Additional File BREASTIFLU-1 Study Protocol

Additional File 2: Study Protocol

Supplementary Methods

This protocol is in accordance with the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidelines [1].

Participants

Table 1. Inclusion and exclusion criteria

Patient selection criteria	
Inclusion criteria	Exclusion criteria
1. Female; 2. Age of ≥18 years; 3. Histological diagnosis of ductal invasive breast cancer; 4. A primary early-stage invasive breast cancer (cT1 and/or cT2, cN0-/N1 assessed clinically and/or radiologically), without prior BC surgery of the affected breast; 5. ECOG Performance Status (PS) 0 or 1; 6. Negative serum pregnancy test for women of childbearing potential (within 7 days before ICG injection) 7. Signed informed consent form (ICF) obtained prior to any study related procedure.	1 Advanced invasive breast cancer cT3 and/or cT4; 2 In situ breast cancer disease; 3 Lobular invasive breast cancer at histology; 4 Invasive breast cancer treated with neoadjuvant chemotherapy and/or endocrine therapy; 5 Prior history of invasive or breast cancer of the affected breast in the past; 6 History of allergy or hypersensitivity to investigational product (active substance or excipients); 7 History of allergy to iodine or to shellfish; 8 Have apparent hyperthyroidism, autonomous thyroid adenoma, unifocal, multifocal, or disseminated autonomy of the thyroid gland; 9 Documented coronary disease 10 Advanced renal insufficiency (serum creatinine >1.5 mg/dL); 11 Chronic liver disease with Child-Pugh class B or C; 12 Concurrent medication which reduces or increases the elimination of indocyanine green dye (i.e., anticonvulsants, haloperidol, and heparin) during the 2 weeks before the expected operation; 13 Pregnant or lactating women; 14 Inability to give informed consent.

Schedule of assessments

Screening period

Patients will be screened for eligibility within 4–5 weeks before surgery. Trial information and invitation to participate will be given by a physician at a preoperative visit at the department of surgery. All patients will be made aware that participation is voluntary and that they are allowed to refuse further participation in the trial whenever they want. The overall trial assessment procedure is shown in Figure 1AF2.

Figure 1AF2. Trial schedule of enrolment, interventions, and assessments procedures

Assessment	**Screening** (+/- 28 days before enrolment)	**IMP time frames**[6]		**Surgery and Fluorescence imaging**[7]	**End of study treatment visit** (7 days +/- 3 days after surgery)
		D-1	**D0**	**D0**	
ICF signature	X				
Demographic data	X				
Eligibility criteria	X				
Diagnosis, past medical, cancer and surgical history,	X				
Concomitant medications, therapies & procedures	X (from 14 days prior to enrolment)				
ECOG PS	X	X	X	X	X
Physical examination & vital signs[1]	X	X	X	X	X
Surgical site examination				X	X
Laboratory tests[2]	X				
Pregnancy test[3]	X (serum)				
IMP treatment administrations[4]					
Indocyanine green 0.125 mg/kg[4a]			X		
Indocyanine green 0.25 mg/kg[4b]			X		
Indocyanine green 0.5 mg/kg[4c]		X			
Indocyanine green 1 mg/kg[4d]		X			
Indocyanine green 2 mg/kg[4e]		X			
Safety[5]					
AEs/SAEs		X			

[1]Vital signs to be measured: body weight, blood pressure and pulse (heart rate), height only at screening.
[2]Laboratory tests include: Biochemistry including serum creatinine or estimated creatinine clearance and liver function tests (e.g., total serum bilirubin, aspartate and alanine aminotransferase, alkaline phosphatase), and fasting glucose. Should not be repeated for surgery if done within 28 days prior to D0.

[3]Serum pregnancy test only at screening (performed within 7 days prior to ICG administration) for all women of childbearing potential.
[4]IMP treatment administration according to five different ICG doses as a single-dose patient arm.
[5]All AEs and SAEs related to indocyanine green and surgery must be collected and reported on the AE page of the CRF and SAE form, respectively, from the administration of indocyanine green and up to 7 days after the surgery.
[6]Two preoperative timeframes will be used for the administration of five different ICG doses as a single-dose patient arm.
[7]After lumpectomy (and/or additional tumor bed re-excision) the breast surgical specimen and the breast surgical cavity will be systematically exposed and explored with the near-infrared (NIR) camera, as well as the freshly resected breast operative specimen, serially sliced into approximately 3-4 mm thick tissue slices, will be additionally imaged with the NIR camera.
IMP, investigational medicinal product; D, day; ICF, informed consent form; ECOG PS, Eastern Cooperative Oncology Group Performance Status; AE, adverse event; SAE, serious adverse event; ICG, indocyanine green.

Time plan

Enrollment of patients started in February 2024 and will stop after the randomization of the targeted population (+/-230 patients), which is expected to be complete Q1 2026. Postoperative therapy will follow local practice. A postoperative visit is provided in the current practice of Institut Jules Bordet (IJB) at 7 days (+/- 3 days) after the breast surgery and can be considered the end of treatment (EOT) visit. The trial will be terminated after the last visit of the last patient, which is expected to be at the beginning of 2026.

Interventions

Surgical procedure

Non-palpable breast tumors will be preoperatively localized with carbon particles injected under ultrasonography as an in-hospital clinical practice. Depending on preoperative TNM staging, a sentinel lymph node biopsy (SLNB) (lymph node mapping using the peritumoral administration of Technetium-99m-labelled nanocolloid one day prior to surgery and subsequent intraoperative detection using a gamma-probe) or an axillary lymph node dissection (ALND) will be performed. Patients will undergo a BCS procedure with SLNB, ALND, or both, performed according to standard breast surgery techniques. Intraoperative margin assessment of breast surgical specimens will be done by pathological macroscopic evaluation following the current institutional practice, and additional tumor bed re-excision will be done in cases of suspected

tangent margins after this pathological evaluation [2]. All breast surgical specimens will undergo classic pathological analysis to determine the type and grade of breast tumors, their size, the histo-prognostic factors, and the definitive resection margins. No further excision of the tumor bed based on ICG-FI findings will be allowed during the surgical procedure.

Fluorescence imaging procedure

After lumpectomy (and/or additional tumor bed re-excision), the breast surgical specimen and the breast surgical cavity will be systematically exposed and explored with the near-infrared (NIR) camera for the detection of fluorescent signals on the operative specimen and/or in the breast surgical cavity. There will be no analysis (interpretation) of the fluorescence images performed during the surgical procedure in order to avoid further influence of the ICG-FI exploration findings on the surgical team and avoid any additional tumor bed re-excision based on ICG-FI findings. So, the standard surgical procedure will be respected.

The freshly resected breast operative specimens (will be inked to mark resection margins on final histopathology with black ink, then serially sliced into approximately 3-4 mm thick tissue slices) will be additionally imaged with the NIR camera, to evaluate the primary breast tumor avidity for ICG and fluorescence patterns.

The working distance of the NIR camera for video recording will be at 20-25 cm (a fixed arm will be used for holding the camera for the ex-vivo ICG-FI). The FI procedure will be done under the supervision of the principal investigator for all of the procedures.

Investigational medicinal product: indocyanine green

Drug information, formulation, and preparation

Indocyanine green (Verdye®, Diagnostic Green) is a water-soluble, tricarbocyanine dye with a peak spectral absorption at 800 nm. Each vial of Verdye® contains 25 mg or 50 mg of indocyanine green as a sterile lyophilized powder. Under sterile conditions, the indocyanine green for injection powder should be dissolved with the sterile water for injection provided.

Exactly 5 ml or 10 ml of sterile water for injection should be added to the 25 mg or 50 mg vial, giving 5 mg of dye per ml of solution.

Therapeutic regimen and dose of ICG

A specific dose (0.125 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 1 mg/kg, and 2 mg/kg) will be administered by slow intravenous injection by peripheral venous catheter in each of the five study arms according to the timing of ICG-FI of each arm. For the first time frame (intraoperative arms), doses of 0.125 mg/kg and 0.25 mg/kg of ICG will be administered at induction anesthesia of the surgical procedure (at least 20 minutes before the BCS) in two subgroups (arms). For the second time frame (preoperative arms), doses of 0.5 mg/kg, 1 mg/kg, and 2 mg/kg of ICG will be administered 24 hours before surgery in three subgroups (arms).

Non-investigational medical device: PDE Neo II near-infrared fluorescence camera

Camera overview

The PDE-Neo II® (Hamamatsu Photonics K.K) is a medical infrared camera designed to observe the fluorescent images from indocyanine green (ICG) in the patient's body. It is equipped with light emitting diodes (LEDs) used to excite the fluorescence from ICG. Observation of normal color images is also possible in the same field of view as that of the fluorescence image by using the image switching function.

Camera operations

In order to observe the images in focus, the distance between the camera window and the area of the patient's body to be observed should be 25 cm to 30 cm. It is recommended that the PDE-Neo II is operated under general indoor fluorescent lamps. Therefore, it is further recommended that infrared light sources, such as surgical lights and halogen and tungsten lamps, are turned off. The room should also be shielded from sunlight. A sterilized drape specifically designed for the PDE-Neo II will be used for intraoperative use. The PDE-Neo II

camera will be connected to a high-definition video recorder in order to capture videos of the fluorescence imaging.

Concomitant treatments

Therapies considered necessary for the subject's well-being (e.g., therapies to manage concomitant chronic pathologies or therapies required for life-threatening medical conditions) may be administered as per the treating physician's discretion. Preparations containing sodium bisulfite, including some heparin products reduce the absorption peak of Indocyanine Green for injection in blood and, therefore, should not be used.

Endpoints

Primary endpoint

The primary endpoint of the trial is the accuracy of the ICG-FI technique for the detection of positive (involved) surgical margins:

- The sensitivity (Se), specificity (Sp), negative predictive value (NPV), false-negative rate (FNR), and false-positive rate (FPR) of the ICG-FI technique will be computed at the patient level

Secondary and exploratory endpoints

The secondary endpoints of the trial are:

- Comparison of ICG-FI technique accuracy at different doses and timings
- Characterization of the breast tumor fluorescence (tumor-to-background fluorescence ratio)

The exploratory endpoints of the trial are:

- Evaluation of fluorescence intensity of axillary lymph nodes

Assessment of efficacy

Efficacy parameters for the primary endpoint:

In order to calculate the accuracy of the ICG-FI technique for the detection of positive (involved) surgical margins, the recorded FI videos will be used to calculate the following parameters:

- The sensitivity (Se)
- The specificity (Sp)
- The negative predictive value (NPV)
- The false negative rate (FNR)
- The false positive rate (FPR)

Efficacy parameters for the secondary endpoints:

- The accuracy of ICG-FI technique for breast surgical margin evaluation will be compared at different doses and timings.
- The breast tumor fluorescence signals with tumor-to-background fluorescence ratio will be characterized.
- The fluorescence intensity of axillary lymph nodes will be evaluated.

Methods for assessing efficacy parameters

The fluorescence intensity of each primary BC tumor, breast surgical specimen, and breast surgical tumor bed will be evaluated according to two methods, a visual evaluation (clinical scale) and a semi-quantitative analysis of the fluorescence intensity (quantitative scale).

In order to calculate the accuracy of the ICG-FI technique for the detection of positive (involved) surgical margins, the recorded videos will be used to calculate the fluorescence intensity of all primary tumors, breast surgical specimens, and breast surgical tumor beds, according to the following parameters (Figure 2AF2):

- Fluorescence intensity, expressed in arbitrary units will be measured with a dedicated

program for FI quantification, IC-Calc® (version 2.0, Pulsion Medical Systems SE)

- The tumor-to-background (TBR) and signal-to-background (SBR) fluorescence ratios will be calculated for each primary tumor and fluorescent spots on the breast surgical specimens, and in the breast surgical cavity.
- The findings of these images will be correlated with definitive pathological reports of the operative breast specimen.

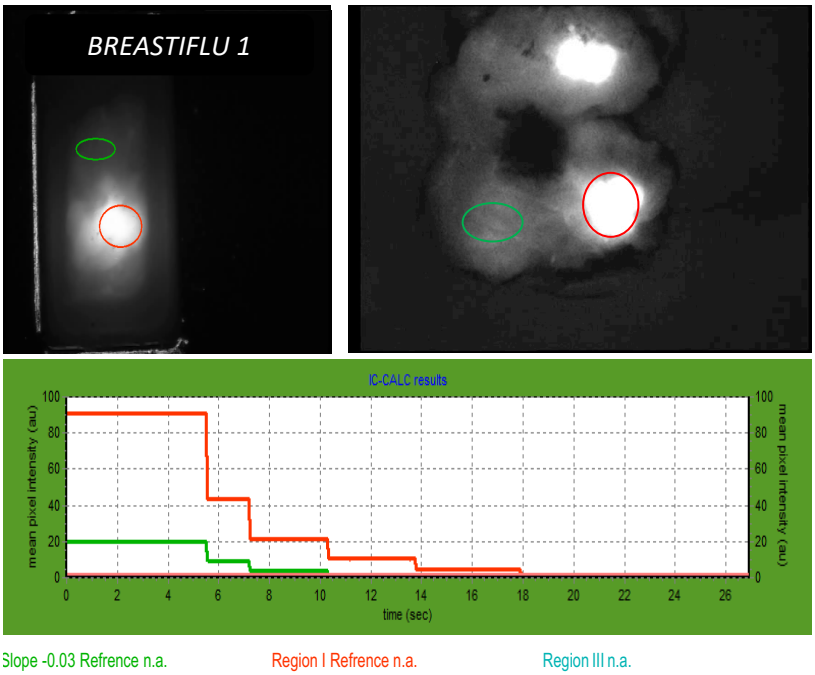
For the clinical scale (visual evaluation), two experienced breast surgeons will separately evaluate all of the recorded FI in order to classify them in cases with or without fluorescence. In cases of discordance, a consensus will be established by consulting a third surgeon with a lot of experience in fluorescence imaging.

The accuracy of the ICG-FI technique for breast surgical margin evaluation will be calculated at different doses and timings and will be compared between the different ICG-dose arms

The breast tumor fluorescence signals with their tumor-to-background fluorescence ratios will be characterized according to the histological appearance of the breast cancer tumors, as well as the ICG distribution at the cellular/extracellular level.

The fluorescence intensity of the axillary lymph nodes (with signal-to-background ratios) will be correlated with the final pathological findings.

Figure 2AF2. Overview of the quantification procedure of the fluorescence intensity



Measurement of fluorescence intensity recorded by NIR camera is illustrate here. The maximum fluorescence signal represented by the white bright spot in our figure, is marked with a red circle. Subsequently, an area considered as normal tissue around the fluorescence spot is selected and marked with the green oval in the figure, by means of the IC-Calc® program. Once the markings are made, the fluorescence signals are measured by running the recorded ICG-FI video. The fluorescence intensity expressed in arbitrary units (AU), and by graphical representations is thereby registered.

Statistical Analyses

The subjects' baseline characteristics will be reported using summary statistics and distributions. Nominal variables will be reported as numbers and proportions. Continuous variables will be reported as the number of subjects, mean and standard deviation values, as well as minimum, median, and maximum values.

The accuracy of ICG-FI for the assessment of margin status will be evaluated by reviewing intraoperatively recorded videos, correlated with the final anatomopathological reports. The fluorescence intensity of each primary tumor, breast surgical specimen, and breast surgical tumor bed will be evaluated according to two methods, a visual evaluation (clinical scale) and a semi-quantitative analysis of the fluorescence intensity (quantitative scale).

The sensitivity (Se), specificity (Sp), NPV, false negative rate (FNR), FPR of the technique

with 95% confidence intervals (CIs) will be computed at the patient level, on the total, and in different subgroups based on ICG dose administered and/or delay between injection of indocyanine green dye and fluorescence examination.

Concordance between visual scale and tumor-to-background fluorescence ratios will be evaluated with the kappa statistic.

Ethics and safety considerations

The study will be conducted in accordance with the principles of the Declaration of Helsinki and the Medical Research Involving Human Subjects Act. The study protocol has received medical-ethical approval by the Belgian Ethics Authority (3608 from 2023/12/21)

There will not be a direct benefit to the patients participating in this study because their surgical treatment (and surgical resection margins) will not be influenced or adapted by the results of the ICG-FI during this study.

The expected risks and toxicities of the ICG dye are those related to all medicines that can cause side effects, although not everybody gets them. Safety issues could arise, but published data demonstrate that ICG IV injection, both in current practice and in clinical trials, is generally safe. Severe allergic reaction is very rare (less than one in every 10,000 patients). The near-infrared fluorescence imaging technique used for this study is a non-invasive imaging technique, no particular risk related to the technique is to be expected. For the patient, the only difference will be this intravenous injection given the day before the surgery or right before the surgery, although vascular access (by peripheral venous catheter) is necessary for the part of general anaesthesia that precedes the breast surgical procedure. There will be no other invasive or diagnostic procedures, not even blood tests, physical exams, or hospital visits, other than the usual ones used for patients who benefit from a BCS-type surgical intervention in our clinical practice.

The sponsor shall set-up and maintain an e-Trial Master File (e-TMF) containing documents as the essential documents and written communications relating to that Study which allow verification of the conduct of a Study and the quality of the data generated.

Data management and availability

The study complies with the General Data Protection Regulation (European Regulation 2016/679), which requires data to be de-identified as soon as it is practical to do so. All data samples to be collected as part of this study will be anonymized with participants being assigned a unique study code. Any information pertaining to personal details will be kept by the principal investigator in a safe and secure location at the sponsor site (Institut Jules Bordet, Rue Meylemeersch 90, 1070 Brussels, Belgium) and is only available to research staff directly running the study. All data will be entered into an electronic database using unique study codes for each participant and securely stored on a password-protected computer database. Important protocol modifications will immediately be communicated to relevant parties (e.g., investigators, trial participants, trial registries, regulators). Data will be available from the study, once conducted, upon reasonable request to the principal investigator.

Safety reporting

The intensity of all adverse events (AEs*)* will be graded according to the NCI-CTCAE version 5.0 on a five-point scale (Grade 1 to 5) and reported in detail on the Case Report Forms (CRF). The annual safety report will be submitted to the Belgian Federal Agency for Medicines and Health Products in accordance with the General Data Protection Regulation (European Regulation 2016/679)

Study oversight

An Independent Data Monitoring Committee will be not needed for this study considering that this is a phase IB/II cross-sectional diagnostic study with no interim analysis and with a reliable safety profile. There is no monitoring for the subjects' compliance as long as the administration of IMP (ICG) is done only once, and it represents the selection criterion for the inclusion of patients in the final analysis. A monitoring for the conduct of the trial will be done by the sponsor of the study by their representatives based on an assessment that takes into consideration all characteristics of the clinical trial.

References

1. Chan AW, Tetzlaff JM, Altman DG, Laupacis A, Gotzsche PC, Krleza-Jeric K, et al. SPIRIT 2013 statement: defining standard protocol items for clinical trials. Ann Intern Med. 2013;158(3):200-207. doi: 10.7326/0003-4819-158-3-201302050-00583.
2. El-Helou E, Eddy C, Picchia S, Van de Merckt C, Radermeker M, Moreau M, et al. Effectiveness of Carbon Localization for Invasive Breast Cancer: An Institutional Experience. Breast J. 2023;18::4082501. doi: 10.1155/2023/4082501.