Project Protocol

1. Title

Exploring biomarkers in the heat and moisture exchange filters

2. Purpose, rationale, hypothesis and background

Purpose: To investigate if standard heat and moisture exchange (HME) filters contain endogenous proteins, that can be used for diagnostic purposes in patients undergoing elective major non-malignant surgery. Pulmonary complications are common in these patients and this pilot-study will investigate if human proteins are contained in the HME filters.

Rationale for the project: Patients undergoing major surgery are invasively mechanical ventilated during anaesthesia. In the ventilatory circle, a HME filter is inserted near the tracheal tube to warm and humidify the inhaled air to prevent drying of the airway mucosa. The HME filter also traps the exhaled heat and moist from the patient's lungs and releases the stored heat and moisture. The HME filters contains an electrostatic filter, which might catch exhaled human proteins without releasing them during ventilation. Previous studies have explored the potential for diagnosing medical conditions based on exhaled proteins collected by special devices, and together they prove the principle of exhaled breath diagnostics.[1–5]

The HME filters are routinely discarded when the patients are extubated, but maybe they contain a goldmine of information about the patients. This project investigates if exhaled breath proteins can be collected using standard HME filters.

Project hypothesis: The HME filters from surgical patients on mechanical ventilation contains endogenous proteins and extracellular vesicles and can be a non-invasive source for diagnostics and monitoring.

Project objective and aims: The project will investigate the exhaled proteins and extracellular vesicles from HME filters from patients undergoing elective surgery after invasive mechanical ventilation during anaesthesia.

Primary aim:

- Measure total amount of human protein in HME filters and compare to total protein in blood.

Secondary aims:

- Assess variation in exhaled breath protein concentration.
- Measure HME filter protein contents according to length of invasive mechanical ventilation.
- Investigate if protein molecular weight and isoelectric point has influence on representation in HME filters.
- Investigate if extracellular vesicles from the exhaled breath can be captured and analyzed from HME filters.
- Investigate if the HME filter protein content differs between intravenous versus inhalation anaesthesia.

Background: Postoperative pulmonary complications (PPCs) are common and associated with increased morbidity and mortality. The diagnoses varies from atelectasis, pneumonia, acute lung injury, pneumothorax, and pulmonary embolism among others. Several perioperative therapies have been evaluated to decrease the risk of PPCs, but the evidence is still sparse. The major challenge is to identify patients at risk as there is no specific biomarker for acute lung tissue injury. The clinic-pathological understanding of PPCs., and hence precise diagnosis and effective prevention and treatment are challenged because it is hard to get representative samples from the lungs of patients with acute respiratory failure without use of invasive methods such as bronchoalveolar lavages (BAL) or biopsies. However, these methods itself increases the risk of PPCs. During a BAL, the surfactant of the lungs is washed out, which increases the risk of alveolar collapse and lung biopsies increase the risk of pneumothorax, which highlights the unmet need for techniques for non-invasive biospecimen sampling from the airways. This was recently addressed in a new working group report from the American Thoracic Society, with emphasis on HME filter fluid, non-bronchoscopic BAL, endotracheal aspirates and sputum samples.[6]

One of the authors of the working group report have specifically investigated the proteins in the fluid, that can be retained from some HME filter types.[7,8] Our group has demonstrated diagnostic potential of exhaled breath proteins[1,3,9] and efficient exhaled breath protein-capture by electrostatic filter, which is the matrix in HME filters (manus in preparation). We therefore envision, that analysis of the proteins in the filter matrix, not restricted to the HME filter fluid, will identify significantly more protein and thus serve as an efficient new non-invasive source for biomarkers.

A recent study has detected lung tissue derived extracellular vesicles in exhaled breath condensate.[10] Extracellular vesicles (EVs) are nano-sized, membrane-bound particles secreted by all cell types, ranging from 30-5000 nanometers in diameter. These lipid bilayer-enclosed structures carry diverse molecular cargo including proteins, RNA, and DNA that directly reflects the physiological state of their parent cells.[11]

3. Methods, Statistics and Study Subjects

Study subjects and design:

This study is a prospective clinical study addressing the objectives and aims. We will include adult patients who are scheduled for elective surgery for non-malignant conditions under general anesthesia with invasive mechanical ventilation. The written and oral study information will be provided at the standard preoperative consultation with the anesthesiologist the day before elective surgery. We will include 10 patients, in- and exclusion criteria are elaborated in section 5.

Protein measurements and detection of extracellular vesicles:

HME filters: Total protein concentration will be estimated by elution of the proteins from the dismantled HME filters using SDS buffer solutions and fluorescence analysis. Single proteins, as indicated in table 1, will be measured by standard biochemical analysis at the Department of

Clinical Biochemistry. Besides, we will use flowcytometry to check potential presence of extracellular vesicles using CD9 as specific marker of this.

Blood samples: Standard clinical biochemical analysis for the proteins in table 1.

Table 1. List of proteins to measure in HME filters (mg/10 Liter exhaled air) and blood.

Protein	MW (kDa)	Isoelectric Point (pI)	Plasma Concentration (g/L)
IgM	~900	5.0	0.5–2
Fibrinogen	340	5.5	2–4
IgA (dimer)	160–400	6.0	1–4
Complement C3	190	5.5	0.8–1.8
Haptoglobin	45–100	6.0	0.3–2
α1-antitrypsin	52	5.5	0.9–2
Ceruloplasmin	132	5.5	0.2–0.6
IgG	150	7.0	7–16
Transferrin	80	6.5	2–3
Albumin	66	4.7	35–50
Apolipoprotein A-I	28	5.2	1–1.5
Apolipoprotein B-100	550	6.0	0.9–1.2
IgD	180	7.0	<0.05
IgE	190	7.0	<0.05
Complement Factor H	155	5.0	0.2–0.4
β2-microglobulin	12	11	0.002–0.003
Cytokines (IL-6, TNF-α)	21–25	6–7	<0.001
α2-macroglobulin	720	5.3	1–3
Plasminogen	92	6.0	0.2–0.4
Fibrinogen Fragments	50–250	5–6	variable

4. Sample size and statistics

We cannot perform a regular power calculation before we have the results from this study, which will form the basis for new studies of exhaled breath proteomics based on collection from HME filters. Based on experience from the project collaborators at Department of Clinical Biochemistry, who as a daily task perform test of biochemical analysis validity and variation, 10 study subjects is a suitable sample size to investigate the project aims.

Spaghetti -and box plots will be used to depict the protein concentrations, microdata from spaghetti plots will not be published. To compare means and inter-individual, we will apply the t-test if underlying assumptions are met, and report interclass correlation coefficients as well as coefficients of variation. The statistical analyses will be supervised by biostatistician from the department of hematology.

5. Inclusion and Exclusion criteria

Inclusion criteria	Exclusion criteria
• ≥18 years, able to understand study information in Danish. • Admitted for elective surgery for non-malignant conditions with either intravenous or inhalation anaesthesia and invasive mechanical ventilation according to standard practice. • Expected invasive mechanical ventilation > 1 hour. • Possess legal capacity. • Informed, signed consent is obtained.	• Patients who are transferred to the ICU for continuous invasive mechanical ventilation after end of elective surgery. Known chronic lung disease.

6. Risks and adverse events

Since this project uses HME filters, that are normally discarded, we don't expect that the patients will experience discomfort because of collection of the exhaled breath proteins in this project.

Arterial blood sample will be collected from the arterial line placed during anaesthesia according to standard practice. The volume of blood (4 mL EDTA blood + 3,5 mL citrate blood) is minimal and does not give rise to any risk for the participants.

7. Biological data

We will collect the HME filter and ask for two arterial blood samples (4 mL EDTA blood + 3.5 mL citrate blood) from each study participant. See Figure 1 for overview of samples. The HME filter is inserted in the respiratory circuit as part of the standard procedure in patients being invasively mechanically ventilated. At the time of extubation, we will collect the blood samples and the HME filter and store them in the projects research biobank until analysis, as all samples must be analyzed simultaneously. Blood samples will be double centrifuged to obtain platelet poor plasma supernatants (whole blood: 2500 g for 15 minutes, supernatant: 2500 g for 15 minutes) before transfer to the project's research biobank.

The complete amount of exhaled breath proteins captured in the HME filters will be used in analysis, no material will be left after analysis. A minor amount of platelet poor plasma will probably be left after analysis. These remains will be destructed after all the planned protein analysis are finished.

The laboratory data (protein measurements) will be stored at the logged Q-drive at Department of Hematology, Aalborg University Hospital, North Denmark Region.

Data will be deleted after project end, which is defined in the Article 30 registration as 31. December 2031.

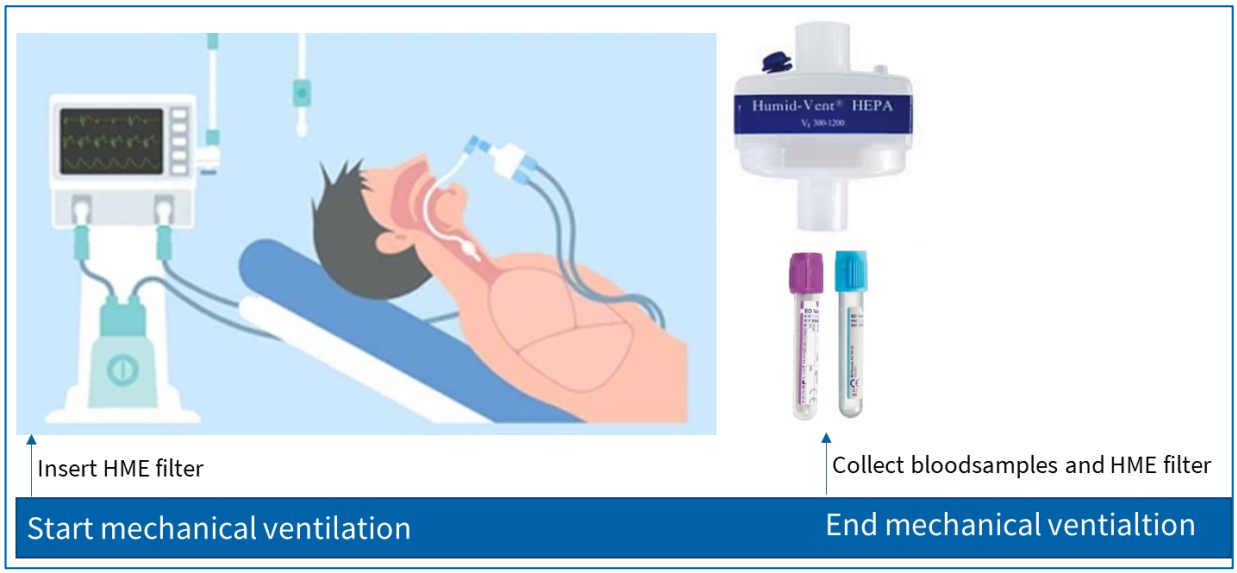

Figure 1. Overview of biological samples per patient.

8. Data from electronic medical records

Patients who are scheduled for elective major surgery will be identified at the weekly list of surgical patients, which is preplanned Thursday or Friday the week before surgery. Based on the type of surgery and thereby the type of anaesthesia, the anaesthetists who is member of the project team, will identify patients who fulfill the inclusion criteria. The data below will be transferred from the surgical lists to the project anaesthetists to identify potential project participants. We expect that these informations will be transferred to researchers from a maximum of 25 potential project participants as we expect that most invited patients will participate in the study:

- CPR number, name and age.
- Type of elective surgery.
- Length of invasive mechanical ventilation (minutes).

The following data will be transferred after informed consent from included project participants. Data are obtained directly from the patients at the conversation with the project worker, obtained directly from the mechanical ventilator during surgery or electronic medical records:

- Date of elective surgery.
- Total liters of exhaled air per HME filter during invasive mechanical ventilation.
- Ventilatory parameters: Tidal volume (ml/kg), respiratory frequency/minute, peak pressure (cmH_2O), positive end-expiratory pressure (cmH_2O), end-tidal CO_2 (kPa).
- Length of invasive mechanical ventilation.
- pH, pO2 and pCO2 from arterial blood gas analysis, if analyzed during surgery.

The clinical data will be entered into the projects REDcap database at Aalborg University Hospital.

An informed consent gives the investigator and her delegates direct access to obtain relevant information from medical records, including electronic medical records, to see information regarding the study participants' health conditions, which is necessary for the projects' execution – hereunder quality control and monitoring, cf. *Komitéloven §3, stk. 3.*

9. Handling of personal data

In this study, the law of handling of personal data will be followed, cf. *Komitéloven §20, stk. 1, nr. 4 and 6*. The project will include collection of personal data and analyses performed on the exhaled breath referable to the patients and control subjects included. The project is following the General Data Protection Regulation and the project has been registered at North Denmark Region's record of processing activities (F2025-193). Published data will be anonymized.

10: There are no external partners in this project.

11. Economy

Inger Lise Gade is the project principal investigator. She has taken initiative to this study, which is designed and conducted in a collaboration between the Department of Hematology, the Department of Anesthesia and Intensive Care and the Department of Clinical Biochemistry at Aalborg University Hospital. The project will be financed by internal funds at the involved departments. Two medical students will contribute to the participant recruitment and laboratory work as part of their master thesis. Blood collection tubes and expenses to laboratory analysis are covered by the Department of Clinical Biochemistry. Companies are not involved in the project. Inger Lise Gade's salary for research time is covered by a career grant for clinical research from the Novo Nordisk Foundation (Grant ID: NNF24OC0089034), but the project expenses are not covered by this grant.

12. Participant compensation

The study subjects will not receive any economic compensation for their participation in the study.

13. Recruitment of study subjects and informed consent

Study subjects will be recruited at the Department of Anesthesia at Aalborg University Hospital.

The subjects matching the inclusion criteria will receive oral introduction and written information about the study during the routinely preoperative anaesthesia assessment by an anesthetist the day before surgery. Subsequent oral, structured information about the study will be given after the routine anesthesia work-up either in the examination room or in a nearby meeting room, in quiet surroundings. The principal investigator or one of the projects team members (team anesthesiologists or the involved medical students) will give the structured, oral information. No actions related to the scientific project will take place before informed consent is achieved. The oral study information will be given in quiet surroundings with the room door closed and in the presence of an assessor in form of a relative/other person accompanying the patient to the hospital, if the patient wishes. The patient will not be included in the study if the assessor is unwilling or unable to accompany the patient. The patients can ask clarifying questions and will be offered time for deliberation. The patient will be informed the day before surgery and as early as possible enabling

sufficient time for the patients to consider participation and to bring a relative. The signed informed consent can be given at the day of surgery. In the informed consent, the patients can specify if he/she wants to be informed about the outcome of the study. The patients can withdraw their consent at any given time. If an unexpected complication should occur, the patient will be excluded from the study and will be informed about this.

14. Dissemination of results

The study will be registered at Clinicaltrial.gov before study start. Besides the master thesis, we plan to submit an abstract for the American Thoracic Society annual meeting 2027 (deadline for abstract submission is medio November 2026). The master students will present the abstract, in case they wish to participate in the congress. The findings in the master project are expected to form the basis for a scientific publication afterwards. If the master students wish to draft the manus, they will be first authors, otherwise the involved collaborator from Department of clinical Biochemistry will be first author. Inger Lise Gade will be the last author of the scientific publication. Both positive, neutral and negative results will be published.

15. Research Ethics

The study will be performed according to the ICH/GCP guidelines and in agreement with the Helsinki declaration. The study must be approved by the regional Committee on Health Research Ethics before patients can be enrolled. Patients must receive all relevant information and have signed an informed consent before initiation of exhaled breath sample collection. The patients can at any time point withdraw his or her informed consent without giving a reason and leave the study. This will have no impact on future treatment. Participation in the study will not benefit the patients directly in any way.

The overall consequences and discomfort related to participation in this study are considered minor and hence minimal compared with the potential benefit for future patients, if this study shows that we can search for non-invasive biomarkers of life threatening conditions in patients on mechanical ventilation.

16. Insurance

Study participants are insured by Patienterstatningen.

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