Development and validation of a pan-cancer neutrophil biomarker test for predicting clinical benefit from immunotherapy based on flow cytometry analysis of blood samples

NeutroFlow

OBSERVATIONAL STUDY

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Protocol Synopsis

Full Title	Development and validation of a pan-cancer neutrophil biomarker test for predicting clinical benefit from immunotherapy based on flow cytometry analysis of blood samples
Study Code	NEUTROFLOW
Study Objectives	Primary objective 1: To develop a kit and flow cytometry assay for detecting and quantifying the level of Ly6Ehi neutrophils in blood samples. Primary objective 2: To develop a model for predicting clinical benefit from treatment with PD-(L)1 inhibitors based on the level of Ly6Ehi neutrophils in patient blood samples.
Sample Size	A minimum sample size of N=600 was determined with up to 350 NSCLC, 180 TNBC, 60 RCC, 50 melanoma and 120 HNSCC patients.
Interventions	N/A (observational study)
Number of Sites	1. Virgen Macarena University Hospital (Clinical coordinator site) 2. European Institute of Oncology 3. Heidelberg University Hospital
Date and version	12/06/2025 v1.0

Version control

Date	Version	Changes
12/06/2025	v1.0	Original study protocol

Protocol Signature page

The present study protocol was subject to critical review and has been approved in the current version by the persons undersigned. The information contained is consistent with:

- the current risk-benefit assessment of the study intervention(s),
- the moral, ethical, and scientific principles governing clinical research as set out in the latest relevant version of Declaration of Helsinki, the principles of the guidelines of International Council on Harmonization (ICH) Good Clinical Practice and the applicable legal and regulatory requirements.

The investigators will be supplied with details of any significant change of the benefit-risk-assessment of the study.

It will be ensured that the first subject is enrolled only after all ethical and regulatory requirements are fulfilled. Written informed consent will be obtained from all subjects after detailed oral and written information. It will be confirmed that all study subjects are informed about the type of encoding of their personal data (pseudonymization) and about who receives or has access to such data. Subjects who do not agree to this data encoding and transfer will not be enrolled into the study. In this context it will be assured that all investigational sites comply with the local regulatory requirements for data protection.

Via current versions of the study protocol, it will be ensured that all principal investigators are informed about results regarding the benefits and risks of the study.

Date: 12/06/2025	Signature:	
	Name (Print Name): Function:	Dr. Alberto Moreno Conde Coordinating Investigator
Date: 12/06/2025	Signature:	
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Principal Investigators from the participating sites are:

- Virgen Macarena University Hospital: Dr. Alberto Moreno Conde
- European Institute of Oncology: Prof. Dr. Francesco Bertolini
- Heidelberg University Hospital: Prof. Dr. Petros Christopoulos

List of Acronyms

Table 1. List of acronyms

Acronym	Definition
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
AUC	Area Under the Curve
C-index	Concordance Index
CB	Clinical Benefit
CI	Confidence Interval
CRP	C reactive protein
EC	Ethics Committee
ECOG	Eastern Cooperative Oncology Group
eCRF	Electronic Case Report Form
EIC	European Innovation Council
GDPR	General Data Protection Regulation
GEAR	Gender Equality in Academia and Research
HIPPA	Health Insurance Portability and Accountability Act
HNSCC	Head and Neck Squamous Cell Carcinoma
HR	Hazard Ratio
ICF	Informed Consent Form
ICIs	Immune Checkpoint Inhibitors
IEO	Istituto Europeo di Oncologia
irAEs	Immune-related Adverse Events
IRB	Institutional Review Board
KOL	Key Opinion Leader
LDH	Lactate Dehydrogenase
MSI	Microsatellite Instability
NSCLC	Non-Small Cell Lung Cancer
NSE	Neuron-Specific Enolase
OH	OncoHost
OS	Overall Survival
PBMC	Peripheral Blood Mononuclear Cells
PFS	Progression Free Survival
PI	Principal Investigator
RBC	Red Blood Cells

RCC	Renal Cell Carcinoma
RECIST	Response Evaluation Criteria In Solid Tumors
RNA-seq	Ribonucleic Acid sequencing
ROC	Receiving Operating Characteristic
SAS	Servicio Andaluz de Salud
SOC	Standard of Care
TMB	Tumour Mutational Burden
TNBC	Triple Negative Breast Cancer
TNM	TNM Malignancy Classification System
UKHD	Universitätsklinikum Heidelberg
WBC	White Blood Cells

1. Background

1.1. Description of the clinical study

1.1.1. Study rationale

Immunotherapy based on immune checkpoint inhibitors (ICIs) has revolutionised the cancer therapy field. ICIs harness the patient's own immune system to fight cancer, producing unprecedented durable remissions in a subset of patients. Over the last decade, multiple ICIs in the form of therapeutic monoclonal antibodies targeting PD-1 (e.g., Pembrolizumab, Nivolumab, Cemiplimab), PD-L1 (e.g., Atezolimumab, Durvalumab, Avelumab) or CTLA-4 (e.g., Ipilimumab) have been approved for treating a broad range of solid cancer types. Currently, they are widely used as standard-of-care in first- and advanced-line settings (either as monotherapy, ICI-ICI combination, or combined with chemotherapy/other agents) for lung cancer, melanoma, head and neck cancer, kidney cancer and others [1]. However, despite the remarkable promise of ICIs, several clinical challenges exist: (i) Response varies widely between patients, with the majority experiencing resistance to therapy. It is estimated that only 10-40% of patients respond to treatment across different cancer types [2,3]; (ii) There is a risk of serious side effects known as immune-related adverse events (irAEs) [4]; (iii) Treatment costs are high at around €100,000 per patient annually [5].

Currently, there are three types of clinically endorsed predictive biomarkers to determine eligibility for ICI therapies: (i) tumour expression of PD-L1; (ii) tumour mutational burden (TMB); and (iii) tumour microsatellite instability (MSI) status [6,7]. Although these biomarkers are routinely used in the clinic for multiple cancer types, they are considered imperfect for several reasons. Their predictive performance is not optimal, and their utility is limited by tumour heterogeneity, differences in defining biomarker thresholds, and variability in sensitivities of detection platforms [8]. Additionally, these tests generally require tumour biopsies sampled by an invasive procedure that is not always possible.

Thus, there is an urgent unmet need for novel biomarkers with superior predictive performance. Ideally, such biomarkers would be minimally invasive (e.g., measured in a blood sample). Powerful predictive biomarkers would expand the benefit of ICIs to more patients, improve therapy outcome and quality of life, avoid the risk of irAEs in ineligible patients and reduce economic burden.

Through the proposed EIC Transition project, the consortium aims to develop and validate a novel flow cytometry-based diagnostic test (termed NeutroFlow) intended for detecting a specific neutrophil subtype in the blood that serves as a predictive biomarker for therapeutic benefit from ICIs in patients across different tumour types. The neutrophil biomarker is termed '$Ly6E^{hi}$ neutrophil'. For this purpose, a clinical study will be conducted for the collection of pretreatment blood samples and clinical data from at least 600 cancer patients across 5 cancer types: non-small cell lung cancer (NSCLC); melanoma; head and neck squamous cell carcinoma (HNSCC); triple negative breast cancer (TNBC); renal cell carcinoma (RCC).

The blood samples and clinical data will be used to: (i) develop a kit and flow cytometry assay for detecting and quantifying Ly6E^{hi} neutrophils in blood samples; (ii) determine the optimal threshold of Ly6E^{hi} neutrophils that distinguishes between patients with clinical benefit (CB) versus without CB.

1.1.2. State of the art

The NeutroFlow diagnostic test (to be developed through the proposed EIC project) will be based on a biomarker in the form of a neutrophil subtype termed 'Ly6E^{hi} neutrophil'. The discovery of the Ly6E^{hi} neutrophil as a potential pan-cancer biomarker for ICI response stems from a proof-of-concept study performed in the laboratory of Prof. Yuval Shaked (Technion, Israel Institute of Technology). The work was recently published in Cancer Cell - a leading peer-reviewed scientific journal [9]. The relevant markers for the detection of Ly6E^{hi} neutrophils by flow cytometry were previously defined in the proof-of-concept study and are protected by patent application (WO2022/091097). The Ly6E^{hi} neutrophil biomarker has shown strong predictive performance (validated in 109 blood samples from NSCLC and melanoma patients, AUC~0.9; validated in 1440 publicly available RNA-seq samples across six cancer types) [9]. It thus shows potential for development into a highly accurate clinical test.

The NeutroFlow test will be designed as a simple-to-use, rapid, point-of-care test. To perform the test, a blood sample will be drawn from the patient before beginning treatment. Peripheral blood mononuclear cells (PBMCs) will be isolated and analysed on-site using the NeutroFlow kit consisting of a specific monoclonal antibody panel for the detection of Ly6E^{hi} neutrophils by flow cytometry.

The patient blood samples and clinical data (including clinical response data) collected through the clinical study will be used for the development and validation of the NeutroFlow test. The flow cytometry protocol will be optimised on at least 30 patient samples. A proprietary computational model that predicts clinical benefit from ICI therapy based on the level of Ly6E^{hi} neutrophils in the blood will be developed using the flow cytometry results and clinical data from a development cohort (~300 patients), and validated in a blinded manner using data from an independent cohort of ~300 patients.

1.1.3. The NeutroFlow project

The recent introduction of cancer immunotherapy based on immune checkpoint inhibitors (ICIs) has revolutionised the treatment landscape for a broad range of cancer types. However, response to ICIs varies widely between patients, with the majority experiencing resistance to therapy. Moreover, the increasing use of these costly drugs coupled with management of ICI-related toxicities creates a substantial economic burden. Current biomarker tests for determining eligibility for ICIs have limited predictive performance, and many require invasive tumour biopsies. Thus, novel (and preferentially non-invasive) biomarkers for predicting ICI clinical benefit are desperately needed for better guiding clinical decisions.

The NeutroFlow project directly addresses this unmet need. We build on comprehensive academic research describing a flow cytometry assay for measuring a novel predictive biomarker in the blood – Ly6E^{hi} neutrophil – that accurately predicts therapeutic benefit from ICIs, outperforming the approved

PD-L1 biomarker. The NeutroFlow project aims to upgrade the research-level assay to an industry-level, point-of-care prototype test based on a kit consisting of an antibody panel for biomarker detection by standard flow cytometry (FC). Specifically, we will: (i) develop a FC assay according to clinical standards; (ii) develop and clinically validate a computational model that translates FC readout into a prediction of clinical benefit; (iii) evaluate analytical validity of the prototype across three medical centres (i.e., at the point-of-care); and (iv) prepare for future market introduction. The future product represents a minimally invasive, simple, rapid, and cost-effective test performed using standard hospital equipment.

As a pan-cancer blood test for accurately predicting ICI therapeutic benefit, the product will potentially expand the benefit of ICIs to more patients, improve quality of life, limit the risk of ICI-related toxicities and reduce economic burden.

2. Objectives of the Clinical Study

Primary objective 1:
To develop a kit and flow cytometry assay for detecting and quantifying the level of Ly6E^{hi} neutrophils in blood samples.

Primary objective 2:
To develop a model for predicting clinical benefit from treatment with PD-(L)1 inhibitors based on the level of Ly6E^{hi} neutrophils in patient blood samples.

Secondary objectives:

1. Evaluate the capability of the model to predict clinical benefit from treatment with PD-(L)1 inhibitors for each of the investigated indications: non-small cell lung cancer (NSCLC), melanoma, head and neck squamous cell carcinoma (HNSCC), renal cell carcinoma (RCC), triple-negative breast cancer (TNBC).
2. Evaluate the capability of the model to predict the efficacy difference between different approved PD-1/PD-L1 inhibitor-based treatments.
3. Evaluate the correlation between the level of Ly6E^{hi} neutrophils in the blood to PD-L1 staining (TPS / CPS).
4. Evaluate the correlation between the level of Ly6E^{hi} neutrophils in the blood to other clinical parameters associated with response to PD-(L)1 inhibitors.

3. Study design

3.1. Study population

At least **600 treatment-naïve patients** due to be treated with a first line regimen that includes PD-1/PD-L1 inhibitors as shown in Table 2 with the following diagnoses are planned to be enrolled in the study over a period of 3 years or as needed:

1. Stage IV NSCLC or Stage III-unresectable NSCLC
2. Stage IV or Stage IIIb-d malignant melanoma
3. Stage IV HNSCC
4. Stage IV RCC
5. Stage IV TNBC

Table 2. Approved first-line treatments based on PD-1/PD-L1 inhibitors

Indication	PD-1/PD-L1 inhibitors - monotherapy	PD-1/PD-L1 inhibitors with another agent
NSCLC	Pembrolizumab Atezolizumab Cemiplimab	Pembrolizumab + Chemotherapy Nivolumab + Ipilimumab Cemiplimab + Chemotherapy Atezolizumab + Chemotherapy + Bevacizumab
Melanoma	Nivolumab Pembrolizumab	Nivolumab + Ipilimumab Nivolumab + Relatlimab
HNSCC	Pembrolizumab Cemiplimab	Pembrolizumab + Chemotherapy
RCC	N/A	Nivolumab + Ipilimumab; Nivolumab + Cabozantinib Pembrolizumab + Lenvatinib or Axitinib Avelumab + Axitinib
TNBC	N/A	Pembrolizumab + Chemotherapy

Inclusion criteria:

1. Patients newly diagnosed with advanced-stage/metastatic NSCLC, melanoma, HNSCC, RCC, or TNBC, who are due to be treated with first-line PD-(L)1 inhibitors (as monotherapy or combined with other agents, as listed in Table 2).
2. Male or female aged at least 18 years.
3. ECOG PS: 0/1-2.
4. Normal hematologic, renal and liver function.
5. Absolute neutrophil count > 1500/mm^3, platelets > 100,000/mm^3, hemoglobin > 9 g/dL.
6. Creatinine concentration ≤ 1.4 mg/dL, or creatinine clearance > 40 mL/min.
7. Total bilirubin < 1.5 mg/dL, ALT+ AST levels ≤ 3 times above the upper normal limit.

Exclusion criteria:

1. Any concurrent and/or other active malignancy that has required systemic treatment within 2 years of the first dose of treatment.
2. For the NSCLC: presence of activating EGFR, ALK, ROS1, RET, NTRK alterations linked to an approved first-line targeted drug.

Participation in NeutroFlow study will be compatible with other research studies as long as the inclusion and exclusion criteria described above are met.

The study is neither gender-blinded nor gender-biased (as defined by the GEAR tool). The gender dimension is considered in the trial's selection criteria for patient recruitment. Since breast cancer mostly affects women, recruitment of participants for this indication is expected to be focused on biological sex. However, a balanced cohort with respect to gender and sex will be recruited for all other cancer indications.

3.2. Sample size and power calculation

A sample size of **N=600** was determined with up to 350 NSCLC, 180 TNBC, 60 RCC, 50 melanoma and 120 HNSCC patients.

Minimal sample size per indication was determined assuming predictive capability of AUC ≥ 0.7 per indication. Under this assumption, and in order to have 95% power to detect a similar AUC at an alpha of 0.05, a cohort of at least 50 patients per indication is required.

Sample size per largest indication (i.e., NSCLC) was determined assuming an Overall Survival Hazard Ratio of 0.5 between patients with positive to negative NeutroFlow prediction. Under this assumption, and assuming a 55% event rate, a validation cohort of 166 patients is required to have 90% power to detect a similar (0.5) HR at an alpha of 0.05. Further assuming a 1:1 ratio between development and validation cohort, a total sample size of 332 patients is required.

Sample size calculations were conducted using pROC package V1.18.4 for R.

3.3. Design of the clinical study

This is an uncontrolled (observational) clinical study. The study will prospectively collect patient blood samples and clinical data from patients diagnosed with NSCLC, melanoma, HNSCC, RCC or TNBC for the development of an in vitro diagnostic medical device.

3.4. Type of intervention

Study is a non-interventional. The patients are treated with the standard of care treatment for their disease and stage. The study serves to collect patient blood samples and clinical data for the development of an in vitro diagnostic medical device.

3.5. Description and timing of study procedures

Patients will participate in the study for at least 2 years. The study schedule per patient is described below:

1. Patient consent for participation in the clinical study (after diagnosis and before commencement of treatment).
2. Blood withdrawal at baseline before treatment initiation (any time from 1 month before treatment up to the day of first treatment).
3. Blood sample analysis at the point of care (i.e., flow cytometry assay to measure the level of $Ly6E^{hi}$ neutrophils in the blood sample).

4. Up to 2 years of follow up, monitoring disease progression, death events and adverse events.
5. Statistical analysis of interim results once every 4 months.

Table 3. Schedule of procedures and follow-ups

Procedure	**Screening / Before 1st treatment**	**Every 3 months following treatment or according to the SOC**	**End of Study (up to 2 years from screening)**
Clinical data collection	X	X	X
Blood collection	X	-	-
Blood sample analysis (flow cytometry)	X	-	-
Response assessment by RECIST 1.1	-	X	-
Total volume of blood collected	up to 34 ml	-	-

3.6. Study variables

Data will be collected by a qualified personnel into a secure, de-identified (without personal identifying details) electronic database as Electronic Case Report Forms (eCRF) and will include the following information (according to the time points):

Screening/Prior to the first blood collection:

- Demographic information (ethnicity, year of birth, gender)
- Medical history (including smoking history, hypertension, diabetes, ischemic heart disease, renal insufficiency, chronic obstructive pulmonary disease, asthma, liver insufficiency, Inflammatory Bowel Disease, autoimmune diseases, endocrine diseases, and others)
- Concomitant medication (prescribed and non-prescribed, including vitamins and herbal supplements) taken at the time of blood sampling (name and dosage)
- Hematology (RBC, WBC, neutrophils count, platelets count, lymphocytes count, % lymphocytes, Absolute eosinophils count, % eosinophils, Monocyte count, % monocytes, hemoglobin)
- Renal function (Creatinine concentration or creatinine clearance)
- Liver function (Total bilirubin, ALT, AST)
- Specific biomarkers (LDH, CRP, albumin, alkaline phosphatase, NSE where applicable)
- Chemistry (Calcium, Sodium)
- Cancer information: Date of cancer diagnosis, stage, primary cancer type and location, molecular type (if exists), history of the cancer treatments (if relevant), histology (if exists), Biomarkers (including mutation if exist) including PD-L1 status, MSI (Microsatellite Instability) and TMB (Tumor Mutational Burden), TNM stage, assessment of measurable lesions (at least

one), metastasis location, date of metastatic disease diagnosis (if relevant), site of metastasis (if relevant, local, distant: liver, lung, brain, bone, lymph nodes, other).
- Intended treatment protocol (drug/treatment and dosages, regimens)
- ECOG status
- Vital signs (Temperature, Pulse, Weight, Height, blood pressure)
- Date, time, and volume of sample collection
- Date and time of first treatment

Clinical Benefit (CB) evaluation (Every 3 months or according to SOC until progression):
- CB using the imaging modality of RECIST 1.1 to assess response. If imaging is used, it should be the same modality used to assess the measurable lesion at screening.
- Date of CB evaluation
- Compliance with the planned treatment (which is defined as compliance with the treatment up to the point of current CB evaluation)
- Current anti-cancer treatment and the date and reason of change or stop, if relevant
- Data on patients' outcome (including first event of recurrence/progression, survival status and OS date, PFS date, date of last treatment given)
- In case of patient death - cause of death
- Side effects to the cancer treatment, and treatment given to treat these side effects
- Received concomitant medications
- In case of stopping/changing the treatment protocol - the proposed treatment protocol and the reason for changing
- Adverse events related to the study procedures

End of study (2 years after screening or earlier in case of death or early termination):
- Data on completion or reason for early termination of the study, if relevant.
- Data on patients' outcome (survival status and OS or last follow-up date, PFS date, date of last treatment given)
- In case of patient death - cause of death

4. Preparedness status

4.1. Development of the clinical study protocol

4.1.1. Scientific advice from regulatory and health technology assessment bodies

This clinical study protocol has been established by the NeutroFlow project consortium made up of four members:

1. OncoHost Ltd (**OH**)
2. European Institute of Oncology – Istituto Europeo di Oncologia SRL (**IEO**)
3. Heidelberg University Hospital – Universitätsklinikum Heidelberg (**UKHD**)

4. Virgen Macarena University Hospital – Servicio Andaluz de Salud (**SAS**)

The consortium has consulted regulatory consultants regarding regulatory issues (e.g., GDPR requirements, ethics), and a KOL committee established from representatives of each clinical partner involved in the project regarding clinical issues (e.g., response labelling, time-points per indication).

4.1.2. Clinical efficacy, safety, and methodological guidelines (including guidelines on statistics)

As the study is non-interventional, efficacy and safety considerations have a limited effect on the study design. Dr. Anat Reiner Ben-Naim (Department of Public Health, Ben Gurion University, Israel) will supervise statistical analysis through the study. Depending on the statistical and clinical characteristics of the cohort and the study objective, the following metrics may be used to evaluate the model predictive capability:

a. Area Under Curve (AUC) of the Receiving Operating Characteristic (ROC) curve.
b. R^2 (goodness-of-fit).
c. Concordance Index (C-index).
d. Hazard Ratio (HR) of Overall Survival and Progression Free Survival (PFS) distribution between different treatment modalities.
e. Statistical significance, as expressed by Cox-regression p-value, and 95% Confidence Interval (CI).

4.1.3. Involvement of citizens / patients, carers in drawing up the clinical study protocol

Citizens and patients are not involved in the clinical study protocol design. Physicians from SAS, IEO and UKHD, serving as the principal investigators, have participated in the protocol design. A KOL committee established from representatives of each clinical partner have been consulted regarding clinical issues (e.g., response labelling, time-points per indication).

4.1.4. Regulatory intelligence to ensure timely regulatory approval and ethics clearance of the clinical study in all jurisdictions where its implementation is planned

For each participating site, an experienced investigator (who has experience with initiation and approval of clinical trials in the specific site) will be assigned to support regulatory approval and ethics clearance. Three clinical sites will participate in the study: IEO, UKHD, SAS.

Dr. A. Moreno (SAS) will lead the clinical trial and co-ordinate between all participants involved (IEO, UKHD, SAS). Dr. D. Vicente-Baz, Coordinator of the Andalusian Integral Oncology Plan at SAS, will lead NSCLC & HNSCC indications. Dr. L. de la Cruz-Merino, Director of the Clinical Oncology Unit at SAS, will lead TNBC & Melanoma indications. Dr. M. Colleoni will lead breast cancer patient recruitment at IEO.

The assigned investigator will submit the study protocol, informed consent form (ICF) and any required study documents to the Ethics Committee (EC) / Institutional Review Board (IRB) for review and approval. Any amendment to the protocol will require review and approval by the EC/IRB before the changes are implemented to the study. All changes to the consent form will be EC/IRB approved. An assessment will be conducted to decide if re-consent from participants is required.

4.2. Ethics and regulatory compliance

Regulatory requirements in all jurisdictions will be learned and applied. If necessary, the consortium will contact the regulatory bodies for clarifications or will use regulatory advisors. The study will be conducted in compliance with the HIPPA (Health Insurance Portability and Accountability Act) legislation and the GDPR (General Data Protection Regulation). Only the PIs, site personnel and study monitors will have access to the medical records of the patient. In order to maintain confidentiality of the patients, patients will be identified using a designated code, and the key that relates the code with the subject will be kept by the PI. Clinical information will be entered to the eCRFs using the coded subject number. The ICF will incorporate wording that complies with relevant data protection and privacy legislation. All relevant software that may contain patient's information will be compliant with HIPPA/GDPR.

The study will be performed in accordance with ethical principles that have their origin in the Declaration of Helsinki and are consistent with ICH E6 (GCP), and in full conformity with the regulations for the protection of human subjects of research codified in 45CFR part 46, 21CFR part 50, 21CFR part 56, and applicable local regulatory requirements whichever provides most protection to the human subjects.

Ethical requirements in all jurisdictions will be learned and applied to the study proceedings and documentation. In case of misunderstanding, the consortium will contact the ethics committee or an advisor for clarification.

4.3. Scientific and operational governance

The proposed NeutroFlow project will be conducted by a consortium made up of four members:

- OncoHost LTD (OH) – SME, Israel
- European Institute of Oncology (IEO) – hospital, Italy
- Heidelberg University Hospital (UKHD) – hospital, Germany
- Virgen Macarena University Hospital – Servicio Andaluz de Salud (SAS) – hospital, Spain

The clinical trial (which is an integral part of the NeutroFlow project) will be led by SAS.

OH will be responsible for:
(i) Overall EIC project coordination.

(ii) Development and clinical validation of a prototype NeutroFlow test including: Diagnostic-grade kit; Flow cytometry assay protocol; Computational predictive model that translates flow cytometry readout into a prediction of durable clinical benefit.
(iii) Business development activities.

IEO will be responsible for:
(i) Leading flow cytometry-related activities and analytical validation of the prototype to ensure reproducibility, standardisation, precision and accuracy.
(ii) Providing blood samples and clinical data from ~250 patients (NSCLC, TNBC).
(iii) On-site flow cytometry analysis.

UKDH will be responsible for:
(i) Leading communication and dissemination activities.
(ii) Providing blood samples and clinical data from ~250 patients (NSCLC, RCC, HNSCC).
(iii) On-site flow cytometry analysis.

SAS will be responsible for:
(i) Leading the collection of patient blood samples and clinical data.
(ii) Providing blood samples and clinical data from ~250 patients (NSCLC, TNBC, melanoma, HNSCC).
(iii) On-site flow cytometry analysis.

5. Operational feasibility

5.1. Availability of resources throughout implementation phase

The NeutroFlow prototype kit for the flow cytometry assay will comprise a panel of fluorophore-conjugated monoclonal antibodies, each specifically directed to a marker expressed on the surface of Ly6E^{hi} neutrophils (CD45, HLA-DR, Lin-CD3/14/16/19/20/56, CD11b, CD33, CD14, CD15, Ly6E). All antibodies will be GMP-compliant, diagnostic-grade monoclonal antibodies that comply with requirements for in vitro diagnostic (IVD) device components. The relevant antibodies for the above-listed markers (excluding Ly6E) are commercially available as IVD-grade off-the-shelf products, and will be acquired from specialist providers (e.g., BD Biosciences). The Ly6E antibody will be available as a proprietary, monoclonal Ly6E antibody that will be custom produced by a contractor specialising in clinical products. All antibodies will be shipped frozen to each medical centre.

Patient blood samples will be analysed on-site by the clinical flow cytometry facility available at each medical centre. Disposable and non-disposable equipment required for blood collection, PBMC isolation and storage will be provided by each of the participating clinical centres. All antibodies required for the flow cytometry assay will be shipped frozen to the analysis sites. All clinical data will be documented in a secure, de-identified (without any personal identifying details) electronic database as Electronic Case Report Forms (eCRF) hosted by SAS and made accessible to the rest of recruitment sites.

5.2. Recruitment of study population

PI and sub-investigators from SAS, IEO and UKHD will identify potentially eligible patients from their practices. They will discuss participation in the study with patients and their families during the consent discussion and address any questions or concerns they may have about the study. Informed consent will be obtained from all patients being considered for this study prior to the initiation of any study-specific procedure, including screening. Only patients meeting the eligibility criteria will participate in the study.

During the consent discussion, all potential risks and benefits associated with this study will be explained to the patient in their language as objectively as possible by one of the study investigators, and an Informed Consent Form approved by the Institutional Review Board / Ethics Committee will be provided. In the informed consent form, the patient will be required to provide consent for the collection of biological samples and clinical information. Potential subjects will be given an opportunity to review the consent form at their leisure. Once all their questions have been addressed and the individual is willing to participate in the study, informed consent to proceed with the study procedures will be obtained from the patient using the IRB-approved form (a template of the ICF to be translated and adapted for each clinical site is provided in Annex 1) and co-signed by one of the study investigators.

Recruitment progress will be monitored to identify delays or underperformance. We have set goals of reaching interim targets of 75 recruited patients by M12, 250 patients by M18, and a final target of 600 patients by M30. The targets are in line with the timeline of the EIC project as a whole (specified in clause 5.5), ensuring successful completion of the project.

Three clinical sites will contribute to patient recruitment – IEO (Italy), UKHD (Germany) and SAS (Spain). These clinical centres are part of the NeutroFlow consortium applying to the EIC transition program. All clinical centres have on-site flow cytometry facilities required for sample analysis.

Patient recruitment will not be of competitive nature. The estimated number of recruited patients per centre is summarised in Table 4. Numbers are based on patient throughput per centre in recent years. Samples will be collected over 2 years (M6 to M30, allowing at least 6-month follow up by project end at M36).

Table 4. Estimated number of recruited patients

	SAS	UKHD	IEO	Total
NSCLC	150	100	100	**350**
TNBC	40	-	140	**180**
RCC	-	60	-	**60**
Melanoma	50	-	-	**50**
HNSCC	16	100	-	**116**

Total	**256**	**260**	**240**	**756**

Mitigation of underperformance:
The trial recruitment potential is 25% higher than the requested number of participants (n=600). We have set a conservative end goal of reaching 600 samples by M30, and interim targets of 75 samples by M12, and 250 samples by M18. If we do not reach the interim targets, we will purchase retrospective, frozen samples (in accordance with trial protocol and inclusion/exclusion criteria) from blood banks to further increase the sample size. In case of major underperformance, additional medical centres will be approached.

As this is an uncontrolled study, the recruitment capability is estimated based on the average annual number of patients (per indication) that are treated in each of the participating sites, and an estimation of the percentage of patients that will provide their consent for participation (based on performance in previous trials).

5.3. Data Management

The study will be conducted in compliance with the HIPPA (Health Insurance Portability and Accountability Act) legislation and the GDPR (General Data Protection Regulation). Patient clinical data will be collected by qualified personnel into a secure, de-identified (without personal identifying details) electronic database as Electronic Case Report Forms (eCRF) hosted by SAS and made accessible to the rest of recruitment sites.

Data integrity, accuracy, and completeness will be verified in comparison to the source data by a qualified person. All project-related data (including clinical data and flow cytometry data) will be stored in an encrypted database restricted to the data owners on SAS' secure IT infrastructure with daily back-ups. Access to the database will not be made public, and all connections to the database will be performed via VPN connection. No information concerning the study, collected data or generated data will be released to any unauthorized third party without prior written approval from study PIs.

5.4. Reporting and monitoring obligations

Reporting and monitoring obligations will be met by the consortium clinical partners (UKHD, IEO, SAS). Study monitoring will be performed in compliance with recognized Good Clinical Practices. The monitor's duties include on-site or remote visits and review of study documents. Monitoring visits are intended to assess the investigators' adherence to the investigational plan and study protocol, maintenance of the study records, and review of the source documents for accuracy, completeness and legibility. During these visits, the monitor will assess the progress of the study towards meeting the study objectives and identify any concerns that stem from reviewing of the patient records, study management documents and informed consent documents. The monitor will report the findings of each visit to the study personnel. The final monitoring visit is intended to assure that all the data have been properly completed, clinical study documentation is complete, and to perform a closing meeting

with the investigator and the staff members. Reports of the visits will be written by the monitor and should include resolution of concerns, completion of follow up activities, completion of assigned tasks and any necessary corrective actions. At study completion, the monitor will prepare a final report.

The study protocol, documentation, data and all other information generated will be held in strict confidence. Only the study monitors, other authorized representatives of the clinical sites or representatives of the EC/IRB/competent authority may inspect documents and records required to be maintained by the investigator. No information concerning the study, or the data will be released to any unauthorized third party without prior written approval of the study PIs.

All regulatory requirements, clinical site monitoring and data management activities will be answered directly by the consortium partners (OH, UKHD, IEO, SAS). No responsibilities will be supported by entities that are not part of the NeutroFlow consortium partners.

5.5. Study milestones

The study schedule will follow the timeline (evaluating time in months from day of proposal approval):

1. Compilation of required regulatory and ethics submission package – M1-M3
2. Receipt of regulatory and ethics approval - M6
3. Initiation of clinical sites – M6
4. Recruitment of the first 200 patients - M15
5. Clinical response data (6 months clinical benefit) collected for 50 patients - M15
6. Mid-term recruitment report (300 recruited patients) – M21
7. Blood samples collected from a total of 600 patients across 5 tumour types – M30
8. Final assessment of all study participants – M36
9. Analysis and reporting of study results (including flow cytometry data analysis and performance of final predictive model) – M36
10. Completion of last patient follow-up (post-project) – M54

The plan is based on previous experience of the involved parties (OH, UKHD, IEO, SAS) in the initiation and execution of multicentre observational trials.

6. References

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Annex 1. ICF template

PATIENT INFORMATION SHEET

Title: Development and validation of a pan-cancer neutrophil biomarker test for predicting clinical benefit from immunotherapy based on flow cytometry analysis of blood samples (NeutroFlow)

This form is part of the informed consent process. You do not have to take part in this study if you do not want to, and your medical care will not depend on whether or not you take part. This study may not directly help you, but we hope that it will allow us to expand our knowledge so that we can help other patients in the future.

In accordance with the provisions of the General Data Protection Regulation and applicable regulations for clinical and biomedical research, we ask you to take the necessary time to read this document, consult with your relatives and clarify with your doctor any possible doubts you may have in this regard. This research study fully respects your data-related rights, such as access, rectification, cancellation, the right to limitation of processing, portability, the right to withdraw consent to data processing and the right to complain to your data privacy agency.

GENERAL INFORMATION

We are reaching out to you to inform you about a research study in which you are invited to participate. You are being invited to participate in this research study because you have been diagnosed with any of the following diseases: Stage IV Non-Small Cell Lung Cancer or Stage III-unresectable Non-Small Cell Lung Cancer; Stage IV or Stage IIIb-d malignant melanoma; Stage IV Head and neck squamous cell carcinoma; Stage IV Renal Cell Carcinoma; or Stage IV Triple-Negative Breast Cancer; and your doctor considers that you are eligible for this research study. Your inclusion in the study is subject to the screening procedures described below, and other entry criteria. Before you can take part in this study, it is important that you understand what is involved. Please read this document carefully and ask any questions you may have, and consult with others as you feel appropriate. The study has been approved by the relevant Clinical Research Ethics Committee.

OBJECTIVE

The study objective is two-folded:

1. To develop a kit and flow cytometry assay for detecting and quantifying the level of Ly6E^{hi} neutrophils in blood samples.
2. To develop a model for predicting clinical benefit from treatment with PD-(L)1 inhibitors based on the level of Ly6E^{hi} neutrophils, a specific type of white blood cells in patient blood samples.

In this study, the Ly6E^{hi} neutrophils will be assessed as potential biomarkers to predict whether a type of treatment (Immunotherapy based on immune checkpoint inhibitors) will have a clinical benefit in patients like you.

VOLUNTARY NATURE OF PARTICIPATION AND THE POSSIBILITY OF WITHDRAWAL

Your participation in this study is voluntary. If you decide to participate, but later change your mind, you are free to do so without explanation. You may withdraw from the study at any time without explanation and without affecting your medical care.

STUDY PROCEDURES

- To take part in this study, we'll need to take a sample of your blood of up to 34 ml to analyse your Ly6E^{hi} neutrophils level. This sample will be taken before you start your treatment, and will be followed by standard procedures for blood extraction in your clinical center.
- During the study you will receive the standard of care treatment from your doctor, and no additional study-related extra tests beyond those considered necessary by your clinician to monitor and follow-up your health status will be performed.
- In addition to the blood sample, we ask you to give us permission to collect information from your health record, such as demographic information; medical history, concomitant medication, lab tests, and specific information about your disease. In addition, throughout the next 2 years, we will also collect information about your evolution, treatment efficacy, and potential adverse events that you may experience.
- The clinical information and results generated as part of the study may be shared with other researchers and doctors, national and international regulatory agencies as well as in scientific repositories, always ensuring the necessary conditions of quality, security and confidentiality. In case of transfer of your data to third countries, the Research Group will ensure that a level of data protection at least equivalent to that granted by Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on Data Protection (GDPR) is maintained. The Research Group is obliged to retain the data collected for the study for at least 25 years after its completion. Thereafter, your personal information will only be retained by the centre for your health care and by the sponsor for other scientific research purposes if you have given your consent to do so, and if permitted by applicable law and ethical requirements.

POTENTIAL BENEFITS

There may be no potential benefit to you from participating in this study. However, a better understanding of the potential clinical benefit related to immunotherapy based on immune checkpoint inhibitors and the role that Ly6E^{hi} neutrophils may have in this regards may make it easier to provide individualised treatment for each patient in the future, minimising the impact of side effects and increasing the likelihood of treatment success.

POTENTIAL HAZARDS

To participate in the study we need you to allow us to obtain peripheral blood samples which will be collected in two EDTA tubes. Drawing blood is a routine medical procedure, but like any procedure, it may involve some risks and discomfort. These may include:

- Mild pain or discomfort: You may feel a brief pinch or stinging sensation when the needle is inserted.
- Bruising or swelling: It is common to experience some bruising, swelling, or redness around the site where the blood is drawn.
- Bleeding: Occasionally, there may be slight bleeding at the puncture site. Rarely, some people may experience more prolonged bleeding, especially if they have a bleeding disorder or take blood-thinning medications.

- Fainting or dizziness: Some individuals may feel lightheaded or faint during or shortly after the procedure.
- Infection: There is a small risk of infection any time the skin is broken. All necessary precautions are taken to keep this risk very low.
- Nerve injury: Rarely, the needle may come close to a nerve, which can cause temporary pain or tingling.

All these side effects are usually minor and go away on their own.

COMMERCIAL INTERESTS AND PATENTS DERIVED FROM THE STUDY

OncoHost, Ltd., an SME based in Binyamina, Israel, coordinates the NeutroFlow project, and it has declared its interest in the commercialization of the results derived from the research study. You will not receive any benefit derived from any potential commercial and/or industrial patent derived from this research study.

PERSONAL DATA PROCESSING AND CONFIDENTIALITY

Your personal data, as well as clinical data and any data obtained from the analysis of samples, will be processed in accordance with the General Data Protection Regulation (GDPR) 2016/679 on the protection of personal data and any other applicable regulation.

In accordance with current law, you have the right to access your personal data, and to request its rectification, cancellation, or objection. You may exercise these rights at any time by contacting your attending physician or the principal investigator of this study. If you choose to withdraw from the study, the research team will continue to use any data already collected about you, unless you specifically request that such data not be used.

You may also exercise your rights to limit processing, withdraw consent (exclusively in relation to the processing of personal data), and file complaints by contacting the designated data protection agency.

Samples will be coded and identified using a combination of numbers and letters, which will not allow your identification except by the principal investigator of the study. Your name, initials, or medical record number will not appear in the databases generated by the study. However, if the information obtained in the study is deemed by your physician to be relevant to your health or that of your biological relatives, you may be informed of this if you so wish. In any case, communication will be limited strictly to the data necessary for these purposes.

USE OF BIOMEDICAL SAMPLES

Your blood samples and associated data will be used only by the researchers of the project. The data and samples will be used only for the purposes of the study. After the end of the project, surplus samples may be stored in the Biobank if you give your express consent. In this case, you will sign the biobank consent form in force at the time. Otherwise, the samples will be stored until the project has been completely concluded and will be subsequently destroyed in accordance with the regulations applicable in the centre.

Should you have further doubts or questions about your participation in the study and your rights, please contact with _____ phone number: _____

INFORMED CONSENT FORM (Date: 12/06/2025; VERSION: v1.0)

Title: Development and validation of a pan-cancer neutrophil biomarker test for predicting clinical benefit from immunotherapy based on flow cytometry analysis of blood samples (NeutroFlow)

I, _____

Hereby declare that:

YES ☐ NO ☐ I've read the patient information sheet attached to this consent form.

YES ☐ NO ☐ I've been able to ask questions and have received enough information about the NeutroFlow study.

YES ☐ NO ☐ I understand that my participation in the study involves giving blood sample and information from my clinical records.

YES ☐ NO ☐ I accept that my blood samples can be analysed for the purposes described in the information sheet.

YES ☐ NO ☐ I understand that I participate on a voluntary basis and that I'm free to participate or not in the study, being able to withdraw whenever I want, without further explanations, and that this will not affect my medical care.

YES ☐ NO ☐ I authorize that the results obtained from this research study can be reused in future research projects.

YES ☐ NO ☐ I authorize that the surplus of my biological samples, data, and any derivatives from this project, if any, may be stored in the Biobank, to be used in future projects within the same line of Oncology research.

YES ☐ NO ☐ I wish to be informed of any personal information obtained during the course of the research, including any unexpected findings that may arise, provided that this information is necessary to prevent serious harm to my health or that of my biological relatives.

YES ☐ NO ☐ I freely give my consent for my data to be transferred to other public or private institutions, individuals and legal entities, within the European Union or outside it, that will use them in studies aimed at acquiring or expanding knowledge related to cancer treatment and research.

I freely give my consent to participate in the study entitled: Development and validation of a pan-cancer neutrophil biomarker test for predicting clinical benefit from immunotherapy based on flow cytometry analysis of blood samples (NeutroFlow).

Signed in _____ , at _____

Study participant	Informing clinician	Legal representative (when needed)