Statistical learning for complex data to enable precision medicine strategies

Zwep, L.B.

Chapter 4

Longitudinal metabolomics of community-acquired pneumonia

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Abstract

Longitudinal biomarkers in patients with community-acquired pneumonia (CAP) may help monitoring of disease progression and treatment response. The metabolic host response could be a source of such biomarkers since it closely represents the current state of the patient. To this end, we performed longitudinal metabolic profiling for a comprehensive range of metabolites in patients with CAP. Previously collected serum samples from 25 patients with CAP with a confirmed Streptococcus pneumoniae infections were used. Samples were collected at multiple time points after hospital admission and up to 30 days after admission. A wide range of metabolites was measured, including amines, acylcarnitines, organic acids, and lipids. The associations between metabolites and C-reactive protein (CRP), procalcitonin, the CURB disease severity score (CURB) at admission, and total length of stay were examined. Distinct longitudinal profiles of metabolite profiles were identified, in particular for cholesteryl esters, diacyl-phosphatidylethanolamine, diacylglycerols, lysophosphatidylcholines, sphingomyelin, and triglycerides. Positive correlations were found between CRP and Phosphatidylcholine (PC) (34:1) (cor = 0.63) and negative correlations were found for CRP and nine lysophosphocholines (cor = 0.57 to 0.74). The CURB disease severity score was negatively associated with six metabolites, including acylcarnitines (tau = 0.64 to 0.58). Finally, we found negative correlations between the length of stay and six triglycerides (TGs), especially TGs (60:3) and (58:2) (cor = 0.63 and 0.61). In conclusion, the identified metabolites may provide inside into biological mechanisms underlying disease severity and may be of interest as potential biomarker to monitor treatment response.

4.1 Introduction

Community-acquired pneumonia (CAP) is a lower respiratory tract infection with a high incidence and is associated with the hospitalization of approximately one million adults per year (Battleman et al., 2002). The most common cause of CAP is *Streptococcus pneumoniae* (Meijvis et al., 2011). In hospitalized CAP patients, there is a need to monitor the antibiotic treatment response to optimize the treatment strategy (Pletz et al., 2022). In addition, there is a need for guidance on decisions about earlier termination of antibiotic treatment to minimize the risk of antimicrobial resistance. Monitoring of treatment response is currently achieved through observation of clinical symptoms and with inflammatory markers such as C reactive protein (CPR) and procalcitonin (PCT) (Aulin et al., 2021; Karakioulaki & Stolz, 2019). In particular, PCT is relevant for informing early treatment termination decisions but lacks predictive performance for CAP prognosis (Guo et al., 2018). Therefore, there is a need for biomarkers that give early insights into the clinical course of CAP.

Biomarkers that reflect the current physiological state of the patient have the potential to accurately monitor and predict the treatment response in CAP patients. Because the metabolome closely represents this physiological state, metabolomics-

techniques may enable discovery of relevant novel biomarkers. Indeed, for CAP and sepsis, the potential for metabolomics-based biomarkers measured at a static time point has been demonstrated (Seymour et al., 2013). However, the longitudinal monitoring of metabolic changes within patients may allow for an improved characterization of treatment response (Kohler et al., 2017). For example, CAP patients show a change in lysophosphatidylcholines that mirrors the transition from acute illness to recovery after starting antibiotic treatment (Müller et al., 2019). Further systematic characterization of longitudinal metabolic changes in CAP patients may thus be of relevance for identification of metabolic biomarkers that can predict and monitor the treatment response in these patients.

To this end, in this study, we aimed to comprehensively characterize the change of longitudinal metabolite profiles in hospitalized CAP patients with a confirmed *S. pneumoniae* infection using metabolomics, and relate these changes to disease severity, inflammation markers, and treatment response outcomes.

4.2 Materials and methods

4.2.1 Patient cohort

In this study, we utilized serum samples from 25 hospitalized CAP patients with an *S. pneumoniae* infection. These samples were previously collected as part of a larger clinical study that was performed between November 2007 and September 2010 (Meijvis et al., 2011). We selected samples from patients that had a confirmed infection with *S. pneumoniae*, while we excluded patients with a mixed infection or multiple pathogens. All patients that died within the study time were removed (one patient). Samples were collected at five times: on the day of admission (day 0), and days 1, 2, 4, and 30 after admission. CRP and creatinine were measured in the hospital setting at the same time points as blood samples were obtained. Not all time points were available for each patient, resulting in 115 samples over the 25 patients.

On the day of admission, disease severity was determined using the CURB score, which is a scoring system based on confusion, blood urea > 7 mmol/l, respiratory rate (RR) ≥ 30/min; systolic BP < 90 mmHg or diastolic BP ≤ 60 mmHg (Neill et al., 1996). A score of two or higher is classified as severe CAP.

4.2.2 Bio-analytical procedures

Serum samples were analyzed using five targeted LCMS methods and one targeted GCMS method by the Biomedical Metabolomics Facility of Leiden University, Leiden, The Netherlands, as described previously (den Hartog et al., 2021). A total of 369 unique metabolites was measured as relative levels, of which 6 metabolites were removed due to high missingness ($\leq$20%), resulting in 363 metabolites being evaluated in data analysis. Biochemically-selected sums and ratios of metabolites were calculated and added to the data (Table S4.1).

PCT was measured in the same serum samples used for the metabolomics analysis. PCT analysis was performed using the human procalcitonin CLIA kit from Abbexa (abx190129). Samples were measured in duplicate if sample volumes were sufficient (95% of samples).

4.2.3 Data analysis

The metabolite levels were scaled through log-transformation and standardization. To explore the variability of the high-dimensional metabolomics dataset, the dimension reduction method principal component analysis (PCA) was used. The PCA was used on the scaled metabolomics data over the different time points, with the metabolites as variables and each observation being a sample from a patient for a specific time point (Ham et al., 1997). As part of PCA, missing values were imputed through multiple imputation using expectation maximization (EM-PCA), which iteratively calculates the principal components and imputes the missing values (Josse et al., 2011).

To evaluate how much of the variation in the metabolites could be explained by the change over time, the first two principal components were related to time using a polynomial regression model. The importance of the metabolites to explain the variation between the patients over time was evaluated by evaluating the squared variable loadings. Specifically, the squared variable loadings within and between biochemical metabolite classes were evaluated to study similarities within classes and see which biochemical classes vary more between the patients.

To characterize the metabolic time profiles and profiles of current inflammation

Table 4.1: Patient characteristics

	CAP patients (N=25)
Age (years)	
Median [Min, Max]	67.0 [18.0, 98.0]
Sex	
Male	12 (48.0%)
Female	13 (52.0%)
CURB score	
Median [Min, Max]	1.00 [0, 3.00]
Duration of symptoms before admission (days)	
Median [Min, Max]	3.00 [1.00, 14.0]
Missing	15 (60.0%)
Antibiotic treatment before admission	
No	8 (32.0%)
Yes	2 (8.0%)
Missing	15 (60.0%)
Length of stay (days)	
Median [Min, Max]	7.50 [2.50, 24.5]

markers for different patients, we estimated the correlations between the scaled metabolite levels and the CRP, PCT and creatinine over time. Next, we evaluated which metabolites could be of interest for the prediction of the clinical course, by estimating the correlations between the scaled metabolite levels and a clinical disease severity marker (CURB score (Neill et al., 1996)) at hospital admission, and the outcome length of stay (LOS) in the hospital. Since the CURB and LOS are static values, while the metabolites changed over time, the correlations between these outcomes and the change in metabolite levels from baseline ($m_{t=k} - m_{t=0}$) at each time point (k) were calculated. The metabolites with the largest correlations were further evaluated in literature research to assess their biological function.

All analyses were performed in R. The scripts and data used for the analyses were deposited on GitHub (`github.com/vanhasseltlab/LongitudinalMetabolomicsCAP`).

4.3 Results

4.3.1 Metabolite time profiles

Metabolic profiling was performed for 25 patients and resulted in 363 metabolite levels on five time points. The patient characteristics are displayed in Table 4.1. Comorbidities in patients included kidney disease ($n = 1$), cardiovascular disease ($n = 4$), malignancy ($n = 2$), COPD ($n = 1$, $n_{missing} = 15$), diabetes ($n = 3$, $n_{missing} = 15$). No patients were using corticosteroids before admission ($n_{missing} = 15$).

Metabolite profiles within all CAP patients shifted over time, as shown in the PCA over all time points (Figure 4.1). The close relationship between metabolite levels and time is reflected in the results from the polynomial regression model which showed that 45% of the metabolite variation captured in these first two principal components

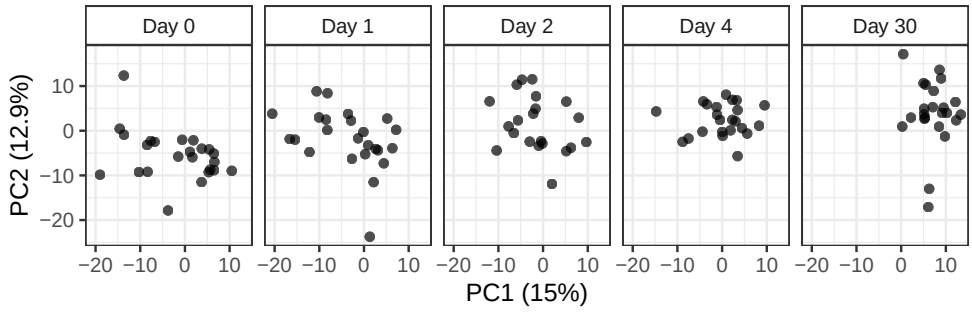

Figure 4.1: Patient metabolite profiles over time, in PCA scores. Every point represents the scores of an individual patient at a certain time point, in two dimensions based on the metabolite values. The panels show a trend over time of the metabolite profiles. Abbreviations: PC: principal component.

could be explained by time.

The metabolites that were targeted in this study were categorized into different biochemical classes. Metabolites from different biochemical classes showed distinct contributions to the total variation between the patients over time as was expressed in the variable loadings and directionality of the principal components (Figure 4.2). The squared PCA loadings represent the weight that the different metabolites in the biochemical class have in explaining the variation between patients over time. Of the variation in principal component one and two, 48% was explained by metabolites of the classes of cholesteryl esters, LPC's, sphingomyelins, diacylglycerols, and triglycerides (Figure 4.2A). The metabolites were categorized in classes based on their biochemistry and not based on their biological functions. The PCA results showed that metabolites that are categorized in the same class do not necessarily behave similarly (Figure 4.2B). For example, amino acids behave very differently from each other. Metabolites that do behave similarly in their biochemical class are for example triglycerides and sphingomyelins.

For each patient, the metabolic time profiles were shown as the two first components from the PCA (Figure 4.3, Figure S4.1). Generally, a shift from low to high principal component values was seen over time, corresponding to the shift in metabolite levels for the different metabolites (Figure 4.2B). The large variability in the time profiles, indicates a large interpatient variability in metabolic levels and changes over time.

4.3.2 Inflammation marker associations

To explore associations between metabolite profiles and inflammation, the metabolite values were compared to currently used inflammation biomarkers. Correlations were found between CRP and PCT and several metabolites. For example, phosphocholine (PC) (34:1) showed a positive correlation with CRP (cor = 0.63). Several individual lysophosphocholines (LPCs) and the sum of all LPCs showed a negative correlation with CRP (cor = -0.57 to -0.74, Figure 4.4A). PC (34:1) was found to decrease over time and several LPCs showed an increase over time, thereby mirroring the clinical disease progression (Figure 4.4B). Positive correlations with CRP and PCT were reported for the short-chain acylcarnitines (SCACs) tiglylcarnitine, 2 methylbutyroylcarnitine, and isovalerylcarnitine (cor with PCT = 0.61, 0.58, and 0.57; cor with CRP = 0.54, 0.64, and 0.51, respectively). Negative correlations were seen between the long-chain acylcarnitine (LCAC) stearoylcarnitine and CRP (cor = 0.62). This trend for decreasing SCACs over time is also represented by the positive correlation of CRP and PCT with the sum of all SCACs (cor = 0.55 and 0.53, respectively).

Correlations between metabolite levels and creatinine, a marker of renal failure, were also found. The same trends were seen for creatinine as for CRP and PCT (Figure S4.2). Also, strong positive correlations were found between creatine and 1-Methylhistidine, SDMA, inositol, homoserine, methionine sulfone, and octanoylcarnitine (cor > 0.7)

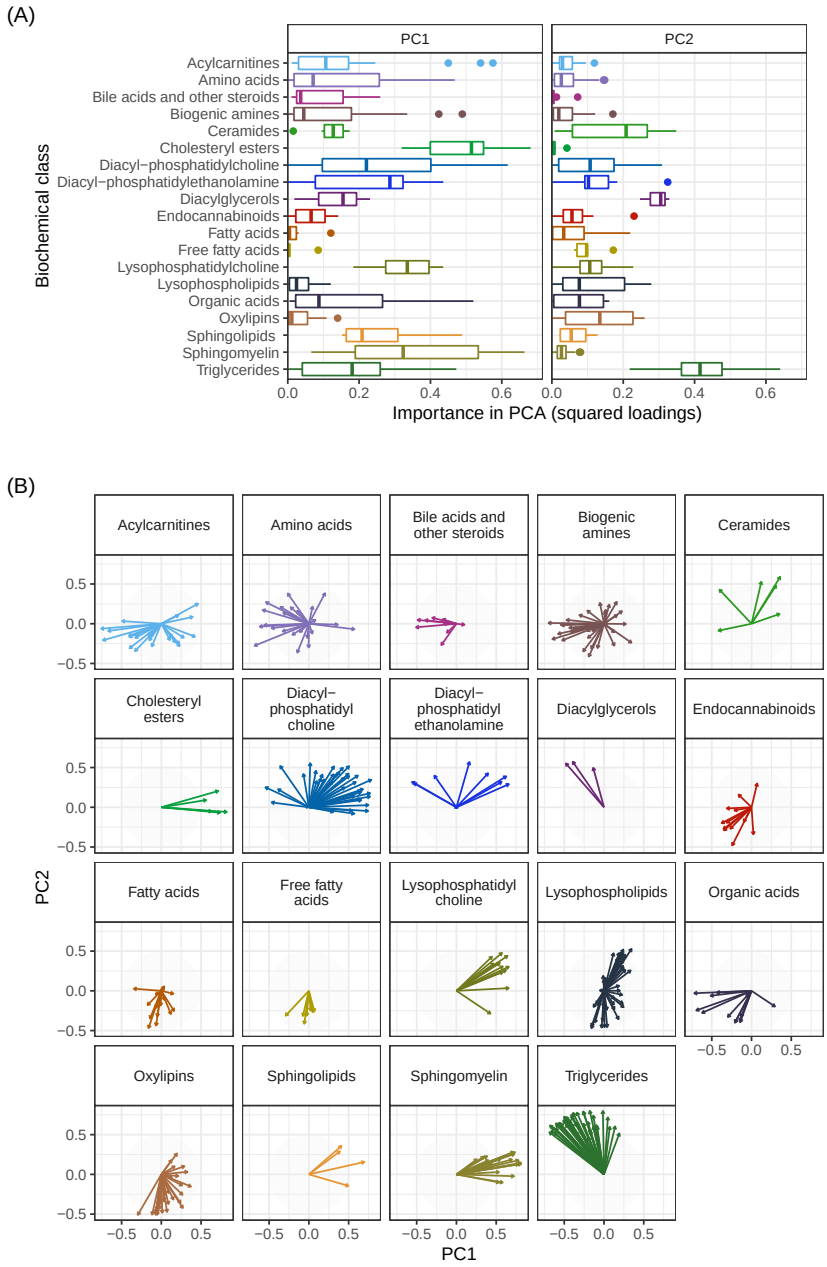

Figure 4.2: Metabolite contributions to the two dimensions of the PCA as variable loadings. A) The importance of each biochemical class for the different principal components (PCs), expressed by their squared metabolite loadings. Each box represents the squared loadings of the metabolites within a metabolic class. High squared loadings indicate a larger contribution to explaining the variation between patients. B) The loading plots for each biochemical metabolite class. The arrows indicate the importance (length) and direction of the metabolites in the principal component space. For example, high PC1 values correspond to high metabolite levels for metabolites with right pointing arrows, and low metabolite levels for metabolites with left pointing arrows. Arrows with a similar direction have similar metabolite patterns. Abbreviations: PC: principal component.

4.3.3 Disease severity score associations

To identify possible metabolic biomarkers for indication of disease severity, associations between the CURB disease severity score at admission and the change in metabolite levels on from day 0 to days 1, 2, 4, and 30 were evaluated (Figure S4.2). Negative associations were found between the CURB score and the change of metabolite levels (m) between day 0 and day 30 ($m_{t=30} - m_{t=0}$) of tiglylcarnitine, isovalerylcarnitine, 3 hydroxyisovaeric acid, carnitine, N6,N6,N6 trimethyl lysine, and isobutyryl carnitine (tau = 0.64 to 0.58, Figure 4.5). Patients with higher CURB scores showed decreasing levels of these metabolites.

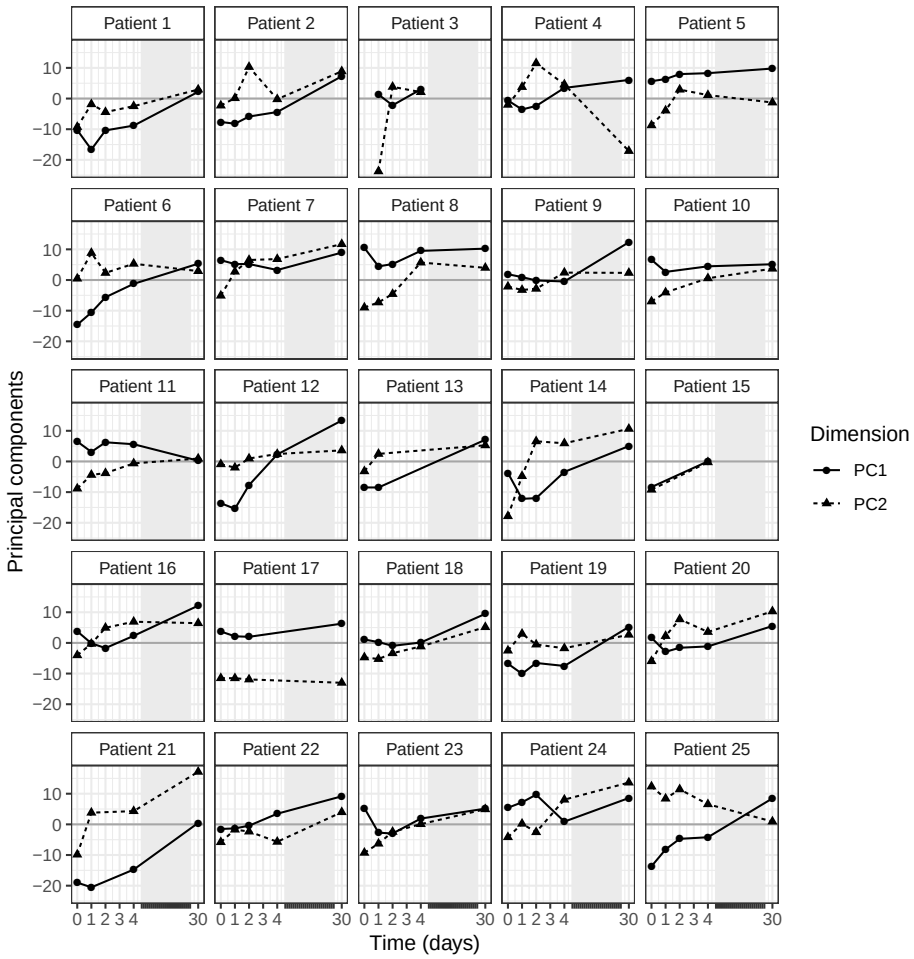

Figure 4.3: Individual metabolite profiles over time, expressed in PCA scores. The lines PC1 (solid) and PC2 (dashed), indicate the change in the corresponding principal component over time. Changes in PC values correspond to changes in metabolite levels according to their respective loadings. Abbreviations: PC: principal component.

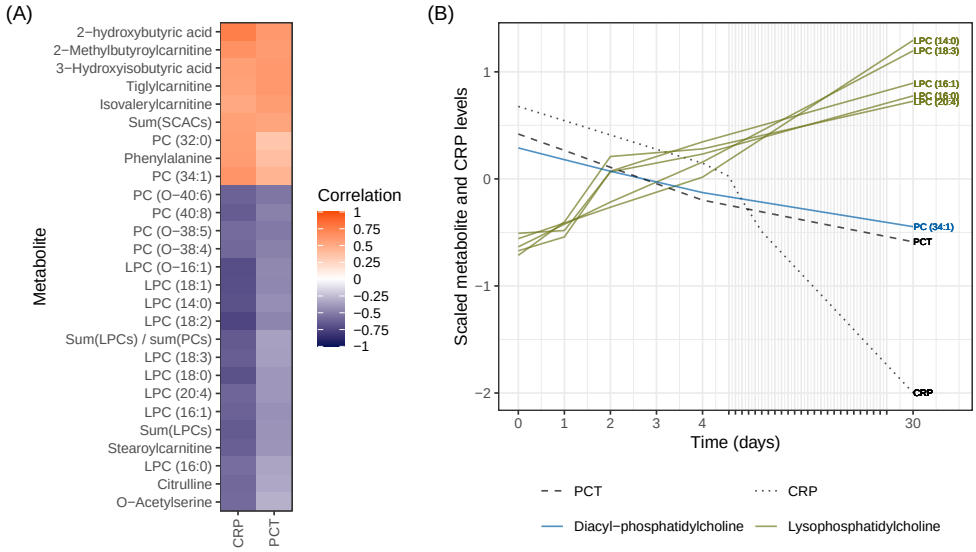

Figure 4.4: Correlations between inflammation markers CRP and PCT, and metabolites. A) The correlations between metabolites and CRP or PCT. Metabolites with a correlation >0.55 or <-0.55 for at least one marker are shown. A positive correlation (orange) indicates that a higher CRP or PCT level corresponds to an increase of that metabolite over time, while a negative correlation (blue) indicates a decrease over time for patients with a higher CRP or PCT level. B) Average CRP, PCT, PC (34:1), and LPC levels over time over all patients. Metabolite and CRP data were scaled. Abbreviations: see the abbreviation list.

4.3.4 Hospital length of stay associations

We evaluated the association between metabolites and clinical outcomes using the length of stay (LOS) as a potential surrogate endpoint. The strongest negative correlations to LOS were reported for the metabolite change over the first two days of admission ($m_{t=2} - m_{t=0}$, Figure 4.6), especially for the triglycerides (TGs) (60:3) and (58:2) (cor = 0.63 and 0.61 respectively). The correlations of these metabolites to LOS were much stronger than to CRP and PCT (cor = 0.08 and 0.25 respectively). Positive correlations were most pronounced when analyzing the metabolite change from the day of admission to day 30 ($m_{t=30} - m_{t=0}$). In the case of fatty acid (FA) (22:1) the day after admission ($m_{t=1} - m_{t=0}$) was the most strongly positively correlated to the LOS (cor = 0.58).

4.4 Discussion

In this study, we characterized the dynamics of the serum metabolites and their biochemical metabolite classes in pneumococcal CAP patients. We found that a large part of the variation in the metabolite values could be explained due to the changes over time within the patients. Several groups of metabolites were found to correlate with inflammation markers, CURB score, and length of hospital stay.

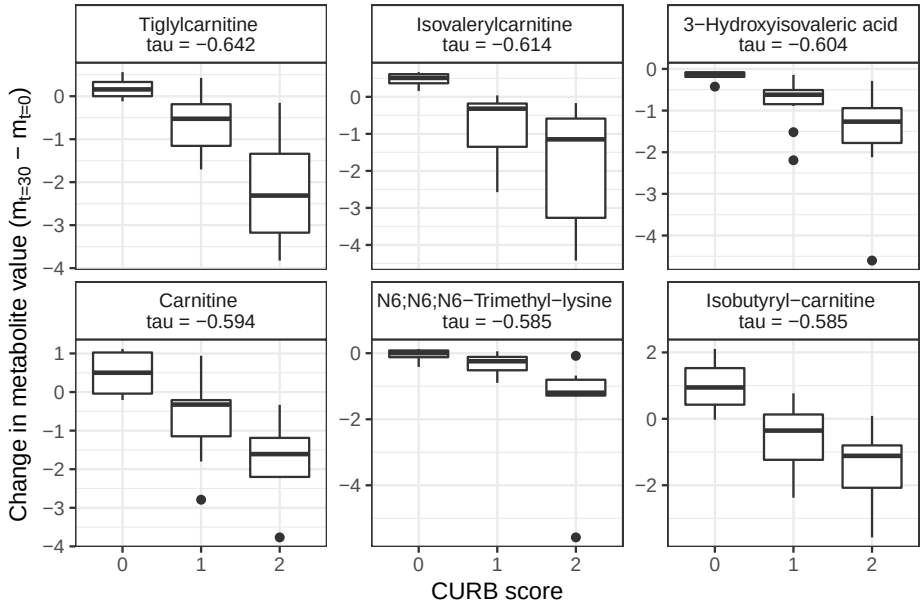

Figure 4.5: The correlation between the CURB score and six metabolites with highest associations. The change in metabolite level is the difference between the scaled metabolite level at day 30 and scaled metabolite level at admission (y-axis). These six metabolites all show a negative correlation with the CURB score (tau). This means, for patients with a CURB score of 0 the metabolite change between day 30 and day 0 is positive, so their metabolite levels were increasing over time. For patients with a CURB score of 2, the metabolite levels decreased over time.

The length of stay in the hospital was negatively correlated with the triglycerides, TG (60:3) and TG (58:2). Since these TGs are not highly correlated to CRP, PCT, or the CURB score, they explain a part of the variability of the disease progression that has not been studied before. Decreasing levels of TG (60:3) or (58:2) could be predictive for length of hospital stay. These results may not be specific for patients with *S. pneumoniae* infections. Triglycerides have not been found in metabolomics studies to etiological diagnosis of CAP, indicating its use for multiple infections, not just for pneumococcal CAP (den Hartog et al., 2021). TGs are also known to vary with diet, which could explain a negative correlation to disease severity (Parks, 2001).

PC (34:1) and LPCs (14:0), (16:0), (16:1), (18:0), (18:1), (18:2), (18:3) and (20:4) correlated to inflammatory markers, which also corresponds to previous findings (Banoei et al., 2020; Müller et al., 2019). PC (34:1), a ligand of nuclear receptor PPARα30, showed a positive correlation with CRP, which was previously associated with an anti-inflammatory response (Colombo et al., 2018). LPC (14:0) has been recently identified as a biomarker for disease severity in CAP patients (Nan et al., 2022). These metabolites could be of interest as treatment response biomarkers, not only in pneumococcal CAP patients, but also in other infections, because CRP and PCT are clinically used for many infections (Saleh et al., 2019). The CURB score was negatively associated with six metabolites, including some acylcarnitines. One of these acylcarnitines, tigylcar-

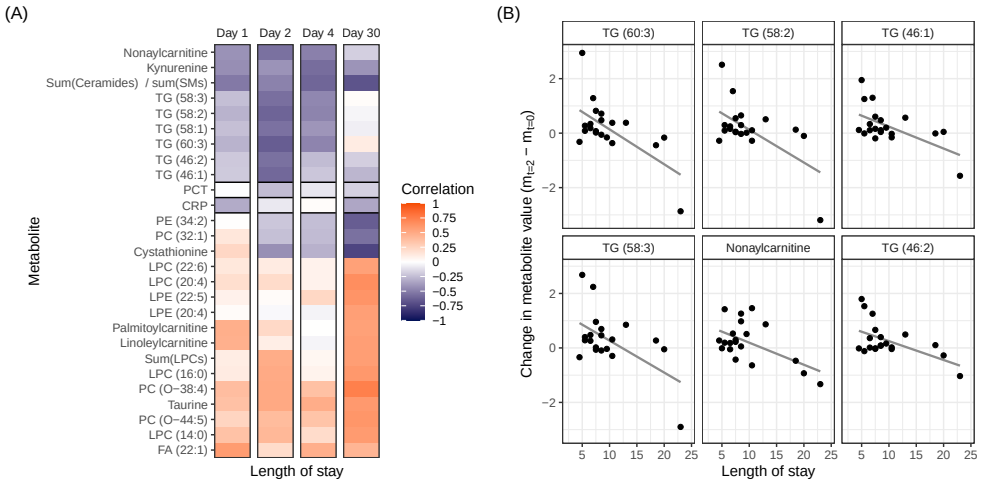

Figure 4.6: Associations between metabolites and length of stay. A) The correlations between the LOS and metabolite change from baseline at days 1, 2, 4, and 30 after admission ($m_{t=k} - m_{t=0}$). CRP and PCT are added as a reference. A positive correlation (orange) indicates that a longer stay in the hospital corresponds to an increase of that metabolite over time, while a negative correlation (blue) indicates a decrease over time for patients with longer stay. B) Metabolite levels over time for individual patients for metabolites with large negative correlations (cor < -0.55) over the first two days after admission. Abbreviations: see the abbreviation list.

nitine, has previously been found to be increased in non-survivors of CAP and could be considered a marker for disease severity (Banoei et al., 2020). Isovalerylcarnitine and isobutyrylcarnitine have, to our knowledge, not been studied as disease severity marker before, but may show a comparable performance to tigylcarnitine as their direction on the first principal component is similar.

We showed which biochemical metabolite classes explain most of the variation between individuals and over time. Triglycerides and LPCs were important for explaining the variation over time in the principal component analysis (PCA) and correlated with LOS and inflammatory markers. Within the biochemical classes, not all metabolites showed similar patterns, indicating that metabolites in some biochemical classes behave similarly during the infection, while metabolites in other classes behave differently (Figure 4.2B). The amino acids behave very differently, which could be expected since they are involved in a wide variety of biological functions (Wu, 2009). The longitudinal analysis of the metabolomics data enabled us to gain insight into acute and longer-term changes in the metabolome during the clinical course of CAP. The differences in metabolite levels are largely explained by changes over time, which could not have been evaluated without longitudinal data. Further studies using a longitudinal approach in this field could tackle long-existing issues in determining the appropriate empirical antibiotic therapy and guiding early targeted small spectrum antibiotic treatment or discontinuation.

This study was conducted in a well-characterized set 25 CAP patients with *S. pneumoniae* infections. The addition of patients with other causes of CAP is of in-

terest to compare metabolic time profiles for different treatment strategies based on the causative pathogen. Early recognition of a pathogen-drug mismatch using metabolomics could make antibiotic therapies more targeted and shorter. This study shows that mainly TGs, LPCs, PCs, and acylcarnitines are of interest for the disease severity and the length of stay for patients with CAP. By focusing on these metabolite classes, the number of metabolites that has to be measured for every patient can be reduced.

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Supplementary material

Table S4.1: Metabolite ratios and sums

Metabolite sum or ratio name in R	Metabolite sum or ratio formula
BCAA_sum	isoleucine + leucine + valine
TCA_cycle_sum	Citric acid + lactic acid + malic acid + fumaric acid
urea_cycle_sum	Citrulline + arginine + ornithine + fumaric acid
lc_Carnitines_sum	Myristoilcarnitine + Hexadecenoylcarntine + Palmitoyl-carnitine + Stearoylcarnitine + Dodecenoylcarnitine + Tetradecenoylcarnitine + Linoleylcarnitine + Oleylcarnitine + Tetradecadienylcarntine
mc_Carnitines_sum	Hexanoylcarnitine + Octanoylcarnitine + Octenoylcarnitine + Decanoylcarnitine + Lauroylcarnitine + Nonaylcarnitine + Pimeylcarnitine + Decenoylcarnitine
sc_Carnitines_sum	Acetylcarnitine + Propionylcarnitine + Isobutyrylcarnitine + Butyrylcarnitine + Tiglylcarnitine + Methylbutyroylcarnitine + Isovalerylcarnitine
Cer_sum	Cer(d18:1/22:1) + Cer. (d18:1/24.1. + Cer(d18:1/24:0) + Cer(d18:1/16:0) + Cer(d18:1/23:0) + Cer(d18:1/24:0)
SM_sum	Sphingomyelin (d18:1/14:0) + (d18:1/15:0) + (d18:1/16:0) + (d18:1/16:1) + (d18:1/17:0) + (d18:1/18:0) + (d18:1/18:1) + (d18:1/18:2) + (d18:1/20:0) + (d18:1/20:1) + (d18:1/21:0) + (d18:1/22:0) + (d18:1/22:1) + (d18:1/23:0) + (d18:1/ 23:1) + (d18:0/24:0) + (d18:0/24:1) + (d18:0/24:2) + (d18:0/25:0) + (d18:0/25:1)
LPC_sum	Lysophosphatidylcholine (14:0) + (16:0) + (16:1) + (18:0) + (18:1) + (18:2) + (18:3) + (20:4) + (20:5) + (22:6) + (O-16:1) + (O-18:1)
PC_sum	Diacyl-phosphatidylcholine (32:0) + (32:1) + (32:2) + (34:1) + (34:2) + (34:3) + (34:4) + (36:1) + (36:2) + (36:3) + (36:4) + (36:5) + (36:6) + (38:2) + (38:3) + (38:4) + (38:5) + (38:6) + (38:7) + (40:4) + (40:5) + (40:6) + (40:7) + (40:8) + (O-34:1) + (O-34:2) + (O-34:3) + (O-36:2) + (O-36:3) + (O-36:4) + (O-36:5) + (O-36:6) + (O-38:4) + (O-38:5) + (O-38:6) + (O-38:7) + (O-40:6) + (O-42:6) + (O-44:5)
HT5_Trp_ratio	Serotonine / Tryptophan
ADMA_Arg_ratio	ADMA / Arginine
SDMA_Arg_ratio	SDMA / Arginine
Carnitine_sum_lc_Carnitines_ratio	Carnitine / LCAC sum
Carnitine_sum_mc_Carnitines_ratio	Carnitine / MCAC sum
Carnitine_sum_sc_Carnitines_ratio	Carnitine / SCAC sum
DCA_CA_ratio	DCA / CA
FA_14.1_14.0	FA (14:1) / FA (14:0)
FA_16.1_16.0	FA (16:1) / FA(16:0)
Gln_Glu	Glutamine / Glutamic acid
Kyn_Trp	Kynurenine / Tryptophan
sum_BCAA_sum_Phe_Tyr_ratio	BCAA sum / (Phenylalanine + Tyrosine)
sum_CER_sum_SM_ratio	Cer sum / SM sum
sum_LPC_sum_PC_ratio	LPC sum / PC sum

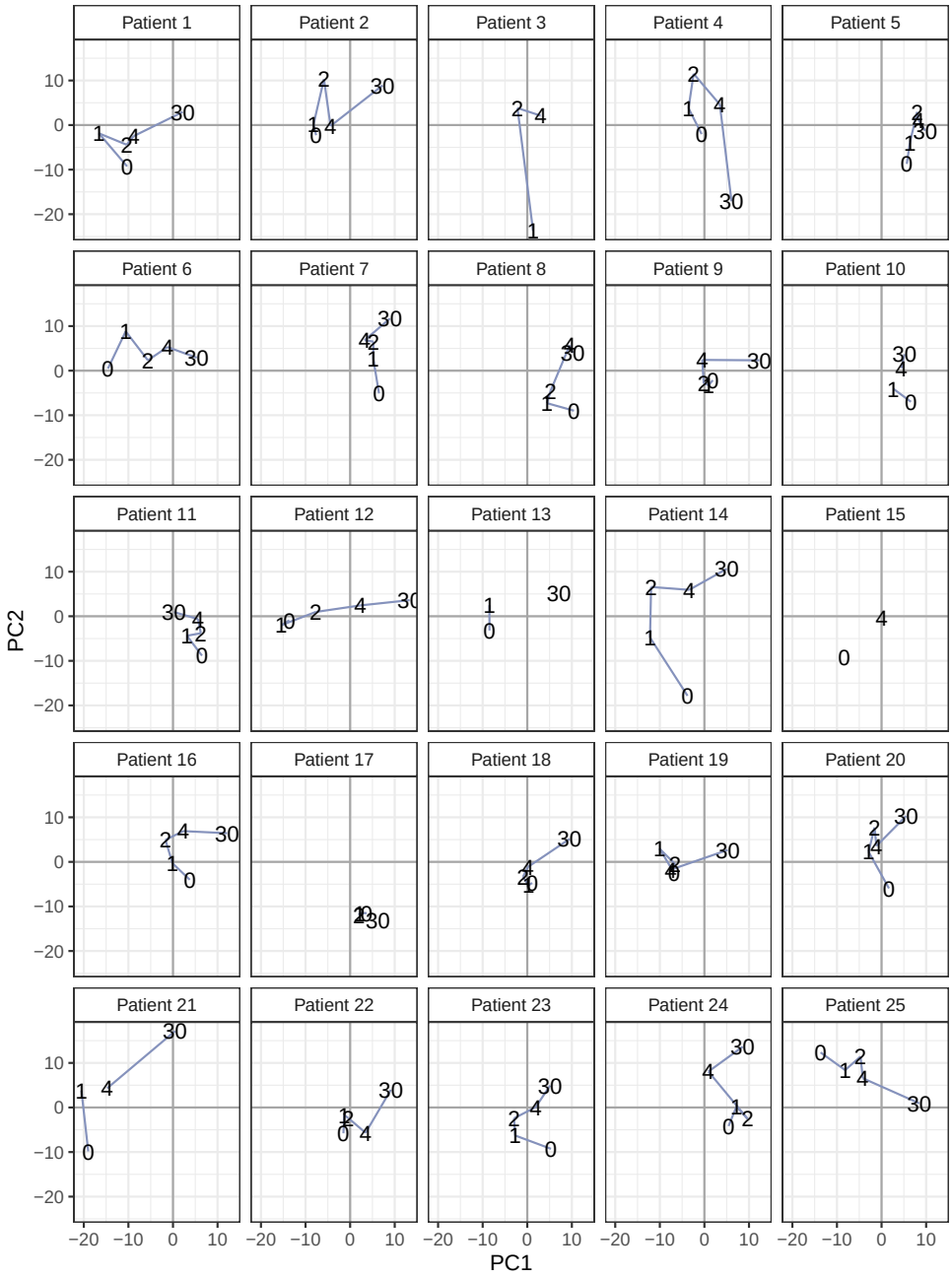

Figure S4.1: PCA score plots for each patient. For each patient, the time points are labelled and connected with lines. Abbreviations: PC: principal component.

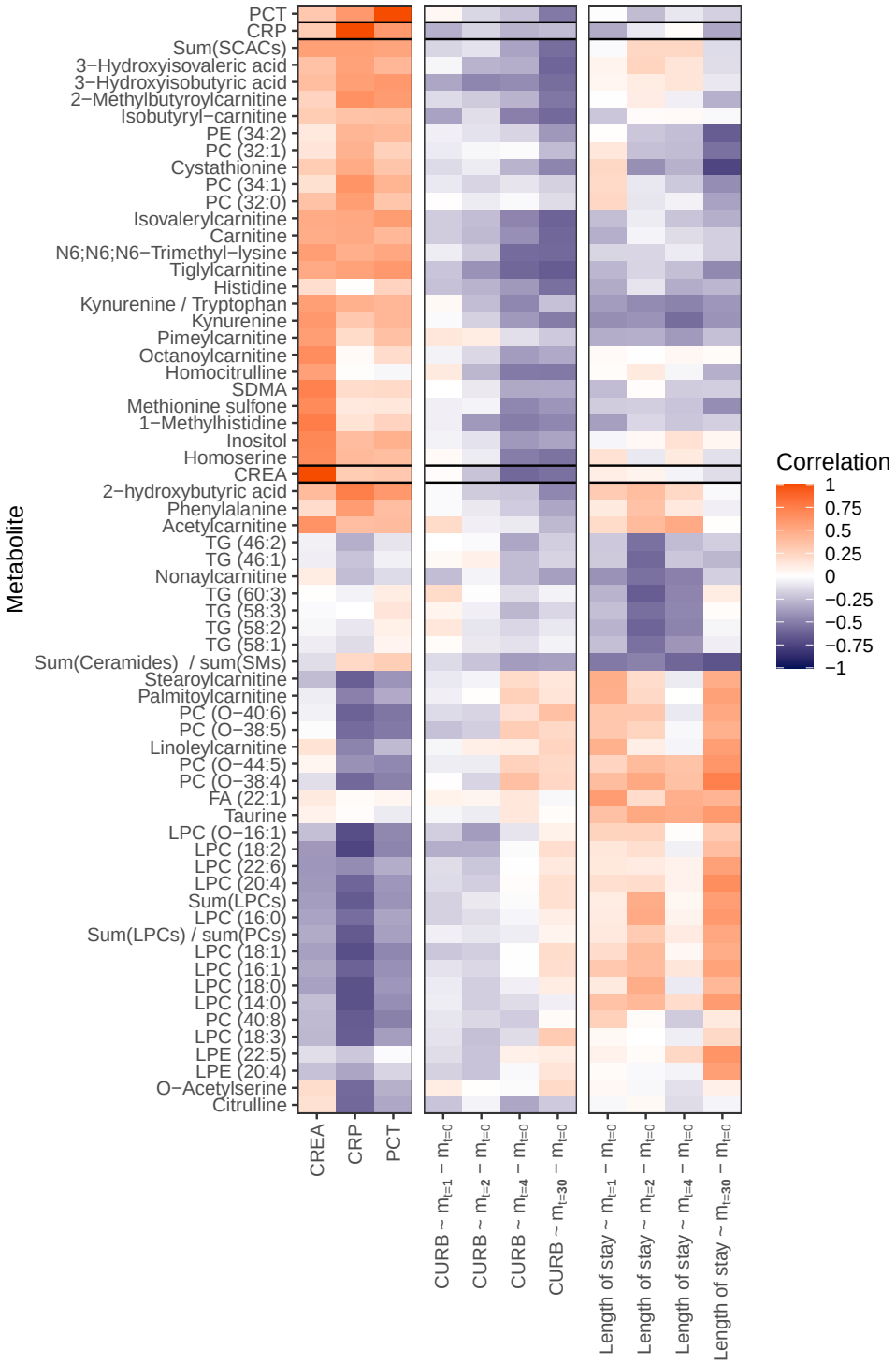

Figure S4.2: The correlations between metabolites and creatinine, CRP, and PCT over time; and the correlations of the CURB score and length of stay with a change of the metabolites between day k and day 0, where the change in metabolite levels is denoted by $m_{t=k} - m_{t=0}$.