ARTICLE

Surfactant proteins gene variants in premature newborn infants with severe respiratory distress syndrome

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Abstract

Objective Genetic surfactant dysfunction causes respiratory failure in term and near-term newborn infants, but little is known of such condition in prematures. We evaluated genetic surfactant dysfunction in premature newborn infants with severe RDS.

Patients and methods A total of 68 preterm newborn infants with gestational age ≤32 weeks affected by unusually severe RDS were analysed for mutations in *SFTPB*, *SFTPC* and *ABCA3*. Therapies included oxygen supplementation, nasal CPAP, different modalities of ventilatory support, administration of exogenous surfactant, inhaled nitric oxide and steroids. Molecular analyses were performed on genomic DNA extracted from peripheral blood and Sanger sequencing of whole gene coding regions and intron junctions. In one case histology and electron microscopy on lung tissue was performed.

Results Heterozygous previously described rare or novel variants in surfactant proteins genes *ABCA3, SFTPB* and *SFTPC* were identified in 24 newborn infants. In total, 11 infants died at age of 2 to 6 months. Ultrastructural analysis of lung tissue of one infant showed features suggesting ABCA3 dysfunction.

Discussion Rare or novel genetic variants in genes encoding surfactant proteins were identified in a large proportion (35%) of premature newborn infants with particularly severe RDS. We speculate that interaction of developmental immaturity of surfactant production in association with abnormalities of surfactant metabolism of genetic origin may have a synergic worsening phenotypic effect.

Introduction

Physiologic transition from foetal to neonatal period requires production of pulmonary surfactant, a complex mixture of phospholipids and proteins that reduces alveolar collapse by decreasing surface tension within the alveoli. Deficiency of pulmonary surfactant due to immaturity is considered the main cause of respiratory distress syndrome (RDS), a multifactorial disease characterised by respiratory failure and altered gas exchange [1]. However, lethal mutations in surfactant genes in term and near-term newborns support the notion of a genetic contribution to RDS [2, 3]. Surfactant proteins represent a small percentage in surfactant composition but they are essential for surfactant homeostasis. In particular, surfactant protein B (SP-B) and C (SP-C) are essential for tensioactive properties of pulmonary surfactant, whereas ATP-binding cassette 3 (ABCA3) participates in the transport of phospholipids and cholesterol to lamellar bodies, the organelles where surfactant is stored before secretion in the alveolus. Pathogenic variants in genes encoding SP-B and ABCA3 result in severe and often fatal lung disease transmitted as a recessive trait in neonates [4–8]. In addition, recessive and dominant pathogenic variants in *ABCA3* and *SFTPC* genes, respectively, have been associated with chronic interstitial lung disease (ILD) in term newborns, children and adults [9, 10].

Little is known about the correlation of variants in genes encoding surfactant proteins in premature newborn infants with severe RDS, since the majority of genetic tests are

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requested on term newborns with unexplained RDS. In this study we analysed the genes encoding protein B (*SFTPB*), C *(SFTPC)* and ABCA3 (*ABCA3)* in premature infants with gestational age ≤32 weeks presenting a particularly severe course of RDS.

Patients and methods

We retrospectively reviewed molecular analyses of premature newborn infants presenting particularly severe RDS referred to our laboratory of Molecular Biology from 2005 to 2016, performed for diagnostic purposes. All the examined subjects gave their written informed consent for genetic testing, data treatment and storage, approved by the Institutional Quality Assessment Committee.

Sixty-eight preterm newborn infants with gestational age ≤32 weeks and birth weight ranging from 550 to 1900 g, affected by particularly severe RDS, were analysed by Sanger sequencing in the *SFTPB, SFTPC* and *ABCA3*. Treatments included prolonged oxygen supplementation, respiratory support with CPAP, conventional mechanical ventilation, high frequency oscillatory ventilation (HFOV), exogenous surfactant administration, inhaled nitric oxide, postnatal steroids administration and home oxygen supplementation. Severity of the disease was based on the clinical judgement of the treating neonatologist.

Molecular analysis

Molecular analyses were performed on genomic DNA extracted from peripheral blood leukocytes by a Maxwell 16 extractor (Promega Co., WI USA) or by conventional phenol–chloroform method. All exonic and flanking regions of *SFTPB*, *SFTPC* and *ABCA3* genes were amplified by polymerase chain reaction (PCR) and directly sequenced using the Sanger method on a 3730 Applied Biosystems automatic sequencer. The PCR purification and dye removal steps were performed on a Biomek FX Laboratory Automation Workstation (Beckman Coulter) with AmPure and CleanSeq kit (Agencourt-Beckmann Coulter) respectively.

Using the Gene Codes Sequencer software (V.5.0), called sequences were aligned to each gene reference sequence: *SFTPB*: ENSG00000168878 and ENST00000409383; *SFTPC*: ENSG00000168484 and ENST00000318561; *ABCA3*: ENSG00000167972 and ENST00000301732.

All identified variants were described in accordance with the 'Human Genome Variation Society' nomenclature. Gene variants were evaluated by their absence or frequency in the dbSNP, 1000 Genomes, ExAc, gnomAD databases. The HGMD professional 2016.4 database and PubMed were interrogated to check for already described variants. The

PolyPhen2, SIFT, Grantham distance, Align GVGD, were used to predict the functional effect of missense changes. Human Splicing Finder, Gene Splicer, MaxEntScan, NNSplice and Splice Site Prediction by Neural Network were used to predict the potential effect on splicing. Classification of the variants was performed based on the ACMG-AMP criteria [11].

Immunohistochemical analysis

Immunohistochemical analysis was carried out on 4 micron, formalin-fixed, paraffin-embedded sections. Samples were deparaffined in xylene and grades of alcohol, then rehydrated in water and were incubated whit a polyclonal antibody to ABCA3 at a dilution of 1:30 (Atlas Antibodies, St Louis, MO,USA). Antigen retrieval was performed by heating in a pressure cooker with Citrate buffer for 15 min. A polymer detection methods (Thermo Fisher Scientific, Fremont,CA,USA) followed by a brown chromogen solution was used for detection.

Electron microscopy

In one infant, who deceased at the age of 4 months, lung tissue was examined with electron microscopy. For ultrastructural studies, lung fragments were either fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), postfixed in osmium tetroxide and processed into epoxy resin. Ultrathin sections were obtained from several blocks, stained with lead citrate and uranyl acetate, and observed with a Philips CM10 transmission electron microscope.

Results

Overall, 28 rare or novel variants of *ABCA3, SFTPB* and *SFTPC* were identified in 24 infants (Tables 1 and 2), with the exclusion of common variants. In particular, 7 were novel while the remaining were rare variants. *ABCA3* was the most frequently affected gene with 21 variants in 18 neonates. In the six newborns without variants in ABCA3, variants in *SFTPB* or *SFTPC* were present. In case n.2 variants in both *ABCA3* and *SFTPB* were identified. By looking at the variants, one in-frame deletion, 9 missense, 4 synonymous, 12 intronic and 2 variants in the 3′UTR were found. By applying ACMG-AMP criteria for interpretation of sequence variants, all of them have been classified as variants of unknown significance. Nevertheless based on the nature and location of the variant, the in silico analysis, the population frequency and critical analysis of the literature we can speculate on which variants may have

Table 1 Patients characteristics

n	Sex	GA (w)	BW (g)	Therapies	Outcome	SFTPB	SFTPC	ABCA3
1	F	25	860	Oxygen, CPAP, surfactant, CMV, HFOV, Steroids	Died at 5 months	wt	wt	p.(Ala501Glu) [12] c.991-94 G > C c.3279-39 G > A
2	M	24	660	Oxygen, CPAP, surfactant, CMV, HFOV, Steroids	Died at 2 months	p.(Ala295Thr)	wt	c.3483 + 133 g > A
3	F	27 + 3	800	Oxygen, CPAP, surfactant, CMV, HFOV, Steroids	Died at 6 months	wt	wt	c.2513 + 23_2513 + 24insGT c.2513 + 28 T > A
4	M	31	1180	Oxygen, CPAP, surfactant, CMV, Steroids	Died at 6 months	p.(Pro181_Leu184del)	wt	wt
5	M	29	1180	Oxygen, CPAP, surfactant, CMV, HFOV, Steroids	Alive at 6 years	wt	wt	p.(Asp1374Glu)
6	M	29	1290	Oxygen, CPAP, surfactant, CMV, HFOV, Steroids	Died at 4 months	wt	wt	c.3704-5 C > A
7	M	29 + 5	1260	Oxygen, CPAP, surfactant, CMV, HFOV, Steroids	Alive at 5 years	wt	wt	p.(Tyr1390 =)
8	F	26	650	Oxygen, CPAP, surfactant, CMV, NIV, HFOV, Steroids	Alive at 2 years	wt	wt	p.(Arg288Lys) [13, 15]
9	M	26	881	Oxygen, CPAP, surfactant, CMV, HFOV, Steroids	Alive at 4 years	wt	wt	p.(Arg661Gln)
10	M	30	1900	Oxygen, CPAP, surfactant, CMV	Alive at 10 years	wt	wt	p.(Ile703Thr)
11	M	27	950	Oxygen, CPAP, surfactant, CMV	Alive at 2 years	wt	c.43-7 G > A [14] c*12 T > A	wt
12	M	28	1050	Oxygen, CPAP, surfactant, CMV, HFOV, Steroids	Alive at 5 years	c*9 C > T	wt	wt
13	M	30	1300	Oxygen, CPAP, surfactant, CMV, HFOV, Steroids	Alive at 6 years	wt	wt	c.613 + 18 G > T
14	F	26	850	Oxygen, CPAP, surfactant, CMV, HFOV, Sildenafil, Steroids	Died at 5 month	p.(Pro306 =)	wt	wt
15	M	27	1240	Oxygen, surfactant, CMV, HFOV	Died at 2 months	wt	wt	p.(Ala756 =)
16	M	24	800	Oxygen, surfactant, iNO, CMV	Died at 5 months	wt	wt	c.3483 + 26 G > A
17	M	30	1100	Oxygen, surfactant, CMV, HFOV	Alive at 3 months	p.(Phe132Leu)	wt	wt
18	M	28	960	Oxygen, surfactant, CMV	Alive at 1 year	p.(Glu109 =)	wt	wt
19	M	32	850	Oxygen, CPAP, CMV, Steroids	Alive at 7years	wt	wt	p.(Phe1077Leu)
20	F	32	1725	Oxygen, CPAP, surfactant, CMV, Steroids	Died at 5 months	wt	wt	p.(Pro249Thr)
21	M	26	1056	Oxygen, surfactant, CMV, HFOV, Steroids	Died at 2 months	wt	wt	c.3862 + 40 T > C
22	M	26	949	Oxygen, CPAP, Steroids	Alive at 1 year	wt	wt	c.613 + 11 C > T
23	F	32	980	Oxygen	Alive at 3 years	wt	wt	c.2053-65 G > A
24	M	25	550	Oxygen, surfactant, CMV, HFOV, Steroids	Died at 2 months	wt	wt	c.2053-65 G > A

CPAP continuous positive airways pressure, CMV conventional mechanical ventilation, HFOV high frequency oscillatory ventilation, NIV non-invasive ventilation, iNO inhaled nitric oxide

Table 2 Rare and novel variant identified

Gene	Region	Variant		rs	Frequency		n. of homoz.	Protein prediction		Grantham distance	Align GVGD	Splicing prediction
		Coding DNA	Putative protein		1000 G	ExAC/ gnomAD		PP2	SIFT			MaxEnt, NNSPLICE, GeneSplicer, HSF, SSPNN
ABCA3	Coding	c.745 C>A	p.(Pro249Thr)	Novel	0	0	0	Benign (0.063)	Deleterious (0.03)	38	C0	–
		c.863 G>A	p.(Arg288Lys) [13, 25]	rs117603931	0.002	0.0059	10	Benign (0.002)	Tolerated (1)	26	C0	–
		c.1502 C>A	p.(Ala501Glu) [12]	rs141621969	0.0034	0.0038	17	Benign (0.001)	Tolerated (1)	107	C0	–
		c.1982G>A	p.(Arg661Gln)	rs376672600	0	0.00003	0	Benign (0.054)	Tolerated (0.4)	43	C0	–
		c.2108 T>C	p.(Ile703Thr)	novel	0	0	0	Benign (0.001)	Tolerated (0.37)	89	C0	–
		c.2268 C>T	p.(Ala756 =)	rs143552557	0.0006	0.0017	1	–	–	–	–	No difference
		c.3231 C>A	p.(Phe1077Leu)	rs143240655	0	0.0003	0	Benign (0.003)	Tolerated (0.34)	22	C0	–
		c.4122 C>G	p.(Asp1374Glu)	–	0	0.00001	0	Benign (0.001)	Tolerated (1)	45	C0	–
		c.4170C>T	p.(Tyr1390 =)	rs532847219	0.0002	0.00001	0	–	–	–	–	IVS27-1 new donor
	INTRONIC	c.613 + 11 C>T	p.?	rs370890203	0	0.00006	0	–	–	–	–	No difference
		c.613 + 18 G>T	p.?	rs201364163	0	0.0019	2	–	–	–	–	IVS7 + 12 new donor
		c.991-94 G>C	p.?	Novel	0	0	0	–	–	–	–	No difference
		c.2053-65 G>A	p.?	rs147395547	0.0034	0	0	–	–	–	–	No difference
		c.2513 + 23_2513 + 24insGT	p.?	Novel	0	0	0	–	–	–	–	No difference
		c.2513 + 28 T>A	p.?	Novel	0	0	0	–	–	–	–	No difference
		c.3279-39 G>A	p.?	rs374377819	0	0.00001	0	–	–	–	–	No difference
		c.3483 + 26 G>A	p.?	rs183047748	0.0002	0.00015	0	–	–	–	–	No difference
		c.3483 + 133 G>A	p.?	Novel	0	0	0	–	–	–	–	IVS23 + 135 new acceptor
		c.3704-5 C>A	p.?	Novel	0	0	0	–	–	–	–	Affecting splice at exon 25 acceptor
		c.3862 + 40T>C	p.?	rs369424856	0.0006	0.003	5	–	–	–	–	No difference
SFTPB	Coding	c.327 G>A	p.(Glu109 =)	rs34682912	0.0028	0.0036	11	–	–	–	–	No difference
		c.394 T>C	p. (Phe132Leu)	rs138729391	0	0.00006	0	Benign (0.018)	Tolerated (1)	22	C0	No difference
		c.541_552del12	p.Pro181_Leu184del	–	0	0.0004	0	–	–	–	–	–

Table 2 (continued)

Gene	Region	Coding DNA	Putative protein	rs	1000 G	ExAC/gnomAD	n. of homoz.	PP2	SIFT	Grantham distance	Align GVGD	Splicing prediction (MaxEnt, NNSPLICE, GeneSplicer, HSF, SSPNN)
		c.883 G > A	p.(Ala295Thr)	rs36210375	0.0034	0.0016	1	Benign (0.000)	Tolerated (0.43)	58	C0	Affecting splice at exon 5 donor
		c.918 G > A	p.(Pro306 =)	rs750280427	0	0.00002	0	–	–	–	–	No difference
	3′-UTR	c*9 C > T	p.?	rs771704983	0	0.000017	0	–	–	–	–	No difference
SFTPC	INTRONIC	c.43-7 G > A [14]	p.?	rs79440568	0.0086	0.0027	12	–	–	–	–	No difference
	3′-UTR	c*12 T > A	p.?	rs200469074	0.0004	0.00008	0	–	–	–	–	No difference

been likely to play a role in the pathogenesis of lung disease. In one infant (case n.6) who died at 4 months, an intronic heterozygous variant in *ABCA3* (c.3704-5 C > A) was identified; the variant was novel and predicted to affect the splicing at exon 25, thus supporting a possible pathogenic role. Lung tissue of this infant was available for histological and ultrastructural analyses. Histology showed marked hyperplasia of type II pneumocytes and chronic inflammatory cells in alveolar lumen, and immunohistochemical analysis showed expression of ABCA3 in tissue sampling; at electron microscopy peripheral dense inclusions were observed in lamellar bodies, suggestive for ABCA3 dysfunction [12] (Fig.1). Among the other novel or very rare variants, three ABCA3 missense (p.Pro249Thr; p.Ile703Thr, p.Arg661Gln) were found, predicted as benign; interestingly the variant p.Ile703Thr as well as the p.Arg661Gln are located in the ATPase AAA + domain, suggesting that they could determine a functional change in the ATPase activity, as it was showed for the p.Arg288Lys [13].

In cases n. 1, 8 and 11, three variants (*ABCA3* p. Ala501Glu [14]; *ABCA3* p.Arg288Lys[13–15]; *SFTPC* c.43-7 G > A [16] have been already described in affected subjects, as pathogenic. Data supporting this conclusion are debatable for two of these: (i) the *ABCA3* p.Ala501Glu was previously identified in an infant who also had findings of alveolar capillary dysplasia (ACD), with *FOXF1* not already known as a disease gene for that condition [17]; the variant was not so rare in the general population and all predictions were consistent for the absence of a functional effect; (ii) for the SFTPC c.43-7 G > A, the evidence of pathogenicity is weak mainly because no effect on the splicing is predicted and its frequency in some populations makes it very unlikely as a truly pathogenic variant [16, 18, 19].

Additional rare missense variants in ABCA3 or SFTPB were found and always predicted as benign (Table 2). By looking at intronic or at exonic variants close to the intronic junctions, only in few cases a change in the splicing was predicted: this was the case for the *SFTPB* in-frame deletion p. Pro181_Leu184del, predicted to affect splicing at exon 5; or for the ABCA3 p.Tyr1390=, c.613 + 18 G > T, c.3483 + 133 G > A predicted to activate cryptic splice sites (Table 2).

For those infants in whom multiple sequence variants were identified, parental studies were not feasible. Only two infants had familiarity for lung disease: RDS in the family of patient case n.6 and pneumothorax in the family of case n.17; no parents had consanguinity. All infants were of European origin except one who was of African descent. In total, 11 infants died between 2-to-6 months, 13 are alive and 2 of them were oxygen-dependent up to 1 and 5 years.
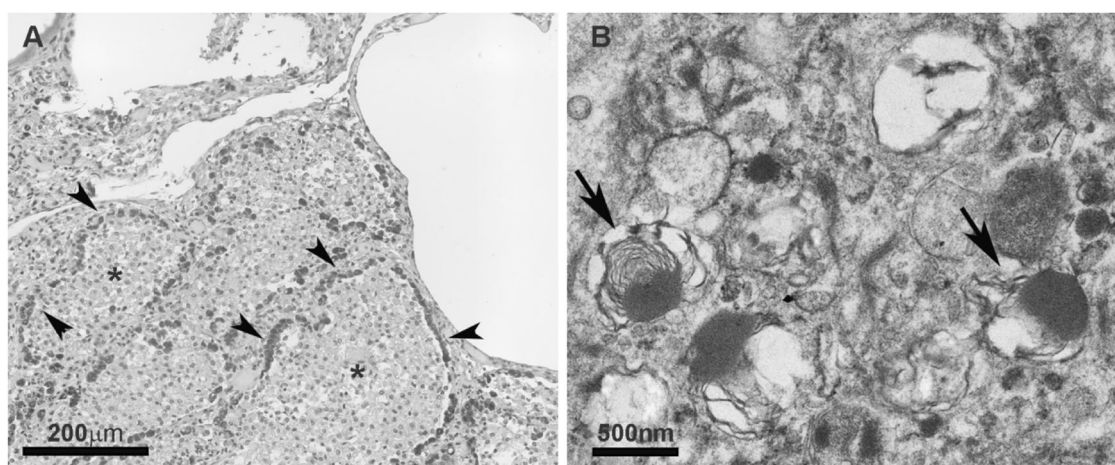
Fig. 1 Immunohistochemistry and EM analysis of case N°6 **a** representative immunohistochemical staining with ABCA3 antibody (brown colour) showing aType II pneumocyte hyperplasia (arrowhead). Lumen contains chronic inflammatory cells (asterisk). **b** Electron microscopy shows lamellar bodies electron-dense aggregates in the form of 'fried egg' with an asymmetric core (arrows)

Discussion

RDS is a complex disease and several factors may contribute to its aetiology. The major pathogenic factor of RDS is a lack of pulmonary surfactant due to developmental immaturity of the lungs. However, genetic mechanisms may contribute significantly to development of neonatal RDS [2]. Pathogenic variants in *SFTPB, SFTPC* and *ABCA3* transmitted in a Mendelian pattern cause severe or lethal RDS in term newborn infants. Sequencing of *SFTPB, SFTPC* and *ABCA3* allowed us to identify 28 single base substitutions or small indels in 24 preterm newborn infants, while we did not excluded the presence of large structural variants or the presence of pathogenic variants in other genes, not exclusively associated with Respiratory Distress (i.e., NKX2-1, OMIM600635).

In relation to the rare and novel variants reported in the present study, we referred our comparison to the data on the general population frequencies, available in the genomic public databases (Table 2). The ideal control group would have be a population of infants matched for ethnicity and gestational age without lung disease or with mild RDS, but unfortunately this group was not available.

Overall, rare or novel gene variants were found in 24 patients out of the 68 examined (35.3%). By focusing on variants with a lower frequency in the general population, a predicted effect or located in a functional domain, we can restrict to 11 stronger candidate variants, corresponding to 16.2%. None of the study subjects had a diagnosis of a surfactant dysfunction disorder clearly established by genetic testing, nevertheless our results, indicating an over-representation of possibly deleterious alleles, support the findings of previous studies. Partial SP-B deficiency due to a single SP-B gene variant was reported in a term neonate

who survived developing chronic lung disease with prolonged oxygen-dependency [20]. Experimental data indicate that partial SP-B deficiency increases susceptibility to lung injury. Animal studies in genetically engineered mice, in which SP-B can be reduced, indicated that a level of SP-B production of 20–30% of control level is needed for normal lung function [21]. Mice heterozygous for a null SP-B allele showed an increased susceptibility to oxygen-induced lung injury: a decrease in lung compliance was associated with increased severity of pulmonary oedema, haemorrhage and inflammation, lung permeability, and protein leakage into the alveolar space. It was likely that partial SP-B deficiency resulted in instability of affected alveoli, leading to micro-atelectasis, a decrease in residual lung volume and air space enlargement detected in histological sections [22, 23]. Therefore, infants who are heterozygous for pathogenic variants can be at risk for lung disease if other environmental factors such as prematurity further reduce SP-B expression.

Moreover, Wambach et al. showed that single *ABCA3* mutations are overrepresented among European descent infants ≥34 weeks of gestation with RDS and account for 10.9% of the attributable risk among term and late preterm infants. Because ABCA3 expression is developmentally regulated, a single mutation coupled with developmental immaturity could reduce ABCA3 expression below a functional threshold that results in RDS [24]. One specific single mutation of *ABCA3* (p.Glu292Val) was found to be at increased risk for developing pneumo-thoraces and the phenotype of ILD. The authors hypothesised that the p. Glu292Val change could in part increase sensitivity to the developmental risk of RDS [25]. Index patient n. 6 presented a single intronic variant in *ABCA3* and died at 4 months; ABCA3 was detected at immunohistochemical

staining of the lung tissue, however type II pneumocytes hyperplasia, inflammatory cells infiltration and the presence of peripheral dense aggregates in lamellar bodies at electron microscopy may suggest a disorder in surfactant metabolism [26, 27]. (Fig. 1). *SFTPC* mutations are responsible of ILD in infants and children with onset of symptoms after the neonatal period; however, sometimes they become symptomatic in the neonatal period [3].

Our study shows that candidate variants in surfactant protein genes were identified also in newborn infants of gestational age ≤32 weeks with particularly severe RDS, even without consanguinity and positive family history, suggesting that prematures could be affected not only by an insufficient amount of pulmonary surfactant due to pulmonary immaturity, but also by abnormalities of surfactant metabolism and function of genetic origin. In our cohort, a common clinical characteristic of infants was the perception that the clinical course of RDS was more severe, complicated or prolonged than usual. Infants showed poor response to surfactant administration, difficulty of weaning from mechanical ventilation, need of unconventional ventilatory support or prolonged oxygen dependency. In total, 11 infants died within the first 6 months, 13 survived and 2 were oxygen dependent after discharge from the hospital for 1 and 5 years (Table 1). Our study is not population-based and we do not know if all neonates with very severe respiratory failure were referred for genetic testing, precluding estimates of the incidence of these disorders.

We speculate that premature newborn infants with a single pathogenic variant in surfactant protein genes may have a reduced production or impaired function of the protein that, in conjunction with pulmonary immaturity, increases severity of the lung disease, thus behaving as a multifactorial disorder.

Testing for surfactant dysfunction is currently recommended in term neonates of more than 37 weeks gestation who develop unexplained respiratory distress, or of earlier gestational age when there is a positive family history and consanguinity [28, 29]. Extension of genetic testing for surfactant dysfunction in premature newborn infants with a worse phenotype of RDS, regardless of familiarity for lung diseases or consanguinity, to allow better prognostication, a more conscious choice of therapeutic options and eventually genetic counselling, seems to be useful but more challenging. Our findings outlined our still limited knowledge and difficulty in interpretation of genetic variants, since genetic testing was not conclusive and, as previously reported, this point is one of the main issues of genetic testing. Moreover due the high cost of molecular testing, it is not sustainable by health care systems for all the patients. Further studies are needed to improve our knowledge on genetic variants and understanding of the mechanisms that regulate surfactant metabolism in term and preterm newborn infants with unexplained and severe RDS.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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