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Immune Response and Exhaled Breath Profile Changes after Initiation of CFTR Modulator Therapy in Children with CF

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Abstract: Background: In recent years, cystic fibrosis transmembrane regulator (CFTR) modulating therapy has made it possible to treat the underlying pathophysiological defect in children with cystic fibrosis (CF). Response to therapy varies among patients. We investigated the immune responses and exhaled breath profile changes after the initiation of CFTR modulator therapy to explore their potential as markers of therapy response. Methods: We performed a prospective, longitudinal proof-of-principle study, investigating immune responses and exhaled breath volatile organic component (VOC) profiles prior to and during the initiation of therapy with Lumacaftor/Ivacaftor in a cohort of 17 patients with CF aged 2 to 6 years old. Response to therapy was assessed based on clinical markers and the decrease in sweat chloride. Whole blood stimulation assays were performed at t = 0, 6 and 18 weeks, while VOC analysis was performed at t = 0 and 18 weeks. Results: A pattern of immune reconstitution was found in the first 4 months of therapy. The same pattern was found in responders and non-responders. Exhaled breath VOC profiles were significantly affected by therapy. A trend toward a significant difference was found between responders and non-responders. Conclusions: Pediatric CF patients show a pattern of immune reconstitution after the initiation of CFTR modulating therapy. We hypothesize that this could be explained by the need for a pro-inflammatory profile for a more effective clearance of latent airway pathogens in the initial phase. The exhaled breath profile also clearly changes after the initiation of therapy, indicating the therapy's influence on airway inflammation and oxidative stress; thus, it might predict the response to therapy.

Keywords: pediatric; cystic fibrosis; exhaled breath; volatile organic components; gas chromatography time-of-flight mass spectrometry; immune response

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1. Introduction

CFTR modulation therapy with Lumacaftor/Ivacaftor (Orkambi®, Vertex Pharmaceuticals) has become available for children with CF and homozygous F508del mutations after pivotal phase III trials showed its safety and effectiveness in this patient group [1,2]. The response to treatment, however, seems to vary amongst patients [3]. Researchers have tried to predict the responsiveness to treatment in various ways. Studies investigating transcriptomic changes in whole blood or peripheral blood mononuclear cells found that prediction of the clinical response might be possible based on gene expression [4,5]. Dekkers et al. showed that the clinical response to treatment may be predicted by the effect of CFTR modulator therapy on rectal organoids of the patient in vitro [6].

In a cohort of 17 children with CF, we investigated the potential of two innovative methods to assess the clinical response to treatment with Lumacaftor/Ivacaftor: whole blood stimulation assays and exhaled breath analysis. Predicting clinical response can eventually help clinicians and patients make decisions about personalized treatment.

We chose to investigate whole blood stimulation assays as one of our readouts because it is known that CFTR dysfunction causes an impaired innate immune response, which may contribute to infection and (hyper)inflammation in CF patients. In a thorough review, Cohen and Prince summarized evidence that CFTR (dys)function goes well beyond viscous mucus and that it is involved in the innate immune response [7]. In vitro studies have shown abnormalities in the innate immune response in children with CF, leading to excessive inflammation [8,9]. Research from our center showed pathogen-specific cytokine signatures in CF patients, e.g., a *Mycobacterium abscessus*-specific IL-17 deficiency [10].

Several studies, mostly performed in adults, have investigated the effect of CFTR modulation on the innate immune response. Although different approaches were used, they generally show that the pro-inflammatory profile seems to return to baseline levels and that phagocytosis and the intracellular killing of pathogens improve after treatment with CFTR modulators [11–16].

The other investigation we used to predict the clinical response to therapy was exhaled breath analysis. Analyzing exhaled volatile organic compounds (VOCs) is a way to evaluate or diagnose a variety of pulmonary diseases such as asthma, chronic obstructive pulmonary disease and CF [17]. In CF, several studies have shown that analyzing VOC profiles can discriminate the exhaled breath of CF patients from healthy controls [18–20]. In the context of CFTR modulator therapy and exhaled breath research, the fraction of exhaled nitric oxide (FeNO) has been investigated, with conflicting results showing either a transient rise in FeNO after treatment or a more persistent rise in levels [21,22]. Another study showed a significant difference in the metabolic composition of breath after 3 months of treatment, which remained different from baseline until 12 months after treatment [23].

We performed a prospective, longitudinal proof-of-principle study investigating the immune response ex vivo and exhaled breath VOC profiles prior to and during therapy with Lumacaftor/Ivacaftor in 17 CFTR modulator treatment-naïve children with CF homozygous for the F508del-CFTR mutation, aged 2 to 6 years old. Age-matched healthy control subjects were recruited for analysis of the immune response.

We aimed to answer the following four research questions:

(1) What are the characteristics of the immune response prior to treatment with CFTR modulator therapy with Lumacaftor/Ivacaftor? (2) What is the effect of treatment on the immune response after 6 and 18 weeks? (3) Is there a change in the exhaled breath VOC profile from baseline to after 18 weeks of treatment? (4) Are either of the immune response changes or VOC profile changes associated with the response to treatment?

2. Methods

2.1. Patients and Control Subjects

Children aged 2 to 6 years old with CF homozygous for the F508del-CFTR mutation and starting CFTR modulator therapy with Lumacaftor/Ivacaftor were eligible for inclusion. They were recruited between May and October 2019 from the pediatric CF clinic at Amalia Children's Hospital, Radboudumc, Nijmegen. Five aged-matched healthy subjects were recruited as normal controls for the immune response analysis. These were recruited from the pediatric endocrinology outpatient clinic. Exclusion criteria for these subjects were any underlying immunological disease or a history of fever or any signs of infection in the last 4 weeks.

2.2. Measurements

As part of routine care, blood samples were taken prior to and at 6 and 18 weeks after the initiation of treatment. Clinical endpoints were measured before and after the initiation of treatment: sweat chloride (at baseline and at 6 weeks), weight and height (at baseline, 6

and 18 weeks), exacerbation rate (in the year before and after the initiation of therapy) and a non-validated therapy improvement questionnaire, scoring improvement on 8 different clinical domains (at 6 and 18 weeks; questions on improvement of cough, fatigue, and exercise intolerance, amongst others). We also collected microbiology results of cough swab and/or sputum cultures.

2.3. Responders/Non-Responders

To categorize patients into responders versus non-responders, we took the difference in sweat chloride, difference in BMI-for-age Z-score from baseline to 18 weeks, exacerbation rate and response to the therapy improvement questionnaire as outcome measures. Based on earlier studies, response to therapy in our study was defined as a decrease in sweat chloride of >30 mmol/L, an increase in BMI-for-age Z-score of 0.25 (only for patients with baseline Z-score < 0), a response of improvement in at least of 4/8 items in the therapy improvement questionnaire and fewer exacerbations in the year after versus before the initiation of therapy [2]. If 2/4 of these items (or 2/3 if the BMI-for-age Z-score is not included) were checked, the patient was considered a responder.

2.4. Collection and Processing of Samples

After informed consent, additional blood samples (3 mL Lithium–Heparin tube) were taken from control patients (once) and CF patients (prior to treatment and at 6 and 18 weeks after the initiation of treatment). This blood sample was only drawn when technically possible and after all the required blood samples needed for regular care were collected.

Breath samples were collected prior to and 18 weeks after the initiation of treatment. We used a tailored breath sampling system for children that had been developed by our departments [24]. Children breathe tidally through a silicone oro-(mouth) mask (Hans Rudolph, Inc., Shawnee, KS, USA) that separates nasal- and bronchial-expired air and that is connected to a Y-shaped, non-rebreathing two-way valve system (Hans Rudolph, Inc., KS, USA). At the inhalation port of the two-way valve, a VOC filter (Honeywell, Charlotte, NC, USA) is attached, which ensures the inhalation of environmental air free from exogenous VOCs. On the expiratory port of the valve, a custom-made 3 L polycarbonate bag (Tedlar® bag, MediSense, Groningen, The Netherlands) is connected to collect the exhaled breath. The child was first asked to breathe tidally for 3 min to ensure that their entire lung capacity was refreshed with environmental VOC-free air. After 3 min, a sampling bag was attached to the exhalation port. After the bag was filled up to a maximum of 80%, the bag was disconnected from the sampling device. The bag was emptied across a stainless steel, two-bed sorption tube filled with Carbograph 1 TD/Carbopack™ X (Markes International, Llantrisant, UK) for the rapid adsorption and stabilization of volatile compounds. The tubes were airtight-capped and stored at 4 °C before analysis by means of GC-TOF-MS. At first, VOCs were released from the tube using thermal desorption (Unity desorption unit; Markes International, Llantrisant, UK). Next, the sample was injected into the gas chromatography (GC) capillary (Trace GC; ThermoFischer Scientific, Austin, TX, USA). In the GC capillary, VOCs were separated and subsequently detected and identified using time-of-flight (TOF) mass spectrometry (MS) (Tempus Plus; ThermoFischer Scientific, Waltham, MA, USA). Detailed information about the conditions and settings of the GC-TOF-MS measurements has been provided previously [25]. The preprocessing of the raw GC-TOF-MS spectra consisted of noise removal, baseline correction, alignment, and peak detection. Thereafter, complementary compounds in different samples were linked based on the similarity of their retention times and mass spectra. The area under the peak was calculated for each compound. To make the spectra comparable, normalization to the total area was performed [26].

2.5. Whole-Blood Stimulation Assays

For the whole-blood stimulation assay, the following stimuli were used: RPMI medium, purified *Escherichia coli* lipopolysaccharide (LPS) 10 ng/mL, Pam3CysSK4 (P3C) 10 µg/mL,

polyinosinic:polycytidylic acid (poly(I:C)) 10 μg/mL and the following heat-killed pathogens: *Staphylococcus aureus* 10^7/mL, *Haemophilus influenzae* 10^6/mL, *Candida albicans* 10^6/mL, *Pseudomonas aeruginosa* 10^7/mL, and *Streptococcus pneumoniae* 10^7/mL. After drawing blood, 200 μL of fresh blood was incubated with 800 μL of the stimulus at 37 °C for 24 h (all stimuli), 48 h (RPMI, LPS, *Candida albicans* and *Haemophilus influenzae*) or 7 days (RPMI, *Candida albicans* and *Staphylococcus aureus*). After incubation, the supernatant plasma was divided over 96-well plates and stored in a −80 °C freezer until further analysis.

For the 24 h samples, multiple cytokines and chemokines were analyzed using a Luminex assay. Plasma samples were thawed, and debris was removed using a filter plate (Multiscreen, Merck KGaA, Darmstadt, Germany). The Luminex assay was performed according to the manufacturer's instructions (Merck KGaA). The following assay kit was used: HCYTOMAG-60K-05. In short, 25 μL of plasma was used to determine the concentrations in pg/mL of 5 cytokines and chemokines, namely Tumor Necrosis Factor alpha (TNF-α), IL-1β, IL-6, IL-8 (CXCL8) and IL-10. The mean fluorescence intensity of samples was measured with a Flexmap 3D System (Luminex Corp, Austin, TX, USA), and concentrations were calculated using Bio-Plex Manager Software (Bio-Rad Laboratories, Veenendaal, The Netherlands). When cytokine levels were undetectable or above the maximum concentration of the standard curve, respectively, the lowest level of detection (around 3 pg/mL) or the highest level of detection (which differed per cytokine) was used.

For the 48 h and 7-day samples, a single ELISA was performed according to the manufacturer's instructions to measure the concentrations of interferon gamma (IFNγ), using kit M9333 from Sanquin, Amsterdam, The Netherlands, and IL17, using kit DY317 from R&D systems, Minneapolis, MN, USA. When cytokine levels were undetectable or above the maximum concentration of the standard curve, respectively, either the lowest level of detection or the highest level of detection was used.

2.6. Statistical Analyses

Clinical data were entered into an online Castor database and exported in SPSS version 25 for further analysis. For clinical data, mostly descriptive statistics were used. ELISA data were entered into GraphPad Prism version 9. As we assumed a non-normal distribution of data, we used a Wilcoxon signed-rank test to compare the ELISA data among the 3 different time points. A two-tailed p-value of 0.05 was considered to be statistically significant.

The extensive VOC data derived from the GC analyses were utilized in an algorithm using mathematical models (for instance, neuronal networks, random forest, support vector machine, or principle component analysis) for the analysis of sensor signals, as described previously [26]. The two groups of samples being compared were the pre- and post-treatment samples and the samples from responders versus non-responders.

The training set was used for the optimization steps (i.e., variable selection and selecting the model complexity) and for developing a classification RF model. The validation set was subsequently used to validate the constructed model. In the case of the RF model, a validation was performed using the so-called out-of-bag (oob) error and cross-validation. For each RF tree, one-third of the training samples were left out and not used in the construction of the classification model. These left-out cases were next used to establish the prediction error. Variable selection was based on the variable importance as assessed by RF.

To visualize the results, principal coordinate analysis (PCoA) was performed on the proximity matrix obtained from the RF model. The model performance was demonstrated using a receiver operating characteristic (ROC) curve using the validation sample.

2.7. Ethical Considerations

Our institutional ethical review board waived approval for this study as they considered the study procedures to be of negligible burden and not harmful for the patients. The blood samples for the purpose of this study were only taken after the material needed for clinical follow-up was obtained during the blood sampling performed as part of the routine clinical care. Furthermore, we acted in accordance with the "Code verzet" (code resistance)

set by the Dutch Pediatric Society, and no extra blood samples were taken for the purpose of this study.

Prior to participation, informed consent was requested after informing the parents and patient. All participating patients were given a unique study number, and all the clinical data were collected using a Castor database. Hence, the patient data were coded, and the key for patient identification was kept by the principal investigator.

3. Results

All 17 patients included in the study completed the follow-up. A blood draw for the study was (partially) successful in 47/51 (92%) of the time points. The blood draw was successful in all control subjects. Exhaled breath collection was carried out in 30/34 (88%) of the time points (see Table 1).

Table 1. Patient characteristics and treatment responses of the study subjects.

	All (SDS)	Responders (SDS)	Non-Responders (SDS)
Gender (male/female)	11/6	5/4	6/2
Age (years)	3.8 (1.3)	4.1 (1.5)	3.3 (0.8)
BMI Z-score			
t = 0	−0.23 (0.8)	−0.62 (0.7)	+0.21 (0.7)
t = 18 weeks	−0.11 (0.8)	−0.42 (0.7)	+0.23 (0.8)
Delta Z-score	+0.12 (0.4)	+0.20 (0.3)	+0.02 (0.4)
Sweat chloride (mmol/L)			
t = 0	91 (6.6)	92 (7.4)	91 (5.4)
t = 18 weeks	66 (13)	63 (14)	69 (10)
Delta sweat chloride	−25 (10)	−29 (9)	−22 (9)
Clinical improvement score (n/8 items)			
t = 6 weeks	2.5 (1.9)	2.9 (2.1)	2.2 (1.6)
t = 18 weeks	2.6 (2.2)	3.0 (1.8)	2.0 (2.6)
Exacerbation difference (year after vs. year before)	−0.6 (1.0)	−1.1 (0.6)	0.0 (1)

3.1. Treatment Response

Concerning the response to the Lumacaftor/Ivacaftor treatment: all patients showed a decrease in sweat chloride with delta values varying from 6 to 41 mmol/L. A total of 6/17 (35%) of patients had a delta value of more than 30 mmol/L. Of the patients with a BMI-for-age Z-score < 0 at baseline, 7/11 (63%) showed an increase in their Z-score and 5/11 (45%) showed an increase of 0.25 or more at t = 18 weeks. Looking at the difference in exacerbations in the year after compared with before initiation of the Lumacaftor/Ivacaftor treatment, 10/17 (59%) had fewer exacerbations. On the therapy improvement questionnaire, in 7/33 (21%) of the timepoints (being 5 patients), ≥4/8 items were filled in as positive. A total of 7/33 of the timepoints were missing. Using the algorithm described in the Section 2.3, 9/17 (53%) of the patients were categorized as responders. A summary of these results can be found in Supplementary Table S1.

3.2. Stimulation Assays

In the 24 h stimulation assays, a clear pattern in cytokine levels over time was observed with an initial, often significant, rise in levels from baseline to 6 weeks and a return to baseline at 18 weeks. The baseline (t = 0) and 18-week levels corresponded to the levels found in control patients. This pattern was observed most clearly for IL1β, IL6, IL8 and, to a lesser extent, for TNFα and IL10 and was not dependent on the stimulus used. Results for the more general stimuli, LPS, P3C and Poly(I:C), are shown in Figure 1A.

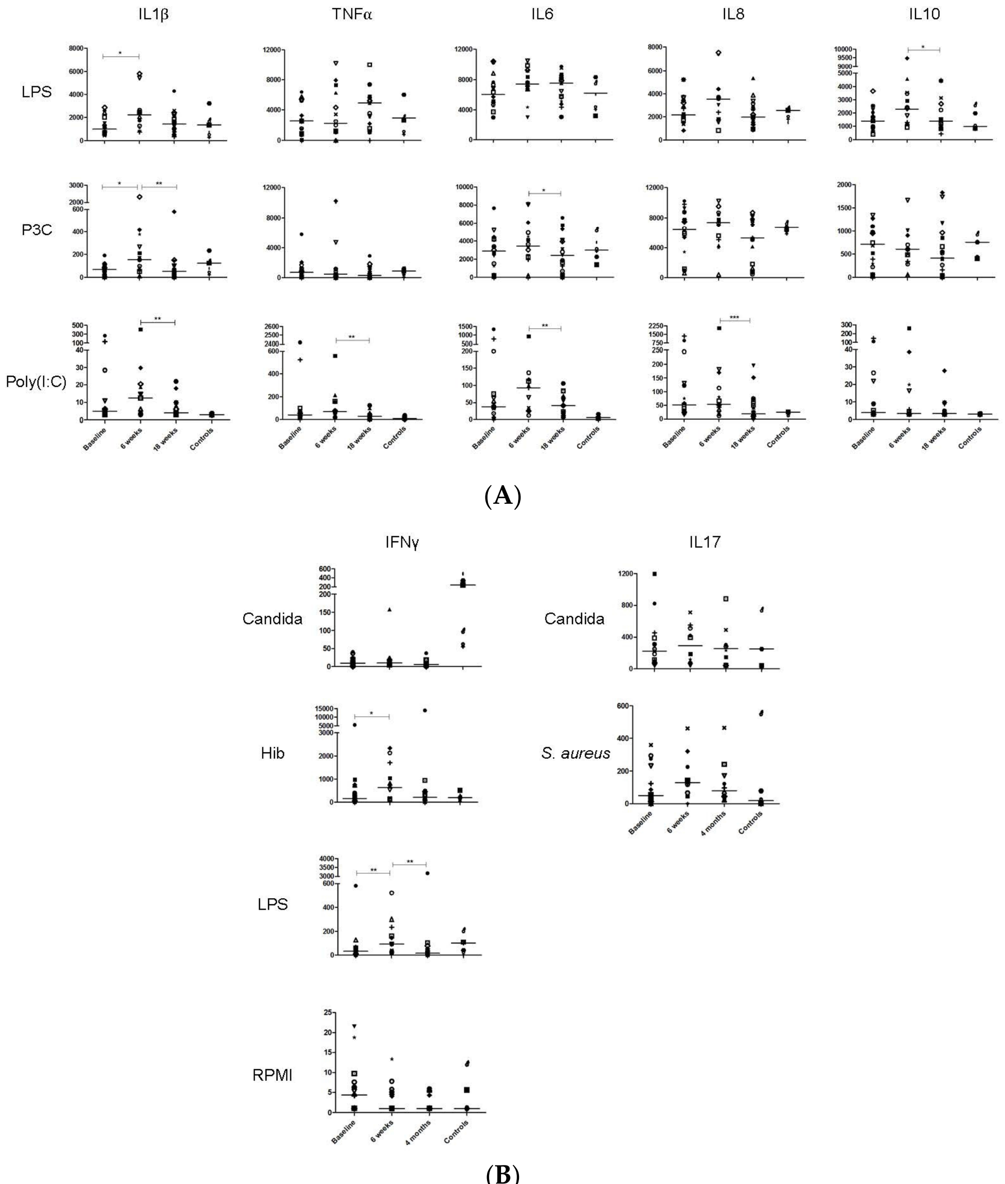

Figure 1. (**A**) Results of the 24 h whole-blood stimulation assay at different time points. Each figure (triangle, circle, square) represents an individual study subject. (**B**) Results of the 48 h and 7-day whole-blood stimulation assays at different time points. Each figure (triangle, circle, square) represents an individual study subject. (**A**): ELISA data for 5 cytokines/chemokines after 24 h stimulation with LPS, P3C and poly(I:C). X-axis represents different time points for patients and data of controls, Y-axis the concentration found in pg/mL. Each special represents one case. Significance: * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$. (**B**): ELISA data for IFNγ and IL-17 stimulation with different stimuli after respectively 48 h and 7 days. X-axis represents different time points for patients and data of controls, Y-axis the concentration found in pg/ml. Each special represents one case. Significance: * = $p \leq 0.05$ and ** = $p \leq 0.01$.

For IFNγ (48 h) and IL17 (7 days), the same pattern was seen after stimulation using different stimuli, with significant changes mainly for IFNγ, which rose from baseline to 6 weeks and then returned to normal (Figure 1B). For RPMI, some patients showed a raised level of IFNγ that returned to normal at later time points. In control subjects, a strikingly high production of IFNγ was seen after stimulation with Candida compared with the patients.

The same described pattern was seen in patients who were classified as responders and non-responders, with no significant differences between these groups.

3.3. Exhaled Breath Analysis

Explorative analysis of data based on the whole breath profile (360 compounds included) aimed for the identification of natural groupings. Data are presented in a score plot, where each point is the measurement of an individual (Figure 2A). The grouping of points seems to indicate two clusters: T0 (baseline before treatment) and T1 (18 weeks after initiation of treatment). Subsequently, a discriminatory model was built to differentiate between T0 and T1. The validation was performed via cross-validation. The ROC curve is shown, with an AUC of 0.85 (Figure 2B). A set of 18 of the most discriminatory compounds was selected. Supplementary Figure S1 shows the VOCs' importance in differentiating pre- and post-treatment individuals. The magnitude of the bars indicates the importance of the compounds, with higher numbers representing more important compounds. This set of 18 compounds was further investigated by looking at natural groupings in the data. The result is shown in the scatter plot (Figure 3A). Two distinct clusters are visible, indicating that the breath profile at T0 and T1 is different. Figure 3B shows the same figure, but with the points color-coded with respect to responders and non-responders. As shown from Figure 3B, the VOC profiles of responders and non-responders are not significantly different and partially overlap.

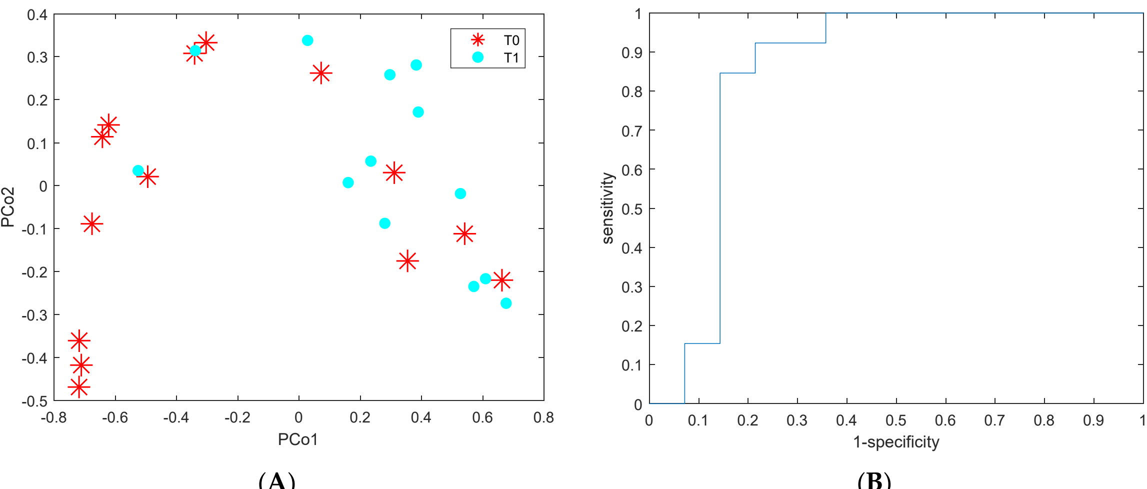

Figure 2. (**A**) The explorative analysis of the whole breath profile (360 compounds included). The corresponding score plot demonstrates the grouping of the points into 2 clusters: T0 and T1. (**B**) ROC curve for the validation set of the random forest model comparing individuals before and post-treatment. The AUC of the ROC curve is 0.85.

The putative identification of the relevant VOCs was carried out using the National Institute of Standards and Technology (NIST) library in combination with expert interpretation.

Of the 18 discriminatory compounds analyzed, 12 showed a decrease in concentration in the exhaled breath of individuals at T1, while the remaining 6 compounds exhibited an increase. The putative identification of the five most discriminatory compounds was performed, revealing 2-pentanone, heptane, 2-butanone, nonanal and 3-methylpentane. Interestingly, the concentration of these compounds showed downregulation in the exhaled breath samples collected at T1, i.e., post-treatment. Since these compounds are generally associated with inflammatory and reactive oxygen species (ROS)-related processes, their decrease in amount might suggest a reduction in inflammation.

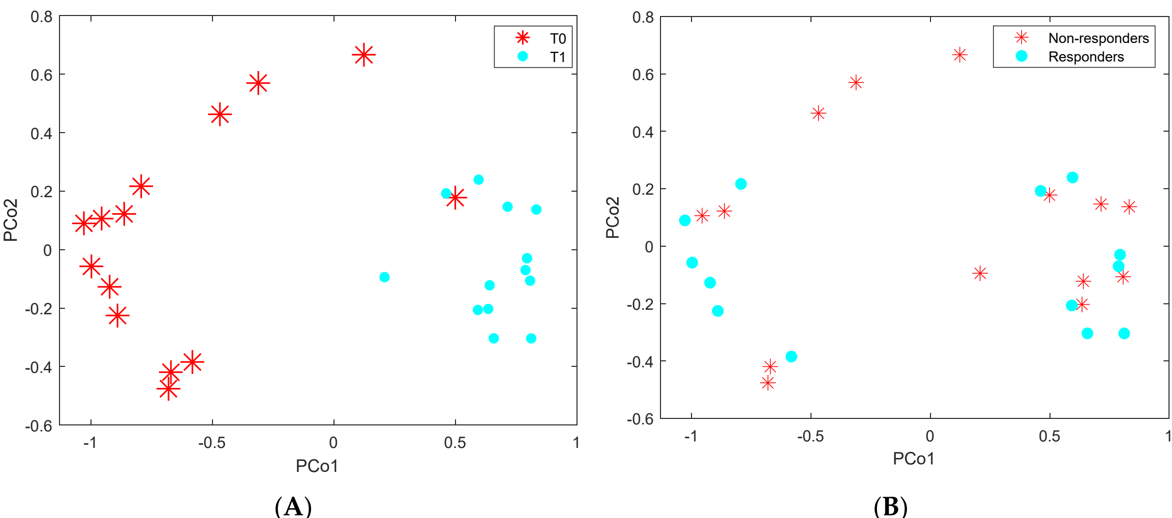

Figure 3. (**A**) Principal coordinate analysis score plot for PCo1 and PCo2, performed on the data containing the 18 most discriminatory compounds of T0 compared with T1, showing that the breath profile at the 2 time points is different. Each point corresponds to a single breath fingerprint. (**A**) the samples are color-coded with respect to pre- (T0) and post-treatment (T1). (**B**) The samples are color-coded with respect to responsiveness to the therapy (Lumacaftor/Ivacaftor responders and non-responders).

To provide a more comprehensive understanding, additional data on the concentration of other exhaled compounds at various time points and their corresponding biological significance would be valuable. Moreover, it would be beneficial to include statistical analyses and comparisons with baseline levels to better illustrate the trends and to validate the significance of the observed changes. Further insights into the pathways and mechanisms through which these compounds are modulated could also enhance the interpretative depth of the findings.

4. Discussion

A few interesting trends were observed in our study. Firstly, when investigating the immune response by the whole-blood stimulation assay, a clear trend was seen where cytokine/chemokine levels increased from 0 to 6 weeks and returned to baseline at 18 weeks. The levels found at t = 0 and 18 weeks in the CF group corresponded with the levels found in control subjects. This seems to suggest some sort of immune reconstitution inflammatory syndrome in vitro. This syndrome is observed in patients recovering from immunodeficiency, mainly patients with HIV and low CD4 counts who have initiated antiretroviral therapy. A possible explanation for our findings could be because phagocytosis and the killing of pathogens by macrophages seem to improve with CFTR modulation [11,16]. It might be that in our patients, in the initial phase after the initiation of CFTR modulation, "latent" infections are more effectively cleared, leading to a pro-inflammatory profile returning to baseline after 18 weeks. This hypothesis is supported by the results of studies that have found lower density levels of *Pseudomonas aeruginosa* or lower total bacterial load in patients after the initiation of CFTR modulation [13,27].

In our study, the immune response was not associated with the clinical response to treatment: in responders and non-responders, the same pattern was observed, with no significant differences. It might be that other factors play a more significant role, like comorbidities, past infections and microbiome changes.

Barnaby and colleagues investigated cytokine production in monocyte-derived macrophages (MDMs) from age-matched healthy controls and CF patients after stimulation with *Pseudomonas aeruginosa* [11]. They found no differences in the level of 30 cytokines between CF patients and controls. However, when the MDMs were exposed to rising concentrations of

Ivacaftor combined with two concentrations of Lumacaftor, a reduction in cytokines in CF patients was found compared with controls. This panel included the cytokines we studied.

Jarosz-Griffiths and colleagues investigated cytokine production in NLRP3-stimulated (LPS followed by the addition of ATP) PBMCs in CF patients before and 1 and 3 months after the initiation of either Lumacaftor/Ivacaftor or Ivacaftor/Tezacaftor treatment [15]. For Lumacaftor/Ivacaftor, they found a significant decline in the level of IL18 and TNFα at all time points, no significant changes in the levels of IL1β and IL6 and an increase in the level of IL10 from baseline to 3 months. Treatment with Ivacaftor/Tezacaftor showed a similar pattern.

Zhang and colleagues described changes in macrophage function in adult CF patients after the initiation of CTFR modulating therapy [16]. They performed different experiments with monocyte-derived macrophages (MDMs) from adult non-CF and CF patients without or with treatment with Ivacaftor or Lumacaftor/Ivacaftor. In one of these experiments, MDMs from the above-mentioned groups were stimulated with *Burkholderia cenocepacia*, and the cytokine levels of IL1β, IL6, IL8, IL10, IL12 and TNFα were measured after 24 h. They found a significantly higher cytokine level after stimulation for all the cytokines, except IL8, in CF patients compared with non-CF patients. In the group treated for 3 months with Ivacaftor and, to a lesser extent, in the group on Lumacaftor/Ivacaftor, these levels returned to baseline for IL6 (both groups), TNFα and IL12 (Ivacaftor only).

More recent studies investigating the effect of Elexacaftor/Tezacaftor/Ivacaftor on the innate immune response show a similar pattern as described above: better functioning of immune cells (phagocytosis, intracellular killing) and reduced levels of pro-inflammatory cytokines following the initiation of treatment [28–30].

In summary, other studies investigating the immune response after CFTR modulation therapy did not find the immune reconstitution pattern of pro-inflammatory cytokines we found. One explanation could be that we did not investigate specific immune cells like PBMCs or MDMs but used whole-blood stimulation assays. We hypothesize that although individual immune cells might show better function and decreased levels of pro-inflammatory cytokine production, they still need to deal with a higher (pulmonary) bacterial load in the initial phase of treatment. Our whole-blood stimulation assay might reflect the immune response evolving after the initiation of therapy in vivo.

With respect to exhaled breath analysis, we found a significantly altered VOC profile after 18 weeks of treatment with Lumacaftor/Ivacaftor compared with the profiles before the start of treatment. We putatively identified the five most discriminatory compounds; these were compounds associated with inflammation and oxidative stress and all decreased, suggesting favorable changes in these processes in the lung after the initiation of Lumacaftor/Ivacaftor treatment. Periodic and seasonal changes in the breath profiles in study design cannot be fully ruled out.

We observed a trend toward different VOC profiles between Lumacaftor/Ivacaftor responders and non-responders, which may indicate that exhaled VOCs may be helpful for the prediction of the clinical response to Lumacaftor/Ivacaftor in CF patients. This is an interesting observation because with the increased number of CFTR potentiators and correctors, it has become increasingly important to find diagnostic methods to predict the clinical response to these kinds of important drugs. This will help clinicians and patients to make decisions about personalized treatment.

5. Strengths and Limitations

By performing the same analyses at different time points we were able to obtain unique longitudinal data for each patient. By adding a control group, the immune response findings could be compared to a healthy population without CF.

The relatively small size of our cohort might have influenced the statistical power; for example, when zooming in on responders versus non-responders.

For the control group, new stimuli had to be produced. Although the same protocol was used to produce the stimuli, a clearly stronger stimulation in the controls was seen for Candida.

6. Conclusions

CF patients from 2 to 6 years old, homozygous for the F508del-CFTR mutation, showed a pattern of immune reconstitution in the first 4 months of the initiation of CFTR modulation with Lumacaftor/Ivacaftor (Orkambi®, Vertex Pharmaceuticals, Boston, MA, USA). We hypothesize that this is explained by a pro-inflammatory profile being needed for more effective clearance of latent airway pathogens in the initial phase, with regulation and normalization of the immune response later. This pattern does not depend on the clinical response to CFTR modulation therapy. Exhaled VOC profiles were significantly affected by treatment with Lumacaftor/Ivacaftor, indicating the influence of this drug on reducing airway inflammation and oxidative stress. A trend toward a significant difference in VOC profiles was seen between responders and non-responders, which needs to be further elucidated.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijtm4030037/s1: Table S1: Baseline characteristics and treatment response of study subjects; Figure S1: Importance of the 18 most discriminatory VOCs.

Author Contributions: Conceptualization, K.J.v.A., G.F. and J.R.; methodology, K.J.v.A., G.F., E.D. and J.R.; formal analysis, K.J.v.A., G.F., A.S.; investigation, K.J.v.A., G.F.; resources, K.J.v.A., G.F., E.D.; data curation, K.J.v.A., A.S.; writing—original draft preparation, K.J.v.A.; writing—review and editing, G.F., A.S., E.D. and J.R.; visualization, K.J.v.A., A.S.; supervision, G.F. and J.R.; project administration, K.J.v.A., J.R.; funding acquisition, J.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Our institutional ethical review board waived ethical review and approval for this study as the study procedures were assessed as being of negligible burden and not harmful for the patients (file no. 2019-5303).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The raw data of this study are available with restricted access in a Data Sharing Collection of the Radboud Data Repository with DOI: 10.34973/0n4p-d459. A request to share data can be addressed at the principal investigator.

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Abbreviations

AUC	Area under the curve
BMI	Body mass index
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
ELISA	Enzyme-linked immunosorbent assay
FeNO	Fractional exhaled nitric oxide
GC-ToF-MS	Gas chromatography time-of-flight mass spectrometry
IFNγ	Interferon gamma

LPS	Lipopolysaccharide
P3C	Pam3CysSK4
Poly (I:C)	Polyinosinic:polycytidylic acid
rMANOVA	Repeated measures analysis of variance
ROC	Receiver operating characteristic
TNF-α	Tumor necrosis factor alpha
VOC	Volatile organic components

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