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Comparison of the Predictive and Prognostic Capacities of Neutrophil, Lymphocyte and Platelet Counts and Tumor-infiltrating Lymphocytes in Triple-negative Breast Cancer: Preliminary Results of the PERCEPTION Study

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Abstract. *Background/Aim:* Triple-negative breast cancer (TNBC) is the most heterogeneous breast cancer subtype, posing numerous challenges in clinical decision-making. Biomarkers are essential to personalize management of TNBC patients. While tumor infiltrating lymphocytes (TILs) are validated prognostic biomarkers, the requirement for tumor biopsy limits their routine use. Therefore, more accessible and reliable quantitative biomarkers are needed. Given the significant role of systemic inflammatory response in tumor onset and progression, assessing inflammatory cells via liquid biopsies emerges as a promising alternative. *Patients and Methods:* The PERCEPTION study, conducted at Centre Jean Perrin in France, aims to determine the correlation between TILs and peripheral blood components at diagnosis. An interim analysis was conducted after enrolling 50% of the estimated population, to evaluate study feasibility and preliminary

correlations between blood cell counts and TILs. *Results:* Sixty-one patients were enrolled over 4.5 years, demonstrating a good inclusion rate with minimal missing data. Preliminary results for 36 analyzable patients showed no correlation between the neutrophil-to-lymphocyte ratio (NLR) and TILs ($r_s = -0.19$, 95%CI = $-0.49-0.16$, $p = 0.3$). However, a moderate, positive, statistically significant correlation was found between NLR and the CD8/FoxP3 TILs ratio ($r_s = 0.36$, 95%CI = $0.03-0.64$, $p = 0.043$). The probabilistic index of 0.7 ($p = 0.06$) between NLR-high and NLR-low groups for this ratio supports the correlation. *Conclusion:* The interim analysis of the PERCEPTION study confirms the feasibility of correlating blood cell counts with TILs in TNBC. Although no significant correlation was observed between NLR and TILs, the moderate positive correlation between the CD8/FoxP3 ratio and TILs suggests a potential link between systemic inflammation and local immune response. These findings underscore the potential of blood-based markers as non-invasive surrogates for TILs, encouraging further research to enhance prognosis and guide treatment strategies in TNBC.

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Key Words: Triple-negative breast cancer, biomarker, tumor-infiltrating lymphocytes, neutrophil-to-lymphocyte ratio, CD8/FoxP3 ratio.

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Triple-negative breast cancer (TNBC) is a subtype of breast cancer (BC) characterized by the absence of hormone receptor expression and human epidermal growth factor receptor 2 (HER2) gene amplification, and it accounts for approximately 15-20% of all BCs (1-7). TNBC is highly aggressive in most cases and is prone to metastatic recurrence, with a high risk of recurrence or death within five years of diagnosis (1-6, 8). Standard treatments include chemotherapy, surgery, and radiotherapy, with emerging options such as immunotherapy and

Table I. Eligibility criteria.

Inclusion criteria	Exclusion criteria
-Female -Age >18 years -Primary (non-recurrent), non-metastatic triple-negative breast cancer at diagnosis -Treated with neoadjuvant chemotherapy, surgery, and adjuvant radiation therapy -Fluent in French -Affiliated to social security -Able to provide signed informed consent	-Unavailable biopsy sample -Unavailable blood cell counts at the time of diagnosis -Pregnant or breastfeeding women, vulnerable persons, adults under legal protection, or unable to give their consent, according to article L.1121-6 of the Public Health Code

PARP inhibitors in certain cases. However, prognosis prediction remains challenging due to intra- and inter- tumor heterogeneity (3, 9). Consequently, there is a need for new biomarkers to better identify high-risk patients and guide treatment decisions.

BC is not often infiltrated by lymphocytes; however, TNBC and HER2-enriched subtypes more frequently contain high levels of tumor infiltrating lymphocytes (TILs) and immune cell infiltrates in their surrounding stroma (4, 6, 10, 11). Several studies have demonstrated that high TIL levels, either before or after neoadjuvant chemotherapy, are associated with a favorable prognosis among early-stage TNBC patients (4-6, 10, 12-15).

TILs are mononuclear cells infiltrating tumor tissue, with CD8+ T cells, FoxP3+ T cells, and CD20+ B cells considered as the main components (16-18). CD8+ TILs are cytotoxic T cells involved in the elimination of intracellular infections and malignant cells, providing long-term immune protection (19, 20). Transcription factor forkhead box protein 3 (FoxP3) belongs to the family of forkhead transcription factors. Mainly localized in the nucleus, FoxP3 is a key marker of regulatory T cells and is known for its role in suppressing CD8+ T cell immunity. FoxP3+ T cells play a crucial role in maintaining immune homeostasis and preventing autoimmune reactions, but a high infiltration in tumors can contribute to tumor immune escape (16, 21). CD20+ lymphocytes are B cells involved in the humoral immune response, producing antibodies that target specific antigens and modulating T cell activity, contributing to tumor cell destruction (22, 23). While CD8+ and CD20+ TILs are associated with a favorable prognosis in early-stage TNBC, FoxP3+ TILs are often correlated with poorer prognosis (17, 24).

The CD8/FoxP3 and CD8/CD20 TILs ratios provide insights into the tumor's immune landscape and its prognostic implications. The CD8/FoxP3 TILs ratio reflects the balance between CD8+ cells and T regulator cells FoxP3, influencing the prognosis of BC patients, with a high ratio often indicating a stronger cytotoxic response compared to regulatory suppression, which is often associated with a favorable prognosis (20, 25, 26). The CD8/CD20 ratio

reflects the number of CD8+ cytotoxic T cells in relation to CD20 B cells. A higher CD8/CD20 ratio suggests a marked cytotoxic T cell presence compared to B cell infiltration, also correlating with favorable prognosis (17).

Systemic inflammatory response plays an essential role in breast cancer progression and development (27-31). In particular peripheral blood inflammatory markers, including neutrophils, platelets, and lymphocytes, are key players in tumor occurrence and development, offering potential applications in cancer treatment and prognosis prediction (30, 32).

Circulating lymphocyte ratios such as the platelet-to-lymphocyte ratio (PLR) and the neutrophil-to-lymphocyte ratio (NLR) are new, reproducible, routinely feasible, inexpensive, and promising biomarkers of immune response reflecting the degree of inflammation (12, 30, 31). NLR is the most widely evaluated on account of its accessibility and ease of calculation (30, 33).

Prospective studies evaluating the relationship between TILs and blood cell counts and their ratios remain rare. That is why we designed the PERCEPTION study, aiming to evaluate the correlation between TILs assessed on diagnostic biopsies and blood cell counts measured at diagnosis. This correlation should enable us to determine whether blood cell counts could be used as quantitative prognostic biomarkers, easily achievable in place of TILs. TIL assessment requires an experienced pathologist or an artificial intelligence algorithm, which are not always available. Additionally, there can be errors in the assessment of TILs, especially in the range between 5% and 30%. Therefore, this study aimed to determine whether peripheral parameters, such as the NLR and PLR, could provide surrogate information. This study also sought to examine the relationship between local tumor immunity, reflected by TILs and their subpopulations, and the ratios between these subpopulations and systemic immunity, reflected by the levels of circulating white blood cells. An interim analysis was conducted after enrolling 50% of the estimated population to evaluate study feasibility and preliminary correlations between blood cell counts and TILs.

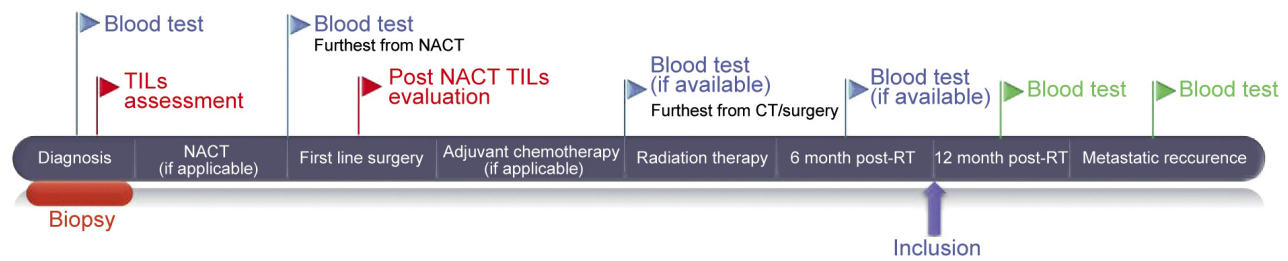


Figure 1. The PERCEPTION study design. The figure is reproduced from Lusho *et al.* (34). NACT: Neoadjuvant chemotherapy; CT: chemotherapy; RT: radiation therapy; TILs: tumor infiltrating lymphocytes.

Patients and Methods

Patients. All patients were enrolled at the Jean Perrin Comprehensive Cancer Center in Clermont-Ferrand, France. The eligibility criteria for patient inclusion are listed in Table I. A total of 90 patients are expected to participate in this study, and the interim analysis was conducted on the first 45 patients enrolled.

Study design. The study design was published in detail by Lusho *et al.* (34). Briefly, this was a prospective, single-center study, approved by the ethics committee “CPP Sud Mediterranean IV” and registered at ClinicalTrials.gov (NCT04068623). A total of 61 patients were enrolled from December 2019 to June 2024.

Patients were enrolled in the study 12 months after completing radiotherapy (Figure 1). At enrollment, the patient’s medical history was reviewed, and blood samples were collected for blood cell counts. Additional blood samples were drawn at the time of metastatic relapse, if it occurred, for further blood cell counts. Blood cell counts and tumor samples obtained at diagnosis were collected retrospectively, as well as blood test results obtained before surgery, before radiation therapy, and six months after radiation therapy (RT). The overall follow-up period is five years.

All PERCEPTION patients were already included in a database that supports the study. The database includes all patients with TNBC who have been followed at the Centre Jean Perrin (CJP) since 2006 (35).

Study endpoints. All study endpoints were detailed in the protocol article published in 2020 (34). In this interim analysis, we focused on the primary endpoint and the first secondary endpoint. The primary endpoint was to evaluate the correlation between TILs in the diagnostic tumor biopsy sample and the NLR, derived from neutrophil and lymphocyte counts at the time of diagnosis. NLR was defined as the ratio of the absolute neutrophil count (ANC) to the absolute lymphocyte count (ALC). The secondary endpoint was to evaluate the correlation between TILs in the diagnostic tumor biopsy and total white blood cell count (WBC), absolute neutrophil count (ANC), absolute lymphocyte count (ALC), absolute platelet count (APC), and PLR routinely assessed among women with TNBC at diagnosis. PLR was defined as the ratio of the absolute platelet count (APC) to the absolute lymphocyte count (ALC).

This interim analysis was conducted to describe and evaluate study feasibility and preliminary correlations between blood cell counts and TILs. In this study, patients are not exposed to additional risks; thus, no interruption in patient enrollment was necessary during this interim analysis.

Tissue analysis. Diagnostic biopsies were obtained either from the Biological Resource Center of the Jean Perrin Comprehensive Cancer Center, identified under No. BB-0033-00075 and registration number DC-2008-339 (Clermont-Ferrand, France) or from the external pathology laboratories, and processed for H&E and immunohistochemistry (IHC) at the center’s Pathology Department.

TILs were assessed on H&E-stained tumor tissue sections, following the recommendations of the International Immuno-oncology Biomarker Working Group (10). Each case was independently reviewed by one of two study pathologists (MK and NRR). In case of discordance, a consensus was reached through discussion. The result obtained is a percentage of TILs ranging from 0 to 100% and classified in 10% increments as per recommendations (10) and as already published in several studies (13, 36).

CD8, CD20, and FoxP3+ TILs were detected by sequential chromogenic IHC (CD8-CD20 and CD8-FoxP3). Chromogenic double sequential IHC was performed on slides. Four-micron formalin-fixed, paraffin-embedded tissue sections were placed on poly-L-lysine coated slides, deparaffinized in xylene and rehydrated in graded alcohol. The antigen retrieval was performed using Cell Conditioning 1 (Ventana/Roche, Meylan, France) by heating tissues to 95°C in 10 mM citrate buffer for 64 min. Endogenous peroxidase activity was inhibited by Dual Endogenous Enzyme-Blocking Reagent for 15 min (Dako, Glostrup, Denmark) and the nonspecific binding was reduced by the serum-free protein blocking (Dako) for 20 min. The sections were then incubated at 37°C with both rabbit monoclonal anti-CD8 (SP16, Thermo Fisher Scientific, Fremont, CA, USA) diluted at 1:100, and rabbit monoclonal anti-CD20 (SP32, Cell Marque) diluted at 1:200 for 60 min. Antibody binding was displayed using the CD8 UltraView DAB detection kit (Ventana/Roche) and CD20 UltraView RED detection kit (Ventana/Roche). For CD8 and FoxP3 detection, the sections were incubated for 60 min at 37°C with both rabbit monoclonal anti-CD8 (SP16, Thermo Fisher Scientific) diluted at 1:100, and rabbit monoclonal anti-FoxP3 diluted at 1:50 (SP97, Invitrogen). Antibody binding was displayed using the CD8 UltraView DAB detection kit (Ventana/Roche) and FoxP3 UltraView RED detection kit (Ventana/Roche).

The stained slides were scanned using a DP200 scanner (Ventana Medical Systems/Roche). An expert pathologist (NR-R) identified two regions of interest (ROI) on each biopsy, chosen in the tumor stromal areas most densely infiltrated by lymphocytes. The IHC-labeled TILs (CD8-, CD20- or FoxP3-positive) were counted using the integrated counting Ventana Image Viewer software version 3.2, by a previously trained PhD student (AG), under the supervision of a board-certified breast cancer pathologist (NRR). The stained TIL count was reported as the number of positive cells per square

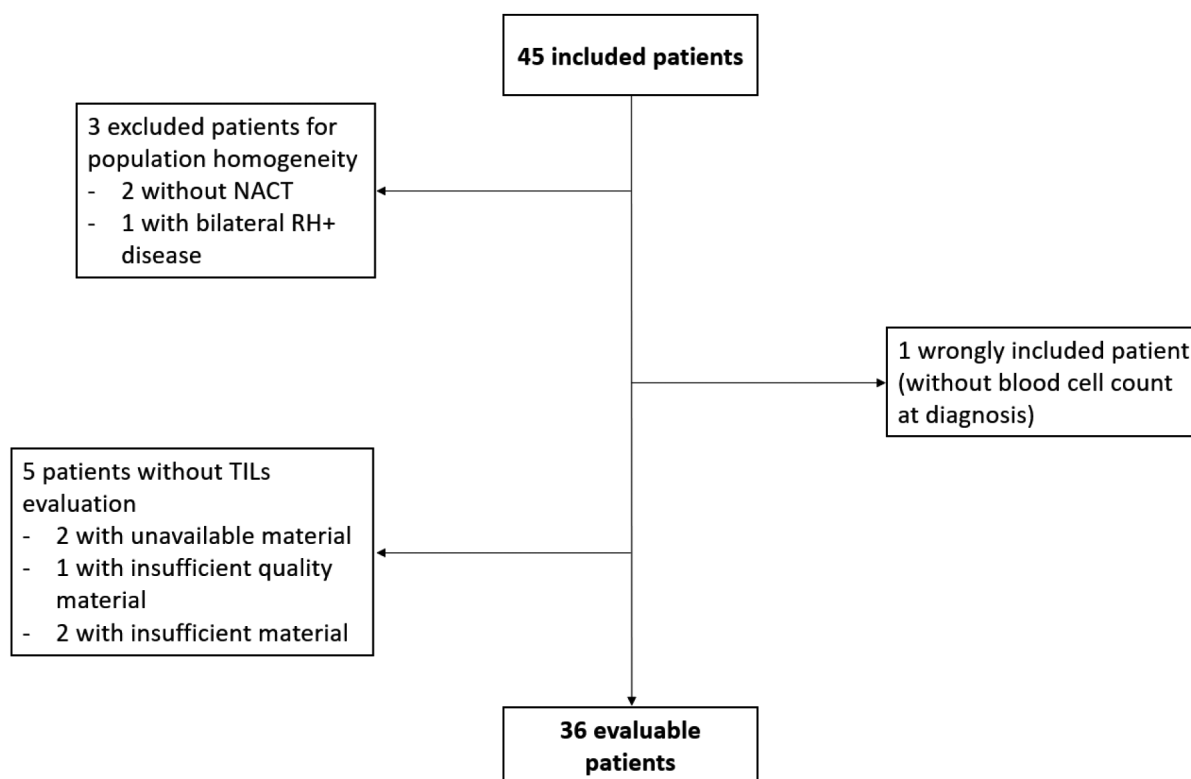


Figure 2. *PERCEPTION* patients flow chart. NACT: Neoadjuvant chemotherapy; HR+: hormone receptor positive; TILs: tumor infiltrating lymphocytes. 1 patient was wrongly included (without blood cell count at diagnosis), 1 patient had bilateral disease, 2 patients did not undergo NACT and TIL evaluation, and the evaluation was not possible for 5 patients (insufficient quality of material, insufficient material or unavailable material).

millimeter. The final ratio calculated was the average of the counts for each type of TILs. In statistical analysis, a value of 0 was replaced by 1 to facilitate ratio calculation, as previously reported in a study directed by the supervising pathologist (37).

Blood analysis. Results of the blood tests performed before inclusion in the study were retrospectively collected from the database. Blood samples collected at inclusion and at the time of metastatic relapse were obtained within the study setting. These samples were used for blood cell counts in a certified University Hospital laboratory, which performs all blood tests for the CJP.

A cut-off value of 2 was determined for NLR to divide patients into NLR-high (NLR >2) and NLR-low (NLR <2) groups, as previously implemented with a good specificity and sensitivity in TNBC studies (28, 38). A cut-off value of 150 was determined for PLR to divide patients into PLR-high (PLR >150) and PLR-low (PLR <150) groups, shown by Qi *et al.* to be a suitable value for PLR and pathological complete response (pCR) association (27).

Statistical analysis. A total sample size of 90 was decided based on a power analysis for an expected effect size of 0.3 (statistical power 0.8, alpha risk 0.05) and recruitment capacity. The interim analysis was pre-planned to be performed on the first 45 patients included (50% of the total planned enrollment) to conduct a preliminary analysis of the study objectives, without early discontinuation criteria. The results presented are preliminary, and no adjustment for multiple testing was

applied (significance level set at 5%). We also examined study feasibility, including the rate of inclusion and missing data.

Since the assumption of normality was not met, the correlation between TILs and different blood cell counts was assessed using Spearman's rank correlation coefficient (r_s) and the Wilcoxon-Mann-Whitney test (with the probabilistic index as the reported effect size). Correlation was considered relevant when the coefficient was larger than 0.2 in absolute value, regardless of statistical significance, since the interim analysis is low-powered. The median follow-up time was estimated using the reverse Kaplan-Meier method. Statistical analyses were performed using R software (v4, R-Project, Vienna, Austria), specifically the *asht* R package for confidence interval calculation for the probabilistic index.

Results

Feasibility and recruitment. A total of 61 patients were enrolled from December 2019 to June 2024, resulting in an average of one new patient per month.

Patient characteristics. Among the 45 patients considered for this interim analysis, nine patients were excluded and the interim analysis was performed on the remaining 36 patients (Figure 2). The median age at enrollment was 58 years (range=26-82 years) and the median follow-up time was 57 months (range=29-76

Table II. Clinicopathological patient characteristics.

Variable	N	Level	Count (frequency)
Age	36	58 years	Range=26-82 years
T	36	T1a	1 (3%)
		T1b	2 (5.5%)
		T1c	14 (39%)
		T2	16 (44.5%)
		T3	2 (5.5%)
		T4	1 (3%)
N	36	N0	25 (69.5%)
		N1	8 (22%)
		N2	1 (3%)
		N3	2 (5.5%)
M	36	M0	36 (100%)
Histology	36	Invasive breast cancer no special type	35 (97%)
		Other	1 (3%)
Scarff-Bloom-Richardson grade	36	1	1 (3%)
		2	17 (47%)
		3	18 (50%)
Germline BRCA mutation	36	BRCA1	1 (3%)
		BRCA2	2 (5.5%)
		No	22 (61%)
		Not known	11 (30.5%)

Table III. Neoadjuvant chemotherapy regimens.

Neoadjuvant treatment	N
EC, taxanes (docetaxel and/or paclitaxel)	23
EC, carboplatin, paclitaxel, pembrolizumab/placebo	6
EC, carboplatin, paclitaxel	5
FEC, docetaxel	1
Docetaxel, cyclophosphamide	1

EC: Epirubicin/cyclophosphamide; FEC: 5-fluorouracil/epirubicin/cyclophosphamide.

Table IV. Tumor infiltrating lymphocyte (TIL) percentage repartition.

Variable	N	Level	Count (frequency)
TIL percentage	35	[0;4]	16 (45.5%)
		[5;9]	6 (17%)
		[10;19]	6 (17%)
		[20;29]	0 (0%)
		[30;39]	3 (8.5%)
		[40;49]	0 (0%)
		[50;100]	4 (11.5%)

months) with a 95%CI=53-64. All clinicopathological details are presented in Table II. Most patients had tumors classified as either T1c or T2, and the majority of these did not show nodal involvement. Most patients presented an invasive breast cancer with no special type, and over 90% were Scarff-Bloom-Richardson (SBR) grade 2 or 3 carcinomas. A germline BRCA1/2 mutation was detected in three patients.

Treatments. Neoadjuvant treatment. All patients included in this interim analysis received neoadjuvant chemotherapy and the regimens are described in Table III. All patients underwent surgery. Conservative procedures were performed on 28 patients (78%), while a mastectomy was performed on 8 patients (22%).

Adjuvant treatment. Adjuvant chemotherapy with capecitabine was administered to 13 patients (36%) and 1 (3%) patient received olaparib. Among the 36 patients, 5 (14%) were also included in the KEYNOTE-522 study (NCT NCT03036488) and received pembrolizumab or placebo. All patients underwent adjuvant radiation therapy.

TIL evaluation. Among the 36 patients included in this interim analysis, 35 (97%) underwent TIL assessments, 32 (89%) underwent CD8+ TIL evaluations, 29 (81%) had FoxP3+ TIL evaluations, and 28 (78%) had CD20+ TIL evaluations. The discrepancy in the number of evaluations is due to the inability to perform all TIL assessments for each patient.

The median percentage of TILs (n=35) was 5% (range=0.5-15%), with the percentage value ranging from 0 to 80%. The TILs percentage distribution is described in Table IV. Median values for individual TILs elements are presented in Table V.

Blood cells counts. Blood component values are described in Table VI. Among the 36 patients, none had lymphopenia (lymphocyte count $<1 \times 10^9/l$), thrombocytosis (platelet count $>450 \times 10^9/l$) or neutrophilia (neutrophil count $>1 \times 10^9/l$). One patient had leukocytosis (leucocyte count $>10 \times 10^9/l$). For PLR, using a cut-off of 150, 29 patients (80.5%) had a low PLR and 7 (19.5%) had a high PLR. With this cut-off, the NLR-high group comprised 14 patients (39%) and the NLR-low group included 22 patients (61%).

Primary endpoint. No significant correlation was observed between NLR and TIL percentages (Spearman's correlation coefficient $r_s = -0.19$, 95%CI=-0.49-0.16, $p=0.3$).

No correlation was found between NLR and CD8+ or CD20+ TILs ($r_s = 0.07$, 95%CI=-0.3-0.4, $p=0.7$; $r_s = -0.06$, 95%CI=-0.4-0.3, $p=0.8$ respectively). Similarly, no correlation was found between NLR and the CD8/CD20 ratio ($r_s = 0.13$, 95%CI=-0.27-0.49, $p=0.5$).

A moderate negative monotonic correlation was found between NLR and FoxP3+ TILs, with a Spearman's correlation coefficient of -0.27 (95%CI=-0.58-0.12, $p=0.2$), indicating that an increase in NLR was associated with a decrease in the FoxP3 count. A moderate positive monotonic correlation was found

Table V. Values of tumor infiltrating lymphocyte (TIL) subpopulation counts as the number of positive cells per square millimeter and the ratios between the subpopulations.

Variable	N	Median	Range
CD8 TILs	32	377	1.6-1,706
FoxP3 TILs	29	43	1-378
CD20 TILs	28	67	0.5-2,249
CD8/CD20 TIL ratio	28	3.7	0.46-269
CD8/FoxP3 TIL ratio	32	6.5	0-294

Table VI. Blood component values for $\times 10^9/l$ and the ratios.

Variable	N	Median	Range
White blood cells	36	6.5	4.4-11
Neutrophils	36	3.7	1.9-6.8
Eosinophils	36	0.15	0.01-0.44
Basophils	36	0.04	0-0.16
Lymphocytes	36	2.2	1-3.7
Monocytes	36	0.53	0.31-0.89
Platelets	36	294	180-405
NLR	36	1.6	0.8-4.4
PLR	36	122	75-304

NLR: Neutrophil to lymphocyte ratio; PLR: platelet to lymphocyte ratio.

between NLR and the CD8/FoxP3 ratio, with a Spearman's correlation coefficient of 0.36 (95%CI=0.003-0.64, $p=0.043$), suggesting that an increase in NLR was associated with an increase in the CD8/FoxP3 TILs ratio. This latter correlation is the only one that was statistically significant. These correlations are presented in Figure 3.

No statistically significant difference concerning TILs was found between the NLR-high and NLR-low group (Table VII). The probabilistic index of NLR-high vs. NLR-low in relation to the CD8/FoxP3 TIL ratio, indicates that the probability of the TIL ratio being higher in a subject with NLR-high compared to one with NLR-low is 0.7 (95%CI=0.049-0.85); this result approaches significance with $p=0.006$.

Secondary endpoints. No significant correlation was found between total white blood cell counts and TILs on diagnostic biopsies.

A moderate negative monotonic correlation was observed between the ANC and the FoxP3 TILs ratio ($r_s=-0.22$, 95%CI=-0.54-0.17, $p=0.3$). Additionally, a moderately positive monotonic correlation was observed between the ANC and the CD8/FoxP3 TILs ratio ($r_s=0.25$, 95% CI=-0.12-0.56, $p=0.2$).

Three moderate monotonic correlations were found between ALC and TILs. The first one is a positive correlation between the ALC and the CD20 TILs ($r_s=0.35$, 95%CI=-0.04-0.64,

$p=0.07$). The second is a negative correlation between the ALC and the CD8/FoxP3 TIL ratio ($r_s=-0.24$, 95%CI=-0.55-0.13, $p=0.2$). The third is a negative monotonic correlation between the ALC and the CD8/CD20 TILs ratio ($r_s=-0.42$, 95%CI=-0.68 - -0.04], $p=0.03$). Only the last correlation is statistically significant.

The APC had moderate negative monotonic correlations with TILs: one with TIL percentage ($r_s=-0.29$, 95%CI=-0.57-0.06, $p=0.09$) and another with the CD8/CD20 TILs ratio ($r_s=-0.23$, 95%CI=-0.56-0.17, $p=0.2$). The only relevant correlation between PLR and TILs was with the TIL percentage ($r_s=-0.21$, 95%CI=-0.51-0.14, $p=0.2$). Using the cut off of 150, the PLR-low group had a higher percentage of TILs compared to the PLR-high group, with an estimated difference of 5% ($p=0.04$). No other significant difference was highlighted between these two groups for other TIL parameters.

Discussion

In this prospective single-center study, we conducted an interim analysis after enrolling 50% of the estimated population to evaluate study feasibility and preliminary correlations between blood cell counts and TILs. We focused on the primary endpoint examining the correlation between TILs and NLR at diagnosis, and the secondary endpoint, analyzing the correlation between TILs and all blood cell counts at diagnosis. With a total of 61 patients recruited over 4.5 years, recruitment of the remaining 39 patients within two years seems feasible.

These preliminary results showed us that the availability and quality of biopsies could be a limitation in evaluating TILs, thus particular attention should be paid to the upcoming inclusions to ensure the availability of biopsy samples before enrollment, in order to avoid missing data.

In this interim analysis, we hypothesized that systemic inflammatory circulating markers could correlate with total TILs on H&E, as well as with the stromal CD8, CD20, FoxP3 positive TILs density and the CD8/CD20 and CD8/FoxP3 TILs ratios.

Correlation between the circulating inflammatory markers and TILs has been inconsistent across studies. Lee *et al.* were the first to study the correlation between NLR and TILs in breast cancer, showing trends for negative correlations but without reaching significance (39). Several retrospective studies did not show any correlation (40-42) whereas one study showed a negative correlation (43). These inconsistent results could be due to small sample sizes and retrospective designs. The preliminary results of the PERCEPTION study show no correlation between NLR and TILs at diagnosis. However, this finding may be influenced by the cohort's low median TIL value and the small sample size used for the statistical analysis. Thus, this result should be confirmed in the final analysis.

The CD8/FoxP3 TIL ratio, indicating a balance between cytotoxic T cells able to induce tumor cell death and Treg

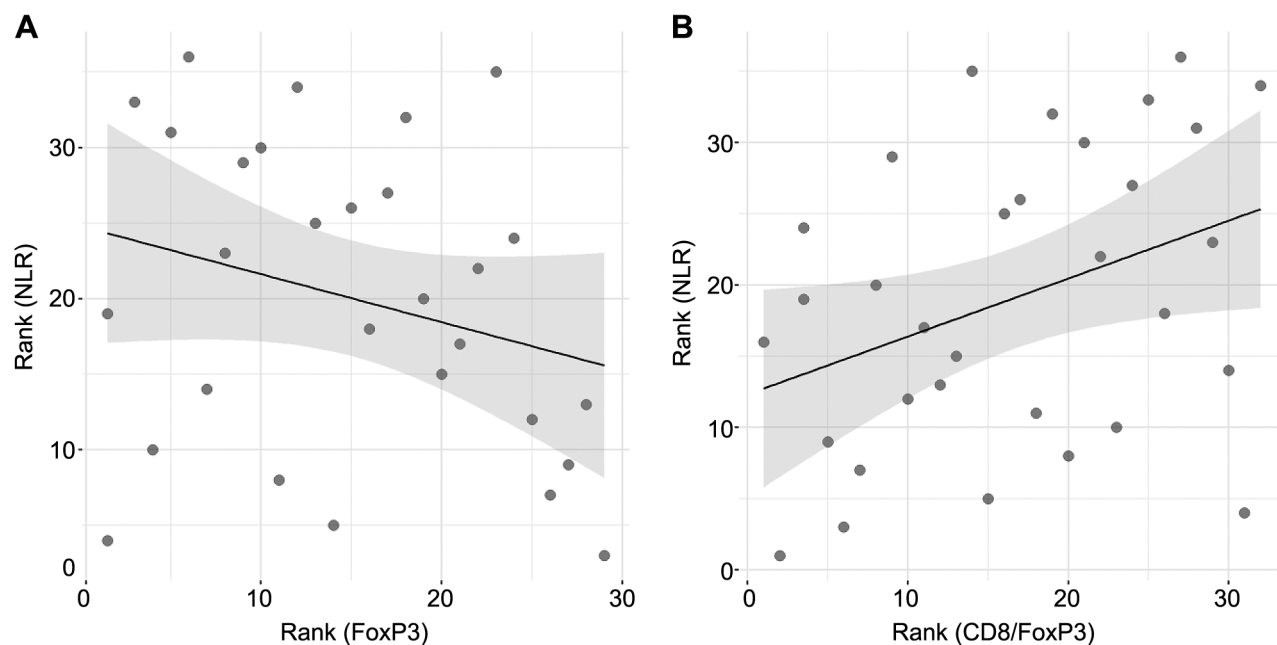


Figure 3. Graphical illustration of correlations between neutrophil-to-lymphocyte ratio (NLR) and FoxP3 tumor infiltrating lymphocytes (TILs) (A) and between NLR and the CD8/FoxP3 TILs ratio (B).

Table VII. Tumor infiltrating lymphocyte (TIL) difference between the neutrophil-to-lymphocyte ratio (NLR)-low and the NLR-high group.

Variable	NLR low Median (IQR)	NLR high Median (IQR)	PI	95%CI	p-Value
TILs (%)	5 (2-15)	4 (0-13)	0.44	0.26-0.63	0.5
CD8 TILs	515 (199-740)	324 (214-425)	0.42	0.24-0.62	0.4
FoxP3 TILs	139 (15-241)	17 (11-53)	0.35	0.18-0.56	0.2
CD20 TILs	170 (21-346)	9 (1-445)	0.39	0.21-0.61	0.3
CD8/CD20 TIL ratio	3 (2-7)	15 (3-73)	0.65	0.42-0.82	0.2
CD8/FoxP3 TIL ratio	4 (2-15)	15 (6-39)	0.7	0.49-0.85	0.06

PI: Probabilistic index of NLR high vs. NLR low (probability that the outcome of a subject with NLR high will exceed the outcome of a subject with NLR low); 95%CI: compatible with the probabilistic index (excludes 0.5 when p-value significant at 0.05 level); p-value: for the Wilcoxon-Mann-Whitney test.

cells able to inactivate cytotoxic T cells, is one of the most studied in breast cancer. A high CD8/FoxP3 TIL ratio is often associated with a better outcome (25, 26, 44-48). Based on this, we expected a negative correlation of this ratio and NLR, where a high level is associated with poor outcome (33, 49, 50) as Zhao *et al.* found in their study (43). We found a moderate positive monotonic correlation between these two parameters. The most significant difference in TILs between the NLR-high and NLR-low groups concerns the CD8/FoxP3 TIL ratio, supporting this correlation. To determine these groups, we used the cut-off value of 2, which has also been used in some studies to predict overall survival (OS) with a good sensitivity and specificity (28, 38). Other studies have

used different cut-off values for NLR, such as 3.16 or 2.85 (31, 51). A previous study on 8,563 patients showed that overall survival was significantly lower when the NLR was greater than a cut-off value of 3.0 (52). As no cut-off value has been validated in literature, the final cut-off value in our study should be further investigated in the final analysis. In our study, a high NLR may be driven by a low ALC, rather than a high ANC, suggesting that increased inflammation could be promoting tumor progression. This could occur because numerous lymphocytes might have left the bloodstream to infiltrate the tumor. This may help explain the positive correlation observed between the CD8/FoxP3 TIL ratio and NLR.

The relationship between FoxP3 TILs and prognosis is unclear, with studies showing both positive and negative associations (26, 44, 53). In the first study showing that FoxP3 TILs were associated with a good outcome in ER-negative breast cancer, this correlation was only significant in high-grade tumors, particularly in TNBC (53). The moderate negative monotonic correlation that we found between the FoxP3 TILs and NLR is thus consistent with the positive prognostic value described in the FoxP3 subpopulation in the BC cohorts containing all molecular subtypes. Nevertheless, another study found that high FoxP3 infiltration negatively affected OS and progression-free survival (PFS) in both the ER-positive and ER-negative BCs (26), whereas other studies did not find any correlation (25, 39). These results reinforce the importance of studying correlations between the FoxP3 TILs and circulating inflammatory cells to gain a better understanding of the interactions between them as well as their respective prognostic values.

Similarly, correlations were found between ANC and FoxP3 TILs, and the other between ANC and the CD8/FoxP3 TIL ratio. These findings, along with the previously mentioned correlations between NLR and FoxP3 expression, suggest a significant interplay between systemic inflammation and the tumor immune environment. In addition, it is known that a high ANC can negatively impact TILs (40) and is considered a poor prognostic marker (54). However, in our study, all patients had ANC levels below 7 g/l, and none presented with neutrophilia. Therefore, correlations between circulating neutrophils or NLR and TILs were not influenced by this parameter.

We did not find any correlation between NLR and CD8, CD20 TILs, and the CD8/CD20 TIL ratio; however, this needs to be confirmed in the final analysis. Lee *et al.* showed that ALC was statistically significantly correlated with CD8+ TILs (39) where a high level was often associated with a good prognosis in breast cancer (25, 26, 53). However, Ladoire *et al.*, did not find any association between the baseline CD8 or FoxP3 TILs and disease-free survival or OS (25).

Lymphopenia has been associated with a generalized suppression of the immune system in patients with cancer, and tends to be associated with a poorer prognosis (55, 56). In our study, no patients presented lymphopenia at diagnosis, which we defined as a lymphocyte count less than $1 \times 10^9/l$ according to the Common Terminology Criteria for Adverse Events (CTCAE) (57). Consequently, the correlation between the ALC and TILs could not be influenced by this factor, as all patients had ALC within the normal range. We found that the ALC negatively correlated with the CD8/FoxP3 and the CD8/CD20 TILs ratio. This could be because a high ALC value could indicate an effective systemic immune response, which might reduce the need for tumoral CD8+ T cell infiltration, thereby affecting the CD8/CD20 and the CD8/FoxP3 TILs ratios.

Leukocytosis is associated with a suppressive immune microenvironment in cancer (58), and studies in digestive cancers have confirmed this by finding a negative correlation between leukocytosis and TILs (59, 60). In our study, only one patient had leukocytosis ($>10 \times 10^9/l$), with a leucocyte count of $11 \times 10^9/l$. Another study found a negative correlation between the ALC and CD8 TILs, but no statistically significant association between FoxP3 TILs and any hematological marker (39). In the preliminary results of the PERCEPTION study, no correlation was found between absolute leucocyte count and TILs, probably because of the lack of statistical power of this analysis. This should be further investigated in the final analysis.

The only correlation between the PLR and TILs was the one with total TILs (on H&E). This correlation was negative and therefore, consistent with the literature, showing that high PLR is associated with poor prognosis and a high TIL percentage with good prognosis. In our previous study performed on 120 patients from our TNBC database, PLR was the only parameter whose predictive value was statistically significant, whereas no correlation was found between PLR and TILs (61). On the other hand, Onagi *et al.* found a significant positive correlation between PLR and TILs in TNBC using multiplex fluorescent immunohistochemistry (12). We used a cut-off value of 150 to form the PLR groups, as Qi *et al.* showed found a significant association between the PLR and pCR with a cut-off value ≥ 150 , but statistically significant association was found with cut-off values <150 (27). Even so, another study that used a cut-off value of 129.52 with good specificity and sensitivity showed that survival was better in the low-PLR group (31). Therefore, further research is needed to determine the optimal cut-off value for PLR for comparisons/correlations of this parameter with TIL-related parameters. In our analysis, negative correlation was also found between the PLR and the CD8/FoxP3 TILs ratio, consistent with the literature (20, 39). The same applies to the negative correlation that we found between the APC and the total TIL count (on H&E). These results could not be influenced by extreme APC values because no patient presented thrombocytosis (APC $>450 \times 10^9/l$). Finally, the negative relationship between APC and the CD8/CD20 TIL ratio requires further investigation for better understanding. In addition, Qi *et al.* obtained different results in the subgroup analysis of the relationship between PLR levels and pCR rates. They suggested that the limited predictive efficacy of PLR in early breast cancer is likely due to the absence of a pronounced inflammatory response in early stage disease (27).

Furthermore, as chemotherapy can modify immune infiltration and inflammation (62), studies that have evaluated TILs and blood cell count both before and after treatment reported contradictory results between these two time points (25, 49). This was recently confirmed by Wang *et al.* who found that post-treatment markers, such as TILs and changes in NLR, have a stronger predictive ability than pre-treatment markers like initial ANC (63). These findings could explain the discrepant

results and should be verified in the final analysis evaluating posttreatment parameters. To achieve this, the final analysis will include both a dynamic examination of the differences between pre- and post-treatment data, in addition to the statistical analysis.

Recently, immunotherapy was added to chemotherapy for early TNBC on the basis of the KEYNOTE-522 study results (64). This means that not all PERCEPTION study patients will receive the same treatment. For this interim analysis, six patients participated in the KEYNOTE-522 study. Although this interim analysis focuses exclusively on diagnostic samples, which are not influenced by the treatment variations, this difference should be considered in the final analysis as treatment could impact biomarker evaluation. Indeed, other parameters can influence systemic inflammation markers (27, 28). For instance, corticosteroid therapy and a concomitant inflammatory or infectious disease can modify these markers. We only used one blood cell count result to evaluate these correlations, without taking the patient's health condition into account. This could modify the results of the analysis. The blood cell counts should be conducted on more than one sample, even before diagnosis, and it is important to ascertain whether a given patient has a concomitant disease that could modify these parameters. This will be assessed in the final analysis.

Assessment of only TILs, their total amounts and their subpopulation ratios, could also explain the discordant results of our study, because it probably does not fully represent the actual tumor immune infiltration. Furthermore, TILs on H&E and the TIL subpopulation ratios (CD8/FoxP3 and CD8/CD20) were not assessed/expressed in the same manner: the first assessed the total TILs in a tumor whereas the second used ratios of densities expressed as cell numbers per surface unit of ROIs (thus, the entire tumor stroma was not assessed). The "hot-spot" approach (using ROIs with the densest lymphocytic infiltration) provides the highest numbers of stained cells, allowing for assessment of their density per unit area and calculation of corresponding ratios. However, having only two ROIs, even if they are large (mean ROI surface: 167,283 μm^2 , data not shown), may be insufficient for accurately evaluating the ratio in a given tumor. The observed weak correlations between the TILs on H&E staining and the ratios assessed on IHC highlight an important consideration for designing similar future studies.

It is important to note that our study focuses exclusively on TNBC, whereas some other studies included all breast cancer subtypes. TNBC has distinct biological and clinical characteristics compared to other subtypes of breast cancer that could influence the relationship between blood cell counts and TILs (27). Some studies, such as the one by Lee *et al.*, found different results in the whole BC population and in molecular subtype analyses (39). These results suggest that the effect of the human immune response on cancer progression could be different according to molecular subtype of breast cancer. Future studies with larger samples and prospective

designs are needed to confirm our findings and further explore this relationship within specific breast cancer subtypes.

The final results will enable comparison between complete blood counts and TILs, and their correlations with PFS and OS. In the final analysis, tissue assessments will also be performed on surgical specimens, enabling the evaluation of the correlation with blood cell counts.

Following the completion of this study, a multi-center study should be conducted to further elucidate the relationships between blood cells and TILs on a larger scale. Therefore, we believe that the final results will enhance our understanding and ability to predict TNBC recurrence.

Study limitations. One of the major limitations of this interim analysis is the small sample size, which results in a lack of statistical power. The effect size of 0.3 was calculated based on the final target population of 90 patients, highlighting the importance of the final analysis to confirm these preliminary results.

In addition, the study is single-center, which may limit its representation of the entire TNBC population. However, this methodology was chosen because it was a feasibility study intended to inform future research on this topic. Finally, each parameter was assessed at only one time point, which may not fully represent the overall health of each patient. Despite these limitations, this interim analysis has highlighted potential correlations and the need for further research to more fully understand the prognostic value of systemic inflammatory markers and TILs in TNBC.

Conclusion

Preliminary results of the PERCEPTION study show some correlations between blood cell counts and the ratios of TIL subpopulations. Although these findings are discordant with previously published literature, they suggest that combining blood cell counts with TIL assessment could lead to more accurate predictions of TNBC patient outcomes. With a larger TNBC patient population analyzed upon study completion, we may be able to determine whether blood counts could replace TIL assessments in some situations and whether this parameter could serve as a simple and rapid prognostic biomarker.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization: Nina Radosevic-Robin, Myriam Kossai, Yannick Bidet, Ioana Molnar, Maureen Bernadach, Frédérique Penault-Llorca, Catherine Abrial, Xavier Durando. Methodology: Nina Radosevic-Robin, Myriam Kossai, Yannick Bidet, Ioana Molnar, Maureen

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