Perioperative metformin treatment for colon cancer, a randomized trial

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Center for Surgical Science

Improving Patient Care

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The trial will be conducted in accordance with the current protocol, ICH-GCP guidelines and the current legislative and administrative demands. Ordinary procedures regarding quality control will be followed, i.e. ICH-GCP guidelines and the investigator will allow direct access to source data and/or patients charts at monitoring point, audits and inspections by the Danish pharmacological agency, the GCP- unit, and all other relevant authorities

BACKGROUND

Colorectal cancer (CRC) is the third most common malignancy worldwide, and more than 5000 patients are diagnosed with CRC annually in Denmark[1]. Surgical removal is the only curative treatment of CRC, but still 30-40% of patients with potentially curable CRC relapses[2].

Emerging evidence suggests that metformin decreases the risk of developing CRC in patients with diabetes. Epidemiologic studies have examined the potential survival benefits in patients with diabetes receiving metformin and shown that metformin users had a decreased all-cause mortality[3–6].

A key factor in tissue homeostasis, especially in the intestinal mucosa, is the balance that exists between the level of cell death and cell proliferation. Disturbance of the balance contributes to the initiation and maintenance of tumor growth and development. Two important hallmarks are deregulation of the proliferative signaling pathway and deregulating of the pathway of apoptosis. Both results in either non- or malfunction of important enzymes or unrestricted release of growth-promoting signals that under normal circumstances are necessary to maintain tissue homeostasis. The proliferation activity and apoptosis of a tumor can be estimated by determining the expression levels of specific cell cycle related proteins such as Ki67 (proliferation) and cleaved caspase-3 (apoptosis) using immunohistochemistry.

Characteristics of the cancer cell as well as the TNM classification of the cancer can to some extend predict the risk of recurrence and death. Growing evidence suggests that the microenvironment of the tumor and the patient's immune response play an important role as well[7]. Presence of cytotoxic T cells, memory T cells and TH1 cells are associated with prolonged survival. The immunoscore which is a qualitative and quantitative measure for the immunogenicity of the cancer has been introduced as a prognostic marker for CRC. The immunoscore is measured by immunohistochemistry. Samples from the core of the tumor and from the invasive margin are stained for CD3 and CD8 positive lymphocytes. A high density is associated with better outcome than a low density[7,8].

mTOR pathway

The serin/threonine kinase mammalian target of rapamycin (mTOR) pathway affects most major cellular functions, giving it a central role in regulating basic cell behaviors such as growth and proliferation. mTOR belongs to the phosphoinositide 3-kinase (PI3K)-related family and interacts with several proteins to form two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 plays a pivotal role in regulating protein synthesis by signaling through its main effectors p70 ribosomal S6 kinase (S6K1) and

eIF4E binding protein 1 (4E-BP1). S6K1 is a major effector of cell growth. Furthermore, mTORC1 promotes lipid synthesis and energy metabolism and suppresses autophagy. mTORC2 phosphorylates protein kinase B (Akt/PKB), serum- and glucocorticoid induced protein kinase 1 (SGK1), and protein kinase C-α (PKCα) regulating cell survival, metabolism and cytoskeletal organization[9–11].

mTORC1 is regulated upstream by the tuberous sclerosis complex 1-2 (TSC1-TSC2). The TSC1/2 complex also responds to diverse stress signals. Upon hypoxia or low ATP state, adenosine monophosphate-activated protein kinase (AMPK) phosphorylates TSC2 and thereby promote mTOR signaling activation.

mTORC1 pathway integrates inputs from growth factors, stress, energy status, oxygen and amino acids. Nutrient signals act mostly through insulin or insulin-like growth factor (IGF) signaling pathways. The PI3K pathway is activated by growth factor receptors (i.e. insulin-like growth factor 1 receptor, IGF-1R) and cell adhesion molecules and promotes cell survival, proliferation and cell growth[9–11].

Metformin

Metformin is the drug of choice for treatment of type 2 DM. Metformin is generally well tolerated with few side effects. Metformin lowers plasma glucose in diabetic patients through a suppression of hepatic gluconeogenesis and also increases glucose uptake by muscle cells[12]. Traditionally the liver has been considered the primary target organ for metformin but studies suggest that metformin exerts part of its effect through direct influence on the intestine[13–15]. Among other effects in the intestine metformin has been seen to change the composition of the gut microbiota[16,17]. Several studies indicate that metformin may decrease the risk of developing some cancers including CRC[3,18–20]. Both in vitro and in vivo studies propose that metformin inhibits cancer cell growth and reduce cancer risk through both direct (insulin independent) and indirect (insulin dependent) mechanisms[3,21,22].

The anticancer molecular action of metformin is mainly associated with the inhibition of mTORC1 by activating AMPK. AMPK phosphorylates TSC2 that inhibits mTORC1 leading to a decrease in protein synthesis and cell growth. Metformin reduces the insulin level, inhibits insulin/IGF signaling pathways among these the IGF-1/PI3K/Akt pathway and is thereby able to inhibit the mTOR pathway without the presence of AMPK[21,22].

Metformin may also inhibit cancer growth through an immune mediated effect. In an animal study metformin has been seen to increase the number of tumor infiltrating lymphocytes in tumors particularly CD8-positive lymphocytes[23]. Treatment with metformin resulted in growth inhibition or complete rejection

of tumor and mice who were afterwards exposed to the same type of cancer cells did not develop a tumor suggesting development of an immunological memory response[23].

The composition of gut microbiota influences the development of colorectal cancer and some studies have focused on whether changing the microbiota can decrease the risk of colorectal cancer and better the prognosis[24–26]. To our knowledge, no research has been made examining the relation between metformin induced changes in microbiota and colorectal cancer.

Cancer cell lines

Cancer cell lines are used to investigate the growth and behavior of cancer cells in vitro when influenced by different stimuli. Several studies have shown that treatment of colorectal cancer cell lines with metformin in combination with different kinds of chemotherapy is more effective in inhibiting proliferation and increasing apoptosis than treatment with chemotherapy alone[27–29]. In most studies the cells are grown in a medium containing bovine serum and metformin is added directly to the assay. In this study we plan to grow the cells in medium containing human serum from our patients in order to subject the cancer cells to a concentration of metformin that is seen in patients treated with metformin.

Surgery is known to induce a surgical stress response with hormonal and metabolic changes. Directly after surgery pro-inflammatory cytokines are released and subsequently this changes to release of anti-inflammatory cytokines. Simultaneously the hypothalamic-pituitary-adrenal axis is activated leading to elevation of cortisol, glucagon, growth hormone and catecholamines[30,31]. The stress response leads to an increased insulin resistance and hyperglycemia postoperatively. The degree of insulin resistance and hyperglycemia is correlated with risk of postoperative complications, reoperation, length of stay and death[32–35].

DESIGN

Objective

The aim of the study is to investigate the effect of treatment with metformin on cell proliferation and on metabolic and immunological changes in non-diabetic patients with colon cancer.

Trial Design

The trial is a randomised, placebo-controlled, double-blinded trial investigating the effect of metformin (intervention group) against placebo (control group) on cell proliferation, metabolic and immunological changes in patients with colon cancer.

Sample size

To our knowledge no trials examining the effect of metformin on changes in expression of Ki67 and cleaved caspase 3 in colon cancer exist. Studies on endometrial cancer patients treated with metformin have shown varying results. One study found a decrease in Ki67 expression of 11,75% in 20 patients[36], another a decrease of 44 % in 31 patients[37] and a third found a decrease of 17,2% in 28 patients[38]. We have chosen a sample size of 24 patients in each group based on these findings. The patients will be randomized in blocks of six and therefore the study will proceed until 24 patients have been enrolled in each arm. The study of the effect of metformin on postoperative hyperglycemia and insulin resistance is exploratory.

TRIAL STRUCTURE AND RESPONSIBILITIES

The principle investigator (EC) is responsible for the preparation of the protocol, CRF, coordinating meetings of the steering committee, and implementing decisions into daily operations of the trial. Furthermore, the principle investigator will along with a team of co-investigators take care of screening, recruitment, data collection, and completion of individual CRF's at the trial location. The steering committee consists of four members (EC, TF, LCT and IG) and its purpose is to overview the progress and safety of the trial. Meetings will be held every 3 months as standard and in case of Severe Adverse Events (SAE) or other serious incidents an extra meeting will be held accordingly. The study will be monitored by the local GCP unit.

EFFECT PARAMETERS

The primary outcome is determination of the difference of the level of proliferation after the intervention (time of surgery) adjusted for the level seen at baseline (time of colonoscopy).
A secondary outcome is the difference in the level of apoptosis after the intervention adjusted for levels seen at baseline. Subgroup analysis in regards to the most common mutations will be performed.
The level of proliferation will be defined as the percentage of tumor nuclei showing Ki67 staining in a specific microscopic field, whereas the level of apoptosis is defined as the absolute number of tumor cells expressing cytoplasmic and perinuclear staining of cleaved caspase-3 at the transition zone[39].

Also the effect of metformin will be measured by examining the difference in proliferation, migration and adhesion of colon cancer cell lines treated with plasma from the patients. Blood samples stored in a biobank will be analyzed for metabolic and immunological changes.

The effect of metformin on enterocytes and hepatocytes will be analyzed on biopsies taken at time of surgery. The effect of metformin on relevant pathways in the tissue specimens will be evaluated using mRNA sequencing, immunohistochemistry and peptide extraction.

The effect of metformin on the composition of the gut microbiome will be analyzed on fecal samples collected before initiation of study medication and before surgery. The composition of the microbiome in blood will be analyzed before and after surgery.

Finally the effect of metformin on postoperative hyperglycemia and insulin resistance will be measured by analyzing the difference in insulin resistance and plasma glucose levels before surgery and on postoperative day 1, 2 and 10.

We hypothesize that treatment with metformin, reduces the post-operative hyperglycemia and insulin resistance and in this way, enhances recovery. Patient perceived quality of recovery should be in focus when implementing new treatments and therefore, we include the widely used and validated "Quality of Recovery" questionnaire in the short 15-item form as an outcome in our study. A 40-item quality of recovery score (the QoR-40 score) was developed by Myles and colleagues in 2000 and has since been widely used and extensively validated[40,41]. The short 15-item score (the QoR-15 score) was derived from the QoR-40 score and has been fully validated and translated to Danish and has shown to perform as well as the QoR-40 score[42,43]. The QoR-15 questionnaire results in a score of 0–150 with a high score indicating a good quality of recovery.

Postoperative complications within 30 days from surgery will be assessed and classified according to the Clavien-Dindo classification[44].

STUDY PARTICIPANTS

A total of 48 patients, 24 in each arm, is needed.

Inclusion criteria:

- Patients with adenocarcinoma of the colon planned for elective curative intended surgery at Slagelse hospital
- Age of 18 or above

- Must be able to understand and sign informed content

- Sufficient amount of representative tumor material from the biopsies taken at the initial colonoscopy must be present

Exclusion criteria:

- Patients diagnosed with diabetes mellitus

- Patients who are receiving or have received metformin or other oral antidiabetics

- Impaired kidney function (eGFR < 60mL/min)

- Severe liver disease (defined as transaminases above X 3 normal levels)

- Participation in another pharmacological intervention trial

- Predictable poor compliance (for instance not speaking fluent Danish, mentally impaired)

- Presenting with metastatic disease

- Patients undergoing neoadjuvant chemotherapy

- Pregnancy or lactation (fertile women must have a negative serum or urine pregnancy test to participate)

- Fertile women who do not use safe contraception during the study period.
  Following contraceptive methods are acceptable when used consistently and in accordance with both the product label and the instructions of the physician are:

  - Oral contraceptive, either combined or progestogen alone

  - Injectable progestogen

  - Implants of levonorgestre

  - Estrogenic vaginal ring

  - Percutaneous contraceptive patches

  - Intrauterine device or intrauterine system with a documented failure rate < 1% per year

  - Male partner sterilization (vasectomy with documented azoospermia) prior to female patient´s entry into the study, and this male is the sole partner for that patient

- Double barrier method: condom with spermicidal agent (foam/gel/film/cream/suppository), condom and occlusive cap (diaphragm or cervical/vault cap) with vaginal spermicidal agent (foam/gel/film/cream/suppository)

- Allergy to metformin or placebo

COURSE OF THE STUDY

Colon cancer is in virtually all elective cases diagnosed through colonoscopy. When colon cancer is suspected, biopsies are taken to confirm pathology indicating adenocarcinoma. After the colonoscopy and when adenocarcinoma is confirmed the patient visits the outpatient clinic for preoperative examination and treatment planning. The study participants for the study will at this time be included in the study. Blood samples will be taken before the patients receive metformin or placebo, before surgery and on postoperative day 1, 2 and 10. Height, weight and BMI are measured at time of preoperative examination and at time of surgery. 30 days postoperatively the patients will receive a phone call asking them about possible complications and the patients' journals will be assessed for complications as well.

Patients will be asked to answer a baseline questionnaire about their well-being after randomization and the QoR-15 questionnaire on postoperative day 1, 2, 10 and 30.

The biopsies taken at the colonoscopy and biopsies from the specimen removed at surgery will be analyzed for Ki67 and cleaved caspase-3 expression through immunohistochemistry (please see below in the "methods" section).

The study participants are randomized to metformin or placebo at the time of preoperative examination. Eligible patients will receive metformin or placebo 500 mg once a day for two days, followed by 500 mg twice a day for two days and then 500 mg three times a day until 10 days after the operation (visit at the out-patient clinic). The treatment is discontinued at the day of surgery and recommenced afterwards with the first meal. The patients will after surgery receive 500mg three times a day again. In summary patients will receive metformin or placebo for 20 days before the operation and again 10 days after the operation.
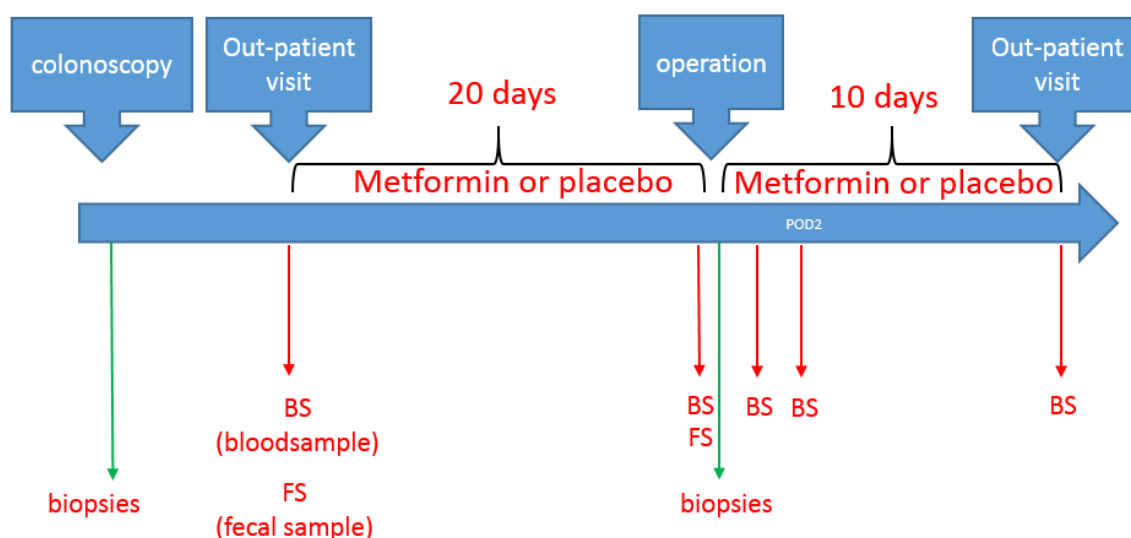

Figure 1: Study course

If a participant withdraws the consent then the participant will leave the study. In case of severe medical emergency (e.g. stroke or severe sepsis) which results in emergency surgery, stay in an intensive care unit, or hospitalization before the planned operation, the patient is excluded from the study. No further data will be collected from these patients after leaving; however, previous collected data will be part of the final data analysis in an intention to treat analysis. If a patient needs a CT scan with intravenous contrast in the treatment period the medication will be stopped.

Each year around 170 patients with colon cancer are undergoing surgery at Slagelse Hospital. In total 48 patients are needed for the study and we expect an inclusion rate of 50%. Approximately 15% are diagnosed with diabetes and are not eligible for the study. The inclusion of patients is therefore expected to last for 8 months. Subsequently, there will be a 4 month time period for data analysis and manuscript preparations. The study will be initiated in the fall of 2017.

RANDOMIZATION, ALLOCATION, CONCEALMENT AND BLINDING

The randomization process will be handled by the regional pharmacy in The Capitol region of Denmark. Block-randomization, in blocks of six patients, will be used. In each block half the patients will receive the intervention drug (metformin 500 mg), and in the other half placebo. The randomization-list will be made before the study starts by using of dedicated online software (http://www.randomization.com/) by the regional pharmacy. Allocation consists of two sets of code-envelopes (opaque, sealed envelope for each patient containing the randomization code for each patient) that will be produced by and sent from the regional pharmacy of the Capitol Region of Denmark, to Slagelse Hospital and will be stored; one in the patients CRF, and the other in the TMF. In this way the blinding will be conserved for both the sponsor/investigator, who informs the patient, the pathologist and the investigator that works with the cancer cell lines. Un-blinding (code-breaking) will be done if an uncertain and unexplainable complication occurs during the treatment period. The code-break for the individual patient should only be performed in case that the complication with high likelihood can be attributed to side-effects to the study drug and knowing allocation is essential to patient safety. The code breaking can be performed by the investigator via access to the code-envelope in the patients CRF. Furthermore, code breaking can be performed by the sponsor (code envelope in TMF). The principal investigator will be available on phone every day and night.

The dataanalysis will be performed before un-blinding of the investigators and a meeting with interpretation of the results will also be held before un-blinding.

METHODS

The patients will initially be diagnosed by colonoscopy, where biopsies will be taken for analysis of tumor cell proliferation and apoptosis. The same analysis will be repeated at the time of surgery on samples from the removed tumor as well as analysis for the most common mutations. The expression of cell proliferation and apoptosis will be compared to results from the preoperative biopsy analysis to examine the effect of a maximum of 20 days of preoperative metformin treatment in patients without diabetes. Six biopsies will be taken at time of colonoscopy and from the removed specimen. The biopsies are taken randomly from the tumor surface and are afterwards fixated according to standard guidelines. Samples from the non-tumor-bearing part of the intestine are taken from the oral and anal end of the removed specimen. Perioperatively, two small biopsies are taken from the surface of the liver (2x2x2mm).Tissue specimens will be distributed into relevant tissue media before storage in the research biobank. The effect of metformin on the enterocytes will be compared to the effects of metformin on hepatocytes.

The immunoscore is measured by immunohistochemistry. From the tumor at time of operation biopsies from the core and from the invasive margin will be taken. These biopsies are stained for CD3 and CD8 positive lymphocytes and the density of these are measured. The density of CD3 and CD8 will also be measured from the biopsies taken at colonoscopy. Part of this analysis will be performed by a laboratory in France under supervision of Jerome Galon who has developed the immunoscore.

The proliferation activity of a tumor can be estimated by immunohistochemical determination of specific cell cycle related proteins. To explore whether treatment with metformin changes the expression of markers for cell proliferation and apoptosis in CRC patients, immunohistochemical staining for Ki67 (proliferation) and cleaved caspase-3 (apoptosis) will be performed. Ki67, a widely used marker for proliferation[45], is present in the nuclei during all active phases of the cell cycle, but is absent during the resting G0 phase. The level of apoptosis can be evaluated by staining of the activated, cleaved form of the pro-apoptotic enzyme caspase-3 in the tumor cell cytoplasm. Caspase-3 is the final enzyme to become activated in the caspase cascade and the level of activated or cleaved caspase-3 is a reliable measure of the level of apoptosis. [45–47]

Immunohistochemical staining and analysis of Ki67 and cleaved caspase-3 tumor cell expression will be performed blinded and in collaboration with experienced pathologists. The immunohistochemical analyses

are performed on formalin-fixed paraffin-embedded tumor tissue. The level of proliferation will be defined as the percentage of tumor nuclei showing Ki67 staining in a specific microscopic field counted at the invasive front, and the level of apoptosis is defined as the absolute number of tumor cells expressing cytoplasmic and perinuclear staining of cleaved caspase-3 at the transition zone[39].

Height, weight and BMI are measured at time of preoperative examination and at time of surgery.

Blood samples from the patient before starting the medication, before surgery and at postoperative day 1, 2 and 10 are taken. Plasma from the patients are added to cultures with cancer cell lines in order to examine the effect on proliferation, migration and adhesion. This will be done in collaboration with researchers at Roskilde University at the department of science and environment, section of eukaryotic cell biology .

Blood samples will be analysed for Hgb, leucocytes and differential counts, CRP, thrombocytes, albumin, creatinine, eGFR, transaminases, glucose, Hba1c, insulin, c-peptide. These are needed to have baseline information about the patients and to be able to adjust for these variables in the multivariate analyses. If the HbA1c is 48mmol/mol or above the patient will be considered to be diabetic and may not be included. In this case the patient will be referred to his or her general practitioner.

Insulin resistance before and after surgery will be measured using the homeostatic assessment model (HOMA) from fasting levels of glucose and insulin. Capillary glucose level will be measured 4 times a day postoperatively on postoperative day 1 and 2 – before the main meals and before sleeping.

Composition of the microbiome in fecal and blood samples will be analysed using 16S rRNA sequencing.

A biobank will be created to store the tumor samples, gut samples, fecal samples, liver biopsies and additional blood samples for later analyses. The purpose of this is to ensure uniform analyses. The bio-bank will be created as soon as the approval from the Danish Data Protection Agency has been achieved, and all requirements concerning creation and maintenance of a bio-bank will be kept.

All samples in the biobank, blood and tumor will be destroyed after 10 years, and participants can have them destroyed earlier, should they wish so.

INTERVENTION

The study medicine will be produced by Teva with each pill containing 500 mg metformin in a quick release preparation. The metformin tablets will be over-encapsulated in a gelatine capsule in order to make identitical placebo. This will be performed by the regional pharmacy of The Capitol Region of Denmark who will also produce the placebo. The placebo will be comparable to study medication except for the active substance and will also consist of a over-encapsulated tablet. The content and chemical composition of metformin and the placebo can be seen in the individual product fact sheets. Pills will be packaged in white Duma cans with a total of 100 pills for each patient. Attached to the Duma can is a, for the purpose designed label, containing the relevant study medication information as well as the identification number for the patient. Metformin has a half-life of approximately 3 hours[48]. When a participant for any reason is terminated in the study the patient will be monitored for side-effects for 24 hours.

The patients' regular medication will not be affected by the current study and patients should take their regular medication as usual.

As mentioned earlier the regional pharmacy will handle the whole randomization process, and handling of medicine before the trial. An independent person from the regional pharmacy (mentioned in the Trial Master File) on the basis of the randomization-list will apply the randomization-code and patient number on the Duma cans. Likewise, batch numbers for the metformin and placebo will be noted on a separate piece of paper correlated to the patient's number. This will all be performed in accordance to "Good Manufacturing Practice" by relevantly trained staff. The randomization-list and the list containing the batch-numbers will be stored by this independent person at a locked box at the regional pharmacy of The Capitol Region of Denmark. The sponsor/investigator will receive two set of code-envelopes from the central pharmacy. Sponsor/investigator does not have immediate access to the medicine or the randomization list, but in case of serious adverse events (SAE) access can be made. In case of urgent need of code breaking this can be performed using the code-envelopes as previously described.

The patients are randomized to metformin or placebo at the time of preoperative examination. Eligible patients will receive metformin or placebo 500 mg once a day for two days, followed by 500 mg twice a day for two days and 500 mg three times a day for the rest of the period except the day of surgery where the medication is paused.
To establish the medicine compliance and the cumulative dosage, pill counts of study medication will be performed at the time of operation and at the visit in the out-patient clinic after surgery. The patient will be asked to bring study medication (empty, open and unopened containers) and missing pill number will be noted in the patient case report form (CRF). The aim of compliance is 75 %. Data on all patients will be

analysed according to the intention to treat principle. Per protocol analyses will be performed on data from participants with a compliance of 75 % or higher.

RISKS, SIDE-EFFECTS AND INCONVENIENCE

Metformin is a safe drug, and the side effects are primarily dose-dependent and limited to mild gastrointestinal symptoms like loss of appetite, diarrhea, vomiting, nausea and mild abdominal pain. Any adverse events will be monitored by weekly contact and graded using Common Terminology Criteria for Adverse Events version 4.0[49]. The participants will be monitored for adverse events from when they start the medication until postoperative day 10 when they have finished the medication. To minimize the side-effects, the dose of metformin is slowly increased, and metformin will be withheld in participants who experience intolerable side-effects until these subsides. Afterwards the participants are recommenced at 500 mg daily, followed by 500 mg twice a day.

In compliance with the new interregional guideline for the Capital Region of Denmark and Region Zealand the treatment is not taken the morning on the day of operation and is recommenced with the first meal after surgery[50]. Some guidelines still recommend that metformin treatment should be suspended for up to 2 days before and after surgery to avoid the risk of lactic acidosis. This risk is however very low and there is limited evidence to support that recommendation[51]. A study of 1284 diabetic patients having cardiac surgery showed that patients treated with metformin up until the day of operation and again when they were able to eat postoperatively did not have worse outcome than other diabetic patients and no difference in serum lactic acid or pH was seen. On the contrary, the rates of infection and overall morbidity were lower in the metformin treated patients[52].

Liver biopsies (2 pieces of approximately 2x2x2 mm) can cause transient bleeding, but they will be taken superficially from the surface of the liver under visual guidance limiting the risk. If bleeding occurs this will be handled by the surgeon immediately, ensuring a non bleeding biopsy surface at the end of surgery. The risk of bleeding when performing an intraoperative biopsy from the liver surface is believed to be less than when performing a transcutaneous needle biopsy.

Blood sampling can cause mild discomfort and may leave a mild hematoma that will disappear within a couple of days.

DATA AND ADVERSE EVENTS

Data is collected on individual case report forms (CRF) and data will be stored for 10 years and then destroyed. Data from these CRF's will be transferred into the database continually as the study progresses and this transfer will be monitored by the GCP unit. In this database patients are coded with a patient number and the database is saved on the hospital's computer server to ensure maximum security. All data will be stored in accordance with all relevant and current legislation. Spot checks to validate data transfer from the CRF's to the electronic database will be completed by The Good Clinical Practice Unit.

Due to metformin's relative non-toxicity no Data Monitoring Committee is initiated and no interim analyses have been planned in the course of the study.

Throughout the trial both the sponsor/investigator, who informs the patient and the patient herself, will be blinded. Furthermore, the pathologist who performs the immunohistochemistry and cell count as well as the investigator working with the cancer cell lines will also by blinded. A code breach will occur if an unexpected event arises which demands urgent treatment where there is a reasonable probability of an adverse reaction related to the study medication. Code breach is possible for investigators in case of emergencies and the principal investigator is available day and night via phone.

The summary of product characteristics (SPC) for metformin will be appended to this document in the form of the product resume of metformin produced by Teva. It will function as reference document in evaluation of adverse events.

All adverse events will be registered and reported to The Danish Medicines Agency and the local ethics committee in the final report. All serious adverse reactions (SAR's) will be reported to the local ethics committee in an annual report together with a report on patient safety. All serious adverse reactions (SAR) will be reported to The Danish Medicines Agency in an annual report together with a report on patient safety. The SPC for metformin will be used to judge whether a serious adverse reaction is expected/unexpected and thereby a possible sudden unexpected serious adverse reaction.

The principal investigator (EC) will make sure that all information about sudden unexpected serious adverse reaction, that are lethal or life-threatening will be registered and reported to The Danish Medicines Agency and the regional ethical committee as soon as possible and at the latest 7 days after investigator has received knowledge of such a reaction. At the latest 8 days after this reporting investigator will inform The Danish Medicines Agency of the follow-up. All other sudden unexpected serious adverse reactions (SUSAR) will be reported to The Danish Medicines Agency and the regional ethical committee at the latest 15 days after investigator has gained knowledge of these. In these situations the patient will be followed until the reaction has terminated – either via contact with the investigator or via the outpatient clinic at the hospital.

Definitions of events, side-effects, unexpected side-effects and adverse reactions are as follows.

- Adverse event (AE): any adverse event in a patient or clinical investigation subject in a clinical trial with administration of a drug whether or not the adverse event is considered related to the drug.

- Adverse reaction (AR): All noxious and unintended responses to an investigational compound at any dose.

- Unexpected adverse reaction (UAR): a side effect the nature or severity is not consistent with the applicable product information (SPC for metformin)

- Serious adverse event (SAE) or Serious Adverse Reaction (SAR): an event or effect that at any dose results in death, is life-threatening, requires hospitalization or prolongation of existing hospitalization, results in persistent or significant disability or incapacity, or is a congenital anomalous or malformation.

ETHICS

Most malignancies have linear or exponential growth models, and onset of symptoms is often preceded by a long asymptomatic phase with increasing degeneration. The accelerating growth and degeneration is what theoretically makes diagnosis-to-treatment delays especially harmful in cancer. Treatment delay is likely to take place during a critical phase with high tumor growth rates, which increase the risk of metastasis. Colon cancer is a slow-growing cancer and is in most cases a result of the adenoma-adenocarcinoma sequence[53], which can take up to 15 years from onset to eventual progression to malignancy.[54]

Treatment delay in colon cancer has previously been controversial; however an American study showed that treatment delay was not associated with worse cancer outcomes for patients with colon cancer even in "high-risk" patients including patients with tumor positive lymph nodes, metastatic disease, extramural vascular invasion and comorbid inflammatory bowel disease[55]. Furthermore, a Danish study showed that treatment delay of at least 60 days had no impact on survival from coloncancer.[56]

Treatment delay, defined as the time lapse between diagnosis and surgery is described in the standardized cancer program[57] ("kræftpakkeforløb") by the Danish Health Authorities and must not be more than 10 days. In the present study we wish to expand the treatment delay to 21 days in order to be able to

investigate the effect of metformin on cell proliferation. As the two studies described before concluded, a reasonable delay between diagnosis and subsequent surgery is not detrimental for the patient and justifies the expansion of the time lapse between diagnosis and surgery in order to examine the effect of presurgical treatment with metformin.

The study will be performed in agreement with the Helsinki II declaration and law 593 of 2011 about the Scientific Ethics Committee System and will be approved by the local ethics committee before initiation. The study will also be approved by The Danish Medicines Agency and The Danish Data Protection Agency before initiation. The project will be registered on www.clinicaltrials.gov as recommended by the International Committee of Medical Journal Editors. The Good Clinical Practice Unit at Copenhagen University will oversee the trial and conduct monitoring visits of the trial periodically. All the information about participants are subjected to "lov om behandling af personoplysninger" and "sundhedsloven", and these will be complied.

If a beneficial effect of metformin is seen in the study it would inspire larger scale intervention studies with metformin and possibly the introduction of metformin as a part of the treatment of colon cancer.

GUIDELINES FOR ORAL INFORMATION AND WRITTEN INFORMED CONSENT

The investigators will screen potential candidates from patient lists of referral for colon cancer patients at Slagelse Hospital. The investigator will contact the candidates at the hospital at the time of their first out-patient visit prior to surgery. The investigator will ask whether or not he / she may present a scientific study that is related to the candidate's forthcoming surgery after the appointment at the out-patient clinic. Only if the candidate has time and interest, the investigator will continue with an information meeting. The candidate will be informed that he / she is allowed to bring assessor for the meeting.

At information meeting the candidate will receive information concerning the study both orally and in writing. The information will be delivered in a room that will only be used for this purpose, to avoid unnecessary disturbances. There will be enough time for the investigator to explain content, extend, purpose, expected risks and advantages of the study in plain Danish. The candidate will have time to ask questions and to read the written information one more time.

The patient will also be given a copy of the pamphlets "***Forsøgspersoners rettigheder i et sundhedsvidenskabeligt forskningsprojekt***" and *"**Før du beslutter dig**"* issued by the Central Committee for Health Research and Ethics.

The patients will be informed that they have at least 24 hours to consider their informed consent, that they can at any time ask for more information about the project and at any time withdraw their consent without having to explain why if they so wish.

Furthermore, the patient will be informed that inclusion in the study will lead to the time delay between diagnosis and surgery being expanded to 21 days compared to the 10 days specified in the standardized cancer program. The patient will be explained that this time delay is not detrimental to patient outcomes and is medically as well as ethical justifiable.

STORAGE OF BIOLOGICAL MATERIAL

In relation to the trial five blood samples each consisting of 54 ml blood will be drawn, of which 39mL is stored in an especially reserved biobank. All blood samples will be kept (without CPR-number, but instead with the patient code), and will be stored until on bloc analysis. The biopsies taken before and after surgery will be kept in a biobank as well. The tumor samples kept in the biobank will be approximately 6 x 0,5 cm. Intestinal samples from the removed specimen will be approximately 2x2 cm and the liver biopsies will be approximately 2 x 2 x 2 mm. The usage of a research biobank and storage is to ensure, that there will not be differences in the analysis run from analysis to analysis and patient to patient. The project will be reported to the data protection agency and all formal requirement and maintenance of the biobank will be performed. The samples will be kept in the research biobank for a maximum of 10 years after collection.

Part of the immunohistochemical analyses well take place in France under supervision of Jerome Galon as previously mentioned. Samples or pictures sent to France will be marked with the patient code. No information with the patient's name or CPR-number will leave the country.

The patients will be asked if they accept that the stored samples in the biobank may be used for future research. If such a study was to be performed it would be with approval of Danish Data Protection Agency and the local ethics committee.

DATA COLLECTION AND HANDLING

Approval from the Danish Data Protection Agency, the Danish Medicines Agency and local ethics committee will be achieved before initiation of the trial. The Trial will be registered at Clinicaltrial.gov, as has been recommended by the ICMJE (International Committee of Medical Journal Editors).

Collected data will continuously be registered and filled into each patient's CRF, which will be ready before the initiation of the trial. Data will be stored confidentially for 10 years, and afterwards destroyed. Investigators will be allowed direct access to source data and documents, among these patients' journals. The information needed from the patients' journals include information regarding current and prior diseases, medical treatment and all information related to their cancer treatment including blood works, histology and imaging. At auditing, monitoring and/or inspection from the Danish Medicines Agency, GCP-unit or from other relevant authorities these will be allowed relevant access to information.

If a patient withdraws from the study the collected data will be used in an anonymous manner, unless the patient requests that the data should be erased. After completion of the CRF the data will be transferred into a predesigned trial database. Double data entry will be performed in order to insure the best possible transfer of data. At the end of the trial the principle investigator will archive all the CRFs in the final database.

QUALITY CONTROL AND QUALITY ASSURANCE

Existing procedures for quality assurance and quality control will be followed. An agreement with the GCP unit will be made to handle monitoring at the beginning, mid-term and at the end of the project.

DATA AND STATISTICAL ANALYSIS

Statistical analysis will be performed using the SAS® Proprietary Software 9.4, SAS Institute Inc., Cary, NC USA. Data will be analyzed via parametric or non-parametric statistic depending on their distribution. Log-transformation will be applied when possible. A two-sided P-value of less than 0,05 will be considered statistical significant. For baseline characteristics, descriptive statistics will be used. In case of multiple comparisons the appropriate statistical correction will be applied. No interim analysis is planned for the study. Intention to treat analyses will be performed.

PUBLICATION

When the last patient has finished the trial the regional ethical committee will be notified within 90 days and a final rapport will be sent within 12 months. The data will be made available on EudraCT in accordance

with the current legislation. Submission of manuscripts for publication will be aimed at an international established journal with high impact and the intended timeframe of publication is within 1 year. The primary outcomes will be reported in a main publication, and the results will be made available at clinicaltrial.gov and on www.clinicaltrialsregister.eu . Both positive, negative and inconclusive trial results will be published.

INSURANCE

There is in relation to this project not taken further assurance measures than those applicable to patients who are part of routine clinical care in hospitals and in clinical trials.

ECONOMY

Initiator of this study is Ismail Gögenur. No private party has any part in the study.

Financial support from grants to cover the expenses will be applied on an ongoing basis.

The responsible investigators are employees of Slagelse hospital and University Hospital Zealand. The investigators have no economic conflicts of interest in this study.
No considerations will be paid to the participating patients.

BUDGET

Salary Ph.D-student Emilie Palmgren Colov 2½ years	1.200.000kr	Money has been granted
Metformin for 24 patients	9000kr	
Placebo for 24 patients	9000kr	
Randomization and handling of study medication	25.000kr	
Cancer cell line examinations	100.000kr	
Immunohistochemistry of tumor samples	90.000kr	

PAX-gene tubes	41.000kr	
Analyses of blood samples	300.000kr	
GCP-monitoring	25.000kr	
Notification to the Danish Medicines Agency (Lægemiddelstyrelsen)	7.734kr	
Total	**1.806.734kr**	

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