Presurgical Treatment with Ribociclib and Letrozole in Patients with Locally Advanced Breast Cancer: the NEOLETRIB study.

Research protocol: October 25th. 2020
Version: 1.0

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SHORT SUMMARY

Aim of the study:

To study the direct and indirect anti-tumor effects of targeted cancer therapy with letrozole and ribociclib given in combination with special focus on the immune system in patients suffering from locally advanced, luminal-A/B breast cancer;

Primary objective:

1. To evaluate the intra-tumor immunological effects of individual patients treated with letrozole and ribociclib in the neoadjuvant setting
   a. Endpoints
      i. Evolution of the intra-tumor immune profile during neoadjuvant therapy of breast cancer [*Time Frame: D1, D21, and at surgery*] determined through IHC or immunoblotting, scRNAseq, and other relevant methods described in the following chapters.

Secondary objectives:

1. To study early and late mechanisms of adaptation and resistance to aromatase inhibitor (letrozole) in combination with ribociclib;
   b. Endpoints
      i. Evolution of the tumor cell profile during neoadjuvant therapy of breast cancer [*Time Frame: D1, D21, and at surgery*] determined through whole tumor sequencing, scRNAseq etc.

2. To evaluate clinical and pathological response of targeted neoadjuvant therapy with letrozole and ribociclib
   a. Endpoints
      i. Determine the on treatment effects assessed by PEPI-0 and CCCA (Ki67<2,7%) at D 21 & at surgery. Change in Ki67 BL-

D 21 to surgery, as well as ROR-score, based on genomic analysis. [*Time Frame: D1, D21, and at surgery*]

ii. Long term effects and outcome will be assessed at yearly intervals in line with clinical routine.

3. To study the effect of ribociclib and letrozole treatment on the gut microbiome.
    a. Endpoints
        i. The longitudinal effect of neoadjuvant treatment will be determined through next generation amplicon sequencing on the V4 region of the fecal bacterial 16S rRNA gene by comparing baseline and follow up samples [*Time Frame: D1, D21, D90 and at surgery (D180)*]

4. To search for surrogate parameters (tumor- or immune related markers etc.) allowing to estimate the anti-tumor effects in liquid biopsies before and during therapy;
    a. Endpoint:
        i. Assess serum cytokine levels, serum metabolite levels and circulating free tumor DNA (cftDNA) [*Time Frame: D1, D21, D90 and at surgery (D180)*]

Exploratory objectives:

1. To study the relationship between cancer treatment effect, the immune system and gut microbiota;

2. To develop optimal methods to evaluate the tumor response by novel CT / MRI techniques (tumor measurements);

Study Design: Multicentre, single-arm, open-label, neoadjuvant study;

Clinical Setting: Collaboration of two Norwegian Cancer Centers:

1. Department of Oncology, Akershus University Hospital, Norway);
2. Department of Oncology, Drammen Hospital (Vestre Viken Health region, Norway);

   (additional breast cancer centers in Norway may be involved if patient recruitment is not reaching the expected pace);

Patients: Patients diagnosed with (biopsy-confirmed) locally advanced (defined as either large T2, or T3/T4, or N2-3), ER-positive ($\geq$50% pos. cancer cells), HER-2 negative, luminal-A/B breast cancer suitable for neoadjuvant anti-hormone therapy will be considered for this protocol;
At least 100 patients should complete all study procedures. Thereafter, the protocol will be closed for further inclusion, but each patient included up to that time will be completed according to the protocol. The patients will be followed by the involved medical oncologists for up to 10 years after surgery or until relapse (whatever comes first).

Statistical considerations:

Based on the results of the CORALLEEN trial [1] investigating neoadjuvant ribociclib treatment of luminal B patients, the spread between level of treatment response (24 weeks of treatment) based on MRI was 14% Complete Response (CR), 43% Partial Response (PR), 33% Stable Disease (SD) and 10% N/A. We propose a minimum sample size in either group to be at least >30 patients, thus an inclusion goal of 100 patients in total would be sufficient for hypothesis generating results. However, it is important to mention that the purpose of this trial is to deliver basic research results and not primarily to evaluate the efficacy or comparisons of treatments. Based on the major endpoints and their anticipated data variation, the data will be sufficient to come to reliable results. Finally, statistical power is added as all patients will serve as their own controls based on sequential intra-patient data obtained over time (in general 180 days all in all).

This is aligned with advice given by gastroenterologists and on par with earlier studies that allowed for characterization of the tumor immune microenvironment and the gut microbiota [2,3] and gives sufficient statistical power to prioritize biomarkers for larger companion diagnostic studies in the future.

Treatment:

Suitable patients will receive neoadjuvant therapy with letrozole (Femar□) in combination with ribociclib (Kisqali□) for at least 6 months followed by surgery; premenopausal women will also be considered for this protocol (in combination with the LHRH-analogue goserelin in addition to letrozole and ribociclib). Indication for additional adjuvant therapy (chemotherapy and/or endocrine therapy) will be considered individually after surgery

(without restrictions and according to national guidelines provided by the Norwegian Breast Cancer Group; www.nbcg.net).

Diagnostics: All patients will perform a general staging prior to inclusion with CT-scans, MRI of the breasts and routine blood samples; some of the procedures will be repeated every 3 months (i.e. blood samples, spectral-CT and MRI of the breast). A clinical tumor evaluation will also be completed every 4 weeks (prior to starting a new ribociclib-cycle).

Samples: Blood samples/liquid biopsies, tissue samples and feces samples will be collected before (baseline) and during treatment (day 21, day 90 and day 180) according to the protocol (specified in details in: Appendix B: "laboratory procedures").

BACKGROUND AND AIMS OF THE STUDY

Neoadjuvant endocrine therapy (NET) offers a good treatment option to down-size large (inoperable) primary breast tumors and / or advanced axillary lymph node metastasis prior to definite surgery. For selected patients (postmenopausal women with ER-positive breast cancer), primary endocrine therapy has been shown to be as effective as standard neoadjuvant chemotherapy [4]. It is the general opinion that aromatase inhibitors of the "third-generation" (letrozole, anastrozole, exemestane) are the preferable drugs for neoadjuvant endocrine therapy in ER-positive, postmenopausal breast cancer patients [5]. Anastrozole, letrozole and exemestane have shown to suppress total body aromatisation as well as plasma and tissue estrogen levels by > 90% in vivo [6-10]. All three drugs are currently used as standard care in the neoadjuvant, adjuvant and metastatic setting worldwide, including Norway.

In 2015, a new class of anti-cancer drugs was introduced, known as cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors [11,12]. Following pivotal trials showing dramatic effects when combined with aromatase inhibitors [13-15] or steroidal antiestrogens like fulvestrant [16-20], these compounds are now well established in distinct combinations during therapy of metastatic breast cancer.

All in all, the addition of a CDK4/6 inhibitor to standard anti-hormone therapy of breast cancer, doubled the time to disease progression and caused significant improvements in overall survival [1,21-23]. Based on these findings, CDK4/6 inhibitors have also been tested in the neoadjuvant setting in clinical trials with very promising results [24-29]. However, the combination of an aromatase inhibitor and a CDK4/6-inhibitor is currently not (yet) approved as standard neoadjuvant treatment in the algorithms for patients with locally

advanced breast cancer (LABC) in Norway. Thus, the study described here, will make this highly promising drug combination available for all patients who may participate in this study. At the same time, the protocol will allow us to optimize the selection of patients benefitting from this treatment, highlight relevant biomarkers for personalized medicine and treatment, evaluate predictive markers and study the basic biology underlying treatment effects and resistance.

In the recent years we have seen a growing body of evidence concerning the involvement of the immune system in the onset and prognosis of breast cancer both locally (in the tumor or connected tissues [30]), but also through the involvement of the immune system as a whole [31]. Interestingly, the gastrointestinal (gut) microbiota seems to play an important role in determining whether the immune system is able to fight against several cancer types [2,32-37]. Pre-clinical studies have shown that the microbes in the gut may influence the repertoire and activity of immune cells, such as T cells, potentially tailoring the immune system to allow cancer cell recognition and killing. This hypothesis is supported by the finding that treatment responses in a variety of cancer types to immune checkpoint inhibitors (ICIs) is depended on the distinct gut microbiome of an individual patient, as the efficacy of the ICIs rely on the presence of cancer-recognizing T cells [3,38,39].

In addition, recent findings indicate, that the emerging group of small molecules / targeting cancer therapies have immune altering properties. The MAPK kinase (MEK) inhibitors exemplify this by contributing to anti-tumor immunity through increasing the levels of cytotoxic T lymphocytes in preclinical model systems [40]. Similar findings have recently been reported for both CDK4/6 inhibitors and PI3K-inhibitors [41-43]. Thus, CDK4/6 inhibitors are believed to

increase the antigen presentation of cancer cells, to increase the activity of tumor infiltration by CD45+ cells and effector T cell activation, as well as to decrease the Treg cell proliferation [42,44,45]. While these mechanisms have been well described in *in vitro* and animal models, they have to be studied in humans as soon as possible to understand them and to allow to take advantage of these novel aspects in clinical decision making. In addition, an immunomodulatory effect of e.g. CDK4/6 inhibitors warrants further investigation and confirmation to allow for optimal drug combinations and enable concomitant anti-tumorigenic effects of drugs. These findings may also pave the way for microbiome and immune-related data as potential biomarkers for patient selection and response evaluations during therapy in the long run.

In addition to the clinical effects as standard care for breast cancer patients suffering from locally advanced breast cancer, neoadjuvant therapy is widely used to study the endocrinology of breast cancer in general [46] and is recognized as one of the best model systems to predict responses in other clinical settings (early breast cancer or metastatic breast cancer) as well [47].

Tumor biopsies obtained before initiation of treatment and following six months on therapy will allow correlations to the individual type of clinical response (partial responses vs complete responses etc.). We will focus on intra-tumor mechanisms of adaption through tumor characterization using single cell technology which will allow us to follow both (i) the different cancer clones but also (ii) the evolution of different immune cells during therapy. We will use novel approaches to evaluate liquid biopsies (measurement of cytokines levels, metabolites and cell free DNA-fragments etc.) during letrozole and ribociclib using state of the art laboratory methods at our hospital and in the laboratories

of our listed collaborators. Finally, basic information on intra-tumor gene expression and regulation in individual breast cancer patients treated with both drugs in concert will become available in the planned spin-off-studies involving some of the leading cancer research teams in the world.

Thus, all in all, the planned study will be able to contribute to our basic understanding of this very potent new drug combination that may be used in large groups of breast cancer patients in the future, including locally advanced breast cancer, hopefully reducing the use of traditional chemotherapy in this setting.

PATIENTS AND TREATMENT

Patients:

Patients diagnosed with locally advanced, ER-positive (defined by ER-pos. in $\geq$ 50% of cancer cells), HER-2 negative, luminal-A/B breast cancer suitable for neoadjuvant anti-hormonal therapy ("pre-surgical downstaging") will be considered for this protocol. Locally advanced breast cancer according to this protocol is defined as either large T2 (> 3 cm in diameter) or T3-T4, and/or N2-3 primary breast cancer. Patients with primary tumors above 3 cm in diameter („large T2-tumors") may be recruited in accordance with the international trend to provide neoadjuvant therapies to these patients in clinical trials.

At least 100 patients should complete the study, with the aim of having an equal distribution between Luminal A and Luminal B. Thereafter, the protocol will be closed for further inclusion, but each patient included up to that time point will be completed according to the protocol procedures.

All patients have to be postmenopausal (natural status or induced by treatment with the LHRH-analogue goserelin 3.6 mg implant s.c. given every 4 weeks) to be able to benefit from aromatase inhibition (definition of natural postmenopausal status: age above 55 years or age above 50 years and at least 2 years of amenorrhea in addition to LH-, FSH-, and plasma estradiol levels in the postmenopausal range). Patients with HER-2 positive disease, suitable for neoadjuvant therapy with trastuzumab, pertuzumab and taxanes, will be excluded from this protocol. The decision to include patients in this protocol will only be made following a comprehensive evaluation of each individual patient by a multidiscipline team of experienced breast cancer oncologists, pathologists, surgeons and radiologists.

Triple-negative breast cancer is an exclusion criteria. Patients with distant metastasis of any type are also excluded from this trial.

Patients who are in need of therapy with other strong CYP3A4 inhibitors should (if unavoidable additional therapy) reduce ribociclib to 400 mg daily (D1-21). Patients who are using strong CYP3A4 inducers should only be considered if they may use alternative additional therapies with less potential to induce CYP3A4 [48]. Inclusion and exclusion criteria are summarized in table 1.

Table 1: Summary: Inclusion/Exclusion criteria for the NEOLETRIB-trial

Key Inclusion Criteria:

- The decision to include patients in this protocol will only be made following a comprehensive evaluation of each individual patient by experienced breast cancer oncologists

- ≥ 18 years-old at the time of ICF signature

- Histologically confirmed locally advanced breast carcinoma, defined as either large T2 (>3cm in diameter) or T3-T4, and/or N2-3 primary breast cancer.

- ER-positive (defined by ER-pos. in > 50% of cancer cells) and HER-2 negative, luminal A/B breast cancer

- Postmenopausal status (natural status or induced by treatment with the LHRH-analogue goserelin 3.6 mg implant s.c. given every 4 weeks)

  - definition of natural postmenopausal status: age above 55 years or age above 50 years and at least 2 years of amenorrhea in addition to LH-, FSH-, and plasma estradiol levels in the postmenopausal range.

- Patient has adequate bone marrow and organ function as defined by the following laboratory values (as assessed by central laboratory for eligibility):

  - Absolute neutrophil count ≥ 1.0 × 109/L

  - Platelets ≥ 100 × 109/L

  - Hemoglobin ≥ 9.0 g/dL

  - INR ≤1.5 (unless the patient is receiving anticoagulants and the INR is within the therapeutic range of intended use for that anticoagulant within 7 days prior to the first dose of study drug)

  - Estimated glomerular filtration rate (eGFR) ≥ 30 mL/min/1.73m2 according to the Modification of Diet in Renal Disease (MDRD) formula

  - Total bilirubin < ULN except for patients with Gilbert's syndrome who may only be included if the total bilirubin is ≤ 3.0 × ULN or direct bilirubin ≤ 1.5 × ULN.

  - Aspartate transaminase (AST) < 2.5 × ULN,

  - Alanine transaminase (ALT) < 2.5 × ULN,

- Patient must have the following laboratory values within normal limits or corrected to within normal limits with supplements before the first dose of study medication:

  - Potassium

  - Magnesium

  - Total Calcium (corrected for serum albumin)

- Standard 12-lead ECG values defined as the mean of the triplicate ECGs

  - QTcF interval at screening < 450 msec (QT interval using Fridericia's correction)

  - Mean resting heart rate 50-90 bpm (determined from the ECG)

- Performance Status: Eastern Cooperative Oncology Group (ECOG) score 0-1

- Ability and willingness to comply with study visits, treatment, testing and to comply with the protocol.

Key Exclusion criteria:

- Any prior treatment for primary invasive breast cancer.

- Patient with a known hypersensitivity to any of the excipients of ribociclib or letrozole

- Patient with known hypersensitivity to peanuts or soya-products;

- Any evidence of distant metastasis;

- Triple-negative breast cancer

- HER-2 positive disease, suitable for neoadjuvant therapy with trastuzumab, pertuzumab and taxanes etc.

- Other conditions rendering patients in need of other treatment options with immediate effect like chemotherapy

- Concomitant medications that are known strong *inducers* of CYP3A4/5.

- Clinically significant, uncontrolled heart disease and/or cardiac repolarization abnormality, including any of the following:

  - History of documented myocardial infarction (MI), angina pectoris, symptomatic pericarditis, or coronary artery bypass graft (CABG) within 6 months prior to study entry

  - Documented cardiomyopathy

- – Left Ventricular Ejection Fraction (LVEF) < 50% as determined by Multiple Gated acquisition (MUGA) scan or echocardiogram (ECHO)

  – Long QT syndrome or family history of idiopathic sudden death or congenital long QT syndrome, or any of the following:

    - Risk factors for Torsades de Pointe (TdP) including uncorrected hypocalcemia, hypokalemia or hypomagnesemia, history of cardiac failure, or history of clinically significant/symptomatic bradycardia

    - Concomitant medication(s) with a known risk to prolong the QT interval and/or known to cause Torsades de Pointe that cannot be discontinued or replaced by safe alternative medication (e.g., within 5 half-lives or 7 days prior to starting study drug)

    - Inability to determine the QTcF interval

  – Clinically significant cardiac arrhythmias (e.g., ventricular tachycardia), complete left bundle branch block, high-grade AV block (e.g., bifascicular block, Mobitz type II and third degree AV block)

  – Systolic Blood Pressure (SBP) >160 or <90 mmHg

- Patient is currently receiving or has received systemic corticosteroids ≤ 2 weeks prior to starting study drug, or who have not fully recovered from side effects of such treatment. Note: The following uses of corticosteroids are permitted: a short duration (<5 days) of systemic corticosteroids; any duration of topical applications (e.g. for rash), inhaled sprays (e.g., for obstructive airways diseases), eye drops or local injections (e.g., intra-articular).

- Pregnant or breast-feeding (lactating) women or women who plan to become pregnant or breast-feed during the trial

- Women of child-bearing potential defined as all women physiologically capable of becoming pregnant, unless they are using highly effective methods of contraception during the study treatment and for 21 days after stopping the treatment. Highly effective contraception methods include:

  – Total abstinence (when this is in line with the preferred and usual lifestyle of the patient. Periodic abstinence (e.g., calendar, ovulation, symptothermal, post-ovulation methods) and withdrawal are not acceptable methods of contraception

  – Female sterilization (have had surgical bilateral oophorectomy with or without hysterectomy), total hysterectomy or tubal ligation at least

6 weeks before taking study treatment. In case of oophorectomy alone, only when the reproductive status of the woman has been confirmed by follow up hormone level assessment

- Male partner sterilization (at least 6 months prior to screening). For female patients on the study, the vasectomized male partner should be the sole partner for that patient and the success of the vasectomy must be medically confirmed as per local practice.

- Placement of an intrauterine device (IUD).

- Note: Use of oral (estrogen and progesterone), transdermal, injected, implanted, hormone containing intrauterine systems (IUS) or any other hormonal methods of contraception is not allowed in this study

- Autoimmune disorders or significant allergies (i.e. rheumatoid arthritis, asthma, psoriasis etc).

- Known HIV infection, Hepatitis B or C infection (testing not mandatory)

- History of autoimmune celiac, inflammatory bowel disease, or other chronic GI disease

- Recent use (within past month) of more than 3 days of antibiotics use

- Current use of probiotic supplements

- Taking proton pump inhibitors, steroids, other non-steroidal anti-inflammatory drugs such as ibuprofen or acetyl salicylic acid;
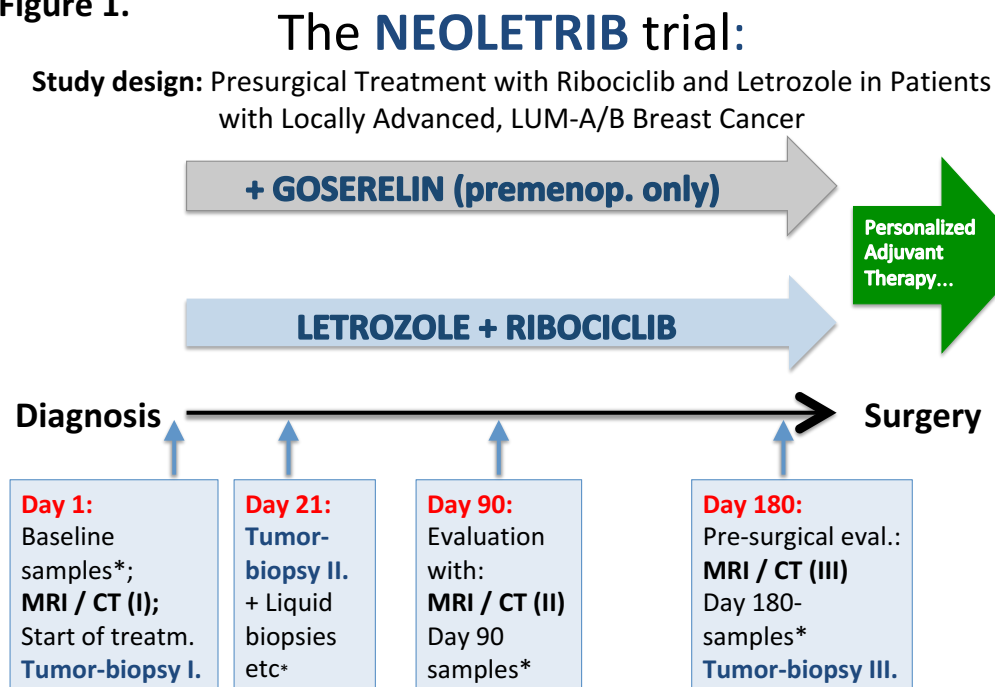
- Past bariatric surgery

Treatment:

Participating patients will receive neoadjuvant therapy with letrozole and ribociclib for at least 6 months. Patients will have a clinical tumor evaluation every 4 weeks and MRI-evaluations etc. every 3 months. The design of this trial is given briefly in figure 1. Letrozole will be given once daily by the oral route in the established dose of 2.5 mg daily. Ribociclib will be given as 600 mg once daily p.o. (standard starting dose) for a period of 21 days followed by 7 days without ribociclib. The general recommendations for drug dose reductions, blood

sampling, ECG assessments etc. provided by the manufacturer (Novartis Pharmaceuticals) will be followed through the entire trial. ECG assessments will be performed at baseline (screening), day 1 and 15 of cycle 1 and 2, on day 1 of all following cycles and at the end of treatment.

Premenopausal women will always start with goserelin from day 1 in addition to the aromatase inhibitor letrozole and ribociclib.

Concerning the timing of surgery, it is planned to do surgery during one of the last days of the ongoing treatment cycle with ribociclib (surgery while on treatment). A check of the hematological profile will, however, be done directly prior to surgery and surgery will be postponed if needed (to guarantee sufficient peripheral blood cell counts on the day of surgery).

Figure 1.

The NEOLETRIB trial:

Study design: Presurgical Treatment with Ribociclib and Letrozole in Patients with Locally Advanced, LUM-A/B Breast Cancer

*Samples: serum, plasma, feces etc. (for details see: laboratory manual);

All cancer drugs used in this trial (letrozole, ribociclib, goserelin) are well-established in MBC and have also proven high efficacy in previous neoadjuvant trials in locally advanced breast cancer, both as monotherapy and in combination [4,49-58]. Thus, it is known that most of the patients will have clinical benefit from these medications and many will be able to avoid neoadjuvant/adjuvant chemotherapy that is associated with considerable more serious side-effects compared to study medication. However, if patients do not profit sufficiently from the medication given in this trial, they will be taken off study medication and be treated with all available types of standard chemotherapy established for these patients (no restrictions caused by the present protocol). In addition, all patients will be considered for adjuvant therapy according to the final pathological report following surgery (yTNM-status) and according to the Norwegian breast cancer guidelines published by the Norwegian Breast Cancer Group (www.nbcg.net).

MEDICAL EVALUATIONS and SAMPLING

Clinical evaluations

In general, patients will be followed at the outpatient clinics (Akershus University Hospital, Department of Oncology & Drammen Hospital, Department of Oncology) by experienced medical breast cancer oncologists at least every 4 weeks during the entire study (including caliper measurements of maximum tumor diameters). In addition, measurement of maximum tumor diameters will be performed at study entry and during therapy every 3 months by advanced MRI (ax T1, T2, DWI, dynamic study and spectroscopy) and Spectral-CT scans at

the Departments of Radiology, Akershus University Hospital and Drammen Hospital. All measurements (MRI, spectral-CT etc.) will be performed always prior to biopsies at baseline and before final surgery as usual to avoid that bleeding etc. may interfere with the diagnostic procedures. Patients will be informed about the opportunity to perform PET-scanning prior to treatment after protocol as a general screening procedure for distant metastasis (not mandatory). All patients will be screened for distant metastasis by CT-scanning (thoracic, abdominal and pelvic CT-scanning as well as a bone-scintigraphy) at study entry. All available additional diagnostic procedures may be used at the discretion of the responsible oncologists / breast cancer surgeons (not mandatory).

Blood samples (scientific samples)

Blood samples will be drawn preferably in the morning following an overnight fast (not mandatory but recommended) at the following time points:

- 1st.. blood sample: on days – 7 to 1 (but before the first treatment after protocol; day 1 = day of the first tablets)
- 2nd. blood samples: day 21;
- 3rd. blood sample: following 3 months on therapy (+/- 7 days);
- 4th. blood sample: following 6 months on therapy (+/- 7 days); (whenever possible during on of the last days of the ongoing ribociclib cycle);
- Additional blood samples: every 3 months (if treatment > 6 months);
- After surgery: once every year for 5 years;
- At relapse/metastasis;

Blood samples per timepoint include: 2 x 10 ml of heparin-plasma; 2 x 10 ml EDTA-plasma (cells will be kept in separate vials); 2 x 10 ml serum; 2 x 10 ml of citrate-plasma; 2 CPT vials and 4 x 10 ml of full-blood (EDTA). These scientific blood samples will be spinned as usual and stored at –80ºC at the individual research departments (EPIGEN-center, Akershus University Hospital; Research unit at Drammen Hospital) until processing.

In addition, all standard established blood samples (liver samples, kidney-function, bone-marrow function etc.) will be taken as usual and recommended for the treatment with aromatase inhibitors in combination with ribociclib.

Detailed instructions for the handling of scientific blood samples are summarized in the appendix of this protocol.

Tissue samples

All in all, tumor tissue samples (each about 500-1000 mg of tumor tissue) will be obtained at three time points (at baseline, after 21 days on treatment and after 6 months of treatment) and either stored or processed immediately for scRNA-seq. The tumor tissue will be divided in four portions as described below. One sample (ca. 250-500 mg) will be stored immediately in liquid nitrogen (transport medium to the research departments and will be primarily used for RNA and DNA extraction) before the sample is stored at –80ºC until processing otherwise. The second sample (ca. 250-500 mg) will be immediately (within 24h) dissociated at the single cell level for single cell RNA profiling and single cell T-cell receptor sequencing. The third tissue sample will be prepared for histology assessments at the departments of pathology, (ca. 100-300 mg, stored in formalin before it will be paraffin-embedded as usual). A fourth sample will be stored in RNA-later over night at 4ºC and then transferred to -80ºC. The tissue samples will be prepared as quickly as possible (max. time: 5-10 minutes) preferably in the operational theatre, trimmed (if necessary) for fat and blood as usual. In general, all biopsies will be obtained as either open (surgical) biopsies or ultrasound guided biopsies using adequate local anesthesia. Vacuum-biopsies are also allowed. During final surgery, the scientific tumor biopsy will also be obtained through the skin whenever possible to avoid manipulation of the posterior surface of the breast (allowing the normal evaluation of free margins, distance to tumor tissue etc.). If patients need additional neoadjuvant treatment

following 6 months of therapy according to this protocol, the biopsy scheduled after 6 months is giving critical information and should therefore be obtained for all patients.

Pathology / Histology

As described above, a baseline tissue biopsy will be used to ensure that the right patients are selected for the protocol and will provide all routinely needed standard information such as subtype of breast cancer, grading, ER-status, PGR-status, HER-2 status and level of Ki67-expression. For this purpose, a formalin-embedded sample will be prepared as usual at baseline and at all subsequent time points. All following (scientific / non-diagnostic) biopsies will be investigated by a pathologist to ensure that the biopsy is consisting of tumor tissue.

Fecal samples

Fecal samples will be collected at baseline and during follow up (day 21, day 90, day 180 and annually yr. 1-5; in addition, in case of relapse/metastasis). The fecal samples are sampled at home, placed in designated containers with DNA and RNA preserving additives (Stool DNA Stabilizer, Stratec Molecular GMBH, Berlin, Germany) and kept refrigerated before being delivered to Akershus University Hospital / Drammen Hospital, the biobank location, where it will be stored at -80°C.

We will use a DNA-based approach to determine fecal microbial composition. DNA purification from fecal samples will be performed using PSP Spin Stool DNA Plus Kit (Stratec Molecular GMBH). Next generation amplicon

sequencing targeting the V4 region of the 16S rRNA gene (DNA) will be applied to detect the members of the fecal microbiota.

Study endpoints

The longitudinal collection of biopsies and blood samples, at different time point of the neoadjuvant treatment regimen and after surgery (follow up with blood samples only) is carefully designed to answer several key questions:

1. Study the microenvironment of the tumor and changes during therapy with the chosen AI/CDK4/6i -combination

The immune microenvironment has been associated with response to neoadjuvant chemotherapy [30]. The single cell RNA-seq profiling of tumors collected in this trial will allow us to accurately profile the composition of the immune microenvironment at diagnosis and after treatment. This will enable us to (i) find possible immune related biomarkers of response at the tumor site, and (ii) link the gut microbiota and the serum cytokines profiling to the immune tumor microenvironment. As described above scRNA-seq will be performed at Ullevål Hospital.

In addition, we will sequence the T-cell receptors of the lymphocyte T cells found at the tumor site. Effective immune induced tumor eradication is conditioned by the recognition by immune cells of neoantigens which are created by the genetic aberration ongoing in tumor cells. Measuring both the tumor neoantigens and the ability of the T-cell receptor to recognize such tumor-antigen will help understanding the 'laws' and molecular events underlying immune-related tumor eradication. T-cell receptor sequencing will be performed
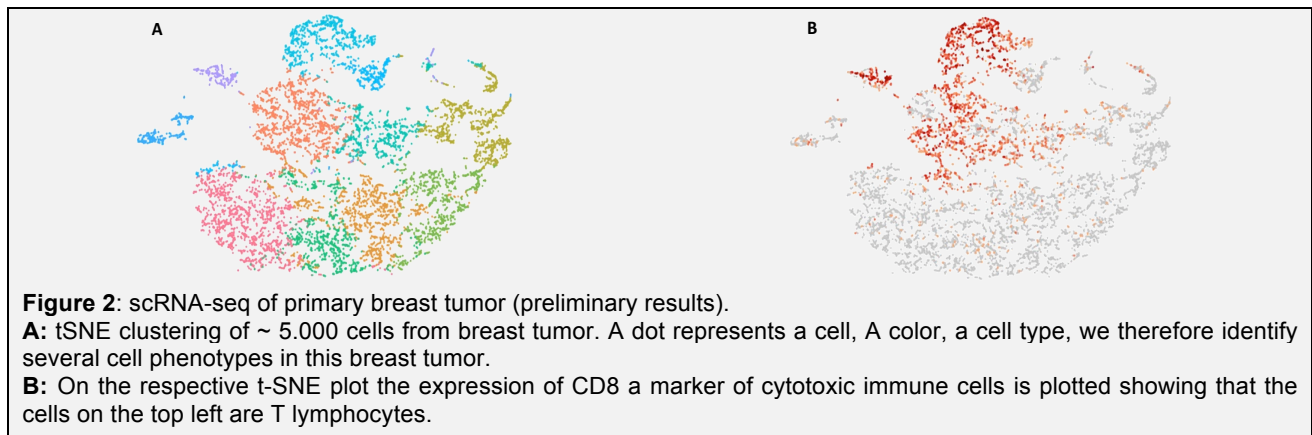
at Ullevål University Hospital at the same time as the scRNA-seq. Neoantigen detection will be performed by collaborators at Karolinska Institute, Stockholm University, Sweden (Prof. Lehtiö's laboratory) using state of the art proteomics quantification and analysis [59].

Based on recent results we will also evaluate whether responders *vs* non responders of this drug combination (AI + CDK4/6i) belong to a newly discovered immune-related breast cancer subtype [30]. Subtyping for these *immune-clusters* will be performed by the inventor of the method.

2. Study the mechanisms of adaptation and resistance to aromatase inhibitor (letrozole) in combination with the CDK4/6-inhibitor ribociclib.

We will use the samples collected at diagnosis and after treatment (at surgery) to follow the changes in tumor and immune cell phenotypes. We will profile each cell of the tumor tissue using single-cell RNA-seq (scRNA-seq). This method allows us to profile thousands of individual cells and to build complex cellular atlases of whole tumors biomarkers for targeted cancer treatments [60]. scRNA-seq will allow us to characterize which exact cancer cell phenotypes (clones) are eradicated by the treatment combination and find biomarkers of response while monitoring which cancer cells survive or are expanding under the treatment pressure. Being able to find the cancer cells surviving treatment will allow to pave the way to designing the next treatment options for the patients not responding to the here propose treatment regimen. We have established the scRNA-seq method on primary breast tumors (Figure 2), these data are the first scRNA-seq data performed on solid tumors in Norway.

The experimentation will be conducted at Ullevål University Hospital using the 10X GENOMICS platform for single cell analysis.

Figure 2: scRNA-seq of primary breast tumor (preliminary results).
A: tSNE clustering of ~ 5.000 cells from breast tumor. A dot represents a cell, A color, a cell type, we therefore identify several cell phenotypes in this breast tumor.
B: On the respective t-SNE plot the expression of CD8 a marker of cytotoxic immune cells is plotted showing that the cells on the top left are T lymphocytes.

Furthermore, to follow clonal evolution of the tumor based on mutations and genetic aberrations, whole genome sequencing of the tumor DNA is planned. Bulk transcriptomic of the tumor tissue will allow accurate molecular subtyping using gene expression and to obtain for example PAM50 subtypes and ROR scores [61]. Such standard molecular profiling of the tumor using next generation sequencing will be performed either at Akershus or Ullevål University Hospital, where such methods are used routinely.

3. To evaluate the immediate effects of letrozole/ribociclib treatment on the clinical presentation of the tumor in addition to surrogate markers (like suppression of Ki-67, influence on PEPI-score etc.)

4. To study the relationship between treatment effect and the gut microbiota
The gut microbiome in women with breast cancer differs from that of healthy women [38]. How the gut microbiome may affect breast cancer risk is as not known and warrants further research. Proposing hypotheses on the relationship

26

between breast cancer and gut microbiota involve both the microbiota/estrobolome (the collection of the enteric bacterial genes whose products metabolize estrogen and its metabolites) where perturbation could lead to elevated levels of estrogens and its metabolites, and the estrogen-independent pathway where bacterial translocation from the gut to the mammary gland via an endogenous route could take place. The microbiota has been found to regulate the response to cancer chemotherapy by affecting mechanisms of action and toxicity, and regulate the effectiveness of ICI by modulating inflammation and adaptive immunity [39]. In the present study we will explore the influence of the gut microbiota on the treatment effect and the immune system, as well as observing the direct effects of ribociclib and letrozole on the gut microbiome. We will perform next generation amplicon sequencing on the V4 region of the bacterial 16S rRNA gene at baseline and follow up. This gives the opportunity to identify and follow the composition and diversity of the gut microbiota associated with disease course, immune response and response to treatment. Changes in the intestinal microbiota during treatment and the possibility of the gut microbiota, as a community or certain members, to be a biomarker and prognostic tool will be explored. The DNA extraction and amplicon sequencing will be performed at Dept of Clinical Molecular Biology, Akershus University Hospital.

5. Non-invasive biomarkers of response or relapse during therapy with the aromatase inhibitor letrozole in combination with the CDK4/6 inhibitor ribociclib

a. Serum cytokine levels

To seek for non-invasive biomarkers of response to therapy in relation to systemic and tumor-related immune reaction, we will measure cytokines serum levels at different time points. Cytokines are small proteins that are important in cell signaling. They are highly inducible peptides and mediate intercellular communication in the immune system [62]. Alterations in specific cytokine expression patters have been detected in patients with breast cancer, and systemic inflammation has been associated with aggressive tumor growth, invasion and metastasis [63]. Therefore, serum cytokines levels represent promising non-invasive biomarkers to monitor patient's response to therapy and will be measured in this study. Measurement of cytokines will be performed at Akershus University Hospital using the Luminex platform as we previously described [64].

b. Serum metabolite levels

Metabolites are small chemical compounds that are intermediates or products of metabolism. It is well known that cancer cells have a reprogrammed metabolism to convert nutrients to biomass while maintaining energy production. This phenomenon is increasingly recognized as a potential target for treatment, but also as a source for biomarkers that can be used for risk stratification, prognostication and therapy monitoring. In breast cancer, significant changes in the metabolism have been described, both in tumor tissue [65] and in biofluids [66]. Our assessment of metabolites in the serum of the patients will therefore allow for the discovery of biomarkers as well as finding a connection between the microbiota and the host / systemic host metabolism. Indeed, the gut microbiota is involved in the regulation of numerous physiological pathways, among others

the energy metabolism is of particular interest. Metabolites will be measured using mass spectrometry as previously by collaborator in Trondheim (NTNU) as previously described [65].

c. Circulating free tumor DNA (cftDNA)

Detection of circulating free tumor DNA (cftDNA) during follow-up after initial treatment for early breast cancer was associated with a high risk of relapse [67]. This gives the rationale to collect blood samples after surgery and end of treatment as it has strong potential to early detect the patients relapsing and or at risk of relapsing. Circulating free DNA will be extracted and sequenced at Akershus University Hospital.

RECRUITMENT TIME

Starting on December 1st. 2020 (or when the protocol is approved by local authorities and at the hospital level) until all patients are recruited (estimated recruitment period: 2 years).

SUBSEQUENT THERAPY

All patients treated following this protocol will be evaluated by an experienced breast surgeon for the best local surgical treatment option (breast conserving surgery or mastectomy including necessary procedures including the ipsilateral axilla).

After surgery, all patients will be considered for adjuvant treatment (both endocrine options and chemotherapy but also zoledronic acid etc.) according to

the national recommendations (Norwegian Breast Cancer Group; www.nbcg.net).

Patients with progressive diseases (at any time point) during neoadjuvant therapy or stable diseases following 3 months of therapy according to this protocol, will be able to obtain additional therapies (chemotherapy etc.) without any restrictions to allow down-staging prior to surgery by other means.

PROTOCOL AMENDMENTS AND SAE REPORTING

If necessary, protocol amendments will be done in collaboration with all involved parties and the regional ethical committee will be informed about all changes prior to implementation.

ADVERSE EVENT (AE) REPORTING

AE definition

An adverse event (AE) is defined as the appearance (or worsening in the case of pre-existing conditions) of any undesirable sign, symptom or disease that arises after having obtained the patient's informed consent, regardless of if it is considered to be related to the study drug. Any abnormal analytical values or test results occurring after obtaining informed consent shall only be reported in the eCRF under adverse events when there are clinical signs or symptoms, they are considered clinically relevant, they require treatment (for example, a hematological abnormality that requires transfusions or supportive therapy with hematopoietic stem cells) or require the study medication to be modified.

AEs that appear or worsen after obtaining the informed consent should be recorded in the eCRF under adverse events. Diseases that were already present at the time of granting informed consent must be recorded in the eCRF under medical history. Continuous monitoring for AEs will be carried out for at least 30 days after the last dose of the study treatment. The AEs (including the abnormal

analytical values constituting AEs) must be described by mentioning a diagnosis to the extent possible, instead of the underlying signs and symptoms. When no clear diagnosis can be identified, every sign or symptom must be reported as an independent AE.

The AEs shall be assessed in accordance with the Common Terminology Criteria for Adverse Events (CTCAE), version 4.03, whenever possible. In the event that a grade for an AE is not included in the CTCAE, the severity of mild, moderate, severe, potentially fatal and death shall be used, corresponding to grades 1 to 5.

The onset of AEs should be investigated by asking the patient open-ended questions during the screening process after the signing of the informed consent and at each study visit. AEs can also be detected by spontaneous reporting by the patient during the screening process or between visits, as well as through the physical examination, analyses or other assessments. To the extent possible, all AEs must be assessed to determine:

1. Grade of severity (CTCAE grades 1 to 5).
2. Duration (dates of onset and finalization).
3. Relationship with the study treatment (reasonable likelihood that the AE is related: No/ Yes; causality will be assessed individually for each study treatment).
4. Measure adopted with regard to the study treatment (none, dose adjustment, temporary discontinuation, permanent discontinuation, unknown, not applicable).
5. Administration of medication or treatment (absence of concomitant medication or nonpharmacological treatment, concomitant medication or non-pharmacological treatment).
6. Outcome (no recovery/no resolution, recovery/resolution, in recovery/being resolved, recovery/resolution with sequelae, fatal, unknown).
7. Seriousness, in accordance with the definition of serious adverse event (SAE) indicated in the section under.

All AEs must be treated appropriately. When a concomitant medication or non-pharmacological treatment is administered, this circumstance must be recorded in the eCRF under AE.

Once an AE is detected, it must be monitored until its resolution or until it is considered permanent; at each of the visits (or more frequently if necessary) the variation in the severity, the supposed relationship with the study treatment, the interventions needed to treat it and the outcome will be assessed.

With regard to determination of causality mentioned in point 3, the sponsor will subsequently classify the adverse event, on the basis of causality with the drug, according to the algorithm of Karch and Lasagna (1977), as:

- Definitive: there is a reasonable temporal sequence between administration of the drug and the appearance of the adverse event. This event coincides with the adverse event described for the drug, improves when it is interrupted and reappears after readministration and cannot be explained by alternative causes.
- Probable: there is a reasonable temporal sequence between administration of the drug and the appearance of the adverse event. This event coincides with the adverse event described for the drug, improves when it is interrupted and cannot be explained by alternative causes.
- Possible: there is a reasonable temporal sequence between administration of the drug and the appearance of the adverse event. This event coincides with the adverse reaction described for the drug, but may be explained by alternative causes.
- Conditional or unlikely: there is a reasonable temporal sequence between administration of the drug and the appearance of the adverse event. This event does not coincide with the adverse reaction described for the drug, and may be explained by alternative causes.
- Unrelated: there is no reasonable temporal sequence between administration of the drug and the appearance of the adverse event. This event does not coincide with the adverse reaction described for the drug, and may be explained by alternative causes.

Serious adverse events

Definitions

Serious adverse event (SAE) is defined as one of the following:

- Is fatal or life-threatening

- Results in persistent or significant disability/incapacity
- Constitutes a congenital anomaly/birth defect
- Is medically significant, i.e., defined as an event that jeopardizes the patient or may require medical or surgical intervention to prevent one of the outcomes listed above
- Requires inpatient hospitalization or prolongation of existing hospitalization,

Note that hospitalizations for the following reasons should not be reported as serious adverse events:
- Routine treatment or monitoring of the studied indication, not associated with any deterioration in condition (i.e. to perform study related assessments)
- Elective or pre-planned treatment for a pre-existing condition that is unrelated to the indication under study and has not worsened since signing the informed consent
- Social reasons and respite care in the absence of any deterioration in the patient's general condition

Note that treatment on an emergency outpatient basis that does not result in hospital admission and involves an event not fulfilling any of the definitions of a SAE given above is not a serious adverse event

Reporting

AEs and SAEs

All reported adverse events and serious adverse events will be reported as defined in the previous section and recorded in the patient's eCRF.

SAEs must be reported by the investigator to national coordinating investigator as outlined in the ISF within 24 hours after the site has gained knowledge of the SAE. Every SAE must be documented by the investigator on the SAE pages in the eCRF, generating an automatic message to the national coordinating investigator and sponsor. The initial report shall promptly be followed by detailed, written reports if necessary. The initial and follow-up reports shall identify the trial subjects by unique code numbers assigned to the latter.

The sponsor keeps detailed records of all AEs and SAEs reported by the investigators and performs an evaluation with respect to seriousness, causality and expectedness.

Suspected Unexpected Serious Adverse Reaction (SUSARs)

SUSARs will be reported to the Competent Authority according to national regulation. The following timelines should be followed:

The sponsor will ensure that all relevant information about suspected serious unexpected adverse reactions that are fatal or life-threatening is recorded and reported as soon as possible to the Competent Authority and Ethics Committee, and in any case no later than seven (7) days after knowledge by the sponsor of such a case, and that relevant follow-up information is subsequently communicated within an additional eight (8) days.

All other suspected serious unexpected adverse reactions will be reported to the Competent Authority concerned and to the Ethics Committee concerned as soon as possible but within a maximum of fifteen (15) days of first knowledge by the sponsor.

SUSARs will be reported using the Council for International Organizations of Medical Sciences (CIOMS) form

The Sponsor is responsible for informing all investigators about SUSARSs occurring in the study on a regular basis.

Pregnancies

To ensure patient safety, each pregnancy in a patient on study treatment must be reported to the Sponsor within 24 hours of learning of its occurrence. The pregnancy should be followed up to determine outcome, including spontaneous or voluntary termination, details of the birth, and the presence or absence of any birth defects, congenital abnormalities, or maternal and/or newborn complications.

Pregnancy should be recorded on a Clinical Trial Pregnancy Form and send to the Sponsor.

Pregnancy follow-up should be recorded on the same form and should include an assessment of the possible relationship to the study treatment of any pregnancy outcome. Any SAE experienced during pregnancy must be reported on the SAE Report Form.

Pregnancy outcomes must be collected for the female partners of any males who took study treatment in this study. Consent to report information regarding these pregnancy outcomes should be obtained from the mother.

Study drug misuse or abuse

Any misuse or abuse of the Study drug must be reported to the Sponsor within 24 hours of learning of its occurrence.

Annual Safety Report

Once a year throughout the clinical trial, the sponsor will provide the Competent Authority with an annual safety report. The format will comply with national requirements.

ETHICAL CONSIDERATIONS AND APPROVALS

The protocol will be submitted to the regional ethical committee for approval prior to initiation of the study. There are no ethical conflicts connected to this protocol. The treatment provided has been tested in previous protocols and is known to be very effective in selected patients (as described in this protocol). The intention of the present protocol is solely to study the unknown mechanisms of tumor cell adaption during therapy in relation to the two drugs involved. As mentioned before, both drugs have been evaluated as highly potent and effective given in combination to breast cancer patients.

The design of the NEOLETRIB-trial has already been discussed with the Norwegian drug approval authority (Statens legemiddelverk – SLV) and we have

received the final evaluation from the responsible committee, confirming that NEOLETRIB may be initiated without further discussions with the SLV (due to the fact that all used drugs are approved in Norway for the treatment of breast cancer and no randomization is planned in NEOLETRIB).

DATA FLOW AND HANDLING

For all analyses to be conducted, patients will be recognized by a code number not allowing personal identification. The manual identifying each patient number by name will be kept locked in the Department of Oncology and is the responsibility of the principal corresponding investigator.

PUBLICATION OF DATA

All results will be published in relevant medical journals. Qualification for co-authorship will be decided by the principal investigator in collaboration with all involved partners in accordance with general practice (Vancouver-guidelines etc.).

FUNDING AND INVOLVED PARTIES

This trial is a research collaboration between the involved parties (UiO, AHUS and Novartis Norway AS), which involves study design, steering group participation and sharing of cost as regulated by the contracted agreement between the parties.

APPENDIX

Single cell RNA and T cell and B cell receptor sequencing

In the surgery theatre needle or open biopsies will be immediately immerged in culture media to allow optimal transport to Ullevål Hospital on ice. At Ullevål, the breast tumor tissue will be dissociated at the single cell level using enzymatic and mechanical means and red blood cells removed. Approximatively 10.000 live dissociated cells will be processed through the 10X genomics chromium controller to embed each cell with a gel bead which contains a unique bar code which will allow to recognize the cell from which the mRNA molecules are captured from. Following library preparation and sequencing, the molecular RNA profiling of each of the 10.000 cells will be obtained to allow in depth characterization of the composition of the tumor tissue. In addition, though enrichment and amplification of the genes coding for T cell and B cell receptors, we will have unprecedent knowledge of the clones of immune cell present at the tumor.

Cytokines measurement

Serum from 4 mL EDTA blood tubes will be obtained by centrifugation the blood 30 min after collection at 15000 x g for 10 minutes in a refrigerated centrifuge. The serum will be aliquoted in clean polypropylene tubes and stored at -80C. Aliquots will be thawed the day cytokines levels need to be measured using the 54-plex cytokine panel (Bio-Rad Laboratories) and will be analyzed with the Luminex xMAP 200 platform (Luminex Corporation, Austin, TX, USA). The assay includes a series of known concentrations to generate standard curves. All samples will be analyzed in duplicates.

DNA and RNA extraction from fresh frozen tissue

Tumor biopsies will immediately be immerged in liquid nitrogen for instant freezing to preserve nucleic acids (DNA and RNA). Fresh frozen biopsies will be stored at -80C until they will be further processed. Fresh frozen tumor biopsies are dissected into small pieces, mixed, and divided into amounts suitable for DNA and RNA extraction. For RNA extraction using the RNeasy kit for RNA extraction (Qiagen) or DNA extraction with the DNeasy for tissue extraction kit (Qiagen).

RNA sequencing for Molecular profiling of biopsies

1 µg of starting total RNA will be processed for library construction and sequencing. poly(A) mRNA is isolated from the total RNA. First strand cDNA synthesis is performed using random hexamers and standard dNTP mix, second strand cDNA synthesis is performed using dUTP. The cDNA is end-repaired and A-tailed, and diluted TruSeq adapters with barcodes are ligated. Adapter-ligated cDNA is then size-selected Typically, 10 to 24 barcoded libraries are included in a pool and each pool is sequenced in at least one lane across dual flowcells. Paired-end sequencing of 50 bp read-length is performed on an Illumina HiSeq 2000 instrument.

DNA sequencing for mutation and clonality analysis

gDNA isolated is normalized to 1-3 µg. Libraries are prepared using the Agilent SureSelectXT Target Enrichment Library protocol and Agilent SureSelectXT Human All Exon v4 enrichment capture library. Captured Whole Exome final libraries passing the final QC step are normalised to 2nM and pooled for

sequencing on the HiSeq 2500 instrument. Dual HiSeq SBS v4 runs at 101bp paired-end reads generated the data for analysis. Target coverage was 400-500x for the tumour regions and 100-200x for the associated normal.

DNA sequencing of the gut microbiota

DNA purification from fecal samples will be performed using PSP Spin Stool DNA Plus Kit (Stratec Molecular GMBH, Berlin, Germany). The bacterial taxonomic composition of the fecal microbiota will be determined using amplicon sequencing of the bacterial 16S rRNA variable 4 (V4) region and the Illumina MiSeq technology following published protocol (ref). A MiSeq instrument is available at the Dept of Clinical Molecular Biology, Akershus University Hospital. The sequencing data will be analyzed using QIIME2 (ref) and the R computing environment (ref).

APPENDIX

A. Detailed study design (see figure 1);

B. Laboratory Procedures

C Primary timelines:

It is planned to start enrolment of patients in December 2020 at two Norwegian Centers. The last patients should be enrolled after ca. 2 years.

D. Budget (separate document has been provided);

REFERENCES

1. Prat, A.*, et al.* Ribociclib plus letrozole versus chemotherapy for postmenopausal women with hormone receptor-positive, HER2-negative, luminal B breast cancer (CORALLEEN): an open-label, multicentre, randomised, phase 2 trial. *Lancet Oncol* **21**, 33-43 (2020).
2. Vetizou, M.*, et al.* Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* **350**, 1079-1084 (2015).
3. Gopalakrishnan, V.*, et al.* Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* **359**, 97-103 (2018).
4. Semiglazov, V.F.*, et al.* Phase 2 randomized trial of primary endocrine therapy versus chemotherapy in postmenopausal patients with estrogen receptor-positive breast cancer. *Cancer* **110**, 244-254 (2007).
5. Smith, I.E.*, et al.* Neoadjuvant treatment of postmenopausal breast cancer with anastrozole, tamoxifen, or both in combination: the Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) multicenter double-blind randomized trial. *J Clin Oncol* **23**, 5108-5116 (2005).
6. Geisler, J.*, et al.* Influence of neoadjuvant anastrozole (Arimidex) on intratumoral estrogen levels and proliferation markers in patients with locally advanced breast cancer. *Clin Cancer Res* **7**, 1230-1236 (2001).
7. Geisler, J., Haynes, B., Anker, G., Dowsett, M. & Lonning, P.E. Influence of letrozole and anastrozole on total body aromatization and plasma estrogen levels in postmenopausal breast cancer patients evaluated in a randomized, cross-over study. *J Clin Oncol* **20**, 751-757 (2002).
8. Geisler, J.*, et al.* Letrozole is Superior to Anastrozole in Suppressing Breast Cancer Tissue and Plasma Estrogen Levels. *Clin Cancer Res* **14**, 6330-6335 (2008).
9. Geisler, J.*, et al.* In vivo inhibition of aromatization by exemestane, a novel irreversible aromatase inhibitor, in postmenopausal breast cancer patients. *Clin Cancer Res* **4**, 2089-2093 (1998).
10. Geisler, J.*, et al.* Influence of anastrozole (Arimidex), a selective, non-steroidal aromatase inhibitor, on in vivo aromatisation and plasma oestrogen levels in postmenopausal women with breast cancer. *Br J Cancer* **74**, 1286-1291 (1996).
11. Gnant, M., Steger, G.G. & Bartsch, R. CDK4/6 inhibitors in luminal breast cancer. *Lancet Oncol* **16**, 2-3 (2015).
12. Lim, J.S., Turner, N.C. & Yap, T.A. CDK4/6 Inhibitors: Promising Opportunities beyond Breast Cancer. *Cancer Discov* **6**, 697-699 (2016).
13. Hortobagyi, G.N.*, et al.* Updated results from MONALEESA-2, a phase III trial of first-line ribociclib plus letrozole versus placebo plus letrozole in hormone receptor-positive, HER2-negative advanced breast cancer. *Ann Oncol* **29**, 1541-1547 (2018).
14. Hortobagyi, G.N.*, et al.* Updated results from MONALEESA-2, a phase III trial of first-line ribociclib plus letrozole versus placebo plus letrozole in hormone receptor-positive, HER2-negative advanced breast cancer. *Ann Oncol* **30**, 1842 (2019).

15.   Finn, R.S., *et al.* Palbociclib and Letrozole in Advanced Breast Cancer. *N Engl J Med* **375**, 1925-1936 (2016).

16.   Slamon, D.J., *et al.* Phase III Randomized Study of Ribociclib and Fulvestrant in Hormone Receptor-Positive, Human Epidermal Growth Factor Receptor 2-Negative Advanced Breast Cancer: MONALEESA-3. *J Clin Oncol* **36**, 2465-2472 (2018).

17.   Turner, N.C., *et al.* Palbociclib in Hormone-Receptor-Positive Advanced Breast Cancer. *N Engl J Med* **373**, 209-219 (2015).

18.   Cristofanilli, M., *et al.* Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. *Lancet Oncol* **17**, 425-439 (2016).

19.   Turner, N.C., *et al.* Overall Survival with Palbociclib and Fulvestrant in Advanced Breast Cancer. *N Engl J Med* **379**, 1926-1936 (2018).

20.   McCaw, Z.R., Vassy, J.L. & Wei, L.J. Palbociclib and Fulvestrant in Breast Cancer. *N Engl J Med* **380**, 796 (2019).

21.   Slamon, D.J., *et al.* Overall Survival with Ribociclib plus Fulvestrant in Advanced Breast Cancer. *N Engl J Med* **382**, 514-524 (2020).

22.   Rocca, A., Melegari, E. & Palleschi, M. Ribociclib and Endocrine Therapy in Breast Cancer. *N Engl J Med* **381**, 1592 (2019).

23.   Im, S.A., *et al.* Overall Survival with Ribociclib plus Endocrine Therapy in Breast Cancer. *N Engl J Med* **381**, 307-316 (2019).

24.   Johnston, S., *et al.* Randomized Phase II Study Evaluating Palbociclib in Addition to Letrozole as Neoadjuvant Therapy in Estrogen Receptor-Positive Early Breast Cancer: PALLET Trial. *J Clin Oncol* **37**, 178-189 (2019).

25.   Gianni, L., *et al.* Neoadjuvant treatment with trastuzumab and pertuzumab plus palbociclib and fulvestrant in HER2-positive, ER-positive breast cancer (NA-PHER2): an exploratory, open-label, phase 2 study. *Lancet Oncol* **19**, 249-256 (2018).

26.   Cottu, P., *et al.* Letrozole and palbociclib versus chemotherapy as neoadjuvant therapy of high-risk luminal breast cancer. *Ann Oncol* **29**, 2334-2340 (2018).

27.   Chow, L.W.C., Morita, S., Chow, C.Y.C., Ng, W.K. & Toi, M. Neoadjuvant palbociclib on ER+ breast cancer (N007): clinical response and EndoPredict's value. *Endocr Relat Cancer* **25**, 123-130 (2018).

28.   Ma, C.X., *et al.* NeoPalAna: Neoadjuvant Palbociclib, a Cyclin-Dependent Kinase 4/6 Inhibitor, and Anastrozole for Clinical Stage 2 or 3 Estrogen Receptor-Positive Breast Cancer. *Clin Cancer Res* **23**, 4055-4065 (2017).

29.   Buyukkaramikli, N.C., *et al.* Ribociclib with an Aromatase Inhibitor for Previously Untreated, HR-Positive, HER2-Negative, Locally Advanced or Metastatic Breast Cancer: An Evidence Review Group Perspective of a NICE Single Technology Appraisal. *Pharmacoeconomics* **37**, 141-153 (2019).

30.   Tekpli, X., *et al.* An independent poor-prognosis subtype of breast cancer defined by a distinct tumor immune microenvironment. *Nat Commun* **10**, 5499 (2019).

31. Jabeen, S.*, et al.* Serum cytokine levels in breast cancer patients during neoadjuvant treatment with bevacizumab. *Oncoimmunology* **7**, e1457598 (2018).

32. Elkrief, A., Derosa, L., Zitvogel, L., Kroemer, G. & Routy, B. The intimate relationship between gut microbiota and cancer immunotherapy. *Gut Microbes* **10**, 424-428 (2019).

33. Routy, B.*, et al.* The gut microbiota influences anticancer immunosurveillance and general health. *Nat Rev Clin Oncol* **15**, 382-396 (2018).

34. Kroemer, G. & Zitvogel, L. Cancer immunotherapy in 2017: The breakthrough of the microbiota. *Nat Rev Immunol* **18**, 87-88 (2018).

35. Derosa, L., Routy, B., Kroemer, G. & Zitvogel, L. The intestinal microbiota determines the clinical efficacy of immune checkpoint blockers targeting PD-1/PD-L1. *Oncoimmunology* **7**, e1434468 (2018).

36. Zitvogel, L.*, et al.* Cancer and the gut microbiota: an unexpected link. *Sci Transl Med* **7**, 271ps271 (2015).

37. Viaud, S.*, et al.* The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Science* **342**, 971-976 (2013).

38. Gopalakrishnan, V., Helmink, B.A., Spencer, C.N., Reuben, A. & Wargo, J.A. The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy. *Cancer Cell* **33**, 570-580 (2018).

39. Routy, B.*, et al.* Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* **359**, 91-97 (2018).

40. Ebert, P.J.R.*, et al.* MAP Kinase Inhibition Promotes T Cell and Anti-tumor Activity in Combination with PD-L1 Checkpoint Blockade. *Immunity* **44**, 609-621 (2016).

41. Deng, J.*, et al.* CDK4/6 Inhibition Augments Antitumor Immunity by Enhancing T-cell Activation. *Cancer Discov* **8**, 216-233 (2018).

42. Goel, S.*, et al.* CDK4/6 inhibition triggers anti-tumour immunity. *Nature* **548**, 471-475 (2017).

43. Zhang, J.*, et al.* Cyclin D-CDK4 kinase destabilizes PD-L1 via cullin 3-SPOP to control cancer immune surveillance. *Nature* **553**, 91-95 (2018).

44. Petroni, G., Formenti, S.C., Chen-Kiang, S. & Galluzzi, L. Immunomodulation by anticancer cell cycle inhibitors. *Nat Rev Immunol* (2020).

45. Goel, S. & Tolaney, S.M. CDK4/6 inhibitors in breast cancer: a role in triple-negative disease? *Lancet Oncol* **20**, 1479-1481 (2019).

46. Wang, L.*, et al.* Functional genetic polymorphisms in the aromatase gene CYP19 vary the response of breast cancer patients to neoadjuvant therapy with aromatase inhibitors. *Cancer Res* **70**, 319-328 (2010).

47. Geisler, J., Smith, I. & Miller, W. Presurgical (neoadjuvant) endocrine therapy is a useful model to predict response and outcome to endocrine treatment in breast cancer patients. *J Steroid Biochem Mol Biol* **131**, 93-100 (2012).

48. Bellet, M.*, et al.* Palbociclib and ribociclib in breast cancer: consensus workshop on the management of concomitant medication. *Ther Adv Med Oncol* **11**, 1758835919833867 (2019).

49. Krainick-Strobel, U.E.*, et al.* Neoadjuvant letrozole in postmenopausal estrogen and/or progesterone receptor positive breast cancer: a phase

IIb/III trial to investigate optimal duration of preoperative endocrine therapy. *BMC Cancer* **8**, 62 (2008).

50. Dixon, J.M*., et al.* Increase in response rate by prolonged treatment with neoadjuvant letrozole. *Breast Cancer Res Treat* **113**, 145-151 (2009).

51. Ellis, M.J. & Ma, C. Letrozole in the neoadjuvant setting: the P024 trial. *Breast Cancer Res Treat* **105 Suppl 1**, 33-43 (2007).

52. Eiermann, W*., et al.* Preoperative treatment of postmenopausal breast cancer patients with letrozole: A randomized double-blind multicenter study. *Ann Oncol* **12**, 1527-1532 (2001).

53. Mustacchi, G*., et al.* Neo-adjuvant exemestane in elderly patients with breast cancer: a phase II, multicentre, open-label, Italian study. *Ann Oncol* **20**, 655-659 (2009).

54. Tubiana-Hulin, M*., et al.* Exemestane as neoadjuvant hormonotherapy for locally advanced breast cancer: results of a phase II trial. *Anticancer Res* **27**, 2689-2696 (2007).

55. Takei, H*., et al.* Multicenter phase II trial of neoadjuvant exemestane for postmenopausal patients with hormone receptor-positive, operable breast cancer: Saitama Breast Cancer Clinical Study Group (SBCCSG-03). *Breast Cancer Res Treat* **107**, 87-94 (2008).

56. Mlineritsch, B*., et al.* Exemestane as primary systemic treatment for hormone receptor positive post-menopausal breast cancer patients: a phase II trial of the Austrian Breast and Colorectal Cancer Study Group (ABCSG-17). *Breast Cancer Res Treat* **112**, 203-213 (2008).

57. Barnadas, A*., et al.* Exemestane as primary treatment of oestrogen receptor-positive breast cancer in postmenopausal women: a phase II trial. *Br J Cancer* **100**, 442-449 (2009).

58. Semiglazov, V*., et al.* Exemestane (E) vs tamoxifen (T) as neoadjuvant endocrine therapy for postmenopausal women with ER+ breast cancer (T2N1-2, T3N0-1, T4N0M0). *Journal of Clinical Oncology* **23**, Abstract # 530 (2005).

59. Johansson, H.J*., et al.* Breast cancer quantitative proteome and proteogenomic landscape. *Nat Commun* **10**, 1600 (2019).

60. Valdes-Mora, F*., et al.* Single-Cell Transcriptomics in Cancer Immunobiology: The Future of Precision Oncology. *Front Immunol* **9**, 2582 (2018).

61. Parker, J.S*., et al.* Supervised risk predictor of breast cancer based on intrinsic subtypes. *J Clin Oncol* **27**, 1160-1167 (2009).

62. Esquivel-Velazquez, M*., et al.* The role of cytokines in breast cancer development and progression. *J Interferon Cytokine Res* **35**, 1-16 (2015).

63. Kawaguchi, K*., et al.* Alteration of specific cytokine expression patterns in patients with breast cancer. *Sci Rep* **9**, 2924 (2019).

64. Jabeen, S*., et al.* Noninvasive profiling of serum cytokines in breast cancer patients and clinicopathological characteristics. *Oncoimmunology* **8**, e1537691 (2019).

65. Bathen, T.F*., et al.* Feasibility of MR metabolomics for immediate analysis of resection margins during breast cancer surgery. *PLoS One* **8**, e61578 (2013).

66.   Furberg, A.S., Veierod, M.B., Wilsgaard, T., Bernstein, L. & Thune, I. Serum high-density lipoprotein cholesterol, metabolic profile, and breast cancer risk. *J Natl Cancer Inst* **96**, 1152-1160 (2004).
67.   Garcia-Murillas, I.*, et al.* Assessment of Molecular Relapse Detection in Early-Stage Breast Cancer. *JAMA Oncol* (2019).