

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

**Human Epididymis Protein 4 in a novel role: a new
prognostic biomarker and pathophysiological
mediator in cystic fibrosis**

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The PhD Defense takes place at:

University of Debrecen, Faculty of Medicine, Department of
Internal Medicine, Building A lecture hall
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1. Introduction and Literature review

Pathomechanism of cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive inherited genetic metabolic disorder caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. More than 2,000 variants of the CFTR gene have been identified, among which the most common is the *p.Phe508del*-CFTR allele. The CFTR protein functions as an ion channel transporting Na^+ , Cl^- , and HCO_3^- ions, thereby regulating ion and fluid transport and maintaining the proper function of the airways, digestive system, pancreas, sweat glands, and reproductive organs. As a result of genetic mutations, the CFTR ion channel is either dysfunctional or not properly expressed. This leads to the production of abnormally thick secretions in exocrine glands, ultimately causing progressive damage to the affected organs. Early mortality in patients is primarily due to progressively declining pulmonary function and the development of pulmonary fibrosis on the basis of chronic inflammation. Pulmonary fibrosis is preceded by the process known as epithelial–mesenchymal transition (EMT); however, the pathomechanism of these processes in CF remains insufficiently understood.

The clinical outcome of CF, patients' quality of life, and mortality risk are primarily determined by the extent of pulmonary involvement, the pathological processes of which begin early in infancy. The viscous mucus promotes chronic bacterial colonization, and recurrent infections trigger a persistent inflammatory response characterized by increased release of pro-inflammatory cytokines, leading to enhanced infiltration of neutrophil granulocytes. These processes result in the release of proteases and reactive oxygen species, which damage the airway epithelium. The NF- κ B pathway remains chronically activated in CF airway epithelial cells, further promoting the production of inflammatory mediators. Ultimately, this cascade may lead to bronchiectasis, airway obstruction, and respiratory failure.

CFTR modulators in the treatment of CF

The treatment of cystic fibrosis (CF) has undergone a significant transformation over the past decade. Previously, therapy primarily focused on symptomatic management, including the control of airway infections, mucus clearance, reduction of inflammation, nutritional and enzyme supplementation, and, in severe cases, lung transplantation. In addition, novel pharmacological agents—so-called CFTR modulators—are now available, which directly target the molecular cause of the disease. CFTR modulators restore the function of the

dysfunctional CFTR protein. Based on their mechanisms they are classified as potentiators, which increase frequency of the channel opening (e.g., *ivacaftor*), or correctors improving protein folding and facilitate the trafficking of the CFTR protein to the cell membrane (e.g., *lumacaftor*, *tezacaftor*, *elexacaftor*). Combination therapies integrating these mechanisms are also available. The first major breakthrough was Kalydeco® (*ivacaftor*), used as a potentiator in patients with class III mutations such as *Gly551Asp* and other gating mutations. Orkambi® (*lumacaftor/ivacaftor*) was the first combination CFTR modulator specifically developed for patients homozygous for the *p.Phe508del* mutation. Kaftrio® (marketed as Trikafta® in USA) represents a new generation triple combination therapy consisting of two correctors (*elexacaftor* and *tezacaftor*) and one potentiator (*ivacaftor*). While Orkambi® has proven effective in patients homozygous for the *p.Phe508del* mutation, Kaftrio® has also been effective even in patients with heterozygous genotypes involving the *p.Phe508del* mutation representing a substantial portion of CF patients. Early initiation of CFTR modulator therapy—prior to the development of significant structural lung damage—substantially slows disease progression, reduces the frequency of acute pulmonary exacerbations, hospitalizations, and the need for antibiotic therapy, and also mitigates extrapulmonary complications. Furthermore, it contributes to improvements in nutritional status and overall metabolic condition in affected patients.

Biomarkers associated with airway inflammation and tissue damage in CF

Since quality of life and mortality in CF are primarily determined by pulmonary status, monitoring has great importance in patient management. This requires a multidisciplinary approach that integrates clinical, functional, microbiological, imaging, and biomarker-based methods. In recent years, the role of biomarkers has gained increasing importance, as they enable the detection of early, subclinical changes when conventional parameters (e.g., FEV1) still remain within normal ranges. However, the detection of airway involvement remains limited, particularly in children. Therefore, there is an increasing need for easily measurable and highly reproducible biomarkers in blood or airway secretions that reliably reflect lung status in patients with CF. Biomarkers may facilitate the early identification of disease activity and progression, monitoring of therapeutic response, and evaluation of the efficacy of novel treatments (e.g., CFTR modulators).

Sputum and bronchoalveolar lavage (BAL) fluid reflect the local environment of airway inflammation and infection, and thus contain numerous inflammatory markers (e.g., IL-6, IL-8, IL-1 β , antiproteases, neutrophil elastase (NE), TGF- β 1, MMP-9), which may serve as potential biomarkers for predicting deterioration in lung function and for monitoring therapeutic

efficacy. However, due to the lack of standardized measurement procedures, difficulties in obtaining high quality sputum samples, and the invasive nature of BAL sampling, these approaches have not been implemented in routine clinical practice.

One of the most practical, analytically well standardized, and reproducible approaches for monitoring airway inflammation is the measurement of biomarkers in blood samples—either plasma or serum. Blood sampling is a routine, minimally invasive procedure that can be performed safely and easily regardless of age or patient group. Systemic biomarkers offer the advantage of reflecting inflammatory and immunological processes occurring in the lungs, and may correlate with clinical status, lung function (FEV1), and the effectiveness of CFTR modulator therapy. However, at present, there are no clearly validated and sufficiently sensitive plasma- or serum-based biomarkers suitable for reliably monitoring treatment efficacy. Consequently, there is a substantial need for the identification and implementation of novel, reproducible, and easily measurable biomarkers for use in the clinical management of patients with CF.

Human Epididymis Protein 4 (HE4) as a potential biomarker in CF

Human Epididymis Protein 4 (HE4), also known as Whey Acidic Protein Four-Disulfide Core Domain Protein 2 (WFDC2), is a low molecular weight glycoprotein of the whey acidic protein (WAP) family. The WFDC protein family comprises small proteins that typically has protective functions, including antiprotease, antibacterial, and anti-inflammatory activities. The protein was originally identified in epithelial cells of the epididymis; however, it has been shown to be widely expressed in epithelial cells of the airways, endometrium, kidneys, and pancreas. In addition, HE4 has inflammatory and immunomodulatory functions. It inhibits proteases of neutrophil origin, such as neutrophil elastase (NE), and may act as an endogenous counter regulatory factor against proteolytic tissue damage in the airways. HE4 expression has been demonstrated in various healthy and malignant tissues. Its most well-established clinical application is in the diagnosis and monitoring of ovarian carcinoma, although it has also been investigated as a novel tumor marker in endometrial carcinoma and lung cancer.

Epithelial–mesenchymal transition (EMT) in CF

EMT is a process of transcriptional reprogramming during which epithelial cells lose their epithelial characteristics, leading to disruption of epithelial integrity and alterations in cellular morphology. In parallel, genes associated with the mesenchymal phenotype become

activated, including those related to cytoskeletal components (e.g., vimentin, α SMA), cell–cell adhesion (e.g., N-cadherin), and extracellular matrix (ECM) proteins (e.g., collagen I, fibronectin). Currently, three types of EMT are distinguished. Type I EMT, characteristic of embryonic development, plays a fundamental role in tissue formation and organogenesis and remains resting in normal, healthy tissues. However, EMT also occurs under pathological conditions, such as in inflammatory diseases (Type II) and cancer (Type III). Type II EMT is a key driver of tissue remodeling and fibrosis in inflammatory diseases and has been demonstrated in several chronic lung disorders, including CF. EMT in CF is often present in a hybrid or partial form, meaning that cells present an intermediate epithelial/mesenchymal phenotype. Accordingly, EMT should be interpreted as a dynamic spectrum encompassing multiple stable intermediate states. The level of TGF- β 1, one of the most important mediators of EMT, is significantly elevated in CF, thereby promoting the development of pulmonary fibrosis, which represents the leading cause of mortality in this disease. However, it remains unclear whether EMT activation is driven by CFTR dysfunction alone or secondary factors, such as bacterial infection and chronic inflammation, also contribute to this process.

Under normal conditions, the CFTR ion channel plays a role in regulating epithelial transport processes and has been implicated in several cellular functions, including epithelial cell differentiation, polarization, and proliferation, as well as in promoting efficient regeneration of the airway epithelium after injury. In contrast, in CF, airway regeneration is delayed and is accompanied by an enhanced pro-inflammatory response and altered expression of matrix metalloproteinases (e.g., increased IL-8 and MMP9 production). Furthermore, an increased risk of malignancies - particularly gastrointestinal cancers - has been reported in CF. These observations support the concept that CFTR may function as a tumor suppressor and raise the possibility CFTR dysfunction may be linked to EMT and oncogenic processes.

In the dissertation, we investigated the role of HE4 as a potential biomarker in CF for monitoring the efficacy of CFTR modulator therapy. In addition, our aim was to gain a better understanding of the complex EMT related cellular processes involved in the regulation of HE4 expression. Therefore, we analyzed changes in HE4 protein expression and its functional relationship with MMP9 in *in vitro* bronchial epithelial cells.

2. Objectives

Our aim was to investigate the HE4 protein as a novel prognostic biomarker and pathophysiological mediator in CF. Therefore, we analyzed changes in HE4 concentration in serum and plasma samples of patients with CF in response to different CFTR modulator therapies to evaluate disease severity and compare the efficacy of therapeutic interventions. Furthermore, to achieve a better understanding of the complex cellular processes involved in the regulation of elevated HE4 expression in CF, we performed *in vitro* studies on bronchial epithelial cells. Specifically, we analyzed changes in HE4 protein expression during CF associated EMT, as well as its functional relationship with MMP9. To address these issues, we sought answers to the following questions:

- Is there a strong correlation between plasma/serum HE4 levels and lung function parameters (ppFEV1), and could HE4 measurement serve as a biomarker for monitoring the efficacy of CFTR modulator (Orkambi[®], Kaftrio[®]) therapies?
- To what extent do plasma/serum HE4 concentrations differ in CF patients treated with the two types of CFTR modulators (Orkambi[®] and Kaftrio[®])?
- How does HE4 expression change during CF related EMT, and does it contribute to elevated MMP9 expression *in vitro* in bronchial epithelial cell cultures?
- Does altered HE4 expression promote the development of an EMT phenotype in bronchial epithelial cells?

3. Materials and Methods

In the three scientific publications forming the basis of this PhD dissertation, a wide range of experimental and analytical methods were applied for the investigation of *ex vivo* clinical samples as well as for conducting *in vitro* cellular experiments. As there are methodological overlaps among the procedures used in the individual studies, the experimental and laboratory methods are presented in this chapter in an integrated and standardized manner, rather than being described separately for each publication.

Patient inclusion and clinical study design

Included patients were categorized into a total of four main groups. In the first clinical trial, 68 patients treated with Orkambi® (selected from the PROSPECT study) were enrolled. In the second clinical study, a total of 123 patients with CF homozygous for the *p.Phe508del* mutation and treated with Orkambi® (n = 43, cohort 3) or Kaftrio® (n = 51, Cohort 1; n = 29, cohort 2) were recruited to compare plasma and serum HE4 levels. Patients were selected from two international centers (Division of Pulmonary and Critical Care Medicine, Johns Hopkins University, Baltimore, MD, USA; and the Department of Pulmonology, Charles University, Prague, Czech Republic) and four Hungarian CF centers (Department of Pediatrics, University of Pécs, Pécs; National Korányi Institute of Pulmonology, Budapest; Pulmonology Hospital of Törökbálint, Törökbálint; Department of Pediatrics, Albert Szent-Györgyi Health Center, University of Szeged, Szeged). The diagnosis of CF was established based on current consensus based diagnostic criteria. All enrolled individuals presented with the classic form of the disease. From the PROSPECT study, 38 children (under 18 years) and 30 adult CF patients were selected retrospectively. CF patients receiving Kaftrio® treatment in cohort 1 (n = 51) were exclusively adults, with a gender distribution of 28 females and 23 males. The median age of the patients was 27 years (range: 20–47 years). Cohort 2 consisted of children and young adult patients (n = 29). Based on gender distribution, there were 17 females and 12 males, with a median age of 12 years (range: 6–50 years). In this patient group, samples were taken prior to the initiation of treatment, as well as at months 1–2 and 3–6 of therapy, allowing for monitoring of therapeutic response over time. To compare the effects of the two CFTR modulators age- and sex-matched Orkambi® treated (n = 43) CF patients were enrolled as control group (cohort 3) with a median age of 14 years (minimum: 11 years, maximum: 53 years). Blood sample collection was performed at three time points according to the protocol used in Kaftrio® treated cohort 2.

Samples were collected during outpatient follow-up visits at the aforementioned time points, when patients were generally in a mild or moderate clinical state.

Subgroups based on ppFEV1 were defined based on the publication by Sagel et al.; accordingly, CF patients included in the first study (Orkambi[®], n = 68) were classified into three subgroups: <50% ppFEV1 (n = 9), 50–89% ppFEV1 (n = 45), and $\geq$ 90% ppFEV1 (n = 14). Participants in both clinical trials were assigned to two subgroups based on therapeutic response: "responders" were defined as patients whose ppFEV1 increased by at least 2.6% (first clinical trial) or 5% (second clinical trial) during the 6-month (Orkambi[®], Kaftrio[®] Cohort 2) or 3-month (Kaftrio[®] cohort 1) follow-up period. In contrast, the "non-responder" group included patients whose ppFEV1 increase was below 2.6% or 5%. It is important to note that no CF patient experienced an acute pulmonary exacerbation during the study period.

Respiratory function was assessed using spirometry based on the ppFEV1 value in accordance with current guidelines. Sweat chloride concentration analysis was performed via sweat conductivity measurement during scheduled study visits. Notably, these analyses were conducted on the same day as the blood sampling.

HE4 levels were also measured in sputum samples from 72 CF patients from a U.S. center, for whom various clinical parameters were also available. We investigated if sputum HE4 concentration correlates with the severity of pulmonary exacerbation and whether it shows an association with classic inflammatory parameters measured in sputum, such as NE, IL-1 β , MPO, and MMP9.

Furthermore, we collected BAL samples from 13 CF patients to determine HE4 levels and to investigate *in vitro* the complex effects of the altered conditions characteristic of CF airways on bronchial epithelial cells. A sample pool was prepared from the BAL samples and then added to the cells at a 20% concentration and incubated for 48 hours. Following treatment, total RNA was isolated from the cells, and RT-qPCR analysis was performed to examine expression changes in EMT markers.

Measurement of HE4 concentration in plasma, serum, sputum, and BAL samples

Blood samples were obtained via venipuncture and centrifuged to separate plasma and serum. Subsequently, samples were stored at -70°C and transported on dry ice to the University of Debrecen for further processing. Sputum and BAL samples were stored and transported in the same manner. HE4 concentrations in patient plasma, serum, sputum, and BAL samples, as

well as in the supernatant of CFBE cells, were measured using a chemiluminescent microparticle immunoassay (CMIA) on Architect® i2000SR and i1000SR analyzers (Abbott Diagnostics, Wiesbaden, Germany).

Following the incubation period, the supernatant of the CFBE cells was aspirated and centrifuged at 10,000 g for 1 minute at room temperature to remove any potential cells or cellular debris. The same procedure was applied to the plasma, serum, sputum, and BAL samples after thawing.

Cell cultures and treatment conditions

The CFBE 41o- cells expressing either the *p.Phe508del*-CFTR mutation, reflecting the cellular conditions characteristic of CF airways, or the wild type CFTR (wt-CFTR), were obtained from the laboratory of Dr. J. P. Clancy (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA). For cell culture, Minimum Essential Medium Eagle (EMEM) (Lonza Inc., Morristown, NJ, USA) containing Earl's BSS (EBSS) and 1% L-glutamine was utilized, supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 5 µg/ml Puromycin (Sigma-Aldrich). Cells were incubated at 37 °C in a 5% CO₂ atmosphere. CFBE cells were seeded into 8-well chambers (PEZGS0816, Merck Group) at a density of 2.5×10^5 cells/well for immunofluorescence (IF) microscopy, and into 6-well plates at 2.5×10^5 cells/well for RNA isolation and supernatant measurements. Seeding was performed 24 hours prior to treatment to allow for cell attachment. To induce EMT, cells were treated with TGFβ1 at 37 °C for 24 hours; a combination of 40 mM acetic acid and 0.1% BSA was used as a control. Additionally, cells were treated with combinations of CFTR modulators: *lumacaftor* (3 µM) and *ivacaftor* (10 µM) (LUM/IVA), or *ellexacaftor* (3 µM), *tezacaftor* (5 µM), and *ivacaftor* (10 µM) (ELX/TEZ/IVA). In these instances, DMSO served as the negative control. CFTR channel activity was stimulated using a combination of FSK (10 µM) and IBMX (100 µM) (FSK/IBMX) in both cell lines (including cells both treated and untreated with CFTR modulators), while CFTR_{inh-172} (20 µM) was administered to wt-CFTR expressing CFBE cells for CFTR inhibition. DMSO was added to the control samples in these experiments as well. The experimental conditions employed for the CFTR modulators are consistent with methodologies described in previous *in vitro* studies. CFBE cells carrying the *p.Phe508del*-CFTR mutation were plated 24 hours before transfection at a density of 2.5×10^4 cells/well in 8-well chambers for IF microscopy, and at 5×10^4 cells/well in 24-well plates for RNA isolation and supernatant measurements. Subsequently, HE4 expression was silenced for 48 hours using a specific siRNA (WFDC2 siRNA) at 37 °C. A negative control siRNA (NEG01 siRNA) was used as a reference. To model the complex CF

airway environment more accurately, a sample pool was prepared from BAL samples of CF patients. The *p.Phe508del*-CFTR expressing CFBE cells were then treated with this pool at a 20% sample concentration for 48 hours.

Cell viability assay

To assess the cell viability after TGF β 1 treatment, a Thiazolyl Blue Tetrazolium Bromide (MTT)-based cell viability assay (Sigma-Aldrich) was performed. CFBE cells (25,000 per well) were sub-cultured into PLL-precoated 24-well plates (SPL Life Sciences, Naechon-Myeon, Korea) and then exposed to TGF β 1 for 48 hours. After treatment, supernatants were removed and 300 μ L of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) solution in Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) was added to each well. After incubation for 3 hours (37 °C, 5% CO₂, humidified atmosphere), the MTT solution was discarded and blue formazan crystals were solubilized in 300 μ L dimethyl sulfoxide (DMSO, Sigma-Aldrich), and optical density was determined at 570 nm. Cells treated with H₂O₂ (50 μ M) for 48 hours were used as positive control, while samples stimulated by TNF- α (100 ng/mL) for the same period was applied as a negative control.

CFTR functional assessment (whole-cell patch clamp)

As in previous publications, Cl⁻ currents in CFBE 41o- cells were measured using the conventional whole-cell patch-clamp configuration. The external (bath) solution contained 145 mM NaCl, 4 mM CsCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM D-glucose, and 10 mM HEPES (pH 7.4 titrated with NaOH, 315 mOsm). The intracellular (pipette) solution contained 113 mM L-aspartic acid, 113 mM CsOH, 27 mM CsCl, 1 mM NaCl, 1 mM MgCl₂, 1 mM ethylene glycol tetra-acetic acid (EGTA), 10 mM HEPES, and 3 mM Mg-ATP (pH 7.2, titrated with CsOH, 285 mOsm). Mg-ATP was freshly diluted in the intracellular solution from 200 mM stock solutions every hour. The intracellular solution was stored on ice before usage. FSK/IBMX and CFTR_{inh172} were diluted from 10 mM stock solutions freshly prepared in water-free DMSO into the extracellular solution before the start of the experiments. Stock solutions were prepared in DMSO. Bath perfusion around the measured cell with different external solutions was achieved using a gravity-flow micro perfusion system at a rate of 200 μ L/min, and the excess fluid was removed continuously. Solutions containing CFTR_{inh172} (Sigma-Aldrich) were also made fresh in the external solution from 25 mM stocks stored at -20 °C. The robust change in the current amplitude upon perfusion with CFTR_{inh172} was an indicator of both the expression of CFTR channels and the proper operation of the perfusion system. Micropipettes were pulled in four

stages using a P-2000 automatic pipette puller (Sutter Instruments, San Rafael, CA, USA) from GC150F-7.5 borosilicate capillaries (GC150-TF10, Harvard Apparatus Co., Holliston, MA, USA) with a tip resistance typically ranging from 3 to 10 M Ω in the bath solution. Cl⁻ currents were recorded using an Axopatch 200B amplifier connected to a personal computer using Axon Digidata 1550A data acquisition hardware (Molecular Devices Inc., Sunnyvale, CA, USA). The holding potential was maintained at -40 mV throughout the experiment, and the cutoff threshold for acceptable series resistance values was 15 MOhm. To monitor the current evolution under drug applications and to confirm the absence of significant leak current, single depolarization pulses from -40 to 0 mV for 1 s were applied every 5 s for 4–5 min. The current–voltage (I–V) relationship was determined by 800 ms long pulses from the holding potential to test potentials between 0 and +60 mV in 20 mV increments every 10 s. Experiments were conducted at room temperature (RT, between 20 and 24 °C). Data were analyzed using the pClamp10.5 software package (Molecular Devices Inc.). Before analysis, current traces were digitally filtered with a three-point boxcar smoothing filter. Before analysis, current traces were corrected for ohmic leak. Dr. Tibor G. Szántó, Dr. Beáta Arnódi-Mészáros, and Prof. György Panyi (University of Debrecen, Faculty of Medicine, Department of Biophysics and Cell Biology) provided technical assistance in the execution of the studies.

Total RNA extraction

According to the manufacturer's recommendations, total RNA from CFBE cell culture samples was isolated using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA). The purity and concentration of the isolated RNA samples were verified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The total RNA samples were stored at -80°C before analysis.

Downregulation of HE4 expression by siRNA transfection in CFBE cells

The *p.Phe508del*-CFTR CFBE cells were seeded at a density of 25.000 per well and cultured for 24 hours before transfection. HE4 (WFDC2) expression in mutant CFBE cells was silenced using a specific siRNA (20 pmol, ID: 4392420, Invitrogen, Carlsbad, CA, United States) for 48 hours in the absence or presence of TGF β 1 pretreatment. Control samples were treated with NEG01 siRNA (Silencer Select Negative Control No.1, 20 pmol, ID: 4390846, Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions. Transfection efficiency was assessed by measuring HE4 protein expression through immunofluorescence (IF) and mRNA levels via RT-qPCR. Additionally, levels of EMT markers

and MMP9 were evaluated. GAPDH mRNA expression was also measured to verify the specificity of the siRNA transfection. Furthermore, MMP9 protein concentrations in cell supernatants were quantified by ELISA to confirm the effect of altered HE4 expression on MMP9 levels. The proliferation rate of CFBE cells treated with HE4 siRNA was compared to cells treated with NEG01 siRNA by analyzing Ki67 positivity through IF.

RT-qPCR analysis

Complementary DNA (cDNA) synthesis was conducted using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's instructions. An initial RNA amount of 1000 ng per reaction was used. Quantitative PCR was carried out on a LightCycler 480 qPCR instrument (Roche Diagnostics, Mannheim, Germany) with LightCycler 480 SYBR Green I Master mix (Roche Diagnostics) and gene-specific primers (10 μ M, Integrated DNA Technologies, Leuven, Belgium). The reactions were incubated at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 1 minute. For normalization, we used the reference gene *RPLP0* (*36B4*) and *GAPDH*. To study EMT development, E-cadherin, N-cadherin, Vimentin, IL1B and TWIST1 mRNA levels were quantified in both CFBE cell lines and in *p.Phe508del*-CFTR CFBE cells after 48 hours of TGF β 1 treatment, while HE4, IL1B, and MMP9 mRNA expression were measured in parallel. RNA expression levels were determined using the following formula, taking HE4 RNA as an example: $\Delta Cq = Cq_{36B4} - Cq_{HE4}$, while the fold change in the target gene relative to the reference gene was converted as follows: $F = 2^{\Delta Cq}$.

Detection of MMP9 and N-cadherin proteins using ELISA

The concentrations of MMP9 protein (DMP900; Human MMP-9 ELISA Kit – Quantikine, R&D Systems, Minneapolis, MN, USA) and N-cadherin protein (EH0050; Human CDH2 (Neural Cadherin) ELISA kit, FineTest, Wuhan, Hubei, China) in the cell supernatant samples were determined by ELISA. These measurements were performed to confirm the direct impact of altered HE4 expression on MMP9 production, as well as the initiation or progression of the EMT process in response to various *in vitro* treatments.

Immunofluorescence (IF) microscopy

Detection of EMT-specific phenotype in CFBE cell cultures was visualized using immunofluorescence staining for EMT markers. For this purpose, wt-CFTR and *p.Phe508del*-CFTR CFBE cells were cultured on 6-well plates for 2 days and transferred onto sterile

uncoated microscope slides at a density of 5×10^4 cells per slide. Basal positivity for EMT characteristics in both cell lines was first observed based on genotype. Simultaneously, the EMT process was further induced by TGF β 1 (15 ng/mL) for 48 hours in p.Phe508del-CFTR CFBE cells, compared to control samples with vehicle (PBS, baseline). When studying the impact of CFTR modulator treatment on EMT, p.Phe508del-CFTR CFBE cells were incubated with ETI or DMSO (baseline) for 48 hours in the absence or presence of TGF β 1 administration. Cells were then fixed with ice-cold methanol-acetone (50:50 v/v) for 10 minutes. Non-specific antibody binding sites were blocked with FBS (Sigma-Aldrich) for 15 minutes. For primary labeling of EMT markers, monoclonal mouse anti-human E-cadherin (610181) and anti-N-cadherin (610920) (250 μ g/mL, BD Biosciences), as well as polyclonal rabbit anti-HE4 (AB273130) and anti-MMP9 (AB73734) (100 μ g/mL, Abcam), were used for 1 hour, followed by secondary staining with FITC-labeled goat anti-mouse (A11029) and goat anti-rabbit (A11034), or rhodamine-conjugated goat anti-mouse IgG (2 mg/mL, dilution 400x, Thermo Fisher Scientific Inc.) for 1 hour. Ki67 protein staining (AB15580) (100 μ g/mL, Abcam) was used to detect actively dividing cells in the growth phase of the cell cycle, with and without HE4 expression, manipulated with siRNA transfection (see below). Cell nuclei were stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, 283 nM), and samples were observed using a Zeiss Axio Scope A1 fluorescent microscope (HBO 100 lamp) (Carl Zeiss Microimaging GmbH, Göttingen, Germany). DAPI: excitation at G 365 nm, emission BP 445/50 nm; fluorescein: excitation BP 470/40 nm, emission BP 525/50 nm; rhodamine: excitation BP 546/12 nm, emission BP 575–640 nm. Images were analyzed with ZEN 2012 software (Carl Zeiss Microimaging GmbH). The mean fluorescence intensity (MFI) was calculated for each marker to quantify positivity levels. The specificity of immunostaining was confirmed by incubating cells with only the secondary antibody, which showed no background staining. Dr. Ferenc Fenyvesi and Dr. Ágnes Rusznyák (University of Debrecen, Faculty of Pharmacy, Department of Pharmaceutical Technology) helped in the execution of the studies.

Ethics statement

This study received approval from Regional Institutional and Research Ethics Committee of the University of Debrecen (License Number: 4813–2017) and the Hungarian Scientific and Research Ethics Committee (permit number: ETT-TUKEB No. IV/10417–3/2020/EKU), with consent granted on December 29, 2020, in accordance with the World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects.

Statistical Analysis

Data are presented as mean $\pm$ 95% confidence interval (95% CI) or, where indicated, as median (1st and 3rd quartiles, IQR) or median (min-max). The normality of the data was assessed using the Shapiro–Wilk and Kolmogorov–Smirnov tests. For comparisons between two groups, the paired t-test was used for normally distributed data, while the Mann–Whitney U-test was employed for variables with non-normal distribution. When comparing results between baseline and follow-up samples, the Wilcoxon signed-rank test was applied. For comparisons involving multiple groups, parametric ANOVA or the non-parametric Friedman test was utilized, followed by Dunn’s post-hoc analysis. Spearman’s rank correlation coefficient (r) was used to examine associations between plasma HE4 and ppFEV1, as well as between sweat chloride concentration and BMI absolute or delta values. Multiple logistic regression analysis was performed to evaluate which covariates most significantly influence the probability of "responder" status at month 6 following the initiation of LUM/IVA treatment. The Area Under the Curve (AUC) of the Receiver Operating Characteristic (ROC) curve was determined for both absolute and delta HE4 values to investigate whether pulmonary function improvement can be detected by these parameters during Kaftrio® or Orkambi® treatment at month 3 or 6 (depending on the subgroup). A threshold for the binary variable was set at a mean change in ppFEV1 of 2.6% (LUM/IVA) and 5% (Kaftrio® or Orkambi®). In the case of delta HE4, the AUC value was also analyzed separately using the 5% delta ppFEV1 threshold, as this degree of pulmonary improvement is expected from a patient responding well ("responder") to treatment. Cut-off or threshold values were determined based on the maximum of the Youden index (sensitivity, specificity, PPV, NPV). A $p < 0.05$ value was considered statistically significant. Data processing and statistical analyses were conducted using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA) and SPSS Statistics 26.0 (IBM Corporation, Armonk, NY, USA) software.

4. Results

Investigation of plasma HE4 concentrations in CF patients homozygous for the *p.Phe508del*-CFTR mutation under Orkambi® treatment

Based on literature data, our research group was the first to investigate plasma/serum concentrations of the HE4 protein for the laboratory assessment of CF patients—not as a tumor marker, as previously utilized in malignant conditions, but as a novel type of inflammatory parameter. We aimed to analyze plasma HE4 for monitoring the efficacy of Orkambi® treatment in patients with CF. At the beginning of our investigations, Orkambi® was the available as a CFTR modulator; subsequently, Kaftrio® also became accessible. Consequently, the results of patients receiving Orkambi® treatment is presented first.

Changes in clinical parameters and plasma HE4 in response to LUM/IVA treatment

Sweat chloride decreased by an average of -18.1 mmol/L (95% CI, -22.3 to -15.0) from baseline by 1 month following LUM/IVA administration and such decreased values remained at this level at 6 months (mean value of -18.9 mmol/L; 95% CI, -24.5 to -14.8) compared to pre-treatment data. In ppFEV1, a moderate, statistically not significant change was observed from baseline to 1 to 6 months, with a mean change at 1 month of 1.6% (95% CI, -0.4 to 3.6) and at 6 months of 2.6% (95% CI, 0.6 to 4.5) in this sub-cohort in contrast to the results of the entire study population. Nutritional status substantially improved, as indicated by steadily increased BMI values throughout the study with a mean change of 0.73 kg/m² (95% CI, 0.42 to 0.98) at 6 months.

Plasma HE4 concentrations before the introduction of LUM/IVA therapy were similar (median, IQR, 68.5 (56.6–87.3) pmol/L) in other CF cases before IVA treatment (63.1 [47.9–97.5] pmol/L) in our previous study. Plasma HE4 levels were reduced at all post-treatment time points showing the largest delta value at 6 months, i.e., -10.7 mmol/L (95% CI, -15.4 to -5.9) vs baseline. Interestingly, the mean change of HE4 (-6.5 pmol/L, 95% CI, -12.4 to -0.5) was smaller at 12 months among these pwCF, which was related to less improved lung function (delta ppFEV1: 1.0% (95% CI, -1.7 to 3.8) by the end of study course.

Using Spearman tests, the comparison of change in ppFEV1 at 1 and 6 months against changes in sweat chloride concentration at 1 month ($r = 0.0595$; $P = 0.6403$) and 6 months ($r = 0.0148$, $P = 0.9117$) did not reveal consistent trends. Thus, we sought to investigate the

potential of the HE4 plasma-based biomarker to monitor the effect of LUM/IVA CFTRm treatment throughout the study visits.

HE4 plasma concentration strongly correlates with ppFEV1

Initially, we correlated the HE4 concentrations with the ppFEV1 values determined at baseline and 6 months after the initiation of LUM/IVA therapy (i.e., when the largest improvement in ppFEV1 was detected) to observe whether absolute values of plasma HE4 reflected the lung function status. A significant inverse correlation was detected between pre-treatment values of plasma HE4 and ppFEV1 ($r = -0.5661$; $P < 0.0001$). Likewise, there was a significant negative relationship between plasma HE4 concentrations and ppFEV1 at 6 months of LUM/IVA therapy ($r = -0.5294$; $P < 0.0001$).

In parallel, the mean change of HE4 from baseline (i.e., delta HE4 of its plasma level) similarly correlated with delta ppFEV1 ($r = -0.5297$; $P < 0.0001$) in all study participants. However, when we separately analyzed this association among pwCF under 18 years of age, a stronger correlation was found in childhood ($r = -0.7053$; $P < 0.0001$). When the absolute HE4 concentrations measured at baseline and 6 months were evaluated using the baseline ppFEV1 categories of $< 50\%$, $50-89\%$, and $\geq 90\%$, respectively, as previously set by Sagel et al., the baseline and 6-month HE4 concentrations were significantly higher in those with $< 50\%$ baseline ppFEV1 compared to the other two sub-groups. More importantly, the largest, statistically significant mean difference in HE4 was determined in these (mostly adult) pwCF with severely impaired lung function before treatment, while the lowest, nonsignificant changes were observed in subjects with baseline ppFEV1 $\geq 90\%$.

Change in plasma HE4 predicts the improvement of CF lung disease under LUM/IVA therapy

We then investigated whether decreasing HE4 concentrations in response to LUM/IVA predicted the improvement of CF lung function as assessed by delta ppFEV1 values. Thus, we calculated the discriminative power of the mean change of HE4 if 2.6% delta ppFEV1 at 6 months of treatment (i.e., the mean value of this pwCF cohort) was used as the binary classifier. Considerable AUC value of delta HE4 (0.7898 [95% CI 0.6823–0.8972], $P < 0.0001$) was found with its cut-off value of -10.7 pmol/L demonstrating 62% sensitivity and 79% specificity for the entire pwCF population. The mean change of HE4 was more powerful in CF children (0.9139 [95% CI 0.8268–1.0000], $P < 0.0001$) and using the same cut-off value sensitivity (80%) and specificity (83%) were much higher. This value for HE4, when 5% delta ppFEV1

was the classifier being the expected lung function improvement from a “good responder”, was 0.6786 (95% CI 0.5523–0.8048), $P = 0.0139$) in all pwCF using a cut-off value of -11.8 pmol/L with a 73% sensitivity and 74% specificity. On the other hand, the AUC value of delta HE4 in children was higher as 0.7913 (95% CI 0.6481–0.9345), $P = 0.0027$), but at the same cut-off value, we achieved 50% sensitivity and 74% specificity. When pwCF were sub-grouped according to their responder status, i.e., with $\geq 5\%$ mean change of ppFEV1 at 6 months of treatment or their non-responder status with lower delta ppFEV1, we found that significantly decreased plasma HE4 concentrations were observed only in case of responders with expected improved lung function by 6 months of LUM/IVA treatment. In contrast, sweat chloride concentrations (measured in mM) and BMI values were significantly altered in both sub-cohorts. These latter values indicate the overall effect of LUM/IVA medication but cannot facilitate the assessment of responsiveness to the combined CFTR modulator treatment. Finally, multiple logistic regression analysis was used to evaluate the likelihood of being a responder against the following covariates: delta HE4, age, sex, baseline ppFEV1, sweat chloride concentration, BMI, and *P. aeruginosa* positivity at 6 months after initiation of LUM/IVA treatment. Delta HE4 (OR: 0.89, 95%CI: 0.82–0.95; $P = 0.001$) and baseline ppFEV1 (OR: 1.09, 95%CI: 1.02–1.16; $P = 0.005$) were independently associated in responders compared to non-responders.

Investigation of serum HE4 concentrations in CF patients homozygous for the *p.Phe508del*-CFTR mutation under Kaftrio® medication

In the second clinical trial of ours, we analyzed the serum HE4 levels of CF patients receiving Kaftrio® treatment, which became available for patients during the period of our Orkambi® measurements. In this study, the Orkambi® treated CF patients were utilized as a comparative control group to evaluate the relative efficacy of the two CFTR modulator therapies. We aimed to determine whether HE4 could be a suitable biomarker for monitoring the efficacy of Kaftrio® treatment in CF patients; additionally, we compared the CFTR-correcting effects of the two CFTR modulators *in vitro*.

Treatment with ETI (Kaftrio®) or LUM/IVA (Orkambi®) lowers serum HE4 levels in CF subjects homozygous for *p.Phe508del*-CFTR variant

Since plasma HE4 levels showed significant changes in response to LUM/IVA treatment, we aimed to investigate the protein concentration in the serum of CF patients changes following ETI treatment. First, we determined serum HE4 levels in two independent cohorts

with pwCF homozygous for the *p.Phe508del*-CFTR variant who were under Kaftrio® medication. In the presence of substantially improving lung function (17.5 [14.5–20.5]% of ppFEV1) in CF adults of cohort 1, there were significantly ($p < 0.0001$) reduced HE4 serum concentrations—regardless of its baseline value—at 3 months of treatment, this being the only follow-up time point of these patients. In cohort 2, pulmonary status also improved in most pwCF, showing a mean value of 4.0 [–3.9–12.1]% of ppFEV1, and serum HE4 was gradually decreased already at 1–2 months of Kaftrio® treatment ($p = 0.0076$), while further reduction was observed by 3–6 months of therapy ($p < 0.0001$ compared to baseline). In parallel, pwCF on Orkambi® also demonstrated significantly lowering serum HE4 values at consecutive visits ($p = 0.0010$ by 1–2 months and $p < 0.0001$ by 3–6 months vs. pretreatment levels). Very importantly, there was no difference in the baseline level of HE4 between the cohorts on Kaftrio® and Orkambi® ($p = 0.3422$). At the same time, HE4 concentrations were significantly lower at each follow-up time point ($p = 0.0158$ at 1–2 months and $p = 0.0474$ at 3–6 months of therapy) in those on ETI compared to those on LUM/IVA, in correlation with the degree of lung function improvement.

Individual alteration in serum HE4 was separately analyzed and was found to be decreased in most study participants observed between the baseline and their last available follow-up time point. In cohort 1, serum HE4 levels were substantially reduced below baseline after 3 months of ETI, with a mean delta HE4 of –45.7 pmol/L when the mean change of ppFEV1 was 17.5%, while ETI pwCF in cohort 2 showed a modest mean value of 4.0% in ppFEV1 by 3–6 months of treatment in the presence of a mean delta value of –20.7 pmol/L HE4. In contrast, only 1.6% of ppFEV1 was measured in those on Orkambi® medication, with a mean change of HE4 of –18.5 pmol/L by the 3–6-month visit. These clinical data underscore the impact of CFTRm therapies in terms of decreasing serum HE4 concentrations in CF. Furthermore, the superior therapeutic effect of Kaftrio® over Orkambi® on pulmonary clinical status could be successfully monitored via serum HE4.

HE4 serum levels strongly correlate with ppFEV1

Serum HE4 levels were compared with ppFEV1 values in the ETI treated study subgroups, where the patient group receiving LUM/IVA treatment served as the control group. We statistically correlated the HE4 levels with the ppFEV1 values determined on the same day when blood sampling occurred to observe if absolute values of serum HE4 reflected lung function status in each study subgroup. A significant inverse correlation was detected between

HE4 and ppFEV1 in all cohorts ($r = -0.422$, $p = 0.0020$ in cohort 1; $r = -0.537$, $p < 0.0001$ in cohort 2; and $r = -0.575$, $p < 0.0001$ in the Orkambi[®] cohort).

In parallel, the mean change in HE4 from baseline (i.e., delta HE4) similarly correlated with delta ppFEV1 ($r = -0.559$, $p = 0.0001$ in cohort 1; $r = -0.591$, $p = 0.0048$ in cohort 2; and $r = -0.511$, $p = 0.0017$ in the LUM/IVA cohort) in all study participants. Serum HE4 strongly correlated with ppFEV1 values; thus, it was likely related to CFTRm-induced lung function improvement. Delta HE4 values were even more reflective of ppFEV1 alterations, especially in cohort 2, consisting of relatively young pwCF on ETI treatment.

Change in serum HE4 levels predicts the improvement of CF lung disease under Kaftrio[®] therapy

We next investigated whether decreasing HE4 levels in response to Kaftrio[®] were associated with improving CF lung function as assessed by ppFEV1 values. Thus, we calculated the discriminative power of the mean change in HE4 if 4.0% of delta ppFEV1 by 3 or 6 months of treatment was used as the binary classifier in ETI cohorts 1 and 2 and in two age groups of all patients on Kaftrio[®]. A considerable AUC value of delta HE4 (0.765 [95% CI 0.633–0.898], $p = 0.0012$) was found in adult cohort 1, with a cut-off value of -42.4 pmol/L, showing 68.0% sensitivity and 69.0% specificity, while in cohort 2, with younger individuals, a similarly high AUC value (0.789 [95% CI 0.668–0.909], $p = 0.0003$) was determined in the presence of its cut-off value of -20.3 pmol/L, showing 77.4% sensitivity and 70.8% specificity.

In parallel, Orkambi[®] treated pwCF showed an AUC value of delta HE4 of 0.809 [95% CI 0.648–0.969], $p = 0.0023$) when the cut-off value was -17.2 pmol/L, with 78.9% sensitivity and 80.0% specificity. Among the Kaftrio[®] treated subjects, the mean change in HE4 was similarly practical in both children (0.791 [95% CI 0.594–0.988], $p = 0.0242$) and adults (0.788 [95% CI 0.624–0.953], $p = 0.0042$) using cut-off values of -14.6 and -21.6 pmol/L, respectively, with sensitivity of 72.7% and 80.0% and specificity of 77.8% and 81.3%, respectively.

We also analyzed the diagnostic characteristics of baseline absolute HE4 levels to predict a satisfactory clinical response to CFTRm with improved lung function of delta 4.0% ppFEV1 by 3–6 months of treatment in any of these cohorts, but no statistically significant relationship was observed under either the Kaftrio[®] (AUC value of 0.618 [95% CI 0.365–0.872], $p = 0.3600$) or Orkambi[®] therapy (AUC value of 0.698 [95% CI 0.513–0.883], $p =$

0.050). Hence, delta—but not absolute—HE4 can be effectively applied to indicate the beneficial effects of Kaftrio®.

Comparison of the effects of the two different CFTRm on *p.Phe508del*-CFTR Cl⁻ currents in CFBE cells

The rescue of the CFTR function by CFTRm treatment was monitored using the whole-cell configuration of the patch-clamp technique in airway epithelial cells. Human CFBE 41o-cell cultures expressing *p.Phe508del*-CFTR were treated with two different combinations of CFTR modulators (LUM/IVA or TEZ/IVA) in vitro. In this study, we compared the effects of Kaftrio® and Orkambi® treatment on Cl⁻ channel function using the same methodology when *p.Phe508del*-CFTR expressing CFBE cells were studied with or without ELX/TEZ/IVA or LUM/IVA pretreatment for 24 h. In parallel, untreated wt-CFTR-expressing CFBE cells were analyzed as controls. One-second-long voltage pulses from the holding potential to 0 mV were applied in the absence of FSK/IBMX to measure the basal Cl⁻ current, in the presence of CFTR activator FSK/IBMX, or when FSK/IBMX treatment was followed by perfusion of the cells with CFTR-selective inhibitor CFTR_{inh172}. For easier comparison, the Cl⁻ current amplitude was normalized to the cell capacitance to obtain the current density value, which, for a particular cell, was defined as the average of current densities obtained for at least three depolarizing pulses repeated at every 5 s in a sequence. FSK/IBMX perfusion elicited a robust, CFTR_{inh172}-sensitive whole-cell Cl⁻ current with a linear and CFTR_{inh172}-sensitive current density–voltage relationship in cells expressing wt-CFTR. Perfusion of FSK/IBMX failed to elicit the Cl⁻ current in CFBE cells expressing *p.Phe508del*-CFTR channels. Importantly, pretreatment of *p.Phe508del*-CFTR-expressing cells with either a combination of CFTR modulators (LUM/IVA or ELX/TEZ/IVA) significantly increased the basal Cl⁻ current. Moreover, the basal current was significantly larger upon ELX/TEZ/IVA pretreatment as compared to LUM/IVA pretreatment; current densities were (in pA/pF) 2.8 ± 1.3 (n = 8), 24.9 ± 5.8 (n = 10, p = 0.0044), and 99.4 ± 14.2 (n = 15, p < 0.0001) for untreated *p.Phe508del*-CFTR cells, cells pre-treated with LUM/IVA, and cells pretreated with ELX/TEZ/IVA, respectively. FSK/IBMX stimulated the Cl⁻ current in both LUM/IVA- and ELX/TEZ/IVA-pretreated *p.Phe508del*-CFTR-expressing cells. The FSK/IBMX-stimulated currents were significantly larger compared to the Cl⁻ current measured in the absence of CFTRm pretreatment. Moreover, the currents were significantly larger upon ELX/TEZ/IVA pretreatment as compared to LUM/IVA pretreatment (138.0 ± 13.8 (n = 16) vs. 31.3 ± 7.7 (n = 10) pA/pF; p < 0.0001). The Cl⁻ currents were sensitive to CFTR_{inh172}, the FSK/IBMX-induced currents were reduced by 20 μM CFTR_{inh172},

to ~10% of the currents measured in the absence of the inhibitor. In summary both CFTRm pretreatments successfully corrected *p.Phe508del*-CFTR function; however, pretreatment with Kaftrio® was more effective than Orkambi®, as expected.

ELX/TEZ/IVA treatment reduced HE4 concentrations in the supernatant of CFBE 41o- cell cultures *in vitro*

To investigate the effects of CFTRm on HE4 protein expression, CFBE41o- cells expressing the *p.Phe508del*-CFTR variant were treated with either the triple combination of ELX/TEZ/IVA (Kaftrio®) or the dual combination of LUM/IVA (Orkambi®) for 24 h. HE4 concentrations were subsequently measured in the culture supernatants. CFBE41o- cells expressing wt-CFTR were used as controls. Consistent with previous findings, basal HE4 secretion was significantly higher in *p.Phe508del*-CFTR cells compared to wt-CFTR controls ($p < 0.001$). Both treatment regimens resulted in a marked reduction in HE4 levels compared to vehicle-treated (DMSO) control samples ($p < 0.001$ for both combinations). Importantly, ELX/TEZ/IVA produced a more pronounced decrease in HE4 concentration than LUM/IVA ($p < 0.01$).

In a complementary experiment, FSK/IBMX agents known to stimulate CFTR channel activity were applied alone or in combination with CFTR modulators in *p.Phe508del*-CFTR cells. Treatment with FSK/IBMX alone led to a significant reduction in HE4 levels ($p < 0.001$), presumably through activation of residual CFTR function. Notably, co-treatment with FSK/IBMX and CFTR modulators resulted in a further decrease in HE4 expression, which was statistically significant compared to FSK/IBMX treatment alone ($p < 0.05$), reinforcing the additive effect of these compounds.

To further explore the inverse relationship between CFTR function and HE4 expression, we examined the effects of CFTR inhibition in wild-type CFTR-expressing CFBE41o- cells. Pharmacological blockade using CFTR_{inh172} resulted in a statistically significant elevation of HE4 levels in the culture supernatant ($p < 0.05$), supporting the hypothesis that reduced CFTR activity contributes to increased HE4 secretion. Conversely, stimulation of CFTR function via FSK/IBMX led to a significant reduction in HE4 concentrations when compared with untreated controls ($p < 0.05$), indicating that upregulated CFTR activity exerts a suppressive effect on HE4 expression.

Surprisingly, both ELX/TEZ/IVA and LUM/IVA treatments were able to reduce HE4 levels, even in CFBE41o- cells with wild-type CFTR expression. The reduction was significant

in both cases ($p < 0.001$) and comparable in magnitude. These data suggest that CFTR function affects basal levels of HE4 expression, and impaired function of CFTR could explain elevated HE4 concentration in CF airway epithelial cells in vitro. Taken together, these data reinforce the concept that baseline HE4 expression is modulated by CFTR activity. Impaired CFTR function, as seen in CF-derived airway epithelial cells, may underlie the elevated HE4 secretion observed in vitro. Moreover, in cells expressing the *p.Phe508del*-CFTR mutation, ELX/TEZ/IVA elicited a more substantial reduction in HE4 levels than LUM/IVA, consistent with its superior efficacy in rescuing CFTR function. This corresponded with a larger shift in extracellular HE4 concentration, mirroring previously reported changes in systemic HE4 levels.

HE4 concentration in the sputum of CF patients in relation to classical inflammatory parameters

In addition to determining plasma and serum HE4 concentrations, we supplemented our clinical investigations with the analysis of sputum samples from CF patients. While the concentrations of several pro-inflammatory cytokines (e.g., IL-8) can be measured in sputum, they can have significant intra-individual variability even in clinically stable CF patients, however, the reasons for this remain not fully elucidated. We aimed to determine whether HE4 levels in the sputum samples of CF patients correlate with the severity of pulmonary exacerbation and various inflammatory parameters.

Investigation of the correlation between sputum HE4 levels and pulmonary exacerbation in CF

We analyzed sputum samples from 72 CF patients, whose primary demographic and laboratory parameters have been previously detailed. During our investigations, CF patients were categorized into three subgroups based on the severity of exacerbation: mild, moderate, and severe. We found that sputum HE4 concentrations were significantly elevated in CF patients presenting with severe exacerbation compared to those with mild or moderate exacerbation, however, no significant difference was observed between the latter two groups. Based on these findings, HE4 levels measured in sputum may correlate with the severity of pulmonary exacerbation.

Correlation between classical inflammatory parameters and sputum HE4 levels in CF patients

Sputum HE4 concentrations were measured in the sample of 72 CF patients who had exacerbation and whose data for various inflammatory parameters were available. One of the markers investigated was neutrophil elastase (NE), produced by activated neutrophils, which is one of the most sensitive biomarkers of airway inflammation in CF. NE demonstrated a significant correlation with sputum HE4 concentration ($r = 0.1905$; $p = 0.0433$). In CF, recurrent infections and chronic inflammation are common, associated with neutrophil activation and the increased production of pro-inflammatory cytokines (e.g., IL-6, IL-8, TNF- α , IL-1 β). These cytokines not only play a functional role in the pathomechanism but are also considered potential biomarkers.

IL-1 β showed an even more significant association with HE4 levels ($r = 0.3005$; $p = 0.0012$). The most significant correlations were observed with two other inflammatory markers: myeloperoxidase (MPO), produced by neutrophils ($r = 0.4022$; $p < 0.0001$), and matrix metalloproteinase-9 (MMP9), an extracellular matrix-degrading enzyme ($r = 0.382$; $p < 0.0001$), which is also released from neutrophils and macrophages during inflammatory processes. In CF, MMP9 levels are frequently elevated in the airways, contributing to tissue damage and airway remodeling. In summary, sputum HE4 concentration correlates with the severity of pulmonary exacerbation in CF and shows an association with classical inflammatory parameters measured in sputum, such as NE, IL-1 β , MPO and MMP9.

Investigation of HE4 expression in the process of EMT induced by abnormal CFTR function *in vitro* in bronchial epithelial cells

Elevated expression of the HE4 protein has been described in CF, and based on *in vitro* studies, it is directly regulated by impaired CFTR via the NF- κ B pathway in CFBE 41o⁻ cells expressing the *p.Phe508del*-CFTR mutation. Furthermore, CFTR dysfunction is associated with the process of EMT in CF. Since there are no data available in the literature regarding this relationship, our investigations aimed to investigate whether HE4 expression is altered and relates to the process of EMT associated with CF.

CFBE cells expressing *p.Phe508del*-CFTR demonstrate EMT characteristics compared to wt-CFTR CFBE cells

Human CF airway epithelial cells, using *p.Phe508del*-CFTR CFBE 41o- cell cultures and control wt-CFTR CFBE 41o- cells, were first analyzed through combined immunofluorescence (IF) and transcript investigations to evaluate the native expression of EMT markers. IF analysis revealed that E-cadherin expression was decreased, while N-cadherin positivity was higher in CF versus normal epithelial cells. Additionally, if HE4 and MMP9 protein expression were detected, the baseline mean fluorescence intensity (MFI) values for both markers were increased in *p.Phe508del*-CFTR CFBE cells compared to wt-CFTR counterparts.

In parallel, mRNAs were quantified, and both cell lines showed similar differences in the mRNA levels of these biomarkers. We also measured the mRNA expression of Vimentin as an additional mesenchymal marker and IL1B, which functions as a pro-inflammatory mediator in these samples, and both mRNA levels were significantly higher in *p.Phe508del*-CFTR CFBE cells compared to controls. Furthermore, we analyzed TWIST1 mRNA level which mediates EMT, and its elevated expression underlined the development of mesenchymal phenotype in basal as well as TGF- β 1 treated mutant CFBE cells. These data suggest epithelial disruption and the induction of mesenchymal-specific cellular features in native CF bronchial epithelial cells.

Rescue of CFTR function results in a restored epithelial phenotype in *p.Phe508del*-CFTR CFBE cells

Next, *p.Phe508del*-CFTR CFBE cells were treated with ETI to revert EMT phenotype, and higher E-cadherin and reduced N-cadherin expression were observed. Then, we aimed to investigate whether TGF β 1 could further enhance EMT in CF airway cells. MTT assay was first performed to assess cell viability following TGF- β 1 treatment at different concentrations and time points, and no cytotoxic effects were observed at 15 ng/mL of TGF- β 1 for up to 120 hours or at even larger concentration for 48 hours. This treatment resulted in even lower E-cadherin levels and increased N-cadherin and MMP9 expression at the protein level in *p.Phe508del*-CFTR CFBE cells compared to untreated cells, as seen in IF images. Surprisingly, HE4 expression at both protein and mRNA levels decreased in response to TGF β 1 relative to its baseline levels induced in *p.Phe508del*-CFTR CFBE cells. These findings suggest that ‘provoked’ EMT by TGF β 1 may be linked to reduced HE4 expression in CF.

We then aimed to demonstrate that these changes under EMT could be reversed using CFTR modulator treatment *in vitro*. For this purpose, *p.Phe508del*-CFTR CFBE cells co-incubated with TGF β 1 and ETI were analyzed for EMT biomarkers. According to IF tests, E-cadherin positivity increased and normalized, while N-cadherin returned to baseline levels or even lower. Importantly, HE4 and MMP9 expression were significantly reduced, reflecting CFTR rescue by ETI. RT-qPCR also measured these biomarkers, and N-cadherin, Vimentin, and IL1B expression returned to their baseline values. Moreover, HE4 and MMP9 mRNA levels were substantially decreased in the presence of TGF β 1 and ETI together vs TGF β 1 alone. N-cadherin was also evaluated at protein level by ELISA and similar changes were observed in response to distinct treatments above. These results confirm that TGF β 1 is the primary trigger to induce EMT with decreasing HE4 levels, and there is a direct relationship between CFTR dysfunction and EMT development in CF.

Lack of HE4 expression among mesenchymal cellular conditions

As HE4 expression was reduced in *p.Phe508del*-CFTR CFBE cells due to TGF β 1 administration for already 24 hours, these epithelial cells were incubated with this mediator to monitor HE4 expression under long-term TGF β 1 treatment. Importantly, HE4 expression gradually and significantly further decreased after 48 hours, continuing up to 120 hours in response to TGF β 1. As controls, other cell lines of non-epithelial origin were analyzed for HE4, and no detectable protein levels were observed in either human pulmonary mesenchymal stem cells or human lung fibroblasts with CF origin.

Furthermore, protein levels of MMP9 were also measured by ELISA in wt-CFTR and *p.Phe508del*-CFTR CFBE cells, as well as in the latter cell line treated with or without ETI in the absence or presence of TGF β 1. Similar to the findings of IF and RT-qPCR measurements, baseline MMP9 concentrations were higher in the supernatant of *p.Phe508del*-CFTR CFBE cells compared to wt-CFTR CFBE cells which could be suppressed by ETI. The protein level was further increased in the mutant cells after activation with TGF β 1; however, TGF β 1-induced MMP9 expression was significantly reduced by ETI.

According to these results, HE4 expression decreased due to prolonged exposure to TGF β 1 during advanced EMT in CF epithelial cells, which is linked to the development of a more severe mesenchymal phenotype, while HE4 is already absent in mesenchymal cells, such as CF fibroblasts.

Downregulated HE4 expression contributes to EMT, elevated MMP9 expression and decreased cell proliferation

HE4-specific siRNA artificially lowered HE4 expression in *p.Phe508del*-CFTR CFBE cells to examine the effects of TGF β 1 during EMT in CF. IF and RT-qPCR analyzed cellular responses by measuring changes in EMT markers and MMP9 levels in HE4 deficiency. After 48 hours, HE4 siRNA significantly reduced HE4 protein and mRNA levels in mutant CFBE cells compared to those transfected with NEG-01 siRNA, confirming transfection success. Notably, the control gene GAPDH mRNA level remained unchanged.

EMT was induced due to downregulation of HE4 expression, showing decreased E-cadherin and increased N-cadherin as well as MMP9 positivity detected by IF. Furthermore, we analyzed the impact of downregulated HE4 in unstimulated and TGF β 1-stimulated cells. For this purpose, HE4 siRNA and TGF β 1 were administrated together in *p.Phe508del*-CFTR CFBE 41o- cells vs cells with NEG-01 siRNA + TGF β 1, and these results have been compared to the data of HE4 siRNA-treated cells without TGF β 1 stimulation. Alterations in studied parameters at mRNA level measured in unstimulated cells were identical to those samples on TGF β 1. Based on these data, we think that reduced HE4 expression in CFBE cells seems to be a key cellular event in EMT, while elevation in MMP9 protein expression is more substantial when decreased HE4 expression was associated with TGF β 1 treatment as well. Finally, the extent of cell proliferation during EMT with reduced HE4 was assessed via Ki67 positivity, which was significantly decreased in *p.Phe508del*-CFTR CFBE cells after HE4 siRNA treatment compared to control cells with NEG-01 siRNA, indicating that EMT status was associated with decreased cell proliferation.

Modeling the effects of CF airway conditions in *p.Phe508del*-CFTR CFBE cells using BAL samples from CF patients

Finally, we aimed to investigate the complex effects of CF airway conditions on bronchial epithelial cells *in vitro*, using BAL samples from CF patients as a treatment. The use of BAL samples is particularly suitable for modeling the CF airway environment, as they originate directly from the lower respiratory tract, thus faithfully reflecting the inflammatory and microbial milieu characteristic of the disease. CF BAL fluid contains high concentrations of inflammatory cytokines (e.g., IL-8), proteases (e.g., NE, MMPs), and bacterial components, which play a key role in altering the function and phenotype of airway epithelial cells, including the EMT process. Consequently, the application of BAL samples enables the modeling of the

complex *in vivo* environment on CFBE cell cultures *in vitro*, specifically in the case of the *p.Phe508del*-CFTR mutation, which is the most prevalent in the disease.

Therefore, a pool was prepared from BAL samples of CF patients, which was then added to the cells at a 20% concentration and incubated for 48 hours. We analyzed expression changes in EMT markers, IL-1 β , and the HE4 protein to examine whether the CF airway environment, modeled with patient-derived BAL samples, contributes to the development of EMT in CF epithelial cells. Similar trends to those observed following TGF β 1 treatment were found for EMT markers and IL-1 β mRNA levels. In response to BAL treatment, E-cadherin expression significantly decreased, while N-cadherin increased, as did Vimentin; although the elevation in the latter was not significant, the trend was evident. IL-1 β levels also showed a significant increase following treatment.

In summary, our preliminary investigations suggest that the complex airway milieu characteristic of CF may contribute to the development of EMT and the initiation of inflammatory processes in the lung; furthermore, HE4, as a potential biomarker, correlates well with respiratory function status in BAL samples from CF patients.

5. Discussion

In our investigations, we have provided the first evidence of an association between plasma and serum HE4 levels and improvements in lung function—specifically delta ppFEV1—following treatment with LUM/IVA (Orkambi[®]) and the ELX/TEZ/IVA (Kaftrio[®]) triple combination of CFTR modulators in patients with CF. The clinical benefits of these therapeutic combinations have been previously investigated across all age groups, largely through the monitoring of ppFEV1, BMI values, CFTR functional tests on airway and intestinal epithelial cells, and sweat chloride concentrations.

However, it is important to note that these functional and laboratory parameters have limitations regarding the monitoring of CFTR modulators. The discriminative power of ppFEV1 may be limited in patients with moderate or near-normal lung function; furthermore, ppFEV1 shows significant variability across different ages and populations. Performing reliable spirometry can also present technical challenges, particularly in younger patients. A limited correlation has been described between absolute or relative changes in sweat chloride concentration and ppFEV1, even when lung function showed clear improvement 1–6 months after LUM/IVA therapy. At the same time, the standardization of sweat testing remains a challenge.

Based on these factors, there remains an ongoing need for other more accessible and robust biomarkers—preferably from peripheral blood—that can more effectively track treatment response in clinical practice. The serum levels of several peripheral blood biomarkers (e.g., CRP, IL-8, TNF- α) have been shown to decrease significantly following LUM/IVA treatment. Significant reductions in neutrophil count, fibrinogen, CRP, and other cytokines, such as GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-8, IL-12p70, IL-17A and TNF- α , have been reported during Kaftrio[®] therapy. Furthermore, significant associations have been demonstrated between these inflammatory biomarkers and ppFEV1, BMI, and sweat chloride levels. The triple combination also has beneficial effects on liver function and nutrient absorption, improving cholesterol and albumin levels after one year of therapy.

In recent years, our research group has demonstrated that the measurement of plasma/serum HE4 concentrations can be useful for assessing the severity of CF lung disease in Czech and Hungarian populations prior to the initiation of CFTR modulator treatment. The positive correlation between HE4 concentration and the severity of CF lung disease was independent of the patients' age. Conversely, in non-CF populations, factors such as patient age,

smoking, and impaired renal function can influence HE4 levels. We successfully applied this marker to monitor changes in lung function when investigating the efficacy of IVA in three independent patient groups, as well as in patients receiving Orkambi® treatment.

Previous results suggest that CFTR dysfunction may contribute to abnormal HE4 expression through the activation of the NF-κB pathway. Nevertheless, the progression of plasma/serum HE4 concentrations during *in vivo* LUM/IVA and ELX/TEZ/IVA treatment has not yet been investigated in a large CF patient cohort to evaluate therapeutic effects. Although the introduction of these CFTR modulators has led to substantial improvements in lung function and quality of life—most notably with the application of ETI—and has reduced the need for lung transplantation, the regular monitoring of CFTRm treatment efficacy in daily clinical practice is still necessary.

Here we analyzed well-selected plasma specimens for plasma HE4 measurements in a sub-cohort of pwCF homozygous for the p.Phe508del-CFTR variant. In total, 68 CF patients were enrolled from the PROSPECT study, who demonstrated a modest mean change in ppFEV1 (2.6%) compared to baseline by the 6th month of LUM/IVA treatment, in contrast to the results of the overall study population (−0.2%). Furthermore, 80 patients receiving Kaftrio® treatment were enrolled across two independent cohorts. These patients demonstrated a significant mean change in ppFEV1 (17.5% and 4.0%, respectively) compared to baseline during 3–6 months of therapy, which is consistent with findings in other study populations that confirmed significant mean ppFEV1 improvements in children (11%) and adolescents (14.3%) by week 24.

In response to LUM/IVA therapy, HE4 concentrations were significantly lower already at the first follow-up examination and remained that way through the end of respective study periods. The changes of plasma HE4 demonstrated the largest delta value (−10.7 mmol/L) with the lowest absolute HE4 levels (61.5 [47.0–79.7] pmol/L) by 6 months, i.e., representing a level which approached - but did not reach - the reference interval of HE4 (36.3 [31.1–43.4] pmol/L) previously determined in another study. This observation was corroborated by our recent *in vitro* experiments in CFBE 41o-cells expressing p.Phe508del-CFTR, where treatment of these cells with LUM/IVA showed significantly decreased HE4 expression compared to untreated cells, but overall HE4 concentrations did not normalize. All these data underline that HE4 do not reach normal (i.e., “wild-type levels”) in pwCF even in the presence of improved CFTR function following administration of CFTRm.

Nevertheless, the mean change in HE4 levels from baseline correlated with delta ppFEV1 values across all study participants. When this association was analyzed separately among CF patients under the age of 18, a stronger correlation was found at younger ages. The largest mean difference in HE4 was measured in patients with severely impaired lung function (< 50%) before starting Orkambi® treatment, while the least significant changes were observed in those with a baseline ppFEV1 $\geq$ 90%. Although there was no difference in baseline ppFEV1 among CF adults and children, a much higher mean delta ppFEV1 was achieved in children (3.7%) versus adults (1.1%) by 6 months, with a higher ratio in responders. Consequently, due to the strong correlation between plasma HE4 and ppFEV1, the HE4 biomarker showed better discriminatory power in children. On the other hand, regardless of age, those study participants who had worse pre-treatment lung function parameters showed a more substantial pulmonary improvement by 6 months of CFTRm therapy leading to higher alterations of HE4 plasma concentration, a finding also confirmed by the PROSPECT study.

In the cohort 1 of our second clinical study, serum HE4 concentrations significantly decreased following 3 months of ETI treatment compared to baseline, with a mean change in HE4 of -45.7 pmol/L. Patients receiving ETI in cohort 2 demonstrated a smaller mean delta value of -20.7 pmol/L. Concurrently, serum HE4 levels also decreased in patients under Orkambi® therapy (mean change: -18.5 pmol/L), however, significantly lower HE4 levels were measured at all follow-up time points in the ETI treated group compared to the Orkambi® treated group showing only moderate improvement in lung function (+1.6%).

Previously, IVA alone (Kalydeco®) could cause a larger alteration in plasma HE4 (-14.4 pmol/L vs -10.7 pmol/L) in a cohort with CF patients bearing at least one p.Gly551Asp-CFTR variant in trans (the Class III CFTR variant). Among these patients in the GOAL study, the mean delta in ppFEV1 was also higher (7.0%) after 6 months compared to participants in the PROSPECT study, who carried the Class II *p.Phe508del* variant with a mean delta ppFEV1 of only 2.6%. These findings are consistent with our original hypothesis that plasma HE4 sensitively tracks even minor improvements in lung function in CF patients receiving CFTR modulator treatment. Furthermore, it can be concluded that the more significant therapeutic effect of Kaftrio® compared to Orkambi® was clearly reflected in plasma/serum HE4 concentrations. Both delta and absolute HE4 levels correlated well with delta and absolute ppFEV1 values, and a significant inverse correlation between HE4 and ppFEV1 was observed across all cohorts.

Recently, Schmidt et al. also analyzed HE4 and matrix metalloproteinase 9 (MMP9) to monitor the effect of ETI, and a significant decrease in the level of both markers was observed by 6 months of ETI treatment. In parallel, MMP9 was utilized in a small group of pwCF on Trikafta[®], and plasma MMP9 expressed via the NF- κ B pathway by circulating mononuclear cells was downregulated in responders. At the same time, non-responders had sustained elevated MMP9 levels under therapy, suggesting the potential role of MMP9 as a new biomarker for treatment efficacy.

The discriminatory power of the mean change in plasma and serum HE4 was determined using ROC analysis for different degrees of lung function improvement during Orkambi[®] (2.6% and 5% improvement at month 6) and Kaftrio[®] (4% improvement at 3–6 months) treatment. For Orkambi[®], the AUC value for delta HE4 was significant in all CF patients (0.9139). This was particularly pronounced in children, showing high sensitivity (80%) and specificity (83%).

In the case of Kaftrio[®] treatment, the mean change in HE4 also resulted in significant AUC values in both cohort 1 (0.765) and cohort 2 (0.789) on ETI, as well as in both children (0.791) and adults (0.788). When a delta ppFEV1 of 5% was used as the threshold, the AUC value was 0.6786 for all patients, while it was also higher in children (0.7913). Patients were categorized as responders ($\geq 5\%$) or non-responders ($< 5\%$) based on the mean change in ppFEV1 at the 6th month of treatment. Significantly decreased plasma HE4 levels were observed only within responders.

Logistic regression analysis was performed to evaluate which factors influence responder status. Both delta HE4 and baseline ppFEV1 were identified as independent variables associated with the responder group (defined as a ppFEV1 change $\geq 5\%$) compared to non-responders. Similarly, baseline variables—including age, sex, sweat chloride concentration, and BMI—showed no statistically significant association with the treatment response. In our previous study, we likewise demonstrated a significant, independent relationship between delta ppFEV1 and delta HE4 in CF patients receiving IVA treatment.

The rescue of CFTR function by CFTRm was observed using the whole cell patch clamp technique, similar to previous publications. Based on our current data, we considered *p.Phe508del*-CFTR function rescued, since Boinot et al. suggested that if the current density is greater than the threshold of 4 pA/pF after treatment with CFTRm, the CFTR activity be considered to be corrected. Consequently, HE4 was significantly reduced by both the ELX/TEZ/IVA and LUM/IVA combinations. Importantly, ELX/TEZ/IVA caused a more

substantial alteration in the Cl⁻ current and the HE4 concentrations than LUM/IVA. This observation was corroborated by our recent *in vitro* experiments in CFBE 41o- cells expressing *p.Phe508del*-CFTR, where treatment of these cells with both TEZ/IVA and LUM/IVA showed significantly decreased HE4 expression compared to untreated cells. Still, overall HE4 concentrations did not normalize. All these data underline that HE4 levels do not reach normal (i.e., “wild-type levels”) in pwCF, even in the presence of improved CFTR function following administration of CFTRm. This finding is consistent with the partial correction of *p.Phe508del*-CFTR by CFTRm. In summary, ETI caused a larger reduction in HE4 expression in the cell cultures *in vitro*, correlating with altered serum HE4 levels observed in *ex vivo* samples.

We complemented our clinical investigations with HE4 levels in sputum samples obtained from CF patients who developed exacerbations following sample collection. Previous studies have shown that serum IL-1 β , MPO, and CRP, as well as plasma IL-8, can predict acute pulmonary exacerbations, but only in the short term. Therefore, we aimed to assess whether sputum HE4 could serve as a predictive biomarker for pulmonary exacerbations. Our results demonstrated that HE4 levels measured in sputum correlate well with the severity of pulmonary exacerbations in CF and are associated with classical inflammatory parameters detected in sputum, such as neutrophil elastase (NE), IL-1 β , MPO, and MMP-9.

In addition to plasma, serum, and sputum samples, we also investigated BAL samples from CF patients to evaluate the relationship between HE4 levels and lung function. HE4 concentrations measured in BAL samples showed a significant correlation with FEV1 values.

Recent investigations have provided strong evidence for the activation of EMT events in chronic lung diseases, such as chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and CF. TGF β 1, a key mediator and powerful inducer of EMT, is significantly overexpressed in these disorders and in the airways of CF patients. Notably, EMT in CF is often partial, resulting in cells with hybrid EMT phenotypes. This aligns with the idea of EMT as a spectrum of flexible, intermediate states rather than a complete epithelial-to-mesenchymal transition.

Furthermore, we examined HE4 and MMP9 expression in CF-related EMT cellular conditions *in vitro*. We aimed to show that altered HE4 levels are linked to EMT development in CFBE cells, which contribute to increased MMP9 expression. To do this, *p.Phe508del*-CFTR CFBE 41o- cell cultures were compared to control wt-CFTR CFBE cells using IF, RT-qPCR and ELISA, based on native EMT biomarker expression. CF epithelial cells showed decreased

E-cadherin and increased N-cadherin, Vimentin, and IL1B expression. Additionally, both HE4 and MMP9 protein levels were higher in CFBE cells carrying *p.Phe508del*-CFTR compared to controls. Consistent with the protein data, transcript analysis revealed more pronounced differences in mRNA levels of these markers between the two cell lines. These native EMT features align with previous findings where E-cadherin appeared weaker in CF bronchial epithelium tissue compared to controls and stained positively for N-cadherin, Vimentin, and Collagen I. Moreover, polarized *p.Phe508del*-CFTR CFBE 41o- cells showed increased expression of these mesenchymal markers compared to wt-CFTR cells without manipulation. Overall, these findings indicate a shift toward a mesenchymal-like and pro-inflammatory phenotype in native CF bronchial epithelial cells, suggesting disruption of epithelial integrity and induction of EMT-related molecular traits under basal conditions.

To explore whether EMT could be further enhanced in CF airway epithelial cells, *p.Phe508del*-CFTR CFBE cells were treated with TGFβ1. As expected, TGFβ1 exposure caused a significant decrease in E-cadherin and an increase in N-cadherin and MMP9 protein levels, as shown by IF and ELISA. Interestingly, HE4 expression was notably reduced after TGFβ1 stimulation compared to its elevated baseline in CFBE cells homozygous for *p.Phe508del*-CFTR, suggesting that activated EMT is linked to HE4 downregulation in CF. These data imply that HE4 is usually expressed in normal epithelial cells, but its level decreases when the epithelium loses its epithelial phenotype.

During the investigation of EMT development, we analyzed the effects of the complex airway inflammatory milieu—well modeled by specific stimuli—on CF airway epithelial cells in the process of EMT. Therefore, airway epithelial cells were treated *in vitro* with BAL samples obtained from CF patients. Following BAL treatment, E-cadherin expression was significantly decreased, while N-cadherin expression was increased, similarly to Vimentin and IL-1β. Based on these findings, we propose that the airway microenvironment characteristic of CF may contribute to the induction of EMT and the initiation of inflammatory processes in the lungs.

Next, we explored whether pharmacological CFTR rescue could reverse these EMT-related changes. Therefore, basal *p.Phe508del*-CFTR CFBE cells were treated with ETI as well as being co-treated with TGFβ1 and the triple CFTR modulator combination. To monitor the restoration of CFTR function by ETI, we recently applied a whole-cell patch clamp technique under the same *in vitro* experimental conditions we also used. The improvement of CFTR function by ETI was acknowledged in these experiments. We here showed a restoration of epithelial features, evidenced by increased E-cadherin and decreased N-cadherin expression by

IF. With these immunostaining data, RT-qPCR confirmed normalization of E-cadherin, N-cadherin, Vimentin, and IL1B mRNA levels. However, HE4 and MMP9 mRNA remained significantly lower in response to ETI and in the presence of TGF β 1 and ETI compared to TGF β 1 alone. ETI treatment partially restored tight junction integrity by increasing claudin-1 levels and the E-/N-cadherin ratio in mutant CFBE cells. In a rat model, the pathways involved in EMT were shown to depend on CFTR mutation. Overall, the level of functional CFTR closely correlates with the epithelial/mesenchymal state of CFBE cells.

We then examined how HE4 is regulated over the long term under continuous stimulation. Continuous exposure to TGF β 1 caused a steady and significant decrease in HE4 levels up to 120 hours. HE4 expression was also checked in non-epithelial cell lines to see if this effect is specific to certain cell types. Interestingly, no HE4 protein was detected in human pulmonary mesenchymal stem cells or in CF-derived human lung fibroblasts, confirming that HE4 is limited to epithelial cells. Wang et al. also observed a reduction in WFDC2 (HE4) in nasal mucosal epithelial cells from people with chronic rhinosinusitis, indicating an abnormal mix of epithelial and mesenchymal cells caused by EMT during chronic inflammation. Importantly, as we found, fibroblasts did not express HE4.

Additionally, MMP9 protein levels were measured in native wt-CFTR and *p.Phe508del*-CFTR CFBE cells, as well as in mutant cells treated with TGF β 1 with or without ETI. Consistent with the immunofluorescence and RT-qPCR data, baseline MMP9 levels were higher in *p.Phe508del*-CFTR CFBE cell supernatants than in wt controls which were lowered by ETI alone. MMP9 expression increased further after TGF β 1 stimulation, but this increase was significantly reduced by co-treatment with ETI. Plasma MMP9 was elevated in CF patients before CFTR-specific treatment and decreased following Trikafta[®] (which is ETI). Since TGF β 1 can induce MMP9 expression in other human cells, such as meningeal cells, through activation of ERK and Smad pathways, it is likely that high levels of TGF β 1 partly regulate the increased MMP9 expression seen in CF.

HE4 expression was artificially silenced in unstimulated and TGF β 1-stimulated *p.Phe508del*-CFTR CFBE cells using a HE4-specific siRNA approach. HE4 knockdown induced EMT-like changes. Additionally, MMP9 mRNA and protein expression was increased, suggesting a direct functional link between HE4 and MMP9 expression. This molecular connection was first shown by others when HE4 directly interacted with MMP9 protein in fibrotic kidney lysates, as demonstrated by immunoprecipitation with an anti-HE4 antibody followed by western blotting analysis.

We systematically analyzed IL1B mRNA level under EMT conditions as well in parallel to EMT characteristics, HE4 and MMP9. Although non-CF bronchial epithelial cells showed that IL-1 β enhanced TGF β 1-induced EMT, this established connection in lung epithelial biology supports the plausibility that IL-1 β might act similarly in inflamed CF epithelia as well. Based on our data, EMT was associated to a higher mRNA level of IL-1 β especially after exposure to TGF β 1 suggesting a link between inflammation and EMT in CF. Finally, assessment of cell proliferation using Ki67 staining revealed a significant decrease in proliferative activity in p.Phe508del-CFTR CFBE cells after HE4 silencing compared with NEG-01 control cells, indicating that EMT induction under reduced HE4 expression is associated with decreased proliferative potential. Similarly, knocking down HE4 decreased EMT development, cell proliferation, and Ki67 expression in ovarian cancer cells *in vitro*.

However, our study has several limitations. Serum HE4 levels were monitored for a maximum of 6 months; therefore, we were unable to assess the long-term effects of CFTR modulator therapy. Furthermore, the relationship between CFTR function corrected by modulators and HE4 expression was not fully elucidated, prompting additional *in vitro* investigations to improve understanding. We did not perform further experiments to more comprehensively characterize EMT in CFBE cells (e.g., wound healing assays). In parallel with HE4 measurements, we also did not examine the direct impact of EMT development on CFTR expression in CFBE cells. Finally, the mechanisms underlying TGF- β 1 mediated modulation of HE4 expression, as well as the detailed relationship between HE4 and MMP9, were not fully clarified; therefore, further studies are required to better define these regulatory pathways in the future.

In summary, plasma/serum HE4 levels more accurately reflect the efficacy of pharmacological treatment in CF patients receiving ETI therapy compared to those treated with LUM/IVA. Based on our findings, monitoring CF lung disease using changes (delta values) in HE4 levels appears to be particularly informative. Continuous measurement of plasma/serum HE4 may facilitate the assessment of CFTR modulator therapy effectiveness in routine clinical practice. Our *in vitro* results confirm that EMT is associated with decreased HE4 expression and increased MMP9 expression in CF airway epithelial cells, and there is a direct link between CFTR dysfunction and the development of EMT in CF. Furthermore, decreased HE4 expression contributes to the development of the EMT-associated phenotype and reduced cell proliferation in airway epithelial cells.

6. Summary

Cystic fibrosis (CF) is the most common autosomal recessive inherited genetic metabolic disorder in the Caucasian population, characterized by the absence or dysfunction of the CFTR ion channel. This results in the production of altered, viscous secretions by exocrine glands, leading to progressive damage of the affected organs. Early mortality in CF patients is primarily caused by the gradual decline in pulmonary function and the development of pulmonary fibrosis on the basis of chronic inflammation dominated by neutrophil granulocytes. Pulmonary fibrosis is preceded by the process known as epithelial–mesenchymal transition (EMT).

Currently available clinical parameters for monitoring the efficacy of novel CFTR modulator therapies in CF are limited. Airway inflammation characteristic of the disease can be detected even at early stages; therefore, measuring the concentrations of mediators involved in these processes in blood or airway samples represents a feasible approach for assessing therapeutic effectiveness. These therapies improve lung function, reduce inflammation, and decrease the frequency of infections. However, there remains a need to identify reliable biomarkers that enable the monitoring of disease progression and treatment efficacy. Plasma and serum HE4 concentrations have shown a strong correlation with disease severity, suggesting their suitability for monitoring the effectiveness of Orkambi® and Kaftrio® therapies. Based on our findings *in vitro*, the CFTR correcting effect of Kaftrio® was significantly greater compared to Orkambi®. Furthermore, a properly functioning CFTR maintains normal ion transport and epithelial integrity in healthy airways, whereas in CF, CFTR dysfunction and inflammation lead to elevated levels of HE4 and MMP9. In the long-standing presence of TGFβ1, the process of EMT progresses, accompanied by increased MMP9 levels and decreased HE4 expression.

In summary, plasma and serum HE4 concentration correlates with improvements in pulmonary function in CF and may serve as novel, reliable, and easily measurable biomarker for monitoring the efficacy of both Orkambi® and Kaftrio® CFTR modulator therapies. Moreover, our findings indicate that EMT-induced downregulation of HE4 expression is accompanied by an increase in MMP9 levels in CF airway epithelial cells, and decreased HE4 expression contributes to the development of an EMT-associated phenotype and reduced cell proliferation.

7. Novel scientific findings of the dissertation and the candidate's own contributions

1. Plasma/serum HE4 levels show a strong correlation with the ppFEV1 lung function parameter and reliably reflect the efficacy of Orkambi[®] and Kaftrio[®] treatments.
2. Orkambi[®] therapy results in a significant decrease in plasma HE4 levels in CF patients, with an even more pronounced reduction observed after Kaftrio[®] treatment.
3. EMT is associated with decreased HE4 expression and increased MMP9 expression in bronchial epithelial cells.
4. Reduced HE4 expression contributes to the development of an EMT phenotype and decreased cellular proliferation in epithelial cells.

8. Keywords

Biomarker research, cystic fibrosis, CFTR, CFTR modulator, ELX/TEZ/IVA, EMT, HE4, treatment efficacy, lung function, LUM/IVA, sweat chloride

9. Publication list



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List of publications related to the dissertation

1. **Pócsi, M.**, Balla, G. J., Fenyvesi, F., Rusznyák, Á., Fejes, Z., Balogh, I., Macek, J. M., Amaral, M. D., Nagy, B. Jr.: Investigation of HE4 expression concerning epithelial-mesenchymal transition (EMT) in cystic fibrosis epithelial cells.
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IF: 6 (2024)
2. **Pócsi, M.**, Fila, L., Péterfia, C., Halász, A., Szántó, G. T., Mészáros, B., Major, J., Laki, I., Szabó, H., Panyi, G., Balogh, I., Amaral, M. D., Macek, J. M., Nagy, B. Jr.: Comparison of the Effect of CFTR Modulators elexacaftor/tezacaftor/ivacaftor and lumacaftor/ivacaftor via Serum Human Epididymis Protein 4 Concentration in p.Phe508del-CFTR Homozygous Cystic Fibrosis Patients.
J Clin Med. 14 (17), 1-18, 2025.
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4. Bekéné Debreceni, I., Krajcsir, B., Mátrai, Á. A., Ujhelyi, B., **Pócsi, M.**, Balla, G. J., Nagy, B. Jr., Deák, Á., Németh, N., Fülesdi, B., Kappelmayer, J.: IgM-enriched immunoglobulin treatment significantly improves coagulation dysbalance in an experimental animal model of fulminant sepsis.
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