On line supplement for *P. aeruginosa* quorum sensing molecules correlate with clinical status in cystic fibrosis

Sample preparation

Extracts of sputum samples for LC-MS/MS analysis were prepared by solvent extraction. Up to 1.0 mL of 50% (v/v) sputum suspension was spiked with 10 µL of an internal standard mix (1.0 µmol/L solution of deuterated PQS (PQS-d_4) and deuterated C5-HSL (C_5-HSL-d_9) in methanol), and extracted in triplicate with 0.5 mL volumes of 0.01% (v/v) acetic acid in ethyl acetate. After the addition of acidified solvent the samples were vortex-mixed for approximately 1 min and centrifuged (3 min at 12,000 g) with the analytes of interest partitioning into the organic phase. The combined organic extracts were dried under vacuum.

Urine and plasma samples were prepared by solid phase extraction (SPE). The SPE cartridges (Waters, Oasis hydrophilic-lipophilic balanced (HLB), 60 mg, reversed-phase sorbent extraction cartridges) were pre-conditioned with 3 mL of methanol followed by 3.0 mL of 1% (v/v) acetic acid. Urine (1.0 mL) and plasma (0.5 mL) samples, diluted and acidified with an equal volume of 1% (v/v) acetic acid, were spiked with 10 µL of PQS-d_4 internal standard solution (1.0 µmol/L in methanol). After loading onto the SPE cartridges, they were washed with 2 x 3 mL of 30% (v/v) methanol. The retained extracts, were eluted from the cartridges with 1.5 mL of methanol, and then dried under vacuum. Dried extracted samples were re-dissolved in 50 µL of 0.1% (v/v) formic acid in methanol prior to LC-MS/MS analysis.

Preparation of calibration and quality control (QC) standards

For the production of urine and plasma matrix matched calibration samples, blank samples (1.0 mL of urine and 500 µL of plasma) from a healthy volunteer donor were spiked with 50 µL of a methanolic mix of all the AQ and AHL standards prepared at a range of concentrations (0, 5, 15, 30, 60 and 100 nmol/L), giving an overall calibration range of 0-5 nmol/L for urine samples and 0-10 nmol/L for plasma samples. Quality

control samples were prepared similarly, spiking blank samples with 50 µL of the analyte mix at 10 and 80 nmol/L, producing plasma QC samples of 1 and 8 nmol/L, 0.5 and 4 nmol/L for urine. In the absence of blank sputa to spike with analytes and produce matrix matched calibration and QC samples, 1.0 mL aliquots of 0.9% (w/v) NaCl were used, spiking with 50 µL of methanolic analyte mix at 0, 5, 25, 50, 100, 200, 400, and 1000 nmol/L. QC samples were prepared at 75 and 800 nmol/L. All calibration and QC samples were prepared in triplicate, extracted and prepared ready for LC-MS/MS analysis as described above.

LC-MS/MS analysis

LC-MS/MS analysis was conducted according to Ortori *et al* 2011[16] on a 4000 QTRAP hybrid triple-quadruple linear ion trap mass spectrometer in tandem with a Shimadzu series 10AD VP LC system. The method is described briefly here. 20 µL of the prepared sputum, urine and plasma extracted samples were injected into the LC instrument for analysis. The chromatographic separation was achieved using a Phenomonex Gemini C18 reversed phase column (3.0 µm, 100 x 3.0 mm) with a constant mobile phase flow rate of 450 µL/min. Mobile phases consisted of aqueous 0.1% (v/v) formic acid (A) and 0.1% (v/v) formic acid in methanol (B). The binary gradient began initially at 10% B and ran isocratically for the first 1 min before increasing linearly to 99% B over 9 min. After a further 5 min at this composition, the gradient was returned to 10% B over the next 1 min and allowed to re-equilibrate for 4 min. The MS, operating in the positive electrospray (+ES) mode, was set up for multiple reaction monitoring (MRM) to constantly screen the eluent from the LC column for all the analytes of interest.

Sample quantification

For each analyte, ratios of LC-MS/MS peak areas to internal standard peak areas were calculated and used to construct calibration lines of peak area ratio against analyte

concentration. Results from the QC samples were used to ensure suitable precision and accuracy at both the high end and low end of the calibration lines. The lower limit of quantification (LLOQ) was established by using serial dilutions of the analyte mix and spiking into blank urine and plasma samples prior to extraction and analysis. The LLOQ was defined as the analyte concentration at which a signal/noise ratio of 10:1 was achieved.

For each analyte, linearity over the calibration ranges used was demonstrated. Results for the QC samples confirmed that analytical precision was <15% (<20% for the low end QC samples), and accuracy was 100±15% (100±20% for the low end QC samples), values that are generally considered acceptable for such analytical methodology. Calculated LLOQs in plasma and urine samples were as follows: (plasma) HHQ, 10 pmol/L; NHQ, 10 pmol/L; HQNO, 30 pmol/L; NQNO, 40 pmol/L; PQS, 100 pmol/L; C9-PQS, 100 pmol/L; and (urine) HHQ, 20 pmol/L; NHQ, 10 pmol/L; HQNO, 30 pmol/L; NQNO, 50 pmol/L; PQS, 50 pmol/L; C9-PQS, 50 pmol/L.

Statistical analysis

The associations between baseline sputum, plasma and urinary QSSM concentrations were compared using Spearman's rank correlations. Comparisons in change of lung function, quantitative microbiology and sputum neutrophil concentration following antibiotic therapy were made using paired t-tests with logarithmic transformation as required. Comparisons in individual QSSM concentrations at differing time points were made using Wilcoxon matched pairs signed-rank tests. We estimated that 53 subjects would provide 90% power to detect a decrease in the logged value of the QSSMs of 0.5 units from baseline after the administration of IV antibiotics (using a standard deviation of 1.1 units derived from pilot data [7]). Data were analysed using STATA 11 statistical software (Texas, USA).

Table S1. Cross-sectional association of QSSMs detected in sputum, plasma and urine using LC-MS/MS in participants whose baseline sputum did not initially isolate *Pseudomonas aeruginosa* using traditional microbiological culture techniques in the hospital laboratory.

QS signal molecule	Number of patients with detectable QSSM in each media with negative hospital sputum culture for *P. aeruginosa*		
	Sputum (N=6)	Plasma (N=6)	Urine (N=7)
HHQ	2/6	3/6	2/7
NHQ	2/6	1/6	1/7
HQNO	3/6	2/6	4/7
NQNO	2/6	1/6	2/7
PQS	1/6	1/6	-
C9-PQS	1/6	-	-
3-oxo-C12-HSL	3/6	-	-
C4-HSL	1/6	-	-

- = QS signal not detectable above threshold levels
3-oxo-C12-HSL = *N*-(3-oxododecanoyl)-L-homoserine lactone
C4-HSL = *N*-butanoyl-L-homoserine lactone
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
N = number of patients

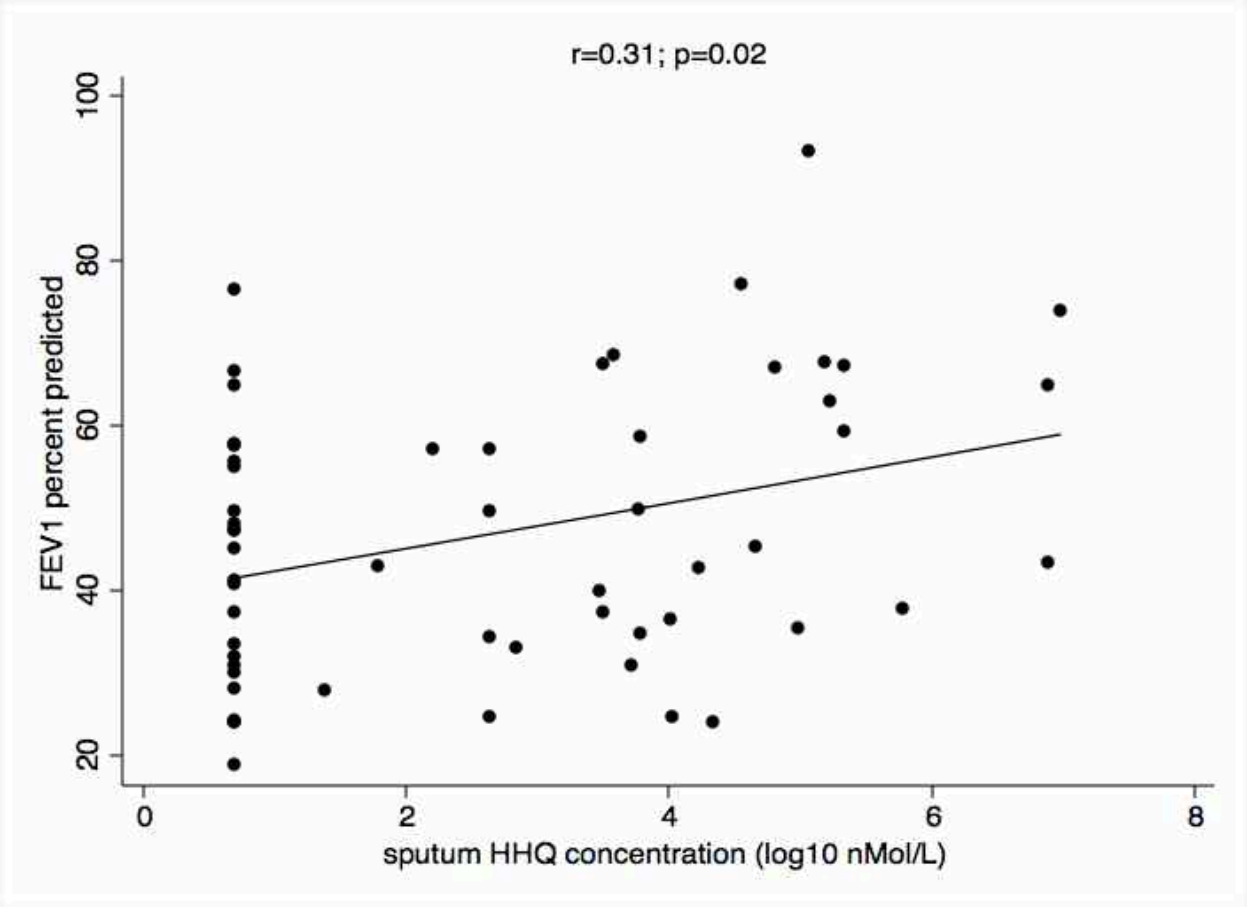
Table S2. Spirometry, sputum cell concentrations and quantitative microbiology at the start and end of antibiotic therapy.

Variable	**Pre antibiotics** (N=58)		**Post antibiotics** (N=58)		**P value**
	Mean	S.D.	Mean	S.D.	
Absolute FEV_1 (L)	1.70	0.70	1.97	0.82	<0.0001
FEV_1 (% predicted)	47.2	16.9	53.5	18.6	<0.0001
Absolute FVC (L)	2.79	1.03	3.08	1.03	0.0001
FVC (% predicted)	65.4	19.2	71.7	18.2	0.0001
Weight (kg)	61.8 (N=60)	12.6	63.1 (N=57)	12.9	0.001
Neutrophil conc. (log_{10} neut/g)	7.1 (N=56)	0.4	6.9 (N=51)	0.4	<0.001
CFU on blood agar (log_{10} CFU/g)	7.7 (N=54)	0.7	7.6 (N=48)	0.8	0.22
CFU on PIA (log_{10} CFU/g)	7.0 (N=52)	1.1	7.0 (N=48)	1.1	0.98

Comparisons made using paired t-tests with corresponding p values shown

FEV_1 = forced expiratory volume in one second
FVC = forced vital capacity
PIA = pseudomonas isolation agar
S.D. = standard deviation
Log_{10} = logarithmically transformed to the base 10
Neut = neutrophil
Conc =concentration
CFU = colony forming units
g = gram
N = number of participants with samples available for analysis

Figure S1. Scatter graph and Spearman rank correlation (r) between sputum 2-heptyl-4-hydroxyquinoline (HHQ) concentration and percent predicted forced expiratory volume in one second (FEV_1).



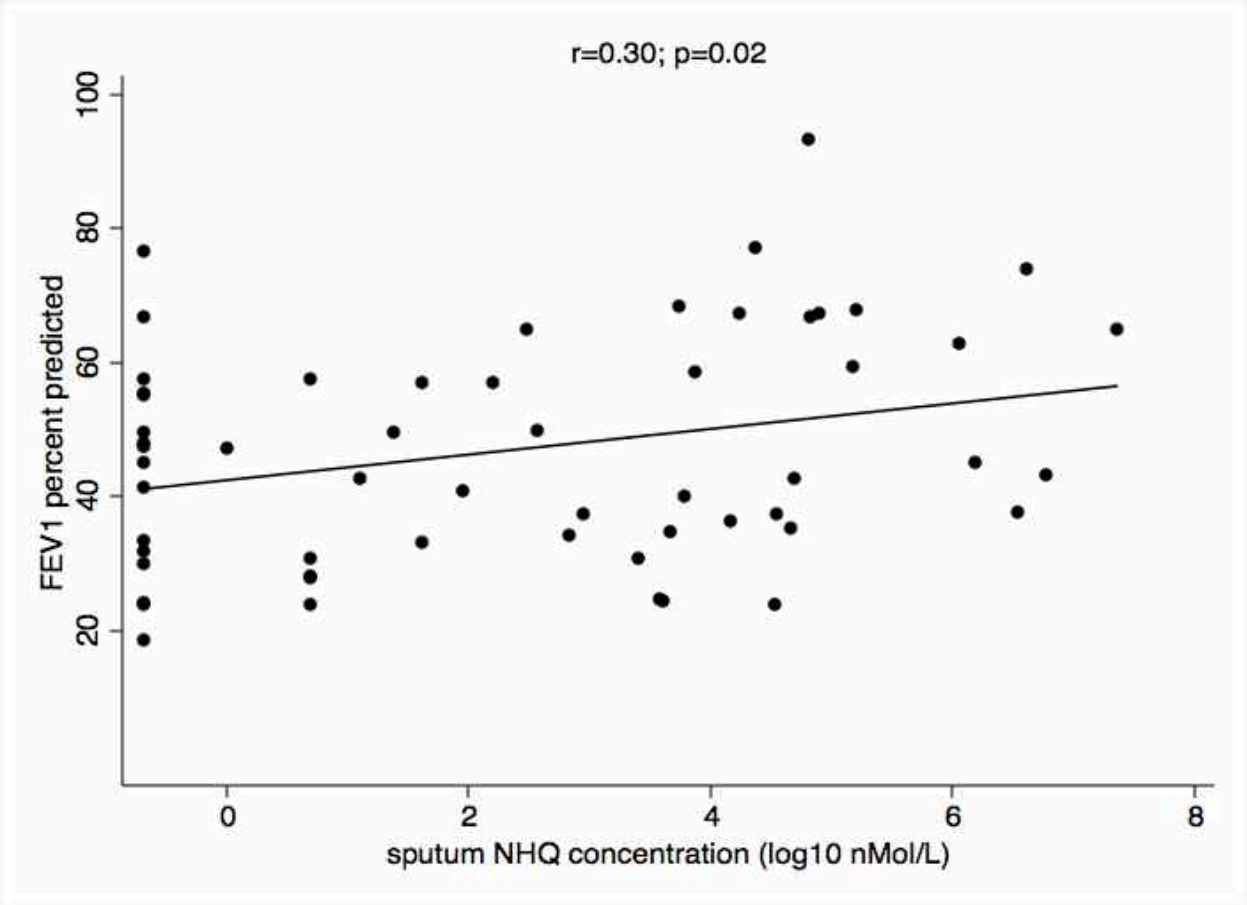
Sputum HHQ concentration was logarithmically transformed for graphical purposes only with values below the threshold level of detected represented as half the threshold value.

HHQ= 2-heptyl-4-hydroxyquinoline

nMol/L= nanomols per litre

FEV_1= forced expiratory volume in one second

Figure S2. Scatter graph and Spearman rank correlation (r) between sputum 2-nonyl-4-hydroxyquinoline (NHQ) concentration and percent predicted forced expiratory volume in one second (FEV_1).



Sputum NHQ concentration was logarithmically transformed for graphical purposes only with values below the threshold level of detected represented as half the threshold value.

NHQ= 2-nonyl-4-hydroxyquinoline

nMol/L= nanomols per litre

FEV_1= forced expiratory volume in one second