Postoperative Lung Injury- The path from Initiation to Clinical Diagnosis

a molecular view on a complex pathophysiological process

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A MOLECULAR VIEW ON A
COMPLEX PATHOPHYSIOLOGICAL PROCESS

BY
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DENMARK

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PREFACE

This PhD thesis is submitted in partial fulfilment of the requirements for obtaining the degree of Doctor of Philosophy. The work was financially supported by the Department of Chemistry and Bioscience, Section of Biotechnology, Faculty of Engineering and Science, Aalborg University and the Department of Anaesthesia and Intensive Care Medicine, Aalborg University Hospital, Denmark. The experimental work was carried out at the NMR Laboratory at the Department of Chemistry and Bioscience, with Professor MSO Reinhard Wimmer as the main supervisor.

The dissertation consists of an introduction summarizing literature relevant to the project, aims of study, methods, results and discussions, two published papers (Study I, II), one manuscript (Study III), and one patent application (Study IV).

I would like to take this opportunity to express my appreciation to all those persons who provided me with invaluable support and assistance. First of all, I would like to thank my supervisors, Professor MSO Reinhard Wimmer and Clinical Professor Bodil Steen Rasmussen for giving me the opportunity to work in such an interesting field. It has been very fascinating and educational to have both a technical and a clinical perspective on my research project.

Special appreciation is also extended to all my co-authors for their helpful discussions and contributions to the studies included in this thesis. I greatly appreciated the help from my colleague MD MSc Munsoor Ali Hanifa, who has introduced me to the world of metabonomics, and has captured my interest within big-data analysis. Appreciation also goes to Professor MD Søren R. Kristensen from the Department of Clinical Biochemistry for his ideas and suggestions, as well as for the excellent comments regarding the molecular interpretation of the data; Senior Researcher Shona Pedersen from the Department of Clinical Biochemistry for her valuable comments, enthusiasm, and help whenever needed; and Associate Professor Sergey Kucheryavskiy from the Department of Chemistry and Bioscience for sharing his chemometrics ideas with me.

Finally, I owe a debt of gratitude to my husband and our children for their understanding, support, and patience during the many hours I missed being in their company.

OBJECTIVES

Respiratory disorders are amongst the most complex and frequently encountered diseases in the intensive care unit (ICU). A wealth of clinical and biological information is available on patients diagnosed with pulmonary dysfunction after cardiac surgery. However, the primary reasons and paths involved in disease progression remain largely unknown. The major reason for this deficiency is that the disease involves complex mechanisms, including the interaction of different cells and molecules.

Early detection of pulmonary dysfunction is the most effective way to improve patients' outcomes postoperatively. To understand its complexity, new ideas and approaches are needed. Metabonomics provides a powerful platform for biomarker discovery and for the detection of altered biochemistry, which could help with early diagnosis. The main objective of this thesis was to investigate whether metabolome analysis could be used to detect early signs of molecular impairments causing progression to postoperative lung injury in patients undergoing cardiac surgery with the use of cardiopulmonary bypass (CPB).

During the course of the study it became clear that nothing about this disease was straightforward. The main problems being: the multiple perturbed mechanisms occurring in parallel or in series; patients' preclinical histories (e.g. lifestyle, prior diseases, etc.); surgical trauma elicited on each individual; and the lack of early biomarkers.

Although my area of research was metabolome analysis, I became involved in the investigation of clinical, biochemical, inflammatory, and coagulation data. After attending four open-heart surgeries, it became clear that all these platforms must be included, to get a more realistic insight into the mechanisms underpinning disease progression. Being a part of different laboratories and units (e.g. NMR Spectroscopy, Anaesthesia and Intensive Care Unit, and Clinical Biochemistry), has given me the opportunity to be involved in investigating disease progression from multiple angles. By integrating the engineering and clinical world, we discovered some interesting results. We found that the early pathological development of lung injury involves alteration in the levels of more than 60 metabolites (Study I, Study III, and Study IV), coagulant and fibrinogen factors (Study II), and pro-inflammatory mediators (manuscript in preparation). Although we have achieved a lot, there is still much to learn.

I have spent the main part of my PhD analysing samples collected on the first morning postoperatively (Study I and IV). The main objective with this analysis was to investigate whether we could detect early signs of subsequent impairment,

and to learn NMR-based metabonomics. The most exciting part was learning how to think 'big-data' and how to analyse complex information. All aspects, from signal processing, the choice of machine learning algorithms, model optimization, feature selection, validation, multivariate data analysis, biostatistics, programming, and to the visual representation of the data, were important tasks that I am grateful to have learn during this PhD. With regards to the biological aspects, I have spent a significant amount of time searching for the 'needle in the haystack' and looking for meaning in the biological information obtained. This involved the identification of metabolites and their complex interactions, as well as the identification of possible biomarkers and the optimal combination that could achieve the highest predictive values. Finding which metabolites were connected to each other and how they may be involved in disease progression was an exciting puzzle, and a valuable learning process.

The main objective of analysing the rest of the time series data (Study III), involving the analysis of nearly 850 samples collected before, during, and after the end of surgery, was to understand how the procedure affected the human metabolome, and how the this may elicit the development of lung injury. The main achievements from this study were finding how surgery affected metabolites, and how the metabolome could predict disease progression while patients were still at the operating room. In addition, this study confirmed and validated our findings from the earlier study conducted on samples from the first morning postoperatively.

While I have achieved the main objective of this study, there is still a long way to go and many questions to answer. Are the identified mechanisms similar in other types of patients undergoing cardiac surgery (e.g. sepsis, chronic obstructive pulmonary disease)? Can these results help to find novel therapeutics to attenuate, or even prevent, disease progression? Can our approach be used in hospitals? …

LIST OF PAPERS

This thesis is based on three scientific papers and a patent application, which are referred to in the text by Roman numerals. These studies have been carried out in the period from 2011-2016:

Study I

Maltesen R.G., Hanifa M.A., Kucheryavskiy S., Pedersen S., Kristensen S. R., Rasmussen B.S., Wimmer R./ *Predictive Biomarkers and Metabolic Hallmark of Postoperative Hypoxaemia*/ Metabolomics. 2016; 12(5):1-15

Study II

Rasmussen B.S., Maltesen R.G., Pedersen S., Kristensen S.R./ *Early coagulation activation precedes the development of acute lung injury after cardiac surgery*/ Thrombosis Research, Vol. 139, 82-84, 2016.

Study III

Maltesen R.G., Rasmussen B.S., Pedersen S., Hanifa M.A., Kucheryavskiy S., Kristensen S.R., Wimmer R./ *Metabotyping Patients' Journeys Reveals Early Predisposition to Lung Injury after Cardiac Surgery*/ Manuscript

Study IV

Maltesen R.G., Wimmer R., Rasmussen B.S., Pedersen S., Kristensen S.R., Hanifa M.A./ *Biomarkers for prediction of development of hypoxemia due to acute lung injury*/ Patent: EP15168879.3, 2015.

Papers not included

Study V

Xu Y., Maltesen R.G., Larsen L.H., Schønheyder H.C., Le V.Q., Nielsen J.L., Nielsen P.H., Thomsen T.R., Nielsen K.L./ *In vivo gene expression in a Staphylococcus aureus prosthetic joint infection characterized by RNA sequencing and metabolomics: a pilot study*/ BMC Microbiology, Vol. 16, No. 1, 80, 2016.

Study VI

Rasmussen B.S. et *al./ Cytokines and acute lung injury after cardiac surgery*/ Manuscript in preparation

I hereby declare that this is my original work. The following laboratory aspects of the study have been undertaken by the following persons:

1. Professor MSO Reinhard Wimmer set up the automated pulse sequences for the NMR experiments.

2. Clinical Professor Bodil Steen Rasmussen provided all blood samples, blood gas measurements, and the cytokine data used in Study VI.

3. PhD student Munsoor A. Hanifa prepared and run 60 samples for Study I.

4. Senior researcher PhD. Shona Pedersen provided albumin and creatinine measurements used in Study I, and the experimental data used in Study II.

Aalborg, June 2016

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ENGLISH SUMMARY

Introduction: Postoperative pulmonary dysfunction after cardiac surgery with the use of cardiopulmonary bypass (CPB) is common, ranging from transient hypoxemia to severe lung injury. Even after uncomplicated surgeries, some patients experience a systemic inflammatory response, a disrupted coagulation, ischemia-reperfusion lung injury, and oxidative stress. Since the nadir values of partial pressure of arterial oxygen (PaO_2) appears on the second to third postoperative day, it is difficult to predict which patients will develop pulmonary dysfunction. Currently, there is no early diagnostic test to detect the progression to hypoxaemia and no molecular-driven intervention to prevent its course.

Hence, we hypothesized that the surgery elicits molecular changes during, or early after weaning from CPB, that can be related to the subsequent hypoxaemia.

Aims: Thus, the aims of this study were to (1) identify possible molecular changes (e.g. metabolites, coagulant and fibrinolytic factors) that occur as a consequence of cardiac surgery; (2) to relate these changes to the later degree of pulmonary dysfunction; and (3) to identify the possible mechanisms that are involved in disease progression.

Methods: Fifty consecutive patients undergoing cardiac surgery with use of CPB were included. Nearly 850 blood samples were collected from the systemic and pulmonary microcirculation at ten different time points: the day before surgery, intraoperatively, and postoperatively for up to three days. Changes in the levels of metabolites were analysed by means of nuclear magnetic resonance (NMR) spectroscopy and multivariate data analyses such as principal component analysis (PCA) and partial least-square regression (PLS) analysis. Changes in the levels of coagulant and fibrinolytic markers were assessed by means of ELISA kits assays.

Results: All patients had normal preoperative PaO_2; 11.3±1.3 kPa (mean±SD). PaO_2 decreased to 7.8±1.4kPa at 72 hours postoperatively. Twenty three patients presented moderate hypoxemia ($8.4 > PaO_2 \geq 6.3$kPa), nine presented severe hypoxaemia ($PaO_2 < 6.3$kPa), while eighteen patients showed no sign of hypoxaemia ($PaO_2 > 8.5$kPa) on the third postoperative day.

There are several significant findings in this present study. Firstly, metabolome analysis by PCA revealed immediate and striking changes in both the pulmonary and systemic samples as a consequence of cardiac surgery. Secondly, prolonged ischemia, and duration of CPB and CABG impacted both the metabolome and the coagulant and fibrinolytic activity, especially in patients developing hypoxaemia. Thirdly, several metabolite markers of surgical trauma were identified and were related to the development of hypoxaemia. Fourthly, the intraoperative metabolome

indicated a possible predisposition to lung injury, since PLS regression analysis detected a 0.9 cross-validated correlation with PaO_2 measured 72 hours post-CPB. Finally, a 'metabolic biosignature' was identified that clearly discriminated unaffected from hypoxaemic patients while patients were still undergoing the surgery. The results suggest that patients progressing to hypoxaemia have a limited metabolic reserve with regard to adenosine triphosphate (ATP) synthesis, deranged antioxidant defence system, excessive activated lipolysis, and more coagulant and fibrinolytic activity, which have probably made them more prone to produce an exaggerated stress response to the cardiac surgery.

Conclusion: To the best of our knowledge, this is the first comprehensive study demonstrating the link between intra- and postoperative time-dependent molecular changes and the later development of postoperative hypoxaemia. The study provides novel insights into the underlying mechanisms that trigger progression into hypoxemia, allowing for new hypothesis and treatment options to come.

Further research is needed in this complex research field to confirm the results and to achieve even better understanding about the molecular mechanisms leading to disease progression.

DANSK RESUME

Baggrund: Postoperativ lungedysfunktion efter hjertekirurgi med anvendelse af kardiopulmonal bypass (CPB) er en almindlig fremkommende tilstand, som spænder fra forbigående hypoxæmi til svær lungeskade. Selv efter relativt ukomplicerede operationer, oplever nogle patienter en systemisk inflammatorisk respons, en ændret koagulationsaktivitet, iskæmi-reperfusion lungeskade, og oxidativt stress. Da de laveste værdier af partieltrykket af oxygen i arteriel blod (PaO_2) oftest forekommer på anden til tredje postoperative dag, er det vanskeligt, at forudsige og udpege de patienter som vil udvikle lungedysfunktion. På nuværende tidspunkt findes der ingen tidlig diagnostisk test som kan detektere progressionen til hypoxæmi. Desuden findes der ingen terapeutisk behandlingsmåde til at forhindre dens progression.

Vores hypotese har været at operationen fremprovokerer molekylære ændringer i kroppen under selve kirurgien eller tidligt herefter, der kan muligvis relateres til den efterfølgende hypoxæmi.

Formålene med denne Ph.d. afhandling var at (1) identificere mulige molekylære ændringer i fx metabolitter, koagulant og fibrinolytiske faktorer, der opstår som følge af hjertekirurgi; (2) at relatere disse ændringer til senere grad af lunge-dysfunktion; og (3) at identificere de eventuelle mekanismer, der indgår i sygdoms-progression.

Metoder: Halvtreds konsekutive patienter, der gennemgik hjertekirurgi med brug af CPB blev medtaget i studiet. Næsten 850 blodprøver blev opsamlet fra den systemiske og pulmonale cirkulation på ti forskellige tidspunkter: dagen før operationen, intraoperativt og postoperativt i op til tre dage efter CPB. Ændringer i metabolitternes koncentration blev analyseret ved hjælp af nuclear magnetic resonance (NMR) spektroskopi og multivariat dataanalyser, så som principal component analysen (PCA) og partial least-square (PLS) regression analysen. Ændringer i koagulant og fibrinolytiske faktorer blev analyseret ved hjælp af ELISA kits analyser.

Resultater: Alle patienter havde normal præoperativ PaO_2 (11.3±1.3kPa; middelværdi±SD). Tre dage efter operationerne faldt deres PaO_2 værdier til 7.8 ± 1.4kPa. Treogtyve patienter udviklede moderat hypoxæmi ($8.4 > PaO_2 \geq 6.3$kPa), ni havde svær hypoxæmi ($PaO_2 < 6.3$kPa), mens de resterende patienter viste ingen tegn på hypoxæmi ($PaO_2 > 8.5$kPa) på tredje postoperative dag.

PCA analysen viste at den pulmonale og systemiske metabolitprofil ændrede sig markant som følge af hjertekirurgi. Derudover fremgik det af resultaterne at langvarigt iskemi-, CPB- og CABG-tid påvirkede både metabolomet og den koagulant og fibrinolytisk aktivitet, især hos de patienter som udviklede hypoxæmi.

Desuden blev der identificeret flere metabolitmarkører for det kirurgiske trauma, og nogle af disse blev relateret til udviklingen af hypoxæmi. PLS regressionsanalysen har vist en 0.9 korrelation mellem metabolomet ved afsluttet CPB og de målte PaO_2 værdier på den tredje dag efter operation. Endelig blev en "metabolisk biosignatur" identificeret som kunne differentiere patienter der udviklede hypoxæmi af patienter som ikke blev påvirket af lungeskade. Denne biosignatur har vist sig at kunne forudsige sygdomsudviklingen allerede under operationen. Resultaterne viste også, at de patienter som udviklede hypoxæmi, havde en begrænset metabolisk reserve i forhold til adenosintrifosfat (ATP) og antioxidantsyntesen, og en højre grad af lipolytisk-, koagulant- og fibrinolytisk aktivitet. Dette gjorde dem mere tilbøjelige til at producere en forhøjet stressreaktion som følge af hjertekirurgi.

Konklusion: Dette er den første omfattende undersøgelse der pointerer sammenhængene mellem molekylære intra- og postoperative tidsafhængige ændringer, som kan føre til senere udvikling af postoperativ hypoxæmi. Undersøgelsen giver nye indsigter i de underliggende mekanismer, som muligvis udløser progression i hypoxæmi, og giver mulighed for nye hypoteser og behandlingsmuligheder fremover.

Der er brug for yderligere undersøgelser indenfor dette komplekse forskningsområde for at bekræfte resultaterne og for at opnå endnu bedre forståelse for de molekylære mekanismer, som kan føre til udvikling postoperative lungeskade.

LIST OF ABBREVIATIONS

ALI	acute lung injury
ANOVA	analysis of variance
ARDS	acute respiratory distress syndrome
ATP	adenosine triphosphate
BCAA	branched-chain amino acids
BALF	bronchoalveolar lavage fluid
CABG	coronary artery bypass grafting
CPMG	Carr-Purcell-Meiboom-Gill
CPB	cardiopulmonary bypass
CV	cross-validation
DAG	diacylglyceride
DAGPL	diacylglycerophospholipid
FA	fatty acid
FID	free induction decay
FiO_2	fraction of oxygen inspired
F12	fragment 1.2
GPC	glycerophosphocholine
^1H *NMR*	proton nuclear magnetic resonance
HMDB	human metabolome database
HSQC	hetero-nuclear single quantum coherence
ICU	intensive care unit
IL	interleukin
LA	left atrium
LV	latent variables
MUFA	monounsaturated fatty acid
N-Ac-Gal	n-acetyl-galactosamine
N-Ac-Glc	n-acetylglucosamine
PA	pulmonary artery
PaO_2	partial pressure of oxygen
PAP	plasmin-antiplasmin
PC	phosphatidylcholine
PC1, PC2	principal component 1 and 2
PCA	principal component analysis
PEP	phosphoenoyl pyruvate
PLS	partial least squares
PLS-DA	partial least squares-discriminant analysis
ppm	parts per million
PUFA	polyunsaturated fatty acid
RF	radiofrequency
ROC	receiver operating characteristic
ROS	reactive oxygen species
SD	standard deviation
SM	sphingomyelin
TCA	tricarboxylic acid
TOCSY	total correlation spectroscopy
1D, 2D	one- and two-dimensions
3-HBA	3- hydroxibutyric acid

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CHAPTER 1. BACKGROUND

Cardiovascular disease is society's number one health problem (1), being the leading cause of death in many countries (2, 3). The vast majority of these patients suffer from coronary heart disease (2). Nearly one million patients undergo coronary artery bypass grafting (CABG) surgery with the use of cardiopulmonary bypass (CPB) each year (4), of which more than 2000 procedures are performed in Denmark (5).

Patients undergoing cardiac surgery are at risk of developing postoperative pulmonary dysfunction (6). Pulmonary dysfunction has been known since the earliest days of cardiac surgery (7-10), yet, predicting which patients will develop postoperative complications is difficult. Several risk factors are known to influence the development of postoperative pulmonary dysfunction, including previous cardiac surgery (11), the surgical procedure itself (6), blood transfusion (11, 12), anaesthesia, and the use of CPB (8, 13, 14). In addition, polymorphisms in the genes encoding some inflammatory mediators (15, 16), and increased circulating free fatty acids (17) have recently been identified as predictors of early pulmonary dysfunction after surgery. While these factors are crucial for the early prognostication of at-risk patients, there is still need for more research-based knowledge to gain a better understanding of the molecular reasons as to why certain patients develop life-threatening complications postoperatively, while others do not.

This PhD thesis will focus on investigating the intra- and early postoperative molecular changes occurring in patients undergoing CABG, and on identifying possible associations with the progression to pulmonary dysfunction. Small molecules (or metabolites) are the end products of genes, transcripts, and protein regulation. Since they are strongly influenced by environmental changes (e.g. disease progression, medication) (18), measuring metabolic variations in response to CABG could potential reveal an individual's risk of developing pulmonary dysfunction. The metabolite studies presented in this thesis will serve as an example of an approach to identify molecular perturbations and predictive markers of the degree of the dysfunction. In addition, one study based on coagulation and fibrinolysis factors will serve as a small example of macromolecular changes occurring in some patients early postoperatively. The findings of the individual studies will contribute to the pool of knowledge upon which future research and possible new treatment options may be based.

1.1. CORONARY ARTERY DISEASE

More than 17 million Americans suffer from cardiovascular diseases, of which, approximately 11 million suffer from coronary artery disease (CAD) (19). CAD is caused by the build-up of cholesterol deposits in the wall of coronary arteries supplying the heart muscle. Accumulation of cholesterol limits the blood flow to the heart and causes poor blood supply (or ischemia), chest pain, and even heart attack. Treatments of CAD include changes in lifestyle (diet, exercise, weight loss, and

smoking cessation), medication to lower cholesterol levels, and surgery for patients with more severe symptoms. Surgical treatments may involve dilatation of the coronary artery with a balloon (percutanaeous cardiac intervention), or CABG performed with, or without, the use of CPB. (20)

Since this thesis is based upon patients undergoing CABG, only this subject will be discussed in the following section.

1.1.1. CABG-SURGERY

CABG is an open-heart surgery. During the procedure new arteries are created to improve blood supply to the heart (21) (Figure 1-1).

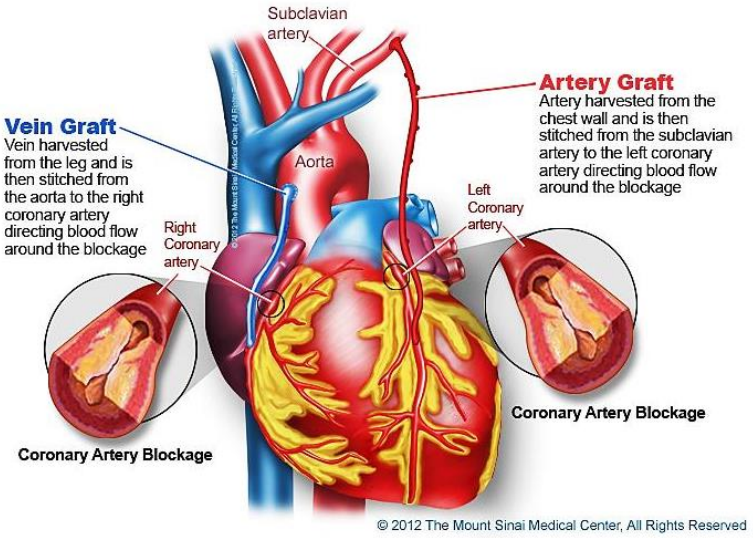


Figure 1-1 Vein and artery bypass grafts are attached to the heart. During CABG, a healthy artery from the forearm, or vein from the leg is grafted (or connected) to the heart and blood bypasses (or goes around) the blocked portion of the coronary artery, creating a new path for oxygen-rich blood to flow to the heart muscle. (Copyright © 2016 Icahn School of Medicine at Mount Sinai)

CABG is performed under anaesthesia. During anaesthesia a tube is inserted through the mouth to help patients breathing during and following hours postoperatively (20). After the induction of anaesthesia, an incision in the middle of the sternum (or sternotomy) is required, and the CABG is then performed either on a beating (off-pump) or a stopped heart (on-pump or CPB). About 80% of all CABG surgeries are performed on-pump (22). During on-pump CABG the pulmonary ventilation is stopped and the lungs are collapsed to ensure stabilization of the heart. Subsequently, the CPB takes over the functions of the heart and lungs, allowing for proper blood oxygenation and oxygen delivery during the surgical procedure. (23)

Postoperatively, patients are admitted to intensive care units (ICUs) for 1-2 days, where their breathing is facilitated by a mechanical ventilator. At the ICUs additional blood transfusions, medications, and drainage of the excessive fluids gained during surgery is often necessary. (20) Patients are usually discharged from the hospital within a week after surgery. However, in some cases, complications may occur (22). Among the most common complications are irregular heartbeat, stroke, infection of the sternum (20), pulmonary dysfunction (6, 8, 12, 14, 24-28), and acute renal failure (21).

Since this thesis is based on postoperative pulmonary dysfunction, only this topic will be discussed further.

1.2. POSTOPERATIVE PULMONARY DYSFUNCTION

Pulmonary dysfunction, defined as the reduced ability of the lungs to exchange gases, such as oxygen, is one of the most common complications following cardiac surgery (8). Up to 90% (29) of patients can develop pulmonary dysfunction for at least one week after uncomplicated surgery (7).

The dysfunction can be caused by many factors and can be divided into poor postoperative lung mechanics and abnormal gas exchange. (30) Alterations in the lung mechanics may be attributed to the surgical factors per se (e.g. impaired movement of the chest after sternotomy, pulmonary oedema drain discomfort, and wound pain), while the abnormal gas exchange (CO_2, O_2) may be attributed to the anaesthesia, lung collapse or atelectasis, and the use of CPB.

Despite improvements in the CPB techniques, the use of the artificial circuit usually leads to a systemic and pulmonary inflammatory reaction. This, in combination with the alveolar collapse, induces dysfunction in the alveolar-endothelial barrier, with subsequent intrusion of oedema fluid in the lungs. As a consequence, lungs are injured and the normal gas exchange is impaired. (8) Clinically, these alterations are indicated by respiratory difficulties, changes on chest radiographs, low arterial oxygen levels, and cough. (6)

1.2.1. GAS EXCHANGE

The gas exchange (Figure 1-2) is determined by quantifying the partial pressure of oxygen (PaO_2) in arterial blood (7) or by taking the ratio between PaO_2 and the fraction of inspired oxygen (PaO_2/FiO_2). PaO_2 is a measure of the amount of oxygen that binds to haemoglobin. The higher the pressure, the more oxygen is bound to haemoglobin, and the more oxygen is transported to the tissues. Under normal conditions, approximately 97-98% of the oxygen is attached to the haemoglobin molecules in the red blood cells, while the remaining oxygen dissolves in the plasma. Depending on the body environment, the tissue demand, and the number of haemoglobin molecules, oxygen dissociates from haemoglobin when needed.(31)

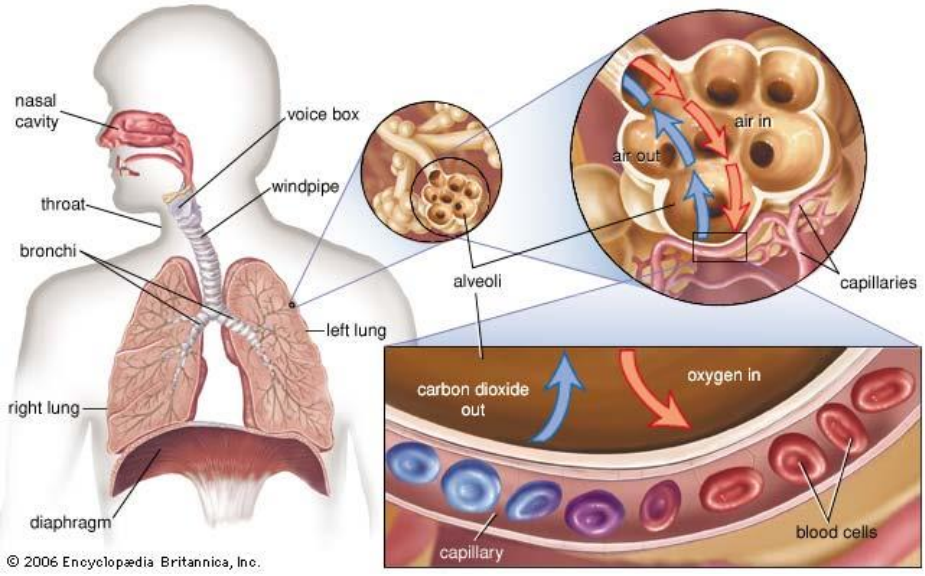


Figure 1-2 The gas exchange across alveoli. Each lung is made up of millions of alveoli. Capillaries surround each cluster of alveoli and ensure proper gas exchange between the air and the blood. Under normal conditions oxygen moves from the alveoli to the bloodstream, and further on to the body tissues and cells in exchange for carbon dioxide. Copyright © 2006 by Encyclopaedia Britannica, Inc.

All patients exhibit some decrease in the alveolar gas exchange immediately after surgery (32). Hence, the percent of O_2 delivered by haemoglobin to tissue is lower than normal (31). Gas exchange is abnormal when the PaO_2/FiO_2 is below 40kPa (or below 300mmHg) or when the PaO_2 is below 8.4kPa (or below 63mmHg). The lower the values, the more affected the lungs. (8, 10, 26)

In order to increase, stabilize, and ensure adequate oxygen delivery to vital organs, supportive oxygen (e.g. mechanical ventilation) is given following surgery. Despite oxygenation, some patients do not respond to the intervention and develop lung injury with compromised oxygen delivery to the pulmonary capillary.(8) As a result, blood oxygen content will drop (***hypoxaemia***) and cells and tissues may experience low oxygen levels (***hypoxia***). If unresolved, it may eventually lead to cell death and organ failure. Evidence suggests that the brain is the organ most exquisitely sensitive to injury from low intracellular PaO_2 (< 3 minutes), followed by kidney, liver, and muscles. (33, 34)

The degree of hypoxaemia is determined by arterial blood gas analysis. The lower the PaO_2 values the more severe the hypoxaemia (Figure 1-3)(35). Hypoxaemia may vary from mild decreases, requiring only a short period of mechanical ventilation, to severe decreases, causing difficulties in breathing, excessive cough, prolonged mechanical ventilation, pulmonary infections, and requiring longer hospitalization

(36). In severe cases, patients may develop acute respiratory distress syndrome (ARDS) or its milder form, previously termed acute lung injury (ALI). Although the occurrence of postoperative ARDS is relatively low (< 3%) (8), the syndrome is devastating, with significant morbidity and mortality (40-80%) (8, 37-39).

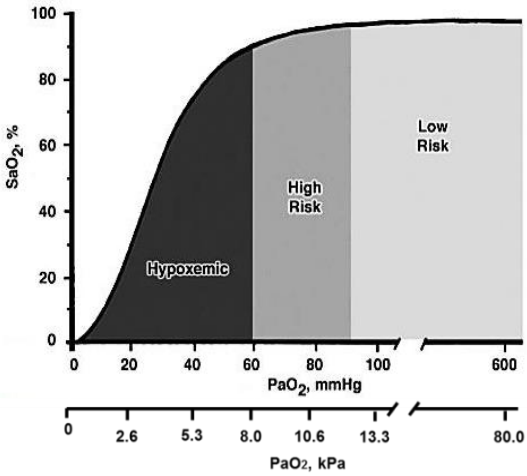


Figure 1-3 The haemoglobin oxygen dissociation curve demonstrates how the oxygen saturation of haemoglobin (SaO_2) varies with the levels of the partial pressure of oxygen (PaO_2). Risk categories are overlaid on the curve. Normal arterial PaO_2 levels are above 80 mmHg, while normal SaO_2 ranges from 93-97%. At low PaO_2 the haemoglobin binds to fewer O_2 molecules, and patients suffer from severe hypoxaemia. As the PaO_2 increases the haemoglobin molecule binds to more O_2 molecules, causing the steep part of the curve as the PaO_2 goes from 15 mmHg to 60 mmHg. As PaO_2 increases, the level of hypoxaemia decreases. From 60 mmHg to 80 mmHg more O_2 binds to haemoglobin and fewer binding sites become available. Above a PaO_2 of 80 mmHg the slope of the curve becomes nearly flat, and at a PaO_2 of 100 mmHg the haemoglobin is 97-98% saturated. The flat upper portion of the curve represents the PaO_2 entering the lungs exceed the oxygen concentration in blood returning to the lungs; and thus, the oxygen binds easy to the haemoglobin. Copyright © 2011 by the American College of Emergency Physicians.

Because all patients present decreased oxygenation right after surgery, it is difficult to recognize progressive hypoxaemia and the severity of forthcoming pulmonary insufficiency. Also, it is still not known why certain patients develop hypoxaemia, whilst others do not. (8) Several studies have shown that patients do not necessarily develop hypoxaemia during the first postoperative hours, but rather during the second to third postoperative day (30, 32, 40-42). In a study conducted by Singh and co-workers (30) serial arterial blood gas measurements obtained on days 1, 2, 4, 6, and 8 postoperatively revealed a drop in the PaO_2 levels at all time points compared to the preoperative values, with the lowest values on the second postoperative day. Same decreases were reported by Rasmussen and colleagues (41, 42), with lowest

PaO_2 values on the third postoperative day. Although PaO_2 improved the following days, impaired oxygenation can persist for up to two weeks postoperatively (43).

Since hypoxaemia extends hospitalization and increases the risk of various complications, as well as mortality, it is important to understand the risk factors and the mechanisms leading to its progression. (26, 44)

1.2.2. RISK FACTORS FOR PULMONARY DYSFUNCTION

Many factors are implicated in the development of postoperative lung injury; hence, knowledge about possible predictors of hypoxaemia is a prerequisite for risk stratification of patients.

1.2.2.1 Preoperative factors

A neglected but crucial factor of postoperative pulmonary dysfunction is patients' medical history before surgery (25, 45). Older (6, 38), sicker (6, 25, 38) and more obese (26) patients, with a history of smoking (6, 26) have a higher risk of developing postoperative pulmonary dysfunction. In addition, some patients are more genetically predisposed to produce greater inflammatory responses to severe trauma such as CPB, and thus, to progress to lung injury. Studies have shown that polymorphisms in the pro-inflammatory interleukin-encoding genes of IL-6 and IL-18 could predispose patients to develop post-CPB ALI/ARDS. (14-16)

1.2.2.2 Intra- and postoperative factors

A combination of many factors unique to cardiac surgery is reported to be attributed to the development of poor lung function and impaired gas exchange. Among the most significant are general anaesthesia (6, 46), sternotomy (6), atelectasis, contact of blood components with the artificial surface of the CPB circuit, blood exposure to the different shearing forces within the CPB (12), ischemia–reperfusion (13, 14), and endotoxemia (47).

Studies have shown that anaesthesia during surgery temporarily decreases PaO_2 due to relaxation of the chest wall, affecting the lung capacity and lung mechanics. In addition, sternotomy is believed to affect muscle tone of the chest wall and proper ventilation. Also, the cold topical solutions given to protect the heart may induce damage to the respiratory nervous system and may cause postoperative respiratory distress. Furthermore, the deflation and the time of inactivity of the lungs may cause postoperative partial atelectasis, causing partial closure of the alveoli, inhomogeneous airway constriction, and concomitant impairment in the alveolar function of gas exchange. (6, 8, 46)

The passage of blood through the circuit induces an acute phase reaction that can lead to a systemic inflammatory response. Since the lungs are the filters of the venous circulation, and since the pulmonary capillaries are smaller in diameter than

the systemic capillaries, substances produced during CPB may aggregate, or may be trapped, when passing through the pulmonary microcirculation. Hence, it may affect the lungs more than other organs, leading to lung injury with subsequent impaired gas exchange caused by: endothelial cell swelling; plasma and proteins intrusion into the interstitial space between capillaries and alveoli; and congestion of the alveoli with oedema fluid.(8)

To reduce the pulmonary dysfunction associated with CPB, the number of off-pump CABG has increased in the last 25 years. Follow-up studies have shown that while the off-pump CABG may attenuate the levels of IL-8 and IL-10, the procedure does not necessarily improve postoperative oxygenation (41), nor does it lower the mortality rate among patients (22, 48). Also, while some studies have shown predictive association between the time on CPB and the later postoperative hypoxaemia (36, 44), others have not found this association (26).

Higher intraoperative use of fluids during CPB has also been associated with an increased risk of pulmonary oedema formation, and thus, increased risk of postoperative hypoxaemia (44). Patients often require blood products during and after surgery to replace their blood loss, and these products carry the risk of transfusion-related ALI. (12)

Furthermore, studies have reported that during surgery microorganisms and their endotoxins enter the blood stream and induce further complications. They may originate from the surgical materials used (instruments, gloves, oxygenator), the fluids used, the blood transfusion, drugs, the ice for external cooling of the heart, or the translocation of bacteria from the intestinal flora into the bloodstream, among others. As a consequence, endotoxins bind to various receptors on inflammatory cells, causing an escalation in the production of pro-inflammatory mediators.(14, 47)

Lastly, for some patients, inappropriate oxygen supplementation and mechanical ventilation given during and after the surgery can be harmful, leasing to oxygen toxicity, rupture of the alveoli during lung stretch, and subsequent ventilator-induced lung injury. (33, 34)

1.2.3. MOLECULAR MECHANISMS OF PULMONARY DYSFUNCTION

Although only a small part of the mechanisms involved in the development of postoperative pulmonary dysfunction is known, current evidence reveals increased damage to alveolar cells, neutrophil infiltration, and increased microvascular permeability. (8, 49)

1.2.3.1 The systemic inflammatory response

Although the surgical procedure saves lives every day, the contact of blood with the CPB circuit activates the inflammatory cascade and initiates a systemic acute phase reaction (8, 25). Cytokines play an important role in this cascade, either as pro-

inflammatory (e.g. tumor necrosis factor-α, TNF-α; IL-2, IL-6, IL-8; and platelet-activating factors, PAF) or anti-inflammatory mediators (e.g. IL-1 and IL-10). Under normal conditions, the balance between pro- and anti-inflammatory cytokines is essential to avoid inflammation. This balance is shifted towards the production and activation of pro-inflammatory mediators after CPB.(14, 50) Pro-inflammatory mediators activate a large variety of cell types in the blood, including neutrophils, leukocytes, monocytes, macrophages, and lymphocytes, recruiting them to the lungs. These cells bind to adhesion molecules on the surface of capillary cells and migrate into the interstitial fluid space of alveoli, where they release proteolytic enzymes and reactive oxygen species (ROS) such as superoxide anions, peroxides, hydroxyl radicals). (49)

1.2.3.2 The ischemia-reperfusion mechanism

During CPB the lungs are almost excluded from the circulation and the blood remains almost stagnant inside them for up to several hours. Consequently, the lungs experience ischemia and an increased demand of oxygen.(8) Since cells cannot maintain the necessary energy supply, they compensate by degrading adenosine triphosphate (ATP) to adenosine, inosine, and hypoxanthine. During ischemia these by-products accumulate. Decreased ATP levels inactivate the cellular membrane pumps and ions are thus freely to move across the membranes. Moreover, due to the anaerobic environment, lactate accumulates and intracellular pH decreases, causing acute cellular swelling and even cell death. (14)

At the end of surgery, lungs are re-expanded and blood flow and oxygen supply returns (reperfusion). Although oxygen is necessary for cell salvage, the reperfusion process creates more injury than ischemia itself, culminating in damage to the DNA, proteins, carbohydrates, and lipids. When oxygen is reintroduced in the lungs, xanthine oxidase converts the accumulated hypoxanthine to uric acid and produces superoxide anions. Superoxide anions are converted by superoxide dismutase to hydrogen peroxides, which bind to free iron, producing hydroxyl radicals. These radicals are highly destructive and potent oxidizing agents that react with almost every molecule in the cell, including lipids. Lipid peroxidation products enhance the ongoing inflammation; damage the cell membrane; lead to the loss of cellular membrane stability; affect proper functioning of the enzyme systems and receptors; alter the ionic channels; and increase cellular permeability, finally leading to cell death and even organ dysfunction. (14, 27, 49)

Hence, the combination of imbalanced inflammatory responses, the adverse effects of pulmonary ischemia-reperfusion, the possible swelling of the capillary cell membrane, the fluid accumulation in the interstitial space, and the intrusion of protein-rich oedema fluid in the lungs leads inevitably to impaired gas exchange and deterioration in the respiratory mechanics of the lungs. (8)

1.2.4. DETECTION OF PULMONARY DYSFUNCTION

Despite the known mechanisms, early detection of patients developing postoperative pulmonary dysfunction can be challenging (39, 51). Currently, there is no early gold standard diagnostic test for the quantification of postoperative pulmonary dysfunction (52), and the PaO_2 values used to assess the degree of lung injury cannot be used to predict the progression to hypoxaemia. Roe and co-workers (53) have associated the PaO_2 values measured before and during surgery with the postoperative PaO_2 values in a group of patients suffering from postoperative hypoxaemia. Poor predictive association was detected, indicating that prior knowledge about the gas exchange is not a good prognosticator of later hypoxaemia.

In addition, there is no treatment option (9) and no molecular-driven intervention (39, 51) to prevent the progression into pulmonary dysfunction. When lung injury is present, supportive care and conservative fluid management is given to the patients (39, 51, 54). If patients do not improve upon management, multiorgan dysfunction may develop, and patients may die. On the other hand, if patients survive, they recover gradually; with many patients continuing to have persistent pulmonary abnormalities for longer periods of time.(55)

1.2.5. BIOMARKERS OF PULMONARY DYSFUNCTION

Early identification and stratification of patients at risk of developing pulmonary dysfunction is an area of great research.

A biomarker can be anything (e.g. vital sign, laboratory value, and finding on an image) that shows association to a disease, is relatively stable, sensitive, specific, and can be useful for screening, diagnosis, and monitoring of the effect of initiated therapies. A predictive biomarker is a marker that can spot at-risk patients before the symptoms are detectable, and that may help in the development of therapeutics targeting disease progression. (52, 56)

Biomarkers, whether measured in blood, urine, or exhaled breath, have found limited use for the early identification of patients developing postoperative pulmonary dysfunction (52). A possible explanation for this might be the complex nature of chain reactions that sequentially or in parallel activate molecules during and after surgery. Since ALI/ARDS is the most severe form of pulmonary dysfunction, studies have focused on identifying markers that could be used for diagnostic purposes and for the prognostication of patients' outcomes.(56) Inflammatory markers (IL-6, IL-8, TNFr-1 and -2, urine nitric oxide NO), epithelial cell markers (receptor for advanced glycosylation end-products RAGE, surfactant proteins), adhesion molecules (intercellular adhesion molecule ICAM-1), markers of endothelial injury (von Willebrand factor antigen), extracellular matrix (desmosine), and coagulation proteins (protein C, plasminogen activator inhibitor PAI-1) were all associated independently with mortality in diagnosed ALI/ARDS patients.(52, 57-62) Procollagen peptide III has been shown to be correlated with patients' outcomes

(62). A few of these markers (SP-D, IL-6, IL-8, protein C, RAGE, and urine NO) had shown modest association with the effect of ventilator treatment, however, they were too weak to be used in monitoring the effects of treatment.(63) Fremont and co-workers (64) have found that by combining seven of the known inflammatory markers (brain natriuretic peptide, RAGE, procollagen peptide III, angiopoietin Ang-2, IL-8, IL-10, and TNF-α) trauma-induced ALI patients could be differentiated from non-ALI patients.

While these markers were found in the general patient population of ALI/ARDS, only soluble sRAGE has shown predictive value in patients undergoing cardiac surgery with the use of CPB (65, 66). Plasma sRAGE was found to be predictive for pulmonary dysfunction immediately after end surgery in young children (65). In an adult population, increased plasma concentrations of sRAGE were found to associate with lower PaO_2/FiO_2 values, longer mechanical ventilation, and longer ICU stay.(66) More recently, small molecules such as fatty acids were also found to be predictive of postoperative pulmonary dysfunction. Sheng and co-workers (17) have reported that increased levels of free fatty acids in blood collected 2 hours after end CABG could predict patients developing hypoxaemia ($r = -0.367$, $p < 0.001$). In addition, they found a positive correlation between free fatty acid and the endothelial activator marker ICAM-1, indicating their possible role as early biomarkers of lung injury.(17)

These biomarkers are crucial for the early identification of patients who are likely to develop lung injury. Recognizing the dysfunction before finding that the patient cannot ambulate due to symptomatic shortness of breath or hypoxaemia would lower mortality rate among patients, and would improve patients' outcomes postoperatively.(6) While these studies are crucial for the early prognostication of at-risk patients, there is still need for more research-based knowledge, to gain a deeper understanding of the molecular reasons for the development of postoperative pulmonary dysfunction.

CHAPTER 2. AIMS OF STUDY

2.1. PREVENTING PULMONARY DYSFUNCTION

Preventing postoperative pulmonary dysfunction is a desirable goal since it can cause increased morbidity and mortality, and its treatments are expensive. Because some degree of postoperative hypoxaemia is expected, the question of which patients will develop pulmonary dysfunction is difficult to answer (6, 8).

To make progress, we need to improve the current knowledge of the molecular mechanisms involved in the development of pulmonary dysfunction. We propose that metabolites may contain valuable information that will help us to answer this question. Since metabolites are the end products of transcripts and protein regulation, they change rapidly upon perturbations such as anaesthesia, sternotomy, the use of CPB, and medication. Therefore, mapping the metabolic changes during and in the early postoperative period could offer new insights into the chemical processes involved in disease progression.

Nuclear magnetic resonance (NMR) spectroscopy has previously been applied to measure the levels of tens to hundreds different metabolites in a biological sample. Since the technique has shown applicability in biomarker discovery (67-72), we hypothesized that NMR could be used to identify possible mechanisms involved in the development of postoperative hypoxaemia.

2.1.1. STUDY DESIGN AND RESEARCH QUESTIONS

This study was designed to address the effect of the surgical procedure on the human metabolome, and to address the metabolic interactions and their changes over time in relation to the progression into hypoxaemia. Thus, this study attempted to build models that would predict outcomes and to answer the following research questions:

1. How does the surgical procedure affect the levels of metabolites?

2. Can metabolites be used to detect early signs of postoperative hypoxaemia?

3. Which mechanisms are involved in disease progression?

2.1.2. POSSIBLE OUTCOMES

By answering these questions, we aim to gain a better understanding of the mechanisms involved in the progression of hypoxaemia. Such knowledge may be valuable to the clinicians to diagnose the dysfunction earlier in its course, may improve morbidity, may lower the mortality among patients, and may improve treatment options.

2.1.3. METHODOLOGY

To answer these questions, fifty patients who underwent CABG with the use of CPB at Aalborg University Hospital were recruited. Thirty two patients showed moderate to severe signs of hypoxaemia ($PaO_2/FiO_2 < 40$ kPa), whilst eighteen showed no sign of hypoxaemia ($PaO_2/FiO_2 \geq 40$ kPa) on the third day postoperatively. Their blood samples were collected at ten different time points before, during, and after end surgery. Nearly 850 blood samples were prepared and more than 1600 spectra were analysed by NMR and multivariate data analysis.

CHAPTER 3. METABONOMICS

As with most conditions, outcomes are influenced by genetic makeup, environmental factors, and treatment options. While several studies have been conducted to detect the risk factors and to identify the affected genes and proteins that may trigger the progression into postoperative pulmonary dysfunction, little is known about the metabolic states in these patients.

3.1. METABOLITES

Metabolites are the endpoint of genes, transcripts, and protein regulation and comprise all the low-molecular weight molecules (<2000 Daltons) present in a cell, tissue, or organism (73). Since metabolites respond to environmental perturbations (e.g. disease progression, toxic exposure, drug treatment, dietary change, and genetic manipulation (73-75)) long before the upstream processes, they carry valuable information regarding what is happening in the biological system (76).

The precise number of metabolites is still unknown, however, over 40,000 metabolites were annotated in 2013 in The Human Metabolome Database (HMDB v.3.0), and the number is expected to grow (73). There are two types of metabolites: primary and secondary. Primary metabolites are the building blocks of DNA, mRNA, and proteins. They are involved in regulatory processes and synthesis of cellular energy, and are thus considered essential for life. Examples of such metabolites are amino acids, carbohydrates, ketones, alcohols, glycoproteins, sugars, lipids, vitamins, antioxidants, organic acids, and nucleotides. Secondary metabolites are important in the defence system. Examples of secondary metabolites are pigments, sterols, toxins, drugs, and chemicals that humans ingest or come into contact with.(73, 77)

Metabolites are very heterogeneous, encompassing both volatile and non-volatile compounds. Their concentrations range from femto- to millimol, and are highly dependent on the physiological state of the biological system, i.e. the environmental perturbations, and the activity of all the enzymes involved in the synthesis and conversion of one specific metabolite. (78) Metabolites are highly interconnected, as one metabolite can participate in only one, or multiple, different reactions. This ensures a highly complex metabolic network of tightly regulated reactions.(79) Even small perturbations in the genome, transcriptome, or proteome can create significant changes in the levels of many metabolites.(76, 79)

Due to this interconnectivity and their prompt response to perturbation, metabolites are considered to be the cellular fingerprints of ongoing processes in our biological system (80), and thus, they might be the best molecules to explain disease mechanisms (75). This makes metabolite analysis attractive for medical purposes,

where the goal is to understand diseases, identify biomarkers, evaluate responses to therapies, and identify potential drug targets (79, 81).

Several studies have demonstrated metabolites' reproducibility and predictive powers in complex diseases. Examples of metabolites routinely used as biomarkers in the clinic include: creatinine to detect kidney disease; glucose to diagnose diabetes; cholesterol and triglycerides to determine the risk of CAD; and thyroxine hormone to detect hypo- and hyperthyroidism.

3.1.1. THE HUMAN METABOLOME

The complete collection of all metabolites in the body represents the human **metabolome**. In comparison, the complete set of genes inside a cell defines the human genome, the RNAs produced by the genome represent the transcriptome, and the set of all proteins in the system defines the proteome. Together, they define who and what we are (Figure 3-1).(73, 76)

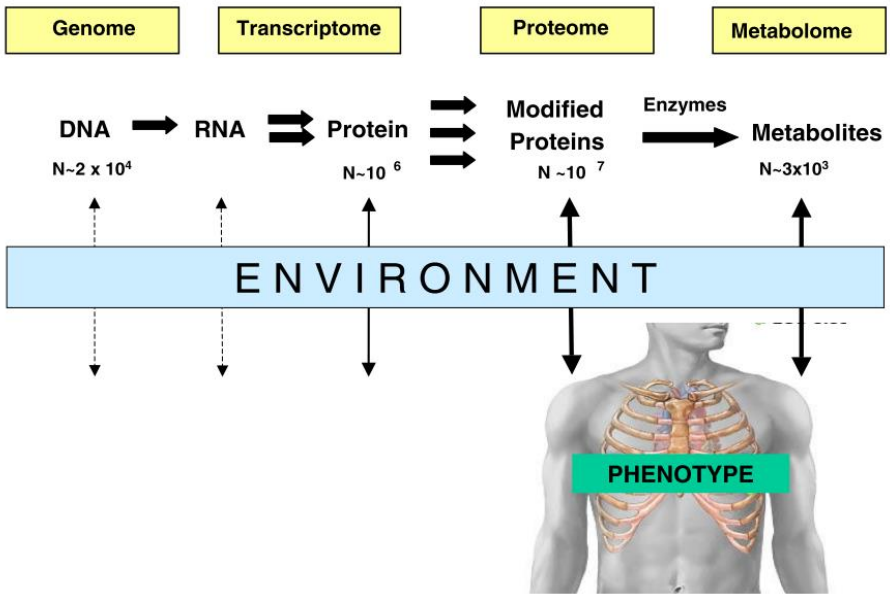


Figure 3-1 Interaction of the metabolome with genome, transcriptome, proteome, and their relationship to the phenotype. Each '–ome', with the exception of the genome, is a complex function of the other -omes, and the amount of integration increases from the genome to the metabolome. In addition, the environmental impact on each –ome increases from the genome to the metabolome (after Lewis et al., 2008 (82)*). Of note, the number of identified metabolites has increased since the publication of this figure (in 2008) by over 1300% (73) (in 2013).*

While the genome can be described by 4-letter codes for genes, and the proteome by 20-letter codes for proteins, the metabolome itself is more diffuse and not easily defined (75). Also, the metabolome is not solely dictated by genes, proteins, and the environment (what we eat, breathe, drink), but also by the microflora that live inside our body. Hence, the contribution of all these compounds together with their heterogeneity makes the characterization of the metabolome challenging.(73)

3.2. METABOLOME ANALYSIS

The approach that quantifies metabolic responses to various biological and/or environmental perturbations is called '**metabo*n*omics**' (83, 84), while the approach that aims at measuring all metabolites under homeostatic conditions is termed "**metabo*l*omics**" (85, 86).

Although the terms are now often used interchangeably, they have slightly different aims and historical origins. While metabo***n***omics was introduced by Prof. Jeremy Nicholson in 1999 (83) and had its foundations in toxicology studies of mammalian systems, metabo***l***omics was launched a year later by Prof. Oliver Fiehn (85) and had its origin in plant and microbial sciences. Metabo***n***omics aims to find insights into the actual phenotype of a disease and the causes of its progression, while metabo***l***omics aims to find the common metabolic phenotypes of biological systems at the time of sample collection.(76) In the last two decades both approaches have evolved and expanded, and nowadays, they are inseparable parts of the integrated systems biology strategies, together with genomics, transcriptomics and proteomics.

Metabonomics has gain significant attention in many areas of medical research (87). Especially, the approach has great potential for intensive care medicine, where diseases are complex, and where understanding the relationship between host factors, disease, and effect of treatments is highly desirable (88). Some of the clinical applications of the approach have been in characterizing the metabolome of patients diagnosed with ALI/ARDS (67, 69, 89, 90), cardio-vascular disease (91), type-2 diabetes (92), liver disease (93, 94), preeclampsia during pregnancy (95), cancer (68, 70, 72, 96), and neurodegenerative disorder (71, 97-99).

3.2.1. ANALYTICAL TECHNIQUES

Metabolomics/metabonomics analysis is highly dependent on the analytical technology used. Due to the large number of metabolites and their heterogeneous nature, there is no single analytical technique that can capture the entire metabolome's complexity. There are, however, several techniques that are applicable for the purpose of characterizing part of the metabolome, as they can measure tens to thousands of metabolites in a sample. Such techniques include nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) coupled to different separation techniques, Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy, and electrochemical array, among others. Each technique has a different selectivity, sensitivity, speed, need for sample pre-treatment, and degree of

difficulty in data pre-processing. Since FTIR, Raman and electrochemical array cannot offer detailed metabolic identification, they are less used in metabolomics/ metabonomics research. On the other hand, NMR and MS offer the capacity to both identify and quantify metabolites, and are thus frequently used for metabolome characterization.(78)

Both NMR and MS offer platforms that obtain rich information from a broad range of biological samples (e.g. serum, urine, saliva, bronchoalveolar lavage fluid BALF, exhaled air, cerebrospinal fluid CSF, tissue) (88).

NMR uses magnetic properties of nuclei to determine the structure and concentration of a metabolite. For metabolome analysis proton NMR (^{1}H-NMR) is typically used. The technique involves the use of a large and powerful magnet (400-1000 MHz) to obtain metabolic information. MS is a technique that identifies metabolites based on the detection of the mass/charge ratio (m/z) of ions produced by ionization of chemical compounds. Molecules in a sample are vaporized before being ionized. Thus, molecules are broken into charged fragment and these can then be sorted and detected based on their m/z. To improve ion separation, MS can be coupled to chromatographic techniques. Typically the separation is performed using gas chromatography (GC), liquid chromatography (LC), high-performance LC (HPLC), ultra-HPLC or capillary electrophoresis.(100) During GC, molecules are separated in gaseous phase before they are sent to the ion source; while during LC molecules are separated in a liquid phase by the chromatogram prior to ionization.(82, 87, 88) A comparison between the advantages and disadvantages of NMR and MS is given in Table 3-1.

With the exception of NMR, most techniques, including MS, need sample preparation prior to analysis. NMR is unique in being suitable for measuring complex samples (e.g. blood, urine, tissue) with little or no prior preparation (101). The technique offers the possibility of measuring a wide range of metabolites arising from very different parts of the metabolic network (e.g. amino acids, lipids, carbohydrates, glycoproteins, ketones, alcohols, etc.) (102). Since most of the central nodes within the metabolic networks are 'NMR visible', and since these nodes are highly interconnected (103, 104), the technique offers the possibility of indirectly detecting a large number of metabolic perturbations (102, 105). The biggest disadvantage of NMR is its poor sensitivity (>1μM (78)) compared to MS (106).

Unlike NMR, sample preparation for MS requires sample separation (e.g. extraction or derivatization), which can introduce laboratory biases. Since MS is usually placed after a chromatography step, some metabolites will not be able to reach the detector and therefore further biases can be introduced in the analysis. However, MS is much more sensitive than NMR, being able to detect very low metabolic concentrations (78).

Table 3-1: A comparison of the NMR and MS techniques used for metabolomics/ metabonomics studies (after ref.(100, 102-107)). Abbreviations: GC, gas chromatography; LC, liquid chromatography; NMR, nuclear magnetic resonance.

Technology	***Advantages***	***Disadvantages***
NMR	Quantitative Good reproducibility Non-destructive No separation needed Fast (2-3 min/sample) Cheap on a per-sample basis Measures liquids and solids Allows identification of new metabolites	Not very sensitive Expensive instrumentation Requires larger sample volume (> 0.3 mL)
GC-MS	Quantitative Good reproducibility Good sensitivity Relatively inexpensive Modest sample size needed	Sample not recoverable Extensive sample preparation Slow (20-30 min/sample) Difficult metabolite identification Only applicable for vaporizable metabolites
LC-MS	High sensitivity Minimal sample volume Potential to detect the largest portion of the metabolome	Sample not recoverable Less reproducible Expensive instrumentation Slow (20-30 min/sample) Difficult metabolite identification

3.2.2. APPROACHES FOR DATA ANALYSIS

Regardless of the technology used, metabolomics/metabonomics data comes as a collection of unassigned peaks with varying intensities and information about chemical shifts (for NMR data), the retention times (for LC or GC data), and masses or mass fragments (for MS data). These peaks must be subjected to different approaches I order to interpret the biological information.

There are two types of approaches: targeted and untargeted. The targeted approach focuses on analysing one or several selected metabolites related to a specific pathway, while the untargeted approach measures all detectable compounds in a sample. The targeted approach does not intend to detect novel metabolites in a sample, and it is thus mainly used to test specific hypotheses. The untargeted approach aims to detect novel biological information and is often used in hypothesis-generating studies.(87) The approach can be further divided into fingerprinting and profiling.

Metabolite fingerprinting is an untargeted, high throughput, global analysis of samples which provides a global snapshot or 'fingerprint' of the metabolome without precisely quantifying or identifying the chemical compounds in the sample. This approach is based on 'chemometrics', a statistical comparison of spectral intensities with the aim of identifying spectral features or patterns that discriminate between samples (e.g. case/control). Metabolite fingerprinting is powerful, rapid, and is suitable as a screening tool for diseases. Metabolic profiling is more challenging than fingerprinting, since it is based on identification and quantification of a set of metabolites in a biological sample. This approach is also called 'quantitative metabolomics/metabonomics', and it can be either targeted or untargeted.

Both approaches have advantages and disadvantages, and their choice depends on the problem to be solved. Although fingerprinting is applied to a wider range of metabolites, a greater understanding of the underlying mechanisms of action can be achieved when combining fingerprinting with profiling. While fingerprinting can be used to investigate different metabolic phenotypes (e.g. healthy/disease states), metabolic profiling can then be used to provide detailed information about the specific differences between the two phenotypes. In this way, the metabolic signature differentiating the experimental groups can be detected, and the metabolites causing this differentiation can be identified and quantified. (75, 78) A combination of both approaches has been chosen for this PhD study. A collection of some terminologies are presented in Table 3-2.

Table 3-2*: Definitions of terms used in metabolomics/metabonomics studies. From ref. (79, 81, 106, 108).*

Terms	**Definitions**
Metabolome	The complete set of all primary and secondary metabolites
Metabonomics	Quantitative analysis of metabolite changes in response to biological and environmental perturbations
Metabolomics	Identification and quantification of metabolites in a biological system under homeostatic conditions
Metabolic profiling	Identification and quantification of a group of predefined metabolites (e.g. carbohydrates)
Metabolic fingerprinting	A global analysis of samples that provides a snapshot or 'fingerprint' of the metabolome without precisely quantifying or identifying the metabolites in the sample
Metabolic footprinting	Analysis of the metabolites secreted or excreted by an organism (i.e. bacteria) in a growth medium or extracellular fluid.
Metabolic phenotypes (metabotypes)	Represent deviations from the optimal condition (i.e. homeostasis, steady state) and can be used as indicators of an individual's health state.

Fluxomics (metabolite flux analysis)	Analysis of labelled metabolites by their conversion through metabolic pathways.
Lipidomics	Analysis of lipids and their interactions in a biological system.

3.2.3. METABONOMICS IN ACUTE LUNG DISEASES

Metabonomics work relevant to lung diseases is in infancy. Only a small amount of work has been carried out focusing on pneumonia and ALI/ARDS (87, 88), and no study has been conducted on patients at risk of developing lung injury after CPB.

3.2.3.1 Pneumonia

Pneumonia is a common acute lung inflammatory disease affecting millions of people every year (87). The disease is the leading cause of death among young children globally (109). Since pneumonia is caused by different microbial organisms (e.g. bacteria, viruses, fungi, and parasites) it can be difficult to identify the pathogen(s) responsible for the disease. Hence, it often leads to delays in appropriate antimicrobial therapies. Metabonomics could possibly help to accurately identify the cause, and to monitor disease progression or regression.(110)

NMR-based metabonomics analyses performed on urine samples from human (110) and mice (111) with pneumonia have identified distinct metabolic profiles based on bacterial strains or viral infections. Patients with *Streptococcus* pneumonia could be differentiated from controls through metabolites such as glucose, lactate, citrate, succinate, acetate, acetone, and several amino acids (110). Serial collection of urine samples over time showed how the metabolic profiles changed upon resolution from the infection (111). A study using the MS approach on plasma and urine collected from children with pneumonia (109) has supported the concept of applying metabonomics analysis to diagnose community-acquired pneumonia. Although the study was small (only 11 children with pneumonia), they found similar results as the previous study.

3.2.3.2 ALI/ARDS

ALI/ARDS is an acute lung injury syndrome that can be triggered by different factors (e.g. cardiac surgery (8, 37-39), pneumonia, sepsis, and severe trauma (87)). Patients with ARDS are critically ill, requiring mechanical ventilation for several days. The mortality rate among patients is highly dependent on the triggering factor, and sepsis-induced ARDS is considered the leading cause of death. Despite the disease' severity, knowledge of the pathogenesis of ARDS is incomplete, and to date there are no predictive biomarkers and no preventive pharmacotherapy.

Only few metabonomics studies have been performed to elucidate the metabolic phenotypes within ARDS, and currently, no study has found predictive biomarker for the development of ARDS.(87)

In a metabonomics study performed on rats with ventilation-induced lung injury (VILI), a research group has found shifts in the energy metabolism (glucose, lactate) and a possible disruption of the cellular membrane integrity (glycine, phospholipids) (112). In a new study performed on serum from a rat model, Naz and co-workers (113) identified 18 metabolites to associate with VILI. In a study performed on male rats with lipopolysaccharide (LPS)-induced ARDS, Bos and colleagues (114) concluded that several exhaled volatile metabolites were predictive of the early development of lung injury (1 hour after the LPS exposure). Serkova and collegues (90) have investigated the metabolome of cytokine-induced ALI in a mouse model, and they found disturbances in the energy balance, decreases in the energy state (ATP), and increases in the glycolytic activity (lactate, glucose) in sick compared to control mice.

The number of clinical studies on human models of ARDS is increasing. As early as 1998, Schubert and colleagues (115) showed the utility of exhaled breath sample for GC-MS based metabonomics analysis in mechanically ventilated ARDS patients. They have found a more than 50% decrease in isoprene from exhaled breath of mechanically ventilated patients with ARDS compared to mechanically ventilated patients without ARDS. The utility of exhaled breath samples for ARDS metabonomics studies have been confirmed by Bos and colleagues (116), however, they could not confirm the results from the previous study. While the authors found three volatile compounds to be discriminative between ARDS and control subjects, they did not find any difference in isoprene levels. Recent data suggests that some metabolic levels vary greatly depending on the breath sampling method and the use of anaesthesia (117), thus, other, more stable, samples have been collected to achieve information of the local lung environment. BALF samples obtained through flashing the airways by a saline liquid were previously analysed. The metabolic profiles of sepsis-induced ARDS patients were compared to that of healthy control subjects, and nearly 50 metabolites were found to different between groups (118). Lastly, a small metabonomics study was also performed on plasma samples. Stringer and co-workers (119) compared plasma metabolic profiles of sepsis-induced ALI patients with healthy individuals, and found differences in the levels of glutathione, adenosine, myo-inositol, phosphatidylserine, and sphingomyelin.

Taken together, these experimental and clinical studies indicate some perturbations in the levels of energy and oxidative stress metabolites, and show the potential of metabonomics to find some of the mechanisms involved in acute lung diseases.(119)

CHAPTER 4. METHODOLOGY, MATERIALS, AND METHODS

Detailed descriptions of materials and methods are included in each paper. A summary and additional theory is provided below.

4.1. METHODOLOGY

The studies for this PhD thesis evolved as a part of continuing efforts to find predictive biomarkers for the progression to postoperative pulmonary dysfunction. This thesis is part of a study (registered at clinicaltrials.gov, NCT02475694) involving the characterisation of inflammatory mediators, coagulation factors, metabolites, and oxygen patterns in patients that have undergone cardiac surgery between September 2008 and March 2009 at Aalborg University Hospital in Denmark.

4.1.1. STUDY POPULATION

After ethical approval, a total of fifty consecutive patients scheduled for elective CABG with use of CPB were included in the study (Table 4-1). Inclusion criteria were adults above 18 years of age, on treatment with statins. Exclusion criteria were treatment with steroids or other immune suppressor therapies.

Table 4-1 Patient characteristics. Annotations: n, number of subjects; SD, standard deviation; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CPB, cardiopulmonary bypass; PaO_2, partial pressure of oxygen in arterial blood; FiO_2, fraction of inspired oxygen (21%); Surgery, from skin incision to last suture.

	All (n= 50)	**Hypoxaemia (n= 32)**	**Unaffected (n= 18)**	**p-value**
Subject characteristics				
Age, mean ± SD, years	65.8 ± 9.7	65.3 (48-83)	66.5 (48-81)	-
Men, n (%)	41 (82)	23 (72)	18 (100)	0.01
BMI, mean ± SD, kg/m^2	27.5 ± 0.2	27.2 ± 0.2	28.0 ± 0.3	-
Diabetes mellitus, n (%)	14 (28)	10 (31)	4 (22)	-
Smokers(yes/no/ unknown)	16/30/4	11/19/2	5/11/2	-
COPD, n (%)	7 (14)	4 (13)	3 (17)	-
Surgical procedure, mean ± SD, min				
Surgery	196 ± 50	207 ± 52	177 ± 38	0.04
Time on CPB	64 ± 29	67 ± 33	57 ± 21	0.23
Cross clamp (Ischemia)	33 ± 19	37 ± 21	25 ± 11	0.03
72 hours post-CPB, mean (range), kPa				
PaO_2	7.8 (10.7-4.9)	6.9 (8.4-4.9)	9.2 (10.7-8.6)	<0.0001
PaO_2/FiO_2	37.0 (51- 23.3)	33.1 (40-23.3)	43.9 (51-41)	<0.0001

4.1.2. OUTCOMES

After cardiac surgery, patients were transferred to the thoracic intensive care unit, under sedation, and mechanically ventilated. Patients were weaned from mechanical ventilation within the first 24 hours. The majority were discharged to the surgical ward on the first postoperative day, while four patients were discharged on the second postoperative day. Physiotherapy and supplementary oxygen were given to all patients on the surgical ward as standard care.

A decrease in PaO_2 was observed in all patients the three days following surgery. On the second and third day, several patients experienced hypoxaemia (48% and 64%, respectively). Hypoxaemia was defined by a PaO_2/FiO_2 below 40kPa or a PaO_2 below 8.4kPa. Because oxygenation worsened in some patients whiles in others improved within the second and third day (r_p= 0.49, p= 0.0003), we defined patients' outcome based on the third days PaO_2 values. A total of thirty-two patients developed hypoxaemia, of which nine had severe hypoxaemia (PaO_2/FIO_2< 30kPa; PaO_2< 6.3kPa), while eighteen showed no sign of hypoxaemia (PaO_2/FIO_2> 40kPa). In addition, the severe hypoxaemic group had more bilateral infiltrates on the chest images.

4.1.3. CHRONOLOGY OF THE STUDIES

Three patients were unable to provide samples at 16, 20, 48, and 72 hours post-CPB. Hence, a total of 832 plasma and serum samples were collected for this metabonomics study; 144 plasma samples were obtained from arterial blood the day before surgery, and 48 and 72 hours postoperatively; 688 serum samples were obtained simultaneously from the catheters inserted in the pulmonary artery (PA) and the left atrium (LA) after induction of anaesthesia. The serum samples were collected at seven different time points: after sternotomy, but before CPB; immediately after weaning from CPB (0 hours); and at 2, 4, 8, 16, and 20 hours after CPB. The aim of their collection was to investigate possible differences between the systemic (PA) and systemic + pulmonary microvasculature (LA).

The overall hypothesis for this study was that *early stages of hypoxaemia may be reflected at the metabolite level*. Due to a lack of previous similar research, and due to the large number of samples to analyse, the first time point to analyse had to be chosen based upon clinical experience. The samples had to be relatively far from the surgical procedure, to avoid possible interference from anaesthesia and the surgical procedure, but they also had to be relatively far from the symptoms of hypoxaemia. Hence, we picked samples collected on the first postoperative morning (precisely 16 hours after weaning from CPB). Because the results were positive, the analysis resulted in one publication (Study I, Appendix A). Also, since a biomarker signature was identified that could predict progression to hypoxaemia, a patent application was filed (Study IV, Appendix D).

The remaining samples, collected from nine different time points, resulted in a manuscript (Study III, Appendix C). The results confirmed the previous findings and identified several deranged mechanisms.

Two additional studies were performed, aiming to *characterize the coagulant and inflammatory mechanisms* in these patients. The coagulation study resulted in a publication (Study II, Appendix B), while the inflammatory study is still ongoing.

4.2. NMR-BASED METABONOMICS SAMPLE PREPARATION

The main goal of NMR-based metabonomics studies is to identify metabolites that correlate to a specific disease. Since metabolites are prone to change even after small perturbations, the success of each study relies on consistency in sample collection and preparation. In the following section, some theoretical and practical aspects are presented concerning sample variation, NMR experiments, spectra processing, data analysis, and data interpretation.

4.2.1. SAMPLE VARIATION

Maintaining consistency in a sample set can be challenging, as several factors can affect the levels of metabolites. Studies have shown that pre-existing health conditions, diurnal variation, gender, age, diet, ethnicity, hormonal status, and exposure to stress factors affect the metabolome. Therefore, matching patients prior to the study is important (74, 120-122). This study was a retrospective study, and hence, selecting patients according to these recommendations was not possible. However, patients were relatively similar with regards to: their medical history (CAD), age, ethnicity (all Danish), body mass index, and the surgical procedure (Table 4-1). Also, since the surgical procedures were performed in the morning on fasting patients, both the variability arising from the diurnal effects and the diet were minimized.

Plasma and serum specimens were obtained according to the routine hospital procedures. During this procedure some metabolic variation is inevitable, since some samples may be kept for a longer time at room temperature than others (123). This variation was minimized by having the same person prepare the samples, and by being consistent with the time a sample was standing at room temperature. Unfortunately, we did not have serum samples for all the time points. Since there are some metabolite differences between serum and plasma, some information was difficult to combine or compare in Study III. Serum is known to contain higher levels of several amino acids, phosphatidylcholines, and triglycerides, while plasma contains higher levels of lysophosphatidyl-inositol.(123, 124)

Storing blood samples at -80^{o}C has been shown to be the optimal choice for metabonomics studies (101, 124). Spectra of fresh plasma samples were compared to spectra of plasma stored for 9-13 months at -80°C, and it was found that sample storage did not influence the metabolite levels. Even a prolong storage of plasma

samples (2-15 years at -20°C) indicated that most metabolites were stable.(125) For this study, blood samples were stored for 2-3 years at -80°C prior to the NMR analysis, and therefore, it is assumed that possible metabolic variation from sample storage may be insignificant.

4.2.2. SAMPLE PREPARATION

One of the major advantages of NMR as a tool for metabonomics studies is its minimal requirements for sample preparation (101). For the metabonomics studies, a deuterated buffer was added to the samples to provide the necessary "deuterium lock signal" for data acquisition.

4.3. DATA ACQUISITION BY NMR

NMR spectroscopy is a powerful analytical tool that is well suited for analysis of different types of sample, including plasma (119, 126, 127), serum (126, 128), BALF (129), cerebrospinal fluid (130), pus (131), saliva (132), faeces (133), urine (126) and tissue (126).

NMR was discovered in 1946 by two research groups (Bloch and Purcell) which, independently from each other, observed nuclear magnetic resonance signals for the first time. Their discovery was the subject of the Nobel Prize for Physics in 1952.(134) After the discovery of the chemical shift (i.e. the observation that the resonance frequency of a nucleus is affected by its chemical environment) in 1950, the technique quickly became significant as an analytical tool in chemistry. The first biological applications began in 1954, when a group at Stanford used proton (^{1}H) NMR spectroscopy to show that DNA strands have large hydration shells (135). Three years later, biomolecular NMR had advanced to the stage where the first intact ribonuclease protein was examined (136). Since then, the field of NMR has evolved rapidly; from the first commercialized 60 MHz magnet capable of detecting only ^{1}Hs, to today's systems equipped with superconducting magnets operating at as high as 1000 MHz.(124) As a result of its continuously increasing application base, two more Nobel Prizes were awarded: one for the methodological development of high resolution NMR spectroscopy (R.R. Ernst in 1991); and the other for the development of NMR spectroscopy for determining the three-dimensional structure of biological macromolecules in solution (Kurt Wüthrich in 2002).(134) Also, a shared Nobel Prize (Paul C. Lauterbur and Peter Mansfield in 2003) was given in the field of Medicine for the development of magnetic resonance imaging (MRI), derived from the NMR spectroscopy principles. Since then, MRI has become an invaluable diagnostic method in medicine.(137) Today, NMR has become one of the most powerful and flexible analytical techniques available in the field of chemistry, biology and medicine, including metabonomics. It is also one of the few methods that gives analytical information from deep within living cells, tissues, and biofluids with minimal or no sample preparation.(138)

4.3.1. THE PRINCIPLES OF AN NMR EXPERIMENT

^{1}H NMR is a spectroscopic method based on the principle that protons resonate in a high magnetic field. The techniques uses radiofrequency (RF) waves to reveal information about magnetic nuclei in a compound. The information received consists of structural data and quantification data of molecules in a sample. (138)

Running an NMR experiment is straightforward. Once the sample is prepared, it is placed in an NMR glass tube and put into an electromagnet, whose large coil generates a strong magnetic field (B_0). Inside the magnet, another smaller electromagnetic coil exists, located at the end of a cylindrical probe, which sustains the sample. A high power, short duration, RF pulse produced by the probe coil irradiates the sample and the data acquisition process begins. The RF waves (or the magnetic field B_1) have electrical and magnetic (electromagnetic) components that can flip nuclear magnetic moments from one spin state (+½) to another (-½). The more power applied to the RF waves, the more nuclei are flipped (a process called excitation). When the irradiating RF waves are turned off, the nuclei relax back to their original equilibrium state. During relaxation, they produce RF waves that provide information about the molecule(s) inside the sample. These RF waves are than collected as Free Induction Decays (FIDs) in the time domain, and are further Fourier transformed into the frequency domain. Here the signals are translated into peaks, displayed across a spectrum with units of parts per million (ppm) to distinguish their positions (i.e. chemical shift). The chemical shifts of these peaks are affected by the neighbouring chemical groups (nitrogen, oxygen, carbonyls, double bonds, halogens, etc.). Depending on the proximal electronegative group, each proton has its own unique position in the spectrum. Each proton resonance is further split by the interaction with protons on neighbouring NMR active nuclei. The area under the peak is directly proportional to the concentration of each metabolite, and hence, besides structural information, information about metabolite concentration is also obtained.(87, 138)

4.3.1.1 One-dimensional experiments

The bedrock of NMR-based metabonomics studies rely on one-dimensional (1D) experiments. These experiments are relatively fast, robust, and easy to implement, providing information about the concentration, the structure, and the position (chemical shift) of a metabolite in a spectrum (139). One-dimensional NMR spectra provide a global snapshot, or fingerprint, of the metabolome state, and are thus, typically used to evaluate metabolite alterations between groups or classes.(140)

In most cases, samples are prepared in the solution state, which allows metabolites to rotate freely. Due to serum and plasma containing high water concentrations (>50 M) compared to the rest of the molecules (µM to mM) (141), the water signal must be suppressed. This is done by irradiating the water resonance, so that the water proton signal vanishes (a process called saturation). Once the water is saturated, other signals are more visible.

Serum and plasma spectra contain both small and large metabolites. Depending on their size, metabolites tumble in solution at different speeds. This influences their relaxation rates and in further consequences, the width of their NMR resonances. Larger metabolites such as proteins, lipoproteins, and fatty acids, have a reduced rotational diffusion, and thus, they tumble more slowly, resulting in faster transverse relaxation and broader signals. On the other hand, smaller metabolites rotate faster and produce sharper and narrower signals. This information can be used to digitally 'filter out' signals of larger molecules from the spectra.(142) The most commonly used pulse sequences in blood metabonomics studies are the spin-echo Carr Purcell Meiboom and Gill (CPMG) (143) and the 'diffusion-edited'(144) methods.

Small metabolites have relatively long relaxation times compared to larger metabolites. Thus, the differences in the relaxation times can be used to manipulate the information recorded by NMR. Using a short spin echo delay in the standard 1D pulse sequence, prior to data acquisition, lets the peak intensities decay proportionally to their relaxation times. Hence, this property contributes to the attenuation of signals from large molecules. Consequently, the spectrum will contain fewer baseline distortions from larger molecules. On the other hand, when larger molecules are of interest, a "diffusion filter" can be applied to eliminate the fast-diffusing molecules.

Due to signal loss during relaxation delay, the sequences cannot be used for absolute quantification, but only for relative metabolite quantification. Figure 4-1 shows how the small metabolites are masked by the large molecule background in a standard 1D ^{1}H pulse sequence. The small metabolites are visible in the CPMG spectrum, while the large molecules are visible in the diffusion-edited spectrum. In this thesis, both CPMG and diffusion-edited pulse sequences have been applied.

Even after the application of these filters, the 1D NMR spectra can still be crowded with extensive peak overlap. This can make it difficult to differentiate between multiple peaks and to assign the metabolites. Most metabolites contain several hydrogen atoms that resonate at different chemical shifts, and since they often overlap with other signals, it complicates the assignment process. To simplify spectral interpretation a common approach is to spread the overlapping resonances into a second dimension (2D).(145)

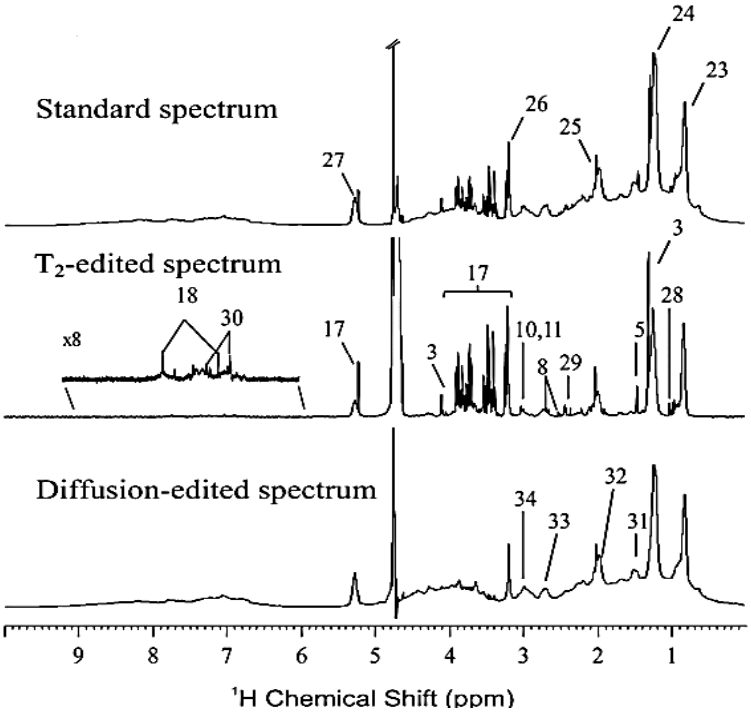


Figure 4-1 ^{1}H NMR plasma spectra (500 MHz): standard 1D (top), CPMG (T_2-edited) (middle), and diffusion-edited (bottom). Legend: 3, lactate; 5, alanine; 8, citrate; 10, creatine; 11, creatinine; 17, glucose; 18, histidine; 23, lipid CH_3; 24, lipid $(CH_2)_n$; 25, glycoproteins; 26, choline; 27, lipid CH=CH; 28, valine; 29, glutamine; 30, tyrosine; 31, lipid CH_2CH_2CO; 32, lipid $CH_2CH=CH$; 33, lipid $CH=CHCH_2CH=CH$; 34, albumin lysyl CH_2. After Diaz Sílvia et al. 2011, (146).

4.3.1.2 Two-dimensional experiments

Two-dimensional homonuclear ^{1}H-^{1}H-TOCSY (TOtal Correlation SpectroscopY) and heteronuclear ^{1}H-^{13}C HSQC (Heteronuclear Single-Quantum Correlation) are commonly used 2D NMR experiments in metabonomics studies. These approaches are mostly used for metabolite identification, since their acquisition time is often long (e.g. 24 hours). TOCSY correlates the entire network of coupled protons in a metabolite and produces cross peaks between each pair of protons. HSQC correlates the ^{1}H and ^{13}C shifts for each C-H pair in a molecule. (145, 147)

4.3.1.3 Metabolite identification

Metabolite assignment is typically done with the help of different databases (e.g. The Human Metabolome Database (HMDB) (148), The Madison Metabolomics Consortium Database (149), and the BioMagResBank(150)). These databases match the experimental chemical shifts (e.g. ^{1}H and ^{13}C) against those in the spectral

database. For this study a list of NMR chemical shifts found in TOCSY and HSQC spectra were uploaded to HMDB (http://www.hmdb.ca/). The database matched the shifts and provided a list of potential metabolites, with the number of matching resonances. Since the confidence level of correct metabolite assignment depends on the number of matching resonances (151), few metabolites with resonances in overlapping regions were difficult to distinguish, and hence, they are annotated as 'unknown' metabolites.

4.4. DATA PROCESSING

Two types of processing exist for NMR spectra; a pre-processing step and a post-processing step.

4.4.1. SPECTRA PRE-PROCESSING

The pre-processing step includes: adding digital data points to the FIDs to enhance the resolution (a process called zero-filling); Fourier transformation; correcting the phase to produce pure absorption line shapes and to remove offsets in the peak shapes; correcting the baseline; and calibrating the spectra to a reference peak. In this thesis, the CPMG spectra were calibrated to the methyl peak of lactate (1.33 ppm) or alanine (1.48 ppm), while the diffusion-edited spectra were calibrated to the methyl signal of N-Acetylglycosamine (2.04 ppm).

4.4.2. SPECTRA POST-PROCESSING

The post-processing step consists of: further spectra alignment (if needed); binning; normalization; exclusion of spectral regions with non-reproducible information; centring; and scaling (if necessary).

4.4.2.1 Spectra alignment

Even after the addition of deuterated buffer, small pH variations may occur within the sample set. Also, random phasing, peak shape, and small baseline distortions may lead to minor differences in the chemical shifts, and to inappropriate peaks alignment. In addition, small changes in the temperature, differences in the salt concentration, and dilution can also cause peaks shifts.(152) These are usually corrected by the calibration and binning (or dividing the spectra into small intervals) procedures. However, if some regions are still not properly aligned, specific alignment algorithms can be applied. In this thesis, after dividing the spectra into equal intervals of 0.001 ppm, some peaks were aligned by using the *I*coshift (153) algorithm implemented in Matlab.

4.4.2.2 Normalization

Normalization is a significant post-processing step that compensates for variations in the RF pulse calibration or dilution of samples. Total intensity normalization is the most commonly used technique, which scales each spectrum to a constant total intensity. During the procedure, the value of each individual bin is divided by the sum of all the bins in the spectrum.(154) In this study, CPMG spectra were normalized either to the concentration of glucose measured by blood gas analyser, the DSA-d_6 peak (4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate), or to the total intensity, while diffusion-edited spectra were normalized to the total intensity.

4.4.2.3 Centring and scaling

Following normalization, data is normally centred. Centring focuses the analysis on the fluctuating part of the data. For example, the commonly used 'mean centring' approach converts the metabolic levels to fluctuations around zero instead of around the mean of each metabolite, by subtracting the mean value from each metabolite. The approach adjusts for differences in the offsets between high and low abundance metabolites, and leaves only the variation between samples in the analysis.

Scaling approaches aim to adjust for the differences between metabolite concentrations by dividing each variable by a unique scaling factor. This often minimizes the influence of large values and inflates the influence of small values. Small values from baseline noise can also be inflated, making the models more prone to overfitting. Therefore, choosing the right scaling is crucial for further analyses.

Auto-, pareto-, vast-, range-, and level- scaling are some of the most common methods used in metabonomics studies. Auto- scaling (or unit variance scaling) divides each variable by its standard deviation. Pareto-scaling divides each variable by the square root of its standard deviation, and is thus an intermediate between auto- and no-scaling (mean centring). Vast scaling (or variable stability scaling) divides each variable by its standard deviation and its coefficient of variation. Range scaling divides each variable by the difference between the minimal and the maximal concentration. Level scaling divides each variable by its average or median concentration.(155) In this thesis all these scaling methods were tried, however, only auto- and no-scaling gave the best results in terms of classification and error of prediction. For some models, data was additionally logarithmically transformed prior to scaling.

4.5. DATA ANALYSIS

After processing, the table of NMR integrals is subjected to different statistical tests. Since a spectrum of biofluids contains many dimensions (each dimension

corresponding to the intensities of individual metabolites), powerful statistical analyses are required to simplify the data (156).

The more simple univariate tests (e.g. Student's t -test, analysis of variance or ANOVA, Wilcoxon, Kruskal–Wallis) focus on finding a few individual metabolites that can separate the samples based on class labels (Figure 4-2a). Metabolites with insignificant discriminative power are removed, and hence, data dimensionality is reduced. The drawback of these tests is that only significant metabolites are taken into account, while combinations of statistically less significant metabolites are not assessed. Hence, valuable information can be lost.(157)

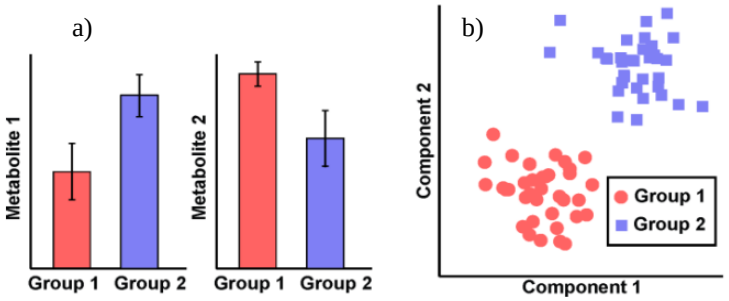


Figure 4-2 Approaches to analyse metabonomics data: A) Differences in the concentration level of single metabolites between two groups (e.g. t-test). B) Multivariate approaches like principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) model the relationships between metabolites and/or samples to detect group differences. Each data point represents one sample. Two samples that are close to each other on the plot are metabolically similar, while two samples that are far away from each other are metabolically different. After Bartel, Jörg et al., 2013 (158).

Multivariate analyses are more complex since they are not limited to a few metabolites, and aim to capture the overall metabolic information, including the correlation or covariance between metabolites. These analyses can identify individual metabolites that are insignificant on their own, but when in combination, can produce powerful discriminant models.(139, 157) Multivariate models are either unsupervised or supervised. Unsupervised methods are based on finding variations in the data without prior knowledge about the class labels, while supervised methods introduce knowledge about sample membership. Supervised methods are especially useful in finding metabolite combinations and biomarker signatures that are discriminative between e.g. case and control subjects. This feature is of particular interest in multifactorial diseases where several compounds are affected and where a single biomarker is not sufficient to detect the condition.(157) By far the most common multivariate methods in NMR-based metabonomics studies are: the unsupervised principal component analysis (PCA) (Figure 4-2b); the supervised partial least squares or projection to latent structures (PLS) regression; and the partial least squares discriminant analysis (PLS-DA) (139, 157, 158).

4.5.1. PRINCIPAL COMPONENT ANALYSIS

PCA attempts to explain the largest variation in the data set. Data is transformed into two matrices: a scores and a loadings (or variables) matrix. The scores matrix contains the positions of the samples in a new, rotated coordinate system, and can be visualized in a scores plot. The coordinate axes of a PCA scores plot are called principal components (PCs). These components represent the best-fit of each spectrum to a series of PCs. The first principal component (PC1) vector describes the largest variation in the data. The second principal component (PC2) is perpendicular to PC1, and describes the second largest variation in the data, and so on.(159) The PCA scores plot summarizes global similarities and differences between NMR spectra based on metabolite concentrations, with each spectrum being reduced to a single point. Also, PCA scores plot reveals sample outliers, trends and possible clusters (Figure 4-2b). The loadings matrix contains the weights for the original variables that are used to calculate samples' positions on the scores plot, and are visualized on a loadings plot.(158, 159) The loadings plot shows the relative contribution of each NMR bin (or metabolite) to the principal components, and hence, they are important in interpreting the data.(139)

4.5.2. PARTIAL LEAST SQUARE REGRESSION AND DISCRIMINANT ANALYSIS

When the largest variation in the data is not correlated to the classes, supervised methods are used.

PLS regression is a supervised linear mixed model that combines PCA with multiple linear regression analysis to predict a set of dependent variables, Y, from the data set, X (NMR spectra). The prediction is achieved by searching for a set of orthogonal components, or latent vectors (LVs), that perform a simultaneous decomposition of X and Y, to obtain components which explain the covariance between X and Y. The LVs obtained from X are then used to predict Y.(159, 160)

When Y is a categorical response (e.g. case-control, time points), the discriminant version of PLS is used. The goal of PLS-DA is to predict the class Y, from X, and to find variables that maximize class separation. By orthogonalizing the information in the scores plot on the first latent vector (LV1), the model can be more easily interpreted. This method is called orthogonal PLS-DA (OPLS-DA), and it highlights the between-class separation while reducing the within-class separation. The quality of the models is assessed by goodness of fit (R^2) and its validated fit (Q^2). A good model has $R^2 > 0.5$ and $Q^2 > 0.4$ (ranges between 0 and 1), and the results can be interpreted as the simple linear regression analysis. To overcome the risk of overfitting (e.g. incorporating noise into the statistical models, or finding random associations), the results must be validated. The validation process can be done by introducing a new set of samples to the model and trying to predict them, or by leaving a subset of samples out, calculating the model, and then, predicting the left out samples. The latter method is called cross-validation (CV).(139)

In this PhD study, PCA, PLS, OPLS, PLS-DA, and OPLS-DA were used. PCA was used to identify trends in the data, and to identify possible outliers. Since the diurnal variation was larger than the metabolite variation corresponding to the progression to hypoxaemia, PCA was only used to assess clusters corresponding to the time of sample collection. For the PLS (OPLS) modelling, the PaO_2/FiO_2 values obtained on the third day postoperatively were used to find possible metabolite associations. The PaO_2/FiO_2 was chosen since it is known to be an indicator of the severity of lung injury (118). For the PLS-DA (OPLS-DA), the Y vector was segmented into two or three classes of zeroes and ones (e.g. $PaO_2/FiO_2 \geq 40$ kPa vs. $PaO_2/FiO_2 < 40$ kPa for unaffected vs. hypoxaemic patients; $PaO_2/FiO_2 \geq 40$ kPa vs. $40 < PaO_2/FiO_2 < 30$ kPa vs. $PaO_2/FiO_2 < 30$ kPa for unaffected vs. mildly vs. severely hypoxaemic patients). To avoid overfitting, the Venetian-Blinds CV, the Monte-Carlo CV with sample resampling, and permutation testing were used. Additional information about these procedures is provided in the attached papers and patent application.

4.5.3. OTHER STATISTICAL ANALYSES

Analyses such as correlation analysis (used to determine metabolites associated with the PaO_2 values); stepwise multi-linear regression analysis (used to define the best combination of predictors of PaO_2); the parametric t– and analysis of variance (ANOVA) tests, and the non-parametric Wilcoxon and Kruskal–Wallis tests (used to quantify the differences between the groups); and the receiver operating characteristic (ROC) curve (used to describe the sensitivity and specificity of identified putative biomarkers) were also applied. ROC curves show the results of two-state cases (e.g. unaffected vs. hypoxaemia). The curve is constructed by plotting sensitivity versus 1-specificity of a hypothetical decision boundary that moves across the total range of the predictive score (0 and 1 corresponding to the unaffected and hypoxaemic patients, respectively). Sensitivity is defined as true positives/(true positives + false negative), while specificity is defined as the true negatives/(true negatives + false positives). True positives represent the number of sick patients correctly identified; true negatives - the number of unaffected patients correctly identified; false positives - the number of unaffected patients incorrectly identified as hypoxaemic patients; and false negatives - the number of sick patients incorrectly identified as unaffected. If the area under the ROC curve is 0.5 the models cannot discriminate between classes. On the other hand, if the area under the ROC curve is 1, there is complete separation between classes, and samples are classified with 100% sensitivity (no false negatives) and 100% specificity (no false positives).(161)

All data analysis was performed in the SPSS (IBM® SPSS® Statistics v.21-23) and Matlab R2011b software packages.

4.6. DATA VISUALIZATION

Due to the vast quantity of data and the complexity of the modelling methodologies, data visualization is an important step in interpreting metabonomics studies. In this

study, the NMR data was graphically compared by overlapping spectra, and by the scores and loadings plots achieved from multivariate analyses. The validation data achieved from (O)PLS-DA models were presented using the multivariate ROC curve, which has similar principles to the univariate ROC curve.

For the visualization of metabolites, several plots were used. When the number of metabolites represented was large, heat maps and correlation maps were utilized. When one, or few, metabolites were represented, scatter plots, histograms, box-plots, and ROC curves were applied. Finally, the interaction between metabolites was shown by visualizing metabolic reactions.

For metabolic pathway analysis, the Metaboanalyst (162, 163) tool (v. 2.0 and 3.0) was used to identify possible connections between metabolites. The list of identified metabolites was mapped into the tool and a corresponding table was produced, containing possible reactions for each of the individual metabolites. To produce the pathways diagram presented in Study I, additional information from the literature was needed (The Medical Biochemistry Page ©1996–2016 themedicalbiochemistry-page.org, and the Biochemical Pathways Atlas of Biochemistry and Molecular Biology (164)).

CHAPTER 5. RESULTS AND DISCUSSIONS

The results for this project are generated based on two papers (Study I, II), a manuscript (Study III), and a patent application (Study IV), presented in the Appendix section. Since Study I and Study III complement each other, they are presented together, while Study II is presented separately.

5.1. STUDY I AND STUDY III

5.1.1.1 Study I

Maltesen R.G. *et al*., *Predictive biomarkers and metabolic hallmark of postoperative hypoxaemia*, 2016, 12, Metabolomics

Aim of Study

No study has characterized metabolic phenotypes in patients undergoing CABG with the use of CPB, a well-established risk factor for the development of postoperative lung injury (27). Hence, the aim of Study I was *to identify early biomarkers of postoperative lung injury*.

5.1.1.2 Study III

Maltesen R.G. *et al*., *Metabotyping Patients' Journey Reveals Early Predisposition to Lung Injury after Cardiac Surgery* (manuscript)

Aim of Study

While several mechanisms were found deranged in patients progressing to hypoxaemia already at the first postoperative morning (Study I), there is still need for a better understanding of the molecular reasons as to why certain patients develop pulmonary dysfunction, while other do not. Hence, the aims of this study were (1) *to investigate the time course of metabolite events, from the start of surgery, to the clinical symptoms of hypoxaemia observed 72 hours postoperatively;* (2) *to investigate whether it was possible to find a characteristic 'metabolic biosignature' that made certain patients prone to develop hypoxaemia*.

RESULTS AND DISCUSSIONS

The metabonomics approach was used since it aims to find insights into the actual metabolic phenotype of diseases, and the causes of their progression (83). NMR spectroscopy combined with complex multivariate statistical data analyses was used as the methodological approach, due to its advantages over classical biochemical assays: rapid, reproducible (165), and able to measure hundreds metabolites simultaneously in a sample (101).

Having samples collected at ten different time points from the pre-, intra-, and post-operative period collected from different places in the body (radial artery, pulmonary artery, and left atrium), allowed us to (1) specifically focus on the immediate metabolic effects of the surgical procedure, and to determine whether a metabolite was produced, consumed, or just transiently passing through the lungs; (2) to create a metabolic journal and follow each patient's journey through surgical trauma towards progression to hypoxaemia or recovery.

The workflow scheme used to answer the aims of both studies is shown in Figure 5-1. The steps included sample preparation, acquisition, data processing, data analysis, and biological interpretation of results.

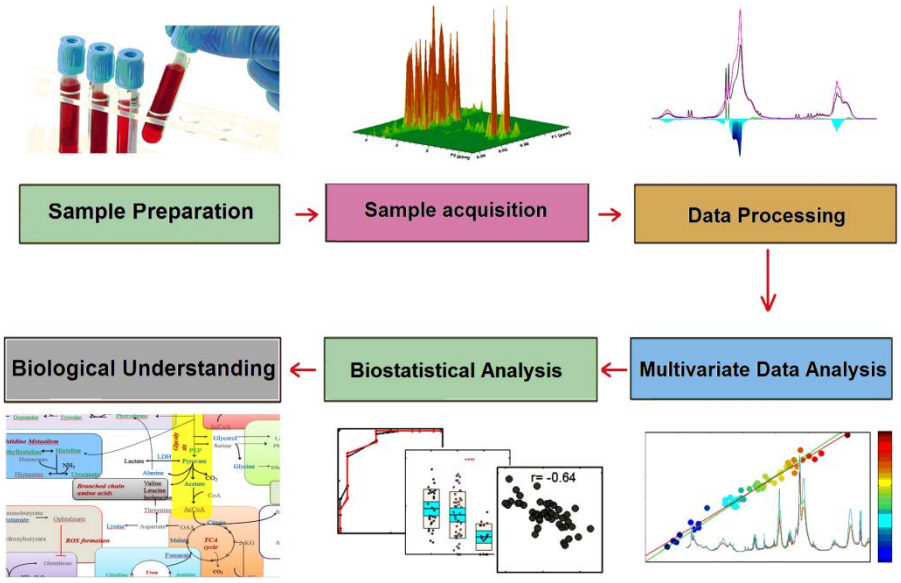


Figure 5-1 Workflow scheme for Study I and Study III

Patient characteristics

The characteristics for patients enrolled in this project are provided in Table 4-1. All patients had normal preoperative PaO_2; (mean± SD, 11.3± 1.3 kPa). PaO_2 decreased to 7.9 ± 1.5 kPa at 48 hours and to 7.8 ± 1.4 kPa at 72 hours, respectively (Figure 5-2). There was no significant difference between the groups in age, body mass index, and smoking habits, however, slightly differences were found in gender ($p= 0.01$) and the duration of CABG and ischemia ($p< 0.04$), but not of CPB ($p= 0.23$).

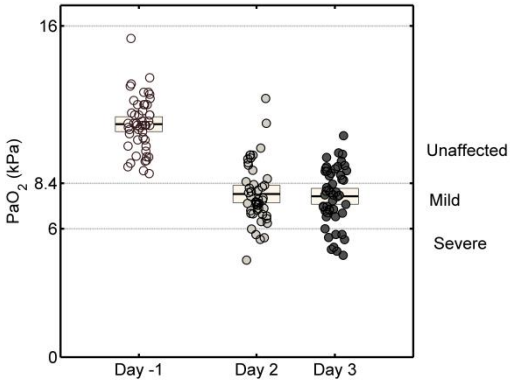


Figure 5-2 Partial pressure of oxygen (PaO_2) measured in arterial blood on fasting patients the day before surgery, and the second and third day postoperatively, to assess the degree of hypoxaemia. Arterial PaO_2 levels showed a 29% decrease on the second (48h) ($p<0.0001$) and a 31% ($p<0.0001$) decrease 72h postoperatively compared to their baseline levels measured the day before surgery (day-1). Based on PaO_2 values obtained on the third postoperative day, patients were divided into: unaffected patients with $PaO_2 \geq 8.4$ kPa and hypoxaemic patients with $PaO_2 < 8.4$ kPa. The hypoxaemic group was further subdivided into 'mild' hypoxaemic ($8.4 > PaO_2 \geq 6.3$ kPa) and 'severe' hypoxaemic patients ($PaO_2 < 6.3$ kPa).

Metabolome changes as a consequence of surgery

Besides oxygenation, the levels of several plasma metabolites were also found to change on the second and third day postoperatively (Figure 5-3a). Decreases in the levels of several amino acids and increases in the levels of ketones, fatty acids, and lipids were observed. Similar trends were observed in serum samples collected intra- and postoperatively the following 20 hours post-CPB (Figure 5-3b).

Since patients received nutritional support on days two and three, and since plasma and serum matrices are biologically different in terms of the levels of some amino acids and lipids (166-168), plasma and serum results were analysed separately.

To systematically analyse metabolome response to surgery, unsupervised PCA was generated on plasma (Figure 5-4a) and serum (Figure 5-4b) samples. PCA recognized patients according to the time at which their samples have been collected, and clustered them accordantly. Both samples collected at 72 and 20 hours postoperatively revealed no overlap with baseline samples (the day before surgery for plasma, and the pre-CPB for serum samples, respectively), indicating that the metabolome was still perturbed at these time points following surgery.

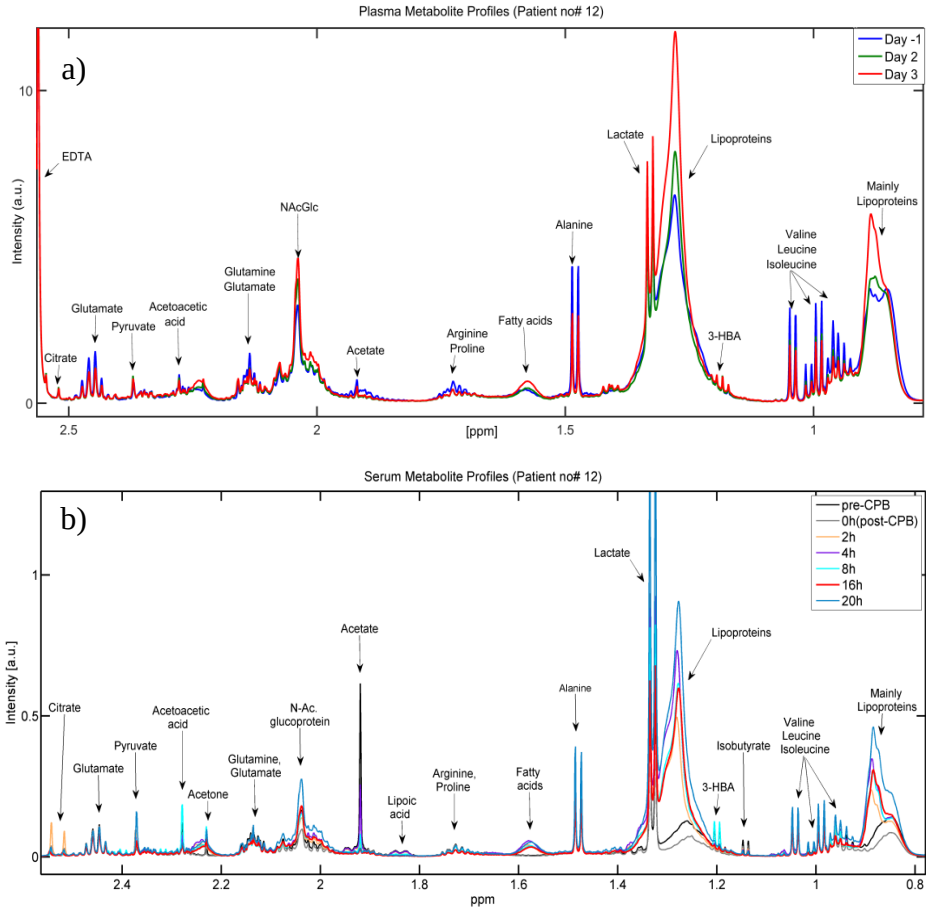


Figure 5-3 The metabolite 'journal' of patient no. 12. a) Changes observed in plasma the second (green) and third postoperative day (red) compared to the day before surgery (blue). b) Changes observed in serum collected during and after the surgery: after sternotomy, but before cardiopulmonary bypass (pre-CPB, black); immediately after weaning from CPB (0h post-CPB, grey); 2 hours post-CPB (2h, orange); 4 hours post-CPB (4h, purple); 8 hours post-CPB (8h, light blue);16 hours post-CPB (16h, red); and 20 hours post-CPB (20h, blue green). Abbreviation: h, hours; a.u., arbitrary units; 3-HBA, 3-hydroxibutyric acid; N-Ac, N-Acetyl; EDTA, ethylene diamine tetraacetic acid.

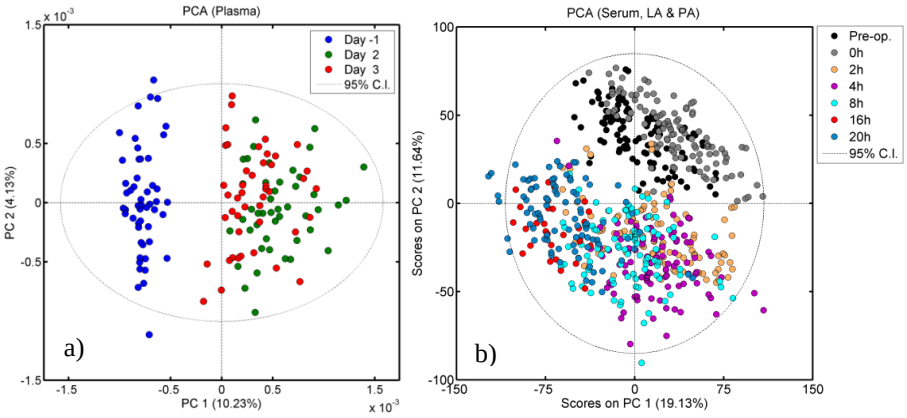


Figure 5-4 Metabolome changes as a consequence of surgery. (a) Principal component analysis (PCA) performed on 144 plasma projected the samples according to their similarities on the first principal components (PC1 and PC2). Clear separation is observed between the time at which each sample has been collected (blue scores: plasma from the preoperative day, 'Day-1'; green and red scores: plasma from day two and tree, respectively). The largest difference between samples is observed the second postoperative day. (b) PCA performed on 688 serum samples collected from the left atrium and pulmonary artery during the surgery (black, pre-CPB; grey, 0h post-CPB) clustered along PC2, while postoperative samples migrated from 2h (orange), to 4h (purple), 8h (light blue), 16h (red), and 20h (blue green) along PC1.

Patients' metabolite journals

At the end of CPB, the levels of glucose, pyruvate, alanine, lactate, citrate, and creatine increased, while the levels of phospholipids, free fatty acids (free FA), polyunsaturated fatty acids (PUFA), choline-containing compounds, lipoproteins, glycerol, ketone metabolites (3-hydroxybutyric acid, acetate, acetoacetic acid), and amino acids, including purine metabolites (inosine, hypoxanthine, uric acid, and xanthine), nicotinic acid metabolites (trigonelline, tryptophan), tyrosine metabolites (tyrosine and L-dopa), branched-chain amino acids (BCAA; valine, leucine, and isoleucine), histidine, and methylhistidine decreased (Figure 5-5). Some metabolites have reached baseline levels within few hours postoperatively; however, most metabolites continued to change even after 72h postoperatively.

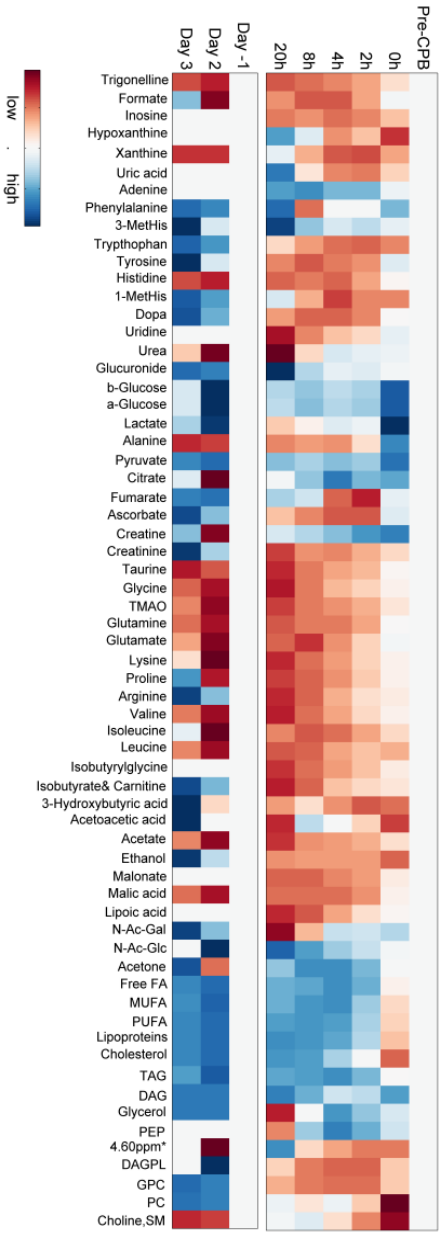


Figure 5-5 Time-dependent metabolic changes found in Study III. Abbreviations: TMAO, trimethylamine-N-oxide; N-Ac-Gal, N-acetyl-galactosamine; N-Ac-Glc, N-acetylglucosamine; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids; DAG, diacylglycerol; PEP, phosphoenoylpyruvate; 4.60ppm, unassigned peak; DAGPL: diacylglycerophospholipid; GPC: glycerolphosphocholine; PC, phosphatidylcholine; SM, sphingomyelin.*

Insights into the systemic and pulmonary phenotypes

While the exact origin of the identified metabolites is unknown, their releases or disappearances from pulmonary (LA) and systemic (PA) vasculature may reflect their potential location.

In general, metabolites showed similar trends in both LA and PA samples, with slight increases in the levels of some metabolites in the LA samples, indicating their production in the lungs (Table 5-1). For example, lactate increased by 33% while pyruvate by 61% in PA samples at the end of CPB, with higher levels in the LA samples, suggesting a more anaerobic environment in the lungs. Additional information is provided in Study III.

Table 5-1 Metabolite changes over time in relation to both left atrium (LA) and pulmonary artery (PA) samples. Significance was assessed by two-way ANOVA. A p-value <0.05 was considered significant. Se Figure 5-5 for abbreviations.

		Percent mean change (%)										Significance	
LA	**Metabolites**	**0h**		**2h**		**4h**		**8h**		**20h**		**p-value**	
		LA	**PA**	**LA**	**PA**	**LA**	**PA**	**LA**	**PA**	**LA**	**PA**	**LA**	**PA**
↑	Lactate	**36**	33	**5**	0	**6**	2	**-1**	-4	**-7**	-11	**<0.0001**	<0.0001
↑	Pyruvate	**67**	61	**36**	29	**38**	35	**32**	28	**41**	33	**<0.0001**	<0.0001
↑	Acetone	**5**	-1	**108**	96	**139**	132	**146**	134	**97**	87	**<0.0001**	<0.0001
↑	Free FA	**-29**	-30	**221**	206	**266**	260	**226**	219	**222**	208	**<0.0001**	<0.0001
↑	MUFA	**-25**	-25	**49**	46	**77**	76	**76**	75	**63**	59	**<0.0001**	<0.0001
↑	PUFA	**-27**	-27	**38**	35	**60**	59	**62**	61	**59**	58	**<0.0001**	<0.0001
↑	DAG	**15**	13	**6**	8	**4**	6	**12**	12	**19**	14	**0.001**	0.02
↑	Glycerol	**11**	10	**26**	20	**34**	32	**1**	-1	**-45**	-46	**<0.0001**	<0.0001
↑	GPC	**-7**	-7	**-14**	-16	**-14**	-17	**-14**	-16	**-10**	-11	**<0.0001**	<0.0001
↑	Lipoprotein	**-30**	-30	**30**	28	**52**	52	**60**	58	**64**	60	**<0.0001**	<0.0001
↑	Cholesterol	**-32**	-35	**1**	0	**20**	18	**33**	30	**38**	28	**<0.0001**	<0.0001
↓	DAGPL	**-25**	-19	**-46**	-48	**-47**	-48	**-41**	-43	**-22**	-16	**<0.0001**	<0.0001
↓	Ascorbate	**2**	10	**-27**	-28	**-27**	-28	**-21**	-22	**-19**	-9	**<0.0001**	<0.0001
↓	N-Ac-Gal	**16**	21	**15**	13	**13**	18	**-22**	-23	**-61**	-56	**<0.0001**	<0.0001
↑	Glucuronate	**5**	6	**15**	13	**9**	9	**30**	26	**100**	94	**<0.0001**	<0.0001
↑	Glycine	**-3**	-4	**-7**	-11	**-12**	-13	**-20**	-21	**-30**	-33	**<0.0001**	<0.0001
↑	Lysine	**-3**	-3	**-9**	-11	**-18**	-19	**-23**	-24	**-30**	-32	**<0.0001**	<0.0001
↑	Proline	**-3**	-3	**-10**	-11	**-18**	-18	**-22**	-23	**-24**	-26	**<0.0001**	<0.0001
↑	Valine	**-3**	-3	**-9**	-11	**-18**	-17	**-25**	-26	**-33**	-35	**<0.0001**	<0.0001
↑	Isoleucine	**-14**	-14	**-28**	-30	**-39**	-39	**-44**	-44	**-33**	-35	**<0.0001**	<0.0001
↑	Leucine	**-7**	-8	**-5**	-7	**-8**	-9	**-11**	-13	**-11**	-14	**0.004**	<0.0001
↑	Trigonelline	**-15**	-17	**-32**	-38	**-45**	-46	**-50**	-53	**-49**	-63	**0.010**	0.001
↑	Adenine	**6**	12	**56**	45	**51**	47	**67**	67	**65**	54	**<0.0001**	<0.0001
↑	Inosine	**-14**	-18	**-27**	-25	**-28**	-29	**-24**	-25	**-24**	-30	**<0.0001**	<0.0001
↑	Hypoxanthine	**-13**	-12	**-6**	-4	**-9**	-7	**4**	0	**12**	8	**<0.0001**	<0.0001
↑	Xanthine	**-16**	-16	**-26**	-26	**-24**	-25	**-14**	-16	**5**	3	**<0.0001**	<0.0001
↑	Uric acid	**-6**	-8	**-13**	-17	**-13**	-16	**-2**	-6	**24**	17	**<0.0001**	<0.0001
↑	Tyrosine	**4**	3	**-13**	-15	**-15**	-16	**-18**	-19	**-13**	-16	**<0.0001**	<0.0001
↑	L-Dopa	**0**	-1	**-8**	-10	**-11**	-12	**-11**	-12	**-7**	-9	**<0.0001**	<0.0001
↑	Fumarate	**2**	1	**-11**	-13	**-9**	-9	**7**	-1	**8**	3	**0.04**	0.21
↑	Histidine	**-1**	-1	**-9**	-11	**-14**	-15	**-13**	-14	**-14**	-16	**<0.0001**	<0.0001
↑	3-Metylhistidine	**4**	4	**11**	9	**6**	6	**14**	15	**36**	32	**<0.0001**	<0.0001
↑	1- Metylhistidine	**-7**	-10	**-7**	-11	**-12**	-14	**-5**	-9	**6**	0	**<0.0001**	<0.0001
↑	Uridine	**4**	3	**-5**	-7	**-9**	-9	**-14**	-15	**-23**	-28	**<0.0001**	<0.0001
↑	Urea	**3**	1	**4**	1	**5**	4	**-4**	-6	**-24**	-27	**<0.0001**	<0.0001

Metabolome screening reveals early signs of lung injury

Since metabolome changed as a consequence of surgery, metabolome's potential diagnostic value for early detection of the progression into hypoxaemia was investigated. Hence, by applying metabolite fingerprinting approach, spectra were screened for possible signals associations with later PaO_2 values.

Patient's history

Since patient's history prior to the surgery (age, lifestyle, smoking habits, etc.) is an important risk factor for the development of postoperative lung injury (6, 26), it was investigated whether such information was embedded in the metabolome. Interestingly, partial least square (PLS) regression analysis identified few metabolic fingerprints that showed weak associations with later outcomes (cross-validated correlation, $R^2_{cv} = 0.43$, Figure 5-6a). Although the association is weak, these results may suggest that the individual metabolome configuration may play a key role in the later development of pulmonary dysfunction.

Surgical procedure

PLS analysis performed on samples obtained after sternotomy, but before CPB showed a 0.71 cross-validated correlation between metabolome and PaO_2 values (Figure 5-6b), suggesting that the general anaesthesia and sternotomy procedure have affected the metabolome, which in turn, showed predisposition to the later development of lung dysfunction. The association increased to 0.92 after weaning from CPB (Figure 5-6c), indicating that the CPB and cross-clamp itself contributed significantly to the later dysfunction. Same high associations were found the following 20 hours postoperatively (Figure 5-6d), indicating that the surgical procedure itself had triggered the major chain of reactions that elicited the progression into hypoxaemia. To the best of our knowledge, this is the first study that demonstrates and quantifies the surgical impact on the metabolome ad it links it to later pulmonary dysfunction.

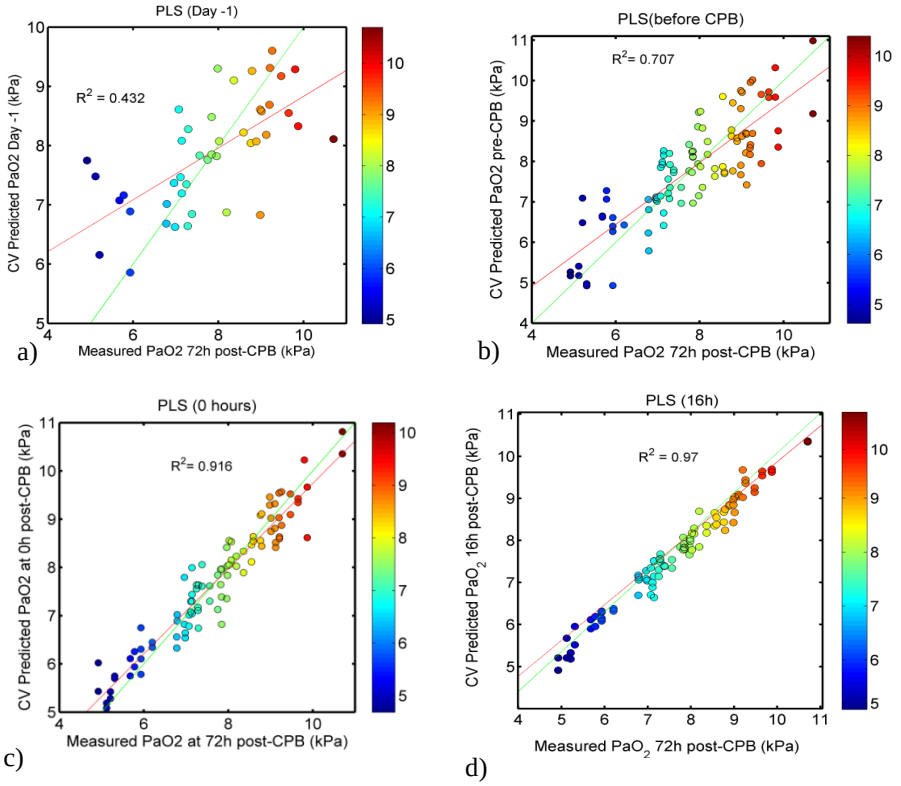


Figure 5-6 Metabolome screening reveals early predisposition to hypoxaemia. (a) PLS regression revealed a weak cross-validated correlation (R^2=0.432)between plasma metabolome measured the day before surgery (Day-1) with arterial partial pressure of oxygen (PaO_2) values measured 72h postoperatively. Cross-validated (CV) was performed using the Venetian-Blinds approach with 5 segments split. (b) PLS reveals a 0.71 predicted correlation between serum metabolome measured after sternotomy, but before CPB, and PaO_2. A Venetian-Blinds CV approach with 10 segments splits was implemented. (c) PLS reveals a 0.92 predicted correlation between serum metabolome immediately after weaning from CPB (0h) and PaO_2 values. Venetian Blinds approach with 10 segments split was performed for validation purpose. (d) PLS reveals a 0.97 predicted correlation between serum metabolome at 16h post-CPB and PaO_2. The model was validated using a Monte-Carlo validation (explained in Study I, Appendix A). In addition, all models were further validated by using 500x permutation testing. Except for model (a), there were no other over-fitted models ($p<0.004$). Green line, the calibrated results; red line, the cross-validated results.

The metabolic path towards disease progression

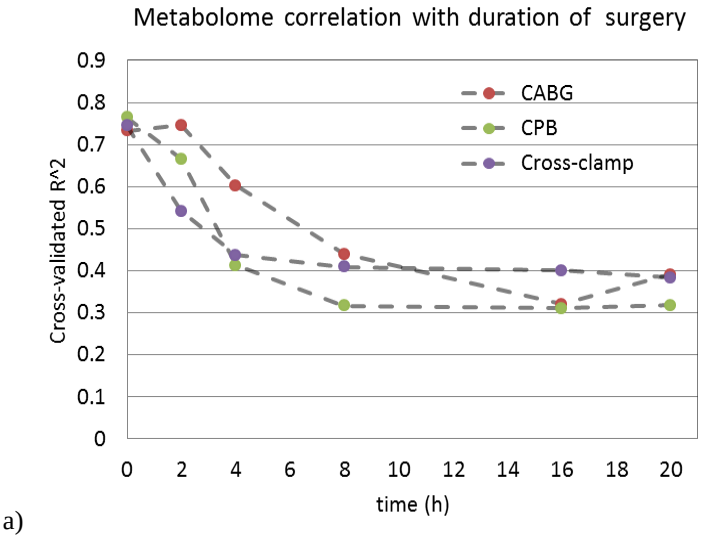
Since the previous results indicated that the CPB procedure itself has contributed to the later development of hypoxaemia, and due to a general longer cross-clamp and CABG time for hypoxaemic patients (Table 4-1), the possible effects of prolonged surgical procedures on human metabolome was further evaluated.

Relatively strong associations were identified by PLS regression analysis between metabolome and the duration of CPB, cross-clamp, and CABG procedures at 0 and 2 hours post-CPB (Figure 5-7a). The associations decreased with time, indicating a normalization of the metabolome after longer surgical stress.

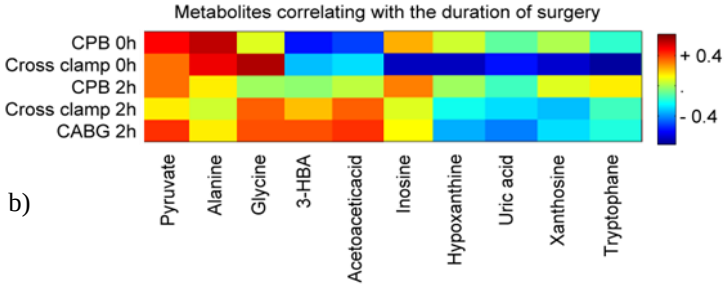
Several changes were identified to occur during the surgical procedure (Figure 1d and Table 2 in Study III, Appendix C). It was found that metabolome shifted towards anaerobic metabolism of glucose by switching from net lactate extraction to lactate release. In addition, an increased tricarboxylic acid (TCA) cycle activity, a fall in ketone extraction, and a dysregulation of fatty acid and lipid metabolisms was observed at the end of CPB.

Pyruvate and alanine levels were found correlating with the duration of cross-clamp and CPB (Figure 5-7b). These metabolites were also found to discriminate between hypoxaemic and unaffected patients at 0h (Table 5-2), indicating their link between surgery and later development of hypoxaemia. Glycine was found correlating with the duration of cross-clamp and with the risk of developing hypoxaemia. In fact, its levels provided evidence for groups' discrimination already before surgery (Table 5-2, Figure 5-8). Ketone metabolites found inversely correlated with the length of CPB, were also found to differentiate between later outcomes. Finally, purine metabolites found negatively correlated with the length of cross-clamp, were found decreased in patients developing hypoxaemia already before surgery (Figure 5-8), indicating a general impairment in their syntheses.

In conclusion, a characteristic metabolite biosignature was identified that made patients prone to develop postoperative lung injury (Table 5-2, Figure 5-8). This signature consisted of metabolites involved in the purine metabolism, nicotinic acid metabolism, methylhistidine metabolism, tyrosine metabolisms, glycine and isobutyrylglycine metabolism, glycolysis and TCA cycle, fatty acids and lipid metabolisms, and glycoprotein metabolism.



a)



b)

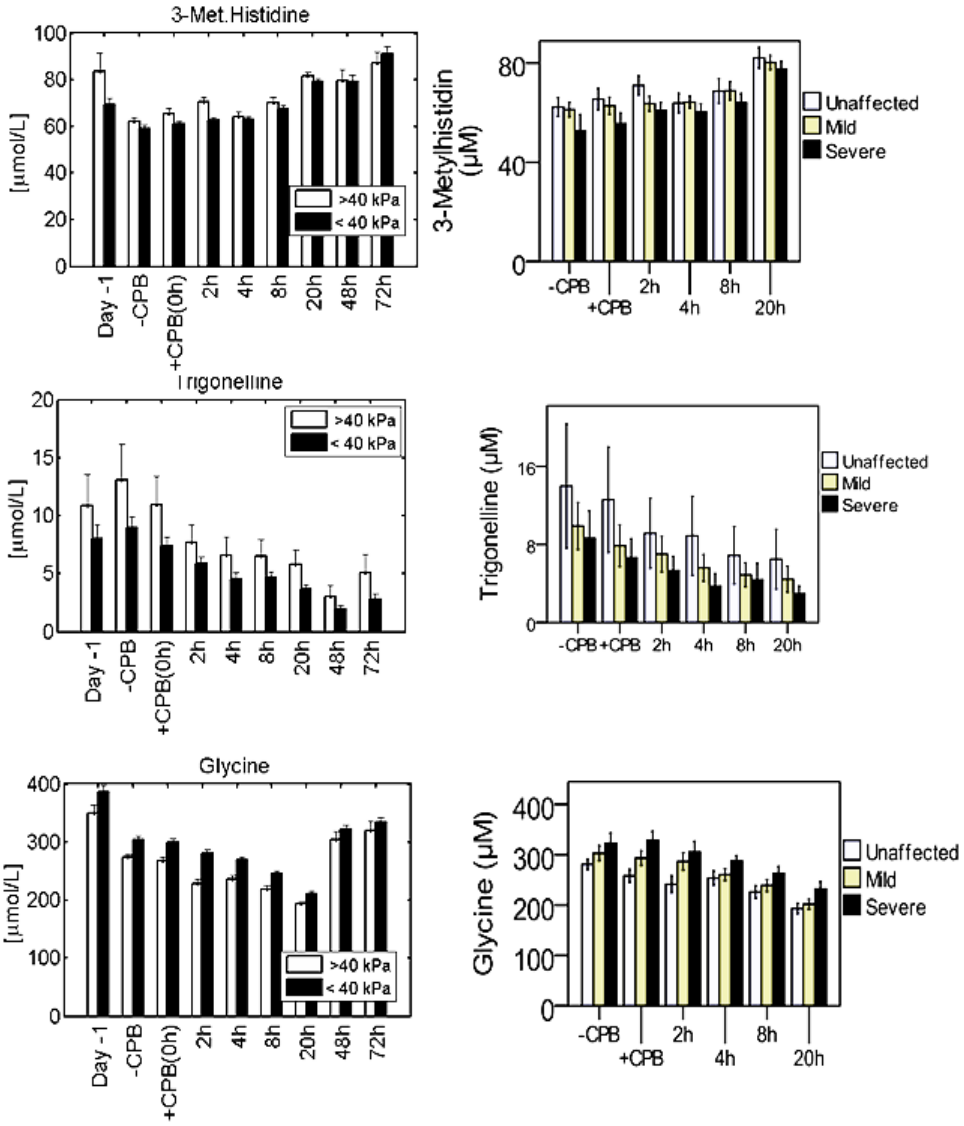
Figure 5-7 The effects of prolonged surgical procedure on the human metabolome. (a) PLS regression analysis performed on serum spectra from samples collected immediately after weaning from CPB (0h) and 2 hours post-CPB showed cross-validated associations (R^2) with the duration of cross-clamp (purple), CPB (green) and CABG (red). Higher correlations were observed immediately after weaning from CPB. All models were cross-validated using the Venetian Blinds approach with 10 segments. (b) Metabolites found to correlate both with the duration of CPB, cross-clamp, and CABG, and the progression into hypoxaemia. Correlation was assessed through the Pearson correlation test (red: positive correlation; yellow: no significant correlation; blue: negative correlation).

Table 5-2 The chronological metabolic events for the progression to postoperative lung injury. Annotation: '√', the time point at which a metabolite was found to discern between patients; '-', metabolites not measured in Study I (16h post-CPB).

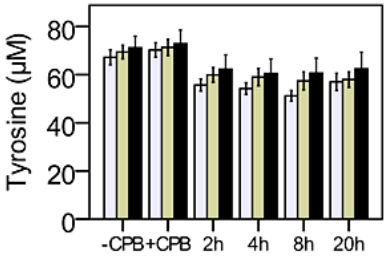
Metabolites	Day -1	Pre-CPB	CPB (0h)	2h	4h	8h	16h	20h
Trigonelline	√	√	√	√	√	√	-	√
3-Methylhistidine	√	√	√	√	√	√	√	√
Glycine	√	√	√	√	√	√	√	√
Inosine		√	√	√	√	√	-	√
Tyrosine		√	√	√	√	√		√
Dopa		√	√	√	√	√	√	√
Isobutyrylglycine		√	√	√	√	√	√	√
Malonate		√	√	√	√	√	√	√
Monounsaturated fatty acids (MUFA)		√			√	√	√	√
Free fatty acids			√		√	√	√	√
PUFA			√	√	√	√	√	√
Glycerol			√	√				
Lipoproteins			√	√	√	√	√	√
Cholesterol			√		√	√	√	√
Phosphatidylcholine			√	√	√	√	√	
Choline			√	√	√			
Hypoxanthine			√	√	√	√	√	√
Xanthine			√	√	√	√	√	√
1-Methylhistidine			√	√				
Uric acid			√	√	√	√	√	√
Isoleucine			√	√			√	√
Leucine			√		√	√	√	√
Glutamate			√	√				
Alanine			√	√	√	√	√	√
Pyruvate			√	√	√	√	√	√
Citrate			√	√	√	√	√	√
N-Ac-Galactosamine			√	√			√	√
3-HBA			√		√	√	√	
Acetoacetic acid			√		√	√	√	
Acetate				√	√	√	√	√
Acetone				√	√	√	√	√
TAG					√	√	√	√
N-Ac-Glucosamine						√	√	√

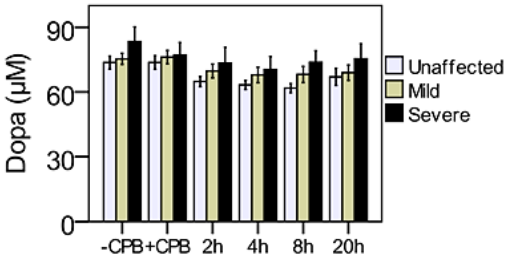
Figure 5-8 Selected metabolites showing time- and phenotype dependent changes. Each metabolite concentration is represented by its mean and 95% confidence intervals.

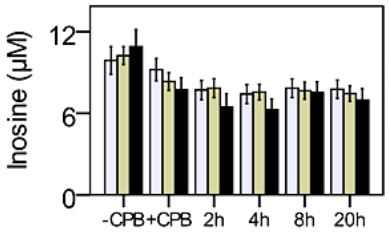
Before surgery

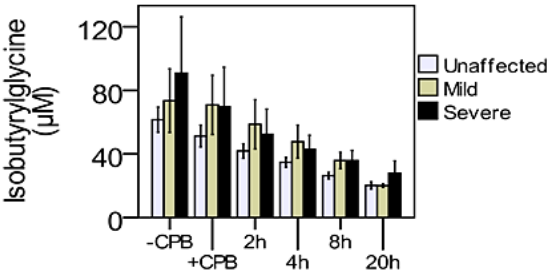


After sternotomy, but before CPB

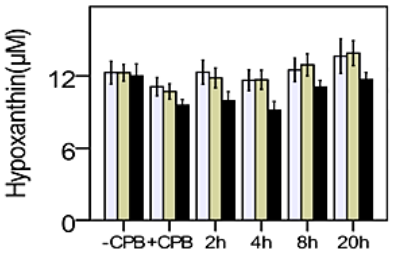


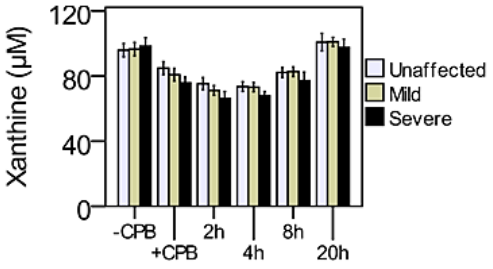


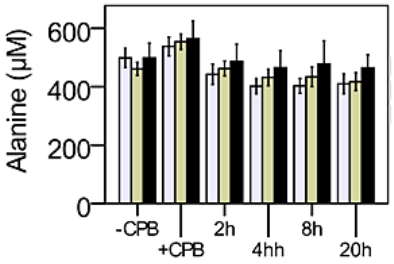


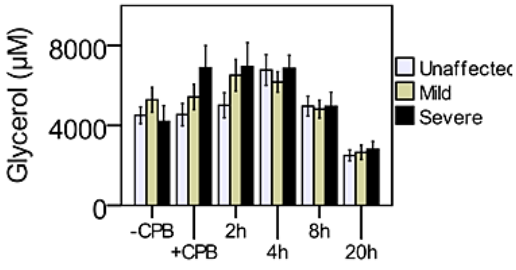


At the end of CPB

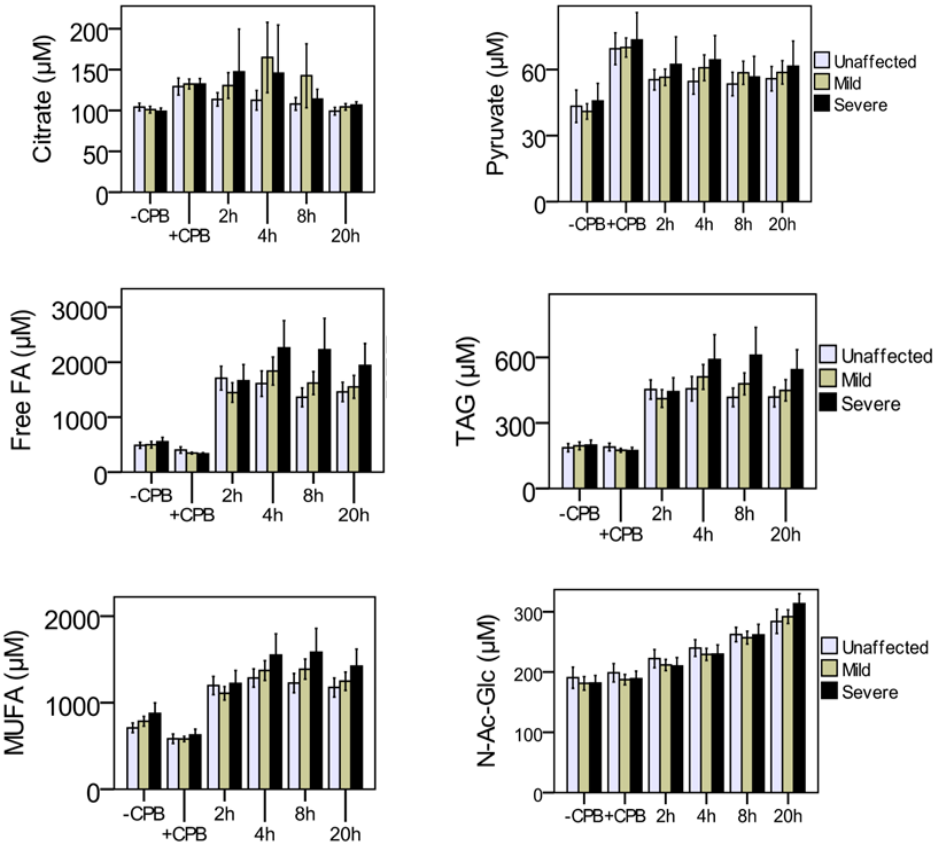








Post-CPB



Possible metabolic interpretation

In plasma samples collected on the day before surgery, we found trigonelline, 3-methylhistidine, and glycine to differentiate unaffected from hypoxaemic patients (Figure 5-8). The question of why these metabolites were different at such an early time point is difficult to answer. The levels of these metabolites are similar in both males and females (169, 170), and hence, the gender imbalance in the study should not have influenced patient grouping the day before surgery. Since metabolites reflect lifestyle differences, an alternative explanation to this early differentiation may be that the overall patients' health status before surgery was weakened in patient developing postoperative pulmonary dysfunction. While this explanation is speculative, further studies are needed to confirm these particular results.

Trigonelline has previous been linked to oxidative stress, and its supplementation has been shown to attenuate oxidative stress (171). Glycine has been reported to have multiple protective effects, and to play central roles in: protection against shock caused by haemorrhage and ischemia-reperfusion injury; inflammatory cell activation; and free radical formation. Moreover, in plasma membranes, glycine has been shown to affect regulation of chloride channels (172) and cell volume (173). Hence, its changes may reflect a higher need for protection in hypoxaemic patients. Finally, 3-methylhistidine, a marker of tissue damage and inflammation (174), was found to increase with time in all patients. However, its levels were relatively diminished in the hypoxaemic group at all time points, probably indicating that these patients experienced impaired synthesis prior to the surgical procedure.

While inosine, nicotinic acid metabolites, and 3-methylhistidine levels were found to be lower, tyrosine, dopa, glycine, and isobutyrylglycine were found to be elevated in hypoxaemic patients after sternotomy, but before CPB, suggesting their potential roles as early markers of lung injury. In samples taken just after weaning from CPB, alanine, pyruvate, citrate, purine metabolites, BCAA, glutamate, taurine, ketones, some fatty acids, and cholesterol could differentiate between later outcomes. At 20 hours, most of these metabolites could still differentiate patients (Figure 5, Study III, Appendix C), signifying their key roles in the early progression of lung injury.

Previous studies conducted on patients diagnosed with ALI/ARDS have shown similar changes with respect to TCA cycle metabolites (89, 175), however, several studies are needed to confirm the newly-identified metabolites. Ketones and cholesterol have previously been related to ischemia-reperfusion injury and to an impaired fatty acid oxidation (176), however, they have not yet been linked to the development of lung injury after ischemia-reperfusion. Isobutyrylglycine, a marker of impaired mitochondrial FA β-oxidation, was significantly increased in patients progressing to hypoxaemia already post-sternotomy, indicating derangements in FA metabolism.

Under homeostatic conditions, various antioxidants are capable of alleviating the damaging effects of ROS from the system (177), however, under stress, the antioxidant barrier is not sufficient to counteract this damage. Sampling at the end of CPB revealed lower levels of the antioxidants inosine and hypoxanthine in patients developing hypoxaemia, which may indicate an inefficient scavenging ability, leading to a continued ROS presence. Interestingly, previous metabonomics work conducted by Evans and co-workers on BALF of patients diagnosed with ARDS revealed a 41 fold increase in hypoxanthine levels compared to healthy subjects (175). Our results may be complementary to their findings. We found a 30% decrease in hypoxanthine levels in patients progressing to severe hypoxaemia at the end of CPB, which could indicate that the metabolite may diffuse into the lungs in the early stages of pulmonary dysfunction, and that it may accumulate in the later stages of full blown disease. To verify this hypothesis, other studies involving both blood and BALF samples collected at different time points during disease progression should be performed.

Glycerol was found to differentiate patients at 0 and 2 hours post-CPB. Glycerol release has previously been potentially linked to cellular ischemia-reperfusion injury and lipolysis (176), however, it has not been linked to progression to lung injury. In addition, free FA, phospholipids, and lipoproteins could discriminate between hypoxaemic and unaffected patients at 4h postoperatively and later time points. In line with these findings, a recently published study conducted by Sheng and colleagues (17) has reported that the levels of circulating fatty acids 2 hours after CABG were predictors of the development of hypoxaemia ($r = -0.367$, $p < 0.001$).

N-Acetylglucosamine and N-acetylgalactosamine could also differentiate patients in at least two simultaneous time points after CPB, indicating their minor, but still significant, roles in ongoing processes. N-acetyl glucosamine has previously been linked to increased inflammation after cardiac surgery (178) and to stress conditions in general (179), but its levels have not yet been linked to hypoxaemia or ALI/ARDS. Finally, several metabolites were found to differentiate between later outcomes at two different time points (e.g. urea, creatinine, creatine, arginine, valine, histidine, uridine, glucose, and lactate). Since their changes did not following a time-line trend, but rather a sporadic trend, they were considered to be less significant for disease progression.

Taken together, these findings suggest that patients progressing to hypoxaemia have limited metabolic reserves with regard to ATP production; excessive activated lipolysis after surgery; and are more prone to produce an exaggerated stress response to CABG.

Although the study may be the largest of its type published to date, sample sizes are still relatively small and involve comparisons between multiple groups. Also, since the study was performed on consecutive patients undergoing CABG, we could not match for sex differences. Therefore, further studies must be undertaken to address these limitations. Another important limitation is the lack of samples collected at the beginning of anaesthesia and before sternotomy. Under normal conditions organisms utilize several metabolites as fuel substrates, including glucose, lactate, amino acids, ketones, and FA. Therefore, having arterial samples just before the induction of general anaesthesia, at the beginning of anaesthesia, and before sternotomy would have provided information about the effects of anaesthesia and sternotomy on the human metabolome. Regarding Study I, only data from samples collected on the first morning postoperatively were presented, and hence a significant amount of information concerning the course of events involved in disease progression could not be investigated. Also, after analysing the time series data, a few metabolites found to differentiate patients in Study I could not be reproduced in Study III. This is due to the fact that some metabolites showed potential discriminative power at only one of the investigated time points. In addition, we mostly used serum samples for the analyses. Since the coagulation processes take some time, the samples were left at ambient temperature before centrifugation. In this period metabolic changes may have occurred. However, all samples were treated equally, and hence the differences between samples can be expected to reflect the real changes in the

patients. Finally, some of the significant metabolites could not be identified because their signals were close to the spectral baseline. In the future, this could be overcome by applying mass spectrometry-based techniques, which are more sensitive than NMR.

In conclusion, the scope of Studies I and III was to elucidate the paths towards progression to postoperative lung injury and to pave the way for future research. The results indicated that metabotyping patients' journeys at the beginning of, or just after the end of CPB, may have potential clinical impact for the early diagnosis of postoperative lung injury, and for monitoring the effect of therapeutics targeting disease progression. In samples taken whilst patients were still undergoing surgery, we found a unique metabolic biosignature that clearly discriminated unaffected from hypoxaemic patients. Hence, these metabolites may have potential as early biomarkers for the diagnosis of hypoxaemia.

To the best of our knowledge, this is the first metabonomics study demonstrating the link between intra- and postoperative time-dependent metabolite changes and the later development of postoperative hypoxaemia. If these results can be translated to the clinical setting, this would provide doctors and nurses with a diagnostic approach that allows for continuous monitoring of the progression and regression of postoperative lung injury.

5.2. STUDY II

Rasmussen B.S. *et al*., *Early coagulation activation precedes the development of acute lung injury after cardiac surgery*; Thrombosis Research Volume 139, March 2016, Pages 82–84

Aim of Study

The aim of this small pilot study was *to investigate potential changes in the coagulant and fibrinolytic activity in cardiac surgical patients at risk of developing postoperative lung injury*.

RESULTS AND DISCUSSIONS

This was a small pilot study including ten patients selected from the whole cohort of fifty patients. Five patients presenting low PaO_2/FiO_2 (all below 27 kPa), and five patients with the highest PaO_2/FiO_2 (all above 40 kPa) were selected. The severe hypoxaemic patients were defined as the acute lung injury ('ALI') group, while the other five patients were defined as the 'non-ALI' or unaffected group. Patient demographic data is shown in Table 1 (Study II, Appendix C).

Two markers of coagulation activity, prothrombin fragment 1.2 (F12) and thrombin-antithrombin complexes (TAT), and two fibrinolytic markers, D-dimer and plasmin-antiplasmin complex (PAP) were measured and their plasma profiles were followed perioperative (after sternotomy and right after weaning from CPB) and during the subsequent 20 hours post-CPB (Figure 5-9a-d).

The coagulation activity was found increased immediately after CPB in patients developing severe hypoxaemia, and reached its maximum within the first 2 hours postoperatively (Figure 5-9a,b). D-dimer increased immediately after CPB in patients developing severe hypoxaemia compared to unaffected patients (Figure 5-9 c), possibly indicating that more fibrin was formed. However, the fibrinolytic activity measured as PAP, was at the same level in both groups (Figure 5-9d), probably indicating that the fibrinolytic activity was insufficient to remove the increased amount of fibrin in severe hypoxaemia patients. Similar patterns were observed in both LA and PA samples; however, a trend towards higher levels of TAT, F12, PAP, but not D-dimer was detected in LA, suggesting that the lungs are actively releasing these molecules in the pulmonary microvasculature.

Since these changes are mostly observed to occur mostly during and after weaning from CPB, the time of CPB and cross-clamp was correlated to the levels of TAT, F12, and D-dimer. A modest correlation between time of CPB or cross clamp and D-dimer ($r= 0.50$, $p= 0.02$ and $r= 0.38$, $p=0.06$, respectively), but no significant correlations were observed with the other markers. This result may indicate that the pro-coagulant response is influenced by the time on CPB and cross-clamp. However, since the number of patients was small, several studies are needed to confirm these findings.

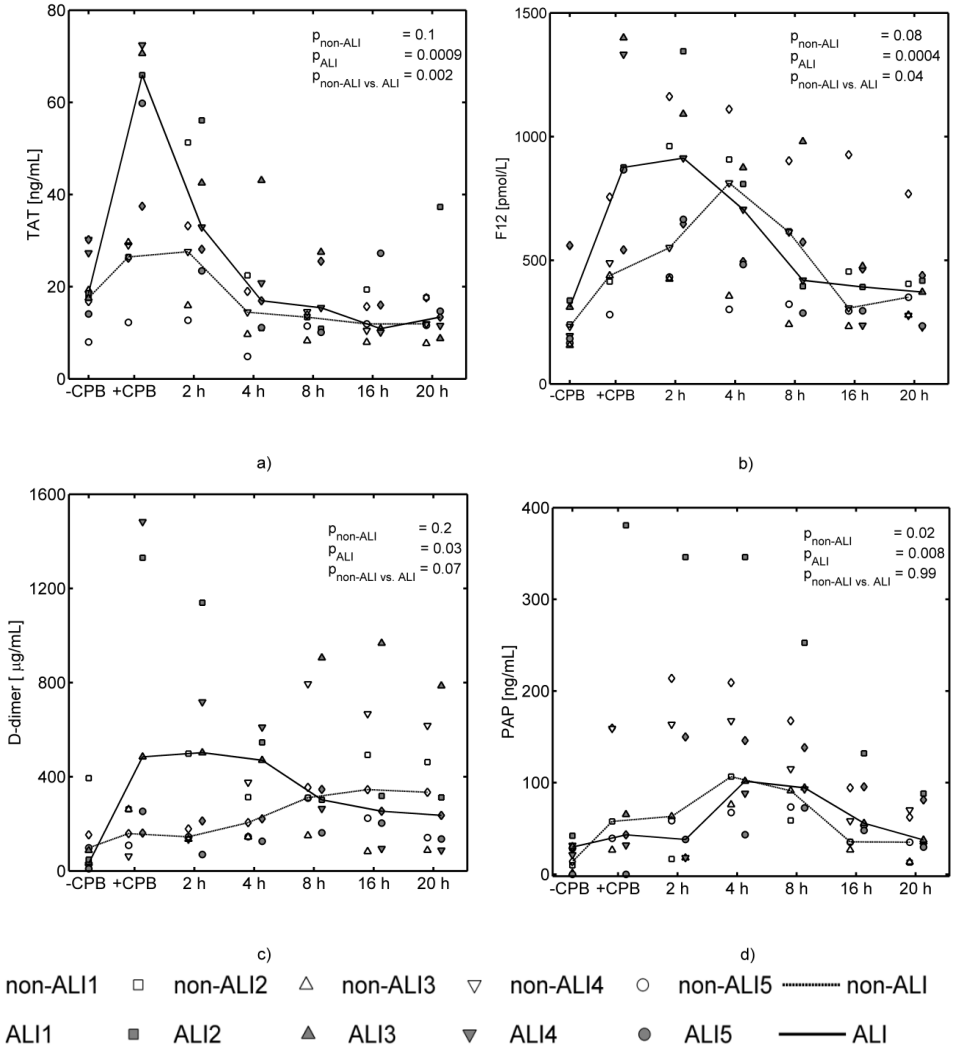


Figure 5-9 A time course comparison of markers of coagulation (a. TAT; b. F12) and fibrinolytic activity (c. D-dimer; d. PAP) observed in patients progressing into severe hypoxaemia (ALI; n = 5) and unaffected patients (non-ALI; n = 5).

These results are in accordance with previous findings which have reported increased a procoagulant activity and a decreased fibrinolytic activity in BALF samples in patients diagnosed with ARDS (180, 181). In comparison, the findings of this study took place immediately after CPB and were related to the later progression into lung injury, while previous results were based on diagnosed patients.

The main limitation of this study was the small number of patients used to envisage trends of potential changes. Larger studies are needed to elucidate whether the procoagulant response found in this study is causal or just an epiphenomenon.

In conclusion, an imbalance between coagulation and fibrinolytic activity following CPB may be part of the complex mechanism leading to development of postoperative lung injury, and thus, to hypoxaemia.

CHAPTER 6. CONCLUSIONS

The aim of this PhD study was to investigate whether metabolomic and coagulant activity can be used to detect early signs of lung injury after cardiac surgery, and to reveal some of the possible mechanisms involved in disease progression.

NMR-based metabonomics and ELISA kit assays were applied to pre-, intra- and postoperative serum and plasma samples, as part of a strategy to analyse the human metabolic, coagulant, and fibrinolytic responses to CABG; to find possible predictive biomarkers; and to identify deranged mechanisms, which could eventually serve as future therapeutic targets.

It was found that surgical trauma induced complex series of molecular responses, probably intended to reobtain body's homeostasis and to ensure patient's survival. Metabolite fingerprinting provided a global overview of surgical trauma and allowed for the prediction of patients progressing into hypoxaemia. Metabolic profiling allowed for the identification and quantification of markers of prolonged surgical trauma. In addition, a metabolic biosignature that made certain patients prone to develop postoperative lung injury has been identified. Activity assays allowed detection of a possible imbalance between coagulation and fibrinolytic activity.

Patients progressing to hypoxaemia were found to have less metabolic fuel, a more anaerobic environment, more impaired fatty acid oxidation, more coagulation activity, and deficient fibrinolytic activity. These complex derangements not only reflect the critical impact of surgery on the human body, but they also reflect the biological system's attempt to restore its homeostasis after traumatic surgery. Also, it was found that patients' histories (life style, smoking habits, prior diseases, etc.) may have amplified the response to the surgery, which in turn, played an important role in the development of postoperative lung injury.

During sternotomy and CPB, tissue trauma, air contact with the heart and lungs, temperature shifts, blood contact with the artificial CPB circuit, blood dilution, and ischemia-reperfusion, amongst other factors, may have caused significant shifts in the metabolome and the coagulation activity. In addition, although similar changes were observed in the systemic (PA) and pulmonary (LA) environment, a trend towards higher molecular levels was found in LA samples, indicating that the lungs are actively involved in the progression of hypoxaemia. Moreover, it was found that events occurring within the first day postoperatively (e.g. chest tube drainage, blood transfusion, and duration of ventilatory support) did not contribute significantly to patient classification, since the associations between the postoperative metabolome to the later PaO_2 values were not markedly different to that observed after weaning from CPB.

As such, this PhD study indicates that surgery is the main triggering factor of the progression to lung injury. In addition, the study provides information about the

molecular mechanisms preceding disease progression, and offers evidence for early markers of the development of hypoxaemia, that could be used for personalized surgical optimization.

Although novel insights have been obtained, there are still several central questions that remain unanswered:

1. How general anaesthesia and sternotomy affect the human metabolome?

2. How much lung injury is related to cross-clamp, and how much to CPB itself?

3. Are there metabolites that are lost to the oxygenator filters during CPB?

4. Are these biomarkers reliable?

To answer these questions and validate the results further research is needed.

CHAPTER 7. PERSPECTIVES AND FUTURE DIRECTIONS

Metabolomics is still a relatively new but rapidly growing field. The approach has the potential to impact our understanding of the molecular mechanism of complex conditions such as postoperative pulmonary dysfunction.

The application of NMR and MS-based metabonomics to investigate diseases has provided better molecular insights into the perturbed mechanisms. However, for application in early diagnosis and prognosis, the technology is still in its evolutionary stages. This longitudinal study was the first of its kind, predicting the development of lung injury after cardiac surgery, and hence, the learning phase of identifying metabolic perturbations may not be finished.

As highlighted throughout this thesis, NMR-based metabonomics offered some unique answers to questions about changes in the human metabolome as a consequence of surgical trauma. Also, it offered the possibility to predict the progression to pulmonary injury while patients were still undergoing surgery (Figure 7-1). Hence, metabonomics had some advantages over the standard clinical approach, which normally detects hypoxaemia at a later time point.

Many confounding factors exist. Although the possible influence of several confounding factors has been investigated in Study I (e.g. previous diseases, smoking hobbits, age, and body mass index), the proper matching of patients with respect to gender should be considered in further studies. In addition, the possible effects of medication should also be investigated, since such information would provide insights into individual responses to therapeutic interventions. Hence, further longitudinal studies are needed to confirm and expand these initial findings.

During this PhD period, increased focused has been placed on applying metabonomics to patients suffering from acute and chronic respiratory diseases at the Department of Anaesthesia and Intensive Care Medicine, Aalborg University Hospital, and several studies are starting to take form. An example of one such study involves patients suffering from chronic obstructive pulmonary disease who underwent the same type of surgery (ClinicalTrials.gov identifier: NCT01614951). This is a blinded study, which aims to validate the identified markers of surgical trauma and of the progression into hypoxaemia. Initial results have identified some of the same markers of surgical trauma.

The main advantage of NMR is its reproducibility (Appendix E), while the clear advantage of MS is its sensitivity. Therefore, by combining both platforms, more advanced and deeper insights into disease progression may be achieved. Hence, MS will also be applied to interpret metabolome perturbations in future studies. The

combined platform may help us to achieve the ultimate goal in personalized medicine: to provide the right medicine to the right patient at the right time.

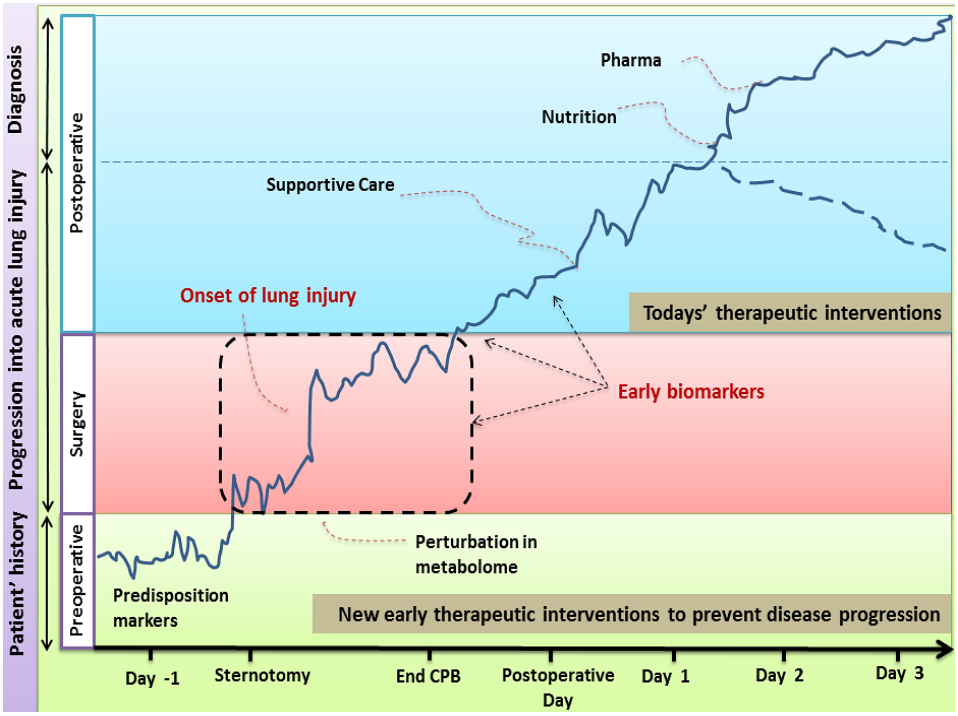


Figure 7-1 The progression of postoperative lung injury from 'healthy' state, to metabolome homeostatic perturbation, to onset of disease, tot todays' diagnosis.

Is there any future for metabonomics in clinical practice?

Before metabonomics becomes a widespread clinical reality, there are two main problems to be solved. The first is to make metabonomics technically straightforward, from both the data acquisition and the processing point of view, so that it can be used in the daily routine by non-specialists. The second problem is related to population variation. In general, studies have focused on small, controlled populations, and after the identification of markers of a disease, few studies have validated the results with new blinded studies. Technical problems will probably be solved sooner than population variation, because of advances in automation, especially in the NMR field, which will soon allow the use of metabonomics routinely for screening purposes. In addition, for hospitals that do not have access to such techniques, once metabolic biomarkers are found by these platforms, their detection may be done using standard analytical methodologies already implemented in the clinical laboratory.

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APPENDICES (PAPERS, PATENT, AND ADDITIONAL RESULTS)

Appendix A. Study I

Maltesen RG, Hanifa MA, Kucheryavskiy S, Pedersen S, Kristensen SR, Rasmussen BS, Wimmer Reinhard. *Predictive biomarkers and metabolic hallmark of postoperative hypoxaemia*. Metabolomics. 2016;12(5):1-15.

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ORIGINAL ARTICLE

Predictive biomarkers and metabolic hallmark of postoperative hypoxaemia

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Abstract
Introduction Pulmonary dysfunctions resulting in postoperative hypoxaemia is a common complication of cardiac surgery. The disease is challenging as it lacks predictive biomarkers. Since a comprehensive metabolic overview of lung microvasculature injury is lacking, we have compared the metabolome of patients undergoing cardiac surgery from blood collected on the first postoperative day from the pulmonary artery and left atrium.
Objectives To identify predictive biomarkers and metabolic hallmark of pulmonary hypoxaemia.
Methods Blood samples collected on the first postoperative morning from 47 patients were analysed by nuclear magnetic resonance and multivariate statistics. Patients' metabolomes were correlated to the level of partial pressure of arterial oxygen (PaO_2) without supplementary oxygen treatment measured on the third postoperative day.
Results Three days postoperatively, 32 patients suffered from hypoxaemia. Spectra recorded on samples collected on the first morning postoperatively revealed metabolic perturbations causing disease progressing. Regression modelling found a 0.97 association between metabolome and PaO_2. Classification modelling distinguished patients according to later hypoxaemia. Sixty-four metabolites were identified as the early hallmarks of disease, of which several showed significant correlations with PaO_2 ($r > 0.55$, $p \leq 0.00001$). The tricarboxylic acid cycle, amino acid and lipid metabolism, together with redox homeostasis were all found affected. An integrated overview reveals complex cross-talk between pathways that can be related to the pathogenesis of hypoxaemia: damaged alveolar-capillary barrier, edema formation, peroxidation, oxidative stress, impaired antioxidant defense, and cell damage.
Conclusion Our results indicate unique phenotypes triggering progression into pulmonary dysfunction resulting in postoperative hypoxaemia. The metabolic hallmarks identified offer important targets for future treatments.

Keywords Cardiac surgery · Pulmonary dysfunction · Hypoxaemia · Metabolome · Pathways · NMR

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1 Introduction

Pulmonary dysfunction remains to be the most common complication to cardiac surgery (Clark 2006; Ng et al. 2002). Disturbance of the alveolar-endothelial barrier affects the pulmonary gas exchange (Ng et al. 2002; Singh et al. 1992) with development of postoperative hypoxaemia with nadir values of partial pressure of oxygen in arterial

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blood (PaO_2) on the third postoperative day (Ng et al. 2002; Rasmussen et al. 2007).

Disturbance of the alveolar-endothelial barrier is part of the acute lung injury syndrome which is a life-threatening condition (Matthay and Zimmerman 2005; Perl et al. 2011; Ware et al. 2010; Wheeler and Bernard 2007), affecting more than one million individuals worldwide every year (Oeckler and Hubmayr 2008). The injury can develop within a week (Stephens et al. 2013) after direct or indirect pathophysiological events such as sepsis, pneumonia, cardiopulmonary bypass (CPB), and severe trauma (Oeckler and Hubmayr 2008; Perl et al. 2011; Reiss et al. 2012; Stephens et al. 2013; Weissman 2004). Regardless of the initial triggering factor, the final result is abnormal gas exchange (Apostolakis et al. 2009) caused by excessive inflammation (Milot et al. 2001) and disordered coagulation (Reiss et al. 2012; Wheeler and Bernard 2007). Endothelial cell swelling, cellular junction widening, edema accumulation, denuded alveolar membranes, increased pinocytosis, and alveolar collapse are the hallmarks of this dysfunction (Rubenfeld and Herridge 2007).

Currently, no therapeutic intervention has proven useful in impeding disease progression into a state of hypoxaemia which is one of the criteria for acute lung injury. (Fremont et al. 2010; Matthay et al. 2012; Perl et al. 2011) As such, identifying patients at risk of developing clinically important hypoxaemia earlier in the course of their illness would enable the development of therapeutic options targeting the disease (Gajic et al. 2011).

Substantial efforts have been made to understand multiple mechanisms involved in acute lung injury (Bhargava and Wendt 2012; Levitt et al. 2009; Matthay et al. 2012; Ng et al. 2002). In general, the research is focused on understanding the delicate balance between protective and injurious immunologic responses. Although little is known about metabolic activity during the injury, recent studies have reported that the sites of inflammation are characterized by significant metabolic shifts followed by fundamental changes in tissue phenotypes (Kominsky et al. 2010). These changes can be revealed by applying metabonomics (Nicholson et al. 1999, 2002), which screens for metabolite perturbations in a biological system as response to pathophysiological modification (Nicholson et al. 1999). Furthermore, it identifies characteristic disease phenotypes that reflect the connection between tissue histology and environmental, genetic, and immunologic factors (Nicholson et al. 2002). Hence, metabonomics is powerful tool for examining disease-related metabolic changes and in discovering biomarkers (Shlomi et al. 2009).

The metabolic shifts during lung injury have partly been revealed (Evans et al. 2014; Rogers and Matthay 2014; Serkova et al. 2008; Stringer et al. 2011). These changes are related to energy depletion (Serkova et al. 2008), oxidative stress, apoptosis (Evans et al. 2014), and endothelial disruption (Stringer et al. 2011). No studies have characterized the metabolic phenotypes in patients undergoing coronary artery bypass grafting (CABG) involving ischemia–reperfusion through the use of CPB, a well-established risk factor for developing this condition due to an indirect pathophysiological event (Ng et al. 2002). As pulmonary dysfunction remains the most common complication of cardiac surgery (Clark 2006; Ng et al. 2002), we intend to identify biomarkers and the metabolic hallmarks of postoperative hypoxaemia. Finding and defining perturbations in the metabolome may explain how inflammatory and coagulation mediators orchestrate the metabolism.

We hypothesized that early stages of pulmonary dysfunction following cardiac surgery may be reflected at the metabolite level and precede the development of hypoxaemia on the third postoperative day. We therefore investigated metabolite changes in blood collected from the pulmonary artery (PA) and left atrium (LA) on the first postoperative morning in order to describe changes in the systemic and pulmonary microvasculature. While both samples reflect systemic changes of the metabolome, we expected that metabolic processes confined to the lungs would be reflected in the difference between the LA and PA samples.

2 Methods

2.1 Patient cohort and diagnosis

Patients scheduled for elective CABG with use of CPB were consecutively included in the study after the informed consent was obtained (study registered at clinicaltrials.gov, NCT02475694). Inclusion criteria were adults above 18 years of age and in treatment with statins. Exclusion criteria were treatment with steroids or other immune suppressor therapies. All patients were monitored with a pulmonary as well as a LA catheter inserted after induction of anesthesia and removed 20 h after weaning from CPB. Of the total cohort of 50 patients, the LA catheter was displaced in three patients at 16 h, giving a cohort of 47 patients in the present study. Information regarding patients enrolled in this study is provided in Table 1 Supplementary.

All patients were spontaneously breathing at all times, but the majority was treated with supplementary oxygen postoperatively to achieve peripheral oxygen saturation (SpO_2) above 95 %. The clinical diagnosis was based on the ratio between partial pressure of oxygen and fraction of inspired oxygen (PaO_2/FiO_2) calculated from PaO_2

measured in arterial blood samples collected from the radial artery 72 h after weaning from CPB and in order to standardize the measurements, arterial blood samples were taken while patients were breathing atmospheric air for at least 10 min.

2.1.1 Samples preparation

To avoid analytical bias due to sample collection, all blood samples were collected and prepared by the same person. Blood samples were obtained exactly 16 h after weaning from the CPB circuit. This time point was chosen because it lies on the first morning postoperatively, while patients still were fasting. Blood samples were taken simultaneously from the tip of a PA catheter placed after induction of anaesthesia and from a catheter placed by the surgeons in the LA after opening of the pericardium. Subsequently, serum was prepared according to standard procedures, where blood samples were allowed to clot at room temperature for 30 min, followed by centrifugation at 3000 rpm for 10 min. Aliquots of LA and PA serum were immediately stored at −80 °C for 3 years.

2.1.2 Biochemical analysis

Blood gas measurements were performed on an ABL837 blood gas analyzer (Radiometer, Copenhagen). Biochemical assays of serum creatinine, albumin, lactate dehydrogenase, and C-reactive protein (CRP) were performed on the multiparametric autoanalyser VITROS FS 5.1 (Ortho Clinical Diagnostics, Raritan, New Jersey, USA) using dedicated reagents. The results are presented in Table 1 Supplementary.

2.1.3 Sample preparation for NMR

Prior to NMR measurements, samples were thawed for 2 h at 4 °C, vortexed, and centrifuged for 5 min at 4 °C and 12,100 g to remove cells and other precipitated materials. Aliquots of 400 µL of supernatant were mixed with 200 µL 0.2 M phosphate buffer (pH 7.4, uncorrected meter reading) in $^{2}H_2O$ (99 % ^{2}H) to minimize variations in pH and to reduce serum viscosity. Throughout the whole process, the samples were kept on ice. The mixture was pipetted into a 5-mm NMR tube and kept at 5 °C until NMR acquisition (not longer than 24 h).

2.1.3.1 NMR measurements Spectra were acquired on a Bruker DRX-600 NMR spectrometer (Bruker BioSpin, Germany and Switzerland) equipped with a TXI (hydrogen, carbon, nitrogen) probe (Bruker BioSpin, Switzerland) operating at 600.13 MHz for ^{1}H. TopSpin 1.3 was used for the acquisition. The experiments were acquired at a constant temperature of 310.1 K (37 °C). For the analysis, a T_2 relaxation-edited Carr–Purcell–Meiboom–Gill (CPMG) (Meiboom and Gill 1958) experiment was used. This experiment attenuates broad signals from slowly tumbling proteins and lipoproteins. 128 free induction decays (FIDs) were collected with 32768 complex data points over a spectral width of 11.97 ppm and an acquisition time of 2.28 s. A relaxation delay of 2 s was applied between each FID during which weak continuous wave irradiation ($\gamma B_1/2\pi = 26.6$ Hz) was applied at the water frequency (presaturation). The total spin-echo relaxation delay was 67.4 ms and consisted of $(\tau–\pi–\tau)$ elements, where τ is a delay of 0.4 ms and π is a 180° pulse of 22 µs length. Spectral processing was carried out in TopSpin version 2.1 (Bruker BioSpin, Germany). Prior to Fourier transformation, exponential multiplication corresponding to a line broadening of 0.3 Hz was applied to the FIDs, which were further zero-filled by a factor of 2 to double the number of points. Spectra were manually phase- and baseline- corrected, and the methyl signal of lactate was used as chemical shift reference at 1.33 ppm.

For metabolite identification, three types of two-dimensional (2D) experiments were acquired. 2D J-resolved ^{1}H-NMR experiments, with water pre-saturation during a 2 s relaxation delay were acquired with 8 FIDs for each of the 80 increments. Spectral width was set to 11.6 ppm and 54.7 Hz in F2 and F1 direction, respectively. Prior to 2D-Fourier transformation, both dimensions were multiplied by an unshifted sine bell function and the number of points was doubled by zero-filling. Further, spectra were tilted by 45° and symmetrized along the F1 axis. After processing, the skyline projections of 2D J-resolved spectra were manually baseline corrected and calibrated using the center of the methyl signal of lactate. 2D homonuclear $^{1}H,^{1}H$-TOCSY (Total Correlation Spectroscopy) and $^{1}H,^{13}C$-HSQC (Heteronuclear Single Quantum Coherence) spectra with presaturation were run on representative samples, with different number of FIDs, increments, spectral widths and mixing times in order to focus on different spectral regions. Information obtained from these spectra was used to find matching metabolites in the Human Metabolome Database (Wishart et al. 2007). Further metabolite assignments were performed using AMIX (v. 3.9.10, Bruker BioSpin), BRUKER bbiorefcode database (v. 2.7.0–2.7.3), and literature.

2.1.3.2 Quantification of NMR data It was performed using the AMIX multi integration tool. Peaks of interest were integrated using the line shape analysis option with a fixed noise factor of 3.5 and a line shape threshold of 0.01. Peaks were integrated using the sum of all points in the region as the integration mode, and normalized to the glucose metabolite region (3.52 ppm (H-5 of β-glucose)

and 5.24 ppm (H-1 of α-glucose)). A 36:64 equilibrium distribution between α- and β-glucose was used in the calculations. To calculate the concentration of a given metabolite, we used the following formula:

$$C_X(\mathrm{mM}) = \frac{I_X}{N_X} \cdot C_{Glucose} \cdot 0.64 \frac{N_{\beta-Glucose}}{I_{\beta-Glucose}}$$

where C_X is metabolite concentration in mM, I_X is the integral of the metabolite ^{1}H peak, N_X. is the number of protons contributing to the metabolite ^{1}H peak, $C_{Glucose}$ is the chemically determined glucose concentration, $0.64N_{\beta-Glucose}$ is the number of protons contributing to the β-glucose signal (at 3.52 ppm) used as reference multiplied by 0.64 (the mole fraction of β-glucose), and $I_{\beta-Glucose}$ is the integral of the β-glucose signal at 3.52 ppm.

2.1.3.3 NMR data preprocessing CPMG spectra were converted into an n-by-m matrix (n = 94 LA and PA samples from 47 patients, m = 8700 equal buckets of 0.001 ppm width) in AMIX (Analysis of MIXtures software package, version 3.9.10, Bruker BioSpin, Germany) using the region between δ 9 and 0 ppm, and excluding the water signal region between 4.80 and 4.5 ppm. Further processing and multivariate modelling were carried out in MATLAB R2011b (Mathworks, Natick, MA) coupled with PLS-Toolbox 6.5 (Eigenvector Research, Wenatchee, WA). Binned data was *i*Coshifted (Savorani et al. 2010), normalized to lactate concentration measured 16 h after surgery, log transformed, and autoscaled.

2.1.4 Multivariate modelling

Several mathematical and statistical modeling approaches were employed to relate the preprocessed NMR data to disease phenotypes. Supervised partial least-squares (Wold et al. 1984) (PLS) regression analysis was used to predict the hypoxaemia score values used in diagnosis on the third day postoperatively. PLS discriminant analyses (Wold et al. 1983) (PLS-DA) was applied to evaluate the diagnostic possibilities of NMR and to discover predictive markers. Due to the large number of variables, several variable selection methods (Li et al. 2009; Norgaard et al. 2000; Wold et al. 2001) were employed. Most important variables obtained by each method were combined into a new data matrix, which was used to build a final PLS and PLS-DA model. For each model, a multivariate mathematical equation was obtained which provided scores for each patient derived from the association between intensities and later outcome. The most significant variables were then identified and quantified. The significance of each metabolite was assessed by the variable importance in projection (Eriksson et al. 2001) (VIP) scores. The sign (positive and negative) of loadings were used to determine which group the corresponding metabolite was a potential marker for.

To establish confidence in our results, we adopted two complementary validation approaches. First, Monte-Carlo cross-validation was applied to reduce the risk of overfitting, by re-sampling the training set (70 % of samples) and cross-validation set (30 % of samples) 5000 times. In each resampling, 14 randomly selected samples were left for prediction, and 33 remaining samples were used to create the model. The omitted samples were then predicted, and a cross-validated (CV) correlation fit (for PLS regression) or classification fit (for PLS-DA) together with a root mean square error of cross-validation (RMSECV) were calculated each time. The final results represent average results of each parameter from the 5000 runs. The average RMSECV was further used to detect the optimal number of latent variables for both PLS and PLS-DA models. This validation procedure gave us an indicator of how well the model might work in predicting future samples (Xia et al. 2013). A second level of model validation was performed by using permutation testing (Good 2011). Here, the models were tested for randomness, to show that no other model performed equally well or better than the main prediction model. After scrambling the group and hypoxaemia score labels 500 times and performing PLS-DA and PLS regression modelling, respectively, we compared the 'true' optimal model with the permuted models. The ''true'' model performance was then statistically compared to the distribution of the permuted models, and a p value was calculated by means of Wilcoxon's test. A p value < 0.05 was considered significant, meaning that the predictive power of our approach was significantly associated with disease phenotypes and hypoxaemia scores, and was not a false-positive association resulting from random prediction.

Additionally, variables found important in the analysis were further used to assess the influence of other potential influencing factors such as gender, age, body mass index, smoking habits, hypertension, and other diseases such as diabetes and chronic lung disease. For each of the potentially influencing factors, we performed a PLS-DA model and assessed its sensitivity, specificity, and accuracy.

As the receiver operating characteristic (ROC) curve analysis is considered to be the standard method for assessing diagnostic tests performance (Obuchowski et al. 2004; Xia et al. 2013), we chose to present all our results using this test. The multivariate ROC curve analysis based on both the calibration and CV set was performed to judge how well PLS-DA models work in predicting new samples. Here, the models' performances were assessed by considering the frequency with which PLS-DA produced: true positives (the number of sick patients correctly identified), true negatives (the number of unaffected patients correctly

identified), false positives (the number of unaffected patients incorrectly identified as hypoxaemia patients), and false negatives (the number of sick patients incorrectly identified as unaffected). By summarizing these values into the proportion of hypoxaemia patients that were correctly classified as positive and the proportion of unaffected patients that were correctly classified as negative, the models' sensitivities and specificities were assessed:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives} + \text{False negatives}}$$

$$\text{Specificity} = \frac{\text{True negatives}}{\text{True negatives} + \text{False positives}}$$

2.1.5 Statistics

The Statistical Package and Services Solutions software v22.0 was used for the univariate statistical analysis. Descriptive statistics were computed for each group and the quantitative data was summarized using mean, median, and standard deviation. Wherever appropriate, data was visualized by using the Matlab script 'notBoxPlot.m' (Campbell 2010) representing the mean, standard error of mean, standard deviation, and outliers.

The Shapiro–Wilk normality test was applied followed by either parametric or nonparametric methods. When comparing metabolite differences between unaffected and hypoxaemia patients ($PaO_2 < 8.4$ kPa), either an independent *t* test or Mann–Whitney U test was used. When comparing differences between the three groups (unaffected, mild, severe), either one way analysis of variance (ANOVA) with Tukey HSD or Dunnett T3 (depending on homogeneity of variance) multiple comparison post hoc tests, or the Kruskal–Wallis test with Dunn's post hoc test were applied. When comparing data between the LA and PA, a paired *t* test or the nonparametric analogue Wilcoxon-signed rank sum test was used. Significance was set at $p < 0.05$.

In order to visualize global changes between groups, the means or medians (depending on data distribution) of each metabolite in the corresponding group were calculated. As such, metabolite fold changes were calculate as the ratio between mild and unaffected and severe and unaffected patients with the formula:

$$FC_{\frac{M(orS)}{U}}(\%) = \frac{M(orS) - U}{U} \cdot 100$$

where FC is fold change in percentage, M is mild, U is unaffected, S is severe.

Because the ratio between two metabolite concentrations may carry more information than the two metabolites alone, the freely available web-based tool ROC Curve Explorer & Tester (ROCCET) was used to compute all possible pairs of metabolite combinations that could be related to later outcomes. Metabolite ratios exhibiting statistical significance ($p < 0.05$) were further used as biomarker candidates.

The association between metabolites and PaO_2 values obtained 3 days postoperatively were assessed by Pearson's correlation analysis. ROC curves were established to determine the prognostic value of each biomarker in future diagnosis. The area under the curve (AUC) and corresponding 95 % confidence intervals are provided.

3 Results

Hypoxaemia is indicative of the severity of lung injury, being measured by the partial pressures of oxygen in arterial blood (PaO_2) or the ration between PaO_2 and the fraction of inspired oxygen (FiO_2) (De Backer et al. 1997; Routsi et al. 1999). Two days postoperatively some patients showed signs of hypoxaemia; however, while several patients' blood oxygenation levels return to normal the following day, others worsened. As lung dysfunction is known to develop within 72 h postoperatively, and due to the PaO_2 changes observed during the second and third postoperative day (Pearson correlation coefficient $r = 0.49$, $p = 0.0003$), the PaO_2 values measured 72 h postoperative were used as end-points in this study. As such, three days postoperatively, 15 patients showed no signs of hypoxaemia and 32 developed hypoxaemia, of which 9 presented severe hypoxaemia (Supplementary Table 1).

3.1 Metabolome screening reveals early signs of disease

The systemic and pulmonary phenotypes were monitored by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. A typical one dimensional (1D) serum NMR spectrum is characterized by broad resonances from lipids and glycoproteins, and narrow resonances from glucose, lactate, and citrate, among others. Spectra of two samples collected on the first postoperative day (exactly 16 h after weaning from CPB), one from a patient not developing hypoxaemia ($PaO_2 = 10.7$ kPa), and one from a patient developing hypoxaemia ($PaO_2 = 4.9$ kPa) on the third day postoperatively, reveal differences in several signals, of which lipids are the most significant (Fig. 1).

Since the metabolome mirrors environmental changes (Nicholson et al. 2002), we hypothesized that the disease could be reflected at the metabolic level on the first day postoperatively. Therefore, we screened for possible associations between the metabolome and the hypoxaemia

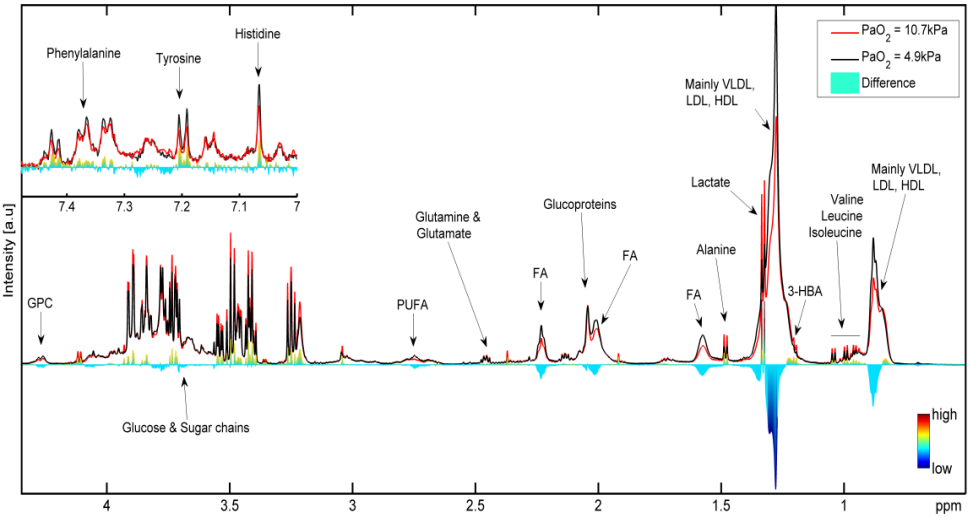

Fig. 1 Serum metabolic fingerprints. Representative 1D CPMG ^{1}H-NMR spectral profiles run on samples collected 16 h postoperatively of a patient not developing hypoxaemia (partial pressure of oxygen in arterial blood, $PaO_2 = 10.7$ kPa) (*black*) and a patient showing hypoxaemia ($PaO_2 = 4.9$ kPa) (red). The diagnosis was based on PaO_2 measured 72 h after weaning from cardiopulmonary bypass (CPB). Spectral difference (unaffected—hypoxaemia) (*red–yellow–blue* colour coded) reveals metabolite fluctuations between the patients. Higher levels of signals from lipoproteins, fatty acids and lower levels of lactate, glucose, and some amino acids are observed in the hypoxaemia compared to unaffected patient. Using a combination of the NMR metabolic profiling database (BBIOREFCODE, Bruker), The Human Serum Metabolome Database (HMDB), and literature references, 63 resonances out of 64 were assigned to metabolites (Table 2, Supplementary). Spectra were internally calibrated to the $-CH_3$ signal of lactate at chemical shift (δ) = 1.33 ppm. *1D* one-dimensional, *CPMG* Carr–Purcell–Meiboom–Gill, *VLDL* very-low density lipoproteins, *LDL* low-density lipoproteins, *HDL* high-density lipoproteins, *Val* valine, *Leu leucine*, *Ile isoleucine*, *3-HBA 3-hyroxybutyric acid*, *FA* fatty acids, *PUFA polyunsaturated fatty acids*, *GPC* glycerophosphocholine, *PaO_2* partial pressure of oxygen, *kPa* kilopascal, *ppm parts per million*, *a.u.* arbitrary units (Color figure online)

scores (PaO_2) used in diagnosis (Table 1, Supplementary). Interestingly, PLS regression analysis revealed a strong correlation between the systemic and pulmonary phenotypes and PaO_2 ($R^2 = 0.97$, Fig. 2a), indicating that serum NMR-based metabonomics can be used to screen for early signs of disease. Based on PLS results (Fig. 2a,b) we reasoned that subgrouping the 32 patients diagnosed with ALI into patients developing mild ($8.4 > PaO_2 \geq 6.3$ kPa) and severe ($PaO_2 < 6.2$ kPa) hypoxaemia, would provide better understanding of underlying mechanisms. Subsequently, the differences between the three groups (unaffected, mild, and severe hypoxaemia) were assessed by PLS-DA. The models displayed >95 % accuracy (Fig. 2c). The models were tested for some potentially influencing factors (Fig. 2d) that could obscure the accuracy of our proposed clinical test, and no significant interference was observed. The duration of anaesthesia, CABG, CPB, cross-clamp time, and fluid administration is known to have an impact on patients' outcome postoperatively. Therefore, we attempted to correlate metabolic information with these intraoperative variables, but did not detect any strong association (cross-validated R^2 values ranging between 0.14 and 0.51).

We also checked, whether common postoperative complications such postoperative systemic inflammatory response syndrome (SIRS) and renal dysfunction, associate with the metabolome and confound the analysis. PLS yielded no association between the observed metabolic changes and indices of SIRS (measured as the levels of leucocytes; cross-validated $R^2 = 0.17$ and CRP; cross-validated $R^2 = 0.04$) and renal dysfunction (measured as creatinine concentration; cross-validated $R^2 = 0.40$) measured 72 h postoperatively. This increases confidence that the metabolic linkage found is specific to hypoxaemia.

In summary, by collecting PA and LA blood samples 16 h after weaning from CPB, applying NMR and

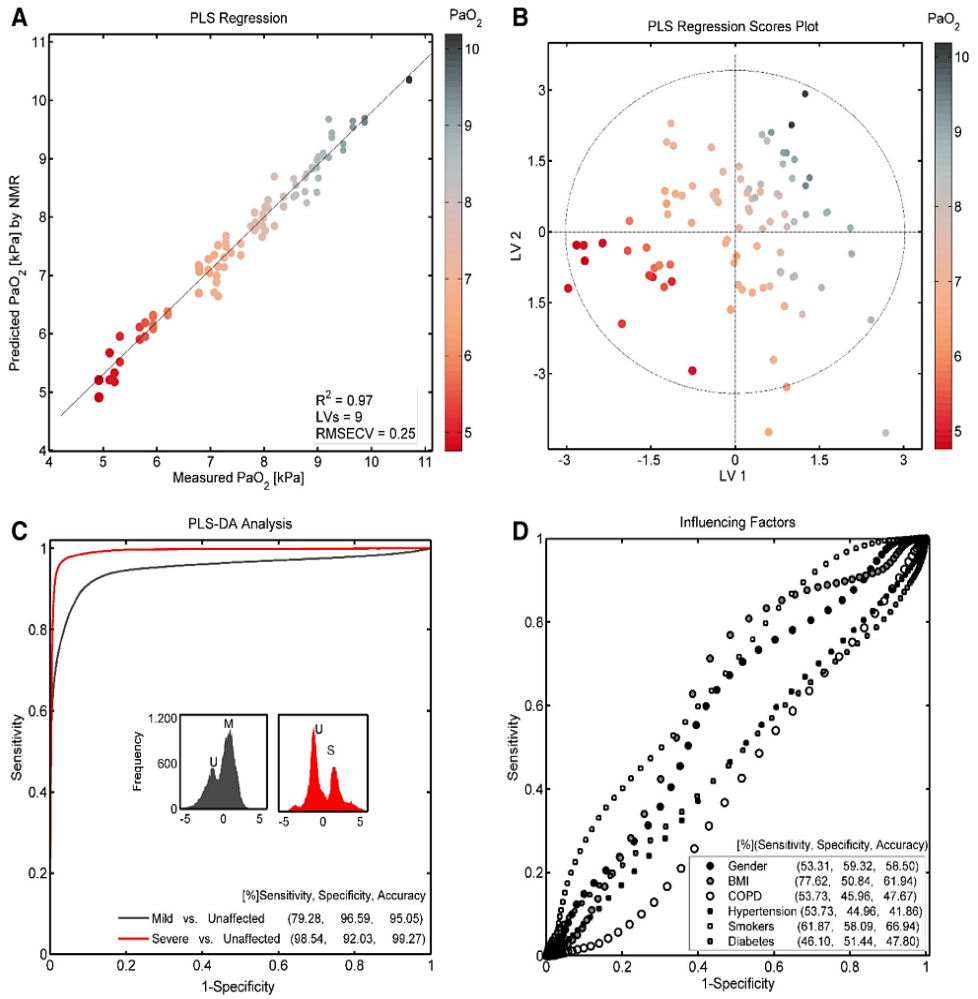

Fig. 2 Metabolome screening reveals early signs of disease. Multivariate analysis was performed on spectra run on serum collected 16 h postoperatively from the left atrium and PA. Monte-Carlo validation was used for validation purpose (Methods 5). **a** Partial least-squares (PLS) regression reveals that NMR predicts the partial pressure of arterial O_2 (PaO_2) measured three day postoperatively already the first morning post-CPB (predicted correlation $R^2 = 0.97$). Model robustness was assessed by repeating PLS modeling six times ($R^2 = 0.97 \pm 0.008$, root-mean-square error of cross-validation RMSECV $= 0.251 \pm 0.034$). **b** The first two latent variables (LVs) show patients clustering according to PaO_2. Patients developing severe hypoxaemia ($PaO_2 \leq 6.2$ kPa) cluster along –LV1; unaffected patients ($PaO_2 > 8.5$ kPa) cluster along +LV1; while mild hypoxaemia patients ($8.4 > PaO_2 \geq 6.3$ kPa) cluster in-between. **c** Separation between Unaffected/Mild and Unaffected/Severe patients was obtained through PLS-discriminant analysis (PLS-DA). Data was randomly split into a calibration (70 %) and validation set (30 %), and PLSDA was performed with 5000 iterations. The frequency plots (*gray* and *pink*) show samples distributions from each group (*U* unaffected, *M* mild, *S* severe) used in the calibration set. Following PLS-DA, each patient's predicted score was used to build the receiver operating characteristic (ROC) curve; each curve contains the results achieved from 5000 runs. Finally, PLS and PLS-DA models were further validated by 500× permuting the labels. No model was found over-fitted ($p < 0.001$). Sensitivity, specificity, and accuracy are given as percentages. **d** The possible influencing factors reveal no interference with the results (AUC < 0.67). *BMI* body mass index, *COPD* chronic obstructive pulmonary disease (Color figure online)

multivariate statistics, the hypoxaemia scores on the third day postoperatively could be predicted with a root-mean-squared error (RMSE) of 0.25 kPa (Fig. 2a).

3.2 Metabolic hallmark of postoperative hypoxaemia

The *fingerprints* or "*snapshots*" (Xia et al. 2013) of metabolism discerned in the screening process were investigated further. A total of 64 different metabolites were analyzed, of which one was unidentifiable (Supplementary Table 2). Perturbations in the levels of metabolites involved in normal cellular functioning (amino acids, carbohydrates, ketones), cellular signaling (1,2-diacylglycerol), inflammation (arachidonic and eicosapentaenoic acid), cell membrane and alveolar surfactant components (fatty acids, cholesterols, phospholipids) (Chen and Kolls 2010; Saxena 2005) were found crucial in the development of hypoxaemia (Fig. 3a). The contribution of each metabolite to modelling PaO_2 values was assessed by variables important in projection (VIPs) scores (Fig. 3b). Carnitine, arachidonic and eicosapentaenoic acid, glycoprotein, citrate, phenylalanine, glycine, plasmalogen, and lysophosphocholine (Lyso-PC), among others, showed highest scores, indicating their key roles in later outcomes. In addition, phospholipids and fatty acids (FA) showed highest fold changes in both hypoxaemia groups compared to the unaffected patients (Fig. 3b), indicating severe derangements in their pathways. Most metabolites showed consistent trends from none-to-mild-to-severe hypoxaemia (Fig. 3c), indicating their correlation to the degree of later pulmonary dysfunction and their possible function as predictive biomarkers. Finally, to understand the metabolic hallmark of hypoxaemia we have provided insights into the complex metabolic crosstalk in some of the affected paths (Fig. 3d), which have the potential to open up for promising therapeutic targets in the future.

3.3 Insights into the systemic and pulmonary phenotypes

While the exact origin of these metabolites is unknown, their release or disappearance from pulmonary (LA) and systemic (PA) vasculature may reflect their potential location. This disease is highly compartmentalized therefore knowing the origin of each metabolite would add new insights into translating its phenotypes.

In general, the same metabolic patterns were observed for both PA and LA samples with some exceptions, particularly for the severe hypoxaemia group (Fig. 4a, b, Supplementary Table 3). The levels of *N*-acetylated carbohydrates involved in the synthesis of glycoproteins and glycolipids (Michal and Schomburg 2012) positively correlated with the severity of developing hypoxaemia. Also, an augmented extracellular supply of pyruvate, alanine, and citrate was observed in patients with hypoxaemia, especially in LA samples. Accumulation of ketone bodies [acetate, acetoacetate, 3-hydroxybutyrate (3-HBA)], carnitine, and FA were detected in hypoxaemia patients compared to unaffected patients. Increased levels of the inflammatory mediators (PUFA) together with accumulation of metabolites involved in cell membrane and surfactant production (phospholipids, cholesterols), seemed to be consistent in patients developing hypoxaemia. While PUFA levels were higher, cholesterol levels were lower in the pulmonary compared to the systemic circulation. Increased levels of triglyceride-rich lipoproteins and their breakdown products (free FA, di-, tri-acylglycerols, and glycerol) were observed in both PA and LA samples of the hypoxaemia patients. Decreased antioxidant levels (histidine, plasmalogen, and hypoxanthine) were observed only in the severe group with a tendency towards consumption by the pulmonary system. Finally, increased extracellular levels of osmoprotectants and cell volume regulators (glycine, glycerophosphocholine (GPC), and glutamate) were found in both PA and LA samples of the hypoxaemia patients. Collectively, these changes reflect a yet unraveled part of the complex pathophysiology and provide new insights into the multiple mechanisms involved in the progression to, or protection from, hypoxaemia post-CPB.

3.4 Early markers for hypoxaemia

A list of several metabolites is not ideal for developing cost-effective diagnostic tests, and due to general lack of hospital NMR instrumentation, diagnosis based on fewer metabolites would be more convenient, if they provide sufficient information to generate robust clinical models. Therefore, we have explored all metabolites individually or as ratios between metabolites and found powerful predictive markers. Several serum metabolites measured 16 h postoperatively were found significantly correlated to PaO_2 measured 72 h after weaning from CPB (Fig. 5a, Supplementary Table 4) and several showed strong predictive power for mild and severe hypoxaemia with high ($AUC > 0.8$, $p < 0.0001$) (Fig. 5b, Table 5 Supplementary).

4 Discussion

This is the first study of its kind, exploring metabolic changes on the first postoperative day following cardiac surgery with the use of CPB in patients at-risk of developing pulmonary dysfunction with hypoxaemia. We have

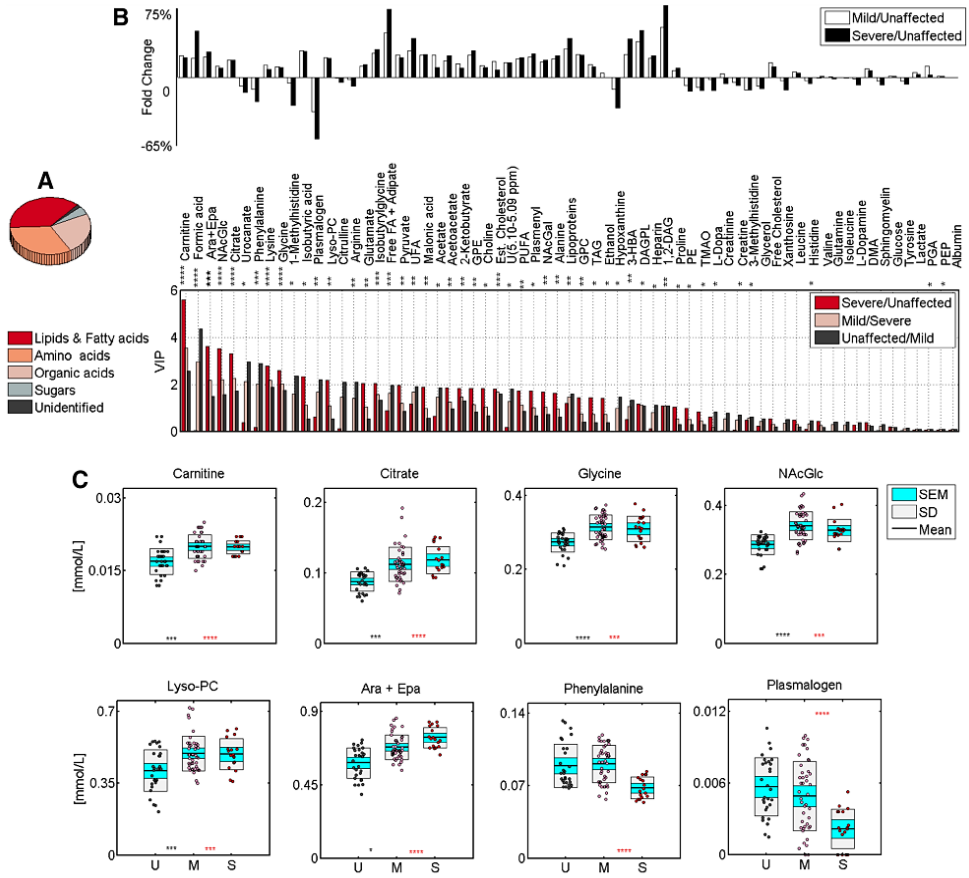

Fig. 3 Metabolic hallmark of hypoxaemia. **a** Metabolites found contributing to the progression into postoperative hypoxaemia. **b** The importance of each metabolite in the progression was assessed by variable importance in projection (VIP) scores. The greater the score, the more influential the metabolite; VIP scores > 1 indicate important metabolites in predicting later outcomes, whereas VIP scores < 0.5 indicate less important metabolites. Metabolic fold changes were calculated as percent change in mean between mild and severe vs. unaffected patients ((M − U)/U)*100), where M is mild, U is unaffected). Metabolite labelled with asterisk were found varying significantly between groups, by using ANOVA or its nonparametric analogue Kruskal–Wallis test (*$0.09 > p \geq 0.01$, **$10^{-2} > p \geq 10^{-3}$, ***$10^{-4} > p \geq 10^{-5}$, ****$p < 10^{-5}$). **c** Concentration distribution of some of the most discerning metabolites; *SD* standard deviation, *SEM* standard error of mean, *U* unaffected, *M* mild, *S* severe, *Asterisks black* significant difference between unaffected and mild, *red* significant difference between unaffected and severe. **d** Simplified metabolite pathways. Only identified and quantified metabolites are colored (*blue* higher concentration, *green* lower concentration in hypoxaemia compared to unaffected patients), whilst unidentified metabolites in the same pathways are in *grey*. *NAcGlc N*-acetylglucosamine, *Ara + Epa* arachidonic acid (Ara, 20:4 ω-6) and Eicosapentaenoic acid (Epa, 20:5 ω-3), *Lyso-PC* lysophosphatidylcholine, *FA* fatty acids; *UFA* unsaturated fatty acids, *GPE* glycerophosphoethanolamine, *Est.* esterified, *U* unidentified, *PUFA* polyunsaturated fatty acids, *NAcGal N*-acetylgalactosamine, *GPC* glycerophosphocholine, *TAG* triacylglycerol, *3-HBA* 3-hydroxybutyric acid, *DAGPL* diacylglycerophospholipid, *1,2-DAG* 1,2-diacylglycerol, *PE* phosphoethanolamine, *TMAO* trimethylamine-*N*-oxide, *PGA* phosphoglyceric acid, *PEP* phosphoenolpyruvate (Color figure online)

demonstrated that the metabolome changes so dramatically that the degree of hypoxaemia can be predicted at least 2 days before the clinical diagnosis.

NMR spectra (Fig. 1) and multivariate modelling accurately related perturbations in the metabolome recorded first morning postoperatively with later diagnostic

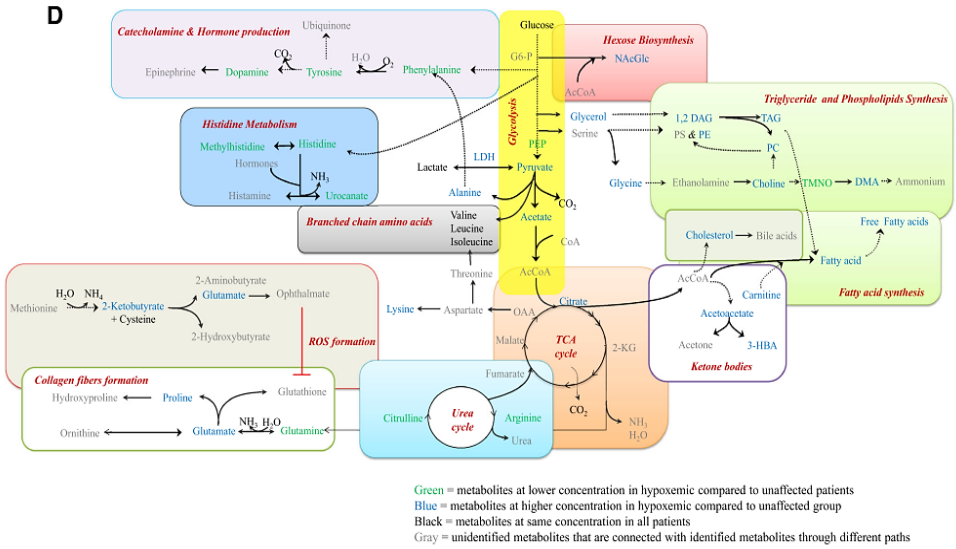

Fig. 3 continued

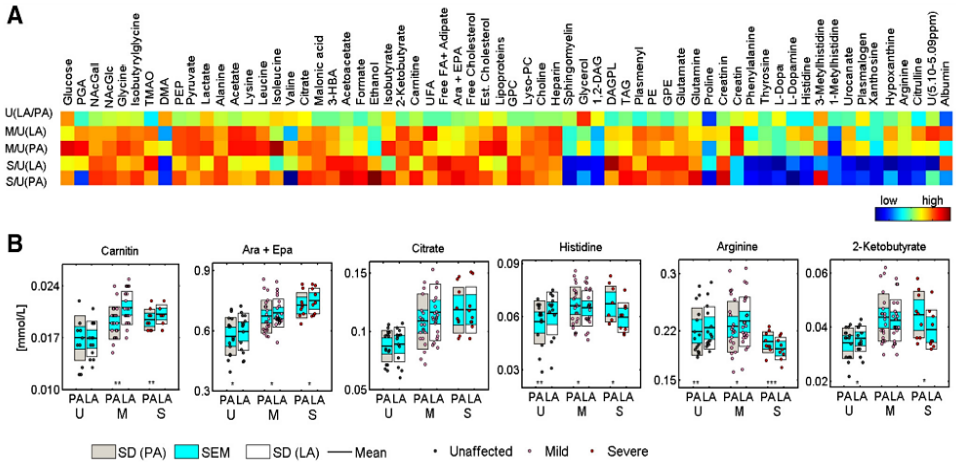

Fig. 4 Systemic versus pulmonary phenotypes. To understand what happens during the short passage from heart-to-lung-to-heart, the pair *t* test or its nonparametric analogue Wilcoxon-signed rank sum test (depending on the data distribution) were used to check for differences between left atrial (LA) and pulmonary artery (PA) samples in each group. **a** Heat map visualization depicts the metabolic abundances between LA and PA samples in the hypoxaemia groups compared to unaffected patients (*blue* higher levels in hypoxaemia compared to unaffected patients; *red* lower levels in hypoxaemia compared to unaffected patients). Of 64 metabolites, 43 varied between LA and PA samples in at least one group (Table 3, Supplementary). **b** For example, higher levels of carnitine and arachidonic acid were found leaving the lungs in both mild and severe hypoxaemia patients; while histidine and arginine were found to be consumed by the pulmonary system (lower levels in LA samples) in the severe acute lung injury group but not in the mild hypoxaemia nor in the unaffected group. Additionally, citrate was significantly increased in both mild and severe hypoxaemia groups compared to unaffected group, however, its levels were found unchanged when LA samples were compared to PA samples. Asterisks: $*0.09 > p \geq 0.01$, $**10^{-2} > p \geq 10^{-3}$, $***10^{-4} > p \geq 10^{-5}$. *SD* standard deviation, *SEM* standard error of mean, *U* unaffected, *M* mild, *S* severe; for metabolite abbreviations see Fig. 3 (Color figure online)

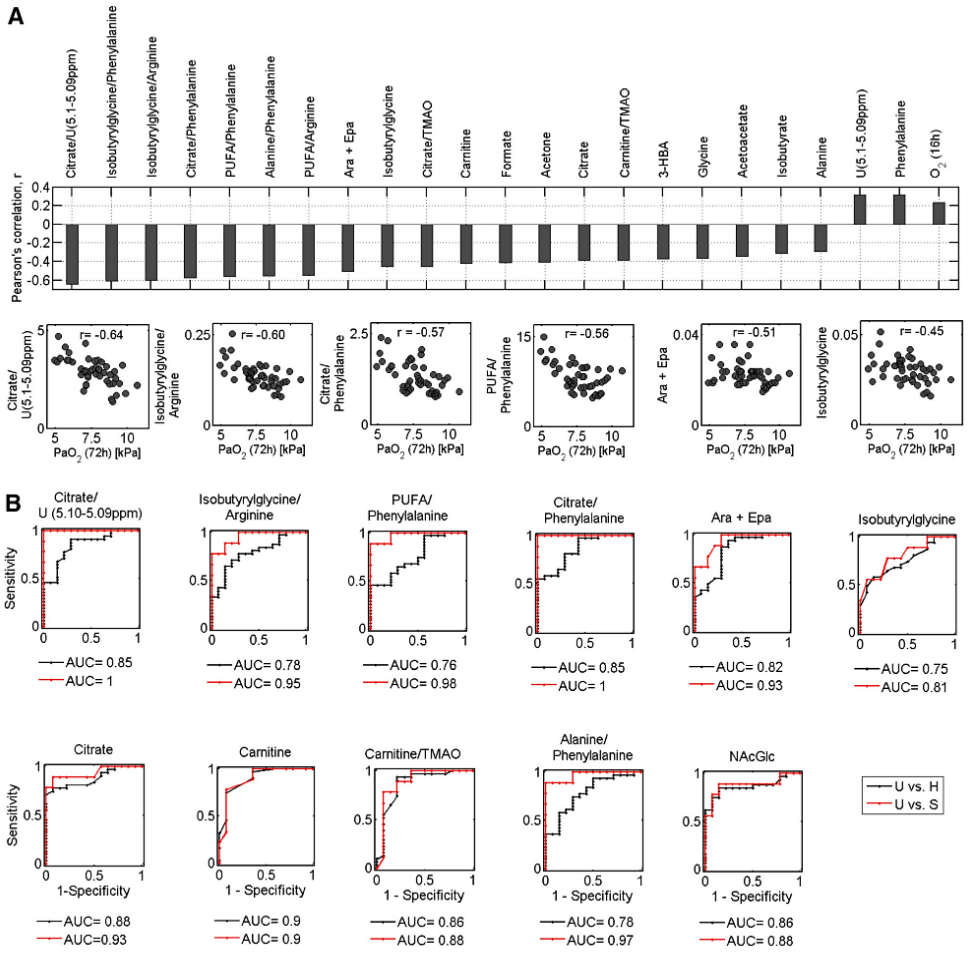

Fig. 5 Biomarkers predict progression into hypoxaemia after cardiac surgery 2 days before clinical diagnosis. **a** The relation between serum metabolites (either alone or as ratio between two metabolites) measured first morning (exactly 16 h) after weaning from CPB and the PaO_2 values measured on day 3 postoperative was calculated by Pearson's correlation coefficient (Pearson's r) (Table 4, Supplementary). Examples of scatter plots are illustrated, showing correlation between left atrial-derived metabolites and PaO_2 (Pearson's $r = 0.45–0.64$, $p < 0.001$). Additionally, the relation between blood O_2 measured 16 h and the PaO_2 measured on day 3 after surgery is shown, indicating no significant correlation (Pearson's $r = 0.264$, $p = 0.08$). (b) We calculated the area under the curve (AUC) for each biomarker candidate (Table 5, Supplementary), and, among others, citrate, phenylalanine, carnitine, TMAO, Ara + Epa, isobutyrylglycine, PUFA, alanine, and NAcGlc were identified as powerful predictive markers of later outcome. *U* unaffected, *H* hypoxaemia (all mild and severe patients), *S* severe. For metabolite abbreviations see Fig. 3

scores (Fig. 2a, b). It is noteworthy that the metabolome not only contained information about the future degree of oxygenation impairment 2 days later, but could also accurately differentiate mild and severe hypoxaemia from unaffected patients with 79.3 and 98.5 % sensitivity, and 96.6 and 92 % specificity, respectively (Fig. 2c). While some of these findings have previously been reported occurring in patients diagnosed with acute lung injury or acute respiratory distress syndrome (Evans et al. 2014; Serkova et al. 2008; Stringer et al. 2011), we report

metabolic changes occurring before the clinical signs of hypoxaemia.

From PA and LA samples, several metabolites were observed to vary between patients (Fig. 3 and 4). Of these, several markers significantly correlated with PaO_2 ($p \leq 0.05$) (Fig. 5a,b). These are markers of antioxidant status and peroxidation (histidine, plasmalogen, PUFA); ATP depletion and reactive oxygen species (ROS) formation (citrate); inflammation [arachidonic and eicosapentaenoic acid, 1,2-diacylglycerol (1,2-DAG)]; changes in osmotic and cell-volume regulation (glycine); membrane injury (phospholipids); and mitochondrial dysfunction (carnitine). The use of metabonomics predicting postoperative hypoxaemia with AUC and detection rates as described here has not been reported before.

The range of changes found in this study, from carbohydrates to amino acids, ketone bodies, FA, and phospholipids adds information to the present knowledge of the pathophysiology leading to hypoxaemia induced by an indirect insult to the lungs by cardiac surgery with use of CPB. The metabolic manifestations of mild and severe hypoxaemia compared to the unaffected patients appear to be similar, indicating the existence of common pathways. Therefore, knowledge of the core pathways and their interconnections may help us understanding the pathogenesis. The role of each metabolite and its connection to other metabolites in the human body is far from being fully understood. Nevertheless, in the following, we try to combine our observed alterations with possible roles of single metabolites into a model of intracellular processes leading up to hypoxaemia.

We observed altered metabolite levels down-stream of glucose metabolism. Pyruvate, alanine, and tricarboxylic acid (TCA) metabolite levels positively correlated with the severity of hypoxaemia. Their levels were higher in the pulmonary circulation (LA) compared to the systemic circulation (PA), indicating that pulmonary cells release these metabolites. This suggests an impaired oxidative phosphorylation in the lung tissue. In line with these observations, our results confirm previous studies conducted on plasma (Stringer et al. 2011) and bronchoalveolar lavage fluid (BALF) (Evans et al. 2014) samples collected from patients diagnosed with acute lung injury, in which the levels of TCA metabolites were found to be increased. Increased TCA levels were also found predicting the progression from SIRS to multiple organ failure in critically ill patients (Mao et al. 2009) and in predicting sepsis outcomes (Langley et al. 2013). Carnitine, normally found in mitochondria, helping FA delivery for β-oxidation, was significantly increased in LA samples of patients with hypoxaemia, which may also indicate impaired oxidative phosphorylation and a possible leakage into the bloodstream. Supporting this, isobutyrylglycine, a marker of impaired mitochondrial FA β-oxidation, was also associated with severity of hypoxaemia. Mitochondrial impairments enhance oxidative stress (Jana et al. 2013), causing alveolar cell death (Chow et al. 2003). Under homeostatic conditions, however, various antioxidants are capable of alleviating the damaging effects of ROS from the system (Scheibmeir et al. 2005), but an insufficient antioxidant barrier cannot counteract this damage. Low levels of the antioxidants histidine, urocanate, and plasmalogen in patients with severe hypoxaemia, in contrast to the mildly affected patients, may indicate an inefficient scavenging ability leading to a continued ROS presence, especially in the pulmonary microenvironment. The lower histidine levels in LA samples of the severely affected group may indicate an increased pulmonary demand to counteract peroxidation events. In addition, we found lower levels of the antioxidant hypoxanthine in patients developing hypoxaemia. Interestingly, previous metabonomics work conducted by Evans and co-workers on BALF of patients diagnosed with the severe lung injury form acute respiratory distress syndrome revealed a 41 fold increased in hypoxanthine levels compared to healthy subjects (Evans et al. 2014). Our results may be complementary to their findings, as hypoxanthine may diffuse in the lungs early in the stage of pulmonary dysfunction, and may accumulate later in the stage of full blown disease. To verify this hypothesis other studies involving both blood and BALF samples collected at different time points during disease progression should be performed.

It has been demonstrated that 2-hydroxybutyric acid is an early marker of increased lipid oxidation (Nishiumi et al. 2012). Membrane bound phospholipids and PUFA are susceptible to oxidation leading to the formation of highly reactive hydroperoxides (Crader and Repine 2012). These react with many biochemical substances having enormous impacts on normal cellular functioning, including endothelial activation and surfactant phospholipid disruption. In this study, the levels of PUFA and adipic acid (a byproduct of peroxidation (Jana et al. 2013) were found correlating with later pulmonary dysfunction, whereas plasmalogen (implicated in protection against ROS and peroxynitrite formation (Zoeller et al. 2002) levels were decreased, especially in the severe hypoxaemia group.

The ketogenic capacity (3-HBA, acetoacetate, and acetate) positively correlated with progression of hypoxaemia, and elevated levels are known to be indices of inadequate energy supply, or poor metabolism, leading to excessive triglyceride and fatty acid breakdown. This may partially explain the increased levels of circulating free FA, glycerol, triacylglycerol, and lipoproteins in both PA and LA samples of patients with hypoxaemia. Choline, phospholipids, 1,2-DAG, PUFA, and cholesterol are essential for structural integrity and cell membrane signaling. An

increase in their levels reflects an activation of the protective mechanism and a possible structural derangement in patients progressing into disease. Lipids are highly interconnected signaling molecules that regulate metabolic, innate immune and inflammatory processes, and alteration in one lipid will automatically trigger major deregulation in several signaling pathways causing profound physiological responses (Wymann and Schneiter 2008). The levels of arachidonic and eicosapentaenoic acids were higher in patients with hypoxaemia, especially in LA samples, indicating an increased inflammatory environment. These PUFA may be released by activation of phospholipase A_2, hydrolyzing membrane glycerophospholipids (Wymann and Schneiter 2008), which may also indicate cell membrane injury. According to Wymann and Schneiter, accumulation of diacylglycerols activates protein kinases, increases production of ROS and oxidative stress, and induces the synthesis of pro-inflammatory cytokines. Because one of the characteristics of this disease is dysfunction of alveolar surfactants, the increased levels of some phospholipids, especially in LA samples of hypoxaemia patients, may also be partly explained by surfactant leakage, which could indicate a damaged endothelial-alveolar barrier.

Interestingly, decreased arginine and citrulline levels were observed in the group developing severe hypoxaemia, especially in the pulmonary circulation. In fact, blood arginine depletion has previously been linked to ALI (Murakami et al. 2007; Naz et al. 2013), sepsis (Murakami et al. 2007) and cystic fibrosis (Luiking et al. 2005), and supplementation of arginine was observed to reduce inflammation (Murakami et al. 2007). The deficiency of arginine was not due to lack of metabolites involved in its synthesis, as lysine, glutamate, and proline levels were increased in these patients. During stress responses, blood lysine levels are known to increase to reduce oxidative stress (Jana et al. 2013). The increased levels of lysine observed especially in LA samples from hypoxaemia patients may reflect an attempt to counteract oxidative stress. In addition, dimethylamine (DMA) was elevated in sick patients. DMA is synthesized from asymmetric dimethylarginine, the endogenous inhibitor of NO synthesis, and an increased level of this metabolite may result in accumulation of ROS and reactive nitrogen species contributing to lung dysfunctions (Naz et al. 2013).

N-Acetylated carbohydrates involved in synthesis of glycolipids and glycoproteins (Michal and Schomburg 2012) were also found positively correlating with the disease. These acids are part of the pentose metabolism, which is known to be more active under stress (Zachara et al. 2004), especially under hypotonic stress conditions. Interestingly, the osmolytes counteracting cellular swelling (GPC, glutamate, and glycine) were elevated in hypoxaemia patients, probably indicating activated mechanisms involved in cell-volume regulation and the prevention of cell death (Naguro et al. 2012).

Thus, our findings suggest increased glucose metabolism, defects in the mitochondrial respiratory system affecting ROS generation, impaired antioxidant state, increased peroxidation and oxidative stress, cell membrane derangements, cellular swelling and cell damage in patients developing hypoxaemia.

It is important to bear in mind that some of above mentioned mechanisms are still not proven beyond doubt, hence the discussed metabolic considerations and consequences are somewhat hypothetical, yet plausible to explain important derangements leading to hypoxaemia. Based on our findings, the serum metabolome seems to reflect not only metabolic changes in the systemic but also in the pulmonary responses to later hypoxaemia. However, as these changes are minimal, they may not fully reflect the impairments occurring in the lungs. Including metabonomics analysis performed on BALF samples may therefore add further insights into the metabolic changes in the lungs.

Regarding our study design, there are some limitations. The small sample size, especially in the severe hypoxaemia patients, may bias the results. The fact that we achieved similar results in LA and PA samples offers to some extent confidence in data validity, however, the results must be verified in larger studies. We used serum samples for the analyses. Since the coagulation processes take some time, the samples were left half an hour at ambient temperature before centrifugation. In this period metabolic changes may have occurred. However, all samples were treated equally and, therefore, the differences between samples can be expected to reflect the true changes in the patients. In addition, blood metabolite levels might not fully reflect changes in intracellular metabolism. Regarding the choice of methodology, despite the robustness and informative nature of NMR, the technique suffers from low sensitivity and signal overlap impeding identification of metabolites occurring at low concentrations, and therefore, some potentially important changes were not detected. In the future, collecting different biological matrices and analyzing samples by both NMR and e.g. mass spectrometry-based approaches would add more insights to the pathogenesis of postoperative pulmonary dysfunctions.

5 Conclusion

In conclusion, by mapping metabolic perturbations at an early stage, we have identified metabolite hallmarks of hypoxaemia, which may bridge the gap between pathogenesis and full-blown disease. Markers such as

arachidonic and eicosapentaenoic acid, citrate, carnitine, glycine, phenylalanine, arginine, and histidine serve as central nodes in their metabolism and therefore have a high impact in predicting disease progression. No single metabolite captured the complexity of hypoxaemia alone, as different pathways simultaneously showed imbalances. Therefore, to prevent early progression into hypoxaemia, therapeutics targeting several pathways may be more effective.

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Compliance with ethical standards

Conflict of interest R.G.M, B.S.R., M.A.H., S.R.K., S.P. and R.W. have filed a patent application for some of the metabolic biomarkers described in the manuscript and for an algorithm predicting the condition from experimental data.

Ethical approval The study was approved by the regional ethical committee (N-20080016).

Informed consent After oral as well as written informed consent was obtained, patients scheduled for elective coronary artery bypass grafting (CABG) were included.

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Supplementary materials at http://link.springer.com/article/10.1007/s11306-016-1018-5 (11306_2016_1018_MOESM1_ESM.docx (75360)) containing additional information regarding: (1) identified metabolites; (2) metabolic differences between unaffected and hypoxaemic patients 16 hours post-CPB; (3) correlation between metabolites and PaO_2 measured 72 hours postoperatively.

Appendix B. Study II

Rasmussen B.S., Maltesen R.G., Pedersen S., Kristensen S.R. *Early coagulation activation precedes the development of acute lung injury after cardiac surgery*; Thrombosis Research Volume 139, March 2016, Pages 82–84

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Letter to the Editor

Early coagulation activation precedes the development of acute lung injury after cardiac surgery

Keywords:
Acute lung injury
Cardiac surgery
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Fibrinolysis

Dear Editors,

The triggers of acute lung injury (ALI) are numerous, and it is a well-known and potentially life threatening complication to cardiac surgery using cardiopulmonary bypass (CPB). Irrespective of the initial triggering factor, the alveolar-capillary barrier becomes compromised thus impairing the gas exchange [1,2]. The pathogenesis of ALI is not fully elucidated, and insight into the processes inducing disease progression is needed [2]. Neutrophils and inflammation have been shown to have an important role in pathogenesis of ALI and several biological pathways may be affected contributing to the development of the condition, e.g. coagulation [2–4]. The injury involves activation of the endothelial cells leading to a pro-coagulant endothelium and of coagulation with fibrin formation. Fibrin is important for tissue repair, but may also be deleterious if excessive [5]. An increased coagulation and decreased fibrinolytic activity in bronco-alveolar lavage of patients with the most severe form of lung injury, the adult respiratory distress syndrome (ARDS) have been described [5–7]. If coagulation is an important pathophysiological factor, markers of coagulation activation could potentially be able to predict progression into ALI.

The aim of this study was to investigate changes in the haemostatic activity in patients at risk of developing ALI, i.e. patients undergoing cardiac surgery with CPB. In order to do this, we measured two markers of coagulation activity, Prothrombin Fragment 1.2 (F12) and thrombin–antithrombin complexes (TAT) and two fibrinolytic markers, D-dimer and plasmin–antiplasmin complex (PAP) in the early postoperative period. F12 is cleaved from prothrombin after thrombin formation, while TAT is formed subsequently when thrombin is inactivated by antithrombin. D-dimer is a product of fibrin degradation, while PAP is a marker for plasmin formation.

Abbreviations: ALI, acute lung injury; ARDS, adult respiratory distress syndrome; CPB, cardiopulmonary bypass; FiO_2, fraction of inspired oxygen; F12, Fragment 1.2; ICU, Intensive Care Unit; kPA, kilopascal; LA, left atrium; mmHg, millimetre of mercury; PA, pulmonary artery; PaO_2, partial pressure of arterial oxygen; PAP, plasmin–antiplasmin complex; TAT, thrombin–antithrombin complex.

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1. Methods

1.1. Patients

From a larger study investigating fifty patients undergoing cardiac surgery with the use of CPB (study no. NCT02475694), ten patients were selected based on their ratio of partial pressure of arterial oxygen and fraction of inspired oxygen (PaO_2/FiO_2) measured 72 h after weaning from CPB and x-ray. All patients were discharged from intensive care within the first 48 h. The five patients with the lowest PaO_2/FiO_2, all below 27 kPa (200 mmHg), were defined as the 'ALI' group and the five patients with the highest PaO_2/FiO_2, all above 40 kPa (300 mmHg), were defined as the 'non-ALI' group. Heparinisation during CPB was followed measuring active clotting time (The Hemochron Signature Elite, Vindmed, Denmark). Patient demographic data is shown in Table 1.

1.2. Blood samples

Blood was sampled and collected in citrated tubes (109 mmol/L) through catheters placed in the left atrium (LA) and pulmonary artery (PA) at seven different time points: before connection to CPB, right after weaning from CPB (0 h), and 2, 4, 8, 16, and 20 h thereafter. Samples were centrifuged at 3220 *g* for 10 min, and plasma stored at −80 °C. Plasma concentrations of TAT, F12, D-dimer and PAP were determined by dedicated ELISA-kits (Siemens, Marburg, Germany) performed on a SpectraMax.

1.3. Statistical analysis

The statistical package SPSS (IBM, Armonk, NY: IBM Corp.) and MATLAB (R2011b) were used for analysis and visualisation. Non-parametric tests were applied due to the low number of patients in each group. Changes in the coagulation markers over time were determined by Kruskal–Wallis test, while changes across the lungs (i.e. PA versus LA) were compared by means of Wilcoxon sign rank test. In addition, Spearman's rank correlation test was used. $p < 0.05$ was considered significant.

2. Results

The demographic data showed more female patients present in the ALI group and a trend towards longer CPB and cross clamp time in ALI patients. There was no significant difference between the two groups regarding the amount of heparin given during CPB. The active clotting times showed complete reversal of the effect of heparin obtained by administration of protamine.

The time courses of the four markers measured in blood from LA are shown in Fig. 1a–d. TAT and F12 (significantly) and D-dimer (borderline significant) showed increases in patients developing ALI compared to non-ALI patients (Fig. 1a–c), whereas PAP profiles are similar in the

Table 1
Patient demographic data. Wherever possible, data is present by the group median and the range values (minimum, maximum). Mann–Whitney U (2-tailed) and chi-square test were used to calculate the significance between groups. Abbreviations: ALI = acute lung injury; n = number of patients; CPB = Cardiopulmonary Bypass; IU = international unit; min = minutes; mg = milligram; ICU = Intensive Care Unit; sec = seconds.

Characteristics	ALI (n = 5)	Non-ALI (n = 5)	*p*-Values
Age (years)	62 (48, 66)	68 (58, 74)	0.17
Gender (male/females)	1/4	5/0	0.01
Body mass index	27.06 (22.5, 37.5)	29.2 (26.9, 30.6)	0.25
Smoking (Yes/No)	2/3	1/4	0.22
CPB time (min)	76 (55, 118)	57 (41, 84)	0.12
Cross clamp time (min)	49 (34, 92)	37 (28, 69)	0.10
Heparin (IU per mL)	41,500 (37,500, 55,000)	48,500 (40,000, 55,000)	0.46
Protamine (mg)	280 (250, 300)	300 (250, 350)	0.21
Active clotting time after Protamine (sec)	116 (96, 125)	106.7 (106, 110)	0.24
Active clotting time at arrival to ICU (sec)	115.7 (107, 123)	113 (108, 133)	0.91
Albumin before surgery (g/L)	26 (23, 32)	27 (20, 30)	0.84
Albumin after surgery (g/L)	16 (14, 17)	18 (11, 20)	0.31
Fresh frozen plasma (Yes/No)	0/5	1/4	0.32
Erythrocytes (Yes/No)	2/3	0/5	0.13
Thrombocytes (Yes/No)	1/4	1/4	1.00

groups. In ALI patients, plasma concentrations of TAT raised steeply immediately after weaning from CPB, declining after 2 h, the F12 concentrations increased immediately after CPB, reaching maximum within the first 2 h postoperatively, and D-dimer also increased significantly immediately after CPB. In non-ALI patients these changes were more moderate. For the PAP measurements a more delayed response was observed, but similar between the groups with a maximum 4–8 h postoperatively. There was a modest correlation between time of CPB or cross clamp and D-dimer (correlation coefficient $r = 0.50$, $p = 0.02$ and $r = 0.38$, $p = 0.06$, respectively), but no significant correlations were observed with the other markers, nor with heparin dosage.

When analysing the differences across the lungs, similar patterns were observed for LA and PA samples. A trend towards higher levels of TAT, F12, PAP, but not D-dimer was detected in LA, indicating their release from the pulmonary microvasculature.

3. Discussion

This small pilot study shows that the coagulation activity, as measured by TAT and F12, was increased immediately postoperatively in patients developing ALI. In addition, D-dimer, as a measure of fibrin degradation, was elevated indicating that more fibrin was formed. However, the fibrinolytic activity measured as PAP, a marker for the amount of

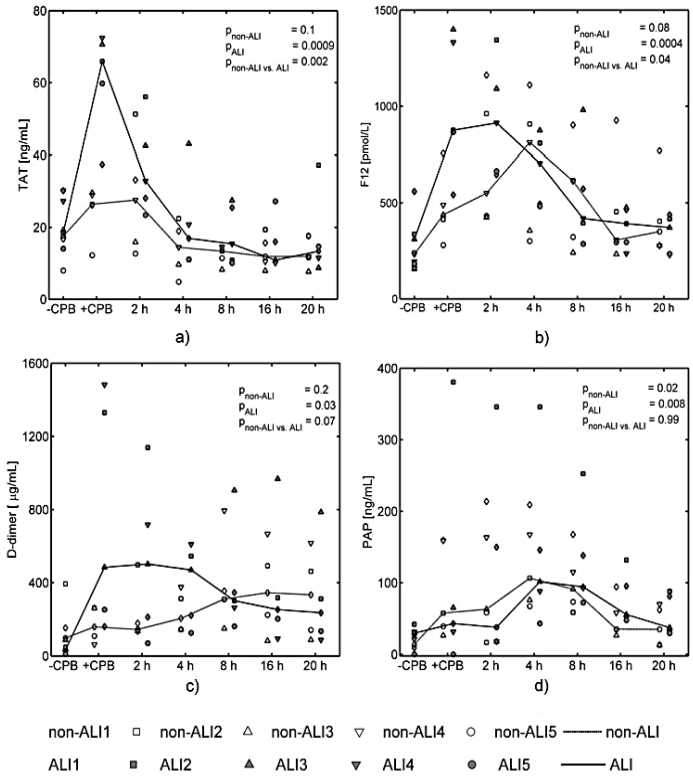

Fig. 1. A time course comparison of markers of coagulation (a. TAT; b. F12) and fibrinolytic activity (c. D-dimer; d. PAP) observed in patients progressing into ALI ($n = 5$) and patients not developing the condition (non-ALI; $n = 5$) compared to their baseline levels measured before cardiopulmonary bypass (CPB). Changes across the seven time points were assessed by means of Kruskal–Wallis test. Abbreviations: TAT = thrombin–antithrombin complexes; F12 = Fragment 1.2; PAP = plasmin–antiplasmin complex; −CPB = before CPB; +CPB = after weaning from CPB (0 h).

plasmin formed, was at the same level in both groups, indicating that the fibrinolytic activity may be insufficient to remove the increased amount of fibrin in ALI patients.

F12 and TAT differ in their half-life in plasma (longer half-life for F12), and this may impact the time course illustrated in Fig. 1a,b. The level of D-dimer reflects both an increased formation of fibrin and a subsequent fibrinolytic cleavage of fibrin. Thus, the increased levels in ALI patients are concurrent with the increase of TAT and F12, since D-dimer is rather a marker of an increased level of fibrin than an increased level of fibrinolysis. The marker of fibrinolytic activity, PAP is not different in the two groups indicating that the coagulation activity in patients developing ALI seems amplified and not counteracted by a simultaneously increase in fibrinolysis. A deteriorated balance between coagulation and fibrinolysis may be present which may lead to an increased amount of fibrin and microthrombi in the capillaries of the lungs causing disturbance of blood flow and subsequently affecting gas exchange [8].

However, there was also a trend towards a difference in CPB and cross clamp times between the groups. It cannot be excluded that the procoagulant response is influenced by this difference, since there was a modest correlation between these durations and D-dimer. CPB times are known to be a risk factor of ALI [9] probably due to increased lungs ischemic time, but the coagulation activity may also be exaggerated during the prolonged surgery. In addition, more female patients were present in the ALI group. However, gender is not supposed to influence the coagulation response. Regarding heparin, no correlation was observed between heparin doses and the coagulation markers. Finally, two ALI patients received blood transfusion indicating more bleeding, but this would probably tend to lower the difference (loss of markers).

The changes shown here took place after CPB, but may probably begin during the surgery, whereas the nadir values of arterial oxygenation will appear on the second to third postoperative day. Moreover, fibrin may serve as a sump for certain anti-inflammatory proteins and thereby activate the inflammatory process further [5]. Idell et al. [6] reported that procoagulant activity was increased and fibrinolytic activity depressed in broncho-alveolar lavage fluid in patients diagnosed with ARDS. A similar finding was observed by Günther and co-workers [7] in a larger group of ARDS-patients. Prabhakaran and co-workers [10] found that an increased level of plasminogen activator inhibitor-1 in lung oedema fluid corresponding to a low level of fibrinolysis identified ALI patients with a high sensitivity and specificity. Hofstra and co-workers [11] found increased TAT and D-dimer and low fibrinolytic activity in lavage fluid from patients after burn injuries and inhalation trauma. Recently Abrams and co-workers [12] found that circulating histones able to activate coagulation may mediate trauma-associated ALI. All these findings are in accordance with our results.

The coagulation activation presented here is in fact discerned at an earlier stage than previously reported; therefore, these markers may have potential in early detection of the development of ALI. Cardiac surgery with CPB is a major trauma to the body and the present study indicates that an imbalance between coagulation and fibrinolytic activity affects the gas exchange in the lung. Perhaps a more aggressive anticoagulant treatment might attenuate the following development of ALI.

A limitation of this pilot study is the small number of patients used to envisage trends of potential changes. Larger studies including more risk factors may elucidate whether the procoagulant response is causal or just an epiphenomenon.

In conclusion, the present results indicate that an imbalance between coagulation and fibrinolytic activity following CPB may be part of the complex mechanism leading to development of ALI. Therefore, markers for these processes may potentially serve as early indicators of an increased risk of ALI.

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22 September 2015

Appendix C. Study III

Metabotyping Patients' Journeys Reveals Early Predisposition to Lung Injury after Cardiac Surgery

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Running Title: Progression to Lung Injury
Key words: cardiopulmonary bypass; surgical stress; predisposition; biomarkers; lung injury; hypoxaemia; time series analysis; NMR; multivariate data analysis.
Reference counts: 57
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Authors' contribution:
RGM executed the experimental part, processed the data, performed data analysis, identified metabolites, interpreted the results, wrote and revised the manuscript. B.S.R., S.P., and R.W. came up with the main research idea. B.S.R. has designed the main study, collected the samples, and performed the blood gas analyses. R.W. set up the NMR experiments and supervised acquisition. All authors assisted in drafting the manuscript and approved the final document.

Abstract

Cardiac surgery with the use of cardiopulmonary bypass is a major risk factor leading to postoperative lung injury. We hypothesized that the surgical procedure elicits metabolic activity that can be related to the development of postoperative hypoxaemia. Here we charted metabolic activity in fifty consecutive patients undergoing cardiac surgery, by analysing nearly 1500 nuclear magnetic resonance spectra run on blood samples collected at nine different time points, from the day before surgery to the third postoperative day. We integrated global systemic and pulmonary metabolic information by analysing samples from the radial artery, pulmonary artery, and left atrium. By metabotyping patients' journeys from before surgery to the third postoperative day, when hypoxaemia was observed, we could determine the impact of cardiac surgery on the human metabolome, and identify some of the triggering mechanisms preceding the progression to lung injury. The surgical procedure induced increases of circulating anaerobic metabolites, and decreases in choline-containing compounds, fatty acids, ketones, and several amino acids, with slight differences between the global and pulmonary metabolite profiles. In addition, the duration of surgical trauma impacted the levels of metabolites produced or consumed by the lungs. The results indicate that patients progressing to hypoxaemia may produce greater metabolic derangements after traumatic stress. Hence, we propose that reduced metabolic adaptability, which cannot meet the metabolic challenges of the surgical procedure, may lead to pulmonary dysfunction and hypoxaemia.

1. Introduction

Cardiovascular disease is society's number one health problem, being the leading cause of death in many countries [1, 2]. Annually worldwide, nearly one million patients undergo coronary artery bypass grafting (CABG) surgery with the use of cardiopulmonary bypass (CPB)[3]. Even after uncomplicated procedures, some patients experience a systemic inflammatory response and ischemia-reperfusion lung injury. Lung injury consists of a series of pathophysiological changes, including pulmonary edema, decreased lung compliance, disturbance in the ventilation-perfusion ratio, and increased pulmonary vascular resistance. Finally, in worse cases, this leads to severe postoperative hypoxaemia and acute respiratory distress syndrome (ARDS).[4-6]

Since postoperative lung injury extends hospitalization and increases various complications, as well as mortality[6, 7], it is of interest to identify at-risk patients early. Determining the progression into hypoxaemia is challenging, as no early diagnostic test exists[8]. Early measurements of the partial pressure of arterial oxygen (PaO_2) - used to assess the degree of lung injury, and thus, of hypoxaemia[9-11]- has shown poor predictive value for later outcomes[12]. Because the nadir values of PaO_2 appear on the second to third postoperative day[13, 14], it is difficult to predict which patients will develop lung injury[15].

Currently, there is no proven treatment option[7, 16] and no molecular-driven intervention[17-19] to prevent disease progression. When lung injury is present, supportive care and conservative fluid management are essential[19]. Therefore, an understanding of the risk factors and molecular mechanisms may help preventing hypoxaemia[6]. A patient's medical history before cardiac surgery (e.g. age, health state, and smoking habits)[4, 20, 21], previous cardiac surgery[22], the surgical procedure itself (general anaesthesia, sternotomy, atelectasis, and the use of CPB)[4, 23-26], and blood transfusion[22, 27] are well known risk factors of postoperative lung injury. Polymorphisms in the pro-inflammatory interleukin-encoding genes of IL-6 and IL-18 have been shown to predispose patients to CPB-induced ARDS[28, 29]. In addition, increased circulating free fatty acids two hours after CABG have been identified as being early signs of postoperative hypoxaemia[6]. In line with these findings, we have recently shown that it is possible to predict PaO_2 measured the third day postoperatively from a blood sample collected on the first postoperative morning[30]. Using nuclear magnetic resonance (NMR) spectroscopy and multivariate data analysis it was possible to predict the degree of hypoxaemia with high accuracy (>79% sensitivity, >92% specificity). A pattern of disturbed metabolism was found, of which changes in ketones, amino acids, and lipid metabolism were dominant. Metabolites were related to the pathogenesis of hypoxaemia: damaged alveolar-capillary barrier, edema formation, peroxidation, oxidative stress, impaired antioxidant defense, and cell damage. [30]

While these molecular mechanisms are crucial for the early prognostication of at-risk patients, there is still need for a better understanding of the molecular reasons as to why certain patients develop lung injury, while others do not. Hence, the aim of this study was to investigate the time course of metabolic events, from the start of the operation, to the clinical symptoms of hypoxaemia observed on the third postoperative day. Furthermore, since a patient's history prior to surgery is an important risk factor[4, 20, 21], we wanted to investigate whether it was possible to find a certain 'metabolic biosignature' that made certain patients prone to develop hypoxaemia.

We have used a metabonomics approach, since it aims to find insights into the actual metabolic phenotype (metabotype[31]) of diseases, and the causes of their progression[32]. NMR spectroscopy combined with complex multivariate data analyses was used as the methodological approach, due to the advantages over classical biochemical assays. First, it is a rapid (within a few minutes[31]), reproducible, and relatively cheap technique per sample basis[33]. Second, it measures multiple metabolites simultaneously, with little or no sample preparation[34]. Finally, NMR provides structural and quantitative metabolic information[35], providing evidence for identifying deranged mechanisms[36]. Since we have serial sampling, each patient served as its own control. This allowed us to create an individual 'metabolite journal' and to follow each metabolite profile from before surgery to the day of diagnosis.

2. Methods

2.1. Patient population and sample collection

Fifty consecutive patients scheduled for CABG with the use of CPB at Aalborg University Hospital, Denmark, were included in this study, after obtaining informed consent (NCT02475694). Inclusion criteria were adults above 18 years of age and on treatment with statins. Exclusion criteria were treatment with steroids or other immune suppressor therapies.

Patients underwent an overnight fast and standardized anaesthetic, surgical and perfusion management. Immediately after anaesthesia, a pulmonary artery (PA) catheter was inserted, and after sternotomy, a left atrium (LA) catheter. Paired blood samples were collected simultaneously at baseline (before CPB), right after weaning from CPB (0 hours), and at 2, 4, 8, and 20 hours after weaning from CPB (when the catheters were removed). Three patients were unable to provide samples at 20h postoperatively since their LA catheters were displaced. A total of 594 blood samples were collected and serum was obtained through standard hospital protocols. In addition, three patients were unable to provide samples the second and third day postoperatively, hence, a total of 144 blood samples were drawn from the radial artery the day before surgery, and at 48 and 72 hours after weaning from CPB, and

plasma was obtained. Both serum and plasma aliquots were stored at -80^{o}C until analysis.

Postoperatively, patients were treated with supplementary oxygen to achieve peripheral oxygen saturations above 95%. The clinical diagnosis of hypoxaemia was based on the ratio between PaO_2 and the fraction of inspired oxygen (PaO_2/FiO_2), calculated from PaO_2 measured in arterial blood samples collected 48 and 72 hours after weaning from CPB. In order to standardize the measurements, arterial blood samples were taken while patients had been spontaneously breathing atmospheric air for 10 minutes.

2.2. Sample preparation

Before NMR analysis, samples were thawed for 30 min. at 4°C, vortexed, and subsequently centrifuged for 5 min at 12100g and 4°C. A total of 400µL of the clear supernatant was mixed with 200µL 0.2M phosphate buffer (pH 7.4, 99% $^{2}H_2O$, 0.3mM DSA-d_6 (1,1,2,2,3,3-hexadeutero-4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate)) in a 5mm NMR tube. Mixture pH was 7.4±0.04. During the whole process samples were kept on ice.

2.3. NMR experiments

Nearly 1500 one-dimensional ^{1}H NMR spectra were recorded on a BRUKER AVIII-600 MHz NMR spectrometer (BrukerBioSpin, Rheinstetten, Germany) equipped with a cryogenically cooled, triple-resonance CPP-TCI probe, at a temperature of 298.1K (25°C). Spectral acquisition was controlled using the TopSpin 3.1 software (Bruker BioSpin).

T2 filtered Carr-Purcell-Meiboom-Gill (CPMG)[37] experiments with water presaturation were acquired with the following parameters: 65536 data points over a spectral width of 20 ppm; 256 scans for serum and 128 scans for plasma samples; 32 dummy scans; a fixed receiver gain (RG) of 203; and a relaxation delay (d1) of 4s, during which presaturation of the water resonance was achieved by continuous irradiation at $\gamma B_1/2\pi$=25 Hz. T_2 filtering was achieved with a repeating τ-180°-τ pulse sandwich with τ=300µs, repeated 256 times for serum samples and 128 times for plasma samples for a total of 80 and 40ms, respectively.

To achieve more in-depth information about sample lipoprofiles, a one-dimensional diffusion-edited pulse sequence was used, with the following parameters: 65536 data points; 30 ppm spectral width; 128 scans; RG= 114; and d1= 4 s, during which, presaturation of the water resonance was achieved by continuous irradiation at $\gamma B_1/2\pi$=25 Hz. Diffusion filtering was achieved by inserting a stimulated-echo element into the pulse sequence between excitation and detection (BRUKER standard pulse program ledbpgppr2s1d). The diffusion time Δ, during which water

presaturation was effective, was 120ms, and bipolar sine-shaped gradients of 52.5 G/cm and 1.5ms length were used (δ=3ms) for diffusion encoding. An Eddy-current delay of 5ms before acquisition was used.

2.4. Data processing

Spectral processing was carried out in TopSpin 3.1. FIDs were exponentially multiplied, corresponding to a line broadening of 0.3 Hz (CPMG) and 1 Hz (diffusion-edited), Fourier transformed, phase and baseline corrected, and calibrated (to the chemical shift of the methyl signal of L-alanine at 1.48 ppm for CPMG spectra, and to the methyl signal of N-acetylglucosamine at 2.04 ppm for diffusion-edited spectra). Spectra were reduced to buckets of 0.001 ppm width, and the water region between 4.65 and 4.95 ppm was excluded, using AMIX software (Analysis of MIXtures, v.3.9.10, Bruker BioSpin, Germany).

Data was then exported to MATLAB R2011b[38]. The binned data was generalized log transformed[39] to enhance small signals in the spectrum, normalized to either the DSA-d_6 peak intensity (CPMG) or to total intensity (diffusion-edited), and mean centred.

2.5. Multivariate data analysis

Data analysis was performed in Matlab R2011b and SPSS (IBM® Statistics v.22).

For multivariate analysis both unsupervised principal component analysis (PCA) and supervised partial least-square regression (PLS) and discriminant analysis (PLS-DA) were applied using the PLS-Toolbox 6.5 (Eigenvector Research, Wenatchee, WA). PCA was applied, to find the main source of variation within the data, to check population homogeneity, and to identify outliers based on samples' metabolic similarities and dissimilarities. PLS regression was applied to establish early metabolome associations with later outcome. NMR data were regressed to PaO_2 values measured 72 hours postoperatively. In order to find spectral regions that correlating significantly with later PaO_2 values, the reverse interval-PLS (riPLS) approach was applied. Regions found to correlate with PaO_2 were then used to classify patients according to their diagnosis. For each classification model, a receiver operating characteristic (ROC) curve, and a sensitivity and specificity were obtained. This information was used to evaluate the metabolome's ability to predict later hypoxaemia.

For supervised modelling, a ten-fold Venetian-Blinds cross-validation (CV) was employed. This validation involves: omitting 10 out of 100 samples from model development; developing parallel models from the reduced data; predicting the omitted samples; and comparing the predicted and actual values to provide an estimate of the model's overall predictive power. For the PLS regression models, the

overall predictive power was assessed by the cross-validated root mean square error (RMSECV) obtained from predicting PaO_2 values. For PLSD-DA, the CV sensitivity and specificity values were used. To ensure that no random model performed equally well, or better, than the main PLS and PLS-DA models, permutation testing was also performed. Here we scrambled the PaO_2 values and group labels (hypoxaemia/no-hypoxaemia) 500 times and subsequently performed multivariate modelling. The 'true' optimal PLS and PLS-DA models were then compared to the distribution of the permuted models, and significances were calculated using Wilcoxon's sign rank test. A p-value < 0.004 was considered to be significant.

Models were visualized using scores and loadings plots. Each score represents a sample, while each loading represents the variation in a specific spectral region. Thus, the sum of all loadings determine the molecular signature or metabolic 'fingerprints' of a patient. The orientation of each loadings variable describes the up- and down-regulation of the corresponding bucket containing metabolite information. Spectral regions contributing to sample clustering were identified and quantified.

2.6. Metabolite identification

For the identification process, ^{1}H shifts and their corresponding ^{13}C signals were analysed by running several 2D ^{1}H-^{1}H total correlation spectroscopy (TOCSY) and ^{1}H-^{13}C heteronuclear single-quantum correlation (HSQC) spectra. These signals were matched to The Human Metabolome Database[40], Bruker Bbiorefcode Database (v. 2.7.0), and literature[30, 41, 42].

For metabolite quantification, the NMR peaks were integrated by using the line shape analysis option in the AMIX Multi Integration tool.

2.7. Further analysis and data representation

Metabolites and PaO_2 levels are presented as mean ± standard deviation (SD) and percent change in tables, and as bar-plots. Percentage changes were calculated by the formula: $(Y-X)/X_{*}100$; where Y represents a sample collected at a time point different from its corresponding baseline sample, and X represents the baseline sample (pre-CPB for serum LA and PA samples; the day before surgery for plasma samples collected from the radial artery).

Several comparisons were undertaken on the data. First, we compared serum metabolic profiles recorded at six different time points (before CPB, 0, 2, 4, 8, and 20 h post-CPB) (Figure 1a,b). Second, the metabolic profiles from before, and right after weaning from CPB (0h) were compared in both PA and LA samples (Figure 1c,d and Table 2). Third, the impact of the duration of the surgical procedure on the

human metabolome was evaluated (Figure 2a-c). Fourth, patients' metabolic journeys, from the day before surgery to the third postoperative day were analysed (Figure 3a,b). Finally, the metabolome's ability to predict hypoxaemia at an early stage was assessed (Figure 4a-f), and the metabolites that were associated with the development of later lung injury were quantified and graphed (Figure 5a,b).

Because patients received nutritional support on days two and three, and since plasma and serum matrices are biologically different in terms of the levels of several amino acid and phospholipid[43-45], we chose to differentiate between plasma and serum results, and to show results as percent changes. Hence, only the significant results obtained from plasma samples are presented in the result section (Figure 3a), while additional results is presented in the supplementary part.

Differences between hypoxaemic patients and unaffected patients were evaluated by the χ^2 test for discrete clinical variables, and by the t-test for continuous variables. Differences in paired LA and PA intensities of each metabolite, and differences in paired samples collected before commencement of CPB and just after weaning from CPB (0 hours), were evaluated with the paired t-test or Wilcoxon signed-rank test, depending on the fulfilment of the normality assumptions. The interactions between time-, disease-, and LA/PA-dependent metabolic changes were determined by factorial analysis of variance (ANOVA), with Tukey's post-hoc test for multiple comparisons.

Tests of correlation were performed by calculating the Pearson correlation coefficient (r_p). The 2-sided Fisher exact test was used to determine differences in frequency distributions. Statistical significance was defined as a p-value ≤ 0.05.

3. Results

3.1. Patient characteristics

Patient characteristics, surgical variables, and the PaO_2 levels are given in Table 1. Decreased arterial PaO_2 levels were observed on the second and third postoperative days compared to the day preceding surgery (Supplementary Figure S1). Forthy eight percent of patients suffered hypoxaemia on the second, and 64% on the third, postoperative day (PaO_2/FiO_2< 40kPa). Because oxygenation worsened in some patients, whilst it improved in others, between the second and third days (r_p= 0.49, p= 0.0003), we defined patients' outcome based on the third day's PaO_2 values. Eighteen patients did not develop hypoxaemia ('unaffected'), while thirty-two patients experienced hypoxaemia, of which nine suffered severe oxygen impairments ($PaO_2/FiO_2\leq$ 30kPa).

There was no significant difference between the groups in age, body mass index, and smoking habits, however, gender was slightly different (p= 0.01). There was no

difference in the perioperative fluid balance (1.97± 0.9L, p= 0.50), or medication, between groups. No patient received glucose infusion during the surgical procedure. The duration of surgery and aortic cross-clamp (ischemia), but not of CPB, was different between groups (p< 0.04).

3.2. Metabotyping the patient journey

Each patient's metabolic journey from the surgical procedure through the intensive care unit (ICU) was phenotyped. Spectra run on serum collected at six different time points, before CPB and through 20h post-CPB, revealed changes in the levels of lipids, ketones, and several amino acids (Figure 1a). These changes were consistently seen across all patients.

PCA performed on LA and PA samples, collected at the six time points, showed patients clustering according to the time at which samples were collected (Figure 1b). Samples collected during surgery (pre-CPB, black; 0h post-CPB, grey) clustered along the second principal component (PC2); while postoperative samples migrated from 2h (orange) to 4h (purple) to 8h (light blue) and to 20h (blue green) along PC1, indicating a possible circadian effect on metabolism. While the 20h sample scores approached perioperative samples, they did not overlap. By 48h, samples were still not co-mapping preoperative values; while by 72h sample scores had moved towards baseline values (Supplementary Figure S2), suggesting that the metabolome had almost recovered from surgery.

3.2.1. The surgical procedure affects the metabolome

To investigate how the surgical procedure influenced the human metabolome, a PCA was performed on 200 LA and PA samples collected during surgery (pre- and post-CPB at 0h). Samples clustered according to the time of collection (Figure 1c), suggesting that CPB induced changes in metabolite levels. Postoperatively, the levels of circulating glucose, pyruvate, alanine, lactate, citrate, and creatine increased, while the levels of phospholipids, free fatty acids (free FA), polyunsaturated fatty acids (PUFA), choline-containing compounds, lipoproteins, glycerol, ketones (3-hydroxybutyric acid, acetate, acetoacetic acid), and several other amino acids decreased (Figure 1d, Table 2).

Similar changes were observed in both PA and LA samples, with a few exceptions (Table 2). Lactate increased by 33% while pyruvate by 61% in PA samples post-CPB; in comparison, their levels were more elevated in the LA samples. The purine metabolites including inosine, hypoxanthine, uric acid, and xanthine decreased, especially in the PA samples, suggesting their release from the lungs. In contrast, citrate and N-Ac-glycoprotein fragment levels were more elevated in the PA samples, suggesting their consumption by the lungs. The levels of ketones, including 3-hydroxybutyric acid (3-HBA), acetoacetic acid and acetate, and the branched-

chain amino acids (BCAA) leucine and isoleucine had similar increases in LA and PA samples. Most lipids and fatty acids showed similar decreases in both PA and LA, except for diacylglycerophosphocholine (DAGPL), which was lower in LA samples, suggesting its utilization by the lungs.

3.2.2. The effects of prolonged surgical stress

Since the time on aortic cross-clamp and CABG was slightly different between hypoxaemic and unaffected patients, we evaluated how prolonged procedures affected the metabolome. PLS regression, analysing samples collected at 0h and 2h against the duration of CPB, and CABG showed moderate cross-validated coefficient of association (R_{cv}) (Figure 2b, Supplementary Table S1), suggesting that prolonged surgical procedures stressed the metabolome.

Lactate, pyruvate, and acetate levels positively correlated with the duration of ischemia and CPB at 0h, and with the duration of CABG at 2h, suggesting their increased production with the length of surgery. Glycine, alanine, and glutamine concentrations correlated with the time of CPB and cross-clamp exclusively at 0h. Arginine, isoleucine, and 3-methylhistidine negatively correlated with cross-clamp and CABG time, indicating their utilization with prolonged surgical stress. Acetoacetate and 3-HBA negatively correlated with the duration of CPB and cross-clamp at 0h, and positively correlated with the duration of CABG at 2h post-CPB. The tricarboxylic (TCA) cycle intermediates fumarate and malate, and the purine metabolites inversely correlated with the surgical time. Finally, ethanol correlated with the duration of CABG, indicating increased antiseptics with prolonged surgical time.

The associations did not persist for more than 2-4 hours post-CPB, suggesting a normalization of the metabolome after longer procedures.

3.2.3. The post-CPB period

Postoperatively, several metabolites recovered their pre-CPB levels within the first two-four hours; however, most metabolites continued changing until 20h. In fact, their levels had not returned to baseline at 48h (Figure 3a). Glycolytic and TCA cycle metabolites were mostly elevated in the early postoperative period, but returned towards baseline levels in the following 20-48h. The levels of purine metabolites, nicotinic acid metabolites (trigonelline and tryptophan), tyrosine metabolites (tyrosine and L-dopa), histidine, and uridine were low post-CPB, and few of them reached their pre-CPB levels within 8-20h. In contrast, adenine, phenylalanine, 3-methylhistidine, glucuronate, and N-Acetyl glucosamine (N-Ac-Glc) were increased postoperatively, and their levels continued to rise, even after 48 and 72h. BCAA, lysine, arginine, proline, glutamine, glutamate, glycine, taurine, and trimethylamine-N-oxide (TMAO) levels were reduced even after 72h.

Most fatty acid and lipid concentrations decreased immediately after surgery; however, steep increases occurred after 2h post-CPB. Free fatty acids (free FA) levels decreased by 30% at the end of CPB, and increased to 2-fold pre-CPB levels after 2h at the time sedation was changed from inhalation anaesthesia to intravenous propofol administration. Increases in their levels were observed even after propofol administration was stopped at 4h postoperatively. The same trends were observed for polyunsaturated fatty acids (PUFA), di- and triacylglycerol (DAG, TAG), and lipoproteins. On the other hand, lipoic acid concentrations decreased continuously after CPB, to 70% at 20h post-CPB, and its signals vanished at 48h. Choline-containing compounds including diacylglycerophospholipid (DAGPL), glycerol-phosphocholine (GPC), and phosphatidylcholine (PC) were all found to be decreased after surgery, even at 72h postoperatively, except for choline and sphingomyelin (SM), which were found to be elevated.

With respect to differences between PA and LA samples, in general there was a similar trend in both samples (Figure 3b). While the levels of some metabolites were different at baseline (pre-CPB), their levels were similar in the following 4-8h, and returned to the baseline differences afterwards. Phenylalanine, nicotinic acid metabolites, most purine metabolites, tyrosine metabolites, histidine, 1-methyl-histidine, uridine, glucuronate, lactate, pyruvate, acetone, cholesterol, and DAG were all elevated in LA samples in at least one time point, indicating their release from the lungs. Tryptophan, hypoxanthine, ascorbate, and DAGPL were elevated in PA samples in at least one time point, indicating their utilization by the lungs.

3.2.4. Phenotyping patients' journeys revealed predisposition to lung injury

Due to the vast changes observed, we explored the potential diagnostic value of the metabolome for the early detection of hypoxaemia.

PLS regression analysis carried out to map metabolome onto arterial PaO_2 values obtained 72h postoperatively showed moderate association (R^2_{cv}= 0.71) already with samples taken after sternotomy but before CPB (Figure 4a, Supplementary Table S2). The validity of using the metabolome for accurately distinguishing hypoxaemic from unaffected patients was investigated by building and cross-validating a discrimination model based on these samples (Figure 4b). PLS-DA revealed 77.8% sensitivity and 84.2% specificity towards differentiating later outcomes (Figure 4c). Model robustness was assessed by randomly permuting each patient label 500 times and performing the modelling. The real model, based on samples collected after sternotomy, outperformed the permuted models (p= 0.004), and gave us confidence in the results.

A larger association, sensitivity, and specificity were observed when analysing post-CPB (0h) samples (Figure 4d-f, Table S2), indicating that the CPB and cross-clamp

procedures played significant roles in the development of lung injury. Models performed on samples collected at later time points confirmed these results (Table S2).

The metabolites found to differentiate hypoxaemic from unaffected patients are summarized in Figure 5a. We divided hypoxaemic patients according to their PaO_2 levels into a mild ($8.4>PaO_2>6.3$ kPa) and a severe group ($PaO_2 \leq 6.3$ kPa) to emphasize the level of impairment and the degree of later lung injury. Most discriminatory metabolites were involved in purine metabolism, nicotinic acid metabolism, methylhistidine metabolism, tyrosine metabolism, glycine and isobutyrylglycine metabolism, fatty acid and lipid metabolisms, and N-Acetyl-glucosamine metabolism (Figure 5b). When analysing samples taken just after sternotomy, purine metabolites, nicotinic acid metabolites, and 3-methylhistidine levels inversely correlated with later PaO_2 measurements; while tyrosine, L-dopa, glycine, and isobutyrylglycine levels positively correlated to the degree of oxygenation. The patterns within most of these metabolites continued to be different between groups, suggesting their potential as early markers of postoperative lung injury. Moreover, the levels of glycerol discriminated patients at time points 0 and 2h, with highest levels in the severe hypoxaemia group. The levels of free fatty acids, phospholipids, and lipoproteins discriminated patients at time point 4h, and their levels were inversely correlated with PaO_2. N-Acetyl glucosamine, pyruvate, alanine, glucuronate, histidine, glucose, lactate, creatine, creatinine, urea, arginine, valine, and isoleucine also differentiated patients in at least two of the measured time points postoperatively.

4. Discussion

The aims of this study were to investigate the impact of cardiac surgery on human metabolome and to highlight possible mechanisms involved in the progression of postoperative lung injury defined by hypoxaemia.

The design of the study allowed us to specifically focus on the immediate metabolic effects of the surgical procedure, and to determine whether a metabolite was produced, consumed, or just transiently passing through the lungs. Analyzing samples from nine time points (before, during, and after the operation) allowed us to create a metabolic journal and follow each patient through recovery from surgical trauma or towards progression into lung injury. Since each patient was their own control, potential confounding factors arising from different study populations and surgical units were minimized.

There are several significant findings of the present study. Firstly, NMR spectroscopy detected immediate and striking changes in the metabolite profiles of both PA and LA samples as a consequence of cardiac surgery (Figure 1, Table 2). Secondly, prolonged ischemia, and duration of CPB and CABG impacted the

metabolome (Figure 2, Supplementary Table S1). Thirdly, postoperatively, the metabolome changed as a consequence of its attempt to reestablish homeostasis (Figure 3). Fourthly, the intraoperative metabolome indicated a possible predisposition to lung injury (Figure 4, Supplementary Table S2), allowing us to identify a 'metabolic biosignature' (Figure 5) that made certain patients more prone to develop hypoxaemia.

4.1. The intraoperative period

During the surgical procedure the beating of the heart and the pulmonary ventilation was stopped, the lungs were allowed to collapse, and the blood flow through the lungs was limited. For this period, CPB took over the functions of the heart and lungs, pumping oxygenated blood through the body. Since we collected blood after sternotomy, but before CPB, and right after weaning from CPB (0h), it was possible to explore the impact of CPB on the human metabolome.

We observed dramatic and immediate shifts in the metabolome. PCA showed clear separation between pre- and post-CPB samples (Figure 1c), indicating a global metabolic stress response to the procedure (Figure 1d, Table 2). Because the surgical procedure was an important trigger for the later progression of hypoxaemia, we focus here on explaining the possible metabolic derangements found during the procedure.

Our findings are consistent with a general reliance on the anaerobic metabolism of glucose to generate ATP; a switch from net lactate extraction to lactate release; a compromised TCA cycle activity; a fall in ketone extraction; and a dysregulation of fatty acid oxidation. Previous smaller studies on surgical ischemia-reperfusion[33, 46, 47] have reported similar responses to surgical trauma. In comparison to these studies, we have detected a wider range of metabolites, have analysed both the systemic and pulmonary responses to CPB, and have linked these changes to the progression to lung injury. Hence, this study gives us new insights into the metabolite responses to surgery.

As patients did not receive glucose under the surgery, increased glucose levels could be due to decreased cellular uptake, a common signature of surgical trauma[23, 33, 46]. During ischemia and hypoxia, intracellular lactate accumulates and pH decreases, and hence, to avoid acute cellular swelling and cell death, lactate must be released into the circulation[33, 46]. Hence, the increased lactate levels indicate that cells experienced a post-CPB ischemic environment, which was more aggravated in the pulmonary microcirculation. Simultaneously increased pyruvate levels suggest that pyruvate was diverted away from the pyruvate-dehydrogenase reaction towards the anaerobic lactate-dehydrogenase reaction, and towards alanine formation. Decreased acetate levels in both PA and LA samples indicate that the acetyl-coenzyme A derived from fatty acid oxidation and ketone biosynthesis was compromised. We

observed a ~17% decrease in the total ketone levels, a ~30% decrease in fatty acids and cholesterol levels, and a 30% increase in citrate levels, indicating that the acetyl-coenzyme A, which was still being produced from the ongoing fatty acid oxidation and ketone production, was being used strictly by the TCA cycle. Decreased ketone levels[46] and inhibition of fatty acids[48] have previously been related to ischemia-reperfusion, suggesting a deficiency in fatty acid oxidation.

We observed that glycerol and DAG levels increased, while several phospholipids levels decreased after weaning from CPB. A trend towards more released glycerol and decreased phospholipids was noticed in the LA samples, suggesting that the lungs were actively involved in these processes. Glycerol release has previous been linked to ischemia-reperfusion injury[48]. Glycerol is the phosphoglyceride fraction of phospholipids and the backbone of DAG, which is stored intracellularly or in the interstitial space. Hence, its release may originate from three different paths: the degradation of membrane phospholipids, hydrolysis of glycerol-3-phosphate produced during anaerobic glycolysis, and/or lipolysis of intracellular DAG. Considering these multiple pathways, increases in glycerol and DAG levels are part of a process that indicates potential cellular ischemia-reperfusion injury during CPB.

A previous study performed on ischemic and hypoxic hearts has shown a 50% reduction of phosphatidylcholine in the ischemic and a 22% reduction in the hypoxic heart[49]. We found a 17% reduction in choline and PC; a 19% reduction of DAGPL in PA, and 25% in LA samples; and a 7% reduction in GPC. These findings suggest severe impairments in their biosynthesis during CPB.

At the end of CPB we also found increased levels of N-acetyl glycoproteins, which have previous been linked to increased inflammation after cardiac surgery[47]. In addition, the levels of several amino acids were deranged at the end of CPB. One of the most interest findings was that the levels of all purine metabolites were decreased. These metabolites are by-products of adenosine triphosphate (ATP) metabolism, and are known to accumulate during ischemia due to reduced oxygen and energy supply. To compensate, cells degrade ATP to adenosine, inosine, and hypoxanthine. During reperfusion, when oxygen is reintroduced, xanthine oxidase converts hypoxanthine to uric acid and produces highly reactive hydrogen peroxides and hydroxyl radicals, which can further react with several molecules in the cell, including membrane phospholipids and PUFA[25, 50]. In another study, increased purine levels were observed during ischemia, and by 10 minutes post-clamping, their levels had decreased significantly[51]. We did not sample at multiple time points during the ischemic period. However, we observed an inverse correlation between purine levels and the duration of cross-clamp (Figure 2c), indicating that shorter ischemic periods may increase their levels, but as the time on cross-clamp continues, purines may be used in ongoing processes. Apart from purine metabolites, we also observed that during prolong surgical procedures more anaerobic glycolytic compounds were produced, while arginine, isoleucine, and 3-methylhistidine were

consumed. Arginine and isoleucine are essential amino acids involved in numerous processes, while 3-methylhistidine is mainly involved in muscle metabolism. Their decreases with the duration of ischemia may indicate an increased demand in their utilization that exceeded their rate of production.

4.2. The postoperative period

Retaining homeostatic metabolism is an energy demanding process involving many metabolites[48]. We observed complex changes in the postoperative period. The striking picture that emerges is one of profound impairment in the levels of lipids and metabolic fuels, likely reflecting a severe deficiency in energy metabolites, impaired TCA cycle, unbalanced pyruvate metabolism, and deranged ketone-, nicotinamide-, purine-, and hyaluronic acid metabolism (Figure 3a).

Some metabolites recovered pre-CPB levels by 2-4h postoperatively; however, most metabolites continued changing until 20-72h. We noticed that several metabolites were elevated in either LA or PA samples during surgery (Figure 3b); however, their levels were similar 2-4h postoperatively, and return to previous differences afterwards, indicating the start of a possible systemic and pulmonary normalization.

One of the most interesting trends was the observation of decreased levels of several lipids and fatty acids at the end of CPB, and their steep increase following surgery, even several hours after propofol administration was stopped. The depletion may be explained by a combination of impaired biosynthesis, hemodilution occurring during CPB, and possible entrapment by the oxygenator filters; while the steep increases postoperatively may partially indicate the effect of propofol, but also possible lipolysis and cell membrane damage. In addition, glucuronate, formed by glucose oxidation, was increased significantly until 72h. Glucuronate is involved in the degradation and elimination of xenobiotics[40] and in the synthesis of hyaluronic acid, along with N-acetyl-glucosamine[52], which has been found to be involved in pulmonary diseases and lung homeostasis[53]. N-acetyl-glucosamine positively correlated with glucuronate, and since their levels were slightly elevated in LA samples, it may indicate a possible hydrolysis of the hyaluronic acid within the lungs. In addition, purine metabolism increased post-CPB, and reached baseline levels at 8-20h, suggesting improved aerobic conditions. Finally, several amino acids changed in the postoperative period. The increases in certain amino acids might reflect impaired protein synthesis and gluconeogenesis, enhanced proteolysis, or decomposition of proteins from skeletal muscles, which are all commonly observed after surgical trauma[54]. On the other hand, the decreases in certain amino acids levels may indicate their consumption as energy metabolites. It is worth mentioning that on the second and third postoperative day patients received nutritional support, and hence, some of the changes observed may have been affected by nutrition.

4.3. The metabolome's link to the development of hypoxaemia

Metabonomics has previously been applied to patients exposed to major surgery[54-56], and the approach has shown potential in predicting: systemic inflammatory responses and multi-organ dysfunction syndromes[54], graft failure after kidney transplant[56], and drug-toxicity after liver transplantation[55]. As with these studies, we showed that metabolome has great potential in predicting the development of postoperative lung injury already during the surgical procedure.

PLS regression analysis performed on samples obtained after sternotomy, but before CPB, showed a 0.71 cross-validated association between the metabolome and arterial PaO_2 values measured 72h postoperatively (Figure 4a). The association increased by 0.2 units (R^2_{cv}= 0.92) when using samples taken after weaning from CPB, indicating that the CPB and cross-clamp itself had significantly contributed to the development of later hypoxaemia.

The metabolites found to be affected by prolonged CPB and cross-clamp time were analysed for possible association to hypoxaemia. At 0h, pyruvate and alanine levels, which correlated with CPB and cross-clamp time, were also found to associate with later outcomes (Figure 5), indicating their links with surgical trauma and the development of postoperative lung injury. Glycine, found to correlate the duration of cross-clamp, had already increased in patients developing hypoxaemia after sternotomy. Interestingly, its levels continued to be discriminative of later outcomes even at 20h postoperatively, indicating that glycine may be a good biomarker candidate of lung injury. The reason for its pre-CPB elevation is still unknown, since we did not collect samples after induction of anaesthesia and before sternotomy. Therefore, further studies are needed to confirm and explain its elevation. In addition, ketone metabolites inversely correlated with the length of CPB and showed decreased levels in hypoxaemic patients. Finally, purine metabolites were negatively correlated to the length of cross-clamp and decreased in patients developing hypoxaemia, indicating impairments in their syntheses.

Several metabolites were found to be different between the groups, regardless of time on bypass. Free FA, MUFA, TAG, PUFA, lipoproteins, and cholesterol had all discriminative value after weaning from CPB. In fact, increased circulating free fatty acids have recently been reported as possible predictors of hypoxaemia at 2 hours after CABG ($r = -0.367$, $p < 0.001$)[6]. We also report these changes straight after weaning from CPB, however, the changes became more significant 2-4h post-CPB, confirming previous findings. N-Ac-glucosamine levels increased postoperatively in all patients, and its levels were positively associated with the development of severe hypoxaemia after 8h. In addition, the nicotinic acid metabolites significantly decreased in hypoxaemic patients. Tyrosine metabolism (tyrosine, L-dopa) was decreased in all patients, however, hypoxaemic patients had smaller decreases compared to unaffected patients. Tyrosine metabolism has previously been related to

ischemia-reperfusion injury and tissue damage[57], and hence, their relatively elevated levels in hypoxaemic patients may indicate more ischemia-reperfusion injury and more tissue damage in these patients. Finally, 3-methylhistidine, a marker of inflammation[57], was found increased with time in all patients, however, its levels were lower in patients in patients developing hypoxaemia at all time points, probably indicating its utilization by the inflammatory cells.

Taken together, these findings suggest that patients progressing to hypoxaemia have a limited metabolic reserve with regard to ATP production, excessive activated lipolysis after surgery, and are more prone to produce an exaggerated stress response to CABG. These results confirm our previous findings regarding predictive biomarkers of lung injury based on samples collected at 16h post-CPB[30]. In addition, we found that metabolites involved in purine-, nicotinic acid-, tyrosine-, hyaluronic acid- metabolism were deranged at an early stage, and that the development of hypoxaemia can be predicted earlier than anticipated.

4.4. Study limitations

Several practical limitations must be acknowledged. Although our study is the largest of its type published to date, sample sizes are still relatively small and involve comparisons between multiple groups. In addition, since the study was performed on 50 consecutive patients undergoing CABG, we could not match for sex differences; hence, other studies must be performed to address gender differences in relation to metabonomics and postoperative lung injury. In addition, since we did not have a new set of samples to validate our results, the validation of the identified metabolic biosignature for the progression into hypoxaemia requires further studies with larger populations.

Another important limitation is the lack of samples collected before sternotomy. Under normal conditions, organisms utilize several metabolites as fuel substrates for ongoing processes, including glucose, lactate, amino acids, ketones, and FA; hence, having arterial samples just before the induction of anaesthesia and sternotomy would provide us with information about how the metabolome reacted to these procedures. In addition, we did not have serum samples on the day before, and the second and third day postoperatively. Because the levels of metabolites such as phospholipids and amino acids are different in serum and plasma, direct comparison could not be achieved for some metabolites, and hence, we reported changes over time in percentages.

5. Conclusion

The present study is an attempt to elucidate the pathways of early progression to postoperative lung injury. It was our aim to improve understanding of the mechanistic underpinnings of lung injury, and to pave way for future research.

To the best of our knowledge, this is the first metabonomics study demonstrating the link between intra- and postoperative time-dependent metabolite changes and the later development of postoperative hypoxaemia. We found a unique metabolic signature that clearly discriminated unaffected from hypoxaemic patients at least 48h in advance. Also, the results indicate that metabotyping patients' journeys early, during or just after the end of surgery, may have potential impact in the hospitals for the early diagnosis of postoperative lung injury, and for the monitoring of therapeutics targeting disease progression.

Figures and Tables

Table 1 Clinical and procedural characteristics of the study population. **Independent t- and chi-square test were used to calculate the significance between groups. Annotations: n, number of subject; SD, standard deviation; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CPB, cardiopulmonary bypass; PaO_2, partial pressure of oxygen in arterial blood; FiO_2, fraction of inspired oxygen (21%); Surgery, from skin incision to last suture.*

	All (n= 50)	**Hypoxaemia (n= 32)**	**Unaffected (n= 18)**	**p-value***
Subject characteristics				
Age, mean (range), years	65.8 (48-83)	65.3 (48-83)	66.5 (48-81)	-
Male, n (%)	41 (82)	23 (72)	18 (100)	0.01
BMI, mean ± SD, kg/m^2	27.5 ± 0.2	27.2 ± 0.2	28.0 ± 0.3	-
Diabetes mellitus, n (%)	14 (28)	10 (31)	4 (22)	-
Smokers(yes/no/unknown)	16/30/4	11/19/2	5/11/2	-
COPD, n (%)	7 (14)	4 (13)	3 (17)	-
Surgical procedure, mean ± SD, min				
Surgery	196 ± 50	207 ± 52	177 ± 38	0.04
Time on CPB	64 ± 29	67 ± 33	57 ± 21	0.23
Cross clamp (Ischemia)	33 ± 19	37 ± 21	25 ± 11	0.03
72 hours post-CPB, mean (range), kPa				
PaO_2	7.8 (10.7-4.9)	6.9 (8.4-4.9)	9.2 (10.7-8.6)	<0.0001
PaO_2/FiO_2	37.0 (51- 23.3)	33.1 (40-23.3)	43.9 (51-41)	<0.0001

Figure 1

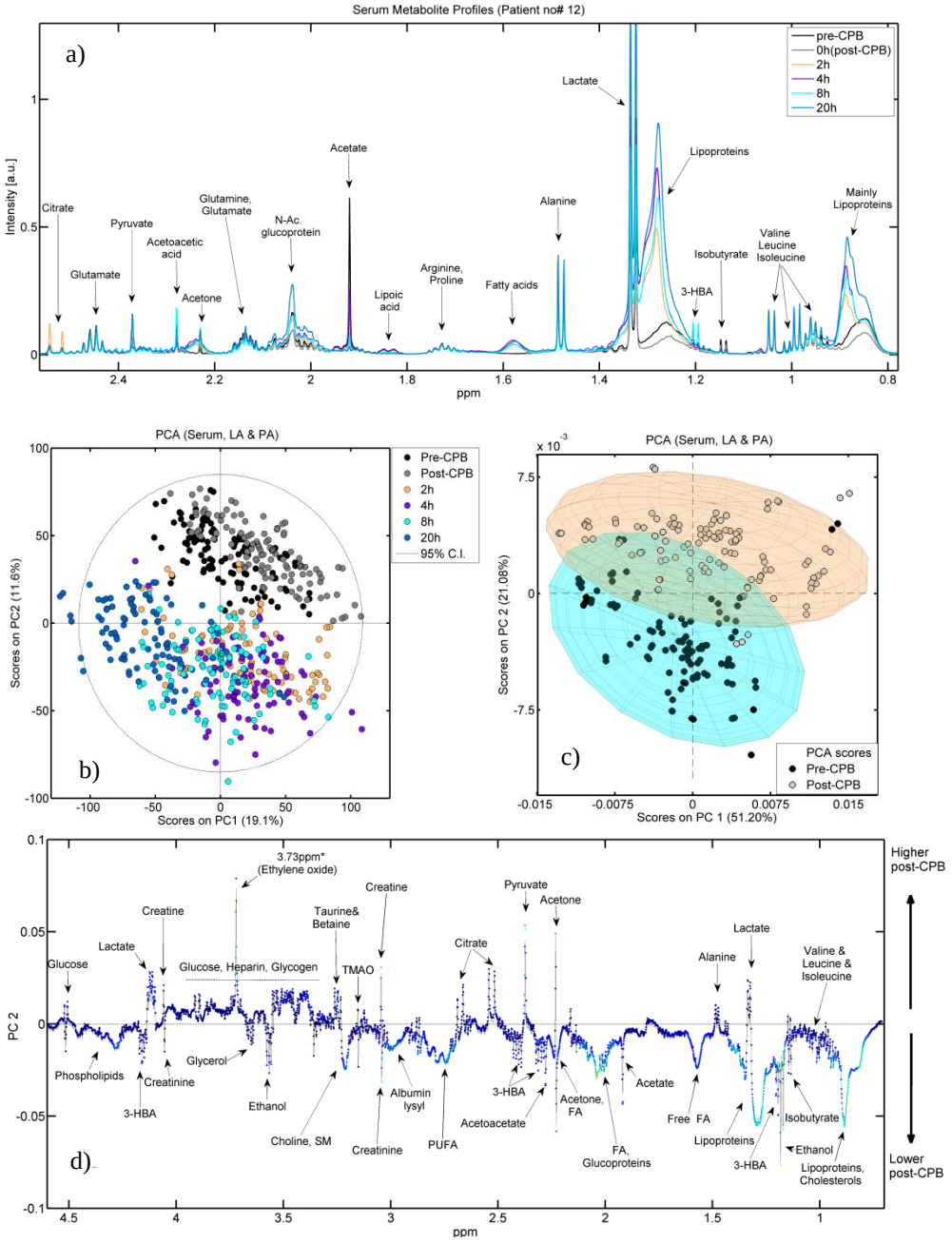

Figure 1 Metabotyping patients' journeys. (a) Representative ^{1}H-NMR CPMG spectra of serum collected after sternotomy but before cardiopulmonary bypass

(CPB), just after weaning from CPB (0 hours), and 2, 4, 8, and 20 hours postoperatively. (b) Principal component analysis (PCA) scores plot of 594 serum samples collected at six different time points. (c) PCA performed on samples collected before and after CPB. (d) The corresponding loadings plot shows metabolite composition on PC2. Abbreviations: 1D, one-dimensional; CPMG, Carr-Purcell-Meiboom-Gill; 3-HBA, 3-hyroxybutyric acid; FA, fatty acids; PUFA, polyunsaturated fatty acids; PaO2, partial pressure of oxygen; kPa, kilopascal; ppm, parts per million; a.u., arbitrary units.

Table 2

Table 2 ***Metabolite changes as a consequence of CPB.*** *Metabolite levels found in pulmonary artery (PA) and left atrial (LA) samples collected before and after cardiopulmonary bypass (CPB). Mean change in percent was calculated using the formula: (postCPB - preCPB) / preCPB)*100. Abbreviations: MUFA: mono-unsaturated fatty acids, DAG: diacylglycerol; PEP: Phosphoenolpyruvate; N-Ac-Gal: N-acetyl-galactosamine; DAGPL: diacylglycerophosphocholine; PC: Phosphatidylcholine; GPC: Glycerophosphocholine; SM: Sphingomyelin; 4.60ppm*: unassigned metabolite; 3-HBA: 3-hyroxybutyric acid.*

Metabolites	**Pre-CPB**						**Post-CPB**	
	PA (µmol/L)		**LA (µmol/L)**		**PA**		**LA**	
	Mean	± SD	Mean	± SD	Change %	p-val	Change %	p-val
Metabolome	648.3	92.5	640.6	91.7	8	0.01	8.1	0.01
Inosine	10.2	2.5	10.1	2.6	-18	0.000	-14.4	0.003
Hypoxanthine	13.9	1.6	13.7	1.9	-21.1	0.000	-20.3	0.000
Tryptophan	140.4	27.6	140.2	26.8	-22.2	0.000	-24.7	0.000
1-Metylhistidine	80.8	13.1	79	14.2	-10.1	0.001	-7.4	0.03
Uric acid	50	9.1	49.1	8.5	-7.9	0.03	-6.2	0.08
Xanthine	96.6	12.6	95.8	12.9	-16.2	0.000	-15.7	0.000
MUFA	786.3	213.3	771.9	202.2	-25.1	0.000	-24.5	0.000
DAG	43.6	8.8	44.5	10.4	12.7	0.003	14.6	0.003
PEP	174.5	54.1	174.2	50.1	7.5	-	10.3	0.060
N-Ac-Gal	209.8	85.3	205.9	98.8	21.3	0.02	15.8	0.10
β-Glucose	5043.2	1616	5015	1554.1	39.5	0.000	38	0.000
4.60ppm*	141.9	42.6	134.7	37.8	-24.9	0.000	-27.2	0.000
DAGPL	222.3	48.9	217.3	47.5	-19.1	0.000	-25	0.000
PC	531.8	73.9	520.4	75.3	-16.6	0.000	-17.7	0.000
GPC	137.4	15.6	134.9	15.8	-7.1	0.001	-7.4	0.001
Lactate	1495.3	585.7	1466.7	557.7	32.8	0.000	35.8	0.000
Glycerol	4870	1820	4763.5	1801	10	0.08	11.3	0.06
α-Glucose	2144.8	643	2127.3	619.9	35.9	0.000	36.1	0.000
Choline, SM	793.4	140.3	788.6	132.9	-17.5	0.000	-17.4	0.000
Creatine	104.5	15.8	105.8	16.3	32.8	0.000	31.4	0.000
PUFA	543.5	145.3	535.5	140.1	-26.8	0.000	-26.7	0.000

Citrate	101.7	13.9	100.9	12.9	30.2	0.000	27.8	0.000
Pyruvate	43.6	16.7	41.9	16.5	61.3	0.000	67.3	0.000
3-HBA	168.3	45.9	167.1	45.7	-17.4	0.001	-17.2	0.001
Acetoacetate	120	50.4	120	50.8	-22.6	0.004	-23.1	0.003
Acetate	100.3	42.5	98.9	36.8	-19.9	0.060	-18	0.080
Lysine	489.2	45.9	484.8	47.5	-3.3	0.10	-3.2	0.12
Arginine	337.4	38.1	334.5	38.4	-6.1	0.009	-5.9	0.010
Free FA	515.2	199.4	494.9	175.8	-30.2	0.000	-29.4	0.000
Alanine	482.8	92.9	475.7	88.8	15.1	0.000	15.8	0.000
Ethanol	492.1	199.3	490.8	223.4	-46.3	0.000	-45.2	0.000
Isobutyrate & Carnitine	32.1	4.7	31.6	5.2	-7.4	0.01	-6.8	0.03
Isoleucine	116.8	17.3	116.2	18.4	-14.4	0.000	-14.2	0.000
Leucine	275.3	37	272.5	38.2	-7.5	0.008	-6.9	0.020
Lipoproteins	3575.7	852.6	3512.4	812.3	-30.4	0.000	-30.2	0.000
Cholesterol	50.6	12.4	50.2	11	-34.5	0.000	-32.2	0.000

Figure 2

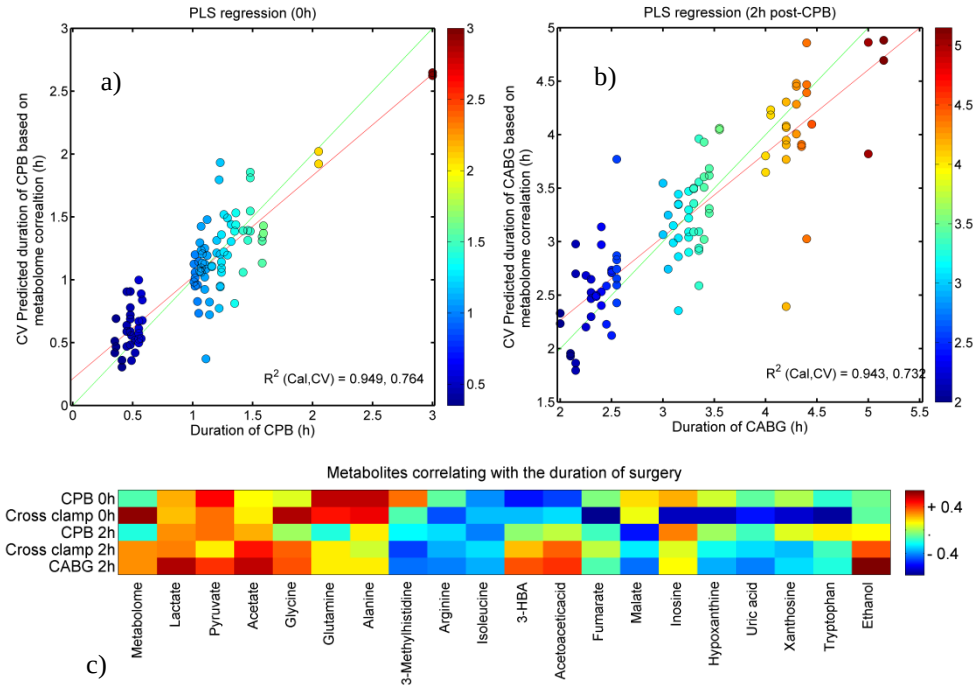

Figure 2 ***The impact of prolonged surgical time on the metabolome***. *Partial least square (PLS) regression analysis performed on serum NMR spectra from samples collected (a) immediately after weaning from CPB and (b) 2 hours post-CPB showed cross-validated (CV) associations with the actual duration of CPB and CABG, respectively. Both the calibrated (Cal) and cross-validated (CV) coefficients of associations (R^2) are provided. (c) Metabolites found to correlate with the duration of CPB, cross-clamp, and CABG, and their time-line trends until 2 hours post-CPB (red: positive correlation; yellow: no significant correlation; blue: negative correlation).*

Figure 3

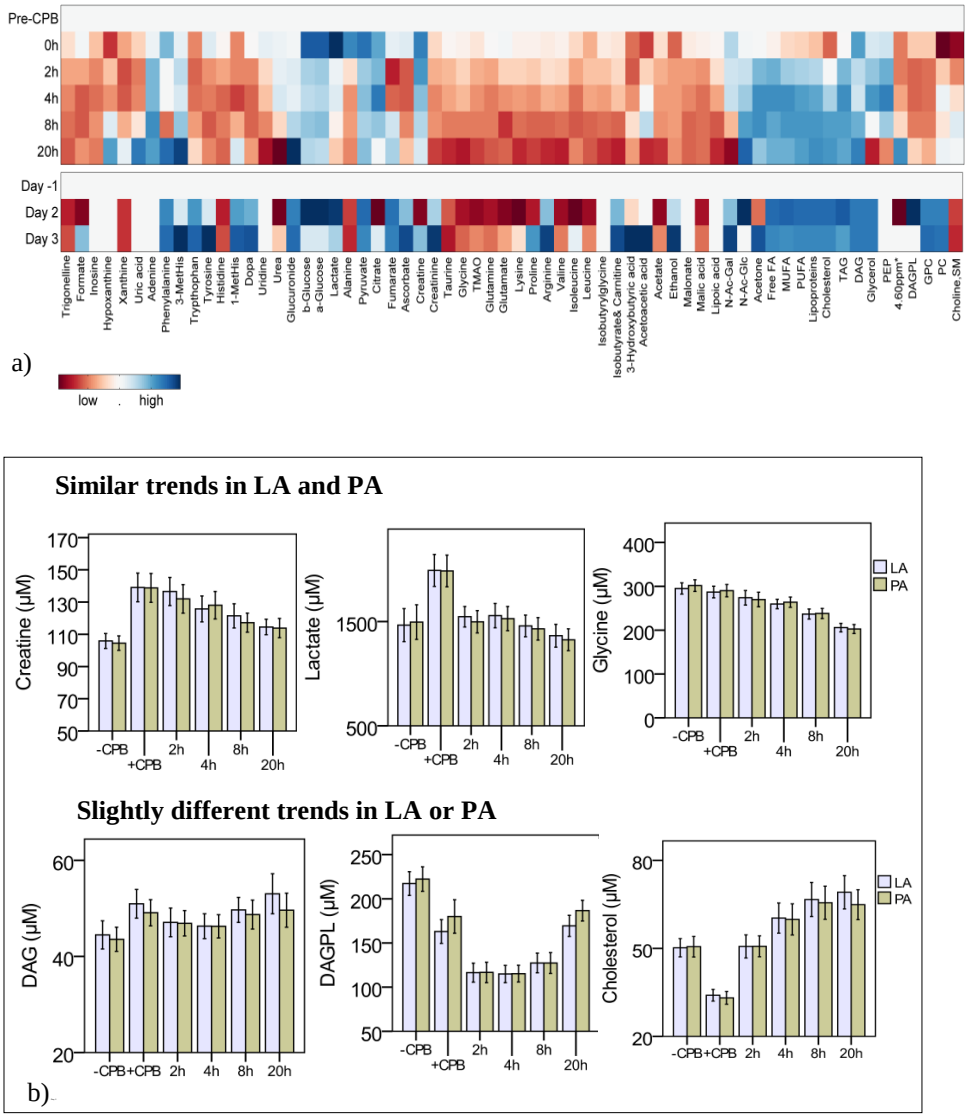

Figure 3 ***Patients' metabolic journeys****. (a) Heat map representation of time-dependent metabolic changes occurring within the nine different time points (the day before surgery, 'Day-1'; before cardiopulmonary bypass, 'pre-CPB', immediately after weaning from CPB '0h', and 2, 4, 8, 20, 48, and 72h post-CPB. (b) Selected metabolites showing similar and slightly different trends in the samples collected from the left atrium (LA) and pulmonary artery (PA). Means and standard deviations are provided. Abbreviations: 1, 3-MetHis., 1- and 3- methylhistidine; TMAO, trimethylamine-N-oxide; N-Ac-Gal, N-acetylgalactosamine; N-Ac-Glc, N-*

acetylglucosamine; FA, fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids; DAG, diacylglycerol; PEP, phosphoenolpyruvate; 4.60ppm, unassigned metabolite; DAGPL, diacylglycerophosphocholine; PC, phosphatidylcholine; GPC, glycerophosphocholine; SM, sphingomyelin;*

Figure 4

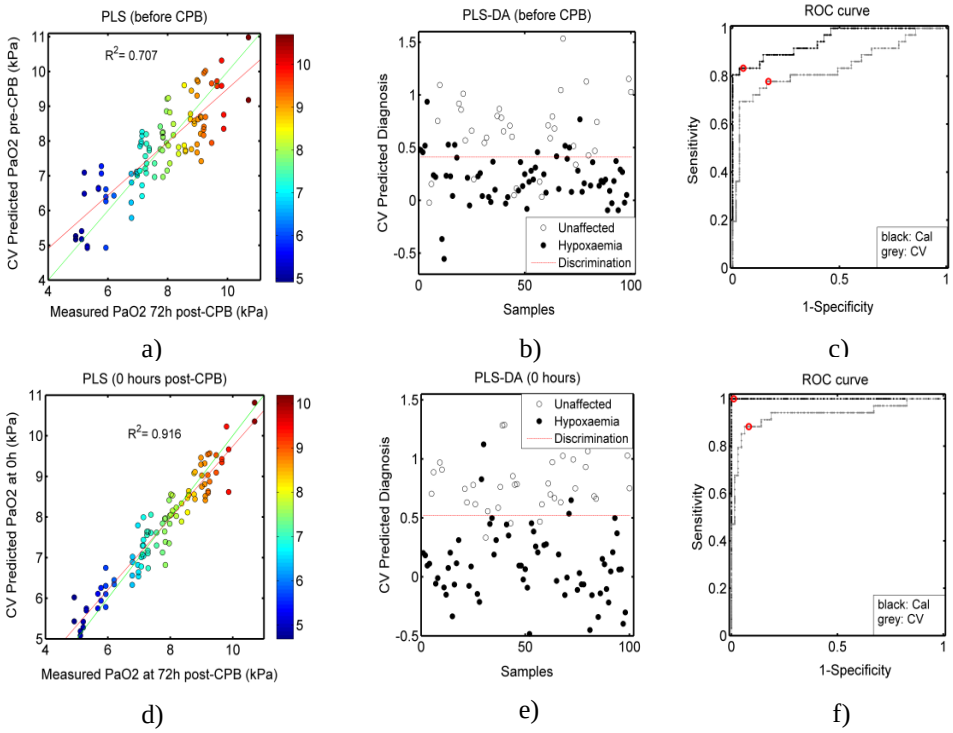

Figure 4 ***Early predispositions to lung injury defined by hypoxaemia****. (a) PLS regression plot shows cross-validated association between the metabolome measured after sternotomy, but before CPB, with arterial PaO_2 values measured 72h postoperatively. (b) PLS-DA prediction scores plot of the cross-validated model discriminating patients who will subsequently develop hypoxaemia (black) from patients who will not be affected by hypoxaemia (white), from serum samples taken after sternotomy, but before CPB. (c) The corresponding receiver operating characteristic (ROC) curve showing the predictive capacity of the model with both calibrated (Cal, black line) and validated (CV, grey line) results for samples collected after sternotomy. (d) PLS regression plot shows cross-validated correlation between the metabolome measured after weaning from CPB with PaO_2 values. (e) PLS-DA prediction scores plot of the model discriminating hypoxaemia (black) from unaffected patients (white) from serum samples taken immediately after weaning from CPB. (f) The corresponding ROC curve.*

Figure 5

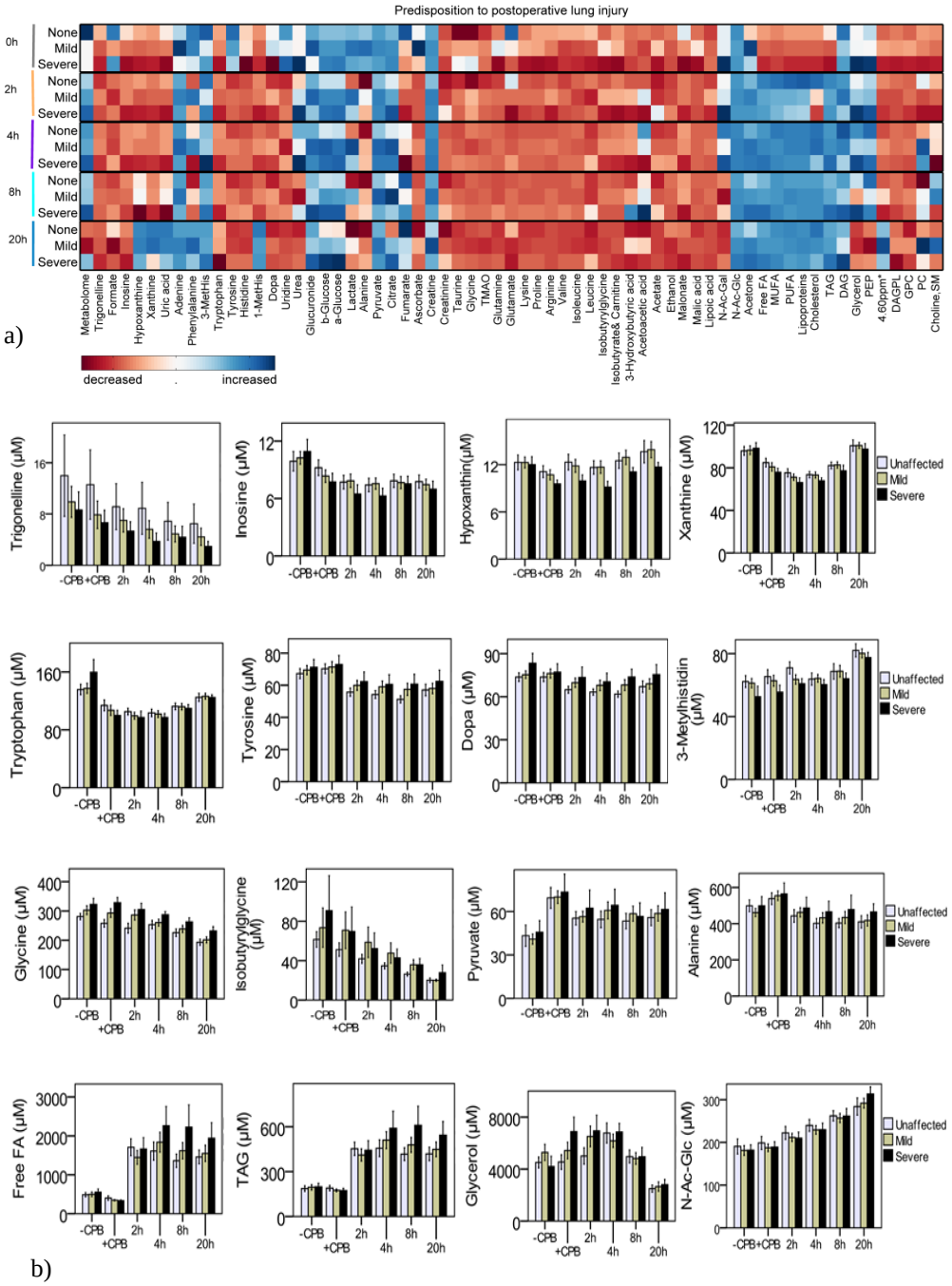
Predisposition to postoperative lung injury
0h
2h
4h
8h
20h
None
Mild
Severe
Metabolome
Trigonelline
Formate
Inosine
Hypoxanthine
Xanthine
Uric acid
Adenine
Phenylalanine
3-MetHis
Tyrosine
Tryptophan
Histidine
1-MetHis
Dopa
Urea
Glucuronide
b-Glucose
a-Glucose
Lactate
Alanine
Pyruvate
Citrate
Fumarate
Asparagine
Creatinine
Taurine
Glycine
TMAO
Glutamine
Glutamate
Lysine
Proline
Arginine
Valine
Isoleucine
Leucine
Isobutyrylglycine
Isobutyrate& Carnitine
3-Hydroxybutyric acid
Acetoacetic acid
Acetate
Ethanol
Malonate
Malic acid
Lipoic acid
N-Ac-Gal
N-Ac-Glc
Acetone
Free FA
MUFA
PUFA
Lipoproteins
Cholesterol
TAG
DAG
Glycerol
PEP
4.60ppm*
DAGPL
GPC
PC
Choline,SM
decreased
increased
a)
Trigonelline (µM)
Inosine (µM)
Hypoxanthin(µM)
Xanthine (µM)
Tryptophan (µM)
Tyrosine (µM)
Dopa (µM)
3-Metylhistidin (µM)
Glycine (µM)
Isobutyrylglycine (µM)
Pyruvate (µM)
Alanine (µM)
Free FA (µM)
TAG (µM)
Glycerol (µM)
N-Ac-Glc (µM)
-CPB
+CPB
2h
4h
8h
20h
Unaffected
Mild
Severe
b)

Figure 5 ***Metabolic signature of lung injury.*** *(a) Heat map representation of the mean percent changes in the unaffected ('None'), mildly affected ('Mild), and severely affected ('Severe') by hypoxaemia patients. (b) Selected metabolites showing time- and phenotype dependent changes. Abbreviations: 1, 3-MetHis., 1- and 3-methylhistidine; TMAO, trimethylamine-N-oxide; N-Ac-Gal, N-acetylgalactosamine; N-Ac-Glc, N-acetylglucosamine; FA, fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DAG, diacylglycerol; PEP, phospho-enolpyruvate; 4.60ppm*, unassigned metabolite; DAGPL, diacylglycerophospho-choline; PC, phosphatidylcholine; GPC, glycerophosphocholine; SM, sphingo-myelin;*

Supplementary materials

Figure S1

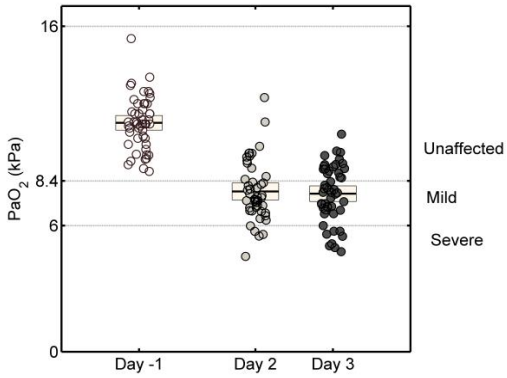

Figure S1 Partial pressure of oxygen (PaO_2) measured in arterial blood on fasting patients the day before surgery, and the second and third day postoperatively, to assess the degree of hypoxaemia. Arterial PaO_2 levels showed a 29% decrease on the second (48h) ($p<0.0001$) and a 31% ($p<0.0001$) decrease 72h postoperatively compared to their baseline levels measured the day before surgery (day-1). Based on PaO_2 values obtained on the third postoperative day, patients were divided in to hypoxaemic patients ('severe' with $PaO_2< 6.3$ kPa, and 'mild' with $8.4>PaO_2\geq 6.3$ kPa) and unaffected patients with $PaO_2\geq 8.4$ kPa.

Table S1

Table S1 ***Prolonged surgical procedure affects the metabolome.*** *Association between metabolome and duration of surgical procedure assessed by partial least square (PLS) regression. Both the calibrated (Cal) and cross-validated (CV) results are provided. At 4h post-CPB the association was weaker, suggesting that the metabolome had begun normalizing after prolonged surgical stress. No significant CV association was observed at 8 and 20h postoperatively. Models with low predicted association are marked in grey.*

PLS regression: Metabolome versus duration of surgical procedure						
	CABG (196 ± 50 min.)		CPB (64 ± 29 min.)		Ischemia (33±19 min.)	
	R^2 Cal	R^2 CV	R^2 Cal	R^2 CV	R^2 Cal	R^2 CV
0 hours	-	-	0.94	0.76	0.92	0.75
2 hours	0.9	0.75	0.87	0.67	0.72	0.54
4 hours	0.82	0.6	0.7	0.41	0.7	0.44
8 hours	0.68	0.44	0.7	0.36	0.61	0.41
20 hours	0.74	0.39	0.55	0.32	0.63	0.38

Figure S2

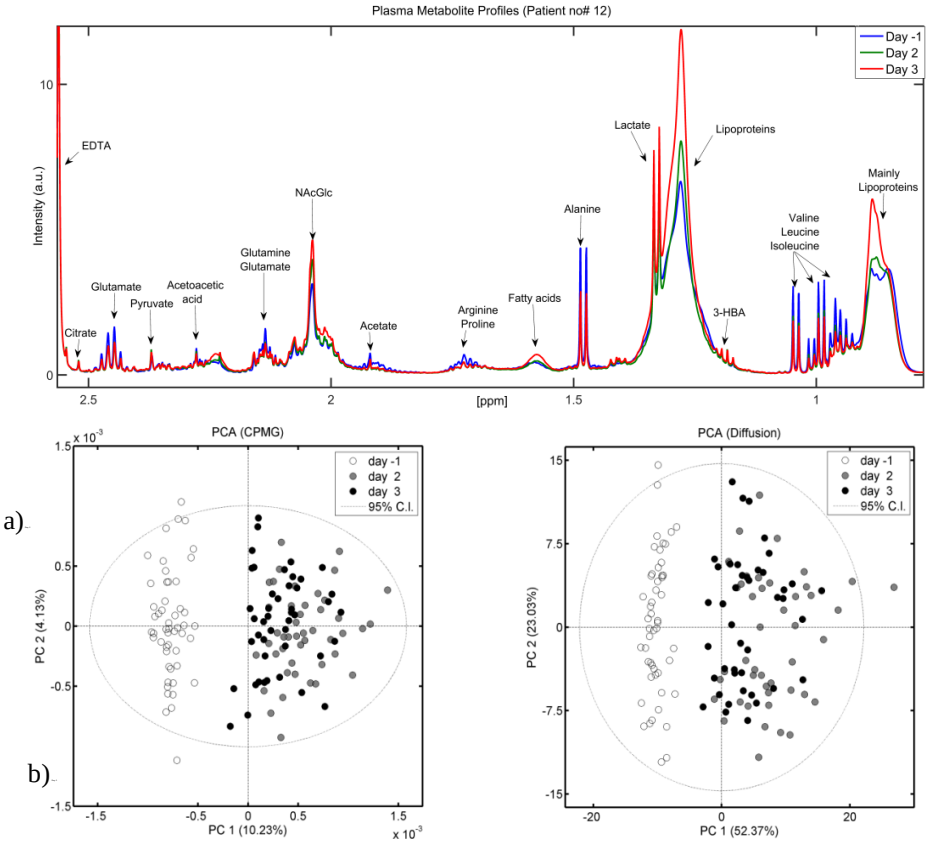

Figure S2 ***Plasma metabolic profiles the day before surgery, and two and three days postoperatively.*** *(a) Metabolite profiles of a patient's plasma samples collected the day before surgery (blue), the second (green) and third day (red) postoperatively. (b) Principal component analysis (PCA) conducted on CPMG (left) and diffusion-edited (right) spectra reveals clear separation between the time at which each sample has been collected (white dots: plasma form the preoperative day; grey and black dots represents plasma from days two and three, respectively.*

Table S2 ***Early prediction of hypoxaemia.*** *Partial least squares (PLS) regression and partial least squares –discriminant analysis (PLS-DA) results. The results show both the calibrated (Cal) and Venetian-Blinds cross-validated (CV) coefficient of association (R^2), specificity, sensitivity, and class error. Each model was built on 100 samples collected from 50 patients from the left atrium and pulmonary artery at one of the mentioned time points. For validation, the Venetian-Blinds cross-validation with 10 segments was applied. 10 consecutive samples were removed and a model was created. Subsequently, the 10 samples were predicted. This procedure was repeated until all samples were removed once. Permutation testing was applied for each model. A p-value <0.001 was considered significant.*

	PLS regression (Metabolome vs. PaO_2)					PLS-DA (Metabolome vs. Outcome (hypoxaemia: yes/no))						
	R^2		RMSE		Permut.	Sensitivity		Specificity		Class error		Permut.
	Cal	CV	Cal	CV	p-value	Cal	CV	Cal	CV	Cal	CV	p-value
Pre-CPB	0.94	0.71	0.33	0.75	0.004	0.83	0.78	0.95	0.84	0.11	0.19	0.004
0 h	0.98	0.92	0.15	0.4	<0.001	1	0.88	0.98	0.92	0.01	0.1	<0.001
2 h	0.98	0.93	0.13	0.36	<0.001	1	0.91	0.98	0.94	0.01	0.1	<0.001
4 h	0.98	0.95	0.12	0.3	<0.001	0.97	0.88	0.92	0.88	0.06	0.12	<0.001
8 h	0.98	0.94	0.13	0.34	<0.001	0.97	0.94	0.97	0.92	0.03	0.07	<0.001
20h	0.98	0.90	0.19	0.44	<0.001	0.96	0.93	0.97	0.91	0.04	0.08	<0.001

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Appendix D. Study VI (Patent)

Patent Application 19. December 2014

Biomarkers for the prediction of developing of hypoxemia due to acute lung injury

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Abstract

The present invention pertains to the prediction and early identification of patients at-risk of developing hypoxemia and related lung dysfunctions such acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Specifically, the invention provides biomarkers to determine whether or not an at-risk patient prior to the admission to the intensive care unit (ICU) will develop hypoxemia and/or ALI/ARDS. The invention is address to patients not diagnosed with hypoxemia and/or ALI/ARDS at the time of ICU admission, but who are at-risk of developing the disease at a later time point during their hospitalization. More specifically, the invention is address to patients that have undergone cardiac -, aortic -, thoracic -, and spinal surgery, and/or patients that have experienced traumatic brain injury, shock, multiple trauma, multiple transfusions, near-drowning, and/or patients that are diagnosed with pneumonia, sepsis, acute pancreatitis, and/or patients that have aspired gastric contents, and/or have inhaled smoke. The method for the prediction and the early identification of hypoxemia and/or ALI/ARDS is based on comparing the level of eleven biomarkers obtained from a blood sample before the disease can be detected by blood gas analyzer (the standard diagnostic tool).

In this particular invention, eleven biomarkers were used to predict the risk of hypoxemia in at-risk patients who underwent cardiac surgery with the use of cardiopulmonary bypass (CPB) at Aalborg University Hospital in Denmark, before the disease could be diagnosed using standard methods (i.e. hypoxemia was predicted the first morning after CPB, two days before the clinical signs appeared).

Summary of the invention

The present invention pertains to the use of eleven biomarkers to predict or detect early signs of hypoxemia and ALI/ARDS in an at-risk human model (cardiac surgery). In particular, the invention pertains to the monitoring, prediction and/or early diagnosis of hypoxemia and ALI/ARDS by distinguishing between at-risk patients developing hypoxemia and at-risk patients not developing hypoxemia. These biomarkers identify early progression into hypoxemia and ALI/ARDS, and enable subsequent intervention to prevent or minimize disease progression and further clinical deterioration (e.g. ALI/ARDS).

For the invention to be fulfilled, one may: a) obtain a blood sample (whole blood, serum, plasma) from an at-risk patient (sample collected first morning postoperatively); and b) assess the levels of the eleven metabolites in the sam-

ple to check the risk of developing the condition.

Problems to be solved by the invention

Due to a delay from the triggering factor (e.g. surgery, trauma, sepsis, etc.) to the onset of hypoxemia and ALI/ARDS, the recognition of at-risk patients and the development of the full-blown condition is not always recognized in time[1]. The main obstacle to early intervention and prevention of ALI/ARDS is the inability to differentiate which of the at-risk patients will develop the disease. At the ICUs, the development of ALI/ARDS after admission is estimated to be between 5-20% in critically ill patients[2], and the highest rates are observed among patients on mechanical ventilation[3]. In the United States alone, approximately 5 million patients are admitted annually to ICUs, of which majority of ICU admissions are due to respiratory insufficiency/failure, postoperative management, ischemic heart disorder, sepsis, and heart failure (SCCM, Critical Care Statistics). It is estimated that these patients receive over 18 million days of care each year, with related health care costs estimated to be almost one percent of United States' gross domestic product.[1] Therefore, differentiating at-risk patients who will develop hypoxemia and ALI/ARDS from at-risk patients who will not develop hypoxemia will have a significant impact on health care costs. Moreover, any intervention decreasing the number of patients developing hypoxemia and/or ALI/ARDS will have a significant impact on morbidity[1] and the high mortality rates (up to 40%)[4, 5].

DEFINITIONS

1. **Hypoxemia** is a dangerous complication where the blood oxygen content drops below normal levels. Without enough oxygen, lungs, brain, liver, and other organs can be damaged within minutes. While hypoxemia can present non-specifically, the main symptoms are acute respiratory distress, cough and chest pain. In severe cases, the disease is fatal if untreated.

2. **ALI/ARDS** (acute lung injury/ acute respiratory distress syndrome) is a heterogeneous disease, which is defined by the acute onset of hypoxemic respiratory failure, with pulmonary infiltrates on chest radiography due to non-cardiogenic pulmonary edema.[6-8]. Patients with ALI/ARDS show low oxygenation in arterial blood (hypoxemia); with PaO_2/FiO_2< 40 kPa (< 300 mmHg); or PaO_2< 8.4kPa (< 63mmHg)); where PaO_2 is the partial pressure of oxygen (O_2) in arterial blood, and FiO_2 is the fraction of inspired O_2 concentration. Patients showing severe hypoxemia with PaO_2/FiO_2 ≤26.6kPa (≤200 mmHg) or PaO_2 ≤5.6 (≤42 mmHg) are considered to suffer from ARDS, while patients with mild to moderate hypoxemia PaO_2/FiO_2 <40 kPa (<300 mmHg); or PaO_2<8.4 kPa (<63 mmHg)) are considered to suffer from ALI.

3. Since the early days of "cardiac surgery", the number of cardiac procedures done worldwide has increased exponentially. "Coronary artery bypass grafting (**CABG**)" with the use of "cardiopulmonary bypass (**CPB**)" remains the gold standard strategy to perform cardiac surgery. CABG is the most common type of open-heart surgery in the United States, with more than 500,000 operations performed each year

(Source: University of Michigan, Cardiovascular Centre, 2014). In Denmark, about 2000 CABG procedures are performed each year, of which over 300 are performed at Aalborg University Hospital. Although this complex surgery saves lives every day, profound inflammation and pulmonary complications[9,10] are associated with the procedure[11]. Despite improvements in intervention and successful reperfusion, all patients experience hypoxaemia, and some experience ALI/ARDS[9] within 72 hours postoperatively[12].

State of the Art:

Because ALI and ARDS are diseases that progress rapidly (within 72 hours after cardiac surgery) and show poor prognosis, no early marker or effective treatment has been established to stop or reverse their progression. Although multiple risk factors for ALI/ARDS are known (e.g. cardiac surgery, aortic surgery, thoracic surgery, spinal surgery, traumatic brain injury, shock, multiple trauma, multiple transfusions, near-drowning, pneumonia, sepsis, acute pancreatitis, gastric contents aspiration, and smoke inhalation), it is widely accepted that no pharmacological intervention has been identified to date. Despite repeated promising pre-clinical and clinical phase I+II studies of therapies, no non-ventilatory strategy has yet proved successful[4].

The current state of the art in the treatment of ALI/ARDS patients in critical care environments is supportive care and mechanical ventilation[5,13]. Despite the high number of cases of ALI/ARDS and mortality rates up to 40%[4,5], preventative therapies for at-risk patients have receive little attention.

Because there are no signs of early hypoxemia before the patients are already affected by the disease, only clinical outcomes are routinely detected. Currently, blood gas analyses are used to monitor hypoxemia and to assist in the diagnosis of ALI/ARDS.

Trials using potential therapeutic targets such as anti-inflammatory agents, anticoagulants, surfactant, vasodilators, and β_2 agonists have shown conflicting results[13]. Early administration of corticosteroids to prevent the development of ARDS showed no beneficial evidence[14]. In other studies corticosteroids were ineffective in preventing the development of ARDS in at-risk patients; however, steroids have been shown to be useful for treating already diagnosed ARDS patients[15].

Several studies suggest that the use of statins in patients with ALI/ARDS is associated with increased ventilator-free days and reduced mortality. The HARP study (ISRCTN 70127774) is investigating the effect of simvastatin in the prevention and treatment of ALI/ARDS and several groups are considering undertaking multicenter studies to address the role of statins in ALI/ARDS[15].

Inflammatory signaling modification agents (e.g. ketoconazole, ibuprofen, complement inhibition, and insulin) have shown conflicting results, however, these anti-inflammatory agents are still under study[15].

Fish oils with the omega 3 poly unsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA), gamma-linolenic acid (GLA) and deocosahexaenoic acid (DHA) reduce the production of the pro-inflammatory arachidonic acid. Studies

administrating fish oils in diagnosed ALI/ARDS patients have demonstrated reduced inflammation and improved outcomes (reduced duration of ventilation and ICU stay, and a lower mortality rate). Further studies of fish oils in ALI/ARDS are underway in Spain (ISRCTN 63673813) and America (NCT00351533)[15].

Antioxidant therapy using glutathione and N-acetylcysteine have had mixed results in small ALI/ARDS studies[15, 16], while the use of the antioxidant procysteine in a lung injury study was stopped early due to increased mortality[15]. Other antioxidant therapies with vitamin C and E have reduced duration of mechanical ventilation and ICU stay, but did not prevent the development of ALI/ARDS[17].

Exogenous surfactant therapy, which is successfully applied in the treatment of neonatal ARDS, has shown no effect on adults suffering from ALI/ARDS[18].

Several immunologic approaches targeting the inflammatory cascade in ALI/ARDS have been described; however, no efficacious therapy has been identified based upon these approaches[15].

Results from several studies on the effects of inhaled nitric oxide (NO) to cause pulmonary vasodilation have shown no positive[5, 19] or even harmful[15] effects on patients with ALI/ARDS.

Prostacyclin (PGE1) derived from arachidonic acid metabolism has shown vasodilatory effects such NO, and shown promising preclinical results, improving oxygen delivery in acute hypoxemic patients. However, further larger studies with either inhaled or intravenous PGE1 administration have produced negative results.[15,20]

Many of the treatments described above have failed partly because of the delayed recognition of at-risk patients, and because the therapies were targeted to patients already diagnosed with ALI/ARDS. Another reason for this failure is that it is difficult to monitor the effect of any treatments, due to the lack of markers for disease progression. Also, other major reasons for failing the translation from bench to bedside are the use of animal models in an attempt to reproduce human models of ALI/ARDS[4]. Much has been learned from animal models, however, these models suffer from numerous limitations, including differences in the immunological, genetic, and host response to injury compared to the human model[15]. While animal studies focus on young, healthy animals, with a defined diet, a defined genetic background and without other diseases before the underlying experimental injury[4], the human models are a direct contradiction to the aforementioned animal models. In fact, patients enrolled in the ICU presenting or developing hypoxemia and ALI/ARDS are generally old, immuno-compromised, have variable genetic makeup and are mostly malnourished[4].

Which aspects do you consider new compared to the existing technology?

What makes this invention novel is that the identified biomarkers provide a means of prediction, and the early differentiation of at-risk patients developing hypoxemia from at-risk patients' not developing hypoxemia. Therefore this invention pertains in part to the early diagnosis of hypoxemia and/or ALI/ARDS, but also to the use of novel pharmacological agents targeting the progression of this condition.

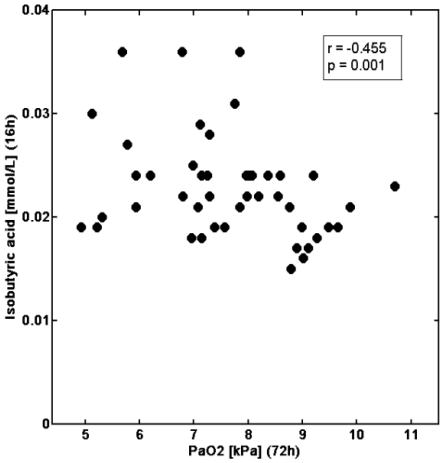
Effect of the Invention

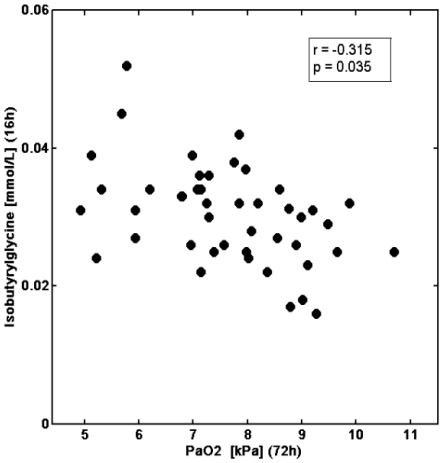
According to the inventors, the presented biomarkers can determine with high sensitivity and specificity whether or not an at-risk patient (e.g. a patient undergoing cardiac surgery) will be affected by hypoxemia and/or ALI/ARDS before the first clinical signs can be detected by the blood gas analyzer (e.g. the next morning after the surgery, which is two days before the clinical diagnosis). This method can provide a powerful diagnostic tool capable of clearly distinguishing at-risk patients developing hypoxemia and/or ALI/ARDS from at-risk patients not developing the condition. Taking into account the number of patients in the ICU every day and the risk of developing the condition, the effects of the invention will have a high impact on morbidity, mortality rates, and health care costs.

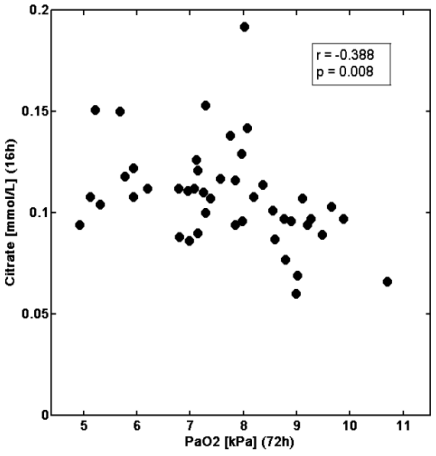
In experiments conducted during the course of discovering these biomarkers, the inventors have found eleven metabolites that showed potential in predicting hypoxemia and/or ALI/ARDS before the clinical diagnosis (two days before the clinical diagnosis in at-risk patients that have undergone cardiac surgery).

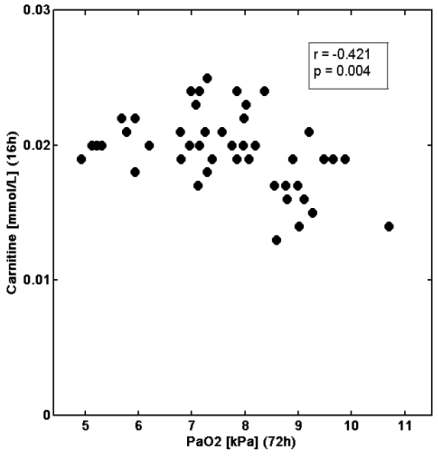
Since the mechanisms involved during ALI/ARDS are believed to be the same no matter what the initial insult is (cardiac surgery with cardiopulmonary bypass, massive blood transfusion, severe non-thoracic trauma, pulmonary infection, aspiration of gastric contents, non-fatal drawing, toxic gas inhalation, hyperoxia, lung contusion, sepsis, pancreatitis, burns, shock), we believe that these present biomarkers are not limited to any particular insult. Also, an understanding of the mechanisms involved in the pathogenesis of this disease is not necessary to practice the present invention. Therefore, it is expected that the levels of the eleven metabolites, of which 6 are single markers (citrate, carnitine, O-N-Acetyl-Glucosamine, arachidonic acid, isobutyric acid, and isobutyrylglycine) and 5 are presented as metabolite ratios (citrate /Trimethylamine-N-oxide(TMAO), carnitine/TMAO, citrate /phenylalanine, isobutyric acid/phenylalanine, alanine/phenylalanine, isobutyrylglycine/arginine, isobutyrylglycine/ phenylalanine, polyunsaturated fatty acids (PUFA)/phenylalanine, and PUFA /arginine), are indicative of the early development of hypoxemia and/or ALI/ARDS in at-risk patients.

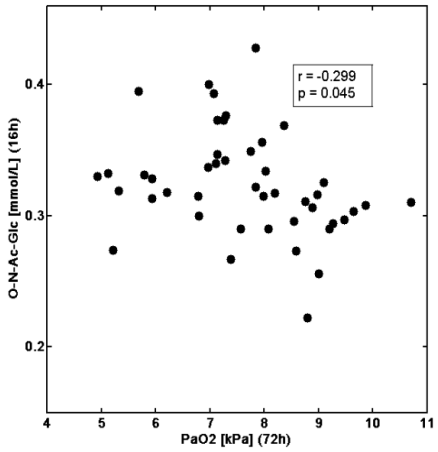
Figures: Metabolites measured 16 hours after weaning from CPB correlate well with the partial pressure of oxygen (PaO_2) measured in arterial blood 72 hours postoperative. PaO_2 values were used to diagnose hypoxemia and ALI/ARDS.







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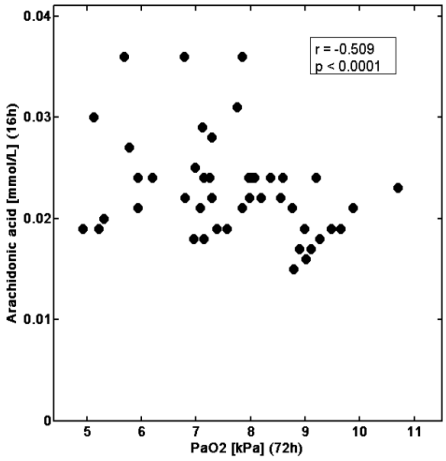
p = 0.045
O-N-Ac-Glc [mmol/L] (16h)
PaO2 [kPa] (72h)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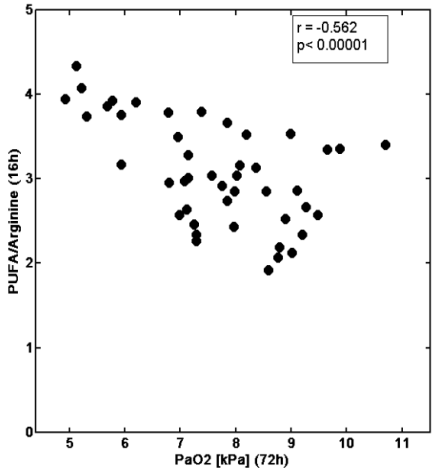
Arachidonic acid [mmol/L] (16h)
PaO2 [kPa] (72h)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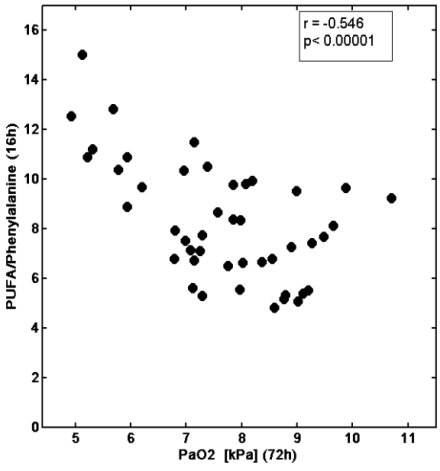
PUFA/Arginine (16h)
PaO2 [kPa] (72h)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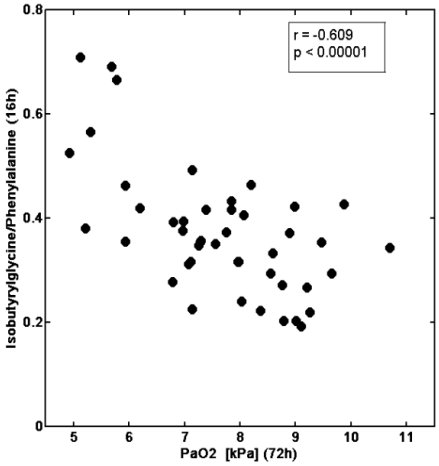
PUFA/Phenylalanine (16h)
PaO2 [kPa] (72h)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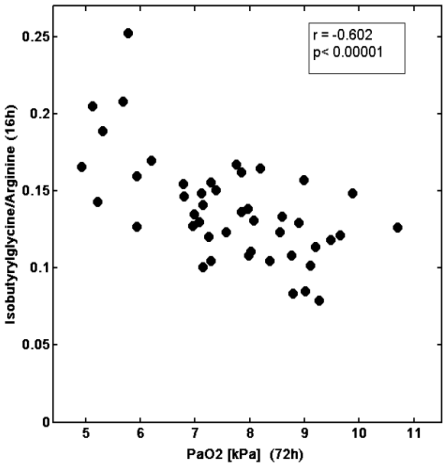
PaO2 [kPa] (72h)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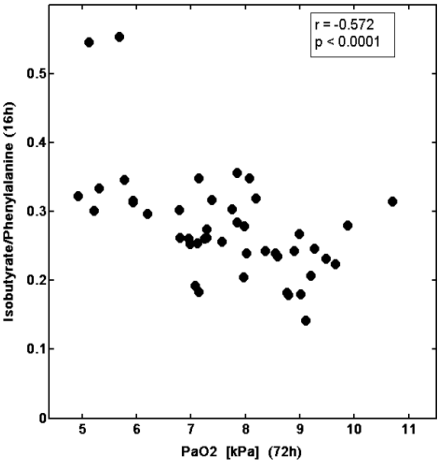
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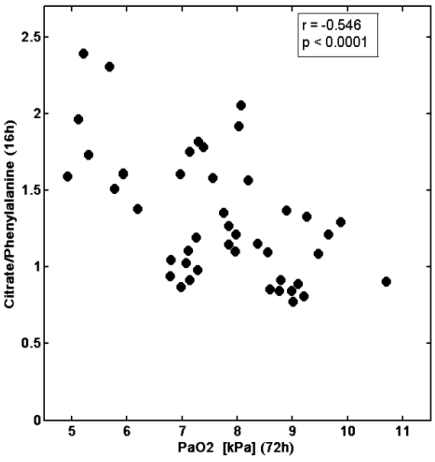
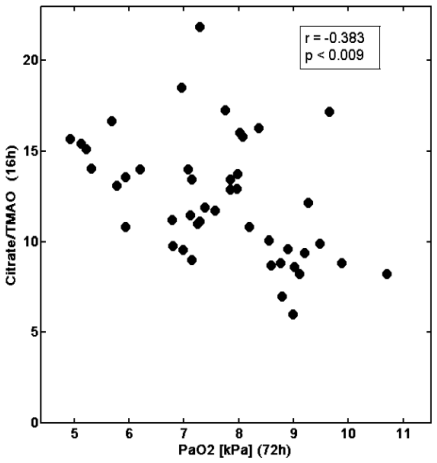
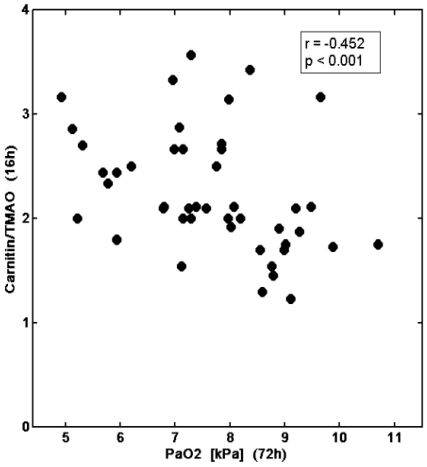
Isobutyrate/Phenylalanine (16h)
PaO2 [kPa] (72h)


r = -0.546
p < 0.0001
Citrate/Phenylalanine (16h)
PaO2 [kPa] (72h)





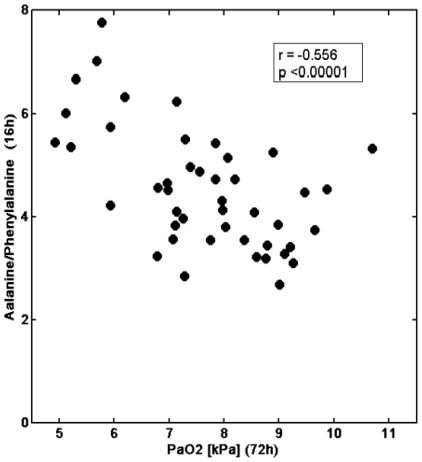


Table 1: Multiple regression analysis test results performed to find the correlation between all eleven blood-derived metabolites measured on the first morning postoperatively and the hypoxemic score values (PaO_2) measured on the 3rd day after cardiac surgery. The results show the correlation score (R) and the F-test for regression.

		Statistics		
Model	R	R Square	F	Sig. F
All metabolites	0.828	0.685	4.212	0.00044

Figures and Table 1 explanations: Eleven biomarkers were identified to assess the risk of developing hypoxemia and/or ALI/ARDS in at-risk patients. These biomarkers (either on their own or in combination (Table 1)) have shown potential in predicting the degree of hypoxemia 16 hours after cardiac surgery, which is 56 hours before hypoxemia was actually diagnosed at Aalborg hospital. These markers show potential in the identification and selection of patients at increased risk of experiencing hypoxemia and ALI/ARDS, for the purpose of initiating earlier and more specific/individualized treatments.

Table 2: Metabolites found significant in predicting hypoxemia in an at-risk patient group by applying the t-test. Abbreviations: AUC= area under the curve, C.l.= confidence level, TMAO= Trimethylamine-N-oxide, O-NAc-Glc= O-N-Acethyl-glucosamine, PUFA= polyunsaturated fatty acids.

Metabolites	Hypoxemia vs. No-Hypoxemia $PaO_2 > 8.5kPa$ vs. $PaO_2 < 8.4kPa$			Severe Hypoxemia vs. No-Hypoxemia $PaO_2 > 8.5kPa$ vs. $PaO_2 < 6.2kPa$		
	AUC	95% C.l. AUC	p-value	AUC	95% C.l. AUC	p-value
Carnitine	0.90	0.790-0.979	6.96E-07	0.90	0.762-1	8.75E-04
Citrate/TMAO	0.89	0.749-0.993	3.59E-06	0.92	0.770-1	8.20E-05
Citrate	0.88	0.764-0.961	2.43E-05	0.93	0.782-1	6.45E-04
Carnitine/TMAO	0.86	0.714-0.971	5.21E-05	0.88	0.714-1	1.59E-03
O-NAc-Glc	0.86	0.748-0.951	2.14E-04	0.88	0.714-1	1.70E-02
Citrate/ Phenylalanine	0.85	0.722-0.942	1.31E-04	1.00	1-1	1.06E-06
Isobutyric acid/ Phenylalanine	0.84	0.676-0.935	5.64E-04	0.98	0.912-1	5.88E-05
Arachidonic acid	0.82	0.688-0.953	5.95E-05	0.93	0.802-1	5.46E-04
Isobutyric acid	0.79	0.630-0.899	2.31E-03	0.78	0.573-0.944	1.40E-02
Alanine/ Phenylalanine	0.78	0.633-0.908	2.94E-03	0.97	0.865-1	1.45E-05
Isobutyrylglycine/ Arginine	0.78	0.635-0.911	1.32E-03	0.95	0.857-1	7.73E-05
Isobutyrylglycine/ Phenylalanine	0.77	0.622-0.917	2.52E-03	0.94	0.833-1	5.44E-05
PUFA/ Phenylalanine	0.76	0.601-0.882	4.83E-03	0.98	0.905-1	2.14E-05
PUFA/Arginine	0.76	0.594-0.894	2.87E-03	0.97	0.881-1	3.85E-05
Isobutyrylglycine	0.75	0.603-0.879	3.65E-03	0.81	0.601-0.962	6.72E-03

Table 2 explanations: The biomarkers show potential in stratifying patients and distinguishing between hypoxemic and unaffected patients, and between severe hypoxemic and unaffected patients. Because postoperative treatments are standardized today, early identification of patients not experiencing hypoxemia (PaO2< 8.5 kPa (63.7 mmHg)) would facilitate the exclusion of these patients from further unnecessary clinical treatments.

Detained description of the invention

The present invention is explained in detail below.

The present invention provides a method comprising eleven biomarkers for determining whether or not a patient will develop postoperative hypoxemia and/or ALI/ARDS, comprising quantification of one or several of the eleven metabolites (citrate, carnitine, O-N-Acetyl-Glucosamine, arachidonic acid, isobutyric acid, isobutyrylglycine, citrate/TMAO, carnitine/ TMAO, citrate/phenylalanine, isobutyric acid/ phenylalanine, alanine/phenylalanine, isobutyrylglycine/arginine, isobutyrylglycine/ phenylalanine, PUFA/phenylalanine, and PUFA/ arginine) in a blood sample (whole blood, serum, or plasma) collected the first morning after surgery (16 hours after the end of surgery).

1. The levels of these biomarkers in a blood sample (whole blood, serum, plasma collected 16 hours postoperatively) derived from patients following CABG was correlated with the possibility of becoming hypoxemic (low PaO_2 values) (see correlation coefficients in the figures). The above-mentioned determination is based on the correlations of these metabolites derived from patients that have undergone CABG and the PaO_2 values measured by the blood gas analyzer on the 3rd day after the surgery. Therefore, for the invention purposes, a correlation diagram of the amount of each metabolite in a blood sample (e.g. whole blood, serum, plasma) and PaO_2 is prepared in advance, and the level of each metabolite in the blood sample collected from an at-risk patient on the first morning following the surgery may be compared to the correlation diagram. Alternately, to enhance the sensitivity of this test, one may use all mentioned biomarkers in the correlation diagram (Table 1).
2. From the comparison results of the levels of each individual metabolite, when the levels of citrate, carnitine, alanine, PUFA, arachidonic acid, isobutyric acid, isobutyrylglycine, and O-N-Ac-Glc in a blood sample (whole blood, serum or plasma) derived from an at-risk patient are relatively high, while the levels of arginine, phenylalanine, and TMAO are relatively low, the patient can be determined to have a relatively high possibility of developing hypoxemia, and thereby have a high risk of developing ALI/ARDS. Conversely, when the levels of citrate, carnitine, alanine, PUFA, arachidonic acid, isobutyric acid, isobutyrylglycine, and O-N-Ac-Glc in a blood sample (whole blood, serum, or plasma) derived from an at-risk patient are relatively low, while the levels of arginine, phenylalanine, and TMAO are relatively high; the patient can be determined to have a relatively low possibility of developing hypoxemia and ALI/ARDS.
3. Alternatively, a cutoff value of the levels of one or a combination of several of the mentioned metabolites in a blood sample (whole blood, serum, or plasma) may be determined in advance, and the cutoff value may be compared with the measured levels of the chosen metabolite(s). The cutoff value is chosen so that it achieves both a high diagnostic sensitivity (true positive rate) and high diagnosis specificity (true negative

rate), when hypoxemic versus no-hypoxemic conditions are evaluated using the value as a standard. For example, when the levels of citrate, carnitine, alanine, PUFA, arachidonic acid, isobutyric acid, isobutyrylglycine, and O-N-Ac-Glc in a blood sample derived from an at-risk patient (16 hours postoperatively) are higher than the chosen cutoff values, the patient can be determined to have a high possibility of developing hypoxemia and/or ALI/ARDS. Conversely, when the levels of citrate, carnitine, alanine, PUFA, arachidonic acid, isobutyric acid, isobutyrylglycine, and O-N-Ac-Glc in a blood sample derived from an at-risk patient (16 hours postoperatively) are lower than the cutoff values, the patient can be determined to have a low possibility of developing hypoxemia.

Which industrial applications do you see for your invention?

The present invention pertains to the monitoring and treatment of hypoxemia and ALI/ARDS in at-risk patients e.g. following cardiac surgery. In particular, the invention pertains to the use of the identified biomarkers for prediction and/or early detection of hypoxemia and ALI/ARDS. Therefore this intervention may result in two outputs: (1) the formation of a commercialized early-stage diagnostic test; (2) the design of more specific medical treatments targeting early stage of the disease.

In the present invention, eleven metabolites were identified as novel biomarkers for the prediction of hypoxemia (herein ALI/ARDS) in an at-risk patient group (e.g. after cardiac surgery), 2 days before clinical signs could be detected. Measurement of these biomarkers in a blood sample (whole blood, serum, or plasma) enabled a convenient and highly accurate diagnosis of whether or not an at-risk patient would progress into hypoxemia and ALI/ARDS.

The hypoxemia and ALI/ARDS related biomarkers refer to a set of eleven metabolites present in a fasting sample collected on the first morning postoperative (16 hours after weaning from CPB) derived from an at-risk patient.

Which advantages/disadvantages does the invention offer?

These metabolites have been identified correlating to the later degree of hypoxemia. Because these metabolites are involved in different pathways, they reveal that more than one path is affected. Therefore, when manufacturing specific therapeutic targets, the chance of developing a successful drug to reverse disease progression will be greater if several of these paths are targeted together.

These metabolites are available in blood, which requires no complicated procedure to obtain a sample.

The disadvantage is that some of the standard techniques and assay kits used to monitor or diagnose other diseases can measure some of our identified markers of hypoxemia (see below).

What measures are required to detect your invention in a competitor's product?

1. A metabolite assay kit containing all eleven metabolites and the corresponding assay buff-

ers. Alternatively, because increasing the number of metabolites would have a positive impact on the sensitivity and specificity of the assay, one may combine the results achieved by using several kits already on the market (e.g. alanine kit, phenylalanine kit, citric acid kit, arachidonic acid kit, O-NAc-Glc kit, carnitine kit, and arginine kit.).

2. A mathematic model containing a specific combination of these metabolites. The mathematical model is adjusted or calibrated to each hospital's existing techniques.

Describe the suggested research and development that is necessary to obtain proof-of-concept

Measuring these metabolite concentrations in new cohort clinical trials of several types of at-risk patients in ICUs.

Has your prior art searches also included patent databases (e.g. uspto.gov/patft; worldwide.espacenet.com; google.com/patents) ?

Yes

What is in your opinion, the closest and most pertinent information in the prior art, related to your invention, that you are aware of?

The study of biomarkers of ALI/ARDS has developed over the past 20 years[21]. Since, numerous studies have focused on finding blood biomarkers for ALI/ARDS[22] and the work is still in progress[23]. Currently biomarkers of ALI/ARDS have been studied in over 2,000 patients, most studies focusing on determining which biomarkers can be used in diagnosing[21] rather than in predicting the disease.

We see two studies that are closest and most pertinent for this invention.

1) Fremont et al studied 192 trauma patients of which some were diagnosed with ALI while others were not suffering from ALI but showed similar signs. In their study, blood samples were withdrawn within 72 hours of ICU admission in order to determine if a panel of biomarkers could improve the diagnostic accuracy in differentiating ALI from non-ALI patients. A combination of seven biomarkers (brain natriuretic peptide, RAGE, procollagen peptide III, Ang-2, IL-8, IL-10, and TNF-α) were found discriminating ALI from non-ALI patients[7]. While the panel of markers showed significant difference between ALI and non-ALI patients, no study has been published yet using these biomarkers in predicting the development of hypoxemia and thereby ALI/ARDS.

2) The second study has been conducted by Stringer and co-workers[24]. They have used nuclear magnetic resonance spectroscopy to examine the metabolite profile in patients with ALI. Plasma glutathione, myo-inositol, adenosine, phosphatidylserine, and sphingomyelin were found differentiating ALI from healthy controls[24]. While these findings are relevant in explaining the pathogenesis of ALI, their approach is also based on already diagnosed patients. Also, these markers have not been tested for predictability in an at-risk patient group prior to any visible clinical signs. Notably, in their study, the healthy control group was younger (mean 31.3± 9.3years, 3 males and 3 females) and medication-free, compared to the ALI group, which was older (age 55.4 ± 16.1, most males), sick, and medicated. Such differences in

the population under study (age, gender, medication) are known to affect the level of metabolites. In our laboratory, we have analyzed phosphatidylserine, sphingomyelin, and 2-ketobutyric acid (the metabolite involved in glutathione pathway), however, none of these metabolites showed significant predictive power to later development of hypoxemia. Also, because in our study the blood samples were collected 2 days before diagnosis, this may indicate that phosphatidylserine, sphingomyelin, and glutathione are not the best markers to predict hypoxemia and ALI/ARDS.

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Appendix E. Additional results

1. Fitting accuracy assessment

To integrate peaks of interest the multi-integration fitting tool implemented in AMIX (v. 3.9.10, Bruker BioSpin) was used. Peaks were integrated using the sum of all points in the specific region as the integration mode.

To find the accuracy of the fitted signals within a compound, two metabolites were compared (Figure E1). Glucose was chosen since its concentration levels were higher than the averaged metabolic concentrations (~ 5mM), while trigonelline was chosen for the opposite reason (~ 10μM).

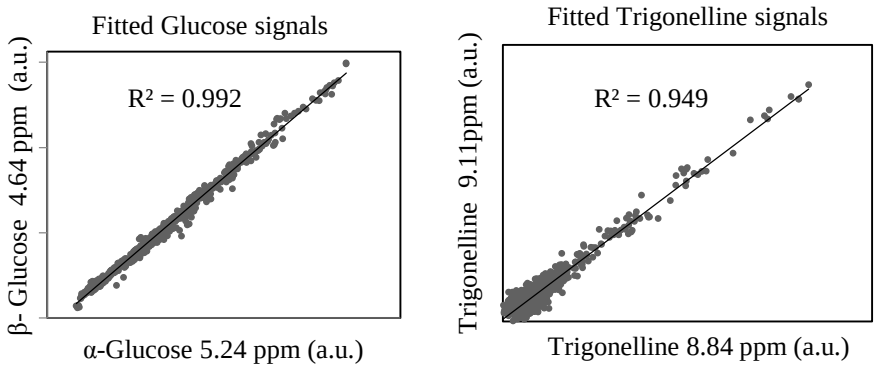

Figure E1 The fitted peak areas obtained for the doublet signal derived from α-glucose at 5.24 ppm were regressed to that of β-glucose at 4.64 ppm (left). Good correlation is observed (R^2 = 0.992). The fitted peak area obtained for the singlet signal of trigonelline at 9.11 ppm was compared to that of the multiplex areas at 8.84 ppm (right). Good correlation (R^2 = 0.949) is observed, indicating high fitting accuracy.

2. Reproducibility assessment

The within and between days reproducibility of NMR was assessed (Figure E2).

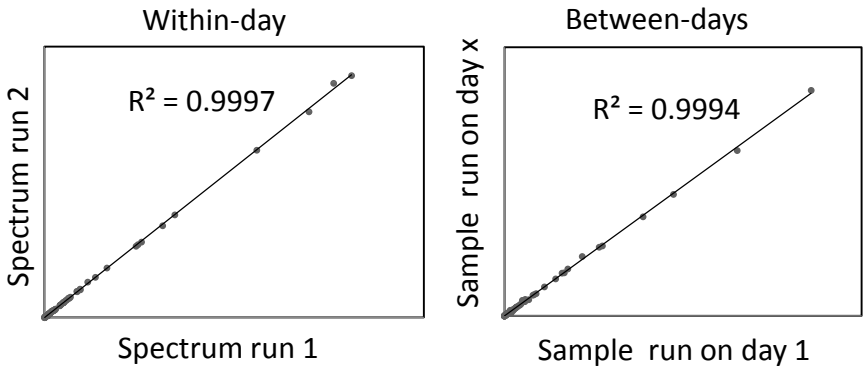

Figure E2: For the within day reproducibility (left), a blood sample collected from patient no. 11 was run twice, within an interval of approximately 12 hours. High correlation is observed between spectra (R^2 = 0.9997), indicating good within-day reproducibility. For the between days reproducibility (right), two different serum samples collected from patient no. 20 (both from the pulmonary artery, and both collected at exactly same time point - 2h post-CPB), were run within an interval of several months from each other. Good correlation is observed between spectra (R^2 = 0.9994), indicating good between-days reproducibility.

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