OXFORD

Human Molecular Genetics, 2023, Vol. 32, 4, 543–550
https://doi.org/10.1093/hmg/ddac221
Advance access publication date 1 September 2022
Original Article

Analysis of CF patient survival confirms STAT3 as a CF-modifying gene with changing impact over time

Inga Dunsche[1,†], Ellen L. Raddatz[1,2,†], Haide Ismer[1,†], Silke Hedtfeld[1], Stephanie Tamm[1,2], Saskia Moser[1], Julia Kontsendorn[1,2], Burkhard Tümmler[1,2], Sabina Janciauskiene[2,3], Anna-Maria Dittrich[1,2,‡] and Frauke Stanke[1,2,‡,*]

[1]Department of Pediatric Pneumology, Allergology and Neonatology, Hannover Medical School, D-30625 Hannover, Germany
[2]Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), German Center for Lung Research, Hannover Medical School, Hannover D-30625, Germany
[3]Department of Respiratory Medicine, Hannover Medical School, D-30625 Hannover, Germany
*To whom correspondence should be addressed at: Hannover Medical School, Department of Pediatric Pneumology, Allergology and Neonatology, OE6710, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. Tel: +49-511-532-6722; Fax: +49-511-532-6723; Email: mekus.frauke@mh-hannover.de
†I.D., E.L.R. and H.I. contributed equally.
‡A.M.D. and F.S. contributed equally.

Abstract

Introduction and aim: The signal transducer and activator of transcription 3 (STAT3) has been identified as one of the cystic fibrosis (CF) modifying genes. In this study, we aimed to assess the association between STAT3 genotype and CF patient survival over several decades and to investigate the effect of STAT3 inhibition on epithelial CFTR expression.
Methods: We analyzed the informative genetic marker STAT3Sat for its association with survival in 174 p.Phe508del-CFTR homozygous CF patients treated at the CF center in Hannover spanning birth cohorts from >3 decades (1959–1994). Furthermore, we treated two epithelial cell lines with STAT3 inhibitors and monitored changes of CFTR protein expression by western blot.
Results: Only for p.Phe508del-CFTR homozygous patients born prior to 1975, survival was significantly influenced by STAT3sat genotype ($P = 0.023$). The expression levels of STAT3 and CFTR positively correlated in epithelial cell lines ($P = 0.01$).
Conclusions: Our results in different birth cohorts identified a time-dependent impact of STAT3 genotype on CF patients' survival and found that improved symptomatic treatment of later-born CF patients obviates STAT3's modifying influence. Consistent with our previous results, STAT3-specific inhibition resulted in increased CFTR expression in the epithelial cell line 16HBE14o-. Thus, care should be taken when CF-modifying genes are studied in cross-sectional cohorts as the impact of modifying genes might not be invariant in the light of changing therapeutic regimens.

Introduction

The signal transducer and activator of transcription 3 (STAT3) translates cytokine signals to changes in gene expression (1,2). STAT3 is known as an acute phase response factor active during the initial stages of infection and inflammation and can act as an adaptor molecule in signal transduction from the type I interferon receptor (2), orchestrating the response to infectious agents (3). In cancer and cancer-derived cell lines, constitutive activation of STAT3 is a well-known characteristic (1) mediating developmental and differentiation signals from growth factors to enable transformation of cell types (1).

In cystic fibrosis (CF), caused by two defective copies of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (4), *STAT3* modifies CFTR expression (5): Genotyping the highly informative intragenic microsatellite marker STAT3Sat among p.Phe508del-CFTR homozygous patients, we have previously identified an association of large STAT3Sat alleles with more than 16 copies of the 35mer repeat motif with CFTR-mediated residual chloride secretion in rectal suction biopsies (5). Furthermore, we were able to show that STAT3 transcript levels of rectal suction biopsies were lower among carriers of such large STAT3Sat alleles (5). While large cohort studies on the association high-resolution CFTR function phenotyping with survival are yet lacking (6), registry studies identified an association of CFTR function via sweat chloride and survival for at least certain phenotypes (7,8). Thus, decreases of STAT3 function, associated with increased CFTR function, might confer a survival benefit to CF patients.

Pathogen defense is a critical component of CF lung disease since chronic bacterial infection largely determines morbidity and mortality of these patients (4). In CF patients, bacterial colonization is accompanied by co-evolution of the immune defense which permits containment of these infections for decades but eventually leads to progressive destruction of lung tissue, respiratory failure and death (4). An increase of STAT3 function confers benefit in the defense against pathogens (1–3), and therefore STAT3 function conferred by different STAT3Sat genotypes might impact beneficially upon CF patients' survival.

In summary, STAT3 function putatively confers two opposing effects on CF patients' survival. To address these opposing roles, we queried the association of STAT3Sat alleles with patient survival in a large group of CF patients spanning several decades of birth years. Furthermore, we directly interrogated the effect of

Received: April 29, 2022. **Revised:** August 16, 2022. **Accepted:** August 30, 2022

STAT3 inhibition on CFTR expression in epithelial cell cultures to validate our previous findings on the association of reduced STAT3 transcript levels with improved CFTR function.

Results

Genotype frequencies for STAT3Sat differ by birth cohort among p.Phe508del-CFTR homozygotes

To assess the effect of STAT3Sat genotypes on CF patients' survival, we first analyzed the distribution of STAT3Sat genotypes among p.Phe508del-CFTR homozygous patients born between 1959 and 1994. As we previously observed that STAT3Sat genotypes are associated with CFTR-mediated residual chloride secretion (5), we have used the very same classification of STAT3Sat genotypes to assess the impact of birth year on the STAT3Sat genotype distribution, contrasting ≥16 ARU and ≤12 ARU repeat lengths, respectively (Fig. 1A). By this approach, we identified that patients born 1959–1977 carry at least one large STAT3Sat allele ≥ 16 ARU more frequently than patients born 1977–1994 ($P = 0.05$).

We have retrieved the year-of-birth distribution of p.Phe508del-CFTR homozygous patients assessed in our previous studies (5). In comparison to our current study, those patients were born later (median 1979, inner quartiles 1971–1985) and the STAT3Sat genotype 'carrying at least one large STAT3Sat allele ≥16 ARU' was associated with the presence of CFTR-mediated residual chloride secretion in that earlier study (5). Likewise, we noted a later year of birth for participants of the earlier study that revealed lower STAT3 mRNA levels in intestinal epithelia among p.Phe508del-CFTR homozygous carriers of at least one large STAT3Sat allele ≥ 16 ARU (median 1982, inner quartiles 1974–1985) (5).

These previous observations thus associate carriage of at least one large STAT3Sat allele ≥16 ARU with decreased STAT3 transcript levels and increased CFTR-mediated residual chloride secretion (5) among patients born in the late 70s and early 80s. In our current study, we observed an increased frequency of larger STAT3Sat alleles ≥16 ARU among patients born earlier than 1977 but being still alive for enrollment in the 1990s. Thus, our data suggest that large STAT3Sat alleles ≥16 ARU confer a survival benefit via decreased STAT3 transcription and increased CFTR-mediated chloride secretion.

Survival of CF patients differs by STAT3Sat genotype in patients born before the early 1970s

To formally assess the impact of STAT3Sat genotype on survival, we used year of birth to define three subcohorts C1, C2 and C3 (Fig. 1B, Table 1). Analyzing these three subcohorts separately, we made the following observations. An impact of STAT3Sat genotype on patient survival was detected for patients from C1 born prior to 1976 ($P = 0.023$). Interestingly, heterozygotes for the two contrasting STAT3Sat alleles (≤12 ARU and ≥16 ARU) had a worse prognosis than either STAT3Sat homozygotes (Fig. 1B) in the subcohort C1. For patients born later than the mid-1970s, no impact of STAT3Sat genotype on survival was detected.

When analyzing all three subcohorts together, survival of STAT3 heterozygotes was similar between C1 (median survival 35 years), C2 (median survival 35 years) and C3 (median survival 37 years; Fig. 1B, Table 1). In contrast, median survival of STAT3 homozygotes who carried STAT3Sat ≤ 12 ARU on both alleles decreased by nearly two decades within the observation period (C1 median survival 50 years; C2 median survival 36 years; C3 median survival 32 years; Fig. 1B, Table 1).

Our previous published data associate carriage of at least one small STAT3Sat allele ≤ 12 ARU with increased STAT3 transcript levels and decreased CFTR-mediated residual chloride secretion (5) among patients born in the late 70s and early 80s. As patients from C1, born prior to the mid-70s, who carry STAT3Sat alleles ≤12 ARU have survived longer than the STAT3Sat heterozygotes who carry one STAT3Sat allele ≥16 we must conclude that roles of STAT3 other than to promote CFTR-mediated residual chloride secretion has outweighed STAT3 function among CF patients who were in childhood during the 1960s and 1970s. Moreover, the increase in survival of homozygotes for two small STAT3Sat alleles ≤12 ARU by nearly two decades when comparing subcohorts C1, C2 and C3 indicates that the role of STAT3 for CF patients' survival has changed along with improvements in symptomatic therapy and overall CF patient survival.

CFTR expression is increased in 16HBE14o- cells by STAT3 inhibitors cps3–2 and cpd3–7

We consecutively used two epithelial model cell lines to assess the effect of STAT3 inhibitors on CFTR protein expression (Fig. 2). We chose four different STAT3 inhibitors whereby inhibitors XII and cpd188 are less specific while cpd3–2 and cpd3–7 confer increased specificity towards binding of the SH2 domain of STAT3 (9). In airway epithelial 16HBE14o- cells, the less specific STAT3 inhibitors XII or cpd188 did not change CFTR expression significantly. Yet, the more specific STAT3 inhibitors cpd3–2 or cpd3–7 increased CFTR expression ($P = 0.05$; Fig. 2A). Conversely, in T84 cells, a cancer cell line with constitutive STAT3 activation, any STAT3 inhibitor decreased CFTR expression (Fig. 2A, $P < 0.05$).

We have noticed that the expression levels for CFTR in 16HBE14o- cells treated with cpd3–2 and cpd3–7 show considerable experiment-to-experiment variation and thus reviewed our approach to compare protein expression directly between treated and untreated sample for technical variability. A subset of cell lysates was analyzed for change in β-actin expression ($n = 13$; 109 ± 25%; n.s., data not shown). In comparison to the heterogeneous expression data obtained from the virus-immortalized respiratory epithelial cell line 16HBE14o-, changes in protein expression observed for the cancer cell line T84 of all proteins were more homogeneous as the standard deviation for CFTR (±23.7%) and STAT3 (±28.7%) among T84 cells were similar to the biological and technical variation of the assay reflected by analysis of β-actin (Fig. 2A and B). In other words, the high experimental variance observed for CFTR expression in 16HBE14o- cells treated with cpd3–2 and cpd3–7 likely reflects the underlying biology and is not a technical artifact.

CFTR expression is positively correlated to STAT3pY705 expression in 16HBE14o- and T84

To further investigate the responses to STAT3 inhibitors in 16HBE14o- cells, we monitored the expression of STAT3pY705 representing the activated canonical STAT3 pathway (10). Upon STAT3 inhibitor application, STAT3pY705 expression was lowered in T84 ($P = 0.05$), but not in 16HBE14o- cells (Fig. 2B). Irrespective of STAT3 inhibitor and cell line, we observed a positive correlation of the change in CFTR protein expression and the change in STAT3pY705 expression upon treatment with STAT3 inhibitors ($P = 0.01$; Fig. 2C).

Discussion

In this study, we demonstrate a birth-cohort dependent benefit of specific STAT3 alleles on CF patients' survival whereby specific

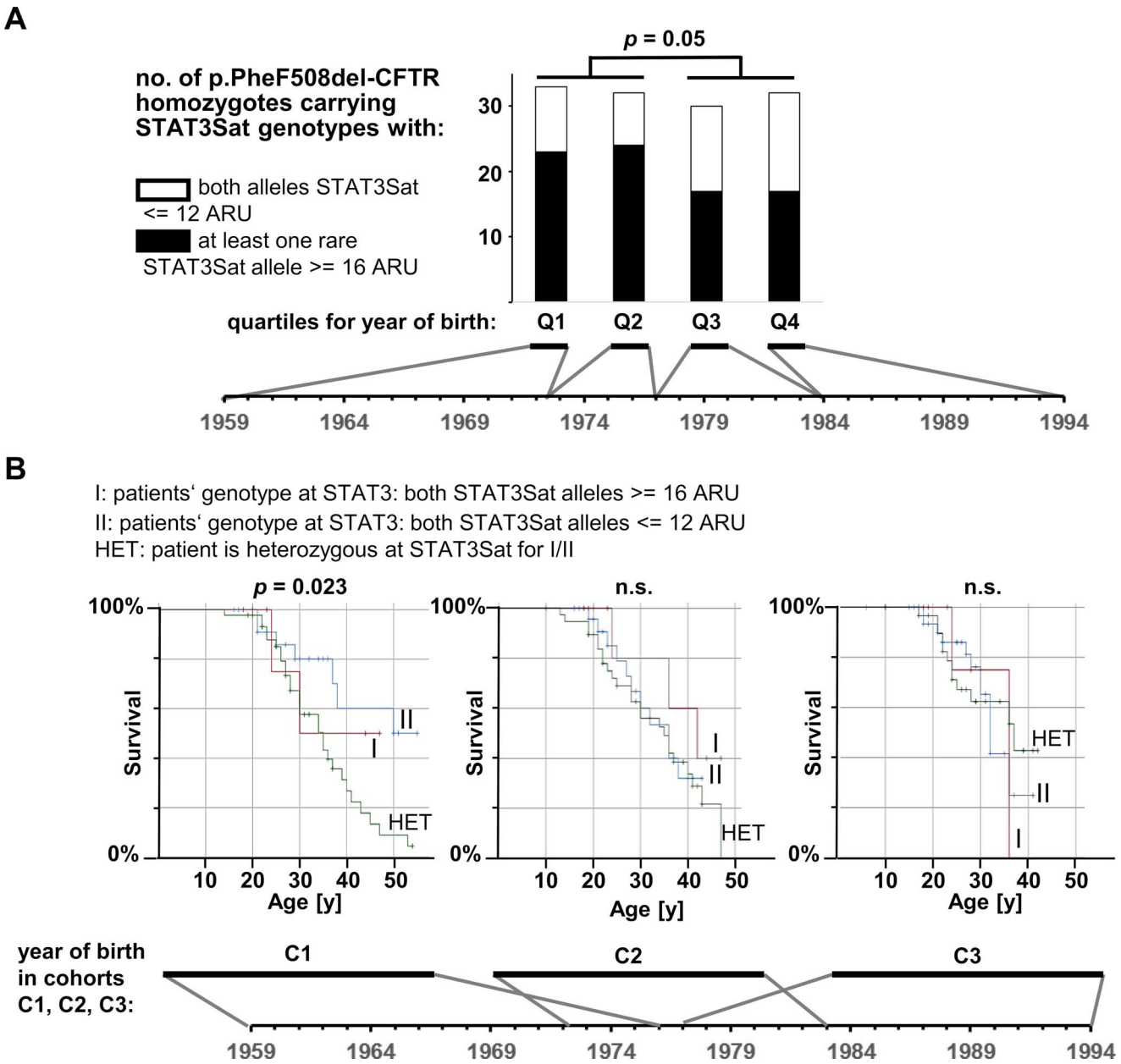

Figure 1. Genotype distribution and patient survival at STAT3Sat. Allele distribution and patient's survival was monitored for unrelated p.Phe508del-CFTR homozygotes who have been recruited for DNA analysis in 1990. To visualize the time span covered by the subsamples, the period of the patient's year of birth is delineated below the STAT3Sat allele frequency distribution (A) and the survival analysis by STAT3Sat genotype (B). (**A**) A subset of 140 CF patients for whom longitudinal clinical data was available were segregated into four equally sized cohorts based on their year of birth (quartiles Q1–Q4, $n = 35$ patients each), and frequency of two contrasting STAT3Sat alleles (≥16 ARU vs ≤12 ARU) was monitored in these four quartiles. Genotype distribution at STAT3Sat differed between patients born 1959–1977 and patients born 1977–1984 ($P = 0.05$). (**B**) A total of 174 CF patients were segregated into three groups of 80 patients each defined by year of birth (C1–C3) and survival statistics were retrieved from our clinical data base. For patients born 1959–1976, STAT3Sat heterozygotes had a significantly worse prognosis than either homozygotes ($P = 0.023$).

STAT3Sat genotypes grouped according to their repeat lengths shown an association with CFTR function (5). Remarkably, this survival benefit was only observed in the oldest patient cohorts but not for CF patients born later (Fig. 1). Moreover, our results validate our previous genotype data (5) in that they demonstrate a direct effect of STAT3 activity on CFTR expression in respiratory epithelial cells (Fig. 2).

Taken together, our data indicate that STAT3 influences the course of CF disease considerably. Our results can be partly explained by STAT3's direct effects on CFTR expression (Fig. 2A) whereby large STAT3Sat alleles ≥ 16 ARU have been previously associated with decreased STAT3 transcription (5) and—as shown here—confer a survival benefit, leading to enrichment of this allele in older birth cohorts ($P = 0.05$; Fig. 1A) and a survival benefit in older cohorts if carried in homozygosity ($P = 0.023$; Fig. 1B). These results recall our earlier observation on the CF modifier gene *TGFB1* and on the frequency of mild *CFTR* mutation genotypes among survivors (11). For the genes *TGFB1* and *CFTR* (11), *IL1R* (12) and *STAT3* (this work), an elevated frequency of mild genotypes has been observed among patients born prior to the mid-70s who have survived until recruitment for DNA analysis in the 90s.

Table 1. Patient survival by STAT3Sat genotype

Year of birth	No of patients	Patients' genotype at STAT3: both STAT3Sat alleles ≥16 ARU		Patients genotype at STAT3: both STAT3Sat alleles ≤12 ARU		Patient is heterozygous at STAT3Sat		*P*
		No of patients, *n* (%)	Median survival	No of patients, *n* (%)	Median survival	No of patients, *n* (%)	Median survival	
1959–1994	165[a]	15 (8.6%)	n.d.	66 (37.9%)	n.d.	84 (48.3%)	n.d.	n.d.
1959–1976	78	7 (8.8%)	30 years	26 (32.5%)	≈50 years	45 (56.3%)	35 years	0.023
1972–1983	75	9 (11.3%)	42 years	27 (33.8%)	36 years	39 (48.8%)	35 years	0.406
1977–1994	75	7 (8.8%)	36 years	35 (43.8%)	32 years	33 (41.3%)	37 years	0.913

[a]Out of 174 p.Phe508del-CFTR homozygotes, the STAT3Sat genotype could not be resolved for eight patients. One patient carried SAT3Sat-14 on both chromosomes and thus could not be classified in either group 'carries at least one allele STAT3Sat-16 or more ARU' or 'both alleles STAT3Sat-12 or less ARU'.

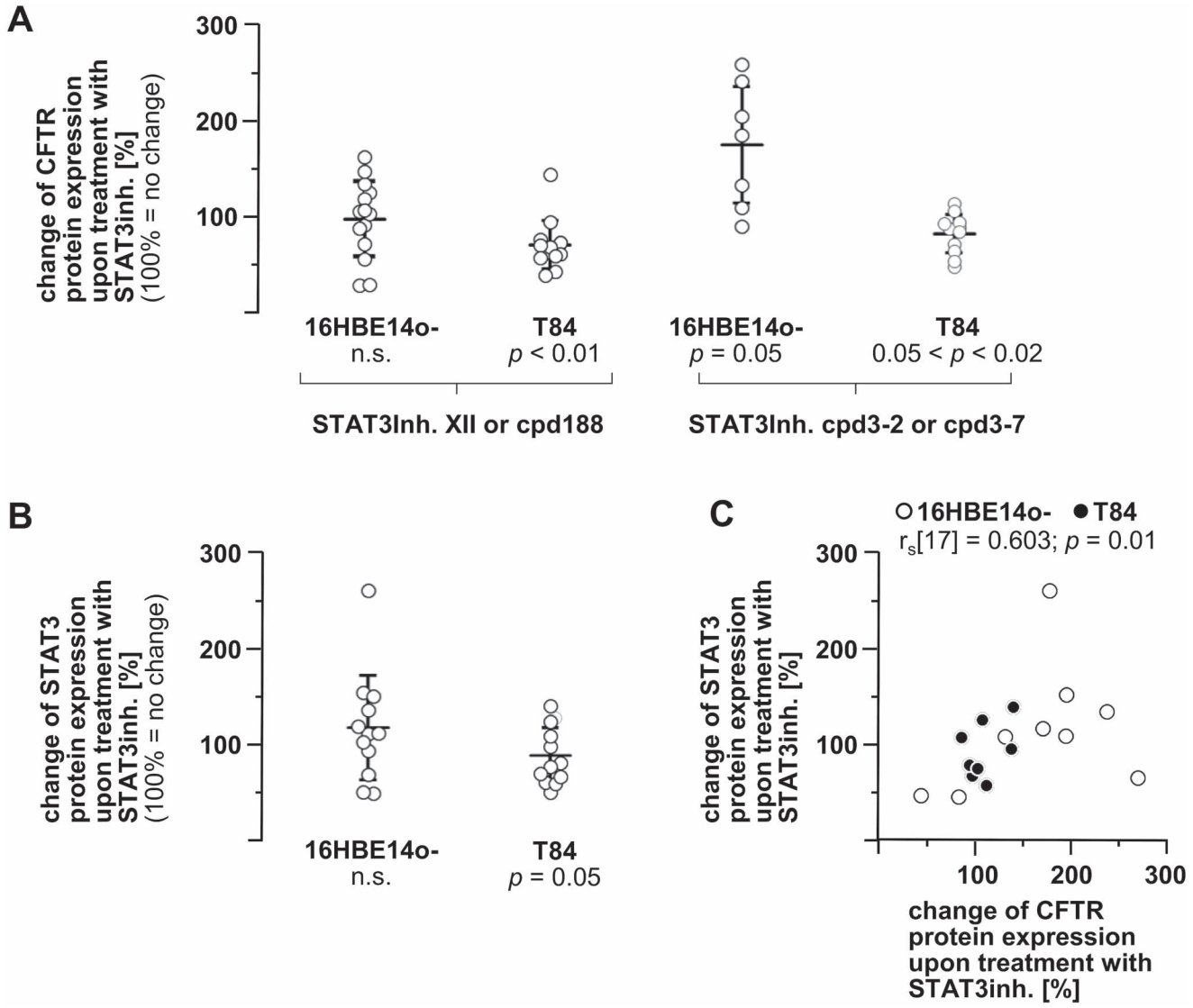

Figure 2. Influence of pharmacological STAT3 inhibitors on CFTR and STAT3 expression. Epithelial model cell lines 16HBE14o- and T84 were treated with STAT3 inhibitors XII, cpd188, cpd3–2 or cpd3–7 and expression of CFTR (A, C) or STAT3 (B, C) in response to the less specific (XII and cpd188) vs the more specific inhibitors (cpd3–2 and cpd3–7) was assessed by western blot. Deviation from the null hypothesis 'no change in protein expression is observed' was judged by Wilcoxon signed rank test. (**A**) Expression of CFTR in 16HBE14o- was increased by inhibitors cpd3–2 and cpd3–7 ($n = 7$; 173 ± 60%, $P = 0.05$) but not by XII and cpd188 ($n = 14$; 96 ± 39%, n.s.). CFTR expression in T84 was reduced by all four STAT3 inhibitors alike (cpd3–2, cpd3–7: $n = 11$; 83 ± 20%, $0.05 < P < 0.02$; XII, cpd188: $n = 13$; 69 ± 25%, $P < 0.01$). (**B**) STAT3-pY705 expression was unchanged in 16HBE14o- cells (inhibitors combined: $n = 12$; 117 ± 53.9%; n.s.) and reduced in T84 (inhibitors combined: $n = 12$; 89 ± 28%; $P = 0.05$). (**C**) Change in expression of CFTR and STAT3-pY705 was positively correlated ($P = 0.01$; Spearman rank correlation test; 16HBE14o-: $n = 9$; T84: $n = 8$).

The microsatellite STAT3Sat is likely functional by itself. STAT3Sat is a 35mer repeat motif present as less than 5 up to more than 25 repeat units (5), thus varying in length between less than 175 bp and more than 875 bp. This repeat is surrounded by less than 300 bp of noncoding sequence in intron 6 of *STAT3*. Hence, intron 6 of *STAT3* will considerably vary in length, which in turn will impact the alignment of the splice site consensus sequences and thus, splicing efficacy. This might explain why STAT3Sat shows an allelic association to STAT3 mRNA levels (5), and it also explains why STAT3Sat is such a powerful marker to detect the influence of STAT3 on CF phenotype.

However, our data also suggest that the penetrance of STAT3Sat alleles that promote CFTR expression and function (5) by decreasing STAT3 amount or function (Fig. 2A) in epithelial cells is incomplete and its effect on patient survival is influenced by environmental, therapeutic changes since the large STAT3Sat alleles are only associated with improved survival in the older cohorts (Fig. 1A and B). Changing environmental, i.e. therapeutic influences seem to obviate the beneficial effects of the ≥16 ARU genotype in patients born later and do not lead to enrichment of this genotype in more recent birth cohorts. For STAT3Sat heterozygotes, accounting for up to 56% of patients in our three subsamples, the median survival of about 35 years was unchanged between cohorts stratified by year of birth. In contrast, median survival of homozygotes for small STAT3Sat alleles (≤ 12 ARU) decreased from 50 years (patients born prior to 1976) to 32 years (patients born after 1977). Thus, our data demonstrate that the impact of small STAT3 alleles (≤12 ARU) has also changed over time: for patients born prior to the mid-1970s, homozygosity for small STAT3Sat alleles posed less of a risk than for patients born after the mid-1970s.

We perceive limitations in our study for homozygotes of two large STAT3Sat alleles (≥16 ARU) as these constitute only 8% to 11% of our subsamples composed of 80 patients. Homozygous carriers of large STAT3Sat alleles and homozygous carriers of the contrasting small STAT3Sat alleles were indistinguishable—contradictory to our annotation of the two contrasting STAT3Sat alleles—in our subcohorts while STAT3Sat heterozygotes had a worse prognosis than either homozygotes (Fig. 1B). We thus cautiously restrict the interpretation of the survival data to the observed contrast between STAT3Sat heterozygotes and STAT3Sat homozygotes of small alleles (≤12 ARU). Our previous studies (5) have been conducted among p.Phe508del-CFTR homozygotes born predominantly in the late 1970s and 1980s. To assess whether this previous data and the present study are compatible, we compare contrasting STAT3Sat genotypes for the cohort C3 building on patients born as well in the late 1970s and 1980s: while only 25% of homozygotes for one small STAT3Sat allele ≤12 ARU were still alive at the end of the observation period, about 40% of STAT3Sat heterozygotes have survived to at least 37 years of age (Fig. 1B). In other words, the impact of at least one mild STAT3Sat allele ≥ 16 ARU manifests in consistency with our previous data (5) as a survival benefit in this replication study when year of birth is adequately reflected.

Few diseases have shown such impressive survival increases as CF since the 1960s, where symptomatic treatment improved the survival of CF patients by several decades of life (13). Key factors that determine CF patients' survival have been firstly, compensation of nutritional deficits due to pancreatic malabsorption, and secondly, the postponement of progressive lung disease leading to respiratory failure by mucolytic and antibiotic treatment to promote airway clearance and combat (chronic) bacterial infections (13). This impressive survival increment, shown strikingly by large epidemiological studies (14,15), infers that only a fraction of patients born prior to the mid-1970s can be recruited into analyses carried out several decades later, such as our patient cohort III where enrollment for DNA sampling took place in the early 1990s. The impact of non-inherited factors such as therapeutic efficacy will differ tremendously across a cross-sectional study population, particularly if sample size maximization is limited by disease prevalence in rare diseases and survival increases to an extent that longitudinal follow-up becomes increasingly difficult. This shortcoming of recruitment or survivor bias is acknowledged for other lethal diseases for which its effects are more obvious (16) than for CF.

By retrospectively evaluating genotype–phenotype relationships in subsamples defined by year of birth, we were also able to identify such a survivor bias where the disadvantage conveyed by STAT3Sat alleles for patients born later did not reach clinical significance (Fig. 1). One explanation would be that the inherited STAT3-mediated disadvantages were successfully evaded by improved symptomatic treatment for later-born CF patients. Alternatively, the genetic impact of STAT3 might manifest later in life for patients born later than 1975, being still undetectable in our subsample as a significant proportion of these patients were still alive by the end of the reporting period. In summary, our results show that the convergence of STAT3's pleiotropic effects in CF—on CFTR expression and hence function on the one hand side and pathogen defense on the other hand side—in patients born across several decades leads to a complex interplay between genetic and environmental factors.

As STAT3 could be confirmed as a modifying gene in this study, we wanted to annotate the risk and the benign allele for STAT3Sat with functional data. Our previous data (5) suggest that carriers of large STAT3Sat alleles will benefit from CFTR-mediated residual chloride secretion in intestinal epithelia, the latter suggested to be caused by lower STAT3 mRNA in epithelia, and consequently, by lower STAT3 protein levels. Thus, we have used STAT3 inhibitors on model epithelial cell lines and monitored CFTR protein expression as outcome (Fig. 2). Furthermore, we have asked whether the heterogeneity among 16HBE14o- cells is due to the pharmacological precision of STAT3 inhibitors XII and cpd188—both known to partially target homologous STAT proteins beyond STAT3 (9)—versus cpd3–2 and cpd3–7, which were designed by a virtual ligand screening approach to selectively target the SH2 domain of STAT3 and thus discriminate between STAT1 and STAT3 (9).

In the model epithelial cell line 16HBE14o-, we could observe an increase in CFTR expression when STAT3-specific optimized inhibitors cpd3–2 and cpd3–7 were employed ($P = 0.05$, Fig. 2A), but not with STAT3 inhibitor XII or cpd188. In other words, less STAT3 function—achieved through blocking the SH2 domain of STAT3 with cpd3–2 or cpd3–7—leads to an increase of CFTR protein in 16HBE14o- epithelial cells. This is consistent with our previous observation among CF patients that low levels of STAT3 mRNA or protein promote elevated levels of CFTR and consequently, CFTR-mediated residual function (5).

Furthermore, the interexperimental fluctuation of CFTR expression observed in 16HBE14o- cells could be partially attributed to the effectivity of pharmacological mediated STAT3 inhibition as expression levels of STAT3 and CFTR were positively correlated in epithelial cell lines ($P = 0.01$, Fig. 2C). This observation, however, does not agree with findings among CF patients that low levels of STAT3 mRNA cause more CFTR-mediated residual function (5) under the logical assumption that less STAT3 mRNA results in less STAT3pY705 protein and ask for additional interrogation beyond the scope of this report.

In the light of this, we want to mention that our study has several limitations, chiefly that in our simple epithelial model cell lines, we did not address any crosstalk between epithelial cells and the immune system in the context of infection and inflammation as this would require a more complex culture and co-culture approach. It is easy to conceive that reduced amounts of STAT3, promoting CFTR expression in uninfected and uninflamed epithelial cells, are a risk for CF patients born prior to the 1970s when infection and inflammation in CF was not yet well-treatable (13). In contrast, the same condition might have been tolerable for CF patients who received increasingly efficient anti-infectious treatment in later decades. Consequently, the association of CFTR-mediated residual chloride secretion and low STAT3 mRNA levels could be observed among patients born mostly in the late 1970s and early 1980s (Fig. 1A and B) (5). Furthermore, inherently, already upon recruitment, the survivor bias might have impacted our results, where only patients could be analyzed that presented themselves alive to our center and consented to usage of their biomaterial and clinical data. Acknowledging this bias, we believe that the latter shortcoming is somewhat balanced by the comparatively large patient number and long time-span, we were able to analyze, which offers an unprecedented illustration of complex interaction of modifier genes, survival and a changing treatment landscape of a progressive disease.

In summary, our data confirm the role of STAT3 as a CF-modifying gene whereby its impact on patient survival changes over time, likely due to its Janus-headed features as active STAT3 can promote epithelial dedifferentiation (which is disadvantageous for CF) as well as stimulate defense against infection and inflammation (which is vital in CF) (1,3). The function of STAT3 is thus context-sensitive, and as therapeutic measures for chronic airway infections in CF have improved survival (13,15), genetic conditions that predispose to promote epithelial CFTR function (17) at the cost of increased vulnerability to inflammation due to low STAT3 activity in non-epithelial cells (3) might have become tolerable for survival. The changing environment thus becomes a framework that determines the impact of modifying genes on the course of the disease. Further investigation of which immune cells are vital for the STAT3-mediated risk might be facilitated by applying specific STAT3 inhibitors to isolated immunologically relevant cells which are challenged with a CF-typical host-defense situation. Furthermore, STAT3Sat can be used as a sensitive biomarker for STAT3 functionality in this context. Thus, the phenotypic monitoring of such experimentally challenged immunologically relevant cells might be carried out using patient's samples. In this scenario, the association of the patient's STAT3Sat genotype and the patient's immune cell phenotype might be employed as a powerful tool to identify the underlying molecular mechanism of the STAT3-mediated risk in CF. In conclusion, care should be taken when CF-modifying genes are studied in a cross-sectional design that summarizes patients across birth cohorts as the impact of a modifying gene might not be constant in the light of changing therapeutic regimens.

Materials and Methods

Patients and analysis of clinical data

We have typed STAT3Sat in a subcohort of 174 CF patients regularly seen at the CF clinic in Hannover, building upon clinical data collected between 1985 and 2015 of more than 297 patients (11,12,18). DNA was obtained for a subset of patients from 1990 to 1994 and genotyped for STAT3Sat (medical ethics approval #3799). For a subset of 140 p.Phe508del-CFTR homozygotes born between 1959 and 1994 for whom longitudinal clinical data were available, STAT3Sat allele distribution was monitored by year of birth (Fig. 1A). For this purpose, patients were segregated into quartiles for year of birth (35 patients each, Q1: 1959–1972, an interval of 13 years, Q2: 1973–1977, an interval of 4 years, Q3: 1977–1984, an interval of 7 years and Q4: 1984–1994, an interval of 10 years). Survival data of 174 p.Phe508del-CFTR homozygotes was retrieved from the clinical documentation system maintained by the CF center in Hannover, the Hannover lung transplant center as well as from the hospital's clinical documentation system and the national CF registry with an observation period until 09/2020 (Fig. 1B). From the 174 p.Phe508del-CFTR homozygotes enrolled for survival analysis, 28 were still alive at the end of the reporting period, as evident from recent visits to the CF center. A total of 78 had either died or had received a lung transplant (LTx 46 of the 78). A total of 70 patients were lost to follow-up and censored for survival analysis, whereby the last known date when these patients were recorded as being still alive into account. Kaplan–Meier survival time analysis was carried out based on birth cohorts whereby 80 individuals each were included and sliding windows with 33 individuals overlap were employed. Survival analysis was done on the entire cohort of 174 p.Phe508del CFTR homozygous patients irrespective of their genotype at STAT3Sat or other CF-modifying genes.

STAT3 genotyping

Genotyping of the STAT3 microsatellite STAT3Sat (5) in intron 6 was carried out using polymerase chain reaction amplification with one biotinylated primer. Products were visualized by direct blotting electrophoresis on a high-resolution polyacrylamide gel and chemoluminescence detection of biotinylated PCR products. Primers for STAT3Sat were 5′-TTCTGCCTGGTCACTGACTG and biotin-5′-GGAGGTACGGGTCCTCAAAG (5). STAT3Sat alleles were classified by size in arbitrary repeat units (ARU) based on an invariant defined set of controls (5).

Cell culture

T84 epithelial cells were grown in Dulbecco's Modified Eagle Medium (DMEM/F12; 21 331–020, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS) and 1% of 100 X penicillin-streptavidin-glutamine-solution (10378–016, Thermo Fisher Scientific) in 100 × 20 mm plates (83.1802, Sarstedt, Nümbrecht, Germany). The respiratory epithelial cell line 16HBE14o- was grown on fibronectin (354008, Corning, Corning, NY, USA) and collagen I (354231, Corning) coated plates (83.3900.002, Sarstedt) with Minimal Essential Medium (MEM; 12 360–038, Thermo Fisher Scientific) supplemented with 10% FCS and 1% of 100 X penicillin-streptavidin-solution (14150–122, Thermo Fisher Scientific) and 1% of 200 mM L-Glutamine-solution (258030–081, Thermo Fisher Scientific).

STAT3 inhibitor compounds

To investigate the effect of STAT3 on CFTR expression, the following inhibitors were used: STAT3 inhibitor XII alias Stattic (6-Nitrobenzo[b]thiophene-1,1-dioxide, 573127, Merck, Darmstadt, Deutschland) (19); STAT3 inhibitor IX alias cpd188 (4-((3-(Carboxymethylsulfanyl)-4-hydroxy-1-naphthyl)sulfamoyl) benzoic acid, 573125, Merck) (9); STAT3 inhibitor cpd3–2 (3-({2-chloro-4-[(1,3-dioxo-1,3-dihydro-2H-inden-2-ylidene)methyl]-6- ethoxyphenoxy}methyl)ben-zoic acid; 6745560, Chembridge Corporation, San Diego, CA, USA) (9); STAT3 inhibitor cpd3–7 (methyl 4-({[3-(2-methoxy-2-oxoethyl)-4,8-dimethyl-2-oxo-2H-chromen-7-yl]oxy}methyl)benzoate; 7682533, Chembridge

Corporation) (9). While STAT3 inhibitors XII and cpd188 retain partial activities towards other members of the STAT family (9), cpd3–2 and cpd3–7 were designed by a virtual ligand screening approach that targets the binding pocket of the tyrosine kinase src homology 2 (SH2) domain of STAT3 (9), allowing it to discriminate between STAT3 and the highly homologous STAT1 (9). For analyses, the less specific inhibitors XII and cpd188 were grouped as were the more specific inhibitors cpd3–2 and cpd3–7.

STAT3 inhibitor assays

Inhibitors were dissolved with isopropanol (STAT3 inhibitor XII), ethanol (cpd188) or ten volume parts ethanol supplemented with one volume part DMSO (cpd3–7, cpd3–2). In other words, we took care to avoid the commonly used solvent DMSO as we could see alterations of CFTR expression induced by DMSO in T84 or 16HBE14o- (data not shown). T84 or 16HBE14o- were treated with STAT3 inhibitors for 16–20 h to a final concentration of 50 μM (STAT3 inhibitor XII), 20 μM (cpd188), 256 μM (cpd3–2) or 137 μM (cpd3–7) which corresponds to half of the maximal inhibitory concentration IC_{50} with respect to binding of the inhibitors in competition to a phosphorylated STAT3-SH2-peptide (9). In all experiments, a specific control using solvent only was incubated in parallel to the cells treated with the STAT3 inhibitor. Change of protein expression upon incubation with STAT3 inhibitors was judged by comparing signal intensities obtained by western blot between adjacently loaded treated and untreated solvent control sample. As the STAT3 pathway is typically constantly activated in cancer (20), T84 mostly served as a technical control for the validity of protein expression monitoring.

Western blot

Cells were lysed using SDS-rich lysis buffer (50 mM Tris pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 100 mM dithiothreitol (DTT), 1:50 proteinase inhibitor cocktail (SRE0055-1BO; Sigma Aldrich, MI, USA), 0.2 mM phenylmethylsulfonylfluoride, 1:10 000 OmniCleave Endonuclease (OC7850K; Biozym, Germany)). Gel electrophoresis was carried out in a Mini-PROTEAN Tetra Cell (#165–8001; Bio-Rad Laboratories GmbH, Munich, Germany) using 6% polyacrylamide (PAA; Rotiphorese Gel 30, crosslink 37.5:1; Roth, Karlsruhe, Germany). To optimize the sensitivity and specificity of the immunechemical CFTR signal, conditions were selected that preferentially resolve proteins within the range of 100–300 kDa, whereby electrophoresis was done until the 72 kDa marker had almost reached the lower edge of the polyacrylamide gel. Respectively, CFTR (fully glycosylated isoform of ~170 kDa) and STAT3 (88 kDa) were visualized by electrophoresis optimized to detect proteins of 70 up to 250 kDa. A subset of cell lysates was analyzed for change in β-actin (42 kDa) expression which requires electrophoresis unfavorable for the detection of high molecular weight proteins.

Proteins were transferred to an uncharged supported nitrocellulose membrane (Amersham Protran Supported Nitrocellulose Blotting-Membrane; 0.45 μm pore size; #10600016; VWR, Darmstadt, Germany) by tank blotting in 125 mM Tris, 950 mM glycine, 0.02% (w/v) SDS in a Mini Trans-Blot Electrophoretic Transfer Cell (#170–3935; Bio-Rad Laboratories GmbH). For detection of protein expression levels, the following antibodies were used: CFTR—equimolar mix of CFTR-AK 596 + 570 + 217 + 660 (Cystic Fibrosis Foundation CFTR Antibody Distribution program; Chapel Hill, NC) diluted 1:400; STAT3pY705—ab76315 (Abcam, Cambridge, UK) diluted 1:6000; β-Actin—ab8226 (Abcam) diluted 1:500. As β-Actin, having a molecular weight of 42 kDa, is incompatible with electrophoresis optimized for CFTR detection including only proteins larger than 72 kDa, a subset of analyses was carried out using altered conditions suboptimal for detection of proteins with high molecular weight. Signals were visualized with horseradish peroxidase coupled to secondary antibodies and substrates SuperSignal West Pico (34078; Thermo Fisher, Darmstadt, Germany) and SuperSignal West Femto Max. Sensitivity (34096; Thermo Fisher).

Densitometry and comparison of protein expression levels

Densitometry of digitized scans was performed with GelAnalyzer 19.1 (www.gelanalyzer.com by Istvan Lazar Jr, PhD and Istvan Lazar Sr., PhD, CSc). Expression was compared to paired samples of adjacently loaded treated and untreated solvent control samples and expressed as % of expression of treated sample (100% = expression in paired control). As lysates can contain extracellular matrix proteins that are detected by protein quantification of the primary lysates but do not migrate into the polyacrylamide gel matrix, we used residual Coomassie staining after transfer onto the membrane to ensure that the amount of proteins that have entered the gel matrix are comparable between STAT3-inhibitor treated sample and solvent control. Data shown are for independent experiments on biological replicates and rely on independent solvent controls.

Data evaluation

Protein expression data was acquired on a DNR-MF-ChemiBIS 3.2 Bio-Imaging System (Berthold Technologies, Bad Wildbad, Germany). Signal intensity within the inhibitor-treated cell culture was normalized to a paired solvent-only control (100% = no change) loaded adjacently on the same gel. Densitometry of digitized scans was performed with GelAnalyzer 19.1 (www.gelanalyzer.com by Istvan Lazar Jr, PhD and Istvan Lazar Sr., PhD, CSc). Deviation from the null hypothesis 'no change in protein expression is observed' was judged by Wilcoxon signed rank test.

STAT3Sat allele distributions between patient subgroups segregated into quartiles for year of birth were compared using Monte Carlo simulation with CLUMP (21).

Kaplan–Meier survival time analysis was carried with the Statistical Package für Social Sciences SPSS on patient subsamples stratified for STAT3Sat genotype. To compare carriers of contrasting STAT3Sat genotypes, non-parametric estimate of the median survival time was obtained.

Conflict of Interest statement. The authors declare that there is no potential financial and non-financial competing interest regarding the content of this manuscript. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Funding

Mukoviszidose e.V. (MI-#1601); German center for Lung Research (82DZL002A1). The funding organizations had no influence on the study design, data analysis and interpretation or intention to publish.

References

1. Yu, H., Pardoll, D. and Jove, R. (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat. Rev. Cancer*, **9**, 798–809.
2. Akira, S., Nishio, Y., Inoue, M., Wang, X.J., Wei, S., Matsusaka, T., Yoshida, K., Sudo, T., Naruto, M. and Kishimoto, T. (1994)

Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell*, **77**, 63–71.
3. Gao, Y., Zhao, H., Wang, P., Wang, J. and Zou, L. (2018) The roles of SOCS3 and STAT3 in bacterial infection and inflammatory diseases. *Scand. J. Immunol.*, **88**, e12727.
4. Shteinberg, M., Haq, I.J., Polineni, D. and Davies, J.C. (2021) Cystic fibrosis. *Lancet*, **397**, 2195–2211.
5. Labenski, H., Hedtfeld, S., Becker, T., Tümmler, B. and Stanke, F. (2011) Initial interrogation, confirmation and fine mapping of modifying genes: STAT3, IL1B and IFNGR1 determine cystic fibrosis disease manifestation. *Eur. J. Hum. Genet.*, **19**, 1281–1288.
6. Simmonds, N.J., D'Souza, L., Roughton, M., Alton, E.W.F.W., Davies, J.C. and Hodson, M.E. (2011) Cystic fibrosis and survival to 40 years: a study of cystic fibrosis transmembrane conductance regulator function. *Eur. Respir. J.*, **37**, 1076–1082.
7. McKone, E.F., Velentgas, P., Swenson, A.J. and Goss, C.H. (2015) Association of sweat chloride concentration at time of diagnosis and CFTR genotype with mortality and cystic fibrosis phenotype. *J. Cyst. Fibros.*, **14**, 580–586.
8. Espel, J.C., Palac, H.L., Bharat, A., Cullina, J., Prickett, M., Sala, M., McColley, S.A. and Jain, M. (2018) The relationship between sweat chloride levels and mortality in cystic fibrosis varies by individual genotype. *J. Cyst. Fibros.*, **17**, 34–42.
9. Xu, X., Kasembeli, M.M., Jiang, X., Tweardy, B.J. and Tweardy, D.J. (2009) Chemical probes that competitively and selectively inhibit Stat3 activation. *PLoS One*, **4**, e4783.
10. Diallo, M. and Herrera, F. (2022) The role of understudied post-translational modifications for the behavior and function of signal transducer and activator of transcription 3. *FEBS J.* https://doi.org/10.1111/febs.16116.
11. Becker, T., Jansen, S., Tamm, S., Wienker, T.F., Tummler, B. and Stanke, F. (2007) Transmission ratio distortion and maternal effects confound the analysis of modulators of cystic fibrosis disease severity on 19q13. *Eur. J. Hum. Genet.*, **15**, 774–778.
12. Stanke, F., Hector, A., Hedtfeld, S., Hartl, D., Griese, M., Tümmler, B. and Mall, M.A. (2017) An informative intragenic microsatellite marker suggests the IL-1 receptor as a genetic modifier in cystic fibrosis. *Eur. Respir. J.*, **50**, 1700426.
13. Elborn, J.S. (2016) Cystic fibrosis. *Lancet*, **388**, 2519–2531.
14. (1988) Cystic fibrosis in the United Kingdom 1977-85: an improving picture. British Paediatric Association Working Party on Cystic Fibrosis. *BMJ*, **297**, 1599–1602.
15. Elborn, J.S., Shale, D.J. and Britton, J.R. (1991) Cystic fibrosis: current survival and population estimates to the year 2000. *Thorax*, **46**, 881–885.
16. Anderson, C.D., Nalls, M.A., Biffi, A., Rost, N.S., Greenberg, S.M., Singleton, A.B., Meschia, J.F. and Rosand, J. (2011) The effect of survival bias on case-control genetic association studies of highly lethal diseases. *Circ. Cardiovasc. Genet.*, **4**, 188–196.
17. Stanke, F., Becker, T., Kumar, V., Hedtfeld, S., Becker, C., Cuppens, H., Tamm, S., Yarden, J., Laabs, U., Siebert, B. *et al.* (2011) Genes that determine immunology and inflammation modify the basic defect of impaired ion conductance in cystic fibrosis epithelia. *J. Med. Genet.*, **48**, 24–31.
18. Stanke, F., Becker, T., Ismer, H.S., Dunsche, I., Hedtfeld, S., Kontsendorn, J., Dittrich, A.-M. and Tümmler, B. (2021) Consistent assignment of risk and benign allele at rs2303153 in the CF modifier gene SCNN1B in three independent F508del-CFTR homozygous patient populations. *Genes*, **12**, 1554.
19. Zhao, W., Jaganathan, S. and Turkson, J. (2010) A cell-permeable Stat3 SH2 domain mimetic inhibits Stat3 activation and induces antitumor cell effects in vitro. *J. Biol. Chem.*, **285**, 35855–35865.
20. Lassmann, S., Schuster, I., Walch, A., Göbel, H., Jütting, U., Makowiec, F., Hopt, U. and Werner, M. (2007) STAT3 mRNA and protein expression in colorectal cancer: effects on STAT3-inducible targets linked to cell survival and proliferation. *J. Clin. Pathol.*, **60**, 173–179.
21. Sham, P.C. and Curtis, D. (1995) Monte Carlo tests for associations between disease and alleles at highly polymorphic loci. *Ann. Hum. Genet.*, **59**, 97–105.