Identification of Potential Urinary Metabolite Biomarkers of *Pseudomonas aeruginosa* Ventilator-Associated Pneumonia

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ABSTRACT

INTRODUCTION: Ventilator-associated pneumonia (VAP) caused by *Pseudomonas aeruginosa* is a major cause of morbidity and mortality in hospital intensive care units (ICU). Rapid identification of *P. aeruginosa*-derived markers in easily accessible patients' samples can enable an early detection of *P. aeruginosa* VAP (VAP-PA), thereby stewarding antibiotic use and improving clinical outcomes.

METHODS: Metabolites were analysed using liquid chromatography-mass spectrometry (LC-MS) in prospectively collected urine samples from mechanically ventilated patients admitted to the Antwerp University Hospital ICU. Patients were followed from the start of mechanical ventilation (n = 100 patients) till the time of clinical diagnosis of VAP (n = 13). Patients (n = 8) in whom diagnosis of VAP was further confirmed by culturing respiratory samples and urine samples were studied for semi-quantitative metabolomics.

RESULTS: We first show that multivariate analyses highly discriminated VAP-PA from VAP–non-PA as well as from the pre-infection groups (R^2 = .97 and .98, respectively). A further univariate analysis identified 58 metabolites that were significantly elevated or uniquely present in VAP-PA compared to the VAP–non-PA and pre-infection groups ($P < .05$). These comprised both a known metabolite of histidine as well as a novel nicotine metabolite. Most interestingly, we identified 3 metabolites that were not only highly upregulated for, but were also highly specific to, VAP-PA, as these metabolites were completely absent in all pre-infection timepoints and in VAP–non-PA group.

CONCLUSIONS: Considerable differences exist between urine metabolites in VAP-PA compared to VAP due to other bacterial aetiologies as well to non-VAP (pre-infection) timepoints. The unique urinary metabolic biomarkers we describe here, if further validated, could serve as highly specific diagnostic biomarkers of VAP-PA.

KEYWORDS: Hospital-acquired pneumonia, VAP, *Pseudomonas aeruginosa*, urine biomarkers, metabolomics, mass spectrometry

Introduction

Ventilator-associated pneumonia (VAP) is the most frequent hospital-acquired infection in the intensive care unit (ICU).[1,2] VAP is generally caused by opportunistic pathogens, of which *Pseudomonas aeruginosa* is one of the major causes.[3] VAP caused by *P. aeruginosa* (VAP-PA) has been associated with higher case fatality rates than VAP caused by other bacterial aetiologies.[4,5] The attributable mortality is even further increased when *P. aeruginosa* is present with other organisms, especially *Staphylococcus* spp.,[6,7] or with multi-drug resistant strains.[6-9] Despite the clinical importance of VAP-PA and the knowledge that timely *P. aeruginosa* specific treatment improves patient outcome, no predictive biomarkers are currently available to diagnose the disease.[10] The lack of rapid diagnostic methods leading to timely treatment, the high mortality associated with *P. aeruginosa* infection, and the high levels of antibiotic resistance in *P. aeruginosa* strains prompts an urgent need for diagnostic markers of VAP-PA to guide pathogen-targeted

therapy.[4,5,8-10] Several targeted/species-specific treatments against VAP-PA are currently in trial, such as a bi-specific antibody targeting *P. aeruginosa* surface markers.[11,12]

Metabolomics is a rapidly advancing technology that can be used for biomarker discovery on a multitude of human samples such as sputum,[13] bronchoalveolar lavage fluid,[14] breath gas/condensate,[15] blood,[16,17] and urine.[18-20] Specifically, urine is an easily accessible body fluid that can be sampled non-invasively and point-of-care urine metabolite tests exist for community-acquired pneumonia caused by *Streptococcus pneumoniae* and *Legionella pneumophila* serogroup 1.[21,22] Moreover, prior urinary biomarker research in pneumonia caused by *S. pneumoniae* or *S. aureus* identified an increase of certain monosaccharides, amino acids, intermediates of glycolysis, fermentation, and tricarboxylic acid cycle intermediates.[18-20]

Currently, 2 techniques are mostly used in metabolite biomarker discovery, namely, 1H nuclear magnetic resonance (NMR) and mass spectrometry (MS). While 1H NMR is a robust method typically utilised in a targeted manner to study a panel of known compounds, semi-quantitative liquid chromatography-mass spectrometry (LC-MS) is an unbiased and more sensitive approach typically used to identify novel targets in a discovery platform.[23,24] Moreover, different ionisation techniques such as analysis in both positive and negative ionisation mode can substantially increase the number of metabolites that can be detected.[25]

In this study, we utilised an untargeted semi-quantitative LC–MS metabolomics approach in both positive and negative ionisation mode to discover biomarkers of VAP caused by *P. aeruginosa* versus other VAP bacterial aetiologies. We show that the global urinary metabolic profile accurately discriminates VAP due to *P. aeruginosa* from VAP due to other aetiologies and describe 3 novel targets that were exclusively present in VAP-PA patients. Additionally, we also identified several other known and unknown metabolites that were significantly elevated in VAP-PA urine samples. While these data have to be further validated, the novel metabolites described here could serve as a basis for developing new diagnostic modalities for VAP-PA.

Materials and Methods

Study design and sample collection

Mechanically ventilated adult patients were prospectively studied at the Department of Critical Care Medicine of the University Hospital of Antwerp (Figure 1). Patients >18 years were included within 24 hours of intubation when expected duration of invasive ventilation was >48 hours. The exclusion criteria for enrolment included infection other than VAP, prior gastric or oesophageal surgery, neutropaenia, unavailability of informed consent, and expected short duration of ventilation such as after cardiac surgery. Patients were excluded when developing urinary infection, receiving <48 hours of mechanical ventilation or withdrew from the study. VAP was clinically diagnosed in patients ventilated for >48 hours, which was essentially based

on a new or progressive consolidation on chest radiology and at least 2 of the following variables: fever greater than 38°C, leucocytosis or leukopenia, and purulent secretions.[26] At this point, bronchoalveolar lavage (BAL) was also performed and BAL fluid was cultured to isolate the causative pathogen. In this study, a definitive diagnosis of VAP was only made when pathogens were cultured from BAL fluid. Written informed consents were obtained from the closest relatives of the patients. Urine samples were collected from patients at the time of mechanical ventilation (hereafter called pre-infection timepoint) and on the day of the clinical diagnosis/suspicion of VAP. For both timepoints, urine was collected for a maximum period of 4 hours and immediately frozen and stored at –80°C. The study was approved by the ethics committee of the Antwerp University Hospital (UZA/EC-12/12/112) and continued from 18/07/2012 through 19/12/2013 when the requested enrolment of patients meeting the study criteria (n = 100) was achieved.

Untargeted metabolomics using rapid resolution liquid chromatography (RRLC) and mass spectrometric (MS) analyses

Urine samples were centrifuged at 15 000 × g for 12 minutes at 4°C to remove cell debris and supernatants were diluted 1:8 with ultra-pure water. Metabolites were separated on a 1200 Rapid Resolution Liquid Chromatography (RRLC) system (Agilent Technologies) using an Acquity HSS T3 C18 column (2.1 × 100 mm, 1.8 µm at 40°C; Waters Corporation) as described previously.[27] A 10 µL injection volume was separated on a linear gradient from 0% to 100% of mobile phase B (0.1% formic acid in acetonitrile) and? in mobile phase A (0.1% formic acid in water) in 20 minutes at flow rate of 0.35 mL/min.

The RRLC system was coupled in-line with a 6530 quadrupole time of flight (Q-TOF) mass spectrometer (Agilent Technologies) using a Jetstream Electrospray Ionisation (ESI) source and operated using MassHunter Workstation Acquisition 5.0 (Agilent Technologies). The Q-TOF was operated in both positive and negative ESI mode. Resolution was set at 10 000 at 1000 mass charge ratio *(m/z)* and mass accuracy ⩽2 parts per million (ppm). Each quality control (QC) sample was made by pooling aliquots of individual urine samples. All separate QC samples then underwent the entire sample preparation process with LC-MS to calculate variability in retention time and peak area. All study samples were analysed in a randomised order, with QC samples analysed after every 5 study samples to control the machine variability. The LC-MS/MS analyses were performed at Research Institute for Chromatography (RIC, Kortrijk, Belgium).

Quality control of untargeted metabolomics approach

The reproducibility of the performed untargeted metabolomic analyses was monitored in a non-targeted manner using the
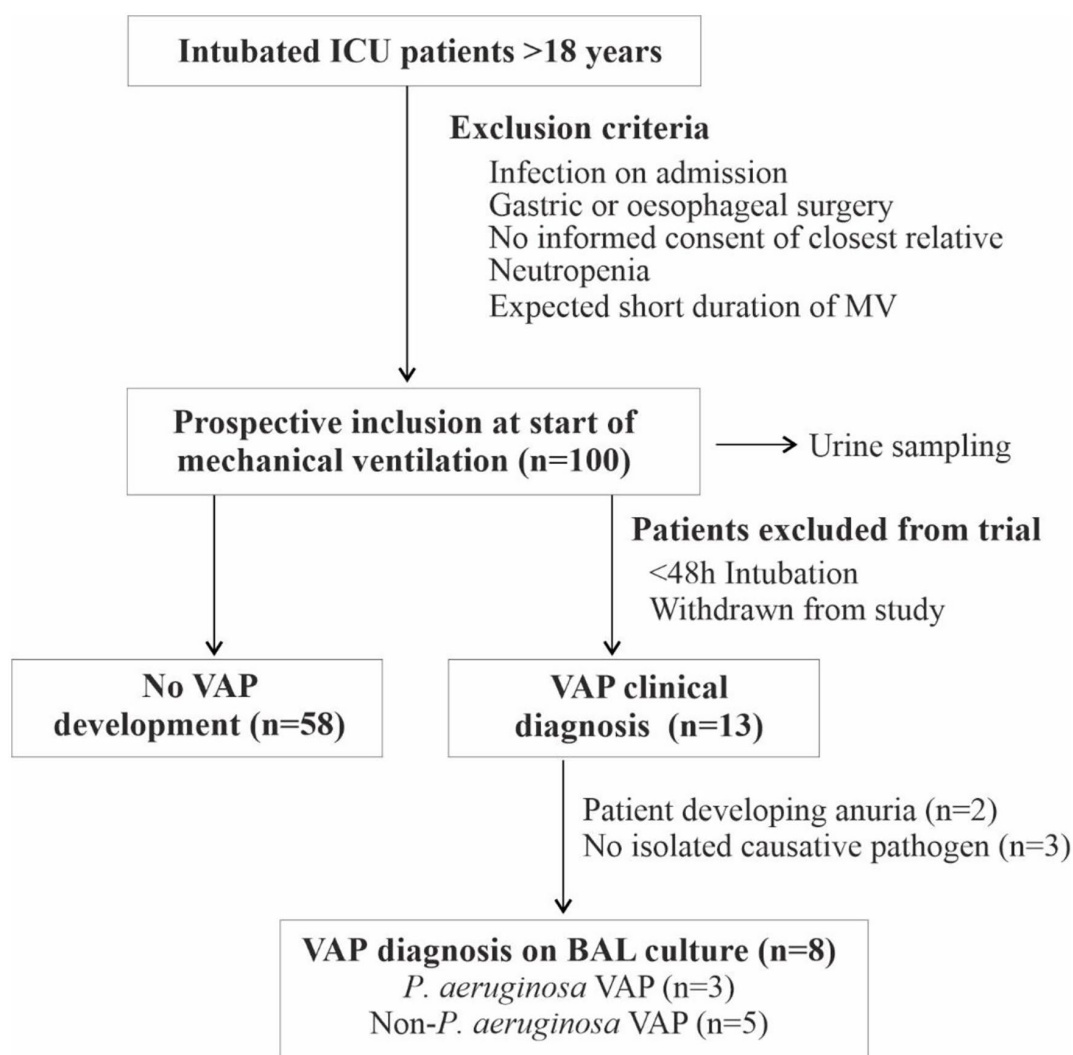
Figure 1. Flow diagram of study design, and patient inclusion and sampling.

QC samples. The error of the measurement of signal intensity (peak area), retention time and mass accuracy for all detected metabolites was calculated to provide a relative standard deviation (RSD) score. An RSD score of 30% is generally considered as the upper limit for untargeted metabolomic analyses.[28] In the positive ionisation mode, a total of 4011 metabolites were detected with 100% frequency in all QC samples. Of these, 1484 (37%) metabolites showed RSD values below 15% while the majority of metabolites (77% or 3088 metabolites) had an RSD below 30%. Negative ionisation mode displayed a total of 1622 metabolites. About 1184 metabolites or 73% of all metabolites had an RSD value below 15%, while 1476 metabolites or 91% of metabolites had an RSD value below 30%.

Data processing and data analyses

Raw LC–MS data files were processed in an untargeted fashion using the Molecular Feature Extraction (MFE; MassHunter Qualitative Analysis 7.0, Agilent Technologies). The resulting feature files were subsequently imported in Mass-Profiler Professional 12.0 (Agilent Technologies) for feature filtration

and alignment. The filtered feature list was exported for recursive peak integration using the Find-By-Ion-Extraction algorithm by applying predefined mass and retention time extraction windows as described previously.[27]

For multivariate analyses, supervised analyses were performed using Partial Least Squares-Discriminant Analysis (PLS-DA) and Orthogonal Projection to Latent Structures (OPLS) on mean centred $\log_{10}$ transformed peak intensities using MetaboAnalyst, a web-based application, as described previously.[29] Additionally, supervised, hierarchical clustering using Euclidean distance and Ward clustering algorithm was performed to generate heat map based on top 50 metabolites, as utilised in previous studies.[17] Each metabolite was scaled by the maximum intensity value of that metabolite in the data set. In addition, parallel univariate analyses were performed for all samples using Mann Whitney-*U* test (SPSS v23, IBM). VAP-PA group was compared to VAP–non-PA group and pre-infection timepoint urine samples of both groups. Upregulations of >8 fold at α level of <0.05 were deemed significant. An 8-fold change threshold is used in these analyses to reduce false positive errors.[30]

Molecular ion identification

A molecular formula based on accurate mass and isotopic distribution was first assigned to the differentially excreted metabolites detected on LC–MS. Based on molecular weight and retention time of metabolites that were present in all VAP-PA samples and absent in VAP–non-PA as well as pre-infection samples of both groups, a further MS/MS fragmentation analysis was performed for possible identification. MS/MS spectra were (i) matched with spectra of standard compounds in the METLIN metabolite–Forensic Toxicology Personal Compound Library (PCDL) (Agilent technologies), (ii) matched with spectra of Pseudomonas Aeruginosa Metabolome DataBase (PAMDB), (iii) interpreted using MassHunter Molecular Structure Correlator (MSC; Agilent Technologies), and (iv) compared to published literature when possible. Molecular structures from Metlin, Human Metabolome Database (HMDB) and ChemSpider were imported into MSC. PCDL spectral library matching was performed using the reverse scoring option, where library spectra were matched against the acquired spectra, after which false positives were removed by manual verification.

Results

Patient inclusion and trial outcome

A total of 100 mechanically ventilated patients were enrolled in this study of which 13 patients were clinically diagnosed with VAP. Of these, causative pathogens were isolated from the BAL of 10 VAP patients, and the 3 patients where no causative pathogen could be isolated were excluded from the study. Of the 10 confirmed VAP patients, 2 had developed anuria and were also excluded from the study. A flowchart of patient enrolment is shown in Figure 1 and a comparison of clinical characteristics between confirmed VAP and non-VAP developing patients is provided in Table 1. Although no significant differences were observed in our study group, an increased ICU stay and intubation time as well as a worse prognosis was noted for patients developing VAP, as has been extensively documented in literature.[1,2] Individual clinical characteristics of the 8 patients included in the study are summarised in Table 2.

Table 1. Comparison of patient characteristics between VAP-developing patients and non-VAP developing intubated patients.

	NO VAP N = 58	VAP N = 10	*P*-VALUE
Age	60.7 ± 14.5	59.1 ± 11.2	.741
Sex (%male)	59%	60%	.950
intubation length	11.5 ± 10.8	15.3 ± 6.1	.436
ICU stay	16.1 ± 13.6	22.0 ± 13.6	.212
APACHE II	27.5 ± 8	23.4 ± 8.4	.152
Prognosis (%deceased)	33 (19/58)	40 (4/10)	.697
MV before VAP (d)		4.9 ± 2.66	

Abbreviations: APACHE II, Acute Physiology and Chronic Health Evaluation II, a marker of disease severity; MV, mechanical ventilation; VAP, ventilator-associated pneumonia.[32]

Table 2. Individual characteristics of VAP patients enrolled in this study.

IBIVAP ID	PATIENT	AETIOLOGY	GENDER	AGE	REASON OF ADMISSION	APACHE II	VENTILATOR DAYS UNTIL VAP*	RELEVANT ANTIBIOTICS RECEIVED DURING TREATMENT
IBIVAP 4-606	Pa 1	*P. aeruginosa*	Female	58	Stroke	24	5	AMC, AMK, FEP, TZP, VAN
IBIVAP 4-609	Pa 2	*P. aeruginosa*	Male	39	Stroke	20	12	AMK, FEP, MEM, TZP, VAN
IBIVAP 4-613	Pa3/Se	*P. aeruginosa; S. epidermidis*	Male	59	Respiratory insufficiency	17	3	AMC, CIP, MEM, PEN, TZP, VAN
IBIVAP 1-010	Sm	*S. marcescens*	Male	71	Cardiac arrest	32	4	AMC, AMK, TZP, VAN
IBIVAP 4-602	Ec	*E. coli*	Male	49	Stroke	14	4	AMB, AMC, CAS, FLC, MEM, MTZ, TMC, TZP, VAN
IBIVAP 1-007	Se 1	*S. epidermidis*	Female	74	Cardiac arrest	27	3	AMC, TZP, VAN
IBIVAP 2-201	Se 2	*S. epidermidis*	Female	55	Stroke	7	3	AMK, FEP, CLR, CRO, TZP, VAN
IBIVAP 4-616	Sa	*S. aureus*	Male	57	Stroke	31	2	AMK, CFZ, TZP, VAN

Abbreviations: AMB, amphotericin B; AMC, amoxicillin-clavulanic acid; AMK, Amikacin; CAS, Caspofungin; CFZ, Cefazolin; CIP, Ciprofloxacin; CLR, Clarithromycin; CRO, Ceftriaxone; FEP, Cefepime; FLC, Fluconazole; MEM, Meropenem; MTZ, Metronidazole; PEN, Penicillin; TMC, Temocillin; TZP, Piperacillin-tazobactam; VAN, Vancomycin.
*All patients were ventilated according to a lung protective ventilation strategy, including tidal volumes between 5 and 8 mL/kg.
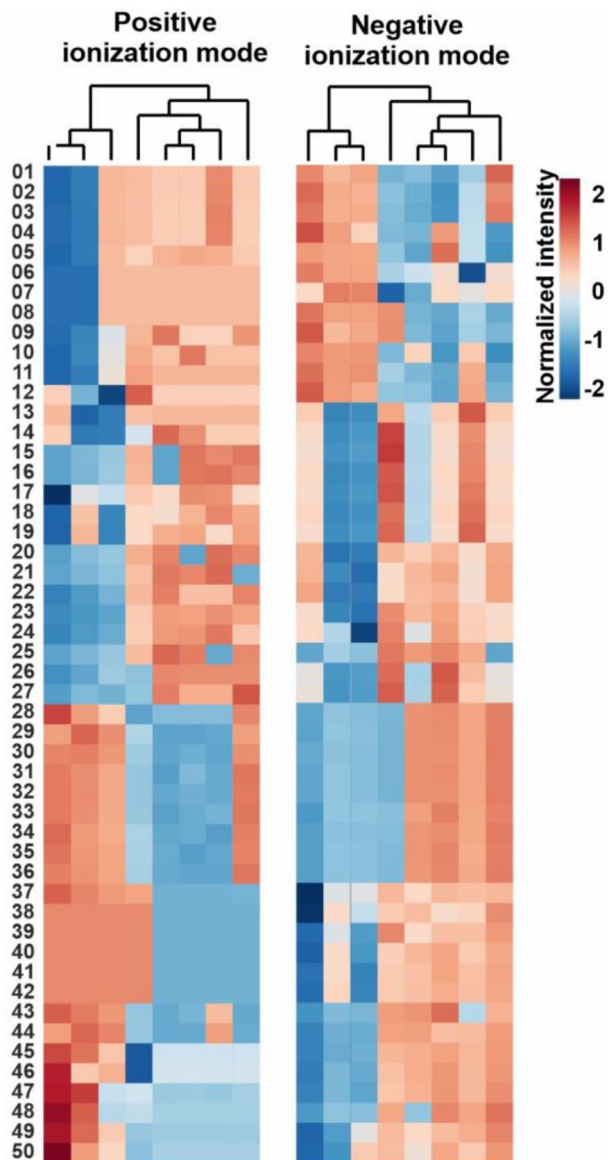
Figure 2. Clustering of VAP patients based on aetiology. Heatmaps for positive electron spray ionisation (left panel) and negative electron spray ionisation (right panel). The heatmaps were constructed based on the top 50 discriminating metabolites using MetaboAnalyst and was based on supervised hierarchical clustering of patients with different aetiologies at presumptive diagnosis of VAP. The discriminating features numbered 1 through 50 on *Y* axis are listed in Supplemental Information, SI Table 2.

Three of the 8 VAP patients included were diagnosed with *P. aeruginosa* VAP (VAP-PA) of which one had a mixed aetiology of *P. aeruginosa* and *Staphylococcus epidermidis*, consistent with recent data where *P. aeruginosa* frequently associates with *Staphylococcus spp.* in causing VAP.[6-8] Five patients were diagnosed with non-*P. aeruginosa* related VAP (VAP–non-PA). Ventilator days until VAP development ranged from 2 to 12 days, the latter being for one of the patients with VAP-PA that generally shows a later time of onset than other aetiologies.[31] Stroke was the most common cause of hospitalisation. Urine samples of pre-infection and VAP development timepoints of VAP-PA and VAP–non-PA were collected and studied. We used 2 control groups. While the pre-infection timepoint group was designed to correct for individual variation of metabolites within each patient, comparisons with non-PA aetiology group ensured specificity of the identified biomarkers for VAP-PA.

Urinary metabolic profile discriminates P. aeruginosa *VAP from other VAP aetiologies*

Patient urine samples were independently analysed in both positive and negative ionisation modes with the RRLC-Q-TOF. Heat map analysis of the top 50 differentially excreted metabolites of both ionisation modes showed clustering of VAP patients based on aetiology (Figure 2). In a supervised PLS-DA analysis, the first PLS factor clearly separated VAP-PA from VAP–non-PA as well as from pre-infection groups (Figure 3). To study how well the PLS-DA model fits the data, a leave-one-out-cross-validation method was performed that showed that with the first 2 components alone, both models displayed a strong fit ($R^2_{\text{Pos ESI}} = 0.97$; $R^2_{\text{Neg ESI}} = 0.98$). An additional OPLS analysis also revealed distinct clustering for VAP-PA, VAP–non-PA, and the pre-infection groups. Interestingly, Neg ESI mode already clustered pre-infection VAP-PA group, demonstrating that early host metabolite profile in certain conditions could already cluster individuals who are more susceptible to *P. aeruginosa* infection (Figure 4A).

S-plots were constructed based on the OPLS data that revealed a significant number of metabolites showing differential urinary excretion in the VAP-PA group compared to VAP–non-PA and the pre-infection groups (Figure 4B). The shift in these metabolic patterns between VAP-PA and VAP–non-PA strongly suggests upregulation of specific infectious pathways in urine samples of VAP patients with *P. aeruginosa* aetiology.

Identification of potential diagnostic markers for VAP-PA

We further performed univariate analyses to study metabolites linked to VAP-PA that could accurately discriminate this group from the VAP–non-PA as well as from the entire pre-infection control group where urine samples were taken at the start of mechanical ventilation. This analysis identified a total of 58 metabolites that were either uniquely present or more than 8-fold significantly elevated in VAP-PA samples compared to the 2 other groups (Supplemental Information, SI Table 1). Several of these metabolites were also positively identified by MS-MS fragmentation analysis.

Targets showing highest excretion in *P. aeruginosa* VAP cases included metabolites related to treatment such as antibiotics (such as vancomycin and piperacillin derivative) and anaesthetics (hydroxypropofol) (Supplemental Information, SI Figure 1A). In addition, we also identified metabolites that have been linked to infection in prior studies, such as hydantoin-5-propionic acid, a histidine derivative that was shown to
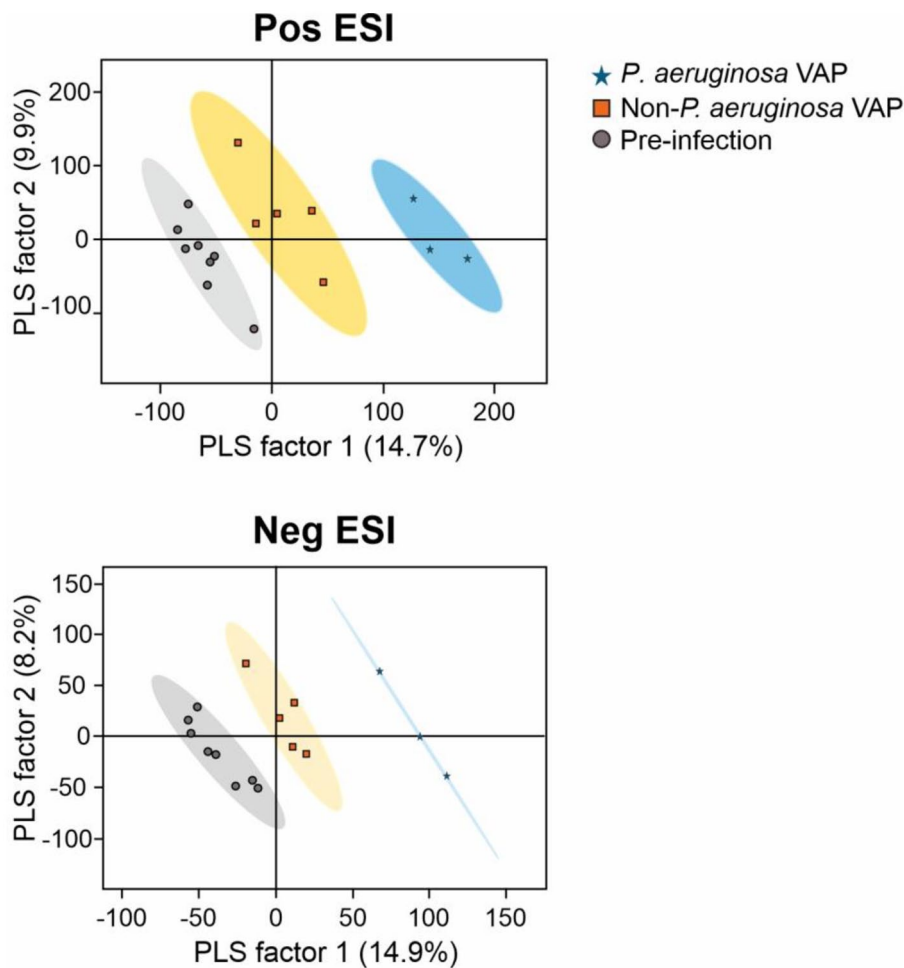
Figure 3. Multivariate analysis shows clear separation of VAP-PA versus VAP–non-PA and pre-infection time-point.
Partial Least Square Discriminant Analysis (PLS-DA) shows clear separation of VAP-PA compared to VAP–non-PA and the pre-infection time-points for both positive and negative ionisation modes (Pos ESI, Neg ESI) ($R^2_{Pos\,ESI}$ = 0.96744; $R^2_{Neg\,ESI}$ = 0.97671).

be elevated in infections due to Gram-negative organisms such as *P. aeruginosa* and *Escherichia coli* but not in Gram-positive *Staphylococcus aureus* and *Streptococcus pneumoniae* infections[20] (Supplemental Information, SI Figure 1B).

Central to the main aim of this study, we identified 3 novel targets to be highly specific to *P. aeruginosa* VAP showing high intensity peaks for VAP-PA samples and being completely absent in VAP–non-PA and the pre-infection timepoints of both groups (Figure 5A). Chemical formula was ascertained for one metabolite: $C_{26}H_{42}N_7O_{19}P_3S$ [MW 881.1538; Rt 13.712 minutes] while the other 2 were identified by their MW and retention time (Rt) as: MW 915.2759, Rt 12.177 minutes and MW 515.1400, Rt 13.750 minutes. In addition, we also identified 2 novel targets that were excreted in high amounts at the infection timepoint in urine samples of all VAP cases caused by *P. aeruginosa* and *Serratia marcescens* while being absent in other urine samples (Figure 5B). *P. aeruginosa* and *S. marcescens* are Gram-negative pathogens that share many functional and metabolic pathways that are not present in other Gram-negative organisms.[32] We also identified a novel target 1,3 dipropyl-6-aminouracil in urine samples of all Gram-negative infected VAP patients (n = 5) which was completely

absent in all Gram-positive infected patients (n = 3) (Figure 5C). Lastly, we identified several glucuronidated metabolites in VAP patients that were completely absent in the pre-infection timepoints (Figure 6A). 3-succinoylpyridine, a metabolite identified earlier in urine of smokers,[33] was also present in 7/8 VAP samples. One of the VAP–non-PA patients not showing expression of 3-succinoylpyridine also did not show expression of several glucuronidated metabolites, and was not due to the causative pathogen as the other *S. epidermidis* patient did show expression of these metabolites. 3-succinoylpyridine was also present in one pre-infection timepoint sample, however, its levels were approximately 400-fold lower than VAP-PA, and 100-fold lower than VAP–non-PA (Figure 6B).

Discussion

Unbiased mass spectrometric platforms are one of the most powerful tools utilised in the biomarker discovery field due to their sensitive and holistic approach. Utilising such an approach on urine samples of VAP patients, we show that the global metabolic profile could accurately discriminate VAP caused by *P. aeruginosa* (VAP-PA) from VAP caused by other aetiologies. We also identified several metabolites specific for
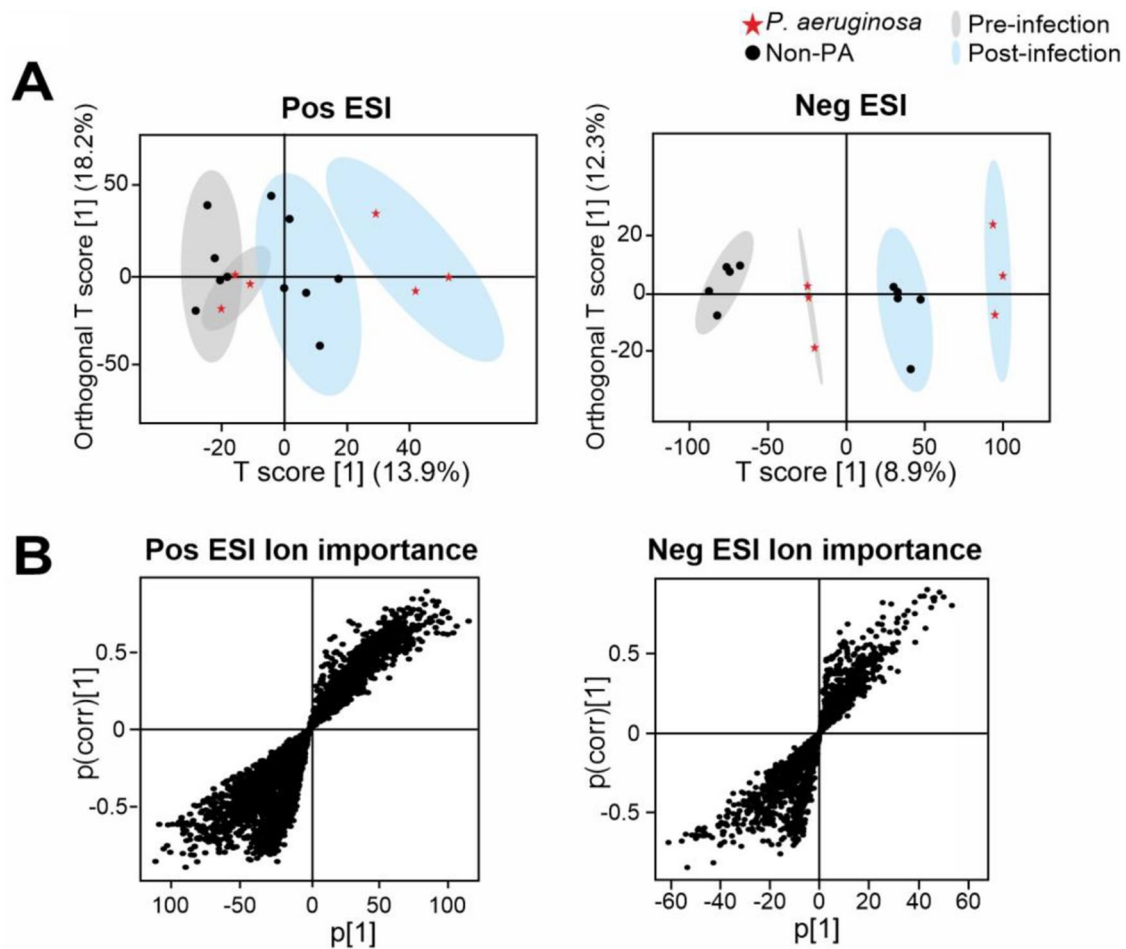
Figure 4. Clustering of VAP patient urine samples by OPLS analyses. (A) Orthogonal Projection to Latent Structures (OPLS) for the separation of spectra from patient urine collected at pre-infection timepoint and at presumptive diagnosis of VAP timepoint. Analyses were performed for datasets of both positive and negative ionisation mode (Pos ESI, Neg ESI) and the shaded area enclosing each group represents 95% confidence interval. (B) S-plots constructed from the supervised OPLS analysis of Pos ESI and Neg ESI respectively. Metabolites with the highest abundance and correlation in the VAP-PA samples populate the upper right quadrant, whereas metabolites with the lowest abundance and correlation in the VAP-PA group are residing in the lower left-hand quadrant.

VAP-PA at the time of the clinical suspicion of VAP prior to definitive diagnosis on BAL culture. Interestingly, in Neg ESI mode, early host metabolite profile can already cluster individuals that would later develop VAP-PA. Several studies have shown that host immune status is extremely important to develop *P. aeruginosa* infection.[34,35] The high robustness of the current approach is also exemplified by identification of antibiotics and anaesthetics that these patients received as well as confirmation of infection-related compounds identified in prior studies, such as the histidine derivative hydantoin-5-propionic acid.[20] Hydantoin-5-propionic acid is part of the L-Histidine degradation VI pathway, which is one of 2 pathways used to catabolise histidine. In mammals, hydantoin-5-propionic acid is the final catabolising step and it is secreted through urine. High levels of urinary hydantoin-5-propionic acid occur in patients with genetic disorders of folate/vitamin B12 metabolism.[36] Some bacteria are capable of using it as a sole source of carbon for growth.[37] An essential metabolite screening test for drug target screening pathogenic bacteria

including hydantoin-5-propionic acid is currently patented [WO-2011034397-A2].

We identified 3 novel metabolites to be highly specific to VAP-PA as these were completely absent for all other aetiologies and from the pre-infection timepoints. Two other metabolites ($C_{39}H_{50}O_{19}$ Rt 13.970; $C_{36}H_{27}N_{26}P_3$ Rt 13.750) were only present in VAP due to *P. aeruginosa* and *S. marcescens* displaying high ion intensities but were absent in all other aetiologies and in the pre-infection timepoints. *P. aeruginosa* and *S. marcescens* are both Gram-negative pathogens and share many functional and metabolic pathways that are not present in other Gram-negative organisms. For instance, the type 6 secretion system (T6SS) of *S. marcescens* is more closely related to the A-type family of *P. aeruginosa* compared to T6SSs in other Enterobacteriaceae.[32]

Similar to histidine and its derivatives as a marker of Gram-negative infections identified in prior studies,[20] we also identified a novel target, 1,3 dipropyl-6-aminouracil that was highly sensitive and specific for Gram-negative infected VAP patients. However, the source of 1,3 dipropyl-6-aminouracil is unknown
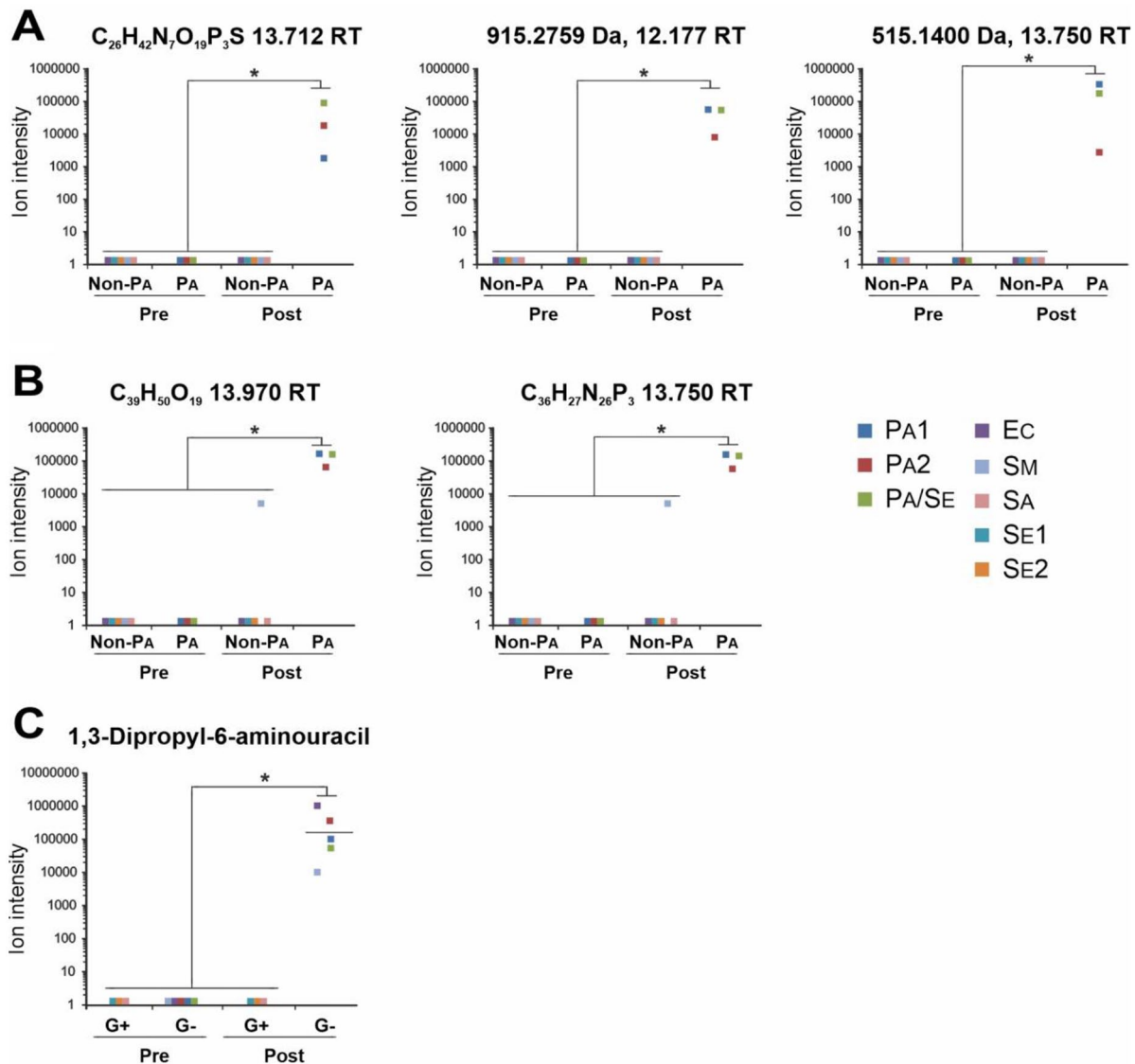
Figure 5. Top discriminating metabolites in univariate analyses. (A) Top 3 targets exclusively present in VAP caused by *P. aeruginosa*. Data is represented as integrated ion intensity extracted through LC-MS data. (B) Top 2 targets exclusively present in *P. aeruginosa* and *S. marcescens* VAP. (C) 1,3-dipropyl-6-aminouracyl showed increased excretion in Gram-negative (G–) VAP and was absent in gram-positive (G+) and pre-infection control samples. *A–C*, data is represented as integrated ion intensity extracted through LC-MS data. $P < .05$ in *A–B* indicates significance between VAP-PA and all other samples and in *C* indicates significance between Gram-negative VAP and all other samples. Statistical differences were calculated using Mann–Whitney test.

and could be both pathogen and host derived. Currently, 1,3 dipropyl-6-aminouracil is commercially produced and used as an intermediate in the production of xanthine derivatives which are under investigation as inhibitors in cancer treatment. We also identified several glucuronidated compounds in VAP due to both Gram-positive and Gram-negative pathogens, but absent in the pre-infection timepoints. Glucuronidation is a process whereby glucuronic acid is transferred to compounds to improve solubilisation and excretion,[38] and therefore could serve as a robust urinary marker. Despite extensive existing libraries of host glucuronidated compounds, our glucuronidated targets did not match any of these known xenobiotics, suggesting that these could be of bacterial origin and then further modified by the human body.

Lastly, we identified 3-succinoylpyridine, a compound which has previously been shown to be excreted in urine of smokers during nicotine breakdown.[33] Interestingly, several prokaryotic organisms, in particular *Pseudomonas spp.*, are known to actively use the pyrrolidine pathway of nicotine degradation.[39] This specific pathway hints at the option that nicotine present in the human body is degraded more effectively through this pathway by the causative pathogen of pneumonia, that is, *P. aeruginosa*. Of the 3 VAP-PA patients, 2 were non-smokers and the third, who was an active smoker, had been critically ill. While 3-succinoylpyridine was also observed in VAP due to other aetiologies and in one pre-infection timepoint sample, levels of this metabolite were fivefold higher in VAP-PA cases compared to other VAP aetiologies and 400-fold higher than the one pre-infection timepoint sample.

While many of the targets identified here in the discrimination of VAP-PA from other VAP aetiologies remain to
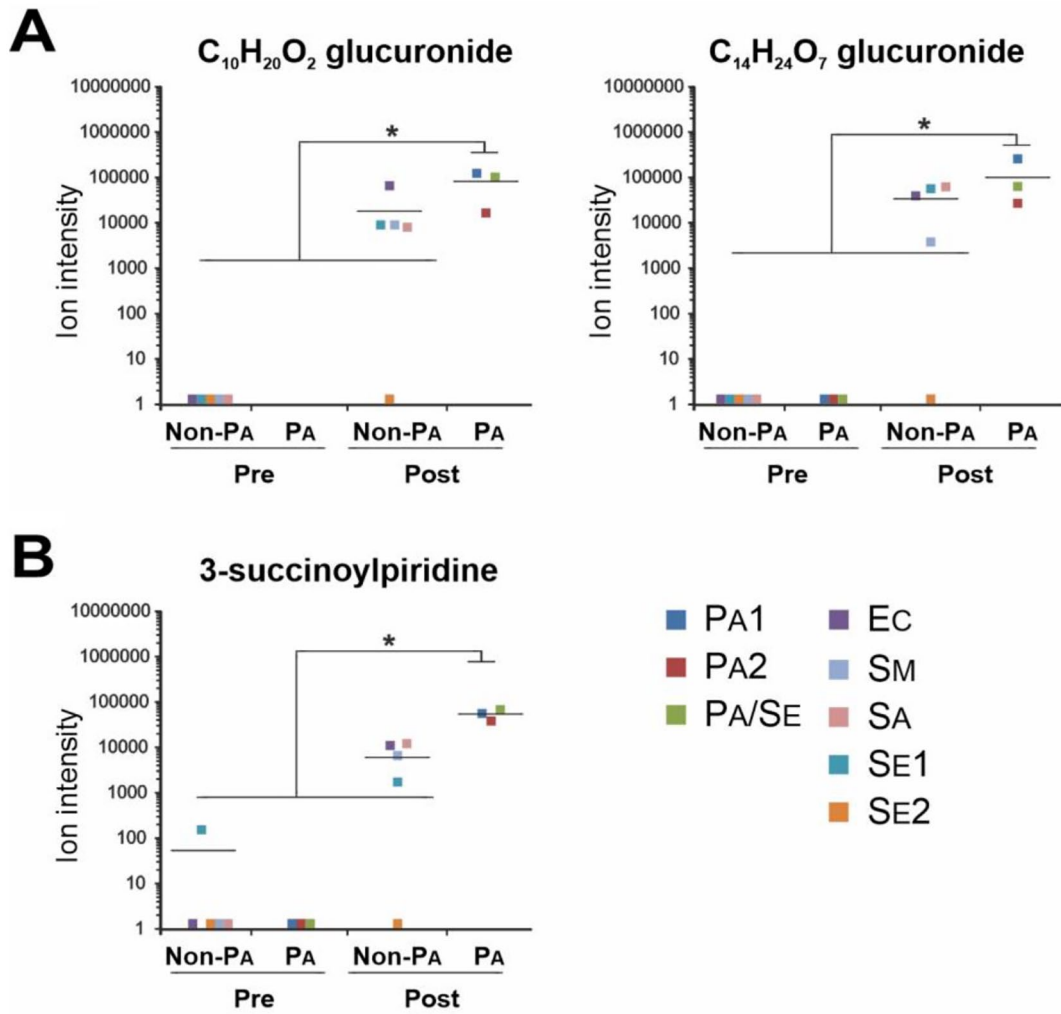
Figure 6. Identified metabolites showing increased excretion in *P. aeruginosa* VAP patients. (A) MS/MS identified glucuronidated targets showing increased excretion in VAP-PA. (B) 3-succinoylpyridine showed increased excretion in all VAP patients, although increase in excretion was remarkably higher in VAP-PA urine samples. *A–B*, data is represented as integrated ion intensity extracted through LC-MS data. $P < .05$ indicates significance between VAP-PA and all other samples. Statistical differences were calculated using Mann–Whitney test.
Abbreviations: EC, *E. coli*; PA, *P. aeruginosa*; SA: *S. aureus*; SE, *S. epidermidis*; SM, *S. marcescens*.

be confirmed in further validation studies, this study already demonstrates that urinary metabolomic biomarkers can differentiate VAP-PA from VAP due to other bacterial aetiologies as early as the time of the clinical suspicion of VAP. A quick identification of the pathogen on the day of the presumptive clinical diagnosis of VAP, which is generally 48 hours before the microbiological confirmation on BAL samples, could effectively eliminate the need of waiting for culture results allowing specific pathogen-targeted or narrow-spectrum treatments to be initiated immediately. Several pathogen-targeted clinical trials are ongoing such as a trial testing a bi-specific antibody targeting 2 virulence factors on the surface of *P. aeruginosa*.[11,12]

Limitations

Even though we identified several interesting biomarkers that can discriminate VAP-PA from other aetiologies, these results were obtained from a limited patient population. A

prospective sample collection study in intensive care unit patients is always difficult to perform and has several reasons. First, the ICU appears as a quite challenging research environment where patients arrive in life-threatening conditions and proper care is an absolute priority. A specific issue of this study group, namely intubated patients, is that they are under general sedation, thus informed consent needs to be given by closest relative. To ensure samples before presence of infection these need to be taken and thus approved <24 hours after intubation, research is not the top priority for relatives available at this point of time. A second difficulty is the complex disease pattern of these patients leading to paired pre-infection samples to be the most fitting control group and requiring strict inclusion and exclusion criteria, specifically absence of infection on admission and urinary infection, severely limiting the available patient population. Third, VAP is known to be frequently caused by more than 1 pathogen. Co-infection by multiple species of bacteria from

different classes complicates both diagnostics and antibiotic treatment leading to an increase in attributable mortality.[6,7] Species-specific biomarkers would thus not completely remove the need for broad-spectrum antibiotics, but more likely aid in combatting specific infections more effectively by allowing rapid implementation of targeted therapies and improve antibiotic stewardship. All these limitations are clearly exemplified in this study where from several 100 ICU admitted patients, prospective sampling could be performed in only 100 mechanically ventilated patients due to strict inclusion and exclusion criteria. From these 100 patients, only 8 fulfilled the criteria of VAP development due to 'defined' bacterial aetiologies and could be included for biomarker analysis. Of these 8 patients only 3 were infected by *P. aeruginosa*, of which 1 patient had a co-infection of *P. aeruginosa* and *S. epidermidis*. Thus while the results of this limited dataset need to be further validated in large multicentre studies, despite the low enrolments for VAP-PA, this study included samples from patients representing all the major VAP aetiologies including *P. aeruginosa*, *S. aureus*, Enterobacteriaceae, and the recently considered commensal pathogen *S. epidermidis*.[7] Most importantly, analysis of pre-infection timepoint samples corrected for individual metabolite variations in patients, thus greatly enhancing the power of this biomarker discovery study.

Besides the sample size limitation, metabolite identifications are also not always possible with MS/MS analysis as the identifications are dependent on the existing database.[25] While the increasing use of metabolomic studies are facilitating construction of more comprehensive and annotated metabolomic databases, identification of targets for their use as biomarkers is not always necessary. For instance, several MS approaches are currently being clinically utilised for microbial identification and diagnosis such as MALDI-TOF fingerprints of spectral patterns of proteins from isolated bacterial colonies.[40] Fully identified metabolites could be used to design among others, rapid antigen tests and would provide more biological information on the origin, function, and modifications of these metabolites and thus a better understanding of disease mechanisms that could be clinically targeted.

Conclusion

Despite these limitations, in this proof-of-principle study we identify several unique urinary metabolic biomarkers for VAP caused by *P. aeruginosa*. If further validated in large multicentre studies for their sensitivities and specificities have the potential to be developed into highly specific early VAP-PA biomarkers that could lead to an improved patient outcome.

Author Contributions

SKS designed and supervised the study. PJ was responsible for the inclusion of the patients and collection of samples. AH, HG and SMK performed microbiological analysis. B'SJ, AH, KB, PV, JB, CL, EF, GB and SKS carried out the experiments and analysed data. B'SJ, KB, AH, GB, AC, SMK, PJ, HG and SKS interpreted data. B'SJ and SKS wrote the manuscript that was edited by all other authors.

Availability of Data and Materials

The datasets generated and/or analysed during this study are included in this article. Further inquiries can be directed to the corresponding author upon reasonable request.

Ethical Approval and Informed Consent

Patient study was approved by the ethics committee of the University Hospital Antwerp (ECD: 12/12/112) and written consent was obtained from the closest relatives of these patients.

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Supplemental Material

Supplemental material for this article is available online.

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