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Inflammation biomarkers in sputum for clinical trials in cystic fibrosis: current understanding and gaps in knowledge ☆,☆☆,★

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ABSTRACT

Rationale: Sputum biomarkers hold promise as a direct measure of inflammation within the cystic fibrosis (CF) lung, but variability in study design and sampling methodology have limited their use. A full evaluation of the reliability, validity and clinical relevance of individual biomarkers is required to optimise their use within CF clinical research.
Objectives: A biomarker Special Interest Working Group was established within the European Cystic Fibrosis Society-Clinical Trials Network Standardisation Committee, to perform a review of the evidence regarding sputum biomarkers in CF.
Methods: From the 139 included articles, we identified 71 sputum biomarkers to undergo evaluation of their clinimetric properties, responsiveness, discriminant, concurrent and convergent validity.
Results: Current evidence confirms the potential of sputum biomarkers as outcome measures in clinical trials. Inconsistency in responsiveness, concurrent and convergent validity require further research into these markers and processing standardisation before translation into wider use. Of the 71 biomarkers identified, Neutrophil Elastase (NE), IL-8, TNF-α and IL-1β, demonstrated validity and responsiveness to

☆ On behalf of the European Cystic Fibrosis Society – Clinical Trial Network: Biomarkers Special Interest Group.

☆☆ "Take home" message of paper:

★ Sputum biomarkers hold promise as a direct measure of inflammation within the cystic fibrosis lung with NE, IL-8, TNF-α and IL-1β, demonstrating evidence of validity and responsiveness to be considered as outcome measures in clinical trials.

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be currently considered for use in clinical trials. Other biomarkers show future promise, including IL-6, calprotectin, HMGB-1 and YKL-40.
Conclusion: A concerted international effort across the cystic fibrosis community is needed to promote high quality biomarker trial design, establish large population-based biomarker studies, and work together to create standards for collection, storage and analysis of sputum biomarkers.

1. Introduction

Cystic fibrosis (CF) therapy is increasingly focused on personalised care, therefore, the ability to accurately monitor disease progression and response to therapy is essential [1]. Starting in infancy, a persistent, progressive and excessive inflammatory state develops within the CF lung, acting as a specific driver of lung damage [2,3]. Defects in the innate and adaptive immune systems [4], neutrophil influx, unbalanced oxidative stress response, and local hypoxia generated by mucus accumulation, drive a vicious pro-inflammatory cycle [5–7]. This is mediated by increased concentrations of pro-inflammatory mediators including nuclear factor kappa B (NF-kB), defective promotion of inflammatory resolution, and an imbalance in protease and antiprotease activity [8]. As inflammation drives disease progression and pulmonary exacerbations [9], therapies targeted at reducing inflammation are an increasing priority for future CF care [4,10,11].

A Biomarker Special Interest Working Group was established within the European CF Society-Clinical Trials Network (ECFS-CTN) Standardisation Committee, to standardise the use of inflammatory biomarkers in clinical trials [12]. The group first evaluated bronchoalveolar lavage (BAL) inflammatory biomarkers, but with BAL sampling limited by bronchoscopic procedural risks, which increase with disease severity, alternatives are required [12,13]. Among these, sputum induction carries minimal risk, is possible in paediatric populations [14] and those unable to expectorate and is increasingly important in the CFTR modulator era [15,16].

Biomarkers have wide roles in clinical research, across biological mechanism studies and all phases of clinical trials. Some biomarkers may be critical for drug development despite lack of correlation with individual clinical outcomes. For example, biomarkers are necessary for evaluation of pharmacokinetics, pharmacodynamics (PK/PD), mechanism of drug action, and safety, as in the case of QT interval monitoring. To date, variability in study design, patient populations and sampling methodology have challenged the translation of sputum biomarkers into wider use [18]. No optimal investigational biomarker has emerged, as a trial outcome measure, in part due to a lack of robust evidence of reliability and validity [17]. To inform drug development in CF, inflammatory biomarkers may be considered independently from clinical response as “pharmacological” indicators of drug efficiency, mainly in proof of concept trials, or as responsive or prognostic biomarkers reflective of clinical response in the short or long term [18]. These 2 conceptual aims may require different validation approaches. This manuscript aimed to identify, through an exhaustive literature search and systematic review of their properties within a validation framework, sputum biomarkers with potential for future use as trial outcomes, highlight gaps in knowledge, areas for future research and provide recommendations for future practice.

2. Methods

A literature search was conducted using the National Centre for Biotechnology Information US National Library of Medicine/National Institutes of Health PubMed database. The search was limited to full text articles in English, with publication ending Feb 2020. For search terms see **Supplemental Figure 1 (S1).** A bibliographic search was conducted of all included articles. Over 3500 references were identified by the initial searches. After review of duplication and abstracts, 187 articles were identified for further review. Further screening for inclusion was conducted by four reviewers (CA, SN, AL and KH). On further full text review, 48 were excluded based on incomplete numerical values, alternative respiratory samples, laboratory studies or animal models, or non-English language papers (**Figure S1**). Variability in study populations, sampling and laboratory methodology, prevented performance of a formal meta-analysis and effective evidence grading, but allowed creation of a consensus guidance document.

In total, 100 papers identified 71 sputum biomarkers for review **(Figure S1).** Investigated biomarkers included: cell counts; pro-inflammatory, anti-inflammatory and lymphocytic signalling/regulatory cytokines; neutrophil related proteases/antiproteases [4,11,19]; growth factors; bioactive metals and co-factors of superoxide dismutase involved in oxidative stress **(Supplemental Table 1: Table S1).** Results from the included studies were collated to provide an overview of clinimetric properties of each biomarker, responsiveness, discriminant, concurrent and convergent validity **(Supplemental Table 2: Table S2)** [20]. Convergent validity was assessed against percent predicted forced expiratory volume in one second ($ppFEV_1$), other clinical measures or other disease biomarkers identified from included papers where possible, and against serum biomarkers. Discriminant validity between people with CF (pwCF), healthy controls (HC) and other respiratory diseases was used to assess biomarkers disease specificity and validity within assessment of inflammatory mechanistic pathways. This clinimetric validation framework allowed assessment of any given biomarkers' validity across the multitude of potential pharmacodynamic, monitoring, predictive and prognostic roles inflammatory biomarkers could play within CF [17,142].

3. Results

3.1. Discriminant validity between people with CF (pwCF) and non-CF subjects

The ability of 23 sputum biomarkers to discriminate between healthy controls (HC) and pwCF is shown in Table 1**.** Interleukin 8 (IL-8) and tumour necrosis factor alpha (TNF-α) were the most frequently assessed, and effectively discriminated between CF and HC in adults and children [21–26]. Neutrophil elastase (NE) also showed significant discriminant validity, with values up to 40 times higher in pwCF than in HC [23,27,28]. Interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and interleukin 10 (IL-10) differed significantly in CF and HC children. High mobility group box protein (HMGB-1), chemokine ligand 18 (CCL-18), chitinase-3-like protein 1 (YKL-40) differed significantly in CF and HC adults. A single study found neutrophil count and percentage could discriminate between pwCF and HC [23].

Supplemental Table 3 (Table S3) shows discriminant validity of biomarkers between CF and other chronic respiratory diseases. IL-8 showed significantly higher levels in pwCF versus asthmatic patients [22,29], while interferon gamma (IFN-γ), interleukin 3 (IL-3), urokinase plasminogen activator (uPA), granulocyte macrophage

Table 1
Discriminant validity of biomarkers between patients with Cystic Fibrosis and Healthy Control patients:

Popn	[Ref.]	CF		HC		BIOMARKER	Biomarker level CF (units stated)	Biomarker level HC	p value
		n	Age (yrs) Mean range	n	Age (yrs) Mean range				
Paediatric	[43]	20	7-10	10	7-10	Nitrites	184.8±11.07 um/L	56.4±5.7	p<0.01
	[24]	25	12 (6-19)	10 BE 5 HC	12.5 (6-16)	TNF-α	448 pg/mL^{-1}	9	p<0.001
						IL-1β	12220	269	p<0.001
						IL-8	71000	2800	p<0.005
						IL-6	26	225	p<0.001
						IL-10	24	45	p=0.02
Adult	[113]	31	21.2± 13.9	30	Age matched	HMGB1	5.1(2.5-25.6) ng/mL	1.1(0.8-1.8)	p<0.0001
	[99]	40	21±14	27	25±9	CCL18	**	**	p<0.0001
						CF chronic Pa vs non-chronic Pa			p<0.05
	[94]	59	22±15	26	25±9	YKL-40	*CF up to 30-fold higher than HC	*	p<0.01
	[25]	18	28 (18-43)	8 BE 8 CB 5 HC	52(31-76) 60(28-73) 31(24-36)	IL-8	7.1±1.0 × 10^{-9} M	1.1±0.3	p<0.05
Mixed	[114]	28	7-41	20	8-35	YKL-40	138.5 ± 132.7 ng/mL	28.2 ± 24.34	p<0.001
	[27]	17	25.1 (8-35)	20	28.5 (23-48)	NE	73.4 ng/mL (median)	1.7	p<0.001
						SLPI	0.01	0.01	NS
						MMP-9	0.9	NA	NS
						TIMP-1	1.8	NA	p<0.036
	[115]	53	≥8	40	≥8	MMP-9	*	*	p<0.001
						MMP-9a	*	*	p<0.001
						MMP-9/TIMP-1;	*	*	p<0.001
						MMP9/TIMP2; MMP9/sput protein MMP9a/TIMP2	*	*	p=0.003
	[21]	24	6-44 (med 20.5)	8	25-38 (med 27.5)	TGFβ-1	76.5 pg/mL (median)	59.1 (median)	p<0.074
						IL-8	5791	28.3	p<0.01
						TNF-α	29.6	16.8	p<0.01
	[28]	33	22.1±1.3 (9-34)	26 HC 26 ARF	7.1±1.3 (9-34)	MMP-8	*	*	p<0.02 CF inpt; p<0.005 CF outpt
						MMP-9	*	*	
						TIMP-1	*	*	
						NE	*	*	p<0.001 CF inpt; NS outpt
									NS
									p<0.01 CF inpt NS outpt

(*continued on next page*)

Table 1 (*continued*)

Popn	[Ref.]	CF		HC		BIOMARKER	Biomarker level CF (units stated)	Biomarker level HC	p value
		n	Age (yrs) Mean range	n	Age (yrs) Mean range				
	[22]	28	23.7±11.1 (12-50)	74 COPD 34 Asthma 44 HC	59.2±9.9 47.4±13.9 20-60	IL-8 IL-10 TNF-α IFN-γ CAP-18 uPA uPAR AI1	29880±4226 pg/mL 386±87 74±31 794±137 79623±18597 59±26 803±112 3812±469	1713±317 72±14 12±6 684±117 39936±3644 18±6 85±11 592±97	p<0.0001 p<0.05 p<0.001 NS p<0.009 similar among grps p<0.001 p<0.05
	[23]	20	11.9 (8.2-19.5) 13.3 (9.4-17.0)	11	11.2 (8.3-17.4)	IL-8 NE Neut counts Neutrophil %	* Non-prod 57.6(0.1-158.6) 10^5/mL Producers 54.3 (3.5-184.0) Non-prod 56.9 (0.5-93.5) Producers 65.6 (28.7-83.3)	* 5.9 (0.1-33.7) – 18.9 (0.7-66) –	p<0.001 p<0.001 p<0.05 – p<0.05 –
	[26]	33	5-31	10	NR	IL-1α IL-1β IL-8 TNF-α	0.45±0.05 ng/mL 18.8±2.6 410.7±61.3 1.08±0.24	0.35±0.11 NR NR NR	NR p=0.0002 p<0.0005 NR

ARF: acute respiratory failure; BE: bronchiectasis; CAP-18: 18-kDa cationic antimicrobial protein; CB: chronic bronchitis; CCL18: C-C Motif Chemokine Ligand 18; CF: cystic fibrosis; COPD: chronic obstructive pulmonary disease; HC: healthy control; HMBG1: high-mobility group box 1; IFN-γ: interferon gamma; IL: interleukin; med: median; mL: millilitres; MMP-9: MMP 9 protein; MMP-9a: MMP9 activity; MPO: myeloperoxidase; n: number; NA: not applicable; NE: neutrophil elastase; neut: neutrophil; ng: nanogram/s; NR: not reported; NS: not significant; p: p value; Pa: pseudomonas aeruginosa; pg: picogram/s; PAI1: plasminogen activator inhibitor 1; prod: producers; SLPI: secretory leukocyte protease inhibitor; sput: sputum; TIMP1: TIMP metallopeptidase inhibitor 1; TGFβ1: transforming growth factor beta 1; TNF-α : tumor necrosis factor α; uPA: urokinase-type plasminogen activator; uPAR: urokinase-type plasminogen receptor; YKL-40: chitinase 3-like 1 protein; yrs: years. *data represented graphically

colony stimulating factor (GM-CSF), eosinophilic cationic protein (ECP) and eosinophil peroxidase (EPX) were unable to discriminate between these two groups (**Table S3**). IL-1β, IL-6, and IL-8 demonstrated variable discrimination between pwCF, versus patients with bronchiectasis [24,25,30] and chronic obstructive pulmonary disease (COPD) [21,22,31]. NE and trace metals were significantly increased in pwCF compared to other respiratory diseases [32,33], but were investigated only in single studies [33].

3.2. Concurrent validity with ppFEV1 and other clincial outcomes

Assessment of concurrent validity with ppFEV$_1$ showed conflicting results across individual biomarkers (Table 2). Total cell and neutrophil count were consistently related to ppFEV$_1$, in five out of six studies [34–38] (**Supplemental Table 5: Table S5**). For IL-8, equal numbers of studies demonstrated both significant negative [22,34,35,40,57,93] and no correlation with ppFEV$_1$ [36,38,39,42-44]. Similarly, for NE, correlation with ppFEV$_1$ was inconsistent, with studies demonstrating no significant correlation [28,34,38,39,43,44] outnumbering those demonstrating significant negative correlation [35,36,41,45]. Importantly however, in two paediatric cohorts, increased levels of NE at baseline were associated with accelerated ppFEV$_1$ decline over the following 3-year period [36]. Moreover, in a larger multicentre study, free elastase was associated with ppFEV$_1$ decline, demonstrating a -2.9% decline (95% CI: –5.0, –0.9) per 1-log increase in elastase [140].

Other cytokines, including IL-1β, IL-6 and growth factors did not demonstrate consistent correlation with ppFEV$_1$ (**Table S5**).

Concurrent validity was inconsistent for a range of clinical outcomes, including survival, bacterial or pulmonary exacerbation (PEx) status, symptoms and radiological appearances (**Table S5**). IL-8 correlated significantly with age [46], chest x-ray (CXR) findings [42] and time to next PEx [43], but not with mutation or survival [46,48] (Table 2). NE was significantly associated with corticosteroid use [43], microbiota diversity and evenness [47], but not with other outcomes including survival [45,48]. *P. aeruginosa* (Pa) load was associated with increased NE, *S. aureus* (SA) load with increased IL-1β and vascular endothelial growth factor (VEGF) [47] (Table 2). In a longitudinal cohort, IL-6 and HMGB-1 were the only other sputum biomarkers to demonstrate significant correlation with survival and PEx [36,48-50] (**Table S5**).

3.3. Convergent validity between sputum biomarkers and with serum biomarkers and clinical measures

Convergent validity between sputum biomarkers was evident across multiple studies and biomarkers (**Supplemental table 6: Table S6**). IL-8 was the most extensively investigated, with twenty studies (Table 3). IL-8 significantly correlated with NE [23,26,36,38,46,51,53] (Table 3), TNF-α [22,54] and IL-1β [26,49,52] (**Table S6**). Similarly, NE showed significant correlation with TNF-α levels [51–53], and with IL-1β [36,52,53] (Table 3). Sputum matrix metalloproteinase-9 (MMP-9a) and MMP-9b showed partial correlation with serum MMP-9a [56] and a negative relationship with SLPI (27).

Convergent validity between blood and sputum inflammatory biomarkers was less well studied. C-reactive protein (CRP) and IL-8 correlated in one study, when measured at the end of PEx treatment [57] but not at the initiation of PEx treatment [38]. No correlation could be demonstrated between sputum and plasma levels of IL-1α [58].

3.4. Responsiveness by therapy

Multiple studies have assessed the responsiveness of sputum biomarkers to investigational medicinal products and clinical interventions including antibiotic (oral, IV and inhaled), anti-inflammatory, cystic fibrosis transmembrane conductance regulator (CFTR) modulator and airway clearance therapies. The majority of studies investigated IL-8, NE, TNF-α and IL-1β. Multiple other biomarkers have been explored in a smaller number of studies such as HMGB-1, IFN-γ, SLPI, TIMP-1, and several cathepsins.

Antibiotic Therapies

Evidence of the responsiveness of IL-8 to antibiotic therapy (oral/IV) is conflicting, with seven studies showing a significant reduction [53,59-64], and five no significant change [36,38,62,65,66]. One of the largest studies was carried out in 55 patients treated with IV antibiotics, demonstrating a decline in NE and IL-8 by 0.4 and 0.5 log respectively, in parallel to a 2 to 4 log reduction in lower airway bacterial density [63].

One study demonstrated a significant increase in IL-8 in response to inhaled tobramycin [67] (Table 4). NE response was slightly more consistent, with nine studies showing significant reductions after antibiotics [39,43,59,60,63,65,66,68,134], one a significant increase [36] and three no significant change [27,38,64] (Table 5). Across the five studies assessing responsiveness of TNF-α to oral, IV and inhaled antibiotic therapy, four showed a decrease after treatment [31,42,62,67], one an increase [36] and one no significant change (38). A similar pattern of discordant responses across studies was observed for IL-1β, [31,36,38,57] and IL-6 [36,66] (**Supplemental Table 7: Table S7**). IL-4, calprotectin and MMP-9 showed a significant reduction in response to antibiotic therapy [27,33,38,57,62], club cell secretory protein (CCSP) and tissue inhibitor of metalloproteineases-1 (TIMP-1) a significant increase [38,52], whilst myeloperoxidase (MPO) demonstrated no significant response [33,38] **(Table S7)**.

Anti-inflammatory therapies

Evidence regarding sputum biomarker response to anti-inflammatory therapies is limited, hampered by short study durations and variation, within and between individuals [98]. Of the seven biomarkers assessed, only IL-6 showed a significant reduction in response to oral Ibuprofen [98] (**Table S7**). IL-8 did not show significant responsiveness to inhaled corticosteroids (ICS) [70] (**Table 4**). Recombinant alpha-1-antitrypsin (rAAT) resulted in a significant reduction in IL-8, NE, IL-1β and TNF-α [53,71] (**Table 4; Table S7**). IL-8, but not NE, demonstrated a significant reduction in response to the inflammatory resolution promoter, Lenabasum [72]. NE did not decrease significantly in response to a neutrophil elastase inhibitor (AZD9668) [73].

CFTR Modulator Therapies

Studies of the impact of CFTR modulation on inflammation are mainly pre-clinical, with some exploration in patient cohorts (74,75,135). Variability in response is evident. One study demonstrated no significant change in sputum biomarkers, including IL-8 and NE, after 60 days Ivacaftor [135], also confirmed in a recent study where no changes were observed [10]. Two studies demonstrated reduced IL-8 after short (day 0-14) [76] and long term (600 days) therapy with Ivacaftor [77]. IL-1β and calprotectin reduced significantly after 7 days of Ivacaftor [77]. Further studies to explore the potential anti-inflammatory aspects of CFTR modulation are needed, as current studies pre-date the introduction of triple CFTR modulator combinations.

Airway Clearance Therapies

One study showed a significant reduction in IL-8 in response to inhaled heparin [78], but this has not been reproduced in other

Table 2
IL-8 and NE concurrent validity for $ppFEV_1$ and other clinical parameters (e.g. infection status, age, disease severity etc.):

Popn	Author [Ref.]	Subjects (n)	Sample type: S/IS	Exacerbation status		Biomarker correlation to $ppFEV_1$	Biomarker correlation to other clinical parameters
				Stable	PEx		
NE							
Paediatric	[39]	17	S	NA	17	NS: NE activity with $ppFEV_1$ changes (r^2=0.2185; p=0.08)	NR
	[36]	35 "main cohort"	IS	45	0	Negative NE change from baseline (r=-1.12; p<0.04) Negative NE change and annual rate of change in $ppFEV_1$ (r=-0.55; p<0.01)	Number of ABx courses over the study period NS
	[46]	20	IS	30	16	NR	Age, mutation severity, *Pa* infection, PEx, azithromycin, inh ABx, Dornase alfa, continuous oral ABx: NS inhaled steroids: data NR
Adult	[47]	16	S/IS	16	0	NR	Shannon diversity index and evenness: negative for both comparisons p=0.06 NE levels: with Inc. biodiversity p= 0.01
	[48]	57 (n=40)	S	57	0	NR	NE not assoc. with survival p=0.186
Mixed	[41]	73 (baseline) 37 (longitudinal)	IS	73	0	Negative NE**: r=-0.42 [-0.69; 0.06]	NR
	[27]	17	S	18	1	Positive NE in LAW: with $ppFEV_1$ (p=0.033, r=0.593)	NR
	[28]	33	S	0	33	NS NE activity	NR
	[34]	269 (4 studies)	IS	0	0	NS Free elastase	NR
	[35]	20 CF	S/IS	20	0	Negative: NE (r=-0.75, p=0.001)	Baseline SaO_2 NS
	[43]	10	S	10	0	NS: NE complex	NE complex with steroid use p<0.001
	[44]	12	S	12	0	NS	NR
	[45]	28	S	28	0	Negative Active elastase (r= -0.57, p<0.01)	Disease severity NS Positive with levels of pulmonary disease p<0.01
IL-8							
Paediatric	[39]	17	S	0	17	NS	FEF NS
	[36]	35 "main cohort" 10 stable	IS	45	0	NS IL-8**	ABx courses over the study period NS
	[46]	20	IS	30	16	NR	Positive with Age p<0.05 Mutation severity, infection with Pa, PEx, azithromycin, inh ABx, dornase alfa, continuous oral ABx NS
Adult	[48]	57 (sputa n=40)	S	57	0	NR	Not assoc. with survival p=0.348
Mixed	[41]	73 (baseline) 37 (longitudinal)	IS	73	0	Baseline r=-0.39; 95%IC[-0.57;-0.17] IL8**: r=-0.22; 95%IC[-0.55; 0.17]	NR
	[21]	24	IS	24	0	NR	NR
	[57]	24	S	0	24	Negative during Rx (r=-0.4578; p=0.0245)	NR
	[93]	19	IS	19	0	Negative (r=-0.64; p=0.001)	NR
	[34]	269 (4 studies)	IS	n/a	n/a	Negative (r=-0.28; p NR)	NR
	[40]	16	S	16	0	Negative (r=-0.4; p=0.003)	Negative with sputum cough transportability p=0.02
	[22]	28	IS	28	0	Negative (r=-0.39; p=0.056)	NR
	[35]	20 CF	S /IS	20	0	Negative (r=-0.72; p=0.002)	Baseline SaO_2 NS
	[42]	58	S	48	10	NS	Severity of pathological findings on x-ray p=0.008 Elevated levels correlated with symptoms of deterioration
	[43]	10	S	21	35	NS	Time to next PEx, with steroid use p=0.0048
	[44]	12	S	12	0	NS	NR
	[54]	40	S	22	18	NR	Brasfield or Shwachman-Kulczycki clinical scores NS
	[26]	33	S /IS	NA	NA	NR	Sig Inc assoc. with Chronic Pa colonization (data NR)

ABx: antibiotic/s; assoc.: associated; bet: between; CF: cystic fibrosis; corr: correlated; FEF: forced expiratory flow; inc: increase/d; inh: inhaled; IS: induced sputum; IV: intravenous; LAW: lower airways; n: number; n/a: not applicable, NR: not reported;, NA: not assessed; NE: neutrophil elastase; NEAPC: neutrophil elastase-α_1-antiproteinase complex; NS: not significant; NR: not reported; Pa: pseudomonas aeruginosa; PEx: pulmonary exacerbation; $ppFEV_1$: percent predicted forced expiratory volume in 1 second; Pa: pseudomonas aeruginosa; PEx: pulmonary exacerbation; Popn: population; Rx: treatment; S: sputum; SaO_2: oxygen saturation; sig: significant; sput: sputum
**changes of the biomarkers assessed

Table 3
Convergent validity for IL-8 and NE with other sputum biomarkers (only significant correlation shown)

Popn	Author [Ref.]	Subjects (n)	Sample type: S/IS	Correlation with other biomarkers/parameters in sputum
IL-8				
Paediatric	[36]	35 "main cohort" 10 stable	IS	NE (r=0.73; p NR) **
	[46]	20	IS	NE comparing all specimens (r=0.85; p<0.001, n=31); one specimen per subject (r=0.79; p<0.001, n=22)
Adult	[51]	36	S	NE (r=0.547; p=0.001)
	[33]	23 CF; 14 (CF PEx) 14 CF (recovery)	IS	Zinc levels (r=0.067; p<0.001) Sputum iron (NR)
	[52]	45	S	MMP-9, IL-6, TNF-α, IL-1β, NE (NR)
	[56]	40	S/IS	Total protein count in IS (r=0.76; p<0.001)
	[99]	40	IS	CCL18 (r^2=0.47; p<0.05)
	[30]	45	S/IS	Mg (r=0.42; p<0.01), Fe (r=0.39; p<0.01) and Zn concentrations (r=0.38; p<0.01)
	[21]	24	IS	TGFβ1 (r=0.549; p<0.0007); TGFβ1 (r=0.593; p<0.007) in infected patients
	[49]	49	S	IL-1β (r=0.56; p<0.0001); MPO (r=0.73; p<0.0001); TGFβ1 (r=0.47; p<0.0001); TCC (r=0.47; p<0.0001)
	[59]	19	S	Desmosine levels (r=0.86; p<0.01) Desmosine (r=0.64; p=0.02)**
	[53]	52	IS	NE (r=0.48; p<0.01)**
	[34]	269 (4 studies)	IS	Free elastase (r=0.72; p NR)
	[22]	28	IS	uPAR (r=0.47; p=0.0001) and uPA (r=0.34; p=0.0001); TNF-α (r=0.31; p=0.0001); IL-10 (r NR; p<0.0001); CAP-18 (r=0.16; p=0.04)
	[105]	16	S	MPO (r=0.45; p=0.01); DNA (r=0.45; p=0.01)
	[23]	20	S/IS	Neutrophil counts IS (r=0.80; p<0.001)
	[42]	58	S	TNF-α (in 9 CF patients with PEx) (data NR)
	[54]	40	S	TNF-α (r=0.878; p<0.0005)
	[26]	33	S/IS	IL-1α (NR); IL-1β (NR); NE activity (p<0.02)
Mixed	[38]	44	S	MMP-9 (r=0.424, p<0.01), calprotectine (r=0.539, p<0.01), NE activity (r=0.752, p<0.0001), MPO (r=0.582, p<0.01)
Neutrophil Elastase (NE)				
Paediatric	[36]	35 "main cohort" 10 stable	IS	Total neutrophil counts (r=0.54; p NR) **; Neutrophils % (r=0.46; p NR) **; IL-8 (r=0.73; p NR) **; IL-1β (r=0.63; p NR) **; MMP-9 (r=0.50; p NR)**; NEAPC (r=-0.53; p NR)**; SLPI (r=-0.58; p NR)**
	[46]	20	IS	IL-8 comparing all specimens (r=0.85; p<0.001; n=31); one specimen per subject (r=0.79; p<0.001; n=22). Neutrophil % (r=0.35; p<0.05) for all specimens but not for one specimen per subject
Adult	[51]	36	S	LL-8 (r=0.547; p=0.001); TNF-α (r=0.515; p=0.001); Cathepsin B (r=0.508; p=0.002); Cathepsin S (r=0.592; p<0.0001)
Mixed	[41]	73 (baseline) 37 (longitudinal)	IS	MPO (r=0.88, p NR)
	[106]	34	S	C5a (β= 1.093; 95% CI [1.051, 1.136]; p<0.001); C3a (β= 0.998; 95% CI [0.984, 0.991]; p<0.001)
	[52]	45	S	CCSP conc (r NR; p=0.0373) ; MMP-9, IL-6, TNF-α, IL-1β, NE (NR)
	[32]	41	IS	Bile Acid in patients with CF with detectable Bile Acid in sputum (r=0.60; p=0.002)
	[59]	19	S	Desmosine levels (r=0.78; p<0.01)
	[28]	33	S	MMP-9 (in-patient samples r^2 =0.74; p NR)
	[53]	52	IS	IL-8 (r=0.48; p<0.01) **; IL-1β (r=0.39; p<0.05) **; TNF-α (r=0.4; p<0.05) **; LTB4 (r=0.45; p<0.05) **; Neutrophils (r=0.52; p<0.01) **; % 54-kDa IgG before and after AAT treatment (r=-0.49, p<0.05) and after 4 weeks of AAT inh (r=-0.43; p<0.05)
	[34]	269 (4 studies)	IS	IL-8 (r=0.72; p NR)
	[23]	20	S/IS	Neutrophil counts in IS (r=0.84; p<0.001)
	[26]	33	S/IS	IL-8 (r NR p<0.02)
	[45]	28	S	Active elastase levels (r=0.49; p<0.05); α1PI levels (r=0.68; p<0.005); α1PI BE (r=0.68; p<0.05)
	[38]	44	S	Calprotectin (r=0.611, p<0.0001), IL-8 (r=0.752, p<0.01), MPO (r=0.646, p<0.0001)

AAT: alpha1 antitrypsin; BE: bronchiectasis; bet: between;; CAP-18: cationic antimicrobial protein 18; CCL18: C-C Motif Chemokine Ligand 18; CCSP: clubcell secretory protein; CF: cystic fibrosis; CI: confidence interval; corr: correlated; conc: concentration; Fe: iron; FEF: forced expiratory flow; IgG: immunoglobulin G; inhl: inhaled; IL: interleukin; inh: inhaled/inhalation; IS: induced sputum; IV: intravenous; LAW: lower airways; LTB4: leukotriene B4; Mg: magnesium; MMP-9: matrix metalloproteinase 9; MPO: myeloperoxidase; n/a: not applicable, NR: not reported, NA: not assessed; NE: neutrophil elastase; NEAPC: neutrophil elastase-α_1-antiproteinase complex; NR: not reported; p: p value; Pa: pseudomonas aeruginosa; $ppFEV_1$: percent predicted forced expiratory volume in 1 second; PEx: pulmonary exacerbation; Popn: population; Rx: treatment; S: sputum; SaO_2: oxygen saturation; sig: significant; sput: sputum; TCC: total cell count; TGFβ1: transforming growth factor beta 1; TNF-α : tumor necrosis factor; uPA/R: urokinase-type plasminogen activator and receptor; Zn: zinc; α1PI: alpha 1 proteinase inhibitor.
**changes of the biomarkers assessed

Table 4
Responsiveness to therapy for IL-8.

Rx	Author [Ref.]	n	Age (yrs) Mean (Range)	Intervention	Baseline Biomarker	Post Treatment biomarker	Response p value	ppFEV$_1$ p value	Did other clinical endpoints detect response?
Antibiotic Therapy									
	[64]	36	23.9 (10.1-49.2)	IV ABx	177.0 (42.7-1193.5) ng/mL	-140.9 (-1106.7-158) -30.1 (-826.9-421.45)	Dec p<0.02	Inc p=0.04	Sputum density NS
	[36]	35 CF 10 stable CF	6-15	IV ABx	4.7 (4.6-4.8) log pg/mL	4.7 (4.6 to 4.9)	NS	Dec p<0.01	–
	[59]	19	19.8 (6.7-36.8)	IV ABx	NR	NR	Dec p<0.01	Inc p<0.01	CRP p<0.01
	[60]	18	59 (32-101)	IV ABx	12.7 ng/mL	8.5	Dec p=0.01	NR	Pa p<0.001 SA (p NR) ppFVC (10%) (p NR)
	[61]	32	18.6 (11.4-35.7)	IV ABx	Start of PEx 7165 pg/mL	5415	Dec p<0.01	Inc p<0.001	ppFVC p<0.0001 WBC p=0.001 CRP p=0.001
	[67]	155	15 (13.5-18.8) 23.1 (21.1-25.9)	Inh ABx	1087 ng/ml	I697	Inc p=0.001	NA	Pa p=0.01
	[62]	27	12±0.6 Mean (SD)	ABx	*	2.7 pg/mg 3 mo 5.94 6 mo 2.3 12 mo	Dec p=0.03 NS Dec p=0.03	Inc p=0.03	FVC p=0.03
	[63]	55	18.2	IV ABx	4.8±0.8 log^{10} pg/mL	4.3±1.5	Dec p<0.05	Inc p<0.0001	Pa density p<0.0005 SA density p<0.0001 WBC p<0.0001
	[42]	10 CF PEx	29.5 (8-33)	IV ABx	166-2403 pg/L	59-2403	NS p=0.054	Inc p NR	Pulse rate p=0.045 Weight p=0.021 ESR (p NR) FVC (p NR)
	[65]	17	12	ABx	7.3 ± 0.6 ng/mL (Chronic Pa) 3.7±0.7 (No Pa) 6.7 ±1.8 (Int Pa)	5.13±0.9 3.8±0.8 8.5± 2.2	NS NS p=0.55	NA	–
	[53]	52	≥8	Inh AAT	1765±867 ng/mL Peripheral dep 1682 ± 988 Bronchial dep	-723 ± 469 -638 ± 336	Dec p<0.05 at 2/52 Dec p<0.001 at 4/52	NS	Pa p<0.05 at 4/52
	[70]	22 CF 22 placebo	6-17	ICS	376 pg/mL	Post Rx: 8 Post placebo: -67	Dec p=0.03 (in favour of placebo)	NS	MEF$_{25\%}$ p<0.009 in favour of placebo
	[38]	44 CF	23(18-28)	IV ABx	13.8(9.2) ng/ml	15.4(13.0)	NS	p<0.001	LCI p<0.01 CT scores p<0.01 Symptoms p<0.001 Air trapping p<0.01 Mucus plugs p=0.001
	[66]	87 CF 98 placebo	20.2(7.9) 20.6(8.6)	Oral ABx	5.2(0.5) log pg/ml	Mean diff post Rx -0.1	NS	Inc p=0.009	Risk of PEx p=0.03 Weight p=0.02

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Table 4
(continued)

Rx	Author [Ref.]	n	Age (yrs) Mean (Range)	Intervention	Baseline Biomarker	Post Treatment biomarker	Response p value	ppFEV$_1$ p value	Did other clinical endpoints detect response?
CFTR Modulator Therapy									
	[77]	12	29.5 (22-57) Median (range)	Ivacaftor	*	*	Dec p<0.05 Day 400 Dec p<0.005 Day 600	Inc p=0.07 Day 7 Inc p=0.09 Day 400 Inc p<0.001 2 yrs	Pa p=0.006 Yr1 Sweat Cl^- p<0.0001 day 7
	[107]	27	25.8±6.0	Sildenafil	NR	-4.1 × 10(4) μg/mL (trend towards improvement mean change)	NS	Dec p=0.04	Pa count NS CFQR Resp domain NS
	[76]	20 17 placebo	>12	aerosolized tgAAVCF	*	*	Dec p=0.03 Day 14	Inc p=0.04 day 30	HRCT NS
	[135]	153	21.1(11.3)	Ivacaftor	*	*	NS	Inc p<0.001 6 mo	FVC p<-.001 BMI p<0.001 6 mo Sweat Cl^- p<0.001 6 mo Hospitalization rate p<0.001 Pa burden p<0.01 Mucociliary clearance p<0.001 GI pH p=0.001
Airway Clearance Therapy									
	[46]	20 CF 8 non-CF resp	3 Median 4.5	inh albuterol	NA	834(81-6920) pg/ml CF 1809 (150-48550) Non-CF	NS	NR	IL-8 with age p<0.05
	[78]	10	21-34	heparin	*	NR	Dec p<0.002	NS	Sputum expectoration p<0.04 Sputum thinner p=0.07
	[80]	14	17-29	rhDNase	121.0 (13.2) ng/mL	104.9 (13.3) 12 wks 111.2 (17.5) 52 wks	NS	NS	–
	[79]	19 59	27.5 (16-55)	rhDNase	140.9(85.6-200.3) ng•mL^{-1}	127.4 (75.6-217.7) 6 mo	NS	NA	–
	[138]	48	5-18	HS/rhDNase	102.4 ng/g	Expressed a geometric mean ratio	NS HS/daily rhDNase Inc free IL8 alternate day rhDNase p=0.03	NS	–

AAT: alpha1 antitrypsin; Abx: antibiotic/s; ACTs: airway clearance therapies; CF: cystic fibrosis; CFQR: cystic fibrosis questionnaire revised; CFTR: cystic fibrosis transmembrane conductance regulator; Cl^-: chloride; CRP: C reactive protein; Dec: decrease; dep: deposition; ESR: erythrocyte sedimentation rate; FVC: forced vital capacity; HRCT: high-resolution computed tomography; HS: hypertonic saline; IL: interleukin; Inc: increase; inh: inhaled; ICS: inhaled corticosteroids; Int: intermittent; IV: intravenous; MEF: maximal expiratory flow; mL: millilitres; mo: months; Mods: modulators; mL: millilitres; MPO: myeloperoxidase; n: number; NA: not applicable; ng: nanogram/s; NR: not reported; NS: not significant; p: p value; Pa: pseudomonas aeruginosa; PEx: pulmonary exacerbation; pg: picogram/s; ppFEV$_1$: % predicted forced expiratory volume in 1 second; Ref.: reference; rhDNase: recombinant human deoxyribonuclease; SA: staphylococcus aureus; ug: micrograms; WBC: white blood cell count; wk/s: week/s; yrs: years.
***data represented graphically**

Table 5
Responsiveness to therapy for Neutrophil Elastase (NE): (data only shown for NE)

[Ref.]	n	Age (yrs) Mean (Range)	Intervention	Baseline Biomarker	Post Treatment Biomarker	Response p value	ppFEV$_1$ p value	Did other clinical endpoints detect response?
Antibiotic Therapy								
[39]	17 CF 11 PCD	12.0 (6.9-16.7) 11.0 (6.8-15.3)	Oral ABx No placebo	46.3 nmol.min^{-1} (4.5-224.5) 91.4 nmol.min^{-1} (11.8-166.4)	*	Dec PCD p<0.05* Dec CF/PCD p=0.07 Dec CF p=0.08*	NS	Dec bacterial counts in CF not PCD
[27]	17 CF 20 HC	25.1	IV ABx No placebo	328.8 (2.3-2366.5) ng/mL	697.4 (2.3-17987.2)	NS	NR	Dec TCC p=0.005
[64]	36	23.9 (10.1-49.2)	IV ABx No placebo	177.0 (42.7; 1193.5) µg/mL	201.2 (42.7-1193.5)	NS	Inc p = 0.04	Dec IL-8 p<0.02 Sputum density dec p=0.06
[36]	35 CF 10 stable CF	6-15	IV ABx No placebo	Total neut 7.6(7.4-7.9) log mg/mL % Neut 63 (57 to 70) Elastase 1.2(1.0-1.4) NEAPC 2.4 (2.2-2.5)	7.4 (7.2-7.7) 64 (57 to 71) 1.6(1.4-1.8) 2.1 (1.9-2.2)	+0.5 (0.2-0.8) linear trend reported	Inc p<0.01	–
[134]	27 10 placebo	17-64	IV ABx Placebo	98.8 (97.2-99.6) % Neut	97.5(95.6-98.7) % Neut	Dec Sputum neutrophils p=0.04	Inc p=0.001	Dec CRP p=0.002 Dec VEGF p=0.013 Dec WCC p=0.004
[59]	19	19.8 (6.7-36.8)	IV ABx No placebo	NR	NR	Dec p<0.01 Dec p=0.05 3-8days	Inc p<0.01	Dec Plasma CRP p<0.01
[60]	18	59 (32-101)	IV ABx No placebo	90% median count	68% median count	Dec p=0.03	Inc 6% median	Inc ppFVC 10% median
[63]	55	18.2	IV ABx No placebo	Neutrophil count 6.9±0.4 Neutrophil% 70.4% ±19.7 Free Elastase 1.9±0.7 log^{10}µg/mL	Neutrophil count 6.6±0.6 Neutrophil% 61.7% ±24.4 Free Elastase 1.5±0.9	Dec p<0.0005 Dec p<0.0001	Inc p<0.0001	Dec density Pa p<0.0005 Dec density SA p<0.0001 Dec WCC p<0.0001
[65]	17	12	ABx No placebo	10.7±1.5 µ/mg (no Pa) 8.1±1.7 µ/mg (int Pa) 34.7±5.5 µ/mg (Chronic Pa)	2.2±0.6 µ/mg 4.7±1.2 µ/mg NR	Dec p<0.03 Dec p<0.02–	NA	–
[43]	10 CF 2 HC	15-44	ABx (oral, IV and neb) No placebo	106.4+12.8ug/l	*	Dec p=0.078	Inc p=0.055	Dec sputum protein p=0.001 Dec a1PI p=0.002
[68]	42 CF 30 HC	0.75-28	ABx No placebo	Neutrophil conc 7020 (1895.7) cells/µL	Neutrophil conc 4420 (1978.2) cells/µL	Dec p<0.01	NR	Inc serum levels MPO p<0.0001 CF NS ECP
[38]	44 CF	23(18-28)	IV ABx No placebo	595(384) U/L	698(574)	NS	p<0.001	LCI p<0.01 CT scores p<0.01 Symptoms p<0.001 Air trapping p<0.01 Mucus plugs p=0.001
[66]	87 CF 98 placebo	20.2(7.9) 20.6(8.6)	Oral ABx Placebo	1.9(0.4) log pg/ml	0(0.5) log µg/ml	Dec p=0.01	Inc p=0.009	Dec risk of PEx p=0.03 Weight inc p=0.02
Anti-inflammatory Therapy								
[73]	55 27 Rx Grp 29 placebo	>16	AZD9668	Rx 154.7 μmol/L^{-1} Placebo 55.72 μmol/L^{-1}	Rx 148.4 μmol/L^{-1} Placebo 106.7 μmol/L^{-1}	NS	NS	Dec Free urine des p<0.01 Dec Total urine des p<0.05 NS Plasma des
[53]	52	≥8	Inh AAT No placebo	Peripheral deposition Free elastase 32.7±31.7 µg/mL^{-1} % neutrophils 39.5±23.4 Bronchial deposition: Free elastase 24.9±20.1 µg/mL^{-1} % neutrophils 41.8±24.1	Peripheral dep (4 wk) Free elastase -7.4±30.0 % neutrophils -8.2±20.9 Bronchial dep (4 wks) Free elastase 6.1±31.3 % neutrophils -20.8±30.3	Dec p<0.05 IS Dec p<0.05 IS	NS	Dec Pa p<0.05 at 4 wks

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Table 5
(continued)

[Ref.]	n	Age (yrs) Mean (Range)	Intervention	Baseline Biomarker	Post Treatment Biomarker	Response p value	ppFEV$_1$ p value	Did other clinical endpoints detect response?
[71]	39 29 Rx Grp 10 placebo	27.5 (16.6-69.2)	Inh rAAT Placebo	500mg 0.72±1.88 (0.004-6.05) 250mg 2.81±4.94 (0.005-16.16) 125mg 0.83±1.66 (0.004-5.15)	*	Dec NE 125mg vs placebo p<0.05	NS	NS Microbiology
Airway Clearance Therapy								
[80]	14	17-29	rhDNase No placebo	Elastase activity: 0.686 (0.131) mU Mean (SE) Total elastase: 41.7(7.1) µg/mL Mean (SE)	12 wks: Elastase activity: 0.435(0.065) mU Total elastase: 39 (5.7) µg/mL 52 wks: Elastase activity: 0.366(0.089) mU Total elastase: 31.5 (SE 8.2)	Dec p<0.05 at 12 wks Dec p<0.005 plasma elastase at 52 wks	NS	–
[44]	12	24±10	rhDNase No placebo	Day 0: Elastase 161±126 U	Day 15: 280±212 Last day: 248±243 Last day+7: 61±72	Inc p<0.05	NS	Dec HLE/cathepsin G within a week stopping Rx after 4-6 mo Rx p<0.05 Inc HLE/HCG after 15 days Rx p<0.05
[81]	18 CF 9 HC	≥10	N-acetylcysteine No placebo	Neutrophil count Elastase activity CD63 neutrophils	Expressed as % change	Dec p<0.003 Dec p<0.006 Dec p<0.005	NS	Inc Blood neutrophil GSH p<0.025 Whole blood GSH p<0.031
[138]	48	5-18	HS/rhDNase No placebo	19.8 units/ml	Expressed a geometric mean ratio	NS	p=0.03	No correlation between ppFEV1 abd changes in total IL8, free IL8, MPO or ECP
CFTR Modulator Therapy								
[77]	12	median 29.5 (22-57)	Ivacaftor No placebo	*	*	Dec p<0.00003 wk 1	Dec day 7 p=0.07 Dec day 400 p=0.09 Rate of change 2 vs 2 yrs preceding Rx p<0.001	Dec Pa conc during 1st yr Rx p=0.006 rebounded yr 2
[107]	27	25.8±6.0	Sildenafil No placebo	NR	-57μg/mL (reported as mean change)	Dec p=0.03	ppFEV$_1$ -2.4% pred mean change pre/post Rx p=0.04	No change Pa count or CFQR Resp domain
[135]	153	21.1(11.3)	Ivacaftor No placebo	*	*	NS	Inc p<0.001 6 mo	FVC inc p<-.001 BMI inc p<0.001 6 mo Sweat Cl^- dec p<0.001 6 mo Dec hospitalization p<0.001 Dec Pa burden p<0.01 Inc mucociliary clearance p<0.001 GI pH inc p=0.001
Other therapy								
[109]	85	>18	SB-656933 Placebo	NR	Expressed as % change	Dec 26% vs baseline Dec 23% vs placebo	NS	Inc serum fibrinogen 11% vs placebo Inc CRP 78% vs placebo

AAT: alpha1 antitrypsin; ABx: antibiotic/s; BA: bronchial asthma; Bac Pneu: bacterial pneumonia; CB: chronic bronchitis;; CF: cystic fibrosis; CFTR: cystic fibrosis transmembrane conductance regulator; CFQR: cystic fibrosis questionnaire revised; COPD: chronic obstructive pulmonary disease; CRP: C reactive protein; dec: decrease; dep: deposition; des: desmosine; ECP: eosinophil cationic protein; HC: healthy control; GSH: glutathione; HCG: human leukocyte cathepsin; HLE: human leukocyte elastase; HMBG1: high-mobility group box 1; HS: hypertonic saline solution; IFN-γ: interferon gamma; IL: interleukin; inc: increase; inpt: in-patient; IS: induced sputum; ml: millilitres; MMP9: MMP 9 protein; MMP9a: MMP9 activity; mo: months; MPO: myeloperoxidase; n: number; NA: not applicable; NE: neutrophil elastase; neb: nebulized antiobiotics; ng: nanogram/s; NR: not reported; NS: not significant; p: p value; ppFEV$_1$: % predicted forced expiratory volume in 1 second; Pa: pseudomonas aeruginosa; PCD: primary ciliary dyskinesia; pre: preceding; rAAT recombinant alpha-1-antitrypsin; RANTES: regulated on secretion normal T-cell expressed and secreted; Resp: respiratory; rhDNase: recombinant human deoxyribonuclease Rx; treatment; SA: staphylococcus aureus; SE: standard error; TCC: total cell count; TGFβ1: transforming growth factor beta 1; TNF-α : tumor necrosis factor α; U: units; µg: micrograms; VEGF: vascular endothelial growth factor; vs: versus; wk/s: week/s; yr/s: year/s.
*data represented graphically

studies using rhDNAse [79,80], hypertonic saline (HTS) [138] or bronchodilators [46] (**Table 4**). Responsiveness of NE to airway clearance therapies was inconsistent, with two studies showing a significant decrease in response to recombinant human dornase alpha (rhDNase) and N-acetylcysteine [80,81] one a significant increase in response to rhDNase [44], and one no change [138] (Table 5). Hypertonic saline may reduce IL-8 after a single inhalation [139] but does not reduce IL-8 or other biomarkers after 12 weeks therapy [138].

3.5. Sputum sampling and processing

Sputum biomarker levels are affected by sampling and laboratory methodology [83,139]. Samples are obtained through spontaneous expectoration (ES: expectorated sputum) or induction (IS: induced sputum) via inhalation of HTS. Four studies compared inflammatory biomarkers in ES and IS from the same patients, all showing no significant difference in biomarker levels [15,23,47,82] (**Table S4**). Among those, one applied a correction factor for dilution by HTS, concluding similarity between sampling processes [15].

To measure biomarkers in sputum, samples must first undergo solubilisation, using mucolytic agents (such as dornase alpha), reducing agents [dithiothreitol (DTT) and dithioerythritol (DTE)] or mechanical dissociation. The use of reducing agents may impact biomarker levels [83]. Myeloperoxidase (MPO), TNF-α, leukotriene B4 (LTB4), NE, IL-6, IL-8 and secretory leukocyte peptidase inhibitor (SLPI) levels were significantly altered by use of stabilising/reducing agents in processing [83].

Sputum samples are frequently frozen, then thawed to facilitate batch analysis in clinical trials. Two studies addressed this question, one showing significant variation in HMGB-1 levels between fresh and freeze-thaw samples [55], whilst the other demonstrated no significant differences in IL-1β, NE and MPO measurements. Nevertheless, NE levels trended higher in immediately processed samples [41]. Prompt low temperature processing (completion within one hour at +4°C), increases accuracy of sputum biomarker analysis, demonstrated by close NE correlation across sputum and BAL samples [84–86].

Collection, storage and processing of sputum is highly inconsistent across studies, impacting biomarker levels [41,86] and generating wide variability in measured values (**Supplemental Table 8**). For IL-8 alone, over 20 assays have been used, with measured ranges varying by a factor of 1000 (**Supplemental Table 9**).

4. Discussion

For sputum biomarkers to have potential as outcome measures in clinical trials, they must reflect physiological or pathogenic processes, be objective, reproducible and quantifiable [18,87]. In CF, sputum biomarkers hold promise as reflections of airway inflammation, with potential to provide mechanistic, safety and efficacy data, to assess disease progression and response to interventions. This study provides evidence on the clinimetric properties of individual biomarkers, identifying those with current and future promise, across a wide range of potential roles for biomarkers within CF.

Classification of sputum inflammation biomarkers

To determine inflammatory pathways and identify targets for drug development, disease specific biomarkers are required. Multiple sputum biomarkers show effective discrimination, with biomarker levels 5-30 times higher in CF populations, compared to HC and other respiratory diseases [22,24,25,29-33,39,45,92,93], providing mechanistic information and evidence of disease specificity. To identify a target sub-population or predict individuals with higher or lower likelihood of response to an intervention, biomarkers which discriminate across the CF population are required. So far evidence about the discriminative ability of sputum biomarkers between pwCF based on disease severity and bacterial status is limited. IL-8, TNF-α and NE have most evidence so far, but other biomarkers including IL-1β, and YKL-40 are worthy of further study [22-26,28,30-32,39,45,93,94].

To monitor disease status, predict disease trajectory and inform therapeutic drug development, biomarker changes must correlate with changes in clinical condition, or a surrogate of the clinical condition, with evidence provided by studies of concurrent validity and responsiveness. To monitor pharmacological drug response and serve as “go/no go” for clinical development, correlation with clinical outcomes or disease status may not be necessary. Concurrent validity of inflammatory biomarkers is limited by inter and intra-individual variability in the CF population, driven by heterogeneity, disease severity and fluctuating stability, including PEx [18,98]. In sputum, additional variability is generated by fluctuating stability during processing, temporal variation from sampling at differing time points, and spatial variation created by samples originating from different parts of the lung, reflecting heterogeneous lung inflammation [87,89-91]. This may explain lack of consistent concurrent validity with clinical outcomes, including ppFEV$_1$, itself only a surrogate biomarker of disease status. Studies do point to a negative association between NE increase and ppFEV$_1$ decline, mainly in the paediatric population [36, 46]. This is supported by BAL studies in infants demonstrating correlation between elastase and IL-8 at 3 months of age and bronchiectasis prevalence [3]. This provides evidence for potential use, at least in young patients, for NE as a predictive biomarker of future disease evolution. Evidence for other sputum biomarkers, to act as predictive or prognostic biomarkers for disease trajectory, through correlation with infection status, bacterial load, lung imaging, pulmonary exacerbation status or survival, is so far inconsistent, with limited longitudinal data [36,42,43,46,47,48-50].

There is more potential for sputum biomarkers within drug development and monitoring of therapeutic response, with NE, IL-8, IL-1β and TNF-α having the greatest body of evidence around responsiveness [31,33,36,42,43,53-68,71,76-78,80,81,95]. Variability in responsiveness between biomarkers and interventions is evident [12,27,33,36,39,43,59,60,63-65,68], with TNF-α and NE more consistent, IL-8 more variable, in response to antibiotic and inhaled airway clearance therapies [31,36,39,42-44,46,59-68,78-81,134,138]. Despite their importance, studies of biomarker responsiveness to CFTR modulation and anti-inflammatory therapies are limited and pre-date triple CFTR modulation, showing inconsistency between biomarkers, and short versus long term response [53,70-73,77,98].

These differences in responsiveness patterns may assist understanding of the physiological effects of therapeutic interventions, how this varies between individuals, and with time. With a longer lifespan and an increasingly heterogeneous population, biomarkers appropriate to the intervention, study design and/or population are increasingly important. Physiologically relevant biomarkers with clinimetric properties specific to groups of patients should be further characterized to guide clinical trials in these populations. For example, biomarkers representative (or predictive of progression) of early disease in infants or children. As an exemplar, IL-1β responsiveness is greater in adults than children [31,57], perhaps related to its inflammatory mediator role, via NF-kB, which may increase as inflammation progresses [96]. The relative importance of specific biomarkers may vary by intervention, sampling type, age and disease stage, reflecting physiological differences in inflammatory pathways and response. With evidence of conver-

gent validity between biomarkers, for example IL-8 significantly correlates with NE [23,26,36,38,46,51,53], TNF-α [22,54] and IL-1β [26,49,52], a smaller, more select, group of biomarkers may also be a future possiblity.

In future, it is important to discriminate between variability in biomarker response and inconsistent correlation with clinical outcomes driven by genuine differences between individuals, defective ability of the drug explored, inappropriate biomarker selection or actual inadequacy of the biomarker. The rise of highly effective CFTR modulating therapies demonstrate how a targeted, appropriately selected, responsive and reproducible biomarker, sweat chloride, can assist in drug development, provide information on CFTR-related pharmacological action and monitor therapeutic response [143]. Biomarker choice should be targeted to the role required, the question asked, the drug and population being studied. More biomarker data and specific studies, reflecting a changing CF population are required before this can become a reality. For example, in an era of triple CFTR modulation, biomarkers able to provide evidence of clinical response, in the short and longer-term, are needed in a population where current clinical outcomes including ppFEV$_1$ are reducing in sensitivity.

Methodological Challenges

Methods of sputum collection, storage, processing and analysis are of critical importance to the validity of sputum biomarker results. Reducing/stabilising agents [83] and freezing may impact biomarker levels, with variable impact between biomarkers [41]. Further work is needed to fully understand their influence [41]. Accuracy of sputum biomarker measurement [84–86] may be improved by robust, standardised methodology, reliant on prompt processing and effective temperature control [84–86].

The CF population able to spontaneously provide sputum, differs from those who may require sputum induction, for example, paediatric populations, patients with less severe disease, or those on CFTR modulator therapy [14]. With widening access to CFTR modulators, spontaneous sputum production is lessening, increasing this challenge. There is limited evidence on congruity in biomarker levels between ES and IS [63,87,89-91]. Levels could vary, driven by the induction process itself [100–102], sputum processing with reducing agents, differences in disease severity and locational variation in the respiratory tract. Further work is mandatory to explore this, and the mixed-methods approach used in previous studies, where spontaneous or induced sputum were used interchangeably, is not recommended.

To address methodological variability, greater standardisation in collection, storage and laboratory processes is necessary. To confirm reliability and reproducibility, sputum processing steps, from collection onwards, should be clearly reported. This may require creation of collective standard operating procedures (SOPs) or reference laboratory standards, such as those created by the ECFS-CTN for IS sampling and collection [103,104,137].

A greater emphasis on data and methodological sharing is necessary. Studies frequently present insufficient data on ranges or biomarker values with results shown graphically, limiting attempts to collate raw data, and subsequent meta-analyses. To overcome this, standards for publication, as well as methods, and a collaborative approach to methodological and data sharing are required.

Use of sputum inflammatory biomarkers in clinical trials

To determine the suitability of sputum biomarkers for any clinical trial, evidence of their physiological relevance, responsiveness to planned intervention and reproducibility over the intended timeline must be assessed [4,8,10,18,87]. From the included studies, biomarker measurement is frequently a secondary outcome, with, in addition to the specific biomarker of interest, multiple biomarkers measured in an exploratory fashion without a specific hypothesis. This “scatter gun” approach of measuring multiple biomarkers generates large volumes of data, obscuring valid signals and limiting assessment of validity and reliability. This is counter-productive to establishing optimal biomarkers for future clinical trial use. Whilst evidence of therapeutic responsiveness exists, the necessary specificity to determine which biomarker is an optimal measure of response to a specific intervention, in a specific population, is lacking. To advance biomarker research, a more targeted approach in larger studies is required. Whilst NE, TNF-α, IL-8 and IL-1β, could be currently considered for wider use, this may relate to historical and publication bias, with further validation, especially around reliability still necessary. Other biomarkers including IL-6, calprotectin (which demonstrates prolonged stability), HMGB-1 and YKL-40 demonstrate potential utility, but lack of reliable data, variable study design and methodological challenges hamper their current usage. They should be included in new studies to speed translation into wider use.

Future studies

Future studies comparing the stability and trajectory of biomarkers in clearly defined populations are urgently needed. The challenges of sputum expectoration in the era of CFTR modulators also require careful assessment. Large population-based studies reflective of the heterogeneity of the CF population are necessary to establish intervention mechanisms, inter and intra-patient variability, temporal variation, reproducibility and reliability. Targeting of biomarker research is required, with study design based on physiological relevance and specificity of biomarker to planned intervention in the study population. Reflecting the approach taken in the US, large population-based biomarker studies should be established to produce evidence of validity and reliability, with consideration given to the creation of local, regional or national biobanks to support this [55].

Such studies could establish standardised practice, provide evidence of convergent validity with clinical outcomes and may mitigate publication bias. This should enable the identification of discriminative biomarker patterns across the spectrum of CF disease, target therapy appropriately and risk-stratify individuals [18]. Such knowledge may be applicable well beyond the remit of CF to other pro-inflammatory lung disease, including Bronchiectasis or COPD, for example, a recent bronchiectasis study successfully demonstrated a reduction in neutrophil serine protease activity with the enzyme inhibitor Brensocatib [141]. For such studies to be successful, an international collective effort to investigate, optimise and standardise the methodology of sputum biomarker measurement would be beneficial.

Conclusion

Sputum biomarkers hold promise as a direct measure of inflammation within the CF lung. To optimise sputum biomarker use in clinical trials, and ultimately CF care, a harmonised effort across the CF community is needed to address biomarker trial design, applicability throughout disease trajectory, establish large population-based studies, and work together to create standards for collection, storage and analysis of sputum biomarkers.

Conflict of Interest Statement

AL, KH, LD, MF, OE, TL, CL, & DGD have no conflicts of interest to declare; CA has received honoraria from Gilead & is Member of the British Thoracic Society Board; P-RB has received grants from Vertex and GSK and consulting fees from Astra-Zeneca, Boehringer

Ingelheim, Chiese, Insmed, Novartis, Pfizer, Teva, Vertex and Zambon; SN is currently an employee of Vertex Pharmaceuticals Inc. and, as such, may own stock or stock options in that company, the submitted work was performed by SN whilst a research fellow at the French Institute for Health and Medical Research (INSERM, Paris, France) and had no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript; SB has received consulting fees from Vertex and Zambon; IS has received grant and consulting fees from Vertex pharmaceuticals.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2021.10.009.

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