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List of Abbreviations

CF: Cystic Fibrosis

CFTR: Cystic Fibrosis Transmembrane conductance Regulator

PI: Pancreatic Insufficiency

PS: Pancreatic Sufficiency

CFLD**:** Cystic Fibrosis Related Liver Disease

$FEV_{1:}$ Forced Expiratory Volume in one second

BMI: Body Mass Index

UDCA: Ursodeoxycholic Acid

US: Ultrasonography

CT: Computed Tomography

MRI: Magnetic Resonance Imaging

WBC: White Blood Cells

SD: Standard Deviation

CI: 95% confidence interval

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Abstract

Background: Cystic fibrosis related liver disease (CFLD) is a common symptom in patients with cystic fibrosis (CF). However, its prevalence, risk factors, and evolution are unclear. We used a large database of patients with CF to investigate the incidence of CFLD and related risk factors.

Methods: We retrospectively analyzed 3,328 CF patients with pancreatic insufficiency born after 1985 and recruited into the French CF Modifier Gene Study since 2004. We determined liver status, age at CFLD and severe CFLD onset, sex, *CFTR* genotype, history of meconium ileus, treatment with ursodeoxycholic acid (UDCA), and respiratory and nutritional status.

Results: The incidence of CFLD increased by approximately 1% every year, reaching 32.2% at age 25. The incidence of severe CFLD increased only after the age of 5, reaching 10% by age 30. Risk factors for CFLD and severe CFLD were male sex, *CFTR* F508del homozygosity, and history of meconium ileus. Increasingly precocious initiation of UDCA treatment did not change the in CF patients. Finally, patients with severe CFLD had worse lung function and nutritional status than other CF patients.

Conclusion: CFLD not only occurs during childhood as often as believed, but also occurs later in the lifetime of patients with CF. Male sex, *CFTR* F508del homozygosity, and history of meconium ileus are independent risk factors for CFLD development. Earlier use of UDCA over the last 20 years has not changed the incidence of severe CFLD, a fact that should be considered when prescribing UDCA in the young children, along with its possible adverse effects.

Cystic fibrosis (CF) is the most common, severe, autosomal recessive genetic disease in Caucasians. It is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel expressed in epithelial cells throughout the body (1). The disease affects several organs such as the lungs, pancreas, intestine, and liver. More than 2000 mutations in the *CFTR* gene have been described, the most frequent being F508del. The *CFTR* genotype strongly influences pancreatic function, which is either deficient (PI for pancreatic insufficiency), or normal (PS for pancreatic sufficiency). It is recognized that patients carrying two severe *CFTR* mutations, also called "CF-causing mutations", have a classical form of CF associated with PI, whereas others have a milder form of disease associated with PS (2).

Cystic fibrosis-related liver disease (CFLD) includes a wide range of hepatobiliary abnormalities. Focal biliary cirrhosis is the most clinically relevant CFLD, since extension of the initially focal fibrogenic process may lead to multilobular biliary cirrhosis with subsequent portal hypertension and related complications (3). Indeed, multilobular cirrhosis is recognized to have a significant impact on morbidity, and accounts for ~2.5% of mortality in patients with CF (third cause after respiratory failure and transplantation-related complications) (4). The prevalence of CFLD remains controversial, with estimates ranging from 2 to 68% in young patients with CF, due to the lack of a consistent definition (4, 5). Identification of patients with CF who are at risk of developing cirrhosis and regular screening for early detection of liver involvement is important as therapeutic intervention is likely to be more effective in patients with early liver disease (6). However, evidence of CFLD is usually subclinical until the disease is advanced, and, therefore, often under-diagnosed. Some risk factors have been recognized as being associated with CFLD, such as severe *CFTR* genotypes and PI, both of which are dependent upon one another. However, others are still controversial such as male sex and meconium ileus, a severe neonatal intestinal obstruction occurring in ~15% of patients with CF (4).

In this study, we aimed to investigate the evolution of liver disease and its related risks factors in a large cohort of patients with CF, using longitudinal clinical data collected in the French CF Gene Modifier Study. CFLD was homogeneously defined in the entire cohort, in accordance with the European best practice guidance (3).

Methods

Since 2001, all patients with CF in France are evaluated, at least once a year, by one of the 45 French hospital-based CF centers according to the national CF care recommendations (https://www.has-sante.fr/portail/jcms/c_2792719/fr/mucoviscidose) (7). Neonatal CF screening was generalized in France in 2002.

Patients

Patients with CF treated in 38 participating CF centers between January 2004 and January 2017 were enrolled in the French CF Modifier Gene Study. As of January 1, 2017, 4,798 patients with CF had been included (corresponding to ~80% of all French patients with CF (8)). Longitudinal data were obtained from electronic medical records or abstracted from the patients' paper records, retrospectively before 2004, and prospectively after January 2004. We analyzed patients born after 1985 with PI (n=3,328) since patients with PS are known to have milder disease (2) (*see* **Supplementary Figure S1** for the flowchart, and **Supplementary Information** for pancreatic status definition). Patients born before 1985 were excluded to limit selection biases due to an over-representation of patients with milder disease in those surviving longer (*see* **Supplementary Information**). The following patient characteristics were analyzed: sex, *CFTR* genotype (described as homozygous for the *CFTR* F508del mutation, heterozygous for this mutation, or others), and history of meconium ileus (**Table 1**). Information on liver status, measurements of the forced expiratory volume in one second (FEV_1), and body mass index (BMI) were collected at each visit. When no history on CFLD was available in the patient's record, he/she was considered not to be affected. Age at onset of liver disease and severe liver disease were determined (*see* definition below). Age at ursodeoxycholic acid (UDCA) treatment initiation was also recorded.

CFLD definition

CFLD was defined according to the European best practice guidance by Debray *et al.* (3) when at least two of the following characteristics were present: 1) abnormal physical examination: hepatomegaly and/or splenomegaly; 2) abnormalities of liver function tests defined as an increase of transaminase (alanine aminotransferase and/or aspartate aminotransferase) and/or gamma-glutamyl transpeptidase levels above the upper normal limits (*see* **Supplementary information**); 3) ultrasonographic (US) evidence of liver involvement (heterogeneous echogenicity, irregular margins, or nodularity), portal hypertension (splenomegaly, increased thickness of the lesser omentum, spontaneous splenorenal anastomosis, large collateral veins, or ascites), or biliary abnormalities (bile duct dilatation). Patients with cirrhosis, diagnosed by US, computed tomography (CT) and/or magnetic resonance imaging (MRI), and/or portal hypertension (splenomegaly, hypersplenism (platelets <150,000 10^9/L and white blood cells (WBC) <3000 10^9/L), and/or spontaneous portosystemic shunts on US), and/or esophageal varices were classified as having "severe CFLD" (9).

Statistical analyses

Descriptive statistics used mean ± standard deviation (SD), percentages, and hazard ratios (HR) with 95% confidence intervals (CI), as appropriate. The cumulative distribution of CFLD with age was computed using the non-parametric maximum likelihood estimator approach for interval censored data (10). All patients were considered to be at risk of CFLD since birth. Patients who did not have a CFLD diagnosis before January 2017 were censored at the last visit. In the other patients, the date of diagnosis was computed as the first date when the diagnostic criteria were met based on medical record data. When this date was not precisely known, we considered that it was "interval censored" between birth and the age of the first report (*i.e.,* CFLD onset could occur at any time in this range – this was the case for 142 out of 605 patients with CFLD [24%]). We defined onset of severe CFLD as the first-time cirrhosis and/or portal hypertension and/or esophageal varices were reported, and applied the same approach regarding uncertainty to this date (the date of severe CFLD

was interval censored in 19 out of 175 patients [11%]). Comparisons between the cumulative incidences curves were done using the log-rank test adapted to interval censored data (10). Factors linked to age at CFLD and the time interval between CFLD and severe CFLD were tested using a Cox-regression model adapted to interval censored data (11). Confidence intervals were computed by the bootstrap method. We also performed a sensitivity analysis including only patients prospectively enrolled since 2004 (*see* **Supplementary Information**).

Age at treatment with UDCA was analyzed as above, with patients not under treatment censored at the age of their last visit. We also tested the impact of treatment on the occurrence of severe CFLD. We used instrumental variable analysis to estimate the true causal association between treatment and disease (12). Indeed, protopathic bias may affect this analysis if UDCA is preferentially prescribed in those who are more likely to develop the disease (13), leading to reverse causality with a higher incidence of CFLD in patients under treatment. A statistical instrument must satisfy: 1) association with UDCA treatment, 2) changing the risk of severe CFLD only through UDCA prescription (i.e. no direct link between instrument and outcome), and 3) not sharing a common cause with treatment (*see* **Supplementary Information**). For each analysis, the patients were divided in two groups (those born before and after 1995, in early/late prescribing centers) (see **Supplementary Information**). The effect of UDCA on severe CFLD incidence was computed as the restricted (at age 20) mean difference in age at severe CFLD onset, using pseudo-observations for survival and the generalized method of moments (12).

We computed percent-predicted FEV_1 (14) and BMI Z-scores (WHO2007) with respect to those of healthy populations, and CF-specific percentiles and Z-scores for BMI and FEV_1 using previously published methodology (15, 16). BMI and FEV_1 Z-scores were averaged from measurements taken in the last 3 years for FEV_1 and the last 2 years for BMI, and compared according to CFLD stage (no CFLD, CFLD, and severe CFLD) using the Kruskal-Wallis test.

Ethics

The study was approved by the French ethical committee (CPP n°2004/15), and the information collection was approved by the Commission Nationale de L'informatique et des Libertés (n°04.404). Informed consent in writing was obtained from each patient and/or guardian.

Results

CFLD cumulative incidence and risk factors

At the time of the study, 18% of the patients had CFLD and 5% had severe CFLD. The incidence of CFLD increased by approximately 1% every year from birth, reaching 32.2% (CI95%: 29.7, 35.2) by age 25 and leveling out thereafter (**Table 2, Figures 1A & S4**). The most frequent factors defining CFLD were the joint presence of clinical and biochemical abnormalities (50% of the cases), clinical and ultrasonographic (US) abnormalities (26%) and biochemical and US abnormalities (24%). The cumulative incidences of individual items defining CFLD (clinical, biochemical, and US abnormalities) are reported in the **Supplementary information** (**Table S2**).

The incidence of CFLD was higher in male patients [HR=1.15; 95% CI=0.99-1.36], in *CFTR* F508del homozygous patients (HR=1.17; 95% CI=1.00-1.37] and in those with a past history of meconium ileus (HR=1.66; 95% CI=1.36-2.01) (**Table 3 & Supplementary Figure S5**). The CFLD cumulative incidence at 20 years was 32% in patients with meconium ileus *vs.* 21% in others, 24% in males *vs.* 21% in females, and 25% in *CFTR* F508del homozygous *vs.* 20% in other *CFTR* genotypes. A multivariable analysis showed that these factors were independently associated with time to CFLD occurrence. Male CFTR F508del homozygous patients with meconium ileus (~4% of the population) were the most at risk of developing CFLD with a 33% cumulative incidence at age 20, while the least at risk were non-*CFTR* F508del homozygous females without meconium ileus (~21% of the population) who reached 17% cumulative incidence at the same age **(Supplementary Figure S6**). The sensitivity analysis of patients prospectively enrolled since 2004 showed similar results (*see* **Supplementary Information, Figure S8 and Table S6**).

Severe CFLD and risk factors

Severe CFLD complications were rare up to the age of 5 (**Table 2**). Overall, the incidence of severe CFLD increased by approximately 0.4% per year after the age of 5, reaching 10.2% (95% CI:

8.5-12.9%) by age **25** **(Table 2, Figures 1A & S4**). Severe CFLD was diagnosed by cirrhosis (56%), portal hypertension (41%) or esophageal varices (3%). Risk factors for severe CFLD were similar to those for CFLD (**Table 3**). At age 20, the incidence of severe CFLD was higher in males (8.5% *vs.* 5.4%), in patients homozygous for the *CFTR* F508del mutation (8% *vs.* 6%), and in those affected by meconium ileus (8% *vs.* 7%) **(Supplementary Figure S5**).

Treatment with UDCA

Treatment with UDCA increased with age (**Figure 1A**). At the age of 30, 38% of all patients were under treatment. At the time of the study, 64% of patients with known CFLD were receiving UDCA treatment. Treatment with UDCA had been initiated in the absence of CFLD in 83% of those treated. UDCA prescriptions changed with time and according to CF center. Patients born after 1995 received UDCA earlier than those born before (**Figure 1B**): at 10 years of age, 7% of patients born between 1986 and 1995 were treated with UDCA compared with 23% of patients born after 1995. CF centers could also be split by early or late UDCA prescribers (**Figure 1C**). Yet, irrespective of the split, the cumulative incidence of severe CFLD remained the same between groups (**Figure 1B & 1C**). The difference in restricted mean age at severe CFLD onset, according to UDCA treatment, was 2 ± 3.5 years (P=0.56 for comparison to 0) with birth cohort as the instrument, 0 ± 1.0 (P=0.98) with CF center as the instrument, and 0 ± 0.8 (P=0.95) with both (*see* **Supplementary Information** for a detailed description of this analysis).

BMI and FEV_1 change according to CFLD and severe CFLD

At the time of the study, BMI and FEV_1 measurements were available for most patients (**Table 4**). As expected, patients had impaired lung function, with percent predicted FEV_1 ($ppFEV_1$) of 75% of the reference population in the non-CFLD group, 71% in those with CFLD, and 63% in those with severe CFLD (P<0.001). With reference to CF patients, those without CFLD had slightly better lung

function (CF-specific FEV_1 percentile 53%) and those with severe CFLD slightly worse (CF-specific FEV_1 percentile 47%) (P=0.04).

BMI measurements showed the same trend, with CF patients having an altered nutritional status compared with the normal population, and increasingly worse performance in those affected with CFLD and severe CFLD (P<0.001 and P=0.01 for BMI Z-score and CF-specific BMI percentile respectively). Similar results were obtained in a subset of patients exactly matched for age and sex (*see* **Supplementary Information**).

Discussion

The French CF Modifier Gene Study provided an unprecedentedly large database of 3,328 PI patients with CF born after 1985, enabling us to study the incidence of CFLD and severe CFLD. We observed that CFLD increased with age up to 32% by age 30, and increased with independent risk factors such as male sex, *CFTR* F508del homozygosity, and a history of meconium ileus at birth. We showed that severe CFLD was rare before age 5, increased to 10.2% by age 30 with risk factors similar to CFLD, and was not modified by UDCA treatment. Interestingly, we also found that liver disease was associated with worse lung function and nutritional status.

While CFLD is often believed to develop during childhood, we observed that the incidence rates continued to increase in young adults. CFLD incidence increased between 1 and 2% every year from birth up to the age of 25, reaching 32.2% at this age, at which it a plateaued, which may be because of fewer participants in our study. So far, few studies have reported on CFLD incidence after infancy. In a cohort of 241 young patients with CF followed up for a mean duration of 9.8 years (5 months to 20.6 years), CFLD was mostly diagnosed before 12 years of age but not thereafter (17). In a cohort of 177 patients, Colombo *et al.* observed a higher incidence of CFLD than that in the present study (1.8% per year overall), but a plateau was reached at 20 years for male patients and at 10 years for female patients (18). These two studies included a limited number of patients, mostly children, whereas we were able to analyze a very large cohort of patients with CF, including children and adults, that is likely more representative of all patients with CF under clinical care. Furthermore, in these two studies, patients were only followed up over a short time, meaning that limited information was available to estimate CFLD incidence over the patients' lifetime. In accordance with our findings, a recent study reported that adult-onset CFLD was likely underestimated (19). The improved survival of patients with CF in the last decade may also explain the change in the incidence profiles of CFLD (20).

Severe CFLD complications were rare before 5 years of age, but increased afterwards by 0.3% per year, reaching 10.2% by the age of 30. The prevalence of severe CFLD was higher in male patients, in patients homozygous for the *CFTR* F508del mutation and with a history of meconium ileus at birth. An international study based on 561 CF patients with severe CFLD (cirrhosis and portal hypertension) showed similar results (21). The complications were mostly cirrhosis and portal hypertension, as shown by others (4, 22-24). As recently described, we also observed a subset of patients with portal hypertension but no cirrhosis (24, 25). Differences in biochemical characteristics in patients with CFLD and severe CFLD were consistent with aggravation of the disease (**Supplementary Table S4**).

We identified three independent risk factors associated with CFLD and severe CFLD: male sex, *CFTR* F508del homozygosity, and history of meconium ileus at birth. One third of the patients at most risk (*CFTR* F508del homozygous male patients with a history of meconium ileus) developed CFLD by the age of 20, while only 16% of the lower-risk factor group (non-*CFTR* F508del homozygous females without meconium ileus) had developed CFLD at the same age. While several studies reported similar associations between CFLD and severe *CFTR* genotype (17, 18, 26, 27), male sex (18, 26-29), and meconium ileus (5, 17, 18, 26, 27, 30), some discrepancies exist (31, 32). So far, the literature is inconclusive about the impact of CFLD on CF evolution. While some studies have shown that patients with CFLD were likely to have a more severe CF phenotype with altered nutrition and lung function statuses (5, 33-36), others did not observe any association (17, 18, 32, 37, 38). We were able to confirm in this large cohort that patients with CFLD had more severe lung disease, with the worst sufferers being patients with severe CFLD complications. Nutritional status showed the same trend. One particularity of this study was the use of CF-specific percentiles and Z-scores for BMI and FEV_1 that allowed referencing of the patients with CF with respect to their peers and, thus, peer to peer comparisons (15, 16).

Finally, we did not observe less severe CFLD as a result of precocious use of UDCA in time or between centers. UDCA efficacy in CFLD remains controversial (39). In sharp contrast with pulmonary

and nutritional CF complications, where new treatments have been shown to improve survival and quality of life, no drugs have demonstrated effect on CFLD. Although a recent Cochrane systematic review concluded that "There is currently insufficient evidence to justify [UDCA] routine use in cystic fibrosis" (39), several guidelines for CFLD patients' care still recommend its use (7, 9). High doses of UDCA treatment have even been detrimental to patients with primary sclerosing cholangitis, as its biotransformation in the colon produced lithocholic acid, a secondary hydrophobic bile acid with potential toxicity (40, 41). However, this was not seen in CF patients in whom the prescribed doses of UDCA are much lower (42). In our cohort, UDCA treatment has been initiated earlier than in CF patients over the last decades, even before the onset of CFLD. In turn, one could expect the occurrence of severe CFLD to either decrease or to be delayed in patients treated with UDCA earlier in life. The same trend would be expected in centers where treatment is given earlier in life. Analyzing the impact of preventive treatments requires taking into account protopathic bias, whereby treatment is preferentially prescribed to those who are at risk of developing the disease. This could cause CFLD incidences to be larger in those treated, reversing the cause and effect in the conclusion (13). To avoid this bias, we utilized an instrumental analysis, with birth cohorts and early/late prescribing centers as instruments. We found no changes between early and late treatment with UDCA, based on birth periods or on centers. These results were expected in the case of a limited effect, or in the absence, thereof, of UDCA treatment in the prevention of CFLD complications. However we acknowledge that a true effect of UDCA could be masked for the following reasons: first, if the signs of severe CFLD were recognized increasingly earlier in the most recent cohort, this could, by chance, balance out a delay in severe CFLD symptoms induced by UDCA. It is, however, unlikely that severe CFLD symptoms would be missed in CF patients born since the 1980s, and this does not explain the absence of a difference between early- and late-treating CF centers. Second, and more subtly, the observed differences in age at UDCA treatment initiation could be irrelevant, if all patients at risk of CFLD were treated early enough in the end. One would only observe the severe CFLD cases of patients who would have progressed to severe CFLD disease

irrespective of UDCA treatment, which does not rule out the possibility that other treated patients were actually protected in both cohorts. In that case, we would only have shown the absence of an effect for early versus later UDCA treatment for severe CFLD.

Other limitations in our analysis mainly concern its design, where data for the years before 2004 were obtained by retrospective examination of medical records. On the basis of CF mortality rate, we have estimated that between 2 and 7% of CF patients born between 1986 and 2004 were not included in our study, which could lead to selection by survival (see **Supplementary Information**). It is also possible that CF clinicians grew more aware of CFLD with time, especially after the study by Colombo et al. in 2002 wherein the diagnostic criteria of CFLD were formalized (18). Analysis of CFLD incidence in the post-2004 cohort hints that the disease is reported earlier, with a cumulative incidence of 13% [10.5, 15.7] at 10 years of age versus 10% [8.7, 11.0] in the whole cohort (**see Figure S8 and Table S6**). As mentioned above, this is less likely to be the case for severe CFLD, because the clinical signs are less dependent on interpretation (*i.e.* cirrhosis, portal hypertension, esophageal varices) and its incidence has not changed over the last 20 years. This is the reason we focused on severe CFLD in the UDCA treatment analysis. Yet, our results will have to be verified by future studies based on prospectively collected data alone.

To conclude, we observed, in this large CF cohort, a high incidence of CFLD. While liver disease is thought to develop in the pediatric age in patients with CF, we were able to show that its incidence continuously increased over time, with a consequential rate of progression to severity. CFLD and severe CFLD were associated with male sex, *CFTR* F508del homozygosity, and history of meconium ileus at birth, as well as with worse lung function and nutritional status. Finally, the absence of effect of UDCA treatment on the incidence of severe liver disease is an important finding, as UDCA is commonly prescribed in young patients. In the future, it will be critical that potential therapeutic agents be evaluated in well-designed randomized clinical studies, to ensure that the patients most likely to benefit from the treatment are identified.

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Figure legends

Figure 1. A) Cumulative incidence of cystic fibrosis related liver disease (CFLD), severe CFLD and ursodesoxycholic acid treatment (UDCA) according to age with 95% confidence intervals. B & C) Cumulative incidence of UDCA treatment (dotted) and of severe cystic fibrosis (CF) related liver disease (plain) in patients with CF born between 1986-1995 and 1995-2005 (B) and in CF centers treating early and late (C).