



## Review

## Laboratory biomarkers for lung disease severity and progression in cystic fibrosis



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## ABSTRACT

Although the clinical outcomes of cystic fibrosis (CF) have been markedly improved through the recent implementation of novel CF transmembrane conductance regulator (CFTR) modulator drugs, robust and reliable biomarkers are still demanded for the early detection of CF lung disease progression, monitoring treatment efficacy and predicting life-threatening clinical complications. Thus, there is an unmet need to identify and validate novel, ideally blood based biomarkers with strong correlations to the severity of CF lung disease, which represents a major contribution to overall CF morbidity and mortality. In this review, we aim to summarize the utility of thus far studied blood-, sputum- and bronchoalveolar lavage (BAL)-based biomarkers to evaluate inflammatory conditions in the lung and to follow treatment efficacy in CF. Measurements of sweat chloride concentrations and the spirometric parameter FEV<sub>1</sub> are currently utilized to monitor CFTR function and the effect of various CF therapies. Nonetheless, both have inherent pitfalls and limitations, thus routinely analyzed biomarkers in blood, sputum or BAL samples are required as surrogates for lung disorders. Recent discovery of new protein (e.g. HE4) and RNA-based biomarkers, such as microRNAs may offer a higher efficacy, which in aggregate may be valuable to evaluate disease prognosis and to substantiate CF drug efficacy.

## 1. Introduction

Cystic fibrosis (CF) is one of the most common life-shortening monogenic disorders amongst European-derived populations currently affecting more than 85,000 patients worldwide, with ~ 45,000 patients listed in the European Registry [1]. The expected lifespan at birth has risen to cc. 30 years by now thanks to recent advantages in its standard and targeted therapies using new small molecules [1]. CF is caused by CF-causing variants in the gene (*CFTR*) ([www.cftr2.org](http://www.cftr2.org)) encoding the CF transmembrane conductance regulator (CFTR) protein, a chloride (Cl<sup>-</sup>)/bicarbonate (HCO<sub>3</sub><sup>-</sup>) channel, which regulates fluid transport

across various epithelial surfaces namely in the lumen of the bronchial tree and pancreatic exocrine ducts [2]. The diagnosis of CF is based on consensus clinical and laboratory criteria [3]. To confirm a diagnosis of CF, it is necessary to obtain evidence of CFTR dysfunction through the identification of two *CFTR* gene mutations *in trans* previously assigned as CF disease causing, and to perform tests showing high Cl<sup>-</sup> concentration in sweat (> 60 mEq/l), distinctive transepithelial nasal potential difference (NPD) measurements and/or assessment of CFTR (dys)function in native colonic epithelia *ex vivo* [4–6]. For individuals with symptoms suggestive of CF, but intermediate sweat Cl<sup>-</sup> values (30–59 mEq/l), the need for additional proof of CFTR function (through

**Abbreviations:** CF, cystic fibrosis; BAL, bronchoalveolar lavage; CFTR, CF transmembrane conductance regulator; NPD, nasal potential difference; NE, neutrophil elastase; NE-APC, neutrophil elastase-antiprotease complex; IL-8, interleukin-8; FEV<sub>1</sub>, forced expiratory volume in 1 s; PEx, pulmonary exacerbation; miRNA, microRNA; CRP, C-reactive protein; MPO, myeloperoxidase; SAA, serum amyloid A; TNF-α, tumor necrosis factor-α; TGF-β1, transforming growth factor β1; G-CSF, granulocyte colony stimulating factor; sTREM-1, soluble triggering receptor expressed on myeloid cells-1; MMP, matrix metalloproteinase; HE4, human epididymis protein 4; WAP, the whey acidic protein; WFDC, WAP four-disulfide-core; sCD14, soluble CD14; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; ROC-AUC, area under the receiver operating characteristic curve; NF-κB, nuclear factor-kappa B; HMGB1, High-mobility group box 1 protein; SLPI, secretory leukocyte protease inhibitor

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**Table 1**

Blood biomarkers in CF with their clinical utilities. Abbreviations: CF: cystic fibrosis, FEV<sub>1</sub>: forced expiratory volume in 1 s, PEx: pulmonary exacerbation, CRP: C-reactive protein, IL-6: interleukin-6, IL-8: interleukin-8, IL-1 $\beta$ : interleukin-1 $\beta$ , TNF- $\alpha$ : tumor necrosis factor- $\alpha$ , TGF- $\beta$ 1: transforming growth factor  $\beta$ 1, NE-APC: neutrophil elastase  $\alpha$ -1 antitrypsin complex, MPO: myeloperoxidase, MMP8: matrix metalloproteinase 8, MMP9: matrix metalloproteinase 9, HE4: human epididymis protein 4, sCD14: soluble CD14, SAA: serum amyloid A. “+” indicates the proved utility of a biomarker, N/A: not applicable. IVA means ivacaftor, LUM means lumacaftor, TEZ means tezacaftor, TOBR means tobramycin, CEFT means ceftazidime, MRP means meropenem, “i.v. ab” means intravenous antibiotics, indicated in case of those studies when the drug was not reported. Ages are expressed in mean  $\pm$  SD or range.

Biomarker	Number of patients	Age group	Differentiation of CF vs. healthy individuals	Correlation with lung disease severity (FEV <sub>1</sub> )	Correlation with <i>P. aeruginosa</i> infection	Significant alteration in PEx	Prediction of PEx	Treatment monitoring (medication, time period)	Refs
CRP	26	13–45 years	+			+		ciprofloxacin, 14 days	41
CRP	63	39.8 $\pm$ 6.3 years		+					42
CRP	55	1–16 years		+	+				43
CRP	51	33 $\pm$ 13.6 years		+			+		45
CRP	88	14 $\pm$ 1.8 years		+					28
CRP	67	6–18 years					+		32
CRP	53	32.8 $\pm$ 1.8 years					+	i.v. ab, 21 days	46
CRP	260	6–18 years		+				azithromycin, 28/128 days	47
CRP	43	33.7 $\pm$ 12.6 years					+	i.v. ab, 5 days	31
CRP	7	9–42 years						IVA, 6 months	37
IL-6	22	34.8 $\pm$ 12.9 years		+				i.v. ab, 14 days	34
IL-6	12	20–26 years		+				i.v. ab, 14 days	49
IL-6	103	23.5 $\pm$ 9.9 years					+	i.v. ab, 28 days	35
IL-8	53	32.8 $\pm$ 1.8 years					+	i.v. ab, 21 days	46
IL-8	103	23.5 $\pm$ 9.9 years					+	i.v. ab, 28 days	35
IL-1 $\beta$	21	23–50 years						IVA/LUM or TEZ, 1–3 months	39
IL-18	21	23–50 years						IVA/LUM or TEZ, 1–3 months	39
TNF- $\alpha$	21	23–50 years						IVA/LUM or TEZ, 1–3 months	39
TGF- $\beta$ 1	40	12.2 $\pm$ 0.7 years			+		+	i.v. ab, 14 days	33
NE-APC	60	17–46 years	+	+					25
NE-APC	88	14 $\pm$ 1.8 years		+					28
NE-APC	26	13–45 years	+			+		ciprofloxacin, 14 days	41
NE-APC	22	N/A				+		i.v. ab, 15 days	51
MPO	55	1–16 years		+	+				43
MPO	260	6–18 years		+				azithromycin, 28/128 days	47
MPO	27	12.2 $\pm$ 4.0 years			+			i.v. ab, 1 year	53
MPO	67	6–18 years					+		32
MMP8	54	31 $\pm$ 8.8 years	+			+			55
MMP8	54	31.6 $\pm$ 6.7 years		+		+			56
MMP9	54	31 $\pm$ 8.8 years	+			+			55
MMP9	54	31.6 $\pm$ 6.7 years		+		+			56
Calprotectin	260	6–18 years		+				azithromycin, 28/128 days	47
Calprotectin	40	9.8 $\pm$ 3.1 years					+	azithromycin, 28 days	36
Calprotectin	27	23.9 $\pm$ 7.6 years		+			+	TOBR + CEFT/MRP, 28 days	57
Calprotectin	57	26.4 $\pm$ 7.7 years		+			+		58
Calprotectin	88	14 $\pm$ 1.8 years		+					28
YKL-40	59	22 $\pm$ 15 years	+	+					26
YKL-40	54	31 $\pm$ 8.8 years	+			+			55
HE4	77	1–17 years	+	+		+			27
HE4	60	20.6 $\pm$ 12.2 years		+				IVA, 1–6 months	38
sCD14	30	20–37 years					+		30
SAA	260	6–18 years		+				i.v. ab, 28/128 days	47
SAA	88	14 $\pm$ 1.8 years		+					28
SAA	67	6–18 years					+		32

NPD measurements or intestinal current measurements) is particularly important [7]. Currently, most new cases of CF are usually identified within newborn screening programs including immunoreactive trypsinogen in bloodspots [8].

More than 2,000 variants have already been identified in the *CFTR* gene, with the major *p.Phe508del-CFTR* mutation accounting for approximately 70% of all CF-causing alleles worldwide, similarly in Hungary and the Czech Republic [9,10]. This class II variant is associated with misfolding, retention of unprocessed CFTR protein in the endoplasmic reticulum and its degradation prior to reaching the cell surface [11]. Impaired ion transport, airway dehydration and thick

mucus secretion with abnormal mucociliary clearance lead to chronic respiratory bronchial inflammation interrelated with pathognomonic pathogens, such as *P. aeruginosa* [11]. Neutrophil infiltration with high intrapulmonary protease levels (e.g. neutrophil elastase, NE) and induced interleukin 8 (IL-8) expression are associated with chronic inflammation leading to progressive damage of the bronchial tree [12]. Due to the *CFTR* allelic heterogeneity, there are various forms of CF, comprising e.g. monosymptomatic, pancreatic sufficient/insufficient forms.

Morbidity and mortality in the classical form of CF is mainly linked to progressive lung- and/or hepatobiliary diseases [2,12]. To monitor

lung disease severity and efficacy of treatment in CF, sweat  $\text{Cl}^-$  concentrations have been applied as an indirect indicator of CFTR-mediated ion transport function reflecting the ‘severity’ of the CFTR molecular defect. Consequently, sweat  $\text{Cl}^-$  effectively responded to treatment with CFTR potentiator *ivacaftor* and CFTR corrector *lumacaftor* [13,14]. The spirometric parameter forced expiratory volume in 1 s ( $\text{FEV}_1$ ; expressed in % predicted values) has become a standard to monitor the improvement of lung function that has been utilized in clinical trials [13–16]. However, both sweat  $\text{Cl}^-$  concentrations and spirometric parameters have inherent limitations. Obtaining sufficient amount of sweat can be challenging, especially in infants and young children, and sweat  $\text{Cl}^-$  concentrations have shown substantial inter-individual and intra-subject variability [17,18]. Spirometry is less sensitive in milder lung disease and some therapeutic agents (e.g. anti-inflammatory drugs or *lumacaftor*) have only little effect on  $\text{FEV}_1$  [14,19]. Moreover, a clinically meaningful change in  $\text{FEV}_1$  has not been established that can be generally applied to CF lung disease treatment [20]. Finally, reduction in sweat  $\text{Cl}^-$  and improvements in  $\text{FEV}_1$  are not always directly correlated in individual patients [21]. Therefore, there is still an unmet need for additional laboratory biomarkers that may be integrated into daily practice to improve the evaluation of early lung disease severity and to monitor treatment efficacy of CFTR modulators in CF [22]. Biomarkers of airway inflammation can be detected via analyzing blood or other body fluid samples from airway surface (i.e. sputum or bronchoalveolar lavage, BAL). However, because of preanalytical conditions and other systemic confounders, these biomarkers possess strength and weakness that may impact their clinical capabilities [22].

In this review, we aim to focus on the utility of various thus far evaluated inflammation dependent biomarkers measured in blood, sputum and/or BAL samples to monitor disease activity and to predict pulmonary exacerbations (PEX), including those applied to follow the efficacy of different therapeutic interventions in CF. Key characteristics and indications of the most widely used biomarkers are introduced in detail based on former clinical experience below. In addition, some advantages and disadvantages of these laboratory parameters are also emphasized. Finally, we also summarize the role of some emergent tools, such as the analysis of intra- and extracellular microRNAs (miRNA) with their potential as future diagnostics in this disease.

## 2. Blood-based biomarkers

Monitoring of pulmonary inflammation via peripheral blood biomarkers is the most common strategy that could be analytically standardized and is generally reproducible. Moreover, blood-based biomarkers are easily and non-invasively sampled in CF patients of practically any age- and disease category [23,24]. There is broad evidence on available blood biomarkers in the current literature, which i) reliably discriminated CF subjects from healthy individuals [25–27], ii) evaluated the severity of CF lung disease [27,28], iii) indicated other systemic conditions [29], iv) predicted the risk of exacerbation [30–32], and v) showed substantial change in PEX [33,34]. Additionally, their rapid alterations in relation to the degree of inflammation enabled assessing the decrease of inflammation following treatment with antibiotics [33,35,36] or CFTR specific agents [37–39]. On the other hand, we have to note that non-pulmonary inflammation may also affect the levels of blood biomarkers that decreases the specificity for lung disease [40]. Furthermore, systemic inflammation, increasing age, reduced body mass index and bacterial colonization are considered as important risk factors for poor health outcomes in CF, hence there was a strong relationship among plasma inflammatory biomarkers (i.e. IL-6, IL-1 $\beta$ ), reduced lung function and prior hospitalization [29]. Reviewed studies with a focus on distinct blood-based biomarkers determined in different patient populations with various clinical utilities are summarized in Table 1.

### 2.1. C-reactive protein (CRP)

Serum or plasma CRP concentrations as indicators of systemic inflammation in CF have been analyzed since the early nineties of the last century when these were first found to be increased in CF [41]. After a 2 week-intravenous *ciprofloxacin* administration, serum CRP was significantly reduced compared to pre-treatment values in CF demonstrating its utility in monitoring treatment efficiency as well [41]. Since then, a large number of clinical data have been accumulated on CRP measurements because of different indications. Elevated serum CRP levels were correlated with lung function parameter  $\text{FEV}_1$  [42] and were associated with *P. aeruginosa* colonization [43,44] and allergic bronchopulmonary aspergillosis [44]. Higher baseline plasma CRP levels ( $\geq 5.2$  mg/L) were found in adult CF patients who had a more severe disease and were at higher risk for PEX [45]. Similarly, serum CRP was among those individual biomarkers, which could effectively differentiate CF children for lung disease severity [28]. In parallel, CRP was applied as a major component of different clinical and laboratory panels and predicted the development of PEX requiring iv. antibiotics within 84 days [32]. Early recurring PEX was also indicated by plasma CRP levels at the end of related antibiotic treatment [46]. Treatment efficacy in CF was monitored by serum CRP together with myeloperoxidase (MPO), serum amyloid A (SAA) and calprotectin levels for a 1-month *azithromycin* administration compared to the placebo group [47]. Alterations in these biomarkers were negatively correlated with changes in  $\text{FEV}_1$  and body weight [47]. Recently, plasma CRP ( $> 75$  mg/L) predicted non-responsiveness to antibiotics with 90% specificity and time until next exacerbation under PEX treatment [31]. Only few data have been published on the role of CRP in monitoring CFTR specific regimen efficacy as yet. CRP plasma levels slightly but significantly decreased by 2 months of *ivacaftor* treatment in a small cohort of patients with at least one non-p.Gly551Asp-CFTR variant, and showed a positive correlation with leukocyte count and sweat  $\text{Cl}^-$  concentrations [37]. In our recent study, serum CRP was measured as an additional biomarker to human epididymis protein 4 (HE4), but a relatively large ratio of CRP values was undetectable ( $< 0.5$  mg/L) in *ivacaftor*-treated CF subjects, thus, here serum CRP could not be efficiently utilized [27].

### 2.2. Cytokines

Airway disease is characterized by chronic infection and recurrent inflammation with neutrophil dominance in association with increased production of a number of pro-inflammatory cytokines, such as IL-6, IL-8 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), etc. [48]. These mediators do not merely have a functional role in the pathomechanism of inflammatory processes but can behave as potential biomarkers in CF [40]. For instance, serum IL-6 separated CF subjects from normal individuals and showed a significant reduction during antibiotic therapy [34,49]. Similarly, plasma IL-8 monitored antibiotic efficacy by 21 days and indicated the onset of early re-exacerbation [46]. Higher plasma IL-6 and IL-8 concentrations at the time of exacerbation were associated with an increased risk for being a non-responder to antibiotics [35]. Very recently, when the anti-inflammatory properties of CFTR modulators was investigated, *ivacaftor/tezacaftor* combined CFTR specific therapy significantly lowered serum IL-1 $\beta$ , IL-18 and TNF- $\alpha$  levels, while *ivacaftor/lumacaftor* decreased only IL-1 $\beta$  over 3 months of treatment [39]. Finally, transforming growth factor  $\beta 1$  (TGF- $\beta 1$ ) is a pleiotropic cytokine with a wide range of cellular functions in CF, such as immunomodulation and airway remodeling [33]. Plasma levels of TGF- $\beta 1$  were increased in *P. aeruginosa* infection and were significantly reduced in response to antibiotic administration to monitor the clinical status [33].

## 2.3. Neutrophil activation-dependent biomarkers

Activated neutrophils produce several proteins under inflammatory conditions, such as NE, MPO, calprotectin, YKL-40, etc., which circulate at a high quantity in CF blood specimens. NE is a ‘destructive’ serine protease impacting extracellular matrix proteins that forms complexes with antiproteases leading to NE-APC [50]. Plasma NE-APC was elevated in persons with CF vs. non-CF controls, and showed continuous inflammation and lung damage between two infective exacerbations regardless of type of bacterial colonization [25]. For discriminating ‘mild’ and ‘severe’ patient cohorts from each other, different combinations of several markers were tested based on the area under the receiver operating characteristic curve (ROC-AUC) analysis [28]. The best panel of two biomarkers (NE-APC + granulocyte colony stimulating factor, G-CSF) had an AUC value of 0.74, a triplet (NE-APC + G-CSF + linoleic acid) showed a similar AUC value of 0.73, while the best individual marker was the G-CSF with an AUC of 0.75 [28]. High plasma NE-APC levels were detected in cases of PEx associated with fever and leukocytosis, and oral *ciprofloxacin* administration resulted in an improvement of spirometric parameters and clinical scores together with significantly reduced plasma NE-APC [41]. When the parallel reduction in the host inflammatory and catabolic responses was followed during PEx in the presence of chronic *P. aeruginosa* colonization, plasma NE-APC levels were decreased in the presence of weight gain and improving lung function due to antibiotic treatment [51].

Next, MPO is the most abundant constituent within neutrophils that catalyzes the formation of hypochlorous acid, a potent reactive oxygen species that has strong bactericidal properties. MPO may be secreted or leaked into the extracellular environment when neutrophils are exposed to certain stimuli [52]. In CF, serum MPO concentrations were elevated in cases with *P. aeruginosa* infection compared to controls [43]. Oral *azithromycin* therapy decreased MPO levels after a 28 day-long regimen [47]. The effect of repeated antibiotic therapy was significant on serum MPO levels and was thus utilized in assessing therapeutic efficacy in PEx [53]. Recently, serum MPO with other biomarkers effectively predicted short-term PEx in children and adolescents with CF [32].

The soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) is a surface receptor on neutrophils that has been recently reported to be constitutively augmented in CF [54]. Surprisingly, no significant alteration in sTREM-1 was observed in PEx or following antibiotic treatment [54]. In contrast, its level highly correlated with plasma NE activity as well as matrix metalloproteinase- (MMP) 8 and 9 levels [54].

In response to TNF- $\alpha$ , neutrophils also release MMPs that potentiate lung inflammation and facilitate airway remodeling in CF [50]. It is supposed that MMPs may be responsible for cleaving sTREM-1 from the surface of neutrophils as well [54]. Serum/plasma MMP-8 and MMP-9 levels were highly elevated in CF compared to controls, especially in those patients who suffered from PEx [54–56]. Furthermore, MMP-8 was associated with abnormal FEV<sub>1</sub>, while reduced expression of MMP-1, MMP-8 and MMP-9 was observed after antibiotic therapy [56].

Calprotectin produced by neutrophils, has an important anti-microbial effect and its circulating levels are elevated upon systemic inflammation and infection [47]. Reduction in plasma calprotectin over time correlated with an improvement in FEV<sub>1</sub> after 28 and 168 days of *azithromycin* treatment vs. placebo in CF children [47], while early changes in serum calprotectin in response to medication for 28 days was predictive for PEx risk by day 168 (ROC-AUC: 0.76) [36]. Similar results were reported on calprotectin in regard to monitoring antibiotic treatment of PEx [57] and prediction for the time to the onset of next PEx (at a cut-off value of 9.1 mg/mL at the end of the exacerbation treatment) [57]. Its serum concentrations also indicated clinically suitable changes in CF disease activity in adults [58] that has been recently confirmed in children as well [28].

The chitinase-3-like protein 1, also known as YKL-40, is secreted from the granules of neutrophils upon activation, hence, its levels are

increased in several human inflammatory diseases [59]. Higher than normal serum YKL-40 levels were detected in CF vs. controls [26] and were significantly associated with the severity of CF lung disease, including PEx [26,55]. Abnormal levels of this serum protein were more pronounced in CF patients with impaired glucose tolerance [60]. In contrast, serum YKL-40 levels were not affected by CF-associated liver disease or pancreatic insufficiency [55].

## 2.4. Human epididymis protein 4 (HE4)

The whey acidic protein (WAP) or WAP four-disulfide-core (WFDC) protein family includes small secreted proteins, which possess multiple functions, such as anti-protease, anti-bacterial and anti-inflammatory properties [61]. Due to their abundance in the lung, they have been proposed to be involved in lung homeostasis and protection of the lung from proteolytic ‘attacks’ [62]. Among 18 different subtypes, Secretory Leukocyte Protease Inhibitor (SLPI or WFDC4) is produced by mucosal epithelial cells and macrophages upon inflammation and blocks inflammatory signals in monocytes after being secreted [63]. In addition, it neutralizes NE and IL-8 as well as controls neutrophil recruitment. Elafin (WFDC14) is expressed by bronchial epithelial cells and macrophages induced by NE or TNF- $\alpha$ . This serine protease can eradicate pathogenic bacteria in CF (e.g. *P. aeruginosa*) via their opsonization [63]. The expression of HE4 was demonstrated in several normal and malignant tissues, and typically overexpressed in reproductive tract and pulmonary malignancies [64,65]. In addition, HE4 is highly expressed in the lower, chronically inflamed airways of CF according to immunohistochemistry findings [62]. The first report about HE4 (also known as WFDC2) as a CF blood biomarker was published by our group when serum HE4 was found to be elevated in CF in comparison to non-CF subjects and clinical controls [27]. Patients suffered from acute PEx showed even higher serum HE4 concentrations [27]. In bronchoepithelial biopsy CF samples, HE4 mRNA levels were also significantly elevated compared with the non-CF control samples. This finding suggests that high serum HE4 concentrations represent its increased production in the CF lung. In parallel, culture of polarized cystic fibrosis bronchial epithelial (CFBE) 410- cells expressing p.Phe508del-CFTR mutation showed elevated HE4 levels in the supernatants compared to cells with wt-CFTR [27]. In a study of gene expression by Clarke *et al.*, WFDC2 was recorded among the upregulated genes in native CF nasal epithelium [66]. HE4 was positively correlated with the severity of CF lung disease and serum CRP levels in two large independent international cohorts consisting of children and adult cases [27]. In order to evaluate the role of HE4 in monitoring therapy efficacy in CF, *ivacaftor*-treated patients carrying at least one p.Gly551Asp-CFTR mutation have been investigated for plasma HE4 concentrations at baseline and after 1–6 months of medication [38]. After 1 month of therapy, HE4 levels were significantly lower compared to baseline and remained decreased for up to 6 months. Moreover, significant inverse correlation between absolute and delta values of HE4 and FEV<sub>1</sub> was observed in cases treated with *ivacaftor*. A ROC-AUC value was determined for plasma HE4 when 7% mean change of FEV<sub>1</sub> was used as classifier (ROC-AUC: 0.72), especially in the first 2 months of *ivacaftor* treatment (ROC-AUC: 0.81) [38]. Impaired renal function may be a confounder of blood HE4 levels, therefore, estimated glomerular filtration ratio needs to be accounted for interpretation of HE4 values, also in CF [67]. Overall, plasma HE4 levels can serve as a sensitive prognostic and predictive biomarker of drug effectiveness in CF patients receiving *ivacaftor* [38]. Therefore, based on these results, HE4 can be considered as a very promising blood-based biomarker in CF. However, extended clinical studies with larger cohorts are required to validate this analyte not only for the follow-up of other forms of CFTR-modulating therapies, but also for the prediction of PEx.



**Table 2**

Sputum-based CF biomarkers measured in previous clinical studies for different utilities. Abbreviations: CF: cystic fibrosis, FEV<sub>1</sub>: forced expiratory volume in 1 s, PEx: pulmonary exacerbation, NE: neutrophil elastase, TLP: trypsin-like protease, HMGB-1: High-mobility group box 1 protein, SLPI: secretory leukocyte protease inhibitor, HE4: human epididymis protein 4, IL-8: interleukin-8, IL-1 $\beta$ : interleukin-1 $\beta$ , IL-6: interleukin-6, YKL-40: chitinase-3-like protein 1. “+” indicates the proved utility of a biomarker, “i.v. ab” means intravenous antibiotics, indicated in case of those studies when the drug was not reported, TOBR means tobramycin, CEFT means ceftazidime, MRP means meropenem, IVA means ivacaftor. Ages are expressed in mean  $\pm$  SD or range.

Biomarker	Number of patients	Age group	Differentiation of CF vs. healthy individuals	Correlation with lung disease severity (FEV <sub>1</sub> )	Correlation with <i>P. aeruginosa</i> infection	Significant alteration in PEx	Prediction of PEx	Treatment monitoring (medication, time period)	Refs
NE	35	11.1 $\pm$ 2.6 years		+					76
NE	36	10.1–49.2 years					+	azithromycin, 14 days	78
NE	87	20.1 $\pm$ 7.9 years		+	+		+	azithromycin, 128 days	79
NE	12	22–57 years		+	+			IVA, 2 years	81
TLP	62	20.5–33.5 years		+					83
SLPI	35	11.1 $\pm$ 2.6 years		+					76
SLPI	17	25.5 $\pm$ 7.1 years	+					TOBR + CEFT/MRP, 14 days	84
HE4	77	1–17 years	+	+					27
IL-8	32	11.4–35.7 years		+		+		i.v. ab, 14 days	86
IL-1 $\beta$	24	18.8 $\pm$ 4.8 years						TOBR + CEFT/MRP, 15 days	87
IL-6	12	20–26 years	+	+		+			49
IL-6	72	23.1 $\pm$ 10.7 years						ibuprofen, 30 days	88
HMGB-1	31	21.2 $\pm$ 13.9 years	+	+		+			89
HMGB-1	97	21.9–30.3 years		+			+		91
YKL-40	59	22 $\pm$ 15 years	+	+					26
YKL-40	28	17.4 $\pm$ 9.4 years			+	+			92
Resistin	32	24–30 years	+	+					93

## 2.5. Other inflammation-related biomarkers

Several other inflammation-associated biomarkers have been thoroughly studied. In this regard, SAA, produced by hepatocytes during the acute phase of inflammation, has been tested as a surrogate of inflammation in CF. Elevated SAA level significantly decreased from baseline to day 28 due to *azithromycin* treatment and highly correlated with improving lung function [47]. Additionally, this biomarker could categorize CF children into two subgroups of ‘mild’ or ‘severe’ patients [28] and predicted PEx in young CF individuals [32].

Plasma soluble CD14 (sCD14) is also promising for CF [30]. The sCD14 is cleaved from its membrane-bound forms on the surface of monocytes and macrophages by proteases, such as NE. Recently, it was revealed that sCD14 within a ‘danger-associated molecular pattern’, stimulates proinflammatory cytokine/chemokine production independently of the bacterial lipopolysaccharide (LPS) via the toll-like receptor 4 (TLR4)/CD14 membrane complex, nuclear factor-kappa B (NF- $\kappa$ B), and the inflammasome that jointly maintain tissue inflammation [68]. Plasma sCD14 demonstrated high ROC-AUC value of 0.91 (at a cut-off value of 1450 ng/mL) with a sensitivity of 100% and specificity of 82% in the prediction of PEx within 4 months of stable CF diseases [30]. However, further studies are required to validate the utility of sCD14 as a biomarker in CF.

## 2.6. Autoantibodies

Colonization with *P. aeruginosa* can induce to the development of certain autoantibodies in CF, which may be useful in the laboratory follow-up of disease complications. Bactericidal/permeability-increasing (BPI) protein is a granule protein of neutrophils with antimicrobial activity [69]. Anti-neutrophil cytoplasmic antibodies (ANCA) with BPI specificity have been identified in CF due to the infection that predicted disease severity and showed decreased levels following lung transplantation [69]. Since then, a prospective 10-year long clinical study of CF adults reported that high serum IgA-BPI-ANCA concentration was associated with adverse outcome after lung transplantation [70]. In parallel, robust formation of neutrophil extracellular traps (NETs) by activated neutrophils was described in response to *P.*

*aeruginosa* in CF airways [71]. Although serum level of NETs was not higher in CF than controls, autoantibody against an extracellular component of NETs (e.g. anti-PAD4) was increased in serum samples of CF patients compared to healthy individuals and showed a positive relationship with *P. aeruginosa* infection and an inverse correlation with FEV<sub>1</sub> [72].

## 3. Sputum-based biomarkers

Since airway inflammation plays a key role in the progression of CF lung disease, direct analysis of biomarkers in sputum is also principal that monitors affected large airways and aids treatment efficiency [73]. Collection of sputum is a non-invasive manner, e.g. via application of hypertonic saline and/or by spontaneous expectoration and is generally feasible in adolescents and adults with CF. Induced sampling by inhaled hypertonic saline is usually exploited in children above 10 years of age and can provide a higher amount of sputum. Essentially, there are insignificant differences in the composition of spontaneous and induced sputum samples, thus both types could be utilized for biomarker studies [73]. Of note, the analysis of sputum samples must be performed immediately after collection and the concentrations of inflammatory biomarkers can be influenced by regional variability in lung disease [40]. Additionally, if there is no way to analyze sputum specimens on-site, sample preparation needs to be minimized and shipping conditions should be carefully standardized to avoid any bias as the concentration of certain biomarkers, e.g. HMGB-1 may be affected [74]. To date, the lack of commercially available equipment and sample collection protocols prevent the use in routine clinical practice [75]. Currently available sputum biomarkers are primarily applied to predict lung function decline [76,83,86] and to monitor therapeutic regimens via [78,79,81]. Below, we summarize the major sputum biomarkers of CF airway inflammation with their potential capabilities (Table 2).

### 3.1. Neutrophil elastase

NE is one of the most sensitive sputum biomarkers to monitor CF airway inflammation that was found to be predictive of long-term lung function decline, however, did not predict patient survival [76,77].

Larger reduction in sputum NE activity independently reflected the positive airway response to antibiotic treatment already at day 14 of treatment (OR: 2.94) vs. non-responders, while its sustained high values were associated with an increased risk of subsequent PEx (HR: 1.71) [78]. Moreover, oral *azithromycin* treatment for 6 months was associated with an improvement in sputum NE activity in the presence of increasing FEV<sub>1</sub> values in subjects chronically infected with *P. aeruginosa* compared to placebo [79]. In contrast, the beneficial effect of *ivacaftor* could not be effectively monitored by sputum NE activity in a p.Gly551Asp-*CFTR* positive population [80]. Recently, sputum NE with IL-1 $\beta$  and IL-8 levels showed a rapid, significant reduction with decreasing bacterium density in the first week of *ivacaftor* treatment. Moreover, among the *P. aeruginosa*-infected patients, these biomarkers continued to decline over the next 2 years monitoring suppressed inflammation by *ivacaftor* [81].

### 3.2. Other serine proteases and antiproteases

Due to the heavy neutrophil burden in CF, the high concentration of neutrophil derived proteases overwhelms antiproteases. This protease/antiprotease imbalance causes abnormal mucus clearance, increased level of inflammation, and impaired immune responses [82]. Sputum trypsin-like protease (TLP) has been investigated as a novel biomarker for CF lung disease [83]. Increased epithelial-derived TLP activity promotes epithelial sodium channel-mediated fluid absorption and activates PAR-receptors causing inflammatory processes. TLP activity in sputum inversely correlated with FEV<sub>1</sub> and was greater in those individuals who did not survive beyond 5-years from analysis [83]. Levels of antiprotease WFDC proteins have been also investigated in CF sputum. Sputum SLPI showed no difference between CF and healthy individuals, however, SLPI had a positive correlation with FEV<sub>1</sub> values [76]. Interestingly, others found decreased sputum levels of SLPI in untreated CF patients vs. controls, which increased following antibiotic therapy [84]. Elafin is also cleaved by NE and showed lower sputum levels in CF cases colonized by *P. aeruginosa* [85]. However, the clinical utility of elafin measurement has not been studied in CF as yet. When we determined HE4 concentrations in sputum from CF or other non-CF diseases, similar to sera, there were significantly higher HE4 concentrations in CF vs. non-CF or healthy sputum specimens [27].

### 3.3. Cytokines

Many pro-inflammatory cytokines, such as IL-1 $\beta$ , IL-6, IL-8, etc. can be reliably measured in CF sputum specimens that generally show elevated levels in CF [73]. For instance, sputum IL-8 levels were significantly decreased after parenteral antibiotic treatment during PEx and were inversely correlated to FEV<sub>1</sub> in young CF patients [86]. The same group later reported that sputum IL-1 $\beta$  levels positively correlated with the respiratory function and serum CRP before therapy, and significantly decreased during antibiotic treatment [87]. IL-6 level in sputum was also abnormal in CF vs. controls, and during PEx, there was a relationship between sputum IL-6 and FEV<sub>1</sub> [49]. Others found that sputum IL-6 was the only biomarker, measured along with IL-8, TNF- $\alpha$ , IL-1 $\beta$  concentrations and NE activity, that was markedly reduced by high-dose *ibuprofen* by the end of 4 weeks of its administration [88]. However, this study demonstrated significant inter- and intra-subject variability in sputum inflammatory markers even in CF patients with stable lung disease. The exact cause of this variability is not known and should be considered when designing CF clinical trials using anti-inflammatory drugs in which outcomes include changes in sputum inflammatory markers [88].

High-mobility group box 1 protein (HMGB1) acts as both a nuclear factor and a secreted cytokine. It is a chromatin-binding factor and a potent mediator of inflammation [89]. HMGB1 is secreted by activated monocytes and macrophages and is passively released by necrotic or damaged cells [89]. This protein was detected in both CF serum and

sputum samples at higher levels than samples from normal individuals [89,90]. Increased serum HMGB-1 levels were associated with 5% increased risk of PEx [89], while abnormal sputum HMGB-1 was related to higher risk for lung function decline [89]. Moreover, the neutralization of this protein by antibody reduced neutrophil influx and chemotaxis, which suggests that HMGB1 contributes to pulmonary inflammation and lung matrix degradation in CF airway disease [90]. Similarly, sputum HMGB-1 had strong associations with concurrent FEV<sub>1</sub> values and the number of PEx suffered within the year preceding sampling of the sputum [91]. Taken together, this biomarker can predict time-to-first PEx, number of future PEx within the upcoming 5 years and time-to-lung transplantation [91].

Sputum YLK-40 was also significantly elevated in sputum samples of CF patients vs. healthy controls and correlated with severity of pulmonary obstruction [26]. Interesting, its levels in the sputum were more correlated with chronic *P. aeruginosa* airways colonization in patients with FEV<sub>1</sub> < 50% than those with FEV<sub>1</sub> values > 50% [92].

Resistin synthesized by monocytes and neutrophils, is a small cysteine-rich inflammatory regulator that acts via binding to the TLR4 receptor, an inducer of the NF- $\kappa$ B pathway [93]. In CF, although plasma resistin was observed to be only marginally higher than in controls, sputum resistin concentration was much higher than its levels in healthy controls. Levels of this biomarker negatively correlated with lung function independently of age, gender and *CFTR* genotype, while were associated with CF-related diabetes mellitus [93].

### 3.4. Bronchoalveolar lavage- (BAL) based biomarkers

BAL performed by bronchoscopy represents another suitable material to investigate lung-derived inflammatory biomarkers in CF lung disease (Table 3). However, BAL due to its invasive nature is being limited to specialized centers and suitable for patients with early and mild CF lung disease [94]. Flexible bronchoscopy with BAL provides a regional measurement of lung inflammation and infection within the respiratory tract, including the small airways and alveoli [95]. Inflammation has been shown to be already present in CF lung disease before pulmonary function changes detected by spirometry. Hence, early airway inflammation and infection biomarkers are of importance in infants and young children [95]. On the other hand, the utility of BAL fluid biomarkers for longitudinal monitoring of CF lung disease is still unproven [96]. Current BAL biomarkers have discriminative capability between CF and non-CF subjects [50,97], between subcohorts of CF patients with different phenotypes [98–101] and are effective to guide different therapeutic regimens via clinically substantial alteration in their level [97,102].

### 3.5. Neutrophilic inflammation specific biomarkers

BAL samples from CF typically contain high levels of different cytokines (e.g. IL-8, IL-6, etc.) [94]. The expression of these proteins is promoted by the NF- $\kappa$ B pathway via cellular interaction with bacteria, bacterial products and proinflammatory cytokines. These mediators are part of the CF pathogenesis, e.g. IL-8, which is a strong chemoattractant for neutrophils [94]. Analysis of BAL samples showed differences for changes in IL-8 and NE levels versus non-CF subjects, and infection was associated with elevated inflammatory mediators in CF BAL fluid [97]. TGF- $\beta$ 1 in BAL was upregulated in CF vs. non-CF subjects and its abnormal level was associated with diminished lung function and recent hospitalization [103]. Similarly, much higher levels of NE and MMP2 and MMP9 in BAL were reported in CF- vs. non-CF-related bronchiectasis that also showed correlation with FEV<sub>1</sub> values [50]. Based on a recent study, IL-1 $\alpha$  measured in BAL had the strongest association with structural damage in the absence of infection in CF children [101]. In the early phase of lung disease, cellular and cytokine alterations in BAL may demonstrate the preclinical pulmonary inflammation. A substantial proportion of infants diagnosed with CF already have active

**Table 3**

Laboratory biomarkers measured in BAL samples of CF subjects. Abbreviations: CF: cystic fibrosis, FEV<sub>1</sub>: forced expiratory volume in 1 s, PEx: pulmonary exacerbation, NE: neutrophil elastase, IL-8: interleukin-8, IL-1: interleukin-1, TGF- $\beta$ 1: transforming growth factor  $\beta$ 1, MMP2: matrix metalloproteinase 2, MMP9: matrix metalloproteinase 9, YKL-40: chitinase-3-like protein 1, rhDNase means recombinant human deoxyribonuclease. “+” indicates the proved utility of a biomarker, “i.v. ab” means intravenous antibiotics indicated in case of those studies when the drug was not reported. Ages are expressed in mean  $\pm$  SD or range.

Biomarker	Number of patients	Age group	Differentiation of CF vs. healthy individuals	Correlation with lung disease severity (FEV <sub>1</sub> )	Correlation with <i>P. aeruginosa</i> infection	Significant alteration in PEx	Prediction of PEx	Treatment monitoring (medication, time period)	Refs
NE	70	1.5–71 months	+	+				i.v. ab, 14 days	97
NE	10	20.5–26.5 years		+	+				50
NE	57	2.3–4.9 months		+	+				99
NE	111	2.9 $\pm$ 1.7 years		+	+				98
NE	46	11.3 $\pm$ 5.1 years		+				rhDNase, 3 years	105
IL-8	70	1.5–71 months	+	+				i.v. ab, 14 days	97
IL-8	111	2.9 $\pm$ 1.7 years		+	+				98
IL-8	46	11.3 $\pm$ 5.1 years		+				rhDNase, 3 years	105
IL-1 $\alpha$	102	1.8–5.3 years		+					101
IL-1 $\beta$	102	1.8–5.3 years		+					101
TGF- $\beta$ 1	29	9.1 $\pm$ 1.2 years		+	+	+			103
MMP2	10	20.5–26.5 years		+	+				50
MMP9	10	20.5–26.5 years		+	+				50
YKL-40	36	3.95 $\pm$ 2.21 years			+				104

pulmonary inflammation: 30% have detectable NE activity, 20% have pulmonary infection and 80% have evidence of structural lung disease on chest computed tomography at 3 months of age [99]. As a result, free NE activity was associated with structural lung disease when most children had no clinically apparent lung disease [99]. Furthermore, Sly *et al.* showed free NE activity in BAL as a predictive biomarker of persistent CF-related bronchiectasis at 3 months of age (OR: 3.02) with the odds 7 times as high at 12 months and 4 times as high at 3 years of age [100]. Young children with CF with both upper and lower airway *P. aeruginosa* infection had higher free NE levels, neutrophil counts and IL-8 with lower Shwachman scores compared with those with positive upper airway cultures only [98]. Moreover, the presence of both *P. aeruginosa* and *S. aureus* had an additive effect on concentrations of these lower airway inflammatory markers [98]. YKL-40 levels in BAL highly reflected the early pulmonary inflammation (i.e. elevated macrophage and neutrophil number) and thus the infection [104].

Biomarkers in BAL samples have demonstrated to be responsive to different interventions in CF subjects. Based on an early clinical study, decreased NE activity was observed in adults after 2–6 weeks of iv. antibiotic therapy [102]. Eradication of infection in young infants was accompanied with sustained decrease in NE and IL-8 levels [97]. Treatment with inhaled recombinant human deoxyribonuclease (rhDNase; dornase alfa; Pulmozyme) attenuated lung inflammation over 3 years leading to lowered neutrophil cell percentage, NE and IL-8 levels in the treated group vs. the placebo cohort [105].

Due to the limitations of current biomarkers, novel proteins are currently under identification for BAL analysis in relation to CF lung disease. Esther *et al.* has recently performed a mass spectrophotometric analysis of metabolomics, and 93 metabolites showed a strong association with neutrophilic inflammation with the prediction of future structural disease [106].

#### 4. Role of miRNAs in the pathogenesis and testing of CF lung disease

There is a significant phenotypic heterogeneity between CF patients, which may be caused by inherent epigenetic regulation to a certain degree, for example via altered miRNA species [107]. Direct effects of miRNAs on *CFTR* expression and modulation of inflammation have been recently revealed. First profiling of miRNA expression in CF was performed a couple of years ago, when 667 miRNAs were analyzed in cells obtained by bronchial brushing [107]. Out of 391 miRNAs detected in all studied CF samples, 56 were downregulated, while 36 were

upregulated in contrast to non-CF individuals. Among significantly decreased miRNAs in CF, miR-126 was the one that increased the expression of *TOM1*, the negative regulator of IL-1 $\beta$ /TNF- $\alpha$ -induced pathway related to the innate immune response [108]. In contrast, miR-155 was significantly increased causing upregulation of IL-8 via direct suppression of *SHIP1* [109]. Among several miRNAs, miR-145 and miR-494 [110], miR-223 as well as miR-144 [111,112] in addition to miR-509 [113] target *CFTR* gene and thus downregulate its expression. According to these favorable preliminary results, miRNAs are implied to behave as regulators (and possibly drug targets) in CF. Our group recently found that increased HE4 mRNA levels were associated with significantly lowered miR-140 expression in CF airway biopsy specimens [27]. However, this interaction was evaluated by bioinformatics analyses and not direct assays, hence it needs to be independently confirmed. Elevated Mirc1 (miR-17–92) cluster expression in CF sputum positively correlated with PEx, while there was a negative correlation with spirometric parameters [114]. However, CF patients treated with CFTR modulator drugs *ivacaftor/lumacaftor* did not demonstrate significant change in this miRNA expression after 6 months of treatment [114]. Recently, a microarray profiling has been performed for circulating plasma miRNAs to identify new biomarkers, and there were 10 miRNAs – 3 of them belong to the let-7 family – which were significantly elevated in CF vs. healthy individuals [115]. Further clinical studies are required to validate these miRNAs in CF lung disease.

#### 5. Summary, future perspectives

This review summarizes the main findings of several potential biomarkers for CF disease activity and progression evaluated in numerous patient studies. Differences in the results among cited clinical investigations may be related to numerous factors, such as diverse study design, various methodologies, local clinical practices with different genetic background and nutritional status of recruited patients. CF itself is a heterogeneous disease with an overall short life expectancy, and that is why the most important outcome in clinical trials is still the mortality of subjects that can be predicted by FEV<sub>1</sub> predictive percentage or the rate of FEV<sub>1</sub> decline [96]. However, survival and referral to lung transplantation have gradually become the new outcomes that can be achieved via complex clinical investigation of CF individuals consisting of predictive laboratory biomarkers [96]. In parallel, the need to detect early lung damage in infants or early childhood has got in focus recently [101]. For this purpose, lung function tests (e.g. lung clearance

index), radiological imaging (e.g. chest CT scores) and different clinical scores (e.g. CF-ABLE score) in combination with laboratory biomarkers can predict and indicate the development of early CF lung disease [75]. In addition, early treatment of inflammation and management of infections can aid to prevent structural changes and lung function impairment with the assistance of regular analysis of laboratory biomarkers. Finally, predictive markers of disease progression may guide targeted CFTR specific therapies and also improves our understanding on CF pathomechanism. These biomarkers have been utilized in parallel and their characteristics were individually analyzed, while some recent studies tried to combine biomarkers in panels in comparison to other clinical investigations or novel parameters [28,31].

Alternatively, the use of models based on patient's own tissue has recently become available to predict responses to CFTR modulators at an individual level [116]. Theranostics is the diagnostic and functional test that targets the individual genetic and functional profile of CF disease to select the best therapy [116]. As such, patient-derived intestinal organoids can act as patient specific surrogate systems based on forskolin-induced swelling [117], which have been used to quantitate individual CFTR-specific drug response *in vitro* [118]. Rectal organoid responses have correlated with both change in FEV<sub>1</sub> and sweat chloride values, and *in vivo* (non)responders were identified with high predictive values [119]. Accordingly, organoids may be used for personalized medicine in CF in the near future.

## 6. Conclusions

In summary, analysis of single or multiple blood, sputum and BAL-based inflammatory biomarkers can aid directing the care of CF individuals, although these biomarkers only complement the currently assessed endpoint parameters at present. For instance, we have recently found the blood-based analyte HE4 as a reliable prognostic and predictive novel biomarker in CF. Moreover, in contrast to other laboratory markers [37,80,114], HE4 could also monitor the effect of *ivacaftor* treatment [38]. miRNAs, which are involved in the modulation of CF lung disease, might also have potential as new laboratory markers via indicating changes in the level of their circulating form [115]. Clinical evaluation of the value of emerging biomarkers will be required in the near future for better laboratory monitoring of disease progress and treatment efficacy. This is particularly relevant to bring new treatments to all individuals with CF, namely by extending the label of CFTR modulator drugs to patients with ultra-rare mutations, in particular for N-of-1 trials and personalized therapies, where benefit needs to be quickly and effectively assessed in sole individuals at a time [116]. Finally, the field of biomarker development is rapidly evolving whereby it is expected that various omics technologies will be utilized together with advanced bioinformatics algorithms in order to grasp the multi-system complexity of CF disease in various stages of its development.

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## Conflict of interest statement

The authors have no direct or indirect conflict of interest which could affect the content and outcomes of this review.

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