Characterization of human intestinal macrophages in metabolic disease – iMAC (pilot) study

Research legislation:	Ordinance on human research with the exception of Clinical trials (HRO)[1].
Type of Research Project:	Research project involving human subjects
Risk Categorisation:	A
Project Leader:	PD Dr. med. Claudia Cavelti-Weder Senior physician and group leader University Hospital Basel, Department of Endocrinology, Diabetes and Metabolism, Petersgraben 4, CH-4031 Basel Email: claudia.cavelti-weder@usb.ch Phone: +41 61 328 63 23 Fax: +41 61 265 51 00

PROTOCOL SIGNATURE FORM

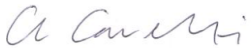
Study Title: Characterization of intestinal macrophages in metabolic disease – iMAC (pilot) study

The project leader has approved the protocol version 2, 9.5.2018, and confirms hereby to conduct the project according to the protocol, the Swiss legal requirements[1,2], current version of the World Medical Association Declaration of Helsinki[3] and the principles of Good Clinical Practice.

Project leader:

Site: University Hospital Basel,
Department of Endocrinology, Diabetes and Metabolism,
Petersgraben 4, CH-4031 Basel

Name: PD Dr. med. Claudia Cavelti-Weder

Date: 12.11.2018 Signature: 

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GLOSSARY OF ABBREVATIONS

BASEC	*Business Administration System for Ethical Committees*
CRF	*Case report form*
HRA	*Human Research Act*
HRO	*Ordinance on Human*

1 BACKGROUND AND PROJECT RATIONALE

Metabolic disease including obesity and diabetes has reached epidemic proportions in the past years. Classical risk factors are unhealthy diet and physical inactivity. However, also smoking and air pollution have emerged as unexpected risk factors for type 2 diabetes[4-6].

Inflammation has been recognized as key characteristic of metabolic disease and is predictive of future cardiovascular events. In the blood, levels of circulating pro-inflammatory cytokines are elevated[7,8]. Additionally, macrophages are increased in adipose and muscle tissue of obese subjects[9,10], the pancreas of diabetic patients[11] and atherosclerotic lesions[12]. However, the starting point of chronic low-grade inflammation is not known.

As the gastrointestinal tract first comes into contact with dietary components, but potentially also air pollution/ smoke particles ingested upon mucociliary clearance from the lung, the gut could be the starting point of inflammation in metabolic disease. Indeed, gastrointestinal changes prevail in both metabolic disease by classical risk factors and air pollution, such as altered gut microbiota and increased gut permeability[13-21], suggesting that inflammation could start in the gut.

In preclinical studies with mice fed high fat diet, we observed a shift in intestinal macrophages towards pro-inflammatory subpopulations (unpublished). These inflammatory macrophages could potentially initiate systemic/ adipose tissue inflammation and impact thereby on insulin secretion.

The aim of our study is to characterize intestinal macrophages in obese versus lean subjects and smokers versus non-smokers to translate our preclinical findings to human disease and assess whether an inflammatory shift prevails in human intestinal macrophages in metabolic disease. Additionally, to assess whether intestinal macrophage subpopulations can be altered deliberately by nutritional intervention, we will assess intestinal macrophages from subjects scheduled for bariatric surgery that will be on a calorie-restricted diet during the last 4 weeks prior to surgery.

2 PROJECT OBJECTIVES AND DESIGN

2.1 Hypothesis and primary objective

We hypothesize that intestinal macrophages shift towards pro-inflammatory subpopulations in metabolic disease, potentially initiating chronic low-grade inflammation. Additionally, it seems conceivable that a reduction of caloric intake could reverse this inflammatory shift. Regarding air pollution, exposure to particulate matter could also alter intestinal macrophages and thereby mediate metabolic disease.

Thus, our primary objective is to characterize intestinal macrophages in obese versus lean subjects and smokers versus non-smokers and to assess whether intestinal macrophage subpopulations become less pro-inflammatory upon nutritional intervention. To our knowledge, this is first study to comprehensively analyze human intestinal macrophages in metabolic disease.

2.2 Primary and secondary endpoints

Primary endpoints: In our preclinical studies, we found that mice fed a high fat diet had a shift of intestinal macrophages towards inflammatory subpopulations, which precedes adipose tissue inflammation. We think that intestinal macrophages could also be involved in human metabolic disease. Therefore, our primary endpoint is to investigate:

1) the frequency & subpopulations of intestinal macrophages from the *colon* in obese versus lean subjects and smokers versus non-smokers,

2) the frequency & subpopulations of intestinal macrophages from the *upper gastrointestinal tract* in obese versus lean subjects,

3) the frequency & subpopulations of intestinal macrophages from the upper gastrointestinal tract in obese subjects *before and after a nutritional intervention.*

Secondary endpoint: Besides macrophages, also other intestinal immune cells could be implicated in metabolic disease. For example, one preclinical study found that adaptive immunity might be involved[22]. In case we do not find clear differences in frequency or subpopulations of intestinal macrophages, we will assess other intestinal immune cells (e.g. B-, T-lymphocytes), and enteroendocrine cells (e.g. L-cells) as a secondary endpoint in an explorative manner. Additionally, we aim to correlate our findings from the biopsy samples with inflammation markers and immune cells in the blood, with microbiota and immune cells in the stool and with the eating habits of the patients.

2.3 Project design

This is a prospective, observational study aiming at improving our understanding of the pathophysiology of metabolic disease. The study will entail qualitative and quantitative analyses of patient-derived gut biopsy samples. To our knowledge, this is the first study to comprehensively analyze human intestinal macrophages in metabolic disease.

3 PROJECT POPULATION AND STUDY PROCEDURES

3.1 Project population, inclusion and exclusion criteria

Study population: To elucidate the role of intestinal immune cells in metabolic disease, human gut samples are necessary, which can be obtained from gut biopsies or surgical samples. As patients will undergo diagnostic endoscopy of the gastrointestinal tract or bariatric surgery for clinical reasons and the standard of care will not be changed by the study, there will be no additional interventions to patients by their participation in the study. Participants fulfilling the following inclusion criteria are eligible for the study:

For *colonic tissue samples*, 10 subjects per group referred to the Department of Gastroenterology at the University Hospital Basel or the doctor's office MagenDarm Basel, for a diagnostic colonoscopy on the basis of symptoms suggestive for irritable bowel syndrome or for a screening colonoscopy for colorectal cancer will be included:

- Obese weight non-smokers (BMI >32kg/m^2), n=10
- Obese smokers (BMI >32kg/m^2, ≥1 pack cigarettes/d), n=10
- Normal weight non-smokers (BMI <27kg/m^2), n=10
- Normal weight smokers (BMI <27kg/m^2, ≥1 pack cigarettes/d), n=10

For *tissue samples of the upper gastrointestinal tract and assessment of nutritional intervention,* 10 subjects per group referred to the Department of Visceral Surgery at the University Hospital Basel, the doctor's office MagenDarm Basel or the Department of Visceral Surgery, Hospital Lindenhof Bern, for bariatric surgery will be included. All bariatric patients receive a screening gastroscopy as standard of care prior to the surgery. Subjects willing to participate will contribute gastric and duodenal biopsies from the preoperative screening gastroscopy as well as an approximately 1cm long piece of the jejunum, which is usually disposed during bariatric surgery. As controls, 10 subjects referred to the Department of Gastroenterology at the University Hospital Basel or the doctor's office MagenDarm Basel, for an upper endoscopy on the basis of dyspeptic symptoms will be included as well.

- Obese non-smokers (BMI >35kg/m^2) undergoing bariatric surgery, n=10
    - a) Pre-diet: gastric and duodenal biopsies from the preoperative screening gastroscopy
    - b) Post-diet: jejunal tissue specimen from bariatric surgery
- Normal weight non-smokers (BMI <27kg/m^2), n=10

Patients will be excluded from the study in the following cases: Inability to provide informed consent, e.g. because of mental impairment or insufficient knowledge of project language; intake of corticosteroids, anti-inflammatory/ immunosuppressive drugs potentially altering immune cells; clinical signs of current infection; known anemia (e.g. hemoglobin < 110g/L for males, < 100g/L for females); known neutropenia (e.g. leucocyte count < 1.5 x 10^9/L or ANC < 0.5 x 10^9/L); known immunodeficiency, e.g. HIV; known vasculitis, collagenosis; known inflammatory bowel disease; known adrenal insufficiency and/or substitution with glucocorticoids; known clinically significant kidney or liver disease (e.g. creatinine > 1.5mg/dL, AST/ALT > 2 x ULN, alkaline phosphatase > x ULN, or total bilirubin > 1.5 x ULN); risky daily alcohol consumption (> 24g/d for males, > 12g/d for females), known liver cirrhosis Child B or C; known uncontrolled congestive heart failure; known uncontrolled malignant disease; currently pregnant or breastfeeding.

Substudy (validation of diet questionnaire): To validate a diet questionnaire called „Healthy Eating Index" (modified after J. Russell[23]), we will perform a substudy in normal- and overweight people from the Department of Endocrinology (n=100).

3.2 Recruitment, screening and informed consent procedure

Eligible patients will be screened by the participating gastroenterologist, endocrinologist or visceral surgeon. Patients planned for diagnostic endoscopy of the gastrointestinal tract or bariatric surgery at the University Hospital of Basel, the doctor's office MagenDarm Basel or the Department of Visceral Surgery, Hospital Lindenhof of Bern will be considered, contacted and informed about the study. Ample time for consideration and the opportunity to ask questions will be given. Written informed consent will be obtained according to pertinent regulations. No laboratory or diagnostic tests are necessary for inclusion of the patient. As the study does not involve any additional procedures for the patient, no compensation will be paid. Substudy (validation of diet questionnaire): Normal- and overweight people from the Department of Endocrinology will be asked to fill out a diet questionnaire (no inclusion or exclusion criteria).

3.3 Study procedures

Screening and inclusion: The study procedure is summarized below. The first patient is planned for September 2018 with a study duration of 2 years. Patients planned for endoscopies of the gastrointestinal tract or bariatric surgeries at the University Hospital Basel, the doctor's office MagenDarm Basel or the Hospital Lindenhof Bern will be screened for inclusion and exclusion criteria and informed about the study by the participating gastroenterologist, endocrinologist or visceral surgeon. Informed consent will be obtained at the latest on the day of the endoscopy or hospital admission for bariatric surgery.

Tissue sampling: Endoscopies or bariatric surgeries will be carried out as per clinical standard of care. Our collaborators are PD Dr. P. Hruz at the Department of Gastroenterology and PD Dr. T. Delko at the Department of Visceral Surgery, University Hospital Basel, Dr. J. Pilz and Prof. Dr. R. Meier at the doctor's office MagenDarm Basel and PD Dr. I. Langer at the Department of Visceral Surgery, Hospital Lindenhof Bern. The samples obtained from endoscopies comprise 10 to 12 biopsies per subject including two biopsies being frozen and later on analyzed for inflammatory markers (e.g. TNFα, IL6). The biopsies will be either collected in the Colon transversum in case of colonoscopy or in the stomach and duodenum in case of gastroscopy as we have seen in our mice study different subpopulations depending on the location. The tissue samples from the bariatric surgery are a 1cm piece of the jejunum that would normally be disposed. Additionally, four EDTA and one serum blood tube for the analysis of inflammatory cells and markers in the blood will be taken as well as a single stool sample.

Data: Medical data will be obtained from the patient's electronic health record. The work-up of the tissue specimen, the stool sample and the EDTA blood samples by flow cytometry will be carried out in the laboratory of PD Dr. C. Cavelti-Weder. Leftover tissue specimen will be snap frozen as well as the serum blood sample after centrifugation. We do not expect biases in our study.

Substudy (validation of diet questionnaire): For validation purposes, normal- and overweight people from the Department of Endocrinology will be asked to fill out a diet questionnaire called „Healthy Eating Index" modified after J. Russell) taking about 15 minutes.

3.4 Withdrawal and discontinuation

No tissue samples will be taken from patients withdrawing consent prior to the endoscopy or bariatric surgery. If withdrawal from the study occurs after tissue sampling but prior to sample analysis, clinical data and the sample will be destroyed and work-up of the tissue not performed. Discontinued patients will be replaced in order to reach the pre-specified number of study participants. If withdrawal occurs after work-up of the sample, clinical and tissue data will be retained in the study and used for analysis in a coded manner. After the analysis, the clinical and tissue data will be irreversibly anonymised.

4 STATISTICS AND METHODOLOGY

4.1. Statistical analysis plan

The study will entail samples collected from patients undergoing endoscopy of the gastrointestinal tract or bariatric surgery at the University Hospital Basel who sign an informed consent allowing to use their

tissue for research purposes. Samples sizes were calculated using G*Power, University Düsseldorf. The primary endpoint will be analyzed by Mann-Whitney U test assuming an alpha-error of 0.05 as limit of significance and a power of 0.95. Based on our preclinical mouse data experiments, we calculated an expected effect size of 2.4 (in terms of absolute numbers of intestinal macrophage subpopulation P1), leading to a necessary group size of 6 subjects per group. However, as we expect greater variance with human data compared to mice due to diet/ lifestyle, we suggest including 10 subjects per group as specified below, accounting for a total of study participants of 60:

Colonic tissue samples:

- Obese weight non-smokers (BMI >32kg/m^2), n=10
- Obese smokers (BMI >32kg/m^2, ≥1 pack cigarettes/d), n=10
- Normal weight non-smokers (BMI <27kg/m^2), n=10
- Normal weight smokers (BMI <27kg/m^2, ≥1 pack cigarettes/d), n=10

Tissue samples of the upper gastrointestinal tract and assessment of nutritional intervention:

- Obese non-smokers (BMI >35kg/m^2) undergoing bariatric surgery, n=10
    - a) Pre-diet: gastric and duodenal biopsies from the preoperative screening gastroscopy
    - b) Post-diet: jejunal tissue specimen from bariatric surgery
- Normal weight non-smokers (BMI <27kg/m^2), n=10

Descriptive statistics will be used for outcome measures. To compare groups (e.g. obese versus lean, smokers versus non-smokers) two-tailed non-parametric tests will be used (Mann-Whitney). A p-value <0.05 will be considered as statistically significant. These statistical analyses will be done with the help of GraphPadPrism.

4.2. Handling of missing data

Missing data will not be imputed.

5 REGULATORY ASPECTS AND SAFETY

5.1 Local regulations / Declaration of Helsinki

This research project will be conducted in accordance with the protocol, the Declaration of Helsinki[3], the principles of Good Clinical Practice, the Human Research Act (HRA) and the Human Research Ordinance (HRO)[1] as well as other locally relevant regulations. The Project Leader acknowledges his responsibilities as both the Project Leader and the Sponsor.

5.2 Notification of safety and protective measures (HRO Art. 20)

The project leader is promptly notified (within 24 hours) if immediate safety and protective measures have to be taken during the conduct of the research project. The Ethics Committee will be notified via BASEC of these measures and of the circumstances necessitating them within 7 days.

5.3 Serious events (HRO Art. 21)

If a serious event occurs, the research project will be interrupted and the Ethics Committee notified on the circumstances via BASEC within 7 days according to HRO Art. 21[1].

5.4 Amendments

Substantial changes to the project set-up, the protocol and relevant project documents will be submitted to the Ethics Committee for approval according to HRO Art. 18 before implementation. Exceptions are measures that have to be taken immediately in order to protect the participants.

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[1] A serious event is defined as any adverse event where it cannot be excluded, that the event is attributable to the sampling of biological material or the collection of health-related personal data, and which:
a. requires inpatient treatment not envisaged in the protocol or extends a current hospital stay;
b. results in permanent or significant incapacity or disability; or
c. is life-threatening or results in death.

5.5 End of project

Upon project termination, the Ethics Committee is notified within 90 days. Coded medical records and biological material will be retained for 10 years at the Department of Endocrinology, Diabetes and Metabolism or the Department of Biomedicine, University Basel, and then destroyed.

5.6 Insurance

In the event of project-related damage or injuries, the liability of the University Hospital Basel provides compensation, except for claims that arise from misconduct or gross egligence.

6 FURTHER ASPECTS

6.1 Overall ethical considerations

We assume that study results will be generalizable to the general population. As study procedures are performed as per clinical standard of care and no additional procedures are planned just for study purposes, burden and time for participants are well justifiable. Project-specific ethical aspects such as incidental findings seem unlikely as our readouts focus on characterization of intestinal macrophages. Therefore, a fair balance for the study participants seems to be granted.

6.2 Risk-Benefit Assessment

As study participants will undergo endoscopies of the gastrointestinal tract and bariatric surgeries per clinical standard care and the study does not involve additional procedures except a simple blood withdrawal and a stool sample, subjects are not exposed to an additional risk.
The study participant has no immediate benefit from the study. However, elucidation of the role of intestinal macrophages in metabolic disease could help to better understand the pathophysiology of this highly prevalent condition with potential development of targeted therapies in the future.

7 QUALITY CONTROL AND DATA PROTECTION

7.1 Quality measures

For quality assurance, we will develop standard operating procedures for handling and analyses of tissues samples.

7.2 Data recording and source data

Available medical data about date of birth, gender, race, weight, height, BMI, waist and hip circumference, blood pressure, heart rate, smoking status, alcohol consumption, diabetes (including date of diagnosis), other relevant diagnoses, diet and drugs within past month before samples collection, family history regarding diabetes, obesity, cardiovascular diseases, laboratory values for Hb, HbA1c, leucocytes and their differentiation, lipids, CRP, creatinine, AST, ALT, AP and total bilirubin will be collected from hospital records and documented in a coded manner in paper Case Report Forms (CRF). Numerical values will be later transferred into GraphPad prism for statistical analysis. The original source data file will be archived. Changes of the data will be saved as separate files with accompanying information why values had to be modified. Upon the participant's permission, medical information may be given to his or her personal physician or other appropriate medical personnel responsible for her or his welfare.
Data retrieved from biological material regarding intestinal macrophages will be stored in a coded manner in GraphPad prism files upon analysis.
Participants will be asked whether the data obtained during this research project may be used for subsequent projects or secondary analyses.

7.3 Confidentiality and coding

Medical data obtained for this study is confidential and is only accessible to authorized personnel who require the data to fulfil their duties within the scope of the research project. Disclosure to third parties

other than those noted below is prohibited. Confidentiality of the subjects will be maintained by assigning subjects an ID number, keeping identifiers separate from the data and storing data in a locked file in the department of Endocrinology, Diabetes and Metabolism. The principle investigator and the co-investigators will have access to the encryption list.

Biological material in this project is not identified by participant name, but by a unique ID number. Biological material is appropriately stored in a restricted area only accessible to authorized personnel.

7.4 Retention and destruction of study data and biological material

Records and documents pertaining the conduct of this study, including CRFs, consent forms, laboratory test results and clinical notes will be retained for 10 years at the Department of Endocrinology, Diabetes and Metabolism, University Hospitals Basel, and then destroyed. Remaining tissue samples will be archived and used for additional analyses if deemed appropriate. The Department of Endocrinology complies with regulations of its own biobank.

8 FUNDING / PUBLICATION / DECLARATION OF INTEREST

PD Dr. Claudia Cavelti-Weder has obtained funding from the Velux-Foundation and from the Swiss National Science Foundation. There is no conflict of interest between the funding bodies and the researchers.

9 REFERENCES

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