**Protocol**

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1. Title page

**Intratumoral Influenza Vaccine for Early Colorectal Cancer**

**Sponsor and primary investigator**

Professor, MD, DMSc., Ismail Gögenur

Department of Surgery, Zealand University Hospital
Lykkebaekvej 1, DK-4600, Koege, Denmark.

**Subinvestigators**

From

Department of Surgery, Zealand University Hospital
Lykkebaekvej 1, DK-4600, Koege, Denmark.

- M.D, research fellow, Mikail Gögenur
- M.D, senior consultant, Mustafa Bulut
- Medical student, Noor Al-Huda Hadi
- M.D., PhD-student, Tobias Justesen

From

Department of Immunology and Microbiology, University of Copenhagen

Blegdamsvej 3B, 2200 København N, Building: 07-11-59

- Professor Ali Salanti

From
Department of Surgery, Sydvestjysk Sygehus
Finsensgade 35, 6700 Esbjerg

- Senior Consultant, PhD, Victor Jilbert Verwaal

From

Department of Surgery, Slagelse Sygehus

Ingemannsvej 30, 4200 Slagelse

- Senior Consultant, Line Rosell Walker

**Monitor**

GCP Unit, Copenhagen University Hospital

Bispebjerg Hospital, Building 51, 3rd floor

Bispebjerg bakke 23, 2400 Copenhagen NV, Denmark

## 2. Introduction

The remarkable results achieved in the past years with cancer immunotherapies and checkpoint inhibitors have revolutionized the field of oncology. However, recent results also show that it is only a subgroup of all patients benefitting from these treatments[1]. Tumor-infiltrating T-cells (TILs) have been highlighted as a paramount factor in the effectiveness of immunotherapies. Patients with tumors with a high amount of TILs, termed “hot” tumors, benefit the most from immunotherapies while patients with a low amount of TILs, termed “cold” tumors, benefit the least[2–4].

The T-cells ability to infiltrate and be effective in the tumor microenvironment is regulated and effected by numerous cells and cytokines[5]. Thus, the research into modulating the tumor microenvironment has started so more patients can be treated with effective immunotherapies. A recent discovery has been the usage of intratumoral interventions as a way to directly affect the tumor and possibly sparing the patients of side effects of systemic treatment[6,7]. An approach have been to use off-the-shelf vaccinations and administer them intratumorally where rotavirus and influenza virus vaccinations have been the most prominent[6,7]. The effects of either vaccine was shown to be substantial when administered intratumorally but not intramuscularly. In vivo models of mice with injected tumors in both flanks where only one flank was treated also showed a systemic response in the form of regression of both the treated and non-treated flank[7].

Both author groups focused on TILs in subsequent analyses of the tumors and both found that T-cell infiltration rose but interestingly also found that tumor cells upregulated PD-1 and CTLA-4 expression. A combination of rotavirus vaccination and anti-CTLA-4 therapy was conducted and showed a more prominent response, thus proving that a combination of the treatments complement each other[7].

Studies investigating the effects of systemic influenza vaccine in patients with colorectal cancer have shown an increase in unspecific NK cell activity[8]. The authors investigating intratumoral influenza vaccine also analyzed the tumors with Nanostring, a multiplex mRNA gene assay providing information on known

inflammatory and immune pathways, where they could show an increase in IFN-gamma, the key stimulator of NK cells[6].

**Aim of the study**

The aim of this explorative phase II clinical trial is to establish the safety and efficacy of intratumoral influenza vaccine in patients with colorectal cancer, as an additive treatment prior to intended curative surgery.

**Previous experience at Zealand University Hospital**

Professor Ismail Gögenur is the founder of Center for Surgical Science and Zealand Surgical Forum. He is one of the leading colorectal surgeons in Denmark. His primary investigational field covers optimization of colorectal cancer treatment and prognosis for the patients. Department of Surgery, Zealand University Department of endoscopy has all the necessary resources to perform this study. Only senior surgeons with high expertise in endoscopy will perform the procedures.

3. Methods

## Objectives

**Primary:**

To investigate if intratumoral influenza vaccine is a safe treatment modality for tumor down staging prior to intended curative surgery in patients undergoing treatment for colorectal cancer.

**Secondary:**

To investigate if intratumoral influenza vaccine will induce immunologic invasion of the primary tumor

**Tertiary:**

To investigate if the treatment will induce a systemic immunologic response.

**Quality of recovery**

To assess quality of recovery for patients recruited into this trial. A quality of recovery questionnaire (QoR-15) will be given to patients pre- and post-treatment.

## Study design

This is an explorative phase 2 clinical trial which will be conducted in two phases. The aim of this study is to establish the safety and efficacy of treating patients with early colorectal cancer with intratumoral influenza vaccine as a down staging and immune response enhancing treatment prior to intended curative surgery.

The first part of the study will be conducted as a pilot study. Six patients with histologically verified or clinically suspicious sigmoid colon cancer who are planned to undergo curative surgery will be included. Patients will be recruited from the Department of Surgery, Zealand University Hospital, Department of Surgery, Slagelse Sygehus and Department of Surgery, Sydvestjysk Sygehus after their case has been reviewed by the multidisciplinary team (MDT). Standard treatment involves intended curative surgery within two weeks after the diagnosis. The treatment will be performed within a few days and it will be ensured that the experimental treatment will not lead to a significant delay of intended curative surgery.

If the pilot study finishes without violating any stop rules and without any serious adverse events the second part of the study will be initiated. This will be conducted as a phase 2 study where 24 patients with histologically verified or clinically suspicious sigmoid colon cancer and rectal cancer will be included. Patients will be recruited from the Department of Surgery, Zealand University Hospital, Department of Surgery, Slagelse Sygehus and Department of Surgery, Sydvestjysk Sygehus after their case has been reviewed by the multidisciplinary team (MDT). Standard treatment involves intended curative surgery within two weeks after the diagnosis. The treatment will be performed within a few days and it will be ensured that the experimental treatment will not lead to a significant delay of intended curative surgery.

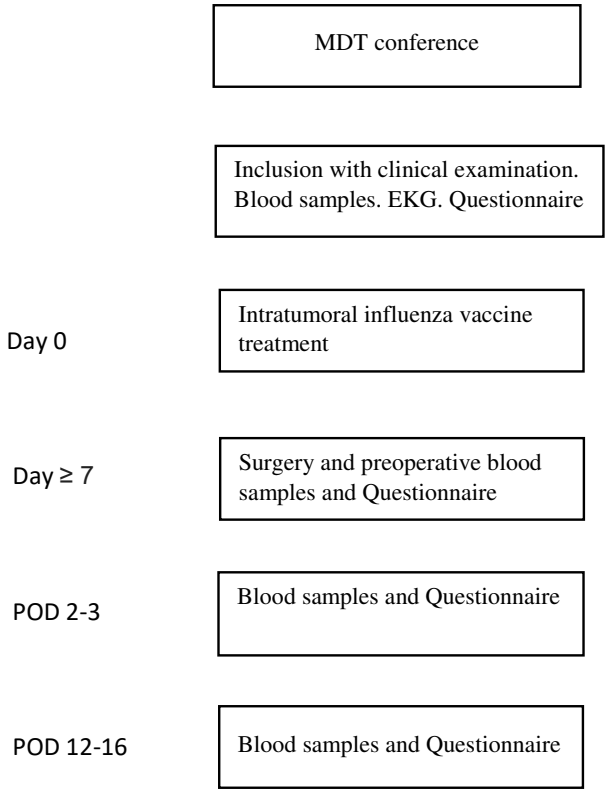
In relation to both parts of the study following samples will be collected:

Blood samples will be collected prior to the treatment with intratumoral influenza vaccine. Furthermore, blood samples will be collected on admission prior to elective surgery, within 3 days after surgery, and when the patients return to the outpatient clinic (approximately 14 days after surgery). The systemic response to intratumoral influenza vaccine will be evaluated through a multiplex gene assay, multiplex cytokine analysis, flow cytometry and NK cell activity analysis. Furthermore, ct/cfDNA will be analyzed and cell adhesion assays will be performed.

In relation to the intervention, eight intraluminal biopsies (8 x 0.5-1.5 cm) from the tumor will be collected and stored in a biobank for later analysis. The samples will be collected just before influenza vaccine is injected. In order to ensure uniformity, all tumor biopsy analyses will be performed when samples have been obtained from all participants. Tumor tissue will be registered and stored in “Dansk Cancer Biobank" according to Danish law. The Biobank will be approved by the Danish Data Protection Agency and subjected to “Persondataloven”. An additional approval will be required for future research with remaining tumor

tissue. Further, after the elective surgery, three samples of 0.5 $cm^3$ will be collected from the tumor and adjacent normal tissue and stored for later analysis. Additionally, if pathological examination reveals positive lymph nodes (eg. lymph nodes with live cancer cells) three formalin fixated specimens of the lymph nodes will be stored in the biobank. In order to ensure uniformity, all tumor biopsy analyses will be performed when samples have been obtained from all participants. Any tumor tissue left from the analyses will be stored in "Dansk Cancer Biobank" for future research according to Danish law. The Biobank will be approved by the Danish Data Protection Agency. Tissue collected before the experimental treatment will be characterized and compared with tumor tissue obtained from the final surgical specimen. Additionally, histopathologic characterization will be performed according to current standards (pTNM staging and tumor regression grade). Specific immunohistochemical staining for PD-1/PD-L1 and conventional prognostic marker analysis will be performed on biopsies and the final surgical specimen. Tumor infiltration of T-cells and subtypes will be characterized according to the immunoscore classification system. Quality of Recovery (QOR15) will be evaluated at baseline, prior to surgery on admission to the Department of Surgery, post-operative day 2-3, and at follow-up day 12-16.

*Study design:*



**Dosage**

A single vial containing 0.5 ml of Influvactetra® influenza vaccine will be suspended in 2 ml of NaCl in one vial. The treatment will be administered in four quadrants where 0.5 ml will be deposited in each location.

**Surgical procedure**

Standard surgical procedure for rectal cancer and sigmoid colon cancer, respectively. Surgical treatment outlined by MDT.

## Study endpoints

**Safety endpoints**

Safety evaluation will be performed via continuous assessment of safety parameters by reviewing events as they arise. We will conduct the pilot study in order to measure potential safety issues in a small cohort of patients before including patients in the phase 2 study.

The investigation will be put on hold if unacceptable safety issues are detected.

Primary safety endpoints:

1. Evaluation of serious adverse events
2. Evaluation of any adverse events reported.

**Efficacy endpoints**

The efficacy of the treatment will be evaluated according to the pathological examination of the surgical specimen. As such, final tumor staging, tumor regression grade (Mandard classification) T-cell subtype infiltration of the primary tumor and PD1/PDL-1 status before respective to after treatment are considered endpoints parameters. Furthermore, systemic immune responses will be evaluated through a multiplex gene assay, flow cytometry and NK cell activity analysis. A detailed evaluation of patient reported quality of recovery will be performed through repetitive administration of standardized questionnaires before the treatment with influenza vaccine (baseline), before surgery, postoperative day 2-3, and at follow-up (postoperative day 12-16).

4. Statistical considerations

## Statistical considerations

The aim of this explorative study is to establish the safety and efficacy of treating patients with primary, rectal cancer and sigmoid colon cancer with intratumoral influenza vaccine prior to intended curative surgery.

### Sample Size

This study will be the first to investigate intratumoral influenza vaccine in patients with colorectal cancer. Therefore, it is not possible to conduct formal sample size calculations. It was agreed at the coordinating investigator´s pre sub-mission meeting that six patients were an appropriate initial sample size for the pilot study.

For the phase 2 study it was agreed that 24 patients were an appropriate initial sample size for this explorative study corresponding to 24 patients with either sigmoid colon cancer or rectal cancer.

### Endpoints

1. **Safety endpoint:** Safety endpoints will be assessed continuously for serious adverse events as they may arise. Review of the adverse event reports for events that may have occurred during the investigation will serve as overall assessment of safety endpoints. Conclusions will be drawn through full analysis of all reports rather than through statistical analysis.
2. **Efficacy endpoint:**
   The Department of Pathology at Roskilde Hospital will objectively assess the surgical specimen after resection in accordance to standard guidelines (Mandard classification).
   Furthermore, our aim is to investigate changes in immunological invasion in tumor tissue before and after intratumoral influenza vaccine. Finally, we intend to investigate the systemic response to intratumoral influenza vaccine.

## Reporting of data and statistical analysis plan

After all participants have undergone treatment, the coordinating investigator will perform the statistical analyses. The analyses will begin with an exploration of the data to check for anomalies that might require data queries to be raised. Data will be presented as mean (95% confidence interval), median (interquartile range [IQR]), or number (%) as appropriate. The level of significance will be set at $p<0.05$. Data will be analyzed via parametric or non-parametric statistic depending on their distribution. Missing values, selection or exclusion of observations and variables and handling of missing values in the statistical analysis will be described carefully. Missing values will be filled in with last value available (Last Observation Carried

Forward – LOCF). A principal analysis will be performed according to the intention-to-treat principle (ITT). A per-protocol analysis will also be performed. For statistical analysis of data derived from the multiplex gene assay normalization and gene expression index calculation of probe intensities will be done, using the robust multi-array average (rma) method. Only perfect match probes will be used for data analysis. Regularized t-test will be used to calculate differences in gene expression between samples taken at different time sets. The Benjamini Hochberg method using the false discovery rate (FDR) will be used to correct for multiple hypothesis testing.

The results of this trial will be published in international or national peer-review journals regardless of negative, positive or inconclusive research results. In addition, the trial will be registered at www.clinicaltrials.gov and will be updated according to the study progress. In the event that part of the analysis is changed from the statistical analysis plan, these changes will be described and justified. Additional analyses that are not specified in the statistical analysis plan will be described at this stage and will be labeled as 'post-hoc' and the reason for their inclusion will be provided.

5. Participants

The participants in this study are individuals over 18 years with histologically verified or clinically suspicious rectal cancer and sigmoid colon cancer, with no indication for neoadjuvant chemoradiotherapy prior to intended curative surgery. The study involves recruitment of 30 patients in total, 6 in the pilot phase of the study and 24 patients with either sigmoid colon cancer or rectal cancer in the second phase of the study.

Estimated time needed to recruit participants: 24 months

Patients will be recruited from Zealand University Hospital in the Region of Zealand, Denmark, Slagelse Sygehus in the region of Zealand, and Sydvestjysk Sygehus in the Region of Southern Denmark

**Inclusion criteria**

- Patients must be mentally capable of understanding the information given.
- Patients must give written informed consent.
- Clinically suspected or histologically verified malignant tumor of the rectum or sigmoid colon.
- Tumor described as passable at index endoscopy.
- Men or women aged at least 18 years.
- Case reviewed by MDT (surgery, radiology, oncology). Case considered curable with standard surgical resection.

**Exclusion criteria**

- Highly inflamed gastrointestinal tissue which is ulcerated and bleeding
- Ongoing immunosuppressive treatment.
- Concurrent treatment with an investigational medicinal product.
- Patients with any other clinical condition or prior therapy that, in the opinion of the investigator, would make the patient unsuitable for the study or unable to comply with the study recruitments.
- Advanced tumor stages, clinical UICC stage IV.
- Indication for neoadjuvant chemoradiation or chemotherapy prior to surgery
- Acute surgical resection.
- Pregnancy
- Any previous allergic reaction to influenza vaccine or constituents, egg and chicken proteins, neomycin, formaldehyde or octoxinol-9
- Acute febrile illness
- Acute infectious disease
- Influenza vaccine administered within 30 days before study inclusion

**Discontinuation criteria**

Trial subjects are withdrawn from the study if:

- The patient withdraws his or her consent
- The disease progresses so that the patient is in need of another treatment
- Investigator deems that withdrawal is in the best interest of the patient

6. Risks and side effects

**Registration of adverse events**

Adverse events / reactions are recorded from day of treatment (Day 0) until the surgery, as it will be difficult to differ between adverse events/reactions to the experimental treatment or surgery. All adverse events / reactions should be described in medical terminology in the patient's file and recorded in case report forms (CRF). The following information must be recorded: start date/date when observed, severity, any initiated treatment, assessment of the AE if it meets the criteria for SAE, end date, and relationship to study drug. For AEs that meet the criteria for SAE, the outcome must be recorded.

The adverse reactions are classified according to CTCAE version 4.0 (Common Terminology Criteria for Adverse Events)

**Reporting adverse events**

Unexpected and serious suspected adverse reactions that are fatal or life-threatening shall be reported to the Medical Products Agency and Science Ethics Committee as soon as possible, and no later than 7 days after the sponsor has knowledge of such suspected side effect. Within 8 days after reporting, the sponsor must notify the National Board of Health and Ethics Committee with all relevant information on the sponsor and the investigator's follow-up report.

All other unexpected serious suspected adverse reactions should be reported to the same authorities within 15 days after the sponsor has been informed of this. Any report must be accompanied by comments on any consequences for the trial. After completion of the trial, all adverse reactions will be reported in the final report to the Board of Health and Ethics Committee.

Sponsor must report all events related to the medical device to the manufacturer.

**Definitions**

- **Adverse Event, AE:** Any adverse events in a patient that occur or worsen during the trial and does not necessarily have a causal relationship to study treatments.
- **Adverse Reaction, AR:** All noxious and unintended reactions to a trial drug at any dose. The term “reaction to a trial drug” means that a possible relation between the study drug and the adverse reaction cannot be excluded.
- **Unexpected Adverse Reaction, UAR:** An adverse reaction with a nature or severity that is not in accordance with the current product information.
- **Serious Adverse Event, SAE or Serious Adverse Reaction, SAR:** an event or side effect that at any dose:
  - Results in death.
  - Is life threatening.
  - Leads to hospitalization or prolongation of hospital stay.
  - Results in persistent or significant disability or incapacity.
  - Leads to a congenital anomaly or birth defect.
  - Is a major medical event.
- **Suspected Unexpected Serious Adverse Reactions, SUSARs:** Unexpected and serious suspected adverse reactions that are not described in the product information for the experimental drug.

The investigator must, based on medical and scientific experience, assess whether it is relevant to report the medical event /reaction that is not immediately life-threatening, but which may bring the patient's health at risk or require medical treatment, to prevent one of the above mentioned events/reactions. Such incidents should usually be classified as serious.

**Other events to be treated as serious adverse events**

If the patient becomes pregnant around the time of treatment or 6 months after treatment, she must immediately be removed from the trial. The patient should be followed throughout the pregnancy. The results of pregnancy and childbirth must be reported, even if the process is normal and without AEs.

**Stop rules**

If a patient previously treated with influenza vaccine experiences anaphylactic shock to the intratumoral application of influenza vaccine the study will be stopped immediately. If a perforation of the bowel happens at the intervention site eg. at tumor level but not in the remaining bowel, as this will be seen as a known side effect to colonoscopies. Additionally, the trial may be interrupted prematurely and before inclusion of all patients if:

- Unexpected, significant or unacceptable risks to patients.
- Failure to enter patients at an acceptable rate.
- Insufficient adherence to protocol requirements
- Unacceptable compliance.
- Decision by sponsor or regulatory authority based on safety evidence.

**Side effects to the influenza vaccine**

| | |
|---|---|
| Very common (> 10%) | Decreased appetite, reactions and discomfort at the insertion site, malaise.<br>Myalgia.<br>Headache, Irritability. |
| Common (1-10%) | Fever, chills. |
| Uncommon (0.1-1%) | Abdominal pain.<br>Lymphadenopathy, Thrombocytopenia.<br>Dizziness. |
| Rare (0.01-0.1%) | Dyspnea.<br>Arthralgia. |

| | |
|---|---|
| | Paresthesia.<br>Allergic reactions, Hypersensitivity. |

**Side effects to applying the influenza vaccine intratumorally using a needle in the colon.**

Injecting a needle into a tumor or normal tissue in the colon is part of many routine clinical procedures. Every patient with an endoscopically identified malignant polyp or tumor will be marked by injecting ink by a needle in the adjacent tissue[9], so that part can be identified during the subsequent surgery. This techniques has been used since the 1980's and has virtually no both short term and long term side effects[9]. Injecting a needle and administering up to 10 ml of saline is also a routine part of endoscopic mucosal resection[10].

**Side effects to the additional endoscopy**

The study necessitates and additional endoscopy of the sigmoid colon. This is associated with the same risk as in a standard endoscopy of the sigmoid colon, ie. a risk of bleeding, infection and perforation of the colon, up to 0.5% overall. These complications are severe and will require hospitalization and presumably surgery. Prior to the endoscopy, the included patient will have to take a laxative to cleanse the bowel. This may be associated with discomfort.

7. Biological material from participants

**Blood samples**

Blood samples will be collected four times during the study period. Before intratumoral influenza vaccine treatment (baseline), on admission to surgery, within 72 hours after the surgical procedure, and when patients return to the outpatient clinic for final evaluation (approximately 14 days surgery). Additionally, one blood sample will be collected at baseline and prior to surgery for flow analysis.

Following blood samples will be collected at four specific time points:

- 1 x 6 ml in serum separator tubes
- 5 x 10 ml in EDTA tubes for full blood, buffy coat, flow cytometry, NK cell activity and ct/cfDNA
- 2 x 2.5 ml in PAXgene RNA tubes

A total of 244 ml will be collected during the entire study period.

Systemic immunological responses will be analyzed when all samples have been collected from all patients. Research shows that immune cells and their invasion of the primary tumor correlate to the patient's prognosis, again suggesting that the immune response is important for cancer growth[11–13], thus we will perform an evaluation of the systemic immunologic response.

Furthermore we plan to analyze circulating tumor DNA (ctDNA) and cell free DNA (cfDNA). Studies have shown elevated levels of cfDNA in cancer patients compared with healthy individuals[14,15]. Studies have considered cfDNA as a prognostic marker for outcome, and high levels of cfDNA have been related to poor survival[16]. Furthermore, evidence suggest that the level of ctDNA is correlated to the tumor burden[17]. Further, we will analyze NK cell activity as this has been shown to increase after systemic influenza vaccine.[18]

Finally we plan to perform cell adhesion assays to analyze the metastatic ability of the cancer cells.

All samples will be stored in a biobank created for this study according to Danish legislation and approval from the Danish Data Protection Agency will be obtained prior to initiation of the study. Study blood samples and multiplex gene assays will be analyzed at Zealand University Hospital. Cell adhesion assays will be performed at Roskilde University. Samples will be shipped in anonymized form by applying ID numbers for all samples and blinding for intervention prior to safe transport. All bio-banked blood samples will be kept (without CPR-number, but instead with the patient code), and will be stored until analysis during the course of the trial. The project will be approved by the Danish Data Protection Agency and all formal requirements and maintenance of the biobank will be performed accordingly.

Upon termination of the current study, the samples will be kept in a research biobank for future studies for 10 years. Any remaining samples after this time point will be destroyed. The research biobank for future studies will be approved by the Danish Data Protection Agency, are subjected toData Protection Act and will follow General Data Protection Regulation (GDPR) guidelines. Any additional analyses require renewed approval from the Ethics Committee and the Danish Data Protection Agency.

**Biopsies and tumor samples**

Final tumor samples, pre-treatment biopsies (8x0.5-1.5 cm) and a sub-specimen (3x0,5 $cm^3$) of the original surgical specimen will be stored in a biobank according to the instructions issued by the Danish Cancer Biobank. Additionally, if pathological examination reveals positive lymph nodes (eg. lymph nodes with live cancer cells) three formalin fixated specimens of the lymph nodes will be stored in the biobank. The biobank will be approved by the Danish Data Protection Agency prior to initiation, and all formal requirements and maintenance of the biobank will be upheld during the study. Standard histopathological

evaluation will be performed at Zealand University Hospital. Immunohistochemistry and the Immunoscore will be performed in department of Pathology, Zealand University Hospital Roskilde. The Immunoscore is measured by immunohistochemistry by applying a novel clinical available and approved assay. Samples from the core of the tumor and form the invasive margin are stained for CD3 and CD8 positive lymphocytes. Tumor samples, including biopsies, and sub specimens will be sent for analysis. The Data Protection Act and GDPR will still be valid when samples are sent and handled. Samples will be anonymized through specific ID numbers and blinded for intervention prior to safe transport. The sponsor will retain a patient identification list. Upon completion of the current study any excess material will be stored in a biobank. From this time point additional analyses require renewed approval from the Ethics Committee and the Danish Data Protection Agency.

**Quality of recovery**

Patient perceived quality of recovery should be in focus when implementing new treatments and therefore, we include the widely used and validated "Quality of Recovery" questionnaire in the short 15-item form as a main outcome in our study. The 15-item QOR questionnaire (QoR-15 score) results in a score of 0–150 with a high score indicating a good quality of recovery. The questionnaire will be applied at baseline, prior to surgery, within 72 hours post-operatively and at 14 days after surgery, four times in total.

**Photografic documentation**

Photografic documentation of tumor and clinical response will be performed.

8. Information from electronic health records

We intend to use patient records of the included participants to register the following items during the admission for surgery.

- Clinical information including birthday, age, ASA score, height and weight
- Procedural details and possible adverse events in relation to the intratumoral influenza vaccine treatment and surgical procedure
- Clinical symptoms and vital signs
- Blood test results
- Photografic documentation

This information is needed in order to provide safety of the participants during the study. We will first obtain permission from the physician that has the responsible to participant's treatment, before asking the participant for consent for assessing this information.

9. Storage of information from patients

All information about participants is subjected to 'lov om behandling af personoplysninger' and ' Sundhedsloven', and these will be complied. The approval from Danish Data Proctection Agency (Datatilsynet) will be applied. Authorized representatives from the Local Ethical Committee are granted inspection of documents related to the study if needed. Each participant will be informed that representatives from the Danish Data Protection Agency and the Local Ethics Committee may inspect their medical journals and trial records, in all confidentiality. The requirements to ensure anonymity of data, data security and confidentiality of data will be explained to the participants and complied. All information will be treated with strict confidentiality and stored as confidential material according to the Data Protection Act and GDPR. TeamSite (a secure website at Zealand University Hospital) will be used for storage of such information. Only the principal- and coordinating investigators will have access to the information. Study results will be reported in anonymous form. The investigator will keep identification lists of all participants including a sample log. This list will include full name and CPR-number (social security number). The recorded data will be kept in an electronical Case Report Form system, EasyTrial, that complies with all data safety requirements in Region Zealand. An individual journal will be created for the study. The information will only be available for inspection for authorized representatives from the relevant authorities upon request (GCP Unit, local ethical committee, Danish Data Protecting Agency, Danish Medicines Authority). The investigators will retain investigational records, copies of CRF and source documentation for the maximum period required by the regulatory authorities.

The informed consent gives the primary investigator, sponsor and sponsor's representatives as well as any control authority (GCP Unit, local ethical committee, Danish Data Protecting Agency, Danish Medicines Authority) direct access to obtain information in the patient's record, including electronic health record, in order to see information on the patient's health conditions which are necessary as part of the implementation of the research project, and for control purposes, including self-control, quality control, and monitoring, which they are required to perform.Changes to the protocol will require written approval from the competent authority prior to implementation except when modifications are needed to eliminate immediate hazard(s) to the patients.

10. Economy

Economy**:** Financial support from private and public grants to cover the expenses will be applied on ongoing basis. The responsible investigators are employees of Zealand University Hospital.

Time plan: Enrolment of the first participant has been set for August 1st 2020. We are expecting the clinical study with patient enrolment to last for approximately 12 months. Data analyses are expected to last for three months. Lastly we are expecting a three-month period for article writing.

11. Payment to patients

The patients will not receive any payment to be part of the study.

12. Inclusion of patients and informed consent

All required regulatory end ethics approval must have been obtained before enrolment in this trial. Each potential participant must be given a patient information sheet and full written informed consent must be obtained from the patient before registration on to the trial. Potential participants will be screened and enrolled on the clinical trial on the basis of the inclusion/exclusion criteria specified in the protocol. Only patients fulfilling all inclusion criteria, and without any exclusion criteria can be registered. Any queries about eligibility should be addressed directly to the coordinating investigator.

The investigators will screen potential candidates for the study at the biweekly MDT colorectal conference at Zealand University Hospital. The investigator will contact the potential participant at the preoperative examination 2-3 weeks before scheduled surgery. Only if the patient has time and interest in participating in the study, the investigator will continue with an information meeting. In addition to an oral and written presentation of the study, the patient will be given a written copy of information concerning the study and copies of the pamphlets "Forsøgspersoners rettigheder i et sundhedsvidenskabeligt forskningsprojekt" and "Før du beslutter dig" issued by the Central Committee for Health Research. The candidate will be informed in advance that he/she is allowed to bring an assessor for the meeting.

At the information meeting the candidate will receive information concerning the study both orally and in writing. The meeting will be held in a room that will only be used for this purpose to avoid unnecessary disturbances. The investigator will explain content, extent, purpose, expected risks and possible advantages

of the study in plain Danish. The candidate will be given time to ask questions and read the information one more time. The following points will also be covered during the interview:

- A description of the purpose with the study and how it will be organized.
- The permission to access his / her patient records to register any adverse event, medication use, blood tests and other basic clinical parameters during his / her admissions.
- The methods used in the study.
- The permission to use his / her clinical data as well as clinical images, scanning images, pathological images for publication in strictly anonymized form, e.g. case reports, posters, lectures, and future publications.
- The right to ask for additional information at any time.
- The patient's right to withdraw from the trial at any time without having to explain why.
- The insurance.
- The contact person.

The informed consent will include all the elements that are required according to Danish legislation. The participant will be given the necessary and needed time for consideration, to determine the participation in the study. The participant will be asked for permission to be contacted by telephone for final decision regarding study participation within 24 hours. A formal inclusion interview will be arranged if the patient accepts inclusion in the trial. All questions will be answered to the candidate's satisfaction. Once the candidate accepts to take part, the participant will be asked to take part in an inclusion interview and sign the informed consent. After the information meeting, all active participants will be updated with any new and important information or change in the study that can have influence on the participants' willingness to participate or safety. The approved documents will be updated accordingly.

13. Publication

All results, including positive, negative and inconclusive will be published in international peer-reviewed journals.

14. Ethical considerations

The only curative treatment for rectal cancer and sigmoid colon cancer is surgical excision. In this study we aim to examine the effect of intratumoral influenza vaccine on tumor response. The study will provide further knowledge to the understanding of the tumor response in colorectal cancers. The effects may possibly lead to better oncological outcome and alterations in the current management towards less invasive treatment. To our knowledge, no one has completed a study of this kind on patients undergoing treatment for colorectal cancer. The participants of the current study may not directly benefit from the participation. However, the knowledge gained from the study will provide knowledge that has vast clinical implications moving forward. A protective effect of the influenza vaccine will mean that the immune system can be strengthened by simple means to increase its role in the killing of circulating tumor cells. Mostly, patients with solid tumors are offered curative surgery, whereby the influenza vaccine will play a crucial role in the course of these patients.

If the influenza vaccine protects against recurrence of cancer, it will be revolutionary in this area. The influenza vaccine causes few side effects and will thus be a patient-friendly alternative or supplement to chemotherapy.

Further, the multiplex gene assay analysis, is focused on the synthesis of proteins and will analyze RNA sequences, thus not having the risk to discover possible unknown genetic diseases in the patients. When analyzing cfDNA, we do not analyze for known genetic variants related to inherited diseases. Analyzing cfDNA is based on a specific sequence that is found in all human DNA and will thus not reveal any genomic variants related to inherited diseases. Nevertheless, if such a variant should be found, its clinical importance will be evaluated by an expert committee, appointed by Department of Surgery.

The committee is appointed when needed and the members will be chosen according to the potential disease. The committee will include a molecular biologist, specialized in genetic sequencing, and a medical doctor specialized in personalized medicine, a clinical geneticist specialized in inherited diseases, and a medical doctor specialized in the disease in question. If deemed relevant other specialists may be included.

This committee will assess if

1) The technological quality of the analysis is sufficient for a reliable result.
2) There is sufficient evidence in the literature for a clinical relevance (e.g. expected penetrance)
3) The sum of information justifies a relevant risk for a genetic disposition
4) The disease, according to current standards, can be treated or prevented

Based on the assessments the committee decides, whether or not the patient (and/or his family) should be informed (by written letter) that the research accidentally has resulted in a finding, with potential influence

on his or hers health, and that further information and advice on the matter is offered to him/her and/or potentially affected family members. If accepted, this will be initiated.

Whether or not to provide feedback to relatives of deceased study participants or to study participants who, themselves, deny information about genetic issues, will be decided based on a medical perspective according to "DNVKs retningslinjer af 29. april 2013, sundhedslovens § 43, stk. 2, nr.2" and in "autorisationsreglerne om lægers omhu og samvittighedsfuldhed".

This study will be conducted according to the principles of the Helsinki Declaration. This protocol will be submitted to the Regional Committee for Health Research and Ethics, the Danish Data Protection Agency, and the Danish Medicines Agency for approval. Cooperation with the regional GCP unit will be initiated before the study is launched to ensure good clinical practice before during and after the study. The head of department for the experiment site has given the approval. The protocol will be registered at clinicaltrials.gov. All participants in this study have to give their oral and written consent before they can be included in the study. It is the investigators duty to inform the participant orally and in writing, so they are thoroughly informed about all aspects concerning participation in the study. The participant can at any point withdraw the consent concerning participation in the study. If a participant decides to do so it will not impair the relationship to the investigator or the doctors involved.

The clinical investigation shall not begin until the required approvals from the Local Ethics Committee, the Danish Data Protection Agency, and the Danish Medicines Agency have been obtained. Any additional requirements imposed by the Local Ethics Committee or regulatory authority will be followed.

15. Compensation to patients

The Department of Surgery, where participants are admitted, assumes the legal responsibility on behalf of the investigators and the investigators' assistants for all participants in the trial concerning any injury that is caused by the study procedures either directly or indirectly, assuming that the investigators and assistants have followed the guidelines given in this protocol and in any eventual additions to this protocol. In addition, it is also assumed that the study is performed scientifically and in coherence with existing rules and accepted techniques. The participants are in the case of injury or death without connection to the completion of the trial insured by the hospitals insurance. The participants will be informed that they are

covered by the public participant insurance, and if the participant wishes to complain, the participant will be informed on how to obtain help for this matter.

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