**Original Research**

**Transforming Growth Factor β1 and Gap Junction Protein Alpha 4 Gene Heterogeneity in Relation to the Severity of Clinical Disease in Cystic Fibrosis**

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**Abstract**

**Background:** High TGFβ1-producing variants cause severe clinical disease in F508del homozygous patients. Lately, we showed that a single nucleotide polymorphism (SNP), rs41266431, in the GJA4 gene modifies the disease severity of cystic fibrosis (CF). Our aim was to investigate whether the clinical phenotype associated with GJA4 variants was independent of TGFβ1 variants. **Methods:** Homozygous F508del patients (n = 115, mean age 27.2 years, m/f (65/50)) were included in this study. A deep sequence analysis was performed for GJA4 and TGBβ1, and disease severity was assessed over 3 years using lung function tests (LFTs), body mass index, diabetes mellitus, colonization with *Pseudomonas aeruginosa*, survival to end-stage lung disease (ESLD), as well as distinct inflammatory biomarkers. **Results:** The analyses revealed that one SNP (rs41266431) in GJA4 may be clinically relevant. Carriers homozygous for the G variant (n = 84; 73%) presented with worse LFTs (forced vital capacity (FVC) % predicted: mean 80/86.6, \(p < 0.035\)) and a lower survival to ESLD (\(p < 0.029\)). For the TGBβ1 variant: 509 carriers of the C variant (CT + CC genotype, n=105, 91.3%) had better LFTs (Forced expiratory flow at 75% of the FVC (FEF75% predicted: median 40/29.5, \(p < 0.015\)), although a similar outcome to ESLD. A gene–gene interaction was not observed between TGBβ1 and GJA4 variants for any clinical measure. **Conclusions:** GJA4 variants are independent of TGBβ1 variants. Both variantshad an impact on the LFTs, although only GJA4 variants were associated with an improved outcome for ESLD. **Clinical Trial Registration:** The study was registered with ClinicalTrials.gov, number NCT04242420, retrospectively on January 24th, 2020.

**Keywords:** cystic fibrosis; transforming growth factor beta1; gap junction protein alpha 4; bronchial inflammation; F508del homozygous; gene–gene interaction; geno-/phenotype relation; precision medicine

1. **Introduction**

Cystic fibrosis (CF) is an autosomal recessive genetic disorder. The basic defect in the cystic fibrosis transmembrane regulator (CFTR) gene disrupts the function of the cystic fibrosis transmembrane regulator protein. CFTR encodes a chloride-conducting transmembrane channel, which regulates anion transport and airway mucociliary clearance [1]. Several bodily systems are thus affected. Co-morbidities caused by the functional failure of CFTR occur in the pancreas (malabsorption and CF-related diabetes), liver (biliary cirrhosis), and sweat glands (heat shock), and can also result in infertility.

The major cause of morbidity and mortality in CF is the progressive pulmonary destruction, mostly due to a leukocyte-driven inflammation in CF lung disease [2]. Previously, we were able to show in F508del homozygous patients that there was a negative correlation between blood cytokines, such as transforming growth factor β1 (TGBβ1), and pulmonary function [3–5].
In CF patients with the same CFTR genotype (e.g., delta F508 (homozygous) [6,7]), there are wide variations in the lung disease phenotypes [8,9]. Large studies analyzing modifier genes provided evidence indicating that these modifier genotypes were more important for the pulmonary phenotype than the CFTR genotype. Severe pulmonary disease in F508del homozygous patients was associated with high TGBβ1-producing alleles [10]. These patients have increased levels of TGBβ1 in biomaterials, such as bronchoalveolar lavage (BAL) fluid (F) [11] and blood [12,13], which is linked to fibrosis, goblet cell hyperplasia, abnormal inflammatory responses, and dysregulated ion transport.

The molecular mechanism of how TGBβ1 modifies CF lung disease remains unclear [11]. It has been shown by studies in vitro using human airway cells that TGBβ1 downregulates CFTR [14–16]. However, the relevance of these findings to CF, where CFTR activity might be minimal or completely absent is interestingly unclear. The relative impact of TGBβ1 expression on critical aspects of lung pathology in the presence and absence of CFTR expression remains unknown [11].

Recently, we demonstrated that carriers of the A allele with a single nucleotide polymorphism (SNP), rs41266431, in the gap junction protein alpha 4 (GJA4) gene are better protected against end-stage lung disease (ESLD) and produce better lung function tests. The explanation is that this SNP causes an amino acid substitution [17]. Moreover, this variant has no effect on the frequency of acquiring Pseudomonas aeruginosa (P. aeruginosa). However, CF patients chronically colonized with P. aeruginosa, and carriers of the A allele have significantly better pulmonary function than those with the G/G genotype.

CFTR modulator therapies targeting the basic defect in CF and restore CFTR function are currently available. Moreover, they can effectively improve nutritional status, as well as the quality of life, via lung function and a reduction in pulmonary exacerbations. Therefore, the question arises, as to whether there remains a need for further genotyping of disease-modifying variants. The most successful CFTR modulator Trikafta (elixacaftor, ivacaftor, and tezacaftor) was firstly licensed in 2019; however, data are lacking as to the evidence of a long-lasting effect (>10 years). Similarly, for Ivacaftor, e.g., the effect on the body mass index (BMI) was no longer statistically significant after 5 years [18]. Moreover, we cannot evaluate the long-term side effects (e.g., to the liver) of using a triple modulator therapy. Thus, precise genotyping might be needed to prevent any unnecessary use in patients, e.g., those exhibiting a mild clinical phenotype but at high risk of liver disease. In addition, there are also countries with a moderate healthcare plan/budget. Precise genotyping to predict the clinical phenotype could help to decide the patients that should be prioritized for treatment with an expensive CFTR modulator.

The aim of this paper was two-fold: Firstly, to examine the impact of a well-documented pulmonary CF modifier, TGBβ1 [10,19], on clinical phenotype in F508del homozygous CF patients in a large German cohort and in the initial Dutch cohort. Secondly, to test for a potential co-modulation of the GJA4 and TGBβ1 genotypes on the clinical disease phenotypes.

2. Material and Methods

2.1 Patients

For the exploratory study, F508del homozygous patients were recruited from the cystic fibrosis ward of the Children’s Hospital Medical Center, University of Bonn, Germany. Patients from the CF centers of the Goethe University Frankfurt, Germany and the Department of Respiratory Medicine, Amsterdam University Medical Center (Amsterdam UMC). Of note, the Netherlands cohort served as the replication study sample.

After approval of the ethics committee of the University of Bonn (178/01 + 092/17), Frankfurt (07/02 + 206/16), and Amsterdam UMC (NL60220.018.16), written informed consent was signed by all the representatives of the patients and/or their legal guardians.

The study was retrospectively registered with ClinicalTrials.gov (NCT04242420), on January 24th, 2020, and updated on November 24th, 2020, and July 7th, 2021.

Subjects were excluded from the study if they were treated with systemic steroids 14 days preceding the trial beginning, had participated in another study within the past 30 days, had been treated with a lumacaftor/ivacaftor combination (Orkambi), or were status after lung transplantation and unable to perform all the procedures required in the study.

Lung function testing (spirometry (forced expiratory volume in one second (FEV1), forced vital capacity (FVC), and forced expiratory flow at 75% of the pulmonary volume (FEF75))), as well as a blood sample, was provided by every subject. Lung function testing was performed in accordance with the recommendations of the American Thoracic Society and the European Respiratory Society [20].

2.2 Laboratory Testing

Serum TGBβ1 was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Wiesbaden, Germany), according to the manufacturer’s manual, and measured as previously described [4]. Serum interleukin-8 was analyzed by chemiluminescence (Immulite, Siemens Healthcare Diagnostics, Eschborn, Germany, formerly DPC Biermann), as described previously [3,4,21].

2.3 Genotyping

2.3.1 Transforming Growth Factor β1

Genetic testing for the SNP-509 (rs1800469) was performed as published previously by Drumm [10]. For
the rs1800469 SNP, no evidence of a deviation from the Hardy–Weinberg equilibrium was detected (p = 0.11016).

2.3.2 Gap Junction A4

For the rs4126431 variant, genotyping was conducted as described previously [17]. In our previous publication [17], the cohort consisted of 116 patients. However, TGBβ1 variants were only available in 115 patients. Therefore, to precisely evaluate these variants, we analyzed the data for these 115 patients. Here, no evidence of a deviation from the Hardy–Weinberg equilibrium was detected for the rs41266431 SNP (p = 0.36816217).

2.4 Data Analysis

For continuous data, a comparison of aggregated outcomes (median over 3 years) was performed. We calculated and tested the parametric data mean, standard deviation, and 95% confidence intervals (CIs) using the Student’s t-tests. Non-parametric data were presented as the median and interquartile range (IQR), while the Mann–Whitney U-Test was used for unpaired samples. To categorize patients as either P. aeruginosa positive or negative individuals, sputum or nasopharyngeal swab culture results were used. Analysis of the binary data was performed by Chi-squared test. Kaplan–Meier analysis was used for survival analysis. In addition, to estimate the effect of the TGBβ1 and gap junction protein alpha 4 genotypes and chronic P. aeruginosa colonization on pulmonary functions, a mixed linear model was performed. Covariates, such as age and BMI were considered for additional multivariate analyses. To estimate the effect of TGBβ1 and gap junction protein alpha 4 genotype and chronic colonization with P. aeruginosa on serum TGBβ1 levels, a general linear model for cross-sectional data was applied.

Statistical significance was assumed if the p-value was <0.05. SPSS (version 26.0, IBM Corp., Armonk, NY, USA) was used for all statistical analyses.

3. Results

3.1 Patient Characteristics

In total, 115 F508del homozygous patients (65 male and 50 female Caucasian subjects, (p < 0.191); 97 German and 18 Dutch patients) were recruited for the study. The mean age was 27.2 years (95% CI (24.8–29.6 years), range 7–60 years). All patients were able to perform pulmonary function testing. The following clinical data were available: Microbiological (swabs or sputum cultures) from 114 study subjects, cystic fibrosis-related diabetes (CFRD) in all German patients (n = 97). A total of 25 patients were recruited from Bonn and used in the exploratory study. Thereafter a total of 72 patients from Frankfurt and 18 Dutch patients from Amsterdam were available for further analyses in the replication study.

3.2 Haplotyping

Genotyping was available for all patients (n = 115). Analysis of the TGBβ1 variant-509 revealed that 55 (47.8%) were homozygous for the CC variant, 50 (43.5%) were heterozygous (CT), and 10 (8.7%) were homozygous for the TT variant. Grouping was conducted as published by Drumm [10]: dominant model (TT + CT genotype, n = 60, 52.2%), codominant model (CC + TT genotype, n = 65, 56.5%), and recessive model (CT + CC genotype, n = 105, 91.3 %). Regarding the gap junction protein alpha 4 variant (rs41266431), the patients were similarly grouped by those homozygous for the G allele (G/G genotype, n = 84, 73%) and as carriers for the A allele (A/G + A/A genotype, n = 31, 27%). Concerning gene–gene interactions, 4 haplotypes were created: TGBβ1 T/T + GJA4 G/G (n = 9, 8%), TGBβ1 carriers of C + GJA4 G/G (n = 75, 65%), TGBβ1 T/T + GJA4 carriers of A (n = 1, 1%), and TGBβ1 carriers of C + GJA4 and carriers of A (n = 30, 26%). The characteristics of the patients with the haplotypes defined above are provided in Table 1.

Furthermore, to create extreme genotypes, we summarized the haplotypes as follows: High risk of severe clinical disease (n = 9; 7.8%), i.e., the carriers of the TGBβ1 risk diplotype (TT genotype) and GJA4 (G/G genotype). Intermediate risk (n = 76, 66.1%), i.e., patients with one protective and one risk variant. Lastly, patients were classified at low risk of more severe disease when they were carriers of protective variants for TGBβ1 and GJA4 (n = 30, 26%). The characteristics of the patients relating to the risk haplotypes are provided in Supplementary Table 1.

3.3 Mortality

3.3.1 Transforming Growth Factor β1

No significant difference was identified between the observation times for the two genotypes (T/T vs. C/T + CC: median 34/27 years; IQR (29.75–41.75/19–38 years); range 20–53/9–62 years, respectively; p < 0.15).

Moreover, the CT/CC genotype was the only genotype related to mortality within this study (for each setting: death without lung transplantation, death after transplantation, lung transplants death, and survivors), with 0 deaths among the T/T genotype but the log-rank test (Kaplan–Meier curves) was not significant (p < 0.296, 0.218, p < 0.178) (Fig. 1).

3.3.2 Gap Junction Protein Alpha 4

Furthermore, we did not observe any significant differences in the observation time between the two genotypes (G/G genotype/(A/G; A/A genotype): mean 29.1/32.2 years; 95% CI (26.5–32.8; 27.1–37.4 years); range 9–58/12–62 years, respectively; p < 0.248). There were nine deaths that occurred among patients homozygous for the G allele, while no deaths occurred among the carriers of the A allele (heterozygous as well as homozygous) among those without lung transplantation.
### Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TGFβ1 (–509 C/T)</th>
<th>GJA4 (rs41266431)</th>
<th>Haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(T/T)</td>
<td>(T/C; C/C)</td>
<td>(T/T+GG)</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 105)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>Age (years)</td>
<td>32.5</td>
<td>24</td>
<td>26.4</td>
</tr>
<tr>
<td>SEX(m/f)</td>
<td>(6/4)</td>
<td>(57/48)</td>
<td>(47/37)</td>
</tr>
<tr>
<td>P. aeruginosa+ (%)</td>
<td>60</td>
<td>67</td>
<td>64</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>22</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td>BMI (%)</td>
<td>53.5</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>FEV1 (%)</td>
<td>67</td>
<td>67</td>
<td>68</td>
</tr>
<tr>
<td>FEF75 (%)</td>
<td>29.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.4</td>
</tr>
<tr>
<td>FVC (%) predicted</td>
<td>75</td>
<td>84</td>
<td>80&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGFβ1 (ng/mL)&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>23.8</td>
<td>25.9</td>
<td>26.4</td>
</tr>
<tr>
<td>Interleukin-8 (pg/mL)&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>15.6</td>
<td>10.8</td>
<td>11.9</td>
</tr>
<tr>
<td>CRP (mg/dL)&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>1.35</td>
<td>2.57</td>
<td>2.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>p < 0.015; <sup>b</sup>p < 0.035, <sup>1</sup>only Germans, <sup>2</sup>median. BMI, body mass index; FEV1, forced expiratory volume in one second; FEF75, forced expiratory flow at 75% of the pulmonary volume; FVC, forced vital capacity.

Thirty years was the median age of death. Carriers of the A allele survived longer (p < 0.044) according to the log-rank test (Kaplan–Meier curves). As there were no deaths in the Dutch cohort, we calculated the log-rank test for the German cohort alone: there was a trend (p < 0.053) for longer survival of carriers of the A allele (G/G genotype/(A/G; A/A genotype): mean age of 30/34 years; 95% CI (26.5–32.5, 27.2–41 years); range 9–58/12–62 years, respectively).

Considering the nine deaths of CF patients who did not receive a lung transplant until 2018, four patients received a lung transplantation. Therefore, we have to consider the 13 total deaths for the G/G genotype vs. none in the carriers of the A allele (p < 0.009). As there were only deaths in German patients, there was also a significant difference, according to the log-rank test (p < 0.013), in the German cohort.

In addition, throughout the whole cohort, only 2 of the 6 patients to receive a lung transplant survived. Thus, we classified 15 as reaching the CF end stage (death or lung transplant). Fourteen had the G/G genotype, while only one CF patient, who was alive following the lung transplant, was a carrier of the A allele. Therefore, for the CF end stage, homozygosity for the G/G genotype was significantly higher (p < 0.029). As there were no end-stage CF patients in the Dutch cohort, the German cohort was analyzed separately (p < 0.039).

Cox regression analysis revealed a seven-fold (CI 0.91–52.6) higher risk for those with the G/G genotype of experiencing severe lung disease than the overall cohort (p < 0.061). For the German cohort, the risk was 6.5-fold higher (CI 0.84–50) for the G/G genotype than for carriers of the A allele (p < 0.073).

### 3.3.3 Haplotypes

There was no difference between the observation time for the four haplotypes in this study ((TGBβ1 T/T + GJA4 G/G) vs. (TGBβ1 carriers of C + GJA4 G/G) vs. (TGBβ1 T/T + GJA4 carriers of A) vs. (TGBβ1 carriers of C + GJA4 carriers of A)), the median years of age was 34/26/53/27.5; IQR: 28.5–39.5/19–38/53/20.5–44.25 years; range 20–44/9–58/53/12–62 years, respectively; p < 0.179).

Among those who did not receive a lung transplantation, there were only deaths (n = 9) among the TGBβ1 carriers of C + GJA4 G/G. The median age of death was 30 years old. The log-rank test (Kaplan–Meier curves) indicated that there were no significant differences between the haplotypes in terms of survival; or death without lung transplantation (p < 0.124).
Table 2. Characteristics of patients with chronic *P. aeruginosa* colonization.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TGFβ1 (−509 C/T)</th>
<th>GJA4 (rs41266431)</th>
<th>Haplotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(T/T)</td>
<td>(T/C; C/C)</td>
<td>(G/G)</td>
</tr>
<tr>
<td></td>
<td>(n = 3)</td>
<td>(n = 45)</td>
<td>(n = 35)</td>
</tr>
<tr>
<td>MEDIAN MEAN MEDIAN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>36 34 34</td>
<td>38 36 33</td>
<td>41</td>
</tr>
<tr>
<td>SEX (m/f)</td>
<td>0/3 14/31 24/11</td>
<td>8/5 3/0 21/11</td>
<td>8/5</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>33 49 51</td>
<td>39 33 53</td>
<td>39</td>
</tr>
<tr>
<td>BMI (%)</td>
<td>56 + 6 + 18</td>
<td>15 56 6.5</td>
<td>4</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>70 49 48</td>
<td>59 70 41.5 &amp; 62</td>
<td>62 &amp; 7</td>
</tr>
<tr>
<td>FEF75 (% predicted)</td>
<td>23 20 26</td>
<td>30 23 19</td>
<td>29 5</td>
</tr>
<tr>
<td>FVC (% predicted)</td>
<td>90 68 65</td>
<td>78 90 59.5 @ 83</td>
<td>83 @ 5</td>
</tr>
<tr>
<td>TGFβ1 (ng/mL)</td>
<td>1,2 25.3 25</td>
<td>26.1 21.2 25.3</td>
<td>21.2</td>
</tr>
<tr>
<td>Interleukin-8 (pg/mL)</td>
<td>15.6 10.6 12.1</td>
<td>7 15.6 11.9</td>
<td>7</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>1,2 1.79 1.67</td>
<td>1.67 1.75 1.8</td>
<td>1.7</td>
</tr>
</tbody>
</table>

(Haplotype TT + AG; n = 0), p† < 0.025, *p < 0.078, † p < 0.025, ‡ p < 0.036, § p < 0.014, ¶ p < 0.021. *only Germans, †median.

In summary, up to 2018, 13 patients (11.3%) who had (n = 4) and had not (n = 9) received a lung transplant among the TGBβ1 carriers of C + GJA4 G/G, unfortunately, died. There were no deaths resulting from the other haplotypes, meaning that death was more commonly associated to this haplotype (p < 0.03) (Fig. 2). Among the Dutch patients, there were no deaths reported; therefore, the German cohort was analyzed separately (p < 0.04).

End-stage lung disease: Fourteen patients were TGBβ1 carriers of C + GJA4 G/G (12.3%) and received a lung transplant. This haplotype presented a trend for significantly more end-stage lung disease (p < 0.066). One patient (1%), who was a TGBβ1 carrier of C + GJA4 carrier of A also received a lung transplantation. There were no deaths in the Dutch cohort, while the German cohort was analyzed separately (p < 0.086). The mortalities in regard to the haplotype risks are provided in the Supplementary Material of this article.

3.4 Influence of Variants on *Pseudomonas Aeruginosa* Colonization

Seventy-six (67%) patients had microbiological evidence of *P. aeruginosa* colonization (chronic n = 57 (50%), intermittent n = 19 (16.7%)).

3.4.1 Transforming Growth Factor β1

Regarding the influence of the TGBβ1 genotype (recessive model) on colonization (= intermittent + chronic): T/T genotype (n = 6 (60%)) vs. carriers of the C allele (n = 70 (67%)) (p < 0.911), as well as chronic (T/T genotype (n = 4 (40%)) vs. carriers of the C allele (n = 53 (51%)) (p < 0.742) with *P. aeruginosa*, our data does not show any impact for the overall cohort (n = 114) (Table 1).

3.4.2 Gap Junction Protein Alpha 4 Variant

For the overall cohort (n = 114), the GJA4 genotype did not have an influence on colonization (= intermittent + chronic): G/G genotype (n = 54 (64.3%)) vs. carriers of the A allele (n = 55 (55.5%)) (p < 0.911), as well as chronic (G/G genotype (n = 40 (40%)) vs. carriers of the C allele (n = 53 (51%)) (p < 0.742) with *P. aeruginosa*, our data does not show any impact for the overall cohort (n = 114) (Table 1).

Moreover, the results were similar for the German cohort (where longitudinal data were also available) for colonization (= intermittent + chronic): T/T genotype (n = 5 (55.5%)) vs. carriers of the C allele (n = 62 (70.5%) (p < 0.653) and for chronic (vs. non-chronic): T/T genotype (n = 3 (33.3%)) vs. carriers of the C allele (n = 45 (51.1%) (p < 0.487).

3.4.2 Gap Junction Protein Alpha 4 Variant

For the overall cohort (n = 114), the GJA4 genotype did not have an influence on colonization (= intermittent + chronic): G/G genotype (n = 54 (64.3%)) vs. carriers of the A allele (n = 55 (55.5%)) (p < 0.911), as well as chronic (G/G genotype vs. carriers of the A allele (n = 62 (70.5%)) (p < 0.653) and for chronic (vs. non-chronic): T/T genotype (n = 3 (33.3%)) vs. carriers of the C allele (n = 45 (51.1%)) (p < 0.487).

End-stage lung disease: Fourteen patients were TGBβ1 carriers of C + GJA4 G/G (12.3%) and received a lung transplant. This haplotype presented a trend for significantly more end-stage lung disease (p < 0.066). One patient (1%), who was a TGBβ1 carrier of C + GJA4 carrier of A also received a lung transplantation. There were no deaths in the Dutch cohort, while the German cohort was analyzed separately (p < 0.086). The mortalities in regard to the haplotype risks are provided in the Supplementary Material of this article.
aeruginosa on the disease clinical phenotypes. The characteristics of patients with chronic P. aeruginosa colonization are provided in Table 2.

3.4.3 Haplotype

For the overall cohort (n = 114), the haplotypes did not have an influence on the colonization (= intermittent + chronic): TGBβ1 T/T + GJA4 G/G (67%), TGBβ1 carriers of C + GJA4 G/G (64%), TGBβ1 T/T + GJA4 carriers of A (0%), and TGBβ1 carriers of C + GJA4 carriers of A (76%) (p < 0.669) with P. aeruginosa (see Table 1).

We evaluated the longitudinal data to analyze the influence of P. aeruginosa on the clinical disease phenotypes. The detailed data on the characteristics of patients with chronic P. aeruginosa colonization are summarized in Table 2. For haplotype risk, the characteristics of the patients are provided in Supplementary Table 2.

3.5 Pulmonary Function

FEV1 was available from all patients (n = 115), and FVC and FEF75 values were provided by 110 patients (Supplementary Table 1).

4. Discussion

We demonstrated that GJA4 variants are independent of TGBβ1 variants. Both variants had an impact on the lung function tests (LFTs), although only GJA4 variants were associated with a better outcome for death as well as for death and ESLD.

Concerning gene–gene interactions, the only haplotype that had a significant impact on survival was the haplotype TGBβ1 carriers of C + GJA4 G/G. However, in the Cox regression analysis, there was no significant augmentation of risk to death, similar to GJA4.

Analysis of the overall cohort revealed a significantly lower lung function was linked to the TGBβ1 (FEF75), as well as the GJA4 (FVC) risk variant. Though evaluating haplotypes and extreme risk haplotypes (see Supplementary Material) for gene–gene interactions did not show any augmentation of these effects. Observation of those chronically colonized with P. aeruginosa (Table 2) revealed a significantly lower lung function in the TGBβ1 carriers of C + GJA4 G/G compared to the TGBβ1 carriers of C + GJA4 A/G. In this regard, it appears that in terms of lung function, the GJA4 genotype was more important than the TGBβ1 for those chronically colonized with P. aeruginosa.

Moreover, in terms of lung function, the high-risk haplotype was not associated with more severe results compared to the other risk variants (Supplementary Table 1). In those chronically colonized with P. aeruginosa, we were able to observe significantly improved lung function values (FEV1 and FVC) in patients with the low-risk haplotype compared to those at intermediate risk. As there was no significant difference between a high- and a low-risk haplotype, it should be discussed whether haplotype risk has an impact on the clinical disease phenotype.

Several studies with large patient numbers have shown that variants of the TGBβ1 gene have an impact on clinical phenotype in F508del homozygous CF patients [10,19]. Most of this data was generated from CF populations in northern America [10]. Currently, there are fewer studies involving German patients [4,22,23], which present smaller numbers of F508del homozygous patients (n < 30) and to the best of our knowledge, Dutch patients. Trojan and coworkers [22] could not find a significant association between lung function and the polymorphisms in the TGBβ1 promoter, which corresponds to our initial study [4]. This might be attributed to the low number of F508del homozygous patients in those previous studies. Corvol et al. [23] could only find an association for the +869 T/C variant but not for the −509 T variant (rs1800469). In this regard, our cohort, which indicated a significant impact of the −509 T variant (rs1800469) on lung function, represents the first study in German CF patients to confirm the data by Drumm and coworkers [10]. They used several models to interpret the −509 variant in the TGBβ1 promoter: The odds ratio was the best for the recessive model (comparison of the T/T variant with the combined C/T and C/C genotypes). In addition to the recessive model, we also evaluated the codominant (comparison of the T/T with the C/C genotype) and the dominant models (comparison between the combined C/T and T/T genotypes and the C/C genotype). Here, we were only able to find significant differences in lung function parameters in the recessive model.

The disadvantage of this is the low incidence (<15%) rates of these variants, which could explain why the expected difference only reached statistical significance for FEF75 and not FVC. Moreover, with absolute numbers of only three patients that were homozygous for the T/T genotype of the −509 variant in chronically P. aeruginosa colonized patients (Supplementary Table 2), we doubt the relevance of a significantly better BMI in those with the risk variant in our study.

Among the 115 patients, there were 3 sibling pairs. In terms of the GJA4 genotype, all 3 were carriers of the protective A allele. For the TGBβ1 genotypes, all were carriers of the protective A allele (C/C genotype n = 5, C/T genotype n = 1). Therefore, in summary, the 3 siblings pairs were not carriers of a risk genotype. Therefore, given that the phenotype was also attributed to environmental factors, the question remains as to whether there were any differences in these sibling pairs. In detail, two pairs of siblings were chronically colonized with P. aeruginosa (18/15 y; 50/47 y). Among those, one male patient received a lung transplant, while the younger sibling of 3 years had a FEV1 of 78% predicted. The extreme differences can be attributed to compliance. For the second pair of siblings with P. aeruginosa colonization, the brother, who was again 3 years younger, had an approximately similar FEV1 (65 vs. 63% predicted). The third pair (18/15 y) also showed some differences (FEV1 80/90% predicted). This can be at-
tributed to the older sibling having intermittently P. aeruginosa, meaning the compliance was slightly better in the younger siblings. In this regard, our data are in keeping with a study on Italian CF sibling pairs [24], which reported on the heterogeneity in CF siblings with the same CFTR genotype. Moreover, our data also support these findings for two CF modifier genes (GJA4/TGBβ1).

In terms of serum TGBβ1, we could not confirm the negative correlation between serum TGBβ1 and lung function, which we found in our previous study [4] or, which was later presented in a German study [22]. Those studies with small numbers of F508del homozygous patients (n < 30) were conducted with younger patients (median age: 20 y [4] / 21 y [22]) and fewer patients. We assume that our cohort (mean age: 27 y) with a higher rate of chronic P. aeruginosa colonization (50%) compared to those studies (Trojan et al. [22]: 37.5%, Eickmeier et al. [4]: 33.9%) other proinflammatory mechanisms might be important: TGBβ1 does not affect the efficiency of phagocytosis [25–28]. Moreover, TGBβ1 can be activated either by an ανβ6 or an ανβ8-mediated pathway. The ανβ6-mediated activation appears to be fundamentally dependent on direct cell–cell contact and does not release any diffusible free TGBβ1 [29]. Such a pathway is ideally suited to the alveolar space. Nevertheless, TGBβ1 activated by this pathway can have an impact on lung function and will be influenced by high-producer genotypes, although it will not be measurable in blood. The release of free TGBβ1 is provided by activation from the ανβ8 integrin in the conducting airways [30]. This mechanism does not depend on direct cell–cell contact and should provide TGBβ1 with the ability to diffuse and translocate, thereby affecting distant cells. Moreover, ανβ8 mRNA is expressed in a variety of leukocytes [31].

We could not provide evidence of a significant impact of the SNP-509 (rs1800469) on the TGBβ1 gene in end-stage lung disease (transplantation or death). This is in keeping with the data reported in previous studies; however, to the best of our knowledge, no study has previously reported on the impact of TGBβ1 variants on ESLD.

5. Conclusions

GJA4 gene variants are independent of TGBβ1 variants. Both variants have an influence on disease phenotypes relating to lung function, although only GJA4 variants are associated with better outcomes in ESLD.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

JPL: investigation, data curation, and writing–original draft and review. ML: conceptualization, methodology, investigation, resources, writing–original draft (lab part) and review, and supervision (genetic lab). TH: investigation (genotyping). OE: investigation (inflammatory markers) and writing, review, and editing. CS: investigation and writing, review, and editing. RS: investigation, methodology (inflammatory markers), supervision (lab), resources, and writing–review and editing. SZ: conceptualization (inflammatory markers), writing, reviewing, and editing. CM: investigation, writing, review, and editing. MA: writing, review, and editing. AS: analysis of the data, review. SSG: conceptualization, methodology, data curation, formal analysis, funding acquisition, writing–original draft and review, supervision, and project administration. All authors have contributed to the manuscript and approved the submitted version. All authors have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Studies involving human participants were conducted in accordance with the Declaration of Helsinki reviewed and approved by the University of Bonn (178/01 + 092/17), University of Frankfurt (07/02 + 206/16), and Amsterdam University Medical Center (NL60220.018.16). Informed consent was obtained from all subjects involved in the study.

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Conflict of Interest

Dr. Sabina Schmitt-Grohè is on the advisory committee of Vertex and has received a travel grant from Vertex (for ECFS 2019) and ERS (ERS Research seminars). Moreover, she gave a talk to Vertex, participated in studies for the same, received a fee for a congress report (ECFS conference Liverpool 2019), and attended a vertex-sponsored conference (1.CF-Akademie, Schloss Hohenkammer). In addition, she got financial reimbursement for a talk for Deutsche CF Hilfe and received a personal fee for an expert opinion for the Institut für Qualität und Wirtschaftlichkeit im Gesundheitswesen. Dr. Stefan Zielen reports grants and personal fees from Bene-Arzneimittel GmbH, grants and personal fees from Biotest GmbH, grants from Vifor Pharma Deutschland GmbH, grants from ALK Arzneimittel, personal fees from Novar-
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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2807138.

References