Clinical significance of *Pseudomonas aeruginosa* 2-alkyl-4-quinolone quorum sensing signal molecules for long-term outcomes in adults with cystic fibrosis.

Karmel Webb[a], Andrew Fogarty[a], David A. Barrett[b], , Edward F. Nash[c], Joanna L Whitehouse[c], Alan R. Smyth[d], Iain Stewart[e] Alan Knox[e], Paul Williams[f], Nigel Halliday[f], Miguel Cámara[f], Helen L. Barr[g]

[a] *Division of Epidemiology and Public Health, University of Nottingham, City Hospital Campus, Nottingham, UK*

[b] *Centre for Analytical Bioscience, Division of Advanced Materials and Healthcare Technologies, School of Pharmacy, University of Nottingham, Nottingham, UK*

[c] *West Midlands Adult CF Centre, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK*

[d] *Division of Child Health, Obstetrics and Gynaecology, University of Nottingham, Nottingham, UK*

[e] *Division of Respiratory Medicine, University of Nottingham, City Hospital Campus, Nottingham, UK*

[f] *National Biofilms Innovation Centre, Centre for Biomolecular Sciences, School of Life Sciences, University of Nottingham, Nottingham, UK*

[g] *Wolfson Cystic Fibrosis Centre, Department of Respiratory Medicine, Nottingham University Hospitals NHS Trust, Nottingham, UK*

Nottingham NIHR Biomedical Research Centre, Nottingham MRC Molecular Pathology Node

Correspondence and requests for reprints to Dr Karmel Webb: karmel.webb@nottingham.ac.uk

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Abstract

Introduction

Pseudomonas aeruginosa is an important respiratory pathogen in cystic fibrosis (CF), which is associated with an accelerated decline in lung function, frequent pulmonary exacerbations and increased mortality. *P. aeruginosa* produces intercellular signalling molecules including 2-alkyl-4-quinolones (AQs), which regulate virulence factor production and biofilm formation in the CF airways. Studies have shown that AQs are detectable in the sputum and plasma of adults with CF and chronic pulmonary *P. aeruginosa.*

Aim

We tested the hypothesis that the presence of six AQs in plasma or sputum obtained from adults with CF was associated with long term adverse clinical outcomes.

Methodology

We analysed clinical data over an 8-year follow period for 90 people with CF who had previously provided samples for AQ analysis at clinical stability.

The primary outcome was all cause mortality or lung transplantation. Secondary outcomes were rate of lung function decline and number of intravenous (IV) antibiotic days for pulmonary exacerbations.

Results

There was no statistical association between the presence of any of the six measured AQs and the primary outcomes or the secondary outcome of decline in lung function. One of the 6 AQs was associated with IV antibiotic usage. The presence of 2-nonyl-3-hydroxy-4(1*H*)-quinolone (C9-PQS) in sputum was associated with an increase in the number of IV antibiotic days in the follow up period (Mann-Whitney; p=0.011).

Conclusion

Further investigation to confirm the hypothesis that C9-PQS may be associated with increased antibiotic usage for pulmonary exacerbations is warranted as AQ-dependent signalling is a potential future target for anti-virulence therapies.

Introduction

Pseudomonas aeruginosa is a highly successful opportunistic gram-negative bacterium which is well adapted to the airway niche in cystic fibrosis (CF). *P. aeruginosa* is the dominant pathogen in the CF lung and is associated with increased morbidity and mortality in this population [1]. *P. aeruginosa* 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 these virulence factors using a cell-to-cell communication known as quorum sensing (QS) [3]. This allows the whole bacterial population to sense and respond to changes in environmental stimuli and to coordinate gene expression of the community as a whole.

The *P. aeruginosa* QS system consists of 3 interlinking QS circuits, one of which is the *pqs* QS circuit. The *pqs* QS systems uses multiple 2-alkyl-4 quinolones (AQs) as signal molecules, including the pseudomonas quinolone signal (PQS) molecule (2-heptyl-3-hydroxy-4(1*H*)-quinolone) and its precursor HHQ (2-heptyl-4-hydroxyquinoline) [4]. Both PQS and HHQ act as autoinducers to increase AQ biosynthesis [5] . In addition, PQS is crucial for the production of virulence factors and biofilm formation both *in vitro* and in animal models of infection [5]. *P. aeruginosa* produces over 50 AQs, and the roles of many of these AQs are not yet fully understood [6].

Several AQs are detectable in the sputum and plasma of adults with CF and chronic pulmonary *P. aeruginosa*. Higher systemic concentrations of several AQs are associated with higher *P. aeruginosa* loads and lower lung function, in cross section analyses [7, 8]. This suggests that high systemic AQ levels may be associated with an adverse prognosis. In addition, systemic concentrations of several AQs decrease following intravenous anti-pseudomonal antibiotics, [8] suggesting they have potential as biomarkers of change in clinical status.

We hypothesised that higher levels of AQs in the sputum and plasma of adults with CF would be associated with adverse long term clinical outcomes in this patient population.

We investigated whether detection of six individual AQs in the sputum and plasma of people with CF could be linked to long-term outcomes, including death or lung transplantation, as well as annual rate of lung function decline and intravenous (IV) antibiotic use for pulmonary exacerbations. Clinical

data over an 8 year period was retrospectively collected on 90 adults with CF who had previously participated in an AQ biomarker study [7].

Methods

Participants

We studied 90 adults with CF who had previously participated in an AQ biomarker study, the full details of which were previously published [7]. In summary, participants were recruited at clinical stability from two UK adult CF centres between the years 2009 and 2011. Baseline demographic data and data on six AQs measured in both sputum and plasma samples were used [7].

Study design

Follow up clinical data were retrospectively obtained from the UK CF registry. Annual data were collected from the participants from the year of recruitment to the end of the study period in 2017. Data on death, lung transplantation, lung function and the number of IV antibiotic days for pulmonary exacerbations were obtained. The number of IV antibiotic days was measured annually from the year of recruitment to the end of 2017. For lung function data, the best recorded forced expiratory volume in 1 s (FEV_1) of the preceding year was used.

The primary outcomes were death or lung transplantation during the follow up period. Secondary outcomes were the number of IV antibiotic days for pulmonary exacerbations and rate of decline in FEV_1.

Sample processing and AQ analysis

All sample processing and AQ analyses were performed in the initial study as previously described [7]. Sputum plugs were harvested for quantitative AQ analyses [9, 10]. Venous blood samples were centrifuged at 1000 *g* for 15 min at 4°C, plasma was then separated and snap frozen in liquid nitrogen. Sputum samples for AQ analysis were extracted using acidified ethyl acetate (Fisher Chemicals, Loughborough, UK) [9, 10]. Plasma samples were extracted by solid phase extraction and plasma matrix matched samples from a healthy volunteer donor were prepared to allow calibration of samples.

Prepared clinical samples for AQ analyses were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Ortori *et al* 2011 [9]. All samples were analysed once using LC-MS/MS with no replicate analysis performed.

The lower limit of quantification (LLOQ) was established by using serial dilutions of the analyte mix and spiking into blank plasma samples prior to extraction and analysis, The LLOQ for plasma was defined as the analyte concentration at which a signal/noise ratio of 10:1 was achieved. In the absence of blank sputa to produce matrix matched calibration, 1.0mL aliquots of 0.9% NaCl were used and there was no LLOQ defined for sputum samples. A total of six AQs were analysed individually: HHQ (2-heptyl-4-hydroxyquinoline), NHQ (2-nonyl-4-hydroxyquinoline), PQS (2-heptyl-3-hydroxy-4(1*H*)-quinolone), C9-PQS (2-nonyl-3-hydroxy-4(1*H*)-quinolone), HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) and NQNO (2-nonyl-4-hydroxyquinoline-*N*-oxide). Calculated LLOQs in plasma samples were as follows: HHQ 10 pmol/L; NHQ 10 pmol/L; HQNO 30 pmol/L, NQNO 40 pmol/L; PQS 100 pmol/L and C9-PQS 100 pmol/L [7]. Quantitative concentrations for the six AQs both in plasma and sputum are summarised in Supplementary Table 2.

Statistical Analysis

The six measured AQs were analysed individually and combined detectable AQ levels in sputum and plasma were calculated.

For initial analyses, individual AQ levels were classified as detectable or undetectable (concentrations above or below the LLOQ respectively). A binary measure of combined AQs was defined as a detection of at least one individual AQ in sputum or plasma respectively. Two-sample Wilcoxon rank-sum (Mann-Whitney) tests and Pearson's chi-squared tests were used to assess binary AQ levels with the primary and secondary clinical outcomes.

If significant associations were obtained using binary AQ analyses, further quantitative analyses were performed. Individual AQ concentrations were then compared with clinical outcomes using Spearman rank correlation coefficients. Statistical significance was assessed as $p<0.05$. All data were analysed using Stata SE15 statistical software (Texas, USA).

Results

Of the 90 participants in the original study, 7 were lost during follow up and therefore 83 participants were included in the analyses. Baseline characteristics are summarised in Table 1.

The median follow up period was 6.3 years (IQR 5.6 to 6.7 years). During the follow up period, 23 participants (27%) either died or had bilateral lung transplantation. Three people had a bilateral lung transplant and subsequently died during the follow up period. These characteristics are summarised in Table 2.

Table 1. Baseline clinical characteristics and *P. aeruginosa* status of participants.

Variable	Baseline (n=83)
Nottingham University Hospitals NHS trust	42
University Hospitals Birmingham NHS Foundation Trust	41
Age in years: median (range)	28.4 (17.8 to 61.5)
Gender, males (%)	54 (65.1)
FEV_1 % predicted: mean (SD)	58 (±20)
Absolute FEV_1 in L: mean (SD)	2.13 (±0.9)
BMI: mean (SD)	22.9 (± 3.3)
P. aeruginosa status at baseline: n (%)	
Never	0 (0)
Free	1 (1.2)
Intermittent	2 (2.4)
Chronic	80 (96.4)

n = number of participants with data available; SD = standard deviation
P. aeruginosa status of participants defined by Leeds criteria [11]

Table 2. Summary of clinical data during follow up period.

Variable	N	(%)	Outcome
Follow up time¥	83	100.0%	6.3 (5.6-6.7)
Number of deaths/lung transplantation	23	27.7%	
Died during follow up	15	18.1%	
Rate of decline per year§:			
Absolute FEV_1 (ml)	80	96.4%	53.1 (55.5)
Percent predicted FEV_1 (%)	80	96.4%	1.6 (2.0)
Number of IV antibiotic days per year¥:			
Overall	81	97.6%	37.5 (16.4-58.7)
No death/transplant	59	71.1%	31.9 (13.0-43.7)
Death/transplant*	22	26.5%	60.1 (46.5-80.4)

¥: reported as median and interquartile range. § reported as mean and standard deviation. *Mann-Whitney significance p<0.001. IV= intravenous. N= number of participants with data available.

Presence or absence of detectable levels of AQs at baseline on primary outcomes.

Death or lung transplantation during follow up was not statistically different in the presence or absence of detectable levels of six individual AQs at baseline (Table 3), using binary AQ analyses (detected versus not detected). Similarly, there were no statistical associations between the combined AQs measured and primary outcomes, indicating indicating independence (chi-squared p=0.751 and p= 0.351 for total sputum and plasma AQs respectively, Table 3).

Table 3. Individual and combined total AQs with primary outcome of death or lung transplantation.

AQ	Death/transplant: n(%)	No Death/transplant: n(%)	p-value (chi²)
Sp HHQ +	14 (70)	34 (65.4)	0.71
Sp HHQ -	6 (30)	18 (34.6)	
Pl HHQ +	14 (66.7)	35 (58.3)	0.501
Pl HHQ -	7 (33.3)	25 (41.7)	
Sp NHQ +	15 (75)	35 (67.3)	0.526
Sp NHQ -	5 (25)	5 (32.7)	
Pl NHQ +	3 (14.3)	18 (30)	0.157
Pl NHQ -	18 (85.7)	42 (70)	
Sp PQS +	12 (60)	31 (59.6)	0.98
Sp PQS -	8 (40)	21 (40.4)	
Pl PQS +	8 (38.1)	20 (33.3)	0.693
Pl PQS -	13 (61.9)	40 (66.7)	
Sp C9-PQS +	15 (75)	33 (63.5)	0.352
Sp C9-PQS -	5 (25)	19 (36.5)	
Pl C9-PQS +	3 (14.3)	5 (8.3)	0.431
Pl C9-PQS -	18 (85.7)	55 (91.7)	
Sp HQNO +	16 (80)	39 (75)	0.655
Sp HQNO -	4 (20)	13 (25)	
Pl HQNO +	11 (52.4)	28 (46.7)	0.652
Pl HQNO -	10 (47.6)	32 (53.3)	
Sp NQNO +	14 (70)	39 (75)	0.666
Sp NQNO -	6 (30)	13 (25)	
Pl NQNO +	8 (38.1)	21 (35)	0.799
Pl NQNO -	13 (61.9)	39 (65)	
Total Sp AQ+	18 (90)	48 (92.3)	0.751
Total Sp AQ -	2 (10)	4 (7.7)	
Total Pl AQ +	15 (71.4)	36 (60)	0.351
Total Pl AQ -	6 (28.6)	24 (40)	

+ detected; - undetected; Sp=Sputum; Pl=Plasma; AQ=2-alkyl-4 quinolones; n= number; HHQ= 2-heptyl-4-hydroxyquinoline; NHQ= 2-nonyl-4-hydroxyquinoline; PQS=2-heptyl-3-hydroxy-4(1*H*)-quinolone; C9-PQS= 2-nonyl-3-hydroxy-4(1*H*)-quinolone; HQNO= 2-heptyl-4-hydroxyquinoline-*N*-oxide; NQNO= 2-nonyl-4-hydroxyquinoline-*N*-oxide

The presence or absence of detectable levels of AQs at baseline on secondary outcomes

There was no statistical difference demonstrated with rate of FEV_1 decline both with individual and combined AQs detected in plasma or sputum (Supplementary Table 1).

There was no association between five of the individual AQs and number of IV antibiotic days per year (Supplementary Table 1).

The presence of C9-PQS in the sputum was associated with an increase in IV antibiotic days per year during the follow up period (Mann-Whitney p=0.011; Figure 1). The median number of IV antibiotic days per year if sputum C9-PQS was detected was 41.4 (IQR: 26.6 to 60.7) compared with 28.2 (IQR: 4.0 to 44.4) when not detected. A similar finding was observed when follow up was restricted to 3 years; 44.8 (IQR: 25.1 to 62.1) when sputum C9-PQS was detected compared with 29.7 (IQR: 4.7 to 50.4) when not detected (Mann-Whitney p=0.046). The concentration of C9-PQS in sputum was positively correlated with number of IV antibiotics per year but did not reach statistical significance (Spearman rank correlation; r= 0.2, p= 0.09; Supplementary Figure 1). There was no statistical difference in the number of IV antibiotic days when C9-PQS was detected in plasma (Supplementary Table 1; Mann-Whitney p=0.32), nor detectable levels of total combined AQs in plasma or sputum (Supplementary Table 1). The number of IV antibiotic days per year was statistically higher in people who died or had a bilateral lung transplantation in the follow up period (Mann-Whitney p<0.001, Table 2).

Figure 1. Annual number of IV antibiotic days according to C9-PQS detection at baseline.

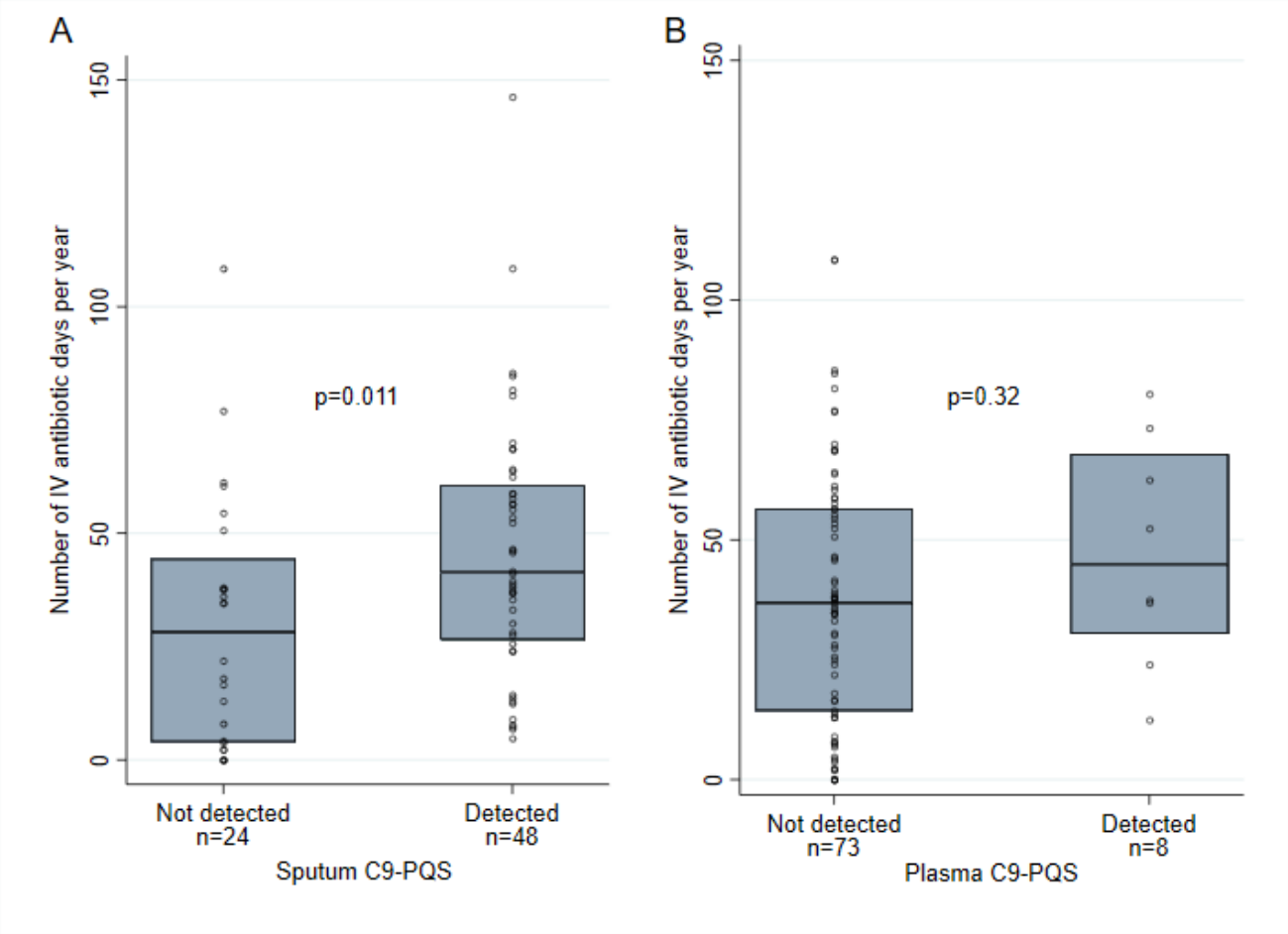

Figure 1 legend. Box plot showing the relationship between the presence and absence of detectable levels of sputum and plasma C9-PQS at baseline with the number of intravenous antibiotics days per year during the follow up period. Box represents interquartile range, Line represents median value. C9-PQS= 2-nonyl-3-hydroxy-4(1H)-quinolone; IV= Intravenous; p value derived from Mann-Whitney test; n= number of observations.

Discussion

This is the first study to explore the relationship between baseline AQ quorum sensing signal molecules measured in sputum or plasma with long term outcomes in adults with CF. There were no

significant difference between the detection of sputum or plasma AQs at baseline with death, lung transplantation or rate of FEV_1 decline over the follow up period. One of the six AQs measured was associated with increased IV antibiotic usage in the follow up period. A higher number of IV antibiotic days for pulmonary exacerbations were observed in the presence of detectable levels of sputum C9-PQS. However, plasma levels of C9-PQS levels were not significantly associated with IV antibiotic usage. This may be explained by the low number of participants with detectable C9-PQS in plasma (eight adults compared to forty eight adults with detectable C9-PQS in sputum).

There are a number of limitations in this study that should be considered when interpreting these data. This is a retrospective analysis and the number of participants who died or had lung transplantation resulted in small numbers. We primarily assessed AQ levels as dichotomous; detectable or not detectable, as the variability across the sample size would have provided low power. Intra-subject variability of AQ concentrations is unknown and a single measure of AQ concentration may not reflect the longer period during which the AQs may influence disease progression. Whilst effect sizes are robust, findings are to be regarded as 'hypothesis generating' as significant *p* values may be a consequence of multiple hypothesis testing.

Despite limitations, these findings provide evidence of a possible association between both sputum and plasma C9-PQS levels and antibiotic usage, which should be confirmed through prospective study design.

P. aeruginosa is the major respiratory pathogen in people with CF and is difficult to eradicate from the CF airways as it is intrinsically resistant to many classes of antibiotics and forms antibiotic resistant biofilms [12]. The AQ class of quorum sensing molecules plays an important role in pathogenicity for *P. aeruginosa* and AQ deficient mutants show reduced virulence in infection models [13-15]. PQS and its immediate precursor HHQ are the major AQ signalling molecules in *P. aeruginosa* [16]. PQS regulates the expression of at least 182 genes including those that code for the iron-chelating siderophores, pyoverdine and pyochelin [17, 18] as well as playing a role in regulating antibiotic resistance and biofilm maturation [19-21]. In addition, PQS regulates the production of key virulence factors that are associated with pulmonary exacerbations such as elastase [22], pyocyanin [23] and cyanide [24]. Both C9-PQS and NHQ are as effective as their C7 congeners PQS and

HHQ at activating the AQ receptor PqsR which further drives the autoinduction of AQ biosynthesis and up regulates key virulence determinants [25]. Furthermore, molecular imaging techniques have shown that initial biofilm formation is marked by a dramatic increase in the production of C9-PQS, suggesting it may be important for the growth of *P.aeruginosa* in communities and early biofilm formation [26]. Although our current understanding of the role of C9-PQS is limited [27], it is biologically plausible that C9-PQS may be associated with increased antibiotic usage due to increased virulence factor production during pulmonary exacerbations.

Recurrent severe pulmonary exacerbations are associated with both increased morbidity and mortality in CF [28]. In recent years, attempts to develop new classes of antimicrobial agents have included targeting of virulence factors or virulence regulatory mechanisms. Consequently, the AQ signalling system is a promising potential target for antimicrobial agents which do not kill the organism but instead block or attenuate the ability to cause disease. This is important as multiple courses of antibiotics are detrimental to the host and contribute to a growing global burden of multi-antibiotic resistance that needs to be addressed urgently.

In conclusion, this hypothesis generating study showed an association between C9-PQS detected in the sputum and increased antibiotic usage in the CF population, which requires more comprehensive investigation to confirm or refute these findings. However, there were no other associations between the five AQs detected and adverse clinical outcomes measured. There is much to learn about AQ regulation in the clinical setting, particularly as development of anti-virulence drugs that target PQS-dependent QS pathways progresses [29].

Author Contributions

The study was designed by KW/HB/AF. KW collected the data. The analysis was performed by KW and IS. All authors contributed to data interpretation, data presentation and writing of the manuscript. All authors approved the final version of the manuscript.

Declarations of Interest

The University of Nottingham has a patent for the use of alkyl quinolones as biomarkers for *P. aeruginosa* infection (PCT/GB2014/051458).

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