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

Investigation of the role of HE4 as a novel inflammatory biomarker and its bronchoepithelial expression in cystic fibrosis

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The PhD Defense takes place at the Lecture Hall of the Institute of Paediatrics, Faculty of Medicine, University of Debrecen, March 17, 2023. 1.30 pm.

Introduction and literature review

Pathomechanism of cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive inherited systemic disease with a life shortening effect. More than 2000 pathogenic defects of the *Cystic Fibrosis Transmembrane Regulator (CFTR)* gene have been identified. As a result of genetic mutations, the CFTR chloride/bicarbonate channel is either not expressed on the epithelial cell surface or is expressed in the cell membrane but becomes dysfunctional, leading to abnormal ion and water transport. In the European patient population, including Hungary, the most common CFTR gene variant is p.Phe508del (F508del, in the old nomenclature delta-F508), which is carried by about 80-85% of CF patients in homozygous or compound heterozygous form. This mutation consists of a deletion affecting 3 base pairs in exon 11 of the gene, which leads to the loss of phenylalanine at position 508 of the amino acid chain. Different mutations can cause different degrees of quantitative and/or qualitative disruption of CFTR, which profoundly affects the spectrum of clinical symptoms and the severity of the disease.

The classic form of CF is a multi-organ disease in which altered, abnormal secretion of endocrine glands leads to pathological processes that cause permanent damage to the affected organs. The lungs and the gastrointestinal system are most severely affected. The latter abnormalities are manifested by severe malabsorption and malnutrition, even as early as infancy. Impaired airway clearance, chronic intrapulmonary inflammation associated with high neutrophil granulocyte burden provoked by infections and characterized by increased expression of interleukin-8 (IL-8), IL-6 and tumor necrosis factor-α (TNF-α), play a fundamental role in the progression of CF airway disease. Toll-like receptors (TLRs) mediate inflammatory activation through the recognition of several bacterial products, such as lipopolysaccharide (LPS) of Gram-negative bacterial origin, in partially through the nuclear factor-kappa B (NF-κB) signaling pathway. Chronic airway inflammation leads to damage of the bronchi and the lung tissue, and consequently to respiratory failure with permanent deterioration of lung function. Convincing evidence suggests that the increased inflammatory response in the airways that is characteristic of CF is not uniquely secondary, i.e., due to infections, but also depends on pro-inflammatory processes associated with defective CFTR. In epithelial cells, increased activity of the mitogen-activated protein kinase (MAPK) protein and NF-κB-dependent signaling pathways are also characteristic. Hunter and his group have

previously shown that the wild-type CFTR channel has an inhibitory effect on basal and TNF-α induced NF-κB pathway activity in airway epithelial cells by a mechanism that is still not well understood, whereas the inhibition of selective function of the CFTR ion channel significantly increased NF-κB signaling activity. Based on these findings, intact channel function of the protein seems to be required for CFTR to elucidate its anti-inflammatory effect. Although only mild inflammation is present in the airways of CF lung at birth, abnormal immunoregulatory processes and ineffective defense against pathogens result in a more intense inflammatory reaction in response to early pulmonary infections. The inflammation of CF lung is characterized by a predominance of neutrophil granulocytes and increased neutrophil elastase (NE) activity associated with lung injury.

The role of mutation specific CFTR modulators in CF therapy

While traditional CF therapy has focused on controlling disease progression and managing its consequences and complications, newer treatment strategies in the last decade have turned towards novel therapeutic options that can either enhance the amount of functional CFTR channel on the cell membrane (correctors) or improve the ion channel function of CFTR expressed on the cell surface (potentiators) for a subset of mutations with residual CFTR activity. The CFTR potentiator *ivacaftor* (Kalydeco®) enhances the opening frequency of the chloride channel and can therefore be used effectively in CF patients carrying a class III so-called gating mutation (e.g. G551D, p.Gly551Asp). CFTR correctors such as *lumacaftor*, *tezacaftor* or *elexacaftor* enhance the intracellular maturation of the channel protein and its release to the cell surface. In the case of p.Phe508del mutations carried by the majority of CF patients, the molecular mechanism of the CFTR defect is more complex, with protein maturation, abnormal channel motility and increased "turnover". In the latter mutation, the combined use of at least a corrector and a potentiator molecule is required for clinical efficacy. *Lumacaftor/ivacaftor* (Orkambi®) or *tezacaftor/ivacaftor* (Symdeco®, Symkevi®) have been shown to be effective in patients homozygous for p.Phe508, while the newest triple combination, *tezacaftor/elexacaftor/ivacaftor* (Kaftrio®), has been shown to be effective in the heterozygous genotype with p.Phe508, which accounts for the majority of CF patients. The early introduction of CFTR modulators, before the onset of pronounced structural bronchopulmonary damage, significantly delays the progression of lung disease, reduces the frequency of acute respiratory exacerbations, hospitalizations, and antibiotic treatment, and also

has a beneficial effect on extrapulmonary sequelae, including improved nutritional status of patients.

The role of inflammatory biomarkers in CF

Follow-up of CF lung disease is a key aspect of care, as the prognosis, quality of life and mortality are mostly determined by the severity of the lung disease beginning as early as infancy. However, the possibilities for detecting airway involvement, especially at a younger age, are still limited today. Measurement of various inflammatory mediators of airway inflammation from blood or airway samples may provide an opportunity to monitor inflammatory processes in the lung.

A variety of inflammatory biomarkers can be determined in sputum or bronchoalveolar lavage fluid (BAL), which are useful for prognostic purposes, predicting deterioration of respiratory function and monitoring the effect of therapeutic regimens (e.g., NE, antiproteases, IL-6, IL-8, IL-1ß, TGF-ß1, etc.). However, due to sampling difficulties, the invasive character of BAL, and the lack of standardized measurement procedures, these biomarkers have not been introduced into routine clinical practice.

Probably the most obvious and analytically standardized and reproducible method of monitoring airway inflammation is the determination of biomarkers from blood samples i.e., plasma, or serum. Blood sampling is generally an easy and non-invasive procedure at any age and in any type of disease. Different blood-based biomarkers carry useful information for clinicians in different aspects of CF lung disease:

1. reliably distinguish a patient with CF from a non-CF person,
2. track the severity of CF lung disease,
3. predict the onset of acute pulmonary exacerbation,
4. show significant concentration changes during pulmonary exacerbation, and
5. monitor the degree of inflammation under antibiotics or CFTR-specific treatment.

Although a considerable number of biomarkers have been investigated to monitor inflammatory processes in CF lung disease, they have not yet become available for routine practice due to analytical limitations or limitations of their assessment. At present, there are no approved and sufficiently sensitive plasma/serum-based parameters for monitoring the efficacy of CFTR modulators or even conventional treatments, therefore there is an unmet need to introduce new, well reproducible, easily measurable, and accessible biomarkers for the regular follow-up of CF patients.

HE4 as a potential CF biomarker

The WFDC family of proteins includes small proteins that have various, usually protective functions, such as antiprotease, antibacterial, or anti-inflammatory properties. They are expressed in high levels in the lung and may therefore play a role in maintaining lung homeostasis and protecting against proteolytic effects. Currently, 18 subtypes are known, of which SLPI, also known as WFDC4, is produced by epithelial cells and activated macrophages and exhibits anti-inflammatory effects on monocytes, neutralizes NE and IL-8 and inhibits neutrophil influx. Elafin (WFDC14) is also expressed in the airway epithelial cells and activated macrophages and can opsonize *P. aeruginosa*.

Expression of HE4 (also known as WFDC2) protein has been detected in a variety of healthy and malignant tissues. In the clinical setting, it was first investigated as a novel tumor marker in ovarian and endometrial carcinoma and lung tumors. The potential association between CF disease and abnormal HE4 expression was brought to our attention by a previous study showing elevated epithelial HE4 expression in lung biopsy specimens from CF patients by immunohistochemical staining. However, the authors did not investigate whether the increased production of intrapulmonary HE4 also results in elevated levels of this protein in the circulation in CF. Furthermore, the cellular mechanism of the increased production of HE4 protein in this disease was not known.

In this dissertation, we investigated the potential role of a novel biomarker called human epididymis protein 4 (HE4) protein for CF in assessing and monitoring the severity of CF lung disease and in the follow-up of CFTR mutation-specific pharmacotherapy. In addition, we aimed to gain a detailed understanding of the mechanism of abnormal HE4 expression in CF airway epithelial cells via studying broncho-epithelial cell cultures maintained and treated *in vitro*.

Objectives

Serum HE4 as a potential diagnostic and prognostic biomarker in CF

Our aim was to evaluate whether serum HE4 has a reliable diagnostic and/or prognostic value in the evaluation of lung disease in CF. For this purpose:

- We measured HE4 concentrations in serum samples from two independent CF patient populations and compared these results with serum HE4 values from non-CF lung disease patients and healthy controls.

- We were curious to see whether serum HE4 levels in CF patients correlated with serum CRP levels, sweat chloride concentrations, or showed any correlation with patient sex and age.

- We analyzed whether differences in HE4 concentrations were associated with lung disease severity or subsequent acute exacerbations.

Analysis of the effectiveness of plasma HE4 levels in monitoring CF treatment

We also aimed to study the hypothesis that HE4 is a novel biomarker for monitoring CF patients during CFTR-specific *ivacaftor* or *lumacaftor/ivacaftor* treatment. For this purpose:

- We measured the evolution of plasma HE4 levels before and during treatment in three independent groups of CF patients with p.Gly551Asp (G551D) mutations carrying at least one allele of the class III CFTR mutation and treated with *ivacaftor* (Kalydeco).

- We examined if changes in plasma HE4 levels correlated well with the improvements in other clinical parameters in these patient groups.

- We assessed whether plasma HE4 concentrations and their change (delta value) showed a predictive role in the improvement of lung function status.

We examined separately the variation of plasma HE4 levels in pre- and post-treatment plasma samples from CF patients homozygous for the p.Phe508del-CFTR mutation under Orkambi (*lumacaftor/ivacaftor*) treatment.

Investigation of the pathomechanism of increased HE4 expression and its association with intraepithelial inflammation in bronchoepithelial cell cultures

A further aim of our experimental work was to gain insights into the pathomechanism of increased HE4 expression and its association with intraepithelial inflammatory processes.

Therefore, we investigated whether increased HE4 expression in CF bronchoepithelial (CFBE) cells could be influenced by alterations in CFTR function and whether it could be associated with the increased pro-inflammatory state characteristic of CF.

- We examined whether CFBE 41o$^-$ cells expressing F508del-CFTR showed an increased HE4 expression compared to CFBE 41o$^-$ cells expressing wt-CFTR.

- We observed how CFTR function directly affected HE4 expression in CFBE 41o$^-$ cell cultures expressing wild-type CFTR or F508del-CFTR.

- We also examined whether HE4 expression can be enhanced by the activation of the NF-κB signaling pathway in CFBE cells in response to inflammatory stimuli (using TNF-α).

- Finally, we examined whether the CFTR channel regulated HE4 expression in CFBE cells via or independently of the NF-κB signaling pathway.

Materials and methods

Patients and controls

In our first study, we compared serum HE4 levels in CF and non-CF patient groups from six CF centres in Hungary (University of Debrecen, Institute of Paediatrics, Debrecen; Kenézy Gyula County Hospital, Debrecen; Szent-Györgyi Albert University, Institute of Paediatrics, Szeged; Heim Pál Children's Hospital, Budapest; Petz Aladár County Hospital, Győr; Markusovszky Lajos County Hospital, Szombathely), a total of 77 Hungarian pediatric CF patients (under 18 years) and, as an independent cohort, a total of 57 Czech adult CF patients from one CF center in Prague (Department of Pulmonology, Charles University, Prague, Czech Republic) were selected. The diagnosis of CF was established according to current CF consensus diagnostic criteria. All participants presented the classic form of the disease. The clinical status of the patients was categorized according to the Shwachman-Kulczycki scoring system, or in some institutions, in the absence of its application, according to the clinician's opinion, considering detailed clinical and laboratory investigations. Based on the Shwachman-Kulczycki scale score, patients were classified into three subgroups: mild (71-100 points), moderate (41-70 points) and severe (≤ 40 points). Blood samples were collected at the outpatient follow-up visits, when patients were usually in mild to moderate condition, or on hospital admission when they were in severe status, sometimes due to acute pulmonary exacerbation (e.g., increased cough with increasing sputum volume, dyspnea, weakness, or severe worsening of the respiratory function score). CRP levels were available for children with severe CF (n=29) and all Czech adult patients.

To investigate the possible non-specific effect of non-CF airway inflammation on HE4 serum concentrations, we also included 64 patients from the above institutions who also had severe non-CF lung disease (e.g., chronic bronchitis, asthma, or pneumonia). Additionally, 12 control patients with other medical conditions were also included, diagnosed as: non-CF bronchiectasis (n=2); congenital diseases (Werdnig-Hoffman syndrome, Williams-Campbell syndrome, microcephaly, n=7); and therapy-resistant epilepsy (n=3). These children showed signs of bronchiectasis due to frequent aspiration and recurrent respiratory tract infections due to bronchial secretions. Eighteen adult patients with non-CF bronchiectasis and chronic obstructive pulmonary disease (COPD) were included as a control group of adult CF patients. The diagnosis of this clinical control group was confirmed because of standard radiological and laboratory investigations. Exclusion criteria were smoking, malignancies, immunodeficiency,

and chronic kidney and liver disease. When young and adult patients were divided into separate groups, there was no significant difference in patient age between the CF and control groups, however, non-CF subjects were younger than CF patients. Finally, 12 healthy, asymptomatic Hungarian CF subjects (mean age 31 [25-37] years) who were carriers of the p.Phe508del CFTR mutation on one allele were tested for serum HE4.

After assessing the utility of serum HE4 measurement for the evaluation of CF patients and the severity of the pulmonary process, we retrospectively analyzed plasma HE4 concentrations in K_3-EDTA-anticoagulated plasma samples in three independent groups of CF patients aged 6 years or older, carrying G551D-CFTR mutation on at least one allele, and receiving CFTR-specific *ivacaftor* (Kalydeco) treatment. A subset of these patients had previously been enrolled in large clinical trials, such as the US GOAL and Irish CORK studies, and we also included a cohort of an Australian center (Adult CF Centre at The Prince Charles Hospital, Brisbane, Australia). The samples of 29 patients treated within the GOAL study (Group 1) were obtained from the Cystic Fibrosis Foundation Therapeutics (CFFT) Biorepository (Bethesda, MD, USA) biobank. HE4 levels were determined from plasma samples taken before treatment (baseline) and 1, 3 and 6 months after initiation of *ivacaftor* treatment. The 12 patients in group 2 had samples taken before treatment and 1 and 2 months after treatment. Finally, 19 CF patients were sampled from the CORK study with samples taken before therapy and at 3 months. Since the drug *ivacaftor* was not yet available in our country within the setting of a clinical study at the time of our study, we obliged to request samples within the framework of a foreign collaboration to further test HE4.

Finally, in parallel to the *in vitro* assessment of the mechanism of HE4 expression in bronchoepithelial cells, we evaluated the effect of *lumacaftor/ivacaftor* CFTR-specific treatment on circulating HE4 levels in 10 CF patients with stable clinical status who were homozygous for p. Phe508del-CFTR mutation (5 females and 5 males, mean age 16.1 ± 4.8 years). Pre-treatment plasma HE4 values were compared with those obtained 1 month after initiation of Orkambi treatment. These plasma samples were also obtained frozen from the CFFT Biorepository biobank and had previously been obtained from patients enrolled in the PROSPECT study. Since samples from patients treated with *lumacaftor/ivacaftor* were not yet available at the time of this study, we again used a foreign sample source for the measurements.

In each CF patient group, sweat chloride concentrations were determined using a Sweat-Chek Conductivity Analyzer (Wescor, Logan, UT, USA) at each center. To detect CFTR mutations in Hungarian patients, genomic DNA was isolated from white blood cells of blood samples using QIAgen Blood Mini Kit (Qiagen, Hilden, Germany). The 30 most common

CFTR mutations in Hungary were identified using the Elucigene CF29v2Kit (Tepnel-Diagnostics, Manchester, UK) at the Department of Molecular Pathology, Institute of Laboratory Medicine, University of Debrecen, Germany, complemented by the analysis of the CFTRdele2,3 (21kb) "Slavic" mutation by Sanger sequencing. Elucigene EU v3 assay (Tepnel-Diagnostics) and MLPA kit (MRC-Holland, Amsterdam, The Netherlands) were used to genotype the selected Czech adult CF patients (Department of Biology and Medical Genetics, Charles University, Prague, Czech Republic). In parallel to serum testing, two other types of samples were also collected: bronchial mucosa biopsies were taken during diagnostic bronchoscopy from three Hungarian CF patients (2 boys and 1 girl; 3, 4 and 9 years old) and three patients with recurrent episodes of bronchitis (2 boys and 1 girl; 2, 4 and 10 years old) with non-CF pulmonary abnormalities (University of Debrecen, Institute of Pediatrics). In the control group, bronchoscopy was performed to clarify the background of wheezing episodes and to exclude central airway obstruction.

Reagents for cell culture experiments

CFTR correctors lumacaftor (VX-809, LUM) (S1565) and tezacaftor (VX-661, TEZ) (S7059), CFTR potentiator *ivacaftor* (VX-770, IVA) (S1144), voltage-independent selective CFTR inhibitor $CFTR_{inh172}$ (S7139), CFTR activator Forskolin (FSK, S2449), and NF-κB pathway inhibitor BAY 11-7082 (S2913) were purchased from Selleck Chemicals (Houston, TX, United States). cAMP phosphodiesterase inhibitor IBMX (3-isobuthyl-1-methylxanthine, I5879) was ordered from Sigma-Aldrich (St. Louis, MO, USA). Except for recombinant TNF-α (Gibco, Carlsbad, CA, USA), all reagents were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich).

Cell cultures and treatment conditions

Cell Culture CFBE $41o^{-}$ cells cultures stably expressing F508del-CFTR or wt-CFTR were grown in Minimum Essential Medium Eagle (EMEM) with Earle's BSS (EBSS) and 1% L-glutamine (Lonza, Walkersville, MD, USA), 10% fetal bovine serum (FBS, Sigma-Aldrich) and 5 μg/ml Puromycin (Sigma-Aldrich) at 37°C, 5% CO2. These cells were obtained from Dr. J. P. Clancy's lab (Cincinnati Children's Hospital Medical Center, OH, USA). CFBE cells were seeded in 6-well plates (250.000 cells per well/ sample). Supernatants for the analysis of HE4 and IL-6 protein levels were collected after CFBE cells were treated with TNF-α or phosphate buffer solution PBS, (i.e., at baseline) and combined CFTR modulators: corrector *lumacaftor* (3 μM) with potentiator *ivacaftor* (10 μM) (LUM/IVA) or corrector *tezacaftor* (5 μM) with

ivacaftor (10 μM) (TEZ/IVA) or DMSO vehicle alone (thus representing the baseline) were administered for 24 h. For the activation of CFTR function, FSK (10 μM) with IBMX (100 μM) (FSK/IBMX) were added to both types of CFBE cells, while CFTR inhibition was carried out by $CFTRinh_{172}$ (20 μM) in wt-CFTR CFBE cells vs. control samples with DMSO with or without CFTR modulators for 24 h. CFTR modulators were applied under similar experimental conditions as in comparable *in vitro* studies (Hunter et al., 2010; Wang et al., 2016; Pranke et al., 2017; Kmit et al., 2019). To investigate the role of NF-κB pathway in HE4 expression *in vitro*, BAY 11-7082 (5 μM) or DMSO (baseline) was used for 24 h to inhibit pro-inflammatory signaling in both unstimulated and TNF-α activated CFBE cells both in the presence or absence of LUM/IVA or TEZ/IVA molecules.

Peripheral blood samples were obtained by venous puncture and stored at -70°C after centrifugation. Plasma samples from clinical studies conducted in foreign countries were shipped by international courier service on dry ice to the Institute of Laboratory Medicine, University of Debrecen.

HE4 concentrations were measured in serum or plasma samples from patients and controls as well as in supernatants of the CFBE cells by chemiluminescent microparticle immunoassay on Architect i2000SR and i1000SR automated instruments (Abbott Diagnostics, Wiesbaden, Germany). Serum CRP levels in Hungarian pediatric and adult CF patients and IL-6 concentrations in the supernatant of CFBE cell cultures were determined by electro-chemiluminescent immunoassay (Cobas e411, Roche Diagnostics). To avoid operator-related measurement bias, these measurements were performed in an analyst-blinded way.

Since it was hypothesized that sputum samples from CF patients would have even higher HE4 concentrations than blood samples due to the pulmonary origin of this protein, spontaneous sputum samples were collected from 6 CF patients. Furthermore, we also collected sputum samples from non-CF (bronchitis) lung patients (n=8) and healthy individuals (n=5) as controls. To homogenize the sticky, viscous sputum, dithiothreitol (DTT) was added to the samples for 15 min on room temperature, then they were centrifuged at 180 g for 5 min at 25 °C to measure HE4 levels in the supernatant.

Electrophysiology to measure chloride current through the CFTR channel

Cl^- currents in CFBE $41o^-$ cells were measured in the whole-cell patch-clamp configuration similar to former publications. The external (bath) solution contained 145 mM NaCl, 4 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 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 tetraacetic acid (EGTA), 10 mM HEPES, and 3 mM Mg-ATP (pH 7.2 titrated with CsOH, 285 mOsm). Mg-ATP was freshly diluted into the intracellular solution every hour. The intracellular solution was stored on ice before usage. FSK/IBMX and $CFTR_{inh-172}$ were freshly diluted into the extracellular solution before the start of the experiments, respectively. Micropipettes were pulled in four stages by using a Flaming Brown automatic pipette puller (Sutter Instruments, San Rafael, CA, USA) from Borosilicate Standard Wall with Filament aluminum-silicate glass (GC150- TF10, Harvard Apparatus Co., Holliston, MA, USA) with tip diameters between 0.5 and 1 μm and heat polished to a tip resistance ranging typically 3–10 MΩ in the bath solution. All measurements were carried out using an Axopatch 200B patch-clamp amplifier (Molecular Devices Inc., Sunnyvale, CA, USA) in voltage-clamp mode, which allows the membrane potential of the analyzed cell to be kept constant while the current across the cell membrane can be determined. The measurements were performed using a Burleigh PCS5000 (Thorlabs Inc., Newton, New Jersey, USA) micromanipulator connected to a personal computer using Axon Digidata 1,550 A data acquisition hardware. (Molecular Devices Inc.). The holding potential was maintained at −40 mV throughout the experiments, and two voltage-clamp protocols were used to measure whole-cell CFTR currents. First, a single depolarization from −40 to 0 mV was applied every 5 s for 4–5 min to monitor the current evolution and to confirm the absence of significant leak current. For determining the current–voltage (I–V) relationship the cells were held at a holding potential of −40 mV and depolarized to test potentials between −80 and +80 mV in steps of 20 mV increments every 10 s. Experiments were done at room temperature (RT) ranging 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. Prior to analysis, current traces were corrected for ohmic leak. Excitation voltage pulses and data acquisition were analyzed via Axon Digidata 1440 A data acquisition hardware (Molecular Devices Inc.) driven by an IBM compatible personal computer using the pCLAMP10 software package (Molecular Devices Inc.). Current traces were digitally filtered with a three-point boxcar smoothing filter. The holding potential was maintained at −40 mV throughout the experiments, and two voltage-clamp protocols were used to measure whole-cell CFTR currents. First, a single depolarization from −40 to 0 mV was applied every 5 s for 4–5 min to monitor the current evolution and to confirm the absence of significant leak current. For determining the current–voltage (I–V) relationship the cells were held at a holding potential of −40 mV and depolarized to test potentials between −80 and +80 mV in steps of 20 mV increments every 10 s. Experiments

were done at room temperature. Dr. Tibor Szántó and Prof. György Panyi (University of Debrecen, University of Debrecen, Institute of Biophysics and Cell Biology) assisted in these studies.

Total mRNA extraction

Total mRNA from CFBE cell culture samples and bronchial mucosa biopsy tissue samples were isolated by TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's recommendations. The biopsy samples were kept in isotonic saline at 4°C in sterile plastic tubes and centrifuged at 1,500 g for 5 min, and the pellets were stored at –70°C until analysis. The purity and the concentration of separated mRNA samples were verified by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Extracted mRNA samples were stored at −80°C before further analysis.

Real-Time quantitative PCR analysis

Complementary DNA (cDNA) synthesis was performed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Vilnius, Lithuania) according to the manufacturer's protocol on extracted mRNA samples. The initial amount of RNA was 1,000 ng per reaction. Real-time quantitative PCR (RT-qPCR) was performed on a LightCycler 480 qPCR instrument (Roche Diagnostics, Mannheim, Germany) with LightCycler 480 SYBR Green I Master mix (Roche Diagnostics) including WFDC2-specific oligonucleotide primers (10 μM, Integrated DNA Technologies, Leuven, Belgium). The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95° C for 10 s and 60° C for 1 min. All measurements were run in triplicate. For normalization, we used the reference gene RPLP0 (36B4). HE4 expression level was determined using the formula Cq=Cq36B4-CqHE4, while the rate of change in the target gene relative to the reference gene was converted as F=2Cq. HE4 expression in TNF-α stimulated CFBE cells was monitored from 1 h up to 1 week vs. the baseline (using PBS) via measuring mRNA concentrations, while induced cellular activation was followed by IL6-, IL8- and IL1B-specific mRNA levels.

Immunofluorescence staining

Detection of the NF-κB pathway activation in CFBE 41o$^-$ cells with or without CFTR dysfunction was visualized via p65 nuclear immunofluorescence staining. For this purpose, F508del-CFTR and wt-CFTR CFBE cells were cultured in 12-well plates on sterile glass microscope slides at a density of 5×10^4 cells/slide for 2 days. Cells were then treated with

TNF-α (100 ng/mL) or vehicle (PBS, baseline) for 4 h. When the impact of CFTR modulator treatment on activation level of NF-κB pathway was studied, CFBE cells were preincubated with *lumacaftor* (3 μM) with *ivacaftor* (10 μM) or *tezacaftor* (5 μM) with *ivacaftor* (10 μM) or DMSO (baseline) for 24 h, and with TNF-α (100 ng/ml) or PBS (baseline) was added for 4 h. After these pretreatments, cells were fixed with ice-cold methanol-acetone (50 v/v %) for 10 min. Nonspecific antibody binding sites were blocked with fetal bovine serum (FBS, Sigma-Aldrich) for 15 min. For primary labeling of NF-κB p65 subunit, polyclonal rabbit anti-human p65 antibody (100 μg/ml, Sigma-Aldrich) was used for 1 h followed by secondary staining with Alexa Fluor 488-conjugated goat-anti-rabbit IgG (5 μg/ml, Sigma-Aldrich) for 1 h. Cell nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA, United States), and samples were observed by Zeiss Axio Scope. A1 fluorescent microscope (HBO 100 lamp) (Carl Zeiss Microimaging GmbH, Goettingen, Germany). DAPI: excitation at 365 nm, emission BP filter 445/50 nm; fluorescein: excitation of BP filter at 470/40 nm, emission BP filter 525/50 nm. Images were analyzed with ZEN 2012 v.1.1.0.0. software (Carl Zeiss Microimaging GmbH). The ratio of nuclear and perinuclear (cytosol) fluorescence intensity was calculated for NF-κB p65 staining. The specificity of immunostaining was checked by incubating the cells with the secondary antibody only, and where very limited background staining was seen. Dr. Ferenc Fenyvesi and Dr. Judit Váradi (University of Debrecen, Faculty of Pharmacy, Department of Pharmaceutical Technology) assisted in these studies.

Ethics statement

Parents or legal guardians of all participating children and adult participants gave written informed consent to participate in the study before the study started. The study was approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen (licence numbers 3777-2012; 4813-2017) and the local ethics committee of Motol University Hospital, Charles University (Prague) for the participation of Czech patients. Large clinical trials were approved by local institutional ethics committees.

Statistical analyses

The Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. Demographic and laboratory parameters and the measured HE4 and interleukin results were expressed as mean SD (standard deviation) or SEM (standard error of mean) for normally distributed data, and median value (interquartile range, IQR) or (min-max) for non-normally distributed data. When comparing pre- and post-treatment samples, paired t-test or Wilcoxon

paired test was used. When comparing more than two sets of data, ANOVA or Friedman's test with Bonferroni or Dunn's post-hoc analysis was used. For comparisons of demographic variables, χ^2 test was used, while for correlation analyses, since the data were non-parametric variables, Spearman's rho test was used. The diagnostic ability of serum HE4 and CRP in CF, as well as the discriminative power of pre-treatment plasma HE4 levels to predict the mean 7% change in FEV_1 % in response to therapy, was assessed by receiver operating characteristic (ROC) curve analysis using area under the curve (AUC). The cut-off or threshold value was determined based on the maximum of the Youden index (sensitivity, specificity, PPV, NPV). Multiple regression analysis was performed to examine the relationship between plasma HE4 level and FEV_1 %, CRP level and other dependent variables before and during *ivacaftor* treatment. For detailed diagnostic analysis of HE4, we also calculated delta values, which were defined as the difference between the HE4 values measured before treatment and those measured at the same study time point for each patient. A probability level of $P < 0.05$ was considered statistically significant. Analyses and graphs were performed using GraphPad Prism 6.01 software (GraphPad Software, USA) and SPSS v.22.0 (IBM SPSS Statistics, IBM Corporation).

Results

Comparison of serum HE4 concentrations in pediatric and adult CF patients with non-CF and normal controls

Our group was the first to consistently measure serum HE4 protein concentrations in CF patients for laboratory evaluation as a new type of inflammatory parameter, and not as a tumor marker as previously used in the clinical practice. We were curious to see whether the increased pulmonary HE4 expression previously observed by immunohistochemistry would also be reflected in CF blood samples.

Main laboratory characteristics of the patient populations included in the first study

The CF patient group had elevated (>60 mmol/L) (median: 107 mmol/L; 90-120 mmol/L) levels of sweat chloride (CF), except for eight patients with borderline levels (30-60 mmol/L). Sweat tests were not performed in the non-CF patient group and clinical control individuals, as CF disease could be excluded with high confidence by other tests. However, in all CF patients included, CFTR variants were detectable on both alleles. The proportions of the major mutation types were as follows: in the pediatric population 43 patients (55.8%) showed a p.Phe508del/p.Phe508del variant, 26 (33.8%) had a p.Phe508del/other mutation and 8 patients (10.4%) did not show a p.Phe508del variant. Among the adult patients, 24 subjects (42.1%) had a p.Phe508del/p.Phe508del variant, 26 (45.6%) were p.Phe508del/other mutation positive and 7 (12.3%) had no p.Phe508del. Serum CRP levels were elevated (reference range < 4.6 mg/L) in young CF patients with severe clinical status (n=29), in all adult CF patients (n=57) and in the two non-CF control groups (n=76+18). Chronic bacterial colonization was detected in 54 cases (70.1%) in the pediatric CF group, while all adult CF patients (100%) were involved. Pancreatic insufficiency (CF child group: 70 (90.9%), adult CF cohort: 46 (80.7%)) and CF-associated diabetes mellitus (CF child group: 8 (10.4%), adult CF cohort: 20 (35.1%)) were diagnosed at different ratio in the two CF populations.

Significantly elevated serum HE4 levels in CF regardless of the CFTR gene variant

Median serum HE4 levels were significantly elevated ($p < 0.0001$) in both children with CF (99.5 73.1-128.9 pmol/L) and adult patients (115.7 77.8-148.7 pmol/L) compared to their healthy controls. Of note, even higher HE4 concentrations were measured in samples from 29 children with severe CF (134.9 [124.5-275.0] pmol/L). In the adult CF group, 13 patients were

classified as severe CF, and had much higher HE4 levels compared to the other patients: 170.8 [146.8-182.9] pmol/L. Neither the sex of the patients (boys: 95.4 [75.0-128.5] vs. girls: 101.5 [72.6-129.6] pmol/L, P = 0.881), nor the CFTR genotype had an effect on the measured HE4 levels: p. Phe508del/p.Phe508del positive patients: 99.5 [73.0-127.5] pmol/L vs p.Phe508del/positive patients with other mutations: 94.6 [66.4-127.8] pmol/L). In addition to clinical controls, we also examined HE4 serum levels in a population of patients with severe non-CF lung disease, such as acute bronchitis, asthma, pneumonia, or bronchiectasis of non-CF origin. In these cases, we also found higher HE4 levels compared to the control group ($p < 0.001$), which were well below those seen in CF ($p < 0.001$). When we further subdivided the children with non-CF lung disease according to their condition, similar HE4 levels were measured in the presence of severe bronchitis or asthma (63.7 [54.1-79.5] pmol/L; n=42) and pneumonia (57.8 [51.2-74.2] pmol/L; n=22). Similarly, samples from 18 adult lung patients without CF (bronchiectasis, COPD) showed moderately elevated HE4 levels (59.2 [41.1-88.6] pmol/L), whereas only carriers of CFTR mutations had completely normal HE4 levels (33.9 [30.1-43.9] pmol/L; n=12). Age did not have a significant effect on serum HE4 (p=0.164).

Similar to serum results, we measured significantly higher HE4 concentrations in sputum from CF patients compared to samples from non-CF lung patients, and especially compared to control samples, despite the absence of significant systemic inflammation at the time of sampling based on serum CRP. When we examined the correlation between serum and sputum HE4 concentrations using Spearman's test, we found a significant positive correlation ($r = 0.9429$; $P = 0.0167$; $n = 6$). These results also support that increased HE4 production and/or release may originate from the lungs in CF and may result in elevated serum concentrations.

High serum HE4 level is strongly associated with CF lung disease severity, abnormal respiratory function and elevated CRP levels

To further investigate the diagnostic value of HE4 as a biomarker, we examined whether there is an association between HE4 levels and the severity of CF lung disease. HE4 levels showed a strong positive association with disease severity in both CF patient groups. Significantly higher HE4 levels were measured even in children with moderate severity CF (108.0 [95.8-133] pmol/L) compared with patients with mild symptoms (60.8 [50.7-73.8] pmol/L). However, even higher HE4 levels were found in patients with severe symptoms (134.9 [124.5-275.0] pmol/L; $p < 0.0001$ compared with mild cases). HE4 levels were also closely related to disease severity in adult CF patients, with lower HE4 levels in mild cases (72.5 [64.1-94.8] pmol/L), much higher in moderate cases (126.5 [104.3-148.3] pmol/L) and very high HE4

levels in the severe group (170.8 [146.8-182.9] pmol/L; $p < 0.0001$ compared with mild cases). However, there was also a significant difference ($P < 0.05$) in serum HE4 between moderate and severe CF patients in both age groups.

Subsequently, we statistically evaluated by Spearman test whether there was a detectable association between HE4 concentration and FEV_1 % as a sensitive indicator of the severity of lung disease. We found a significant inverse correlation between HE4 results and FEV_1 % values (Spearman rho: -0.522; $P < 0.0001$). In conclusion, the worse the pulmonary condition of a CF patient is, the more HE4 is produced, which enters the circulation and becomes measurable in peripheral blood samples.

We also examined whether there was any relationship between changes in HE4 and elevated CRP levels in CF patients for whom the latter data were also available. There was a significant positive correlation between serum HE4 and CRP levels in children with severe CF and in all adult CF patients (Spearman rho: 0.595; $P < 0.001$). In contrast, no correlation between the two parameters was found in non-CF lung patients. Our results, therefore, suggest that serum HE4 may act as a new sensitive airway inflammation parameter in addition to CRP. This is also supported by the comparison of the laboratory characteristics of HE4 and CRP by ROC curve analysis: AUC value (0.724) (95% CI, 0.617-0.830; $P < 0.0001$) for HE4 was much higher than for CRP (AUC: 0.453, 95% CI, 0.347-0.559; $P = 0.435$).

Association of serum HE4 with chronic bacterial colonization and CF-related comorbidities

Children with positive microbiology test results had significantly higher levels of HE4 compared with those without bacterial colonization (106.5 [75.8-133.3] pmol/L vs 78.3 [61.4-96.9] pmol/L; $P = 0.001$). However, since all adult participants with CF had bacterial colonization, we could not analyze the relationship of HE4 with chronic bacterial infection. In the presence or absence of CF-related DM, no substantial alteration was seen in HE4 (DM, 102.6 [97.8-121.5] pmol/L; non-DM, 91.1 [64.7-128.9] pmol/L [$P = 0.645$]). In contrast, pancreatic insufficiency in adults was associated with higher serum HE4 values than those with normal pancreatic function (122.2 [84.1-149.6] pmol/L vs 96.8 [73.8-108.8] pmol/L; $P = 0.037$)

The diagnostic characteristics of HE4 in CF

We studied the potential diagnostic role of HE4 in the overall CF patient population using ROC curve analysis. Based on the values of the entire CF group, the AUC for HE4 was as high as 0.993 (95% CI, 0.986-0.999; $P < 0.0001$) at the 49.3-pmol/L cut-off value for differentiating patients with CF from normal control subjects. The sensitivity, specificity,

positive predictive value (PPV), and negative predictive value (NPV) for HE4 were 97.0%, 96.6%, 66.5%, and 96.6%, respectively. When the AUC for HE4 was calculated for the discrimination of CF from other non-CF pulmonary diseases, this value was slightly lower at 0.778 (95% CI,0.716-0.841; P < .0001), with a modest sensitivity of 56.0%, but a high specificity of 90.0% at the 98.4-pmol/L cutoff value (64.6% PPV and 90.4% NPV).

Finally, we also examined whether sweat chloride results showed any correlation with HE4 values. We found a positive but moderate association between serum HE4 levels and sweat chloride values (Spearman rho: 0.345; P = 0.005). We evaluated HE4 levels separately in patients (n=8) with a borderline range of sweat chloride results (30-60 mmol/L). In this subgroup of CF patients, we observed similarly high HE4 results (100.9 [88.2-115.0] pmol/L) similarly to the entire CF cohort.

Increased level of HE4 mRNA in respiratory epithelium biopsy specimens of CF

Human bronchial epithelium biopsy specimens were obtained via bronchoscopy from three patients with CF, while three patients without CF served as control subjects. HE4 mRNA levels in the patients with CF were significantly upregulated compared with those of the non-CF control subjects (mean ± SEM, HE4/36B4 ratio: 0.354 ± 0.178 vs 0.041 ± 0.017; P = 0.011) n = 3 per group). These results suggest that highly elevated serum HE4 concentrations in CF may be due to its increased secretion by the airway epithelium.

Monitoring plasma HE4 concentrations in cohorts of CF patients treated with *ivacaftor*

In our second clinical study, we examined the ability of HE4 to monitor the clinical and pulmonary status of CF patients who were not only receiving conventional disease management but were already on CFTR-specific therapy. Given that they are currently very expensive drug molecules, it is of great clinical importance to use a relatively inexpensive biomarker that is easy to measure, reproducible and sensitive to changes in respiratory function and clinical status.

Key demographic and clinical characteristics of three independent cohorts of CF treated with ivacaftor

Study samples were requested from three independent centers in a collaborative partnership. Samples were classified into three subgroups according to their origin (Group 1: US GOAL study, n=29; Group 2: Australian study, n=12; and Group 3: Irish CORK study, n=19), from which plasma HE4 concentrations were first determined. Patients treated with

ivacaftor had at least one class III p.Gly551Asp CFTR variant. The groups had different severity of lung disease at the time of sampling. All but four of the other CF patients had blood chloride levels > 60 mmol/L. Australian patients in group 2 were older ($P < 0.010$) compared with patients in the other two groups. It should be noted that none of the patients had impaired renal function as indicated by serum creatinine results, which could influence HE4 values.

Baseline plasma HE4 levels decreased significantly under ivacaftor therapy

All CF patients showed higher HE4 concentrations compared to the normal HE4 values previously described before starting *ivacaftor* treatment. In group 1, plasma HE4 levels started to decrease significantly as early as 1 month after starting *ivacaftor* treatment (60.1 [48.2-77.4] vs. 48.2 [40.8-66.2] pmol/L, $P = 0.023$) and remained well below baseline until the end of the follow-up period (45.1 [38.4-64.2] pmol/L; $P < 0.001$ compared to baseline), in parallel with a slight improvement in respiratory function and BMI and a drastic decrease in sweat chloride concentrations.

As expected, pre-treatment HE4 levels were higher in study group 2, which was characterized by lower mean FEV_1 % (median [min-max] 81.0 [36-137] %). As a result of *ivacaftor* treatment, a significant decrease in HE4 was also seen in this group at 1 month (120.6 [90.9-143.2] pmol/L vs 99.1 [85.1-113.9] pmol/L; $P = 0.039$), accompanied by a further decrease after 2 months (95.6 [90.1-101.9] pmol/L; $P = 0.012$ compared with baseline). Conversely, patients in the third study group had a wider range of FEV_1 % values (median [min-max] 78 [24-119] %) compared with the other two groups, and baseline HE4 concentrations were higher compared with the first group (62.7 [45.4-101.2] pmol/L). In this group, HE4 concentrations were extremely high in some patients: one patient had an HE4 level of 208.5 pmol/L at a very low FEV_1 % of 24% before *ivacaftor* treatment. However, HE4 levels in this group also decreased significantly after 3 months of treatment (47.3 [35.8-71.1] pmol/L; $P < 0.001$).

CRP levels were also determined in all cases. Of note, 26%, 17% and 22% of CRP values were undetectable (<0.5 mg/L) in cohorts 1–3, respectively. In addition, only 15%, 25% and 58% of these data were considered abnormal (above 5.0 mg/L). Mean CRP levels decreased during *ivacaftor* treatment, although these changes did not reach a statistically significant level. Baseline sweat chloride levels were above the diagnostic limit (60 mmol/L) in all but 4 cases, and a rapid and drastic decrease was seen at the first follow-up visit, which further decreased during the study period ($P < 0.001$ and $P = 0.011$). During the clinical trials, patients' nutritional status, monitored by BMI, also improved.

Correlation of plasma HE4 level with FEV1 % and CRP concentration during ivacaftor treatment

Absolute and delta (mean change from baseline) HE4 levels were correlated with absolute and delta FEV_1 % in each patient group separately. A significant inverse correlation was observed between absolute values of HE4 and FEV_1 %. Similarly, the delta HE4 results showed a significant negative correlation with the delta FEV_1 % values in each patient group. When the data from all patient groups were pooled, a significant inverse correlation between absolute and delta HE4 and FEV_1 % values was still detectable ($r = -0.5376$; $P < 0.001$ and $r = -0.3285$; $P < 0.001$, respectively), especially for absolute values.

We also performed multiple regression analyses to determine whether plasma HE4 levels were independently associated with respiratory function and laboratory parameters. HE4 showed an independent correlation with FEV_1 % both before (ß= -0.57; $P = 0.019$) and during (ß= -0.47; $P = 0.043$) *ivacaftor* treatment, whereas an independent association between HE4 and CRP was only demonstrated before drug treatment (ß=0.19; $P = 0.004$). All these results support our preliminary conclusion that blood HE4 levels can closely follow changes in respiratory function and inflammatory conditions in CF lung disease.

Plasma HE4 levels can predict improvement in respiratory function during ivacaftor treatment in CF patients

We calculated the discriminative power of baseline HE4 concentration at 7% FEV_1 % improvement in the pooled patient population. This FEV_1 % value was determined by the mean value shown by the CF patients included in the study. Parallel analysis was used to compare the discriminative power of HE4 with CRP and sweat chloride results. We found that HE4 at a high AUC of 0.722 can predict treatment efficacy as effectively as the currently used sweat chloride test. HE4: 74% sensitivity, 79% specificity (cut-off value: 62.7 pmol/L) CRP: 67% sensitivity, 68% specificity (cut-off value: 7.6 mg/L) sweat chloride: 80% sensitivity, 83% specificity (cut-off value: 99 mmol/L).

Plasma HE4 alters in mild and moderate/severe lung function of CF subjects especially in the early period of ivacaftor medication

Delta HE4 values were pooled for calculation in the early (1–2 months) and mid-term (3–6 months) follow-up periods with the 7% mean change of FEV_1 % as comparator. A substantial AUC value of delta HE4 was determined after 1-2 months of medication (0.806 (95% CI 0.665-0.947); $P < 0.001$) with 81% sensitivity and 89% specificity at the cut-off value

of −15.8 pmol/L. In contrast, delta HE4 was less predictive after 3-6 months of treatment (AUC: 0.648 (95% CI 0.523–0.773); P = 0.020). In the entire population, we found a −12.1 pmol/L mean decrease (with 13.9% rate of decline) in HE4 in the first 2 months, while there was no additional extensive lowering in its concentration after 3-6 months of treatment with a −14.4 pmol/L mean decrease (15.6% rate of decline). That is why HE4 showed a good AUC value in the short-term periods, while due to this "plateau phenomenon of HE4" in these cohorts, its AUC was weaker after 3–6 months. This tendency was similar to sweat chloride showing a massive decline after 1 month and was then sustained decreased at that level. Patients were then divided into 2 sub-cohorts to further analyze the magnitude of mean change of HE4 in the light of the severity of lung disease: i) those who had 70% or higher FEV_1 values (mild subgroup), and ii) cases with <70% of FEV_1 % (moderate/severe subgroup) by the end of these studies. In both subgroups, there was a trend towards lowered HE4 levels due to *ivacaftor* therapy, however, a more robust decrease of HE4 was observed upon improving pulmonary function in those with lower FEV_1 % values along the study period. Thus, it seems that HE4 can indicate any lung function change at a higher extent in moderate/severe CF than in mild disease. Finally, we analyzed the ROC-AUC curve for delta HE4 when absolute FEV_1 % of 70% - determined at the last follow-up time point - was used as the classifier. We found that the AUC value of delta HE4 was 0.704 (95% CI 0.568–0.840), P = 0.003) predicting the outcome of lung function alteration. Based on these data above, plasma HE4 level decreases in both mild and moderate/severe CF lung disease under *ivacaftor* therapy, and its change is highly linked to the improvement of pulmonary disease especially after 1–2 months of therapy.

Evaluation of the mechanism of enhanced HE4 expression in CFBE cells *in vitro*

In our two previous clinical studies, we clarified that circulating HE4 protein levels in the blood are significantly elevated in CF compared not only to healthy individuals but also to other non-CF lung disease samples, and that the airway mucosa may be a major source of this elevation. It has also been found that in case of worse respiratory function and greater the degree of inflammation, higher HE4 levels can be measured. However, the regulation of the protein expression in CF airway epithelial cells was not previously known. Therefore, we performed *in vitro* experiments to understand the cellular mechanism of the elevated HE4 expression and to explain the alteration of HE4 levels during CFTR-specific drug treatment.

CFTR function modulates HE4 concentrations in CFBE 41o− cells culture supernatants in vitro

CFBE 41o- cell cultures expressing F508del-CFTR were treated with CFTR modulators LUM/IVA or TEZ/IVA for 24 h to quantify protein levels of HE4 in the cell culture supernatants. Subsequently, CFBE 41o$^-$ cells with wt-CFTR were utilized as controls for HE4 supernatant concentrations. We consistently found that baseline HE4 concentration was higher in F508del-CFTR CFBE 41o$^-$ cells than normal cells ($p < 0.01$) and was significantly reduced by LUM/IVA ($p < 0.01$) and TEZ/IVA ($p < 0.001$) treatment compared to control samples where the vehicle (i.e., DMSO) was applied. The concentration of HE4 in the supernatant was close to that observed in wt-CFTR CFBE 41o$^-$ cells. Interestingly, TEZ/IVA caused a larger decrease of HE4 concentrations compared to LUM/IVA ($p < 0.05$).

In parallel, CFTR activator FSK/IBMX was used alone and in combination with CFTR modulators in cells with F508del-CFTR. The HE4 protein level was decreased by FSK/IBMX ($p < 0.05$) likely due to the activation of residual CFTR function, while further reduction in HE4 was observed after the combined treatment ($p < 0.05$) vs. using individual CFTR modulator molecules. To provide further evidence of the inverse association between HE4 expression measured by its concentration in cell culture supernatants and CFTR function, we also applied pharmacological inhibition of CFTR by $CFTR_{inh172}$ in wt-CFTR CFBE 41o$^-$ cells, which caused elevated HE4 levels ($p < 0.05$). In contrast, there was a significant decrease in HE4 concentrations ($p < 0.001$) after FSK/IBMX treatment when compared to the controls. Interestingly, LUM/IVA and TEZ/ IVA could downregulate HE4 expression to a certain extent even in CFBE 41o$^-$ cells with normal CFTR expression ($p < 0.05$). 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*.

CFTR modulators partially rescue F508del-CFTR Cl$^-$ currents in CFBE 41o$^-$ cells

To verify that the CFTR modulator molecules used in our experiments in fact improved CFTR function in CFBE cells *in vitro*, we treated the CFBE cell cultures expressing F508del-CFTR with the aforementioned two different combinations of CFTR modulators (LUM/IVA or TEZ/ IVA) for 24 h and analyzed Cl$^-$ current density using patch-clamp. CFBE cells expressing wild-type CFTR showed whole-cell Cl$^-$ currents that could be robustly activated by FSK/IBMX and inhibited by the CFTR selective inhibitor $CFTR_{inh172}$. The peak current-voltage relationship indicated a linear current-voltage relationship, which was most obvious after FSK/IBMX treatment, that reversed around −40 mV, the expected reversal potential of a Cl$^-$ current

calculated from the ionic composition of the pipette-filling and extracellular solutions. The same experiments in CFBE cells expressing F508del-CFTR resulted in miniature currents that were insensitive to either FSK/IBMX activation or inhibition by $CFTR_{inh172}$. Most importantly, both combinations of CFTR modulators significantly increased the basal and the FSK/IBMX-stimulated Cl^- current in comparison to F508del-CFTR basal Cl^- current. Moreover, the currents activated by FSK/IBMX treatment were sensitive to $CFTR_{inh172}$. The statistical analysis of the current densities at +40 mV in confirmed that CFTR modulators corrected F508del-CFTR channel function. The current densities recorded in the presence of FSK/IBMX in cells treated with either LUM/IVA or TEZ/IVA were comparable to the wt-CFTR current density in the absence of the activators (~20 pA/pF) and smaller than wt-CFTR current after stimulation (~80 pA/pF). Of note, TEZ/IVA restored F508del-CFTR Cl^- current density at a moderately higher level than LUM/IVA (9.44 ± 1.01 vs. 8.48 ± 1.14 pA/pF; p = 0.560). In summary, LUM/IVA and TEZ/IVA CFTR modulators partially restored CFTR function in CFBE $41o^-$ cells cultures expressing F508del-CFTR.

Treatment with LUM/IVA lowers plasma HE4 levels in CF subjects homozygous for p.Phe508del-CFTR mutation

To demonstrate that the change in HE4 expression obtained in our *in vitro* experiments can be verified *in vivo* in subjects with the same CFTR mutation and that HE4 may be a suitable biomarker for monitoring not only *ivacaftor* but also other CFTR-specific treatments, we determined HE4 concentrations in plasma samples from 10 Orkambi® (LUM/IVA) treated CF patients homozygous for p.Phe508del-CFTR mutation. Plasma samples were taken before and one month after initiation of therapy. In parallel with the reduced sweat chloride concentration as a result of treatment, we also found significantly lower HE4 concentrations in plasma samples from these patients ($P < 0.01$) after one month of treatment, regardless of baseline HE4 levels. These recent clinical data underline the impact of CFTR modulator treatment on HE4 concentrations in CF blood samples and support the effect of these CFTR-specific drug molecules on HE4 expression *in vitro*.

TNF-α induces increased HE4 mRNA expression in CFBE $41o^-$ cells cultures in vitro

We studied whether HE4 expression could be further enhanced by an artificial inflammatory stimulus *in vitro*. For this purpose, F508del-CFTR CFBE cells were stimulated with recombinant TNF-α applied in the range spanning from 1 h up to 1 week. As a result, HE4 mRNA level quantified by RT-qPCR raised already after 1 h of treatment vs. untreated

(baseline) sample ($p < 0.05$) and was further elevated within the period of 4 h ($p < 0.001$). Surprisingly, HE4 mRNA levels returned to baseline within 24 h. When TNF-α was administered for longer periods (from 48 h up to 1 week), there was a much higher expression of HE4 mRNA ($p < 0.0001$) in TNF-α stimulated CFBE cells. In the same set of samples, mRNA levels of pro-inflammatory cytokines IL-6, IL-8 and IL-1β were also analyzed to ascertain if these mediators were also provoked by TNF-α together with increased expression of HE4. In this regard, IL-6 and IL-8 expression showed similar time-dependent alteration patterns as observed in HE4 mRNA, while elevated IL1-β mRNA was sustained from 1 h without a substantial change throughout this period. Furthermore, the protein concentrations of HE4 and IL-6 were measured in the supernatants of F508del-CFTR CFBE cells at some selected time points (between 4 and 168 h), whereby HE4 level was significantly elevated after 4 h and gradually increased up to 1 week of treatment, while IL-6 concentration was significantly augmented at all pre-selected time points. These results imply that expression of HE4 mRNA thus resulting concentrations of HE4 protein are upregulated following TNF-α administration accompanied by different pro-inflammatory cytokines in CFBE cells.

The pro-inflammatory NF-κB pathway is influenced by the combination of CFTR modulators in F508del-CFTR CFBE 41o⁻ cells in vitro

Evidence for the relationship of the NF-κB signaling pathway to CFTR dysfunction has been published previously. In our present experiments, we investigated whether there is a direct correlation between increased pro-inflammatory activity induced by abnormal CFTR function and elevated HE4 expression. For this purpose, we used two experimental approaches. First, the activity of NF-κB pathway was assessed via the p65 nuclear translocation experiments. We applied fluorescence microscopy-based approaches in wt-CFTR and F508del-CFTR CFBE cells that had been treated with TNF-α vs. untreated controls. Second, the effect of CFTR modulators was studied *in vitro* on basal and induced NF-κB signaling in these CFBE cells after the application of LUM/IVA or TEZ/ IVA treatment via p65 nuclear positivity and IL-6 in the cell line supernatants. There was a significantly higher baseline level of p65 positivity in the nuclei of F508del-CFTR CFBE 41o⁻ cells vs. normal cells ($p < 0.01$). When these cell cultures were exposed to TNF-α, significantly higher p65 positivity was seen in both cell types ($p < 0.05$, $p < 0.0001$, respectively), and the difference in p65 translocation between normal and deficient CFBE cells was more pronounced ($p < 0.0001$). These results indicate that there is a higher baseline and induced level of inflammatory status in CF vs. normal CFBE cells. Secondly, CFTR modulators substantially decreased p65 positivity ($p < 0.05$) not only in

unstimulated F508del-CFTR CFBE cells, but a significant reduction was also observed ($p < 0.001$) after TNF-α treatment compared to baseline (DMSO) sample. In parallel, we determined IL-6 protein levels in the supernatants of studied CFBE cell lines which confirmed the anti-inflammatory effect of applied CFTR modulators via downregulation of basal and TNF-α stimulated IL-6 expression. Hence, our data provide evidence that the generally increased levels of NF-κB pathway activation due to CFTR dysfunction could be efficaciously downregulated by the application of LUM/IVA or TEZ/IVA in CFBE cell cultures *in vitro*.

HE4 concentrations in CFBE 41o⁻ cell cultures are not only upregulated by the NF-κB pathway and pro-inflammatory signaling but are also directly influenced by CFTR activity

Finally, we examined if TNF-α-induced HE4 expression could be decreased by applying CFTR modulators in CFBE 41o$^-$ cells expressing F508del-CFTR. Administration of either LUM/IVA or TEZ/IVA resulted in a significant reduction in HE4 concentration ($p < 0.001$) when measured in the supernatants of TNF-α activated CFBE 41o⁻ cells vs. controls with TNF-α and DMSO. Furthermore, FSK/IBMX alone caused a moderate but still significantly reduced HE4 level in the presence of TNF-α ($p < 0.05$), whereas combined administration of LUM/ IVA or TEZ/IVA with FSK/IBMX lowered HE4 concentrations by a significantly higher degree ($p < 0.01$; $p < 0.001$, respectively). Similar to the unstimulated samples, TEZ/IVA caused a larger change in HE4 compared to LUM/IVA with or without CFTR activator ($p < 0.05$). When TNF-α activated wt-CFTR epithelial cells were treated with CFTR activator FSK/IBMX or CFTR modulators, there were similarly reduced HE4 levels ($p < 0.01$) compared to TNF-α activated samples with vehicle. Thus, observed data provide evidence that corrected and/or potentiated CFTR function has a "protective role" against TNF-α-induced upregulation of HE4 expression in CFBE cells *in vitro*.

As we consistently detected enhanced levels of HE4 in the presence of abnormal CFTR function in CFBE cells bearing F508del-CFTR in contrast to wt-CFTR cells under non-activated and TNF-α activated conditions, we raised the question whether increased HE4 expression in CF is under the regulation of NF-κB mediated pathway directly via impaired CFTR function and related pro-inflammatory stimuli. For this purpose, pretreatment with specific NF-κB pathway inhibitor BAY 11-7082 was used in F508del-CFTR CFBE 41o⁻ cells in the absence or presence of CFTR modulators and TNF-α treatment. In these cell culture samples, supernatant HE4 and IL-6 protein levels were determined. We found that BAY 11-7082 mediated inhibition alone significantly lowered baseline HE4 ($p < 0.05$) and IL-6 levels ($p < 0.01$) after 24 h, while BAY 11-7082 with LUM/IVA or TEZ/IVA resulted in a much

stronger reduction in the levels of both proteins ($p < 0.001$; $p < 0.0001$, respectively). TNF-α-induced HE4 expression was also hindered to a large degree via BAY 11-7082-related inhibition of the NF-κB pathway ($p < 0.05$). Moreover, we noted further decrease in HE4 and IL-6 supernatant concentrations when NF-κB pathway inhibitor and CFTR modulators were administered together vs. control samples with TNF-α and DMSO ($p < 0.001$). In turn, CFBE cells with wt-CFTR were treated with $CFTR_{inh172}$ with or without BAY 11-7082. In contrast to increased HE4 supernatant concentrations, $CFTR_{inh172}$-based inhibition of CFTR was not associated with elevated HE4 concentrations in the supernatant in the presence of the NF-κB pathway inhibitor ($p < 0.05$). Detected changes in IL-6 supernatant concentrations had similar patterns which underscores the close association between CFTR dysfunction and the generally increased pro-inflammatory status in CF. In summary, HE4 concentrations measured in the supernatants are not only modulated via the NF-κB pathway and pro-inflammatory signaling, but also directly influenced by CFTR in CFBE cell cultures *in vitro*.

Discussion

Our studies concluded that serum samples from both children and adult CF patients had significantly higher HE4 concentrations compared to healthy subjects and non-CF lung patients. Occasionally, relatively high HE4 values were also measured in the non-CF lung patient cohort, especially in bronchiectasis, but the median HE4 value and most individual results were much lower compared to the values in the CF population. Similar to the serum results, we also measured higher HE4 concentrations in sputum samples from pediatric CF patients compared to samples from non-CF lung patients and controls. These data also confirm that increased HE4 production in CF may originate from the lungs. We found similarly high HE4 concentrations in the adult CF study population as in pediatric CF patients. Although age influences HE4 levels in general, in these CF patients, it had no influential effect on HE4 levels, in contrast to sweat chloride concentrations, which showed an increasing trend with age in a previous study. The prognostic role of serum HE4 in CF is suggested by its close correlation with lung disease severity, the presence of bacterial colonization and pancreatic insufficiency.

When we compared the diagnostic characteristics of HE4 and CRP, although HE4 showed greater variability, we found a positive significant correlation between HE4 and CRP levels in both the child and adult study groups. However, HE4 had a higher diagnostic efficiency in CF based on ROC-AUC value compared to CRP. Interestingly, this HE4/CRP association was not detected in the non-CF lung patient group. In conclusion, we can consider serum HE4 as a potential CF biomarker that effectively indicates increased airway inflammation and thus deterioration of respiratory function.

The diagnostic power of HE4 was further investigated by ROC-AUC analysis. We obtained a very high AUC value of 0.993 (95% CI, 0.986-0.999) for HE4 to discriminate CF from normal controls with a 'cut-off' value of 49.3 pmol/L. Sensitivity, specificity, PPV and NPV were also significant. When the AUC was calculated to separate CF and non-CF lung patient groups, a slightly lower value was obtained (0.778 [95% CI, 0.716-0.841). The gold standard sweat chloride test was stronger (77.1%) in PPV but weaker (54.8%) in NPV at a cut-off of 39 mmol/L compared with HE4. Serum HE4 in CF patients correlated well with sweat chloride results, and in addition, serum HE4 was abnormally elevated even when the sweat test gave results in the intermediate range (30-60 mmol/L).

The origin and mechanism of the increased serum HE4 concentration in CF have not been previously investigated. We quantified HE4-specific mRNA levels in bronchial mucosal

biopsy specimens obtained from bronchoscopy of some CF patients, which were significantly elevated compared with control samples from non-CF bronchitis. This result suggested us that the elevated serum HE4 levels were due to increased production in the CF airways. Our data were supported by a previous work of Clarke and colleagues, in which the *WFDC2* gene was confirmed to be among the genes with increased expression in CF nasal mucosa samples.

The aim of our experimental work was also to investigate whether the increased expression of HE4, a potential biomarker in CF, could be influenced by improving CFTR function. We found an approximately 1.5-fold higher HE4 concentration in the supernatant of F508del-CFTR CFBE cells compared to normal cells, while both LUM/IVA and TEZ/IVA treatment reduced HE4 levels in these cells. CFTR was subsequently inhibited in wild-type cells using $CFTR_{inh172}$, resulting in elevated HE4 levels. In contrast, treatment with the CFTR activator FSK/IBMX decreased HE4 levels in the same cell types. The change in HE4 levels corresponded to the change in chloride current under these conditions. Our results suggest that impaired CF airway epithelial cells with impaired CFTR function increase HE4 expression, which can be effectively reduced by CFTR modulators that restore ion channel function. Our results can be paralleled with a previous study in which increased NF-B activity and higher IL-8 levels were found following $CFTR_{inh172}$ administration, whereas decreased NF-κB activity was detected in epithelial cell culture following FSK/IBMX treatment.

To explore the possible relationship between HE4 expression and inflammatory cell activation, we further investigated if the elevated HE4 expression level is subsequently enhanced in F508del-CFTR expressing CFBE $41o^-$ cell culture upon inflammatory stimulation. For this purpose, we treated cells with TNF-α for short and long term (1 h to 1 week). HE4 mRNA levels were elevated after 1 h of cell activation and remained high for 4 h but returned to baseline levels after 24 h. After longer TNF-α stimulation, an even greater increase in HE4 expression occurred. In parallel with the increased expression of HE4, TNF-α induced the expected pro-inflammatory effect in CFBE cells, which was monitored by RT-qPCR via IL-6, IL-8 and IL-1ß mRNA levels. In case of IL-6 and IL-8, the time course of change in mRNA levels was in agreement with the change in HE4 expression, whereas high IL-1ß expression was less variable at different time points. Our data suggested that basal expression of HE4 was increased in parallel with elevated levels of inflammatory cytokines in CF bronchoepithelial cells, and thus may be further enhanced under inflammatory conditions.

Using fluorescence microscopy to detect p65 nuclear translocation, consistent with the increased pro-inflammatory activity that is characteristic of CF, we detected significantly higher

basal p65 nuclear positivity in F508del-CFTR positive CFBE cells compared to wild-type cells even in the absence of TNF-α stimulation. When cells were activated with TNF-α, the difference in p65 translocation between the two CFBE cell cultures was even more pronounced. Thus, our results confirm the correlation between baseline and inflammation-induced increased levels of HE4 expression and NF-κB activity *in vitro*. The applied CFTR modulators (LUM/IVA and TEZ/IVA) significantly reduced the nuclear positivity of p65 not only in unactivated CFBE cells expressing F508del-CFTR but also in TNF-α stimulated cells. Reduced IL-6 levels were measured in the supernatant of these CFBE cells with corrected CFTR function, suggesting that CFTR modulators may have additional anti-inflammatory properties in addition to their inhibitory effect on HE4 expression, as recently highlighted by other authors. The results suggest that the inflammatory state of the airway epithelial cells in CF can be directly reduced by CFTR modulators.

The NF-κB inhibitor used in our experiments significantly reduced not only basal but also TNF-α induced HE4 and IL-6 concentrations. This result suggests that the NF-κB signaling pathway plays a role in the regulation of HE4 expression. This finding is consistent with our data from the study of CF patients: more severe airway inflammation (clinically defined as airway exacerbation) was associated with elevated CRP levels and higher serum HE4 levels. However, when CFBE cells were also treated with CFTR modulators in addition to NF-κB pathway inhibition, there was a further significant decrease in HE4 and IL-6 levels, suggesting that HE4 expression is also under the direct influence of CFTR.

Our group was the first to investigate the role of plasma HE4 as a potential biomarker to measure the efficacy of CFTR modulator treatments. First, we determined the HE4 concentration in plasma samples from three independent groups of CF patients treated with *ivacaftor* and compared them with typical clinical follow-up parameters such as FEV1 %, sweat chloride levels and BMI. Patients with CF aged 2 years or older who carry one of the class III mutation variants (most commonly p.Gly551Asp CFTR) are candidates for *ivacaftor* treatment. The drug increases the frequency of the opening of the CFTR ion channel in the apical membrane subsequently enhancing chloride transport. Clinical studies have demonstrated the long-term clinical efficacy of *ivacaftor* by improving the patients' FEV_1 % and their nutritional status, reducing the number of *P. aeruginosa* colonization events and the risk of pulmonary exacerbations. All patients in our study groups had elevated plasma HE4 levels before *ivacaftor* treatment compared to the healthy population. As a result of *ivacaftor* treatment, in addition to improved respiratory function and decreased blood chloride levels, HE4 levels were

significantly lower at the first follow-up time point, regardless of baseline concentrations, and gradually continued to decrease until the end of the study period. These data are consistent with our previous observation that HE4 levels correlated with lung disease severity. Moreover, absolute and delta HE4 values were inversely proportional to FEV_1 % and delta FEV_1 % values in all three patient groups. In addition to the in vitro cell experiments, we measured the effect of *lumacaftor/ivacaftor* therapy on HE4 levels. For this purpose, HE4 levels were measured in plasma samples from 10 Orkambi-treated CF patients homozygous for the p.Phe508del-CFTR mutation. Our results showed that Orkambi® therapy significantly reduced plasma HE4 concentrations as early as one month after initiation of treatment, and the extent of the reduction was independent of baseline HE4 levels.

In our studies, absolute and delta values of HE4 showed discriminative power when the mean change in FEV_1 % observed in the patient groups was used as the basis for ROC curve analysis. HE4 at a cut-off of 62.7 pmol/L with a significant AUC (0.722) was able to predict a 7% improvement in FEV_1 % with *ivacaftor* treatment with a sensitivity of 74% and a specificity of 79%. An even higher AUC value (0.806) was found for delta HE4 after 1-2 months of drug treatment with 81% sensitivity and 89% specificity at a cut-off of -15.8 pmol/L, while delta HE4 was less predictive after 3-6 months of treatment (AUC: 0.648).

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Some limiting factors of our studies need be mentioned. We retrospectively analyzed HE4 in plasma samples from CF patient groups receiving IVA or LUM/IVA treatment without a non-treated (placebo) clinical control group. The results may be biased by the different sources of the samples. We were unable to select CF patients receiving TEZ/IVA treatment for the measurement of plasma HE4 levels, as samples from such patients were not available at the time of our experiments, neither from CFFT nor from domestic studies. Therefore, further research is needed to validate the use of HE4 as a biomarker endpoint under different CFTR modulator treatments. In our experiments with CFBE cells, we did not evaluate whether the elevated HE4 levels were due to the lack of CFTR function itself or to the presence of an abnormal, "misfolded" CFTR protein. Finally, the mechanism of the relationship between CF-

specific inflammation and HE4 expression could not be fully investigated, and additional *in vitro* studies are needed to further clarify the pathomechanism.

From the combined results of the three series of studies on which this thesis is based, we believe that there is a significant increase in circulating HE4 protein levels in CF, which is associated with CFTR dysfunction and activation of the NF-κB pathway. This is supported by our clinical data showing that the correcting/modulating effect of CFTR-specific drugs on CFTR channel function significantly reduces HE4 levels, parallel to the improvement of the respiratory function.

Summary

CF is the most common monogenic inherited metabolic disease in the Caucasian population. In this disease with multi-organ involvement, prognosis is largely determined by respiratory consequences. The pathomechanism of the pulmonary disease is essentially characterized by chronic airway inflammation dominated by neutrophil granulocytes, resulting from recurrent respiratory tract infections and immune dysregulation as a consequence of CFTR dysfunction.

The currently available clinical parameters for the follow-up of CF lung disease are limited, especially in juvenile and mild lung involvement. Airway inflammation is detectable early in the disease, thus measuring the concentrations of the mediators involved in the pathological processes, either from blood or airway samples, seems to be a feasible option for monitoring lung disease. Elevated serum HE4 was found to be more effective for monitoring airway inflammation in CF compared to routine biomarkers, such as CRP. Plasma HE4 concentrations correlated well with the severity of CF lung disease and thus seemed to be suitable for monitoring *ivacaftor* treatment. Our experimental studies also highlighted that increased HE4 expression in CF originates from airway epithelial cells. Our *in vitro* results suggest that the increase in HE4 expression in F508del-CFTR positive CFBE cell cultures is partially the result of the increased NF-B pathway activation as a consequence of CFTR dysfunction.

Overall, we conclude that blood HE4 concentrations are significantly elevated in CF compared to healthy individuals and patients with non-CF lung disease. HE4 protein, available in routine clinical setting, may be considered as a novel biomarker in CF that is closely related to inflammatory processes in airway epithelial cells and may be suitable for monitoring CFTR modulator treatments both in clinical trials and in daily patient care.

New scientific results of the thesis

1. Serum/plasma HE4 concentrations are significantly elevated in CF compared not only to healthy individuals but also to non-CF lung patients.
2. Abnormal CFTR function can directly modulate HE4 expression in CF.
3. External inflammatory stimuli, e.g., TNF-α can further induce HE4 expression parallel to the increase of the production of pro-inflammatory cytokines mediated by the NF-κB signaling pathway in bronchoepithelial cells.
4. Plasma HE4 levels highly correlate with the respiratory function parameter (FEV_1 %) and effectively monitor the efficacy of CFTR-specific drug treatment.

Keywords:
Biomarker research, bronchoepithelial cell, cystic fibrosis, CFTR, CRP, HE4, inflammation, respiratory function, sweat chloride

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List of publications related to the dissertation

1. **Bene, Z.**, Fejes, Z., Szántó, G. T., Fenyvesi, F., Váradi, J., Clarke, L. A., Panyi, G., Macek, J. M., Amaral, M. D., Balogh, I., Nagy, B. Jr.: Enhanced Expression of Human Epididymis Protein 4 (HE4) Reflecting Pro-Inflammatory Status Is Regulated by CFTR in Cystic Fibrosis Bronchial Epithelial Cells.
   Front. Pharmacol. 12, 1-16, 2021.
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   IF: 5.988

2. **Bene, Z.**, Fejes, Z., Macek, J. M., Amaral, M. D., Balogh, I., Nagy, B. Jr.: Laboratory biomarkers for lung disease severity and progression in cystic fibrosis.
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3. Nagy, B. Jr., **Bene, Z.**, Fejes, Z., Heltshe, S. L., Reid, D. M., Ronan, N. J., McCarthy, Y., Smith, D., Nagy, A. C., Joseloff, E., Balla, G., Kappelmayer, J., Macek, J. M., Bell, S. C., Plant, B. J., Amaral, M. D., Balogh, I.: Human epididymis protein 4 (HE4) levels inversely correlate with lung function improvement (delta FEV1) in cystic fibrosis patients receiving ivacaftor treatment.
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List of other publications

5. Balogh, E., Nagy, B. Jr., Gyetvai, Á., **Bene, Z.**, Hendrik, Z., Jeney, V., Nagy, P., Papp, Á., Balla, J., Balla, G., Kappelmayer, J., Nagy, B.: Impaired Immunosuppressive Effect of Bronchoalveolar Mesenchymal Stem Cells in Hypersensitivity Pneumonitis: preliminary findings.
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