# Immune-dependent mechanisms of anti-Her2 therapy

## Summary

Understanding the mechanisms governing sensitivity and resistance to therapy will have profound implications to breast cancer treatment and patient care.

Recent data indicate immunoediting, i.e. selection of immune privileged cellular subclones, to play an important role in tumour evolution and probably also towards therapy efficacy. This may be of particular importance to immunotherapy, but recent evidence indicates that anti-Her2 therapy may also act by triggering an immune response, pointing to a role of immunoediting in anti-Her2 therapy.

We hypothesize that a *synergy* between breast cancers' immunogenic mutations and anti-Her2 therapy triggers an immune response and underlies a desired efficacy of the therapy.

Our team has unique therapy-related biobanks suitable for addressing these questions. Thus, in two previous neoadjuvant trials (the PETREMAC- and the DDP-trial), patients with Her2 positive tumours were treated with either dual-drug (PETREMAC) or single-drug (DDP) anti-HER2 therapy in concert with taxane. Snap-frozen biopsies were collected pre- and post therapy, in concert with formal response assessment. Further, we are currently launching a second-generation dual anti-Her2 therapy trial (PETREMAC 2), including longitudinal sampling pre-, inter-, and post therapy, together with response monitoring. These materials provide an ideal setting to explore the mechanisms underlying anti-Her2 therapy sensitivity and resistance, including assessment of the evolution and/or elimination of different subclones of cancer cells as well as the dynamics of immune cells, during treatment.

The results of this investigation may fundamentally change our understanding of the mechanism(s) by which anti-Her2 therapy executes its anti-tumour effects in individual patients and may pave the way both for better personalized treatment and for future therapy developments.

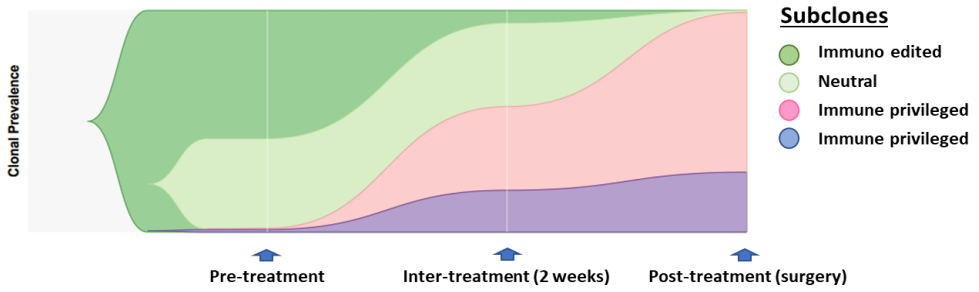


**Figure 1.** Hypothesis: during treatment with anti-Her2 therapy, immune privileged subclones will progress while those with immunogenic mutations will be eradicated (immunoediting).

## 1. About the project

### 1.1 State of the art and knowledge needs

#### 1.1.1 State of the art

Breast cancer is the most frequent malignant disease affecting women in the western world, with an annual incidence of about 3.700 cases in Norway alone. While chemotherapy administered neoadjuvant (before surgery) or adjuvant (after surgery) reduces the relapse rate by about 35% (*1, 2*), the fact that two out of three patients do not benefit from such treatment clearly illustrates the need for improved therapy.

Breast cancers may be classified based on biological parameters. About 15-20% overexpress the human epidermal growth factor receptor 2 (Her2) proto-oncogene (*3*). Historically, before application of anti-Her2 therapy, Her2 amplification was associated with poor prognosis (*4*). Today, applying anti-Her2 therapy (trastuzumab) on top of chemotherapy reduces the hazard ratio for relapse by an additional 40% among patients with Her2 positive tumours (*5*). Despite this, many patients still relapse on contemporary therapy.

Little is known regarding the mechanisms underlying resistance to systemic cancer treatment. Single gene defects associated with reduced or enhanced sensitivity to different drugs have been identified (*6, 7*) but comprehensive mapping of resistance, allowing for personalized therapy selection, is lacking (*8*). Gene expression signatures like the OncotypeDX (*9*) and the MammaPrint classification (*10*) provide prognostic information and may be used to select patients where chemotherapy can be omitted based on a low risk of relapse, but these tests are not predictive for sensitivity to specific therapeutics (*11*). For anti-Her2 therapy, overexpression of Her2 is the obvious predictive biomarker, but little is known regarding the mechanisms of resistance in patients where treatment fails. Several trials on anti-Her2 therapy have assessed different molecular features to identify additional prognostic and predictive factors, including immunological assessments. **However, importantly, most of these studies have been hampered by single timepoint biopsies, lacking the possibility of longitudinal assessments of clonal evolution during the treatment** (*12, 13*). Regarding potentially predictive biomarkers, *in vitro* data has linked loss of *ARID1A* to resistance to the anti-Her2 drug trastuzumab (*14*), while gene expression analyses have found an association between a STAT3-signature in tumours and trastuzumab resistance (*15*). Recently, a gene expression signature related to reactive stroma was found to predict trastuzumab resistance in Her2-positive, ER-negative breast cancers, but the sensitivity and specificity is not high enough for clinical application for therapy selection (*16*).

While these findings are interesting, it is important to note that the currently used anti-Her2 drugs trastuzumab and pertuzumab are both humanized IgG-antibodies that bind to the Her2 protein on the cancer cell surface. Trastuzumab binds to and blocks signaling from the Her2-protein, while pertuzumab binds to another epitope on Her2 and prevents dimerization with Her1/3 (*17*). Blocking Her2 signaling is considered an important mechanism of action. However, emerging evidence shows that both these anti-body-based drugs have the ability to trigger immunological reactions as that this is an (at least) equally important mechanism of action (*18-21*). Thus, both drugs are found to attract immune cells, causing cancer cell death by antibody-dependent cell mediated cytotoxicity (ADCC) (*17*). **This provides a strong rationale for assessing immunologic aspects of anti-Her2 therapy further.**

The level of tumour infiltrating lymphocytes (TILs) has been found to be a general prognostic marker across breast cancers of different subclasses but also predictive for response to trastuzumab (*18-20*). However, the quantity of TILs is a descriptive marker of immune activation, and the underlying molecular aberrations causing TIL infiltration in the tumour tissue remains unclear. The potential importance of tumour genomics to TIL infiltration in breast cancer is indicated by the findings that TIL counts play a key role to prognosis in triple negative and Her2 positive breast cancers. These are two subtypes harbouring the highest level of indels and structural rearrangements, likely to generate neoantigens (*22*) that are able to trigger an immune response. In contrast, TILs seem to play a limited role in breast cancers of the luminal subtypes which, in general, harbour less mutations.

Recent advances in the clinical use of immune checkpoint inhibitors have triggered investigations into the interaction between tumour mutation burden, specific mutation profiles and the immune response. Importantly, tumour mutation burden, as a surrogate marker for immunogenicity, has been linked to response to checkpoint inhibitors, but this association has not been fully explored with respect to anti-Her2 therapy. While it is generally accepted that cancer metastases undergo sub-clonal expansion in a “darwinistic” evolution (“survival of the fittest”), recent data has indicated that this selection to a large extent may be driven through interaction with the immune system (*23*): subclones with a high load of immunogenic mutations are likely to be eradicated (so-called *immunoediting*), while “immune privileged” subclones are more likely to progress (*23*). While this process, so far, is mainly characterized in the metastatic tumour evolution, we believe similar mechanisms may play a role in early cancers as well, in particular in response to therapies interacting with the immune system.

### 1.1.2 Knowledge needs

Breast cancer treatment has improved significantly over the last 40 years, but the fact that 600 Norwegian women die from the disease annually underlines the need for therapy improvement. Breast cancer is a heterogenous disease and therapy improvements must be obtained through more

individualized treatment. This requires refined translational research and clinical trials applying individualized therapy based on appropriate biomarkers. A prerequisite for such trials is to identify the optimal biomarkers that can predict which drug has the best effect for each patient. This need is illustrated by the fact that only about 50% of patients with Her2+ tumours undergoing optimal pre-surgical (neoadjuvant) therapy achieve a pathological complete response (pCR), the best surrogate marker for long-term survival in this patient group (*24*).

While traditionally, sensitivity to treatment has been related to intracellular features in the cancer cells, it is becoming increasingly clear that the immune response plays a significant role to treatment outcome. The aim of the current project is to explore the *interaction* between the individual tumours' genomic alterations and the immune response. This will lead to a better understanding of the mechanisms directing anti-tumour efficacy in Her2 positive breast cancers treated with anti-HER2 therapy.

## 1.2 Project objectives / research questions

**Primary objective:**

To identify the potential **synergistic** role of **immunogenic mutations** and **anti-Her2 therapy** with respect to triggering immunoediting and treatment response in Her2-positive breast cancer.

**Secondary (sub-)objectives:**

A. Define the predictive value of immunogenic mutations in pre-treatment biopsies, with respect to response to anti-Her2 therapy.
B. Pinpoint the subclonal dynamics (longitudinal changes in subclonal composition) in each tumour during neoadjuvant anti-Her2 therapy, with emphasis on identifying immunogenic mutations that are present in tumours / subclones eradicated by anti-Her2 therapy (immunoediting) versus those persisting through the therapy.
C. Explore the role of tumour infiltrating lymphocytes (TIL) and different subtypes of immune cells under treatment, with respect to immunoediting and response to anti-Her2 therapy.

## 1.3 Project plan

### 1.3.1 Hypothesis

We hypothesize that there is an interaction between immunogenic mutations and immune cells during anti-Her2 therapy (immunoediting), governing the response to treatment. This favours the eradication of tumours and subclones with immunogenic mutations by anti-Her2 therapy, while immune privileged tumours and subclones may progress (Figure 1). This contrasts conventional thinking where eradication of cells is assumed to be caused by direct toxic drug-effects on sensitive cells.

### 1.3.2 Theoretical approach and methodology

The objectives set for the project and the research questions raised will be addressed by a structured approach where we will take advantage of a unique set of key resources:

- We will perform initial analyses in the biobank collected in the PETREMAC trial (see Biobanks), a biobank built specifically for projects assessing mechanism of therapy response in breast cancer.
- We will compare data from patients who received dual- versus single drug anti-Her2 therapy, in the PETREMAC- and the DDP-trials, respectively (see Biobanks).
- We will perform validation of findings, in samples from a novel independent in-house biobank to be collected from in the neoadjuvant trial PETREMAC 2 (see Biobanks).
- We will perform structured analyses comparing molecular and immune cell characteristics between patients with good versus poor response to therapy as well as longitudinal intra-patient comparisons (see WPs 1-4), focusing on the interplay between immunogenic mutations and the attraction of specific immune cell subtypes.

**Biobanks**

***Initial (main) samples:*** We will use our own unique, prospectively collected biobank material and clinical data set from the **PETREMAC trial**, from which we recently published our first paper (*25*). This

was a clinical neoadjuvant study (NCT02624973), enrolling patients with large T2 or locally advanced breast cancers. The study and the biomaterial (tumour tissue and blood samples) collected, provides several advantageous features for studying mechanisms of drug sensitivity and resistance in breast cancer: Each patient underwent tumour biopsies (fresh frozen) for research purposes prior to and after treatment. At the same time, each patient had close monitoring of clinical response, scored according to the standardized response evaluation criteria in solid tumours (RECIST1.1) (*26*) and, at surgery, assessment of pathological complete response (pCR). Patients were allocated to eight different treatment arms, depending on biomarker profiles. Regarding Her2-positive breast cancers, today, dual anti-Her2 therapy by application of both trastuzumab and pertuzumab together with taxane chemotherapy is standard-of-care for patients with Her2 positive breast cancers in need of neoadjuvant therapy (*27, 28*). In PETREMAC, for the first time in Norway, this combination was administered to Her2-positive patients (Figure 2), together with docetaxel. For patients with tumours harbouring *TP53* mutations, cyclophosphamide was added in concert. In total, PETREMAC enrolled 60 Her2-positive patients (34 in arm E and 26 in arm F). The material provides an optimal setting for statistical comparison between responders versus non-responders (48.3% obtaining a pCR).

***Validation samples:*** For validation of findings, we will use samples from two others of our own unique prospectively collected biobanks and clinical data sets: the upcoming PETREMAC 2-trial and the completed DDP-trial (NCT00496795). In **PETREMAC 2**, patient inclusion and treatment stratifications are designed on the basis of preliminary molecular analyses of the original PETREMAC-trial. Thus, defects in genes involved homologous recombination repair (HRD) will be used as a biomarker. Patients with Her2-positve and HRD-wild-type tumours will be treated with dual anti-Her2 therapy in concert with docetaxel, while patients with HR-deficient tumours will receive the same therapy, but

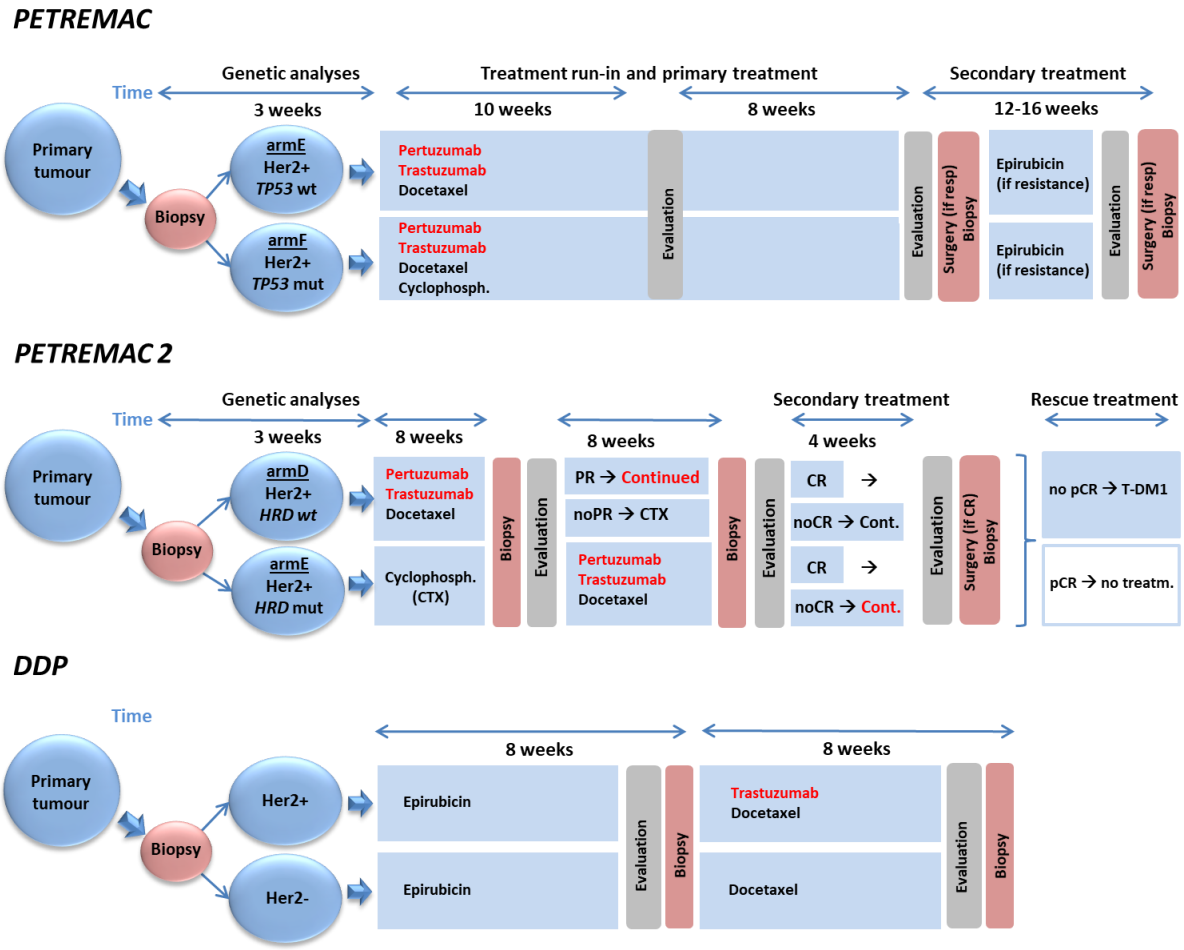


**Figure 2.** Schematic overview of neoadjuvant treatment of patients with Her2-positive primary breast cancers in the PETREMAC-, PETREMAC 2-, and DDP-trials. Biopsies marked by pink boxes and anti-Her2 treatment marked in red font. PETREMAC and DDP are completed trials, while PETREMAC 2 is under initiation.

after a period 8 weeks on high dose cyclophosphamide monotherapy (Figure 2). PETREMAC 2 is designed to enroll 450 patients, out which 185 are estimated to have Her2-positive tumours. From these, 30 poor and 30 good responders will be selected for analyses, to match the 60 from PETREMAC. Based on the inclusion rate from PETREMAC, the inclusion period in PETREMAC 2 is estimated to 30 months. In the **DDP-trial**, patients with locally advanced breast cancer received neoadjuvant treatment administered as sequential monotherapy of dose dense epirubicin followed by docetaxel. Patients with Her2-positive tumours received single drug anti-Her2 therapy (trastuzumab) in concert with the docetaxel (Figure 2). Patients underwent tumour biopsies (fresh frozen) for research purposes prior to treatment, at switch from epirubicin to docetaxel, and (if residual tumour left) at the time of surgery. The number of Her2-positive tumours in the DDP-trial was 24 out of whom eight had a pCR.

***Biomaterial:*** Importantly, **sufficient high-quality DNA, RNA and tissue sections for all molecular analyses** in the present project is already isolated from the samples in both the PETREMAC- and the DDP trials. Identical procedures for sample handling will be followed in PETREMAC 2.

**Approach and Methodology**

The project is organized into the four work packages (WPs) described below. For all WPs, initial analyses will be performed in samples from the PETREMAC-trial (Figure 2), while validations will be performed in samples from PETREMAC 2 and DDP. Notably, data from DDP will also be used to assess any potential difference between single- and dual drug anti-Her2 therapy.

**WP1: Predictive immunogenic mutations in pre-treatment biopsies**

***Research question:*** *What are the differences between patients with good versus poor response to anti-Her2 therapy with respect to molecular characteristic, pre-treatment? (secondary objective A).*

***Approach:*** We will perform whole genome sequencing (WGS) and whole transcriptome sequencing (WTS) of pre-treatment biopsies from the patients described above. Thus, we will provide a comprehensive data set of all mutations, copy number alterations, structural rearrangements and expression levels in each biopsy. In addition to expression levels, WTS will also enable us to detect fusion transcripts, which are typically good candidates / markers for immunogenic proteins as they indicate the generation of fusion proteins. We will then perform ***inter-patient*** comparisons of tumour mutations in treatment resistant versus sensitive tumours. Findings in pre-treatment biopsies will be compared between patients with subsequent response classified as pCR vs non-pCR, and also between clinical response groups (objective response vs non-response; illustrated in Figure 3A). Assessment of predictive value will be performed for mutations affecting single genes and after classifying mutations into functional pathways. Further, particular emphasis will be on predicted immunogenic mutations.

***Methodology:*** The wet-lab procedure of library preparation for WGS will be done by application of Lucigen's NxSeq AmpFREE DNA kit and sequencing will be done on our in-house Illumina NovaSeq high-capacity sequencer (see section 3.3). In order to achieve adequate confidence in our mutation calling, we aim to sequence the tumour samples to an average depth of 60x. In parallel to tumour DNA, we will sequence DNA from white blood cells from the patients, enabling identification of tumour specific (somatic) versus germline variants. This normal DNA will be sequenced to an average depth of 30x. Mapping, mutation calling, copy number analyses and detection of structural rearrangements will be run on our in-house informatics pipeline, mainly based on the Dragen system (illumina).

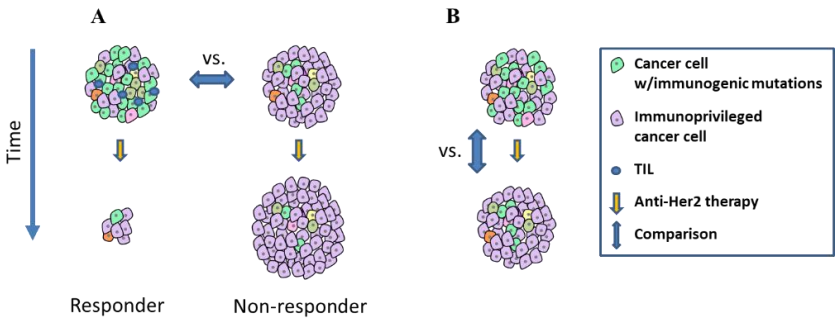


**Figure 3.** Assessment of predictive biomarkers.
**A:** Inter-patient comparison of pre-treatment markers between patients with different responses to the same treatment.
**B:** intra-patient comparison of subclonal composition, at different timepoints.

For the present proposal, it will be crucial to define immunogenic mutations / neoantigens and assign an immunoscore to each sample, based on the total mutational characteristics. Immunogenic mutations will be identified aided by prediction of HLA-binding using the netMHCpan (v3.0) algorithm, and an immunoscore will be assigned to each subclone in each patient based on previous algorithms (*23, 29*). Wet-lab procedures for WTS library preparation will be by application of Illumina's Stranded Total RNA Prep with Ribo-Zero. WTS will be done to a minimum of $100x10^6$ reads per sample. Mapping, quantification and detection of fusions will be run on our in-house informatics pipeline (Dragen).

**WP2: Subclonal dynamics during anti-Her2 therapy**

***Research question:*** *How does anti-Her2 therapy influence the longitudinal composition of subclones with respect to immune privileged subclones versus immunoedited ones, and what type of subclones are present in residual tumour tissue at the time of surgery? (secondary objective B).*

***Approach:*** We will perform WGS and WTS on the biopsies collected **under** and **after** anti-Her2 therapy in the trials described above (Figure 2). We will use this information to assess subclonal composition in each biopsy. We will perform ***intra-patient*** longitudinal comparisons to detect growth or shrinkage of particular subclones in the tumours, with particular emphasis on immunogenic mutations during the course of neoadjuvant therapy (Figure 3B). Both qualitative (mutation-, and expression patterns) and quantitative analysis (fractions of cells harbouring a certain pattern) across multiple patients will indicate types of mutations, genes and pathways associated with treatment resistance or sensitivity.

***Methodology:*** Wet-lab procedures and basic informatics handling will be as described under WP1. *Intra-patient* analyses require comparison of subclonal composition of tumours at different timepoints. This requires a more complex and integrated use of the generated data: The key to assessment of subclones and the fraction of cells from each subclone in a heterogenous tumour lies in estimates of variant allele frequencies (VAF; i.e. the frequency of reads carrying a mutation in a given nucleotide). However, such estimates cannot be made based on VAF alone: it is necessary to adjust all VAFs for copy number state in the region in which each mutation resides. Further, in order to assess any changes in the size of a certain subclone (e.g. between pre- and post-treatment samples), each sample must be corrected for tumour cell fraction which may differ substantially between biopsies. These corrections will be made by use of an integrated approach using both mutations, copy number- and SNP data. The backbone of this work will be the application of the PyClone algorithm and an in-house algorithm based on haplotype phasing to achieve higher resolution (unpublished).

**WP3: Dynamics of immune cells during anti-Her2 therapy**

***Research question:*** *What are the differences between patients with good versus poor response to anti-Her2 therapy with respect to the tumours' immune cell profile, pre-treatment and how does the composition of immune cells change during treatment? (secondary objective C).*

***Approach:*** We will perform immunohistochemical- and Hyperion-based qualitative and quantitative assessments of immune cells, focusing on tumour infiltrating lymphocytes (TIL) and macrophages, in samples from the tree trials described above. We will perform ***inter-patient*** comparisons of immune cell composition pre-treatment, in treatment resistant versus sensitive tumours (Figure 3A) and we will perform ***intra-patient*** longitudinal comparisons to detect the potential dynamics of immune cell composition in tumours / subclones that are shrinking or growing during anti-Her2 therapy (Figure 3B).

***Methodology:*** For the samples in the tree trials, HE-stained sections will be used in order to assess the level of immune cells in the tissue. Both intratumoural- and stromal immune cells will be scored for all breast cancer biopsies. In addition to a crude total TIL-count, we will perform immunohistochemical (IHC) analyses quantifying $CD4^+$ and $CD8^+$ cells to pinpoint the dynamics of subtypes of TILs. Since recent data has indicated macrophages to be the key immune cells in some breast cancers (*30*), we will also stain specifically for $CD68^+$ and CD163+ cells. Further, we will assess NK-cells (Granzyme $B^+$ / $CD3^-C56^+$ cells), as markers for commencing / ongoing antibody-dependent cell mediated cytotoxicity (ADCC), since the anti-Her2 therapies are anti-body-based. To correct for other markers potentially

influencing the immunogenic characteristics of each tumour, we will perform IHC to quantify PD1 and PD-L1 in all samples. For a subset of patients with extreme immunoscores from WPs 1 and 2 (n=20 high vs n=20 low, for all trials taken together), we will perform extended high resolution immune cell characterisation in sections from pre-, under-, and after treatment biopsies. This will be performed by assessing an established panel of 41 antibodies on a Hyperion imaging platform. This will enable us to assess whether specific (non-predefined) subtypes of immune cells may be attracted to tumours with particularly high immunoscores and also to assess the dynamics of such subtypes during treatment.

**WP4: Integrated analyses of immunogenic mutations and TILs during anti-Her2 therapy**

***Research question:*** *What is the synergistic impact of immunogenic mutations and anti-Her2 therapy with respect to triggering immunoediting and treatment response in Her2-positive breast cancer? (primary objective).*

***Approach:*** WPs 1-3 will assess the predictive value and the dynamics of subclones with immunogenic mutations and tumour associated immune cells separately. While we envision those analyses to yield important results, we will go on to assess the potential interactions between immunogenic mutations and immune cells with respect to both predictive value and also how such potential interaction may change during anti-Her2 therapy. In particular, **we will assess whether applications of anti-Her2 therapy attracts immune cells of specific subtypes to the tumour over time and, if so, how such an attraction may differ between tumours with high versus low immunoscores with respect to mutations.** All findings will be correlated to response to anti-Her2 therapy.

***Methodology:*** Integrated analyses, as outlined under "Approach", above, will mainly be performed by multivariable logistic regression models where mutational parameters and immune cell parameters are entered, together with their interactions. The major endpoints used as fixed / dependent variable in these models will be the clinical response groups and/or pathological complete response status (as outlined under WP1).

### 1.3.3 Statistical power

Formal estimates of statistical power are limited by the exploratory nature of the project. However, we have modelled scenarios based on the number of patients potentially having immunogenic mutations among those with pCR versus a non-pCR (Table 1). While our main analyses are limited to 60 patients (PETREMAC trial), the study population provides an optimal (in statistical terms) distribution with respect to pCR, with 29 and 31 out of 60 having a pCR vs non-pCR, respectively, enabling comparison between the two groups. The power estimates (Table 1) indicate that the sample size should be adequate for the project. Notably, these estimates only take into account the number of pre-treatment samples. Analyses of samples from several timepoints, from each patient (Figure 3B), adds substantial strength to the power each patient will contribute to potential identification of predictive markers. Further, substantial strength will be added by validation analyses in the PETREMAC 2- and the DDP trials, where longitudinal assessments in each individual patient also will be performed.

**Table 1.** Statistical power for assumed scenarios. "imm+" indicates positivity for immunogenic mutations.

| n Total | pCR | non-pCR | pCR (imm+) | non-pCR (imm+) | α | 1- β |
|---|---|---|---|---|---|---|
| 60 | 29 | 31 | 20 (70%) | 6 (20%) | 0.05 | 0.97 |
| 60 | 29 | 31 | 23 (80%) | 6 (20%) | 0.05 | 1.00 |
| 60 | 29 | 31 | 20 (70%) | 9 (30%) | 0.05 | 0.83 |
| 60 | 29 | 31 | 23 (80%) | 9 (30%) | 0.05 | 0.97 |

## 1.4 Ethical considerations and data management

The PETREMAC- and the DDP trial, including all molecular analyses described in the present proposal are approved by all regulatory authorities, including the Norwegian Drug Agency (SLV), the regional data handling authority (Personvernombudet) and Regional ethics committee (REC approvals: 2015/1493/REKvest; 06/3077/REKvest). Patients have provided written informed consent and have chosen whether they want to be informed about incidental findings. If pathogenic germline alterations are identified, patients are offered genetic counselling. Tissue samples are stored in the biobank of the

Dept. of Oncology, Haukeland University Hospital (HUH), under Norwegian legislation. For the PETREMAC2 2-trial, approvals are pending, but all ethics aspects are similar as for PETREMAC and DDP.

Sensitive patient information will be stored and handled within the hospital files of HUH. The only exception will be that some de-identified data will be processed within the University of Bergen's IT-infrastructure for sensitive data ("S.A.F.E."; see also section 3.3 Project infrastructure). Linking of genetic- and clinical information will be performed after de-identification. However, the oncologists treating the patients will keep a key for this de-identification, to be able to inform about any germline findings and to link genetic findings to long term survival, in accordance with REC recommendations.

# 2. Impact

## 2.1 Impact for cancer patients and next of kin

Her2-positive breast cancer constitutes about 15-20% of breast cancers in total. An improved understanding of the molecular causes of resistance or sensitivity to neoadjuvant therapy, as outlined in this proposal, will be of great benefit to this subgroup of patients. Firstly, this relates to the improved possibilities to predict which type of therapy will be most efficient for each individual patient and thereby ensure early commencement of effective treatment. Secondly, such knowledge will enable oncologists to avoid therapy predicted not to have effect. The latter will spare patients for side effects of unnecessary treatment and improve their quality of life. In addition to the impact for the patients in question, both more effective treatment as well as the avoidance of unnecessary side effects, will also benefit the quality of lives of the relatives / next-of-kin to the patients.

## 2.2 Societal impact

A long-term aim for improved personalized neoadjuvant therapy is to improve prognosis and curation rates. Such improvement of the number of breast cancer patients that are cured by primary treatment will have massive societal impact as it will relief the workload and financial strains on the health care system, currently related to long term treatment of patients with relapse / metastatic disease. Importantly, improved individualized treatment could reduce application of (expensive) treatments predicted to fail for individual patients, further reducing costs for the health care system.

Improved curation rates in the neoadjuvant setting for this group of patients will also lead to an increase in patients being brought back to regular employment and thereby contribute to society in general, rather than remaining patients in the health care system.

Notably, in an international perspective, applying expensive therapies to only those patients likely to benefit, will reduce total cost and contribute to lowering the threshold for developing countries to apply advanced cancer drugs.

For international research, the present project represents a novel and unique approach to assess mechanisms underlying sensitivity and resistance to anti-Her2 therapy. The results are therefore very likely to be taken forward by other researchers and, as such, generate many high quality spin-off projects within basic-, translational- and clinical research.

## 2.3 Dissemination, communication and exploitation strategy

We will publish data from the project in peer-reviewed scientific journals, aiming at journals with high impact. A minimal requirement is that the journals are listed in the most commonly used databases such as Pubmed and Web of Science. We will seek to make all findings openly available (i.e. publish in open access journals) and data sets will be made openly available in repositories (e.g. GEO and EGA), unless restriction are required due to patient privacy. We regularly attend large international meetings, such as the annual meetings of ESMO, AACR, ASCO etc., and results will be presented here.

For dissemination to stakeholders, we are regularly attending and giving talks at meetings for user organizations and we have a close collaboration with our User's Advisory Board (see online application form). For the general public, we provide regular updates on our website for the team (www.uib.no/en/rg/g18) as well as on the applicant's website (www.uib.no/en/persons/Stian.Knappskog). Data of particular interest to the public will be reported in general media, via the communication departments at our university and hospital. Our findings often receive attention in the

media: over recent years, our research has been communicated in major Norwegian TV channels (NRK and TV2), radio (NRK P1), newspapers (BT, BA, VG), and in international media (Daily Express, RTBF).

The data generated in the present project may have a great innovation potential. Identification of markers predictive of therapy response, is of massive importance in a clinical setting. As a consequence of this, our findings may lead to development of biomarker assays for detection / quantification of such biomarkers. If so, we will enlist help of our Technology Transfer Office (VIS) to develop suitable IPR- and commercialization strategies.

# 3. Implementation

## 3.1 Project manager and project group

The project will be managed by the applicant (Stian Knappskog). After completing a recruitment grant as Senior Research Fellow, I was appointed as a full-time professor of translational cancer research at the University of Bergen in 2019. I have extensive experience in cancer genomics and have worked on similar projects leading to high impact publications both within our own team (*31*) and in international collaborations (*32, 33*), as well as attracted major grants for my research on this topic (see CV).

The project team is listed in Table 2. We see it as necessary to hire a technician to take care of the majority of wet-lab sample preparation and WGS/WTS. If granted, the project will receive an additional PhD-student position financed by the Faculty of Medicine, UiB. This student will work closely together with the PI on both the wet-lab activities and the data handling / interpretation.

The proposed project will benefit hugely from an integrated collaboration within the Bergen Breast Cancer Group (BBCG). Especially the clinical oncologists **Hans P. Eikesdal** and **Per E. Lønning** will be instrumental. Eikesdal and Lønning have been the clinical PIs for the PETREAMAC- and the DDP trials and will also lead the PETREMAC 2-trial. Close collaboration with their teams, in particular with respect to translational and clinical interpretation of the results, will be utterly important. Also, the project will benefit from collaboration with **Emiel Jansen** (Stavanger University Hospital). Jansen has been responsible for centralized histology assessment within the PETREMAC-trial and will have the same role in PETREMAC 2. He will assess immune cells and immune markers in WPs 3 and 4.

A senior technician (Laura Minsaas) will be in charge of the wet-lab NGS work and a senior scientist bioinformatician (Wei Deng) will be in charge of the informatics analyses. The latter field is moving fast and we will adapt new informatics strategies throughout the project. Subsequent to my research stay at the Wellcome Sanger Institute, Cambridge, UK (2011-2012) we have an active collaboration (*32-35*), through which the present project (especially WP2) will benefit from access to new developments in handling and interpretation of massive parallel sequencing data.

## 3.2 Project organization and management

Work in the WPs will be overlapping in time and conducted according to the plan outlined in Figure 4.

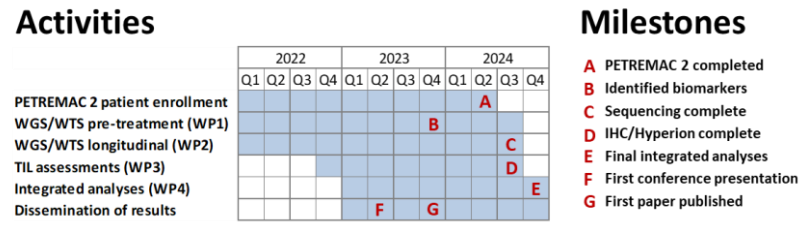


**Figure 4.** Timelines of activities and milestones for the proposed project.

**Table 2.** Contributors and roles

| Contributor | Expertise | Role / contribution | WP |
|---|---|---|---|
| Stian Knappskog | Cancer genomics | Project leader, integrated analyses and interpretation | 1-4 |
| Hans P. Eikesdal | Breast oncology | Clinical and translational interpretation of data | 1-4 |
| Per E. Lønning | Breast oncology | Clinical and translational interpretation of data | 1-4 |
| Emiel Jansen | Pathology/IHC | TIL quantification, IHC of immunomarkers | 3-4 |
| Laura Minsaas | Wet-lab NGS | NGS library-prep and run of sequencing instruments | 1-2 |
| Wei Deng | Bioinformatics | Mutation calling, expression- and epigenetic analyses | 1,2+4 |
| Technician (NN) | Wet-lab NGS | NGS library-prep and run of sequencing instruments | 1-2 |
| PhD-student (NN) | Wet-lab and dry-lab | Wet lab and data handling / interpretation | 1-4 |

The proposed project will be managed by the applicant and performed by my team and collaborators. The contributions from the project participants towards the different WPs are specified in Table 2.

## 3.3 Project infrastructure

The required infrastructure for the present project is already established within the Bergen Breast Cancer Group. We have established a substantial infrastructure and experience in cancer genomics which has resulted in several high impact publications both based on in-house work (*31*) and collaborations with international teams (*32, 33, 35*). **Wet-lab work** will be performed within the Mohn Cancer Research Laboratory, Haukeland University Hospital. This is a modern facility, with state-of-the-art technology for translational cancer research. Importantly, this includes an in-house sequencing facility. We have one NovaSeq instrument, for whole genome- and whole transcriptome sequencing, and two MiSeq instruments for targeted panel sequencing and targeted promoter methylation sequencing. Hyperion analyses will be performed at the Flow Cytometry Core Facility of the University of Bergen. Regarding **data processing**, we currently have dedicated servers for handling of sequencing data. This includes the "Dragen" from Illumina, especially built for rapid mapping and processing of sequencing data. Through previously established custom-made pipelines and the "Dragen", we have a fully operational infrastructure for QC, mapping, variant calling and downstream handling of genetic information from our samples (see WPs 1 and 2). Regarding **data storage**, we have storage domains directly linked to our in-house sequencing infrastructure (within the frameworks of the IT-departments at the University of Bergen and Haukeland University Hospital). These are scalable solutions, and built to take on large data sets, including the data planned for in the present proposal.

## 3.4 Risk assessment

The possibility exists that we will not be able to identify any clear re-distribution of subclones during anti-Her2 therapy that can be linked to immunogenic mutations and/or presence of immune cells. Given contemporary knowledge, we consider such a scenario to be unlikely. However, even such a negative finding would also be valuable since it may point the research field in different directions.

On an operational level, the present project relies on analyses within three different biobanks, one of which is not yet completed. Although it is not likely (based on the experience from the original PETREMAC-trial), there is a risk that completion of the sample collection in the PETREMAC 2 may suffer delays, hampering the present analyses. However, we will run analyses continuously and even if PETREMAC 2 should not be completed, we will analyse a substantial number of patients from this trial within the time frames of the present project. Notably, in a worst-case scenario, the present project could even be run based only on the PETREMAC- and the DDP-trials as there is sufficient samples size (power) in these samples sets, without samples from PETREMAC 2.

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# Addition to project description

**– Specification of the role of the PhD student (Hatice Toprak Dogramaci)**

The PhD project will be conducted within the overarching program "Immune-dependent mechanisms of anti-Her2 therapy". The program, and the previously submitted project description, contains 4 work packages (WPs) that will be conducted across 3 clinical trials. The PhD-student will be in charge of performing the described analyses in all 4 WPs but restricted to one of the trials, the PETREMAC trial.

Among the 3 clinical trials, the DDP trial applied an older regimen with a single anti-Her2 drug and the PETREMAC2 trial will not be completed in the near future. As such, although both these trials are highly relevant for the overall research question, the trial best suited for a restricted PhD-project, both for scientific and practical reasons, is the PETREMAC-trial. In PETREMAC, all patients with Her2-positive breast cancer were treated with dual anti-Her2 therapy; trastuzumab and pertuzumab. Both these drugs are antibodies and even though their main mechanism of action is by blocking the Her2-receptor, our hypothesis is that they also may have an anti-tumor effect by attracting immune cells via general antibody-dependent cell mediated cytotoxicity (ADCC).

Following the original project plan, the PhD-student (Dogramaci) will perform analyses of neoantigens / immunogens in whole genome sequencing and whole transcriptome sequencing data from pretreatment biopsies, in order to assess if the composition of such mutations has a predictive value for response to the anti-Her2 treatment. Further, she will assess a similar question in a longitudinal setting by analysing pre- and post-treatment samples from the same patients. Thereby, she will be able to assess what subclones in any analysed tumor that disappear versus which subclones that grow during the treatment. This will add value to the overall interpretation of predictive markers. A third angle will be to assess the longitudinal evolution, not from the perspective of tumour cells, but by analyses of immune cells. This will be done by assessment of immune cell composition and infiltration in the tumours during treatment and importantly also by assessing whether there may be a *synergism* between the presence of neoantigens and anti-Her2 therapy with respect to the attraction / infiltration of immune cells.

The PETREMAC trial is completed. All biopsies are taken and all clinical data has been recorded, including response evaluation for all patients treated with anti-Her2 therapy. The whole genome sequencing and whole transcriptome sequencing has been performed. The PhD-student has started bioinformatics handling of these data and is currently optimizing and performing neoantigen predictions in the pretreatment samples.

**Publication plan:**

**Paper I (related to WP1 in the original project description)**
Tentative title: Predictive markers for response to anti-Her2 therapy
Research question: What are the differences between patients with good versus poor response to anti-Her2 therapy with respect to molecular characteristic, pre-treatment?
Approach: Identify neoantigens in whole genome sequencing data from anti-Her2 treated tumours in the PETREMAC-trial and compare the number and repertoire of neoantigens across patients (i.e. between different response groups.
Intended role for the PhD-student: First author

**Paper II (related to WP2 in the original project description)**

Tentative title: Subclonal tumour dynamics during anti-Her2 therapy

Research question: How does anti-Her2 therapy influence the longitudinal composition of subclones with respect to immune privileged subclones versus immunoedited ones, and what type of subclones are present in residual tumour tissue at the time of surgery?

Approach: Identify neoantigens in whole genome sequencing data from anti-Her2 treated tumours in the PETREMAC-trial, both pre and post-treatment. Then use this information, along with the overall mutation data, to predict subclones and subclonal evolution during the treatment. By linking neoantigenes to subclones and follow the evolution of subclones one may assess whether specific subclones that are eradicated may have a particular neoantigen/immunogenic profile, making them more susceptible for immune cell attacks.

Intended role for the PhD-student: First author

**Paper III (related to WPs 3 and 4 in the original project description)**

Tentative title: Dynamics of tumor immune cell invasion during anti-Her2 therapy

Research question: What are the differences between patients with good versus poor response to anti-Her2 therapy with respect to the tumours' immune cell profile, pre-treatment and how does the composition of immune cells change during treatment? Is there a synergism between the level of neoantigens and anti-Her2 therapy with respect to the attraction of immune cells during treatment.

Approach: Identify immune cells by a set of approaches such as IHC, Hyperion and RNA sequencing as well as mapping the spatial distribution relative to cancer cells (Hyperion) This will be done from anti-Her2 treated tumours in the PETREMAC-trial, both pre and post-treatment. Then use this information to pinpoint the evolution / invasion of immune cells in the tumor during treatment and, in an integrated analysis, assess whether presence of neoantigens and immune cells have a major impact on final outcome (treatment response).

Intended role for the PhD-student: First author