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Evaluation of trophoblast cell surface antigen-2 (TROP2) protein expression in chemotherapy-resistant and metastatic breast carcinomas

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ABSTRACT

Trophoblast cell-surface antigen 2 (TROP2), a transmembrane receptor expressed in many carcinomas, is a target for novel antibody-drug conjugates such as sacituzumab govitecan. TROP2-targeted therapy is used for unresectable locally advanced or metastatic triple-negative and hormone receptor-positive, HER2-negative breast cancers. The role of TROP2 as a predictive marker is yet unclear. Standardized interpretation criteria for TROP2 immunohistochemistry (IHC) are lacking. Here, we compared three antibody clones and two methods for semi-quantitative assessment, aiming to establish reproducible evaluation criteria. First, TROP2 IHC was performed on normal tissues and nine breast cancers, using the BSB-148, EPR20043 and SP293 clones. EPR20043 was selected for subsequent evaluation in 69 breast cancers without pathological complete response to neoadjuvant chemotherapy (NAC). Four pathologists applied the ASCO/CAP guidelines for HER2 IHC testing (designated as the 'membrane score') and the H-score. All H-scores were categorized as low (0−100), intermediate (101−200) and high (201−300). Although the membrane scores strongly correlated with the categorized H-scores, the latter showed higher interobserver variability. Next, TROP2 IHC was performed on 94 breast cancer metastases and evaluated by six pathologists, confirming the strong correlation between the membrane scores and H-scores. In metastases, the interobserver variability was similar for both methods. Our observations support the application of the HER2 ASCO/CAP guidelines for semi-quantitative evaluation of membranous TROP2 protein expression, as this method strongly correlates with the H-score and is less prone to interobserver variability in post-NAC breast resections. Future studies should investigate the association between the TROP2 membrane score and response to TROP2-targeted therapy.

Abbreviations: ADC, antibody-drug conjugate; CC1, cell conditioning 1; DAB, 3,3'-diaminobenzidine; DCIS, ductal carcinoma in situ; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization; IVD, in vitro diagnostic use; NAC, neoadjuvant chemotherapy; NST, no special type; RTU, ready-to-use; PR, progesterone receptor; RCB, residual cancer burden; SD, standard deviation; TILs, tumor-infiltrating lymphocytes; TNBC, triple-negative breast cancer; TROP2, trophoblast cell surface antigen-2.

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1. Introduction

Antibody-drug conjugates (ADCs) are monoclonal antibodies that are attached to a cytotoxic drug or 'payload' via linker molecules [1]. Their advantages comprise a short systemic half-life of the cytotoxic drug, and its effective targeted delivery to tumor cells [2,3]. This results in higher efficacy and less systemic side effects than classic chemotherapy [4]. ADCs are directed against cell surface antigens (receptors) on tumor cells. Tumor cells internalize the ADC-receptor complex through endocytosis, and lysosomal enzymes cleave the linker, thereby releasing the payload and causing cell death [4,5]. The 'bystander antitumor effects' are an additional advantage, i.e. neighboring tumor cells are killed, even in the absence of the target antigen on those cells, because the cleaved payload easily diffuses from the targeted cell to its surrounding cells [5]. This is useful in tumors with low levels or heterogeneous expression of the target antigen, such as so-called 'HER2 low' breast cancers [6]. Besides HER2, ADCs can target other surface antigens, like trophoblast cell-surface antigen 2 (TROP2). TROP2 is a transmembrane calcium signal-transducing glycoprotein that is highly expressed in many normal epithelia and thus, in many carcinomas, including breast cancer [7]. This makes TROP2 a particularly interesting target for ADC construction, as these novel targeted therapies may become potent all-round 'pan-cancer' drugs, their use not being restricted to breast cancer but extendable to other types of carcinoma as well [1,8].

Data on TROP2 overexpression in breast cancer are limited, and its role as predictive marker for response to TROP2-targeting ADCs, including datopotamab deruxtecan and sacituzumab govitecan, is yet unclear. Biomarker analyses of triple-negative breast carcinomas (TNBCs) in the phase III ASCENT trial suggested higher response rates to sacituzumab govitecan in TNBCs with intermediate to high TROP2 expression, although the number of TROP2-low TNBCs was insufficient for definitive conclusions [9]. In the final ASCENT trial report, an overall improved outcome in favor of sacituzumab govitecan was observed regardless the TROP2 expression, but the TROP2 status was only known for 60 % of the intention-to-treat population [10]. The trial protocols of the TROPICS-02, TROPION-Breast01 and TROPION-Breast02 studies do not provide technical details on TROP2 immunohistochemistry (IHC), and TROP2 positivity is not a prerequisite for eligibility [2,11,12]. Sacituzumab govitecan has FDA- and EMA-approval for treatment of unresectable locally advanced or metastatic TNBCs and hormone receptor-positive, HER2-negative (HR+/HER2-) breast cancers with at least two prior systemic therapies [13,14]. Datopotamab deruxtecan showed promising results in heavily pretreated TNBCs and HR+/HER2- breast cancers in the TROPION-PanTumor01 study and is currently investigated in phase III trials [15].

Standardized protocols and interpretation criteria for TROP2 IHC are lacking. Various commercially available monoclonal and polyclonal anti-TROP2 antibodies are used, without knowledge about their specificity and sensitivity. This might explain different positivity rates among various studies, analogous to the discrete differences observed for anti-HER2 antibody clones, especially in the 'HER2-low' range [16–19]. In most reports so far, TROP2 immunoreactivity was evaluated by a single pathologist, by applying the H-score or similar methods combining proportion and intensity scores [7–9,20,21]. Semi-quantitative IHC assessment is prone to interobserver variability, which was extensively demonstrated for 'HER2-low' breast cancers [22–26]. Ideally, multiple observers should be involved to establish whether TROP2 overexpression is a robust predictor of therapy response. Such an investigation requires reproducible evaluation criteria.

In this study, six pathologists compared three commercially available monoclonal TROP2 antibodies, and two different methods for TROP2 IHC assessment in two different patient cohorts. Since the eligibility for therapy with sacituzumab govitecan is currently limited to breast cancer patients with unresectable locally advanced or metastatic disease, our retrospective analysis focused on (heavily) pretreated breast cancer patients.

2. Materials & methods

2.1. Tissue samples and histopathological data

This retrospective study comprised two patient cohorts. Tissue samples from the first cohort were resection specimens from breast cancer patients who underwent surgery after neoadjuvant chemotherapy (NAC) between 1 December 2020 and 15 October 2022. A residual cancer burden (RCB) score of >1.200 was required for inclusion, and was used as a definition for NAC resistance. At least one residual focus of carcinoma with minimal cellularity of 10 % and a minimum size of 2 mm, either in the breast resection or the axillary lymph nodes, was required for inclusion.

Tissue samples from the second cohort were resection specimens or biopsies from metastatic breast cancer patients who were diagnosed between 1 January 2015 and 15 October 2022. Inclusion was based on the amount of residual tumor in the formalin-fixed paraffin-embedded (FFPE) tissue blocks. Core needle biopsies and decalcified bone biopsies were excluded. Tissue processing was performed as previously described [27]. Information on patient age at diagnosis, histological type, Nottingham grade, estrogen receptor (ER) and progesterone receptor (PR) status, HER2 status, Ki-67 expression, RCB class and score, lymphovascular invasion, and metastatic site were retrieved from electronic histopathological reports. Information on systemic therapies was not retrieved from the patient files.

Histopathological assessment, IHC for ER, PR, HER2 and Ki-67 (Table S1), and *HER2/CEP17 in situ* hybridization were performed as previously described [27,28]. Normal tissue samples consisted of normal tissue surrounding metastases, and leftover human tissues that are routinely used as on-slide controls in the ISO15189-accredited laboratory of pathology of the Cliniques universitaires Saint-Luc (Brussels, Belgium). This study was approved by the institutional ethics committee (file name: 2022/21NOV/436) and performed in accordance with the World Medical Association's Declaration of Helsinki.

Table 1
Protocol details for the immunohistochemical stains with three different monoclonal anti-TROP2 antibodies [a]. Each staining protocol started with automated deparaffinization at 72°C and ended with 8 minutes of haematoxylin II [b] counterstaining.

Protein	Clone	Firm	Species	Dilution	Prim-AB	HIER	Visualisation
TROP2	EPR20043	Abcam	Rabbit	1/500	60 min – 36°C	CC1[b] – 52 min – 95°C	UltraView DAB Detection[b]
TROP2	SP293[c]	Abcam	Rabbit	1/50	52 min – 36°C	CC1[b] – 52 min – 95°C	UltraView DAB Detection[b]
TROP2	BSB−148[c]	BioSB	Mouse	1/25	60 min – 36°C	CC1[b] – 56 min – 100°C	OptiView DAB Detection[b]

Abbreviations: CC1: Cell Conditioning 1 Tris-based buffer [b]; C: Celsius; DAB: 3,3'-diaminobenzidine; HIER: heat-induced epitope retrieval; IHC: immunohistochemistry; min: minutes; Prim-AB: incubation conditions for the primary anti-TROP2 antibody; TROP2: trophoblast cell-surface antigen 2

[a] Performed on 4-μm-thick sections mounted on Superfrost plus slides (Menzel-Gläser, Braunschweig, Germany)

[b] Ventana Medical Systems, Tucson, Arizona (USA)

[c] An amplification step was added to the staining protocol

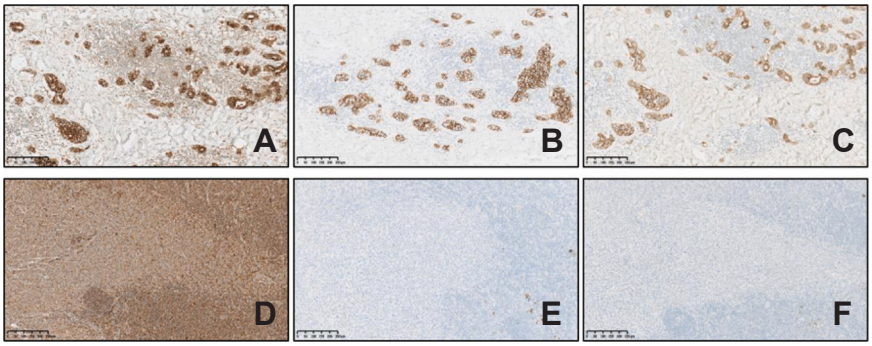

Fig. 1. TROP2 immunoreactivity. Clone BSB-148 (A) showed a weakly to moderately intense cytoplasmic immunoreactivity in lymphocytes and fibroblasts of the breast, which was not observed for EPR20043 (B) and SP293 (C). The invasive breast carcinoma showed similar immunoreactivity patterns (A-C). The TNBC lymph node metastasis showed no or hardly any immunoreactivity with EPR20043 (E) and SP293 (F), whereas BSB-148 (D) showed diffuse cytoplasmic and membrane staining with at least moderate intensity. Original magnification 100x.

Table 2
NAC-treated breast carcinoma cohort. Kappa values[$] for each duo of pathologists, reflecting the degree of inter-observer variability for the evaluation of TROP2 protein expression by using the membrane score[°] and the categorized H-score*.

Membrane score °				
Observer	**P1**	**P2**	**P3**	**P4**
P1	-	0.462	0.551	0.496
P2	0.462	-	0.490	0.318
P3	0.551	0.490	-	0.487
P4	0.496	0.318	0.487	-
*H-score categories **				
Observer	**P1**	**P2**	**P3**	**P4**
P1	-	0.237	0.705	0.425
P2	0.237	-	0.274	0.165
P3	0.705	0.274	-	0.362
P4	0.425	0.165	0.362	-

[°] Membrane score comprises 3 categories: negative (score 0/1+), intermediate (score 2+) and positive (score 3+).
* H-score categories comprise 3 categories: negative (H-score 0–100), intermediate (H-score 101–200) and positive (H-score 201–300).
[$] Colour codes for kappa values are as follows: red = slight agreement; orange = fair agreement; yellow = moderate agreement; green = substantial agreement.

2.2. TROP2 immunohistochemistry (IHC)

TROP2 IHC was performed on 4μm-thick FFPE tissue sections, using an automated slide stainer (Benchmark Ultra, Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. Staining protocols were developed for three monoclonal anti-TROP2 antibodies; their details are shown in Table 1. The BSB-148 clone (BioSB, Santa Barbara, CA, USA) was selected because of its 'in vitro diagnostic use' (IVD) label. The selection of the EPR20043 (Abcam, Cambridge, UK), and SP293 (Abcam) clones was based on their use in previous reports [20,29,30]. As TROP2 expression is strong in squamous epithelia [7,31], normal skin biopsies were applied as positive on-slide controls for optimization of the protocols, aiming to obtain a similar staining intensity of the epidermis for each antibody. Membrane immunoreactivity was quantified by applying the ASCO/CAP guidelines for HER2 IHC [32], which was designated as the 'membrane score'. This membrane score included negative (0/1+), intermediate (2+) and positive (3+) results. The H-score for membrane immunoreactivity ranged from 0 to 300 and was determined as follows: (3 x % tumor cells with strong immunoreactivity) + (2 x % tumor cells with moderate immunoreactivity) + (1 x % tumor cells with weak immunoreactivity) [9].

2.3. Molecular analysis

Data derived from next-generation sequencing were available for twenty tumor tissue samples derived from twenty patients. DNA extraction and NGS were performed in the BELAC-accredited and ISO15189-accredited laboratory of the Department of Molecular Biology (Cliniques universitaires Saint-Luc, Brussels, Belgium). For each tumor, 4–10 FFPE sections were prepared. One section was stained with hematoxylin/eosin (HE) and used to annotate the tumor. A tumor cell percentage of at least 20 % was required for further analysis. Tumor cells were macro-dissected manually. The obtained tissue was digested with proteinase K. DNA extraction was performed using the MagCore® Plus II system (Atrida, Amersfoort, The Netherlands), according to the manufacturer's instructions. DNA concentrations were measured using the

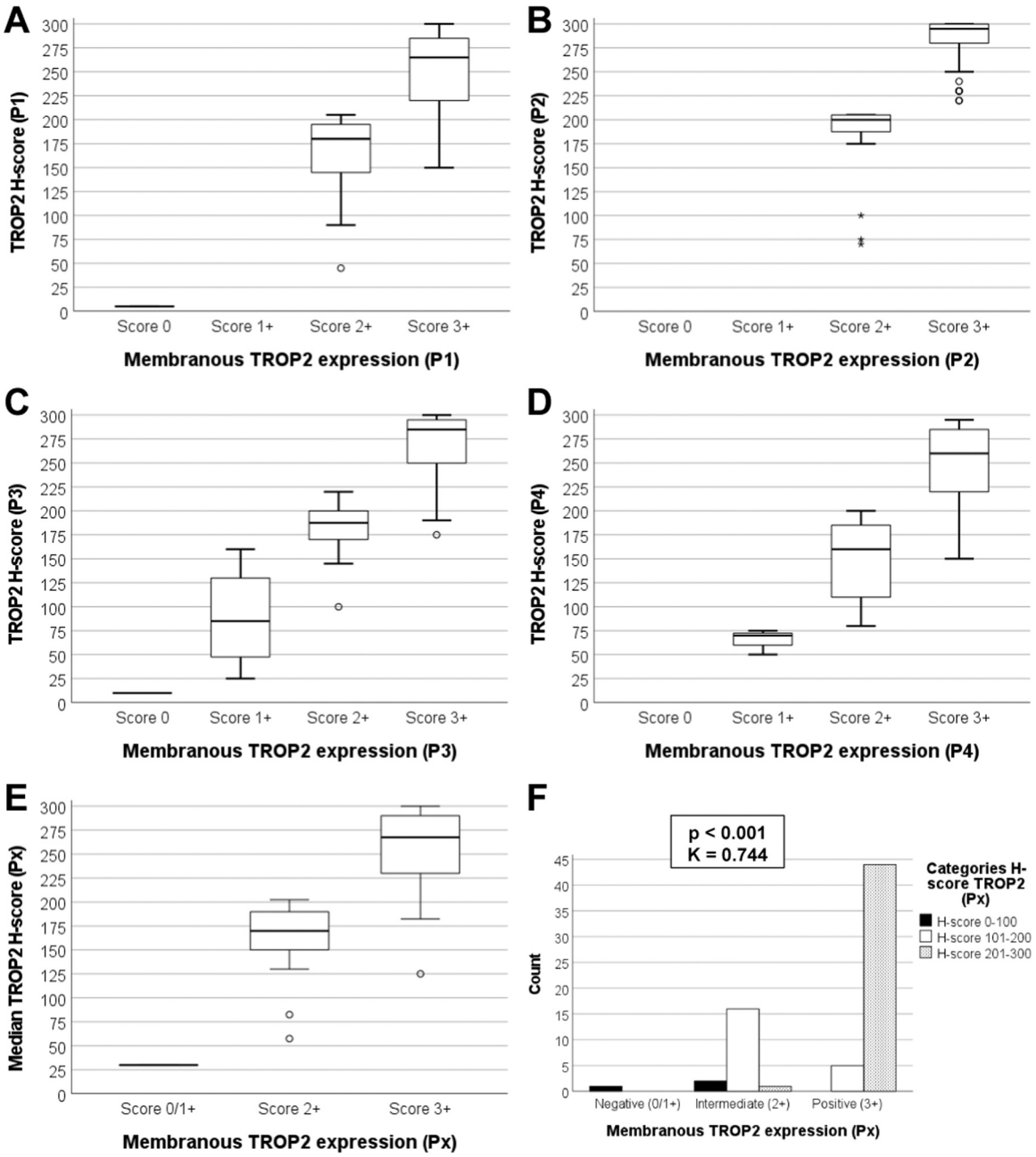

Fig. 2. Box-and-whisker plots (A-E) illustrating the strong correlation between the continuous H-score and the membrane score for each pathologist and for the median 'Px' in the NAC-treated breast cancer patient cohort. Asterisks signify outliers; circles indicate extreme values. Bar chart (F) illustrating the strong correlation between the categorized H-score and the membrane score for the median 'Px'.

Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA). A custom panel for targeted NGS was used (Custom Capture Panel TE-98036185_hg38; TWIST Bioscience, South San Francisco, CA, USA) for the library preparation, comprising exons of 117 genes (Table S2).

The targeted fragments were amplified and sequenced using the Illumina Nextseq500 paired-end sequencing (Illumina, Eindhoven, The Netherlands). This method was optimized to detect single-nucleotide variants (SNVs), as well as small insertions and deletions (indels). The detection limit was 5 % mutant alleles, corresponding to 10 % tumor cells. The Human Genome Variation Society (HGVS) nomenclature was used to report each variant at the cDNA level (c. annotation) and the protein level (p. annotation) [33]. All variants were biologically and clinically classified according to the Belgian quality standards (BELAC 2–405-NGS Rev 3–2021), in accordance with the Belgian guidelines for next-generation sequencing analysis, which are based on the recommendations published by Richards et al. and Li et al. [34–37]. Variant-calling and variant-annotation, visualization and filtering was done by using the SOPHiA DDM™ platform (SOPHiA GENETICS, Inc., Boston, MA, USA). A coverage of at least 500X was considered optimal. Exons with a coverage between 200X and 500X were also analyzed. A coverage below 200X was considered as inferior and these exons were not analyzed. A minimum of 10 reads was required to call a variant. All pathogenic variants, potentially pathogenic variants and variants of unknown significance meeting the aforementioned criteria were reported.

2.4. Statistical analysis

Statistical analyses were performed using IBM SPSS statistics 28.0 software (Chicago, IL, USA). Continuous variables were tested for

Table 3
NAC-treated breast carcinoma cohort. TROP2 expression in association with histopathological features.

	TROP2 negative 0/1+ score n (%)	TROP2 intermediate 2+ score n (%)	TROP2 positive 3+ score n (%)	p-value*
Histological subtype				0.004
No special type (NST)	1 (100)	10 (53)	37 (76)	
Invasive lobular carcinoma	0 (0)	8 (42)	3 (6)	
Other special type	0 (0)	1 (5)	9 (18)	
Nottingham grade				0.807
Well differentiated	0 (0)	1 (5)	4 (8)	
Moderately differentiated	1 (100)	12 (63)	25 (51)	
Poorly differentiated	0 (0)	6 (32)	20 (41)	
RCB class				0.113
RCB-I	0 (0)	3 (16)	3 (6)	
RCB-II	1 (100)	6 (32)	33 (67)	
RCB-III	0 (0)	10 (53)	13 (27)	
Lymphovascular invasion				0.487
Absent	0 (0)	10 (53)	30 (61)	
Present	1 (100)	9 (47)	19 (39)	
Oestrogen receptor status				0.121
Negative (<1 %)	1 (100)	2 (11)	10 (20)	
Positive (≥1 %)	0 (0)	17 (90)	39 (80)	
Progesterone receptor status				0.875
Negative (<1 %)	1 (100)	10 (56)	29 (59)	
Positive (≥1 %)	0 (0)	8 (44)	20 (41)	
HER2 expression				0.835
Score 0	0 (0)	5 (26)	14 (29)	
Score 1+	1 (100)	6 (32)	13 (27)	
Score 2+	0 (0)	6 (32)	19 (39)	
Score 3+	0 (0)	2 (11)	3 (6)	
Immunohistochemical subtype				0.340
HR-positive, HER2-negative	0 (0)	14 (74)	29 (59)	
HER2-positive	0 (0)	3 (16)	10 (20)	
Triple-negative	1 (100)	2 (11)	10 (20)	

HR: hormone receptor; NST: no special type; HER2: human epidermal growth factor receptor 2; RCB: residual cancer burden; TROP2: trophoblast cell surface antigen-2
* Chi-Square test or Fisher's Exact test when appropriate

normal distribution using the Shapiro-Wilk test. Patient age showed normal distribution in both cohorts, with equal variances according to Levene's test. The association between age and TROP2 status was analyzed with one-way ANOVA tests and reported as mean ± standard deviation (SD). All other continuous variables in both cohorts were not normally-distributed and were therefore reported as median (P25, P75). Categorical data were analyzed with Chi-Square tests or Fisher's Exact tests when appropriate, and reported as absolute number with relative percentage. All tests were two-sided, with the significance level set at $\alpha = 0.05$.

The median TROP2 H-score was selected for each sample, based on the assessment of all participants, and was designated 'Px' (i.e. the median pathologist). Associations between the Px TROP2 H-scores and categorical histopathological characteristics were investigated by Mann-Whitney U or Kruskal-Wallis tests, depending on the number of categories. For each observer, box-and-whisker plots visualized the relation between the H-scores and membrane scores. The H-scores were categorized as low (0−100), intermediate (101−200) or high (201−300) [20]. Their correlation with the membrane scores was investigated by Chi square tests.

Interobserver variability was quantified by calculation of the intraclass correlation coefficients (ICCs) for the continuous H-scores, with interpretation according to Koo and Li [38]. ICC settings were: two-way random, single measures, absolute agreement [28]. Kappa values were calculated for the categorized H-scores and the membrane scores, per duo of pathologists, and interpreted according to Landis and Koch [39]. Since both the categorized H-scores and the membrane scores are categorical instead of continuous variables, Bland-Altman plots (i.e. scatter plots to estimate agreement between two paired measurements on a continuous scale) were not appropriate for this study. Additionally, Bland-Altman plots were not constructed for the continuous H-scores, as the differences between the H-scores of two observers were not normally distributed.

3. Results

3.1. Comparison of 3 monoclonal antibodies for TROP2 IHC

Each staining protocol was initially performed on normal tissue, comprising appendix, breast, Fallopian tube, kidney, liver, lung, lymph node, muscle, ovary, pancreas, placenta, prostate, testis, thyroid, tonsil, and uterine cervix (Figure S1). Staining patterns were identical for EPR20043 and SP293, whereas BSB-148 showed non-specific weakly to moderately intense cytoplasmic immunoreactivity in lymphocytes and fibroblasts, despite multiple protocol adjustments (Table S3; Fig. 1A-C).

Each staining protocol was performed on nine breast carcinomas, comprising eight resected metastases surrounded by normal tissue, and one mastectomy specimen. The three antibody clones showed similar immunoreactivity patterns for all but one tumor. This TNBC lymph node metastasis showed no immunoreactivity with EPR20043 and SP293, whereas BSB-148 showed diffuse cytoplasmic and membrane staining with at least moderate intensity (Fig. 1D-F). Taking the aforementioned observations into account, and given its higher dilution (and associated lower cost) as well as its use in previous reports [29,40], the EPR20043 clone was selected for further analyses.

3.1.1. Characteristics of the patient cohorts

The NAC-treated cohort comprised 69 patients with residual tumor after NAC: six patients (9 %) had an RCB-I, forty patients (58 %) had an RCB-II and twenty-three patients (33 %) had an RCB-III response. The mean patient age was 54 (range 29–90). The metastatic breast cancer patient cohort comprised 94 tissue samples from 90 female patients, including 29 biopsies and 65 surgical resections. The mean patient age was 60 (range: 30–88). Details on both cohorts are provided in Table S4.

3.2. TROP2 expression in NAC-resistant breast cancer

NAC-resistance was defined as residual disease after NAC, with an RCB score of >1.200, as mentioned in the Materials & Methods section. The evaluation of TROP2 immunoreactivity as a continuous variable, by applying the H-score, showed variable agreement, ranging from moderate (lowest ICC of 0.593) to excellent (highest ICC of 0.940; Table S5). After categorization of the H-score, agreement among the four observers as determined by kappa values ranged from slight (κ=0.165) to substantial (κ=0.705), with a mean kappa of 0.361 (fair agreement). By applying the membrane score, agreement among the four observers ranged from fair (lowest κ=0.318) to moderate (highest κ=0.551), with a mean kappa of 0.467 (fair agreement; Table 2).

To investigate whether there is a rationale for the membrane score, box-and-whisker plots were constructed (Fig. 2A-2E). For each pathologist, the continuous H-score correlated strongly with the membrane score ($p<0.001$). Categorization of the H-scores as negative (score 0–100), intermediate (score 101–200), and positive (score 201–300; Fig. 2F) corresponded well with the membrane scores ($p<0.001$).

Patient age was not associated with TROP2 expression ($p=0.358$). None of the histopathological features was significantly associated with TROP2 expression ($p>0.05$), except for the histological type ($p=0.004$; Table 3).

Table 4
Metastatic breast carcinoma cohort. Kappa values[$] for each duo of pathologists, reflecting the degree of inter-observer variability for the evaluation of TROP2 protein expression by using the membrane score[°] and the categorized H-score*.

TROP2 Membrane Score °						
Observer	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
P1	-	0.571	0.537	0.398	0.630	0.380
P2	0.571	-	0.600	0.396	0.458	0.551
P3	0.537	0.600	-	0.425	0.512	0.698
P4	0.398	0.396	0.425	-	0.423	0.394
P5	0.630	0.458	0.512	0.423	-	0.453
P6	0.380	0.551	0.698	0.394	0.453	-
*TROP2 H-score categories **						
Observer	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
P1	-	0.537	0.676	0.514	0.653	0.485
P2	0.537	-	0.503	0.323	0.456	0.581
P3	0.676	0.503	-	0.513	0.575	0.601
P4	0.514	0.323	0.513	-	0.519	0.354
P5	0.653	0.456	0.575	0.519	-	0.354
P6	0.485	0.581	0.601	0.354	0.354	-

[°] Membrane score comprises 3 categories: negative (score 0/1+), intermediate (score 2+) and positive (score 3+).
* H-score categories comprise 3 categories: negative (H-score 0–100), intermediate (H-score 101–200) and positive (H-score 201–300).
[$] Colour codes for kappa values are as follows: red = slight agreement; orange = fair agreement; yellow = moderate agreement; green = substantial agreement.

3.3. TROP2 expression in metastatic breast cancer

Given the frequent use of sacituzumab govitecan in the metastatic breast cancer setting, we aimed to validate these initial findings in a patient cohort with breast cancer metastases, with two additional observers. The continuous H-score showed good (lowest ICC=0.797) to excellent (highest ICC=0.946) agreement (Table S6). In this cohort, the interobserver variability among six observers was similar for the membrane score and the categorized H-score (Table 4). Agreement for the membrane score was fair (lowest κ=0.380) to substantial (highest κ=0.698). Agreement for the categorized H-score was fair (lowest κ=0.323) to substantial (highest κ=0.676). The mean kappa values for the membrane score and the categorized H-score were 0.495 and 0.510, respectively. The strong association between the continuous H-score and the membrane score was confirmed for each pathologist in this cohort ($p<0.001$; Figure S2). The categorized H-score also correlated strongly with the membrane score ($p<0.001$; Figure S2).

Patient age at the time of diagnosis was not associated with TROP2 expression ($p=0.393$). Histopathological features were not associated with TROP2 expression ($p>0.05$), except for HER2 expression ($p=0.041$) and the immunohistochemical subtype integrating hormone receptor status and HER2 status ($p=0.034$; Table 5).

3.4. TROP2 expression and mutation analysis

NGS data were available for twenty different tumor samples, of which four (20 %) showed insufficient coverage to allow reliable interpretation. Of the remaining sixteen tumor samples with available NGS data, nine (56 %) showed low TROP2 protein expression (i.e. median H-score <100) and seven (44 %) showed high TROP2 protein expression (i.e. median H-score >200). Nine of sixteen (56 %) tumors were triple-negative, three (19 %) were HER2-positive, and four (25 %) showed a hormone receptor-positive, HER2-negative profile. *TP53* was the most frequently mutated gene: four TROP2-low and two TROP2-high tumors showed a pathogenic or likely pathogenic mutation in *TP53*, although this is most likely linked to the triple-negative profile, as five out of six *TP53*-mutated tumors were triple-negative (Fig. 3). Other pathogenic or likely pathogenic mutations were found in the following genes: *ARID1A, ATM, CDH1, CIC, ESR1, HRAS, NF1, PBRM1, PIK3CA, PIK3R1, POLD1, PTEN, RB1, SDHA, TERT* promoter and *TSC1* (Fig. 3). The limited sample size did not allow to demonstrate a particular mutational signature for either TROP2-low or TROP2-high invasive breast cancers.

4. Discussion

Data about TROP2 overexpression in breast cancer are limited so far. Microarray data from the I-SPY, METABRIC and TCGA studies showed that expression of the TROP2 gene *TACSTD2* is present in all breast cancer subtypes, but it is generally lower in HER2-positive tumors than in luminal-A tumors and TNBCs [41]. However, a tissue microarray study showed that low TROP2 expression was more frequently observed in TNBCs [7]. TROP2-positivity is present in 85 % of high-grade metaplastic breast carcinomas, which are most often TNBC [42]. This percentage seems rather high, but Chartier et al. used a 1 % cut-off for TROP2-positivity with the AF650 clone [42].

In a series of 685 TNBCs, TROP2 overexpression as determined with the SP293 clone did not correlate with patient age, body mass index,

Table 5
Metastatic breast carcinoma cohort. TROP2 expression in association with histopathological features.

	TROP2 negative 0/1+ score n (%)	TROP2 intermediate 2+ score n (%)	TROP2 positive 3+ score n (%)	p-value*
Histological subtype				0.161
No special type (NST)	3 (75)	14 (67)	59 (86)	
Invasive lobular carcinoma	0 (0)	4(19)	4 (6)	
Other special type	1 (25)	1 (5)	4 (6)	
Not specified/unknown	0 (0)	2 (10)	2 (3)	
Nottingham grade				0.196
Well differentiated	0 (0)	0 (0)	0 (0)	
Moderately differentiated	0 (0)	8 (47)	21 (32)	
Poorly differentiated	4 (100)	9 (53)	44 (68)	
Unknown	0 (0)	4 (19)	4 (6)	
Location of metastasis				0.741
Skin	2 (50)	6 (29)	15 (22)	
Brain	0 (0)	5 (24)	21 (30)	
Ovary & peritoneum	0 (0)	3 (14)	7 (10)	
Bone	0 (0)	2 (10)	12 (17)	
Lymph nodes	1 (25)	2 (9)	5 (7)	
Other°	1 (25)	3 (14)	9 (13)	
Oestrogen receptor status				0.098
Negative (<1 %)	3 (75)	5 (25)	31 (45)	
Positive (≥1 %)	1 (25)	15 (75)	38 (55)	
Unknown	0 (0)	1 (6)	0 (0)	
Progesterone receptor status				0.344
Negative (<1 %)	3 (75)	8 (42)	41 (59)	
Positive (≥1 %)	1 (25)	11 (58)	28 (41)	
Unknown	0 (0)	2 (10)	0 (0)	
HER2 expression				0.041
Score 0	4 (100)	7 (39)	18 (27)	
Score 1+	0 (0)	1 (6)	21 (31)	
Score 2+	0 (0)	7 (39)	19 (28)	
Score 3+	0 (0)	3 (17)	10 (15)	
Unknown	0 (0)	3 (14)	1 (2)	
Immunohistochemical subtype				0.034
HR-positive, HER2-negative	1 (25)	11 (52)	33 (48)	
HER2-positive	0 (0)	3 (14)	10 (15)	
Triple-negative	3 (75)	4 (19)	26 (38)	
Unknown	0 (0)	3 (14)	0 (0)	

HR: hormone receptor; NST: no special type; HER2: human epidermal growth factor receptor 2; RCB: residual cancer burden; TROP2: trophoblast cell surface antigen-2

* Chi-Square test or Fisher's Exact test when appropriate

° Other metastatic locations comprise lung, liver, digestive tract, soft tissue, bladder and adrenal gland.

tumor size, Nottingham grade, mitotic activity, or tumor-infiltrating lymphocytes (TILs) [20]. However, TROP2 overexpression in TNBC was associated with the histological type, the presence of lymphovascular invasion, an in situ component, axillary lymph node metastases, and androgen receptor positivity [20]. In contrast, Jeon et al. did not observe a substantial association between the clinicopathological characteristics of 807 TNBCs and their TROP2 status as determined with the EPR20043 clone [40], which is similar to our own observations and those of Gion et al. [43]. Of note, we did not observe any differences in the staining patterns between SP293 and EPR20043, but these contrasting observations might be explained by differences in the analyses of TROP2 immunoreactivity: Izci et al. classified TROP2 expression into three categories [20], whereas Jeon et al. dichotomized TROP2 expression using a 10 % threshold, regardless of its intensity [40]. Moreover, our own study focused primarily on the comparison of two methods for assessment of TROP2 IHC instead of searching for clinicopathological differences between TROP2-negative and TROP2-positive breast cancers. The number of breast tumors included in the present study, and the number of TNBCs in particular, is insufficient for definitive conclusions regarding the potential association of histopathological features and TROP2 status.

In total, four metaplastic carcinomas were present in this study, of which three were metastatic and one was present in the post-NAC patient cohort. One metaplastic spindle carcinoma was TROP-high (H-score >200); one metaplastic spindle cell carcinoma was considered as TROP2-low (i.e. H-score <100). Two metaplastic carcinomas showed intermediate TROP2 expression, comprising one mixed NST/spindle cell carcinoma and one mixed NST/squamous cell carcinoma. Although the number of metaplastic carcinomas is limited in our study, previously published data suggest that 15–25 % of metaplastic carcinomas are TROP2-negative or show TROP2-low expression [21,42]. If future trial results demonstrate a reduced therapeutic response to sacituzumab govitecan in TROP2-low tumors, the use of a reliable anti-TROP2 immunohistochemical assay would be crucial to identify those patients who are less likely to respond to therapy. As stated by Mertens et al., TROP2 IHC could become important to avoid toxicity of an expensive drug in patients who are unlikely to respond [21].

In the post-NAC patient cohort, the histological type is statistically significantly associated with TROP2 expression, but this low p-value is likely due to the very low number of special histological types in this cohort, none of which is TROP2-negative. This observation seems therefore unreliable and should be verified in larger cohorts. Similarly, the HER2 status and the immunohistochemical subtype (based on integrated hormone receptor and HER2 status) were statistically significantly associated with the TROP2 status in metastatic breast cancers, but these associations seem also unreliable: there are only four TROP2-negative tumors, which all show a HER2 IHC score 0 and three of these tumors are TNBCs. The limited number of tumors with available NGS data in our cohort precludes a strong statement regarding a potential association between the TROP2 status and a particular mutational 'signature'. However, our observations suggest that the mutational profile of breast cancer is associated with the hormone receptor and HER2 status, rather than with the TROP2 status. For instance, the *TP53* gene was the most frequently mutated gene in the sixteen breast cancers analyzed here, but the triple-negative subtype is frequently associated with *TP53* mutations and aberrant p53 immunoreactivity [44,45].

The comparison of three monoclonal antibodies showed no apparent differences between SP293 and EPR20043, whereas we did not succeed to establish a staining protocol for BSB-148 without substantial non-specific background and similar membrane staining intensity. This could explain the observed cytoplasmic immunoreactivity in lymphocytes and fibroblasts observed with BSB-148. If future studies are able to demonstrate that TROP2 expression is a predictive marker for therapy response, extensive tests are recommended to investigate the specificity of the companion diagnostic. Ideally, the 'PD-L1 scenario', wherein each anti-PD-1/anti-PD-L1 targeted therapy has its own accompanying anti-PD-L1 clone and scoring method, should be avoided. It is therefore key that pathologists take the lead in establishing a reproducible, reliable and, preferentially, uniform method for TROP2 protein assessment.

The most interesting observation in our study is the strong correlation between the membrane score, based on the ASCO/CAP guidelines for HER2 IHC testing, and the H-score for TROP2 protein assessment. The H-score combines proportion and intensity scores for membrane staining [9]. Although the H-score can be useful for scientific purposes by providing detailed information, its calculation is rather time-consuming in routine practice. We therefore advocate the introduction of a three-tier membrane score, based on HER2 IHC testing. Despite being labor-intensive, the H-score is probably better than the membrane score to capture any heterogeneity of the TROP2 protein expression. The H-scores of the TROP2 2+ cases in our study reflect the presence of substantial heterogeneity, thereby advocating the use of

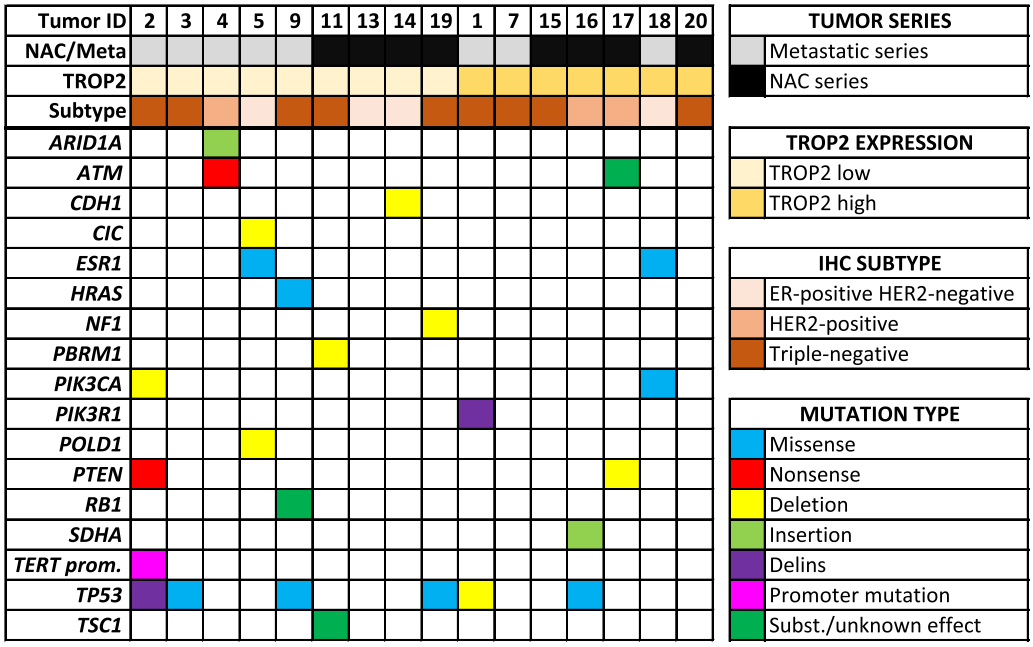

Fig. 3. Mutation plot illustrating the distribution of variants of unknown significance (VUS), and pathogenic or likely pathogenic mutations in sixteen breast cancer samples. Rows represent genes; columns represent patients. Legends indicate the surrogate molecular subtype, the type of cohort (either post-NAC surgical specimen or metastatic breast cancer specimen) and the TROP2 expression status.

whole stained slides instead of tissue micro-arrays, as previously indicated by Mertens et al. [21]. Of note, TROP2 IHC on small biopsies from metastatic breast cancer might be affected by this heterogeneity, analogous to the occasional heterogeneity encountered in HER2 immunoreactivity, especially in the HER2-low range [46].

Additionally, our study shows that the membrane score is somewhat less prone to interobserver variability than the categorized H-score in breast resection specimens. There was no difference in interobserver agreement between both methods in the metastatic breast cancer cohort. This could be due to the presence of other TROP2-positive epithelial structures in breast resection specimens, such as in situ components and benign epithelial hyperplasia, which are absent in metastases.

In conclusion, we demonstrated that the ASCO/CAP guidelines for HER2 IHC testing can be applied for the semi-quantitative evaluation of membranous TROP2 expression, as this well-known method strongly correlates with the H-score and is slightly less prone to interobserver variability in breast resection specimens. We believe this 'TROP2 membrane score' is less time-consuming than the H-score. Nevertheless, both scores could be used for validation and investigation in clinical trials, as the H-score better reflects the presence of heterogeneity. Our study was not designed to and has not demonstrated the biological significance of different levels of TROP2 immunoreactivity. To this purpose, future studies should investigate whether the TROP2 status is predictive of therapy response to TROP2-targeting ADCs, preferentially by taking into account both scoring methods and by involving multiple observers.

Ethics approval statement

This retrospective study was approved by the institutional ethics committee (file name: 2022/21NOV/436). This study was performed in accordance with the latest version of the World Medical Association's Declaration of Helsinki.

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CRediT authorship contribution statement

Dominique Dubois: Writing – review & editing, Resources, Methodology, Data curation. **Anne-France Dekairelle:** Writing – review & editing, Software, Resources, Data curation. **Eléonore Longton:** Writing – review & editing, Data curation. **Cédric Van Marcke:** Writing – review & editing, Data curation. **Hilde Vernaeve:** Writing – review & editing, Data curation. **Mieke R. Van Bockstal:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Francois P. Duhoux:** Writing – review & editing, Data curation. **Giuseppe Floris:** Writing – review & editing, Validation, Resources, Data curation. **Lina Daoud:** Writing – review & editing, Data curation. **Martine Berlière:** Writing – review & editing, Data curation. **Marie-Caroline De Pelsemaeker:** Writing – review & editing, Formal analysis, Data curation. **Aline Francois:** Writing – review & editing, Data curation. **Christine Galant:** Writing – review & editing, Supervision, Project administration, Investigation, Data curation, Conceptualization. **Quitterie Fontanges:** Writing – review & editing, Data curation. **Yves Guiot:** Writing – review & editing, Validation, Resources, Data curation.

Declaration of Competing Interest

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Conflict of interest disclosure

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2024.155724.

Data availability

The datasets generated and/or analyzed during the current study are not publicly available as per institutional policy, but are available from the corresponding author on reasonable request.

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