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2-Alkyl-4-quinolone quorum sensing molecules are biomarkers for culture-independent *Pseudomonas aeruginosa* burden in adults with cystic fibrosis

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Abstract

Introduction. *Pseudomonas aeruginosa* produces quorum sensing signalling molecules including 2-alkyl-4-quinolones (AQs), which regulate virulence factor production in the cystic fibrosis (CF) airways.

Hypothesis/Gap statement. Culture can lead to condition-dependent artefacts which may limit the potential insights and applications of AQs as minimally-invasive biomarkers of bacterial load.

Aim. We aimed to use culture-independent methods to explore the correlations between AQ levels and live *P. aeruginosa* load in adults with CF.

Methodology. Seventy-five sputum samples at clinical stability and 48 paired sputum samples obtained at the beginning and end of IV antibiotics for a pulmonary exacerbation in adults with CF were processed using a viable cell separation technique followed by quantitative *P. aeruginosa* polymerase chain reaction (qPCR). Live *P. aeruginosa* qPCR load was compared with the concentrations of three AQs (HHQ, NHQ and HQNO) detected in sputum, plasma and urine.

Results. At clinical stability and the beginning of IV antibiotics for pulmonary exacerbation, HHQ, NHQ and HQNO measured in sputum, plasma and urine were consistently positively correlated with live *P. aeruginosa* qPCR load in sputum, compared to culture. Following systemic antibiotics live *P. aeruginosa* qPCR load decreased significantly ($P<0.001$) and was correlated with a reduction in plasma NHQ (plasma: $r=0.463$, $P=0.003$).

Conclusion. In adults with CF, AQ concentrations correlated more strongly with live *P. aeruginosa* bacterial load measured by qPCR compared to traditional culture. Prospective studies are required to assess the potential of systemic AQs as biomarkers of *P. aeruginosa* bacterial burden.

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Abbreviations: AQs, 2-alkyl-4-quinolones; CF, cystic fibrosis; DNA, deoxyribonucleic acid; HHQ, 2-heptyl-4-hydroxyquinoline; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; IV, intravenous; LLOQ, lowest limit of quantification; NHQ, 2-nonyl-4-hydroxyquinoline; PMA, propidium monoazide; qPCR, quantitative polymerase chain reaction.
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One supplementary table and three supplementary figures are available with the online version of this article.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium extremely well adapted to the airway niche in people with cystic fibrosis (CF). *P. aeruginosa* is a dominant pathogen in the CF lung and is associated with increased morbidity and mortality in this population [1]. Treatment and eradication of *P. aeruginosa* is a challenge as it is intrinsically resistant to many classes of antibiotics, produces a host of virulence factors, and forms impenetrable biofilms in the CF airways [2].

P. aeruginosa controls the production of virulence factors using cell-to-cell communication mechanisms known as quorum sensing (QS) [3]. One of the *P. aeruginosa* QS systems is based on the production and sensing of 2-alkyl-4-quinolones (AQs), which control virulence in a population dependent manner [4–7]. These AQs are only produced by *P. aeruginosa* and several closely related species [8].

Our previous studies have shown that several AQs are detectable in the sputum, plasma and urine of adults with CF and chronic pulmonary *P. aeruginosa* [9, 10]. Concentrations of two AQs, HHQ (2-heptyl-4-hydroxyquinoline) and HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) were most strongly associated with *P. aeruginosa* load measured by routine sputum culture at clinical stability [10]. Following intravenous anti-pseudomonal antibiotics for a pulmonary exacerbation, the concentrations of NHQ (2-nonyl-4-hydroxyquinoline) and HHQ in the plasma declined significantly, but there was no change in *P. aeruginosa* load, measured by routine culture [9].

In this study, we compared *P. aeruginosa* load measured historically by culture with live *P. aeruginosa* load quantified by polymerase chain reaction (qPCR). By using a photoreactive dye, propidium monoazide (PMA), we were able to differentiate between viable and compromised *P. aeruginosa* cells. With this modification, only viable (live *P. aeruginosa*) cells are amplified and quantified during the qPCR process [11]. We aimed to explore whether three previously defined AQs; HHQ, NHQ, and HQNO correlated more strongly with culture-independent measures of live *P. aeruginosa* load compared to a culture-dependent method. We tested correlations of these three AQs detected in sputum, plasma, and urine with *P. aeruginosa* in the sputum at stability and in response to treatment for a pulmonary exacerbation.

METHODS

Participants and study design

Spontaneous sputum samples were obtained and stored from adults with CF who had previously participated in two AQ biomarker studies, details of which have been previously published [9, 10]. In the original studies, baseline demographic data were collected, and matched duplicate sputum plugs were frozen at −80 °C for future studies. In summary, spontaneous sputum, plasma and urine samples were obtained from 75 adults with CF at clinical stability and from 48 adults at the start and end of intravenous (IV) antibiotic treatment for pulmonary exacerbation, according to Rosenfeld criteria [12]. Adults aged 16 to 60 were recruited from two UK adult CF centres, who were known to have previously isolated *P. aeruginosa* from respiratory samples obtained during routine clinical practice. Spontaneous sputum, 8 ml venous blood and 25 ml urine samples were obtained at stability or within 72 h of the start and end of IV antibiotic therapy [9].

PMA-based qPCR analysis

Prior to DNA extraction, sputum samples were pre-treated with propidium monoazide (PMA) to penetrate dead/compromised bacterial cells and bind to DNA as described previously by Rogers *et al.* [13]. PMA (20 mM in water; Biotium, USA) was added to the samples to a final concentration of 50 µM followed by incubation in the dark on a rotating shaker for 30 min. This allowed the PMA molecules to penetrate only dead/compromised bacterial cells as they were impermeable to intact cell membrane. The PMA molecules were then fixed to DNA by 15 min exposure to LED blue light (IB-Applied Science, Spain). Upon exposure to PMA and light, permanent DNA modification was achieved by formation of stable covalent nitrogen-carbon bond, which prevented amplification (of DNA from dead/compromised cells) during qPCR analysis. Cells were pelleted at 10000 ***g*** for 5 min prior to DNA extraction.

DNA extraction was conducted using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich Co. Ltd., Dorset, UK) with the following modifications. Samples were initially mixed with lysozyme (200 µl; 45 mg ml^{-1}, Sigma-Aldrich Co. Ltd., Dorset, UK) suspended in Gram-Positive Lysis Solution (included in the kit) followed by insertion of glass beads. Cell disruption was then achieved by agitation in a Fastprep-24 Instrument (MP Biomedicals Europe, Illkirch, France) at 6.5 m s^{-1} for 60 s twice and subsequently incubated at 37 °C for 30 min. Further steps remained unchanged, and the DNA was resuspended in 50 µl of elution solution (included in the kit). DNA concentrations were quantified using the Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK). *P. aeruginosa*-specific qPCR assay was performed as described previously [14]. The lower limit of quantification (LLOQ) for *P. aeruginosa* in this assay was 100 c.f.u. g^{-1} of sputum aliquot. Quantitative values were generated by Rotor Gene Q-series software (Qiagen, Crawley, UK) and expressed in c.f.u. g^{-1} of sputum aliquot.

Sample processing for quantitative culture and AQ analyses

All sample processing for quantitative culture, differential cell counts and AQ analyses was performed in the initial AQ biomarker studies as previously described [9, 10]. Prepared clinical samples for AQ analyses were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Ortori *et al.* [15]. Further information regarding the methodology of AQ analyses and the calculated lower limits of quantification (LLOQ) are provided in the online supplementary material. To reduce multiple hypotheses

Table 1. Baseline demographics of participants at clinical stability (*n*=75) and exacerbation (*n*=48)

Variable	Baseline
Clinical stability cohort	*n*=75
Age in years: median (range)	38.6 (17.8 to 61.5)
Gender, males (%)	49 (65.3)
FEV_1 % predicted: mean (SD)	55 (±18)
Absolute FEV_1 in L: mean (SD)	2.0 (±0.8)
BMI: mean (SD)	22.9 (±3.3)
P. aeruginosa status by culture at baseline: n (%)	
Never	0 (0)
Free	1 (1.3)
Intermittent	2 (2.7)
Chronic	72 (96)
Exacerbation cohort	*n*=48
Age in years: median (range)	27.5 (17 to 59)
Gender, males (%)	27 (56.3)
FEV_1 % predicted: mean (SD)*	46.6 (±16)
Absolute FEV_1 in L: mean (SD)*	1.7 (±0.7)
BMI: mean (SD)	21.9 (±3.9)
Diagnostic microbiology results: n (%)	
P. aeruginosa isolated only:	33 (68.8)
P. aeruginosa co-infection with:	11 (22.9)
MSSA	4 (36.4)
MRSA	1 (9.1)
Haemophilus influenza and *MSSA*	1 (9.1)
Aspergillus fumigatus and *MSSA*	1 (9.1)
Candida albicans	4 (36.4)
No *P. aeruginosa* isolated:	4 (8.3)
MSSA	1 (25)
MRSA	2 (50)
Respiratory commensals	1 (25)

*Spirometry at exacerbation. *P. aeruginosa* status of participants defined by *Leeds criteria* [22].
n, number of participants with data available; SD, standard deviation.

testing, only the three AQs which were most strongly associated with *P. aeruginosa* burden from the previous study were analysed [9, 10]: HHQ (2-heptyl-4-hydroxyquinoline), NHQ (2-nonyl-4-hydroxyquinoline) and HQNO (2-heptyl -4-hydroxyquinoline-*N*-oxide).

Statistical analysis

Any value that was under the LLOQ was designated as undetected. AQ levels and *P. aeruginosa* qPCR loads were analysed after the addition of one to each value followed by $\log_{10}$ transformation to scale the data for interpretation. For analysis of absolute difference between two time points (at the beginning and end of IV antibiotics for pulmonary exacerbation), no log transformation was performed on the values. When paired t-tests were used, normality of the data distribution was assessed both visually and using Shapiro-Wilk Test of Normality.

Comparisons of FEV_1 measurements, sputum neutrophil concentrations, *P. aeruginosa* load measured by qPCR and *P. aeruginosa* load measured by culture at the beginning and end of IV antibiotics were analysed using paired t-tests. Quantitative loads of *P. aeruginosa* from culture and qPCR were assessed using Spearman's rank correlations, as well as sputum *P. aeruginosa* qPCR data with HHQ, NHQ and HQNO measured in blood, sputum and urine. Correlation coefficients of *P. aeruginosa* measured by culture and qPCR were transformed from r values to z scores using Fisher Z transformation to identify whether the z test statistic was outside the critical value, defined using two-tailed alpha 0.05 [16]. Post-IV antibiotics changes in absolute FEV_1 measurements, *P. aeruginosa* load (qPCR and culture) and AQ levels were calculated using the values at the end of IV antibiotics minus the values at the beginning of IV antibiotics. All statistical analyses were performed using Stata SE15 statistical software (Texas, USA).

RESULTS

Sputum aliquots from 75 adults at clinical stability and 48 adults at the start and end of IV antibiotic treatment were available and subsequently thawed and processed for qPCR analysis. Based on the 16S rRNA gene sequencing data we obtained, *Burkholderia* sp. and *Acinetobacter* sp. were not present in any of the samples.

Baseline demographics of participants included in the analysis at clinical stability and exacerbation are summarised in Table 1.

Individual AQ molecules are associated with live *P. aeruginosa* qPCR load at clinical stability compared to *P. aeruginosa* culture load

At clinical stability, there was no correlation between live *P. aeruginosa* load measured by qPCR and the load detected by culture (r=0.174, *P*=0.166, Fig. S1, available in the online version of this article). All of the three AQs (HHQ, NHQ, HQNO) consistently correlated with live *P. aeruginosa* load detected by qPCR in all sample types (sputum, plasma and urine, Fig. 1). The strongest relationship with live *P. aeruginosa* qPCR load was observed with plasma HHQ (r=0.550, r^2=0.30, *P*<0.001, Fig. 1).

In contrast, *P. aeruginosa* quantitative load measured by culture only correlated with HHQ and NHQ measured in sputum (r=0.381, *P*=0.002; r=0.296, *P*=0.017) and urine (r=0.264, *P*=0.034; r=0.322, *P*=0.012), respectively (Table 2). No correlations were observed with any AQs measured in plasma. Comparison of the correlation coefficients of *P.*

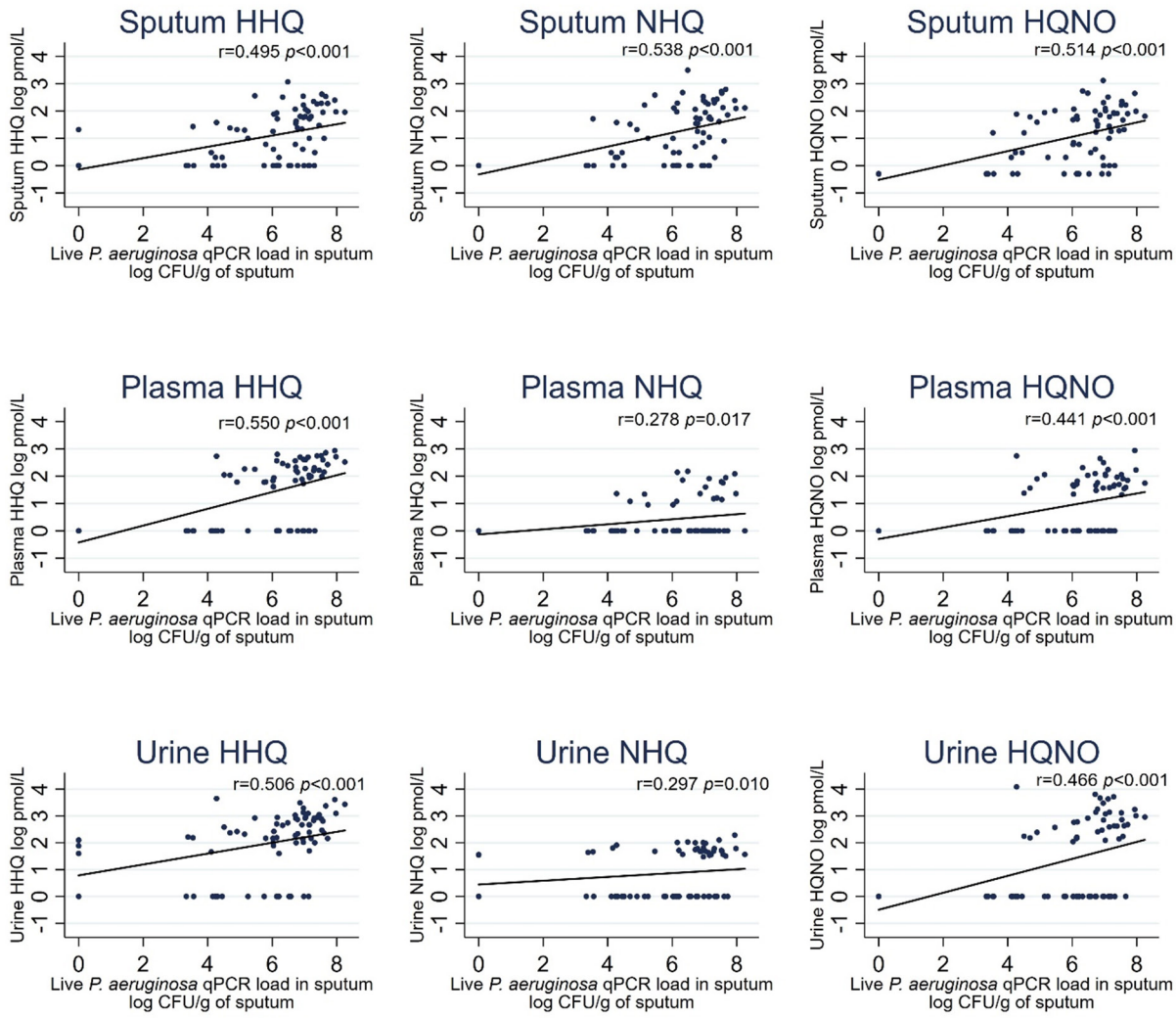

Fig. 1. Correlations of HHQ, NHQ and HQNO detected in sputum, plasma and urine with live *P. aeruginosa* load by qPCR in sputum during clinical stability. Median (interquartile range) for sputum live *P. aeruginosa* load by qPCR was 3.3×10^{6} c.f.u. g^{-1} (1.4×10^{5}–1.4×10^{7} c.f.u. g^{-1}) of sputum. HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline. HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide; Sputum, *n*=75; plasma, *n*=74; urine, *n*=75, where n is the number of participants with data available for statistical analysis. Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol l^{-1}), NHQ (10, 10 pmol l^{-1}) and HQNO (30, 30 pmol l^{-1}). No LLOQ was defined for sputum AQs, The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells g^{-1} of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation.

aeruginosa load measured by qPCR against culture demonstrated significantly higher correlations on qPCR compared to culture in sputum NHQ and HQNO, plasma HHQ and HQNO, and urine HHQ and HQNO (Table 2).

Clinical and microbiological changes in response to treatment for a pulmonary exacerbation

There was a significant correlation between live *P. aeruginosa* load measured by qPCR and the load detected by culture at the beginning of IV antibiotics for pulmonary exacerbation (r=0.520, *P*=<0.001). Correlation was not observed when IV antibiotics were stopped (r=0.258, *P*=0.083, Fig. S1).

Following treatment for a pulmonary exacerbation, the mean absolute FEV_1 increased from 1.68 L (SD ±0.68) at the beginning of IV antibiotics to 1.96 L (SD ±0.82) at the end of IV antibiotics, (*P*=<0.0001). Mean percent (%) predicted

Table 2. Correlations of HHQ, NHQ and HQNO in sputum, plasma and urine with live *P. aeruginosa* loads measured by qPCR at clinical stability compared to historical quantitative *P. aeruginosa* load by culture

		Sputum	Plasma	Urine
	AQ	**Spearman's Correlation Coefficient for qPCR, r (*P*-value)+**		
qPCR	**HHQ**	0.495 (<0.001)*	0.550 (<0.001)*	0.506 (<0.001)*
	NHQ	0.538 (<0.001)*	0.278 (0.017)*	0.297 (0.010)*
	HQNO	0.514 (<0.001)*	0.441 (<0.001)*	0.466 (<0.001)*
		Spearman's Correlation Coefficient for Culture, r (*P*-value)β		
Culture	**HHQ**	0.381 (0.002)*	0.232 (0.065)	0.264 (0.034)*
	NHQ	0.296 (0.017)*	0.121 (0.342)	0.311 (0.012)*
	HQNO	0.203 (0.104)	0.169 (0.183)	0.197 (0.115)
		Fisher Z transformation qPCR vs Culture (*P*-value)†		
qPCR vs culture	**HHQ**	0.818 (0.207)	2.187 (0.014)*	1.661 (0.048)*
	NHQ	1.708 (0.044)*	0.940 (0.174)	−0.088 (0.465)
	HQNO	2.090 (0.018)*	1.733 (0.042)*	1.760 (0.039)*

Median (interquartile range) for sputum live *P. aeruginosa* load by qPCR was 3.3×10^6 c.f.u.g^{-1} (1.4×10^5–1.4×10^7 c.f.u.g^{-1}) of sputum. Median (interquartile range) for sputum *P. aeruginosa* load by culture was 1.2×10^7 c.f.u.g^{-1} (2.8×10^6–3.28×10^7 c.f.u.g^{-1}) of sputum. Analysis with qPCR: Sputum, *n*=75; plasma, *n*=74; urine, *n*=75, Analysis with Culture: Sputum, *n*=65; plasma, *n*=64; urine, *n*=65 where n is the number of patients with samples available for analysis. Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol l^{-1}), NHQ (10, 10 pmol l^{-1}) and HQNO (30, 30 pmol l^{-1}). No LLOQ was defined for sputum AQs. The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells g^{-1} of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation.
*Significant at *P*<0.05.
†γ Z test statistic for difference between spearman rho values.
AQ, 2-Alkyl-4-quinolone; HHQ, 2-heptyl-4-hydroxyquinoline; NHQ, 2- nonyl-4-hydroxyquinoline; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide.

FEV_1 increased from 46.6% (SD ±16) to 52.4% (SD ±17.8), (*P*=<0.0001, Table S1).

The mean number of neutrophils detected in sputum significantly reduced following IV antibiotic treatment for a pulmonary exacerbation, from 7.15 log cells g^{-1} (SD ±0.37) to 6.9 log cells g^{-1} (SD ±0.38), (*P*<0.001). Mean *P. aeruginosa* load detected by culture showed no significant change between the beginning of IV antibiotics; 6.59 $\log_{10}$CFU g^{-1} (SD ±1.83) and the end of IV antibiotics; 6.67 $\log_{10}$CFU g^{-1} (SD ±1.82), (*P*=0.84). Mean live *P. aeruginosa* load measured by qPCR showed a significant reduction from 6.40 $\log_{10}$CFU g^{-1} (SD ±1.52) at the beginning of IV antibiotics, to 5.71 $\log_{10}$CFU g^{-1} (SD ±1.97) at the end of IV antibiotics (*P*=<0.001, Table S1).

Changes in absolute FEV_1 measurements and live *P. aeruginosa* measured by qPCR were negatively correlated (r=−0.415, *P*=0.004, Fig. S2). There was no correlation between changes in absolute FEV_1 and *P. aeruginosa* measured by culture (r=0.115, *P*=0.473, Fig. S2).

HHQ and NHQ concentrations are associated with live *P. aeruginosa* PCR load before and after pulmonary exacerbation

At the start of pulmonary exacerbation HHQ, NHQ and HQNO concentrations measured in sputum, plasma and urine showed positive correlations with live *P. aeruginosa* load measured by qPCR (*P*=<0.001, Table 3). The strongest correlation was with sputum NHQ (r=0.736, r^2=0.54, *P*<0.001). *P. aeruginosa* load measured by qPCR are consistent with *P. aeruginosa* load measured previously by culture with the exception of urinary HQNO. Correlations between HHQ and NHQ measured in the urine were significantly stronger with *P. aeruginosa* qPCR when compared to culture (Table 3).

At the end of antibiotic treatment, HHQ levels were positively correlated with live *P. aeruginosa* load measured by qPCR in all sample types (sputum r=0.527, *P*<0.001; plasma r=0.430, *P*<0.001; urine r=0.509, *P*<0.001, Table 4). Significant correlations of live *P. aeruginosa* load measured by qPCR were observed in sputum NHQ (r=0.554, *P*=0.001), sputum HQNO (r=0.583 *p*<0.001) and urine HQNO (r=0.411 *P*=0.004) but not in plasma (Table 4). These findings are consistent with previous *P. aeruginosa* loads measured in culture with the exception of plasma HHQ and urine HQNO which showed significant correlations with qPCR load but not culture.

Changes in live *P. aeruginosa* load measured using qPCR positively correlated with changes in NHQ signal concentration in plasma

Further analyses were performed to investigate the relationship between absolute changes of live *P. aeruginosa* measured by qPCR with changes in HHQ, NHQ and HQNO concentrations following IV antibiotics for a pulmonary exacerbation. A positive correlation was observed between change in plasma NHQ following systemic antibiotics and changes in live *P. aeruginosa* loads measured by qPCR in sputum (r=0.422, *P*=0.003, Table 4, Fig. S3). No other significant correlations were found between differences in absolute live *P. aeruginosa*

Table 3. Correlations of HHQ, NHQ and HQNO concentrations in sputum, plasma and urine with sputum live *P. aeruginosa* load measured using qPCR at the start and end of IV-antibiotic treatment for a pulmonary exacerbation compared to historical culture data

	Start of IV antibiotics†			End of IV antibiotics†		
	qPCR	Culture	qPCR vs culture	qPCR	Culture	qPCR vs culture
AQ	**Spearman's Correlation Coefficient, r (*P*-value)**		**Z (*P*-value)‡**	**Spearman's Correlation Coefficient, r (*P*-value)**		**Z (*P*-value)‡**
HHQ						
Sputum	0.626 (<0.001)*	0.522 (<0.001)*	0.725 (0.234)	0.527 (<0.001)*	0.345 (0.019)*	1.059 (0.145)
Plasma	0.524 (<0.001)*	0.546 (<0.001)*	−0.141 (0.444)	0.430 (0.003)*	0.121 (0.430)	1.570 (0.058)
Urine	0.607 (<0.001)*	0.306 (0.041)*	1.810 (0.035)*	0.509 (<0.001)*	0.295 (0.047)*	1.207 (0.114)
NHQ						
Sputum	0.736 (<0.001)*	0.532 (<0.001)*	1.627 (0.052)	0.554 (<0.001)*	0.297 (0.045)*	1.486 (0.069)
Plasma	0.452 (0.001)*	0.314 (0.038)*	0.746 (0.228)	0.234 (0.113)	0.103 (0.500)	0.625 (0.228)
Urine	0.636 (<0.001)*	0.350 (0.019)*	1.799 (0.036)*	0.248 (0.090)	0.216 (0.150)	0.157 (0.438)
HQNO						
Sputum	0.600 (<0.001)*	0.431 (0.003)*	1.082 (0.140)	0.583 (<0.001)*	0.294 (0.047)*	1.707 (0.044)*
Plasma	0.577 (<0.001)*	0.479 (0.001)*	0.630 (0.264)	0.206 (0.166)	0.115 (0.453)	0.432 (0.333)
Urine	0.524 (<0.001)*	0.273 (0.070)	1.404 (0.273)	0.411 (0.004)*	0.222 (0.138)	0.988 (0.162)

†Median (interquartile range) for sputum live *P. aeruginosa* load was 8×10^6 c.f.u.g^{-1} (3×10^3–2×10^8 c.f.u.g^{-1}) at pre-antibiotics and 2×10^6 c.f.u.g^{-1} (1×10^3–7×10^7 c.f.u.g^{-1}) at post-antibiotics. ‡AQ: 2-Alkyl-4-quinolone, HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline.HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide. qPCR: Sputum, *n*=48; plasma, *n*=47; urine, *n*=48. Culture: Sputum, *n*=45; plasma, *n*=44; urine, *n*=45, (where n is the number of patients with samples available for analysis). Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol l^{-1}), NHQ (10, 10 pmol l^{-1}) and HQNO (30, 30 pmol l^{-1}). No LLOQ was defined for sputum AQs. The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells g^{-1} of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation. ‡ɣ Z test statistic for difference between spearman rho values. *Significant comparison at $P<0.05$

qPCR load and changes in NHQ, HHQ or HQNO concentrations (NHQ in sputum and urine; HHQ and HQNO in sputum, plasma and urine). There were no correlations with changes in *P. aeruginosa* measured by culture and changes with any AQ in all sample types (Table 4).

DISCUSSION

This is the first study to explore systemic measurements of AQ quorum sensing molecules as biomarkers of live *P. aeruginosa* load measured by qPCR in the sputum in adults with CF. Overall, there were stronger correlations between AQ concentrations and qPCR measures of bacterial load compared to previous culture methodology which is likely to be less standardised therefore contributing to higher levels of measurement error. In contrast to *P. aeruginosa* load measured by culture at clinical stability, qPCR *P. aeruginosa* load showed consistent correlations with HHQ, NHQ and HQNO measured in sputum, plasma and urine. At the beginning of IV antibiotics for pulmonary exacerbation, HHQ and NHQ in sputum, plasma and urine were positively correlated with *P. aeruginosa* load measured by both qPCR and culture. In addition, absolute change of sputum *P. aeruginosa* load measured by qPCR were reflected by an absolute change in plasma NHQ between the start and end of IV antibiotics for pulmonary exacerbation.

We found no consistent correlations between culture and qPCR measures of *P. aeruginosa* load during stability or following IV antibiotic treatment, although correlations were observed at the beginning of IV antibiotics for a pulmonary exacerbation. The *P. aeruginosa* load measured by qPCR reduced significantly after antibiotic treatment whilst there was no significant difference using culture. It is possible that qPCR assays may be more sensitive at detecting *P. aeruginosa* compared to culture [17]. PMA-qPCR may more accurately quantify viable uncultivable bacterial cells which are metabolically dormant and therefore not detected by culture, as suggested by Deschaght *et al.* [18] demonstrating the potential strengths of PMA-qPCR compared to culture. This theory supports our observations that the correlation between qPCR quantification of *P. aeruginosa* load and AQs were stronger compared to culture. However, we found higher levels of *P. aeruginosa* detected by culture compared to qPCR quantification. It is possible that the freeze-thaw cycle may have reduced the viable *P. aeruginosa* in the sputum samples that underwent qPCR analysis. Alternatively, quantification by culture may be subject to individual operator error and inaccuracy [19].

In our study, only *P. aeruginosa* load measured by qPCR was correlated with improvements in absolute FEV_1 following treatment for a pulmonary exacerbation, and not culture. There are conflicting data in the literature regarding the relationship between culture and culture-independent quantitative *P. aeruginosa* load measurements with CF related outcomes [18, 20–22]. For example, McLaughlin *et al.* showed no correlation between

Table 4. Correlations between changes in sputum, plasma and urine AQ concentrations with live *P. aeruginosa* load changes after IV antibiotic treatment

Changes post-IV antibiotics	qPCR	Culture	qPCR vs culture
AQ	**Spearman's Correlation Coefficient, r (*P*-value)**		**Z (*P*-value)†**
HHQ			
Sputum	0.252 (0.083)	0.266 (0.085)	−0.065 (0.474)
Plasma	0.276 (0.061)	0.285 (0.067)	−0.047 (0.481)
Urine	0.182 (0.215)	−0.042 (0.787)	1.044 (0.148)
NHQ			
Sputum	0.237 (0.104)	0.202 (0.193)	0.169 (0.433)
Plasma	0.422 (0.003)*	0.230 (0.143)	0.917 (0.180)
Urine	0.274 (0.059)	0.082 (0.602)	0.982 (0.163)
HQNO			
Sputum	0.230 (0.115)	0.148 (0.345)	−0.001 (0.499)
Plasma	0.125 (0.404)	0.125 (0.431)	0.395 (0.346)
Urine	0.205 (0.163)	−0.109 (0.488)	1.457 (0.073)

Median (interquartile range) for sputum live P. aeruginosa qPCR load was 8 x 10^6 c.f.u. g^{-1} (5×10^5–3×10^7 c.f.u. g^{-1}) at pre-antibiotics and 2×10^6 c.f.u. g^{-1} (2×10^5–1×10^7 c.f.u. g^{-1}) post-antibiotics. Median (interquartile range) for culture *P. aeruginosa* load was 7 x 10^6 c.f.u. g^{-1} (8×10^5–6×10^7 c.f.u. g^{-1}) at pre-antibiotics and 1×10^7 c.f.u. g^{-1} (2×10^6–7×10^7 c.f.u. g^{-1}) post-antibiotics. HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline. HQNO: 2-heptyl-4-hydroxyquinoline-N-oxide. qPCR: Sputum, *n*=48; plasma, *n*=47; urine, *n*=48, Culture: Sputum, *n*=43; plasma, *n*=42; urine, n=48, (where n is the number of patients with samples available for analysis) Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol l^{-1}), NHQ (10, 10 pmol l^{-1}) and HQNO (30, 30 pmol l^{-1}). No LLOQ was defined for sputum AQs. The LLOQ for P. aeruginosa measured by qPCR was 100 cells g^{-1} of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation. †ɣ Z test statistic for difference between spearman rho values. **P* value <0.05.

lung function improvement and a reduction of *P. aeruginosa* load measured by culture after pulmonary exacerbation [22]. However, other studies have shown a significant reduction in culture-dependent and culture-independent measures of *P. aeruginosa* load after treatment for pulmonary exacerbation, and these correlated significantly with lung function improvement [18, 23]. These variable observations are likely to be a result of different methodological approaches in measuring the quantitative load of *P. aeruginosa*. Here, we further demonstrate the dynamic relationship of AQs with longitudinal changes in quantitative *P. aeruginosa* load, which may offer novel opportunities to monitor bacterial burden during clinical stability and acute exacerbation in CF.

Strengths of this study include the large multicentre participant cohort and the pre-treatment of sputum with PMA which enabled only viable *P. aeruginosa* bacterial cells to be amplified and quantified. The sputum samples were collected and processed in line with conventional practice following a rigorous protocol, thereby reducing any variance in the signals generated by random error. In addition, the samples were homogenised and divided into aliquots before storage to avoid multiple freeze-thawing and loss of sample integrity. Furthermore, participants known to have previously isolated *Burkholderia cepacia complex* were excluded from the study as these organisms can also produce AQs [9, 10]. To limit multiple comparisons of different AQs, we assessed the three that were found to have the most promising biomarker potential from previous studies: HHQ, NHQ, HQNO [9, 10], however the type 1 error rate was not controlled.

There are a number of limitations in this study that should be considered when interpreting these data. Duplicate aliquots of sputum were frozen and thawed to perform *P. aeruginosa* qPCR analysis after the time-point of the quantification by culture. Therefore, this exposure to a longer period of frozen storage may limit direct comparisons between the burden of *P. aeruginosa* as measured by qPCR analysis and that quantified by culture colony forming units. This may account for the lack of correlation between the two different measures of P. aeruginosa load at clinical stability. *P. aeruginosa* bacterial load measured using qPCR accounted for up to 30% of the biomarker variability in AQs at clinical stability and 54% at pulmonary exacerbation. Variability in AQ concentrations that was not completely attributable to *P. aeruginosa* load may limit its potential clinical use as a marker of specific bacterial burden in clinical practice. This wide range of variability may be attributable to additional factors not measured in this study that could influence AQ concentration including oxygen gradients and nutrient availability in the lung environment resulting in heterogeneous conditions, which may lead to variability in sputum AQ concentrations [24]. Furthermore, the distribution, half-life and clearance of AQs in different sample types *in vivo* is not known. For example, the collection of urine samples used a random 'catch' method that may be associated with random error in the concentration of molecules being measured. To address the potential of urinary AQs as biomarkers, future studies should correct for differential

dilution with measurement of urinary creatinine, which may enhance the sensitivity to detect effects that were promising but non-significant in the limited longitudinal sample. Whilst analyses do not adjust for demographic or clinical factors, we reduced the effect of between-person variability in paired tests. Lastly, this was a retrospective study limited to adults with CF, further studies are needed to determine whether these findings are applicable to adults without CF and the wider CF population, including the paediatric CF population. Despite these limitations, there were associations between *P. aeruginosa* bacterial load in the sputum and systemic AQ concentrations in plasma and random urine samples, suggesting they may be useful in clinical practice as semi-quantitative, minimally-invasive biomarkers of infection.

Further investigation is needed to determine the relationship between AQs and bacterial burden in newly acquired *P. aeruginosa* pulmonary infection, to determine its potential as a screening tool for early infection. A minimally-invasive diagnostic test for *P. aeruginosa* would be especially useful in young children who cannot always expectorate sputum and may avoid the need for more invasive tests such as bronchoalveolar lavage. In addition, surrogate systemic measures of *P. aeruginosa* burden may prove increasingly useful in the era of highly effective CTFR modulator therapy where spontaneous sputum production is likely to decrease in the longer term [25].

In summary, dynamic changes in the bacterial load of *P. aeruginosa* objectively measured by qPCR were sensitive to the clinical course of exacerbation and intervention, which were not apparent from culture methods. AQs concentrations were more strongly correlated with culture independent methods of *P. aeruginosa* compared to culture dependent techniques. HHQ, NHQ and HQNO measured in sputum, plasma and urine were positively correlated with *P. aeruginosa* load measured by qPCR in sputum at pulmonary exacerbation and at clinical stability. In addition, changes in plasma NHQ reflect a reduction in *P. aeruginosa* after IV antibiotic treatment for pulmonary exacerbation, which has not been previously demonstrated. Further prospective investigation should seek to understand the sources of clinical variability in systemic AQ quorum sensing molecules, and confirm whether detection in plasma or urine can be reliably used as a minimally-invasive biomarker of *P. aeruginosa* load in people with CF.

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Author contributions
All included authors fulfil the criteria of authorship; N.M.Z. and K.W., are joint first authors for this manuscript. N.M.Z., K.W., H.L.B., A.F., M.C., I.S. and K.D.B., had substantial contributions to the study design. The qPCR analysis was performed by N.M.Z., the Q.S., analysis was performed by N.H. The statistical analysis was performed by N.M.Z., K.W. and I.S. All authors contributed to data interpretation, data presentation and drafting of the manuscript. All authors approved the final version of the manuscript.

Conflicts of interest
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Ethical statement
This study was approved by the Nottingham Research Ethics Committee 1(09/H0407/1).

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