Metabolomics in community-acquired pneumonia: exploring metabolomics-based biomarkers for diagnosis and treatment response monitoring of community-acquired pneumonia

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CHAPTER 6

General discussion and summary

Community-acquired pneumonia (CAP) is a lower respiratory tract infection with a high incidence [1, 2]. Hospitalized patients with moderate to severe CAP typically receive empirical broad-spectrum antibiotic therapy, that can be switched to targeted therapy when the microbial etiology is determined. To optimize patient care and to reduce the risk for development of antimicrobial resistance (AMR) in treatment of CAP, there is a need for additional biomarkers to support microbial diagnosis and treatment monitoring of CAP. Metabolomics is a key technology of relevance as a potential source of new biomarkers for CAP. In this thesis, in **Chapter 1**, we first introduced current challenges in diagnosis and treatment of CAP, the basic concepts in the use of metabolomics for biomarkers discovery, and our central hypothesis that changes in the host metabolome in patients with CAP may be a potential source for novel biomarkers. We then described our studies to assess the potential utility of metabolomics-based biomarkers for diagnostic purposes (**Chapter 2 & Chapter 3**) and for the monitoring of the treatment response (**Chapter 4**) in patients with CAP. Finally, we described the development of a computational tool which can help in the design and analysis of metabolomics studies investigating the host immune response (**Chapter 5**)

6.1 Diagnosis of CAP

Currently used approaches for the etiological diagnosis of pathogens in CAP are based on several techniques, including culturing, antigen testing, and PCR [44, 45, 46, 47]. The adoption of PCR-based assays has expanded over the past few years. Due to the high sensitivity and short turnaround times, PCR point-of-care assays have great value for the diagnosis of CAP. Using multiplex PCR respiratory panels, a large number of potential pathogens can be detected [139, 140]. Respiratory PCR assays are typically performed on sputum, which can be a limitation for CAP patients, who are often unable to produce a sputum sample. Nasal or nasopharyngeal swab PCR tests that could overcome this challenge have been proven effective in the diagnosis of Methicillin-resistant

Staphylococcus aureus (MRSA) and COVID-19 [141, 142]. However, the identified pathogens in these PCR tests may not be conclusively the cause of CAP because the sample does not originate from the lower respiratory tract [143]. Blood-based diagnostic assays investigated in this thesis that address these limitations are therefore of potential relevance for diagnosis or prognosis of CAP. Another potential limitation of PCR-based assays relates to their targeted nature, i.e., pathogens for which the associated target sequences are not included, can also not be detected. Potentially, assays which consider the host immune response to specific pathogens could address this limitation.

Evaluation of metabolic biomarkers for etiological diagnosis of CAP

In **Chapter 2**, we aimed to determine if predictive metabolomics-derived biomarkers could be identified to discriminate between key pathogens or pathogen groups in CAP. To this end, we performed extensive metabolomics profiling of serum samples from CAP patients collected at the time of hospitalization. Specifically, we assessed whether patients with a confirmed infection of *Streptococcus pneumoniae*, atypical bacterial pathogens, or viral pathogens could be identified using metabolomics-based biomarkers. The choice for these pathogen groups was made because the identification of either one of these groups drives clinical treatment decision-making. The increased number of patients per group also enhanced the statistical power to detect potential biomarkers.

In our analyses, no metabolites were found to discriminate *Streptococcus pneumoniae* or viral pathogens from the other pathogen groups. However, patients with atypical CAP pathogens, such as *Coxiella burnetii*, *Chlamydophila psittaci*, *Legionella pneumophila,* and *Mycoplasma pneumoniae,* could be discriminated from patients with *Streptococcus pneumoniae* or viral infections using a predictive model. This model included three metabolites: glycylglycine, symmetric dimethylarginine, and lysophosphatidylinositol (18:1). The model showed a predictive performance of 63% sensitivity and 84% specificity. This performance is not superior to the predictive performance of established clinical assays based on culturing, antigen testing, or PCR [44, 45, 46, 47]. Potentially, the use of the identified metabolite-based biomarkers could, however, be further explored to complement established clinical assays, e.g., through combined consideration of both pathogen- and host-response associated characteristics.

Uncovering metabolic differences to CAP-associated pathogens

Whereas in Chapter 2 we focused on groups in pathogens, in **Chapter 3** we further characterized the differential metabolic host response to distinct CAP-associated pathogens. The goal of the analysis was to enhance our understanding of host (or patient)-associated metabolic changes in the pathogenesis of specific pathogens. To this end, we studied associations between metabolite levels and CAP-associated pathogens. While in chapter 2 we did not find metabolites that could discriminate patients with *S. pneumoniae* from all other patients, in chapter 3 we found that patients with *Streptococcus pneumoniae* infections showed high levels of the stress hormone cortisol, high phosphatidylcholines (PCs), and low lysophospholipids (LPCs) in comparison to all

other pathogens, all of which are associated to inflammation [92]. From these results, we can conclude that the pathogens within the viral and atypical pathogen group in chapter 2, are so different from each other, that we should study them individually.

This need for individual assessment of the different pathogens is confirmed by our findings in the group that we defined in chapter 2 as atypical pathogens. In patients with *Legionella pneumophilia* infections, we found the lowest levels of LPCs, the highest levels of cortisol, kynurenine, and phenylalanine, and elevated free carnitine and short-chain acylcarnitines in comparison to the other pathogen groups, and which could be related to inflammation [95] and oxidative stress [100]. In patients with a *Coxiella burnetii* infection, high levels of long-chain acylcarnitines and LPCs, and elevated glutamate compared to other pathogen groups were observed, which can be linked to either inflammation, oxidative stress, or endothelial dysfunction [91, 81, 80, 101, 98].

Within the group of viral pathogens, we found a singular profile for the herpes simplex virus. Despite the small sample size, patients with herpes simplex infection showed a unique disruption in their lipid metabolism. An important limitation of this analysis was the limited sample size per group, the differences in patient age, and the substantial within-group variance of metabolite levels, which could have confounded the results. As such, we view these results as preliminary findings which require further confirmation, but which can still drive hypothesis generation and inform the design of follow-up studies. Also, a larger number of individuals per group would increase the power and change of finding significant differences.

Clinical utility of metabolomics-based biomarkers for etiological diagnosis of CAP

Based on our findings in **Chapters 2 and 3** we conclude that the added value of metabolomics-based blood-based assays for the etiological diagnosis of CAP may be limited. This is because the predictive performance for key pathogens in CAP is so far inferior or, at best similar to current diagnostic assays (**Chapter 2**), and with limitations in pathogen-specificity (**Chapter 3**). However, the information yielded in metabolomics assays could potentially be used to complement information gained from established clinical assays, i.e., through combination of diagnostic assays for host- and pathogen-associated characteristics and/or blood- and sputum-based matrices [144], such as recently has been explored in sepsis patients where combined host and pathogen data improved diagnostic sensitivity to 97-100% [145].

Alternative omics-based biomarkers

Over the last few years, other molecular profiling or "omics" technologies have been explored for the diagnosis of CAP. For example, recent studies suggest potential value of metagenomics next-generation sequencing [146], transcriptomics [147], and proteomics [148] approaches to further enhance the detection rate in pneumonia. Ultimately, we expect that diagnostic strategies which combine several of these technologies and thereby combining predictive biochemical host- and pathogen-associated features may

be most effective in the identification of predictive biomarkers for CAP, and which may be combined in combinatory diagnostic biomarker panels for CAP.

6.2 **Monitoring of treatment response in CAP**

Monitoring the course of disease progression and treatment response in CAP is essential to inform optimal clinical treatment strategies, including the use of effective antimicrobial therapies. To this end, patients are currently monitored based on their clinical symptoms, such as fever. In addition, inflammatory biomarkers in blood, such as C-reactive protein (CRP) or procalcitonin (PCT), are commonly measured longitudinally in patients to determine whether an antimicrobial treatment strategy appears to be effective and to guide when antimicrobial treatment can be terminated, which is relevant to control the risk of AMR emergence. However, current biomarkers have key limitations, i.e., even though CRP is commonly used, it is not specific to infection, and its kinetics are delayed in relationship to the underlying infection, and as such do not directly reflect the current state of the patient [12].

Longitudinal metabolic biomarkers for treatment response monitoring of CAP patients

In **Chapter 4,** we explored the potential of longitudinal metabolomics-based biomarkers for treatment response monitoring in CAP patients. To this end, we measured metabolite profiles in 25 CAP patients with a confirmed *S. pneumoniae* infection during treatment. We aimed to comprehensively characterize the change in longitudinal metabolite profiles of these patients and investigated associations with disease severity, inflammation markers, and treatment response outcomes, quantified using the length of hospital stay. 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. The results showed that the inflammation marker C-reactive protein (CRP) correlated positively with phosphatidylcholine and negatively with nine LPCs. The CURB disease severity score was negatively associated with six metabolites, including three acylcarnitines. Length of stay correlated negatively with six triglycerides (TGs), and especially with TG (60:3) or 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 is not captured by conventional treatment response biomarkers and are therefore of interest as new additional biomarkers associated with length of hospital stay.

To further evaluate the potential of TGs, LPCs, and PCs for treatment response monitoring, a prospective follow-up study is warranted to further characterize the predictive value of these biomarkers and their relationship with disease severity (CURB) and treatment outcomes such as length of stay (LOS). For such a study, the collection of additional frequent blood samples until hospital discharge would be optimal. In this study we have made a deliberate choice to only focus on *S. pneumoniae*-associated infections as the predominant CAP-associated pathogen, and to ensure that a controlled

analysis of the longitudinal profiles could be performed. However, in follow-up studies, assessment of the causative pathogens will be essential to determine whether the identified biomarkers are generalizable to other pathogens besides *S. pneumoniae*.

Beyond the analysis described in this chapter, only very limited research has so far been done investigating longitudinal, treatment response, metabolic biomarkers in CAP. In this context, previous studies investigating procalcitonin in CAP and/or sepsis are the most prominent [149, 108]. When considering the discovery of novel biomarker candidates, several recent studies in CAP patients have focused on prognostic biomarkers for treatment outcome or disease severity, such as studies for serum surviving and Cysteine-rich 61 [150, 151]. However, such prognostic biomarkers which have been determined only at the start of treatment cannot be directly applied as a clinical decision-making tool to adjust treatment strategy during the course of treatment.

6.2 Improving metabolomics study design and interpretation with the Immunometabolic Atlas

The biological interpretation of metabolomics data can be challenging because it is often unclear how certain metabolic biochemical changes associate with immunobiological or inflammatory processes. In addition, during the design of metabolomics studies, choices often need to be made regarding the specific chemical classes of metabolites to be measured, i.e., those which are likely associated with the (immuno-) biological process of interest. There are currently not many computational tools available to guide this association between known metabolic pathways and immune-biological processes.

In **Chapter 5,** we describe the development of the Immunometabolic Atlas (IMA), which can support the interpretation and design of metabolomics studies. In the IMA, we integrated information on metabolites, metabolite-protein interactions and immunobiological processes available in large-scale public databases. Based on these databases, we established a metabolite-immune process interaction network with over 1.4 million metabolite-immune process associations. Through a web interface, this network can be used to infer immunobiological processes from metabolites and vice versa. A current limitation for such analyses remains the lack of uniform metabolite identifiers for many metabolite classes. For example, for 60% of the metabolites that were measured as part of this thesis, a Human Metabolite Database (HMDB) identifier was unavailable. This was in particular these case for lipids, where existing efforts to provide identifiers for lipids [152] should be integrated with platforms such as HMDB. Implementing and integration of such identifiers is crucial to further enable FAIR (re-)use of metabolomics data. Recently, recommendations for metabolite annotations have been made to overcome the inconsistencies in metabolite nomenclature [153]. Furthermore, a new methodology is developed to discover new protein-metabolite interactions that enhances the biological interpretation of metabolomics data [154].

6.3 Discovery and application of clinical metabolomics-based biomarkers

High-quality clinical data

The analyses in this thesis have been performed using patient samples which were collected during previously conducted clinical studies involving hospitalized patients with moderate to severe CAP, which required hospitalization [30, 29]. An important characteristic of these clinical studies was the availability of a confirmed microbial diagnosis, as well as detailed information on patient characteristics, disease severity, antibiotic therapy, inflammation markers measured during treatment, and length of stay in the hospital. As such, these clinical studies provided a unique and relevant set of samples to study the role of metabolomics-based biomarkers in CAP.

Metadata and cofounders

This thesis confirms the importance of complete and extensive data on patient characteristics, treatments administered, and comorbidities, which may all influence metabolite profiles and, therefore, could affect the ability to successfully detect novel biomarkers and should be included to correct for possible confounders in metabolomics data analysis. In our analyses, details such as sex, age, disease severity, comorbidities, and durations of symptoms before admission were already collected as part of the study design. However, several other factors were incomplete or lacking, such as saturation, fever, respiratory rate, food/fasting details, antibiotic dose, and the specific time of drawing a blood sample, and can influence the metabolic profile [155, 156, 157]. In this context, and because of the complexity of design and execution of prospective clinical trials, the role and further establishment of biobanks for patients with CAP or associated respiratory infections with extensive collection of all relevant metadata will be an essential step to aid in future biomarker discovery studies. This is especially a challenge when the number per patient group is as small as in this thesis.

Control groups and longitudinal data

In this thesis we did not incorporate samples from controls, i.e., individuals unaffected by an infection. This has impacted our ability to compare observed metabolite levels in patients with infections to metabolite levels in healthy individuals or in hospitalized patients without active infection. When considering variability in metabolite levels within patients, we did have samples available at 30 days post-admission for a subset of included patients, which can be considered as a within-patient controls. Nonetheless, establishing metabolome-wide large scale public data repositories for such controls could be helpful in future metabolomics-based biomarker discovery studies. However, this would require that metabolomics data can be compared between studies.

In this thesis we have shown that longitudinal metabolite profiles can give more information about the state of the patient and elucidate the effect of comorbidities and

co-medications. Therefore, the design of a new clinical study should include sampling for metabolomics at more than one time point. Since patients are admitted to the hospital in different stages of the disease, diagnostic testing based on the absolute metabolite concentrations at one time point can be challenging. The variable baseline metabolite levels upon admission and the dynamical metabolite profile differs per patient and complicates the development and analysis of longitudinal and diagnostic biomarkers and emphasizes the need for longitudinal biomarkers.

Absolute metabolite levels

In this thesis, metabolite levels were measured as metabolite peak ratio to its internal standard, as is common practice in the metabolomics field. However, for clinical application, metabolite levels should be measured as absolute concentrations so a threshold can be determined that can be easily interpreted by a physician. Measurement of absolute concentrations was not yet feasible for the hundreds of metabolites that were measured in the research in this thesis, as this would require calibration lines for all metabolites. However, if a subset of five to ten metabolites was measured, i.e., in the context of a dedicated clinical metabolomics-derived assay, this would be an achievable and important step. In addition, methods should be developed so that also 100's of metabolites can be reported as absolute concentrations.

Clinical metabolomics assay development

To measure a small number of metabolites with different chemical properties, such as polarity and charge, new analytical methodologies should be developed. Recent research has shown it to be possible to integrate the measurement of metabolites with different chemistries in one methodological platform, and this methodology should be developed further for clinical use [158]. In conclusion, to obtain the integration of metabolomics-based assays in the clinical laboratory, the number of measured metabolites should be minimized to allow fast processing of samples and easy interpretation of the results for the clinician. On the other hand, if a larger number of metabolites are quantified several diseases could be diagnosed (or excluded) in one analysis.

6.4 Conclusion

We have shown that metabolomics-based biomarkers have potential to monitor the treatment response in CAP patients. An important advantage of such biomarkers is their reflection of the host response to infection. Pathogen-specific metabolic responses described may be able to support discrimination between individual pathogens or pathogen groups based on the host response but should be further researched to explore their potential. Ultimately, metabolomics-based biomarkers could in the future be relevant to complement existing diagnostic tools and biomarkers for diagnosis and

treatment monitoring of CAP. For that larger studies that follow the above given recommendations are required.