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ARTICLE

An omic approach to congenital diaphragmatic hernia: a pilot study of genomic, microRNA, and metabolomic profiling

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Abstract

Introduction The omic approach can help identify a signature that can be potentially used as biomarkers in babies with congenital diaphragmatic hernia (CDH).

Objectives To find a specific microRNA (miR) and metabolic fingerprint of the tracheal aspirates (TA) of CDH patients. We conducted a genetic analysis from blood samples.

Methods TA samples collected in the first 48 h of life in patients with CDH, compared with age-matched controls. Metabolomics done by a mass spectroscopy-based assay. Genomics done using chromosomal microarray analysis.

Results CDH ($n = 17$) and 16 control neonates enrolled. miR-16, miR-17, miR-18, miR-19b, and miR-20a had an increased expression, while miR-19a had a twofold decreased expression in CDH patients, compared with age-matched control patients. Specific metabolites separated neonates with CDH from controls. A genetic mutation found in a small subset of patients.

Conclusions Specific patterns of metabolites and miR expression can be discerned in TA samples in infants with CDH.

Supplementary information The online version of this article (https://doi.org/10.1038/s41372-020-0623-3) contains supplementary material, which is available to authorized users.

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Introduction

Congenital diaphragmatic hernia (CDH) is a developmental abnormality in which the diaphragm fails to form properly during embryogenesis. Consequently, the developing abdominal viscera invade the thoracic cavity, inhibiting pulmonary growth. Furthermore, the loss of a continuous diaphragmatic muscle markedly impairs fetal breathing movements that are necessary for proper stretch-induced lung maturation. As a result, newborns with CDH suffer from a combination of severe pulmonary hypoplasia and pulmonary hypertension (PH). The incidence of CDH is about 1 out of 2500 live births [1]. Treatment strategies include fetoscopic endoluminal tracheal occlusion (FETO), nitric oxide (NO) administration, high-frequency oscillatory ventilation, surgical repair of the diaphragm, and extracorporeal membrane oxygenation [2–4]. Despite advances in treatment, mortality and morbidity rates remain high [5]. Morbidities linked to CDH include pulmonary disorders, cardiovascular and gastrointestinal diseases, failure to thrive, neurocognitive defects, and musculoskeletal abnormalities [6]. Long-term pulmonary morbidity is present in 30–50% of the infants and consists of obstructive and restrictive lung function

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impairment due to altered lung structure and prolonged respiratory support [7, 8].

Potential therapies could be developed if the molecular mechanism underlying pulmonary hypoplasia and the development of PH could be better understood. Genomics, transcriptomics, proteomics, and metabolomics are today's promising approaches to better identify the pathways involved in patients affected by CDH [9, 10].

Genetic abnormalities account for less than 10% of the cases of CDH. In fact, most complex diseases involve gene–environment interactions and single-gene studies do not have the ability to analyze the interactions among different genes and the environment, whereas pathway-based approaches could help in understanding the interactions between genetics and environment.

MicroRNAs (miRNAs), a class of 21–25 nucleotide long noncoding RNAs, inhibit protein expression at the post-transcriptional level; the dysregulation of proteins by miRNAs can contribute to the pathogenesis of various diseases [11, 12] and can be used as biomarkers to predict the severity of disease. In experimental models of PH the cluster miR-17–92 has been found to be significantly altered [13, 14]. Moreover some miRs have been found to be altered in survivors of FETO performed for severe CDH [15].

Metabolomics, the quantitative analysis of many low molecular mass metabolites involving substrates or products of a defined metabolic pathway allows us to identify changes in the composition of metabolites caused by the interaction between specific pathophysiological states, gene expression, and environment.

In the present pilot study, we hypothesized that there would be specific metabolomic and miR profiles that would be associated with CDH, and that could be used as potential biomarkers for the development of PH in such patients. Specifically, regarding transcriptomics we focused on the miR-17–92 cluster in the tracheal aspirate (TA) of neonates with CDH. Hence, we performed a study of metabolomic on TA samples with the aim of determining a specific metabolic lung signature of patients with CDH developing PH. In addition, we performed a genetic analysis using peripheral blood samples, to check if we could find specific contributing genes leading to the development of CDH.

Material and methods

Patients

We performed a pilot prospective study involving all neonates with CDH hospitalized in the Department of Medical and Surgical Neonatology of the Bambino Gesù Children's Hospital, Rome, Italy, in the period from October 2014 to February 2016. The study was approved by the institutional review board and informed consent was obtained from one or both parents. All the measurements were conducted at the Yale Child Health Research Center and the MS and Proteomics Resource Center, New Haven, USA.

TA was collected on the first 48 h of life in patients with CDH and in control neonates, who were intubated for reasons not related to pulmonary diseases (for e.g., perinatal asphyxia, surgical conditions). Clinical characteristics studied included lung–heart ratio, mode of delivery, gestational age at birth, birth weight, type of ventilation, age of surgery, echocardiographic diagnosis of PH, pharmacologic therapy administered during hospitalization, and age at death (Table 1). PH was diagnosed by serial echocardiograms demonstrating elevated right ventricle pressure using the following criteria: velocity of tricuspid valve regurgitation ≥3 m/s in the absence of pulmonary stenosis and flat or leftward deviated interventricular septal configuration.

TA was collected by introduction of two aliquots of 1 mL/kg normal saline at room temperature in the lower airways through the tracheal tube followed by immediate removal [16]. The samples were stored at −80 °C till analysis.

All infants were followed during the first month of hospitalization, allowing us to divide the patients in two groups.

In all the neonates involved in the study we performed a karyotype and an array comparative genomic hybridization (array CGH). We extended the genetic assessment to majority of neonates hospitalized for CDH after the study period, from March 2016 to December 2018, in order to identify some de novo variants as a potential etiological mechanism for CDH.

miR real-time PCR

TA samples were centrifuged at $1250 \times g$ for 5 min to recover cells for RNA extraction, and the supernatants were collected and stored at −80 °C for further analysis.

Total RNA was extracted for RT-qPCR using the miRNeasy Mini Kit (Qiagen). To assess the quality of the samples, the concentration of the RNA in each sample was determined using the spectrophotometer (Beckman Coulter DU640). One patient with CDH was excluded from further analysis because of low quality of the sample.

Total RNA was reverse-transcribed by the QuantiTect Reverse Transcription Kit (Qiagen), amplified using SYBR Green PCR Master Mix (Qiagen). The cDNA was diluted 1:40 µL in nuclease free water to be used as a PCR template. PCR reactions for quantification of miR-16, miR-17, miR-18, miR-19a, miR-19b, miR-20a, and miR-92 were performed in triplicate and 25 µL was used as the reaction volume. We included U6 snRNA as endogenous control.

Table 1 Clinical characteristics: patients with CDH.

GA	BW	Mode of delivery	LHR	OE%	Side	Herniated organs	Age at surgery	Type of ventilation	Echocardiographic PH	iNO	Treatment	Age at death
38	2860	Vaginal			Left	Bowel	3	SIPPV	0	0	0	–
37	2510	C section			Left	Bowel, stomach, pancreas, spleen, upper hepatic lobe	ND	HFOV	1	1	Sildenafil	2
37	2400	C section	1.57	54	Right	Bowel, left hepatic lobe	3	SIPPV	0	0	0	–
38	2660	C section	1.06	35.5	Left	Bowel, stomach, spleen	12	SIPPV	1	1	Sildenafil	–
38	3570	C section	2.1	40	Left	Bowel, spleen	2	SIPPV	1	1	0	–
37	2760	C section	1.29	32.8	Left	Bowel, stomach, spleen	12	HFOV	1	1	Sildenafil	76
38	3540	C section			Left	Bowel	2	SIPPV	0	0	0	–
38	3300	C section	1.90	57.7	Left	Bowel	3	SIPPV	0	0	0	–
38	3030	C section			Left	Stomach, liver	3	SIPPV	0	0	0	–
39	3300	C section			Left	Stomach, liver	3	SIPPV	0	0	0	–
34	2170	C section			Left	Bowel, stomach, spleen	4	SIPPV	1	1	Sildenafil	–
36	1865	C section			Left	Bowel, spleen	5	HFOV	1	1	Sildenafil	–
36	2000	C section	2.09	52	Left	Bowel, stomach, spleen	6	HFOV	1	1	Sildenafil	–
40	3590	C section		69	Left	Bowel, spleen	3	SIPPV	0	0	0	–
38	3000	C section	2.2	56	Left	Stomach, liver	1	SIPPV	0	0	0	–
31	1700	C section	1.4	44	Left	Liver	ND	HFOV	1	1	Sildenafil	7
39	2840	C section	2.2	51	Left	Bowel, stomach, spleen	3	SIPPV	0	0	0	–
Controls												
37	2750	C section						SIPPV	0	0		–
40	3950	Vaginal						HFOV	0	0		–
39	3200	Vaginal						SIPPV	0	0		–
38	3270	C section						SIPPV	0	0		–
39	3450	C section						SIPPV	0	0		–
38	3350	Vaginal						SIPPV	0	0		–
37	3000	Vaginal						SIPPV	0	0		–
36	2950	C section						SIPPV	0	0		–
37	2690	C section						SIPPV	0	0		–
34	2300	Vaginal						SIPPV	0	0		–
38	3260	C section						SIPPV	0	0		–
40	3920	Vaginal						HFOV	0	0		–
38	2970	C section						SIPPV	0	0		–
38	3400	C section						SIPPV	0	0		–
39	3280	Vaginal						SIPPV	0	0		–
38	3160	C section						SIPPV	0	0		–

GA gestational age (weeks), *BW* birth weight (g), *LHR* lung area to head circumference ratio, *OE%* observed/expected lung-to-head ratio, *Age* days, *ND* not done, *SIPPV* synchronized intermittent positive pressure ventilation, *HFOV* high-frequency oscillatory ventilation, *PH* pulmonary hypertension, *iNO* inhaled nitric oxide, 0 = no, 1 = yes.

The RT-qPCR reactions were performed using the StepOnePlus™ real-time machine (Applied Biosystems) as recommended by the manufacturer. Relative changes in miRNA expression in different samples were quantitatively analyzed using the comparative ΔCT ($2^{-\Delta\Delta CT}$) method and RNU6 (RNA U6 snRNA) was used as endogenous control for normalization.

Metabolomics

Extraction method

We prepared TA samples according to the modified AbsoluteIDQ p180 (Biocrates Life Sciences AG) MS-based assay kit. Briefly, to ensure good enrichment of targeted metabolites in the assay, we used 500 µL of TA for extraction, which was carried out utilizing a Waters Oasis HLB 1 mL cartridge. Captured compounds on the cartridge were eluted with methanol (50–100 µL). We then used a nitrogen evaporator to dry the cartridge. We resuspended the dried pellet (TA extract) into 30 µL methanol just prior to the AbsoluteIDQ p180 kit preparation [17].

Mass spectrometry method

Before we carried out the targeted metabolomic based multiple reaction monitoring of the AbsoluteIDQ p180 assay, we added 10 µL of internal standards of the p180 Kit

to each TA extract sample. We then randomized the prepared samples using the List Randomizer (https://www.random.org/lists/) and carried out a direct electrospray flow injection analysis (FIA), to quantify 40 different acylcarnitines, hexoses, 90 different glycerophospholipids, and 15 different types of sphingolipids. In the FIA MS/MS method we injected twice for each sample (one in positive mode and one in negative mode). A liquid chromatography (LC) method was used to measure the abundances of 21 amino acids and 12 biogenic amines, and each sample was injected once. Supplementary Table 1 list all the compounds quantified in both the FIA MS/MS and LC method. The LC system we used was a FLEXAR Ultra High-Pressure Liquid Chromatography System (Perkin Elmer, USA) coupled inline to a 4000 Q-Trap LC MS/MS system (AB Sciex, USA), where the direct injection FIA MS/MS method was also carried out. A ZORBAX Eclipse XDB-C18 (Agilent Technologies, USA; 3.0 × 100 mm, 3.5 µm pore size) column coupled to an analytical Security Guard trap (Phenomenex, USA; C18, 4 ×3.0 mm) was utilized for the metabolite separation in the LC assay portion. A 9.5-min isocratic gradient was utilized where a ramp from 100% Buffer A (99.5% water with 0.5% formic acid) at 0.5 min to 95% Buffer B (99.5% acetonitrile with 0.5% formic acid) at 5.5 min was carried out. It was maintained for 1 min at this point prior to dropping back to 100% Buffer A at 7 min and re-equilibrated for 2.5 min at a 0.5 mL/min flow rate. We used Analyst software (v1.5.2) to collect the data and then analyzed these data with Biocrates MetIDQ software (v4.6.1). An internal calibration on QC samples of the p180 Kit was also performed to benchmark the quality of the assay and robustness of the data, as well as to ensure robustness of the chromatography, mass spectrometer, extraction method, and reagents utilized in the experiment. We finally normalized the calculated concentrations of all the metabolites to the predetermined protein weight of the starting TA sample.

Genomics method

Genetic analysis was performed at the Genetics laboratory of the Bambino Gesù Children's Hospital in Rome. Karyotype analysis from peripheral blood and the chromosomal microarray analysis test were performed with the SNP-array technique (single nucleotide polymorphism array) or CGH array, with analysis aimed at identifying variants of the number of copies (microdeletions/microduplications) involving a minimum of consecutive SNP probes greater than ten. The CGH-Array method is based on the competition for the binding at specific loci of two genomic nucleic acids, labeled with different fluorochromes (Cy3–Cy5): the sample DNA and a reference genomic DNA. The two DNAs are mixed and hybridized simultaneously on the array slide; for each locus the fluorescence corresponding to the most represented DNA is thus detected. The result is expressed by the relationship between the two fluorescences. The fluorescence intensity emitted for each chip locus is detected by computerized image analyzers (scanners) and quantified by calculating the deviation from the expected values of the ratio between the signals emitted by the sample DNA and the reference DNA.

Statistics

miR

As appropriate, groups were compared with the two-way ANOVA and corrected for multiple comparisons by the Tukey test and the logrank test (for the survival analysis), using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA, USA). In all analyses, $p < 0.05$ was considered statistically significant.

Metabolomics

Principal component analysis (PCA) biplot was computed on metabolites using *prcomp* and *ggbiplot* libraries in R software v.3.4.2 [18]. Hierarchical cluster analysis was performed using *hclust* function with ward.D method on Manhattan distance, while package *made4* was used to make up heatmap plot based on Spearman correlation [19].

Genomics

We did not perform any statistical analysis for genomics as we found a genetic mutation only in a small subset of patients

Results

During the study period 17 patients with CDH and 16 control neonates (five infants with perinatal asphyxia without PH, six with esophageal atresia, and five with gastroschisis) were recruited. We excluded neonates with moribund clinical conditions and those with major malformations. TA was always collected within the first 48 h of life. We eliminated one patient with CDH due to poor quality of the sample.

miR

The groups of patients and controls did not present statistical differences regarding gestational age (37.17 ± 2 vs 38.2 ± 1.8; $p = 0.23$) and birth weight (2.770 ± 599 vs 2.965 ± 470; $p = 0.33$). The proportion of male infants was similar in both groups (58.8% vs 68.7%; $p = 0.56$).

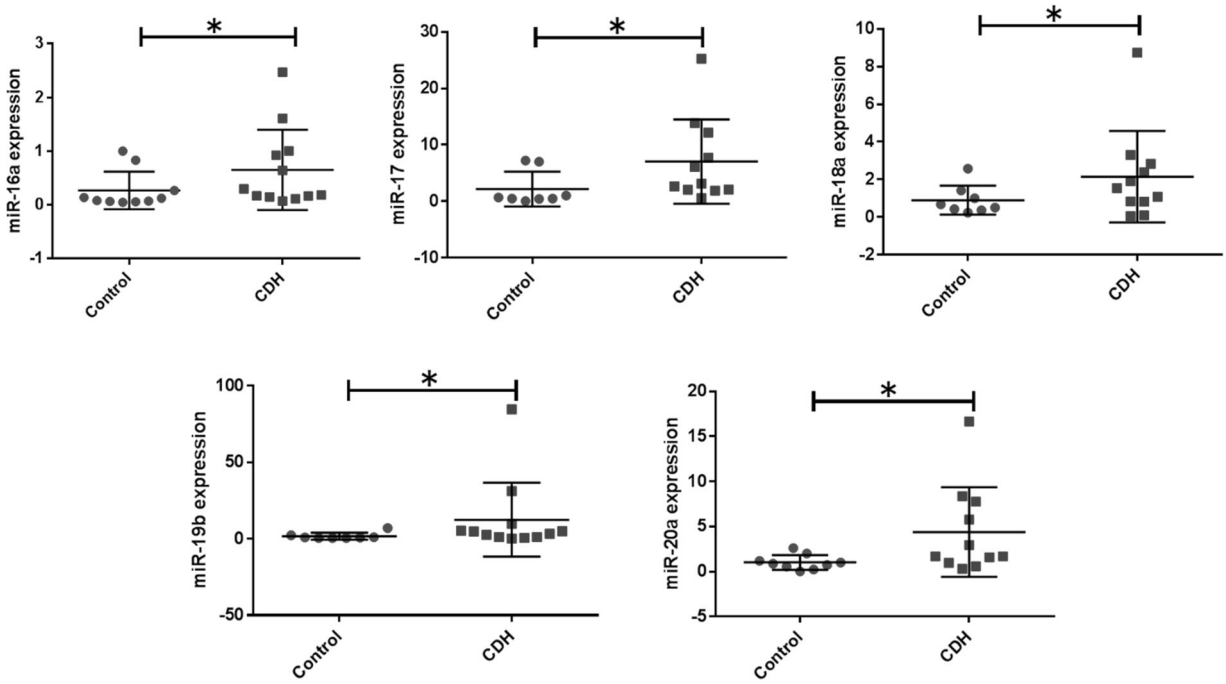

Fig. 1 miRs with increased expression in CDH. miR-16a, miR-17, miR-18a, miR-19d, and miR-20a had an increased expression in patients with CDH compared with controls.

Cesarean section was performed in one neonate with CDH and in nine controls (6% vs 56%, $p < 0.05$).

miR-17–92 cluster and miR-16 had an altered expression in CDH patients compared with age-matched normal control patients. miR-16, miR-17, miR-18, miR-19b, and miR-20a had an increased expression in CDH patients compared with age-matched control patients (Fig. 1). Contrariwise, miR-19 had an approximately twofold decreased expression in CDH patients compared with age-matched control patients (Fig. 2). MiR-92 had an increased expression in CDH patients compared with control patients but this did not reach statistical significance ($p > 0.05$) (Fig. 2).

We found no statistical difference in miR expression between CDH patients with and without PH. This could be related to the small sample of patients who did not develop significant PH.

Metabolomics

We performed a preliminary metabolomics study on five patients with CDH and five controls. One control sample was eliminated for low quality. Cesarean section was performed in one neonate with CDH and in two controls (20% vs 50%, $p = 0.4$). The multivariate data analyses were performed to characterize the distinct composition of metabolites from neonates with CDH without PH (CDH), neonates with CDH who developed PH (CDHPH) and control subjects (CTRLs). We used principal component model for exploratory purposes; in particular, we performed the projection of the samples onto the first two PCs, accounting for 70% of the total variance (51.2% and 23.8%, respectively). Therefore, even if the first two components explain a high percentage of internal variability of the dataset, only the subjects belonging to CDHPH group were well-differentiated from the other two groups (CDH and CTRLs). In particular, the metabolites (loadings superimposed on the PCA) carnosine, valine (Val), glucose (Glu), alpha AAA, lysine (Lys), tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), glycine (Gly), SM (OH) C22:2, ornithine (Orn), methionine (Met), serine (Ser), and proline (Pro) were involved in this separation (Fig. 3).

Finally, the Spearman's correlation analysis highlighted different interactions among metabolites (Supplementary Figs. 1 and 2; Supplementary Tables 2 and 3). In Supplementary Fig. 1, for CTRLs, there was a greater number of positive correlations, in which both variables move in tandem, that is, in the same direction and low number of negative correlations, in which one variable increases as the other decreases, and vice versa. The positive correlation were highlighted between metabolites such as C12, C12. DC, C14.2, C14.2.OH, C16.1.OH, creatinine, and spermine; on the contrary, for patients with CDH, there was a high number of negative correlations and fewer positive correlations than healthy subjects (Supplementary Fig. 2). In particular, the negative correlations were evidenced for specific molecules: glutamine, asparagine, glycine, phenylalanine, tryptophan, carnosine, and serine. Both figures show two opposite trends inside the same map.

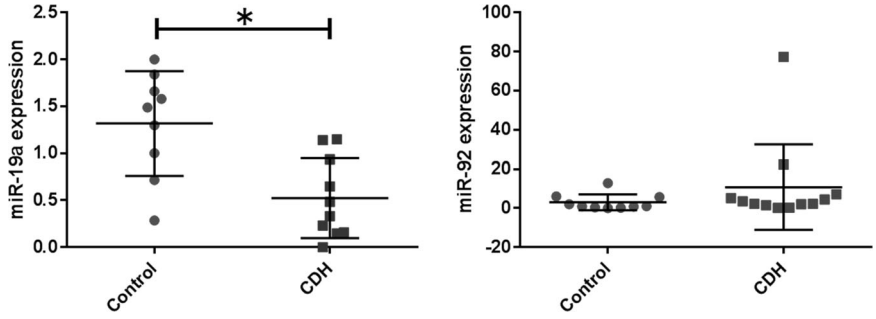

Fig. 2 miRs with decreased expression in CDH. miR-19a had a decreased expression in CDH patients, whereas difference of expression of miR-92 did not reach significance.

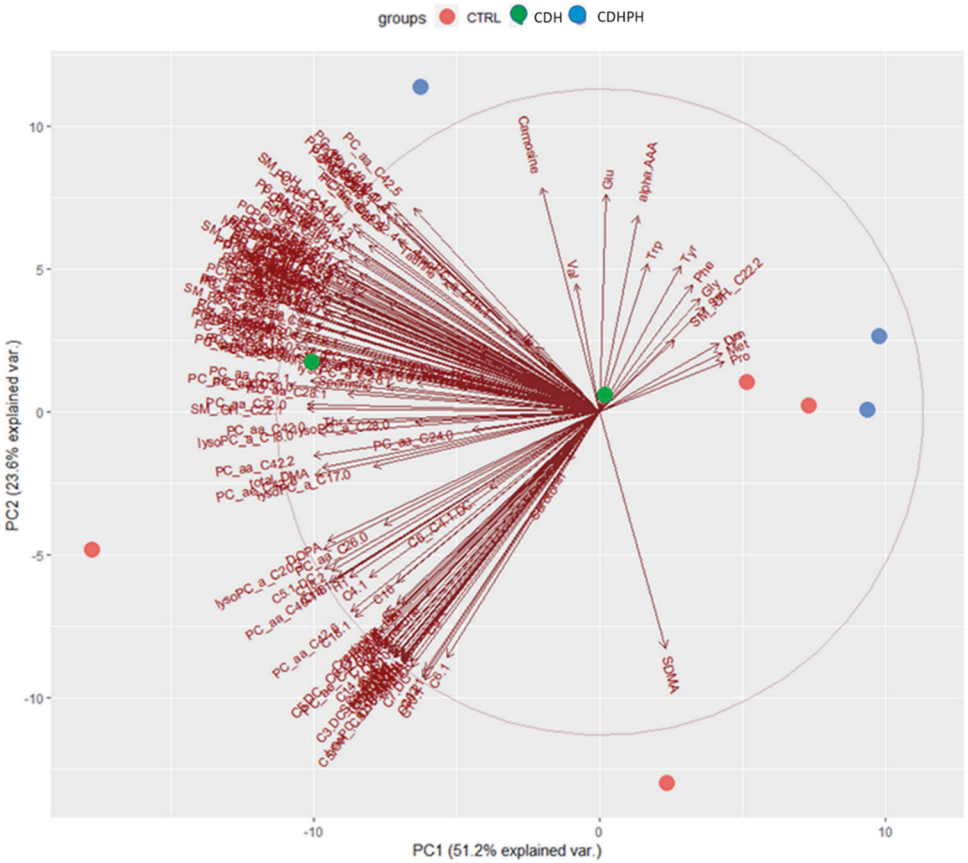

Fig. 3 Distinct composition of metabolites from CDH, CDHPH, and control (CTRLs) subjects. The patients with PH are clearly separated from patients without PH and controls. The present PCA plot combines both subjects score (colored dots) and metabolites (loadings represented by the red arrows). The score plots (red, blue, and green) represents the group of subjects and loading plots of contributing metabolites are superimposed. CTRL controls, CDH neonates with CDH without PH, CDHPH neonates with CDH and PH, carnosine, valine (Val), glucose (Glu), alpha AAA, lysine (Lys), tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), glycine (Gly), SM (OH) C22:2, ornithine (Orn), methionine (Met), serine (Ser), and proline (Pro) are the metabolites involved in this separation.

Genomics

From October 2014 to December 2018, we hospitalized 55 neonates with CDH who survived the first 3 months of life. Genetic analysis was performed in 32 neonates (58.2%). We found a genetic alteration in 28.1% ($n = 9$) of the neonates. No trisomy or monosomy were identified. The results of the mutations that were identified are listed in Table 2.

Discussion

The exact molecular mechanism is poorly understood in CDH patients. Our main results highlight the fact that metabolomics and miRNA profiles are different in CDH cases and controls.

Since miRNAs are known to regulate multiple signaling pathways, our data are innovative and have translational significance for two main reasons. One, it provides human data on an altered distribution of miR, which could inform us of which signaling pathways could be regulated by these involved in CDH. Second, the miR profile, if confirmed with a larger sample size, could be useful as biomarkers for prognostication.

Our main findings are that miR-16, miR-17, miR-18, miR-19b, and miR-20a had an increased expression in CDH patients, while miR-19a expression was significantly decreased, compared with age-matched control patients. This miR-17–92 cluster consists of six miRNAs: miR-17,

Table 2 Abnormal chromosomal results in individuals with CDH.

Patient	Sex	Side of lesion	Karyotype	CGH array	Inheritance	Additional anomalies
1	F	Left	46, XX	LCSH, long contiguous stretches of homozygosity	De novo	
2	F	Left	46, XX	Duplication of the long arm of the chromosome 11–11q14.3 genomic variant c.746C>A in the gene **GDF6**	Paternal segregation	
3	F	Left	46, XX	Microdeletion of the long arm of chromosome 14, region 14q21.1, extension 233.2 kb, including the gene **LRFN5** (variant of unknown significance)	Paternal segregation	ASD, PDA
4	M	Left	46, XY	Two consecutive but not adjacent microduplications on the long arm of a chromosome 3, in regions 3q27.3 and 3q28, extension respectively 297 and 414 kb. The first involving the gene OMIM disease causing **CRYGS** and the second involving the gene OMIM disease causing **CCDC50**	Maternal segregation	
5	M	Right	46, XY	Long contiguous stretches of homozygosity	De novo	
6	M	Left	46, XY	Duplication Xp22.31 (7530741–8164803), extension 600 kb, including the genes **VCX**, **PNPLA4**, and **VCX2**	Maternal segregation	Dextroposition of the heart, pulmonary valve stenosis
7	F	Left	46, XX	Genomic variant c.965G>A in condition of heterozygosity in the gene **ARID1B**. Genomic variant c.13630G>A in condition of heterozygosity in the gene **KMT2D** (variant of unknown significance)	Paternal segregation of the genomic variant KMT2D	ASD
8	F	Left	46, XX	Genomic variant c.1591G>C in condition of heterozygosity in the gene **MCTP2** (variant of unknown significance)	De novo	Chylothorax
9	F	Left	46, XX	Microdeletion of the short arm of a chromosome 2, region 2p22.2, extension 138 kb, including part of the gene OMIM **RMDN2**	Maternal segregation	Cleft palate

miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1. It is known to be located in the locus of the nonprotein-coding gene MIR17HG. cMYC can activate MIR17HG transcription by binding to the promoter [20].

So far only Pereira-Terra et al. has studied miR expression in neonates with CDH [15]. The authors studied the miR composition of hypoplastic lungs of fetuses with CDH and of TA collected in patients with CDH that underwent the FETO procedure. They found that homogenates of CDH hypoplastic lungs display an increased expression of miR-200b and miR-10a compared with control lungs. The TA collected in the FETO responders showed a higher miR-200b expression compared with nonresponders, suggesting that stimulated lungs have an increase in miR-200 expression.

On the other hand, in adult patients the miR-17–92 cluster has been shown to regulate some important pathways [21]. Each miR has specific targets, but different miRNAs can cooperate in specific pathways.

An important pathway involved is the transforming growth factor (TGF)-β signaling pathway, which plays a critical role in guiding early lung branching, morphogenesis, and alveologenesis [22].

The TGFβ2 signaling pathway was also explored by Pereira-Terra et al. [15]. TGFβ2 expression was lower in CDH lungs; therefore, the authors speculated that the miR-200 family functions by inhibiting several genes involved in the TGFβ-Smad signaling pathway. Similarly, other authors have demonstrated an increased TGFβ2 expression in the hypoplastic lungs of a surgical sheep model of CDH [23] and in the nitrofen-induced rat model of CDH [24]. In conclusion both increased and decreased TGFβ signaling can lead to abnormal lung development [25]. In the fetal lung, TGFβ inhibits early morphogenesis and blocks hormone-induced type II cell differentiation [26]. This could explain the reason why neonates with CDH show an abnormal surfactant production.

Our preliminary metabolomics analysis allowed us to well differentiate neonates with CDH who later developed PH (CDHPH group) from controls and neonates with CDH but without PH, within the first 24 h of life. Some metabolites (carnosine, glucose, SM(OH)C22:2, ornithine) and amino acids (Val, Lys, Trp, Tyr, Phe, Gly, Met, Ser, and Pro) were involved in this separation. Metabolites that contribute to the distinction between the groups indicate the involvement of specific metabolic pathways, like those for amino acid biosynthesis.

In adult patients with PH several metabolomics studies were performed, showing the activation of different metabolic pathways. Rhodes et al. performed a comprehensive study of 1416 plasma metabolites in patients with PH and healthy controls [27]. The authors identified 20 metabolites that discern PH from controls. Patients had increased levels of tRNA-specific modified nucleosides (N2, N2-dimethylguanosine,

N1-methylinosine), tricarboxylic acid cycle intermediates (malate, fumarate), glutamate, fatty acid acylcarnitines, tryptophan, and polyamine metabolites and decreased levels of steroids, sphingomyelins, and phosphatidylcholines. Zhao et al. [28] also studied patients with PH and they found metabolomic profiles of disrupted glycolysis, increased TCA cycle, and fatty acid metabolites with altered oxidation pathways in PH lungs.

Some authors [29–31] found a particular metabolic signature in adult patients with PH: a disruption in the arginine–nitric oxide metabolic pathway. In fact, plasma levels of arginine-NO metabolites (arginine, ornithine, citrulline, asymmetric dimethylarginine, and symmetric dimethylarginine) were significantly altered in patients with PH and heart failure.

In our study we did not find an alteration of these previously described metabolites, probably because in the previous studies there was preexisting PH when the studies were done, whereas in our study we were looking for a metabolic biomarker to forecast which babies would develop PH.

Nevertheless, we have been able to differentiate, from a metabolomic point of view, neonates with CDH who developed PH from controls and patients without PH. But given the small dataset, is not possible to make a conclusion on which metabolites could be used as biomarkers. The importance of these preliminary data is that it seems that neonates with CDH who will later develop PH have already in the first 24 h of life a different metabolic fingerprint. This supports the hypothesis of a general modification of metabolic pathways already in the fetal period.

Thus, metabolomics can be used to define detectable, metabolic fingerprints of neonates developing PH and perhaps point to pathways key to development or maintenance of PH. TA and, as an alternative source, exhaled breath condensate (EBC) are important sources to obtain biosamples for OMICS-based approaches to study high-risk groups such as neonates in the neonatal intensive care unit. Noninvasive methods based on EBC are especially important in neonatology in the scenario of premature infants receiving intensive care [32–34].

Most CDH cases are sporadic, but the finding of a causative cytogenetic aberration is becoming more and more frequent, thanks to the improvement of genetic techniques.

In the literature the incidence of a causative cytogenetic aberration varies from 2 to 33% of patients with CDH [35, 36]. The commonest chromosome aberrations are diagnosable with a karyotype, but to date array CGH is almost always performed and some authors also performed studies on full exome sequencing to better identify some new candidate genes. Some familial cases of CDH have been described in the literature, mostly in association with Marfan syndrome [37]. The causative alteration seems to be a deleterious sequence change in some genes—FREM1, DES, PAX3, and MET—that have all been shown to play a role in diaphragm development in mice [38]. Nevertheless, majority of genetic alterations associated with CDH are sporadic. Yu et al. [39] performed one of the largest array CGH study on 256 probands with CDH and found chromosome abnormalities in 6.3% of affected individuals [39]. They found a set of candidate genes including FOXC1, FOXF2, PDGFA, FGF6, COL4A1, COL4A2, SOX7, BNC1, BID, and TBX1.

More recent studies performing whole exome sequencing (WES) support the evidence that de novo variants contribute significantly to the development of CDH [40, 41]. A new diagnostic challenge lies in the interpretation of the presence of benign copy number variations (CNVs) compared with the analysis of parental DNA. CNVs are likely to be the cause of the phenotype when a de novo origin is proved. Conversely, inherited CNVs of unclear clinical significance open new diagnostic challenges, as on one hand the genetic abnormalities can be insufficient to explain the phenotype of the patient, but on the other hand they can be related to a milder phenotype in the parents.

Although many neonates with CDH had some associated phenotypic alteration (long tapered fingers, thin lips, dysmorphic ears, ligamentous hyperlaxity), our genetic analysis highlighted a genetic mutation only in a small subset of patients. A majority of the genetic alterations that we were able to identify are of little significance. Three rearrangements were inherited from the mother and three from the father. We could find only one genetic alteration already described in the literature and associated with the development of CDH (ARID1B) [41].

In our opinion WES should be performed on all babies with CDH to better understand which genes and chromosome regions are involved in the pathogenesis of this multifactorial complex disease. In fact, a high number of neonates with CDH show signs of dimorphism, which are not always associated with a karyotype or CGH-Array alteration. We believe that WES could help us identify some de novo mutations especially in these patients in whom the alteration of the phenotype leads us to hypothesize a genetic alteration that we have not been able to identify.

Conclusions

In our pilot study, we differentiated, from a metabolomic point of view, controls and CDH patients not developing PH from patients developing PH, as early as the first 24 h of life. Given the small dataset it is not possible to make a definitive conclusion on which metabolites could be used as biomarkers; however, our data showed that CDH patients have an altered expression of the miR-17–92 cluster; specifically, an increased expression of miR-17, miR-18,

miR-19b, and miR-20a. We speculate that these alterations are involved in the pathogenesis of the pulmonary remodeling that is caused by CDH. There is a need for additional studies with a larger sample size for confirmation and potential utility of our results, as a combination of miR and metabolites could be used as biomarkers in CDH patients.

The progresses of our understanding of the actual biological roles of miR in CDH patients, the expansion of metabolomics techniques, and the availability of WES technique to detect minor genetic mutations allow us to keep an optimistic approach to the expansion of the "omic" approach for the management of neonates with CDH.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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