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Predictive markers for pathological complete response after neo-adjuvant chemotherapy in triple-negative breast cancer

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ABSTRACT

A combination of Sox10 and GATA3 was previously identified as a marker for metastatic triple-negative breast cancer (TNBC), but it is uncertain whether their expression is associated with pathological complete response (pCR) after neoadjuvant chemotherapy (NAC). This study investigates the predictive value of clinicopathological characteristics, as well as protein expression of Sox10, GATA3, p53 and p63, in a consecutive series of TNBC patients treated with NAC.

Archived hematoxylin & eosin stained slides of core biopsies and resection specimens from 35 TNBC patients were reviewed. The following clinicopathological characteristics were determined at the biopsy level: age at diagnosis, cancer type, Nottingham grade, lympho-vascular invasion, syncytial growth, necrosis, clear cell differentiation, myxoid peritumor stroma, stromal tumor-infiltrating lymphocytes (sTILs) and presence of an in situ component. The MD Anderson residual cancer burden (RCB) score and corresponding RCB class were determined. Immunohistochemistry for Sox10, p53, GATA3 and p63 was performed at the biopsy level.

sTILs, either as a continuous or as a dichotomous variable, were the only parameter that was significantly associated with pCR in univariable and multivariable analyses. Assessment of sTILs showed moderate to good interobserver agreement. High sTILs (≥40%) were significantly associated with increased pCR rates, and this association was observer-independent.

This retrospective study of a consecutive community-based cohort of TNBC patients confirms that sTILs are a robust, observer-independent predictor for therapeutic response after NAC. The combination of Sox10, GATA3 and p53 immunoreactivity is unlikely to harbor any predictive value for pCR in TNBC.

1. Introduction

Triple-negative breast carcinomas (TNBCs) are defined as estrogen receptor- (ER), progesterone receptor- (PR) and HER2-negative breast cancers. This surrogate molecular subtype accounts for around 12–20% of invasive breast cancers and is frequently associated with adverse prognosis [1]. Most TNBCs (80–88%) are invasive carcinomas of no special type (NST) [2]. The most frequently diagnosed triple-negative special histological subtypes comprise metaplastic carcinoma (4%) and pleomorphic invasive lobular carcinoma (pILC; 3%) [2]. Neoadjuvant chemotherapy (NAC) is standard of care for TNBC patients, allowing the monitoring of treatment response [3]. Pathological complete response (pCR), i.e. the

Abbreviations: ASCO/CAP, American Society of Clinical Pathology/College of American Pathologists; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; FFPE, formalin-fixed paraffin-embedded; GATA3, GATA-binding protein 3; GCDFP-15, gross cystic disease fluid protein 15; HE, hematoxylin and eosin; HER2, human epidermal growth factor receptor 2; ICC, intraclass correlation coefficient; IHC, immunohistochemistry; NAC, neoadjuvant chemotherapy; NST, no special type; pCR, pathological complete response; pILC, pleomorphic invasive lobular carcinoma; PR, progesterone receptor; RCB, residual cancer burden; SISH, silver in situ hybridization; Sox10, SRY-Box transcription factor 10; sTILs, stromal tumor-infiltrating lymphocytes; TNBC, triple-negative breast carcinoma; TTF1, thyroid transcription factor 1

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Table 1
Information about the immunohistochemical stains[a].

Protein	Clone	Firm	Species	Dilution	HIER	Visualization
ER	SP1	Roche	Rabbit	RTU	CC1	UltraView DAB Detection kit[b]
GATA3	L50-823	Bio SB	Mouse	1/50	CC1	UltraView DAB Detection kit[b]
HER2	4B5	Roche	Rabbit	RTU	CC1	UltraView DAB Detection kit[b]
Ki67	MIB-1	Dako	Mouse	1/90	CC1	UltraView DAB Detection kit[b]
p53	DO-7	Biocare Medical	Mouse	1/200	CC1	UltraView DAB Detection kit[b]
p63	4A4	Bio SB	Mouse	1/50	CC1	UltraView DAB Detection kit[b]
PR	1E2	Roche	Rabbit	RTU	CC1	UltraView DAB Detection kit[b]
Sox10	EP268	Bio SB	Rabbit	1/50	CC1	UltraView DAB Detection kit[b]

Abbreviations: CC1: cell conditioning 1 tris-based buffer[b]; ER: estrogen receptor; IHC: immunohistochemistry; PR: progesterone receptor; RTU: ready-to-use.
[a] Performed on 4-μm-thick sections mounted on Superfrost plus slides (Menzel-Gläser, Braunschweig, Germany).
[b] Ventana Medical Systems, Tucson, Arizona (USA).

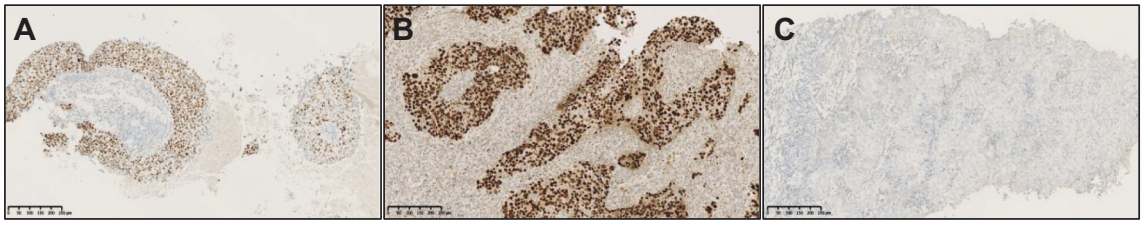

Fig. 1. Immunohistochemistry for p53, illustrating the three immunoreactivity patterns encountered in this study. Wild-type staining (A) was characterized by nuclear expression of variable intensity, resulting in a mixture of negative tumor nuclei and weakly to strongly staining tumor nuclei. Mutation-type staining was characterized by strong nuclear immunoreactivity in ≥70% of tumor cells (B) or by complete loss of nuclear immunoreactivity in all tumor cells (C). Original magnification: 100×.

combined absence of residual invasive carcinoma and nodal metastases after NAC, is associated with a better long-term outcome [4]. In TNBC, achieving a pCR is the most important prognostic factor for distant relapse-free and overall survival [3]. However, the number of predictive markers for pCR is limited. At present, stromal tumor-infiltrating lymphocytes (sTILs) constitute the only robust histopathological predictive marker that has emerged from multiple randomized clinical trials [5-8]. Accurate prediction of pCR would allow studies of omission of surgery, treating TNBC solely based on chemotherapy and active surveillance of the tumor bed by control biopsies. Additionally, future studies could explore low-dose NAC regimens in order to further de-escalate TNBC treatment.

Recently, the combination of Sox10 and GATA3 was identified as an efficient marker for the diagnosis of metastatic TNBC [9-14]. These proteins seem especially useful for the distinction of metastatic TNBC from TTF1-negative pulmonary adenocarcinoma [9]. Although not specific for breast cancer, GATA3 is expressed in > 90% of metastatic invasive breast cancers, rendering it a more sensitive marker than GCDFP-15 [15]. Since GATA3 is a transcription factor involved in the ER pathway, some TNBC lack GATA3 expression [16]. Sox10 was originally identified as a modulator of transcription factors involved in the development and maturation of glial cells [17]. Sox10 expression was observed in normal breast myoepithelial cells, as well as in TNBC and more specifically, in so-called basal-like breast cancers [18,19]. Other molecular subtypes only rarely express Sox10 [18-21].

The predictive value of GATA3 and Sox10 immunohistochemistry for pCR after NAC in TNBC is uncertain. At present, only a limited amount of contradictory evidence is available for GATA3 as a predictive marker [22,23]. Here, we explored the value of GATA3 and Sox10 immunoreactivity in TNBC as predictive markers for pCR. We also performed immunohistochemistry for p53 and p63, as TNBC frequently present with mutant p53 staining and a basal-like TNBC subgroup often expresses p63 [11,24]. Immunohistochemistry was complemented with the assessment of various clinicopathological characteristics.

2. Materials & methods

2.1. Patients and tissue samples

The included patients were diagnosed with TNBC and underwent subsequent post-NAC surgery between 1 January 2015 and 1 March 2020. The standard NAC regimen comprises anthracyclines and cyclophosphamide followed by paclitaxel. In case of poor response after anthracyclines and cyclophosphamide, paclitaxel is complemented by carboplatin. Only those patients whose biopsy and resection specimen were both present in the archives of the Department of Pathology of the Cliniques Cliniques universitaires Saint-Luc (Brussels, Belgium) were included. Our laboratory receives samples from multiple hospitals. Surgery was performed in two hospitals, and some patients received chemotherapy in outpatient clinics: therefore, data on chemotherapy regimen deviations and initial staging were not collected. Information on type of surgery, macroscopic tumor bed size, patient age at diagnosis, post-NAC nodal status, and mean *HER2* and CEP17 copy numbers (if dual-probe silver-enhanced in situ hybridization (SISH) had been performed prior to NAC) was retrieved from electronic histopathological reportsLLIS (LIS DaVinci, MIPS, Ghent, Belgium). This retrospective study was approved by the institutional ethics committee (file number: RETRO-TNBC-15-2019/03JUL/297).

2.2. Histopathological review

Biopsy tissue cores were immediately fixed in 10% neutral-buffered formalin for 6–72 h. All resection specimens were received freshly, sliced at 5 mm intervals, and fixed in 10% neutral-buffered formalin for 6–72 h, in accordance with the ASCO/CAP guidelines [25]. Macroscopic examination was performed according to the MD Anderson residual cancer burden (RCB) protocol [4]. Hematoxylin and eosin (HE) stained slides of the core biopsies were retrieved from the archives and reviewed by one pathologist (MRVB). Grading of invasive breast cancer was performed as previously described [26]. Each constituent of the Nottingham grade was noted separately. Mitotic activity was assessed in ten high-power fields (HPF; i.e. at 400× magnification) with a field diameter of 0,55 mm, corresponding to the following cut-offs: low (≤8 mitoses), moderate (9–17 mitoses), and high (≥18 mitoses). The presence or absence of the following histopathological features was assessed as a dichotomous variable: any in situ component regardless of its extent, syncytial growth pattern, clear cell differentiation (i.e. tumor cells with clear cytoplasm), tumor necrosis, unequivocal lympho-vascular invasion, and myxoid peritumor stroma (i.e. myxoid stromal changes), as previously described

Table 2
Patient characteristics of a real-world cohort of 35 patients with triple-negative breast carcinoma, treated by neoadjuvant chemotherapy and surgery.

	Mean (± SD) or n (%)
Age (years)	55,8 (± 13,3)
Sex	
Male	0 (0)
Female	35 (100)
Type of surgery	
Lumpectomy	11 (31)
Quadrantectomy	7 (20)
Mastectomy	17 (49)
Laterality	
Left breast	19 (54)
Right breast	16 (46)
Cancer type	
Invasive carcinoma NST	33 (94)
Pleomorphic lobular carcinoma	2 (6)
Tumor stage (post-NAC)	
ypT0	8 (23)
ypTis	5 (14)
ypT1	18 (51)
ypT2	3 (9)
ypT3	1 (3)
ypT4	0 (0)
Nodal stage (post-NAC)	
ypN0 and ypN0(i-)(sn)	23 (66)
ypN1	7 (20)
ypN2	4 (11)
ypN3	1 (3)
Nottingham grade (pre-NAC)	
Grade 1	0 (0)
Grade 2	9 (26)
Grade 3	26 (74)
RCB class (post-NAC)	
Class 0 (pCR)	13 (37)
Class 1	3 (9)
Class 2	13 (37)
Class 3	6 (17)
Time interval diagnosis – surgery (months)	5,7 (± 1,2)
Lympho-vascular invasion (pre-NAC)	
Absent	32 (91)
Present	3 (9)

Abbreviations: NAC: neoadjuvant chemotherapy; NST: no special type; pCR: pathological complete response; SD: standard deviation.

[27,28]. sTILs were assessed as percentage by three independent observers (MRVB, FN, CG), according to the International TILs Working Group 2014 methods [29]. The arithmetic mean of the three sTILs scores was calculated and used for the final analyses. sTILs were dichotomized as low (≤40%) versus high (> 40%), as previously described [29]. HE stained slides of all resection specimens were reviewed by one pathologist (MRVB). The therapeutic response was determined by using an online calculator for the RCB score (http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3) [4]. pCR was defined as an RCB score equaling 0.

2.3. Immunohistochemistry

Archived slides stained for Ki-67, HER2, ER, and PR were reviewed by one pathologist (MRVB). Ki-67 expression was assessed as the percentage of tumor nuclei showing immunoreactivity, regardless of the intensity. HER2 protein expression was assessed according to the updated ASCO/CAP guidelines as 0/negative, 1+/negative, 2+/equivocal, or 3+/positive [30]. The amplification status of equivocal tumors was investigated by SISH, using the INFORM *HER2* Dual ISH Cocktail probe on an automated slide stainer, according to the manufacturer's instructions (Benchmark XT, Ventana Medical Systems, Arizona, USA). ER and PR expression were assessed according to the Allred score [31]. A proportion score (as part of the Allred score) of < 1% was considered as negative in accordance with the ASCO/CAP guidelines [25]. ER-negative HER2-negative carcinomas with weak PR immunoreactivity in < 25% of tumor cells were also regarded as TNBC, as Li et al. have shown that the prognosis for this rare tumor subtype is as poor as for TNBC [32]. Sox10, GATA3, p53 and p63 immunohistochemistry was performed on the biopsy specimens using an automated slide stainer (Benchmark XT, Ventana Medical Systems) according to the manufacturer's instructions (Table 1). Stained slides were evaluated jointly by three observers (MRVB, FN, CG) using a multi-head light microscope. Immunoreactivity in the entire biopsy was evaluated, regardless of the amount of tumor present. p53 expression was assessed as wild-type or mutation-type (Fig. 1): mutation-type staining comprised overexpression (i.e. strong nuclear immunoreactivity in ≥70% of tumor cells), total absence of immunoreactivity, or a cytoplasmic staining pattern, as previously reported for high-grade serous tubo-ovarian carcinoma and endometrial carcinomas [33]. TNBCs were considered as positive for Sox10, GATA3, and p63 when ≥10% of nuclei presented immunoreactivity, regardless of the intensity. The presence of p63-positive myoepithelial cells confirmed the presence of an in situ component.

2.4. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 26.0 software (Chicago, IL, USA). Outcome variables comprised pCR and sTILs. Continuous parameters were tested for normal distribution by the Shapiro-Wilk test. Patient age at diagnosis (in years) and the time interval between biopsy diagnosis and surgery (in months) were normally-distributed data. All other continuous variables were not-normally-distributed. Normally-distributed continuous parameters were analyzed with the Student *t*-test (reported as mean ± standard deviation). Not-normally-distributed continuous parameters were analyzed with the Mann-Whitney *U* test (reported as median with 25th and 75th centiles). Categorical data were analyzed with Fisher's Exact test (reported as absolute number and relative percentage). Intraclass correlation coefficients (ICC; two-way mixed model for consistency) were determined to assess interobserver agreement for sTILs and RCB scores [34]. Multivariable logistic regression analysis with backward selection was performed to explore which variables were independently associated with pCR. The time interval between the biopsy diagnosis and post-NAC surgery was used to correct for any toxicity-related NAC regimen interruption. The statistical significance level was set at $p < 0,05$. All tests were two-sided.

3. Results

3.1. Clinicopathological characteristics and pCR

Patient characteristics are provided in Table 2. The average time interval between biopsy diagnosis and post-NAC surgery amounted to 5,7 months (range 4,6–7,8 months). None of the clinicopathological features was associated with pCR, either alone or in multivariable logistic regression analysis, except for sTILs (Table 3). sTILs assessed as a percentage were significantly associated with the chance of achieving a pCR ($p = 0.013$). After dichotomization of sTILs, TNBC with > 40% sTILs showed pCR significantly more often ($p = 0.002$). For the tumors without pCR, none of the clinicopathological characteristics was related to RCB class.

3.2. Reproducibility of RCB and sTILs

To exclude a coincidental association between sTILs assessment by a single observer (P1) and pCR in this community-based cohort, sTILs were evaluated independently by two other observers (P2 and P3). The percentages of sTILS assessed by each observer, as well as their arithmetic mean, were significantly associated with pCR (P1: $p = 0,010$; P2: $p = 0,036$; P3: $p = 0,007$; mean: $p = 0,013$). Concordance for sTILs assessment was moderate to good for each observer duo: the ICC was 0,621 for P1 versus P2, 0,901 for P1 versus P3, and 0,668 for P2 versus P3 [34]. Similarly, the reproducibility of RCB assessment was

Table 3
Clinicopathological differences between TNBC patients with and without pCR after neoadjuvant chemotherapy and surgery.

	pCR	No pCR	*p*-Value
	Mean (± SD)	Mean (± SD)	
Age (years)	55,4 (± 11,8)	55,3 (± 14,4)	0,981
Time interval diagnosis – surgery (months)	6,2 (± 0,9)	5,4 (± 1,2)	0,052

	pCR	No pCR	n (%)
	Median (P25, P75)	Median (P25, P75)	p-Value
Nottingham score (range 3–9)	8 (8,9)	8 (7,9)	0,601
Mitotic count (per 10 HPF)	13 (10,20)	15 (7,24)	0,880

	n (%)	n (%)	p-Value
Type of surgery			0,253
Lumpectomy	6 (46)	5 (23)	
Quadrantectomy	3(23)	4 (18)	
Mastectomy	4 (31)	13 (59)	
Cancer type			0,519
Invasive carcinoma NST	13 (100)	20 (91)	
Pleomorphic lobular carcinoma	0 (0)	2 (9)	
Nottingham grade			0,431
Grade 1	0 (0)	0 (0)	
Grade 2	2 (15)	7 (32)	
Grade 3	11 (85)	15 (68)	
Glandular differentiation			1,000
≥75%	0 (0)	0 (0)	
< 75% and ≥10%	0 (0)	1 (4)	
< 10%	13 (100)	21 (96)	
Nuclear atypia			1,000
Low	0 (0)	0 (0)	
Moderate	0 (0)	1 (4)	
High	13 (100)	21 (96)	
Mitotic activity			0,376
Low	2 (15)	7 (32)	
Moderate	7 (54)	7 (32)	
High	4 (31)	8 (36)	
DCIS (confirmed by IHC for p63)			0,248
Absent	10 (83)	12 (60)	
Present	2 (17)	8 (40)	
Tumor necrosis			1,000
Absent	8 (62)	13 (59)	
Present	5 (39)	9 (41)	
Clear cells			0,726
Absent	5 (39)	7 (32)	
Present	8 (62)	15 (68)	
Syncytial growth pattern			0,157
Absent	6 (46)	16 (73)	
Present	7 (54)	6 (27)	
Lympho-vascular invasion			0,541
Absent	11 (85)	21 (96)	
Present	2 (15)	1 (5)	
Myxoid peritumour stroma			0,724
Absent	7 (54)	14 (64)	
Present	6 (46)	8 (36)	
TILs (assessed as percentage)	45,5 (± 26,1)	21,9 (± 21,6)	0,013*
TILs (2-tier)			0,002*
Low TILs (< 40%)	5 (39)	20 (91)	
High TILs (≥40%)	8 (62)	2 (9)	

Abbreviations: DCIS: ductal carcinoma in situ; HPF: high power fields; IHC: immunohistochemistry; NST: no special type; pCR: pathological complete response; SD: standard deviation; TILs: tumor-infiltrating lymphocytes; TNBC: triple-negative breast carcinoma.
* Statistically significant association.

investigated. The originally reported RCB scores and revised RCB scores were highly concordant (ICC = 0,982).

3.3. Immunohistochemistry and pCR

Table 4 contains data on the association between pCR and immunohistochemistry. Immunohistochemistry was not available for one TNBC due to tissue block exhaustion. Most TNBC presented with a Sox10-positive, GATA3-positive, p53-mutation type profile (Figs. 2-3). Although TNBC with pCR only rarely presented with a wild-type p53 pattern as compared with TNBC without pCR (8% versus 32%), this association was not statistically significant (p = 0,210). Expression of Sox10, p63 and GATA3 was not significantly associated with pCR. ER expression was subsequently classified as completely absent (Allred score = 0) versus focally present in < 1% of tumor cells, but did not correlate with pCR (p = 0,157). Similarly, absent PR expression versus weak PR expression

Table 4
Immunohistochemical and SISH differences between TNBC patients with and without pCR after neoadjuvant chemotherapy and surgery.

	pCR	No pCR	p-Value
	Median (p25;p75) or n (%)	Median (p25;p75) or n (%)	
Estrogen receptor status			0,157
Allred score 0	6 (46)	16 (73)	
Allred score > 1	7 (54)	6 (27)	
Progesterone receptor status			1,000
Allred score 0	8 (62)	14 (64)	
Allred score > 1	5 (39)	8 (36)	
HER2 immunohistochemistry			0,322
0	5 (39)	14 (64)	
1+	5 (39)	5 (23)	
2+	3 (23)	3 (14)	
Mean HER2 copy number (*n* = 22)	2,20 (1,6; 2,79)	2,13 (2,01; 2,76)	0,625
Mean CEP17 copy number (n = 22)	1,90 (1,58; 2,41)	1,88 (1,74; 2,01)	0,886
Mean HER2/CEP17 ratio (n = 22)	1,15 (1,05; 1,23)	1,14 (1,05; 1,17)	0,752
Ki-67 immunohistochemistry (%)	90 (70; 93)	85 (60; 95)	0,960
Ki-67 immunohistochemistry (2-tier)			1,000
Negative (< 20%)	0 (0)	1 (4)	
Positive (≥20%)	13 (100)	21 (96)	
Nuclear Sox10 expression (*n* = 34)			1,000
Absent	3 (25)	6 (27)	
Present	9 (75)	16 (73)	
p53 protein expression (n = 34)			0,191
Wild-type pattern	1 (8)	7 (32)	
Mutation-type pattern (complete loss)	5 (38)	5 (23)	
Mutation-type pattern (overexpression)	6 (46)	10 (45)	
Two-tier p53 protein expression (n = 34)			0,210
Wild-type pattern	1 (8)	7 (32)	
Mutation-type pattern	11 (92)	15 (68)	
Nuclear GATA3 expression (n = 34)			1,000
Negative (< 10%)	2 (17)	4 (18)	
Positive (≥10%)	10 (83)	18 (82)	
Nuclear p63 expression (*n* = 32)			0,338
Negative (< 10%)	9 (75)	18 (90)	
Positive (≥10%)	3 (25)	2 (10)	

Abbreviations: pCR: pathological complete response; SD: standard deviation; SISH: silver in situ hybridisation; TNBC: triple-negative breast carcinoma.

in < 25% of tumor cells was not associated with pCR, nor were Ki-67 and HER2 protein expression. Mean *HER2* and CEP17 copy numbers were available for 22 tumors and did not correlate with pCR. For the tumors without pCR, none of the immunohistochemical characteristics was related to RCB class.

3.4. Characterization of TNBC with low and high sTILs

Since sTILs were the only parameter associated with pCR, we explored the clinicopathological and immunohistochemical characteristics of TNBC with low versus high sTILs (Supplementary Tables 1-2). None of the morphological features was significantly associated with the presence of sTILs, although low sTILs tended to present more often with clear cell differentiation as compared with high sTILs (76% versus 40%; $p = 0{,}059$). As normal myoepithelial cells of the breast can present with clear cytoplasm, the clear cell differentiation in TNBC might indicate a basal-like phenotype. However, clear cell differentiation did not correlate with Sox10 or p63 immunoreactivity.

TNBC with high sTILs were significantly less often completely negative for ER as compared to TNBC with low sTILs (30% versus 76%; $p = 0{,}020$). We observed a weak but statistically non-significant association between sTILs and low (1+ or 2+ immunohistochemical score) HER2 expression: 70% of TNBC with high sTILs presented with low HER2 expression, whereas this was the case in only 36% of TNBC with low sTILS (p = 0,160). Mean *HER2* and CEP17 copy numbers were not significantly associated with sTILs, nor were Ki-67, GATA3, p53, p63 or Sox10 immunoreactivity.

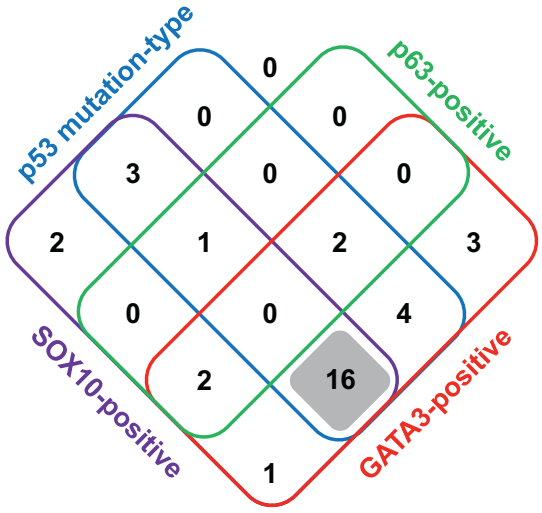

Fig. 2. Venn diagram illustrating the mutual interrelationships between protein expression of Sox10 (purple), p53 (blue), p63 (green) and GATA3 (red). The most common immunohistochemical profile within this cohort was Sox10-positive, GATA3-positive TNBC with a mutation-type p53 staining pattern (marked in grey). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In this study, we showed that immunoreactivity for Sox10, GATA3, p53 and p63 in TNBC, either alone or in combination, is not associated with achieving a pCR after NAC. There is not much evidence regarding these proteins with respect to prediction of treatment response, with the exception of p53. Within the TNBC subgroup of the GeparSixto trial, both p53 protein overexpression and the presence of *TP53* mutations were not associated with treatment response [35], which is corroborated by our findings. However, p53 overexpression was associated with improved survival in TNBC [35]. As for Sox10, Yoon et al. recently showed that Sox10 is frequently co-expressed with p16 in TNBC, but their expression is not associated with overall survival [36]. Nevertheless, others have reported that the combination of Sox10 and GATA3 immunohistochemistry is useful to differentiate metastatic TNBC from TTF1-negative pulmonary adenocarcinoma [9]. Since most TNBC in our series were Sox10-positive and GATA3-positive, this indirectly confirms their utility for differentiating metastatic TNBC from other malignancies. Sox10 expression is associated with increased stem/progenitor activity and plays a role in epithelial-to-mesenchymal transition (EMT) [37]. This might explain its high prevalence in TNBC, which is a molecular subtype frequently associated with a mesenchymal or partial EMT phenotype [38].

None of the histopathological features was associated with pCR, except for sTILs. Despite the moderate to good inter-observer agreement for sTILs assessment, the association between sTILs and pCR was observer-independent in this real-world patient cohort. This contradicts the observations of O'Loughlin et al., who state that the utility of sTILs assessment in clinical practice is restricted by its poor reproducibility [39]. Our findings suggest that sTILs assessment is sufficiently robust to predict pCR in clinical practice, and is not hampered by the limited to moderate inter-observer variability. Nevertheless, subtle differences in sTILs assessment for borderline TNBC cases might have an impact at the individual patient level. Future studies should explore the extent of this impact in larger patient cohorts. Since all observers in our study belonged to the same

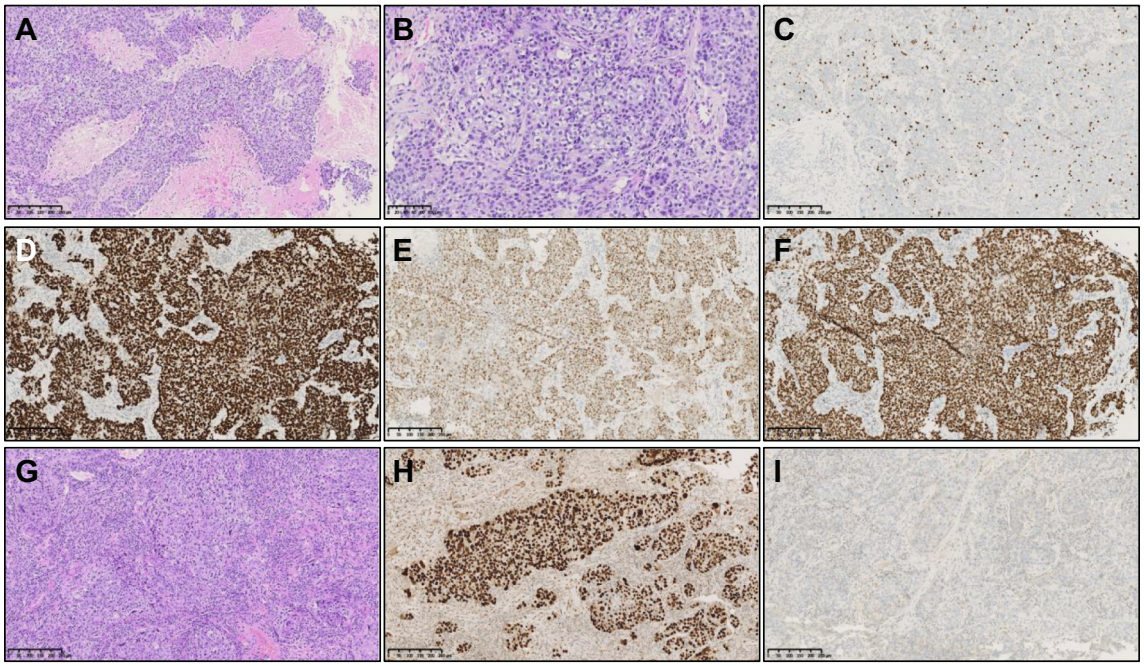

Fig. 3. Core biopsy of a grade 3 TNBC with presence of high sTILs, extensive tumor necrosis and syncytial growth pattern (A), and clear cell differentiation (B). This tumor showed focal p63 immunoreactivity (C), diffuse strong Sox10 positivity (D), a wild-type p53 staining pattern (E) and diffuse GATA3 positivity (F). A second case of grade 3 TNBC with high sTILs (G) showed a mutation-type p53 staining pattern (H), GATA3 negativity (I) and diffuse strong Sox10 positivity (not shown). Both patients achieved a pCR after NAC. Original magnification: 200× (B) and 100× (A, C-I).

institute, the moderate to good interobserver agreement might be due to a similar educational background. Future studies should therefore investigate whether the lack of predictive impact related to inter-observer variability can be confirmed across different institutions.

The limited number of predictive markers identified here might be due to a lack of power, since this cohort only contains 35 patients. However, statistically significant results in large cohorts do not necessarily reflect biologically important differences [40,41]. Strong discriminators do not require large study cohorts, which is illustrated in the present consecutive community-based cohort: 80% of TNBC patients with high sTILs achieved a pCR after NAC, whereas 80% of patients with low sTILs did not achieve a pCR. The two patients with high sTILS without pCR both presented with wild-type p53 staining. One tumor was Sox10-negative and GATA3-positive; the other tumor showed a reverse Sox10/GATA3 pattern. The limited number of patients with high sTILs without pCR in this cohort does not allow to analyze their immunohistochemical and/or molecular profile in-depth. However, future studies should investigate the characteristics of TNBC patients with high sTILs who do not achieve a pCR, as well as patients who achieve a pCR despite low sTILs, as this might allow further finetuning of predictive markers for pCR.

Our findings suggest that tumor cell characteristics are less likely to accurately predict pCR in TNBC, whereas the immune response seems to play an important role. Immunohistochemical subtyping of sTILs might therefore offer valuable insights as to which type of immune cell enhances an anti-tumor response during NAC. Previous studies showed that patients with high CD8-positive sTILs less often present with lymph node metastases and have a better disease-free and/or overall survival, whereas patients with many Foxp3-positive sTILs more often present with lymph node metastases and adverse clinical outcome [42-44]. Similarly, high levels of CD4-positive sTILs in TNBC seem associated with better distant-recurrence free survival [45]. PD-L1 expression is frequently observed in both tumor cells and immune cells in TNBC, and both are positively associated with pCR [46,47]. It might thus be worthwhile to explore the combined value of sTILs and PD-L1 expression as a predictive marker. Immune checkpoint inhibitors might play a major role in future metastatic and non-metastatic TNBC treatment [48]. Our study also demonstrates that around 46% of TNBC present with a negative/1+ or equivocal/2+ immunohistochemical HER2 score in the absence of *HER2* amplification, which is of potential clinical significance. Since the treatment armamentarium of medical oncologists will expand with new anti-HER2 therapies for so-called "HER2-low" breast cancer, around half of TNBC patients might benefit from these novel agents, potentially further increasing the chance of achieving a pCR [49].

A higher pre-NAC tumor stage is associated with a lower pCR rate [50]. A major limitation in the present study is the lack of information concerning pre-NAC tumor staging, as well as any deviations from the standard NAC regimen due to toxicity or non-response. This hampers the correction for cTNM-stage and NAC regimen deviations in multivariable logistic regression analysis. However, this limitation can also be considered as a strength: numerous randomized clinical trials have shown that sTILs are predictive for pCR after NAC [5-7], but this (potentially heterogenous) real-world patient cohort confirms that sTILs still remain a robust predictor outside the randomized trial setting. Moreover, others have shown that larger pre-NAC tumor size is significantly associated with low sTILs, which precludes the introduction of both parameters in a single multivariable logistic regression model due to multicollinearity [51].

We conclude that the combination of Sox10, GATA3 and p53 has no value as a predictor for pCR after NAC in TNBC. This community-based retrospective cohort of TNBC patients confirms that sTILs are a robust predictor for post-NAC therapeutic response. This offers perspectives for research exploring the possibility of reducing NAC regimens and/or omitting (extensive) surgery in TNBC patients with high sTILs, in an attempt to further de-escalate therapy. Future studies should investigate the immune responses in TNBC patients with high sTILs without pCR and TNBC patients with low sTILs with pCR, since this might facilitate finetuning of sTILs as a predictive marker.

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CRediT authorship contribution statement

Mieke R. Van Bockstal: Conceptualization, Methodology, Investigation, Visualization, Data Curation, Formal Analysis, Writing – Original Draft.

Fanchon Noel: Data Curation, Formal Analysis, Writing – Review & Editing.

Yves Guiot: Project Administration, Resources, Data Curation, Writing – Review & Editing.

Francois P. Duhoux: Resources, Data Curation, Writing – Review & Editing.

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Declaration of competing interest

None.

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