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ORIGINAL ARTICLE

In-hospital metabolite changes in infective endocarditis—a longitudinal ^{1}H NMR-based study

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Abstract

Treatment of infective endocarditis (IE) is a 4–6-week provided course of intravenously administered antibiotics. The aim of this study was to investigate how serum metabolites as measured by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy are changing over time during the active phase of IE, and to see whether these metabolite changes might be used to monitor recovery in these patients. Patients hospitalized with first-time IE at Herlev Hospital, Denmark, from September 2015 to June 2017 were included. Longitudinal blood sampling was performed and serum was analyzed using ^{1}H NMR. Orthogonal projection to latent structures discriminant analysis (OPLS-DA) was used to separate sample groups and analyze differences in metabolite profiles. Thirteen patients were included in the study (77% men, median age 62 years (IQR 53–77)). All patients were cured during the hospitalization without any relapse during 6 months of follow-up. We analyzed 61 serum samples (median 5 samples, range 2–8 per person) drawn in the treatment period after IE diagnosis. The main changes during the in-hospital period were decreased levels of glucose, mannose, leucine, isoleucine, phenylalanine, tyrosine, and signals from polyols and N-acetylated protein. The metabolomic changes could in contrast to the routinely used parameters CRP and leucocyte levels distinguish between the early and late stages of disease treatment. We present the first longitudinal study of ^{1}H NMR metabolomics in patients with infective endocarditis. The metabolomic changes show a promising strength compared to routinely used clinical parameters.

Keywords Infective endocarditis · Antibiotic treatment · Metabolomics · NMR · Longitudinal study

Longitudinal NMR metabolomics in 13 patients with infective endocarditis (IE) showed a downregulation of sugars and amino acids during hospitalization and cure of IE. This downregulation was more robust than changes in CRP and leukocyte count.

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Background

Infective endocarditis (IE) is a rare disorder (estimated incidence of 3.6 cases/100000 persons per year [1, 2]). In IE, bacteria circulating in the blood stream adhere to the endothelial surface and/or prosthetic material of the heart [3]. Production of a biofilm on the heart valves adds to the persistence of bacteria and contributes to antibiotic tolerance [3]. The clinical course of IE ranges from a mild disease course to a life-threatening condition. The diagnosis of IE is based on the modified Duke Criteria [4] and treatment includes up to 6 weeks of intravenously administered antibiotic treatment [5, 6]. Most complications, such as stroke, occurs during the first week of antibiotic therapy, and the rate declines thereafter [7]. Today, the treatment length of IE is standardized, and individual clinical evaluation or biomarker response such as decreasing markers of inflammation (i.e., CRP and leucocyte count) are generally not used to determine treatment duration. A poor decline in CRP during the first week of antibiotic treatment is associated with poor outcome [8], but whether it is possible to end antibiotic treatment when CRP is normalized is not investigated. Discontinuation of antibiotics when the infection is cleared would be advantageous, but methods to evaluate when this occurs are lacking. Measurement of metabolite variations may reveal information on the host response to a systemic infection, which potentially could be used to indicate when the infection is cleared.

Metabolomics may be defined as the quantitative measurement of the dynamic multiparametric response of a living system to a pathophysiological stimuli or genetic modification [9]. Untargeted metabolomics provides information about a wide range of metabolites in a single measurement, without having to preselect which analytes to detect [10]. It can be used to monitor phenotypic changes as a function of disease progression [11] and regression [12]. Nuclear magnetic resonance (NMR) spectroscopy is one of the most versatile biophysical techniques for molecular and functional studies in life science [13, 14]. It is rapid and cheap, highly reproducible, non-destructive, and quantifies all the most abundant metabolites: aminoacids, organic acids, and carbohydrates, and larger units such as albumin and lipoproteins [15] with minimum of sample preparation [16]. By using multivariate statistics in combination with NMR, different groups of samples can be established based on the entire metabolite profile. Metabolites that are responsible for clustering or separation of groups, and changed by individual treatments or other parameters, can be identified.

The aim of this study was to investigate how serum metabolites are changing over time during the active phase of IE, and to see if NMR metabolomics might be used as a tool to monitor recovery in the patient during ongoing infection and individualize the antibiotic treatment length in IE patients.

Methods

Inclusion and exclusion criteria

Patients hospitalized with newly diagnosed first-time IE at Herlev Hospital, Denmark, in the period from September 2015 to June 2017 were screened for inclusion (Fig. 1). Patients included in the study should meet the following criteria: Definite IE according to the modified Dukes criteria, age ≥ 18 years and CRP ≥ 30. Patients, who had known rheumatic disease, immunosupression (disease or medication-induced), cancer, and other simultaneously infection at IE diagnosis which required separate medication, were operated during the last 14 days, or were unable to provide informed consent, were excluded (Table 1).

The protocol for the study was approved by the Data Protection Agency (HGH-2015-010, I-suite nr: 03923 and the Scientific Ethics committee (H-15009681), and all patients gave written informed consent.

Samples

From each patient included in the study, blood samples were drawn at least at three different times during the hospitalization period. All samples were collected when the patients were fasting and at the same time of the day. At each sampling time, 12 ml venous blood were drawn after overnight fasting. Sample clotting time was 30 min in room temperature, whereafter they were centrifuged at 3.900×*g* for 10 min. The supernatant serum was aliquoted into cryo tubes, marked with labels and immediately frozen at − 80 °C. Additional blood samples were routinely sent to the clinical chemistry laboratory for evaluation of the standard parameters of CRP and leucocyte count. To clarify presentation of data, sampling times (after IE diagnosis) of 0 to 2 days were termed "early" samples (meaning newly diagnosed IE), 8 to 17 days "intermediate" samples (the period where the risk of complications is reduced compared to the first week), and 25 days to discharge were termed "late" samples (the period where the patients are stable and might be switched to oral antibiotic therapy and discharged [17]) (Table 2).

Sample preparation and ^{1}H NMR experiment

At the day of analysis, all the samples were thawed and prepared using standard Bruker methods [18] where 300 μL of serum were added to 300 μL of phosphate buffer DSS using a robotic system. Standard ^{1}H NMR spectra: NOESYGPPR1D and CPMG were recorded on a 600 MHz Avance III Bruker NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.13 MHz as described previously [19].

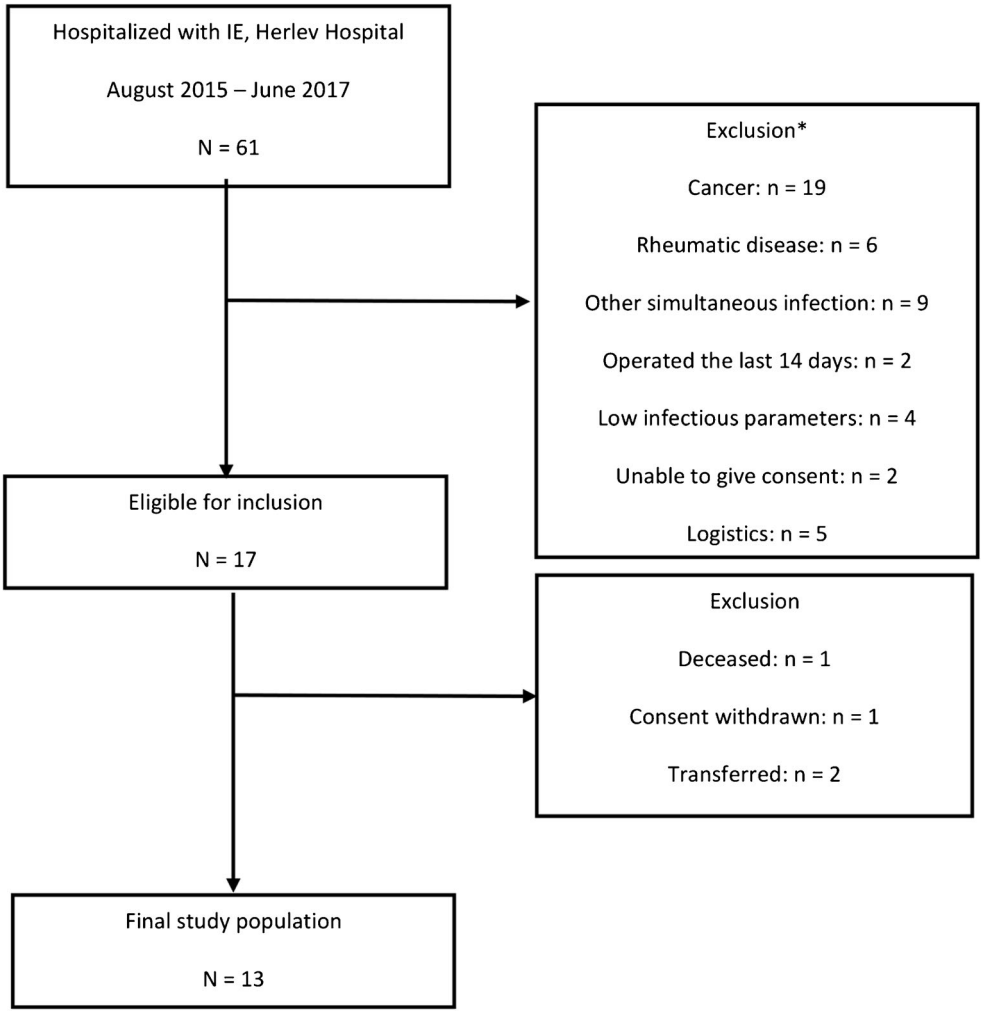

Fig. 1 Flowchart showing patient inclusion and exclusion. *Patients can have more than one exclusion criteria. IE indicates infective endocarditis

Table 1 Baseline patient characteristics showing gender, age, comorbidities and bacteria species for the included IE patients

	N	
	Total *n*: 13	
Male, *n* (%)	10	(77%)
Age in years, median (IQR)	62	(53–77)
Comorbidities		
COPD, *n* (%)	1	(8%)
Diabetes Mellitus, *n* (%)	2	(15%)
Dialysis, *n* (%)	1	(8%)
Hypercholesterolemia, *n* (%)	2	(15%)
Heart valve disease, *n* (%)	1	(8%)
Bacteria species		
Streptococcus species, *n* (%)	6	(46%)
Staphylococcus aureus, *n* (%)	4	(31%)
Coagulase negative staphylococcus, *n* (%)	1	(8%)
Blood culture-negative IE, *n* (%)	2	(15%)

NMR data and analyses The spectra were processed using iNMR [20]. An exponential line-broadening of 0.5 Hz was applied to the free-induction decay prior to Fourier transformation. All spectra were referenced to the DSS signal at 0 ppm, automatically phased and baseline corrected. The spectra were aligned using *i*coshift [21]. Spectra of insufficient quality due to poor water suppression were excluded from the analysis.

Multivariate analysis was carried out on spectra and not on integrated metabolite signals. The region around the residual water signal (4.85–4.6 ppm) was removed not to compromise the analysis. The high- and low-field ends of the spectrum, where no signals except the reference signal from DSS appear, were also removed (i.e., leaving data between 9.5 and 0.5 ppm). The resulting spectrum was reduced to a total number of 14,316 points. In order to focus on variations with time rather than patient specific offsets in metabolite levels, the mean spectrum of sample groups 2, 4, and 5 for each patient was subtracted from all spectra for that patient before multivariate analysis. Groups 2, 4, and 5 were chosen because samples from these groups were available for all patients. In

Table 2 Treatment phase, sample group number, sample day after IE diagnosis, and number of samples

Days after IE diagnosis	0	1–2	3–4	5–7	8–10	11–17	18–24	25–31	32–38	39–45/discharge
N, samples	1	7	6	11	9	10	5	5	4	3
Sample group	1	2	3	4	5	6	7	8	9	10
Treatment phase	Early				Intermediate			Late		

Samples in group 1–10 were drawn during hospitalization for IE

IE, infective endocarditis; *N*, number

order to further focus on the changes with time, we used orthogonal projection to latent structures discriminant analysis (OPLS-DA) [22] to separate early from late phase samples (Table 2). OPLS-DA models predict group membership based on a multivariate input, in this case, the NMR spectra. The model separates variations because of group membership from other (orthogonal) variations. Thus, the first (predictive) component describes variation dependent on group membership while the remaining (orthogonal) components describe variation independent of group membership. The OPLS-DA models were carried out on unit variance scaled spectra. Since samples of the same person in the same physiological conditions tend to cluster together [23], the reproducibility of the models where tested with leave-one-out cross validation where all samples from one patient were left out at a time, and models were calculated to predict group membership for the excluded samples. The model used here only included one predictive and one orthogonal component in order to minimize the risk of overfitting. The predictability, Q^2, was calculated. Significant changes in metabolites were identified using false discovery rates with p values < 0.05, with false discovery rates based on the correlation between the input spectral data and the OPLS-DA predictive scores. Assignment of significantly changing metabolites was done based on chemical shifts only, using spectral databases as previously described [20, 21]. SIMCA15 (Umetrics, Malmö, Sweden) was used for OPLS-DA modeling and MATLAB was used for all other data treatment and plotting. In Figs. 2 and 3, the displayed metabolite scores for early and late phase samples (sample groups 1–2 and 8–10) were cross-validated scores (see above), and for the rest of the samples (that were not used to make the model), the full OPLS-DA model was used to predict scores. "Metabolite score" was defined as the variation of the resulting OPLS-DA predictive scores.

Results

Patient inclusion and sample analyzation

In total, 13 patients were included in the study (Fig. 1). Median age was 62 years (IQR 53–77) and 77% were men. The patients had a variety of comorbidities, including diabetes mellitus and hypercholesterolemia (Table 1). Hospitalization time before IE diagnosis varied from 0 to 21 days. Sixty-one samples were analyzed (median 5 samples per person, range 2–8). All patients were cured during the in-hospital treatment period without any recurrent infections during 6 months of follow-up after discharge.

CRP and leucocyte count

CRP and leucocyte count values at the early, intermediate, and late treatment phases can be seen in Fig. 2a and b. The figure shows the diversity of the levels of these biomarkers in the IE patients, and that there is a great overlap between the three groups. Leucocyte count and CRP changes in all 10 sample groups can be seen in Fig. 3a and b.

Metabolite changes

NMR spectra were recorded for all serum samples (Fig. 4a), and an OPLS-DA model was made to distinguish between early (n = 8) and late (n = 12) phase samples after IE diagnosis. A significant change in metabolite concentrations between the two groups is manifested by a predictability, Q^2, of 0.65. The model used here only included one predictive and one orthogonal component in order to minimize the risk of overfitting. Much better predictability can be achieved by including further components. The variation of the resulting OPLS-DA predictive scores, for simplicity called "metabolite scores," for these phases as well as the intermediate phase are shown in Fig. 2c, and for all sample groups in Fig. 3c. The individual traces for all patients are shown in Supplementary Fig. 1.

Notably, all the significantly changing metabolites were decreasing from early to late samples; glucose, mannose, leucine, isoleucine, phenylalanine, tyrosine, signals from polyols and N-acetylated protein, and a number of unidentified metabolites were all lower in the late samples (Fig. 4b, supplementary Table 2).

Differentiation between treatment phases using metabolomics relative to CRP and leucocyte count

To test how well the metabolomic scores can distinguish between treatment phase compared to CRP and leucocyte count, we made an OPLS-DA model based on CRP and leucocyte

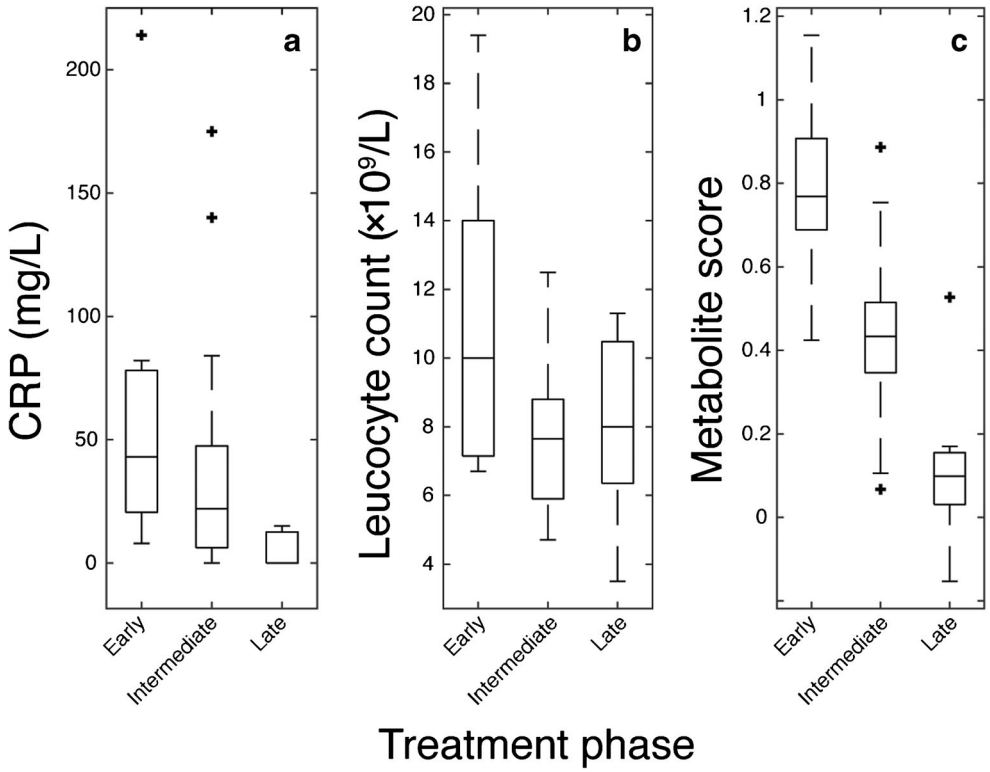

Fig. 2 CRP (A), leucocyte count (B), and cross-validated or predicted OPLS-DA predictive component scores based on the metabolite concentrations (C) for early, intermediate, and late samples (Table 2). The central mark indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points within ± 2.7 standard deviations, and values outside that range are plotted individually using the plus symbol

count only, and on CRP and leucocyte count plus metabolites. Notably, the model including only CRP and leucocyte count had no predictive power ($Q^2 = 0.04$), while the model which also included metabolites had the same predictability as for metabolites only (supplementary Table 1). Thus, only the metabolite scores could be used to distinguish between different treatment phases.

Discussion

The present study represents, to our knowledge, the first analyses of changes in longitudinal NMR metabolomics in serum during the course of the disease in patients with a bacterial infection, in this case IE. We found that metabolites such as sugars and amino acids seen in the NMR spectra decrease over

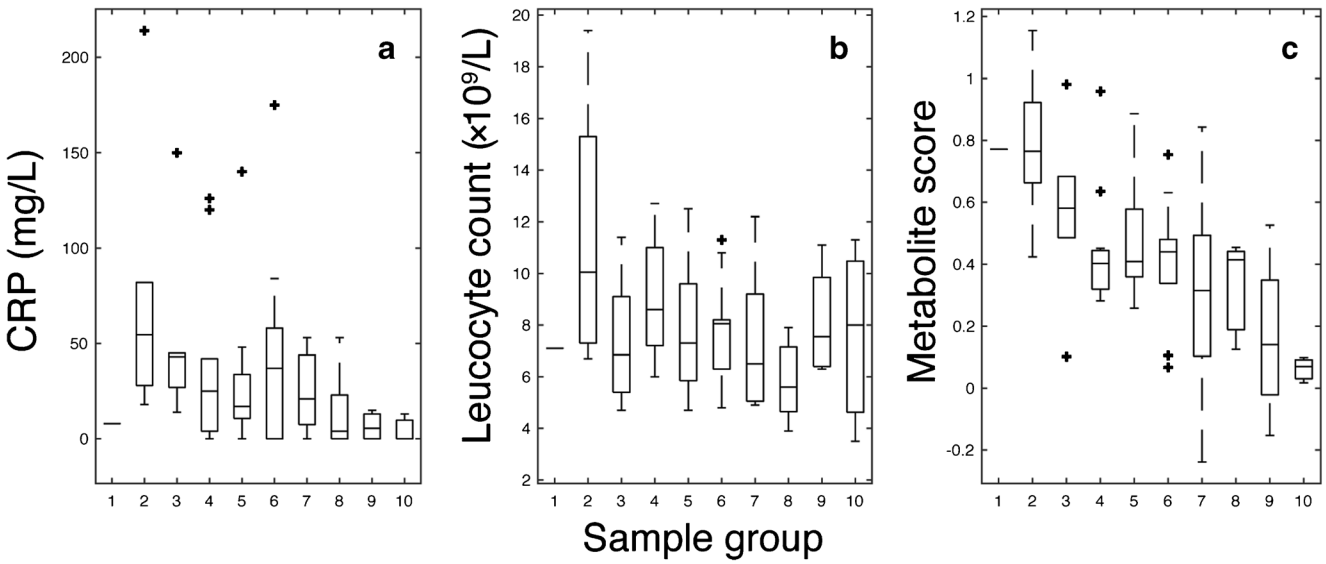

Fig. 3 CRP (A) and leucocyte count (B) and cross-validated or predicted OPLS-DA predictive component scores based on the metabolite concentrations (C) in sample group 1–10 (Table 2). The central mark indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points within ± 2.7 standard deviations, and values outside that range are plotted individually using the plus symbol

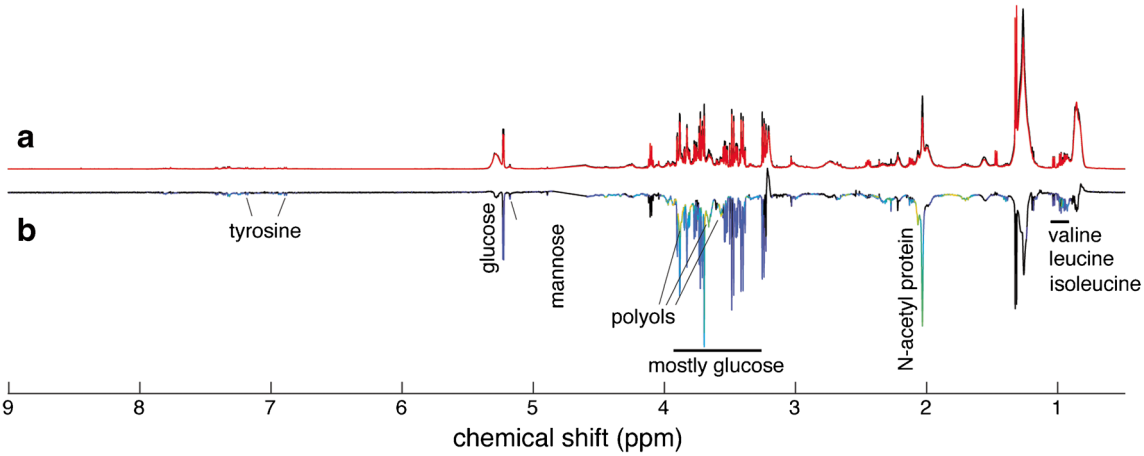

Fig. 4 Median spectra for early (black) and late (red) phase serum samples (A) and OPLS-DA loadings showing significantly changing NMR signals (B). In (B), significant alterations were color coded on a temperature scale, with yellow representing the highest correlation between metabolite and treatment phase

time during the treatment, i.e., during the cure of IE. Our results indicate that a metabolomic fingerprint of IE treatment progression might be established and used to predict which phase the IE patient is in, potentially helping clinicians to individualize the duration of the antibiotic treatment.

Today, temperature and the biomarkers CRP and leucocytes are often used to monitor antibiotic treatment response, but a definite cut-off value of these parameters defining when the bacteria are cleared from the body has not been identified. The use of different biomarkers in deciding when to initiate and withdraw antibiotic treatment however is already debated, e.g., in the critical care setting [24, 25]. Metabolomics show a great potential to assist the decision-making when individualized treatments are wanted.

We do not know whether the identified changes in metabolite concentrations are causally linked to the recovery from IE. However, fever and inflammation generally lead to an increased metabolism of several inflammatory markers (such as antibodies, antigens, cytokines, and interleukins), and patients who are immobilized (as during hospitalization for IE) often loses muscle mass. These conditions might contribute to a high turnover of proteins, breaking down into small molecules (amino acids), which might explain why a higher number of metabolites are circulating in the blood during the early phase of IE, and less metabolites can be measured when the inflammation/infection is recovering. Whatever their cause, metabolomic results like these have the potential to be used as a predictor of treatment response and survival [26]. Two longitudinal NMR metabolomics studies of infective disorders have previously been published, but they were both based on urine metabolomics [12, 27]. In a study investigating patients with pneumococcal pneumonia [12], urine metabolomics could separate patients with pneumonia due to pneumococci from healthy persons, patients with other pulmonary comorbidities, and other types of pneumonia (other bacteria, viruses). In the longitudinal part of the study, each subject changed from pneumococcal to normal metabotype over time. In a study investigating *Schistosoma mansoni* infection [27], it was possible to discriminate infected from uninfected children (7–15 years) and adults (20–40 years), based on differences in their urinary metabolite profile, primarily due to changes in the gut microflora, energy metabolism, and liver function. A recent study showed that nine metabolomic biomarkers were superior to clinical parameters (carcinoembryonic antigen (CEA) and carbohydrate antigen 199 (CA199)) in predicting response to neoadjuvant chemotherapy for colorectal cancer (AUC 0.83 for metabolites compared to 0.54 and 0.59 for CA199 and CEA, respectively) [28]. In our study, what we know are that the patients went from being newly diagnosed IE patients (early phase) to "healthy" patients (late phase), but whether the combination of high levels of the eight metabolites means "diseased" and low levels means "healthy" is needed to be investigated in future studies.

It is also conspicuously that all the significantly changing metabolites are decreasing over time. However, we also found metabolites increasing over time (these changes were not statistically significant)—why we do not believe this is a dilution phenomenon.

Strengths and limitations

We chose to apply a longitudinal design where the patients served as their own controls [29], enhancing the power to identify statistically meaningful metabolite changes over time and avoiding potentially confounding inter-individual clinical variabilities [30]. All the samples were collected after fasting and at the same time during the day. However, no special diet or lifestyle restrictions were applied, and the etiology of IE and thus the antibiotic treatment during the hospital stay was different for different patients. Also, no control group was included, so the absolute concentrations of metabolites for

IE patients at time of hospitalization (baseline) is unknown. Gender and age differences, co-infections, and biological variability reflect the complexity of our dataset. The patients also had been hospitalized for different durations when getting the definite IE diagnosis. Therefore, antibiotic treatment often had been given for a varying duration of time—which also affects the metabolomic results [31].

Metabolomic research of infectious diseases requires special attention, since the metabolomic signature of an infected host potentially reflects the interaction between the host and the pathogen; there is an enormous diversity regarding which pathogen that is considered, and the genetic variance that affects the susceptibility of the host to an infection may affect the results [32]. The specificity of our findings is therefore difficult to evaluate, and additional studies are required to further investigate the metabolomic fingerprint of IE and treatment regime hereof. Also, the population analyzed is very small, which prevent any conclusive result. Therefore, further research in larger IE populations is needed.

Conclusion

In this first longitudinal study of ^{1}(H) NMR metabolomics on human serum in patients with a serious bacterial disease, we identified significant metabolite changes during the active phase of IE. These changes could in contrast to the routinely used parameters CRP and leucocyte levels distinguish between the early and late stages of the in-hospital period of the disease.

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Author contributions HB, KI, AM, and CFK conceived and designed the study. CFK, SLKH, and MHA included the patients, and collected and prepared the blood samples. CFK, AM, and AP made the NMR spectral analyses. AM made the statistical analyses. CFK drafted the manuscript, and SLKH, MHA, AP, HB, KI, and AM revised the manuscript critically for intellectual content, and have read and approved the final manuscript for submission.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the Danish Data Protection Agency (j.nr.: 2012-58-0004, local j.nr.: HGH-2015-010, I-suite number: 03923) and the Danish Scientific Ethics Committee (protocol number: H-15009681).

Informed consent All patients included in this study participated after having given informed consent.

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