GZA vzw

Clinical Protocol

Prospective complete histopathological characterization of liver metastases from colorectal and breast carcinoma to predict the histopathological growth patterns by medical imaging

Study number:	CTOO20084GZA
Protocol version and date:	4.0 – 17 DEC 2024
EudraCT number:	Not applicable
Sponsor:	Ziekenhuis aan de Stroom (ZAS) vzw Kempenstraat 100 2030 Antwerpen

This study will be conducted in compliance with the protocol, International Conference of Harmonization – Good Clinical Practices (ICH-GCP), in agreement with the Declaration of Helsinki (version 2002) and any applicable regulatory requirements.

Confidentiality Statement

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SIGNATURE PAGE

Protocol title: "Prospective complete histopathological characterization of liver metastases from colorectal and breast carcinoma to predict the histopathological growth patterns by medical imaging"

The signatory agrees to the content of the final clinical study protocol as presented.

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Investigator agreement

Protocol title: “Prospective complete histopathological characterization of liver metastases from colorectal and breast carcinoma to predict the histopathological growth patterns by medical imaging”

I confirm that I have read this protocol, I understand it and I shall work according to this protocol and to the ethical principles states in version of 2002 of the Declaration of Helsinki, the applicable guidelines for good clinical practices (GCP) or the applicable laws and regulations of the country of the study site, for which I am responsible, whichever provides the greater protection of the individual.

I am aware of my responsibilities as an Investigator/Representative under the good clinical practices national regulations and trial protocol. I agree to appropriately direct and assist the staff under my control, who will be involved in this clinical trial. This is documented in a training log.

Signature: _____ Date: _____

Name: _____

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LIST OF ABBREVIATIONS

CRF	case report form
CT	computer tomography
EC	Ethics Committee
EOS	end of study
GCP	Good clinical practice
HGP	Histopathological growth pattern
ICF	informed consent form
ICH	International Council for Harmonisation
MRI	magnetic resonance imaging
RFA	radiofrequency ablation
RNA	ribonucleïnezuur
SnRNA-Seq	Single nucleus RNA-sequencing
TACE	transarterial chemoembolization

STUDY SYNOPSIS

Title of clinical trial	Prospective complete histopathological characterization of liver metastases from colorectal and breast carcinoma to predict the histopathological growth patterns by medical imaging
Short title/acronym	POEM – PrOspEctive Metastases Imaging
Sponsor name	ZAS vzw Kempenstraat 100, 2030 Antwerpen, Belgium
Study responsible physician	Dr. Peter Vermeulen
Protocol number	CTOO20084GZA
Eudract number	Not applicable
Medical condition under investigation	Patients with liver metastatic colorectal and breast cancer planned for hepatic surgery
Purpose of trial	To evaluate medical imaging as a means to identify the histopathological growth patterns of liver metastases.
Primary objective	To develop a radiomic signature of the histopathological growth patterns of liver metastases based on standard-of-care CT and MR images and full histopathological sampling
Secondary objective(s)	In-depth molecular characterization of the biology of the growth patterns by single-nucleus RNA-sequencing
Trial design	A multicentric prospective observational study working with remnant tumor tissue from patients with liver metastatic colorectal or breast carcinoma undergoing a partial hepatectomy. Optional biosampling is performed for later research purposes.
Summary of eligibility criteria	• Patients with liver metastatic colorectal or breast carcinoma undergoing a surgical excision of the liver metastases and with MRI (magnetic resonance scan) or CT (computer tomography) of the liver prior to surgery • Time interval between medical imaging, CT and / or MRI is is more than 12 weeks (preferably less than 4 weeks). • No prior radiofrequency ablation (RFA) or transarterial chemoembolization (TACE) on the liver metastasis that would be operated.
Version and date	4.0 – 17 DEC 2024
Trial registration	Not applicable

ADMINISTRATIVE STRUCTURE

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AMENDMENTS

Protocol version	**Issue date**
Original protocol	02-MAR-2020
Amendment 1	16-DEC-2020
Amendement 2	23-JUN-2021
Amendement 3	17-DEC-2024

Amendment 1:

The purpose of this amendment is to:

-switch the trial from a unicentric trial to a multicentric one

Changes to the protocol:

You can find all adaptations in this document hereafter (separate comments) as well as below on this page.

-adjustment of protocol version and date

-adjustment of

- Trial design (multicentric)

-2.1 Study design: 2.1: specifying CT **OR** MRI (one of the two is mandatory)

-2.2.2: total number of study sites/total number of subjects projected: **50 patients** instead of 25 due to expansion to multicentric trial

-4.2: Subject exclusion criteria: Time interval between medical imaging, CT and/or MRI **is more than 12 weeks** (nb: preferably less than 4 weeks but this is not an exclusion criterium) – instead of 4 weeks before

-addition of

- 1.1 Risks and benefits*

-5.1 Screening and case ascertainement*

-5.2 Data sources*

-5.3 Data elements to be abstracted + addition of Table 1: Tabulated overview on data collected during the clinical study and Table 2: Case Report Form

-5.4 Histopathological analysis

-5.7 Biobank sampling

-5.8 Subject discontinuation*

-5.9 Study and site discontinuation*

-6 Safety Monitoring and Reporting*

-7 Data Management and Quality Control*

-9.1 Ethics and regulatory approval*

-9.3 Informed consent*

*= these points were rewritten in a structured way or placed in a different order in the protocol

A copy of the amended protocol will be sent to the Ethics Committee of the GZA Hospitals, Belgium. The changes described in this amended protocol require EC approval prior to implementation.

Amendement 2:

The purpose of this amendment is to:

-include Ziekenhuis Netwerk Antwerpen (ZNA) as a participating center in the POEM-study

Changes to the protocol:

You can find all adaptations in this document itself in red (separate comments):

- adjustment of protocol version and date
- addition of co-investigators of Ziekenhuis Netwerk Antwerpen: Dr. Willemsen, Dr. Gypen, Dr. Buelens
- Interventional biobanking part is limited to patients included in GZA Hospitals

Amendement 3:

The purpose of this amendment Is to:

-Prolong the duration of the study with 3 years, until Dec 2027, for a total inclusion of 50 patients.

Changes to the protocol:

- 2.2.1. Date range of study: we anticipate this study to take up to 7 years to complete instead of 4 years.
- Addition of surgical co-investigators: Dr. P. Boons and Dr. T. Apers as well as PhD-researcher Yasmine Sillis and laboratory technologist Bart Neetens
- Change of sponsor from GZA vzw to ZAS vzw

1. INTRODUCTION

1.1. Background & rationale

Colon cancer is a major cause of death among different cancer types. The tumor in the colon can often be treated with curative intent (by surgery, possibly in combination with medication (chemotherapy)). However, in the vast majority of patients who die from colon cancer, metastases are the real cause of death. This usually involves liver metastases. These metastases can be surgically removed and patients are often treated peri-operatively with chemotherapy or other medication although this treatment is not always successful. The existing method, eg a clinical risk score[1], that is being used to predict which patient will benefit from a specific treatment of liver metastases is inaccurate.

Over the past fifteen years, the team of the Translational Cancer Research Unit has conducted research that explains, at least partly, why not all liver metastases from colon cancer behave in the same way. These differences can be observed by histopathological examination. Surprisingly, liver metastases behave in two very different ways, or growth patterns, and this information is currently not being used in clinical practice. However, the biological differences in the behavior of the cancer cells between these two growth patterns are pronounced. In one growth pattern - we call this "desmoplastic" - the cancer cells behave as in the primary colon cancer itself: they form groups and create new supportive tissue - or “desmoplasia” - and blood vessels – or "angiogenesis".

Inflammation is often present in this type of metastases. In the other growth pattern - we call this 'replacement' - the cancer cells behave in a special way: they take the place of, or 'replace', the normal cells of the liver, the hepatocytes, and use the available supporting tissue and blood vessels of the host. There is no formation of new blood vessels and there is often little or no inflammation. When more than one growth pattern is present in a liver metastasis, the relative contribution of each growth pattern is estimated (percentage).[2]

These different growth patterns contain important information on the success of the treatment of the individual patient[3]. A large retrospective study of 732 patients, where liver metastases from colon cancer were surgically removed, identified a group of patients (19%) with an exclusive desmoplastic growth pattern in all sections of the resected liver metastasis.[4] This subgroup is associated with good survival and progression-free survival. In contrast, patients with any amount of replacement growth, have a significantly shorter overall survival. However, large retrospective studies have shown that these patients with a 'replacement' growth pattern have a survival advantage of adjuvant treatment, i.e. after surgery for liver metastasis, often a combination of chemotherapy and a treatment that inhibits angiogenesis (for example bevacizumab) is given. This is in contrast to patients with the "desmoplastic" growth pattern, the intrinsically more favorable pattern, where this treatment has no survival benefit and is therefore probably not necessary after surgery. These patients may be the so-called "oligometastatic" patients who may benefit from repeated local targeted therapies. The prognostic and predictive value of the growth patterns has been demonstrated not only in patients with colorectal cancer but also in patients with uveal melanoma, skin melanoma and breast carcinoma.

The concept of tumor growth patterns is innovative (and still underexposed in the scientific literature) and offers important perspectives for clinical application. Anti-angiogenic treatment as well as immunotherapy achieved poor results in clinical studies of metastatic colon cancer. We are convinced that a better selection of patients based on characteristics of the liver metastasis, and not only based on characteristics of the primary colon tumor, could lead to success in clinical studies.

Thus, the growth patterns have an important clinical value and provide the treating physician with yet unknown information about the behavior of a liver metastasis in an individual patient. The limitation, of course, is that we can only determine the growth pattern after hepatic surgery. It would be much more useful to be able to identify the growth patterns by means of medical imaging, allowing for the treatment to be tailored to the patient even before surgery. (for example; is there an additional benefit of starting chemotherapy prior to surgery?)

Therefore, the aim of the current project is to ascertain whether we can identify the growth patterns of these tumors by means of imaging with CT (computed tomography) or MRI (nuclear spin tomography). Some published studies suggest that medical imaging can capture the histopathological growth patterns, but these are all retrospective studies with a small number of patients and incomplete pathological sampling of the liver metastases. One study in 2007 correlated the early peri-lesional gadolinium enhancement of the liver metastasis with the presence of the desmoplastic reaction with vascular proliferation and inflammation.[5] More recently, Cheng and colleagues claimed that the prediction of the growth patterns would be possible with radiomics based on CT images.[6]

By identifying the growth patterns in a non-invasive manner, by means of medical imaging, we will be able to use the growth patterns in prospective clinical studies to truly test the predictive power.

This project concerns liver metastases of primary colorectal carcinoma as well as primary breast carcinoma. The research will therefore mainly, but not only, help patients with liver metastases of colorectal cancer and breast cancer when a translation of this project from the pre-clinical phase (explorative study) to the clinical phase (randomized studies with patients) is done.

1.2. Risks and benefits

1.2.1. Risks for subjects

There is no potential for physical risks to subjects. There is a minimal risk of breaches of confidentiality since patient information will be collected and analysed for the proposed study. However, appropriate measures will

be taken to minimize the risk as much as possible. All information entered into the central database will be pseudonomised. This study will abide by all regulations related to protecting human subjects and health information.

1.2.2. Potential Benefits to Subjects

There is no direct benefit to the subjects. However, future patients with liver metastases of colorectal cancer and breast cancer may benefit from improved care as a result of this study.

2. STUDY DESIGN

2.1. General scheme of study design

Patients with liver metastatic colorectal or breast carcinoma who undergo abdominal contrast-enhanced multidetector CT or magnetic resonance imaging followed by partial hepatectomy will be included in this prospective clinical study (see Figure 1). Liver metastases will be sampled completely while respecting the orientation used for medical imaging, in order to correlate the histopathological growth patterns in a 3D-map with the imaging characteristics. A remnant tissue fragment may be snap-frozen to perform single nucleus RNA sequencing. The full pathological sampling will also only use remnant tissue.

This study also has an additional scientific aspect involving the sampling of blood and urine pre- and post-surgery. This aspect of the study protocol is optional and agreement to participate is for the sites to decide. Explicit informed consent from subjects for collecting blood and urine samples must be obtained.

The study design schematic is presented in Figure 1.

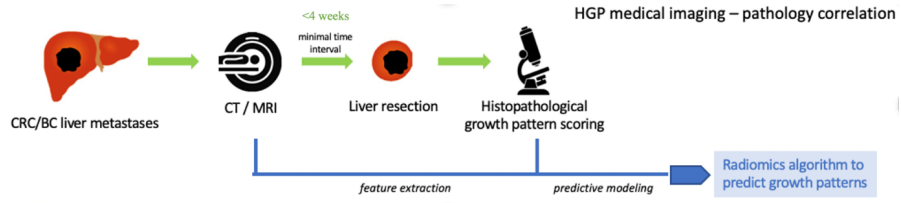


Figure 1: Overview of study design

2.2. Study duration, enrolment and number of sites

2.2.1. Date range of study

We anticipate this study to take up to 7 years to complete, so until 31 DEC 2027.

Met opmerkingen [EL1]: 7 years? (+3 jaar)

2.2.2. Total number of study sites/total number of subjects projected

Approximately 30-40 patients with colorectal cancer liver metastases and about 10 patients with breast cancer liver metastases will be included.

3. STUDY OBJECTIVES AND ENDPOINTS

Liver metastases have essentially two growth patterns. It seems of primordial importance to know the type of growth pattern of the liver metastasis since treatment and survival chances of the patient depend on these growth patterns of the metastasis based on retrospective research in large patient groups. Until now it has been necessary to use surgical resection specimens to evaluate the growth patterns by light microscopy. However, in this project we want to investigate whether and how exactly we can distinguish the growth patterns of metastases in the liver in a non-invasive way (i.e. via medical imaging). The two most important growth patterns are "desmoplastic" and "replacement".

This is the first prospective clinical study to evaluate the growth patterns comprehensively throughout the entire tumor-liver interface of the metastases and to correlate the resulting 3D-map with imaging characteristics (in particular MRI and / or CT) to end up with growth pattern-specific radiomic signatures.

3.1. Primary objective

The primary objective is to be able to identify the different growth patterns in a non-invasive manner, by means of either MRI and/or CT : by means of description of qualitative characteristics on the one hand and on the other hand with development of a radiomic tool that collects quantitative data from medical imaging using algorithms.

3.2. Secondary objective

In addition, given that cancer cells of the two most important growth patterns have a fundamentally different way of communicating with the liver parenchyma, a tissue fragment may be snap-frozen to perform single nucleus RNA sequencing in the lab of Christine Desmedt, head of the Translational Breast Cancer Research at KU Leuven.

4. STUDY POPULATION

4.1. Subject inclusion criteria

Each potential subject must satisfy all of the following criteria to be enrolled in the study.

1. ≥ 18 years old.
2. Patients with liver metastatic colorectal or breast carcinoma undergoing a surgical excision of the liver metastases
3. Therapy-naive patients as well as patients with neo-adjuvant therapy are eligible for inclusion
4. Willingness of the patient to participate in this study demonstrated by signed written informed consent

4.2. Subject exclusion criteria

Each potential subject must not satisfy any of the following criteria to be enrolled in the study.

1. Patients without MRI or CT of the liver prior to surgery
2. Time interval between medical imaging, CT and/or MRI is more than 12 weeks (nb: time interval is preferably less than 4 weeks but this is not an exclusion criterium)
3. Prior radiofrequency ablation (RFA) or transarterial chemoembolization (TACE) on the liver metastasis that would be operated

5. STUDY PROCEDURES

The study procedures (see Figure 2) are limited to review of records and use of leftover tissue specimens.

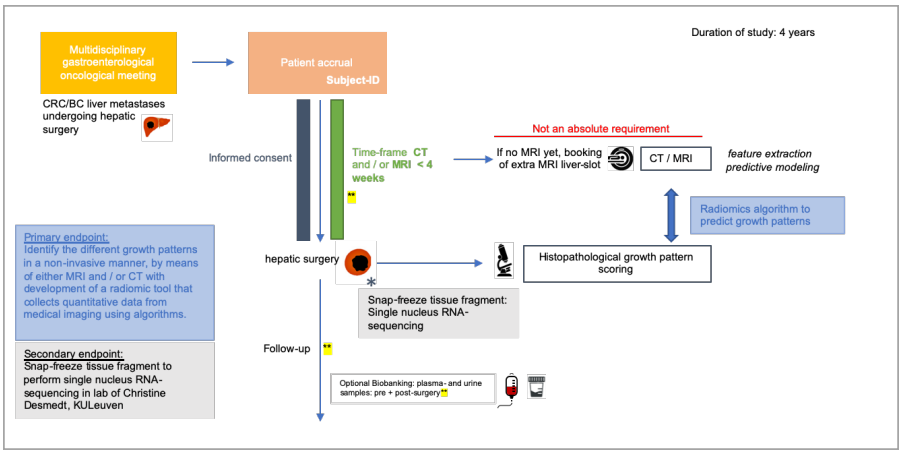


Figure 2: Study flow chart

5.1. Screening and case ascertainment

All subjects must sign an ICF prior to conduct of any study-related procedures (see also section 9.3). Patient's eligibility will be determined by the investigator according to the inclusion and exclusion criteria as detailed in section 4.

5.2. Data sources

The investigator collects historic data (e.g. *tumor information,imaging details, …*) from medical records.

Each patient will be identified in the study by a subject number (subject-id) and this is retained as the primary identifier for the patient throughout his entire participation in the study. The subject-id consists of the site number (site-id) (as assigned by the sponsor to the investigative site) with a sequential patient number suffixed to it, so that each subject is numbered uniquely across the entire database. Upon signing the informed consent form, the patient is assigned to the next sequential subject-id available to the investigator through the electronic data capture system.

5.3. Data elements to be abstracted

5.3.1. EPD

Table 1: Tabulated overview on data collected during the clinical study

Parameter	**Data**
Inclusion	Date of eligibility criteria assessment
Tumor information	Primary tumor, mutation status (NRAS, BRAF, KRAS, MSI)
Imaging details	Dates of MR and CT
Medical history	Systemic therapy pre-surgery details
Surgery	Date and type of surgery

Tabel 2: Case Report Form (CRF)

	Case Report Form (CRF)					
	Patient	Example	1	2	3	4
General	Informed consent procedure (date)	01/01/2021				
	Eligibility criteria assessment (date)	01/01/2021				
	Primary tumor (colorectal ca= 1, breast ca= 2)	1				
Sampling	Plasma-sampling preoperatively	01/01/2021				
	Plasma-samplings postoperatively	01/01/2021				
	Urine-sampling preoperatively	01/01/2021				
	Urine-sampling postoperatively	01/01/2021				
	Snap-freeze (Y= 1, N=0)	1				
Imaging	CT abdomen (date)	01/01/2021				
	MR abdomen (date)	01/01/2021				
Mutations	KRAS (Y=1, N=0)	1				
	NRAS (Y=1, N=0)	0				
	BRAF (Y=1, N=0)	0				
	MSI (Y=1, N=0)	1				
Systemic therapy	Systemic therapy pre-operatively (Y=1, N=0)	1				
	Type of systemic therapy	bevacizumab				
	Anti-angiogenic therapy pre-operatively(Y=1, N=0)	1				
	Date of ending of systemic therapy pre-surgery	01/01/2021				
	Number of days (n) between end of systemic therapy and day of surgery	30				
	Time interval between diagnosis of primary tumor (radiological diagnosis) and the development of liver metastases (radiological diagnosis) (n=days)	150				
Surgery	Date of surgery	01/01/2021				
	Type of surgery	lobectomy				

5.4. Histopathological analysis

Liver metastases will be sampled completely to enable HGP assessment in the entire lesion while respecting the spatial orientation, the orientation used for medical imaging. The full pathological sampling will only use remnant tissue.

Pathological processing:

The surgeon will apply marks to orientate the surgical specimen to enable the pathologist to make slices in transverse (axial) planes that run parallel with the transverse planes of the medical imaging. The following tissue sampling protocol will be used. (Fig 3)

The marks of the surgeon are used to orientate the surgical specimen. Slices are made in the transverse plane (corresponding to the transverse (axial) planes of the MRI and CT images) with a thickness of 0.5 to 1cm and put on a tray together with slice number marks. All slices are photographed when put on a tray.

Clockwise tissue sampling for formalin-fixation and paraffin-embedding is done at the tumour-liver interface (one sample/cassette), applying a dot of ink as shown in figure 3. The last sample of each slice is the central sample. The number of samples will depend on the size of the metastasis, but each quadrant should be sampled at least once (so a minimum of 4 peripheral and 1 central sample should be collected per slice). Each sample will be indicated on the print-out of the photographed slices as demonstrated in figure 3 and cassettes are marked accordingly (slice number – sample number). The tissue samples are fixed in neutral buffered formalin-solution and paraffin blocks and H&E-sections are prepared.

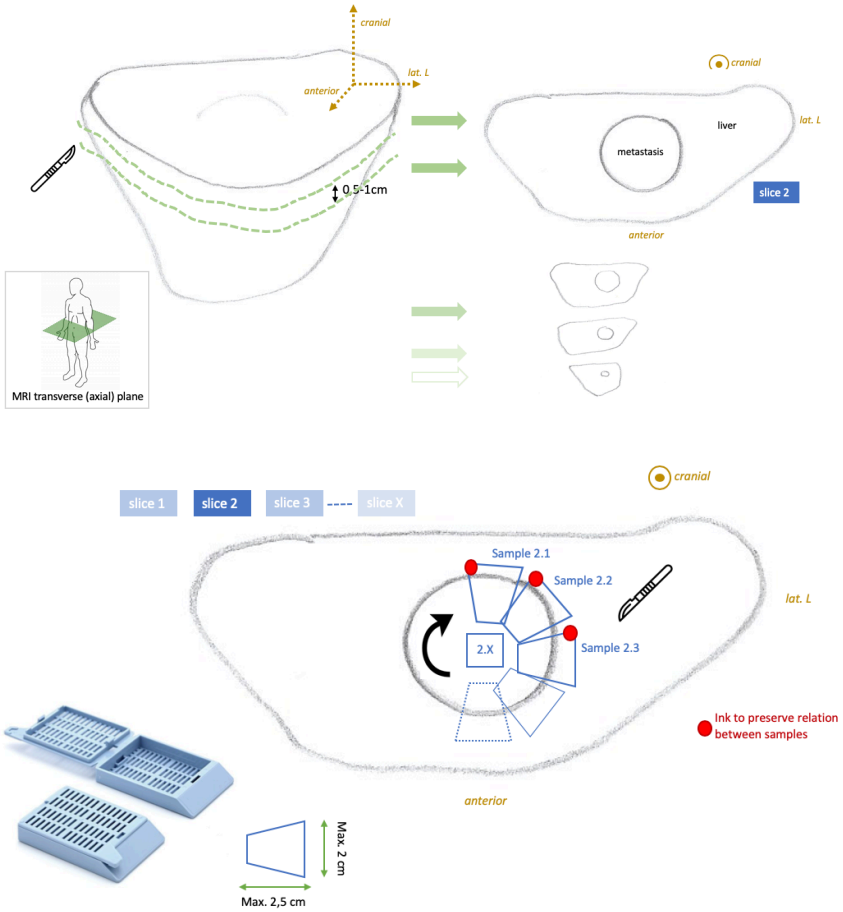


Figure 3: Histopathological processing of liver metastasis

5.5. MDCT image acquisition

CT and MRI prior to surgery:

5.5.1. CT Image Acquisition

- Qualitative and quantitative analyses will be performed on pre- and post-contrast (arterial and portal venous phase) images.
- Standard of care

  or

  Scanning parameters:

  - 120kV, 240-400mA, slice thickness of 5mm
  - Protocols for contrast agent: 100 mL Iopromide 370mg/mL at a rate of 2.5mL/s

- MDCT scans in arterial and portal venous phases will be initiated at 10 and 45s

5.5.2. MRI Acquisition

Standard of care

or

The MRI examination will be performed by using 750W system (GE Healthcare, Milwaukee, WI, USA) and 8-channel phased array torso coils in both institutions. All patients were positioned supine and feet-first.

(1) **T2-weighted image** of fast recovery fast spin-echo (FRFSE) with fat saturation, TR/TE, 5000/96 msec; slice thickness, 7mm; gap, 1mm; matrix, 320×320; FOV, 38cm;

(2) **Diffusion-weighted imaging (DWI)** and the Apparent diffusion coefficient (ADC) quantification, TR/TE, 6000/58 msec; slice thickness, 7mm; gap, 1mm; matrix, 160×160; FOV, 38cm; b value, 0 and 800;

(3) **T1-weighted image**, the (4) arterial phase (AP) and (5) portal venous phase (PVP) image of the dynamic contrast-enhanced T1-weighted liver acquisition volume acceleration (LAVA) with chemically selective fat saturation, TR/TE, 5.116/1.674 msec; slice thickness, 5mm; gap, 2.5mm; matrix, 280×170; FOV, 36cm. The enhanced images will be acquired after the intravenous administration of gadopentetate dimeglumine (Magnevist; Bayer HealthCare Pharmaceuticals, Berlin, Germany) in the arterial phase (46 seconds after contrast injection) and portal venous phases (79 seconds after contrast injection). The contrast administration consisted of 0.25mmol/kg gadopentetate dimeglumine at a flow rate of 2.5 mL/s, followed by a flush of 20 mL of saline (2 mL/sec) with a power injector (Spectris Solaris Injection System; Bayer HealthCare Pharmaceuticals, Berlin, Germany).

5.6. Snap frozen tissue

According to the procedure of the local pathology lab.

Single nucleus RNA-sequencing (snRNA-Seq).

Fresh-frozen samples for single-nucleus RNA-sequencing of approximately 0.125 cm^3 will be isolated from different regions from the same resection specimens (tumour-liver interface and tumour core), and rapidly flash-frozen prior to storage at -80°C. These samples will also be marked on the print-out of the photographed slices.

Frozen samples will be further processed after careful selection on the corresponding H&E-slide. In brief, for each metastatic lesion and for each HGP within the lesion, we will further process samples from the core of the lesion and at the tumour-liver interface. On the day of analysis, samples will be defrosted in lysis buffer and homogenized using a gentleMACS dissociator (Miltenyi) with gentleMACS M tubes (Miltenyi). The flow-through will be centrifuged (1000 x g, 5min), and the resulting pellet resuspended in 2 mL centrifuge buffer. After overlaying this onto 2 mL fresh centrifuge buffer, samples will be centrifuged at 1000 x g, 5 min and nuclei will be redissolved in PBS with 1% BSA and 10U/mL SUPERase. Following nuclei counting, approximately 10,000 nuclei will be converted to barcoded scRNAseq libraries by using the Chromium Single Cell 3' Library, Gel Bead & Multiplex Kit and Chip Kit (10X Genomics; Pleasanton, California, USA), yielding an estimated 6,000 nuclei per sample, of which we will sequence about 3000 per sample. For each patient, all samples will be processed in parallel in the same thermal cycler to avoid experimental bias. Libraries will be sequenced on an Illumina NextSeq and mapped to the human genome using CellRanger (10X Genomics). This workflow on average yields >700 uniquely tagged transcripts (UMIs) originating from >300 genes per nucleus. Raw gene expression matrices will be generated per sample using CellRanger and converted to a Seurat object using the Seurat R package. Gene expression matrices will be normalized to total nuclear read count and to mitochondrial read count using linear regression as implemented in Seurat's RegressOut function. Principle component analysis will be done on variably expressed genes, and to reduce dimensionality of this dataset, the informative PCs will be summarized using algorithms such as t-Distributed Stochastic Neighbour Embedding (tSNE) dimensionality reduction and Uniform Manifold Approximation and Projection (UMAP) (Becht E et al., 2019). Nuclei clusters will be determined using a graph-based clustering approach and annotated to known biological cell types using canonical marker genes. Composition profiles of the different cell types will be compared by Kruskal–Wallis tests. A set of gene

expression signatures retrieved from literature will be computed for each cell and statistical differences between the growth pattern groups will be addressed by Wilcoxon signed-rank test. Difference in cell dynamics between growth patterns will be investigated using pseudo-time reconstruction with the acknowledged monocle R package (Trapnell C et al., 2014). Prediction of cell velocities will also be investigated using the R package velocito (La Manno G et al., 2018). For these experiments we will benefit from the expert guidance from Profs. B. Thienpont and D. Lambrechts (KU Leuven).

5.7. Biobank sampling

Preoperative and postoperative blood- and urine sampling are optional. The postoperative sampling is preferably performed 4-6weeks postoperatively. This optional biobanking is limited to patients included in GZA Hospitals.

5.7.1. Urine:

A midstream urine sample (50 ml) is collected from the patient. Store the sample as 10 ml aliquots in 15ml BD Falcon tubes at -80°C within 4 hours of sample withdrawal.

5.7.2. Serum Separator Tube (SST):

Processing

- Centrifugation 2000g
- Time 10 minutes
- Temperature Room temperature

Storage

- Aliquotisation Material is stored in 1 ml aliquots
  - Preferably 3 aliquots of 1 ml are stored. If there is more material, more aliquots are stored.
- Type of tube PP, 1000 µl, screw cap, sterile, 2D code
- Temperature Material is stored at -150°C in polypropylene tubes

5.7.3. Cell Preparation Tube (CPT):

Definitions and synonymes

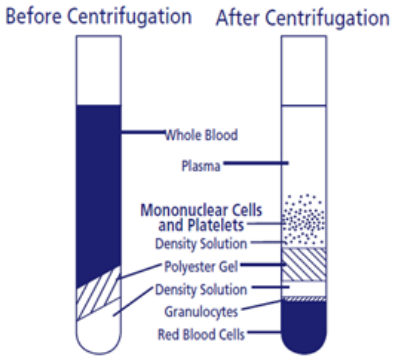
CPT	Cell Preparation Tube
DMSO	Dimethylsulfoxide
FBS	Fetal bovine serum
FSC	foward scatter
MNC	mononuclear cell
RPMI	Roswell Park Memorial Institute
PBMC	Peripheral blood mononuclear cells
Pp	polypropyleen
SSC	side scatter
7-AAD	7-aminoactinomycin D
RT	Room Temperature
RPM	Revolutions per minute

Method

Principle:

A CPT tube is used for this PBMC isolation procedure. This CPT tube contains a coagulant (to prevent clotting), but also contains a blood secretion gel and a ficoll solution. When a CPT tube filled with blood

is centrifuged, a separation occurs between the plasma, the PBMCs (= buffy coat), the gel and the red blood cells and granulocytes, as indicated below.



Sample:

CPT tube filled with blood

Appliances and aids:

- Centrifuge
- 5ml pp-tubes
- 15ml pp-tubes
- 2ml cryovial
- Miltenyi MACSQuant Analyzer 10
- Mr. Frosty

Reagentia:

- RPMI 1640 medium
- CountBright absolute counting beads
- FBS
- DMSO
- 7-AAD
- Miltenyi Calibration beads

Execution:

- Centrifuge the CPT tube: 1600g, 20 minutes, room temperature, **brake.**
- Pipette the plasma into XXX cryovials (1ml / cryovial, 5 aliquots)
- The plasma is stored in the -150 ° C mechanical cryo-freezer
- Isolate the buffy coat (= mononuclear cells and plates, see image above) and transfer it to a 15ml pp tube
- Add RPMI to a volume of 10ml, gently invert the tube 3x
- Transfer 100µl to a 5ml pp tube and add 50µl CountBright Absolute Counting beads and 2µl 7-AAD. Mix up
- Measure on the MACSQuant Analyzer instrument:
    - Calibration must be performed first (see BVS: ”MACSQuant Analyzer 10”)
    - Enter
    - ✓ File :
    - ✓ Mix sample : mix gentle

- ✓ Uptake volume : 100µL
- ✓ Sample volume : 150µl
- ✓ Instrument settings: "setup" today's date "PBMC count"
- ✓ Events : 75.000

- Analysis template : PBMC count
- Click on the big "A" (so that the gates can be adjusted)
- When starting the measurement, check whether the cells are fully visible on the FSC-SSC plot. Pause the measurement, adjust the FSC and / or SSC if necessary (FSC: 240, SSC: 460, Trigger: 150), press "Clear" and restart measurement
- Plot FSC versus SSC: : gate around MNCs
- Plot V1 versus SSC: zet gate around beads
- Plot B3 versus SSC: zet gate around viable cells
- Adjust gates : MNC, beads, viable
- Calculate absolute number of cells (MNCs) :

(events MNC/events beads) x 0.52 x 100.000 / 100 x 10.000 = ??



- Centrifuge residual 10ml (5min, 400g)
- Remove the supernatant by means of decant
- Resuspend in pre-prepared medium (90% FBS and 10% DMSO i.e.for 1 ml: 900 µl FBS + 100 µl DMSO)
- Transfer the cell suspension directly to a cryovial ⇨ in mister frosty to - 80°C
- Transfer to -150°C mechanical cryo-freezer after 24 hours

5.8. Subject discontinuation

Patients have the right to voluntarily withdraw from the study at any time for any reason. In addition, the investigator has the right to withdraw a patient from the study at any time. Reasons for withdrawal from the study may include, but are not limited to, the following:

- Patient withdrawal of consent at any time
- Investigator or sponsor determines it is in the best interest of the patient (when surgery is abrupted either beforehand or during surgery due to intra-operative findings, then this will be the end of study for these patients).

In case a patient would like to withdraw his/her consent, the patient should inform the investigator and the site should document the withdrawal in the patient medical records and in the eCRF. If a patient wants to terminate the participation in the clinical study, no further data will be collected.

5.9. Study and site discontinuation

The sponsor has the right to terminate this study at any time. Possible reasons for termination include, but are not limited to, the following:

- Safety reasons
- New scientific knowledge becomes known that makes the objectives of the study no longer feasible/valid.
- Unsatisfactory enrollment of participants.

In terminating the study, the sponsor will notify the investigator and will ensure that adequate consideration is given to the protection of the subjects' interests.

The sponsor has the right to close a site at any time. Reasons for closing a site may include, but are not limited to, the following:

- Excessively slow recruitment
- Poor protocol adherence
- Inaccurate or incomplete data recording
- Non-compliance with the International Council for Harmonisation (ICH) guideline for Good Clinical Practice
- No study activity (i.e., all patients have completed and all obligations have been fulfilled)

Should the study be terminated, and/or the center closed for whatever reason, all documentation pertaining to the study must be returned to the sponsor or its representative.

6. SAFETY MONITORING AND REPORTING

The PI will monitor and review the study progress and the accuracy and security of the emerging data. The only potential risk for participants is the loss of confidentiality (see safeguards in section 7.1).

This protocol presents minimal safety risk to subjects since it works with remnant tumor tissue and optional biosamples are collected according to standard practice in clinical diagnostics. Adverse events or other problems are therefore not anticipated.

7. DATA MANAGEMENT AND QUALITY CONTROL

7.1. Data protection

The collection and processing of personal data from subjects enrolled in this study will be limited to those data that are necessary to fulfil the objectives of the study.

These data must be collected and processed with adequate precautions to ensure confidentiality and compliance with applicable data privacy protection laws and regulations. Appropriate technical and organizational measures to protect the personal data against unauthorized disclosures or access, accidental or unlawful destruction, or accidental loss or alteration must be put in place. Sponsor personnel whose responsibilities require access to personal data agree to keep the identity of subjects confidential.

The informed consent obtained from the subject includes explicit consent for the processing of personal data and for the investigator/institution to allow direct access to his original medical records (source data/documents) for study-related monitoring, audit, IEC/IRB review, and regulatory inspection.

The subject has the right to request through the investigator access to his or her personal data and the right to request rectification of any data that are not correct or complete. Reasonable steps will be taken to respond to such a request, taking into consideration the nature of the request, the conditions of the study, and the applicable laws and regulations.

7.2. Electronic case report forms

The investigator should maintain adequate and accurate source documents and study records that include all pertinent observations on each of the center's study participants. Source data should be attributable, legible, contemporaneous, original, accurate, and complete.

The designated investigator staff will enter the data required by the protocol into Excell. Investigator site staff will not be given access to the electronic data capture (EDC) system until they have been trained. Data must be entered into Excell in English. The eCRF must be kept current to reflect participant status at each part during the course of the study. The eCRF will not capture personalized data. The investigator must make a separate confidential record of personalized details (name and initials) on the participant identification and enrollment log. All changes to data are done by the investigator or designated site personnel through the electronic data capture system.

7.3. Data monitoring and quality assurance

The sponsor will implement a system to manage quality throughout the design, conduct, recording, evaluation, reporting and archiving of the study with a focus on study activities essential to ensuring protection of participants and the reliability of study results. The quality management system will use a risk-based approach.

This study will be monitored for quality assurance at all stages of its development by the clinical research personnel of the sponsor. Monitoring will be based on a systematic approach and will include site visits and telephone communication to assure that the investigation is conducted according to the protocol, standard operating procedures, Good Clinical Practice guidelines, and applicable regulatory requirements. Quality control procedures will be applied to each stage of data handling to ensure that all data are reliable and have been processed correctly. On-site review of eCRFs will include a review of forms for completeness and clarity, and consistency with source documents available for each participant. Note that a variety of original documents, data, and records will be considered as source documents in this study.

The eCRF itself is not to be used as a source document under any circumstances. The study may be audited by the sponsor or by regulatory authorities. If such an audit occurs, the investigator must agree to allow access to required participant records. By signing this protocol, the investigator grants permission to personnel from the sponsor, its representatives, and appropriate regulatory authorities, for on-site monitoring of all appropriate study documentation, as well as on-site review of the procedures employed in eCRF generation, where clinically appropriate. Relevant data will be transcribed by the center personnel into the eCRF.

7.4. Record retention

In compliance with the ICH/GCP guidelines, the investigator/institution will maintain all Excell files and all source documents that support the data collected from each subject, as well as all study documents as specified in ICH/GCP Section 8, Essential Documents for the Conduct of a Clinical Trial, and all study documents as specified by the applicable regulatory requirement(s). The investigator/institution will take measures to prevent accidental or premature destruction of these documents.

Essential documents must be retained for 20 years.

8. STATISTICAL CONSIDERATIONS

To confront the HGP information with the radiomic features to develop HGP-specific radiomic signatures, we will have established a matrix-like data set consisting of at most 120 radiomic features (i.e. rows) for each of the sampled regions (i.e. columns). Furthermore, each sampled region will be annotated with information related to the growth patterns. Using this matrix, a radiomic signature will be developed using various algorithms (i.e. lasso and elastic-net regularized generalized linear models, kernel-based support vector machines and random forests) available through the classification and regression training (caret) package in R. Prior to the application of these algorithms, data will be pre-processed and the set of sampled regions will be split into a training and validation set. Given the expected number of patients included in the study (i.e. 40-50; We plan to include 30-40 patients with colorectal cancer liver metastases and about 10 patients with breast cancer liver metastases) and the expected regions per metastasis (i.e. 10-30), we will end up with at least 400 regions if we only analyze 1 metastasis per patient. To obtain a validation set of decent sample size, a training/test split ratio of 75:25 will be applied. We will end up with a set of parameters constituting models that allow discrimination of images of the two growth patterns.

In a second approach, data that are obtained as part of the radiomic analyses of animal models of liver metastasis with different growth patterns (sponsored by Kom op tegen Kanker: 'Identification of the histopathological growth patterns by computed tomography scanning and magnetic resonance imaging in colorectal cancer liver metastases in mice'), will be used to refine the models constructed with patient data. To that end, the analysis described above will be repeated using only the discriminatory features identified in the animal models.

9. ADMINISTRATIVE REQUIREMENTS

We will use the electronic patients file for source documentation whereafter we will use Excel to document. We will ensure the confidentiality of the data by using a password-protected file in Excel. In terms of security, we will have a copy of the password-protected file on the office computer, with the original in one of the Hospital's secure servers.

9.1. Ethics and regulatory approval

The trial will be conducted in compliance with the principles of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013), the principles of GCP and all of the applicable regulatory requirements.

A favorable opinion from the relevant IEC(s) will be obtained before the start of the study. The IEC will be notified about the EOS and a report summarizing the study results will be sent to the IEC within 1 year after the EOS. If the study is terminated early, the IEC will be notified within 15 days.

Favorable opinion is required for the study protocol, protocol amendments, ICFs, participant information sheets, and advertising materials if any.

9.2. Insurance/Indemnity

In accordance with the Belgian law relating to experiments on human persons dated May 7, 2004, the sponsor shall assume, even without fault, the responsibility of any damages incurred by a study patient and linked directly or indirectly to the participation to the study, and shall provide compensation therefore through its insurance.

9.3. Informed consent

For each study participant, informed consent will be obtained in writing before any protocol-related activities commence. As part of this procedure, the investigator or a designated representative must explain orally and in writing, by means of the ICF, the nature, duration, the purpose of the study, the number of visits, the assessments, procedures to undergo, and the action of the treatment in such a manner that the participant is aware of the potential risks, inconveniences, or adverse effects that may occur. Participants should be informed that they may withdraw from the study at any time without any resulting disadvantage and prejudice to their standard treatment care. They will receive all information that is required by national regulations and ICH guidelines.

The participant and the investigator will sign the ICF. A copy will be provided to the participant. The originally signed ICF will remain at the study center and filed in the participant's medical records.

All participants will be insured against injury caused by their participation in the study according to the legal requirements. They will be informed about the insurance and the resulting obligations on their part.

9.4. Publication policy

Publications will be coordinated by the study responsible physician. Authorship to publications will be determined in accordance with the requirements published by the International Committee of Medical Journal Editors and in accordance with the requirements of the respective medical journal.

10. REFERENCES

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