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Lipid metabolites as potential diagnostic and prognostic biomarkers for acute community acquired pneumonia

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Abstract

Early diagnosis of acute community-acquired pneumonia (CAP) is important in patient triage and treatment decisions. To identify biomarkers that distinguish patients with CAP from non-CAP controls, we conducted an untargeted global metabolome analysis for plasma samples from 142 patients with CAP (CAP cases) and 97 without CAP (non-CAP controls). Thirteen lipid metabolites could discriminate between CAP cases and non-CAP controls with area-under-the-receiver-operating-characteristic curve of $>0.8$ ($P \leq 10^{-9}$). The levels of glycosphingolipids, sphingomyelins, lysophosphatidylcholines and L-palmitoylcarnitine were higher, while the levels of lysophosphatidylethanolamines were lower in the CAP cases than those in non-CAP controls. All 13 metabolites could distinguish CAP cases from the non-infection, extrapulmonary infection and non-CAP respiratory tract infection subgroups. The levels of trihexosylceramide (d18:1/16:0) were higher, while the levels of lysophosphatidylethanolamines were lower, in the fatal than those of non-fatal CAP cases. Our findings suggest that lipid metabolites are potential diagnostic and prognostic biomarkers for CAP.

1. Introduction

Acute community-acquired pneumonia (CAP) is one of the most common causes of hospitalization and death (Musher and Thorner, 2014). In particular, emerging viral infections including the pandemic and avian influenza viruses, and the SARS and MERS coronaviruses, are associated with mortality rates ranging from 10–60% (Chan, et al., 2015; Meng, et al., 2007; Cheng, et al., 2012; To, et al., 2013a). Early recognition and treatment of CAP improve outcome (Li, et al., 2010; Musher and Thorner, 2014). The diagnosis of CAP depends on compatible symptoms and signs, together with new pulmonary infiltrates on chest imaging (Musher and Thorner, 2014). However, distinguishing CAP from other pulmonary pathologies can be difficult (Wunderink and Waterer, 2014). Some patients with CAP may not have obvious respiratory symptoms. Non-CAP causes of pulmonary infiltrates on chest radiograph can mimic those due to CAP. Therefore, alternative modalities of diagnosing CAP are important.

Biomarkers in body fluids have been increasingly used in determining the diagnosis and predicting the prognosis in lung diseases (Upadhyay and Niederman, 2013; Wheelock, et al., 2013). Procalcitonin is a sensitive biomarker for bacterial pneumonia, but the level is much lower in pneumonia caused by viruses or atypical bacteria (Muller, et al., 2010; Upadhyay and Niederman, 2013). Adenosine deaminase has been used as a biomarker for tuberculosis pleural effusion (Chen, et al., 2004). Prognostic markers include C-reactive protein, procalcitonin, midregional proadrenomedullin, midregional pro-atrial natriuretic peptide, copeptin, endothelin-1 precursor, kallistatin, and cytokines (Christ-Crain and Opal, 2010; Lin, et al., 2013).

Metabolomics study, which is a global strategy to detect low-molecular weight components (Wheelock, et al., 2013), has been increasingly used to identify biomarkers associated with infections (Lam, et al., 2014; Lam, et al., 2015; Langley, et al., 2013; Lau, et al., 2015; Tam, 2013). Using metabolomic analysis, we have previously identified 5 metabolites which could distinguish patients with bacteremia from those without bacteremia (To, et al., 2015). In a small study involving 11 children with severe pneumonia, plasma and urine metabolites were found to be significantly different from healthy community controls (Laiakis, et al., 2010). Plasma metabolites were able to
discriminate between patients with tuberculosis and healthy controls (Frediani, et al., 2014). Urine metabolites were found to be different between patients with pneumococcal pneumonia and those with other causes of pneumonia (Slupsky, et al., 2009). Metabolic profiles may also predict the prognosis of patients with CAP (Seymour, et al., 2013).

In this study, we used a metabolomic approach to identify biomarkers that can differentiate between patients with CAP and those without CAP. Furthermore, we determined whether these biomarkers can predict the prognosis of patients with CAP.

2. Materials and methods

2.1. Study design and definitions

This study included patients admitted to Queen Mary Hospital in Hong Kong between 2007 and 2013. Archived plasma samples collected at admission were retrieved for metabolomics study. CAP cases were hospitalized adult patients 18 years or above with radiological evidence of consolidation within 48 hours of admission and at least one of the following symptoms (cough with or without sputum production, dyspnea, tachypnea, pleuritic chest pain) plus one auscultatory finding or one sign of infection (core body temperature >38 °C, shivers, leukocyte count >10,000 cells/µl or <4000 cells/µl (Muller, et al., 2010). Non-CAP controls were hospitalized adult patients not fulfilling the inclusion criteria for cases, and these included 3 subgroups: the non-infection subgroup consisted of patients without any clinical evidence of infection; the extrapulmonary infection subgroup consisted of patients with infection outside the respiratory tract; and the non-CAP respiratory tract infection subgroup consisted of patients with respiratory tract infections but without radiological evidence of pneumonia. Patients were excluded if they were aged <18 years or had radiological evidence of consolidation occurring >2 days after hospital admission, their clinical records were not accessible for review, or if archived plasma samples were not available. Clinical and microbiological data were retrieved from the Clinical Management System. Pneumonia Severity Index was used to assess the clinical severity (Fine, et al., 1997). Fisher’s exact test and Student’s t test were used in the comparison of categorical and continuous variables, respectively. This study was approved by the Institutional Review Board (HKU/HA HKW IRB; reference number UW 11-360).

2.2. Untargeted plasma metabolomics profiling and identification of metabolites

Untargeted plasma metabolomic profiling and identification of metabolites were performed as we described previously with modifications (To, et al., 2015). Ultra-high-performance liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry (UHPLC-ESI-Q-TOF-MS) analysis was used to identify the metabolites in the plasma (To, et al., 2013b; To, et al., 2015; Woo, et al., 2014). Details of the sample preparation, UHPLC-ESI-Q-TOF-MS and identification of metabolites are provided in the supplementary information.

The metabolomic data was analyzed and reported according to the Metabolomics Standards Initiative (https://msi-workgroups.sourceforge.net/). To identify molecular features (MFs) that could discriminate CAP cases with non-CAP controls, both multivariate and univariate analyses were performed. For multivariate analyses, principal component analysis (PCA) and partial-least squares discriminant analysis (PLS-DA) were performed for unsupervised and supervised analyses, respectively. For univariate analysis, volcano plots were constructed and analyzed using Student’s t-test. Significant MFs with variable importance in projection (VIP) score of >1 in PLS-DA and volcano plot analysis of fold-change >1.5 and P value <0.05 were included for univariate receiver operating characteristic (ROC) curve analysis. Significant MFs with area-under-the-ROC curve (AUC) ≥0.8 were identified and box-whisker plots were generated. Heatmap based on significant metabolites were constructed using hierarchical clustering analysis (HCA). The identities of significant MFs were characterized.

3. Results

3.1. Baseline characteristics of patients

A total of 239 patients were included in this study, including 142 CAP cases and 97 non-CAP controls. The mean age was 76.7 years. There were no statistically significant differences in terms of age, gender and in-hospital mortality during hospitalization between CAP cases and non-CAP controls (Table 1). There were no differences in the underlying diseases except that chronic lung disease was more common among the CAP cases (28.9% vs 15.5%; P = 0.019). Among the CAP cases, the most commonly identified respiratory pathogens were Haemophilus influenzae (n = 10) and Streptococcus pneumoniae (n = 8) (Table S1), and blood culture was positive in 5 patients, including 3 patients with S. pneumoniae (1 patient with Escherichia coli and 1 patient with Corynebacterium species). Among the 97 non-CAP controls, 49 did not have infection, 26 had extrapulmonary infections, and 22 had respiratory tract infections without evidence of pneumonia (Table S2).

3.2. Global metabolome changes associated with CAP

Global metabolome was determined using UHPLC-ESI-Q-TOF-MS. A total of 2960 and 1796 MFs were found in positive and negative modes, respectively. Unsupervised PCA without bias in positive mode showed that 36.5% of the total variance was represented by the first 2 principal components (Supplementary Fig. S1A). PCA in negative mode showed that 30.4% of the total variance was represented by the first 2 principal components (Supplementary Fig. S1B). PCA score plots of all MFs identified showed that samples within the same group were closely clustered in both positive and negative modes.

Supervised PLS-DA score plots after data training were constructed to select MFs that can discriminate CAP cases from non-CAP controls. The score plot in positive mode showed an accuracy of 97.5%, a multiple correlation coefficient (R2) of 90.4% and a cross-validated R2 (Q2) of 83.9% (Supplementary Fig. S1C), while the score plot in negative mode showed an accuracy of 96.3%, a R2 of 86.9% and a Q2 of 79.3%.
The parameters indicated optimal fitness and prediction performance of the PLS-DA models in both ionization modes. More detailed analysis of the MFs using volcano plot analysis showed that 368 MFs in the positive mode and 448 MFs in the negative mode were significantly different between the CAP cases and non-CAP controls (Supplementary Fig. S1E and F). Among these MFs, 274 MFs in positive mode and 180 MFs in negative mode had VIP scores >1. MFs were manually inspected, and those that were not related to interference were included in univariate ROC curve analysis.

The univariate ROC curve analysis showed that 13 metabolites had AUC ≥0.8. The metabolites were identified as acylcarnitine, sphingolipids (glycosphingolipid and sphingomyelin) and glycerophospholipids (lysophosphatidylcholine [LysoPC] and lysophosphatidylethanolamine [LysoPE]) (Supplementary Table S3, Supplementary Fig. S2). CAP cases had higher levels of acylcarnitine, glycosphingolipids, LysoPC and sphingomyelins, but lower levels of LysoPE, than non-CAP controls (Fig. 1). HCA of the 13 differential metabolites separated the CAP cases and non-CAP controls into 2 major clusters (Supplementary Fig. S3). Univariate ROC curve analysis showed that LysoPC(18:1[9Z]) had the largest AUC (0.905), and highest sensitivity (86.6%) and specificity (92.3%) (Table 2). The 13 metabolites were further compared between CAP cases and each of the control subgroups (non-infection subgroup, extrapulmonary infection subgroup and non-CAP respiratory tract infection subgroup). All 13 metabolites were significantly different between CAP cases and all these subgroups (Supplementary Fig. S4, Table S4).

The 13 metabolites were also compared between different subgroups of the CAP cases. The levels of trihexosylceramide(d18:1/16:0) were higher in the 16 fatal cases than those of the 126 non-fatal cases, while the levels of LysoPE(22:6[p4Z,7Z,10Z,13Z,16Z,19Z]/0:0), LysoPE(18:2[9Z,12Z]/0:0), LysoPE(20:4[8Z,11Z,14Z,17Z]/0:0) and LysoPE(18:1[9Z]/0:0) were lower in the fatal than those of non-fatal cases (Table 3). LysoPC(18:1[9Z]), LysoPE(22:6[p4Z,7Z,10Z,13Z,16Z,19Z]/0:0), LysoPE(18:2[9Z,12Z]/0:0) and LysoPE(20:4[8Z,11Z,14Z,17Z]/0:0) were also significantly lower in CAP patients with PSI class 5 than those with PSI class 4 or below. The levels of these 13 metabolites were not significantly different between CAP patients with bacterial and viral/mycoplasma pneumonia (P > 0.1 for all 13 metabolites) (Supplementary Fig. S5).

To assess whether the 13 metabolites can be biomarkers for pneumonia caused by classical pathogens that are commonly identified CAP in other case series, we have compared CAP cases with these classical pathogens (excluding Enterobacteriaceae and glucose non-fermenters) with non-CAP controls. All 13 metabolites remained significantly different between non-CAP controls and patients with CAP due to these classical pathogens (Table S5). LysoPC(18:1[9Z]) had the highest AUC (0.982), with a sensitivity of 94.4% and a specificity of 97.9%.

4. Discussion

In this study, we have used an untargeted global metabolomics approach to identify metabolites which have high sensitivities and high specificities in discriminating CAP cases from non-CAP controls. The levels of 5 sphingolipids (3 glycosphingolipids and 2 sphingomyelins), 1 glycerophospholipid (a LysoPC) and 1 acylcarnitine (L-palmitoylcarnitine) were higher, while the levels of 6 glycerophospholipids (all LysoPEs)
were lower in the CAP cases than those in non-CAP controls. The changes in plasma lipid metabolite levels in CAP patients may be related to several reasons. Firstly, lipids make up 90% of surfactant, and disturbance in surfactant during pneumonia may cause changes in lipid metabolism (Wheelock, et al., 2013). Secondly, lipids are important inflammatory mediators during infection, and changes in lipid metabolites are observed during conditions such as sepsis, bacteremia, and viral infection (Cui, et al., 2013; Langley, et al., 2013; To, et al., 2015). Thirdly, lipids are important in the structure and function of bacteria, and lipid metabolites are produced by bacteria (Kondakova, et al., 2015; Lau, 2014). In our study, the plasma levels of LysoPC were significantly lower in high-risk CAP cases, and were also lower in fatal CAP cases than those of non-fatal CAP cases, although not reaching statistical significance (Table 3). The lack of statistical significance may be related to the relative small number of fatal cases in our cohort. Further studies are needed to assess whether LysoPC is a favorable prognostic factor.

In this study, 7 of the 13 lipid metabolites that discriminated CAP cases from non-CAP controls were glycerophospholipids (1 LysoPC or 6 LysoPEs), which are components of the pulmonary surfactant and are important in modulating in inflammation (Makide, et al., 2009). LysoPC(18:1[9Z]) had the highest sensitivity and specificity. The level of LysoPC was significantly lower in high-risk CAP cases, and were also lower in fatal CAP cases than those of non-fatal CAP cases, although not reaching statistical significance (Table 3). The lack of statistical significance may be related to the relative small number of fatal cases in our cohort. Further studies are needed to assess whether LysoPC is a favorable prognostic factor.

On the other hand, the plasma levels of all LysoPEs were lower in CAP cases than those of non-CAP controls. In a mouse peritonitis model, LysoPE has been shown to increase the level of the anti-inflammatory cytokine interleukin-10, and reduces plasma leakage (Hung, et al., 2011). The levels of 4 LysoPEs were significantly lower in the fatal CAP cases than the non-fatal CAP cases. Therefore, a higher LysoPE level in CAP may improve survival via the amelioration of excessive inflammation.

In this study, the levels of 5 sphingolipids were higher in the CAP cases than in the non-CAP controls. Sphingomyelins are associated with many pulmonary diseases (Uhlig and Gullbins, 2008). Sphingolipids are critical in the structure and function of the plasma membrane, and are involved in many cellular processes. Lactosylceramide, trihexosylceramide and tetrahexosylceramide are metabolites from ceramide (Fig. 2), and ceramide is important in regulating immune cell

### Table 2

| Metabolite Class             | Metabolites                                      | AUC    | 95% CI       | Sensitivity (%) | Specificity (%) | VIP Score | Fold-change | P value        |
|------------------------------|--------------------------------------------------|--------|--------------|----------------|----------------|-----------|-------------|----------------|
| Acylcarnitine                | L-palmitoyl carnitine                            | 0.834  | 0.783–0.886  | 69.1           | 83.1           | 1.62      | +2.98       | 8.31E-10       |
| Glycospingolipid             | Tetrahexosylyceramide (d18:1/16:0)               | 0.802  | 0.742–0.861  | 71.1           | 79.6           | 1.52      | +2.06       | 5.46E-13       |
| Glycospingolipid             | Trihexosylyceramide (d18:1/16:0)                 | 0.828  | 0.773–0.882  | 70.1           | 82.4           | 1.62      | +2.18       | 5.46E-16       |
| Lysopephosphidylinole        | Lactosyleramide (d18:1/16:0)                     | 0.847  | 0.797–0.897  | 78.4           | 75.4           | 1.68      | +2.32       | 6.35E-16       |
| Lysopephosphidylinole        | LysoPC(18:1[9Z])                                  | 0.905  | 0.854–0.955  | 86.6           | 92.3           | 1.71      | +2.42       | 4.18E-09       |
| Lysopephosphidylinole        | LysoPE(18:0/0:0)                                  | 0.86   | 0.807–0.913  | 75.3           | 89.4           | 1.69      | +2.34       | 1.81E-27       |
| Lysopephosphidylinole        | LysoPE(18:0/0:0)                                  | 0.802  | 0.743–0.860  | 74.2           | 73.9           | 1.39      | +1.95       | 5.41E-18       |
| Lysopephosphidylinole        | LysoPE(18:1[9Z])/0:0                              | 0.802  | 0.745–0.859  | 73.2           | 73.9           | 1.48      | +2.20       | 1.61E-17       |
| Lysopephosphidylinole        | LysoPE(18:2[9Z,12Z])/0:0                         | 0.829  | 0.775–0.884  | 76.3           | 78.2           | 1.58      | +2.89       | 1.60E-21       |
| Lysopephosphidylinole        | LysoPE(20:4[8Z,11Z,14Z,17Z,19Z])/0:0             | 0.824  | 0.766–0.882  | 70.1           | 87.3           | 1.55      | +2.74       | 4.86E-23       |
| Lysopephosphidylinole        | LysoPE(22:6[4Z,7Z,10Z,13Z,16Z,19Z])/0:0          | 0.831  | 0.774–0.887  | 72.2           | 78.9           | 1.48      | +2.78       | 1.88E-21       |
| Sphingomyelins               | SM(d18:0/16:0)                                    | 0.861  | 0.812–0.911  | 74.2           | 81.7           | 1.76      | +2.78       | 8.69E-11       |
| Sphingomyelins               | SM(d18:1/16:0)                                    | 0.861  | 0.814–0.908  | 78.4           | 82.4           | 1.69      | +1.54       | 7.97E-24       |

AUC = area-under-the-receiver-operating-characteristic curve; CI = confidence interval; LysoPC = lysophosphatidylcholine; LysoPE = lysophosphatidylethanolamine; VIP = variable importance in projection.

a VIP score in PLS-DA analyses.
b Fold change of CAP cases relative to non-CAP controls. (+) indicates increased level while (-) indicates decreased level in CAP cases when compared to non-CAP controls.
c Student’s t-test.

### Table 3

Comparison of the levels of the 13 metabolites between fatal (n = 16) and non-fatal (n = 126) CAP cases, and between patients in the high risk class (n = 34) and those in low risk class (n = 108).

| Metabolites              | Fold differences between fatal and non-fatal CAP casesb | P value | Fold differences between high risk and low risk CAP casesb,c | P value |
|--------------------------|--------------------------------------------------------|---------|------------------------------------------------------------|---------|
| L-palmitoylcarnitine     | +1.17                                                  | 0.524   | +1.38                                                      | 0.0736  |
| Tetrahexosylyceramide    | +1.22                                                  | 0.136   | +1.14                                                      | 0.211   |
| Trihexosylyceramide      | +1.35                                                  | 0.0188  | +1.09                                                      | 0.425   |
| Lactosyleramide          | +1.26                                                  | 0.114   | –1.01                                                      | 0.942   |
| LysoPC(18:1[9Z])         | –1.30                                                  | 0.246   | –1.47                                                      | 0.0234  |
| LysoPE(16:0/0:0)         | –1.29                                                  | 0.0634  | –1.12                                                      | 0.225   |
| LysoPE(18:0/0:0)         | –1.20                                                  | 0.177   | –1.13                                                      | 0.198   |
| LysoPE(18:1[9Z])/0:0     | –1.52                                                  | 0.0396  | +1.19                                                      | 0.185   |
| LysoPE(18:2[9Z,12Z])/0:0 | –1.68                                                  | 0.0190  | –1.43                                                      | 0.054   |
| LysoPE(20:4[8Z,11Z,14Z,17Z,19Z])/0:0 | –1.42 | 0.0370   | –1.47                                                      | 0.00116 |
| LysoPE(22:6[4Z,7Z,10Z,13Z,16Z,19Z])/0:0 | –1.47 | 0.0357   | –1.63                                                      | 0.000253|
| SM(d18:0/16:0)           | +1.32                                                  | 0.195   | +1.06                                                      | 0.743   |
| SM(d18:1/16:0)           | +1.05                                                  | 0.443   | –1.02                                                      | 0.668   |

a “+” indicates the level is higher in fatal cases than that of non-fatal cases. “−” indicates that the level is lower in fatal cases than that of non-fatal cases.
b High risk = Pneumonia Severity Index class V; Low risk = Pneumonia Severity Index class 4 or below.
trafficking and the vascular and epithelial integrity (Maceyka and Spiegel, 2014). For example, lactosylceramide is a mediator of inflammatory response triggered by *Pneumocystis* (Evans, et al., 2012). In *vivo*, influenza virus infection induces elevated levels of hexosylceramides (Tanner, et al., 2014). Inhibition of glycosphingolipid synthesis has been associated with reduced pulmonary inflammation in mice challenged with lipopolysaccharide (LPS) (Dechecchi, et al., 2011). Sphingomyelin is a structural component of cell membranes (Maceyka and Spiegel, 2014). In mice, sphingomyelin synthase 2 (SMS2), which catalyzes the synthesis of sphingomyelin, is activated during LPS-induced lung injury, and inhibition of SMS2 attenuated lung injury and endothelial barrier disruption (Anjum, et al., 2012). This mice study suggested that sphingomyelin is important in lung injury, and the higher level of sphingomyelins in our CAP cases is compatible with this hypothesis. However, other studies suggest that high levels of sphingomyelin may be protective. Stringer *et al.* showed that patients with acute lung injury had lower levels of sphingomyelin when compared to healthy controls, although the absolute difference is small (Stringer, et al., 2011).

The levels of L-palmitoylcarnitine were significantly higher in CAP cases than non-CAP controls. L-palmitoylcarnitine facilitates the transport of long-chain fatty acids from the cytoplasm into the mitochondria, where fatty acid beta-oxidation takes place. During acute lung injury, fatty acid metabolism is disturbed (Guo, et al., 2014). A previous study also showed that high levels of L-palmitoylcarnitine were associated with poorer prognosis in patients with chronic heart failure (Ueland, et al., 2013). In our study, there is no significant difference in the proportion of patients with heart disease.

These metabolic pathways are interconnected (Fig. 5). Fatty acid beta-oxidation is important in providing the substrate for the generation of sphingolipids and glycerophospholipids via ceramide and phosphatidic acid, respectively (Ecker and Liebisch, 2014; Maceyka and Spiegel, 2014). Ceramide is converted to glucosylceramide, which is then converted to lactosylceramide. Phosphatidic acid is converted to diacylglycerol, which is then converted to phosphatidylcholine and phosphatidylethanolamine. The sphingolipid pathway is also connected to the glycerophospholipid pathway via sphingosine 1-phosphate (Kihara, 2014; Maceyka and Spiegel, 2014).

There are several limitations in this study. Firstly, we have only tested the metabolome profile of the first available plasma samples of each patient. Serial monitoring of these metabolites may help to assess the disease progress. Secondly, we did not analyze the relationship between the specific pathogens and each metabolite because of the limited number of patients infected by each pathogen. Thirdly, a higher proportion of CAP cases had underlying chronic lung disease than non-CAP controls. Previous studies showed that the levels of plasma or serum metabolites are different between patients with chronic lung diseases and healthy controls. When compared with healthy controls, patients with chronic obstructive pulmonary disease had higher levels of glycerophosphocholine but lower levels of lipoprotein and amino acids, while patients with asthma had higher levels of taurine, lathosterol, bile acids, nicotinamide and adenosine-5'-phosphate than healthy controls (Comhair, et al., 2015; Wang, et al., 2013). Further studies are required to determine whether the levels of the metabolites identified in this study are different between patients with chronic underlying diseases and healthy controls. Fourthly, in our cohort, there were no patients with non-infective etiologies which can lead to pulmonary infiltrates, such as interstitial lung disease. Further studies should include patients with non-infective pulmonary disease without evidence of infection.

The detection of low-molecular-weight molecules is increasingly important in the diagnosis of infections. Using a global metabolomics approach, we have identified 13 lipid metabolites which may be used as rapid diagnostic or prognostic biomarkers for CAP. It would be important to verify these biomarkers in larger cohorts and in different populations. Furthermore, the role of these lipid metabolites in the pathogenesis of CAP deserves further studies.

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