TITLE: 'Smart measurement of circulating tumor DNA: a tumor-agnostic computational tool to improve colorectal cancer care'

Short title	The SMART study
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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**General Assessment and Registration form (ABR form), the application form that is required for submission to the accredited Ethics Committee; in Dutch: Algemeen Beoordelings- en Registratieformulier (ABR-formulier)**
AE	**Adverse Event**
AR	**Adverse Reaction**
CA	**Competent Authority**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
cfDNA	**Cell-free DNA**
ctDNA	**Copy Number Variation**
CNV	**Circulating Tumor DNA**
CRC	**Colorectal Cancer**
CV	**Curriculum Vitae**
DNA	**Deoxyribonucleic Acid**
DSMB	**Data Safety Monitoring Board**
EC	**Endometrial Cancer**
EU	**European Union**
EudraCT	**European drug regulatory affairs Clinical Trials**
GCP	**Good Clinical Practice**
GDPR	**General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)**
IB	**Investigator's Brochure**
IC	**Informed Consent**
IMP	**Investigational Medicinal Product**
IMPD	**Investigational Medicinal Product Dossier**
LS	**Lynch Syndrome**
LUMC	**Leiden University Medial Centre**
METC	**Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)**
MMR	**Mismatch Repair**
MRD	**Minimal Residual Disease**
MSI	**Microsatellite Instability**
NKI	**Dutch Cancer Institute (in Dutch: Nederlands Kanker Instituut)**
PIF	**Patient Information Form**

(S)AE	**(Serious) Adverse Event**
SDR	**Source Data Review**
SDV	**Source Document Verification**
SNV	**Single Nucleotide Variant**
SPC	**Summary of Product Characteristics; in Dutch: officiële productinformatie IB1-tekst**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
StOET	**Dutch Foundation for Detecting Hereditary Tumors (in Dutch: Stichting Opsporing Erfelijke Tumoren)**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
sWGS	**Shallow Whole Genome Sequencing**
TMF	**Trial Master File**
UAVG	**Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG**
WMO	**Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale: Lynch Syndrome carriers have a predisposition to develop various types of cancer, especially colorectal cancer (CRC) and endometrial cancer (EC). LS patients are advised to undergo surveillance by colonoscopy every 2 year and gynaecological surveillance. This surveillance is deemed burdensome and fails to detect a small part of the developing CRCs and the majority of extra-colonic cancers. To ensure prevention and early detection of cancer, a reliable and accessible test is needed.
Recent studies have shown the potential of the detection of tumor-derived DNA fragments (circulating tumor DNA; ctDNA). Various molecular characteristics can be used to discriminate ctDNA from healthy circulating cell-free DNA. Current ctDNA assays with the highest sensitivity and specificity to detect for example minimal residual disease (MRD) after surgery are mostly tumor-informed, which means prior information is needed from the tumor tissue about the molecular alterations present. As this information is not available for the detection of newly arising tumors, the aim of this study is to evaluate the use of an optimized combination of tumor-agnostic ctDNA characteristics for the detection of newly developing tumors.
Objective: To evaluate the feasability of a new tumor-agnostic ctDNA assay, the ctDNA estimator, to detect newly developing tumors in Lynch carriers.
Study design: case-control study.
Study population: For validation of the ctDNA estimator, we will include: (1) LS carriers, whom at time of inclusion, have a newly diagnosed tumor and have not received any therapy yet. (2) Samples from the control population (LS carriers without a tumor diagnosis), that have already been collected according to the CATCA study (METC:2019-0119).
Intervention: LS carriers will be asked to participate at time of a new cancer diagnosis. After informed consent is given, blood (3 x 10 mL) will be obtained for ctDNA analysis to compare the ctDNA estimates to that in the control population.
Main study parameters/endpoints: The agreement between estimated ctDNA fractions and the presence of cancer.
Nature and extent of the burden and risks associated with participation, benefit and group relatedness: Blood will be drawn (3 x 10 mL) from LS carriers with a recently diagnosed cancer, which will be combined with a routine hospital visit when possible. The risk of blood collection by venepuncture is negligible.

1. INTRODUCTION AND RATIONALE

1.1 Lynch Syndrome

Over 15,000 individuals are diagnosed with colorectal cancer (CRC) in the Netherlands each year, of which 3-5% can be attributed to Lynch Syndrome (LS) [1]. LS is a genetic predisposition for various types of cancer next to early-onset CRC, including cancer of the endometrium, ovaries, small bowel, stomach, urothelium, biliary tract, pancreas, brain and skin [2]. It is caused by a germline mutation in one of the DNA mismatch repair (MMR) genes; *MLH1*, *MSH2*, *MSH6*, *PMS2,* or in the 3'end of the EpCAM gene [3-7]. Consequently, microsatellite instability (MSI-H) is a hallmark of LS associated tumors [8].
In the Netherlands in all newly diagnosed CRC and endometrial cancer under the age of 70 immunohistochemical staining of the MMR proteins is performed to evaluate the microsatellite status of the tumor and improve LS diagnosis [9]. Overall, about 10% of all CRC display an MSI-H phenotype, of which about a third is due to LS [10]. LS inherits in an autosomal dominant way. Once a germline mutations in *MSH2*, *MSH6*, *PMS2,* or in the 3'end of the EpCAM gene is detected in a patient, presymptomatic testing is available to at risk relatives and carriers are offered colorectal and/or gynecological surveillance to decrease their risk of cancer [11].

1.2 Preventing cancer through surveillance programs

LS carriers have a lifetime risk up to 70% to develop CRC, and female carriers additionally have a lifetime risk of up to 60% to develop EC [12]. Due to these high risks, LS carriers are advised to undergo biennial colonoscopy from 25-35 years of age – and females additional yearly gynecological surveillance between 40 and 60 years of age – to reduce this cancer risk. However, colonoscopies and endometrial screening are burdensome to the patient and fail to detect part of the newly developing tumors [13, 14]. Furthermore, the use of surveillance programs for other LS-associated tumors especially tumors of the ovaries, small bowel, stomach, urothelium, biliary tract, pancreas, brain and skin remain controversial, as no added value to the detection of carcinomas has been shown [15].

1.3 ctDNA analysis in cancer detection

Cell-free DNA (cfDNA) is shed into the circulation via apoptotic and necrotic cells, which means it can contain a fraction of circulating tumor DNA (ctDNA) in case a tumor is present in the body. CRC has a particularly high shedding rate of tumor material into the bloodstream [16]. This provides a promising future for minimally invasive assays for diagnosis, aiming at detection of tumor-derived circulating DNA fragments (ctDNA) in the blood [17]. Recent results show that the detection of minimal residual disease (MRD) - using ctDNA in stage II

CRC patients following surgical removal of the tumor – can be used to reduce the number of patients treated with adjuvant chemotherapy, without negatively impacting the clinical outcome [18]. However, current assays to detect MRD often use tumor-informed designs. This means that patient-specific assays are developed, based on available genetic information of the tumor tissue to maximize sensitivity and specificity for this assay. However, this assay design is complicated and expensive, as sequencing of tumor tissue is needed in combination with development of patient-specific assays that need to be tested. This is a time-consuming process and impedes implementation into the clinical setting. Additionally, tumor-informed assays cannot be used to detect de novo tumors, as no prior information about the molecular landscape is available in this setting.
Based on aforementioned information, there is an unmet need for a sensitive and specific assay to detect ctDNA in individuals, without the need for prior information from the tumor tissue (i.e. tumor-agnostic). The implementation of such an assay would simplify MRD detection after surgery and may improve current surveillance programs as well.

1.4 Cell-free DNA based surveillance for LS-associated cancers

To detect ctDNA in the total pool of cfDNA, numerous tests can be performed. This includes assessment of chromosomal copy number variations (CNVs), microsatellite instability (MSI), single nucleotide variants (SNVs), and DNA methylation assays. The presence of ctDNA is often determined by a combination of aforementioned assays and can be used in various settings [19]. Recent technological advances underline the promise of certain tumor-agnostic (i.e. without prior tissue-based information) ways to detect ctDNA. Schrag et al. have demonstrated the feasibility of tumor-agnostic assays, which can predict both presence of a tumor and its origin [20]. Circulating cfDNA methylation analyses for both early detection and classification of various cancer types have been proven useful [21, 22]. Two recent studies show this potential of cfDNA methylation profiling to detect CRC at an early stage [23, 24].
The recently developed field of ‘fragmentomics’ (i.e. the investigation of fragmentation patterns in cfDNA) shows promise for sensitive detection of cancer. Circulating DNA fragments of cancer patients have a differential size profile compared to cfDNA fragments in healthy individuals. This difference can be used in assays to increase the sensitivity of chromosomal copy number profiling in cfDNA [25]. Additionally, these differential cfDNA fragmentomic profiles can directly distinguish cancer patients from healthy individuals with high sensitivity and specificity [25, 26]. Lo et al. have shown that these fragmentomic features can be retrieved via various paired-end sequencing methods, including methylation sequencing [27]. Features from different molecular read-outs (e.g. methylation and copy number profiles) from both cfDNA methylation profiling and fragmentomics have a

complementary value [28, 29]. Combining cfDNA methylation profiling and fragmentomics will capture information of the circulating tumor genome on a broader spectrum, permitting a higher analytical sensitivity for the detection of minute traces of ctDNA.

Current literature indicates that cfDNA methylation profiling, fragment size-enriched chromosomal copy number profiling, and fragmentomics – or combinations of these – may have the potential to provide a tumor-agnostic assay for sensitive detection of newly developing tumors. Recently we showed that cfDNA methylation profiles could discriminate microsatellite instable advanced colorectal tumors from microsatellite stable tumors [30]. Here, we propose to evaluate the potential of a new ctDNA estimator to detect newly developing tumors and to improve current surveillance programs for LS carriers.

2. OBJECTIVES

Primary objective:

This project aims to deliver a tumor-agnostic ctDNA estimator and provide proof-of-concept of its value for early diagnosis of new colorectal tumors in high-risk individuals.

Secondary objective(s):

Obtain proof of principle that the ctDNA estimator can also play a role in the early detection of newly developing non-colorectal tumors in LS carriers.

3. STUDY DESIGN

This is a monocenter (with potential to be multi-center) case-control study in which we will test the ability of the constructed ctDNA estimator to detect newly developing tumors. We will do this by comparing cfDNA data extracted from already collected blood from LS carriers without a newly developed tumor (controls) to that from LS carriers with a newly developed tumor, whom have not had any form of therapy yet (cases).

3.1 Patient inclusion

Due to their high risk for CRC cancer, LS carriers are under regular surveillance. LS carriers with a newly diagnosed cancer will be asked for informed consent in the period between the diagnosis and treatment.

Also, patients suspected to have Lynch based on routinely performed immunohistochemical staining of the MMR proteins on their newly diagnosed tumor will be invited to participate in the study. MSI is a molecular hallmark of Lynch-associated tumors. About half of patients with a suspect LS tumor will have underlying LS.

3.2 Sample collection

If informed consent is given, blood (3 x 10 mL) will be collected for this study. Blood will be drawn via venipuncture. If possible, this will be combined with routine blood sampling prior to treatment. Blood will be collected in blood-stabilizing tubes and sent to the laboratory of Translational Cancer Genomics at the Medical Oncology Department. Plasma will be processed within 96 hours and stored at -80 degrees according to standard protocol. Blood from controls is readily available at the Erasmus MC from the previous CATCA study (METC:2019-0119).

Cell-free DNA will be isolated from 2-4 mL of plasma using the qiaAMP circulating cell-free DNA kit (Qiagen). The obtained cell-free DNA will be quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific). In addition, we will evaluate the ratio of cell-free DNA (cfDNA) to higher molecular weight genomic DNA using the ProNex DNA QC assay (Promega).

3.3 cfDNA methylation and sWGS analysis

cfDNA will be isolated out of plasma using the QiaAmp circulating nucleic acid kit (Qiagen) according to the manufacturer’s instructions. This will be done for the samples of both the 50 cases and 50 controls included in this study. Ten ng of this isolated cfDNA will be analyzed via MeD-seq, whereas another 5-10 ng cfDNA will be analysed via sWGS.

The required features for the cfDNA estimator will be extracted from the data, using various in-house or publicly available pipelines for MeD-seq and sWGS data.
For DNA methylation profiling we will use the MeD-seq assay [31] , which was shown to have important advantages over other genome-wide methylation assays, such as high CpG coverage (~50% of CpGs in the genome), low costs, no conversion of the DNA, and no dependency on DNA-binding antibodies. Therefore, this assay is ideally suited for the profiling of small amounts of DNA and we have recently shown that we can indeed use the MeD-seq assay to yield reliable cfDNA methylation profiles [32] .
For fragmentomics analyses, we will use 5-10 ng of cfDNA to directly generate sequencing libraries without fragmenting the cfDNA. Libraries will be quantified using the Tapestation (Agilent) and pooled equimolarly. The resulting pool will be sequenced on the NextSeq 500 system (Illumina) generating 150 bp paired end reads. In total we will aim to generate >10M reads per sample (>0.5x coverage). The resulting data will be analyzed with in house sequencing data pipelines to obtain a range of fragmentomic features. In short, adapters and indexes will be trimmed from the reads and the trimmed reads will be mapped to the GRCh38 human genome assembly using burrows-wheeler alignment [33]

Mapped data will be used to extract:
1) ctDNA-enriched chromosomal copy number profiles, which will be summarized into an tumourfraction estimate using IchorCNA [34]
2) genome-wide fragmentation patterns
3) cfDNA fragment size distributions
4) the fraction of fragments derived from open and closed chromatin
5) the diversity of the fragment ends, which will be summarized into a single metric by first calculating a normalized Shannon entropy and subsequently combining this with the proportions of 29 selected trinucleotide fragment-endings into a recently developed FrEIA score [35].

The independently developed multi-omic ctDNA estimator will be used to estimate the ctDNA fraction estimates in cases (i.e. LS carriers with a tumor) and controls (i.e. LS patients without a tumor). The sensitivity and specificity of the ctDNA estimator will be determined in these 50 cases and 50 controls.

3.4 Workflow

The workflow of this study is depicted in **figure 1 and 2**. In case a tumor is detected in a (suspect) LS carrier, consent to be contacted for further information on the cfDNA estimator

study by the researcher is asked by the collaborating physician and written information on the study is supplied to the patient. Informed consent is asked during a live or telephone consult by the researcher and blood drawing is organized in the interval between cancer diagnosis and therapy. As blood from 50 healthy LS carriers (controls) is readily available, we only need to collect blood samples of 50 LS carriers with a newly diagnosed tumor (cases). cfDNA will be isolated from both cases and controls, and the previously described assays will be performed. After data is obtained, the independently developed multimodal ctDNA estimator will be used and its sensitivity and specificity will be determined in our case-control setting.

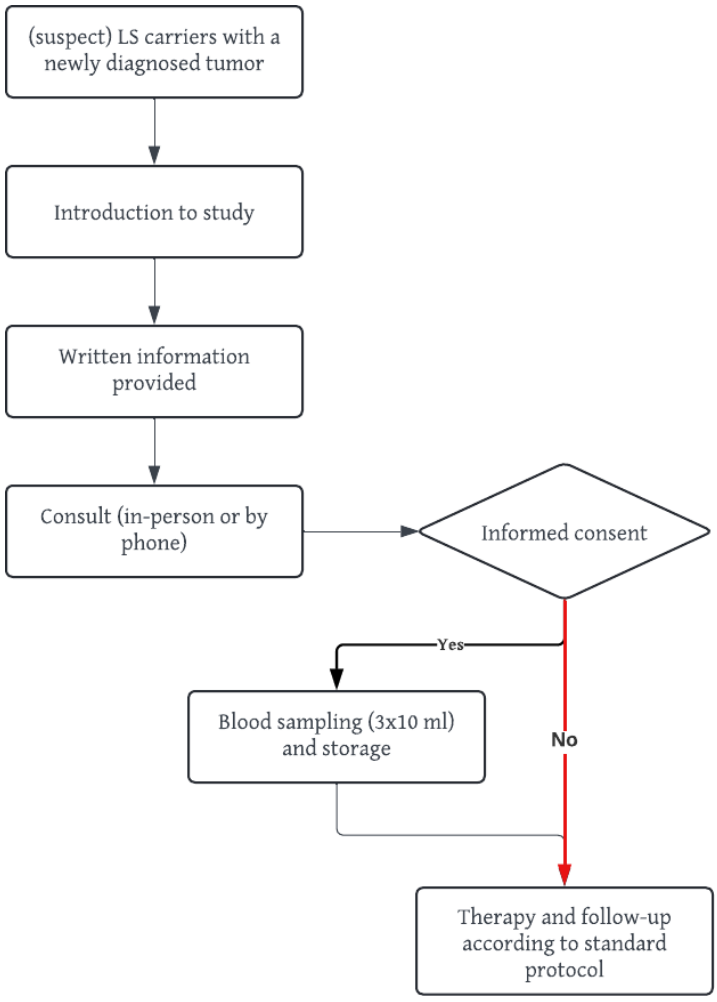

Figure 1. *Flowchart for patient inclusion and sample collection.*

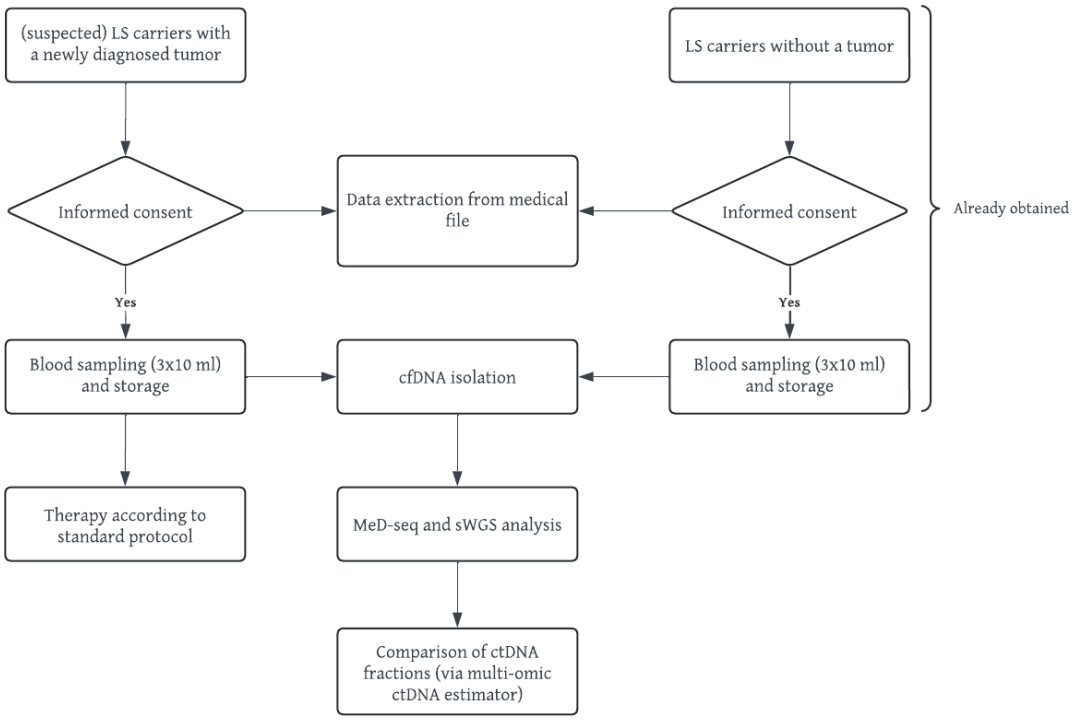

Figure 2. *Workflow for sample and data collection and analysis.*

3.5 Timetable

This project will be carried out in 4 years by a dedicated PhD student. Techniques and methods used for this study are already up and running. In the first year focus is on preparing the study (e.g. developing patient information folders, obtaining METC approvals, other administrative work), followed by the start of patient inclusion. We estimate we will reach our goal of 50 patient inclusions in the third year. Synchronously to patient inclusion, blood sampling will be carried out, along with the necessary pre-processing to plasma. We expect our data to be complete and ready for analysis by the end of the third year, after which the ctDNA estimator will be evaluated. The results will be presented at (inter)national meetings and will be published in peer-reviewed, open-access scientific journals.

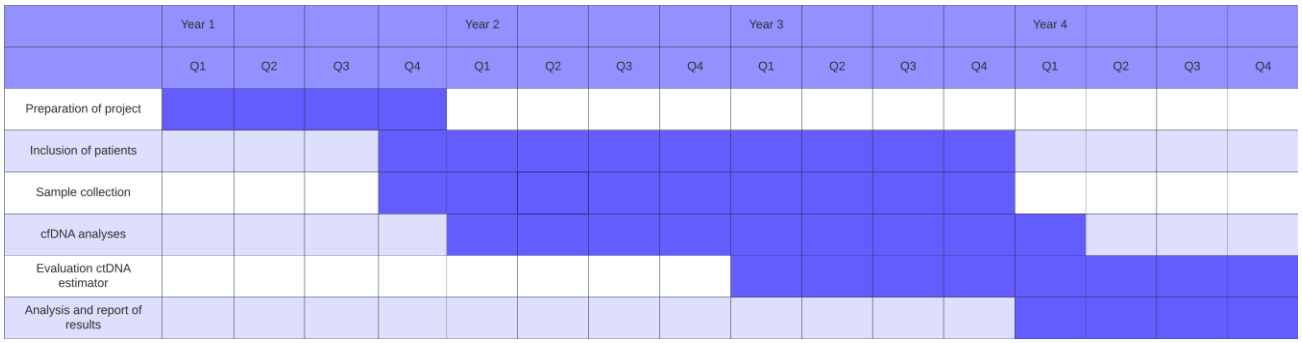

Figure 3. *Gantt chart depicting the timeline of this study.*

4. STUDY POPULATION

4.1 Population (base)

We will include individuals with molecularly proven Lynch Syndrome. In total, we need to analyze 50 cases (as specified in the power calculation below). Based on the StOET (the Dutch Foundation for detection of Hereditary Tumors) database, we expect that an average of 70 LS carriers under surveillance are diagnosed with an LS-associated tumor per year in the Netherlands. Apart from that, about half of newly diagnosed MSI-H CRC and EC cases suspect for LS will turn out to be LS-associated by following diagnostic testing. These are about 15 newly diagnosed LS carriers with a tumor at our center per year. Including also these patients will facilitate the inclusion of 50 cases within the inclusion time.
Next to our own regional LS-task force, participation of the clinical geneticists and gastroenterologists with expertise on LS in the LUMC, and if needed the other Dutch academic centers, will be asked to participate. To ensure sufficient recruitment, patient awareness will also be created via the Lynch Polyposis foundation.

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- (suspect) LS carriers who:
    - Have proven Lynch Syndrome (MMR gene or EpCAM mutation), or have a proven MSI-H tumor;
    - Are ≥ 18 years of age;
    - Have been diagnosed with any form of cancer at time of inclusion, but have had no treatment yet;
    - Have granted informed consent to participate in this study.

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Case (suspect) LS carriers who:
    - Are unwilling to undergo extra blood sampling;
    - Are <18 years;
    - Have no newly diagnosed tumors at time of inclusion;
    - Have been treated for their tumor at time of inclusion;
    - Not able to read or understand Dutch language or mentally not capable.

4.4 Sample size calculation

This study is a case control study in order to test the ctDNA estimator in a relatively small cohort of high-risk carriers. Since most surveillance colonoscopies performed every two years in LS carriers turn out negative and ctDNA estimator could be performed yearly we judge the ctDNA estimator to be successful if it detects at least 60% of all tumors at a maximal false-positive rate of 25% in healthy carriers. With a power of 80% and at an alpha of 0.05, we need to include at least 36 carriers with a tumor and 36 healthy carriers, according to the Chi-squared statistic with continuity correction to compare proportions with a dichotomous outcome between two samples. To ensure sufficient individuals with good quality data and adequate follow-up, we therefore propose to include 50 carriers with and 50 carriers without a tumor. In order to obtain sufficient LS carriers with a newly diagnosed tumor we expect to include an additional 30-50 suspect LS carriers, who will not be analysed for this study, since the diagnosis LS is not confirmed.

5. TREATMENT OF SUBJECTS

Not applicable.

6. INVESTIGATIONAL PRODUCT

Not applicable.

7. NON-INVESTIGATIONAL PRODUCT

Not applicable.

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

The main study endpoint is to evaluate the ability of an independently developed ctDNA estimator to detect newly developing tumors in LS carriers. Sensitivity and specificity of this new detection method will be established in a case-control set-up where we aim to detect at least 60% of all tumors at a maximal false-positive rate of 25% in healthy carriers.

8.1.2 Other study parameters

These data are needed as background information to interpret results of the molecular analyses:

- Demographics:
    - Age;
    - Gender;
    - Genetic predisposition (i.e. which MMR gene mutation);
- Comorbidities;
- Detected malignancies and their characteristics, such as: size, differentiation, grade, and stage.

8.2 Randomisation, blinding and treatment allocation

Not applicable.

8.3 Study procedures

An overview of assessments for (suspect) LS carriers in this study is depicted in **figures 1 and 2**. After informed consent is obtained, baseline characteristics and information on comorbidity will be collected from the patient's medical file.

8.3.1 Inclusion of participants

LS carriers under surveillance and suspect LS patients based on their tumor profile will be informed about this study at moment of cancer diagnosis by their physician and given an informative letter. When the patient gives permission to be contacted, the researcher will contact the patient for further information within one week. Informed consent will be asked for participation in this study and for blood sampling. Blood sampling will be scheduled,

preferably in combination with routine sampling for the patient's cancer treatment, giving the patient enough time to (re)consider participation. In case of suspect LS, the department of Clinical Genetics will communicate the subsequent DNA results from genetic testing. Based on these results, patients with proven LS will be analyzed.

8.3.2 Blood collection

As per protocol, blood will be sampled before start of treatment, regardless of tumor type. After consent is given for collection and storage of blood for further molecular analysis, extra blood sampling (3 x 10 mL) will be ordered preferably at their routine blood-sampling appointment before therapy. This is to minimize extra burden of an additional hospital visit for this blood sampling. Blood will be collected in cell stabilizing blood collection tubes and send to in the laboratory of Translational Cancer Genomics at the Medical Oncology department.

8.3.3 ctDNA analysis

Molecular analyses in cfDNA/ctDNA obtained via blood will be performed according to already established protocols within the Erasmus MC. These analyses do not interfere with routine diagnostics. If participants give consent, unused plasma will be stored for 15 years for possible related and/or subsequent studies.

8.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

8.4.1 Specific criteria for withdrawal (if applicable)

Not applicable.

8.5 Replacement of individual subjects after withdrawal

Not applicable.

8.6 Follow-up of subjects withdrawn from treatment

Not applicable.

8.7 Premature termination of the study

Premature termination of the study is not likely.

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to trial procedure. Adverse events will not be recorded, because the risk of adverse events after blood withdrawal by venepuncture is negligible.

9.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

Serious adverse events will not be recorded, because the risk of serious adverse events after blood withdrawal by venepuncture is negligible.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Not applicable.

9.3 Annual safety report

Not applicable.

9.4 Follow-up of adverse events

Adverse events will be handled as usual practice, according to standard of care.

9.5 Data Safety Monitoring Board (DSMB) / Safety Committee

Not applicable.

10. STATISTICAL ANALYSIS

10.1 Primary study parameter(s)

The primary objective of this study is to provide proof-of-principle that the blood-based ctDNA estimator is able to detect newly developing tumors in LS carriers. The primary study parameter is therefore the distinction between ctDNA estimates between LS carriers with and without cancer, according to the ctDNA estimator. This is determined by analyzing total cfDNA of both cases and controls, as described in chapter 3. Per sample a ctDNA estimate will be calculated (value between 0-1). This resulting value will be compared between the cases and controls with a Mann-Whitney U test. These calculations will be done by using R (version 4.4.1).

As this is a proof-of-principle study, the focus of analysis will be on correctly detecting ctDNA in cases and controls. An ROC-curve will be constructed on the basis of the test-performance and evaluated for possible clinical applications. Further optimization of the test regarding sensitivity and specificity will need to be done in a bigger prospective cohort study. Also, the optimization of sensitivity and/or specificity depends on how this test could be implemented. Likely, the test will be an addition to the normal colonoscopy surveillance, thus higher specificity would be preferred.

10.2 Secondary study parameter(s)

As a secondary objective we focus on expanding the ctDNA estimator beyond colorectal cancer diagnostics. ctDNA analyses will incorporate several parameters to distinguish tissue-of-origin from cfDNA/ctDNA fragments. These analyses will include, for example, differential-methylated regions (DMRs), which are tissue/cell-specific.

10.3 Other study parameters

Baseline patient characteristics are needed to interpret the results of the molecular analyses. These characteristics include:

- Demographics:
    - Age;
    - Gender;
    - Genetic predisposition (i.e. which MMR gene mutation);
- Comorbidities;
- Detected malignancies and their characteristics, such as: size, differentiation, grade, and stage.

Demographics are needed to match the cases to controls, to ensure baseline characteristics are evenly distributed. Information on (possible) comorbidities are needed to interpret cfDNA analyses, as there may be DNA abnormalities that are not related to the tumor. Tumor characteristics are also needed to interpret cfDNA analyses and relate the outcomes of the analyses to the tumor status. If this tumor-data is not present within the Erasmus MC, data and material will be collected via PALGA.

10.4 Interim analysis (if applicable)

Not applicable.

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

This study will be conducted according to the principles of the Declaration of Helsinki (version of 2024) and in accordance with the Medical Research Involving Human Subjects Act (WMO).

11.2 Recruitment and consent

Eligible patients, being (suspect) LS carriers, will be informed by their physician about this study at moment of cancer diagnosis, given an informative letter and asked permission to be contacted by the researcher for further information. In a live or telephone contact, the researcher asks informed consent for participation in this study. Upon consent extra blood sampling will be organized. Patients will follow routine diagnostic work-up and treatment according to their cancer diagnosis, thus time between cancer diagnosis and subsequent blood sampling may vary. Regardless of the duration of this period, participants are free to (re)consider their participation during this time. Furthermore, patients can approach involved researchers/clinicians or the independent expert to ask questions any time prior or during participation. Patients have the right to withdraw their consent at any time during the study.

11.3 Objection by minors or incapacitated subjects (if applicable)

Not applicable

11.4 Benefits and risks assessment, group relatedness

As surveillance programs for LS are considered burdensome and adenomas and CRC are sometimes missed [36], we aim to develop a more sensitive and less invasive tool for the detection of newly developing tumors in high-risk individuals.
We believe there are no relevant risks associated with participation. It is necessary for evaluation of this ctDNA estimator that blood is collected for further molecular work-up and analysis. Blood will be collected (3x 10ml) via routine venepuncture, which we do not consider as a large burden to patients, especially since we aim to combine it with already scheduled blood drawing needed for their treatment. As cancer diagnosis precedes participation, the cfDNA analysis will be based on information from the tumor. Thus, there is no extra burden of unforeseen health problems or unprecedented findings.

11.5 Compensation for injury

We believe participation in this study is without any risks.

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO.

11.6 Incentives

Not applicable.

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

Participant data will be stored in a password-protected database according to the General Data Protection Regulation (in Dutch: '*Algemene Verordening Gegevensbescherming*', AVG). All patients will be asked informed consent for processing and usage of personal data (additional to asking informed consent to participate in the study). This database will be secured with a password and safeguarded by the investigator. Individuals are given a study identification number and personal information will be stored separately from the study data. This study will be reported to the Privacy Knowledge Office of the Erasmus MC, to be included in the Register of Processing Operations (in Dutch: *verwerkingsregister*). Patients will be informed about the handling of their personal records; additionally, they are provided with the contact details of the Data Protection Officer of the Erasmus MC (dhr. P. Van Hoogdalem), the controller (in Dutch: *verwerkingsverantwoordelijke,* dr. A. Wagner) and where to find additional information regarding the rights of the data subjects (Privacy Knowledge Office, Erasmus MC). Data and human material used in this study will be stored for 15 years for possible related and/or subsequent studies. Human material that is not used by the end of the study will be destroyed, unless patients grant informed consent for storage and use in future studies.

12.1 Monitoring and Quality Assurance

Monitoring of the study will be done via the Research Agency Sophia (Onderzoeksbureau Sophia). This will be done yearly, as in compliance with the WMO guidelines. The following will be monitored:

- Patient Information Forms (PIFs)
- Informed Consent Procedure
- Compliance with in- and exclusion criteria
- Qualifications PI and team
- Safety
- Compliance with research procedures
- Collection and storage of samples
- Site File (TMF)
- Source Data Review (SDR)
- Source Document Verification (SDV)
- SAEs

12.2 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion.

12.3 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.4 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit.
The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

12.5 Public disclosure and publication policy

When the study has ended, the principal investigator will be responsible for the public disclosure and publication of study outcome(s). Results will be presented on (inter)national professional meetings/symposia and submitted to journals for publication.

13. STRUCTURED RISK ANALYSIS

Not applicable.

14. REFERENCES

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