Study Protocol

Project title:

Skin tumor biomarkers by mass spectrometry imaging

Sponsor and principal investigator

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2. **Aim**

2.1 Problem definition

More than 1 million patients were diagnosed with non-melanoma skin cancer (NMSC) worldwide in 2018 and this number could be underestimated due to the lack of registries or incomplete registration of this type of cancer [1]. Timely detection of microscopic tumors is of utmost importance in cancer diagnostics. We believe that mass spectrometry imaging (MSI) can successfully locate microscopic aggregates of a common skin cancer, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), and distinguish them from adjacent normal skin ex-vivo. MSI unveils an altered chemical profile in BCC and SCC region (lipid patterns) and does not rely on visual identification of histopathologic features.

2.2 Hypothesis

We hypothesize that NMSC cells exhibit specific, endogenous biomarkers (lipid patterns) that differ from healthy skin tissue in sections of skin biopsies. If that hypothesis is correct it will be possible in the future to develop real-time tissue diagnosis and treatment of NMSC using mass spectrometry guided surgery.

2.3 Outcome measures

- Identification of masses and distributions of selected lipid biomarkers in sections from removed BCC and SCC tissue from Mohs surgery.
- Identification of masses and distributions of selected lipid biomarkers in sections from biopsies with actinic keratoses (pre-malignant lesions).

2.4 Background

The incidence of non-melanoma skin cancer (NMSC) was of 1 million people in 2018, according to the Skin Cancer Report of 2019 by World Cancer Research Fund, being classified as the more commonly occurring cancer worldwide [1]. The NMSC englobes primarily basal cell carcinoma (BCC) and squamous cell carcinoma (SCC).

In Denmark and other Western countries the occurrence of these tumors is also high [2], and Danish incidence continue to rise, amounting to 15.000 per year. The main risk factor to NMSC is exposure to ultraviolet radiation (UVR). Light skin color, light or red hair, light eye color and immunocompromised people are other established causes to NMSC [1,3].

The Danish population is predominantly fair-skinned, geographically mobile and exceedingly sun-loving, which increases the risks of developing skin cancer. Daily, ~44 Danes receive a NMSC diagnosis, consisting of either BCC or SCC, with 40% developing new tumors in the following years [4]

NMSC has a low mortality, however it has a notable patient morbidity due to its high prevalence and recurrence [2], costing more than 19 million Euro yearly to Denmark, exceeding the cost of health management and health care costs of melanoma [5]. Care for NMSC is primarily by dermatologists, and multiple modalities can be used on the treatment of NMSC, such as Mohs micrographic surgery, excision, cryotherapy, and different topical modalities [2].

The lowest recurrence rates of Mohs micrographic surgery make this excisional surgery the first choice of treatment for high risk NMSC [6]. During this microscope-guided surgery, tissue is excised, sectioned, stained, and evaluated by a trained pathologist. The patient waits on the operating table while tissue margins are inspected for cancer cells, a finding that prompts further tissue removal from the treatment area. Accordingly, Mohs surgery is time-consuming and expensive, due to limited number of patients that can be treated per day and need for highly trained laboratory staff.

For decades, laboratories have benefitted from the impressive sensitivity and specificity of mass spectrometry (MS). A well-established analytical technique, MS ionizes compounds and sorts them based on their molecular mass. MS imaging (MSI) visualizes the spatial distribution of molecules in a similar way. Scanning entire tissue sections (e.g. skin) and generating a mass spectrum for each unit area, a map of a compound's tissue distribution is provided (Figure 1).

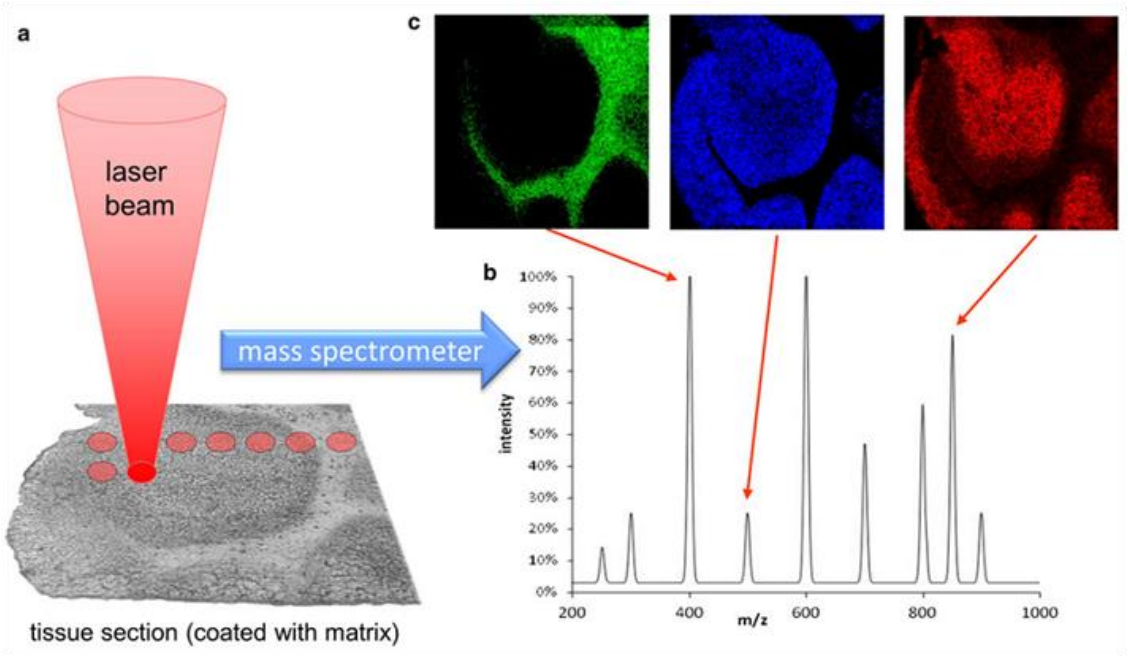

Figure 1. The tissue is sampled point by point with the laser (a). Desorbed material from each point is transfered to the mass spectrometer and a spectrum is recorded (b). Signal intensity of a component is then extracted from each mass spectrum and are transformed into gray scale values of a pixel image (c). In this way the distribution of all compound detected in the mass spectrum can be displayed [7]

MSI can detect many endogenous molecular species simultaneously, including lipids, small proteins, as well as exogenous pharmaceutical drugs distributed in minute concentrations within analyzed tissue [8]. Figure 2 shows a MSI picture of bleomycin after laser-assisted drug delivery in skin [9].

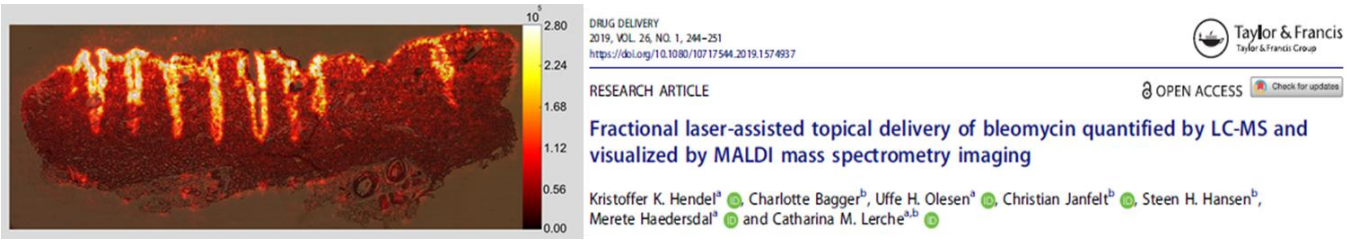

Figure 2. Mass spectrometry imaging. MSI of bleomycin (m/z 1425.56323) with laser channels. (A) Vertically cut skin cryosection after 24 h of topical drug exposure. Laser channels are easily

seen with high concentrations of bleomycin (yellow) in the coagulation zones and drug dissemination into the surrounding tissue.

Janfelt and colleagues [10] were able to distinguish between epidermis and dermis using MSI on phospholipids. We will use this technique to identify endogenous tumor biomarkers present in skin tumours but not present (or in different intensities) in adjacent normal skin. Integrating MSI into the diagnostic arsenal for NMSC, using the technology to differentiate in real-time, specific membrane lipid signatures of malignant and benign tissue.

The long-term goal beyond this project, if it is possible to identify lipid patterns able to distinguish malignant and healthy skin, is to introduce Rapid Evaporation Ionization Mass Spectrometry (REIMS), also now as iKnife [11]. This method has the potential to be far more sensitive by directly identifying chemical tumor signatures rather than simple tissue anatomy, in addition to be less labor-intensive and time-consuming. REIMS technique consists of an electrosurgical handpiece connected to a mass spectrometer, capable of performing real-time tissue identification using the aerosolised tissue produced during electrosurgery. Tissue identification is achieved by automated matching of mass spectra recorded during surgery, with a preexisting "reference library". The modality provides the surgeon with immediate information on what type of tissue is being cut, guiding treatment by delineating cancer margins with astounding precision. Lipidomic profiles showed variation between histological tumor types, enabling distinction of tissues by acting as biomarkers [12]. REIMS has also proven efficacious in other fields as a way of affirming product authenticity and quality. Thus, in the food industry, REIMS is used to distinguish between beef and horse meat in <3 seconds, boasting an accuracy of 100 % [13].

3. Method

3.1 Study design for patients with BCC and SCCs

When patients are referred for Mohs surgical procedure at the Department of Dermatology, Bispebjerg Hospital, BCCs or SCCs are removed and the tissue is embedded, sectioned and stained for the Mohs surgeon to evaluate whether more tissue is needed to be removed. Three skin sections (5-10 um thick) of this tissue there is already removed will be use in our study.

One section will be HE stained so we know exactly where the regions of interest are. Two sections will be used for MS analysis (MSI spectrum and REIMS spectrum).
Currently, we are doing Mohs surgery on high-risk BCCs at Department of Dermatology, Bispebjerg Hospital, but Mohs Surgery of SCCs will be included within a year.

3.2 Study design for patients with actinic keratoses

When patients are referred for a procedure to have treated several actinic keratoses (grade 1, 2 or 3) at Department of Dermatology, Bispebjerg Hospital we will take an extra punch biopsy (2-4 mm) depending on the size of the lesion. The biopsy is embedded and sectioned. We will use 3 skin sections (5-10 um thick). One section will be HE stained so we know exactly where the regions of interest are. Two sections will be used for MS analysis at Department of Pharmacy, University of Copenhagen. We will include actinic keratoses from up to 100 patients.

3.3 Tissue microarray

The above-mentioned sectioning for MSI will provide knowledge about a single tumor in comparison with the adjacent normal tissue. However, we also need to compare patients with each other and therefore we need to make a tissue microarray (Figure 3).

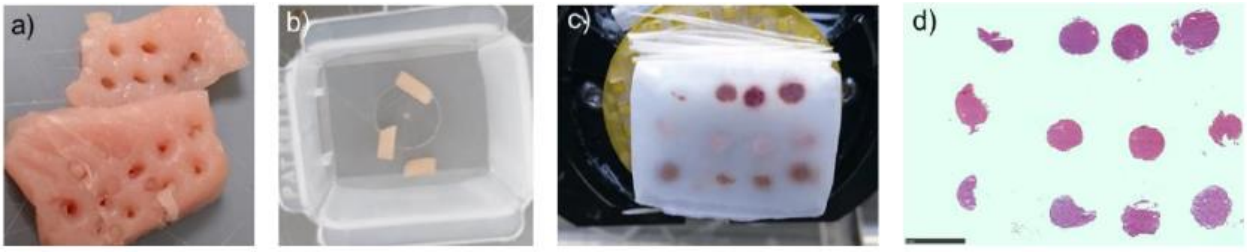

Figure 3. Example of a tissue microarray made from artificial tissue.

We will make the tissue microarray from the removed and embedded Aktinic keratoses/BCC/SCC tissue. One section will analysed by MSI with the same procedure as described above and extra sections will be HE stained for pathology evaluation and traditional immunohistochemistry markers as Ki67 and p53.

4. Statistical calculations

Multivariate statistical analysis will be performed on all mass spectra using Matlab or similar program. Linear discriminant analysis will be used to identify spectral differences between pre-malignant, cancer and normal tissue. Classification performance will be recorded with a leave-

one- patient- out cross- validation scheme. The number of samples is based on sample size calculation and previous publications on other cancer forms and estimated to be sufficient to generate a reference library. We are planning a study of a continuous response variable from matched pairs of study subjects. Prior data indicate that the difference in the response of matched pairs is normally distributed with standard deviation 8. If the true difference in the mean response of matched pairs is 3, we will need to study 58 pairs of subjects to be able to reject the null hypothesis that this response difference is zero with probability (power) 0,8. The Type I error probability associated with this test of this null hypothesis is 0,05. (programmed used for sample size calculation; Power and Sample Size Calculation, PS, Vanderbilt University; version 3.1.2). Based on these calculations will we recruit between 60 and 100 patients with BCCs, AKs and SCCs. Minimum will be 180 patients and maximum 300 patients.

5. Participants

In total maximum 300 patients will be recruited to enter the study. Men and women aged 18 years or older who meet the inclusion and exclusion criteria are included. All potential patients will be recruited from the Department of Dermatology, Bispebjerg University Hospital.

Interested patients will be invited to a preliminary information screening visit at which they will receive comprehensive oral information on the study by the primary investigator, Catharina Lerche, Mohs surgeon Martin Gluud or project nurse June Svendsen, and written information will be handed out. The meeting will take place in a separate office to ensure a safe and calm environment. The patients are specifically informed that they can bring one or more assessors to all meetings and interventions if they so choose. Written informed consent will be secured at inclusion on the surgical procedure day of which will take place at least 24 hours after the screening visit.

Each patient will be evaluated by the investigator to assess the suitability of entering the study. To ensure a homogeneous group of optical comparison, recruitment will be performed according to the following criteria:

5.1 Inclusion criteria

- Patients above 18 years of age.

- Patients presenting with AKs, SCCs and BCCs.
- Written informed consent obtained from patient.

5.2 Exclusion criteria

- Immunosuppressed patients

6. Potential risk and adverse effects

All patients will be informed both verbally and in written form about risks and possible side effects.

6.1 Punch biopsy

For patients with BCC and SCC we will only use tissue there is already removed by the standard Mohs Procedure. For patients with actinic keratoses, a biopsy will be obtained under local anesthesia, with 2, 3 or 4 mm in diameter and 3 mm in depth. Each biopsy will leave a round scar of 2 mm or 4 mm in diameter that hills within 2 weeks. Injection of local anesthesia may cause pain and a tingly/prickling sensation at the injection site. There is a minimal chance for infection, but we will treat that if it occurs.

7. Biobank

A research biobank will be established in accordance to the committee-law §2 nr. 13. All potential biopsies/sections will be kept in a locked -80 °C freezer at the Department of Dermatology, Bispebjerg University Hospital until analysis at the Department of Pharmacy, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen Ø. We cannot always do the analysis within 5-7 days because it takes up to 20 hours to make one MSI picture and if we have several BCC/SCC/Actinic keratoses patients in one day there will be a delay. When making the tissue microarray we will gather tissue from 6-8 patients before analysis. After MSI analysis the samples will be destroyed. Destruction of remaining parts of biopsy blocks and tissue microarrays blocks will be no latter than 1. January 2025. No tissue will be kept for unspecific research.

8. Information from patient journals

Health personnel will use information regarding the pathology description of the suspected lesion from the patient journal when screening possible patients to be included in this study. After the informed consent is signed by the patient, information from the patient journal concerning description of tumor type, location, size and whether it is recurrent lesion will be used.

9. Data protection, information storage & quality control

9.1 Quality control

The declaration of Helsinki II will be respected, as well the standards of good clinical research. Respect of privacy as well as physically and mentally integrity of patients will be maintained. The study will be registered with the Committee on Health Research Ethics and The Danish data Agency protection.

9.2 Data protection and storage

General Data Protection Regulation (Danish: Databeskyttelsesloven/Persondataforordningen) will be respected. Research using Capital Region of Denmark's data is considered public research. Use and distribution of data collected in this study will be discussed with patients during the consent process. The project will be reported to the Danish Knowledge Center on Data Protection Compliance (Danish: Videncenter for Dataanmeldelse, Region Hovedstaden). List of screened and patients including names, study ID number, and date of birth will be devised. All data collected will be anonymized and protected by Danish law regarding management of personal information and the Danish Health Act (Danish: Sundhedsloven). Data will be registered and stored for 5 years after study termination at the Dermatological Department, Bispebjerg Hospital.

10. Financial considerations

The study is conceived by principle investigator Catharina M. Lerche. No financial support has been conceived by commercial companies. The department of Dermatology, Bispebjerg University Hospital will provide salary for the principal investigator Catharina M Lerche. Other

expenses related to project are covered by the Danish Cancer Society (Knæk Cancer midler 1.6 mill DKK) and the Lundbeck Foundation (10 mill DKK). Principle investigator Catharina M. Lerche and the collaborators do not have any personal relation or economic stake in the Danish Cancer Society or the Lundbeck Foundation.

11. Reimbursements

There will be no reimbursements to the patients.

12. Information to patients

The primary investigator, Catharina M. Lerche, is responsible for giving clear verbal and writing information about aim, design and risks of the study, as stated in "Information og samtykke til deltagelse I sundhedsvidenskabelige forskningsprojekter" by Danish Ministry of Healthy (Danish: Sundheds- og Ældreministeriet). In an undisturbed setting, the participant will be made aware of their right to have an/or more assessor(s) present, that participant is voluntary, and that withdrawal is possible at any time during the study. Patients will be given adequate consideration time (minimum 24 hours). Patients will be asked to sign a consent form.

The patient will be urged to read enclosed material on study participant's right. No study procedures will be started before the participants has signed the informed consent. Additional questions from patients will be addressed by the contact person, Catharina M. Lerche

13. Publication of results

Positive, negative and inconclusive results will be published. The aim is to publish and present the results in a peer-reviewed international dermatology journal or/and at dermatological conferences. The intellectual property rights to the results belong to Bispebjerg University Hospital. Publications will be in accordance to the Vancouver guidelines.

14. Ethics

The biopsies will be taken at Department of Dermatology, Bispebjerg University Hospital. Personal information and samples are treated under the Act on Personal Data and Health Act. The project is furthermore carried out with minimal health and safety risks to the participants.

Surgical procedures are daily events at hospitals and clinics all over the world. This study of NMSC biomarkers and development of new treatment of skin cancer based on mass spectrometry has the potential to reduce tumor recurrence, morbidity and costs. A study of NMSC biomarkers and clinical implementation of REIMS in the treatment of skin cancer has not yet been performed. It is anticipated that the potential risks for adverse effects in this study are small and that there is a potential benefit of improvement in tumor recurrence, morbidity, costs, less labor-intensive and time-consuming. The potential of evidence -based future gains and the future perspectives this study may provide, should be held against the participants discomfort during the biopsy, and extra visit to Bispebjerg Hospital. Danish laws regarding patients' rights and compensation will be followed.

15. Insurance

Bispebjerg Hospital

16. References

1. *Diet , nutrition , physical activity and skin cancer*. *2018*.
2. Lamberg, A. L. *et al.* The Danish nonmelanoma skin cancer dermatology database. *Clin. Epidemiol.* 8, 633–636 (2016).
3. Jensen, A. *et al.* Intake of alcohol may modify the risk for non-melanoma skin cancer: Results of a large danish prospective cohort study. *J. Invest. Dermatol.* 132, 2718–2726 (2012).
4. Kyrgidis, A., Tzellos, T. G., Vahtsevanos, K. & Triaridis, S. New Concepts for Basal Cell Carcinoma. Demographic, Clinical, Histological Risk Factors, and Biomarkers. A Systematic Review of Evidence Regarding Risk for Tumor Development, Susceptibility for Second Primary and Recurrence. *J. Surg. Res.* 159, 545–556 (2010).
5. Bentzen, J. *et al.* Costs of illness for melanoma and nonmelanoma skin cancer in Denmark. *Eur. J. Cancer Prev.* 22, 569–576 (2013).
6. Smeets, N. W. J. *et al.* Mohs' micrographic surgery for treatment of basal cell carcinoma of the face - Results of a retrospective study and review of the literature. *Br. J. Dermatol.* 151,

141–147 (2004).

7. Römpp, A. & Spengler, B. Mass spectrometry imaging with high resolution in mass and space. *Histochem Cell Biol* 139, 759–783 (2013).

8. Nilsson, A. *et al.* Mass spectrometry imaging in drug development. *Anal. Chem.* 87, 1437–1455 (2015).

9. Hendel, K. K. *et al.* Fractional laser-assisted topical delivery of bleomycin quantified by LC-MS and visualized by MALDI mass spectrometry imaging. *Drug Deliv.* 26, 244–251 (2019).

10. Sørensen, I. S. *et al.* Combination of MALDI-MSI and cassette dosing for evaluation of drug distribution in human skin explant. *Anal. Bioanal. Chem.* 409, 4993–5005 (2017).

11. Balog, J. *et al.* Identification of biological tissues by rapid evaporative ionization mass spectrometry. *Anal. Chem.* 82, 7343–7350 (2010).

12. Balog, J. *et al.* Intraoperative tissue identification using rapid evaporative ionization mass spectrometry. *Sci. Transl. Med.* 5, (2013).

13. Balog, J. *et al.* Identification of the Species of Origin for Meat Products by Rapid Evaporative Ionization Mass Spectrometry. *J. Agric. Food Chem.* 64, 4793–4800 (2016).