The ISCOLIM trial: Improving Survival of Colo-rectal Liver Metastases by RFA-mediated Immuno-stimulation

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Trial administrative structure

This is a sponsor-initiated study. Professor Frank Viborg Mortensen is the sponsor and primary coordinating investigator (PI). Professor Claus Lindbjerg Andersen is the coordinator of the translational analyses, and Associate Professor Bjarne Kuno Møller is the coordinator of the immunological analyses. The decision-making body of the study is the steering committee (SC), which will have representation from all participating departments. Professor Frank Mortensen is chair of the SC.

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Study summary

Title:	Improving Survival of Colo-rectal Liver Metastases by RFA-mediated Immuno-stimulation
Acronym:	The ISCOLIM trial: **I**mproving **S**urvival of **CO**lo-rectal **LI**ver **M**etastases by RFA-mediated Immuno-stimulation
Principal investigator:	Frank Viborg Mortensen, Aarhus University Hospital
Funding organizations:	Novo Nordisk Fonden Neye Fonden
Study subjects:	Patients with colo-rectal liver metastases (CRLM)
Study design:	Multi-centre Randomized Controlled Trial
Aim:	1. To examine the effect of radio-frequency ablation (RFA) on the immune system in patients undergoing curative-intent surgery for CRLM. 2. To characterize whether alterations to the peripheral T-cell receptor repertoire during the disease course reflect immune cell recognition of cancer neoantigens. 3. To examine the effect of RFA-mediated immune-stimulation on survival in patients undergoing curative-intent surgery for CRLM. 4. To monitor disease recurrence with circulating cell-free DNA (cfDNA) biomarkers, specific for CRLM.
Endpoints:	1. Overall and disease-free survival for CRLM patients. 2. Describe effect of innate and adaptive immune system on survival. 3. Lead time of cfDNA biomarkers in prediction of recurrence compared to conventional imaging technique (CT scans). 4. Description and characterization of T-cell receptor changes on a molecular level.
Intervention:	Intervention arm: RFA combined with liver surgery. Standard-of-care arm: liver surgery alone.
Number of Subjects:	220 patients (110 in each arm) enrolled consecutively.
Selection Criteria:	Inclusion: 1. Patients diagnosed with metachronous or synchronous CRLM planned for liver resection 2. At least one tumor with size ≥3 cm 3. Performance status 0-1 4. Age ≥18 years Exclusion: 1. Liver cirrhosis

	2. Non-curative extrahepatic metastases 3. Other cancer within 5 years prior to CRLM diagnosis 4. Earlier RFA treatment 5. Patients who are unlikely to comply with the protocol
Duration of study:	The study duration is expected to be 18 months for subject recruitment and additional 5 years of follow-up. Interim data analyses will be performed after inclusion of the first ten patients in each arm (expectantly at 6 months), and then after 12 and 24 months. The study will commence on September 1, 2021.
Statistics:	Cox proportional hazards model will be used to compare survival in the two arms.
Power calculations:	Assuming that the 1-year survival rate is 90% in the experimental arm and 75% in the control arm, at a 0.05 significance level with a desired power of 80%, 100 patients are needed in each arm (200 in total). To account for an expected loss-to-follow up of ~10%, 220 patients (110 in each arm) are included.

1. Introduction

In Denmark, colo-rectal cancer (CRC) represents a significant health burden with ~5,000 new cases and ~2000 CRC related deaths in 2018*(1)*. Death of CRC is primarily associated to distant metastases occurring at the time of diagnosis (synchronous) or appearing later in the course of the disease (metachronous), and those are most frequently found in the liver.

Each year 1,600 Danish individuals are diagnosed with colo-rectal cancer liver metastases (CRLM)*(2)*. For these patients, curative treatment options have evolved dramatically over the last 10-20 years*(3)* and includes liver surgery, radio-frequency ablation (RFA), radiation therapy, and chemotherapy, and the relative 5-year survival approaches 50-60%*(4-8)*. However, if CRLM metastases are detected too late for intended curative treatment, the patients instead receive palliative chemotherapy or best-supportive care, and the 5-year survival rate decreases to <10%*(4-6, 8)*. As the ability to offer curative treatment is influenced by the number of metastatic sites and the size of the metastases, early detection of CRLM is of paramount value.

Minimally invasive blood-based analysis of circulating tumor DNA (ctDNA) in CRC has been extensively studied in recent years and has been shown to increase especially in metastatic CRC*(9-11)*. It is also a tool which potentially can identify microscopic cancer early*(12-14)*. The ctDNA analyses is based on the finding that cancer cells shed DNA fragments into the blood*(15)*. The half-life of ctDNA is less than two hours*(16)* so cancer-free patients will expectantly have no remaining ctDNA in the blood during follow-up after CRLM treatment, while patients with residual disease or recurrence are likely to be ctDNA positive. In follow-up studies of patients with locally advanced CRC, it has been shown that the risk of relapse in patients with postoperative ctDNA was close to 100%, while the risk of relapse in ctDNA negative patients was as low as 10% (HR=37.66; 95% CI, 4.23 to 335.49; P<0.0001)*(13, 14, 17)*. Therefore, ctDNA could be a predictor for either residual CRLM or CRLM recurrence, and followingly guide treatment decisions.

Surgical liver resection remains state-of-the-art for treatment of CRLM. However, liver resection can only be performed if patients have a good performance status, and when at least 30% of the liver parenchyma can be preserved, which is unfortunately only possible in 25% of the patients*(3)*. As an alternative, RFA is a low-risk procedure that can be performed safely in most patients, including those with a poor performance status. By thermal ablation, RFA causes a localized coagulation necrosis in the targeted tumor tissue: when the necrosis affects all cancer cells, and no

viable cancer cells remain, the treatment is curative. RFA is a parenchymal-sparing treatment for hepatic malignancies including CRLM and is particularly effective for tumors <3 cm*(18, 19)*. For multiple or larger tumors it can be combined with liver resection, as a parenchymal-sparing strategy that couple effective thermal ablation of small tumors with surgical resection of the larger tumors, for which ablation is less effective *(20)*.

Recently, significant attention has been given towards understanding the immune system's role in cancer development, metastasis, and response to therapy. Here, especially the adaptive immune system is paramount, as it recognizes tumor neoantigens with which to eradicate cancer cells. Further, the microenvironment and supportive stroma surrounding the tumor actively participates in the tumor development over time*(21)*. Thus, it is to be expected that the immune response towards CRLM will change during the disease course in response to treatment, recurrence, or remission, and that it may be prognostic for the clinical outcome, as well as predictive for the response to a given curative treatment. As an example of the effect of the immune system on cancer development, it has been shown that colon tumors with abundant infiltration from cytotoxic T lymphocytes (CTL's) and NK cells have better prognosis than the tumors, which lack such immune response*(22)*. Another example is when the patient's adaptive immune system is activated by tumor vaccines. A tumor vaccine functions by activating the patients' own adaptive immune system against the cancer*(23)* and thereby facilitates tumor death by T-cell mediated apoptosis, and can be used as immune therapy in oncological treatment strategies*(24)*.

During RFA, necrosis of CRLM cells is induced, and their tumor antigens are released to the microenvironment, which has been shown to elicit an immune response in the patient*(25)*. Therefore, the partial destruction of CRLM by RFA may possibly represent a method equal to a tumor vaccine, where tumor antigens are exposed and activates the host immune system and enhance immunogenicity, thereby increasing overall survival. Nevertheless, the effect of RFA on immunogenicity and the following impact on survival in patients with CRLM has never been examined in the clinic.

2. Objectives

Overall, we aim to conduct a clinical randomized controlled trial (RCT) of patients with CRLM with a longitudinal follow-up. Within this RCT, we will study the effect of RFA and liver resection on immunogenicity and CRLM survival. The central hypothesis is that patients treated with RFA will elicit an increased immune response because of the RFA induced partial tumor necrosis that will increase overall survival and reduce cancer recurrence. We further hypothesize that 1) cancer vaccination using the ISCOLIM procedure will lead to alterations in the peripheral T-cell receptor (TCR) repertoire, reflecting immune cell recognition of cancer neoantigens released by the procedure, 2) the induction of T-cell repertoire changes will predict the benefit of the ISOCOLIM procedure 3) characterizing the T-cell receptor repertoire provides an attractive solution with a potentially amplified signal (due to T-cell expansion against the cancer) for assessing response, 4) the longitudinal durability of the T-cell repertoire changes will predict the durability of the clinical benefit of the ISCOLIM procedure, and 5) longitudinal monitoring of the T-cell receptor repertoire will facilitate early detection of relapse.

Finally, we hypothesize that 1) ctDNA are a better diagnostic marker for residual disease after CRLM surgery than CT scans or CEA, that 2) ctDNA are a diagnostic marker of CRLM recurrence equal to CEA or CT scans, that 3) ctDNA are prognostic for overall survival, and 4) that ctDNA are predictive of response to oncological treatment better than CT scans.

2.1 Primary aim

We want to examine the effect of RFA-mediated immune-stimulation in patients undergoing curative-intent surgery for CRLM. We hypothesize that patients treated with RFA and liver resection have improved survival compared to patients treated only with liver resection, due to an increased immune response.

Endpoints

1. Increase in 3-year survival rate from 55% in the control arm to 75% in the experimental arm.
2. Increase in 1-year survival rate from 75% in the control arm to 90% in the experimental arm.
3. Statistically significant increase in recurrence free survival (RFS) in the experimental arm compared to the control arm.

2.2 Secondary aims

2.2.1 T-cell response to tumor development

We aim to characterize the longitudinal changes in T-cell in response to CRLM and the following surgical/RFA treatment. We hypothesize, that RFA treated patients will develop different immune cell characteristics than patients treated with liver surgery only. Further, we expect patients with recurrence during follow-up to present a change in immune response in comparison to patients with no recurrence. By T-cell receptor sequencing and RNA sequencing (RNA-seq), we will identify changes in the T-cell receptor repertoire.

Endpoints

1. Pre- to post treatment change in T-cell receptor repertoire
2. Time to T-cell receptor recurrence (significant change in T-cell receptor repertoire)
3. Durability of the change in T-cell receptor repertoire
4. T-cell receptor repertoire change and correlation to RFS

2.2.2 ctDNA as biomarker of residual disease or recurrence

We will explore the use of ctDNA as a biomarker for prediction of incipient recurrence. The assessment will be made both at milestone time-points and by serial analysis. We will explore if ctDNA can detect recurrence before standard-of-care CT imaging and before CEA and T-cell receptor repertoire analysis (lead-time analysis). We will explore if ctDNA can help guide clinical decision making at time-points where the CT-imaging result are indeterminate (~10% of all CT-scans).

Endpoints

1. Time to radiological recurrence
2. Time to ctDNA recurrence
3. Time to CEA recurrence
4. Time to T-cell-receptor recurrence
5. Overall survival

2.2.3 Gene expression signatures

With DNA and RNA sequencing, we will examine CRLM genes and their expression signatures. Further we will investigate changes in gene expression signatures associated to ISCOLIM treatment response, CRLM recurrence, and overall survival.

Endpoints

1. Gene expression signatures and correlation to overall survival and RFS.

3. Investigational plan

3.1. Overall study design

The present study will establish the sponsor-initiated, randomized prospective clinical trial ISCOLIM. The RCT will investigate the benefit of RFA on survival for CRLM patients and characterize changes in the immune system in response to treatment on a molecular level. Patients will be randomized within blocks of 10, such that an equal number are assigned to each treatment. Patients will be randomized to either the experimental arm or the control arm (*Figure 1*).

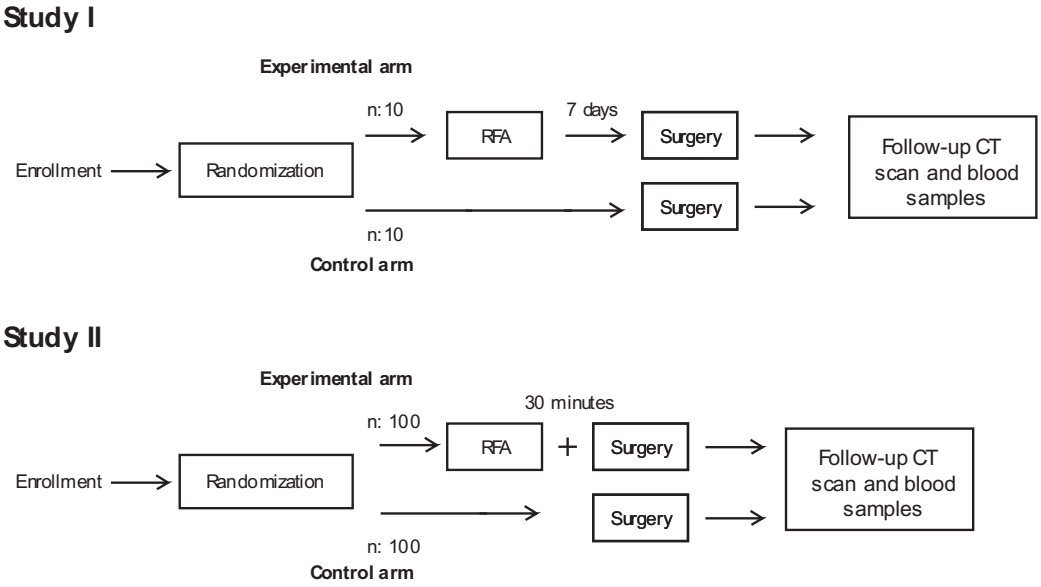

Figure 1. ISCOLIM Study design. Randomization for study I and II.

The study is designed to enrol 220 patients consecutively, where the first 20 patients will partake in as a small sub-study at Aarhus University Hospital (study I, the immunological study) and the remaining 200 patients will be participating in a larger follow-up study (study II, the survival study).

In both studies I and II, CRLM patients will be randomized into two arms:

- The experimental arm: Patients will be randomized to RFA in addition to liver surgery for CRLM in study I and II (*Figure 2*).
- In study I, percutaneous RFA will be performed in general anaesthesia one week prior to surgery. Blood samples for immune cell analyses as well as translational analyses will be collected prior to RFA treatment and again prior to liver surgery in study I, and then post-operatively on day 1, day 7, and day 28. Surgical biopsies of CRLM and normal tissue will be collected peri-operatively.
- In study II, RFA will be performed peri-operatively 30 minutes before liver surgery. Tissue samples from the CRLM and normal liver tissue will be collected peri-operatively, after RFA treatment. Blood samples will be collected before the combined RFA and liver surgery begins, and then post-operatively at day 1, day 7, and 28.
- The control arm: Patients will, as standard care, be randomized to liver surgery for CRLM. Tissue samples from the CRLM will collected peri-operatively. Blood samples for immune cell analyses and translational analyses will be collected prior to surgery and post-operatively at day 1, day 7, and 28.

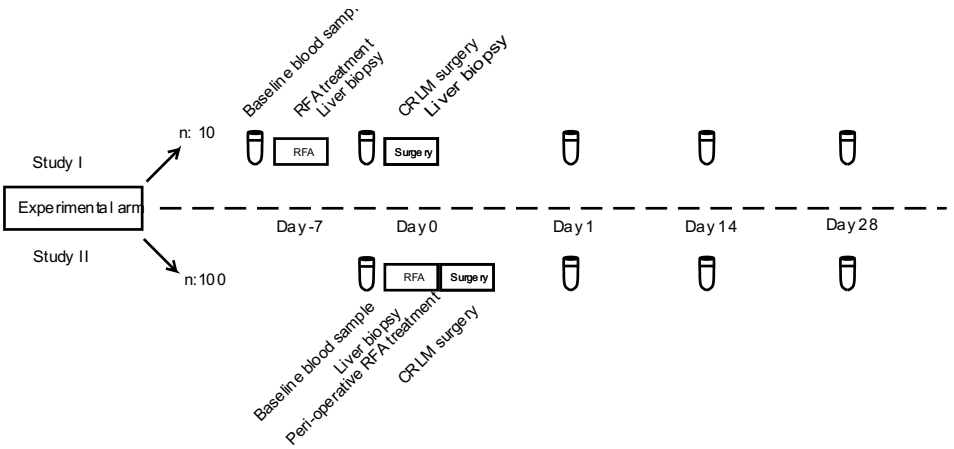

Figure 2. Randomization for study I and study II in ISCOLIM trial.

All 220 included patients will receive the same follow-up 1 month after surgery (*Figure 3*): Blood samples will be collected every 3 months until 3 years of follow-up is concluded; previous studies have shown that most patients with recurrence will have tested positive for ctDNA before 18 months*(12, 13)*. Hereafter, blood samples will be collected each year concomitantly with CT scans. Patients will be followed until 5 years of follow-up is concluded. The first interim molecular and immunological analyses will be performed when ten patients have been included in each arm, (expectantly at 6 months) and then after 12 and 24 months. Concurrently, all included patients will undergo standard radiological follow-up with CT scans at 3, 6, 12, 18, 24, 36, 48, and 60 months. Translational analyses are explorative, and only CEA results will be available to the treating clinicians, unless interim analyses clearly find that translational analyses will improve patient treatment

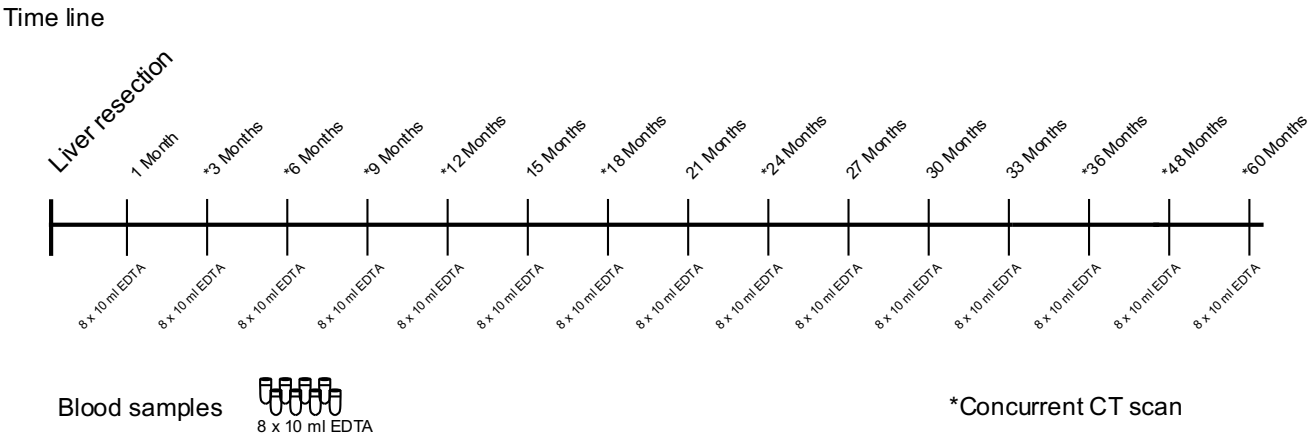

Figure 3. Timeline for follow-up program with CT scans and blood samples.

3.2 Study timetable

Start inclusion: September 2021

End inclusion: February 2022

End data collection and follow-up: February 2027

Interim analyses: 1) After 6 months, 2) September 2022, and 3) September 2023

Primary endpoint reached, ready to publish: July 2027

Follow-up: 5 years from last patient included

3.3 Setting

ISCOLIM is a clinical prospective RCT, initiated at Aarhus University Hospital. During 2021 to 2022, 220 patients eligible for the trial will be recruited in the treatment responsible hepato-pancreatico-biliary surgical departments of Aarhus University Hospital and Rigshospitalet.

3.4 Sample size

In assessment of the sample size needed to meet the primary objective, we used a 0.05 significance level and a desired power of 80%. Assuming that the 1-year survival rate is 90% in the experimental arm and 75% in the control arm, we need 100 patients in each arm (200 in total). Estimates are based on clinical experience, as no literature on this subject has been published yet. To be able to deal with a total drop-out of ~10%, we aim to include 220 patients (110 in each arm). For potential subsequent analyses of 3-year survival, we need 89 patients in each arm, assuming 3-year survival rates of 75% and 55% in the experimental and control arm, respectively. Thus, our study is sufficiently powered to achieve its primary aim.

3.5 Study feasibility

We need to enrol 220 patients during a period of 18 months, equalling to 12 patients each month. We expect to include 4 patients/month at Aarhus University Hospital and 8 patients/month at Rigshospitalet. Under normal circumstances, our institutions perform approximately 10 and 30 operations for CRLM each month, respectively. Thus, we need to include between 30-50% of the patients treated at our institutions. From previous research in the departments, we have already established the infrastructure and experience with inclusion of patients in clinical trials, and we consider the above inclusion rate realistic. The blood and tissue samples will be handled in co-operation with Regionernes Bio- og Genom bank (RBGB). The collaboration with RBGB is well established at both clinical departments.

3.6 Inclusion and exclusion criteria for patients

All patients with resectable CRLM, planned for liver surgery, will be eligible for inclusion. Prior to inclusion, tumor volume will be estimated by CT-volumetry. We will also assess whether the CRLM are synchronous or metachronous.

3.6.1 Inclusion criteria

1. Patients diagnosed with metachronous or synchronous CRLM planned for liver resection
2. At least one tumor with size ≥3 cm
3. Performance status 0-1
4. Age ≥18 years

3.6.2 Exclusion criteria

1. Liver cirrhosis
2. Non-curative extrahepatic metastases
3. Other cancer within 5 years prior to diagnosis
4. Earlier RFA treatment
5. Patients who are unlikely to comply with the protocol, unable to return for subsequent visits and/or otherwise considered by the PI to be unlikely to complete the study

3.7 Time of inclusion and allocation procedure

Before arriving to the outpatient clinic, we will evaluate patient's possibility to participate in the study in relation to the department's diagnostic MDT conference. Data on age, diagnosis, comorbidity, and tumor imaging material, which is necessary for patient treatment, will be assessed to evaluate if the patient is eligible for inclusion in the study. Potential study subjects will then be given the option to participate. CPR numbers will be registered on all eligible patients, also patients who decline to participate in the study. Further whether they were asked to participate in the study, and whether the accepted or not will be registered. This will be done to ensure the quality of the randomisation process, to control for selection bias, and enable future cost-benefit analyses. No additional personal information will be obtained before patient consent.

3.8 Procedures for patient inclusion and informed consent

Patients, who meet the inclusion criteria are approached in person at the treating surgical department. Here, the patients are given written information and verbal information about the project. Patients are encouraged to bring an aide to this session. The oral information is given by trained

health care professionals (either a liver surgeon or a project nurse with special training). Oral information is given in an enclosed room to ensure a private conversation. Further, patients are informed about their juridical rights as participants in a research project. Furthermore, as the project involves genomic sequencing, which might disclose genetic variants predisposing to specific diseases, the participants are also offered genetic counselling before making their decision to participate.

After the oral information has been given, the patients are allowed at least 24 hours to consider their decision to participate, before they can provide a written informed consent form. Informed consent is obtained prior to any study-related procedures. The signed and dated consent forms are stored in a locked room at the trial office and are available for audit and inspection at any time.

The written participant information contains contact information to the person being primary responsible for the project, and to the persons being responsible at the participating departments. The information clearly states that these people can be contacted in case of further questions.

It also mentions that the participant at any time may withdraw his or her consent, causing immediate destruction of all their individual data collected for the project. The consent withdrawal has no influence on their relationship to the department or their current/future treatment.

The written information also mentions that the study involves a biobank, and that residual biological material as well as clinical and sequencing data will be transferred to the Colorectal Cancer Research Biobank at Aarhus University Hospital for future research. The research biobank is approved by the Danish Data Protection Agency (j. no. 1-16-02-27-10). All clinical information will be obtained and processed in compliance with The Danish Data Protection Act.

When the residual biological material is used in future research in will be in accordance with current data protection rules. Participants can at any time withdraw their consent to storage of residual material for future use.

Further the patient information states that biological samples and data may be shared with other international research collaborators, including companies, in a pseudonymized form (non-personalized format) for important research purposes, but only if the research purpose is approved according to European ethical guidelines.

3.9 Patient information and consent

The patient information and consent form are available as Appendix A.

The patient information states that when the patient gives consent to participate, he or she also accept that the PI, and representative of the PI, have direct access to collect the necessary information regarding treatment and clinical outcome from medical records and health registries to complete the study. This information is required to conduct the study, and for monitoring and quality control of the study. All clinical information will be obtained and processed in compliance with The Danish Data Protection Act.

3.10 Randomization

Randomization will be performed using a concealed web-based randomization service managed by Aarhus University (REDcap). Randomization will be stratified for centre and performed in blocks of ten. Patients will be randomized into two groups (*Figure 1*):

Group 1. Additional RFA therapy with liver surgery for CRLM, where the first 10 patients will be included in study I and the subsequent 100 patients will be included in study II (experimental arm).

Group 2. Standard-of-care liver surgery for CRLM, where the first 10 patients will be included in study I and the subsequent 100 patients will be included in study II (control arm).

3.10.1 Study Flow

Gantt chart	Day -14 Baseline	Day -7	Day 0	Day 1	Day 14	Day 28	Mo 3/6/9/12	Mo 15	Mo 18	Mo 21	Mo 24	Mo 27/30/33	Mo 36/48/60
Patient inclusion													
Informed consent													
Registration of baseline data													
Randomization													
RFA		Study I	Study II										
Liver surgery													
CT scan													
CEA													
Blood sampling		Study I	Pre-surgery										
Liver biopsy		Study I											
Interim analyses							~ 6 & 12 months						

Longitudinal data collection													
Blood sample analysis													

Figure 4. Gantt chart with time-schedule and data collection. Blue: Collection from all participants, Green: Collection from experimental arm only.

3.10.2 Baseline visit (-14 days from operation)

- **Record basic information on all patients eligible for participation:**

  -CPR number and surgical centre

  -Patients fulfilling the inclusion criteria are informed about ISCOLIM and asked for consent to participation:

  -register whether the patient accepted or declined participation

- **The following baseline information is collected on participating patients**

  -Whether the patients accepted or declined to receive information about incidental genomic findings with potential health consequences

  -WHO performance status

  -Height, weight, and Body Mass Index (BMI)

  -Smoking and alcohol use

  -Synchronous or metachronous CRLM

  -Tumor size and histology

  -Number and size of all metastases present

  -Diagnosis of other cancers within 5 years from inclusion

  -Chronic diseases

  -Relevant medication

  -Imaging information

- Information on randomization result and treatment plan

3.10.3 Month 3 to 60

- Collection of clinical data (surgical and oncological intervention, and radiological- and biochemical evaluations. Specified in *3.15 Collection of clinical information*)

- End-of-study CT scan and blood samples
- Collection of radiological, biochemical, and pathological reports on CRLM retrieved for use in the statistical analyses
- Recurrence or residual disease recording

3.11 Radio-frequency ablation (RFA)

Under guidance of ultrasonography, a single-electrode RFA-needle is placed in the target CRLM, with a diameter of a least 3 cm, which is planned for subsequent liver surgery. A coagulation necrosis of ~2 cm is created. The RFA needle is removed under the creation of a tract necrosis to avoid tumor seeding (tract ablation). At our departments, we have extensive experience with RFA-treatment of the liver, performing ~150 procedures each year. In the literature, complication rates following stand-alone RFA-treatment are low, making it a very feasible procedure*(26)*. The RFA-treatment will be performed with the patient in general anaesthesia.

3.12 Collection, handling, and biobanking of biological samples

The biological material is collected and stored in the clinical biobank, Bio and Genome Bank Denmark (Danish Cancer Biobank). When this study needs the samples for analysis etc. the project will transfer the samples to the "Colorectal Cancer biobank MOMA, AUH". At the end of the study, left-over biological materials, clinical data, and sequencing data will be transferred to the "Colorectal Cancer biobank MOMA, AUH" with the aim to facilitate future research projects. Patients may at any time request to have his or her samples and data removed from the clinical biobanks or destroyed. New projects based on the biobank material will only be initiated after the pertinent permissions have been obtained from the relevant authorities and will be conducted in compliance with the Danish Data Protection Act. At each blood draw, a total of 80 mL blood is collected in eight 10 ml Potassium ethylenediaminetetraacetic acid (EDTA) tubes. For each patient in the control arm, 80 ml of blood will be sampled 19 times. For patients in the experimental arm, blood will be collected 19 times (study II) or 20 times (study I) (*Figure 2 and 3*). The blood samples will be collected pre- and postoperatively to get a baseline biomarker status and then repeatedly every three months until 36 months. The last two years of follow-up, blood samples will be collected concomitantly with control CT scans. From the samples, CEA are measured, and plasma

and buffy coat (nucleated blood cells) are extracted. Drawing, handling, and storage of the plasma/buffy coat for analyses is done in accordance with a standard operating procedure (SOP).

From the isolated plasma, cfDNA will be extracted, analysed for presence of tumor associated epigenetic and genetic changes. From the white blood cells of the buffy coat DNA and RNA will be extracted and analysed for T-cell receptor changes (see 3.14.). The buffy coat DNA will furthermore be used as a normal reference (patient specific normal DNA) in our efforts to determine the tumor specific DNA changes, and methylation patterns in the ctDNA. We will also use the buffy coat cells to monitor the tumor associated immune cell changes during the disease course. The cells will be used for immune cell characterization with flow cytometry and immune-histochemical assays (see 3.14). DNA and RNA will be extracted from the tumor and normal liver biopsies and used to determine tumor specific mutation- and methylome-profiles. The tissue biopsies will also be used to examine the immune cell infiltration in the CRLM at a histological level.

3.13 Research biobank and biobank for future research

When the study is completed, left-over biological materials, clinical data, and sequencing data will be transferred to the "Colorectal Cancer biobank MOMA, AUH" with the aim to facilitate future research projects. The "Colorectal Cancer biobank MOMA, AUH" has been approved by the Danish Data Protection Agency (jrl n.1-16-02-27-10). The biological materials, clinical and sequencing data will remain in the biobank, until the approval expires (currently 1 March 2030) or until the patients request their tissue and/or data removed. Patients may at any time request to have his or her samples and data removed from the "Colorectal Cancer biobank MOMA, AUH" and destroyed. The storage of tissue and data in the research biobank is done in accordance with the Danish Health Act and the General Data Protection Regulation. New projects based on the biobank will only be initiated after the pertinent permissions have been obtained from the relevant regulatory committees and will abide current law for data protection. Patients will only be included in the study if they consent in writing to collection and biobanking of their blood and clinical data.

3.14 Molecular analyses

3.14.1 CEA analysis

CEA will be measured in all blood samples by standard laboratory procedure.

3.14.2 Immunological analyses

Immunological analyses in study I will be performed on peripheral blood mononuclear cells (PBMCs) isolated from the blood sample's buffy coat and tissue biopsies in a single proliferation assay set-up. Flow cytometry will be done using CellTracker labelling with fluorescent monoclonal antibodies on a Quanteon flow cytometer (NovoCyte). Analysis of flow cytometric data will be performed by comparing the proliferation as measured by CellTracker labelling for every stimulation after gating for cytotoxic effector T lymphocytes (CD3+CD4+CD8+CD44+CD62L-CCR7-CD45RA-CD45RO-CD95+ cells), for activated and degranulated cytotoxic T-cells (CD3+CD4-CD8+CD62L-CCR7-CD107a+), for central memory effector T cells (CD3+CD4-CD8+CD27-CD45RA-CCR7+), and for antigen experienced effector memory T cells (CD3+CD4-CD8+CCR7-CD45RA+CD95+), respectively. In addition, a functional cytotoxic test is undertaken on fractions of suspended tumor and normal cells. Flow cytometric evaluation of the lymhocytotoxic mediated cell death is made by staining tumor cells with (PRF1, CRC_marker(s)) and normal cells with (PRF1, hepatocyte_marker(s)).

3.14.3 ctDNA analyses

ctDNA will be isolated from the longitudinally collected plasma samples using standard methods. The presence of ctDNA in the patient's plasma samples will be used to identify residual disease, monitor treatment response and for studies of tumor evolution. The following approaches for detection and quantification of ctDNA will be used:

1. The TriMeth test: a digital PCR test for detection of circulating DNA fragments with CRC specific methylation*(27, 28)*}. In house, but yet unpublished data, indicate that approximately 98% of all CRLM patients are TriMeth positive pre-operatively. Postoperatively, 65% of patients are TriMeth positive, and their 1-year recurrence-free survival is 12%, compared to 60% for TriMeth negative patients (P<0.001).

2. Targeted DNA sequencing of cell free plasma DNA to investigate if the plasma contains DNA fragments of tumor origin. As marker of the tumor DNA we use tumor specific mutations, copy number changes, and DNA fragmentation patterns. From the sequencing data we will quantify the fraction of ctDNA in each sample and correlate this level to treatment

response and clinical outcome. We will also explore if the sequencing data can be used to assess to tumor evolution during treatment and surveillance. Illumina TruSeq DNA Kit and NimbleGen SeqCap EZ v3.0 (or similar) will be used for generation of sequencing libraries and capture. Data will be generated on the Illumina NovaSeq platform. The target sequence coverage is >1,000x.

3.14.4 T-cell receptor analyses

T-cell Receptor sequencing (TCR-Seq) will be performed to identify the role of the adaptive immune system stimulated by RFA exposure in the ISCOLIM trial. We will do TCR-Seq on DNA and RNA extracted from tumor tissue and from the longitudinally collected blood samples to 1) determine the TCR profiles in the tissues, 2) establish the TCR profiles of the blood, 3) monitor changes in the TCR repertoire over time and in response to treatment, and 4) to compare the profiles of blood and tissue.

For TCR-seq we will use the AmpliSeq for Illumina Immune Repertoire Plus, TCR beta Panel, which is a highly multiplexed targeted resequencing panel to measure T cell diversity and clonal expansion by sequencing T cell receptor (TCR) beta chain rearrangements. Data will be generated on the Illumina NovaSeq platform. Target coverage is >1,000x.

3.14.5 DNA sequencing

We will apply whole genome sequencing, as well as targeted sequencing of smaller cancer panels to tumor and germline DNA to identify tumor specific genomic changes, clonality, and neoantigens. Illumina TruSeq DNA Kit and NimbleGen SeqCap EZ v3.0 (or similar) will be used for generation of sequencing libraries and capture. Data will be generated on the Illumina NovaSeq platform. Target coverage is <100x.

3.14.6 DNA Methylation analyses

We will extract DNA from the CRLM and normal liver tissue and establish DNA methylation profiles of the two tissues by analysing the DNA with the Infinium Methylation EPIC Array, 850K CpG sites (or a similar approach). We will explore if specific methylation patterns are associated

with response to ISCOLIM treatment. We will also explore if the immune cell compositions can be inferred from the DNA methylation profiles.

3.14.7 RNA sequencing

Total RNA-seq will be used for identification of composition and gene expression of tumor-infiltrating immune cells associated with treatment and tumor evolution. Further, RNA-seq will be used to investigate T-cell receptor clonality. The ScriptSeq protocol (or similar) will be used for generation of libraries. Data will be generated on the Illumina NovaSeq platform. As coverage varies with expression level, there is no target coverage. However, we aim to generate 100 mio sequencing reads per sample.

3.14.8 Bioinformatics and Data analyses

Multi-dimensional omics data from ctDNA, DNA, and RNA analyses on tumor and blood will be analysed and integrated in collective, predictive models for treatment response and survival (recurrence free-, disease free- and overall survival). Specifically, we will process the DNA and RNA sequencing data through MOMA's well-established analytic pipelines. For DNA, data will be aligned and calibrated by picard and GATK suites. SNPs will be identified by GATK HaplotypeCaller, and somatic mutations (SNVs) and indels will be identified with Mutect2 and Strelka2 (or similar software).

3.15 Collection of clinical information

To enable recruitment, patient's age, diagnosis, comorbidity, and tumor imaging material will be evaluated on all patients eligible for participation. Specifically, information about surgical intervention, pathology reports, oncological intervention, and radiological evaluations aiming at detecting disease recurrence or assessing changes in tumor burden (e.g. during surveillance and treatment) will be collected. Data will be collected for up to ten years after the last protocolled patient visit, and is required to conduct the study, to use in the statistical analyses, and for monitoring and quality control of the study.

For participating patients, the following information will be obtained from medical records; CPR number, surgical centre, age, gender, height, weight, BMI, WHO performance status, smoking/alcohol, and relevant medication use. Further, tumor size and histology, synchronous or metachronous CRLM, number of metastases, diagnosis of other cancers within 5 years, and chronic diseases will be recorded. Patients who consent to participate, further accept that a central element of the project involves comparison, correlation, and assessment of ctDNA and immune responses (gathered from the blood samples) to cancer treatment and clinical outcome. All clinical information will be obtained and processed in compliance with data protection laws.

3.16 Definitions of predictor variables, co-variates, and endpoints

3.16.1 Primary endpoint

- Fraction of patients with recurrence receiving RFA and curative resection, or curative resection only.
- Overall survival at 1, 3, and 5 years.

3.16.2 Secondary endpoints

- DFS
- RFS
- Time to clinical recurrence (TTCR)
- Time to molecular recurrence (TTMR) by ctDNA, TCR, or CEA measures
- Adherence rate

3.16.3 Safety Endpoints

- Frequency and severity of adverse events (AE) in relation to the per protocol blood draws.
- Allergic reaction to contrast material in relation to CT imaging.
- Complications in relation to RFA treatment (bleeding, hepatic abscesses, hepatic infarcts, and bile duct injuries).
- Complications in relation to general anaesthesia twice in two weeks (Study I).

3.16.4 Definition of endpoints

- Overall survival is defined as the time from surgery to death from any cause.
- Time to clinical recurrence is calculated from date of surgery until loco-regional recurrence or distant metastases, or sudden death from CRLM.
- Time to molecular recurrence is calculated from first time of no detectable ctDNA until detectable ctDNA.
- Adherence rate is defined as the proportion of patients adhering 100% to the protocol.

3.17 Statistical analyses

3.17.1 Demographics and baseline data

All data will be presented using descriptive statistics. Continuous variables will be summarized using mean, standard deviation, median, minimum, and maximum. Categorical variables will be summarized using the number and percentage of patients.

3.17.2 Analysis of OS, TTCR, and TTMR

Data will be analyzed as intention-to-treat. Kaplan-Meier estimates will be used for the estimation of median times to clinical recurrence, disease or death, and their confidence intervals stratified according to follow-up intensity. Endpoints will be assessed using the log-rank test or a Cox regression model, with time to event (clinical or molecular recurrence or death) as response variable and RFA as a factor. The difference in clinical recurrence versus molecular recurrence will be compared using paired t-tests and regression analyses.

We will follow the patients from the date of surgery until disease recurrence, death, or censoring within the five years follow-up. Patients will undergo standard postoperative surveillance CT scans every 3 months the first year and every 6 months hereafter until 36 months (*Fig.3*). Beyond the third year, CT scans will be performed annually.

To avoid unintentional bias, the demographics of patients not willing to participate in the trial will be compared to the study population. Statistics will be performed in close collaboration with professional health research statisticians.

3.17.3 Analysis of Safety

AEs will be reported to the PI. All AEs will be coded according to the standardized Medical Dictionary for Regulatory Activities (MedDRA). The coding will be performed by a researcher appointed by the PI. AEs will be summarized by presenting their incidence of AEs, based on the numbers and percentages of patients with AEs.

4. Data management

Data from the study will be collected from medical records and from the Danish Colo-rectal Cancer Group database (DCCG database). The information from the medical records will be entered into the study database, e.g. baseline information, pathology data, date of visits, and radiological control visits. Other information will be imported to the study database from the DCCG database e.g. tumor characteristics. Other external data e.g. laboratory data will be entered or transferred into the study database.

4.1 Quality Control

A quality control (QC) of data will be performed to ensure that data entry and verification have been performed correctly in accordance with pre-defined instructions. The QC will be performed before data is declared clean.

The trial sites will be visited by the Clinical Trial Manager (Monitor) appointed by the PI. The Monitor will visit periodically at times agreed with the PI. It is the function of the Monitor to ascertain that all aspects of the protocol are complied with and that the conduct of the trial conforms to applicable regulatory requirements and established rules for Good Clinical Practice (GCP). Preferably at the time of each monitoring visit, the Monitor will review the collected data to ascertain that items have been completed and that the data provided are accurate and obtained in the manner specified in the protocol. Further, the Monitor will verify that the data from external databases are consistent with the clinical records or other relevant records (Source Data Verification) and that trial results are recorded completely and correctly. For this purpose, the Monitor must be given direct access to clinical records, original laboratory data, etc., as far as these relate to the trial and without jeopardizing patient integrity. The investigators and other relevant personnel should be available during the monitoring visits and should devote sufficient time.

All investigators and staff carrying out observations of primary or other major efficacy variables involved in the trial should provide a curriculum vitae. The PI will keep a list of all personnel involved in the trial together with their function and trial-related delegated duties. He will ensure that appropriate trial-related training is given to all staff, and that any new information of relevance to the performance of this trial is forwarded to the staff involved. Before inclusion of patients, the Monitor will perform a trial initiation visit to inform and train relevant trial staff.

Any substantial change to the approved Final Trial Protocol will be documented in a written and numbered Protocol Amendment. Any proposed substantial change to the Final Trial Protocol must be discussed with and approved by the Sponsor before submitted to the relevant Regulatory Authority for approval. The study will be registered at ClinicalTrials.gov and monitored by the GCP Unit at Aarhus University Hospital.

5. Processing of personal data in the ISCOLIM trial study

This project is registered at the internal list of research projects in the Central Denmark Region. We will obtain approval from the local scientific ethics committee prior to inclusion of the first patient. We will adhere to the Helsinki declaration, and all data will be handled in accordance with The Danish Data Protection Act ("Databeskyttelsesforordningen" and "Databeskyttelsesloven").

Before study initiation processing of the patient's personal data will be registered in the appropriate system at the Central Denmark Region. Further, the trial will be approved by the Central Denmark Region Ethics Committee. No patient will be included before all permissions are granted. The study is covered by the Danish Patient Compensation Authority.

Throughout the study, all clinical data and samples will be labelled with non-personal identifiers. Information to identify individual patients will only be available to the PI (Prof. Frank Viborg Mortensen) and the TI (Prof. Claus Lindbergh Andersen) and will only be used when collecting clinical information from the patient. The molecular and clinical data produced in the study will be stored on keyword protected and log-file operated servers operated by Aarhus University and Aarhus University Hospital. In accordance with good academic practice and the requirements of the bodies funding the study and the scientific journals publishing the study results, the study data (health data and genomic data), in pseudonymized format, will be transferred to the secure database "European Genome-Phenome Archive, (EGA)" (https://ega-archive.org). This will happen after the study has been completed. The purposes are to enable sharing of the data with other research groups, inside and outside Denmark, confirmation of the study findings as well as future research. The EGA provides the necessary security required to control access, and maintain patient confidentiality, while providing access to those researchers and clinicians authorised to view the data. In all cases, data access decisions will be made by the ISCOLIM steering committee and not by the EGA. Data sharing will be conducted in accordance with the European data protection regulations, including The Danish Data Protection Act and the General Data Protection Regulation (GDPR). Before making a data access decision, the ISCOLIM steering committee will consult the Central Denmark Region Legal Office and data sharing will only take place after all necessary approvals have been obtained from the relevant Danish authorities.

The EGA is part of the European ELIXIR research infrastructure. ELIXIR is an intergovernmental organization that unites Europe's leading life science organisations in managing and safeguarding

the increasing volume of data being generated by publicly funded research. It coordinates, integrates and sustains bioinformatics resources across its member states and enables users in academia and industry to access services that are vital for their research. These resources include databases, software tools, training materials, cloud storage and supercomputers (https://elixir-europe.org/about-us). ELIXIR is partly funded by the European Commission within the Research Infrastructures programme of Horizon 2020. Denmark is an ELIXIR node. An ELIXIR Node is a collection of research institutes within a member country. ELIXIR nodes run the resources and services that are part of ELIXIR. The EGA is hosted by the ELIXIR Nodes of Spain and United Kingdom. Within the EU it is the Spanish ELIXIR node that runs the EGA, specifically it is the Centre for Genomic Regulation (CRG), C/ Dr. Aiguader 88, PRBB Building, 08003 Barcelona, Spain. CRG is a non-profit foundation funded by the Catalan Government through the Department of Business & Knowledge and the Department of Health, the Spanish Ministry of Science & Innovation, the "la Caixa" Banking Foundation, and includes the participation of Pompeu Fabra University.

6. Ethics and risks

6.1. RFA

The RFA-treatment used in this study is a minimally invasive procedure, with a low risk of complications. Thus, it is feasible in most patients. Potential RFA-related risks are bleeding and infection (1-2%). Extremely rare complications are hepatic abscesses, hepatic infarcts, and bile duct injuries. In ten patients, RFA will be performed separately from liver surgery (Study I). Followingly, these patients will undergo general anaesthesia twice. However, general anaesthesia in elective surgery is a very safe procedure with limited risks. The most common side effect is nausea and vomiting, which will be treated with antiemetics. As the potential clinical impact of this study is a possible increased long-term survival due to the reduced risk of cancer recurrence, the benefits of participating in the experimental arm outweigh the few potential side effects.

6.2. Blood samples

Clinical blood samples will be drawn 19 times for patients in the control arm and likewise for patients in the experimental study II. In study I, blood samples will be drawn 20 times. Per draw, 80 ml of blood will be collected. The blood samples will be used to measure CEA, a biomarker that guides clinical treatment decisions, as well as explorative translational biomarkers, that could improve onco-surgical treatment of CRLM patients.

The lost blood volume, contributed to each blood draw, is clinically irrelevant (<2% of the total blood volume) for the patient's health, especially in patients with performance status 0-1. Blood sampling is associated with minimal risk and discomfort to the patients, and rarely causes side-effects (hematoma, rash, or infection). To limit the inconvenience as much as possible, the blood samples will be drawn as part of the routine management, whenever this is possible.

6.3 Tissue samples

Tissue samples from CRLM and normal liver tissue are collected from the resected part of the liver, as needle biopsies and as surgical biopsies. The needle biopsies will be collected peri-operatively with ultrasound guidance concurrently with the RFA treatment before surgery and does not add to the overall procedural risk.

Liver biopsy is a safe procedure that causes few adverse effects and can be performed in an out-patient setting. Risks includes infection and bleeding (1-2%). To avoid the risk of tumor seeding, needle biopsies are taken through a sheet. Both needle biopsies and surgical biopsies are taken peri-operatively and does not affect the patient or the pathological evaluation of the CRLM.

6.4 CT scans

The patients included in ISCOLIM will undergo standard post-operative surveillance CT scans. No additional CT scans will be performed due to this study and thus, there is no additional radiation risk in participating in the study.

6.5 Genomic sequence analysis

In this study, we will DNA and RNA sequencing primarily to determine: 1) the TCR repertoire, 2) establish the somatic tumor mutation profile of each tumor, 3) detect and quantify ctDNA, and 4) establish gene expression profiles. We will not search the data for germline genetic variation (e.g. SNPs) s. Consequently, the risk of incidental finding a potential clinically relevant, genomic variant related to inherited diseases is extremely low, and practically hypothetical. Nevertheless, in the unlikely event that we do identify a genetic variant with potential clinical relevance, then we will have its importance evaluated by an expert committee, appointed by MOMA.

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, 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 the patient should be informed (by written letter) that the research accidentally has resulted in a finding, with potential influence on his or her health. The patient will also be informed that further information and advice on the matter will be offered to him/her and/or potentially affected family members.

If the patient in the consent form opted out of receiving important health-related findings or has died, the committee will assess whether to contact relatives, with purpose of saving lives and preventing disability.

7. Publication and reporting from the trial

The results of the ISCOLIM trial are expected to be published in international scientific journals. The reporting will follow the CONSORT guidelines for reporting randomized controlled trials (http://www.consort-statement.org/). Positive, negative, and non-definable findings will be published and presented at international scientific conferences as soon as at it is considered professionally sound. We will aim for publications in high-impact journals with focus on oncology, radiology, and liver surgery. If results unexpectedly cannot be published in journals, other methods of publication will be sought, such as the collaborators' institutional websites.

8. Economy

This study is sponsor-initiated. The study is supported by two research grants given to prof. Frank Viborg Mortensen (Novo Nordisk Fonden [2.982.000 DKK] and Neye Fonden [675.000 DKK]). Grants from these foundations are administered by Aarhus University Hospital. The grants support running costs related to the project (blood samples, RFA needles, project nurses etc.). The investigators and collaborators have no financial relation to the grant providers, nor any financial interest in the trial. Salaries to the research staff are already financed by their respective employers.

8.1 Remuneration to participants

There is no remuneration to the study participants.

8.2 Patient compensation scheme (patienterstatningen)

The study is covered by the Danish Patient Compensation Scheme (patienterstatningsordningen).

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