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RESPIRATORY RESEARCH

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Measurement of C-reactive protein, procalcitonin and neutrophil elastase in saliva of COPD patients and healthy controls: correlation to self-reported wellbeing parameters

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Abstract

Background: Saliva is increasingly promoted as an alternative diagnostic bio-sample to blood; however its role in respiratory disease requires elucidation. Our aim was to investigate whether C-reactive protein (CRP), procalcitonin (PCT) and neutrophil elastase (NE) could be measured in unstimulated whole saliva, and to explore differences between COPD patients and controls with normal lung function. We also determined the relationship between these salivary biomarkers and self-reported COPD-relevant metrics.

Methods: Salivary CRP, PCT and NE levels were measured at each of 3 visits over a 14-day period alongside spirometry and a daily self-assessment dairy in 143 subjects: 20 never-smokers and 25 smokers with normal spirometry; 98 COPD patients [GOLD Stage I, 16; Stage II, 32; Stage III, 39; Stage IV, 11]. Twenty-two randomly selected subjects provided simultaneous blood samples.

Results: Levels of each salivary biomarker could distinguish between the above cohorts. Significant differences remained for salivary CRP and NE ($p < 0.05$) following adjustment for age, gender, sampling time, gum disease and total co-morbidities; but not for BMI except for salivary NE, which remained higher in smokers compared to non-smokers and stable COPD subjects ($p < 0.001$). Patients with acute COPD exacerbations had a median increase in all 3 salivary biomarkers ($p < 0.001$); CRP: median 5.74 ng/ml, [interquartile range (IQR) 2.86–12.25], PCT 0.38 ng/ml, [IQR 0.22–0.94], and NE 539 ng/ml, [IQR 112.25–1264]. In COPD patients, only salivary CRP and PCT levels correlated with breathing scores ($r = 0.14$, $p < 0.02$; $r = 0.13$, $p < 0.03$ respectively) and sputum features but not with activities of daily living. Salivary CRP and PCT concentrations strongly correlated with serum counterparts [$r = 0.82$, (95 % CI: 0.72–0.87), $p < 0.001$ by Spearman's; and $r = 0.53$, (95 % CI: 0.33–0.69), $p < 0.006$ respectively]; salivary NE did not.

Conclusions: CRP, PCT and NE were reliably and reproducibly measured in saliva, providing clinically-relevant information on health status in COPD; additionally NE distinguished smoking status. All 3 salivary biomarkers increased during COPD exacerbations, with CRP and PCT correlating well with patient-derived clinical metrics. These results provide the conceptual basis for further development of saliva as a viable bio-sample in COPD monitoring and exacerbation management.

Keywords: Salivary biomarkers, C-reactive protein, Procalcitonin, Neutrophil elastase, COPD, COPD exacerbation, wellbeing parameters

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Introduction

Saliva is increasingly used as a non-invasive easily accessible bio-sample for point-of-care diagnostics instead of blood [1, 2] to inform on infection [3–7], drugs [8] and disease states [9–18], including airways inflammation. Salivary eosinophil cationic protein can differentiate between asthmatic and healthy subjects [19]. Increased salivary CRP and haptoglobin levels are demonstrated in childhood allergic asthma [20]; raised salivary leukotriene levels differentiate aspirin-intolerant asthmatics from tolerant counterparts [21].

Biomarkers in various body fluids have been associated with Chronic Obstructive Pulmonary Disease (COPD) pathogenesis and clinical outcome [22, 23]. Serum and sputum CRP are elevated in COPD patients and healthy smokers [24, 25], with moderate inverse correlation of serum CRP to Forced Expiratory Volume in 1 s (FEV_1) [26]. Serum CRP increases during exacerbations [27–32]; with high levels at 14 days post-exacerbation predicting re-exacerbation within 50 days and poor outcome [33]. Serum Procalcitonin (PCT) shows strong correlation to bacterial exacerbations [34], guiding antibiotic prescriptions [29, 35]. Neutrophil elastase (NE), mediator of airway pathogenesis [36, 37], is known to be elevated in smokers [38] and COPD patients [39], has a negative correlation with FEV_1 in patients with expiratory volumes below 40 % predicted [40] and increases further during exacerbations [41].

The importance of CRP, PCT and NE in COPD has been clearly demonstrated. Yet despite the merits that saliva could offer to practical monitoring of COPD and its exacerbations, only two studies have explored its potential clinical role [42, 43]. The aim of our study was therefore to investigate levels of CRP, PCT and NE in unstimulated whole saliva using commercially-validated and modified enzyme-linked immunoassays (ELISA) and to determine differences between patients with COPD and controls with normal lung function. Target biomarkers were measured at 3 time points within a 14-day period. As smoking can influence steady-state biomarker levels [44], control groups included life-long never-smokers and current smokers. COPD data were analysed relative to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage (percentage predicted FEV_1) [45], which alongside MRC scores and self-assessment scores provided information for correlations between target salivary biomarkers and COPD-relevant clinical metrics. For further validation, randomly chosen participants also provided simultaneous blood samples.

Materials & methods

Subject characteristics and study design

From January 2010 to March 2012, individuals were recruited consecutively from our research and outpatient clinic databases to one of 3 cohorts: life-long never-smokers (NS group); current smokers (S group; with > 20 pack years); or COPD, confirmed by spirometry according to GOLD criteria [45]. Patients with other respiratory disorders were excluded. All NS and S subjects had normal lung function. Participants were monitored over 14 days (3 visits, one week apart). At visit 1, demographic details were recorded (Table 1) (Additional file 1: Table S1); participants with any infection or unstable illness in the preceding 6 weeks were excluded. On each visit, the Medical Research Council (MRC) dyspnoea score was recorded [46], spirometry (Koko Legend, nSpire, USA) performed and unstimulated whole saliva collected (2 ml). Participants completed a daily self-assessment diary (Additional file 2) [47], incorporating scores on breathing, activities of daily living (ADL), sputum features and cough presence. In-between scheduled visits, patients were asked to contact the researchers on developing any change in symptoms. An exacerbation was defined as an increase in respiratory symptoms for two consecutive days, with at least two major symptoms (dyspnoea, sputum purulence, sputum volume) or a major plus a minor symptom (wheeze, cold, sore throat, cough) [48]. Randomly-selected subjects provided simultaneous saliva and blood samples. The study was approved by the local research ethics committee [REC project reference: 09/H1203/77]; all participants gave informed written consent.

Unstimulated whole saliva collection protocol

Participants were asked to abstain from alcohol for at least 12 h; fast for 2 h; refrain from brushing their teeth and smoking for 30 min, prior to providing saliva samples. Oral hygiene was checked and mucosal examination performed at each visit. All visit samples were collected at same time of day for each subject.

Immediately before collection participants rinsed their mouths with 10mls water; they then sat in an upright position, tilted their heads forward, and allowed saliva to pool in the mouth before passively drooling into an ice-cooled marked sterile tube (Nunc, Denmark) up-to a total of 2mls.

Collected saliva samples were transported on ice and stored at – 80 °C until analysis. Prior to analysis, thawed saliva was centrifuged at 3000 revolutions per minute (RPM) for 15 min. Sample measurements were undertaken within 3 months of storage; all biomarker assays were performed in duplicate. All saliva samples were tested for blood contamination using an 8-parameter urine dip test strip (Bayer AG, USA). Briefly, 10ul of saliva was aliquoted onto the reagent square for blood, with the colour change after 5 s being read on the key and documented.

Analysis of biomarkers in saliva

CRP was measured in 15ul of saliva using a salivary ELISA kit (Salimetrics Europe, UK) with a detection limit

Table 1 Subject Demographics, Salivary Biomarker & Symptom Profiles

	Control Subjects (n = 45)		Stable COPD Subjects (n = 62)					P value
	NS	S		I	II	III	IV	
	(n = 20)	(n = 25)	(n = 62)	(n = 12)	(n = 19)	(n = 25)	(n = 6)	
Demographics								
Age, [a] years	53 ± 17	42 ± 12	67 ± 7	65 ± 10	64 ± 8	68 ± 5	72 ± 4	<0.001
Gender, male (female)	7 (13)	17 (8)	34 (28)	4 (8)	12 (7)	13 (12)	5 (1)	n/a
$FEV_{1,}$ [a] % predicted	98.1 ± 3.7	99.7 ± 4.7	55.7 ± 22.0	90.4 ± 9.2	64.4 ± 7.6	44.1 ± 3.7	25.1 ± 4.2	<0.001
BMI, [a] (kg/m^2)	29.8 ± 3.6	25.4 ± 3.3	27.3 ± 7.8	28.0 ± 7.0	28.6 ± 2.6	27.3 ± 1.9	19.8 ± 3.6	<0.379
Co Morbidities								
Nil	15	20	27	9	10	5	3	n/a
Gum Disease	2	0	1	0	0	1	0	n/a
Cardiac	3	3	31	2	7	19	3	n/a
Type 2 Diabetes	1	2	10	1	2	7	0	n/a
Treatment								
B2 Agonists (Short Acting)				9	19	25	6	
B2 Agonists (Long Acting)				8	16	25	6	
Anticholinergic (Short Acting)				1	2	3	0	
Anticholinergic (Long Acting)				3	8	18	5	
Inhaled Steroid				8	17	25	6	
Oral Theophyllines				0	1	7	2	
Symptom & Sputum Metrics[b]								
MRC Score	1.00, 0.25	1.00, 0.25	4.00, 1.67	3.00, 2.25	4.00, 1.50	5.00, 1.00	5.00, 0.00	<0.001
Breathing Score	2.00, 1.00	2.00, 0.25	3.00, 0.00	3.00, 1.00	3.00, 0.00	3.00, 0.00	3.00, 0.75	<0.001
ADL Score	1.00, 0.00	1.00, 0.00	3.00, 2.00	1.00, 2.00	3.00, 2.00	4.00, 2.33	3.00, 1.50	<0.001
Sputum Amount	1.00, 0.00	1.00, 1.00	2.00, 2.00	1.50, 1.00	2.00, 1.84	3.00, 1.00	2.50, 2.50	<0.001
Sputum Texture	1.00, 1.00	2.00, 0.00	2.00, 0.00	2.00, 0.50	2.00, 0.00	2.00, 0.00	2.00, 0.00	<0.001
Sputum Colour	3.00, 0.00	3.00, 0.00	3.00, 1.00	3.00, 0.75	3.00, 0.83	3.00, 1.00	3.50, 1.00	<0.001
Salivary Biomarkers, [b]								
CRP, ng/ml	0.89, 0.35	1.70, 1.07	1.66, 2.30	1.62, 1.36	2.44, 2.63	1.45, 2.34	2.34, 5.94	<0.002
PCT, ng/ml	0.09, 0.03	0.13, 0.09	0.09, 0.04	0.10, 0.06	0.09, 0.04	0.09, 0.04	0.11, 0.03	<0.012
NE, ng/ml	152, 96	408, 748	189, 508	227, 104	161, 491	189, 687	163, 181	<0.001

I = GOLD stage I, II = GOLD stage II; III = GOLD stage III, IV = GOLD stage IV; ADL = Activity of Daily Living, COPD = Chronic Obstructive Pulmonary Disease; GOLD = Global Initiative for Chronic Obstructive Lung Disease; NS = healthy non-smoker; S = healthy smoker; FEV_1 = Forced Expiratory Volume in 1 s; BMI = Body Mass Index; ex = ex-smokers; CRP = C-Reactive Protein; PCT = Procalcitonin; NE = Neutrophil Elastase. Data are presented as: a, Mean ± standard deviation; b, Median, inter-quartile range. Exacerbation frequency is divided into 3 groups: Group 1 = 1–3, Group 2 = 4–6, Group 3 = >6. P values represent the difference between controls and stable COPD subjects. No significant difference was found across COPD severity defined by GOLD for: Age, BMI, CRP, PCT, NE, Breathing Score, Sputum Texture and Sputum Colour: ($p < 0.379$; $p < 0.403$; $p < 0.559$; $p < 0.946$; $p < 0.620$; $p < 0.127$; $p < 0.228$; $p < 0.824$). FEV_1 significantly decreased as COPD severity increased ($p < 0.001$), whilst MRC, ADL and Sputum Amount significantly increased: ($p < 0.001$; $p < 0.002$; $p < 0.011$ respectively)

of 0.90 ng/ml; lower concentrations were assigned as 0.89 ng/ml.

Levels of PCT and NE in saliva were measured following in-house modification of commercially-available ELISAs [49].

Briefly, for adapting the VIDAS® BRAHMS PCT (bioMérieux, France) for use in saliva, pre-study experiments involved spiking saliva from non-smoker healthy subjects with PCT Control (provided by the manufacturer for assay calibration) in varying concentrations: 0.09–20 ng/ml. These spiked samples were analysed neat and in varying defined dilutions: 1:1, 1:2, 1:4, 1:8 using Phosphate Buffered Saline - Tween 20–0.05 % (PBS-T). Optimal recovery of PCT (85 %) at all concentrations occurred when saliva was diluted 1:2 in PBS-T. The manufacture's test procedure for performing the assay was not altered. Thereafter, PCT was determined in 100ul of saliva diluted 1:2 in Phosphate Buffered Saline-Tween 20 – 0.05 % (PBS-T)

using VIDAS® BRAHMS PCT (bioMérieux, France) with a detection limit of 0.10 ng/ml; lower concentrations were assigned as 0.09 ng/ml.

For adapting the PMN-Elastase ELISA Kit (Immundiagnostik AG, Germany) for use in saliva, we first spiked non-smoker healthy saliva with NE (provided by the manufacturer to calibrate the assay) in varying concentrations: 115–1000 ng/ml. These spiked samples were analysed in varying dilutions: 1:100, 1:200, 1:400, 1:800 using manufacturer-supplied ELISA wash buffer. Recovery of NE (90 %) was consistent across all 4 dilutions; for the study we elected to use a 1:200 dilution. The manufacture's test procedure for performing the assay was not altered. Thus, NE was measured in 7.0 ul of saliva diluted 1:200 in ELISA wash buffer using PMN-Elastase ELISA Kit (Immundiagnostik AG, Germany), with a detection limit of 70 ng/ml; lower concentrations were assigned as 69 ng/ml.

Analysis of biomarkers in blood

Peripheral blood was collected in supplement-free tubes and ethylene diaminetetra-acid vacutainer tubes (BD Bioscience, New Jersey, USA). Samples were then centrifuged at 2000 RPM for 15 min; retrieved serum was stored at 80 °C until analysis. Serum CRP was measured using ADVIA 2400 Chemistry System (Siemens AG, Germany) with detection limit of 0.3 mg/L. Serum PCT and NE were quantified using same assay kits as for saliva, but following manufacturers' protocols. Serum levels were expressed as ng/ml except for CRP (mg/L). All assays were performed in duplicate.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics Version 19 (SPSS Inc, IBM, USA). Parametric data are expressed as mean ± standard deviation (SD) and non-parametric as median, (inter-quartile range [IQR]). Between-group comparison was performed using Mann–Whitney *U* test, Kruskal-Wallis one-way analysis of variance (ANOVA) and for paired data, Wilcoxon Signed Ranks test. A Bonferroni Correction was applied when undertaking multiple comparison testing. Biomarker data were logarithmically transformed to allow Univariate Analysis and determination of covariate effect. Correlations were assessed by Spearman's Rank correlation co-efficient (r). The reproducibility of salivary biomarker levels was explored using Bland-Altman plots expressing the change within a subject. A p value of <0.05 was considered significant.

Results

In total 143 individuals were recruited: 20 never-smokers (NS) and 25 current smokers, over 20 pack year history, (S) with normal lung function; 98 COPD patients (GOLD Stage I, 16; Stage II, 32; Stage III, 39; Stage IV, 11), all ex-smokers (>20 pack year history). Thirty-six COPD patients experienced an exacerbation during the course of the study; all controls remained clinically stable. Salivary CRP, PCT and NE were measured in all participants (Table 1) (Additional file 1: Table S1), with an intra-and inter-assay co-efficient of variances of <7 % and <12 % respectively for all 3 assays.

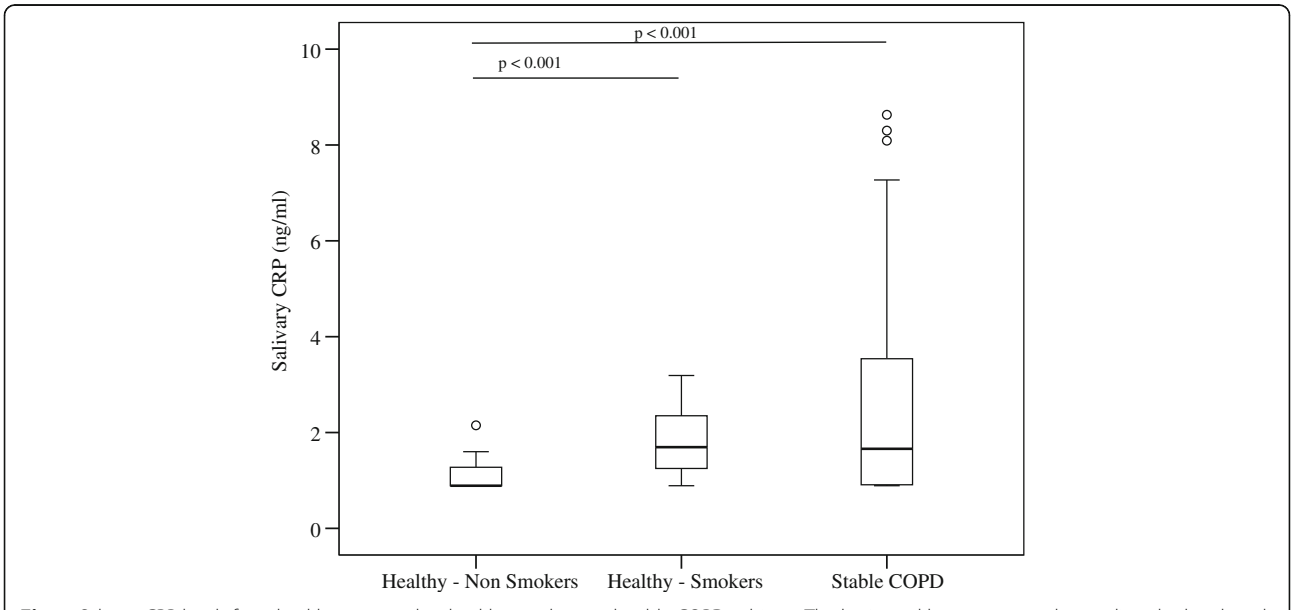

Fig. 1 Salivary CRP levels from healthy non-smoker, healthy smokers and stable COPD subjects. The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ° (1.5 x the interquartile range)

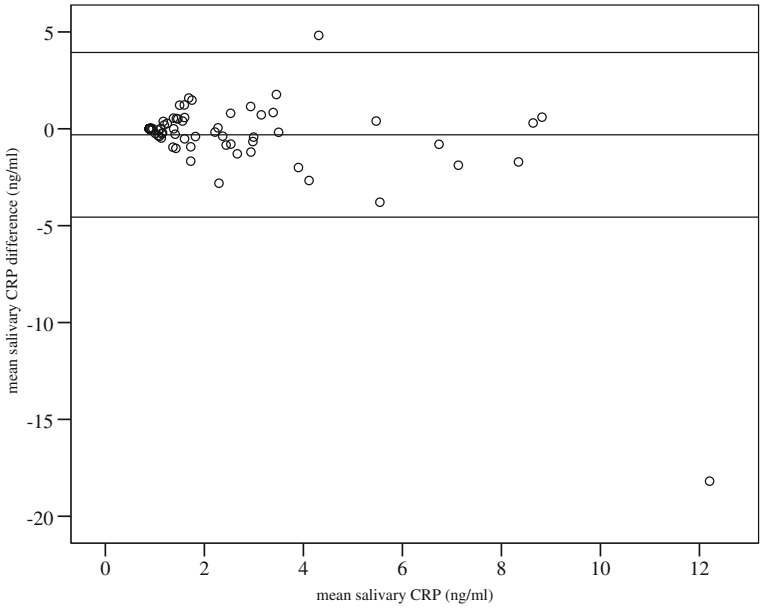

Fig. 2 Bland-Altman plot of baseline salivary CRP levels across healthy non-smokers, health smokers and stable COPD. The upper and lower bars represent 1.96 Standard Deviations (SD) from the mean. Almost all replicates fell within the 95 % limits of agreement (difference between stable baseline values), with only 2 outliers

Biomarkers across health status

Only stable COPD patients (n = 62) were included in the between-group analyses. Salivary CRP differed between the 3 groups ($p < 0.002$ by ANOVA), with significant increase in COPD (median: 1.66 ng/ml; IQR: 2.55 ng/ml) compared to NS (0.89 ng/ml; 0.35 ng/ml, $p < 0.001$ by Mann Whitney U), but not to smokers (1.70 ng/ml; 1.07 ng/ml, $p < 0.605$). Smokers had higher salivary CRP than never-smokers ($p < 0.001$) (Fig. 1). These differences remained statistically significant ($p < 0.05$ by Univariate Analysis) following adjustment for age, gender, sampling time and total co-morbidities; but not for BMI ($p < 0.402$). The coefficient of variance for CRP variability within subjects was 13 %, 27 %, 15 % for NS, S and stable COPD respectively. The Bland-Altman plot with upper and lower limits (1.96 Standard Deviation (SD))

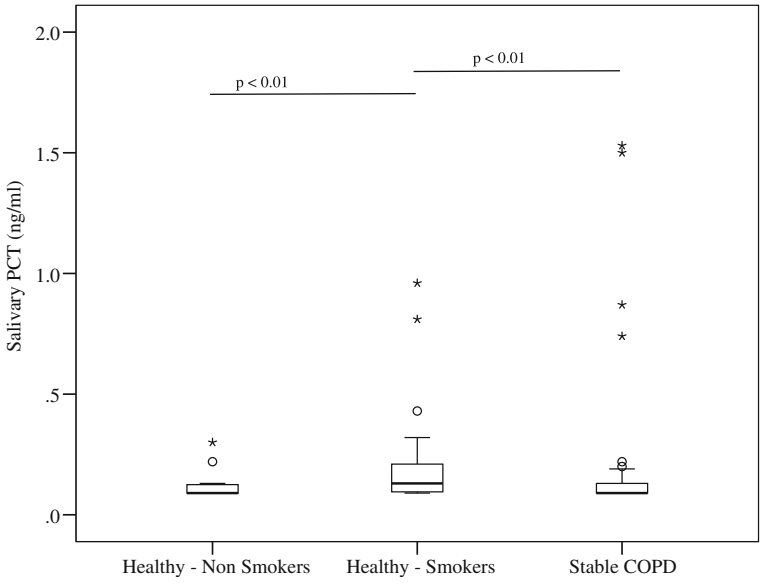

Fig. 3 Salivary Procalcitonin (PCT) levels from healthy non-smokers, healthy smokers, stable COPD subjects. The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ° (1.5 x the interquartile range) and * (3 x the interquartile range)

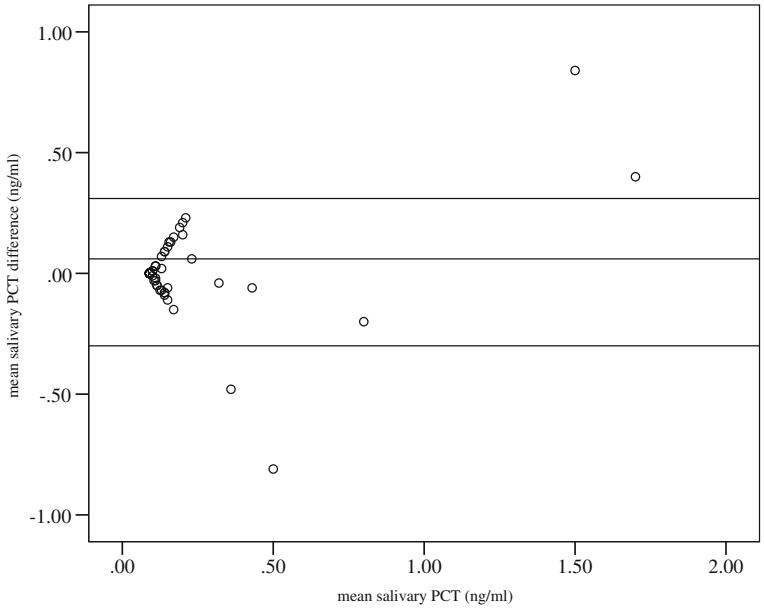

Fig. 4 Bland-Altman plot of baseline salivary PCT levels across healthy non-smokers, health smokers and stable COPD. The upper and lower bars represent 1.96 Standard Deviations (SD) from the mean. Almost all replicates fell within the 95 % limits of agreement (difference between stable baseline values), with only 4 outliers

combing all 3 groups (n = 107) showed good data consistency (difference between stable baseline values), with only 2 outliers (Fig. 2).

Salivary PCT differed between groups ($p < 0.012$). Salivary PCT was significantly elevated in smokers (0.13 ng/ml; 0.09 ng/ml) compared to NS (0.09 ng/ml; 0.03 ng/ml, $p < 0.011$) and COPD (0.09 ng/ml; 0.04 ng/ml, $p < 0.01$); but not between COPD and NS ($p < 0.363$) (Fig. 3). Following covariate adjustment, there was no significant difference ($p < 0.564$) between cohorts. Gender adjustment showed salivary PCT was generally lower in females, (0.11 vs. 0.14 ng/ml [males]: $p < 0.05$). The coefficient of variance for PCT variability within subjects was 19 %, 15 %, 14 % for NS, S and stable COPD respectively. The Bland-Altman plot with upper and lower limits (1.96 SD) combing all 3 groups

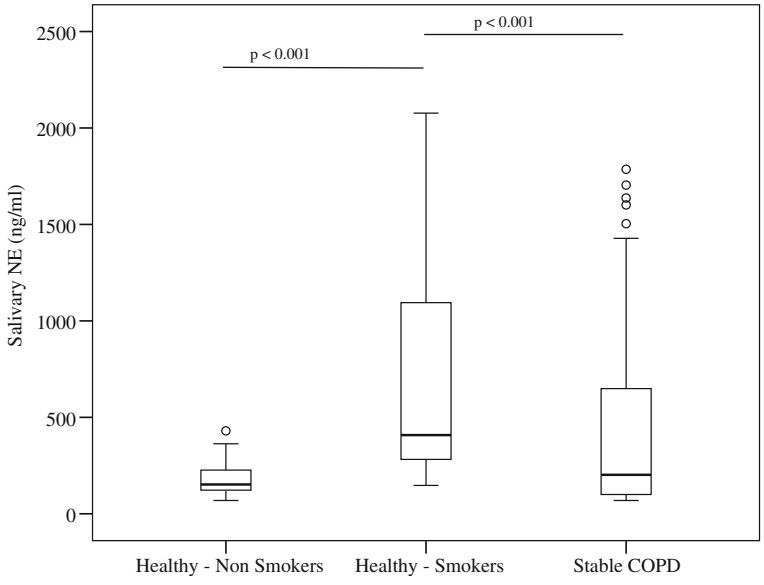

Fig. 5 Salivary Neutrophil Elastase (NE) from healthy non-smokers, healthy smokers, stable COPD subjects. The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ° (1.5 x the interquartile range)

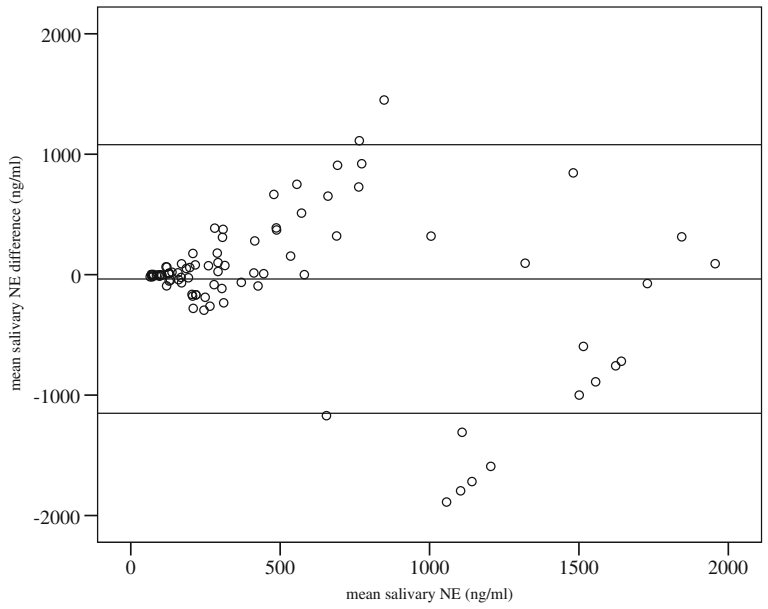

Fig. 6 Bland-Altman plot of baseline salivary NE levels across healthy non-smokers, health smokers and stable COPD. The upper and lower bars represent 1.96 Standard Deviations (SD) from the mean. Almost all replicates fell within the 95 % limits of agreement (difference between stable baseline values), with only 7 outliers

(n = 107) showed good data consistency, with only 4 outliers (Fig. 4).

Differences in salivary NE were observed between cohorts ($p < 0.001$), irrespective of covariate adjustment ($p < 0.011$). Smokers had significantly raised NE (408 ng/ml; 748 ng/ml) compared to NS (152 ng/ml; 96 ng/ml, $p < 0.001$), and COPD patients (189 ng/ml; 508 ng/ml, $p < 0.001$); with no significant difference between NS and COPD ($p < 0.235$) (Fig. 5). Age appeared to affect salivary NE levels ($p < 0.04$), with around 60 ng/ml decline for every increasing decade in COPD patients, regardless of treatment. The coefficient of variance for NE variability within subjects was 32 %, 41 %, 37 % for NS, S and stable COPD respectively. The Bland-Altman plot with upper and lower limits (1.96 SD) combing all 3 groups (n = 107) showed good consistency of data (difference between stable baseline values) with only 7 outliers (Fig. 6).

No association was observed between salivary biomarkers and COPD severity, as determined by FEV_1; CRP: $p < 0.559$; PCT: $p < 0.946$; NE: $p < 0.620$.

Subject-completed symptom scores

All participants completed a daily self-assessment symptom diary (Additional file 2), MRC scores significantly correlated with breathing scores ($r = 0.55$; 95 % Confidence Interval (CI): 0.34–0.70) and ADL ($r = 0.47$; 95 % CI: 0.25–0.64); $p < 0.001$ by Spearman's.

Inter-group analysis demonstrated differences in all symptom scores between stable COPD, NS and S ($p < 0.001$). There was no significant difference between NS and S for any symptom metrics.

Analysis between salivary biomarkers and clinical metrics across participants (n = 143) revealed correlation of salivary CRP with ADL ($r = 0.23$, $p < 0.02$); sputum amount ($r = 0.23$, $p < 0.02$) and texture ($r = 0.24$, $p < 0.02$). Salivary PCT did not significantly correlate with any symptom. Salivary NE only correlated with MRC score ($r = 0.29$, $p < 0.01$).

Separate sub-analysis on all COPD patients (n = 98) (Tables 2 and 3) (Additional file 3: Table S2 & Additional file 4: Table S3) demonstrated salivary CRP correlated with MRC score ($r = 0.16$, $p < 0.01$), breathing score ($r = 0.14$,

Table 2 Correlations of All COPD Subjects (n = 98) Symptom Scores vs. Salivary Biomarker Levels

	Symptom Scores		
Salivary Biomarkers	Breathing Score	ADL Score	MRC Score
CRP	r = 0.142, p <0.02	r = 0.105, p <0.08	r = 0.164, p <0.006
PCT	r = 0.125, p <0.04	r = 0.115, p <0.06	r = 0.04, p <0.444
NE	r = 0.105, p <0.082	r = 0.028, p <0.647	r = -0.074, p <0.222

COPD = Chronic Obstructive Pulmonary Disease; FEV_1 = Forced Expiratory Volume in 1 s; MRC = Medical Research Council; ADL = Activity of Daily Living; CRP = C-Reactive Protein; PCT = Procalcitonin; NE = Neutrophil Elastase. Data are presented as the Spearman's correlation coefficient: r value

Table 3 Correlations of All COPD Subject (n = 98) Sputum Metrics vs. Salivary Biomarker Levels

	Sputum Metrics		
Salivary Biomarkers	Sputum Amount	Sputum Texture	Sputum Colour
CRP	r = 0.148, p <0.013	r = 0.130, p <0.032	r = 0.324, p <0.001
PCT	r = 0.130, p < 0.033	r = 0.107, p < 0.078	r = 0.229, p < 0.001
NE	r = 0.075, p < 0.219	r = -0.118, p < 0.051	r = 0.068, p < 0.266

COPD = Chronic Obstructive Pulmonary Disease; FEV_1 = Forced Expiratory Volume in 1 s; MRC = Medical Research Council; ADL = Activity of Daily Living; CRP = C-Reactive Protein; PCT = Procalcitonin; NE = Neutrophil Elastase. Data are presented as the Spearman's correlation coefficient: r value

p < 0.02) (Fig. 7a, 7b) sputum amount (r = 0.15, p < 0.01), texture (r = 0.13, p < 0.03) and colour (r = 0.32, p < 0.001). Salivary PCT correlated with breathing score (r = 0.13, p < 0.04) (Fig. 7c), sputum amount (r = 0.13, p < 0.03) and colour (r = 0.23, p < 0.001). Salivary NE did not correlate with any clinical features. Sputum amount and colour correlated with breathing (r = 0.34, p < 0.001) and ADL scores (r = 0.34, p < 0.001); texture correlated only with ADL (r = 0.24, p < 0.001) (Table 4) (Additional file 5: Table S4).

COPD Stable vs. Exacerbation

Thirty-six COPD patients experienced an exacerbation at day 11 ± 3 (Table 5) (Additional file 6: Table S5). There was no difference in the median baseline exacerbation frequency (1–3 episodes per year) between these patients and those COPD patients that remained stable throughout the study. Comparison of their paired stable and pre-treatment exacerbation samples demonstrated significant elevation in all target salivary biomarkers at exacerbation (p < 0.001) (Figs. 8, 9, 10). Levels of CRP increased by

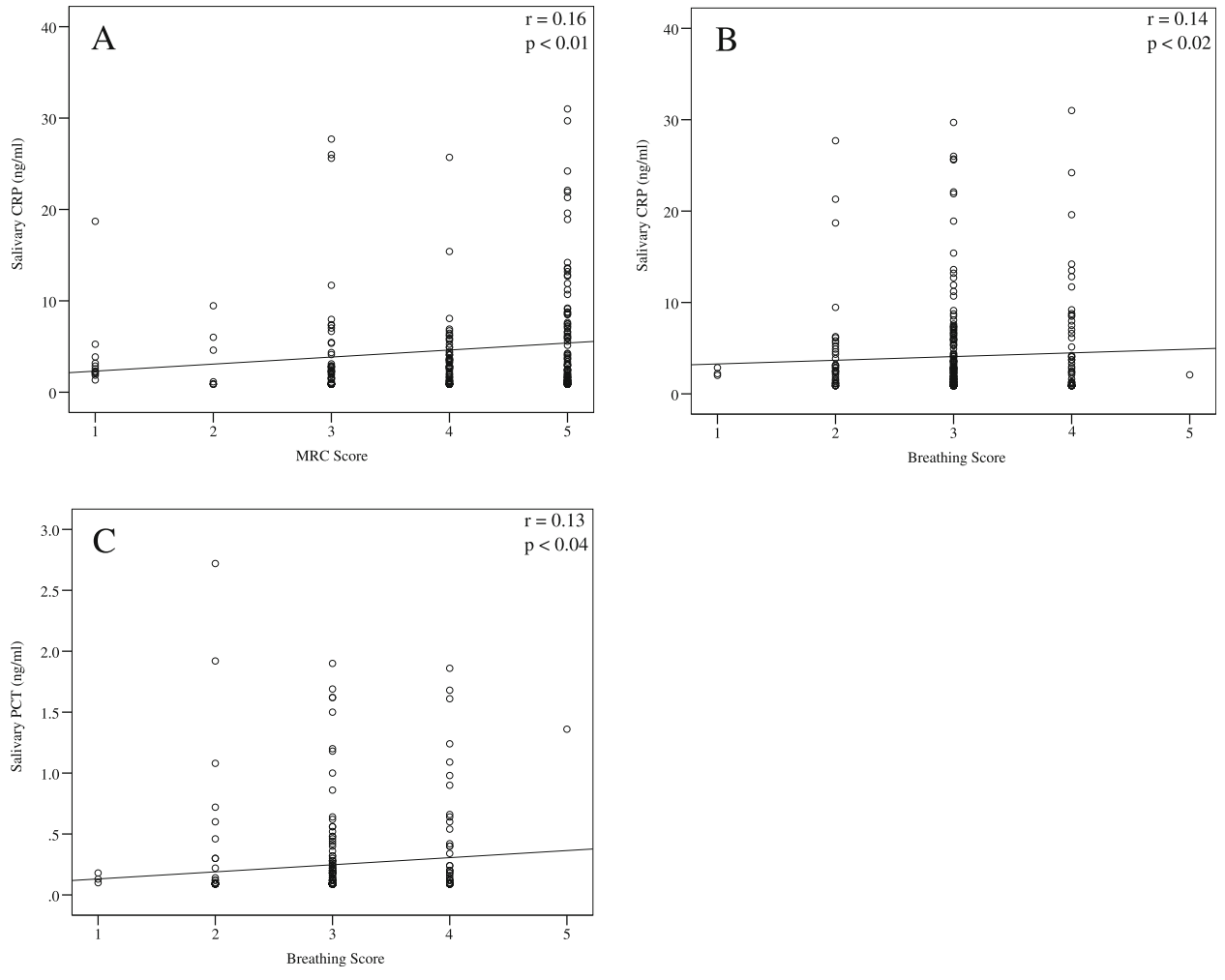

Fig. 7 Scatter plot with a best fit line between COPD subject symptom scores and salivary biomarkers. **a** Salivary CRP vs. MRC score; **b** Salivary CRP vs. breathing score; **c** Salivary PCT vs. breathing score

Table 4 Correlation of All COPD Subject (n = 98) Symptom Scores vs. Sputum Metrics

	Symptom Scores		
Sputum Metrics	Breathing Score	ADL Score	MRC Score
Amount	r = 0.34, p < 0.001	r = 0.34, p < 0.001	r = 0.24, p < 0.001
Texture	r = 0.07, p < 0.24	r = 0.24, p < 0.001	r = 0.31, p < 0.001
Colour	r = 0.26, p < 0.001	r = 0.19, p < 0.001	r = 0.28, p < 0.001

COPD = Chronic Obstructive Pulmonary Disease; FEV_1 = Forced Expiratory Volume in 1 s; MRC = Medical Research Council; ADL = Activity of Daily Living; CRP = C-Reactive Protein; PCT = Procalcitonin; NE = Neutrophil Elastase. Data are presented as the Spearman's correlation coefficient: r value

Table 5 Same COPD Subjects in Stable and Exacerbation phase (n = 36) Demographics, Salivary Biomarker & Symptom Profiles

	Stable	Exacerbation	P value
Demographics			
Age, [a] years	68 ± 9		
Gender, male (female)	17 (19)		
FEV_1[a], % predicted	53 ± 23	48 ± 19	< 0.001
BMI, [a] (kg/m^2)	24.0 ± 6.3		
Co Morbidities			
Nil	5		
Gum Disease	2		
Cardiac	30		
Type 2 Diabetes	4		
Treatment			
B2 Agonists (Short Acting)	35		
B2 Agonists (Long Acting)	32		
Anticholinergic (Short Acting)	5		
Anticholinergic (Long Acting)	25		
Inhaled Steroid	31		
Oral Theophyllines	6		
Symptom & Sputum Metrics[b]			
Increased Cough, n	0	10	< 0.001
MRC Score	5.00, 1.25	5.00, 1.25	< 0.16
Breathing Score	3.00, 0.00	4.00, 1.00	< 0.006
ADL Score	3.00, 1.00	4.00, 2.00	< 0.014
Sputum Amount	2.00, 2.00	3.00, 2.25	< 0.001
Sputum Texture	1.94, 0.33	2.06, 0.41	< 0.001
Sputum Colour	3.00, 1.00	4.00, 0.41	< 0.05
Salivary Biomarkers[b]			
CRP, ng/ml	1.61, 1.10	7.35, 10.04	< 0.001
PCT, ng/ml	0.09, 0.06	0.50, 0.71	< 0.001
NE, ng/ml	128, 190	769, 1680	< 0.001

COPD = Chronic Obstructive Pulmonary Disease; FEV_1 = Forced Expiratory Volume in 1 s; BMI = Body Mass Index; ex = ex-smokers; MRC = Medical Research Council; ADL = Activity of Daily Living; CRP = C-Reactive Protein; PCT = Procalcitonin; NE = Neutrophil Elastase. Exacerbation frequency is divided into 3 groups: Group 1 = 1–3, Group 2 = 4–6, Group 3 = >6. Data are presented as: a, Mean ± standard deviation; b, Median, inter-quartile range. P values represent the difference between stable and exacerbation phases

5.74 ng/ml (95 % CI: 3.72–11.47); PCT by 0.38 ng/ml, (95 % CI: 0.31–0.54) and NE by 539 ng/ml (95 % CI: 169–982); alongside a reduction in FEV_1 ($p < 0.001$) and patient-recorded changes in sputum (amount: $p < 0.001$, texture: $p < 0.05$, colour: $p < 0.001$); ADL and breathing scores ($p < 0.014$ and $p < 0.006$ respectively) (Table 5) (Additional file 6: Table S5).

Comparison of subject-matched saliva and serum biomarker levels

Relationships between saliva and serum biomarkers were studied in 22 randomly-selected subjects, providing a total of 66 paired saliva-serum samples. Salivary CRP was approximately 200 times lower than serum; salivary PCT and NE were about two-fold higher. Salivary CRP and PCT correlated with serum equivalents, r = 0.82, (95 % CI: 0.72–0.87), $p < 0.001$ by Spearman's; and r = 0.53, (95 % CI: 0.33–0.69), $p < 0.006$ respectively (Fig. 11a, 11b). Salivary and serum NE did not correlate (r = −0.24, $p < 0.25$).

Biomarker cross-analysis demonstrated salivary PCT correlated with serum and salivary CRP, r = 0.53, (95 % CI: 0.33–0.69), $p < 0.006$; and r = 0.73, (95 % CI: 0.59–0.83), $p < 0.001$ respectively (Fig. 11c, 11d). Salivary NE correlated with both salivary CRP, r = 0.45, (95 % CI: 0.23–0.63), $p < 0.001$, and salivary PCT, r = 0.58, (95 % CI: 0.39–0.72), $p < 0.001$ (Fig. 11e, 11f).

Discussion

As disease management shifts increasingly towards point-of-care, there is urgency to develop easier, less stressful sampling methods especially for monitoring chronic conditions. This is the first study to explore the potential role of salivary CRP, PCT and NE in COPD. Whilst a validated CRP saliva-based assay is available (Salimetrics®), we have also demonstrated that modification of existing body-fluid assays (PCT and NE) provides reproducible results for saliva [50].

Salivary biomarker targets in COPD patients of varying severity were compared to controls (never-smokers and smokers) under real world/working conditions; hence study participants with co-morbid conditions were included provided these were clinically stable at time of enrolment. To minimise across-cohort demographic variations and circadian influences, analysed measurements

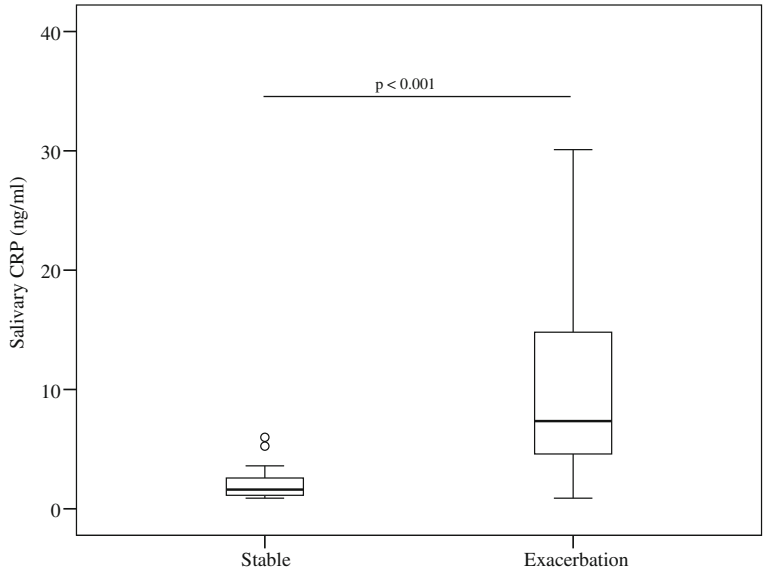

Fig. 8 Salivary CRP levels from stable and the same COPD subjects during an exacerbation. The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ° (1.5 x the interquartile range)

were then adjusted for potential covariate bias [51], including sampling times [52]. Non-smoker salivary CRP levels at 0.89 ng/ml (IQR 0.35 ng/ml) compared favourably to previous observations showing a healthy CRP range of 0.02–2.5 ng/ml in saliva [52–54]. Serum CRP has been shown to distinguish between COPD and controls [55], but not healthy smokers from non-smokers [24]. However no difference in salivary CRP was demonstrated between our study cohorts following all-covariate adjustment, possibly because our controls had relatively high BMIs; indeed significant differences emerged when adjustment excluded BMI. In support, strong correlations between serum CRP and BMI have been previously demonstrated [56]. Whilst correlations between serum CRP levels and FEV_1 have been reported [26, 57], we found no association between salivary CRP and FEV_1 in stable COPD; this possibly reflects the inhaled corticosteroid usage in our COPD patients [24].

We are the first to explore the presence of PCT in saliva. There was no difference in salivary PCT levels between

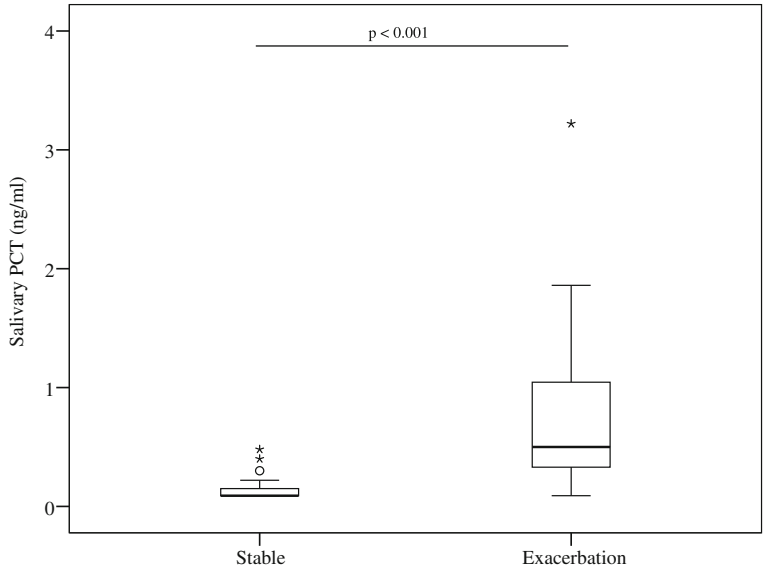

Fig. 9 Salivary PCT levels from stable and the same COPD subjects during an exacerbation. The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ° (1.5 x the interquartile range) and * (3 x the interquartile range)

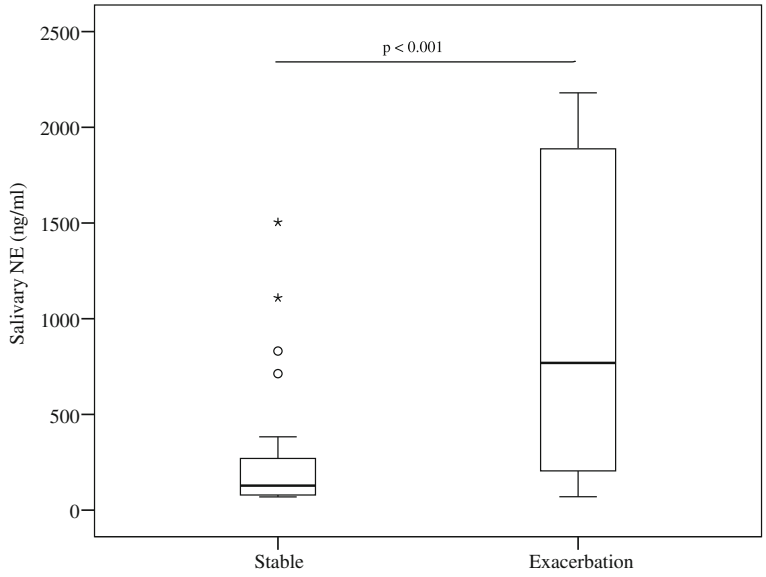

Fig. 10 Salivary NE levels from stable and the same COPD subjects during an exacerbation. The horizontal bar represents the median; the box length represents the interquartile range. Outliers are identified by ° (1.5 x the interquartile range) and * (3 x the interquartile range)

stable COPD patients and healthy controls following covariate adjustment. This is not surprising as PCT is normally hardly detectable in blood (below 0.05 ng/ml) unless there is presence of bacterial infections and sepsis or following trauma [58]. In agreement with previous observations [38, 59] salivary NE was found to be higher in smokers, but not in stable COPD patients [60]; possibly because all study COPD patients were ex-smokers.

The observed increases in salivary CRP, PCT and NE during COPD exacerbations reflect the well-documented elevated CRP and PCT in blood [29, 57, 58] and NE in sputum [41, 59], and have clinical implications. Whilst salivary CRP (or any of the other analytes) may not be sufficiently sensitive for evaluating COPD risk and outcome, it could serve as a potential surrogate for determining exacerbation onset. However, evidence for CRP or any biomarker in isolation to confirm an exacerbation is minimal. On the other hand, our results give support to future development of single-platform immunodiagnostics for near-patient measurement of salivary CRP alongside other readily available biomarkers e.g., PCT to enable sufficient confidence for exacerbation prediction and stratified intervention.

Alongside such developments, we also need to improve understanding of the association between biomarker/physiological measurements and patient-reported outcomes (PRO) in COPD [61]. This is particularly crucial as no one parameter appears to be sufficiently sensitive or specific in monitoring disease status or predicting exacerbation onset. Our study reveals significant differences in self-assessed symptom scores and sputum metrics in COPD patients, similar to studies using SGRQ and CAT [62]. Furthermore, significant correlations were observed between salivary levels of CRP and PCT and breathing scores, with simultaneous changes occurring in both target analyte levels and patient-reported breathing and ADL assessments during exacerbations of COPD. As other PRO instruments have shown similar correlations [47, 63], it is likely that particular COPD symptoms will be shown to be driven by underlying inflammatory events, with those very severe COPD exacerbations requiring hospitalisation possibly exhibiting different clinical and inflammatory profiles [64].

Thus, biomarkers or symptoms in isolation will not be sensitive or specific enough to monitor longitudinal wellbeing in COPD, and combined bio-clinical profiling is essential, particularly if the long-term goal is to enable patient-led prediction of exacerbations and prompt intervention. Indeed, combining serum CRP with one increased major exacerbation symptom (dyspnoea, sputum volume or purulence) was found to be more sensitive than CRP alone in diagnosing exacerbations [30]. Of 36 biomarkers analysed, none were sensitive or specific enough to diagnose exacerbations without symptom assessment [30].

Most serum components are present in saliva, although compositional differences show that saliva is not a passive ultra-filtrate of serum [65]. Biomarkers can enter saliva by cellular diffusion or active transport, ultra-filtration within salivary glands and/or via the gingival sulcus [66]. The precise mechanisms explaining CRP, PCT and NE presence in saliva are unclear. Whilst blood contamination via micro-

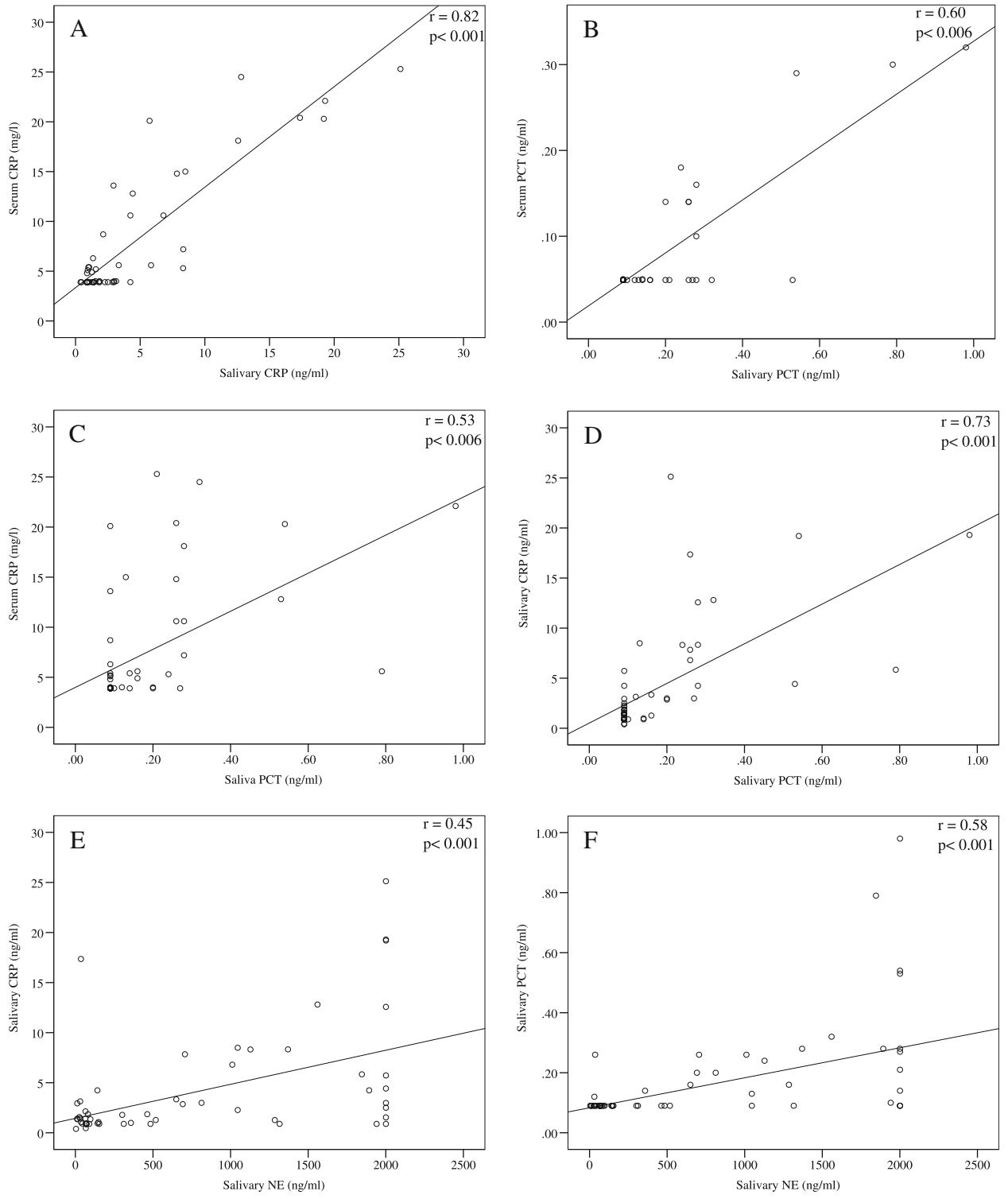

Fig. 11 Scatter plot with a best fit line between same subject serum and salivary biomarkers. These subjects provided both serum and saliva samples on the same visit. **a** Salivary vs. serum CRP; **b** Salivary PCT vs. serum PCT; **c** Salivary PCT vs. serum CRP; **d** Salivary PCT vs. salivary CRP; **e** Salivary NE vs. salivary CRP; **f** Salivary NE vs. salivary PCT

leakages, crevicular fluid overflow from micro-injuries or poor oral health is plausible, biomarker measurements in our study were not affected by adjustment for gum disease; samples also tested negative for blood.

Both salivary CRP and PCT levels correlated with serum counterparts. Saliva-serum CRP correlations have been previously established [54, 67]. Although Ouellet-Morin et al. observed a moderate to strong association between saliva and serum CRP, lower correlations were found at serum CRP below 2.0 mg/L compared to higher CRP (≥2.0 mg/L) [67]. However, Punyadeera et al. demonstrated saliva to serum CRP correlation at concentrations above 5 mg/mL. Whilst these studies suggest that prediction of serum CRP from saliva CRP is more accurate at higher serum concentrations, our study demonstrated strong correlations at both low and high CRP levels. The only study on saliva to serum PCT relationship [68] showed no significant correlation between the two fluids; however saliva samples were stored at – 27 °C rather than the recommended – 80 °C [69].

No correlation was found between saliva and serum NE levels. Whilst one possible explanation could be localised NE production not manifesting systemically, this contradicts the observed moderate to strong correlation of salivary NE to both salivary CRP and PCT levels. An alternative explanation could be the rapid inactivation of NE *in vivo* [70], leading to comparatively slower inactivation in saliva than serum.

Some study limitations need to be considered. Although subjects had three assessments over 14 days, longitudinal studies are required to establish steady-state baselines for the target salivary analytes. These would offer precise correlations of biomarker changes to patient-reported outcomes, specifically in the important prodromal period leading to an exacerbation. Furthermore, as BMI-matched cohorts appear to influence salivary CRP between-group differences, BMI status may need consideration in future larger studies. Another possible shortfall is that our study did not specifically exclude for potential microbial airway colonisation in the COPD group, although we did ensure that participants were excluded in the event of any infection or unstable illness in the preceding 6 weeks to enrolment. We appreciate that the presence of lower airway bronchial colonisation can be associated with elevated serum CRP levels in stable COPD patients [71], and with increased exacerbation frequency [72]. In mitigation, we have provided separate analysis for the COPD subjects that underwent an exacerbation and for those who remained stable throughout the study; thus minimising bias on target biomarker level results. Furthermore, there was no difference in median exacerbation frequency between the exacerbation group and the stable group, which may indirectly indicate that airway microbial colonisation was not significantly different between the 2 groups.

Conclusions

We have established that levels of CRP, PCT and NE can be reliably and reproducibly measured in saliva, providing useful clinical information as blood. All 3 target salivary biomarkers increased during COPD exacerbations, with CRP and PCT correlating with patient-derived metrics. These findings provide the conceptual basis for the further development of salivary biomarkers, alongside PROs, for practical point-of-care monitoring of COPD and prediction of exacerbations.

Additional files

Additional file 1: Table S1. Post Review. The subject demographics and salivary biomarker profiles for the healthy non-smokers, healthy smokers and stable COPD subjects (n = 107).

Additional file 2: Wellbeing Diary & Life Impact Scores. The set of self-assessment questions on symptoms that was provided to and completed by each study subject.

Additional file 3: Table S2. Correlations of all COPD subjects (n = 98) symptom scores vs. salivary biomarker levels.

Additional file 4: Table S3. Correlations of all COPD subjects (n = 98) sputum metrics vs. salivary biomarker levels.

Additional file 5: Table S4. Correlations of all COPD subjects (n = 98) symptom scores vs. sputum metrics.

Additional file 6: Table S5. The subject demographics, salivary biomarker and symptom profiles for the same COPD subjects (n = 36) in stable and exacerbation phase.

Abbreviations

ADL: Activities of Daily Living; ANOVA: Analysis of variance; BMI: Body Mass Index; CAT: COPD Assessment Test; CI: Confidence Interval; COPD: Chronic Obstructive Pulmonary Disease; CRP: C-Reactive Protein; ELISA: Enzyme-linked immunoassay; EXACT: Exacerbations of chronic pulmonary disease tool; FEV1: Forced expiratory volume in 1 s; GOLD: Global initiative for chronic Obstructive Lung Disease; MRC: Medical Research Council; NE: Neutrophil Elastase; NS: Never-smokers (normal spirometry); PBS-T: Phosphate Buffered Salina - Tween; PCT: Procalcitonin; RPM: Revolutions per minute.; S: Smokers (normal spirometry); SD: Standard Deviation; SGRQ: St George's Respiratory Questionnaire

Competing interests

The authors declare that they have no significant conflicts of interest with any companies/organisations whose products or services may be discussed in this article.

Authors' contributions

MS oversaw all activities related to the conduct of the study and contributed to the study idea, and the discussion, writing and editing of the manuscript. NP contributed to the study idea, data collection, statistical analysis and the discussion, writing and editing of the manuscript. GT contributed to the study idea, discussion and editing of the manuscript. JB supervised all statistical analysis and contributed to the discussion of the results. NR contributed to the discussion and editing of the manuscript. All authors accepted the final version. All authors read and approved the final manuscript.

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