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# Nonsense mutations accelerate lung disease and decrease survival of cystic fibrosis children

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Premature termination codon (MeSH: DO:18389) Cystic fibrosis (MeSH: DO008550) Cystic fibrosis transmembrane conductance regulator (MeSH: DO19005)

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# a b s t r a c t

## ***Rationale:***

Limited information is available on the clinical status of people with Cystic Fibrosis (pwCF) carrying 2 nonsense mutations (PTC/PTC). The main objective of this study was to compare disease severity between pwCF PTC/PTC, compound heterozygous for F508del and PTC (F508del/PTC) and homozygous for F508del (F508del + / +).

## ***Methods:***

Based on the European CF Society Patient Registry clinical data of pwCF living in high and middle income European and neighboring countries, PTC/PTC ( $n = 657$ ) were compared with F508del + / + ( $n = 21,317$ ) and F508del/PTC ( $n = 4254$ ). CFTR mRNA and protein activity levels were assessed in primary human nasal epithelial (HNE) cells sampled from 22 PTC/PTC pwCF.

## ***Main results:***

As compared to F508del + / + pwCF; both PTC/PTC and F508del/PTC pwCF exhibited a significantly faster rate of decline in Forced Expiratory Volume in 1 s (FEV<sub>1</sub>) from 7 years (-1.33 for F508del + / +, -1.59 for F508del/PTC; -1.65 for PTC/PTC,  $p < 0.001$ ) until respectively 30 years (-1.05 for F508del + / +, -1.23 for PTC/PTC,  $p = 0.048$ ) and 27 years (-1.12 for F508del + / +, -1.26 for F508del/PTC,  $p = 0.034$ ). This resulted in lower FEV<sub>1</sub> values in adulthood. Mortality of pediatric pwCF with one or two PTC alleles was significantly higher than their F508del homozygous pairs. Infection with *Pseudomonas aeruginosa* was more frequent in PTC/PTC versus F508del + / + and F508del/PTC pwCF. CFTR activity in PTC/PTC pwCF's HNE cells ranged between 0% to 3% of the wild-type level.

## ***Conclusions:***

Nonsense mutations decrease the survival and accelerate the course of respiratory disease in children and adolescents with Cystic Fibrosis.

# 1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The most frequent *CFTR* gene mutation, generating the F508delCFTR protein, is found in over 80% of people with CF (pwCF) in Europe, and causes retention of a mis-folded CFTR protein in the endoplasmic reticulum and its subsequent degradation [1]. CFTR modulators have been proven to clinically benefit in pwCF carrying at least one copy of F508del [2, 3]. Nearly 5% of pwCF worldwide carry a nonsense mutation on both alleles resulting in a premature termination codon (PTC) [4, 5]. For those patients, no efficient targeted therapy is available yet and there is a high unmet need for novel therapeutic strategies aiming to “read-through” the PTC [1]. Indeed, CFTR mRNA transcripts carrying a PTC are generally targeted for degradation by nonsense-mediated mRNA decay (NMD) [6]. However, there are some PTC mutations with high basal read-through that can produce full-length CFTR proteins and moreover, truncated CFTR proteins may display residual activity, such as C-terminal truncated proteins after codon position 1162 [7–9]. Both situations, only shown *in vitro*, should be associated with a less severe disease and a response to read-through treatment.

Limited information is available on the clinical status of pwCF carrying nonsense mutations, apart from those carrying specific mutations such as W1282X [6, 9, 10]. The main objective of this study was to perform a phenotype genotype study of pwCF carrying *CFTR* nonsense mutations resulting in PTCs on both alleles (PTC/PTC) or compound heterozygous for F508del and PTC (F508del/PTC) compared with pwCF homozygous for F508del (F508del + / +), based on the European CF Society Patient Registry (ECFSPR) database, in association with functional analyses of basal CFTR activity and transcripts for specific PTC/PTC genotypes.

## 2. Methods

### 2.1. Study settings and data collection

The European Cystic Fibrosis Society Patients Registry (ECF-SPR) annually collects data on pwCF from 40 countries in Europe and neighboring countries and contains now data from 2008 to 2021. Data are provided to ECFSPR under existing ethical approvals and data governance structures. The present study was approved by the ECFSPR Scientific Committee and Steering Committee on 13/12/2019, data used in this paper were extracted from ECFSPR on 18 October 2022.

Analysis for this study was based on data from 23 countries collected in the ECFSPR between 2008 and 2019 (11,12). The list of countries is shown in Supplemental Table E1, in **online data supplement** according to socio-economical income. The study was restricted to middle-income and high-income European countries to avoid bias of analysis due to geo-economical disparities since the PTC/PTC genotype was significantly more frequent in South Eastern low-income countries compared to the F508del + / + genotype mostly found in the Northern Europe high income countries (Supplemental Fig. E1) (5). Three groups of countries were defined according to terciles of current health expenditure in 2019 in countries included in ECFSPR. Middle-income countries were defined by the current health expenditure per capita between \$2167 and \$5087 in 2019 (<https://databank.worldbank.org/home.aspx>) (Supplemental Table E1).

Considering coverage, i.e. the percentage of pwCF living in a country who are included in the national registry or national data presented by the country, it was estimated to be in 2019 above 80% for all countries included in this study, with the exception of Greece (> 70%), Lithuania (52%) and Poland (> 60%) [11].

### 2.2. Participants and clinical outcomes

Genotypes were identified from the Cystic Fibrosis Mutation database (CFTR1, <http://www.genet.sickkids.on.ca/>), or from CFTR2 [4] or CFTR-France (<https://cftr.iurc.montp.inserm.fr/cftr>) if the mutation was not present in CFTR1. PTC resulted from nonsense mutations and not as a secondary consequence of frame-shift mutations. Variables considered for the analysis followed the ECFSPR definitions, as detailed in the **online data supplement** [12–15].

Methods for primary cultures and study of Human Nasal Epithelial (HNE) cells obtained after nasal brushing of patients are available in **online data supplement** .

### 2.3. Statistical analysis

Categorical variables were expressed as number and percentage of subjects, and numerical variables as mean  $\pm$  standard error of the mean (SEM) or median and quartiles when the distributions were not normally distributed. Missing data were excluded when computing descriptive statistics. To compare the different characteristics between the three mutation groups, Fisher exact test with multiple comparison option was used for categorical variables, and Mann Whitney or Kruskal-Wallis nonparametric test with multiple comparison option for numerical variables. The most recent data for each subject was used in these descriptive analyses.

To test whether the genotype variable remained significantly associated with specific endpoints, after accounting for other explanatory variables, multiple regression models were fitted for *Pseudomonas aeruginosa* colonization, percentage of predicted of Forced Expiratory Volume in 1 s (ppFEV<sub>1</sub>) and death. The adjusting factors included in the models were variables already known from the literature to be associated with clinical evolution of the disease: gender, age, country income group, ppFEV<sub>1</sub> (< 30, 30–70,  $\geq$ 70), Body Mass Index (BMI) (underweight, normal weight), *Pseudomonas aeruginosa*, *Burkholderiacepacia complex*, and insulin-dependent diabetes. This enabled to test whether the effect of genotype (e.g. carrying 1 or 2 nonsense mutation or *F508del* on both alleles), was still independently accounting for specific endpoints, when other variables known to impact on clinical evolution were considered.

To investigate the specific impact of the PTC/PTC genotype on the *Pseudomonas aeruginosa* colonization, a mixed-effect multiple logistic regression model was used, including individual and country as random effects and all adjusting effects previously listed as fixed effects.

Evolution of ppFEV<sub>1</sub> and its decline over time could be quantified from the age of 7 years and was modeled using a mixed effect multiple linear regression model, considering all FEV<sub>1</sub> measurements available from 2008 to 2019. In this model, the explanatory variables were age at FEV<sub>1</sub> measurement, included with a restricted cubic spline with 3 knots, the interaction between age and mutation group and all adjusting factors previously listed. Random effects for individual and for country were included to take into account the correlation between different measurements in the same individual and in the same country. The results were presented graphically as the prediction of ppFEV<sub>1</sub> and of the yearly ppFEV<sub>1</sub> decline obtained from the mixed effect model at specific ages.

The probability of survival at a specific age according to the mutation group was computed by Kaplan-Meier nonparametric estimation and log-rank test was used for comparison. A Cox multiple regression model was fitted, using age as a timescale, to evaluate the effect of the mutation group on survival probability, taking into account all adjusting factors previously listed and including country variable as a cluster. In the Cox model each individual accounts only for the years in which he/she is included in ECFSPR. This primary analysis on pwCF from high and middle income countries was completed by 2 other exploratory analyses restricted to (i) high income countries and (ii) to pwCF not treated with CFTR modulators, to confirm that the observed differences were due to the genotype only and not jeopardized by the socio-economical level or the advent of CFTR modulators. All analyses were performed using R software, version 4.0.0, with packages *fifer*, *FSA*, *lmerTest*, *rms*, *survival* added.

## 3. Results

### 3.1. CFTR genotypes and their geographic distribution

Data of 26.228pwCF from the ECFSPR with a genotype of interest (F508del + / + , F508del/PTC or PTC/PTC) were studied, including 15.768 (54.3%) adults, 3803 adolescents (14.5%) and 6658 (25.3%) children. Of these, 21.317 (81.3%) were F508del + / + , 4254 (16.2%) were F508del/PTC and 657 (2.5%) were PTC/PTC ( Fig. 1 ). A total of 5568 PTC alleles and 130 different PTCs were identified, with the 10 most frequent being G542X (35.2%), W1282X (12.4%), R553X (11.9%), R1162X (7.2%), E60X (2.8%), Q493X (2.1%), Y1092X (2.1%), R1158X (2%) and E585X (1.9%). Their repartition according to the genotype is shown in Supplemental Table E2.

Large variations in the frequency of pwCF from each mutation group were observed between countries ( Fig. 2 and Supplemental Table E3).

### 3.2. Demographic and main clinical characteristics of pwCF

The diagnostic, demographic and clinical characteristics of the study population are presented according to their mutation group in Table 1 . Data per age group ( < 12, 12–17 and  $\geq$ 18 years) are presented in Supplemental Tables E4, E5 and E6. Overall, pwCF with a PTC/PTC genotype were significantly younger than those with a F508del/PTC or F508del + / + genotype, with unexpectedly more male pwCF, and less frequent diagnosis by neonatal screening among F508del + / + patients. They had a trend to higher prevalence of haemoptysis and more diabetes in the adult group but this did not reach the significance level. The carriage of at least 1 PTC was associated with a lower BMI as compared to F508del homozygotes. Use of pancreatic extracts was significantly more reported at all age group in F508del homozygous pwCF as compared to those carrying at least 1 nonsense mutation ( Table 1 , Supplemental Tables E4, E5 and E6). 4% of the PTC/PTC pwCF did not take pancreatic extracts, mainly pwCF carrying E831X (  $n = 9$  ) or mutations in NBD2 (W1282X,  $n = 2$ ; Q1313X,  $n = 1$ ) and the last exon (S1455X,  $n = 1$ ; Q1476X,  $n = 1$ ). As a whole, the number of lung transplants were not significantly different, apart in the PTC/PTC children, who underwent significantly more transplants, but the number is too low to be reliable.

In 2019, very few pwCF had access to Kaftrio® (26 F508del + / + and 58 F508del/PTC). 6376 F508del homozygotes were treated with CFTR modulators, including 4565 with Lumacaftor/Ivacaftor, 1146 for Tezacaftor/Ivacaftor 622 changed from Lumacaftor/Ivacaftor to Tezacaftor/Ivacaftor and 17 changed from Tezacaftor/Ivacaftor to Lumacaftor/Ivacaftor.

### 3.3. Respiratory status

Considering the whole population, ppFEV 1 was not significantly different among the genotypes. However, the 6 to 11 year-old PTC/PTC children had a significantly lower median ppFEV 1 (92.1%) versus (vs) F508del + / + (97.4%) and F508del/PTC children (99%) (Supplemental Table E4). A similar profile was observed in the 12 to 17 year-old adolescents (median ppFEV 1 78.8% in the PTC/PTC versus 86.7% in the F508del/PTC and 88.7% in the F508del + / + adolescents, all comparison significant) (Supplemental Table E5). In adult pwCF, no difference was found between the 3 mutation groups (Supplemental Table E6).

The evolution of ppFEV 1 over age was then modeled using a mixed effect linear model. The results showed a time-dependent decline of ppFEV 1 with a statistically significant difference in ppFEV 1 values between the 3 mutation groups (  $p < 0.001$  ) ( Fig. 3 A). The same results were obtained for the ppFEV 1 yearly decline (  $p$ -value  $< 0.001$  ) ( Fig. S2-A). As compared to F508del + / + pwCF, PTC/PTC and F508del/PTC pwCF exhibited a significantly faster rate of decline from 7 years (  $-1.33$  for F508del + / + ,  $-1.59$  for F508del/PTC,  $-1.65$  for PTC/PTC,  $p < 0.001$  ) until respectively 30 years (  $-1.05$  for F508del + / + ,  $-1.23$  for PTC/PTC,  $p = 0.048$  ) and 27 years (  $-1.12$  for F508del + / + ,  $-1.26$  for F508del/PTC,  $p = 0.034$  ). This resulted in sustained significantly lower ppFEV 1 in comparison to F508del homozygotes, a difference which became

significant from the age of 17 years for PTC/PTC (74.9% vs. 77% in F508del + / + at this age  $p = 0.036$ ), and from the age of 21 years for F508del/PTC pwCF (70.7% vs. 71.9% in F508del + / + at this age,  $p = 0.022$ ) ( Fig. 3 -A and B). Although this never reached the significant level, the PTC/PTC pwCF tended to have a more rapid ppFEV<sub>1</sub> yearly decline than the F508del/PTC pwCF. They had a significantly lower ppFEV<sub>1</sub> than F508del/PTC from 21 years (68.6% vs. 70.7% in F508del/PTC,  $p = 0.043$ ). Apart from genotype (carrying at least 1 PTC vs. F508del homozygous state), the factors independently associated with ppFEV<sub>1</sub> decline in the multiple regression analysis were age, gender (female), middle income country, underweight, diabetes and chronic colonization with *B.cepacia* complex and *P.aeruginosa* (Supplemental Table E7). As a matter of fact, annual decline of ppFEV<sub>1</sub> was faster in pwCF infected with *P.aeruginosa* until early adulthood (Supplemental Fig. E2B and C). These data are summarized in Supplemental Table E8.

### 3.4. Lung infection

*P. aeruginosa* chronic infection was more prevalent in PTC/PTC pwCF than in F508del + / + and F508del/PTC pwCF ( $p = 0.030$  for both comparisons, Table 1 ). A significant difference between the 3 genotype group was observed for children less than 12 years of age ( $p = 0.019$ ) and adolescents ( $p = 0.001$ ) (Supplemental Tables E4 and E5, Fig. 4 ). Further analysis showed an increased prevalence of

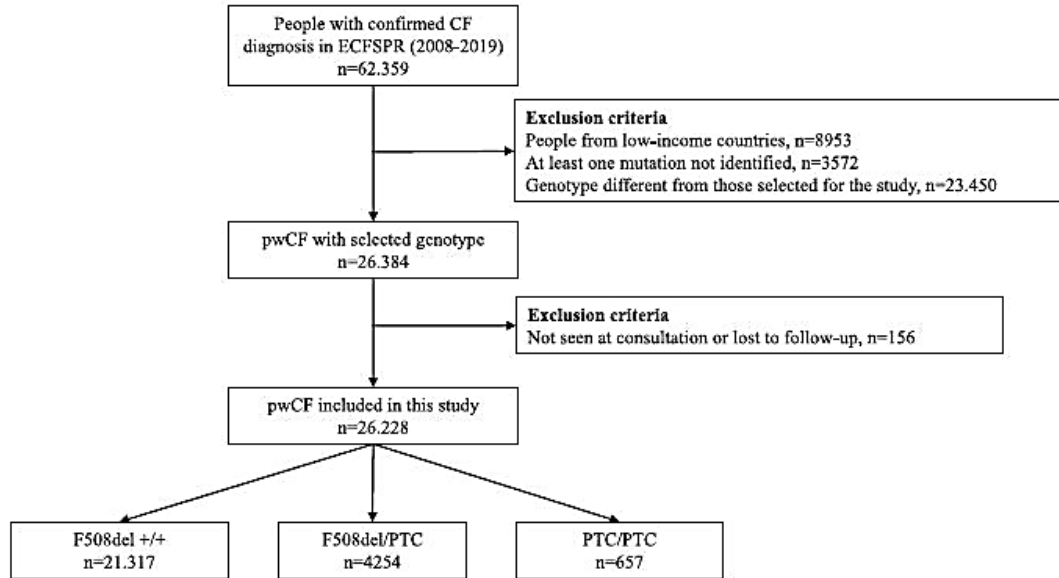
*P.aeruginosa* chronic colonization in both PTC/PTC and F508del/PTC adolescents as compared to F508del homozygotes ( $p = 0.024$  for both comparisons). *P.aeruginosa* chronic colonization rate remained significantly higher in PTC/PTC versus F508del homozygotes after accounting for all potential confounding variables in a multiple logistic regression analysis (Odds Ratio = 1.32,  $p$ -value = 0.011) (Supplemental Table E9). *P.aeruginosa* colonization was also independently associated with age, gender (female), country group (middle vs. high), ppFEV<sub>1</sub> < 70%, underweight status, insulin dependent diabetes and chronic infection with *B.cepacia* complex . Colonization was slightly higher in patients from middle income countries, independently of the genotype and other variables of severity (Odds Ratio = 1.15,  $p$ -value < 0.001).

*Staphylococcus aureus* infection was significantly more frequent in pwCF with one or two PTC alleles than in pwCF with the F508del + / + genotype (Table 1 ), particularly amongst children younger than 12 years (46% of the PTC/PTC subjects vs. 35.8% of the F508del/PTC and 31.2% of the F508del homozygotes, all comparison significant) (Supplemental Table E4).

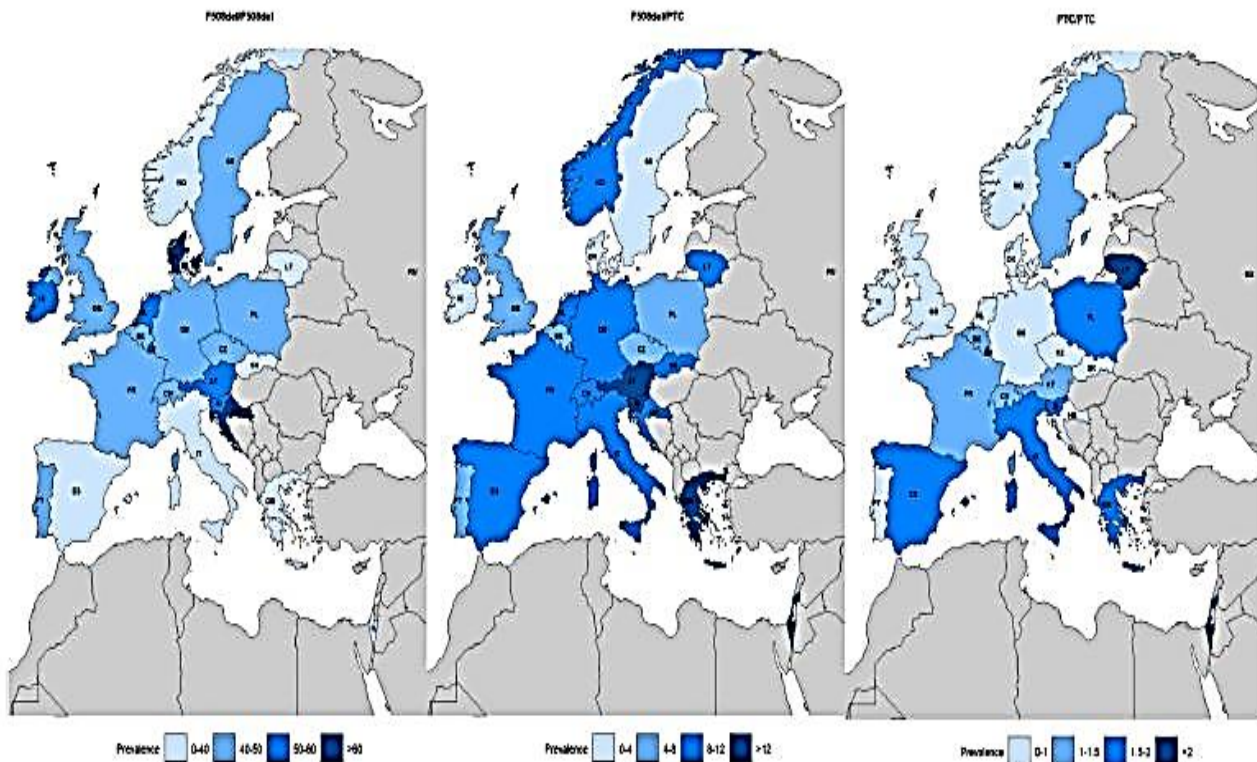
### 3.5. Survival analysis

1894 deaths were reported for F508del homozygotes (166 children) and 348 among F508del/PTC pwCF (50 children). Fifty-four deaths were reported in ECFSPR in PTC/PTC pwCF, 10 occurred before 18 years of age. This included 3 deaths before 5 years of age, among those 2 because of meconium ileus, the other one was unknown. Five other pwCF died of respiratory complication, and 1 after lung transplant.





**Fig. 1.** Flowchart of pwCF from the European CF Patient Registry included in the study  
CF, Cystic Fibrosis; ECFSPR, European CF Patient Registry; PTC, premature termination codon.

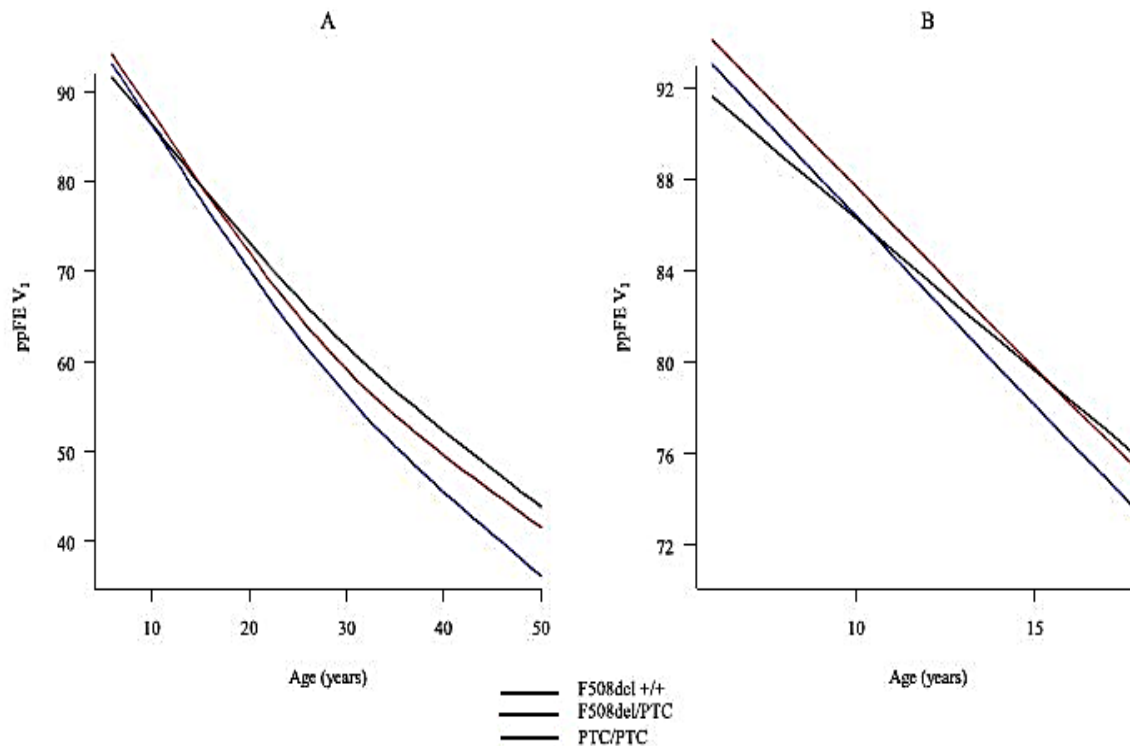


**Fig. 2.** Geographic distribution of the pwCF from the European CF Patient Registry according to mutation group in middle and high income European countries. The abbreviations of the countries refer to Table E1. Percentages represent the number of pwCF in each mutation group per country out of the total number of pwCF registered in the ECFSPR for each country.

The frequency of F508del/++ was over 40% of the number of pwCF in most countries, with the highest frequencies in Croatia, and Denmark (67.9% and 67.9%, respectively). The highest frequencies of F508del/PTC were found in Slovenia (14.9%), Israel (14.1%), Austria (12.9%) and Greece (12.8%). The PTC/PTC genotype was found in less than 2% of pwCF in most countries, except in Israel, (15.6%), Luxembourg (4.9%), Lithuania (2.9%), and Italy (2%).

The most common PTC alleles were G542X with high frequencies in Greece, Israel, Italy, Luxembourg, Spain (4.7%, 6.4%, 4.8%, 4.9%, 6.2% respectively), W1282X in Israel (24.2%), R553X in Lithuania (7.35%), and R1162X in Slovenia (4.6%). The Y122X allele was mainly found in the Reunion Island (French overseas territory).

CF, Cystic Fibrosis; ECFSPR, European CF Patient Registry; PTC, premature termination codon.

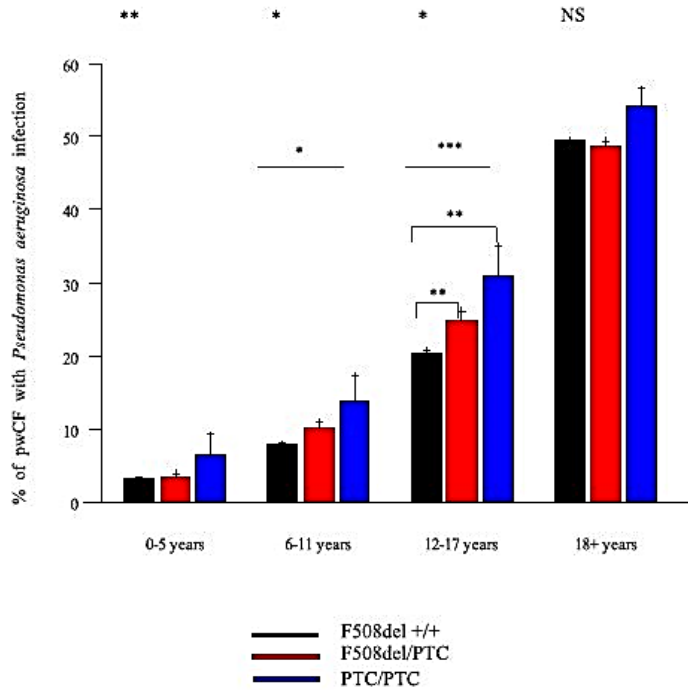


**Fig. 3.** Percentage of predicted FEV<sub>1</sub> according to age and mutation groups, overall (A) and zoom on children between 6 and 18 years (B) in middle and high income European countries.

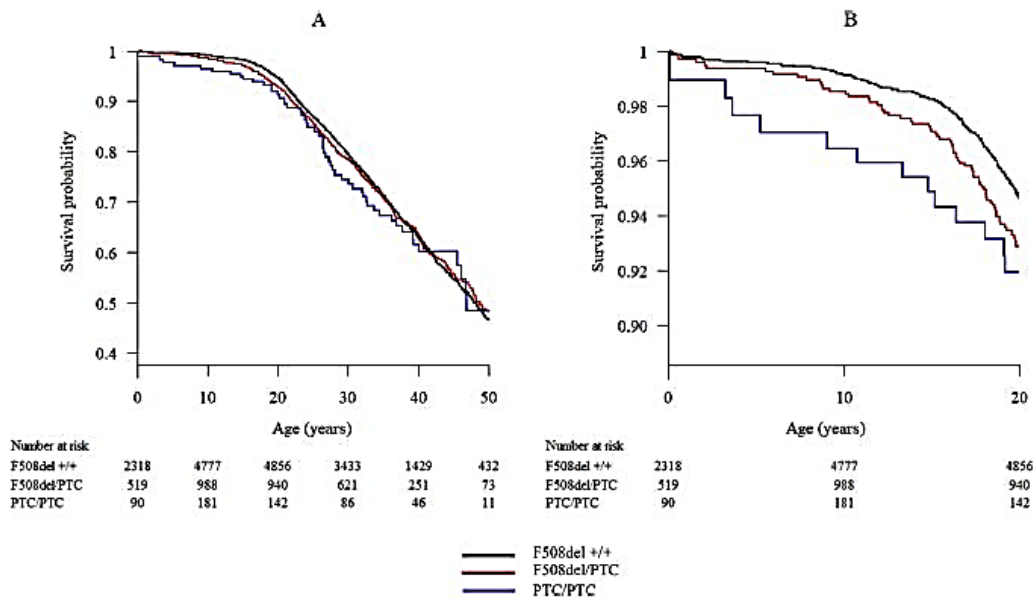
The ppFEV<sub>1</sub> was significantly different between the three mutation groups ( $p < 0.001$ , ANOVA of the mixed effects model).

Age threshold for significance: PTC/PTC vs. F508del+/+: 17 years ( $p = 0.036$ ); PTC/PTC vs. F508del/PTC: 21 years ( $p = 0.043$ ); F508del/PTC vs. F508del homozygotes: 21 years ( $p = 0.022$ ). ppFEV<sub>1</sub>, percentage of predicted forced expiratory volume in 1 s; PTC, premature termination codon.

PTC/PTC pwCF tended to have a lower survival probability than F508del + / + and F508del/PTC pwCF resulting in a median survival age of 48.9 for F508del/PTC, 47.9 years for F508del + / + pwCF, and 46.9 for PTC/PTC subjects ( Fig. 5 -A); however, between-group differences were only significant for pwCF younger than 18 years ( Fig. 5 -B). To adjust for possible confounding factors, a multiple Cox regression model was fitted to estimate the effect of various cofactors on survival probability. The multiple Cox regression model was fitted on 22,508 pwCF, after excluding observation with at least one missing value, being the majority of exclusions due to ppFEV<sub>1</sub> not computed before 6 years of age. The final results are reported in Table 2 . Importantly, it outlined that pwCF younger than 18 years carrying at least 1 PTC mutation had a significantly higher mortality risk than those with the F508del + / + genotype (F508del/PTC risk increase (HR) by 2.35[1.47–3.74],  $p < 0.001$ ) and PTC/PTC risk increase by 2.55[1.00–6.51];  $p = 0.05$ ). Indeed, in the PTC/PTC group there was a higher percentage of pwCF less than 20 years (51.2%, versus 44.9% in the F508del + / + ) and a lower percentage of pwCF having more than 20–30 years (48.7%, versus 55% in the F508del + / + ), meaning that part of the PTC/PTC pwCF died before becoming adults. Even in pwCF older than 18 years the difference was nearly significant for PTC/PTC in comparison to F508del + / + pwCF (HR = 1.25[0.98–1.60],  $p = 0.073$ ). This analysis did not identify country income (middle versus high) as a potential risk factor of death but confirmed that gender (female), ppFEV<sub>1</sub> < 70%, underweight, insulin-treated diabetes and infection with *P.aeruginosa* or *B.cepacia* also impacted independently survival probability.



**Fig. 4.** *Pseudomonas aeruginosa* infection in pwCF in middle and high income European countries. Prevalence of chronic *Pseudomonas aeruginosa* sputum infection according to age group and mutation group (mean, SEM). Comparisons between the mutation groups were analyzed using the Fisher exact test: 0 to 5 years,  $p = 0.304$ ; 6 to 11 years,  $p = 0.031$ ; 12 to 17 years,  $p = 0.001$ ; and  $\geq 18$  years,  $p = 0.179$ . \* $p$  value = 0.031, \*\* $p$  value = 0.024, \*\*\* $p$  value = 0.001; PTC, premature termination codon.



**Fig. 5.** Kaplan Meier survival curves according to mutation group in the whole population (A), and in pwCF < 18 years (B) in middle and high income European countries. A. PTC/PTC pwCF tended to have a lower survival probability than F508del+/+ pwCF and F508del/PTC pwCF in childhood, while no significant differences in survival were observed between genotypes in adult pwCF. B. As compared to F508del+/+, PTC/PTC pwCF and F508del/PTC pwCF under 18 years old had a significantly lower probability of survival ( $p = 0.05$  and  $p = 0.02$  respectively; log-rank unadjusted test). PTC, premature termination codon.

**Table 2**

Multivariable Cox regression model analysis of the impact of cofactors on the survival probability of the pwCF from the European Cystic Fibrosis Society Patient Registry in middle and high income European and neighboring countries.

Variable	HR (95% CI)	p value
Age group (<18 y vs ≥18 y)	3.81 (1.67 - 8.66)	0.001
Mutation group PwCF <18 y		
F508del/PTC vs F508del+/+	2.35 (1.47 - 3.74)	<0.001
PTC/PTC vs F508del+/+	2.55 (1.00 - 6.51)	0.050
PTC/PTC vs F508del/PTC	1.09 (0.46 - 2.58)	0.850
PwCF ≥18 y		
F508del/PTC vs F508del+/+	0.98 (0.80 - 1.20)	0.861
PTC/PTC vs F508del+/+	1.25 (0.98 - 1.60)	0.073
P/PTC vs F508del/PTC	1.28 (0.93 - 1.75)	0.131
Gender (female vs male)	1.37 (1.19 - 1.57)	<0.001
Country income group (high vs middle)	0.87 (0.69 - 1.10)	0.235
ppFEV <sub>1</sub>		
30–70% vs ≥70%	4.48 (3.38 - 5.94)	<0.001
<30% vs 30–70%	5.84 (4.99 - 6.82)	<0.001
BMI (underweight vs normal weight)	2.19 (1.85 - 2.58)	<0.001
Chronic <i>Pseudomonas aeruginosa</i> (yes vs no)	1.11 (0.88 - 1.40)	0.368
Chronic <i>Burkholderiacepacia</i> complex species (yes vs no)	2.54 (2.05 - 3.14)	<0.001
Insulin-treated diabetes (yes vs no)	2.22 (1.99 - 2.49)	<0.001

Abbreviations: pwCF, People with Cystic Fibrosis; HR, hazard ratio; CI, confidence interval; vs, versus; y, years; PTC, premature termination codon; ppFEV<sub>1</sub>, percentage of predicted forced expiratory volume in 1 s; BMI, Body mass index.

### 3.6. Functional analysis of PTC mutants and relationship with respiratory status

To get more insight in the impact of specific nonsense mutations on the phenotype, we investigated whether the PTC/PTC patients carrying at least 1 mutation after position 1162 were less severe, assuming that a truncated protein retaining partial NBD2 may keep some functional CFTR activity. PTC/PTC pwCF with a nonsense mutation after 1162 were significantly older and had a trend to a better ppFEV<sub>1</sub> and more frequent infection with *P.aeruginosa*, but this did not reach the significance level (Supplemental Table E10). The 4 PTC/PTC pwCF carrying mutations located in the last exon (S1455X, *n* = 3 and Q1476X, *n* = 1), had a normal respiratory status with a median ppFEV<sub>1</sub> of 112% (108–118.5).

Second, we investigated CFTR dependent Cl<sup>-</sup> transport in HNE carrying a subset of 13 nonsense mutations frequent amongst PTC/PTCpwC. For reported to positively impact on mRNA abundance in comparison with F508del(7) (Supplemental Fig. E3). Most PTC mutations were associated with reduced mRNA abundance of less than 10% of the WT level, with an activity lower than 1.5% of WT-CFTR as compared to a mean of 2.5 ± 0.6% obtained for HNE cells from F508del + / + pwCF (*n* = 10) (Supplemental Fig. E3). The E585X/R1066C and Y275X/S466X genotype had the highest Cl<sup>-</sup> channel activities (Supplemental Fig. E3-B).

### 3.7. Analysis restricted to the high income countries

To avoid a potential bias due to social economic status on analysis, we performed an exploratory analysis restricted to pwCF from high income countries (260 PTC/PTC pwCF, 2162 F508del/PTC pwCF and 11,519 F508del + / + ) (Supplemental Figs. E4-E6, Supplemental Tables E11-E14). This



analysis, even in a lower number of pwCF confirmed the data obtained in the middle/high income country population. Additional description is provided in **Online data Supplement** .

### **3.8. Analysis restricted to the patients not treated by CFTR modulators**

Analysis of the data excluding pwCF taking CFTR modulators confirmed data obtained in the whole population (for detailed analysis see Online data Supplement and Supplemental Table E15 to E18).

## **4. Discussion**

This study demonstrates the intrinsic deleterious effect starting from childhood of the carriage of 1 PTC in pwCF. This is supported by a significantly faster annual respiratory decline in PTC/PTC and PTC/F508del children and adolescents when compared to F508del + / + , resulting in a lower ppFEV<sub>1</sub> in adults. Furthermore, *P.aeruginosa* infection is significantly more frequent in patients carrying 2PTCs vs F508del/PTC and F508del + / + . Most importantly, PTC/PTC and F508del/PTC children and adolescents have a higher rate of mortality than F508del homozygotes. Those differences remained when the analysis was restricted to the high income countries.

While most studies that characterized disease severity of PTC mutation reported a younger age, worse lung function and a decreased survival probability [ 5 , 16–19 ], 2 studies showed a similar clinical severity between G542X or W1282X homozygotes and F508del/G542X or W1282X heterozygotes [ 10 , 19 ]. The study of Koch was the only one to observe earlier *P. aeruginosa* primary infections and more severe pulmonary involvement [20] .

Our study has the inherent limitations of a retrospective registry study from different countries. The impact of differences between country such as delay in diagnosis or genotyping, neonatal screening implementation, heterogeneity in treatment practices cannot be excluded in interpretation of childhood survival and ppFEV<sub>1</sub> rates of change. This is counterbalanced by the very large number of pwCF carrying PTC mutations, the high quality of data collection, the rigorous analytic adjustment strategy, and the fact that we only considered high and middle income countries with homogeneous access to diagnosis, genetic screening and to treatment. All variables have less than 10% of missing data, with the exception of Neonatal Screening, Sweat chloride value, Chronic Staphylococcus aureus, Nontuberculous mycobacteria, ppFEV<sub>1</sub> (but for this last variable this is due to the fact that it is not performed in children < 6 years). This is the largest study until now comparing the disease severity of pwCF carrying 1 or 2PTC to F508del homozygotes.

Our results provide evidence that lung disease severity starts early in children with at least 1 PTC, based on a more rapid yearly decline of ppFEV<sub>1</sub> from 7 years of age, and very probably early on, but reliable measurements are lacking to support this assumption. Furthermore, children carrying at least 1 PTC had a higher rate of chronic infection with *P.aeruginosa* and most importantly a higher mortality than their F508del homozygous pairs.

We confirm that older age, sex, underweight, ppFEV<sub>1</sub> < 70%, chronic lung infection and CF related diabetes account independently for a worse survival [21] , an accelerated lung decline [22] , and chronic *P.aeruginosa* colonization [ 23 , 24 ]. Nevertheless, after accounting for all these confounding

variables, genotype remained significantly associated with *P.aeruginosa* colonization, accelerated ppFEV1 decline in the whole sample, and worse survival in the pwCF below 18 years of age.

The deleterious impact of the genotype on survival remained when the analysis was restricted to the high income population, as well as for *P.aeruginosa* infection and the respiratory decline, although it was not significant anymore. This result, probably due to a lack of power, also highlights that geoeconomic disparities amplify the effect of the genotype, as a result of differing access to care services and key treatment [ 25 , 26 ].

To get further insight into phenotype-genotype correlation, assuming that PTC read through might be different according to the genetic context, we specifically focused on incomplete NBD2 CFTR polypeptides at, or downstream of codon 1162, which have been shown in heterologous expression systems to traffic to the plasma membrane and be partially functional [ 7 , 27-30 ]. Our results do not support this hypothesis. PwCF listed in ECFSPR carrying at least one mutation at or downstream of 1162 and before the last exon (e.g.; located between codons 1162 and 1415) did not present with a less severe phenotype. This was associated to a very low CFTR activity and transcript level in HNE. Interestingly pwCF carrying mutations in the last exon had normal ppFEV 1 and were pancreatic sufficient, probably due to the absence of NMD and the residual activity of the terminally truncated protein [ 6 , 9 ]. Interestingly, primary cells expressing Y275X/S466X and E585X/R1066C exhibited a level of transcript above 30% and a CFTR activity above 3% of the WT, reflecting low residual levels of PTC read-through. As R1066C-CFTR is a severely misfolded protein [31] , it can be anticipated that the residual CFTR activity stems from residual E585X read-through. Remarkably in ECFSPR, PTC/PTC pwCF carrying S466X ( *n* = 13), Y275X ( *n* = 3), E585X ( *n* = 18) on at least 1 allele had all a ppFEV 1 above 85%, apart from 3 patients carrying the S466X-R1070Q complex allele associated to a severe phenotype, as previously reported [32] . This suggests that a basal level of read-through that generates residual activity as low as 3% is sufficient to mitigate CF respiratory disease. Because they carry a residual amount of CFTR due to basal read through, those variants may be functionally misclassified and might benefit from CFTR modulators. Therefore, the possibility of a positive response to modulators has to be considered for a limited number of PTC variants.

This observation is important because it was recently shown that pharmacologically-induced small increases in CFTR function (e.g.; 2 to 3%) are likely to be clinically relevant [ 33 , 34 ]. This includes combination of small molecules that suppress PTCs and inhibit NMD, in combination with highly effective (HEMT) CFTR modulators as well as other strategies beyond read-through, such as cell based therapies, gene or mRNA editing [ 1 , 35–37 ].

Altogether, our findings support implementation of a proactive and aggressive treatment from birth in patients carrying a non-sense mutation. The clinical impact of these variants make it urgent to focus research on alternative treatments to modulators. Nevertheless the possibility of a positive response to CFTR modulators needs to be investigated for specific PTC variants. It also points to early initiation of HEMT CFTR modulators in PTC/F508del children, aiming to slow down the progression of the disease. It is important to emphasize that the current study included mainly pwCF not yet treated with CFTR modulators. It is likely that the gap in disease severity will increase in the next future since patients carrying F508del are now treated with Trikafta® whereas PTC/PTC patients have no targeted therapies yet.

## 5. Conclusion

Our study unambiguously show that the carriage of 1 or two PTC mutations accelerates CF lung disease from childhood. These data represent a public health challenge for the CF community and highlight the necessity to design early targeted interventions for these patients.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Author's contribution

ISG: designed the study, coordinated acquisition, analysis, and interpretation of data for the work, drafted the manuscript IP: performed the experiments on the primary cells, analyzed, and interpreted data for the work, participated to manuscript drafting and approved final version AO: performed the statistical analysis, participated to manuscript drafting and approved final version JV, MTC, EG, AHi: performed transcript analysis on the primary cells, analyzed, interpreted data for the work, participated to manuscript drafting, and approved final version CF, AHa, AG, AL, ED, DT, BC: collected data, participated to primary cell experiments, revised the manuscript, and approved final version CD, LL: participated to the statistical analysis, participated to manuscript drafting and approved final version ID, PR, PRB, DG, CT, EG, CP, LW, SK, ML, RC, EK, CM: performed nasal brushing, participated to data analysis, participated to manuscript drafting and approved final version BK, AE: participated to manuscript drafting, and approved final version AJ, LN: participated to study design, data interpretation, manuscript drafting and approved final version Funded by the Cystic Fibrosis Foundation (grant number: SERMET1710 ) and Mucoviscidose ABCF2 (Post doctoral fellow ship for I Pranke).

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