Fetal production of growth factors and inflammatory mediators predicts pulmonary hypertension in congenital diaphragmatic hernia

Shannon Fleck1, Geoanna Bautista2, Sheila M. Keating3, Tzong-Hae Lee3, Roberta L. Keller1,4, Anita J. Moon-Grady1,4, Kelly Gonzales2, Philip J. Norris3,5,6, Michael P. Busch3,6, CJ Kim7, Roberto Romero7, Hanmin Lee1,2,5, Doug Miniati1,2,5, and Tippi C. MacKenzie1,2,5

1Department of Pediatrics, University of California, San Francisco (UCSF), San Francisco, CA
2Department of Surgery, UCSF, San Francisco, CA
3Blood Systems Research Institute, San Francisco, CA
4Fetal Treatment Center, UCSF, San Francisco, CA
5Department of Medicine, UCSF, San Francisco, CA
6Department of Laboratory Medicine, UCSF, San Francisco, CA
7Hutzel Women's Hospital, Detroit, MI

Abstract

Background—Congenital diaphragmatic hernia (CDH) represents a spectrum of lung hypoplasia and consequent pulmonary hypertension is an important cause of postnatal morbidity and mortality. We studied biomarkers at the maternal-fetal interface to understand factors associated with the persistence of pulmonary hypertension.

Methods—Maternal and cord blood samples from fetuses with CDH and unaffected controls were analyzed using a human 39plex immunoassay kit. Cellular trafficking between the mother and the fetus was quantified using quantitative real-time PCR for non-shared alleles. Biomarker profiles were then correlated with CDH severity based on the degree of pulmonary hypertension.

Results—Cord blood levels of epidermal growth factor, platelet-derived growth factor, and several inflammatory mediators increased significantly as the severity of CDH increased, while maternal levels growth factors and mediators decreased significantly with CDH severity. Maternal cells were increased in fetuses with severe CDH compared to controls, with elevated levels of the chemokine CXCL-10 in patients with the highest trafficking.

Conclusion—Patients with CDH demonstrate pro-inflammatory and chemotactic signals in fetal blood at the time of birth. Since some of these molecules have been implicated in the development...
of pulmonary hypertension, prenatal strategies targeting specific molecular pathways may be useful adjuncts to current fetal therapies.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) remains one of the most challenging congenital anomalies. While most CDH patients are now diagnosed before birth, there is a wide spectrum of disease severity, which requires accurate perinatal prognostic indicators for appropriate counseling and management. For patients at the most severe end of the spectrum, in utero tracheal occlusion may promote lung growth and improve postnatal outcomes (1–3). However, in spite of sophisticated fetal and neonatal management, severely affected newborns have a high mortality and those who survive often have long-term morbidities (4).

Liver position and lung-to-head ratio (LHR) (5) are widely used to measure the degree of pulmonary hypoplasia and predict prognosis in fetuses with CDH. The degree of pulmonary hypertension is associated with poor outcome in CDH (6, 7) but has been more challenging to predict prenatally. Measurement of pulmonary artery Doppler indices (8) and maternal hyperoxygenation testing (9) have had some encouraging results but require considerable expertise. A prenatal, minimally invasive biomarker to predict the degree of pulmonary hypertension would greatly benefit patient counseling and management.

In inflammatory disease states associated with pulmonary hypertension such as systemic sclerosis, there is evidence that abnormal levels of growth factors and pro-inflammatory cytokines lead to vascular remodeling (reviewed in (10)). Growth factors signal through tyrosine kinase receptors to induce abnormal proliferation and promote the migration of smooth muscle cells, endothelial cells, and fibroblasts (10). While the precise mechanisms leading from inflammation to pulmonary hypertension continue to be defined, it is likely that vascular injury resulting from ongoing inflammation leads to pulmonary vascular remodeling (11). The possible contribution of similar pathways in the development of pulmonary hypertension in patients with CDH has not been explored.

We performed an unbiased analysis of growth factors, cytokines, and chemokines in maternal and cord blood to understand the molecular environment contributing to the persistence of pulmonary hypertension in CDH. We also studied the trafficking of cells between the mother and the fetus to determine whether particular cytokines/chemokines secreted in the context of CDH may alter trafficking. We report that infants with CDH with persistence of pulmonary hypertension at 2 weeks of age have elevated levels of growth factors and pro-inflammatory cytokines as well as increased numbers of maternal cells in their cord blood at the time of birth. These findings indicate that molecular signals leading to the development of pulmonary hypertension are present before birth and suggest that targeted therapies to block inflammation in utero may be beneficial in patients with severe CDH.
RESULTS

Patient Demographics

Maternal and cord blood samples were collected from 19 patients with CDH and 23 unaffected controls (Table 1). The only significant demographic differences between the groups were that mothers of fetuses with CDH were more likely to be nulliparous than controls (p=0.01) and were more likely to deliver earlier than controls (median gestational age in weeks (interquartile range, IQR): CDH: 38.1(37.1–38.9) vs. control: 39.0 (38.6–40.0), p<0.01).

CDH Clinical Features

Seventeen of 19 hernias were left-sided (Table 2). The median LHR was 1.00 (IQR: 0.76–1.35) and the median age of LHR measurement was 27 weeks (IQR 24 1/7–30 weeks). Nine of 14 fetuses with liver herniation into the chest had LHR < 1.0. There were no survivors among three patients who received extracorporeal membrane oxygenation (ECMO) support. All six neonatal deaths were within the first week of life. The patient with late demise expired at three months of age secondary to severe pulmonary hypertension refractory to medical management.

Three patients with LHR < 1.0 and liver herniation into the chest underwent fetal endoscopic tracheal occlusion at 27 weeks’ gestation. Of these, one patient was delivered via Cesarean section at 38 weeks after the balloon was retrieved by a second fetal intervention at 32 weeks. The two other patients required an ex-utero intrapartum treatment (EXIT) procedure (12) at delivery for balloon retrieval at 29 and 36 weeks.

Severity Classification

We applied the CDH severity classification using the degree of pulmonary hypertension measured on echocardiogram performed at 2 weeks after birth as described. Under the dichotomous classification, there were six mild and 13 moderate-to-severe patients. Under the three-level classification, there were six mild, three moderate, and 10 severe patients. All patients with prenatal liver herniation and LHR <1 exhibited “severe” pulmonary hypertension at 2 weeks. We evaluated the relationship of pulmonary hypertension to cytokine levels and PCR for microchimerism. Results were similar regardless of which classification scheme was used; 2-level results are presented for clarity.

Cytokine Profiles in Cord Blood

We used a panel of 39 cytokines to analyze whether patients with CDH have a different cytokine profile in the cord blood plasma compared to unaffected controls. Cytokine data were available for 17 CDH patients and 22 controls. We found that patients with CDH overall had elevated levels of EGF (epidermal growth factor), eotaxin, IL-3 (interleukin-3), MIP-1β macrophage inflammatory protein-1β), PDGF-AA (platelet-derived growth factor-AA), and IL-1α in the cord blood compared to controls (Table 3).

We next performed analyses by CDH disease severity as well as pairwise comparisons. We found that levels of EGF, IL-3, MCP-3 (monocyte chemoattractant protein), MIP-1β PDGF–
AA, IFN-α2 (interferon-α2), and IL-1α increased significantly across severity groups (Control<Mild CDH<Moderate-to-Severe CDH, p<0.05 by Kendall’s Tau-c test) (Table 3 and Figure 1A). Further pairwise comparisons suggested that the most consistent differences were between control and mild CDH compared to moderate-to-severe CDH (Table 3). After the Bonferroni adjustment for multiple comparisons, levels of EGF, MIP-1β and IFN-α2 remained significantly elevated in moderate-to-severe CDH compared to controls (p<0.017). Importantly, a comparison between controls and patients with mild CDH did not yield any significant differences (Table 3), suggesting that the biomarker profiles seen above are unique to patients with more severe CDH, as measured by the persistence of pulmonary hypertension or demise.

Cytokine levels were also assessed with supervised PCA (sPCA), which resulted in the reduction of EGF, IL-3, MCP-3, MIP-1β, PDGF-AA, IFNα2, and IL-1α into two principal components (PCs) that together described 54.7% of the total variance of the cord blood cytokines. IL-3, MCP-3, MIP-1β, IFNα2, and IL-1α contributed the most to PC1, while EGF and PDGF-AA contributed the most to PC2. The score plot (Figure 1B) shows clustering of patients into the three severity groups (Control, Mild CDH, Mod/Severe CDH), with separation of control and mod/severe patients, with mild CDH found in between the two clusters. Ordinal logistic regression was performed to investigate whether these two principal components were significant predictors of severity. PC1 was significantly associated with higher odds of being classified as more severe (OR= 3.67, 95% CI=1.74–7.73, p=0.001), whereas PC2 was not (OR= 1.37, CI=0.67–2.76, p=0.381). Thus, an increase in biomarkers related to PC1 is associated with a greater likelihood of increased CDH severity.

**Cytokine Profiles in Maternal Blood**

In maternal plasma, we first compared all controls to all mothers with CDH fetuses and found decreased levels of FGF-2 (fibroblast growth factor-2), MDC (macrophage derived chemokine) and VEGF (vascular endothelial growth factor) in mothers carrying fetuses with CDH (Table 4). We also found that maternal levels of FGF-2, MDC, and VEGF decreased across groups with increasing severity of CDH (Control>Mild CDH>Moderate-to-Severe CDH, p<0.02 by Kendall’s Tau-c test) (Table 4 and Figure 2A). In pairwise comparisons, FGF-2 was significantly decreased in mothers carrying fetuses with moderate-to-severe CDH compared to controls (p=0.014).

We performed a sPCA for maternal cytokines, which resulted in reduction of FGF-2, MDC and VEGF biomarkers into two principal components that explained 90.3% of the total variance. VEGF contributed the most to PC1, while FGF-2 and MDC contributed the most to PC2. The three severity groups are less differentiated in the maternal score plot (Figure 2B); however, with ordinal logistic regression, PC1 was found to be significantly associated with a decrease in the odds of carrying a fetus with a mod/severe CDH (OR=0.41, 95% CI=0.22–0.75, p=0.001).
Maternal-Fetal Cellular Trafficking

Given the increased levels of several chemokines in the cord blood of patients with CDH, we next examined whether there was increased trafficking of maternal cells into the fetuses in this setting. PCR testing was performed for 18 controls and 19 patients with CDH. Two patients with CDH and one control had no informative (non-shared) allele for detection of maternal cells in fetal blood (“maternal microchimerism”) and two patients with CDH had no informative allele for detection of fetal cells in maternal blood (“fetal microchimerism”).

We first analyzed maternal microchimerism (MMc) levels by amplifying for non-shared maternal alleles in fetal blood. This analysis showed that there is a range of trafficking in unaffected term infants, as has been reported (13). Among CDH patients, the median (IQR) levels of MMc (expressed as the percentage of maternal cells in cord blood) were significantly higher than in controls (control: 0.004(0.0008–0.015) vs. CDH: 0.037(0.013–0.061), p<0.004 by Wilcoxon rank-sum). The levels of MMc tended to increase across severity groups (Kendall’s Tau-c coefficient: 0.506, p<0.002) and were significantly higher in the moderate-to-severe group compared to controls, whereas there was no difference between mild CDH and controls (Figure 3A; moderate-to-severe CDH: 0.047(0.020–0.102), mild CDH: 0.019(0.011–0.02); p=0.003 between moderate-to-severe CDH and control; p=0.2 between mild CDH and control by Wilcoxon rank-sum). These findings are consistent with the cytokine analysis results in which the main differences were those between controls and patients with moderate-to-severe CDH and not between controls and patients with mild CDH. The levels of MMc in the three patients who underwent fetal tracheal occlusion were similar to that seen in the other patients with CDH (0.004, 0.037, 0.06; depicted as light gray circles in Fig 3A).

We next quantified levels of fetal microchimerism (FMc) by amplifying for non-shared fetal alleles in maternal blood. There were no significant differences between median FMc levels between patients with CDH and unaffected controls (control: 0.003(0.0006–0.0091) vs. CDH: 0.007(0.002–0.038), p=0.36 by Wilcoxon rank-sum). No significant differences were seen when patients with CDH were stratified into severity groups (Figure 3B; mild CDH: 0.006 (0.0007–0.077), moderate-to-severe CDH: 0.0067 (0.002–0.038), p=NS with Kendall’s Tau-c test).

We next asked whether patients with the highest levels of MMc expressed any cytokines or chemokines that may be implicated in the trafficking of maternal cells across the placenta. We compared the cytokine profiles from cord blood of CDH patients with high levels of trafficking (MMc level > 80th percentile, n=4) to those with lower levels of trafficking (MMc level ≤ 80th percentile, n=13). Consistent with our hypothesis, patients with high MMc had significantly elevated levels of the chemokine CXCL-10 compared to those with lower trafficking (726.6 (263.3–1468.8) vs. 173.4 (129.0–271.6), p=0.027).

DISCUSSION

This is the first study to directly examine the fetal environment for the presence of biomarkers that may correlate with the persistence of pulmonary hypertension in CDH. We found abnormal levels of several growth factors and cytokines that have been implicated in the development of pulmonary hypertension in other diseases, suggesting that the fetal
milieu contains critical molecular signals that lead to vascular changes resulting in pulmonary hypertension, even before respiratory effort has begun. Thus, prenatal therapies to block key events in these molecular pathways may be beneficial in fetuses with CDH.

Our study suggests similarities between the pathophysiology of pulmonary hypertension in CDH and other diseases. For example, growth factors such as EGF and PDGF have been implicated in the development of pulmonary hypertension in adults (10) and were increased in cord blood of patients with CDH. Although our study does not establish a causal role for these factors in the onset of pulmonary hypertension in CDH, it is interesting to note that blockade of EGF and PDGF have both been successful in animal models of pulmonary hypertension. For example, EGF blockade may be a useful strategy to treat monocrotaline-induced pulmonary hypertension (14). PDGF blockade has also proven beneficial in animal models (15) and may be used clinically in select patients (16).

Inflammatory cytokines implicated in the development of pulmonary hypertension in adults are also increased in fetuses with CDH. For example, IFN-α, given to treat hepatitis C, reportedly causes irreversible pulmonary hypertension in some patients (17) and was increased in our cohort of patients with moderate-to-severe CDH. Increased levels of IL-1, IL-6, and TNF-α have been described in patients with primary pulmonary hypertension (18, 19) and our study indicated some increases in these cytokines with severe CDH.

Conversely, several cytokines and/or chemokines described in the pathogenesis of pulmonary hypertension in other disease settings, such as fractalkine (20), were not increased in our CDH patients. In addition, we did not find elevated levels of anti-inflammatory cytokines such as IL-10 in patients with CDH, as has been reported in adults with idiopathic pulmonary hypertension (19). This discrepancy may be because our assay could not detect small changes in IL-10 since the baseline levels are low, or because of the inability of the fetus to compensate, unlike adults with pulmonary hypertension.

We also analyzed maternal plasma in an effort to define a biomarker that may be followed non-invasively and found significantly decreased levels of FGF-2 in mothers of fetuses with moderate-to-severe CDH. FGF-2 has been implicated in the pathogenesis of pulmonary hypertension and its blockade may ameliorate disease in an experimental model (21) and we speculate that our findings may indicate a compensatory mechanism.

A recent study examined the levels of various cytokines in the blood of neonates with CDH, drawn shortly after birth and longitudinally over 4 days (22). The authors detected elevated levels of several pro-inflammatory cytokines in infants with CDH compared to unaffected controls, although blood from patients with CDH was drawn after the initial resuscitation while control blood was obtained from the umbilical cord. The authors also compared patients who did or did not require ECMO and found that levels of IL-8, IL-10, and MIP-1α were increased in neonates with more severe CDH. Our study adds to these observations by directly comparing both maternal and cord blood levels prior to any respiratory effort or mechanical ventilation and by including other analytes known to be involved in pulmonary hypertension such as EGF, PDGF, and FGF. In addition, we have directly correlated cytokine levels with the degree of pulmonary hypertension measured on echocardiogram to
show that the differences between controls and patients with CDH are most pronounced in patients with moderate-to-severe pulmonary hypertension and that patients with mild CDH exhibit little inflammation. Cellular trafficking between the mother and the fetus has been described in normal pregnancies (23, 24) and may be a mechanism for the induction of maternal-fetal tolerance (25). The observation that patients with more severe CDH have higher levels of MMc compared to controls is intriguing and suggests that molecular signals present in some CDH patients may lead to the recruitment of maternal cells or to the proliferation of maternal cells that have already crossed into the fetus. The finding of increased levels of the chemokine CXCL-10 in patients with the highest levels of trafficking supports this hypothesis. It has been reported that endothelial progenitor cells are recruited from the bone marrow to the pulmonary vasculature in animal models of pulmonary hypertension (26) and similar signals may lead to the recruitment maternal cells in patients with CDH. Interestingly, we did not find elevated MMc after fetal intervention, contrary to what we have seen in our mouse model of fetal intervention (27) or after open fetal surgery for spina bifida (28). It is possible that the minimally invasive nature of the current approach to tracheal occlusion does not lead to increased trafficking, or, signals leading to increased trafficking are different between patients with spina bifida and those with CDH.

The main strengths of our analysis are the study of both maternal and cord blood in patients with CDH and our unbiased approach of testing for multiple cytokines without predicting which ones may be changed based on published data. We are also aware of several weaknesses in our study. The mean gestational age in patients with CDH was lower than that for our controls, which may be one confounding factor in our analysis. The ideal control group would be age-matched unaffected controls, but patients born at 36 weeks usually have other abnormalities prompting delivery such as infection or maternal comorbidity and therefore would not be an appropriate comparison group. In addition, our hospital referral patterns led to a CDH cohort with fewer patients with mild CDH. Finally, longitudinal assessments of biomarkers in maternal blood or amniotic fluid may lead to identification of prognostic factors prior to 24 weeks, when such information may inform the course of prenatal care and such a study may be designed in the future.

Tracheal occlusion is currently the mainstay of fetal intervention but is likely more effective for treating pulmonary hypoplasia than pulmonary hypertension (29). Our finding that prenatal inflammatory signals correlate with the severity of postnatal pulmonary hypertension suggests that prenatal strategies to block molecular pathways may be beneficial in severe CDH, similar to the targeted therapies currently being developed for pulmonary hypertension in other settings (30). Animal studies of prenatal medical therapies to address pulmonary hypertension in CDH have shown encouraging results. For example, prenatal steroids improved pulmonary vascular remodeling in the lamb model of CDH (31) and antenatal sildenafil had beneficial effects on lung microvascular development in a rat model of CDH (32). Our results, in conjunction with other reports describing increased EGF in patients with CDH (33), point to EGF inhibition as another potential therapeutic target. PDGF was recently reported to reduce pulmonary vascular remodeling in a rat model of CDH (34) and was used clinically in one patient with CDH (35). However, potential toxicities such as impairment in alveolar development due to inhibition of PDGF receptor signaling must be considered.
This is the first study to demonstrate an association between maternal and cord blood biomarker profiles and the persistence of pulmonary hypertension in CDH. Our results lend insight into the fetal onset of pulmonary hypertension and suggest that prenatal therapies to block particular molecular pathways may be useful in prenatally diagnosed CDH.

METHODS

Subjects

Mothers carrying unaffected fetuses or those with CDH were prospectively enrolled (8/2009–6/2011). Inclusion and exclusion criteria for all healthy control patients were term pregnancies without preterm labor, preeclampsia, or fetal congenital anomalies. All consenting patients with fetal CDH were prospectively enrolled. Matched maternal and cord blood samples were obtained at birth and only patients with a matched pair of samples were included in the study. Written informed consent was obtained under UCSF Institutional Review Board approval (10-00350). All patients with CDH were evaluated by fetal ultrasound and echocardiogram. Fetal tracheal occlusion was performed in three patients with severe CDH (liver herniated into the thorax and LHR < 1.0). All neonates with CDH were managed as previously described (7). Surgical repair was performed after the patient was stabilized and repair type was dependent on the size of the diaphragm defect and surgeon preference.

Postnatal classification of severity in infants with CDH

We have previously shown that the severity of pulmonary hypertension (PH) at 2 weeks of age (based on an estimate of pulmonary artery pressure (P\textsubscript{PA}) from the echocardiogram) is associated with poor neonatal outcome (7). A single cardiologist (AMG), blinded to patient condition, reviewed echocardiograms performed at two weeks after birth and classified P\textsubscript{PA} relative to systemic blood pressure (7). In a three-level classification system, infants were classified as “mild CDH” if there was no/mild PH (< 2/3 systemic pressure), “moderate CDH” if there was moderate PH (≥ 2/3 systemic pressure) and “severe CDH” if there was severe PH (systemic-to-suprasystemic pressure) or demise prior to two weeks. Alternatively, in a dichotomous classification system, infants were classified as “mild CDH” versus “moderate-to-severe CDH” (≥ 2/3 systemic pressure or demise).

Sample Processing

Cord blood samples were collected at the time of birth and maternal blood was collected within 24 hours of delivery and processed within 36 hours. Whole blood and plasma were stored separately for analysis of cellular trafficking and cytokines, respectively. Blood obtained from patients delivering in Detroit (11/23 controls) was shipped on ice on the day of delivery by overnight mail and processed immediately upon arrival, such that the timing and method of processing of all samples was identical.

Cytokine Assay

Cytokine profiles in the maternal and cord blood plasma samples were assayed using the standard-sensitivity Millipex Map kit (Millipore, Billerica, MA) as previously reported (36). Samples were acquired and analyzed on a Labscan 100 analyzer (Luminex, Austin, TX)
using Bio-Plex manager 6.0 software (Bio-Rad, Hercules, CA). Standard curves were run in duplicate wells and each run included internal controls. Cytokines were excluded from the tables and figures if their levels were below 10 pg/ml for at least 80% of both maternal and cord blood samples.

Quantitative PCR

Maternal and fetal microchimerism were quantified from whole blood by researchers blinded to patient groups using a qRT-PCR assay to amplify nonshared HLA-DR or Insertion-Deletion alleles between the mother and the fetus (37). This assay has a lower limit of detection between 0.001–0.0001% (37) and has been used previously to quantify maternal blood in fetal samples (25, 28). Microchimerism levels in two CDH patients who underwent fetal intervention and seven controls have been reported as control data in the context of an analysis of the effects of open fetal surgery on trafficking (28).

Statistical Analysis

The Wilcoxon rank-sum test with Bonferroni-adjusted p-values for multiple comparisons was used where appropriate. Chi square tests were used to assess the differences in proportions between groups. The Kendall’s Tau-c statistic was calculated to test the magnitude and direction of increases or decreases in cytokine levels or microchimerism across groups. Cytokine levels were also assessed by supervised Principal Component Analysis (sPCA), which uses only a subset of cytokines most associated with the severity of outcome (p<0.05 by Kendall’s Tau-c) for reducing the dimensionality of the data. The principal components identified were then entered as predictors of severity in an ordinal logistic regression model. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using Stata version 12 (Stata Corp LP, TX) or R software (Vienna, Austria).

Acknowledgments

We would like to thank the physicians and nurses at the UCSF Labor and Delivery Unit and the Fetal Treatment Center for their assistance with sample collection; Qizhi Tang, Peter Oishi, and Jeff Fineman for helpful discussions; and our patients for their gracious participation in this research project.

STATEMENT OF FINANCIAL SUPPORT: This work was supported by a March of Dimes Basil O’Connor Award, White Plains, NY (to TCM), and grants from the University of California, San Francisco Resource Allocation Program (to TCM), the California Institute for Regeneration Medicine (to TCM), the National Heart, Lung, and Blood Institute (R01-HL-088388, to MPB), and the Pulmonary Hypertension Association, Silver Spring, MD (to RLK).

References

1. Deprest JA, Gratacos E, Nicolaides K, et al. Changing perspectives on the perinatal management of isolated congenital diaphragmatic hernia in Europe. Clin Perinatol. 2009; 36:329–347. ix. [PubMed: 19559323]
2. Jelin E, Lee H. Tracheal occlusion for fetal congenital diaphragmatic hernia: the US experience. Clin Perinatol. 2009; 36:349–361. ix. [PubMed: 19559324]
3. Ruano R, Duarte SA, Pimenta EJ, et al. Comparison between fetal endoscopic tracheal occlusion using a 1.0-mm fetoscope and prenatal expectant management in severe congenital diaphragmatic hernia. Fetal Diagn Ther. 2011; 29:64–70. [PubMed: 20389048]
4. Jancelewicz T, Vu LT, Keller RL, et al. Long-term surgical outcomes in congenital diaphragmatic hernia: observations from a single institution. J Pediatr Surg. 45:155–160. discussion 160. [PubMed: 20105597]
5. Hedrick HL, Danzer E, Merchant A, et al. Liver position and lung-to-head ratio for prediction of extracorporeal membrane oxygenation and survival in isolated left congenital diaphragmatic hernia. Am J Obstet Gynecol. 2007; 197:422e421–424. [PubMed: 17904987]
6. Dillon PW, Cilley RE, Mauger D, Zachary C, Meier A. The relationship of pulmonary artery pressure and survival in congenital diaphragmatic hernia. J Pediatr Surg. 2004; 39:307–312. discussion 307–312. [PubMed: 15017543]
7. Keller RL, Tacy TA, Hendricks-Munoz K, et al. Congenital diaphragmatic hernia: endothelin-1, pulmonary hypertension, and disease severity. Am J Resp Crit Care. 2010; 182:555–561.
8. Fuke S, Kanzaki T, Mu J, et al. Antenatal prediction of pulmonary hypoplasia by acceleration time/ejection time ratio of fetal pulmonary arteries by Doppler blood flow velocimetry. Am J Obstet Gynecol. 2003; 188:228–233. [PubMed: 12548222]
9. Broth RE, Wood DC, Rasahen J, et al. Prenatal prediction of lethal pulmonary hypoplasia: the hyperoxygenation test for pulmonary artery reactivity. Am J Obstet Gynecol. 2002; 187:940–945. [PubMed: 12388982]
10. Schermuly RT, Ghofrani HA, Wilkins MR, Grimminger F. Mechanisms of disease: pulmonary arterial hypertension. Nat Rev Cardiol. 2011; 8:443–455. [PubMed: 21691314]
11. Hassoun PM, Mouthon L, Barbera JA, et al. Inflammation, growth factors, and pulmonary vascular remodeling. J Am Coll Cardiol. 2009; 54:510–19. [PubMed: 19555853]
12. Mychaliska GB, Bealer JF, Graf JL, Rosen MA, Adzick NS, Harrison MR. Operating on placental support: the ex utero intrapartum treatment procedure. J Pediatr Surg. 1997; 32:227–230. discussion 220–221. [PubMed: 9041277]
13. Hall JM, Lingenfelter P, Adams SL, Lasser D, Hansen JA, Bean MA. Detection of maternal cells in human umbilical cord blood using fluorescence in situ hybridization. Blood. 1995; 86:2829–2832. [PubMed: 7545474]
14. Merklinger SL, Jones PL, Martinez EC, Rabinovitch M. Epidermal growth factor receptor blockade mediates smooth muscle cell apoptosis and improves survival in rats with pulmonary hypertension. Circulation. 2005; 112:423–431. [PubMed: 16027270]
15. Schermuly RT, Dony E, Ghofrani HA, et al. Reversal of experimental pulmonary hypertension by PDGF inhibition. J Clin Invest. 2005; 115:2811–2821. [PubMed: 16200212]
16. Grimminger F, Schermuly RT. PDGF receptor and its antagonists: role in treatment of PAH. Adv Exp Med Biol. 2010; 661:435–446. [PubMed: 20204747]
17. Dhillon S, Kaker A, Dosanjh A, Japra D, Vanthiel DH. Irreversible pulmonary hypertension associated with the use of interferon alpha for chronic hepatitis C. Digest Dis Sci. 2010; 55:1785–1790. [PubMed: 20411421]
18. Humbert M, Monti G, Brenot F, et al. Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. Am J Resp Crit Care. 1995; 151:1628–1631.
19. Soon E, Holmes AM, Treacy CM, et al. Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension. Circulation. 2010; 122:920–927. [PubMed: 20713898]
20. Balabanian K, Fousat A, Dorfmuller P, et al. CX(3)C chemokine fractalkine in pulmonary arterial hypertension. Am J Resp Crit Care. 2002; 165:1419–1425.
21. Iizuki M, Guignabert C, Fadel E, et al. Endothelial-derived FGF2 contributes to the progression of pulmonary hypertension in humans and rodents. J Clin Invest. 2009; 119:512–523. [PubMed: 19197140]
22. Schaible T, Veit M, Tautz J, et al. Serum cytokine levels in neonates with congenital diaphragmatic hernia. Klin Padiatr. 2011; 223:414–418. [PubMed: 22116781]
23. Bianchi DW, Zickwolf GK, Weil GI, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci USA. 1996; 93:705–708. [PubMed: 8570620]
24. Maloney S, Smith A, Furst DE, et al. Microchimerism of maternal origin persists into adult life. J Clin Invest. 1999; 104:41–47. [PubMed: 10393697]
25. Mold JE, Michaelsson J, Burt TD, et al. Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. Science. 2008; 322:1562–1565. [PubMed: 19056990]
26. Hayashida K, Fujita J, Miyake Y, et al. Bone marrow-derived cells contribute to pulmonary vascular remodeling in hypoxia-induced pulmonary hypertension. Chest. 2005; 127:1793–1798. [PubMed: 15888860]
27. Nijagal A, Węgorzewska M, Jarvis E, Le T, Tang Q, MacKenzie TC. Maternal T cells limit engraftment after in utero hematopoietic cell transplantation in mice. J Clin Invest. 2011; 121:582–592. [PubMed: 21245575]
28. Saadat F, Lee TH, Bautista G, et al. Alterations in maternal-fetal cellular trafficking after fetal surgery. J Pediatr Surg. 2012; 47:1089–1094. [PubMed: 22703775]
29. Danzer E, Davey MG, Kreiger PA, et al. Fetal tracheal occlusion for severe congenital diaphragmatic hernia in humans: a morphometric study of lung parenchyma and muscularization of pulmonary arterioles. J Pediatr Surg. 2008; 43:1767–1775. [PubMed: 18926205]
30. O’Callaghan DS, Savale L, Montani D, et al. Treatment of pulmonary arterial hypertension with targeted therapies. Nat Rev Cardiol. 2011; 8:526–538. [PubMed: 21769113]
31. Davey M, Shegu S, Danzer E, et al. Pulmonary arteriole muscularization in lambs with diaphragmatic hernia after combined tracheal occlusion/glucocorticoid therapy. Am J Obstet Gynecol. 2007; 197:381e381–387. [PubMed: 17904968]
32. Luong C, Rey-Perra J, Vadiel A, et al. Antenatal sildenafil treatment attenuates pulmonary hypertension in experimental congenital diaphragmatic hernia. Circulation. 2011; 123:2120–2131. [PubMed: 21537000]
33. Guarino N, Solari V, Shima H, Puri P. Upregulated expression of EGF and TGF-alpha in the proximal respiratory epithelium in the human hypoplastic lung in congenital diaphragmatic hernia. Pediatr Surg Int. 2004; 19:755–759. [PubMed: 14714133]
34. Chang YT, Ringman Uggla A, et al. Antenatal imatinib treatment reduces pulmonary vascular remodeling in a rat model of congenital diaphragmatic hernia. Am J Physiol-Lung C. 2012; 302:L1159–1166.
35. Frenckner B, Broome M, Lindstrom M, Radell P. Platelet-derived growth factor inhibition: a new treatment of pulmonary hypertension in congenital diaphragmatic hernia? J Pediatr Surg. 2008; 43:1928–1931. [PubMed: 18926235]
36. Keating SM, Golub ET, Nowicki M, et al. The effect of HIV infection and HAART on inflammatory biomarkers in a population-based cohort of women. AIDS. 2011; 25:1823–1832. [PubMed: 21572306]
37. Lee TH, Chafets DM, Reed W, et al. Enhanced ascertainment of microchimerism with real-time quantitative polymerase chain reaction amplification of insertion-deletion polymorphisms. Transfusion. 2006; 46:1870–1878. [PubMed: 17076840]
Figure 1.
Fetal biomarkers in CDH. A. Levels of growth factors, chemokines, and inflammatory mediators in cord blood of unaffected controls (white bars), patients with mild CDH (light gray bars), and patients with mod/severe CDH (dark gray bars). The horizontal line represents the median for each group. * = p<0.05 with the Kendall’s Tau-c test for trend across groups. Control: n=22, Mild CDH n=6, Moderate-to-Severe CDH n=11. EGF: epidermal growth factor; IL: interleukin; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; PDGF: platelet-derived growth factor; IFN: interferon.

B. Supervised principal component analysis (sPCA) of biomarkers in cord blood. Biomarkers found to be significantly correlated with severity using the Kendall’s Tau-c test were entered into a sPCA. PC1 comprised 35.4% of the total variability and PC2 comprised 19.3%. Open circles: control; dark gray squares: mild CDH; black squares: mod/severe CDH. The central point of each cluster is represented by filled circles (light gray: control; dark gray: mild CDH; black: mod/severe CDH).
Figure 2.
Maternal biomarkers in CDH. A. Levels of growth factors and chemokines in maternal blood in unaffected controls (white bars), patients with mild CDH (light gray bars), and patients with mod/severe CDH (dark gray bars). The horizontal line represents the median for each group. * = p<0.02 with the Kendall’s Tau-c test for trend across groups. Control: n=22, Mild CDH n=6, Moderate-to-Severe CDH: n=12. FGF: fibroblast growth factor; MDC: macrophage derived chemokine; VEGF: vascular endothelial growth factor. B. Supervised principal component analysis (sPCA) of biomarkers in maternal blood. Biomarkers found to be significantly correlated with severity using the Kendall’s Tau-c test were entered into a sPCA. PC1 comprised 35.4% of the total variability and PC2 comprised 19.3%. Open circles: control; dark gray squares: mild CDH; black squares: mod/severe CDH. The central point of each cluster is represented by filled circles (light gray: control; dark gray: mild CDH; black: mod/severe CDH).
Figure 3.
Maternal-fetal cellular trafficking. Percentage of maternal cells in cord blood (A) and of fetal cells in maternal blood (B) in unaffected controls (white circles) and patients with CDH stratified by disease severity (mild CDH: middle column, gray circles; mod/severe CDH: third column, black circles). The horizontal line represents the median for each group. Samples with no microchimerism detected were graphed as $10^{-4}$ (the lower limit of detection) to allow visualization on the log scale. Control n=17; Mild CDH n=5 in A, n=6 in B; Moderate-to-Severe CDH n=12 in A, n=11 in B. *: p<0.002 with Kendall’s Tau-c for trend across groups; p<0.003 between control and moderate-to-severe CDH by Wilcoxon rank-sum. Light gray circles in the moderate-to-severe group in panel A represent patients who underwent tracheal occlusion.
Table 1

Demographics

|                                | Control (n=23) | CDH (n=19) | p-value |
|--------------------------------|----------------|------------|---------|
| Maternal age (yrs)            | 28 (21–34)     | 31 (22–35) | 0.72    |
| First pregnancy               | 2 (8.7%)       | 8 (42.1%)  | **0.01**|
| Labor                         | 14 (60.9%)     | 14 (73.7%) | 0.38    |
| Mode of delivery              |                |            |         |
| Vaginal                       | 10 (43.5%)     | 12 (63.2%) | 0.20    |
| C-section/EXIT                | 13 (56.5%)     | 7 (36.8%)  | 0.20    |
| Gestational age (wks)         | 39.0 (38.6–40.0) | 38.1 (37.1–38.9) | **0.001** |
| Male fetus                    | 11 (47.8%)     | 12 (63.2%) | 0.32    |
| Fetal weight (g)              | 3480 (3275–3850) | 3000 (2000–3580)$^a$ | **0.007** |

Data presented as median (interquartile range, IQR) or n (%); significant p-values are boldfaced.

$^a$Weight in many CDH patients was estimated since patients were too unstable to weigh.
EXIT= ex-utero intrapartum treatment procedure
Table 2

Patients with CDH

| CDH Characteristics (n=19) | Median (IQR) or n (%) |
|---------------------------|-----------------------|
| Left sided                | 17 (89.5%)            |
| LHR                       | 1.00 (0.76–1.35)      |
| Liver herniation          | 14 (73.7%)            |
| Prenatal tracheal occlusion| 3 (15.8%)            |
| ECMO                      | 3 (15.8%)             |
| Neonatal demise           | 6 (31.6%)             |
| Late demise               | 1 (7.7%)              |

**Repair Type (n=13)**

|                  | n (%)   |
|------------------|---------|
| Primary          | 4 (30.8%) |
| Muscle flap      | 5 (38.5%) |
| Patch            | 4 (30.8%) |

IQR: interquartile range
LHR: Lung-to-head ratio; ECMO: extracorporeal membrane oxygenation
## Table 3

**Cord Blood Cytokines – Median (IQR)**

| Chemokines & Growth Factors (pg/ml) | Control vs All CDH<sup>a</sup> | Mild vs Mod/Severe<sup>b</sup> | Control vs Mod/Severe<sup>b</sup> | Tau Test<sup>c</sup> | Tau Coefficient |
|------------------------------------|-------------------------------|-----------------|-----------------|-----------------|-----------------|
| **EGF** 203 (0.1–37.1)            | 0.008                         | 0.064           | 0.002           | 0.450           |
| **Eotaxin** 64.8 (34.8–96.5)      | 0.041                         | 0.108           | 0.093           | 0.007           | 0.260           |
| **FGF-2** 113 (82.0–153)          | 0.977                         | 0.930           | 0.867           | 0.999           | 0.000           |
| **hFG-3 Ligand** 1.6 (1.6–20.5)   | 0.948                         | 0.471           | 0.311           | 0.064           | 0.061           |
| **Fractalkine** 83.8 (57.2–106)   | 0.650                         | 0.158           | 0.240           | 0.037           | 0.130           |
| **GRO (CXCL-1)** 515 (273–862)    | 0.497                         | 0.531           | 0.088           | 0.000           | 0.024           |
| **IL-3** 3.8 (1.6–7.4)             | 0.043                         | 0.068           | 0.048           | 0.286           |
| **IFN-10** 184 (133–243)          | 0.713                         | 0.408           | 0.614           | 0.576           | 0.083           |
| **MCP-1** 167 (121–371)           | 0.308                         | 0.789           | 0.304           | 0.504           | −0.099          |
| **MCP-3** 10.5 (1.6–160)          | 0.100                         | 0.028           | 1.000           | 0.042           | 0.296           |
| **MDC** 2361 (2026–3047)          | 0.910                         | 0.731           | 0.780           | 0.047           |
| **MIPα<sub>α</sub>** 18.1 (10.6–35.8) | 0.380                       | 0.302           | 0.823           | 0.347           | 0.138           |
| **MIPβ<sub>β</sub>** 73.4 (52.7–111) | 0.384                       | 0.573           | 0.958           | 0.006           | 0.037           |
| **TGFβ** 11.2 (5.9–160)           | 0.854                         | 0.775           | 0.980           | 0.130           |
| **VEGF** 123 (56.9–295)           | 0.412                         | 0.655           | 1.000           | 0.361           | 0.134           |
| **PDGF AA** 2713 (2013–4405)      | 0.041                         | 0.030           | 0.401           | 0.040           | 0.300           |
| **PDGF AB/BB** 38789 (25851–78740) | 0.445                       | 0.268           | 0.911           | 0.376           |
| **RANTES** 113551 (74513–14100)   | 0.126                         | 0.285           | 0.146           | 0.169           | −0.201          |

<sup>a</sup> Control vs All CDH

<sup>b</sup> Mild vs Mod/Severe

<sup>c</sup> Tau Test
|                  | Control (n=22) | All CDH (n=37) | Mild CDH (n=6) | Mod/Severe CDH (n=11) | p-values |
|-----------------|----------------|----------------|---------------|-----------------------|----------|
| IL-1β           | 9.3 (1.6–35.1) | 157 (3.5–49.6) | 10.0 (1.6–41.8) | 29.7 (3.5–62.8) | 0.376    |
| IL-1ra          | 75.8 (34.5–229) | 118 (65.8–217) | 135 (81–177)  | 94.7 (61.1–289) | 0.412    |
| IL-6            | 1.6 (1.6–8.7)  | 4.6 (1.6–33.1) | 1.6 (1.2–2.0)  | 22.1 (43–614)  | 0.436    |
| IL-8            | 30.3 (7.4–421) | 45.7 (21.3–147) | 35.6 (21.3–136) | 129 (20.8–251) | 0.445    |
| IL-9            | 1.6 (1.6–2.8)  | 5.4 (1.6–14.9) | 2.6 (1.3–13.7) | 6.9 (1.6–14.9) | 0.100    |
| IL-10           | 4.0 (1.6–9.3)  | 10.2 (1.6–16.8) | 2.6 (1.6–16.8) | 11.0 (1.6–28.8) | 0.502    |
| IL-12(p40)      | 40.9 (29.3–52.5) | 40.5 (16.3–67.5) | 33.2 (21.3–99.9) | 45.5 (15.4–80.5) | 0.887    |
| IL-12(p70)      | 3.6 (1.8–5.0)  | 2.6 (2.3–6.5)  | 2.4 (2.0–2.5)  | 3.6 (2.6–10.2) | 0.876    |
| sIL-2Rα         | 158 (1.6–289)  | 211 (84.6–408)  | 225 (8.5–319)  | 211 (84.6–305) | 0.191    |
| TNFα            | 14.8 (11.8–36.5) | 15.5 (11.0–21.5) | 12.9 (9.6–15.0) | 18.3 (11.0–25.5) | 0.388    |
| sCD40L          | 24315 (13530–32544) | 23902 (11568–4298) | 17775 (10252–25502) | 30964 (11568–48564) | 0.821    |

p-values

- Control vs All CDH
- Mild vs Mod/Severe
- Control vs Mod/Severe
- Control vs Mild
- Tau Test
- Tau Coefficient

- Wilcoxon rank-sum test;
- Wilcoxon rank-sum test with Bonferroni-adjusted alpha=0.017;
- Kendall's tau-c test between Control, Mild CDH and Mod/Severe CDH groups.

IQR: interquartile range; EGF: epidermal growth factor; FGF: fibroblast growth factor; GRO (CXCL-1): chemokine (C-X-C motif ligand)-1; MCP: monocyte chemotactic protein; MDC: macrophage derived chemokine; MIP: macrophage inflammatory protein; TGF: transforming growth factor; VEGF: vascular endothelial growth factor; PDGF: platelet-derived growth factor; RANTES: regulated and normal T cell expressed and secreted.

Data not shown for IL-2, IL-4, IL-5, IL-7, IL-13, IL-15, IL-17 and TNFα.

Cytokines with significant p-values are boldfaced.
# Table 4

Maxterial Blood Cytokines – Median (IQR)

|                  | Control (n=22) | All CDH (n=18) | Mild CDH (n=6) | Mod/Severe CDH (n=12) | p-values | Control vs All CDH<sup>b</sup> | Mild vs Mod/Severe<sup>b</sup> | Control vs Mod/Severe<sup>b</sup> | Control vs Tau<sup>b</sup> | Tau Test<sup>c</sup> | Tan Coefficient |
|------------------|----------------|----------------|----------------|------------------------|----------|------------------|----------------|----------------|----------------|----------------|----------------|
| **Growth Factors & Chemokines (pg/ml)** |                |                |                |                        |          |                  |                |                |                  |                |                |
| EGF              | 53.3 (19.1–144) | 62.3 (24.4–90.6) | 62.7 (24.3–89.3) | 62.3 (34.7–160) | 0.828 | 0.512 | 0.665 | 0.821 | 0.705 | 0.056 |
| Eotaxin          | 63.7 (42.3–83.3) | 46.1 (31.5–64.7) | 36.5 (31.5–48.8) | 50.3 (31.7–68.5) | 0.174 | 0.454 | 0.331 | 0.198 | 0.279 | −0.158 |
| FGF-2            | 87.7 (54.6–139) | 42.3 (23.7–94.9) | 67.7 (31.4–130) | 38.3 (23.2–78.8) | **0.018** | 0.454 | **0.014** | 0.287 | **0.014** | −0.356 |
| Flk-3 Ligand     | 1.6 (1.6–137) | 1.6 (1.6–8.8) | 1.6 (1.6–8.8) | 1.6 (1.6–5.3) | 0.247 | 0.906 | 0.303 | 0.743 | 0.360 | −0.143 |
| Fucosamine       | 87.3 (49.8–111) | 65.8 (24.0–86.5) | 75.8 (67.3–82.3) | 43.1 (197–92.8) | 0.082 | 0.349 | 0.077 | 0.408 | 0.055 | −0.278 |
| GRO/CXCL-1       | 299 (147–844) | 302 (373–590) | 215 (805–453) | 350.7 (177–671) | 0.664 | 0.303 | 0.829 | 0.238 | 0.907 | −0.019 |
| IL-3             | 1.6 (1.6–116) | 1.6 (1.6–3.4) | 1.6 (1.6–1.6) | 1.6 (1.6–3.8) | 0.041 | 0.780 | 0.541 | 0.926 | 0.572 | 0.069 |
| IFN-α            | 286 (176–407) | 330 (192–501) | 170 (142–274) | 369.1 (303–512) | 0.350 | 0.061 | 0.801 | 0.935 | 0.866 | 0.023 |
| MCP-1            | 122 (86.4–139) | 140 (95.8–310) | 146 (75.5–310) | 133.5 (98.7–284) | 0.028 | 0.051 | 0.801 | 0.935 | 0.928 | 0.013 |
| MCP-3            | 11.4 (3.3–15.8) | 12.9 (7.9–17.9) | 15.1 (7.9–17.9) | 12.2 (7.6–20.3) | 0.377 | 0.963 | 0.449 | 0.599 | 0.410 | 0.120 |
| MDC              | 834 (567–900) | 532 (379–704) | 474 (323–568) | 534.0 (392–787) | **0.004** | 0.512 | 0.028 | **0.014** | **0.011** | −0.368 |
| MIP-1<sub>c</sub> | 16.2 (1.6–463) | 10.5 (1.6–15.0) | 7.2 (1.6–13.8) | 12.1 (1.6–15.1) | 0.124 | 0.705 | 0.203 | 0.234 | 0.174 | −0.195 |
| MIP-1<sub>f</sub> | 42.1 (29.0–96.1) | 39.8 (31.2–55.2) | 54.3 (25.6–91.8) | 36.3 (317–46.1) | 0.913 | 0.303 | 0.614 | 0.578 | 0.706 | −0.036 |
| TGFα             | 11.9 (7.0–28.7) | 12.4 (9.2–17.5) | 10.7 (9.1–12.3) | 14.6 (10.4–24.4) | 0.724 | 0.160 | 0.407 | 0.576 | 0.473 | 0.105 |
| VEGF             | 60.9 (42.6–86.2) | 32.7 (7.6–47.8) | 32.0 (10.8–43.5) | 32.7 (1.6–75.2) | **0.009** | 0.925 | 0.061 | **0.014** | **0.014** | −0.356 |
| PDGF-AA          | 1939 (1042–2784) | 1589 (1267–2689) | 1589 (880–2127) | 1575 (1313–3120) | 0.910 | 0.399 | 0.773 | 0.454 | 0.924 | 0.016 |
| PDGF-AB/BB       | 36237 (1664–7132) | 31357 (22955–5505) | 31357 (16252–39799) | 32290 (23839–60022) | 0.865 | 0.598 | 0.885 | 0.533 | 0.989 | −0.004 |
| RANTES           | 80305 (41946–137800) | 41484 (37907–67606) | 38303 (30878–38057) | 58660 (39085–8003) | 0.113 | 0.035 | 0.449 | 0.029 | 0.332 | −0.142 |
| **Inflammatory Mediators (pg/ml)** |                |                |                |                        |          |                  |                |                |                  |                |                |
| G-CSF            | 21.8 (8.1–48.5) | 307 (1.7–46.6) | 21.3 (9.0–69.4) | 36.4 (1.6–44.6) | 0.765 | 0.925 | 0.773 | 0.867 | 0.764 | −0.045 |
| GM-CSF           | 33.3 (20.2–51.1) | 28.1 (10.3–39.1) | 25.4 (207–36.0) | 28.1 (100–48.9) | 0.158 | 0.779 | 0.280 | 0.218 | 0.208 | −0.184 |
| IFN-α            | 27.1 (21.1–51.4) | 34.5 (22.1–41.2) | 26.6 (189–34.2) | 35.9 (275–42.2) | 0.654 | 0.190 | 0.482 | 0.860 | 0.433 | 0.114 |
| INF-γ            | 6.8 (5.1–134) | 4.9 (3.0–8.4) | 4.4 (3.8–6.0) | 5.2 (2.5–8.7) | 0.135 | 0.999 | 0.207 | 0.198 | 0.133 | −0.278 |
| IL-1<sub>c</sub> | 3.5 (1.6–173) | 153 (1.6–28.0) | 7.0 (1.6–18.5) | 17.8 (1.6–31.1) | 0.224 | 0.504 | 0.187 | 0.637 | 0.179 | 0.188 |
| Cytokine   | Control         | All CDH (n=18) | Mild CDH (n=6) | Mod/Severe CDH (n=12) | Control vs All CDH<sup>a</sup> | Mild vs Mod/Severe<sup>b</sup> | Control vs Mod/Severe<sup>b</sup> | Control vs Mild<sup>b</sup> | Tau Test<sup>c</sup> | Tau Coefficient |
|------------|-----------------|----------------|---------------|-----------------------|-----------------------------|-------------------------------|-------------------------------|-------------------------|------------------|------------------|
| IL-1β      | 6.7 (1.6–43.1)  | 8.6 (1.6–21.6) | 8.6 (1.6–25.5) | 8.6 (1.6–20.8)        | 0.580                       | 0.772                         | 0.770                         | 0.495                   | 0.661            | −0.064           |
| IL-1α      | 47.5 (19.7–385) | 118 (15.4–161) | 80.5 (14.6–118) | 128 (37.1–171)        | 0.407                       | 0.512                         | 0.358                         | 0.779                   | 0.334            | 0.141            |
| IL-6       | 5.1 (1.6–110)   | 9.6 (1.9–26.5) | 10.5 (2.0–26.5) | 7.3 (1.7–26.9)        | 0.200                       | 0.888                         | 0.316                         | 0.269                   | 0.235            | 0.171            |
| IL-8       | 27.6 (7.5–160)  | 19.5 (5.1–78.8) | 17.9 (8.2–45.7) | 23.2 (4.8–90.6)       | 0.581                       | 0.472                         | 0.353                         | 0.744                   | 0.470            | 0.094            |
| IL-9       | 1.6 (1.6–16)    | 1.6 (1.6–4.4)  | 1.6 (1.6–1.6)   | 1.6 (1.6–2.5)         | 0.811                       | 0.472                         | 0.353                         | 0.744                   | 0.470            | 0.094            |
| IL-10      | 3.4 (1.6–98)    | 1.6 (1.6–14.3) | 1.6 (1.6–2.3)   | 6.2 (1.6–18.4)        | 0.809                       | 0.368                         | 0.679                         | 0.235                   | 0.999            | 0.002            |
| IL-12(p40) | 14.9 (1.6–377)  | 1.6 (1.6–53.7) | 1.6 (1.6–21.3)  | 21.1 (1.6–57.0)       | 0.909                       | 0.537                         | 0.654                         | 0.350                   | 0.907            | 0.008            |
| IL-12(p70) | 4.7 (1.6–137)   | 2.9 (1.6–8.2)  | 2.9 (2.3–19.8)  | 3.2 (1.4–7.7)         | 0.549                       | 0.303                         | 0.330                         | 0.779                   | 0.386            | −0.124           |
| sIL-2Rα    | 1.6 (1.6–16)    | 1.6 (1.6–1.6)  | 2.7 (1.6–27.5)  | 1.6 (1.6–1.6)         | 0.795                       | 0.062                         | 0.266                         | 0.341                   | 0.453            | −0.081           |
| TNFα       | 7.2 (5.4–11)    | 5.8 (4.6–7.6)  | 6.3 (4.8–9.1)   | 5.8 (4.5–7.5)         | 0.809                       | 0.454                         | 0.105                         | 0.433                   | 0.083            | −0.251           |
| sCD40L     | 12885 (6443–27625) | 11475 (674–15551) | 12222 (8877–14548) | 10604 (5697–18095)     | 0.462                       | 0.833                         | 0.540                         | 0.574                   | 0.520            | −0.095           |

<sup>a</sup>Wilcoxon rank-sum test;  
<sup>b</sup>Wilcoxon rank-sum test with Bonferroni-adjusted alpha=0.017;  
<sup>c</sup>Kendall’s tau c test between Control, Mild CDH and Mod/Severe CDH groups

IQR: interquartile range; EGF: epidermal growth factor; FGF: fibroblast growth factor; GRO (CXCL-1): chemokine (C-X-C motif ligand)-1; MCP: monocyte chemotactic protein; MDC: macrophage derived chemokine; MIP: macrophage inflammatory protein; TGF: transforming growth factor; VEGF: vascular endothelial growth factor; PDGF: platelet-derived growth factor; RANTES: regulated and normal T cell expressed and secreted.

Data not shown for IL-2, IL-4, IL-5, IL-7, IL-13, IL-15, IL-17 and TNFβ.

Cytokines with significant p-values are boldfaced.