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Human epididymis protein 4 (HE4): a novel serum inflammatory biomarker in cystic fibrosis

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Abstract

Background: Increased expression of the human epididymis protein 4 (HE4) was previously described in lung biopsy samples from cystic fibrosis (CF) patients, but it has remained unknown whether serum HE4 concentrations are elevated in CF.

Methods: Seventy-seven children with CF from six Hungarian CF centers and 57 adult CF patients from a Czech center were enrolled. In addition, 94 individuals with non-CF lung diseases, and 117 normal controls without pulmonary disorders were analyzed. Serum HE4 was measured by an immunoassay and its expression was further investigated via the quantification of HE4 mRNA using RT-qPCR in CF versus non-CF respiratory epithelium biopsies. The expression of the potential regulator miR-140-5p was analyzed using an UPL-based RT-qPCR assay. HE4 was measured in the supernatants from unpolarized and polarized cystic fibrosis bronchial epithelial (CFBE) cells expressing wt- or F508del-CFTR.

Results: Serum HE4 levels were significantly elevated in children with CF (99.5 [73.1-128.9] pmol/L) compared to controls (36.3 [31.1-43.4] pmol/L; P<0.0001). This observation was replicated in CF adults (115.7 [77.8-148.7] pmol/L; P<0.0001). In contrast, abnormal but lower HE4 concentrations were found in cases of severe bronchitis, asthma, pneumonia or bronchiectasis. In CF patients, the concentrations of HE4 were positively correlated with overall disease severity and C-reactive protein concentrations, while a significant inverse relationship was found between HE4 and the spirometric FEV_1 value. Relative HE4 mRNA levels were significantly upregulated (P=0.011) with a decreased miR-140-5p expression (P=0.020) in the CF versus non-CF airway biopsies. There were 2-fold higher HE4 concentrations in the supernatant of polarized F508del-CFTR CFBE cells compared to wt cells.

Conclusions: HE4 serum levels positively correlate with the overall severity of CF and the degree of pulmonary dysfunction. HE4 may thus be utilized as novel inflammatory biomarker and possibly also as measure of treatment efficacy in CF lung disease.

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorders and is linked to a disease-causing variation in the CF transmembrane conductance regulator (*CFTR*) gene [1]. CF is a multisystem disorder in which abnormal ionic transport mediated by CFTR may lead to alteration of properties in the airway surface fluid and mucus [2]. The diagnosis of CF is based on typical clinical features associated with elevated sweat chloride concentrations, and the detection of 2 CF-causing *CFTR* mutations in *trans* [3,4]. More than 2000 genetic alterations have already been identified in the *CFTR* gene with the major p.Phe508del mutation accounting for approximately 70% of all CF alleles in Hungary and the Czech Republic [5-7]. The inflammatory response in response to chronic pulmonary infections is related to a number of overexpressed cytokines and metalloproteinases that are considered to be major pathogenetic components in CF [8-12].

Although several diagnostic guidelines have been published for CF within the last two decades [3,4,13], few studies have employed potential serum biomarkers. These biomarkers may provide the desired quantifiable "endophenotype" which could be utilized to monitor disease severity. The clinical course of CF could then be indirectly assessed using e.g., increased serum concentrations of the C-reactive protein (CRP), interleukin (IL)-1β, and myeloperoxidase [14] that is linked to neutrophilia [15], while the measurement of plasma soluble CD14 [16], as well as CRP and plasma IL-8 [17] concentrations might be predictive of pulmonary exacerbations. Cytokine concentrations were also thoroughly analyzed in the bronchoalveolar lavage fluid (BALF) [9,18] and in sputum samples from CF patients [19]. Moreover, tumor markers, such as cancer antigens (CA) 125 and CA 19-9, were connected with *Pseudomonas aeruginosa* lung colonization and impaired lung function [20], while CA 19-9 may discriminate cases with borderline sweat chloride

concentrations [21]. However, assessment of various biomarkers has been primarily considered within the scientific domain, rather than as part of routine CF laboratory diagnostics.

The human epididymis protein 4 (HE4) is encoded by the *WFDC2* gene [22] and is known to be a consistent tumor marker in epithelial ovarian cancers [23,24], lung malignancies [25,26] and endometrial carcinomas [27]. This protein is a member of the whey acidic protein (WAP) family that is homologous to other serine proteinase inhibitors, comprising e.g., elafin and secretory leukocyte protease inhibitor (SLPI) [28,29]. However, in these mediators different control mechanisms, compared to HE4, have been suggested [14]. Elafin and SLPI proteins have a major role in host defense of the respiratory tract via inhibition of neutrophil elastase (NE) [29] and by NFκB activation [30]. In this regard, increased SLPI levels were observed in acute respiratory distress syndrome [31], and in CF without any concurrent infection. However, SLPI is cleaved and down-regulated by NE in the presence of chronic bacterial infections [32,33]. Similarly to the WAP protein family, HE4 also displays a variety of functions. It acts as an anti-proteinase in the frame of epithelial host defenses of the respiratory tract [32,33], and it is involved in sperm maturation [22]. Although HE4 is overexpressed in the aforementioned tumors, it is also produced by other "normal" tissues (e.g., epididymis, salivary glands, prostate, etc.) at baseline levels [32,34]. The only report on a possible association of HE4 with CF utilized immunohistochemistry for the assessment of *WFDC2* gene expression (hence HE4) in CF lung biopsy samples containing mainly tracheobronchial epithelial cells [14]. However, the authors did not analyze HE4 concentrations in blood specimens.

Here, we carried out a multicenter, analyst-blinded, case-control study of two unrelated CF populations of various age groups in which we analyzed serum HE4 concentrations. We also investigated the possible origin of abnormally high HE4 secretion into the blood via expression

analysis of the *WFDC2* gene in respiratory epithelium samples by measuring HE4-specific mRNA using RT-qPCR. Finally, we examined the level of miR-140-5p as one of the regulatory microRNAs of the *WFDC2* gene according to a miR database (www.microrna.com). To prove the overexpression of HE4 in connection with the CFTR-F508del genotype, supernatant culture medium samples from cystic fibrosis bronchial epithelial (CFBE) cells expressing wt- or F508del-CFTR were obtained for HE4 analysis as an *in vitro* complementation of the *ex vivo* measurements.

Material and Methods

Study participants

We summarized the main characteristics of all study groups (Table 1), and the demographic and laboratory parameters of CF study populations were presented in detail in the Supplementary Tables 1 and 2. Seventy-seven young CF patients were consecutively recruited from six Hungarian CF centers between October 2012 and February 2015. An independent adult cohort, comprising 57 adult CF patients was recruited from an adult CF centre in Prague. All CF subjects were diagnosed in accordance with the CF consensus diagnostic criteria [2] and exhibited the classic form of the disease. The overall clinical status was assessed by detailed clinical- and laboratory examination and "graded" by the Shwachman-Kulczycki (SK) score [8] in those centers where it was used, or based on the opinion of clinicians. Accordingly, we categorized the patients into these 3 subcohorts. CF individuals with available SK scores of 71-100 were classified as 'mild', those with 41-70 scores were in the 'moderate' category, and subjects with ≤40 scores were recruited into the 'severe' group. Serum samples were collected either during routine outpatient clinic visits (these CF patients had predominantly mild to moderate clinical course of CF) or during hospitalization due to e.g., acute pulmonary exacerbations

demonstrating increased cough with increased sputum production, difficulty in breathing, fatigue, and marked decline in lung function results; these criteria were based on Fuchs *et al* [35]. CRP concentrations were only analyzed in children with a severe disease course (n=29, 37.6%) and in all Czech adults. We also analyzed HE4 after medication at improved clinical states in 10 CF subjects who appeared at the Outpatient Clinic for a check-up after the recruitment into this study. These CF patients were treated by two types of intravenous antibiotics, mucolytic drugs, and other anti-inflammatory agents during hospitalization with an average period of 14 days. After discharge, these subjects took oral antibiotics for 3 weeks plus inhaled antibiotics for 3 months.

In order to assess the underlying non-specific effect of inflammation in other "non-CF" pulmonary diseases on HE4 serum concentrations, 64 patients with severe non-CF lung diseases (with chronic bronchitis, asthma or pneumonia). Furthermore, 12 other individuals who had non-CF bronchiectasis (n=2) or congenital disease (e.g. Werdnig-Hoffman-syndrome, Williams–Campbell syndrome, microcephaly, etc., n=7) or treatment resistant severe epilepsy (n=3), were also studied. These subjects suffered from severe recurrent lung infections leading to bronchiectasis due to repetitive aspiration and bronchial discharge retention. In addition, 18 non-CF adult subjects who had bronchiectasis or chronic obstructive pulmonary disease (COPD), were also recruited. These "clinical controls" were diagnosed using standard X-ray examinations and relevant laboratory tests. Exclusion criteria comprised a history of smoking, malignancy, immunodeficiency, chronic kidney, or liver disease. There was no significant difference in age between the CF and the clinical control cohorts, respectively; however, non-CF patients were younger than CF patients ($P < 0.05$) with a similar gender ratio (Table 1). Bronchial epithelial biopsies were obtained via diagnostic bronchoscopy from 3 Hungarian CF subjects (2 M/1 F; 3, 4, 9 years of age), and 3 other non-CF patients (2 M/1 F; 2, 4, 10 years of age) suffering from

repeated episodes of bronchitis (data not shown). Bronchoscopy was performed in the latter patients in order to reveal a background of wheezing and to exclude congenital stenosis of the main bronchi. Age- and sex-matched children and adult individuals without any apparent pulmonary disorders or chronic inflammatory processes were enrolled as random controls (Table 1). Finally, 12 apparently healthy parents of Hungarian CF patients (31 [25-37] years of age), who were all carriers of p.Phe508del mutation, were also examined for serum HE4 concentrations (data not shown).

Ethics statement

Written informed consent was obtained from the parent(s) or legal guardian of each child and adult carriers before the study. The study was approved by the Regional Ethics Committee of the University of Debrecen (DEOEC-RKEB/IKEB 3777-2012) and by the Local Ethics Committee of the Motol University Hospital, Prague.

Laboratory diagnosis of CF and the analysis of serum parameters

Sweat chloride values were analyzed using a Sweat Check Conductivity Analyzer (Wescor, USA). Genomic DNA isolation from blood leukocytes was performed with a QIAgen Blood Mini Kit (Qiagen, Germany). The 30 most common CFTR mutations in Hungary were tested using a Elucigene CF29v2 Kit (Tepnel-Diagnostics, UK) as well as an allele-specific PCR for the 'Slavic' CFTRdele2,3 (21kb) mutation, as previously described [6]. The Czech adult patient cohort was genotyped by the Elucigene EU v3 assay (Tepnel-Diagnostics, UK) and the MLPA kit (MRC Holland, The Netherlands). Blood samples for HE4 were obtained by venous puncture, then centrifuged, and stored at -70 °C until analysis. Serum HE4 was determined through

chemiluminescent microparticle immunoassay (Architect i2000SR, Abbott, USA). Serum CRP was measured through electro-chemiluminescent immunoassay (Cobas e411, Roche, Germany).

Quantification of HE4 mRNA level in airway epithelium biopsies

Respiratory epithelial cells were kept in isotonic saline at 4 °C in sterile plastic tubes, and centrifuged at 1500 g for 5 min, and pellets were stored at -70 °C until analysis. RNA isolation was done using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized using 200 ng of total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). RT-qPCR (Light Cycler 480, Roche) was used with the SYBR Green PCR Master Mix (Invitrogen) for quantification of the relative expression of the HE4 gene in airway epithelial samples. For further details, see Supplementary data.

Quantification of miR-140-5p expression in airway epithelium biopsies

MicroRNAs (miRNA) targeting WFDC2 (HE4) gene function were searched by using a prediction program (http://www.microrna.org). Since hsa-miR-140-5p was predicted by this algorithm as a putative regulator of HE4 gene, this miRNA was selected for further experiments. MiRNA was transcribed into cDNA and quantified via miRNA specific RT-PCR reaction using a TaqMan MicroRNA Reverse Transcription Kit (Life Technology, USA) according to the manufacturer's instructions. For further details, see Supplementary data.

HE4 analysis in the supernatants of CFBE cell cultures expressing wt or F508del CFTR

CFBE 41o- stably expressing either wt-CFTR or F508del-CFTR (a kind gift from Dr. JP Clancy, Dept. of Pediatrics, Cincinnati Children's Hospital, OH) were seeded in triplicate, either in plastic wells (250k cells per 3.8 cm^2 growth area) or on 12 mm collagen IV-coated Transwell permeable

supports (Corning Costar 3460: 250k cells per support) for monolayer formation and polarization, which was assessed by daily measurements of transepithelial resistance (TEER). Supernatant culture medium samples were taken when confluence (for unpolarized samples) or polarization (TEER > 600 Ω/cm2) was reached. The same cells were then treated with LPS (L2880, Sigma: 10 µg/ml, 24 h), following which supernatant was once more collected.

Statistical analysis

The Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. Demographic and laboratory parameters were non-normally distributed thus being expressed as median (range), and were analyzed using the Mann-Whitney U test. Normally distributed data of RT-qPCR analyses were expressed as mean±SEM and were analyzed by Student's independent *t*-test analysis. The Chi-square test was used to compare categorical variables. The Spearman's rho was calculated for correlation analysis. The discriminative power of HE4 and CRP was evaluated using receiver operating characteristics (ROC) curve analysis. P values < 0.05 were considered to be statistically significant. All analyses were performed using SPSS Statistics software, version 19.0 (IBM Corps., Armonk, NY, USA).

Results

Altered laboratory parameters of study populations

Sweat chloride levels were over 60 mmol/L in most CF individuals (median [range] 107 [90-120] mmol/L) except for 7 patients. Of note, this test was not performed in clinical controls and in the non-CF group since CF could be conveniently excluded using other tests. Serum CRP was increased (reference range: <4.6 mg/L) compared to normal in those young CF subjects with a severe clinical status at admission (n=29), in all CF adults and in the groups of non-CF controls

(Table 1). In addition, chronic bacterial colonization was detected in 54 young CF and in all adult CF individuals. Pancreatic insufficiency and CF-related diabetes mellitus (DM) were diagnosed in variable percentages of CF cohorts (Supplementary Tables 1 and 2).

Highly increased level of serum HE4 in CF regardless of genetic alteration compared to controls and non-CF subjects

Median serum HE4 levels were significantly (P<0.0001) elevated in the 77 CF children and 57 adult patients with CF compared to normal controls (Table 1 and Fig. 1A). Twenty-nine CF young subjects in severe condition had even higher HE4 concentrations (134.9 [124.5-275.0] pmol/L). In the adult CF cohort (n=57), there were 13 patients suffering from severe status (170.8 [146.8-182.9] pmol/L). Gender did not affect HE4 levels in either CF group: in children 95.4 [75-128.5] pmol/L in males vs. 101.5 [72.6-129.6] pmol/L in females; P=0.881, while in adulthood median HE4 was 119.4 [85.2-150.6] pmol/L in males; 114.8 [75.3-139.1] pmol/L in females; P=0.290 (data not shown). In terms of CF genetic background, we did not detect differences with variable genotypes among children: homozygous for p.Phe508del median HE4 was 99.5 [73.0-127.5] pmol/L vs. in the presence of other mutations 94.6 [66.4-127.8] pmol/L, and there was no change in HE4 of CF adults between those with p.Phe508del/p.Phe508del (123.1 [74.1-151.1] pmol/L) and others with a distinct genetic background (108.3 [83.9-140.0] pmol/L; P=0.348) (data not shown). We also analyzed HE4 concentrations in additional patients who suffered from severe non-CF lung diseases, such as bronchitis, asthma, pneumonia or bronchiectasis. Increased median HE4 level was measured in these cases (P<0.001) compared to normal controls, however, these data were much below the results observed in CF (Fig. 1A). More importantly, there was a statistically significant difference (P<0.001) in serum HE4 between CF and non-CF patients regardless of age. When we sub-grouped non-CF pulmonary young patients

based on the types of lung disease, similar changes were seen in 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) (data not shown). Furthermore, we enrolled children suffered from non-CF bronchiectasis due to different clinical conditions showing similar HE4 values (80.6 [68.1-134.5] pmol/L) than CF; however, median HE4 level was much higher in CF patients. For CF adults, 18 non-CF patients were recruited with moderately elevated HE4 (59.2 [41.1-88.6] pmol/L). In contrast, the carriers of CF mutations had normal HE4 levels (33.9 [30.1-43.9] pmol/L; n=12). When we determined HE4 concentration in sputum from CF or other non-CF diseases, - similarly to sera - there were significantly higher HE4 concentrations in CF versus non-CF or healthy sputum specimens (see Supplementary data and Table 3).

Serum HE4 in CF is not affected by age and strongly correlates with disease severity

No significant difference in HE4 was observed among different age groups (P=0.164) (Fig. 1B). Age did not seem to have an impact on HE4 levels in these CF patients. In order to investigate if serum HE4 can be used as a prognostic disease marker in CF, the relationship between HE4 levels and disease severity was investigated. HE4 showed a positive trend that followed the degree of impairment in both disease cohorts (Figs. 2A and B). Significantly higher HE4 levels were seen in CF subjects with a moderate status (108.0 [95.8-133.0] pmol/L; P<0.001) compared to those with a mild status (60.8 [50.7-73.8] pmol/L). Even higher HE4 values were measured in those in severe condition with an acute exacerbation (134.9 [124.5-275.0] pmol/L; P<0.0001) (Fig. 2A). In adult CF, there was a positive correlation between HE4 and disease status: mild (72.5 [64.1-94.8] pmol/L), moderate (126.5 [104.3-148.3] pmol/L), and severe clinical symptoms (170.8 [146.8-182.9) pmol/L (Fig. 2B). As a preliminary evaluation of HE4 in the follow-up of CF, we analyzed HE4 after medication at improved clinical states in 10 CF subjects. Accordingly,

there were lower concentrations of serum HE4 (92.5 [87.5-106.2] pmol/L vs. 125.8 [111.8-153.9] pmol/L at baseline; P=0.105) after treatment, though results did not reach the range of normal controls (data not shown). Of special interest, we recognized a significant negative association between HE4 and FEV_1 in CF, which was used to evaluate residual lung function capacity (Spearman's rho=-0.522; P<0.0001) (Fig. 3A).

The association between serum HE4 and CRP in CF

We sought to analyze if changes in HE4, acting as an additional inflammatory parameter, was closely related to the increased levels of CRP. There was a significant and positive correlation between serum HE4 and CRP levels in CF children in severe condition plus all CF adults (Spearman's rho=0.595; P<0.001) (Fig. 3B); while no association was observed in patients with non-CF lung diseases (Spearman's rho=0.168; P=0.074) (data not shown). Based on these data, increased HE4 can signal massive pulmonary inflammation in CF similarly to CRP. When we further compared the laboratory characteristics of HE4 statistically to that of CRP in CF, HE4 showed a substantial AUC value of 0.724 (95% CI: 0.617-0.830) (P<0.0001), while CRP had an average AUC value of only 0.453 (95% CI: 0.347-0.559) (P=0.435). Overall, serum HE4 is able to track the development of clinically significant lung inflammation in CF with much higher accuracy than CRP.

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

All adult CF participants had bacterial colonization, thus we could not analyze the relationship of HE4 with chronic bacterial infection, while children with positive microbiology tests had significantly higher levels of HE4 as compared to those without bacterial colonization (106.5 [75.8-133.3] pmol/L vs. 78.3 [61.4-96.9] pmol/L; P=0.001). 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 vs. 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) (Supplementary Tables 1 and 2).

The diagnostic characteristics of HE4 in CF

We also studied the potential discriminative power of HE4 in the entire study population using ROC analyses. Based on the values of the entire CF group, the AUC for HE4 was 0.993 (95% CI: 0.986-0.999) (P<0.0001) at the 49.3 pmol/L cut-off value for differentiating CF patients from normal controls (Fig. 4). 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<0.0001) with a modest sensitivity of 56.0%, but a high specificity of 90.0% at the 98.4-pmol/L cut-off value with 64.6% PPV and 90.4% NPV (Fig. 4). In addition, we intended to observe if sweat chloride test results showed any relationship with serum HE4. Interestingly, a positive and significant correlation was seen between the actual level of serum HE4 and the sweat chloride values that were previously measured at the diagnosis of CF children (Spearman's rho=0.345; P=0.005) (data not shown). In a subgroup analysis, we analyzed HE4 in those patients (n=8) who had borderline sweat chloride test results (30-60 mmol/L) with predominantly mild/moderate clinical conditions. We found similar serum HE4 values in these cases (100.9 [88.2-115.0] pmol/L) as seen in the entire CF cohort. In summary, serum HE4 was already abnormal when the 'gold standard' sweat chloride test was only intermediate. Since this was a relatively low number of cases, further evaluation of HE4 is necessary in CF with borderline chloride results.

Increased level of HE4 mRNA was detected in respiratory epithelium biopsies of CF

Next, we wanted to investigate the origin of elevated HE4 in CF sera. The goal was to determine if HE4 was a consequence of the destruction of the airway epithelium causing an extensive release of HE4 due to the massive inflammation, or this protein was produced at a higher quantity in the airways. For this purpose, human bronchial epithelium biopsies were obtained via bronchoscopy from 3 CF and 3 non-CF patients for controls. HE4 mRNA level in CF was significantly upregulated compared to that of non-CF control subjects (HE4/36B4 ratio: (mean±SEM) 0.354±0.178 vs. 0.041±0.017; P=0.011; n=3 per group) suggesting that highly elevated serum HE4 concentrations in CF may result from its increased secretion by the airway epithelium (Fig. 5A).

The relationship of miR-140-5p with the expression of HE4 in CF

The level of miR-140-5p was analyzed in the same bronchial epithelial biopsies mentioned above from CF and non-CF individuals, and was normalized to RNU43. We observed that miR-140-5p expression in CF was significantly decreased compared to non-CF control subjects (miR-140-5p/RNU43 ratio: (mean±SEM) 0.033±0.008 vs. 0.443±0.076; n=3 per group; P=0.020) (Fig. 5B). In order to recognize if there was a strong association with HE4 mRNA and miR-140-5p expression, a Spearman's correlation was performed, which revealed a significant inverse correlation between miR-140-5p and HE4 mRNA levels (Spearman's rho: -0.744, P=0.006) (data not shown).

F508del-CFTR expressing CFBE cells produced markedly higher HE4 level than those expressing wt-CFTR

HE4 levels in the supernatants of unpolarized and polarized wt- and F508del-CFTR expressing CFBE cells were compared. We found that there were about 2-fold higher HE4 concentrations in

the supernatants of F508del-CFTR CFBE cells, and the difference was more pronounced in polarized cultures compared to unpolarized cells (Fig. 6). Moreover, these cultures were treated with LPS (10 μg/mL) for 24 hours in order to analyze the direct effect of bacterial infection on HE4 production. We did not, however, observe any induction in HE4 secretion by this stimulation (data not shown).

Discussion

The lungs are usually most affected in CF, and the patient suffers from recurrent bacterial infections and severe inflammation causing destruction in the bronchi, which leads to a reduced life expectancy [9]. The criteria for the diagnosis of this disease are a positive family history and/or abnormal newborn screening test results, typical clinical features, increased sweat chloride test, and the identification of 2 CFTR mutations in trans [4]. However, there are challenging cases that have borderline sweat chloride values or when the genetic analysis reveals zero or only one CFTR mutation or mutation(s) with unknown clinical significance. To solve this situation, Sermet-Gaudelus *et al.* suggested the use of the nasal potential difference diagnostic score, which was found to be a useful tool for inconclusive CF patients [36]. Although there are several potential blood-based biomarkers for monitoring CF subjects and to identify those who are at a risk of acute pulmonary exacerbation, none of them, as yet, have been integrated into routine clinical practice [37]. Serum MPO, IL-1β, and CRP [15], plasma soluble CD14 [16], and plasma IL-8 [17] can predict pulmonary exacerbation effectively, but only at early time points. No significant association between CRP and the severity index of the disease was observed [38].

HE4 was suggested as a tumor marker for epithelial ovarian cancer [23]. Since then, a large number of papers supported the superior diagnostic characteristics of HE4 versus CA 125 in this particular malignancy [24,39,40] with other possibilities we summarized in a recent review [41]. In

addition, HE4 may be elevated in certain non-cancer diseases, such as chronic kidney disease [42], or in pulmonary tuberculosis where only moderately increased HE4 concentrations were reported [43]. HE4 was formerly investigated but only in regard to one aspect of CF, i.e., when high HE4 expression was described in CF lung biopsies by immunohistochemistry [14]. No evidence regarding serum HE4 level changes relative to this disease was provided.

We found that serum HE4 levels were significantly elevated in both young and adult CF patients compared to normal controls. There was no difference in HE4 levels between males and females, similarly to sweat chloride values as previously described [44]. There were a number of increased HE4 values in non-CF patients especially in bronchiectasis that partially overlapped with a ratio of CF results measured in CF patients, however; the median HE4 concentration was much lower than that of CF (Fig. 1A). Within the non-CF group, no significant difference was observed when patients were further separated based on subtypes of lung disease. Since the non-CF children cohort consisted of younger patients as compared to CF children, we reanalyzed the difference in HE4 between CF children and a subgroup of age-matched non-CF children cohorts (7.5 [5.0-14.8] years of age), and there were significantly lower HE4 results (56.7 [46.4-71.6] pmol/L, $P<0.0001$) in this control group (n=49) in contrast to CF. Very importantly, heterozygous healthy parents of some of the studied CF patients showed similar HE4 levels to the controls, which is in good agreement with the recessive inheritance of the disease.

In the adult CF study group, CF patients showed the same HE4 results. We then investigated the effect of age on HE4 levels since age could have been a major factor for HE4 [41,45]. Among our CF patients, there was no substantial impact of age on HE4 results (Fig. 1B) in contrast to sweat chloride test results that undergo an age-dependent elevation, as exposed in an earlier study [46].

Notably, serum HE4 level tightly correlated with bacterial colonization, and pancreatic insufficiency.

HE4 was able to act as a prognostic disease marker in CF when patients were categorized by variable degree of severity (Figs. 2A and B). HE4 showed a positive correlation with the degree of lung impairment. In this aspect, HE4 was proportionally elevated due to advanced inflammatory events. It is noteworthy to mention that this phenomenon is similar to pathologic stages in cancers [26,40].

Previous reports discussed the potential role of CRP in the prediction and detection of pulmonary exacerbations with relatively good efficacy [15,17,38]. However, this parameter has yet to become a routinely used examination in CF. CRP results were abnormal but with a relatively modest change in most cases. As most adult patients were infected with *P. aeruginosa* or *B. cepacia complex*, higher CRP levels were expected even in the stable phase of the disease in these patients [35]. Then we compared the diagnostic characteristics of HE4 to CRP. Although HE4 showed a larger variation, a positive significant correlation was analyzed between serum HE4 and CRP levels in both children and adult study groups. Notably, HE4 in CF had a better diagnostic power according to their AUC values in contrast to CRP. In non-CF subjects, this HE4-CRP association could not be detected. Therefore, serum HE4 can be considered as another candidate biomarker for the indication of the accumulation or recurrence of pulmonary inflammation and pulmonary exacerbation of CF.

Treatment options for CF are limited; however, one drug, ivacaftor, has become clinically important and has been shown to be very beneficial in some mutations. In clinical studies, treatment efficacy was measured using FEV_1 and sweat chloride [47]. Testing would also benefit

from the use of a biomarker. We believe that measurement of HE4 might serve as a potential biomarker for the analysis of treatment in patient follow-up.

The diagnostic characteristics of HE4 were also evaluated using ROC analyses. The AUC for HE4 was 0.993 (95% CI: 0.986-0.999) at 49.3 pmol/L cut-off value for differentiating CF patients from controls with substantial values of sensitivity, specificity, PPV, and NPV. When the AUC for HE4 was calculated for the discrimination of CF from other non-CF pulmonary diseases, this value was slightly lower 0.778 (95% CI: 0.716-0.841). If one compares HE4 to sweat chloride analysis, the 'gold standard' test had a much higher PPV (77.1%), but worse NPV (54.8%) at 39 mEq/L based on Seia *et al.* [44]. We were curious about the relationship between sweat chloride test results and the concentrations of serum HE4. There was a positive significant correlation between these parameters in all CF subjects. Serum HE4 was already abnormal when the 'gold standard' sweat chloride test was only intermediate.

The origin of increased serum HE4 in CF and other non-cancer diseases has not been studied previously. Therefore, HE4 specific mRNA levels in bronchial epithelial biopsies from CF were analyzed. In CF, HE4 mRNA levels were significantly elevated compared to non-CF control samples; suggesting that high serum HE4 concentrations were a manifestation of increased production in the lungs. We recently found WFDC2 among the upregulated genes in a study of gene expression in native nasal epithelium of CF that supports the validity of this present data [48]. Similarly, there were several previous reports on modulated gene expression in mild and severe CF due to inflammation with or without bacterial infection or the presence of CFTR mutations [49-51]. Up-regulated genes in mild CF targeted lipid metabolism, G-protein coupled receptor expression and ion transports, while in severe CF altered genes were involved in oxidoreductase activity and the ubiquitin cycle [50]. In terms of classic CF mutations, the p.Phe508del mutation

was shown to have a minor effect on other gene expression profiles in cell cultures of CF airway epithelium cells compared to non-CF cells [51]. However, these authors used very stringent cut-off statistics as compared to other studies to choose their gene list.

HE4 levels in the culture medium supernatants of unpolarized and polarized wt and F508del-CFTR CFBE cells were compared. We found that there were about 2-fold higher HE4 concentrations in the supernatant of F508del-CFTR CFBE cells in case of polarized cultures as compared to wt cells (Fig. 6). Moreover, these cultures were treated with LPS (10 µg/mL) for 24 hours in order to analyze the direct effect of bacterial infection on HE4 production. We did not observe any induction in HE4 secretion by this stimulation. This later data are in agreement with the results of Bingle *et al.* [14] who did not find an alteration in HE4 expression of LPS-treated tracheobronchial epithelial cells or type II pneumocytes. Overall, in CF endogenous CFTR deficiency may be the main factor for HE4 overexpression, while intrapulmonary inflammation/infection may be supplemental in HE4 secretion.

Certain cytokines such as IL-8 are present with increased concentrations in the BALF obtained from CF patients, while others (e.g. IL-10) are at relatively lower quantity versus non-CF samples [52]. For instance, elevated levels of IL-8 have been shown to be selectively produced by airway epithelium and recruited neutrophils in CF [53], with neutrophil elastase being a key mediator of its production [54]. A quantitative gene expression analysis was processed where IL-8 mRNA level was increased in *ex vivo* bronchial epithelial cells [49]. By measuring the expression of other genes, additional explanations for the development of CF phenotype could be revealed in connection with e.g. airway defense [50]. Recently, miRNAs with aberrant expression seem to be potential regulators of the disease pathomechanism [55]. Out of hundreds of miRNAs, 56 showed decreased levels, while 36 were up-regulated in CF versus non-CF samples [55]. Notably, miR-126 was found

to be down-regulated in CF airway epithelial cells that regulate the innate immune (TH_2) response via TOM1 expression in the TLR2/4 signaling pathway [56]. miR-155 was responsible for the overexpression of IL-8, and bacterial infection promoted the expression of other miRNAs such as miR-215 [57].

In this study, we analyzed the level of miR-140-5p in connection with CF. This particular microRNA showing a high nucleotide complementarity to the HE4 specific mRNA target, has been recently studied in several types of malignancies, such as in non-small cell lung cancer [58]. Based on those data, the monocyte to macrophage differentiation-associated (MMD) gene was targeted by miR-140-5p that enhanced proliferation of lung tumor cells via ERK signaling [58]. According to a miRNA-related database, miR-140-5p was predicted to be associated with WFDC2/HE4 gene expression. We found that the level of miR-140-5p in respiratory epithelium biopsies was significantly decreased compared to non-CF control individuals, and was negatively associated with HE4 mRNA levels. Overall, lower levels of miR-140-5p - at least in part - may contribute to the exaggerated production of HE4 protein via higher HE4 mRNA expression in airway epithelium. Further investigations are needed to prove this relationship.

HE4 exhibited a proteinase inhibitory activity towards trypsin, elastase, matrix metallopeptidase 9, and the secretory proteinases from *Bacillus subtilis* [59]. Accordingly, we suppose that HE4 as an immunological response to bacterial/inflammatory events may show an inhibitory effect in this disease. Moreover, HE4 was described as a fibroblast-derived mediator of kidney fibrosis [60]. Accordingly, we speculate that increased HE4 may also contribute to fibrotic degradation of the lung in CF. Further experiments are required to support these theories.

The finding that HE4, a well-described tumor marker, is found up-regulated in CF, may be linked to a less differentiated epithelial state previously described in CF versus non-CF cells [61]. Indeed,

our own meta-analysis of data of gene expression profile revealed that CF nasal cells' have a significant overlap with genes related to undifferentiated epithelial phenotype [62].

In conclusion, serum HE4 is elevated in CF and correlates with the severity of the disease, thus it has the potential to be utilized as a novel prognostic biomarker that detects pulmonary inflammation. However, further studies are needed to clarify if released HE4 plays any functional role relative to IL-8, NE or any metalloproteinases in the regulation of local inflammatory lung events of CF patients.

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Author contributions. Béla Nagy Jr takes responsibility for the content of the manuscript, including the data and analysis. BNJr designed and performed experiments and wrote the manuscript, BN, LF, FG, OB, DN, RU, AS, AA, MM, and ZB provided human samples and analyzed data, ZF, LAC performed experiments, PAS designed experiments, HBP statistically analyzed data, JK, GB, MDA, MMJr, and IB provided overall direction, critical revisions for intellectual content, and providing final approval of the version to be published. The authors are grateful to Róza Földesi and Imréné Gém for their excellent technical assistance in RT-qPCR experiments and HE4 measurements, respectively. The authors thank Ines Pankonien for technical assistance with cell culture.

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Figure legends

Fig. 1. Serum HE4 levels in CF, non-CF, and normal controls (A). There were significantly increased HE4 concentrations in CF children compared to both controls and non-CF individuals; however, some elevation was also seen in non-CF subjects versus controls. Investigation of the effect of age on HE4 levels (B). The following HE4 concentrations were found in these sub-cohorts: 1-4 years of age (n=11): 122.7 [80.7-130.8] pmol/L; 5-9 years of age (n=19): 113.7 [82.6-190.3] pmol/L; 10-14 years of age (n=21): 91.1 [75.0-121.6] pmol/L; 15-18 years of age (n=26): 83.0 [64.4-112.1] pmol/L; 19-22 years of age (n=20): 107.6 [80.5-142.1] pmol/L; 23-29 years of age (n=24): 120.7 [81.1-120.7] pmol/L; 30-40 years of age (n=13): 115.7 [77.8-146.9] pmol/L. We did not find a significant difference in HE4 between these age groups.

Fig. 2. Correlation of HE4 with the clinical status of CF patients. CF children were categorized into 3 groups at the time of study recruitment showing mild (n=30), moderate (n=18), and severe (n=29) symptoms (A). Similar categorization was done in adults with CF with mild (n=22), moderate (n=22), and severe (n=13) states (B). Serum HE4 gradually increased in relative worsening conditions.

Fig. 3. The relationship between HE4 and FEV_1 (A) and serum CRP (B) values. There was a statistically significant inverse association ($P<0.0001$) between HE4 and FEV_1 values suggesting that HE4 was highly correlated with the degree of lung dysfunction (A). In contrast, HE4 was strongly associated wit CRP parameters in CF (B).

Fig. 4. ROC analysis of HE4 in the differentiation of CF from normal controls (with triangles) and CF from other non-CF lung diseases (with full circles). Substantial AUC values were calculated, and the 49.3 pmol/L cut-off value differentiated CF patients from normal controls, while the 98.4 pmol/L cut-off value was determined to distinguish CF from other pulmonary conditions.

Fig. 5. Analysis of the level of HE4 mRNA in bronchial epithelium biopsies obtained via bronchoscopy from 3 CF and 3 non-CF patients (A). RT-qPCR was assessed to quantify and compare HE4 mRNA expression. We found that HE4 mRNA concentrations were significantly up-regulated in CF versus non-CF control samples. HE4 mRNA level was normalized to the expression of the reference gene 36B4. Data are expressed in this ratio in mean±SEM. Analysis of relative miR-140-5p expression in respiratory epithelium biopsies obtained from CF and non-CF patients. The miRNA level was normalized to RNU43. We observed a significantly decreased level of miR-140-5p in CF compared to non-CF control samples. Data are expressed as a ratio to RNU43 in mean±SEM (B).

Fig. 6. The measurement of HE4 level in the supernatants of unpolarized and polarized wt and F508 deficient CFBE cells. There was about 2-fold higher HE4 level in the supernatant of F508del-CFTR cells in polarized cultures as compared to wt cells. n=3 per each type of sample.

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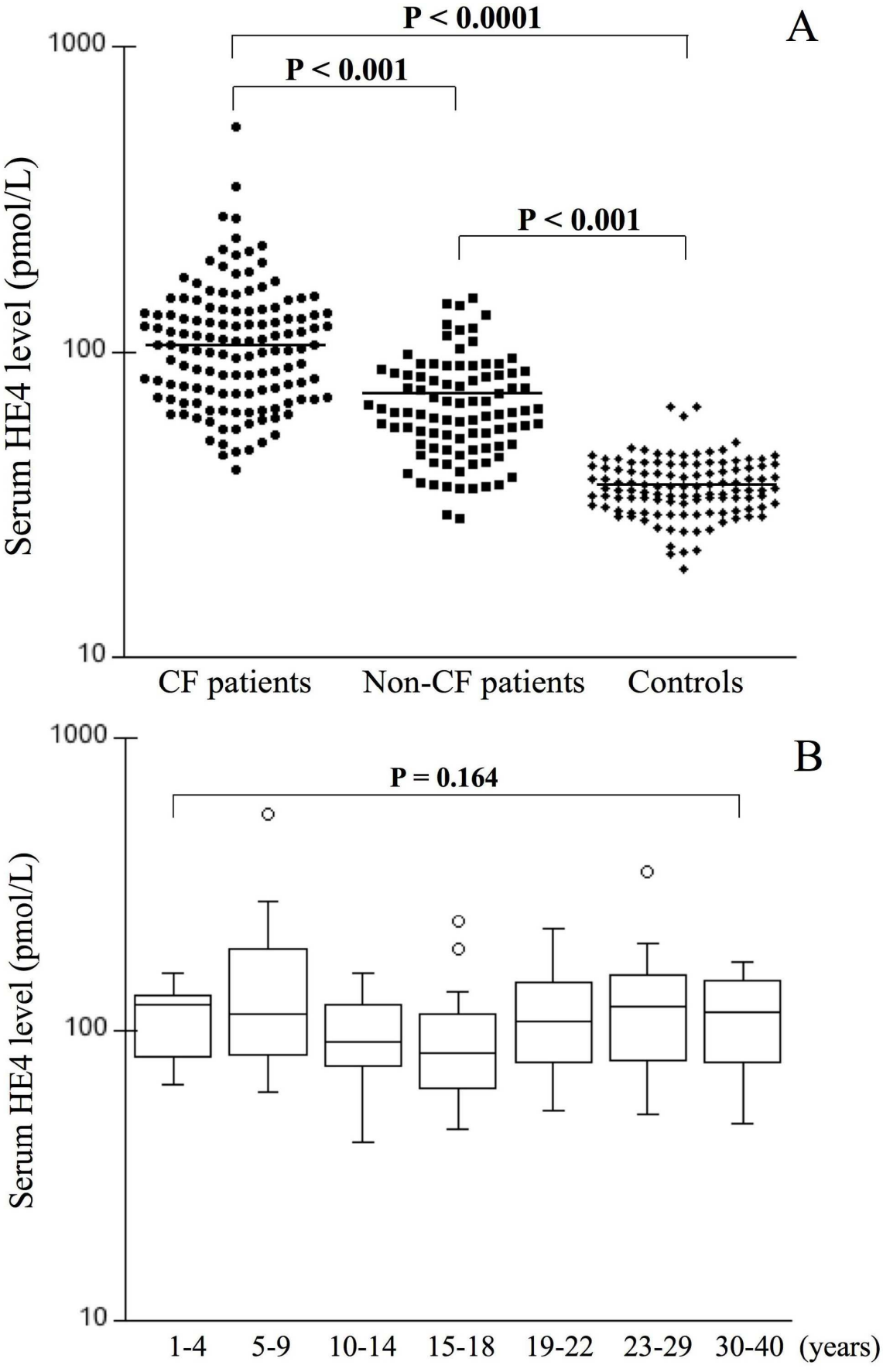
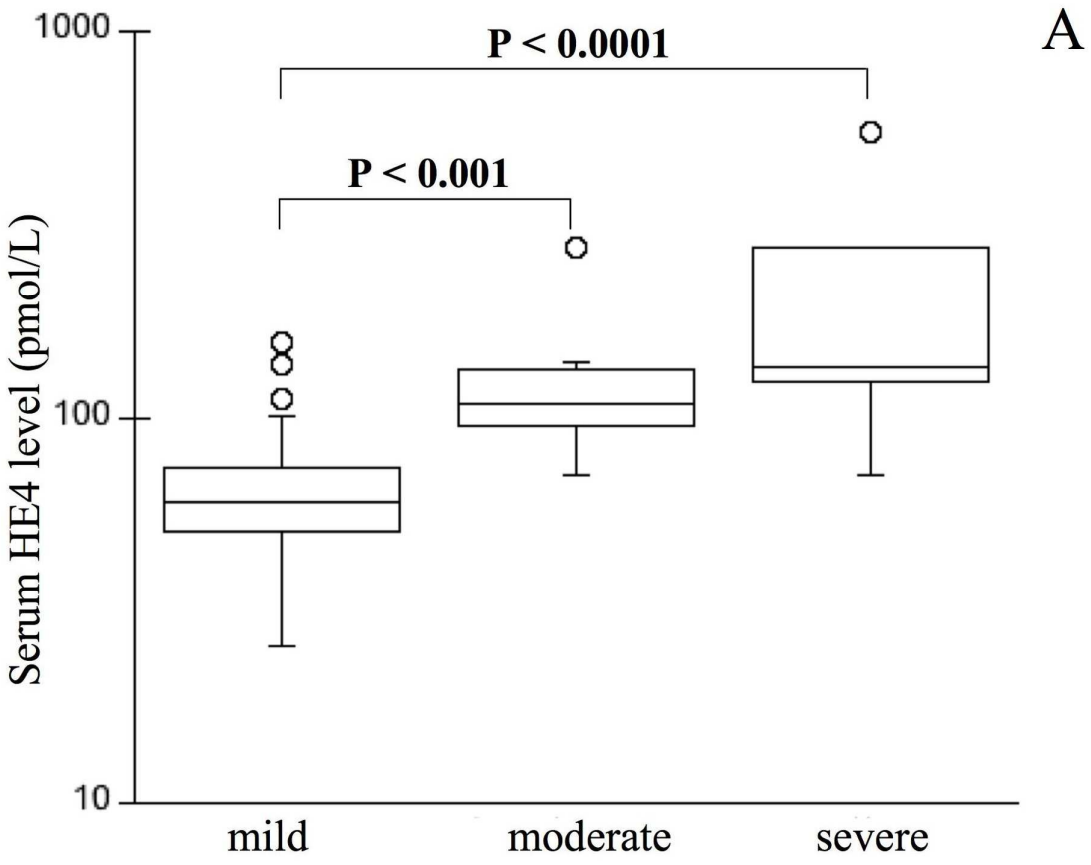
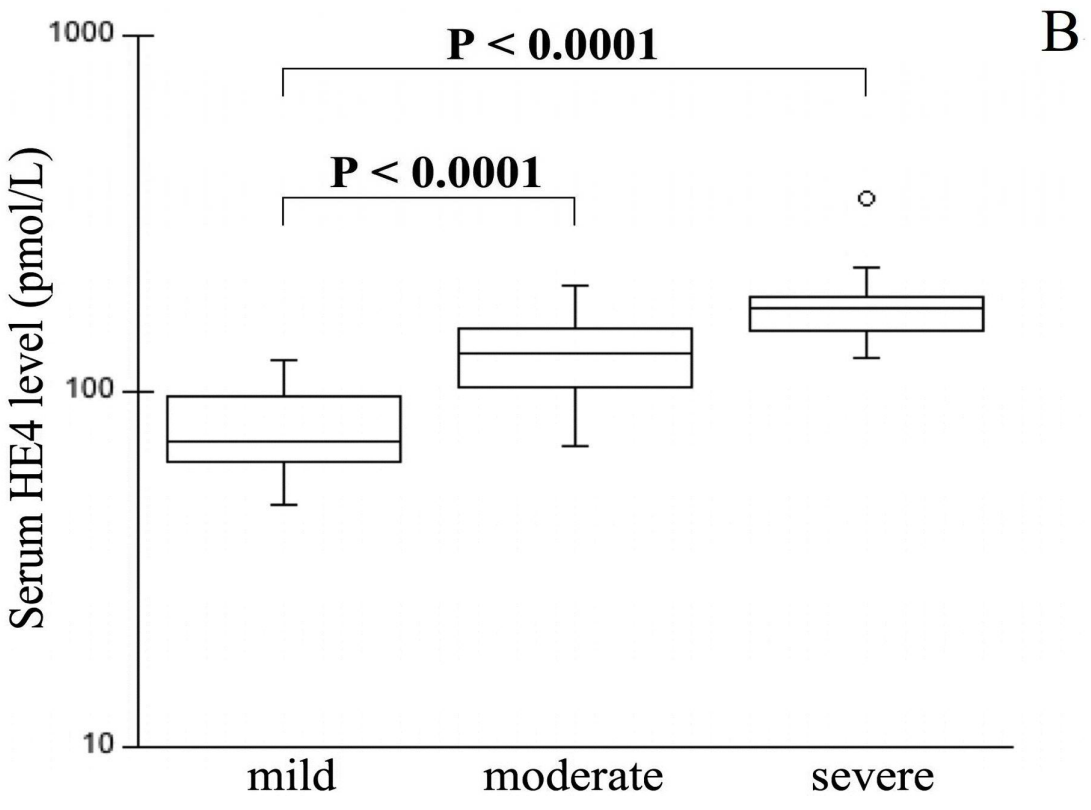
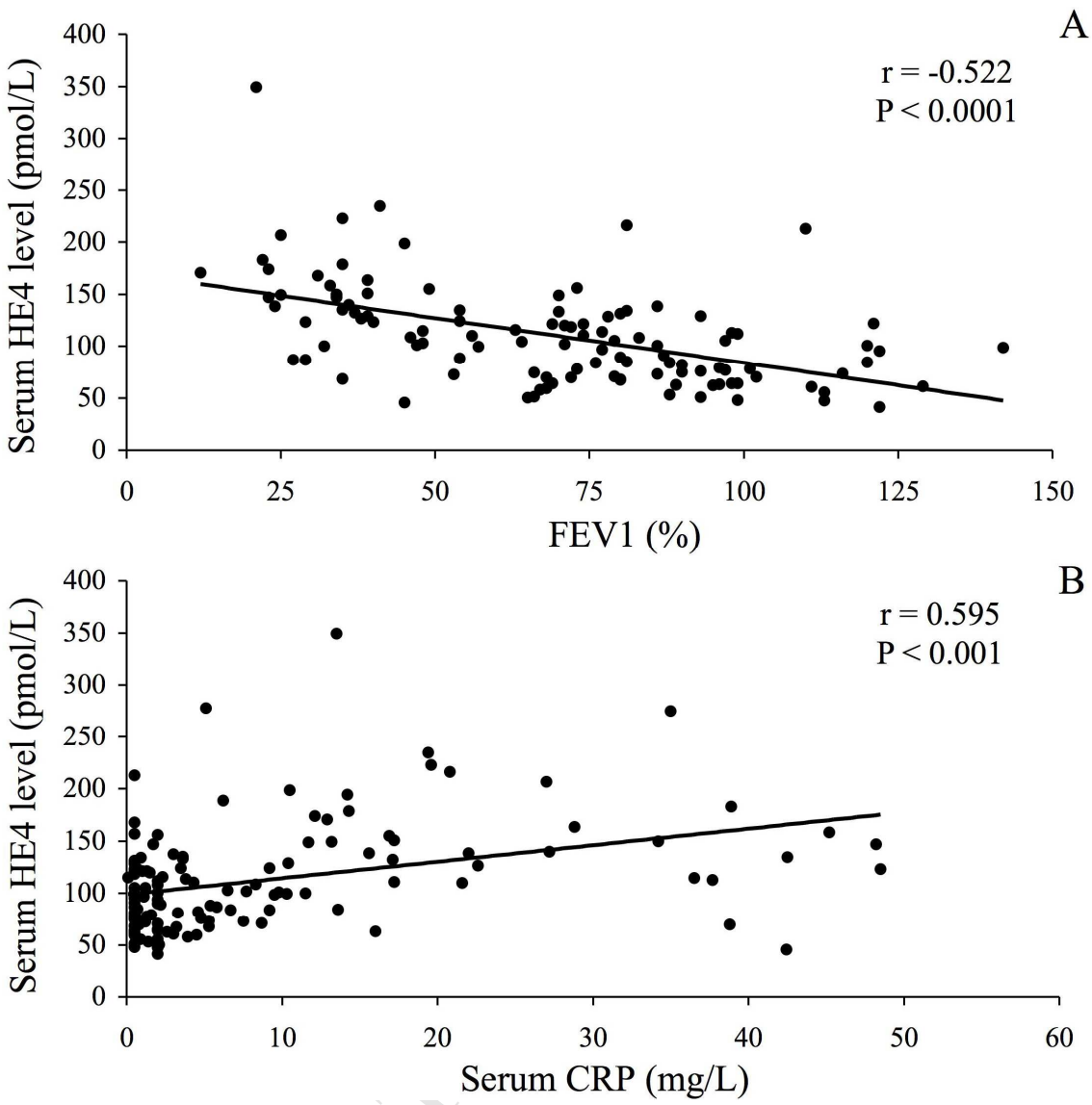
	Age (years)	Gender (m/f; n)	Serum HE4 (pmol/L)	Serum CRP (mg/L)
Young CF patients (n=77)	11 (7-17)	37/40	99.5 (73.1-128.9)	5.3 (2.0-9.3)[#]
Adult CF patients (n=57)	25 (21-29)	26/31	115.7 (77.8-148.7)	14.3 (12.9-27.0)
Non-CF young patients (n=76)	6 (3-14)	32/44	63.9 (54.3-83.4)	7.5 (1.1-18.2)
Non-CF adult patients (n=18)	26 (23-38)	7/11	59.2 (41.0-88.6)	8.9 (3.1-15.7)
Young controls (n=77)	13 (10-15)	33/44	36.3 (31.1-43.4)	nm
Adult controls (n=40)	25 (23-38)	17/23	33.8 (29.8-38.6)	nm

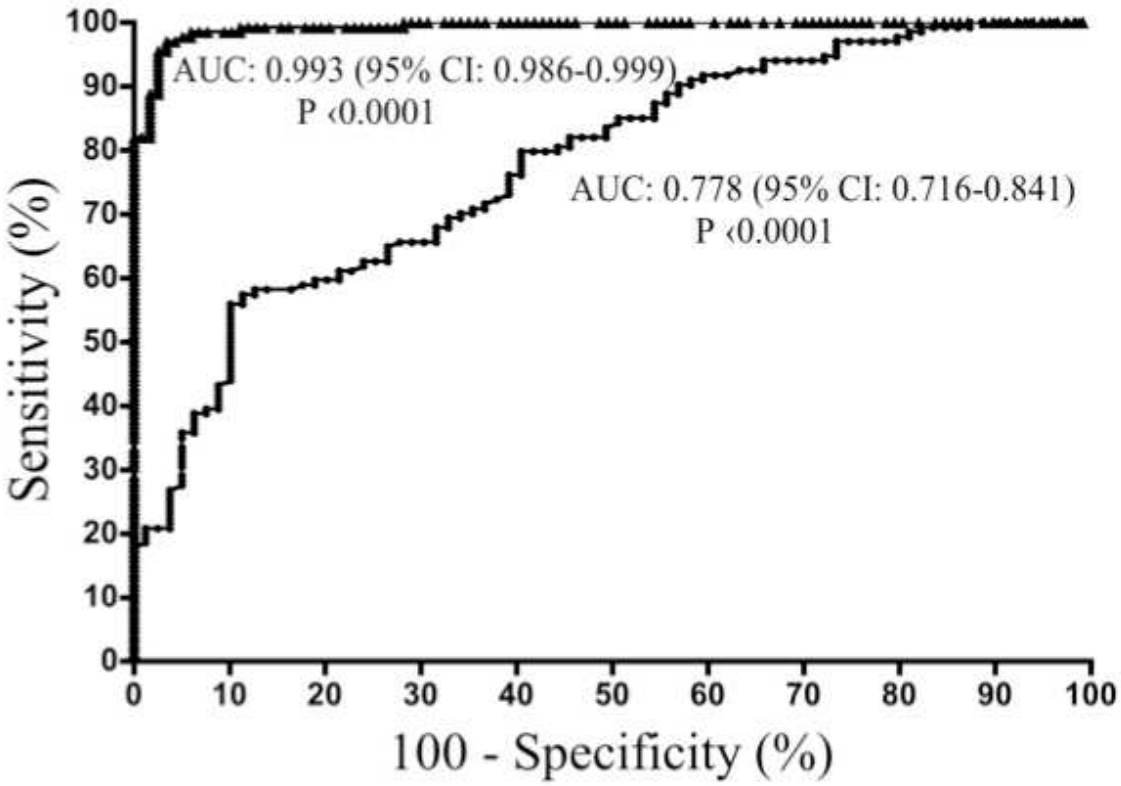
Table 1. Demographic and laboratory parameters of study populations. Data are expressed as median value (range). Serum CRP was measured in 29 individuals with severe clinical conditions out of 77 young CF-subjects marked with #. nm means 'not measured'.

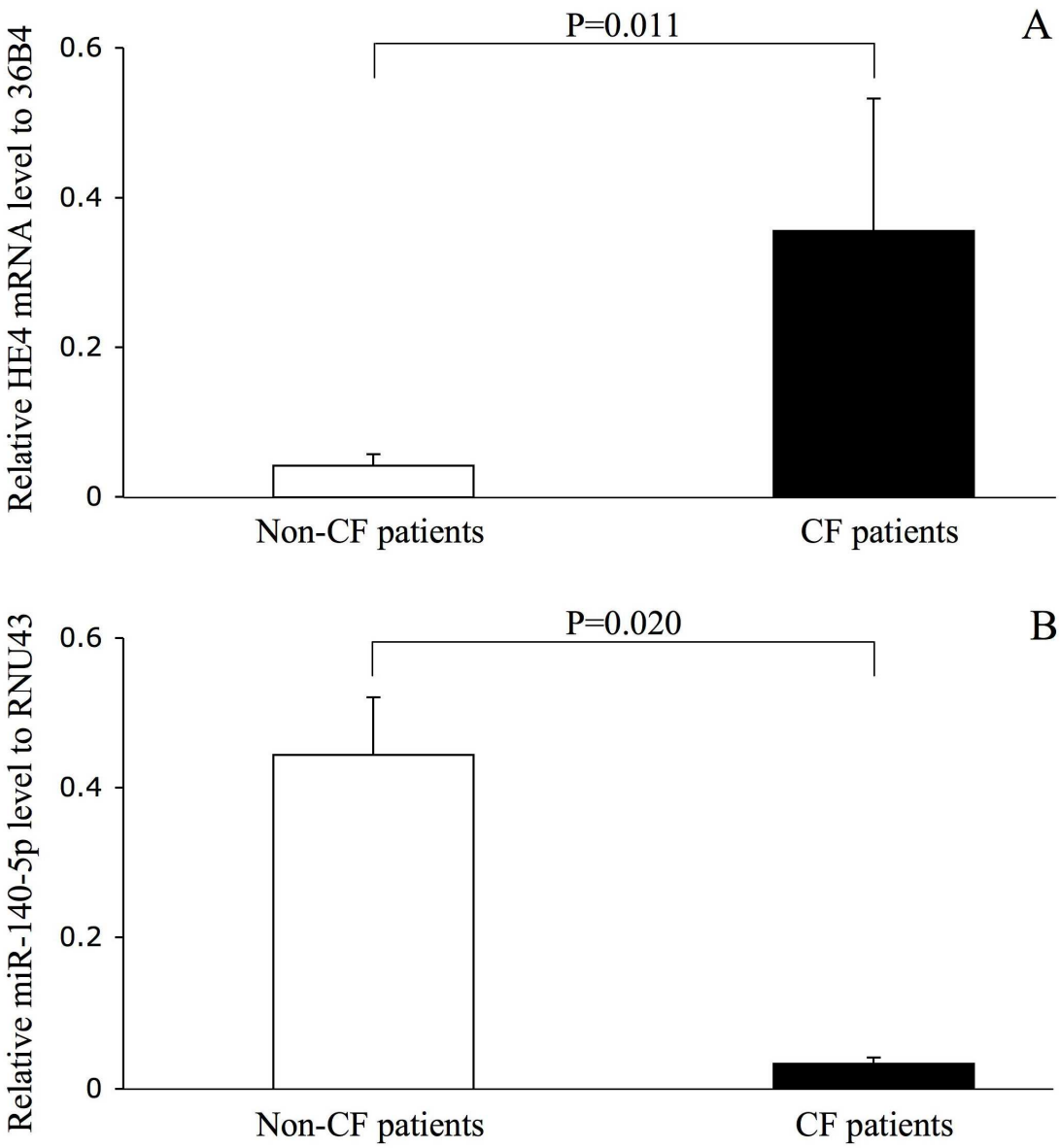

A
P < 0.0001
P < 0.001
P < 0.001
1000
100
10
Serum HE4 level (pmol/L)
CF patients
Non-CF patients
Controls
B
P = 0.164
1000
100
10
Serum HE4 level (pmol/L)
1-4
5-9
10-14
15-18
19-22
23-29
30-40
(years)

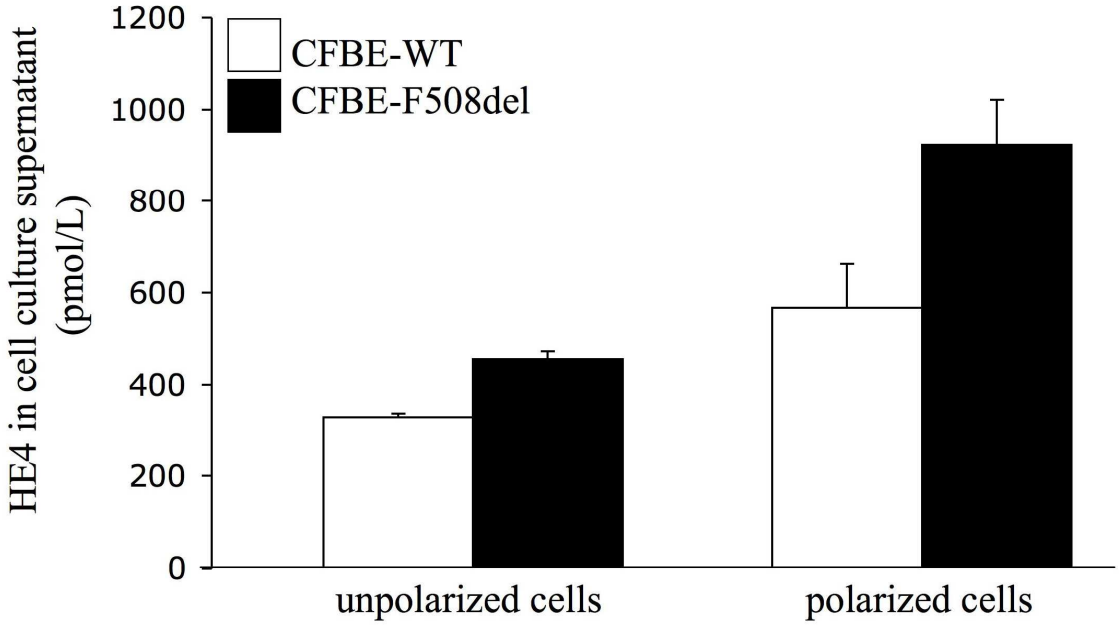

A
1000
100
10
Serum HE4 level (pmol/L)
P < 0.0001
P < 0.001
mild
moderate
severe


B
1000
100
10
Serum HE4 level (pmol/L)
P < 0.0001
P < 0.0001
mild
moderate
severe


A
r = -0.522
P < 0.0001
Serum HE4 level (pmol/L)
FEV1 (%)
B
r = 0.595
P < 0.001
Serum HE4 level (pmol/L)
Serum CRP (mg/L)




A
P=0.011
Relative HE4 mRNA level to 36B4
0.6
0.4
0.2
0
Non-CF patients
CF patients
B
P=0.020
Relative miR-140-5p level to RNU43
0.6
0.4
0.2
0
Non-CF patients
CF patients



List of abbreviations: CF, cystic fibrosis; HE4, human epididymis protein 4; CFTR/*CFTR*, cystic fibrosis transmembrane conductance regulator/gene; CFBE, cystic fibrosis bronchial epithelial; CRP, C-reactive protein; BALF, bronchoalveolar lavage fluid; SK-score, Shwachman-Kulczycki score; FEV_1, forced expiratory volume in one second; NE, neutrophil elastase, TEER, transepithelial resistance