the FFPE blocks and RNA was extracted following manufacturer's instructions using a 2 hour proteinase K digestion at 50°C. RNA yield and integrity were quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). RNA expression of Leptin, VEGF-A and FLK-1 was analyzed by real-time PCR as previously described [37]. Briefly, 1 µg of purified RNA was reverse transcribed into cDNA using Hi-Cap cDNA Conversion Reaction on MJ Research PTC 200 Thermal Cycler (MJ Research Inc., St. Bruno, Canada) following manufacturer's protocol. Primers for human VEGF-A, FLK-1, PlGF and leptin were designed using Primer-BLAST (NCBI.com) for specificity (Hu Flk-1 F GCT GAA AAC TTT GGA AGA CAG A, Hu Flk-1 R CTT CAG ATG CCA CAG ACT CC; Hu VEGFa F GAG GGC AGA ATC ATC ACG AA, Hu VEGFa R TCT CGA TTG GAT GGC AGT AG; Hu PlGF F TGC CGG TCA TGA GGC TGT T, Hu PlGF R GTA CCA CTT CCA CCT CTG A; Hu LEP F TTG TCA CCA GGA TCA ATG AC, Hu LEP R AAA CCG GTG ACT TTC TGT TT). Gene expression was assayed with a melt curve, in duplicate, using Power SYBR Green PCR Master Mix reaction in the Applied Biosystems StepOne-Plus Real-Time PCR System. cDNA in each well was normalized to reference genes Human b-Actin (Hu ACTB) and Human TATA Binding Protein (Hu TBP). Relative quantification expression levels were calculated by comparative CT method [38] via StepOne Software v2.3 (Life Technologies, Grand Island, NY).

Statistical analysis

Data were explored using tests of normality and evaluation of skew and kurtosis to determine whether variables were normally distributed. Normally distributed data are expressed as mean ± standard deviation (SD) for normally distributed data and analyzed with Student's T-test and ANOVA as appropriate. Non-normally distributed data are expressed as median and range and analyzed by Mann Whitney U nonparametric test for non-normally distributed data. Chi square analysis (or Fisher's exact test where appropriate) was utilized for categorical data. All statistical analyses were performed using the Statistical

Package for the Social Sciences (SPSS) version 12.0.1 for Windows, 2003, Chicago, SPSS Inc.

Results

Study cohort

We previously described a cohort of 38 HLHS fetuses [7, 8]. From this cohort, pathology evaluation and archived placenta tissue was available in 16 (42%) cases. All numerical variables were normally distributed except birth weight percentile, gestational age, placenta weight percentile, placental thickness, SNAs/HPF, SNAs/mm² and vessel density.

Maternal health parameters did not differ between HLHS cases and controls

Maternal health parameters are presented in Table 1. The relative frequency of maternal smoking during pregnancy, chorioamnionitis and Cesarean section was not significantly different when comparing the HLHS cohort with the control group.

Birth weight is reduced in HLHS

Among HLHS cases, the average birth weight and percentile was significantly reduced, including 31% of cases that were SGA, when compared with reference standards and the control group (Table 1). There were no statistical differences for gender, gestational age, or placental-fetal weight ratio between the sub-population of HLHS with SGA birth weight and the sub-population of HLHS with appropriate for gestational age (AGA) birth weight.

Placental weight is reduced in HLHS

There was no evidence of gross placental malformation, consistent with previous studies [11]. However, placental weight and percentile were significantly less for HLHS cases than controls (Table 2). Placental weight was positively correlated with birth weight in both the control and HLHS cohorts. Gross pathological examination noted that increased subchorionic fibrin deposition, a non-specific finding, was present in 40% of HLHS cases but there was no evidence of increased chorioamnionitis, inflammation, thrombi, or infarction. No differences were seen in placental thickness or site of cord insertion. There was no evidence of inflammation or immune cell infiltration in any of the HLHS placentas (data not shown).

Intraparenchymal Fibrin Deposition present in HLHS

In addition to the common and variable presence of subchorionic fibrin, as reported by gross pathology, additional microscopic evaluation revealed the presence of fibrin within the parenchyma of the HLHS cases but not in the controls (Figure 1).

Syncytial Nuclear Aggregates are increased in HLHS

A significant increase in the number of SNAs was seen in the HLHS cases compared to controls ($p < 0.001$, Table 3 and Figure 1). In HLHS cases, Ki67 positive nuclei number was significantly increased in the syncytium compared to controls and seen within and adjacent to SNAs (Figure 2). There was no difference in staining between cases and controls when

sections were probed with an anti-Caspase-3 or an RNA Polymerase II antibody (data not shown), suggesting these SNAs represented proliferative syncytial sprouts and not areas of syncytium undergoing cell death and to be sloughed off into the maternal circulation.

Villous assessment indicates reduced terminal villi, vasculo-syncytial membranes and vascular density in placentas from HLHS

Stereological assessment between HLHS cases and controls demonstrated that the number of terminal villi (villi smaller than 80µm in diameter) was significantly reduced ($p < 0.001$) in the HLHS population compared to control (Table 3). However, no difference was seen in villous area. Furthermore, following CD31 staining to identify vasculature (Figure 3), a significant reduction in vascular density ($p = 0.001$) and vascular area ($p = 0.001$) was seen in the HLHS cases compared to controls (Table 3). Vasculo-syncytial membrane counts were also significantly reduced ($p < 0.05$) in the HLHS cases compared to the control group (Table 3). When all placental measurements were plotted against birthweight (Figure 4) no distinct sub-populations were found within the HLHS group, suggesting the identified changes are correlated with the occurrence of HLHS and not altered birthweight.

Syncytial leptin and villus vessel leptin receptor expression is altered in HLHS

Leptin is a known trophic and angiogenic factor in the placenta, and altered leptin expression has been established in several pathologies including SGA. Leptin RNA expression was significantly increased in the HLHS cases compared to controls (4.04 ± 1.54 vs. 1.14 ± 0.75 , $p < 0.05$, $n = 6$ per group) and both Leptin and Leptin-Receptor staining was increased in the syncytium of HLHS cases with an increase in Leptin-receptor staining also seen in the endothelium of the villus vasculature compared to minimal staining in the controls (Figure 5).

Placentas from HLHS cases show differential regulation of angiogenic factors at RNA and protein levels

The expansion of the placental villous structure is regulated by several angiogenic factors including VEGF and PlGF, if perturbed placental capacity for appropriate nutrient and oxygen transport may be reduced. PlGF RNA expression was significantly ($p < 0.05$) reduced in placentas from HLHS cases compared to controls (0.14 ± 0.05 vs. 1 ± 0.42 , $n = 6$), whereas VEGF-A and FLK-1 RNA expression was the same as in the control group (Table 4). VEGF expression was localized to the syncytium and fetal endothelium in both cases and controls with no apparent difference in staining. In contrast, FLK-1 expression was only localized to the fetal endothelium and protein expression appeared reduced in HLHS cases when compared with controls (Figure 6), PlGF protein expression could not be assessed due to lack of antibody specificity.

Discussion

This is the first study to systematically investigate the placenta in detail in HLHS. We identified both structural and vascular changes in the villous tree, consistent with placental immaturity. In addition, the increase in intraparenchymal fibrin deposition indicates abnormal trophoblast secretion and could lead to a reduction in available surface area for

nutrient and oxygen exchange. We demonstrate an increase in leptin expression in HLHS, which may represent a compensatory mechanism to maintain normal placental function. Together, these findings identify significant abnormalities that advance our understanding of pathogenesis of growth abnormalities associated with HLHS and ultimately may impact our approach to clinical care.

Previous studies have established that HLHS newborns are born smaller than their anticipated birth weight based on ultrasound growth trajectory [8]. Despite a lack of abnormal ultrasound Doppler, poor fetal growth was seen in 64% of HLHS cases with only 6% having a birth weight greater than their estimated fetal weight. Hangge et al similarly discovered poor head growth in 32% of the HLHS population studied and poor weight gain in 55%, independent of Doppler flow abnormalities [7]. This suggests a potential role for placental insufficiency with impaired transfer of nutrients and/or oxygen from the maternal circulation by the placenta, an area of research not previously reported in HLHS or CVM more generally.

Analysis of the placental structure in the current study indicates that reduced villous vasculature and reduction in arborization (lower numbers of terminal villi) of the maternal-fetal interface occurs in cases of HLHS and a more severe vascular deficit may lead to SGA within this cohort. The reduction in the number of terminal villi and vasculo-syncytial membranes present in the HLHS placenta and the observed structure is reminiscent of a placenta from earlier in the third trimester rather than one at term [39]. This is an interesting observation because the placenta is often hyper-mature in the context of FGR, suggesting novel mechanisms contribute to pathogenesis in this context, and because there are reports of brain immaturity of approximately 4 weeks in cases of HLHS [40], consistent with late gestation growth failure, suggesting prenatal or mid-gestation interventions may positively impact clinical outcomes. The increase in Syncytial Nuclear Aggregates (SNAs) seen in HLHS cases may also indicate a placenta that has failed to expand its villous tree sufficiently in the third trimester and if they represent sprouts which have failed to develop into terminal villi [41], as indicated by no increased apoptosis, and evidence of proliferation give further support to failure in branching and expansion of the fetal vasculature within the villi. In the normal placenta, terminal villus formation which occurs predominantly in the third trimester to massively increase the placental exchange surface area and capacity in order to accommodate exponential fetal growth is in response to vessel expansion by non-branching angiogenesis including longitudinal growth and coiling of fetal capillaries. However, the normal expansion of fetal vasculature in the placenta may not be triggered when the heart is underdeveloped, leading to a potentially reduced peripheral vasculature.

Furthermore, the lack of abnormal Doppler results may indicate a less compromised villous tree than seen in severe, early-onset FGR with abnormal Doppler [42] and may explain while small, not all HLHS newborns are SGA. As highlighted in our placental structural data, the current cohort of HLHS samples demonstrates a range of impairments from the most severe reduction in villous development through to placental structures that resemble control. Since HLHS fetuses typically demonstrate normal growth and development to mid-gestation [7, 8], and some of the findings of the current study, including the presence of syncytial sprouts and the reduced terminal villi and vasculo-syncytial membrane numbers in

term placentas, it appears impairment of fetal growth in this context occurs during late gestation, consistent with placental villous immaturity.

Our proposal of reduced angiogenesis and therefore villous tree maturation is supported in a recent publication by Llubra et al., which highlighted both maternal and fetal angiogenic imbalance in CVMs and suggested, but did not study, what we now demonstrate, that this angiogenic imbalance might impact placental vascular and villous development [43]. Interestingly in the HLHS cases, increased leptin was detected in the fetal endothelium and villous stroma but appeared to have no effect on VEGF expression levels, perhaps due to the dominance of PlGF and non-branching angiogenesis during late gestation. The effect of Leptin on PlGF expression is not currently known, however, our data demonstrating significantly reduced PlGF expression and the apparent reduction in FLK1 expression in the microvasculature (as the expression of FLK1 in human umbilical vascular endothelial cells (HUVECs) in umbilical cord samples from the cases demonstrated staining comparable to control (data not shown)) would indicate significant imbalance in the angiogenic mechanisms within the placental microvasculature.

Placental adaptation and compensation mechanisms exist to try and maintain optimal blood flow and sufficient nutritional and oxygen supply to the growing fetus. In the HLHS cases we propose the increased fibrin may be the result of altered blood flow around the villi due to reduced numbers of terminal villi to control flow and potentially help support the villous structure [44], however its presence in and around terminal and intermediate villi may also reduce the surface area available for exchange, resulting in impaired placental nutrient and oxygen transport. Similarly, the increase in leptin expression seen in the HLHS placentas may be an attempt to compensate for placental insufficiency as recently, suggested by Schrey et al. in the case of impaired placental transfer capacity in discordant growth in twins [45]. In the normal placenta there is some leptin secretion from the basal compartment, but most leptin secreted by the syncytiotrophoblast is thought to be secreted into the maternal circulation [46] and therefore would have no effect on the villous vasculature but further investigation will be needed to identify the impact of increased leptin levels on placental nutrient transfer in placentas from HLHS cases.

Whilst placental insufficiency may account for fetal somatic and head growth abnormalities in HLHS, the reduction in placental vasculature and alteration to the villous tree may indicate a greater link between the placenta and HLHS. The concept of the Heart-Placenta axis proposes that primary placental abnormalities may be associated etiologically with primary CVMs [16, 17], suggesting the structure and function of the placenta and heart may be predisposed to malformation by shared genetic factors and environmental factors impacting either the mother or fetus, such as smoking [47–49], may represent necessary second insults to realize disease. In addition to important pre-pregnancy maternal health factors, early perturbations in the placental microenvironment may contribute to the development of FGR in the context of associated CVM with or without maladaptive physiology (Figure 7). The recent identification of a bona vide mouse model of HLHS [50] suggests the mechanisms underlying these observations may be delineated. Previous studies have highlighted the involvement of angiogenic pathways such as VEGF in the development of CVM [51, 52], and future studies into other angiogenic pathways, including Placental

Growth Factor and FLT-1, and metabolic/growth factors will aid in elucidating mechanisms involved in the altered placental vasculature and structure. While the findings of this study support the general idea that there may be a shared etiology for placenta and cardiac defects, or a placental cause for some cases of HLHS or other CVM, the generalizability of this concept is limited by disease heterogeneity. Because we are underpowered to stratify cases of HLHS in this study, e.g. aortic atresia vs. aortic stenosis, larger multisite studies are needed to determine those cases that are associated with placental abnormalities.

A better understanding of how common and discrete aberrations in vasculogenesis and angiogenesis impact the manifestation of CVM and associated placental abnormalities will improve our ability to predict those fetuses that will have significant growth abnormalities. Ultimately, elucidation of the pathogenesis of placental vasculature abnormalities may have potential clinical utility beyond improved diagnosis, prognosis, such as new approaches to treating SGA midgestation.

Acknowledgements

We thank Michelle Faust, Peggy Walsh, Karen Henderson and Holly Poling for their assistance. This project was supported by a summer research fellowship from the Society of Pediatric Research through the National Institute of Child Health and Development T35-HD-7446 (SKO), and the Cincinnati Children's Research Foundation (HJ, RBH).

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Highlights

- Placentae from Hypoplastic Left Heart Syndrome cases are immature compared to control
- Leptin expression is up regulated in the Hypoplastic Left Heart Syndrome placenta
- Villous vasculature is reduced in placenta from Hypoplastic Left Heart Syndrome cases

Figure 1. Placenta tissue in HLHS demonstrates morphologic abnormalities

Hematoxylin and Eosin stain of representative placenta parenchyma of control (A) and HLHS cases (B–E), 40×. HLHS placenta (B) contains increased syncytial nuclear aggregates (arrows), increased fibrin deposits (outlined in black) and reduced fetal blood vessel density and area (yellow asterisks). In addition to subchorionic fibrin, as reported by gross pathology, microscopic fibrin is present within the parenchyma of the HLHS cases but not controls (outlined in black).

Figure 2. Proliferative activity is increased in placental tissue from HLHS cases

Ki67 expression in control (A, C) and HLHS cases (B, D). In control placenta, Ki67 staining (an indicator of proliferative activity) was sparse throughout the parenchyma, however, in HLHS cases, positively stained nuclei were present in syncytium, SNAs, and throughout the parenchyma.

Figure 3. Placenta tissue in HLHS is characterized by maladaptive angiogenesis
CD31 staining of representative placenta parenchyma in control (A) and HLHS cases (B), 40 $\times$. CD31 is limited to endothelial cells allowing analysis of vessel number, density and identification of vasculosyncytial membranes to aid villous maturation assessment (See Tables 3 and 4).

Figure 4. Placental weight is correlated with birthweight

Distribution of placental parameters vs. birthweight in control (A) and HLHS (B) cohorts.

Placental weight is positively correlated with birthweight in both control (C, R square = 0.4951) and HLHS (D, R square = 0.3011) cohorts.

Figure 5. Placenta tissue in HLHS demonstrates leptin dysregulation

Leptin expression in control (A & C) and HLHS (B & D) placenta parenchyma. In control placenta leptin expression was localized in the syncytium (arrows). Leptin expression was increased in HLHS cases and localization expanded to the fetal endothelium and mesenchymal core of the villi as demonstrated by the brown staining.

Figure 6. Representative micrographs of FLK-1 expression in human placental parenchyma
Flk1 expression was localized to the fetal endothelium (arrowheads) in both control (A) and HLHS (B) with a reduction in Flk1 expression in the HLHS cases (B).

Figure 7. Model figure of the relationship between CVM, placental abnormalities and FGR

Multiple causative factors including genetics, environmental and nutrition may contribute to initial abnormal placental and/or cardiovascular formation and development. As gestation progresses, abnormal fetal cardiac output may impact the expansion of the peripheral fetal vasculature, including the placental villous vasculature, thought to branch in response to increased cardiac output as the fetus grows in utero. Consequently, associated growth abnormalities predispose the newborn with CVM to adverse outcomes. Placental responses to this hypoxemia and/or impaired villous tree expansion may include compensatory mechanisms to increase nutrient or oxygen transfer, such as increased leptin expression. Taken together, in some cases a combination of adverse factors may lead to a spectrum of placental abnormalities that contribute to FGR.

Table 1

Study Population Demographics

	HLHS cases (<i>n</i> = 16)	Controls (<i>n</i> = 18)	Significance
Maternal pre-pregnancy BMI Mean $\pm$ SD	24.7 $\pm$ 5.2	28.3 $\pm$ 7.4	NS
Maternal weight gain (pounds) Mean $\pm$ SD	23.8 $\pm$ 13.1	23.6 $\pm$ 13.3	NS
Smoking during pregnancy (%)	21	25	NS
Gestational age (weeks) Median (range)	38 (37–39.6)	38.6 (36.8–41.6)	NS
Caesarean-section rate (%)	19	28	NS
Birth weight (grams) Mean $\pm$ SD	2993 $\pm$ 496	3341 $\pm$ 517	<i>p</i> = 0.05
Birthweight Percentile Median (range)	16 (2–84)	37 (18–73)	<i>p</i> = 0.02
Proportion SGA (%)	31% (5)	0	

Table 2

Gross Placental Characteristics by Pathology in HLHS

	HLHS (<i>n</i> = 16)	Controls (<i>n</i> = 18)	Significance
Placental weight (grams) Mean $\pm$ SD	447 $\pm$ 104	538 $\pm$ 122	<i>p</i> = 0.02
Placental weight percentile Median (range)	11 (1–36)	18 (6–94)	<i>p</i> = 0.02
Placental thickness (cm) Median (range)	2.5 (2.0–3.0)	2.5 (2.0–3.5)	NS
Cord Insertion (number)			
Centric	7 (44)	5 (28)	NS
Pericentric	4 (25)	8 (44)	NS
Eccentric	3 (19)	4 (22)	NS
Marginal	2 (13)	1 (6)	NS

Table 3

Morphological Analysis of HLHS Placenta Tissue

	HLHS cases (<i>n</i> = 11–16)	Controls (<i>n</i> = 18)	<i>p</i> -value
SNAs/hpf	5 (1.5–7.2)	1.5 (1.0–2.1)	<0.001
SNAs/mm² villous tissue	0.14 (0.05 –0.19)	0.04 (0.02 –0.06)	<0.001
Vascular Area (mm²)	12.9 ± 6.1	24.8 ± 5.0	<0.001
Vessel Density/hpf	35 (11 –62)	69 (51 –81)	<0.001
Villi <80um (Number)	10.0 ± 3.4	21.3 ± 3.9	<0.001
Vasculo-syncytial membranes/hpf (Number)	3.42 ± 2.27	10.4 ± 2.5	<0.001

SNA syncytial nuclear aggregates; hpf high power field

Table 4

Angiogenic/Mitogenic Factor Expression in HLHS Placentas Compared to Control

Factor	RNA	Syncytial protein	Endothelial protein
Leptin	↑	↑	↑
Leptin Receptor	—	↔	↑
VEGF-a	↔	↔	↔
Flk-1	↔	—	↓
PlGF	↓	—	—