

1 LUNG GROWTH FACTORS IN THE AMNIOTIC FLUID OF NORMAL PREGNANCIES AND  
2 WITH CONGENITAL DIAPHRAGMATIC HERNIA

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4 Running head: Prenatal lung growth factors in CDH

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## 25 ABSTRACT

26 Aim: Respiratory failure secondary to pulmonary hypoplasia is the main cause of death in  
27 congenital diaphragmatic hernia (CDH). Lung growth is regulated by growth factors (GFs),  
28 whose imbalances is reported in pathological conditions. We measured amniotic fluid levels of  
29 GFs, regulating lung development, in pregnancies with CDH and compared them with normal  
30 gestations.

31 Methods: Amniotic fluid was collected at amniocentesis and delivery from 4 women carrying  
32 fetuses with CDH and 12 with normal pregnancy. GFs were isolated and quantified. Same GFs  
33 were measured in lung biopsies collected during autopsy of 3 newborns dead of CDH.

34 Results: Impairment expression of lung GFs in the amniotic fluid of CDH pregnancies in  
35 comparison with normal was found. FGF10, FGF7, VEGF and TGF $\beta$  were decreased at  
36 amniocentesis, while PDGF increased. While FGF10 and PDGF tended to normalize at delivery,  
37 EGF increased and TGF $\beta$  was still decreased. Same GFs were similarly expressed in both lungs  
38 of babies dead of CDH.

39 Conclusion: Anomalies in lung GFs expression of embryos and fetuses with CDH can be  
40 detected by measuring their levels in the amniotic fluid during pregnancy. Further investigation  
41 would help to correlate prenatal expression of GFs and clinical outcome of babies with CDH  
42 after birth.

43

## 44 INTRODUCTION

45 Congenital diaphragmatic hernia (CDH) is a human malformation that causes high mortality in  
46 newborns, because of severe respiratory failure secondary to pulmonary hypoplasia and long-  
47 term pulmonary morbidity in survivors. Although widely studied in the experimental model of  
48 CDH [1-4], the pathogenesis of lung hypoplasia associated with CDH remains unclear. The  
49 studies based on teratogenic animal models of malformations may be criticized because it is  
50 not known whether the anomalies found are to direct effects of the chemical used or to the  
51 malformations induced by it. On the other hand, human studies are difficult to realize for ethical  
52 and technical reasons. Lung development is regulated by complex tissue interactions mediated  
53 by growth factors that promotes cell proliferation and differentiation, such as FGF, EGF, VEGF  
54 and PDGF, and others that oppose these effects, such as TGF $\beta$  [5]. Their imbalances have  
55 been reported in various pathological lung conditions [6]. As growth factors are diffusible  
56 proteins, we hypothesized that abnormal expression of growth factors that regulate lung  
57 development may be reflected in a different concentration of the same proteins in the amniotic  
58 fluid during pregnancy.

59 The purpose of this study is to gain further insight the role of lung growth factors in the  
60 pathogenesis of pulmonary hypoplasia in CDH and to explore the possibility of identifying  
61 prenatal predictive values of the clinical outcome of newborns with CDH.

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## 64 MATERIAL AND METHODS

65 Study groups: After approval by the Institutional Research Committee (licence number:  
66 22-11), all women with a spontaneous pregnancy, in which an advanced-level ultrasound (US),  
67 performed in our Institution between August 2011 and December 2012, revealed CDH and  
68 amniocentesis was indicated, were enrolled in the cases group (A). Twelve women with a  
69 spontaneous pregnancy and a routine anatomy US without anomalies who performed  
70 amniocentesis, as per advice based on maternal age or as per maternal decision, in our  
71 Institution in the same period of time, were randomly enrolled in the controls group (B).  
72 Demographic characteristics, obstetrical variables such as parity, women habits during  
73 pregnancy, mode of delivery, obstetrical complications, indication of amniocentesis and  
74 gestational age when it was accomplished, fetal karyotype, newborn data such as weight and  
75 general conditions at birth were collected.

76 ELISA analysis in the amniotic fluid: The supernatant of the centrifuged amniotic fluid  
77 collected at amniocentesis was snap-frozen and stored at  $-80^{\circ}\text{C}$ .

78 At delivery, 20 ml of amniotic fluid were collected in both groups from the amniotic cavity without  
79 contamination with maternal blood. The supernatant was snap-frozen and stored at  $-80^{\circ}\text{C}$ . The  
80 samples contaminated with blood were discarded.

81 After assessing the protein content of the amniotic fluid collected during amniocentesis and  
82 delivery with the Lowry protein assay (Sigma, Trieste, Italy) and calibrating the samples to  
83 enable a balanced comparison between groups, fibroblast growth factor 10 (FGF10), fibroblast  
84 growth factor 7 (FGF7), epidermal growth factors (EGF), vascular endothelial growth factor  
85 (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor  $\beta$  (TGF $\beta$ ) levels  
86 were quantified using ELISA kits (Tema Ricerca ref: CSB-E14965H, RAY-ELH-FGF7-001, RAY-  
87 ELH-EGF-001, EH2VEGF, CSB-E04701H, EIA-1864, Bologna, Italy) in both groups of pregnant  
88 women. Concentration of amniotic fluid growth factors were expressed in pg/ml.

89 ELISA analysis in lung biopsies: Biopsies from both lungs were collected during autopsy  
90 of newborns who had died of CDH. The lung tissue was homogenized in lysis buffer (1 mmol/l  
91 sodium vanate; 1% SDS; 0.01 mol/l Tris-HCl, pH 7.4) with protease inhibitors. The protein  
92 content was assessed using the Lowry protein assay (Sigma, Trieste, Italy). FGF10, FGF7,  
93 EGF, VEGF, PDGF and TGF $\beta$  levels were quantified by ELISA (Tema Ricerca ref: CSB-  
94 E14965H, RAY-ELH-FGF7-001, RAY-ELH-EGF-001, EH2VEGF, CSB-E04701H, EIA-1864,  
95 Bologna, Italy). All procedures were carried out strictly following the instruction offered with the  
96 ELISA kit. Concentration of lung growth factors were expressed in pg/ml.

97 Statistical methods: The results were expressed as percentages or as means $\pm$ SD and  
98 the groups were compared by Anova tests with a threshold of significance at  $p < 0.05$ . Tukey's  
99 range test was used as post-hoc test.

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## 102 RESULTS

103 Study groups data: Between August 2011 and December 2012, the advanced-level US  
104 confirmed in 4 women the diagnosis of left CDH.

105 The mean maternal age of the study group (A) was 30.25 years, being two of them 22 and 23  
106 years old, while the others 38 years old. All women were Caucasian. Three of them were  
107 nulliparous, two of them had one previous miscarriage. None of them smoked or drank alcohol  
108 during pregnancy. None of them had problems of obesity. All of them had unlabored elective  
109 cesarean deliveries at term (mean 38 weeks) and the procedure was performed without  
110 complications. Gestational age mean at the time of amniocentesis was 18 weeks. The fetal  
111 karyotype was 46,XY in all cases without any chromosomal abnormalities. The mean weight of  
112 the newborns was 2.8 kg (Table 1). In all newborns the diagnosis of left CDH was confirmed at  
113 birth. They were intubated immediately after birth and high-frequency oscillatory ventilation was  
114 started.

115 The mean maternal age of the control group (B) was 37.5 years. All women were Caucasian.  
116 Five of them were nulliparous, four of them had previous miscarriages (3 in one woman, 2 in  
117 another and 1 in the remaining two). None of them smoked or drank alcohol during pregnancy.  
118 None of them had problems of obesity. All of them had unlabored elective cesarean deliveries at  
119 term (mean 39 weeks) and the procedure was performed without complications. Advanced  
120 maternal age was the most frequent indication for amniocentesis. Gestational age mean at the  
121 time of amniocentesis was 17 weeks. The fetal karyotype was 46,XY in 6 cases and 46,XX in  
122 the remaining, without chromosomal abnormalities in all of them. The mean weight of the  
123 newborns was 3.3 kg (Table 1). All the newborns were healthy at birth and had no respiratory  
124 problems.

125 No significant differences were found among the groups with respect to parity, habits during  
126 pregnancy, mode of delivery, obstetrical complications, gestational age at amniocentesis and  
127 newborn weight.

128 ELISA analysis in the amniotic fluid: Growth factors were detectable in most but not all  
129 amniotic fluid samples. All samples of amniotic fluid collected at delivery in women with a fetus  
130 with CDH had no detectable levels of FGF7 and VEGF. Also the samples of amniotic fluid from  
131 delivery of normal pregnancies showed no VEGF (Figure 1 B and E).

132 When FGF10 was quantified, decreased levels were noted in amniotic fluid of CDH  
133 pregnancies at both endpoints, even if the difference was not significant. FGF10 was the most  
134 concentrated growth factor both at the time of amniocentesis and delivery in all pregnancies  
135 (Figure 1 A).

136 A similar trend of decrement was seen in FGF7 levels of amniotic fluid from CDH  
137 pregnancies at amniocentesis. At the end of the pregnancy, FGF7 was lower in control amniotic  
138 fluid and no detectable in CDH pregnancies (Figure 1 B).

139 The levels of EGF were very low in both groups at the time of second trimester  
140 amniocentesis. At delivery, EGF significantly increased in pregnancies with CDH in comparison  
141 with controls (Figure 1 C).

142 PDGF was significantly increased in the second trimester CDH amniotic fluid in  
143 comparison with controls, while its levels decreased at the end of the pregnancies and were very  
144 similar in CDH and control amniotic fluid (Figure 1 D).

145 VEGF significantly decreased in the CDH second trimester pregnancies. VEGF was the  
146 less concentrated growth factor both at the time of amniocentesis and delivery in all pregnancies  
147 (Figure 1 E).

148 TGF $\beta$  levels were significantly decreased in the amniotic fluid of CDH pregnancies both  
149 at amniocentesis and at delivery (Figure 1 F).

150 The levels of the growth factors showed neither any consistency nor any statistically  
151 significant association with parity, gestational age at amniocentesis and newborn weight.

152 ELISA analysis in lung biopsies: Three of the babies born with left CDH died in the first  
153 week of life due to the impossibility to achieve the physiological stabilization. Lung biopsies were

154 collected at autopsy. Growth factors were detectable in all lung samples. Interestingly, no  
155 significant differences were found in the expression of lung growth factors in the right and the left  
156 lung in all cases. EGF had the lowest concentration in both lungs, while VEGF the highest.  
157 FGF10 and FGF7 had concentration lower than 100 pg/ml (Figure 2).

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## 160 DISCUSSION

161 Congenital diaphragmatic hernia is a malformation that still causes high mortality in newborns  
162 mainly because of severe respiratory failure secondary to pulmonary hypoplasia. The  
163 pathogenesis of lung hypoplasia has not been fully understood. It has been suggested that the  
164 abnormalities in bronchial innervation described in infants with CDH [1] might contribute to the  
165 pulmonary developmental deficiency. The role fulfilled by neural tissue in early gestation seems  
166 to be secretion of trophic factors for the smooth muscle that indirectly contributes to lung  
167 development. In fact, it has been proposed that the smooth muscle spontaneous contractions  
168 produce a rhythmic mechanical stimulus across the airway wall and the adjacent parenchyma  
169 that contributes to normal airway differentiation and branching by inducing expression of growth  
170 factors [7,8].

171 Given these observations, we expected that the expression of the growth factors that regulate  
172 branching and control airway size and cell fate in the developing lung may be impaired in  
173 fetuses and infants with CDH. Lung development is the result of the balanced action of growth  
174 factors that promotes cell proliferation and differentiation, such as FGF, EGF, VEGF and PDGF,  
175 and others that oppose these effects, such as TGF $\beta$ . At the end of development, these signals  
176 maintain cellular activities at equilibrium and preserve normal lung structure and function [5,9].  
177 Abnormal expression of these factors and their inappropriate signalling activation cause  
178 pathological lung condition. Growth factors are diffusible proteins and we hypothesized that their  
179 abnormal expression may be reflected in a different concentration of them in the amniotic fluid  
180 during pregnancy. Therefore, we determined the concentration of lung growth factors in the  
181 amniotic fluid at amniocentesis and at delivery in pregnancies with a diagnosis of CDH and  
182 compared them with normal pregnancies. We considered a panel of growth factors involved in  
183 all phases of lung development in an attempt to highlight specific abnormalities in the process of  
184 lung formation. We could enroll only four cases in the study group during the study period.  
185 Despite this, the two groups were not significantly different with respect to parity, habits during

186 pregnancy, mode of delivery, obstetrical complications, gestational age at amniocentesis and  
187 newborn weight.

188 Growth factors were not detectable in all amniotic fluid samples. Surprisingly, we could not find  
189 VEGF in the amniotic fluid of delivery both in CDH and normal pregnancies. The absence of  
190 VEGF in samples of amniotic fluid has been previously reported [10]. VEGF is a strong promoter  
191 of angiogenesis and its signalling is responsible for the differentiation of embryonic  
192 mesenchymal cells into endothelial cells; the interaction between the epithelium and the  
193 mesenchyme contribute to lung neovascularisation that is crucial in normal lung development  
194 [9,11,12]. The concentration of VEGF was significantly decreased in the amniotic fluid from  
195 amniocentesis performed in women carrying a fetus with CDH. It is well known that the  
196 development of vascular bed is defective in CDH [13]. Although VEGF is not only expressed in  
197 the lung, its lower levels could account for the decreased number of vessels per unit in CDH  
198 lung.

199 FGF10 is essential for lung branching morphogenesis having a central role in inducing the  
200 spatial coordinates for patterning the epithelial tubules [5]. The FGF10 levels were decreased in  
201 the amniotic fluid of CDH pregnancies at the time of amniocentesis and tended to normalize at  
202 the end of pregnancy. FGF7 promotes epithelial growth and citodifferentiation and it is  
203 expressed at highest levels at late stages [5]. FGF7 had decreased levels at amniocentesis and  
204 was not detectable at delivery in the amniotic fluid of pregnancies with CDH. Both findings were  
205 consistent with the hypothesis that the lung development is delayed in animals and infants with  
206 CDH [1]. On the other hand, EGF levels were significantly increased at term in pregnancies with  
207 CDH. EGF positively modulates embryonic lung branching morphogenesis and regulates type 2  
208 alveolar cell proliferation [5] and our result could be either the consequence of the delayed in  
209 lung development or an attempt to compensate the lower levels of FGF family proteins. A similar  
210 intent to counteract the decreased expression of growth factors which promote branching, could  
211 be seen in the increased values of EGF and PDGF at the time of delivery. PDGF signalling

212 regulates epithelial DNA synthesis and early branching during embryonic life and it is essential  
213 for the ontogeny of pulmonary alveolar myofibroblasts, elastin synthesis and hence  
214 alveolarization in postnatal life [5].

215 TGF $\beta$  opposes FGF and EGF effects preventing local budding and maintaining proximal airways  
216 in an unbranched form by suppressing epithelial cell proliferation and by promoting synthesis of  
217 extracellular matrix components around airways. Its levels were significantly decreased in the  
218 amniotic fluid of CDH pregnancies at both endpoints. This finding could support the idea that  
219 lung development is delayed in CDH and therefore TGF $\beta$  concentration is still low when a  
220 normal lung development needs it to increase.

221 In conclusion, the expression of lung growth factors is impaired in the amniotic fluid of CDH  
222 pregnancies in terms of decreased levels of proteins that regulate the branching or high levels of  
223 proteins that modulate early branching when the corresponding control values have already go  
224 down. At the same time the levels of growth factors were similar in both lungs of newborns dead  
225 of CDH. The fact that the lung ipsilateral and the contralateral to the hernia were equally affected  
226 is consistent with the pattern of lung innervation abnormalities found in infants with CDH [1] and  
227 further supports the concept of pulmonary hypoplasia as a primary developmental defect rather  
228 than the result of lung compression during development.

229 The present study has several weak points that should be acknowledged. In the first place, the  
230 reduced number of CDH pregnancies weakens our conclusion and invites further confirmation of  
231 our findings in the future. Moreover, the lack of a control group to compare the results of lung  
232 biopsies analysis led us to hypothetical speculations about their being different from the control.  
233 Third, the management during resuscitation and ventilation of CDH newborns might have  
234 affected the expression of lung growth factors. Forth, we were not able to correlate prenatal  
235 expression of the growth factors and clinical outcome of the babies after birth because the  
236 management was different for each patient. Collection of more specimens is desirable to

237 investigate if the levels of some of the growth factors could be a predictor of the entity of  
238 postnatal lung deficiency in babies with CDH.

239 Nevertheless, our results demonstrate an impairment in lung growth factors expression of  
240 embryos and fetuses with CDH, which can be detected by measuring their levels in the amniotic  
241 fluid during pregnancy.

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244 DECLARATION OF INTEREST STATEMENT

245 The authors report no declarations of interest, nor financial assistance.

246

247	LIST OF ABBREVIATIONS
248	CDH: congenital diaphragmatic hernia
249	EGF: epidermal growth factors
250	ELISA: Enzyme-Linked ImmunoSorbent Assay
251	FGF7: fibroblast growth factor 7
252	FGF10: fibroblast growth factor 10
253	GF: growth factors
254	PDGF: platelet-derived growth factor
255	VEGF: vascular endothelial growth factor
256	TGF $\beta$ : transforming growth factor $\beta$
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294 LEGEND FOR THE TABLE

295 Table 1

296 Control and study groups data.

297

298 LEGEND FOR THE FIGURES

299 Figure 1

300 Amniotic fluid levels of FGF10 (A), FGF7 (B), EGF (C), PDGF (D), VEGF (E) and TGFb (F) at  
301 the time of amniocentesis and delivery in pregnancies with a fetus with CDH and in normal ones  
302 (#  $p < 0.05$  vs control of the same endpoint).

303 Figure 2

304 Lung levels of FGF10 (A), FGF7 (B), EGF (C), PDGF (D), VEGF (E) and TGFb (F) in right and  
305 left lung of babies dead of CDH (#  $p < 0.05$  vs control).

306

307 Vanessa Candilera performed the experiments and wrote the draft of the manuscript.

308 Carlo Bouchè collected the amniotic fluid and analyze the data.

309 Jurgen Schleef analyzed the data.

310 Federica Pederiva designed the study, analyzed the data and supervised the experiments.

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