INTRODUCTION

Cystic fibrosis (CF; MIM#219700) is an autosomal recessive disorder caused by mutations in CFTR (CF transmembrane conductance regulator; MIM#602421; GenBank: NM_000492.3), the gene encoding the homonymous chloride-bicarbonate channel (Shteinberg et al., 2021).

Nowadays, CF is one of the most common life-threatening diseases with an incidence of 1/3000 in the European population (Scotet et al., 2020; Southern et al., 2007).
making a CF diagnosis is a delicate multi-stage process that includes evaluation of typical clinical picture and CFTR dysfunction (typically via sweat chloride test, SCT), as well as identification of biallelic causal CFTR variants (Farrell et al., 2017). Even in case of a positive SCT in newborn screening (NBS) (Farrell et al., 2008), all CF subjects should be genotyped, and their variants studied by segregation analysis (Castellani et al., 2009).

Despite well-established directives, the clinical diagnosis and prognosis of CF may still be hampered by the extensive phenotypic heterogeneity, mirroring the high number of CFTR variants (466 variants annotated hitherto, https://cftr2.org/mutations_history [last update: 24 September 2021]) and, especially, of their combinations. In these terms, the existence of alleles with two (or more) variants in cis, named complex alleles, further challenges the establishment of a clear genotype–phenotype correlation (El-Seedy et al., 2012; Terlizzi et al., 2017). Unless the variants of a complex allele have been found separately, their single functional and phenotypic effect (worsened or ameliorated) or their potential synergistic action could indeed be evaluated only through in vitro studies (El-Seedy et al., 2012; Terlizzi et al., 2017).

Herein, we report a young girl affected by an undefined syndrome, who resulted to have CF. Segregation analysis within her family was central to unveil three CFTR variants, including two known CF-causing variants on the same allele and a novel intragenic microdeletion. This case represents a good example to discuss pitfalls in the clinical and molecular diagnosis of CF.

2 RESULTS

A 6-year-old girl of Albanian descent (II-1, Figure 1) and with an unremarkable family history was referred to the medical geneticists for short stature with some dysmorphic physical features. All the methods and materials used in this study are reported as Supporting Information.

Born at term after a pregnancy complicated by an increased nuchal fold, she presented meconium ileus at the first day of life, but negative CF NBS with only one disease-causing mutation reported (c.1521_1523del; p.Phe508del). Despite the first suspicion of CF, specific clinical evaluations were performed just after two episodes of intestinal sub-occlusions.

The girl had also a history of chronic constipation, cough, and growth delay. The investigations revealed a multifaceted clinical picture, including short-limb dwarfism, delayed bone age, hepatomegaly with mildly elevated transaminases, malabsorption of pancreatic origin, and some lung atelectasis. Morphologic features shared with both the father and the paternal grandfather included midface hypoplasia, macrocephaly, mild frontal bossing, and arched tibia.

Standard karyotyping, as well as molecular karyotyping of the proband and her parents by SNP-array did not identify any potential chromosomal alteration.

The wide plethora of proband’s clinical features led us to hypothesize a dominantly inherited skeletal dysplasia (SD) and/or CF. However, it was not possible to determine the SD causative gene neither sequencing the strong candidate FGFR3 (fibroblast growth factor receptor 3) nor analyzing the other candidates enlisted in Mortier et al. (2019) by whole exome sequencing (WES).

On the contrary, the CF diagnosis was supported by positive SCT (100 mEq/L, 241 mg). Indeed, targeted next generation sequencing (NGS) (detection rate of CF alleles about 97%) revealed two heterozygous variants: the already identified c.1521_1523del (p.Phe508del; MAF = 0.007068) and c.1420G>A (p.[Glu474Lys]; MAF = 0.000004). Representing 66% of CFTR alleles worldwide, p.Phe508del has an unquestionable pathogenic effect. The other variant, c.1420G>A, is rare among the CF affected individuals and, in-line with bioinformatic predictions, it is reported as deleterious in databases (see Supporting Information).

However, the segregation analysis revealed that the two mutations were in cis, maternally inherited as a complex allele. The data were confirmed by cloning assay (Figure 1) and allelic-specific PCR, ruling out, albeit very rare, a recombination event between the two variants at least in the proband (we could not exclude a recombination occurred in the ancestors of the maternal family branch). Therefore, we hypothesized that the paternal CFTR allele could be a variant (e.g., small copy number variation [CNV], deep intronic variant) not explored by the previous analyses. We thus performed a statistical evaluation of the ampli-con coverage from the NGS data, which allowed us to predict a deletion of CFTR exons 20 and 21 (Figure 2a). GAP PCR (Figure 2b, Table S1) and long-range PCR followed by Sanger sequencing (Figure 2c,d, Table S1) consistently confirmed the paternal inheritance of a novel CFTR deletion (c.:3158_3468+3219del).

3 DISCUSSION

The case reported is a good example to discuss pitfalls in the clinical and molecular diagnosis of CF, which traditionally relies on the coexistence of clear clinical picture, positive SCT and identification of biallelic disease-causing CFTR variants.

The proband had CF features since the newborn period, including meconium ileus, regarded as the earliest manifestation of CF (20% of infantile cases) (Sathe & Houwen, 2017) and a poor prognostic factor (Gorter...
et al., 2010), and reduced weight gain due to pancreatic insufficiency. Nevertheless, she tested negative for NBS (protocol: single immunoreactive trypsinogen [IRT] assay on blood spots+ DNA analysis), thus leading clinicians to focus on the SD signs and preventing CF diagnosis. Our patient fell within the several cases of false-negative CF
NBS documented, mainly attributable to the strategy adopted (e.g., NBS algorithm, cutoff, and sensitivity) and various conditions at birth (Steven et al., 2006; Taccetti et al., 2020). In this situation, a SCT could have been decisive to resolve the CF suspicion even in concomitance with other diseases; but it was performed only at patient’s age of six, indeed providing a positive result.

Consistently, proband’s mutational screening revealed two known CFTR mutations (p.[Glu474Lys] and p.Phe508del), whose parental origin was ascertained by segregation analysis within the family (Castellani et al., 2009). The study was critical to unveil the maternal inheritance of both anomalies as a CFTR complex allele (c.[1420G>A;1521_1523del]). This suggested the presence of another disease-causing variant on the paternal chromosome, which was confirmed as a deletion affecting exons 20 and 21 and characterized as c.3158_3468+3219del. Taken together these results stress the importance to perform segregation analysis, and to consider CNVs among the disease-causing mutations for accurate CFTR genotyping.

The identification of a complex allele lead us to question on the pathogenetic role of the single variants, which might have different effects when combined in cis (Lucarelli et al., 2010). In the case of c.[1420G>A;1521_1523del]), the deleterious effect of p.Phe508del is unquestionable. For p.(Glu474Lys), we queried CFTR2 database (https://cftr2.org/) and found that it was reported in five subjects. In addition to p.(Glu474Lys), two of the five affected individuals are heterozygous for known missense or frameshift variants, and three—like our proband—for the p.Phe508del mutation. Segregation analysis is not available for any case, as the CFTR2 database is built on genetic information of CF registries assuming that the two variants identified are in trans phase. We questioned if any or all the three subjects with p.Phe508del and p.(Glu474Lys) carried the variants in cis as a complex allele, together with an unidentified third causative one on the other chromosome.

The detection of c.1420G>A (p.(Glu474Lys)) in cis with a known CF-causing mutation raises question about its deleterious effect. However, p.(Glu474Lys) is likely to be pathogenic for the following aspects: (i) damaging effect predicted by bioinformatic tools; (ii) functional assay based on chloride conductance (1.2% compared to wild-type as reported in CFTR2 database; (iii) found in individuals with mutations other than c.1521_1523del (as reported in CFTR2); (iv) rarity in the general population (not reported in gnomAD).

Although the disease-causing role of p.(Glu474Lys) seems not to be in doubt, additional CF subjects with the same c.[1420G>A;1521_1523del] complex allele would be
need to establish any genotype–phenotype relationship, as well as more in-depth study to investigate the concerted effect of the two mutations.

The characterization and determination of the exact prevalence of complex alleles (including those with the well-described p.Phe508del variant) (Baatallah et al., 2018) still remain a thorny issue. Mutational search protocols are generally designed on panels including only the most common CFTR anomalies and/or end following the detection of two variants on different alleles, often excluding additional in cis mutations (Lucarelli et al., 2010). This represents a severe limitation for an accurate genetic testing, which could be instead solved by scanning the entire CFTR gene (Lucarelli et al., 2010). Providing a complete CFTR genotypization appears to be even more important today in view of the recent CF etiological therapies; these approaches are mainly directed to c.1521_1523del (p.Phe508del), given its major frequency, but their effectiveness on this variant within a complex allele is rarely proven (Baatallah et al., 2018; Chevalier & Hinzpeter, 2020). The detection of a further in cis variant could indeed improve patients’ recruitment, expected response to current treatments, and combinatorial targeting of distinct defects.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

ETHICS STATEMENT
All experimental protocols were approved by the ethical committee of IRCCS “Burlo Garofolo” hospital. All subjects provided written informed consent for the study, which was conducted in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS
IP and AF collect and analyze data, review the literature, and drafted the manuscript. RB and AS organized this study, reviewed clinical and laboratory data, and finalized this manuscript. FS, FF, and MMA performed genetic counselling, patient management and reviewed clinical data. KSR revised the draft critically for important intellectual content. MF and GF performed Sanger sequencing and analysis of deletion. MLB, SS, MMO and APD performed NGS and SNP array. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES
Baatallah, N., Bitam, S., Martin, N., Servel, N., Costes, B., Mekki, C., Chevalier, B., Pranke, I., Simonin, J., Giordon, E., Hoffmann, B., Morron, J. P., Callebaut, I., Sermet-Gaudelus, I., Fanen, P., Edelman, A., & Hinzpeter, A. (2018). Cis variants identified in F508del complex alleles modulate CFTR channel rescue by small molecules. Human Mutation, 39(4), 506–514.
Castellani, C., Southern, K. W., Brownlee, K., Dankert Roelse, J., Duff, A., Farrell, M., Mehta, A., Munck, A., Pollitt, R., Sermet-Gaudelus, I., Wilcken, B., Ballmann, M., Corbetta, C., de Monestrol, I., Farrell, P., Feilcke, M., Férec, C., Gartner, S., Gaskin, K., … Elborn, S. (2009). European best practice guidelines for cystic fibrosis neonatal screening. Journal of Cystic Fibrosis, 8(3), 153–173.
Chevalier, B., & Hinzpeter, A. (2020). The influence of CFTR complex alleles on precision therapy of cystic fibrosis. Journal of Cystic Fibrosis, 19(Suppl. 1), S15–S18.
El-Seedy, A., Giordon, E., Norez, C., Pajaud, J., Pasquet, M. C., de Becdelièvre, A., Bienvenu, T., des Georges, M., Cabet, F., Lalau, G., Bieth, E., Blayau, M., Becq, F., Kitzis, A., Fanen, P., & Ladeveze, V. (2012). CFTR mutation combinations producing frequent complex alleles with different clinical and functional outcomes. Human Mutation, 33(11), 1557–1565.
Farrell, P. M., Rosenstein, B. J., White, T. B., Accuro, F. J., Castellani, C., Cutting, G. R., Durie, P. R., Legrys, V. A., Massie, J., Parad, R. B., Rock, M. J., Campbell, P. W., 3rd, & Cystic Fibrosis Foundation. (2008). Guidelines for diagnosis of cystic fibrosis in newborns through older adults: Cystic Fibrosis Foundation consensus report. The Journal of Pediatrics, 153(2), S4–S14.
Farrell, P. M., White, T. B., Howenstine, M. S., Munck, A., Parad, R. B., Rosenfeld, M., Sommerburg, O., Accuro, F. J., Davies, J. C., Rock, M. J., Sanders, D. B., Wilschanski, M., Sermet-Gaudelus, I., Blau, H., Gartner, S., & McCollery, S. A. (2017). Diagnosis of cystic fibrosis in screened populations. The Journal of Pediatrics, 181S, S33–S44.e32.
Gorter, R. R., Karimi, A., Sleeboom, C., Kneepkens, C. M., & Heij, H. A. (2010). Clinical and genetic characteristics of meconium ileus in newborns with and without cystic fibrosis. Journal of Pediatric Gastroenterology and Nutrition, 50(3), 569–572.
Lucarelli, M., Narzi, L., Pierandre, S., Bruno, S. M., Stamato, A., D’Avanzo, M., Strom, R., & Quattrucci, S. (2010). A new complex allele of the CFTR gene partially explains the variable phenotype of the L997F mutation. Genetics in Medicine, 12(9), 548–555.
Mortier, G. R., Cohn, D. H., Cormier-Daire, V., Hall, C., Krakow, D., Mundlos, S., Nishimura, G., Robertson, S., Sangiorgi, L., Savarirayan, R., Sillence, D., Superti-Furga, A., Unger, S., & Warman, M. L. (2019). Nosology and classification of genetic
skeletal disorders: 2019 revision. *American Journal of Medical Genetics: Part A*, 179(12), 2393–2419.

Sathe, M., & Houwen, R. (2017). Meconium ileus in cystic fibrosis. *Journal of Cystic Fibrosis*, 16(Suppl. 2), S32–S39.

Scotet, V., Gutierrez, H., & Farrell, P. M. (2020). Newborn screening for CF across the globe—Where is it worthwhile? *International Journal of Neonatal Screening*, 6(1), 18.

Shteinberg, M., Haq, I. J., Polineni, D., & Davies, J. C. (2021). Cystic fibrosis. *Lancet*, 397(10290), 2195–2211.

Southern, K. W., Munck, A., Pollitt, R., Travert, G., Zanolla, L., Dankert-Roelese, J., & Castellani, C. (2007). A survey of newborn screening for cystic fibrosis in Europe. *Journal of Cystic Fibrosis*, 6(1), 57–65.

Steven, L. C., Gavel, G., Young, D., & Carachi, R. (2006). Immunoreactive trypsin levels in neonates with meconium ileus. *Pediatric Surgery International*, 22(3), 236–239.

Taccetti, G., Botti, M., Terlizzi, V., Cavicchi, M. C., Neri, A. S., Galici, V., Mergni, G., Centrone, C., Peroni, D. G., & Festini, F. (2020). Clinical and genotypical features of false-negative patients in 26 years of cystic fibrosis neonatal screening in Tuscany, Italy. *Diagnostics (Basel)*, 10(7), 446.

Terlizzi, V., Castaldo, G., Salvatore, D., Lucarelli, M., Raia, V., Angioni, A., Carnovale, V., Cirilli, N., Casciaro, R., Colombo, C., di Lullo, A. M., Elce, A., Iacotucci, P., Comegna, M., Scorzà, M., Lucidi, V., Perfetti, A., Cimino, R., Quattruccì, S., ... Amato, F. (2017). Genotype-phenotype correlation and functional studies in patients with cystic fibrosis bearing CFTR complex alleles. *Journal of Medical Genetics*, 54(4), 224–235.

**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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