Research Article

Evidence of Elevated CXCR2 Agonists in Stable Outpatients with Cystic Fibrosis Compared with Healthy Controls

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Abstract

Introduction: Recruitment and activation of inflammatory cells contributes to the pathophysiology of Cystic Fibrosis (CF). Recruitment is initiated, in part, by engagement of the CXCR2 receptor on neutrophils and other inflammatory cells.

Materials and Methods: Levels of CXCR2 agonists (CXCL 1, 3, 5, 7, 8), and systemic biomarkers of inflammation (fibrinogen, CC-16, SP-D, IL-1β, and MMP-9) were measured in the serum of stable outpatients with cystic fibrosis compared to normal healthy age-, gender, and racially-matched non-smoking control subjects. Secondary objectives included characterization of pulmonary function assessed by spirometry, Impulse Oscillometry (IOS), R5-R15 peripheral resistance, R5 total resistance and R25 large airway resistance; frequency dependent reactance as indicators of reactive capacitance properties of the lung (X5, resonant frequency, Fres, AX) in patients with CF compared with controls. Health outcomes scores in patients with CF were assessed using the Cystic Fibrosis Questionnaire.

Population: Single center, prospective, two visit study. 48 subjects were enrolled. Forty-two subjects (28 patients with CF and 14 healthy controls matched 2:1 (age, gender, and race) were evaluable.

Results: Blood fibrinogen levels in CF patients were significantly elevated compared with controls (3.9 g/L vs. 3.0 g/L [p=0.0004]); similar differences were seen in levels of CXCL8 (CF patients=10.2 pg/mL and controls=5.9 pg/mL [p=0.0065]). A trend was noted for CC-16, CXCL1, and SP-D. As expected, lung functions assessed by spirometry and IOS values were significantly different in the patient population compared to the control group. Mean FEV1 and mean reactance were 2.4L (67% predicted) vs. 3.5L (99% predicted) and 0.174 kPa/(L/sec) vs. –0.085 kPa/(L/sec) in CF patients vs, controls, respectively(p=0.0002 and p<0.0001)). No age or gender trends were noted among CF patients or controls.

Conclusions: The study demonstrated elevation of CXCR2 agonists in stable patients with CF. We also confirmed the potential utility of IOS, an effort-independent lung function test. Finally, the study suggests that subject matching for age and gender may not be necessary in future CF trials assessing these biomarkers.

Keywords: Cystic Fibrosis; Biomarkers; Inflammation; CXCR2

Abbreviations: CF**:** Cystic Fibrosis; CXC: Chemokines; IOS: Impulse Oscillometry

Introduction

Cystic Fibrosis (CF) is the most common, life-limiting, autosomal recessive disease among Caucasians. It is characterized by airway obstruction, bronchiectasis, lung infection, and exocrine pancreatic insufficiency. Median survival is approximately 37 years with over 70% of patients dying from respiratory failure and pulmonary disease [1]. Recruitment and activation of inflammatory cells in the lung contributes significantly to the pulmonary pathophysiology of CF, increasing lung damage [2]. Therefore, it is hypothesized that decreasing recruitment of inflammatory cells into the lung will prevent ongoing tissue destruction and be beneficial to patients with CF.

Small studies in CF have revealed that there is a significant inverse relationship between sputum levels of pro-inflammatory cytokines such as CXCL8 (IL-8), neutrophil elastase, total cell counts, neutrophil count, and myeloperoxidase, with lung function [3-6] CXCL-8 was the first identified member of a growing family (currently more than 40 members) of proinflammatory chemokines that attract and activate immune and inflammatory cells. The chemokines (CXC) function by acting at G-protein-coupled transmembrane cell-surface receptors. The CXC receptors (CXCR1 and CXCR2) are located on neutrophils, subsets of T-cells, macrophages, dendritic cells, and mast cells. CXCR1 is selectively activated by CXCL6 and CXCL8, while the CXCR2 receptor

is stimulated potently by CXCL1-3, and CXCL5-8 [1]. Activation of the CXCR2 receptor is thought to be important for neutrophil migration into the lung and formation of neutrophil extracellular traps, while agonism of the CXCR1 receptor appears to be associated with neutrophil degranulation and microbial killing [7,8].

A key objective of this study was to assess the levels of several endogenous CXCR2 agonists (CXCL 1, 3, 5, 7, 8) and inflammatory markers in the serum in a "real-world" population of stable outpatients with CF to strengthen human target validation in this disease. The study was designed to provide support for testing the hypothesis that a CXCR2 antagonist will reduce the recruitment of neutrophils and prevent airway damage in CF. This is important because patients with CF are now routinely provided with anti-inflammatory medications,

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including inhaled corticosteroids and macrolide antibiotics which are known to affect the levels of CXCL8.

CF is a rare disease and development of endpoints to assess biologic activity as well as response to therapy is of paramount importance. This study also included secondary endpoints that focused on method development/feasibility assessment to aid in the design and implementation of subsequent clinical trials [9,10]. Exploratory assessments included blood inflammation markers, lung function measurements (using effort-independent impulse oscillometry, and spirometry); and a subject questionnaire using the CFQ (CF specific health outcomes questionnaire) [11,12]. We report elevation of CXCR2 agonists, markers of systemic inflammation and altered expression of lung-derived blood biomarkers in stable outpatients with CF.

Materials and Methods

Study design

This was a single center, prospective, two visit study. Subjects with CF were recruited from the Adult Cystic Fibrosis Program of the University of Pennsylvania Medical Center. Control subjects were recruited from the general population in Philadelphia, Pennsylvania between February and July 2008. The study population consisted of a total of 48 subjects, consisting of one healthy control subject for every two subjects with CF, matched for age [+/- 5 years], gender, and race. Control subjects were enrolled from healthy subjects in the area. They had no other pulmonary disease.

Patients enrolled into the study had to have a confirmed diagnosis of cystic fibrosis (ΔF508 homozygote, or ΔF508 heterozygote with a second allele known to cause the disease, or two alleles known to cause a class I, II, or III mutation) and had to be at least 18 years of age in order to participate. In addition, patients must have had colonization of at least one typical CF organism (i.e. *Pseudomonas spp.*, *Staphylococcus aureus, Stenotrophomonas, B. Gladioli*) as evidenced by identification in sputum culture within the past year. Colonization with *Burkholderia cepacia* was exclusion for this study as these subjects would not be included in the initial treatment with a CXCR2 antagonist. The patients had to be clinically stable with no change in symptoms or medication, no hospital admissions, and no intravenous antibiotic therapy for at least two months prior to screening. Subjects with CF were required to have a forced expiratory volume in one second (FEV1) between 40% and 100% of predicted.

Due to the concern of the effect of active cigarette smoking on biomarkers all participants were non-smokers or former smokers by history (defined as those who have not smoked for ≥6 months). Due to the concern of the effect of anti-inflammatory medications on biomarkers, the use of oral or parenteral corticosteroids or the regular use (>3 x/wk) of high dose non-steroidal anti-inflammatory drugs (e.g. ibuprofen >1.6 g/day on a regular basis), within two months of screening was prohibited. Patients with CF were not on an inhaled antibiotic during the study (i.e. must have been in an "off-TOBI" month, >1 week after cessation of Tobi or other inhaled antibiotics). Patients on maintenance therapy with hypertonic saline solution or inhaled DNase were able to continue these therapies. Patients on stable chronic azithromycin therapy were eligible to participate in the trial. Because of the pilot nature of this study, a wide variety of stable CF patients were chosen to participate.

Inflammatory Biomarkers

Whole blood was collected by venipuncture into vacutainer tubes. Serum was prepared by allowing the blood to clot for 30 minutes at room temperature followed by centrifugation at 1500 g for 10-15 minutes. Plasma was obtained by centrifugation of vacutainer tubes at 2000 g for 10 -15 minutes. Serum and plasma were stored at -80°C until analyzed. CCL-18 (PARC), surfactant protein D (SP-D), CXCL1 (GROα), CXCL8 (IL-8), Clara cell secretory protein 16 (CC-16), and Matrix Metaloproteinase 9 (MMP9) were measured in serum samples. Fibrinogen was measured in plasma samples. All protein biomarkers were measured with validated immunoassays. Biomarkers were measured by ELISAs: CC-16, SP-D, Fib-c1 and Fib-c2 were measured using kits from HFL Contract Research and CXCL1, CXCL3, CXCL5, CXCL7, CXCL8, IL1b and MMP9 were measured with kits from Pierce Biotechnology.

Lung Function

Spirometry and impulse oscillometry (IOS) were performed in the following order: IOS, slow vital capacity (SVC), then spirometry. Pre- and post-bronchodilator (400 μg of albuterol) IOS, SVC, FEV1, FVC, and FEV1/FVC were performed with the Jaeger MasterScope CT Impulse Oscillometry System (Hoechberg, Germany). Spirometry was performed according to American Thoracic Society (ATS) guidelines. Percent predicted FEV1 was determined using NHANES criteria [13]. IOS was performed seated using a noseclip and a mouthpiece that stabilized the tongue position cheek support. Impulses were delivered for 20 seconds during tidal breathing. A minimum of three maneuvers were performed. Specified IOS parameters for analysis included: Rrs at 5, 15 and 20 Hz (R5, R15 and R20); reactance at 5 Hz (X5) and the integrated area of low-frequency reactance (AX) from 5 Hz to resonant frequency (FRes). In addition, R5–R20 was determined as an index of peripheral airway resistance. Patients were brought to a quiet room and were given ample time to relax for the maneuvers. IOS was reproducible in all subjects.

Ethics

The study protocol, any amendments, the informed consent, and other information that required pre-approval were reviewed and approved by the Investigational Review Board of the University of Pennsylvania. The study was conducted in accordance with Good Clinical Practice (GCP) and all regulatory requirements, including, where applicable, those originating from the Declaration of Helsinki. All subjects provided written informed consent before treatment.

Statistical methods

The sample size was based on feasibility and no formal power analysis was used to calculate the original sample size for the study. For all measurements CF patients were compared to healthy volunteers using a Wilcoxon (non-parametric) test to avoid making any distributional assumptions. In order to also provide estimates of means in each group and differences between groups all measurements were also Analyzed By Analysis Of Variance (ANOVA) following log-transformation where deemed appropriate to improve normality of residuals. The model included a random effect to account for the matching of subjects, which allowed estimation of the percent variance explained by the matching. Since this was an exploratory, hypothesis-generating study, no adjustments were made for multiplicity of statistical tests. P-values, where presented, are used as a guide the magnitude of differences relative to the underlying variation. Pearson's Rank correlation coefficients were calculated between all pairs of measurements.

Results

Forty-eight (48) subjects were enrolled in the study and forty-two (42) were evaluable. Six (6) subjects (5 CF patients and 1 healthy control)

were not evaluated due to ineligibility based on FEV_1 (three) and no match found (three). Evaluable subjects formed the following groups: Cystic Fibrosis patients (Group A; n=28) and Healthy subjects (Group B; n=14). Table 1 reveals the demographics of the study participants.

Blood inflammatory markers

Serum fibrinogen and CXCL8 in patients with CF were elevated and significantly different than the healthy control subjects. Mean plasma fibrinogen levels were 3.9 g/L in the CF cohort and 3.0 g/L among the healthy controls (p=0.0004) and mean serum CXCL8 was 10.2 pg/mL among the CF patients and 5.9pg/mL in the control group (p=0.0065). Of the biomarkers, MMP9 showed the second largest fold-difference (1.57-times higher in CF than controls) but demonstrated high variability (CV 67.7%). Table 2 provides a summary of the most significant CXCR2 agonists and inflammatory markers observed in the study.

Lung function

As expected, mean FEV1 in the CF group was 2.4L (67% predicted) and was 3.5L (99% predicted) in the healthy population (p=0.0002), while mean reactance as measured with IOS in the CF group was –0.174kPa/(L/sec) and –0.085kPa/(L/sec) in the healthy controls
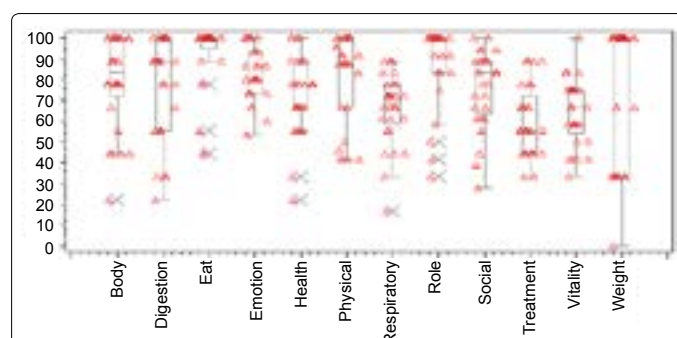

Figure 1: Boxplots for Cystic Fibrosis Questionnaire domains.

Figure 1 shows box-plots for the scaled scores for each of the Cystic Fibrosis Questionnaire (CFQ-R) domains. The y-axis is the CFQ-R scale. Variability can be seen to be large within this relatively small study population. X shows outlier values
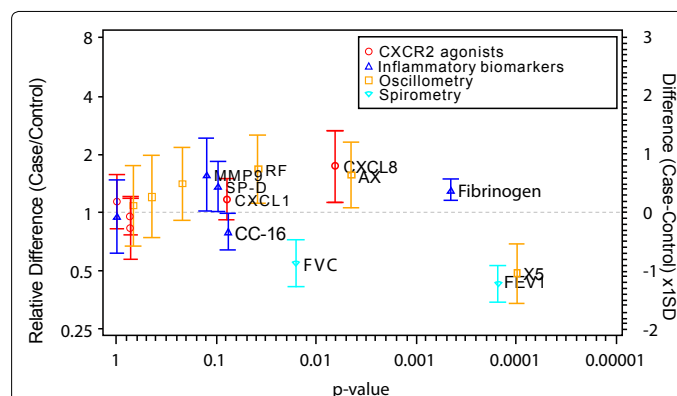

Figure 2: Volcano plot summarizing univariate analyses

For each endpoint the significance of the non-parametric comparison between the two groups is plotted on the horizontal scale, with the corresponding estimate and 95% confidence interval from the ANOVA for the ratio or standardized-difference between the groups on the vertical scale. Variables with p-values <0.1 have been labeled.

(p<0.0001). Table 3 describes the differences in lung function between patients with CF and healthy controls.

Health outcomes

Figure 1 shows box-plots for the scaled scores for each of the Cystic Fibrosis Questionnaire (CFQ-R) domains. Variability was large within this relatively small study population, suggesting a larger population would be needed to discern differences in this domain.

Correlation and impact of subject matching on variability

Correlations between biomarkers and CFQ-R scales or oscillometry and spirometry endpoints were not strong. However, in a post-hoc analysis, a larger number of moderate correlations (magnitude greater than 0.4) were observed than would be expected purely due to chance, in particular between inflammatory blood markers and measures of lung function (Spearman correlations: SP-D vs. R25: -.58; CXCL3 vs. R25: -0.42; CC-16 vs. X5: 0.41).

Table 4 provides the estimated percentage of variability explained by subject matching for all endpoints. This indicates the reduction in variance which would be expected for a comparison between age, gender and race matched subjects in contrast to one between unmatched subjects. The proportion of variability explained by the matching was generally low or zero for the biomarker data, whereas

Demographics of Evaluable Matched Population	CF Patients[1]	Healthy controls
Age in Years, Mean (Range)	33 (20 – 55)	33 (20 – 51)
Height in cm, Mean (Range)	166 (149 – 188)	168 (154 – 183)
Weight in Kg, (Mean (Range)	61.5 (43.9 – 93.4)	72.1 (56.1 – 93.1)
Sex, n (%)		
Female:	16 (57%)	8 (57%)
Male:	12 (43%)	6 (43%)
Race, n (%)		
White – White/Caucasian/European Heritage	28, 100%	14, 100%

[1]All patients had colonization with at least one typical CF organism (except *B. cepacia*) in sputum culture within the past year.

Table 1: Study demographics.

	Geometric mean (%CV)	Ratio A / B (95% CI)	p-value
	Group A - CF	**Group B – healthy controls**	
Fibrinogen [G/L]	3.93 (21.9%)	3.00 (13.6%)	1.31 (1.16, 1.49)	0.0004
CXCL8 [PG/ML]	10.2 (66.1%)	5.9 (81.6%)	1.74 (1.14, 2.66)	0.0065
CC–16 [NG/ML]	4.60 (42.9%)	5.78 (28.7%)	0.80 (0.64, 0.99)	0.0760
CXCL1 [PG/ML]	47.3 (38.3%)	40.5 (41.6%)	1.17 (0.91, 1.50)	0.0782
SP–D [NG/ML]	110 (38.6%)	80 (63.3%)	1.37 (1.02, 1.85)	0.0954
MMP9 [NG/ML]	299 (67.7%)	190 (88.4%)	1.57 (1.02, 2.44)	0.1250

Table 2: Summary of CXCR2 agonists and serum inflammatory endpoints.

	Arithmetic mean (SD)	Difference A – B (95% CI)[2]	p-value[3]	
	Group A - CF	**Group B – healthy controls**		
X5 [kPa/(L/sec)][1]	–0.174 (0.082)	–0.085 (0.062)	–0.090 (–0.135, –0.045)	<0.0001
FEV1 [L] % predicted	2.37 (0.81) 67%	3.53 (0.74) 94%	–1.16 (–1.46, –0.86)	0.0002
AX [kPa/L] [1]	0.835 (1.017)	0.290 (0.116)	0.566 (0.069, 1.063)	0.0044
FVC [L]	3.44 (1.00)	4.36 (0.90)	–0.91 (–1.34, –0.49)	0.0157
RF [Hz] [1]	15.8 (5.8)	12.1 (2.6)	3.9 (0.8, 7.0)	0.0385

Table 3: Summary of spriometric and IOS values.

		Percent of variance explained by matching[1]	Oscillometry		Percent of variance explained by matching[1]
CXCR2 agonists	CXCL1	5.6	**Oscillometry**	AX	35.2
	CXCL3	0.0		R5	22.0
	CXCL5	32.5		R15	5.1
	CXCL7	8.1		R25	12.6
	CXCL8	0.0		RF	28.2
Inflammatory biomarkers	CC-16	25.7		X5	34.3
	Fibrinogen	0.0	**Spirometry**	FEV1	67.9
	IL-1B	0.0		FVC	57.9
	MMP9	4.0			
	SP-D	0.0			

[1]Calculated as $\sigma_b^2/(\sigma_b^2 + \sigma_w^2)$ where σ_b^2 and σ_w^2 are respectively the between- and within-matched-pair variance components from the ANOVA

Table 4: Variability Estimates.

matching was seen to typically reduce variability for oscillometry and spirometric measures by around 30%.

The results of the univariate analyses are summarized in Figure 2. It shows relative differences between CF patients and controls, while taking into account the statistical difference between the groups (p-value on the X-axis), which takes into account the variability of the measurements between CF patients and controls. The endpoints showing the most significant differences between groups are labeled.

Discussion

This is the first study to systematically evaluate lung function (spirometry and IOS), CXCR agonists, and inflammatory biomarkers in the blood of stable patients with cystic fibrosis and compare them healthy non-smoking controls.

The study provides evidence that CXCR2 chemokines associated with neutrophil recruitment are elevated in the serum of stable CF patients. Interference with neutrophil recruitment in the lungs of patients with CF could reduce pulmonary inflammatory burden and have a beneficial effect on clinically important and relevant outcomes, such as lung function and risk of exacerbation. The elevation of systemic markers of inflammation in the peripheral blood of CF patients demonstrates the systemic nature of the disease, and potentially provides justification for extra-pulmonary inhibition of inflammatory cells.

Our study also offers further information regarding potential markers that may be useful to follow in intervention studies in patients with CF. Elevation in systemic markers of inflammation such as plasma fibrinogen, provide evidence for systemic inflammation that may be observed with relatively small sample sizes. Similarly, lung derived markers of inflammation such as SP-D and CC-16 demonstrated a trend toward statistical significance, however, variability in the biomarkers suggests that a study with a larger sample size than ours would be necessary to achieve statistical significance in patients with CF. This will be an important consideration for powering future studies that measure these biomarkers.

Based on pre-clinical evidence and activity in human ozone challenge studies, a CXCR2 receptor antagonist could specifically inhibit the recruitment and activation of neutrophils and other inflammatory cells into the lung in patients with CF and therefore potentially reduce microbial trapping in the airways; selective antagonism of CXCR2, by inhibiting neutrophil recruitment but not neutrophil degranulation and microbial killing (mediated primarily by CXCR1) would potentially restore the balance between host defense and tissue damage mediated by neutrophils [14-19]. The current study provides additional target validation for assessing CXCR2 antagonists in CF. Based upon this information GlaxoSmithKline initiated a study with SB-656933, a novel selective CXCR2 antagonist in adult patients with CF (NCT00903201) [20]. This study was completed recently and showed only trends towards statistically significant differences between patients receiving the antagonist vs. placebo. The variability of inflammatory biomarkers in patients with CF, potentially different responses to inflammatory stimuli based on other genetic profiles, the short length of the study might all have led to the negative results in that study.

The study demonstrated that subject matching had a much greater impact in terms of reducing variance for IOS and spirometry than for the biomarkers. This suggests that subject matching may not be necessary in future CF trials using biomarkers as outcome measures. Interestingly, the serum biomarkers measured in this study were generally less sensitive to differences between clinically stable CF patients and healthy controls than IOS and spirometric endpoints. The study was too small to further explore IOS and spirometric correlations. It was also demonstrated that the correlation of biomarkers, lung function and CFQ-R was weak, suggesting that inflammation is present even in stable CF patients. This finding may describe typical CF phenotypes (i.e. stable patients with diminished lung function and sicker patients with preserved pulmonary function). This observation might also be useful in the future to stratify patients with a greater potential for impending exacerbations.

Our study provides further evidence that biomarkers of inflammation are elevated in CF, a prior study by Eickmeier et al. [21] suggested that patients with CF and COPD have elevated biomarkers of inflammation in their sputum compared to control subjects [21]. CF patients had elevated Th-1 chemokines similar to our study. This study provided further evidence that blocking CXC receptors could potentially lead to decrease in inflammatory biomarkers in patients with CF; this could potentially lead to decreased immune responses to infection. However, blocking of the CXCR2 is attractive, since it does prevent neutrophil migration, but not microbial killing.

This study does have several limitations. It was a small, exploratory, single center pilot study and only included adult subjects over eighteen years of age, who may have a greater degree of systemic inflammation compared to children. Future studies will need to include children to determine levels of CXCR2 agonists in their serum. The study also did not assess bronchoalveolar lavage samples and thus, may underestimate,

or overestimate, the degree of inflammation that is actually observed in the lungs of patients with CF. The lungs are the major areas of infection and inflammation in CF and therefore, even significant inflammation present might have been underestimated. Finally, we have no data on the longitudinal assessment of inflammation over time. Future studies would be helpful to assess CXCR2 agonists and inflammatory marker variability over a longer time course. In addition, many biomarkers showed trends in differences and there multiple comparisons between CF patients and controls and therefore the differences might have been obtained by chance alone.

Conclusions

In summary, biomarkers of inflammation are elevated in stable adult patients with CF compared with matched healthy controls. Biomarkers correlate weakly with lung function and CFQ-R and show almost no variability attributable to age and gender. Future studies will assess variability of these biomarkers over time in patients with CF. It remains to be seen whether modulation of CXCR2 can lead to decreases in inflammation and improved outcomes in patients with CF.

Competing Interests

DAL, SM, and RT-S are full-time employees of GlaxoSmithKline. GlaxoSmithKline financially supported this research and publication of the manuscript. D Holsclaw, GI, MF, MS, and D Hadjiliadis declare that they have no competing interests.

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