CLINICAL TRIAL PROTOCOL

Prostate Androgen Response Investigation using a Stratification BIOmarker

Predicting prostate cancer downstaging by neoadjuvant Darolutamide with PCAI ImmunoScore in a non-randomised open label prospective trial

PARIS-BIO

Trial ID:	PARIS-BIO
EU Trial number:	2025-520639-17-00
Version number:	Version 3.0
Date:	16 Feb 2026
Sponsor:	Karolinska University Hospital
Sponsor representative:	Peter Wiklund

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Revision history

Protocol version	**Date of Issue**	**Summary of changes** *Describe all changes since the first final protocol.*
1.0	25-March-2025	First final protocol
2.0	28-May-2025	PSA assessments added on day 30 and day 60 to enable detection of non-responders with rapid progression. The population for the analysis of side-effects and adverse events has been changed from a per protocol to a more appropriate intention to treat population. A page header with trial information has been added and an incorrect reference changed
3.0	16-Feb-2026	Statistical analysis populations updated: primary and secondary efficacy analyses will be conducted in both the Intention-to-Treat (ITT) and Per-Protocol (PP) populations. Implementation of central pathology review of biopsy and prostatectomy specimens. Implementation of central MRI review. Addition of a new secondary endpoint: association between PCAI ImmunoScore and Residual Cancer Burden (RCB), defined as residual tumour volume in mm^3 corrected for tumour cellularity. Updated inclusion criterion to “A clinically relevant Prostate MRI”. Correction of references

Signature page

Sponsor

I am responsible for ensuring that this protocol includes all essential information to be able to conduct this trial. I will submit the protocol and all other important trial-related information to the responsible investigator(s) so that they can conduct the trial correctly. I am aware that it is my responsibility to hold the staff members who work with this trial informed and trained.

Signature of sponsor's representative Date

Peter Wiklund

Principal Investigator

I have read this protocol and agree that it includes all essential information to be able to conduct the trial. By signing my name below, I agree to conduct the trial in compliance with this clinical trial protocol, the EU Regulation on clinical trials of medicinal products for human use (EU 536/2014), the Declaration of Helsinki, ICH-GCP (Good Clinical Practice) guidelines and the current national regulations governing the conduct of this clinical trial.

I will submit this protocol and all other important trial-related information to the staff members and investigators who participate in this trial, so that they can conduct the trial correctly. I am aware of my responsibility to continuously keep the staff members and investigators who work with this trial informed and trained.

I am aware that quality control of this trial will be performed in the form of monitoring and eventual audit and inspection.

Principal Investigator’s signature Date

Printed name

Contact information

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Study coordination, safety reporting and clinical monitoring organisation	Clinical Trials Office (CTO), Center for Clinical Cancer studies, Theme Cancer, Karolinska University Hospital, Stockholm

List of used acronyms and abbreviations

Abbreviation	Term/Explanation
ADT	Androgen deprivation therapy
Adverse Event (AE)	Any untoward medical occurrence in a subject to whom a medicinal product is administered and which does not necessarily have a causal relationship with this treatment.
AR	Adverse Reaction = any unfavourable and unintended reaction to an investigational medicinal product, regardless of dose
AR	Androgen receptor
ARPI	Androgen-receptor pathway inhibitor
ASR	Annual Safety Report = the annual safety report for reporting to authorities. In Sweden this is the Swedish Medical Products Agency via CTIS.
AUROC	Area under the receiver operating characteristics curve
BCR	Biochemical recurrence
CTIS	Clinical Trials Information System = Centralized EU database/portal for application and communication with authorities concerning clinical trials. In Sweden this includes the Swedish Medical Products Agency and the Swedish Ethical Review Authority.
CTR	EU Regulation 536/2014, also called CTR, Clinical Trials Regulation
ECOG	Eastern Cooperative Oncology Group
eCRF	Electronic Case Report Form
EPE	Extraprostatic extension (on MRI)
ePROM	Electronic patient-reported outcome measures
FFPE	Formalin-fixed paraffin-embedded
GCP	Good Clinical Practice
GnRH	Gonadotropin-releasing hormone
HRQoL	Health-related quality of life
IB	Investigator’s Brochure
ICH	International Council for Harmonization
IMP	Investigational medical product
ISUP	International society of urological pathology

Läkemedelsverket	Swedish Medical Products Agency – the national authority responsible for regulation and surveillance of the development, manufacturing and sale of medicinal products.
Member State	European Union (EU) Member state where an application for authorisation of a clinical trial or of a substantial modification has been submitted.
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
NGS	Next generation sequencing
PCa	Prostate cancer
PCAI	prostate cancer artificial intelligence
pCR	Complete pathological response
PI-RADS	Prostate imaging reporting & data system
PP	Per Protocol analysis = including only data from subjects who have completed the trial completely in accordance with the protocol, with no deviations from the protocol
PSA	Prostate specific antigen
pT stage	(final) pathology tumour stage
RCB	Residual cancer burden
RNAseq	Ribonucleic acid sequencing
RP	Radical prostatectomy
RSI	Reference safety information. A list of all known serious adverse reactions for the investigational medicinal product, including severity and frequency of the adverse reaction. The RSI is contained in the Summary of Product Characteristics or IB and is used to determine which adverse reactions should be reported as suspected unexpected serious adverse reactions (SUSARs).
Serious Adverse Event (SAE)	Any untoward medical occurrence that at any dose requires inpatient hospitalisation or prolongation of existing hospitalisation, results in persistent or significant disability or incapacity, results in a congenital anomaly or birth defect, is life-threatening, or results in death
SPC or SmPC	Summary of Product Characteristics
SUSAR	Suspected Unexpected Serious Adverse Reaction. This is an event that is likely related to the investigational medicinal product but with unexpected occurrence. An adverse reaction is unexpected if its nature or seriousness is not consistent with the information on the product in

	the RSI.

1. Synopsis

EU Trial number:	2025-520639-17-00
Title:	Prostate Androgen Response Investigation using a Stratification BIOmarker
Trial ID:	PARIS-BIO
Short background/ Rationale/Aim:	High-risk prostate cancer patients are at substantial risk for biochemical recurrence and metastatic progression after local therapy. The presence of micro-metastatic disease, undetectable by conventional imaging, likely contributes to the poor prognosis. Neoadjuvant hormonal therapy, particularly with the advent of ARPIs like Darolutamide, has shown promise, but patient selection remains crucial for optimizing outcomes. The study hypothesizes that the PCAI ImmunoScore biomarker will be an effective predictor of response to neoadjuvant Darolutamide treatment for prostate cancer, as determined by the presence of remaining cancer on pathology following radical prostatectomy The aim of the study is to evaluate whether PCAI ImmunoScore can predict patient response to neoadjuvant Darolutamide, potentially paving the way for more personalised treatment and improved clinical results.
Primary objective:	To evaluate the predictive value of the pre-treatment genomic biomarker PCAI ImmunoScore for pathologic minimal residual disease (MRD) in patients undergoing neoadjuvant Darolutamide treatment before radical prostatectomy.
Secondary objectives:	To evaluate the predictive value of the pre-treatment genomic biomarker PCAI ImmunoScore for other pathologic and radiologic outcomes To evaluate hormonal side effects and functional outcomes
Primary endpoint:	The association between the pre-treatment PCAI

	ImmunoScore and the occurrence of minimal residual disease (MRD). This will be assessed on final pathology after the radical prostatectomy
Secondary endpoint:	Assessed on final pathology after the radical prostatectomy: Complete pathologic response. Final pathology pT-stage. Size of any residual tumour. MRI after treatment with Darolutamide but before prostatectomy: Changes in tumour size. Change in the risk of tumour growth through the prostate capsule. Correlation between the MRI response and tumour response on final pathology. Assessed through questionnaires and safety reporting: Hormonal side effects during and after treatment. Functional outcomes.
Trial design:	Open label prospective trial where subjects are treated with Darolutamide for 90-120 days and undergo radical prostatectomy between day 90 and day 120 after start of the drug. Before radical prostatectomy, Darolutamide treatment is discontinued.
Trial population:	Patients with biopsy-verified high-risk prostate cancer as defined below.
Number of subjects:	100
Inclusion criteria:	• Patients must be >= 18 years of age and able to understand the written study information • A clinically relevant Prostate MRI • Biopsy-confirmed high-risk prostate cancer defined as a global ISUP ≥4 or global ISUP=3 with an MRI PIRADS score = 5 • Candidate for radical prostatectomy • ECOG Performance Status score of 0 or 1 • Able to receive Darolutamide for 90-120 days • Signed informed consent form
Exclusion criteria:	• M1 or N2 disease on diagnostic workup. • Prior hormone treatment for prostate cancer. • Prior systemic or local therapy for prostate cancer, including pelvic radiation to the prostate.

	• Major surgery <=4 weeks prior to inclusion.
Intervention:	• Darolutamide treatment for 90-120 days until radical prostatectomy. • Study-specific MRI after day 90 but before radical prostatectomy • Study specific blood samples at six different time-points • HRQoL questionnaires at five time-points during the 16 months trial period
Investigational medicinal product(s), dosage, administration:	Darolutamide (Nubeqa): 2x300 mg tablets orally twice daily for 90-120 days
Ethical considerations, benefit/risk:	For the patient there is a potential benefit with the use of the drug that may reduce the risk of prostate cancer relapse, The risks include hormonal side effects.
Planned duration of the trial:	Q3 2025 – Q1 2030

2. Background and rationale

Patients with high-risk prostate cancer (PCa) face a significant likelihood of experiencing biochemical recurrence (BCR) and metastatic progression after receiving local therapy [1, 2]. Despite accounting for only 20% of all localized PCa cases, this subgroup is responsible for nearly 70% of PCa-related deaths within 10 years of diagnosis [3]. This prognosis is likely due to the presence of micrometastatic disease, which is undetectable by conventional imaging at the time of diagnosis [4-9].

Hormone treatment for prostate cancer has been used since the 1940s. Prostate cancer cells typically rely on androgens to grow. Initially, surgical castration was the standard treatment for these patients. Today, however, there are medications that target androgens to manage the disease [10, 11]. These treatments include:

Androgen Deprivation Therapy (ADT): Reduces the patient's androgen levels (medical castration).

Anti-androgens: Block the effects of androgens on prostate cancer cells.

Combination Therapy: Uses both approaches to enhance treatment effectiveness.

The use of neoadjuvant hormonal therapy before surgery in PCa is supported by two key rationales: it may reduce tumour volume, thereby downstaging the disease and decreasing the likelihood of positive resection margins, and it could also eradicate micro metastases that are not yet visible in preoperative imaging [12].

Since the 1990s, several prospective studies have explored the impact of neoadjuvant hormonal therapy prior to radical prostatectomy (RP) in localized PCa. Although these studies showed significant reductions in rates of positive surgical margins, node-positive disease, in addition to tumour downstaging, they did not demonstrate an improvement in survival when compared to surgery alone [13]. However, these studies primarily involved patients with low- and intermediate-risk PCa, lacked long-term follow-up, and were not designed to detect potential survival benefits [13]. Additionally, these trials used older hormonal therapies, mainly ADT such as luteinizing hormone-releasing hormone agonists and antagonists, but also antiandrogens, sometimes in combination with ADT, or estrogen-derived therapies [13]. With the advent of more potent androgen receptor signalling inhibitors such as the second and third generation anti androgens (ARPIs), there has been renewed interest in neoadjuvant hormonal therapy for high-risk PCa.

ARPIs, such as Darolutamide, when given in combination with androgen deprivation therapy (ADT) have shown significant overall survival benefits over ADT alone in both

metastatic castration-resistant and metastatic hormone-sensitive settings [14]. This raises the question if similar benefits can be observed when ARPIs are used as a neoadjuvant approach before RP in high-risk PCa setting.

However, these drugs are expensive [15, 16] and previous studies have reported a median response rate – defined as complete pathological response or MRD in a neoadjuvant setting – of approximately 21% across nine trials in this field, with a range of 4 to 44% [17]. It is assumed that patients with pCR and/or MRD will benefit most from the neoadjuvant treatment regime. Thus, there is a need to enable selection of patients with a strong response to pre-surgical neoadjuvant ARPI to potentially enrich for patients that will have longitudinal benefit, including improved post-surgical progression-free survival outcomes.

Prostate Cancer Artificial Intelligence (PCAI) immunoscore

The Prostate cancer AI (PCAI) ImmunoScore is a genomic biomarker signature comprising the gene expression of the following 8 genes: ABCC5, CUX2, KIAA1549, RAP1GAP2, PDE4D, SLC39A11, TDRD1, VWA2. The bioinformatics partner in this trial, Philips Electronics, has previously developed a logistic regression model which calculates the PCAI ImmunoScore based on the expression of the 8 genes based on RNA sequencing (RNAseq) [18, 19]. Based on preliminary studies and studies in later stages of the prostate cancer disease there is evidence that the PCAI ImmunoScore biomarker can be employed to select ‘exceptional’ responders to pre-surgical, neo-adjuvant ARPI treatment based on the analysis of pre-treatment tissue samples.

Three data sets from studies where patients have been treated 3-6 months with ARPI before undergoing radical prostatectomy have been analysed for the feasibility to demonstrate whether the PCAI ImmunoScore biomarker signature can predict complete response after RP pathology or BCR after RP using NGS transcriptomics data from the study subjects. The subjects included in these cohorts (30-50 patients per study) were all patients with localized or locally advanced, high-risk prostate cancer and were eligible for surgery [20-21]. In all studies pre-treatment needle biopsy samples were collected before patients were subjected to 3 or 6 months of neo-adjuvant combination therapy with ARPI and ADT. RNAseq was performed on selected biopsy samples. For two studies the endpoint was defined as complete pathology response (pCR) and/or minimal residual disease (MRD) on pathology after RP. Patients achieving pCR and/or MRD on post-surgical pathology were defined as exceptional responders. All other patients were defined as non-responders.

In two of the studies with complete remission (pCR) or MRD on pathology as endpoint the PCAI ImmunoScore was able to separate responders from non-responders (AUROC 0.85 and 0.92, respectively; see Figure 1 below).

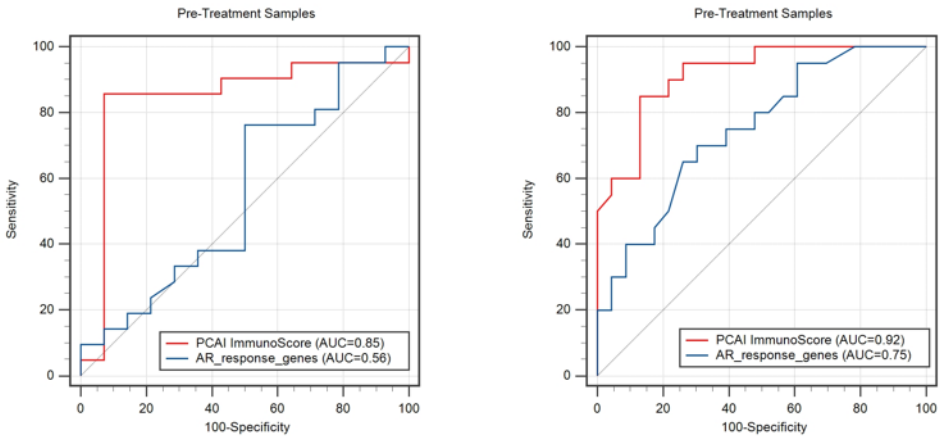

Figure 1: AUROC (area under the ROC curve) of the PCAI ImmunoScore biomarker for prediction of treatment outcome in two cohorts. Left picture: RNA seq data from Wilkinson S et al [20] Right picture: RNA seq data from Tewari AK et al.[21]

The PCAI ImmunoScore biomarker was tested on 2 existing cohorts (30-50 patients per study) with high-risk, localized or locally advanced, prostate cancer. All patients were treated for 6 months with ARPI before undergoing surgery [20-22]. Approx. 30% of the patients in each cohort reached the endpoint of pCR or MRD. These patients were defined as “exceptional responders”. A pre-treatment FFPE biopsy sample collected by needle biopsy of the prostate was used to create RNA sequencing data. These data were used to determine the ImmunoScore based on a model that was trained on external prostate cancer RNAseq data set [23]. In both data sets, the PCAI ImmunoScore did separate “exceptional responders” from “non-responders” with high accuracy (AUROC >=0.85) compared to a model where 15 known AR response genes were combined.

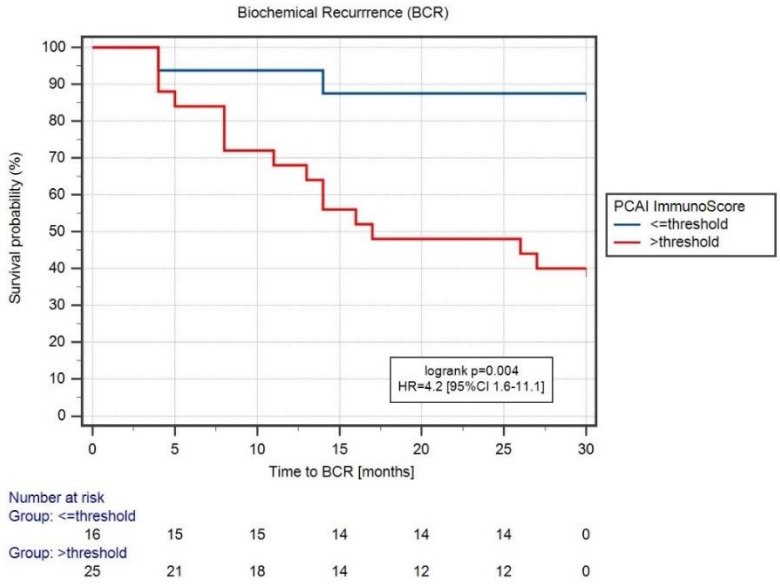

Figure 2: Kaplan Meier survival analysis of 41 patients included into the DARANA trial.

In the DARANA study, patients with high-risk, localized/locally advanced prostate cancer were treated for 3 months with Enzalutamide before undergoing surgery [22]. Patients were monitored after surgery for PSA recurrence. A pre-treatment FFPE biopsy sample collected by needle biopsy of the prostate was used to create RNA sequencing data. This data were used to determine the ImmunoScore based on a model that was trained on an external prostate cancer RNAseq data set [23].The PCAI ImmunoScore did separate “responders” (i.e., low risk of PSA progression) from “non-responders” (i.e., significant risk of post -surgical disease progression) with high accuracy (HR=4.2; logrank p=0.004) [22] (Figure 2).

The proposed study herein, PARIS-BIO, is a retrospective analysis of patient samples collected during diagnosis and prior to treatment to validate a predictive biomarker (PCAI ImmunoScore) potentially associated with response to Darolutamide treatment. Unlike the previously described validation of the biomarker, this will be the first study where the patient population is not retrospectively identified. Although the objective of PARS-BIO is to evaluate the association between the biomarker and response to Darolutamide, we believe that the study does not fall under the scope of the In Vitro Diagnostic Regulation (IVDR) for the following reasons:

- The biomarker is not used to stratify patients. All patients in the trial will receive the same treatment
- This trial will not be used for CE-marking or to guide treatment decisions in the future. Should this study establish an association between the biomarker and the treatment response, Philips will undertake a properly designed Performance study.

Darolutamide as neoadjuvant treatment before radical prostatectomy

Darolutamide currently does not have an approved indication for neoadjuvant treatment before radical prostatectomy according to the European Medicines Agency, the Swedish national prostate cancer guidelines, or the European Association of Urology guidelines [24-26]. However, the drug is approved for use in the later stages of PCa, and a recent meta-analysis has shown that it has a similar safety profile to placebo when given together with ADT, and despite lack of direct head-to-head comparisons with other ARPI compounds, it concluded Darolutamide the preferred ARPI compound in regards to the side effect profile [27]. There are also other ARPIs recommended in the Swedish national prostate cancer guidelines as adjuvant treatment to radiotherapy for very high-risk PCa [25].

To reduce the amount of side effects, Darolutamide will not be administered in combination with ADT and the duration of neoadjuvant treatment will be limited to the shortest time necessary to achieve downstaging [28, 29]. The documented use of Darolutamide as monotherapy is limited. However, as it has similar antiandrogen mechanisms as first-generation antiandrogens, such as bicalutamide, where monotherapy is associated with better quality of life, especially in regard to fitness, sexual activity, and hot flashes compared to combination therapy with ADT, a similar side-effect profile is expected [30]. First generation anti-androgens have also been shown to have less serious long-term side effects and lower decline in comorbidity compared to ADT [31, 32]. Furthermore, in a phase II study exploring safety and efficacy of Darolutamide as monotherapy compared to ADT alone in patients with hormone-sensitive prostate cancer, no specific safety signals were found, side effects were considered mild, and quality of life in the Darolutamide group was higher [29]. Other second and third generation ARPIs have also been evaluated as monotherapy and generally found to be well tolerated and without any specific safety signals [33-36].

The estimated delay of surgery of up to 120 days due to the additional treatment is not expected to impact the future risk of recurrence, as studies have shown that surgical treatment can be postponed for up to 180 days in high-risk prostate cancer without compromising short-term or long-term outcomes [37, 38].

3. Benefit-risk evaluation

As the study is designed to evaluate if the PCAI ImmunoScore biomarker can predict tumour response to Darolutamide treatment, the potential benefit of the biomarker is only for future patients. A biomarker capable of predicting treatment response to

antiandrogen receptor signaling inhibition in high-risk PCa would aid patient selection and reduce both the personal and societal costs of ineffective treatment. PARIS-BIO represents the next crucial step towards the goal of establishing such a biomarker.

There are theoretical benefits from neoadjuvant Darolutamide treatment for the PARIS-BIO patients, including a decreased risk of tumour recurrence and improved functional outcomes due to the tumour shrinkage. There is also a chance of decreased sequalae such as erectile dysfunction and urinary incontinence from the surgery procedure if the tumour shrinkage allows for increased preservation of nerve bundles and urinary sphincters.

The potential benefits of the Darolutamide treatment are for all participating patients, although our assumption is that the biomarker-positive patients will benefit more. With the design of the study the biomarker positivity or negativity will not influence the study treatment, nor the surgeon's or the patient's attitude since the result of the PCAI Immunoscore will not be known to anyone until after the surgery.

Risks and inconveniences from combination treatment with Darolutamide and ADT are considered to be mild and are described in the Darolutamide registration study, most commonly as fatigue, pain in extremities, skin rash, diarrhea and decreased appetite when used in combination with ADT [26]. In the PARIS-BIO study Darolutamide will be used as monotherapy. The most common side effects of Darolutamide as monotherapy are gynecomastia, fatigue, hot flashes, and hypertension and are generally considered grade 1 or 2 [27]. Darolutamide is also considered the ARPI with the most favorable side effect profile [27]. We also aim to avoid excess hormonal side effects, especially post treatment, by excluding ADT and treating with Darolutamide alone. By using Darolutamide as monotherapy, we expect a faster testosterone recovery, and thereby reduced long-term side effects compared to combination therapy [37]. The short-term treatment of 90-120 days is selected to allow time for the shrinkage of tumour while limiting side effects.

The 90- 120 days delay of surgery is a potential risk but is not very different from the within 68 days of diagnosis stated in the clinical routine guidelines in Sweden. Moreover, studies have shown that prostate cancer treatment can be delayed for up to 180 days in high-risk prostate cancer without compromising short-term or long-term outcomes [35, 36].

One could also consider the possibility of increased surgical margins, that potentially could lead to worse oncological outcomes due to that the surgeon after reviewing the study MRI would underestimate the tumour and perform a less radical surgical approach in order to reduce poor functional outcomes. This has however not been the

case in previous neoadjuvant hormone treatments [38] and the surgical setting will be with only a limited number of surgeons performing the surgeries at only two sites.

Overall, the potential benefits of neoadjuvant hormonal treatment prior to radical prostatectomy in terms of improved oncological and functional outcomes significantly outweigh the adverse risks, especially for responders. Further, the clinical and health economic benefits of finding a biomarker for patient selection of antiandrogen receptor signalling inhibition treatment is substantial.

We conclude that the benefit-risk assessment for this trial is positive.

4. Trial objectives

4.1. Primary objectives

The primary objective of this trial is to assess the utility of the pre-treatment genomic biomarker PCAI ImmunoScore in predicting the pathologic response, as measured by MRD, to neoadjuvant Darolutamide.

4.2. Secondary objectives

The secondary objectives of this trial are:

- To assess the association of the genomic biomarker PCAI Immunoscore with specific variables of response to neoadjuvant Darolutamide (see 4.5).
- To assess the correlation between Pathological response and MRI response after neoadjuvant Darolutamide.
- To assess hormonal side effects of Darolutamide during treatment and up to 12 months after treatment start (as reported, not compared)
- To assess functional outcomes after radical prostatectomy, such as incontinence and impotence (as reported, not compared)

4.3. Exploratory objective

In addition to the primary and secondary objectives, the study also has two exploratory objectives: 1) to generate hypothesis for future clinical studies to identify treatment options for non-responders to Darolutamide, based on DNA sequencing data. 2) to investigate whether there are morphological features on digitized images from prostate biopsy material that predict response to Darolutamide and PCAI ImmunoScore.

4.4. Primary endpoint

The primary endpoint is the association of MRD with the pre-treatment genomic biomarker PCAI ImmunoScore.

Minimal Residual Disease (MRD) is defined as <0.05 cm³ residual tumour volume after RP.

4.5. Secondary endpoints

The secondary endpoints of this trial are:

- The association of the PCAI ImmunoScore with:
    - Residual Cancer Burden (RCB), defined as residual tumour volume in mm³ corrected for tumour cellularity.
    - Pathologic complete response (pCR).
    - pT-stage at final pathology.
    - Size of largest cross-sectional dimension of residual tumour on pathology.
    - Number of resection specimen slides in which the tumour can be seen on pathology.
    - MRI assessment of changes in tumour size, cross sectional dimension, volume, and EPE.
    - Blood PSA concentration during treatment

- Correlation between Pathological response and MRI response.
- Hormonal side effects during and after treatment, measured through AE/SAE registration and repeated validated questionnaires (EPIC-26SF – Expanded Prostate Cancer Index Composite, short form, appendix I) as well as changes in blood testosterone concentration
- Post- surgical functional outcomes (erectile function and urinary continence) in the cohort measured through questionnaires (Swedish national ePROM, appendix II)

4.6. Exploratory endpoints

- The association of known genomics prostate cancer risk features (such as TMPRSS2-ERG fusion, AR splice variants, TP53/SPOP mutations, RB1/PTEN/NKX3.1 deletions, MYC amplification, defects in genomic DNA repair genes or homologous recombination repair deficiency, or alterations in PIK3 signalling pathway) to response to Darolutamide and PCAI ImmunoScore.
- The association of morphological features on digitized images from prostate biopsy material to response to Darolutamide and PCAI ImmunoScore.

5. Trial design and procedures

5.1. Overall trial design

This trial is a prospective, single-arm, open-label phase II trial in high grade PCa where all patients meeting the inclusion and exclusion criteria at Karolinska University hospital and Sahlgrenska University hospital will be offered participation in the study. All included patients will, as part of the study, be provided Darolutamide 2x300 mg tablet orally twice daily for 90-120 days (until the day before radical prostatectomy). After radical prostatectomy, the study subjects will be followed for 12 months.

5.2. Procedures and flow chart

Flow chart

Table 1 Flow chart

Procedure	*Screening* *Day/Week x Inclusion visit*	*Visit 1* *Baseline* *Day 0* *(bloodwork can be done up to 30 days before the visit)*	*Visit 2* *Day 30 (±10 days)*	*Visit 3* *Day 60 (±10 days)*	*Visit 4* *Day 90 (±10 days)*	*Radical Prostatectomy* *After Prostate MRI (any time between day 91- day 120)*	*As part of clinical routine* *3 months postop*	*As part of clinical routine* *12 mån postop*
Incl/exclusion criteria	√							
Informed consent	√							
Medical history/ concomitant medications	√				√			
Blood tests		√ + Testo	√ +PSA	√ +PSA	√ +PSA		Testo	Testo

Instructions for handling the medicinal product(s)		√						
Fill out Questionnaires		√		√			√	√
Prostate MRI					√			
Adverse Events (AE & SAE)			√	√	√		√	
Blood pressure		√	√	√	√			
Radical Prostatectomy						√		

Study visits

Screening

The following procedures are included in the screening:

- Eligibility and informed consent
- Medical history
- Concomitant medication

Baseline visit Day Zero

- Clinical laboratory assessments (blood) (up to 30 days before visit)
- Questionnaires (up to 30 days before or at visit)
- Blood pressure (at visit)
- Instructions for handling medicinal product

Visit Day 30 (± 10 days)

- Clinical laboratory assessments (blood)
- Adverse Events (AE & SAE)
- Blood pressure
- Treatment compliance

Visit Day 60 (± 10 days)

- Clinical laboratory assessments (blood)
- Adverse Events (AE & SAE)
- Blood pressure
- Treatment compliance
- Questionnaires

Visit Day 90 (± 10 days)

- Clinical laboratory assessments (blood)
- Adverse Events (AE & SAE)
- Blood pressure
- Treatment compliance
- Prostate MRI (after day 90)

Radical Prostatectomy (day 91 – 120, after Prostate MRI)

Visit at 3 months postoperatively (±1,5 months)

- Clinical laboratory assessments (blood)
- Questionnaires

Visit at 12 months postoperatively (±1,5 months)

- Clinical laboratory assessments (blood)
- Questionnaires

Study Procedures

The study procedures outlined in the flow chart and study visits are explained in more detail below.

- Blood samples (Hb, LPK, TPK, Na, K, Ca, creatinine, ASAT, ALAT) will be taken after inclusion and at one, two and three months after start of treatment (Study nurse visit, contact with site PI as needed). If side effects ≥ grade 3 the dose will be reduced to 300 mg twice daily until the symptoms improve ≤ grade 1.
- Serum testosterone levels will be measured at baseline and at 3 and 12 months post-operatively.
- Study specific PSA will be measured at one, two, and three months to observe for potential non-responders showing signs of rapid progression. This will be evaluated by the investigator to decide if a patient should be scheduled for an earlier Radical Prostatectomy or other treatments as per clinical routine.
- Blood pressure will be measured at inclusion and at the one-, two-, and three-month study nurse visits. If blood pressure is elevated, the patient will be referred to specialist for treatment of hypertension.
- At each study visit while on Darolutamide the patients will be asked if he is experiencing bothersome gynecomastia. If yes, the patient can be prescribed tamoxifen 10-20 mg daily for the remainder of the study
- An MRI is performed between day 90 and 120 and after this the patient goes for surgery before day 120.
- The patient will be asked to fill out a questionnaire – EPIC-26SF – before start of Darolutamide treatment and at each study visit.

After Darolutamide treatment, the patients are handled according to standard clinical routine including RP and follow-up by the treating physician and contact nurse. Patients will be asked to fill out the Swedish national ePROM questionnaire at specific intervals (appendix II). Blood tests, i.e. PSA, will be performed as part of clinical routine.

5.3. Biological sampling procedures

5.3.1. Handling, storage, and destruction of biological samples

Samples for the calculation of the PCAI ImmunoScore biomarker will be collected from pre-treatment prostate biopsies, taken before study inclusion, and will therefore not entail any additional procedures for the patient. Eight tissue sections à 5-10 µm from blocks containing cancer will be obtained from the Regional Biobanks and sent to OHMX.bio in Gent, Belgium, for biomolecular analysis as described in Appendix III.

In short, tissue sections one and eight will be stained with hematoxylin and eosin and digitized for pathology review (encircling of tumour areas). The images will be further analysed using image recognition software for extraction of morphological features. The stained tissue sections will be stored at OHMX.Bio for a maximum of 10 years after end of study.

Nucleic acids (RNA/DNA) will be co-extracted from tumour scraped from slides 2-7, but RNA and DNA will be stored, sequenced and analysed separately. After the analysis, the genomic material will be destroyed. A material transfer agreement (MTA) will be in place before any biopsy sample is sent for analysis.

The prostatectomy specimen will be processed according to clinical routine, and the evaluation of pathological response will be performed *in silico* on digital whole-slide images.

Blood samples will be taken for clinical laboratory assessments as described herein (study procedure 5.2). The analysis will be performed at the local accredited laboratory and the study-specific samples will be destroyed after analysis.

5.3.2. Total volume of blood per subject

The volume of blood taken from each subject during the trial is a maximum of 30 ml per study visit (at six different visits over a period of 16 months).

5.3.3. Biobank

All samples taken in this trial, covered by the Biobank Act, are registered in a biobank at Region Stockholm (Stockholms medicinska biobank, IVO reg. No 914) and handled according to the current biobank laws and regulations. The Biobank Act (2023:38) is not applicable for the samples for clinical laboratory assessments since these samples are not stored and are destroyed immediately after the analysis. All biobank samples are coded to protect the subject´s identity and the samples and the identification/code list are stored securely and separately to prevent access by unauthorised persons.

5.4. Start, end, temporary halt and early termination

5.4.1. Start of the clinical trial

The start of the trial is defined as the first study visit of the first subject. The sponsor will report the start of the trial in the Clinical Trial Information System (CTIS) within 15 days.

5.4.2. Temporary halt or early termination

The trial may be prematurely terminated for safety reasons affecting the risk-benefit balance or if the recruitment of subjects cannot be completed within reasonable time. Decisions on premature termination are taken by the sponsor. If this should happen, the Competent Authorities will be informed as soon as possible via CTIS, but no later than 15 days after trial suspension.

If the trial is prematurely terminated or suspended, the investigator should immediately inform the subjects and ensure appropriate treatment and follow-up.

5.4.3. End of the clinical trial

The trial ends when the last subject has completed the 12-month postoperative follow-up visit.

6. Subject selection

Men, ≥ 18 years old, with high-risk localized or locally advanced prostate adenocarcinoma eligible for radical prostatectomy included from Karolinska University Hospital, Stockholm and Sahlgrenska University Hospital, Gothenburg. All should be able to give informed consent and understand the patient information given in Swedish.

6.1. Inclusion criteria

To be included in the trial, subjects must meet all of the following criteria:

1) Patients must be >= 18 years of age
2) A clinically relevant Prostate MRI
3) Biopsy confirmed high-risk prostate cancer defined as a global ISUP-score ≥4 with any MRI PIRADS score or global ISUP=3 with any MRI PIRADS score =5
4) Candidate for radical prostatectomy
5) Eastern Cooperative Oncology Group (ECOG) Performance Status score of 0 or 1
6) Able to receive Darolutamide for 90-120 days
7) Signed informed consent form
8) A participant who is sexually active is eligible if he is willing to use a condom from the screening visit up to 1 week after last dose of Darolutamide except if the male participant is sterile (e.g. vasectomised); the unique female sexual partner is postmenopausal, is permanently sterilized (e.g. hysterectomy or tubal ligation) or use a highly effective method of contraception (< 1% documented failure rate).
9) Able to understand and comply with planned study procedures and willing to be available for all study-required procedures, visits and calls for the duration of the study.

6.2. Exclusion criteria

Subjects must not be included in this trial if any of the following criteria are met:

1) ≥M1 or ≥N2
2) Prior treatment with androgen receptor antagonists
3) Treatment with gonadotropin-releasing hormone (GnRH)
4) History of prior systemic or local therapy for prostate cancer, including pelvic radiation to the prostate
5) Major surgery <=4 weeks prior to inclusion

6.3. Screening and inclusion

Informed consent must be provided before performing any protocol-specific procedure and subject eligibility (that subjects fulfil all inclusion criteria and do not meet any exclusion criteria) is established before inclusion.

6.4. Withdrawal criteria

Subjects can discontinue their participation in the trial at any time without any consequence to his/her continued treatment. The investigator/sponsor can at any time terminate the trial for a subject due to, e.g., unacceptable adverse events/adverse reactions or because the subject does not follow procedures in the clinical trial protocol. If the subject discontinues the trial, follow-up of this subject will be performed according to the clinic's routine.

7. Trial treatments

All subjects included in the trial will be given Darolutamide (Nubeqa), 2x300mg tablet orally twice per day for 90-120 days. The patients will be treated until the day before their radical prostatectomy, which will happen day 90-120.

7.1. Description of investigational medicinal product

Darolutamide (Nubeqa) is approved by EMA for patients with non-metastatic castration resistant prostate cancer who are at high risk of developing metastatic disease and for patients with metastatic hormone-sensitive prostate cancer in combination with docetaxel and androgen deprivation therapy. The use of Darolutamide in hormone sensitive localized or locally advanced high-risk PCa (the patient population in PARIS-BIO) is currently not authorized. The safety profile and efficacy of Darolutamide as monotherapy was evaluated in a randomized phase 2 trial including hormone-sensitive, metastasized and non-metastasized, patients, with a treatment duration of 24 weeks. In this study, 48% of patients experienced at least one treatment-related adverse event of grade 1 or higher. The most commonly reported adverse events were

gynecomastia (29%), fatigue (13%), hot flashes (13%), and breast pain (7%). A single grade 3 treatment-related adverse event was observed—an increase in serum amylase levels (3%). Furthermore, the study shows that Darolutamide induce a profound suppression of PSA in all patients [30].

7.1.1. Dose and administration

Darolutamide (Nubeqa) will be supplied to the patients in blister packs with 112 x 300 mg tablets. Two tablets (600 mg) should be swallowed whole and together with food twice daily. The chosen dose, dosing regimen, and administration route are according to the EMA approval for Nubeqa. If a dose is missed, the dose should be taken as soon as the patient remembers prior to the next scheduled dose. The patient should not take two doses together to make up for a missed dose. The maximum duration of the study medication is 120 days. If the patient experiences a toxicity reaction of grade 3 or higher (CTCAE v5.0), or an intolerable side effect related to Darolutamide, the dose should be reduced to 300 mg twice daily until symptoms improve. Treatment may then be resumed at a dose of 600 mg twice daily. If the symptoms do not improve to a satisfactory degree, the investigator can decide to discontinue the treatment and terminate the study for that subject (see also 6.4).

7.1.2. Packaging, labelling, and handling of investigational medicinal products(s)

Nubeqa is supplied by the manufacturer (Bayer) in the local commercial package (blister) containing 112 filmcoated 300 mg tablets. It is provided free of charge and will be sent to ApoEx AB (Eugeniavägen 23, 171 64 Solna) where it will be additionally labelled with text in Swedish stating name and telephone of local PI, and “Endast för klinisk prövning, PARIS-BIO”). Distribution of the IMP to the two sites will be arranged by ApoEX.

The IMP is stable between 2 and 30°C and should be stored in the original package at room temperature. Any temperature deviations outside of the specified range should be logged by the study personnel and reported to Bayer who will decide if the IMP should be replaced.

7.1.3. Drug accountability and treatment compliance

The principal investigator is responsible for ensuring Darolutamide accountability. Upon receipt of the IMP the investigator or designated site staff will check for accurate delivery and acknowledge receipt by signing and dating the documentation provided by the central pharmacy and delivery company. Copies of each document will be filed in the TMF and in the ISF.

The dispensing of the IMP to study participants will be recorded at the site on the appropriate drug accountability form and an accurate accounting will be available for verification by the monitor at each monitoring visit. Study participants will return unused Darolutamide to the study site where it will be logged and subsequently destroyed according to the hospital routine.

IMP accountability records will include:

- Confirmation of IMP delivery to the trial site
- The inventory of IMP at the site provided by the sponsor
- The destruction of unused IMP
- Dates, quantities, batch numbers, expiry dates and the patient IDs assigned

Treatment compliance will be checked at the 30-, 60-, and 90-day visits when side-effects are also assessed. Patients will be asked if they have taken the medication according to instructions or not and any deviations will be recorded in the eCRF.

IMP which has been dispensed to a subject must not be re-dispensed to a different subject. Unused IMP must not be used for any purpose other than the present trial.

7.1.4. Randomisation

PARIS-BIO is a single arm trial and therefore no randomization will be performed.

7.1.5. Blinding

The trial is an open label, single arm trial. However, the patients' PCAI ImmunoScore will not be known at the time of treatment and will therefore not affect treatment and/or other clinical decisions.

7.1.6. Destruction

The subjects will return any unused Darolutamide to site where it will be logged in the drug accountability form and destroyed according to the local hospital routine

7.2. Concomitant use of other medicinal products and treatments

Medications considered necessary for the safety and well-being of the subject may be provided at the discretion of the investigators, unless otherwise specified in the exclusion criteria. If tamoxifen is given to reduce gynecomastia side effects this should be specified in patient records and in the eCRF. No mamillar radiation should be provided.

7.3. Treatment after trial end

At end of study, participants will return to treatment with standard of care according to

clinical routine.

8. Methods for measurement of endpoints for clinical efficacy and safety

8.1. Primary endpoint

The primary endpoint is the association of MRD with the pre-treatment genomic biomarker PCAI ImmunoScore.

The PCAI ImmunoScore is calculated based on the log2 transformed TPM (transcript per million) gene expression values for each of 8 specific genes based on RNA sequencing (RNAseq) of the diagnostic biopsy tissue (for details, see Statistics section). MRD is defined as < 0.05 cm3 residual tumour on final pathology after RP. The MRD analysis will be performed centrally by a uro-pathologist upon examination of digital whole-slide images of the final radical prostatectomy specimen. The volume of remaining viable prostate carcinoma following neoadjuvant treatment will be estimated based on its shape and three-dimensional measurements (all prostate tissue is embedded for histological analysis), as detailed in Appendix IV.

8.2. Secondary endpoints

Overall, secondary endpoints are divided into three categories:

- final pathology assessment
- non-invasive assessment – MRI and PSA
- side effects from the treatment.

First, the association between PCAI ImmunoScore and the following endpoints from the final pathology specimen will be evaluated:

- Residual Cancer Burden (RCB), defined as residual tumour volume in mm^3 corrected for tumour cellularity, assessed in the radical prostatectomy specimen
- Pathologic complete response (pCR) defined as no residual tumour visible at final pathology.
- pT-stage at final pathology.
- Size of largest cross-sectional dimension of residual tumour on pathology.
- Number of resection specimen slides in which the tumour can be seen on pathology.

All pathology assessments will be performed centrally to support quality assurance and ensure consistency in endpoint determinations.

Second, the association between PCAI ImmunoScore and the following endpoints from centrally reviewed MRI images will be evaluated:

- Changes in tumour size,
- Changes in EPE (assessment of risk of extraprostatic extension on a Likert scale 1-5)
- Correlation between Residual Cancer Burden (RCB) and MRI-derived response measures (tumour volume change and EPE change).

MRI data will be transferred securely for evaluation by designated central radiologists to ensure consistency of imaging endpoints, support quality assurance, and align radiologic assessments with centrally reviewed pathology outcomes.

In addition, the association between PCAI ImmunoScore and changes in blood PSA concentration during treatment will be evaluated.

The fraction of subjects experiencing treatment side effects will be assessed through AE/SAE reporting and questionnaires (see appendix I) provided to the patients before treatment start, at 60 days into Darolutamide treatment, before surgery and at 3 months and 12 months after surgery.

Further, we will make comparisons between the predictive value provided by PCAI ImmunoScore and available risk prediction nomograms (e.g. the MSKCC pre-radical prostatectomy nomogram).

8.3. Exploratory endpoints

As exploratory, hypothesis-generating endpoint for future clinical studies, molecular analysis of tumour tissue will be performed, to investigate whether ARPI non-responders are candidates for alternative treatment options as predicted by the analysis of DNA sequencing data with a NGS RNA/DNA sequencing-based biomarker panel of previously described transcriptomics and genomics prostate cancer risk features such as TMPRSS2-ERG fusion, AR splice variants, TP53/SPOP mutations, RB1/PTEN/NKX3.1 deletions, MYC amplification, defects in genomic DNA repair genes or homologous recombination repair deficiency, or alterations in PIK3 signalling pathway.

Furthermore, AI-supported image analysis of tumour tissue will be performed on digitized histology slides from pre-treatment biopsy cores. The analysis aims to identify morphological features associated with PCAI ImmunoScore and the study outcomes.

9. Handling of Adverse Events

9.1. Definitions

9.1.1. Adverse Event (AE)

Adverse Event (AE): Any untoward medical occurrence in a subject to whom a medicinal product is administered and which does not necessarily have a causal relationship with this treatment.

9.1.2 Adverse Reaction (AR)

In the pre-approval clinical experience with a new medicinal product or new use of a medicinal product, and particularly as the therapeutic dose(s) may not be established, all noxious and unintended reactions to the medicinal product related to any dose should be considered an adverse reaction (AR). The phrase “reaction” to a medicinal product means that the causal relationship between the medical product and an adverse event is at least a reasonable possibility, that is the relationship cannot be ruled out.

9.1.3 Serious Adverse Event (SAE)

Serious Adverse Event (SAE): Any untoward medical occurrence that at any dose requires inpatient hospitalization or prolongation of existing hospitalisation, results in persistent or significant disability or incapacity, results in a congenital anomaly or birth defect, is life-threatening, or results in death.

Medical and scientific assessment will be made to determine if an event is serious.

9.1.4 Suspected Unexpected Serious Adverse Reaction (SUSAR)

SUSAR: An adverse reaction/event that is unexpected, serious, and suspected to be caused by the treatment, i.e. adverse reactions/events that are not included in the RSI section of the Investigator’s Brochure (IB) or SmPC.

9.2. Assessment of Adverse Events (AE)

9.2.1. Assessment of causal relationship

The investigator is responsible for determining whether there is a causal relationship between the AE/SAE and use of the investigational medicinal product.

Consideration should be given to whether there is a reasonable possibility of establishing a causal relationship between the adverse event and the investigational medicinal product based on the analysis of the available evidence.

All AE can be categorized as either likely related, possibly related, unlikely related or not related, in accordance with the definitions below:

Likely related: Clinical event, including abnormal results from laboratory analyses, occurring within a reasonable time after administration of the intervention/investigational medicinal product. It is unlikely that the event can be attributed to underlying disease or other medications but is most likely caused by the investigational medicinal product and its emergence is reasonable in relationship with use of the investigational medicinal product.

Possibly related: Clinical event, including abnormal results from laboratory analyses, occurring within a reasonable time after administration of the intervention/investigational medicinal product. The event could be explained by the investigational medicinal product and its emergence is reasonable in relationship with use of the investigational medicinal product, but there is insufficient information to determine the relationship. The event could be explained by an underlying disease or other medications.

Unlikely related: Clinical event, including abnormal responses from laboratory tests, unlikely to be related to the intervention/investigational medicinal product and can be reasonably explained by other medication or underlying disease.

Not related: Clinical event, including abnormal results from laboratory analyses, that is not reasonably related to the use of the intervention/investigational medicinal product.

Those AEs which are suspected of having a causal relationship to the investigational medicinal product will be followed up until the subject has recovered or is well taken care of and on the way to good recovery (see also section 1.9.4, Follow-up of Adverse Events).

If the reporting investigator does not provide any information on causality, the sponsor should consult with the reporting investigator and encourage the expression of a position on this issue. The sponsor must take into account the assessment of causality provided by the investigator. If the sponsor disagrees with the investigator's assessment of causality, both the investigator's and the sponsor's views should be included in the report.

9.2.2. Assessment of intensity

Each adverse event shall be classified by an investigator using the Common Terminology Criteria for Adverse Events (CTCAE v5.0):

Grade 1 Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.

Grade 2 Moderate; minimal, local or non-invasive intervention indicated; limiting age-

appropriate instrumental activities of daily living.

Grade 3 Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care.

Grade 4 Life-threatening consequences; urgent intervention indicated.

Grade 5 Death related to AE.

9.2.3. Assessment of seriousness

The investigator is responsible for assessing the seriousness (serious or non-serious). If the adverse event is considered serious, this should be reported as a serious adverse event (SAE) by the investigator to the sponsor. See also section 1.1.9.3.2, Reporting of Serious Adverse Events (SAE).

9.3 Reporting and registration of Adverse Events

At each trial visit, adverse events (AE) are registered, starting from start of treatment with Darolutamide, up to the first post-operative visit. All AE that occurs during the trial and which are observed by the investigator/trial nurse or reported by the subject will be registered in the CRF regardless of whether they are assessed as related to the investigational medicinal product or not. Assessment of causal relationship, severity, and whether the AE is considered to be an SAE will be made by the investigator directly in the CRF. At minimum for each AE/SAE, a description of the event is recorded (diagnosis/symptom if diagnosis is missing), start and stop dates, causal relationship, severity, if the AE is considered to be an SAE, measures and outcome.

9.3.1. Reporting of Adverse Events (AE)

All AEs reported spontaneously by the trial participant or observed by the investigator will be registered in the eCRF.

9.3.2. Reporting of Serious Adverse Events (SAE)

Serious Adverse Events (SAE) are reported to the sponsor on a special SAE form within 24 hours of the investigator being informed of the SAE. Follow-up information describing the outcome and handling of the SAE is reported as soon as this information is available. The original SAE form should be kept in the Investigator Site File.

SAEs are reported using the study specific SAE report form submitted by e-mail to Clinical Trials Office, Department of Clinical Cancer Studies, Karolinska University Hospital:

SAE Email: ctosafety.karolinska@regionstockholm.se

The Sponsor must evaluate each SAE and decide whether the event is a SUSAR or not.

SAEs will also be reported (de-identified) to Bayer AB within 4 business days from date of awareness of the SAE.

9.3.3. Reporting of Suspected Unexpected Serious Adverse Reactions (SUSAR)

Those SAE which are assessed by sponsor to be SUSAR are to be reported to the EudraVigilance database. The completed CIOMS form will be the basis for the reporting, by Swedish Medical Products Agency in the EudraVigilance database according to the specified time frames.

SUSAR that are fatal or life-threatening are reported as soon as possible and no later than 7 days after the SAE has become known to the sponsor. Relevant follow-up information is sent thereafter within an additional 8 days. Other SUSAR are reported as soon as possible and no later than 15 days after they have come to the sponsor's knowledge. Information about SUSAR occurring during the trial is compiled by the sponsor and sent to the principal investigators at all participating sites.

9.4. Follow-up of Adverse Events

After the initial AE/SAE report, the investigator is required to proactively follow each study subject at subsequent visits/contacts. For each event, the investigator must pursue and obtain adequate information until resolution, stabilization, the event is otherwise explained, or the participant is lost to follow-up. The investigator is obligated to perform or arrange for the conduct of supplemental measurements and/or evaluations as medically indicated or as requested by the sponsor to elucidate the nature and/or causality of the AE or SAE as fully as possible.

In case of unacceptable adverse events study participants will either be candidates for dose adjustments, treatment interruption or withdrawn from the trial.

9.5 Annual Safety Report (ASR)

The sponsor will prepare an annual safety report on the investigational medicinal product/products for EU/EEA Member States. The reporting period for the first ASR starts with the original approval date of the clinical trial and ends after one full year. The sponsor then has 60 days to prepare and submit the ASR via CTIS. The ASR will include an anonymized list of all SAE and possible SUSAR that have occurred, a summary assessment of the safety situation and a benefit/risk evaluation. The ASR shall also be accompanied by the Reference Safety Information (RSI) in force at the start date of the report. Significant changes that have occurred in the RSI during the

reporting period should be listed in the ASR.

Since the sponsor is non-commercial and the marketing authorization holder of the investigational medicinal product is within the EU/EEA the SPC is used as RSI. The simplified template of Annual Safety Report will be used for the ASR reporting.

9.6 Procedures in case of emergencies or overdose

Medication errors and use of other medicinal products than those specified in the protocol, including misuse and abuse of the investigational medicinal product, shall be subject to the same reporting obligations as adverse reactions.

If an unforeseen event is likely to have a serious impact on the benefit/risk relationship of the trial, the sponsor and investigator should take appropriate Urgent Safety Measures (USM) necessary to protect the subjects. Examples of such measures are to temporarily halt the clinical trial or to introduce supplementary monitoring measures. The sponsor should, via CTIS, inform the concerned Member States about the event and the measures taken. Notification must be made as soon as possible, but no later than seven days after the measures have been taken.

10 Statistics

10.1. Analysis populations

An intention to treat (ITT) population, including all trial participants, will be used when analysing treatment side effects and adverse events.The primary and secondary analyses will be performed in both the intention-to-treat (ITT) population and a per-protocol (PP) population. The PP population includes subjects without major protocol deviations related to the IMP treatment and with complete endpoint data. All data will be pooled across trial centres.

10.2. Statistical analyses

10.2.1. Statistical methods

The cohort will be reported with baseline characteristics at start of study and with cohort characteristics for adverse events, side effects, and functional outcomes for the whole patient group. Categorical variables will be presented as frequencies and percentages, while continuous variables will be summarized using means and standard deviations or medians and interquartile ranges, as appropriate. Data visualizations such as histograms and boxplots will be used to illustrate distributions.

Any deviations from the original statistical plan will be reported and motivated in peer-

reviewed scientific publications as well as in the final study report.

10.2.2 Analyses addressing the primary objective

The study endpoint MRD will be dichotomized into responder (if MRD is met) or non-responder (if MRD is not met) as input for the statistical data analysis.

A logistic regression model will be used to calculate the PCAI ImmunoScore based on the log2 transformed TPM (transcript per million) gene expression values for each of the 8 genes (ABCC5, CUX2, KIAA1549, RAP1GAP2, PDE4D, SLC39A11, TDRD1, VWA2) based on RNA sequencing (RNAseq). The logarithmic transformation of TPM gene expression values is used to reduce skewness and reduce the impact of extreme TPM expression values.

Model specification: $\text{Logit}(P) = \ln(P / (1 - P)) = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_6X_6 + \beta_7X_7 + \beta_8X_8$ [+ covariates]where P is the probability of treatment response and Covariates are not used on the baseline model but only in the extended model. Covariates used in the model are: baseline tumour volume derived from MRI at diagnosis; log2(TPM) PTEN and log2(TPM) ERG expression derived from the RNAseq data). The regression coefficient β for each gene represents the log-odds change in treatment response per unit change in gene expression and X represents the log2(TPM) for each of the 8 input genes[18, 19].

Association of PCAI ImmunoScore with probability P of ARPI treatment response: for each of the models (i.e., baseline, and extended model) the probability P of ARPI response is calculated via the Logit(P) function of the regression models. The probability of response is positively associated with increasing residual tumour burden, i.e., the larger the PCAI ImmunoScore the higher the likelihood of residual tumour volume 0.05 cm^3 (and vice versa).

AUROC analysis: based on the determined residual tumour burden during pathology assessment after 3-4 months neoadjuvant Darolutamide treatment followed by surgical prostate resection each patient will be classified as Darolutamide responder (MRD met) or Darolutamide non-responder (MRD not met). This classification (ground truth) will be tested in AUROC analysis against the calculated PCAI ImmunoScore-based probability (baseline model) of Darolutamide response. The extended model including the additional covariates will be tested equivalent to the baseline model. The AUROCs of the baseline and extended models will be evaluated for statistically significant difference in the AUC between the two models using De Long's test.

Hypothesis testing:

- **Null Hypothesis (H_0):** The model's AUROC is 0.5, indicating it does not perform better than random guessing.
- **Alternative Hypothesis (H_A):** The model's AUROC is significantly greater than 0.5 (one-sided testing).

Justification for use of one-sided test of statistical significance for the primary outcome: Based on our preliminary data, the calculated probability P is associated with higher residual tumour burden after treatment. The dichotomized patient groups (responders vs non-responders) are assigned such that the resulting AUROC is >0.5; consequently, we would expect the same result again, i.e., an AUROC>0.5 as an outcome of this study. Since an AUC below 0.5 is not practically relevant in this situation, a two-sided test would be inefficient and unnecessary. Additionally, using a one-sided test increases statistical power by allocating the entire significance level to one tail of the distribution, making it easier to detect a meaningful improvement while reducing the required sample size. This approach aligns with standard statistical practices in diagnostic and predictive modelling. Unless there is a specific reason to test for deviations in both directions, such as when comparing two diagnostic models where performance shifts in either direction matter, a one-sided AUROC test remains the most appropriate and efficient choice. For all other comparisons, two-sided tests will be used.

Key results derived from the hypothesis testing:

- The De Long's test will be used to determine the statistical significance (threshold $p<0.05$) of AUROC>0.5 (H_0). If $p<0.05$ we will reject H_0 and accept that AUROC>0.5, i.e., the PCAI ImmunoScore models (baseline, extended) have discriminatory power to separate ARPI responders from non-responders.
- The Area Under the Curve (AUC) will be determined (De Long) (including standard error and 95% confidence interval) to provide a measure of the potential clinical impact of the PCAI ImmunoScore (baseline and extended) models. Generally, an AUC of 0.7-0.8 is regarded as clinically useful. AUCs between 0.8-0.9 are seen as very clinically useful and any AUC>0.9 qualifies as excellent result. Based on the previous data we expect an AUC>0.75 as a result from the data analysis.
- The Youden Index (including 95% confidence interval, associated cut-off criterion, associated sensitivity and specificity) representing the point within the AUC where the sensitivity and specificity of the true positive and true negative classification is maximized will be determined and compared to the previously analysed data.
- Criterion values and coordinates of the ROC curve: This section of the results will list the different filters or cut-off values with their corresponding sensitivity and specificity of the test, and the positive (+LR) and negative likelihood ratio (-LR). When the disease prevalence is known, the program will also report the positive predictive value (+PPV) and the negative predictive value (-NPV).

10.2.3 Analyses addressing the secondary objectives

- To assess the utility of the pre-treatment genomic biomarker PCAI ImmunoScore in predicting the pathologic complete response (pCR) to 3 months neoadjuvant treatment with the Androgen Receptor Pathway Inhibitor (ARPI) Darolutamide. pCR is defined as no visible tumour at pathological assessment of the surgical specimen, and its association to PCAI ImmunoScore will be evaluated similarly as for MRD (see above).
- To assess the association between PCAI ImmunoScore and pT stage at final pathology, non-parametric (e.g. Kruskal-Wallis test) testing will be used for analysing the continuous variable PCAI ImmunoScore against the ordinal variable pT stage. If pT stage is significantly associated with PCAI ImmunoScore, Dunn's post-hoc test will be performed to determine which pairs of pT stages are distinguishable by PCAI ImmunoScore.
- To assess the association between PCAI ImmunoScore and size of largest cross-sectional dimension of residual tumour on pathology. Penalized spline regression in combination with cross-validation or a similar flexible modelling approach will be applied.
- To assess the association between PCAI ImmunoScore and Residual Cancer Burden (RCB), defined as residual tumour volume in mm^3, corrected for tumour cellularity. Penalized spline regression in combination with cross-validation or a similar flexible modelling approach will be applied.
- To assess the association between PCAI ImmunoScore and change in tumour volume from diagnostic, pre-ARPI, MRI to final resection pathology. Penalized spline regression in combination with cross-validation or a similar flexible modelling approach will be applied.
- To assess the association between PCAI ImmunoScore and number of resection specimen slides in which the tumour can be seen on final pathology, non-parametric (e.g. Kruskal-Wallis test) testing and Dunn's post-hoc test will be used.
- To assess the association between PCAI ImmunoScore and changes in lesion size on repeated MRI, Penalized spline regression in combination with cross-validation or a similar flexible modelling approach will be applied.
- To assess the association between PCAI ImmunoScore and changes in EPE (assessment of risk of extraprostatic extension on a Likert scale 1-5) on repeated MRI, non-parametric (e.g. Kruskal-Wallis test) testing will be used for analysing the continuous variable PCAI ImmunoScore against the ordinal variable EPE. If EPE is significantly associated with PCAI ImmunoScore, Dunn's post-hoc test will be performed to determine which pairs of EPE scores are distinguishable by PCAI ImmunoScore.
- To assess the association between PCAI ImmunoScore and $\log^2$ ratios of pre- and post-treatment PSA levels, penalized spline regression or a similar flexible modelling approach will be applied.
- In addition, as a quality metric, we will assess the correlation between pathologic response (MRD and pCR), Residual Cancer Burden (RCB), and changes in lesion size on repeated MRI, similar to previously reported by Fenessy et al [39]. Non-parametric (e.g. Kruskal-Wallis test) testing will be used for analysing the continuous variable change in MRI lesion size against the categorical variables MRD and pCR.

- Treatment side effects will be reported using descriptive analytics for the cohort.

10.3 Drop-outs

Drop-outs will be reported in the baseline characteristics as well as in reports of adverse events and functional outcomes. A subject who discontinues the study will not be replaced.

10.4 Adjustment of significance and confidence interval

For the secondary outcomes, Benjamini-Hochberg corrections will be employed when appropriate, to reduce the rate of false positive observations.

10.5 Sample size calculations

Based on previous studies [20-21] a response rate in terms of MRD of 20-30% after 3-6 months of Darolutamide is expected. Our preliminary data indicate an AUROC of 0.75 to separate responders from non-responders by the PCAI ImmunoScore. Assuming type 1 error alpha 0,05 and type 2 error beta 0,2 requires 13 responders and 65 non-responders (total 78 patients). Assuming a drop-out rate of 10% in the treatment phase and 13% in the tissue acquisition and molecular analysis phases, the total number of patients to be included in the study is n=100 to achieve 80% power at a significance level of p=0,05. Assumptions and inputs for the sample size calculation were made using the Hanley & McNeil method [40].

11 Quality Control and Quality Assurance

The Clinical Trials Office (CTO) at Theme Cancer, Karolinska University Hospital, serves as sponsor support and will coordinate the study. The study will be monitored by staff authorised by CTO. A monitoring plan is set up to specify monitoring steps in detail. To ensure accurate, complete and reliable data according to GCP rules, the CTO is responsible for:

- Supply of instructional material to the study sites as appropriate
- Organization of start-up meetings to instruct the investigators and research nurses on the protocol, the completion of the case report forms, handling and transport of biological material and other study procedures
- Performance of periodic central and on-site monitoring visits to the study sites
- Consultation and continuous contact with the study site personnel by email and / or telephone
- Review and evaluation of case report form data by use of standard computer edits to detect errors in data collection.

11.1. Quality Assurance and Sponsor oversight

The sponsor's quality-related work is based on a risk analysis of the trial as a whole: design, conduct, data collection, evaluation, reporting and archiving. The sponsor will perform quality assurance and quality control activities for the trial; however, responsibility for the accuracy, completeness, and reliability of the trial data presented to the sponsor lies with the PI (and delegate(s)) generating the data.

11.2. Monitoring

The trial will be monitored by an independent monitor before the trial begins, during the conduct of the trial, and after the trial has been completed. This is to ensure that the trial is carried out according to the protocol and that data are reliably and robust and are collected, documented, and reported according to ICH-GCP and applicable ethical and regulatory requirements. Monitoring will be risk-based, which means that the extent of the monitoring is based on the sponsor`s risk-assessment and is performed as per the trial's monitoring plan, The monitoring is intended to ensure that the subject's rights, safety, and well-being are met and that data in the CRF are complete, correct, and consistent with the source data.

11.3. Source data

The investigator must keep source documents for each subject in the trial. A document describing what has been classified as source data in the trial (source data reference document) should be included in the Investigator Site File (ISF). The investigator must ensure that all source documents are accessible for monitoring and other quality control activities.

Source data is defined before trial start at each individual site and can, in cases where source data is not registered in another document, consist of the CRF. This should be decided in consultation with the monitor and clearly stated in the source data reference document.

Access to trial-related documentation, such as subjects' medical records, CRFs, other source data and other trial documentation will be provided for monitoring and auditing purposes. Access to subjects' medical records will require a confidentiality agreement to be signed by the person in charge of the medical records at the trial site and by the monitor and auditor. Access will also be granted in the context of regulatory inspections.

11.4. Deviations, serious breaches and other reporting obligations

The responsible investigator and/or any involved service provider shall, without delay, report to the sponsor any suspected serious breaches from the trial protocol, the CTR,

ICH-GCP and other regulations that are likely to affect the safety, rights of the subjects and/or the data reliability and robustness to a significant degree. The sponsor should assess the suspected serious breach, the consequences of the deviations and without undue delay, but no later than 7 days (from knowledge), report these to the Competent Authorities via CTIS.

Other unexpected events that may affect the benefit/risk relationship for the clinical trial must be reported via CTIS without undue delay, but no later than 15 days after the sponsor becomes aware of the event.

Minor deviations that do not affect subjects' integrity or safety, nor significantly affect the trial's scientific value, are documented in the trial documentation of the principal investigator and the sponsor and appropriate measures shall be taken. The deviations, including minor deviations, must be recorded in the clinical trial report.

11.5. Audits and inspections

The purpose of an audit or inspection is to review trial-related activities and documents systematically and independently, to determine whether these activities were performed, registered, analysed and reported correctly according to the protocol, ICH-GCP and applicable regulations.

Authorized representatives for the sponsor and Competent Authorities (CA) may carry out audits or inspections at the trial site, including source data verification. The investigator must ensure that all source documents are available for audits and inspections.

12 Ethics

12.1. Compliance to the protocol, ICH-GCP and regulations

The trial will be performed in compliance with this clinical trial protocol, the EU regulation on clinical trials on medicinal products for human use (536/2014), the Declaration of Helsinki, ICH-GCP (Good Clinical Practice), and current national regulations governing this clinical trial. This is to ensure the safety and integrity of the trial subjects as well as the quality of the data collected.

12.2. Ethical review of the trial

The final protocol for clinical trials on medicinal products must be approved, as a part of the application for a permit for clinical trials via CTIS, by the Competent Authorities,

before the trial can be conducted. The authorities must be informed via CTIS of any changes in the trial protocol in accordance with current requirements.

12.3. Procedure for obtaining informed consent

The principal investigator at each site shall ensure that the subject is given full and adequate oral and written information about the trial, its purpose, any risks and benefits as well as inclusion and exclusion criteria. Subjects must also be informed that they are free to discontinue their participation in the trial at any time without having to provide a reason. Subjects should be given the opportunity to ask questions and be allowed time to consider the provided information. If the subject chooses to participate, both the subject and the investigator shall sign the informed consent form. A copy of the subject information as well as the informed consent form shall be provided to the subject. The subject's signed and dated informed consent must be obtained before any trial-specific activity is performed. Each subject who participates in the trial will be identified by a subject number on a subject identification list. The subject agrees that monitors, auditors, and inspectors may have access to the subjects' medical records and other source data. If new information is added to the trial, the subject has the right to reconsider whether he/she will continue their participation.

12.4. Data protection

If any part of the trial data is processed by another organization, inside or outside the EU, appropriate agreements and/or other appropriate protective measures will be taken to ensure that the data processing is performed in accordance with the provisions of the General Data Protection Regulation (GDPR) and other relevant legislation, before any data transfer takes place.

In the information provided to subjects, subjects will be fully informed about how their trial data will be collected, used and disclosed. The content of the informed consent form complies with relevant integrity and data protection legislation. The subject information and the informed consent form will explain how trial data are stored to maintain confidentiality in accordance with national data legislation. The study data will be stored in a database located on a server at Region Stockholm with a back-up performed every 24 h. All information processed by the sponsor will be pseudonymized and identified with a study/participant ID.

The informed consent form will also explain that for verification of the data, representatives delegated by the sponsor, as well as relevant authorities, may require access to parts of medical records or trial records that are relevant to the trial, including the subject's medical history.

12.5. Insurances

All subjects participating in the trial are covered by the Swedish Patient Insurance (patientförsäkringen) through Regionernas Ömsesidiga Försäkringsbolag, LÖF, and by the Swedish Pharmaceutical Insurance (Läkemedelsförsäkringen) through LFF.

13 Substantial changes to the trial

Substantial changes to the signed clinical trial protocol are only possible through approved protocol amendments.

In the event that substantial changes to the protocol which may affect the safety, rights of subjects or the reliability and robustness of data generated need to be implemented during the course of the trial, permission from the relevant authority via application in CTIS should be obtained before implementing the change. This includes the addition of a new trial site or a change of the principal investigator at the trial site.

Non-substantial amendments are entered into the CTIS in the next substantial amendment application concerning the same part. If the non-substantial change is relevant to the Authority's oversight (e.g. contact details), the CTIS should be updated on an ongoing basis.

14 Collection, handling, and archiving of data

It is the principal investigator’s responsibility to keep a record, i.e. a screening log, of all patients that were considered for enrolment even if they were not subsequently enrolled. This information is necessary to verify that the patient population was selected without bias. The reasons for non-eligibility are to be defined in terms of one or more of the eligibility criteria.

Subjects who participate in the trial are coded with a trial-specific identification number. All subjects are registered in a subject identification list (subject enrolment and identification list) that connects the subject’s name and personal number with a subject number/trial identification number.

All data will be registered, managed, and stored in a manner that enables correct reporting, interpretation, and verification. The complete Trial Master File with essential documents will be archived for at least 25 years. Source data in the medical records system are stored and archived in accordance with the respective hospital regulations.

14.1. Case Report Form

An electronic Case Report Form (eCRF) is used for data collection. A data management plan is delivered by the Clinical Trials Office at Karolinska University Hospital, documenting all procedures for the data management. eCRFs are required

to be completed for each subject enrolled in the trial and all continuously collected data, both study-specific and routinely collected clinical data, will be entered in the eCRF within 1 month. The PI is responsible for ensuring the accuracy, completeness and legibility of the data recorded in the eCRFs. A copy of the completed eCRF will be archived at the study site.

Any changes or corrections to the eCRFs will be captured in the eCRF audit trail. The Investigator will review the eCRFs for completeness and accuracy and will sign and date the appropriate eCRF page as indicated. On completion of the subject's eCRF, the responsible Investigator will provide a digital signature on the appropriate eCRF. eCRFs will be reviewed at the trial site during regularly scheduled visits by the trial monitor for completeness, consistency and adherence to the trial protocol. Before database lock, the investigator and monitor will have viewing access to the eCRF but only a limited number of delegated investigators will have access to enter data. The sponsor, PI and the study monitor will have full viewing access. Each subject will receive a unique trial identification number via the electronic system, which will be linked to the eCRF. The code key (ID log) will be kept in the Investigator's Site File.

15 Notification of trial completion, reporting, and publication

End of recruitment of subjects and end of the trial is reported in CTIS, within 15 days from occurrence in the Member state. Within one year of trial completion in all Member states a summary of the clinical trial results must be reported in CTIS, including a summary for lay people. In addition, a full clinical trial report with individual data is to be completed and archived in the trial master file by sponsor and in the investigator site files at each site.

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17 Attachments

17.1 Appendix I – EPIC26-SF

EPIC-SF-6 (2002)

Detta frågeformulär är utformat för att mäta livskvalitetsaspekter hos patienter med prostatacancer.
För att hjälpa oss att få den mest exakta mätningen är det viktigt att du besvarar alla frågor ärligt och fullständigt.

Kom ihåg att, precis som med alla medicinska journaler, kommer informationen i denna undersökning att förbli strikt konfidentiell.

Datum för ifyllande av formuläret:
Månad _____ Dag _____ År _____

Namn: _____

Personnummer:_____

EPIC-26

Expanded Prostate Cancer Index Composite – Kort version

1. Hur ofta har du läckt urin under de senaste fyra veckorna?

(Ringa in ett nummer)

- Mer än en gång om dagen → **1**
- Ungefär en gång om dagen → **2**
- Mer än en gång i veckan → **3**
- Ungefär en gång i veckan → **4**
- Sällan eller aldrig → **5**

2. Vilket av följande beskriver bäst din urinvägskontroll under de senaste fyra veckorna?

(Ringa in ett nummer)

- Ingen urinvägskontroll alls → **1**
- Frekvent droppande → **2**
- Tillfälligt droppande → **3**
- Total kontroll → **4**

3. Hur många inkontinensskydd eller vuxenblöjor per dag använde du vanligtvis för att hantera urinläckage under de senaste fyra veckorna?

(Ringa in ett nummer)

- Inga → **0**
- 1 skydd per dag → **1**
- 2 skydd per dag → **2**
- 3 eller fler skydd per dag → **3**

4. Hur stort problem har följande varit för dig under de senaste fyra veckorna?

(Ringa in ett nummer på varje rad)

Problem	Inget problem (0)	Mycket litet problem (1)	Litet problem (2)	Måttligt problem (3)	Stort problem (4)
a. Droppande eller urinläckage	0	1	2	3	4
b. Smärta eller sveda vid urinering	0	1	2	3	4
c. Blod i urinen	0	1	2	3	4
d. Svag urinstråle eller ofullständig tömning	0	1	2	3	4
e. Behov av att urinera ofta under dagen	0	1	2	3	4

5. Hur stort problem har din urinvägsfunktion varit för dig under de senaste fyra veckorna?

(Ringa in ett nummer)

- Inget problem → **1**
- Mycket litet problem → **2**
- Litet problem → **3**
- Måttligt problem → **4**
- Stort problem → **5**

Tarmfunktion

6. Hur stort problem har följande varit för dig?

(Ringa in ett nummer på varje rad)

Problem	Inget problem	Mycket litet problem (1)	Litet problem	Måttligt problem (3)	Stort problem

	(0)		(2)		(4)
a. Brådskande behov av tarmtömning	0	1	2	3	4
b. Ökad tarmtömningsfrekvens	0	1	2	3	4
c. Förlorad kontroll över avföring	0	1	2	3	4
d. Blod i avföringen	0	1	2	3	4
e. Buk-, bäcken- eller rektalsmärta	0	1	2	3	4

7. Hur stort problem har dina tarmvanor varit för dig under de senaste fyra veckorna?

(Ringa in ett nummer)

- Inget problem → **1**
- Mycket litet problem → **2**
- Litet problem → **3**
- Måttligt problem → **4**
- Stort problem → **5**

Sexuell funktion

8. Hur skulle du betygsätta följande under de senaste fyra veckorna?

(Ringa in ett nummer på varje rad)

Funktion	Mycket dålig (1)	Dålig (2)	Ganska bra (3)	Bra (4)	Mycket bra (5)
a. Din förmåga att få erektion	1	2	3	4	5
b. Din förmåga att få orgasm (klimax)	1	2	3	4	5

9. Hur skulle du beskriva kvaliteten på dina erektioner under de senaste fyra veckorna?

(Ringa in ett nummer)

- Ingen erektion alls → **1**
- Inte tillräckligt fast för sexuell aktivitet → **2**
- Tillräckligt fast för onani och förspel → **3**

- Tillräckligt fast för samlag → **4**

10. Hur ofta hade du en erektion när du ville ha en under de senaste fyra veckorna?

(Ringa in ett nummer)

- Aldrig → **1**
- Mindre än hälften av gångerna → **2**
- Ungefär hälften av gångerna → **3**
- Mer än hälften av gångerna → **4**
- Alltid när jag ville → **5**

11. Hur skulle du bedöma din sexuella funktion under de senaste fyra veckorna?

(Ringa in ett nummer)

- Mycket dålig → **1**
- Dålig → **2**
- Ganska bra → **3**
- Bra → **4**
- Mycket bra → **5**

12. Hur stort problem har din sexuella funktion eller brist på sexuell funktion varit för dig under de senaste fyra veckorna?

(Ringa in ett nummer)

- Inget problem → **1**
- Mycket litet problem → **2**
- Litet problem → **3**
- Måttligt problem → **4**
- Stort problem → **5**

Allmänna symtom

13. Hur stort problem har följande varit för dig under de senaste fyra veckorna?

(Ringa in ett nummer på varje rad)

Problem	Inget problem (0)	Mycket litet problem (1)	Litet problem (2)	Måttligt problem (3)	Stort problem (4)

a. Värmevallningar	0	1	2	3	4
b. Ömhet/förstoring av bröst	0	1	2	3	4
c. Känsla av nedstämdhet	0	1	2	3	4
d. Brist på energi	0	1	2	3	4
e. Förändring i kroppsvikt	0	1	2	3	4

Tack så mycket för din medverkan!

17.2 Appendix II – Swedish National PROM form

NPCR Nationella prostatacancerregistret — REGIONALA CANCERCENTRUM I SAMVERKAN

IPSS – symtomskattning vid prostatabesvär (modifierad av styrgruppen för NPCR)
De första åtta frågorna i den här enkäten handlar om hur du kissar och används som en del i beslutsunderlaget för att rekomendera vilken behandling som passar just dig bäst .

Sätt kryss i den ruta som som bäst stämmer överens med hur du upplevt din situation under den senaste månaden.

		Inte alls	Mindre än 1 gång av 5	Mindre än hälften av gångerna	Hälften av gångerna	Mer än hälften av gångerna	Nästan alltid
1. Hur ofta har du en känsla av att blåsan inte har tömts helt när du kissat?		0	1	2	3	4	5
2. Hur ofta har du varit tvungen att kissa oftare än varannan timme?		0	1	2	3	4	5
3. Hur ofta har du avbrott i urinflödet, dvs urinflödet satte igång, stannade upp och satte igång igen?		0	1	2	3	4	5
4. Hur ofta har du haft svårt att vänta med att kissa efter det att du känt behov av att kissa?		0	1	2	3	4	5
5. Hur ofta har du tyckt (upplevt) att strålen varit svag?		0	1	2	3	4	5
6. Hur ofta har du haft behov av att krysta eller ta i för att komma igång att kissa?		0	1	2	3	4	5
		Aldrig	1 gång per natt	2 gånger per natt	3 gånger per natt	4 gånger per natt	5 gånger eller fler
7. Hur ofta har du vanligtvis behövt gå upp för att kissa från det du lagt dig på kvällen tills du stiger upp på morgonen?		0	1	2	3	4	5
	Mycket nöjd	Nöjd	Ganska nöjd	Blandade känslor	Ganska miss-belåten	Olycklig	Frukt-ansvärt
8. Om dessa besvär skulle vara oförändrade under resten av ditt liv, hur skulle du uppleva det?	0	1	2	3	4	5	6

De här frågorna används för att jämföra din situation före och efter behandling

NPCR version 2021-05

NPCR Nationella prostatacancerregistret — REGIONALA CANCERCENTRUM I SAMVERKAN

Sätt kryss i den ruta som bäst motsvarar din upplevelse.

Frågor om urinvägarna, ***den senaste månaden***

	Inte alls	Lite	Måttligt	Mycket
1. Har du svag urinstråle?	☐	☐	☐	☐
2. Har du ett trängande behov att gå och kissa direkt?	☐	☐	☐	☐

3. Har du urinläckage?	☐ Aldrig	☐ Läcker ibland vid hosta, nysning, **och**/eller använder droppskydd vid speciell fysisk ansträngning, t.ex. sportaktivitet, trädgårdsarbete	☐ Bär droppskydd hela tiden (utom möjligtvis nattetid) men de är inte alltid våta	☐ Bär droppskydd hela tiden som måste bytas pga. att de är våta	☐ Läcker kontinuerligt och behöver blöjor som kontinuerligt bytes

	Inte alls	Lite	Måttligt	Mycket
4. Hur mycket urin läcker du?	☐	☐	☐	☐

5. Hur många skydd använder du per dygn på grund av urinläckage?	☐ Jag har inget skydd ☐ Mindre än 1 per dygn ☐ Ungefär 1 per dygn ☐ Ungefär 2 per dygn ☐ Ungefär 3-4 per dygn ☐ Ungefär 5 eller fler per dygn

6. Om du skulle leva resten av ditt liv med din urinvägsfunktion som det är just nu, hur skulle du uppleva detta?	☐ Det skulle inte besvära mig alls ☐ Det skulle besvära mig lite ☐ Det skulle besvära mig måttligt ☐ Det skulle besvära mig mycket

Frågor om tarmfunktion, ***den senaste månaden***

	Inte alls	Lite	Måttligt	Mycket
7. Har du ett trängande behov till att tömma tarmen direkt?	☐	☐	☐	☐
8. Har du slem i avföringen?	☐	☐	☐	☐
9. Har du blod i avföringen?	☐	☐	☐	☐

NPCR version 2021-05

NPCR Nationella prostatacancerregistret — REGIONALA CANCERCENTRUM I SAMVERKAN

10. Har du avföringsläckage?	☐ Aldrig	☐ Läcker ibland vid hosta, nysning, skratt, lyfter tungt eller när du reser dig från sittande till stående	☐ Vid gasavgång	☐ Bär läckageskydd hela tiden som måste bytas pga. att de är våta/smutsiga	☐ Läcker kontinuerligt och behöver blöjor som kontinuerligt bytes

	Inte alls	Lite	Måttligt	Mycket
11. Hur mycket avföring läcker du?	☐	☐	☐	☐

12. Om du skulle leva resten av ditt liv med din avföringsfunktion som det är just nu, hur skulle du uppleva detta?	☐ Det skulle inte besvära mig alls ☐ Det skulle besvära mig lite ☐ Det skulle besvära mig måttligt ☐ Det skulle besvära mig mycket

Frågor om sexuallivet, *den senaste månaden*

13. Har du någon partner?	☐ Ja ☐ Nej
14. Är du sexuellt aktiv (med eller utan partner)?	☐ Ja, fortsätt till **fråga 16** ☐ Nej
15. Om du inte är sexuellt aktiv, vad beror det på? Besvara frågan och fortsätt sen till **fråga 19** (Flera svarsalternativ är möjliga)	☐ Liten eller ingen lust ☐ Erektionsbesvär (problem med ståndet) ☐ Min partner har liten eller ingen lust ☐ Annan eller andra orsaker
16a. Använder du några potenshjälpmedel vid sexuell aktivitet? (Flera svarsalternativ är möjliga) 16b. **Om Ja, hur ofta**? ☐ Ibland ☐ Oftast ☐ Alltid	☐ Nej ☐ Ja, tabletter ☐ Ja, injektion/sprutbehandling ☐ Ja, stift eller gel som förs in i urinröret ☐ Ja, vacumpump ☐ Ja, annat
17. Hur brukar din erektion (ditt stånd) vara? (om du använder hjälpmedel så beskriver du ditt stånd med hjälpmedel. Om du inte använder hjälpmedel så beskriver du ditt stånd utan hjälpmedel. Om du inte har någon sexuell aktivitet men morgonstånd, beskriv den)	☐ Ingen fyllnad eller styvnad alls ☐ Viss fyllnad, men penisen blir inte styv ☐ Måttlig styvnad/hårdhet ☐ Full styvnad/hårdhet
18. Om du skulle leva resten av ditt liv med din	☐ Det skulle inte besvära mig alls

sexualfunktion som det är just nu, hur skulle du uppleva detta?	☐ Det skulle besvära mig lite ☐ Det skulle besvära mig måttligt ☐ Det skulle besvära mig mycket

Allmänna frågor om din hälsa

19. Hur skulle du beskriva din hälsa?	Mycket dålig	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	☐ 6	☐ 7	Utmärkt
20. Hur skulle du beskriva din livskvalitet?	Mycket dålig	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	☐ 6	☐ 7	Utmärkt

	Inte alls	Lite	Måttligt	Mycket
21. Hur mycket påverkar din prostatacancersjukdom eller dess behandling din dagliga aktivitet?	☐	☐	☐	☐
22. Känner du dig orolig?	☐	☐	☐	☐

Här frågor som handlar om din upplevelse av sjukvården

Frågor om information/delaktighet

	Inte alls	Lite	Måttligt	Mycket
23. Känner du dig delaktig i beslut om din vård och behandling, så mycket som du önskar?	☐	☐	☐	☐
24. Har du en kontaktsjuksköterska?	☐ Ja	☐ Nej	☐ Vet ej	

Under din aktuella sjukdom eller behandling, hur mycket information har du fått om:

	Inte alls tillfredsställande	Lite tillfredsställande	Måttligt tillfredsställande	Mycket tillfredsställande
25 Eventuella biverkningar av din behandling?	☐	☐	☐	☐
26. Inverkan av behandlingen på sexlivet?	☐	☐	☐	☐

NPCR Nationella prostatacancerregistret — REGIONALA CANCERCENTRUM I SAMVERKAN

HÄR KOMMER FRÅGOR SOM INGÅR I "MIN VÅRDPLAN"

Har din cancersjukdom påverkat nedanstående områden? Din beskrivning kan underlätta att du får rätt rehabilitering. Sätt kryss i den ruta som bäst motsvarar din upplevelse.

	Inget problem	Litet problem	Besvärande problem	Mycket besvärande problem
1. Trötthet	☐	☐	☐	☐
2. Sömn	☐	☐	☐	☐
3. Nedstämdhet/depression	☐	☐	☐	☐
4. Fysisk aktivitet	☐	☐	☐	☐
5. Familj/relationer	☐	☐	☐	☐
6. Existensiella funderingar (funderingar om livet och döden)	☐	☐	☐	☐
7. Ekonomi	☐	☐	☐	☐
8. Arbete/sysselsättning	☐	☐	☐	☐

Andra problem eller frågor kan du skriva om här:

TACK FÖR DIN MEDVERKAN!

NPCR version 2021-05

17.3 Appendix IIIa – RNA sequencing SOP

Your partner for
Innovative OMICS
solutions

TOTAL RNA SEQUENCING
WET-LAB + Bioinformatics

Project number : BRECISE
Customer : Philips Eindhoven - Karolinska
Date : 18/12/2024

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1 WET-LAB PROCEDURE

1.1 DNA EXTRACTION

The annotated tumor tissue regions from FFPE tissue sections (5-10µM) are first scraped, 5 slides per sample + 1 control slide with tumor sections indicated by a pathologist. These are collected in 1.5ml Sarstedt tubes, upon which both these source tubes (Sarstedt) and collection tubes (1,5ml LoBind Eppendorf tubes) are barcoded. Afterwards, an automated extraction of TNA (DNA/RNA) by Siemens VERSANT extraction method is performed.

1.2 DNase TREATMENT

A total of 200 total DNA/RNA samples originating from FFPE material were received and directly stored at -80°C.
DNase treatment was performed with the VERSANT Tissue Preparation Reagents Kit, Box 3 (Siemens) according to the manufacturer's protocol.

1.3 RNA QUALITY CONTROL

RNA concentration was determined on a Qubit 3 Fluorometer (Thermo Fisher Scientific) with the Qubit RNA HS assay kit (Thermo Fisher Scientific).
The quality of RNA was determined through the RIN score estimated on Agilent 4200 Tapestation with the RNA HS ScreenTape (Agilent).

1.4 FRAGMENTATION, PRIMING AND rRNA BLOCKING

Fragmentation, priming and rRNA blocking were performed in a single step, combining the NEBNext Ultra II fragmentation (NEBNext Ultra II Directional RNA library prep kit, NEB) and FastSelect hybridization protocol (FastSelect rRNA HMR, QIAseq).

Prior to mRNA isolation and library preparation, 200 ng of total RNA per sample is transferred from individual 1.5 ml Eppendorf tubes to a 96-well plate. Due to variable concentrations, achieving 200 ng in 25 µl total volume per sample requires transferring variable volumes. Subsequently, the volumes of all samples corresponding to 200 ng total RNA are evaporated at 8 mbar for 15 minutes using the GeneVac EZ-2 Plus vacuum centrifuge (Sysmex). The evaporated samples are then resuspended with 25 µl of RNase-free water wherein 4µl of a 1/1000 dilution of ERCC Mix 1 is spiked, according to the manufacturer's protocol, ensuring equal starting volumes and input amounts per sample.

Mitochondrial and cytoplasmic rRNA fragments were blocked by the LNA oligos. Depending on the RIN value of the sample, the fragmentation lasted:

* 7.5 min for samples with RIN < 6
* 15 min for samples with RIN > 6

After heat fragmentation was performed, the reaction was gradually cooled to room temperature.

1.5 cDNA SYNTHESIS

First and second strand cDNA synthesis were performed with reagents included in the NEBNext Ultra II Directional RNA library prep kit (NEB). A purification step with AMPure XP beads (Beckman Coulter) followed, for the separation of the ds cDNA molecules from the reagents.

1.6 LIBRARY PREPARATION

Libraries were prepared with the NEBNext Ultra II Directional RNA library prep kit (NEB) following the manufacturer's instructions.
Right after end prep reaction, Unique Dual Index primers found in the NEBNext Multiplex Oligos kit for Illumina (Unique Dual Index Primers DNA Set 1, NEB) were ligated and samples were purified with AMPure XP beads. To enrich the libraries, a total of 12 PCR cycles were performed with index primers from the NEBNext Multiplex Oligos kit for Illumina. PCR products were purified twice with AMPure XP beads and their quality was estimated.

1.7 FINAL LIBRARY QUALITY CONTROL

The concentration and quality of the final libraries was determined on an Tapestation with the High Sensitivity D1000 ScreenTape (Agilent).

2 SEQUENCING

2.1 ISEQ LIBRARY REBALANCING

Pooling/library rebalancing was performed following an optimized sequencing strategy including two low coverage iSeq100 runs as described below:

First iSeq100 run

Without considering library concentration, an equal volume of each library (2 µL) was transferred and pooled into a new 1.5 mL LoBind Eppendorf tube and mixed by vortexing. This pooled sample was then diluted to a concentration of 60 pM and sequenced on an iSeq100 using the i1 reagent cartridge. Automated demultiplexing of the data resulted in a list of percentages of passed filter reads per library.

Second iSeq100 run

Based on the percentages of passed filter reads, new pooling volumes per library were calculated, resulting in a pool theoretically providing an equal number of sequencing reads per library. A second iSeq100 run with the i1 reagent cartridge was performed to verify the pooling.

The final pool is diluted to a concentration of 60 pM and spiked with 2% PhiX loaded onto the iSeq100 i1 reagent cartridge for low coverage sequencing according to the manufacturer's instructions.

2.2 AVITI SEQUENCING

The final library pool was supplemented with 2µl of a 50pM Cloudbreak FS Phix Control and sequenced on the AVITI Benchtop sequencer using the paired-end 2x150 Cloudbreak FS High Output sequencing kit. An average of 40 million paired-end reads per sample is generated.

3 DATA PROCESSING AND ANALYSIS

Data processing is done with the nf-core rnaseq pipeline (version 3.13.2, Figure 1) [1]. Briefly, first strandedness of the library is inferred on a subset of 1M reads per sample, to be compared to the expected strand as a sanity check. Next, a first round of quality control with fastqc is run. Subsequently, adapters are trimmed and low-quality reads are filtered out with fastp using the default settings of the nf-core rnaseq pipeline, followed by a second round of fastqc on the trimmed fastq files. Reads originating from rRNAs are identified and removed, prior to alignment to the GRCh38 reference genome (Ensembl version 111). To allow for quantification of the ERCC spike-ins, the 92 ERCC sequences are added to the genome fa file and gtf file using a custom script. Aligned reads are deduplicated and read counts per gene and per transcript are generated with STAR and salmon, followed by the generation of extra QC metrics using RSeQC (bam_stat, inner_distance, infer_experiment, read_duplication).

This is achieved by running the pipeline with the following parameters:

```
--trimmer fastp \
--aligner star_salmon \
--remove_ribo_rna TRUE \
--seqc_modules bam_stat,inner_distance,infer_experiment,read_duplication \
--skip_qualimap TRUE \
--skip_stringtie TRUE \
--skip_dupradar TRUE \
--skip_deseq2_qc TRUE \
--skip_biotype_qc TRUE
```

[1] https://nf-co.re/rnaseq/3.13.2

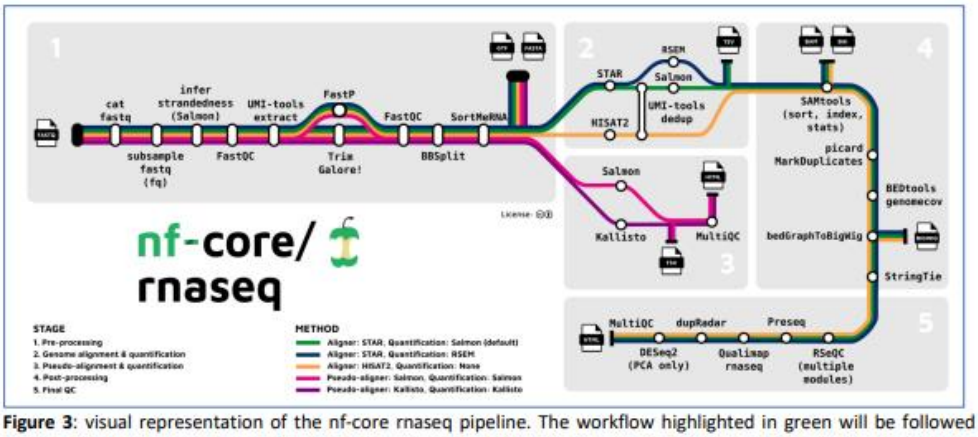

Figure 3: visual representation of the nf-core rnaseq pipeline. The workflow highlighted in green will be followed (alignment with STAR and quantification with Salmon). Read trimming is done with fastp. The bbsplit, Markduplicates, stringtie, preseq, qualimap, dupradar and deseq2 modules are skipped for the analysis of this clinical study.

17.4 Appendix IIIb – DNA Sequencing SOP

Your partner for

Innovative OMICS solutions

NANOPORE DIRECT DNA SEQUENCING
WET-LAB + Basic data analysis

Project number : BRECISE
Customer : Philips Eindhoven - Karolinska
Date : 23/12/2024

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2 WET-LAB PROCEDURE

2.1 INITIAL QUALITY CONTROL

Human DNA samples are received and directly stored at 4°C. DNA concentration is determined on a Qubit Fluorometer (ThermoFisher Scientific) with the Qubit dsDNA High-Sensitivity (HS) assay (ThermoFisher Scientific). The size distribution of the DNA samples was estimated on an Agilent Tapestation 4200 with the Genomic DNA ScreenTape (Agilent Technologies).

2.2 FRAGMENTATION AND QUALITY CONTROL

The whole amount of DNA sample is mechanically fragmented with g-Tubes (Covaris) at approximately 16 kb.
Sample concentration is determined with the Qubit dsDNA High-Sensitivity (HS) assay and the size distribution is estimated on an Agilent Tapestation 4200 with the Genomic DNA ScreenTape.

2.3 LIBRARY PREPARATION

The library is prepared with the Ligation Sequencing kit V14 (SQK-LSK114, ONT) based on the manufacturer's protocol. In brief, the whole amount of fragmented DNA sample is subjected to DNA repair and End prep with the addition of the following reagents: NEBNext FFPE DNA Repair Buffer, NEBNext FFPE DNA Repair Mix, Ultra II End-prep reaction buffer, and Ultra II End-prep enzyme mix (NEB). Following incubation, the end prepped DNA fragments are purified with 1X AMPure XP beads. Then, DNA fragments are adapter ligated with the addition of the following reagents: Ligation Adapter (LA), Ligation Buffer (LNB), and Quick T4 DNA Ligase (NEB). The mix is incubated at room temperature for 10 min and the adapter-ligated fragments are purified with 0.5X AMPure XP beads. The library is quantified with the Qubit dsDNA HS assay and prepared for sequencing with the addition of Sequencing Buffer (SQB) and Library Beads (LIB). A total of 20 fmol library is loaded on a R10.4 PromethION flow cell each time.

3 SEQUENCING

The library is sequenced on a PromethION R10.4.1 flow cell on PromethION2. The flow cell is initially primed with the priming mix containing Flow Cell Tether (FCT) and Flow Cell Flush (FCF), and the library is loaded on the inlet port and run for a total of 48 hours.
Following this run, a nuclease flush is performed and another 20 fmol of prepared library is loaded on the same flow cell and the run lasted for 24 hours. This procedure is repeated one more time for a run of 72 hours. High-accuracy basecalling is performed using MinKNOW and all basecalled raw data is stored as FASTQ formatted files.

4 BIOINFORMATICS

4.1 Reference data

The GRCh38 human reference is downloaded from Ensembl.

4.2 Quality control

For general quality control, PycoQC is applied. From the PycoQC reports, some general statistics can be extracted: (i) the read length distribution, a N50 value, (ii) the mean read quality, Phred score, (iii) the yield, in gigabases (Gbases) of data.

4.3 Alignment

Sequencing data is first mapped to the default human GRCh38 genome using Minimap2. Afterwards variant calling based on Sniffles2 is applied on the alignment output against the default human GRCh38 reference, producing a VCF file with structural variants. For SNV detection DeepVariant is applied.

17.5 Appendix IV – Resection pathology SOP

Instructions for Pathology Evaluation of Prostate Resection Specimen in PARIS-BIO

All handling of the prostatectomy specimen should be performed in alignment with the Swedish National Care Program for Prostate Cancer Kvalitetsdokument för patologi - RCC Kunskapsbanken Version 9.0 or later.

(https://kunskapsbanken.cancercentrum.se/diagnoser/prostatacancer/vardprogram/bilaga-5.-patologi/)

Specifically in PARIS-BIO:

1. Measurements

The prostate is weighed and sectioned horizontally into 5 mm thick slices and divided into the apex (slice 1), midportion (slices 2 to N), and base (slice N+1), where N represents the total number of horizontal slices in the midportion.

Processing of slices:

Apex and base (slices 1 and N+1): Processed sagittally, enabling precise measurements of tumor depth (apical-base plane) and tumor height (ventral-dorsal plane). However, the tumor's width (lateral dimension) in these sections is less exact.

Midportion slices (slices 2 to N): Embedded in their entirety (whole-mount sections) and processed horizontally. In these sections, the tumor's height (ventral-dorsal plane) and width (lateral dimension) can be measured accurately, while the depth (apical-base plane) measurement is less precise.

Considerations:

The accuracy of measurements varies depending on the plane of the section:

Horizontal sections from the midportion provide better measurements of tumour width (lateral dimension) and tumour height (ventral-dorsal plane).

Sagittal sections from the apex and base provide better measurements of height (ventral-dorsal plane) and depth (apical-base plane).

Any sections for histological analysis are cut in 5 µm. The thinness of these sections means that some dimensions (primarily depth in horizontal sections) are less accurate due to their representation of a "cross-sectional view" rather than the full extent of the tissue.

2. Approximation of tumor volume:

The tumor can be modelled as a sphere, ellipsoid, or cylinder, depending on the assumption about its shape.

All measurements, assumptions, and observations are put in the eCRF.