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PML localization in tumor associated macrophages as a prognostic marker in triple negative breast cancer

Bianca Vezzani[1,2], Sonia Missiroli[1,3], Massimo Pedriali[4], Patrizia Querzoli[5], Iva Maestri[8,9], Paolo Carcoforo[6,7], Giovanni Lanza[8,9], Roberta Gafà[8,9], Paolo Pinton[1,3,10] & Carlotta Giorgi[1,3,10]✉

Triple-negative breast cancer (TNBC) is a particularly aggressive and metastatic subtype, characterized by the absence of estrogen, progesterone, and human epidermal growth factor 2 receptors. Outcomes for TNBC patients vary widely, suggesting this classification encompasses different cancers with distinct histological, genomic, and immunological profiles, leading to variable prognoses. The tumor microenvironment, particularly the expression and localization of promyelocytic leukemia protein (PML) in tumor-associated macrophages (TAMs), can influence patient outcomes by modulating inflammation. The beneficial prognostic role of increased tumor-infiltrating lymphocytes (TILs) in TNBC is well-established. In this retrospective study, we found that PML expression in tumor cells is inversely related to the presence of TILs and is associated with poorer outcomes. Patients with disease recurrence exhibited higher levels of TAMs with predominantly nuclear-localized PML, in contrast to patients who showed complete recovery. The accumulation of PML in the nucleus reduces its presence at ER-mitochondria contact sites, impairing its interaction with the NLRP3 inflammasome and leading to increased IL-1β secretion. This promotes a pro-inflammatory tumor microenvironment as seen in patients with adverse outcomes. Our findings suggest that both PML expression in cancer cells and its localization in TAMs can serve as additional prognostic factors, highlighting the potential of PML as a therapeutic target in TNBC.

Keywords Breast cancer, Triple negative breast cancer, Prognostic markers, Tumor-infiltrating lymphocytes, Tumor-associated macrophages, PML

Triple-negative breast cancer (TNBC), a term established in the mid-2000s, categorizes a distinct subtype of breast cancers that lack expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2)[1]. Clinically, TNBC is characterized by its high invasiveness, significant metastatic potential, tendency for relapse, and generally poor prognosis[2]. Since this classification, numerous studies have uncovered the inherent heterogeneity within TNBC, revealing fundamentally distinct cancers with varied histologic, genomic, and immunologic profiles[3–6]. This diversity, coupled with the aggressive nature of TNBC due to the absence of specific therapeutic targets and a high likelihood of metastasis, contributes to the wide variation in patient outcomes. The underlying causes of this heterogeneity are attributed to genomic and proteomic variations, with significant influence exerted by the tumor microenvironment (TME)[1]. The TME encompasses cells, metabolites, the extracellular matrix, cytokines, and chemokines within the tumor boundary, not all of which are directly related to the tumor itself. Key components of the TME include tumor-infiltrating

[1]Department of Medical Sciences, Section of Experimental Medicine, University of Ferrara, Via Fossato di Mortara, 70, c/o CUBO, 44121 Ferrara, Italy. [2]Department of Medicine and Surgery, University of Parma, 43125 Parma, Italy. [3]Laboratory of Technologies for Advanced Therapy (LTTA), Technopole of Ferrara, 44121 Ferrara, Italy. [4]Integrated Activity Department of Onco-Hematology, University Hospital of Ferrara, 44124 Cona, FE, Italy. [5]University of Ferrara, 44121 Ferrara, Italy. [6]Department of Medical Sciences, Section of General Surgery, University of Ferrara, 44121 Ferrara, Italy. [7]Surgery Unit, University Hospital of Ferrara, 44124 Cona, FE, Italy. [8]Department of Translational Medicine, University of Ferrara, 44121 Ferrara, Italy. [9]Anatomic Pathology Unit, University Hospital of Ferrara, 44124 Cona, FE, Italy. [10] Biomedical Research Center, Kansai Medical University, Osaka, Japan. ✉email: grgclt@unife.it

lymphocytes (TILs) and tumor-associated macrophages (TAMs), representing the host's immune cells that influence tumor progression. Several studies have established a positive correlation between the abundance of TILs and favorable prognoses in TNBC patients; however, the specific molecular mechanism linking TIL abundance to patient outcomes remain to be fully defined[7–9]. As we navigate through the complexities of TNBC and its variable outcomes, the pursuit of novel biomarkers for TNM staging underscores a broader shift toward personalized medicine. This context highlights the growing emphasis on prognostic and targetable markers, especially since patients with early-stage TNBC can experience promising outcomes through personalized treatments.

In this scenario, the promyelocytic leukemia protein (PML) emerges as a potential target of interest. PML, known for its role as a tumor suppressor and its function as the organizer of nuclear bodies within the nuclear matrix, is implicated in a variety of cellular processes[10,11]. Intriguingly, studies on TNBC have revealed that PML is overexpressed in the nuclei of TNBC cells, where it exhibits oncogenic characteristics. This overexpression aids ATP production and boosts cell survival by enhancing fatty acid oxidation through the activation of PGC1A and PPAR genes[12,13]. Additionally, it increases tumor aggressiveness by upregulating the expression of the stem cell gene SOX9, underscoring PML's complex role in TNBC pathology. Furthermore, PML is targeted by Hypoxia-Inducible Factor 1A (HIF1A), with its increased expression in TNBC cells partly driven by HIF1A[14].

Our research group has unveiled new tumor-suppressing functions of PML that extend beyond its nuclear localization, notably at the specialized contact sites between the endoplasmic reticulum and mitochondria known as mitochondria-associated membranes (MAMs). We propose that PML forms structures similar to nuclear bodies at these sites, which we termed PML MAMs-bodies[15]. In these sites, PML is crucial in modulating calcium (Ca^{2+}) signaling between the ER and mitochondria, specifically regulating ER Ca^{2+} release via IP3R3, and orchestrating autophagosome formation through the AMPK/mTOR/Ulk1 pathway. Our findings emphasize how PML's displacement from MAMs contributes to tumor growth and resistance by reducing Ca^{2+}-induced apoptosis and promoting autophagy as a survival strategy in hostile environments[10,11]. Moreover, within macrophages, PML at MAMs modulates the inflammatory response through its interaction with the NLRP3 inflammasome. Its delocalization from these sites results in increased IL-1β levels, further promoting tumor growth and progression due to the absence of PML's inhibitory effect on NLRP3[16,17]. Collectively, these findings indicate a versatile, dual role for PML as either a tumor suppressor or promoter, depending on its intracellular localization.

In this study, we confirmed a positive correlation between the expression of TILs and the outcomes of TNBC patients, demonstrating an inverse relationship with PML expression within tumor cells. Furthermore, our evaluation of TAMs revealed that TNBC patients with poor prognosis exhibited an increased number of macrophages at the tumor site, which predominantly showed nuclear localization of PML, in contrast to the good prognosis group, where a primarily cytoplasmic localization of PML was observed. This was numerically correlated with decreased IL-1β levels. Notably, we also identified a positive correlation between the presence of PML in cancer cells and the quantity of TAMs. Consequently, our findings suggest that the expression and localization of PML could serve as a valuable prognostic marker in the diagnostic assessment of TNBC.

Results

Cohort description

In this retrospective study, a total of 20 patients were enrolled. We selected 10 patients diagnosed with TNBC who experienced disease recurrence within a 10-year follow-up period; this group is henceforth referred to as the NEG cohort. As summarized in Table 1, the average time to recurrence was 1.56 ± 0.96 years, with variations in secondary tumor localization. The overall survival rate was 2.33 ± 0.82 years, as also depicted in the Kaplan–Meier curve (Fig. 1a).

Conversely, the positive (POS) cohort consisted of 10 TNBC patients who did not experience any disease recurrence during the 10-year follow-up period. Notably, one patient from this cohort dropped out of follow-up after 7 years (Fig. 1a). However, given that the standard follow-up duration for breast cancer recurrence is typically 5 years, we decided to include this patient in our study.

Inverse correlation of PML expression and TILs predicts poor prognosis

It is well-established that the presence of TILs is directly linked to the prognosis of TNBC[7,8]. This correlation was also observed in our cohort, where a higher density of TILs at the tumor margins was noted in patients who achieved complete recovery and maintained disease-free survival for 10 years (indicative of a positive prognosis). In contrast, patients who experienced a recurrence of the pathology (indicative of a negative prognosis) exhibited fewer TILs, as shown in Fig. 1a–c. Interestingly, this pattern was mirrored in the distribution

N. of patients	Prognosis	Age at diagnosis	Recurrence	Time rec	Overall survival (years)
10	NEG	56.20 ± 15.45	YES Brain n = 3 Lungs n = 3 Ovary n = 2 Liver n = 1 NAL n = 1	1.56 ± 0.96	2.33 ± 0.82
10	POS	53.50 ± 9.66	NO	N/A	10

Table 1. Epidemiologic data of enrolled subjects. All the reported times have to be considered as the average of years ± standard deviation. *NAL* non axillary lymphnodes.

of CD8 + lymphocytes, which were significantly more prevalent in the TILs of patients with a positive prognosis compared to those with a negative prognosis, as illustrated in Fig. 1d,e. These findings underscore a direct correlation between an increase in TILs and enhanced recruitment of CD8 + lymphocytes at the tumor site, as depicted in Fig. 1h. Subsequently, we investigated whether PML expression within cancer cells could predict patient outcomes. Our analysis confirmed that PML expression is significantly elevated in the TNBC tumor cells of patients with a poor prognosis compared to those with a favorable prognosis, as illustrated in Fig. 1f,g. This observation aligns with previously reported findings[12,14,18]. These results suggests that PML expression within tumor cells could serve as a viable prognostic marker. Consistently, we noted an inverse correlation between PML expression in cancer cells and the quantity of TILs, as shown in Fig. 1h,i. This relationship further underscores the potential of PML as a predictive marker for TNBC.

Increased tumor-associated macrophages (TAMs) reflect the tumor's inflammatory state and correlate with poor patients' prognosis

Beyond TILs, TAMs emerge as crucial modulators of the tumor microenvironment's inflammatory state. Figure 2a,b, demonstrate a marked increase in TAMs, identified as CD68-positive cells per high power field (HPF), in TNBC patients with unfavorable outcomes. This observation is consistent with the elevated levels of IL-1β detected in the same cohort, as depicted in Fig. 2c,d. These findings bolster the notion that TILs and TAMs occupy divergent roles in TNBC progression: TILs act as the host's primary defense against the tumor, whereas TAMs, aligning with the tumor, contribute to a harmful pro-inflammatory state that promotes cancer proliferation. Correlation analyses reveal a significant positive relationship between TAMs and IL-1β levels. In contrast, a notable inverse correlation exists between TILs and both TAMs and IL-1β, as shown in Fig. 2e.

No correlation between PML expression in TAMs and patient outcomes

Although we've established a link between PML expression within tumor cells and patient outcomes, we extended our analysis to PML expression in TAMs to determine whether the tumorigenic process influences PML expression in this specific cell subset. However, as depicted in Fig. 3a,b, no variation in PML expression in TAMs was observed in relation to patient prognosis. Furthermore, PML expression levels in TAMs did not correlate with the number of infiltrating TAMs (identified as CD68 + cells) or with IL-1β levels (Fig. 3c,d).

Differential cellular localization of PML in TAMs reflects patient outcomes

Our research has recently highlighted the pivotal role of PML in modulating NLRP3-mediated inflammation by directly interacting with this inflammasome at MAMs. We have demonstrated that within TAMs, the shift in PML localization of from the nucleus to the cytoplasm—and subsequently to the MAMs—is a critical determinant of patient outcomes in various cancers, including melanoma and NSCLC[17].

This differential localization of PML has also been observed in our current cohort of TNBC patients. As depicted in Fig. 4, PML exhibits a varied distribution: it is predominantly localized within the nuclei of infiltrating macrophages (panel b) or more diffusely throughout the cytoplasm (panel a). Upon analyzing the relationship between PML localization and patient outcomes, we found a direct correlation between the nuclear presence of PML and poor prognosis in TNBC patients (Fig. 4c). Conversely, patients with a favorable prognosis exhibited higher levels of cytoplasmic PML (Fig. 4d).

Further analysis through interpolation revealed a positive correlation between the nuclear localization of PML and an increased count of CD68 + macrophages infiltrating the tumor (Fig. 4e). Similarly, a significant direct correlation exists between nuclear PML and elevated IL-1β production (Fig. 4f).

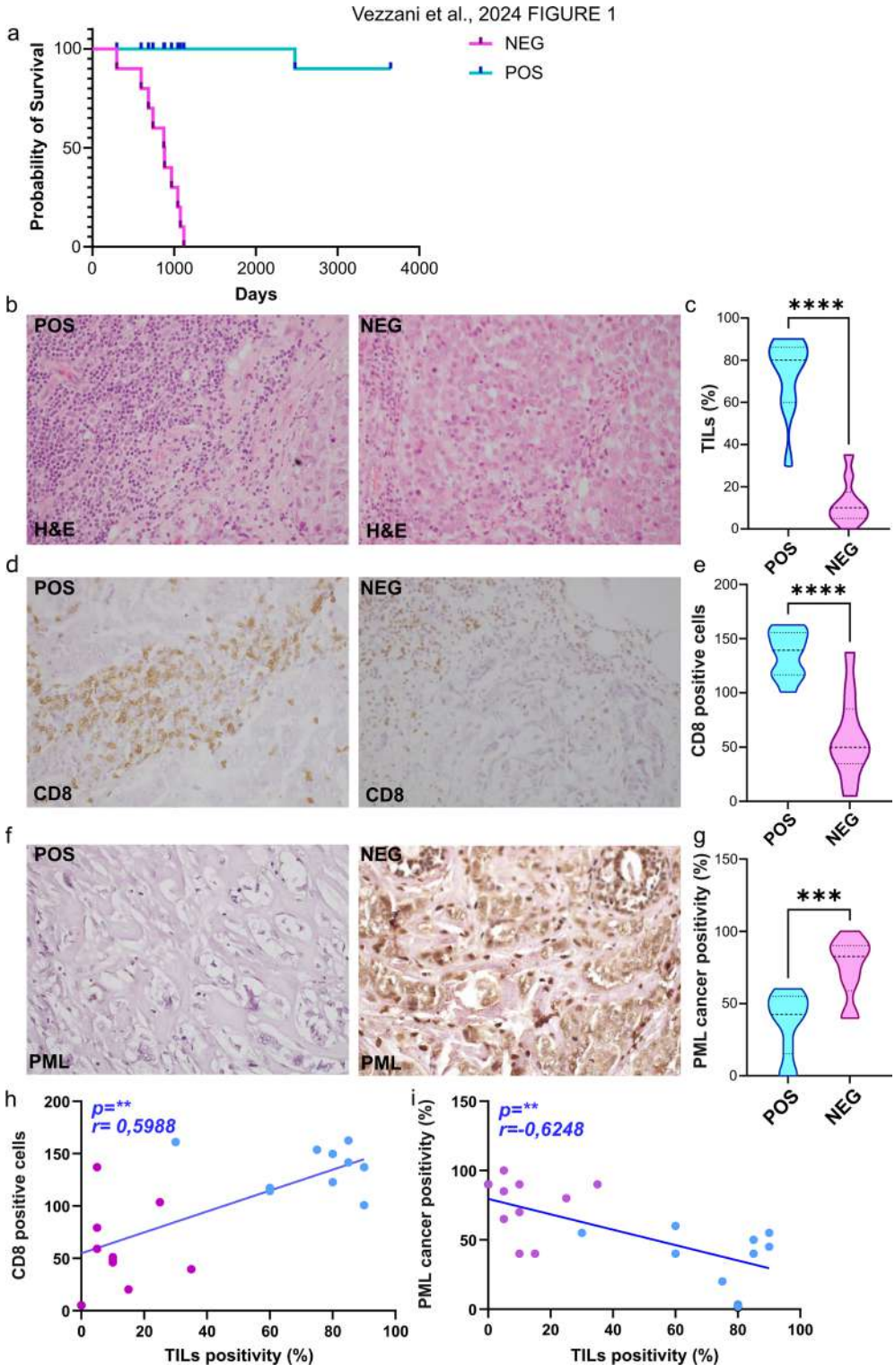
Discussion

Triple-negative breast Cancer (TNBC) remains one of the most aggressive and challenging types of breast cancer to treat. Our study aimed to explore the tumor microenvironment (TME), focusing on tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs), to understand their roles in TNBC progression and to uncover novel insights that could enhance outcome prediction and therapeutic strategies, particularly through the lens of inflammation.

TILs are recognized as significant prognostic markers in breast cancer, highlighting the importance of the local anti-tumor immune response[19]. Our findings corroborate existing literature, confirming that higher TIL counts are associated with improved outcomes[20,21]. Additionally, our analysis revealed that TNBC patients with elevated TIL levels also had a greater number of infiltrating CD8 + T cells, indicative of a favorable prognosis[22].

In the spirit of exploring novel insights within the TME, our study has emphasized the role of PML, a multifaceted protein for which we recently identified a role in modulating the TME. PML is a tumor suppressor known for regulating gene expression and its association with nuclear bodies. Previous research from our laboratory has demonstrated PML's localization at mitochondria-associated membranes (MAMs), where it influences cell metabolism and death by modulating calcium transfer and directly affecting the NLRP3 inflammasome. Despite its traditionally recognized tumor-suppressive role, PML's upregulation and its interaction with HIF1A have been specifically documented in TNBC, collectively portraying PML as an oncogene in this cancer type[14,23]. We confirmed that PML expression within breast cancer cells acts as a negative prognostic factor, being inversely correlated with survival probability. Moreover, we found that an elevated amount of PML was inversely related to the number of TILs infiltrating the tumors.

We further investigate PML's contribution to regulating the TME and the inflammatory response, which is known to sustain tumor progression. Given the critical role of macrophages in shaping the inflammatory state of the TME, we first assessed TAMs' abundance in our cohort. Higher TAM infiltration is known to be associated with more invasive breast cancer; indeed, we observed an increased number of CD68 + TAMs in the TNBC cohort with a poor prognosis. Furthermore, we discovered a direct correlation between the presence



of CD68 + TAMs and IL-1β expression, indicating increased NLRP3 activity. Considering our group's recent discovery of PML's inhibitory role on NLRP3, we closely examined PML's function within TAMs across two patient cohorts. Despite consistent PML expression levels in TAMs across varying patient outcomes, we noted a distinct variation in its localization. Specifically, we observed cytoplasmic localization of PML in patients with a positive prognosis versus predominantly nuclear localization in those with negative outcomes. This shift in PML localization was closely linked to the differential activation of the NLRP3 inflammasome and prognosis: low IL-1β levels and cytoplasmic localization (presumably at MAMs) were associated with a positive prognosis, whereas increased IL-1β levels and nuclear localization were observed in patients with a negative outcome. Therefore, the dynamic localization of PML directly influences the inflammatory response and, by extension, the progression and prognosis of these cancers. PML properly localized at the MAMs correlates with reduced NLRP3 activation; conversely, its nuclear concentration leads to heightened NLRP3 activity, fostering tumor

◀**Fig. 1**. Evaluation of TILs, CD8+, and PML expression in tumor tissue of TNBC patients. (**a**) Kaplan–Meier survival curve for the enrolled subjects, divided according to prognosis: positive (POS, n = 10) and negative (NEG, n = 10). (**b**) Two representative images showing high (left) and low (right) levels of TILs within the tumor margin, obtained from patients in the POS and NEG prognosis groups, respectively. Images acquired at ×20 magnification. (**c**) Violin plots depicting the distribution of qualitative percentages of TILs observed in the 20 TNBC patients, divided according to prognosis: POS (n = 10) and NEG (n = 10). Statistical test: one-tailed unpaired Mann–Whitney, $p < 0.0001$. Dashed line: median, dotted lines: quartiles. (**d**) Two representative images showing high (left) and low (right) levels of CD8 + lymphocytes within TILs, obtained from patients in the positive and negative prognosis groups, respectively. Images acquired at ×20 magnification. (**e**) Violin plots presenting the distribution of average counts of CD8 + lymphocytes in 4 independent high magnification fields for each of the 20 TNBC patients, divided according to prognosis: POS (n = 10) and NEG (n = 10). Statistical test: two-tailed unpaired t-test, $p < 0.0001$. Dashed line: median, dotted lines: quartiles. (**f**) Two representative images showing low (left) and high (right) levels of PML expression in cancer cells, obtained from patients in the positive and negative prognosis groups, respectively. Images acquired at ×20 magnification. (**g**) Violin plots depicting the distribution of qualitative percentages of PML expression observed in the 20 TNBC patients, divided according to prognosis: POS (n = 10) and NEG (n = 10). Statistical test: one-tailed unpaired t-test, $p = 0.0004$. Dashed line: median, dotted lines: quartiles. (**h**) Correlation between the percentage of TILs and CD8 + lymphocytes, with the blue line representing simple linear regression. The p-value is calculated using the two-tailed Spearman correlation coefficient, $p = 0.0053$. Some patient data points may overlap. (**i**) Correlation between the percentage of PML positivity within tumor cells and TILs, with the blue line representing simple linear regression. The p-value is calculated using the two-tailed Spearman correlation coefficient, $p = 0.0032$. Some patient data points may overlap.

growth and progression. These findings underscore the significance of PML's localization in reflecting the TME's inflammatory state and its impact on TNBC patient outcomes[17].

Interestingly, our research revealed a significant inverse correlation between TILs and TAMs, further supported by the inverse relationship between IL-1β expression and TILs. This contrast highlights the dual nature of the immune response within TNBC, where TILs promote beneficial anti-tumor activity, while TAMs contribute to pro-tumoral inflammation.

In summary, our study highlights the importance of evaluating PML expression in both cancer cells and TAMs within TNBC patients. Advocating for the routine evaluation of PML not only provides a nuanced understanding of disease progression but also highlights the potential for anti-inflammatory treatments.

Despite the advancements made, we recognize limitations in the present study. First, we had access to a limited patient cohort, mainly due to the retrospective nature of this study. Secondly, several aspects of our study require further research:

- How does the expression and localization of PML specifically impact the treatment outcomes of TNBC?
- How do TILs and TAMs interact with each other and with other components of the TME such as cancer cells in TNBC, and what implications does this have for disease progression and therapeutic targeting?
- What specific therapeutic strategies or targeted interventions, based on the assessment of PML expression and localization, could significantly improve the management and outcomes of patients with TNBC?
- Are the reported results linked to a specific TNBC subclass, identifiable by specific breast oncogenic driver mutations?

These questions not only suggest directions for future research but also emphasize the need for a deep understanding of PML's complex roles in TNBC. By addressing the open questions outlined above, future research can build on our findings to develop more effective prognostic tools and therapeutic strategies for TNBC.

Methods

Patients' enrollment

The study was conducted by identifying a total of 20 female patients diagnosed with TNBC at the Section of Pathological Anatomy and Biomolecular Diagnostics of the University of Ferrara, who underwent surgical resection between 2005 and 2011. These patients were selected based on the primary inclusion criterion of negativity for the estrogen receptor (ER < 1%), progesterone receptor (PR < 1%), and the HER2 proto-oncogene (score 0, 1+, 2+ N.A. in ISH), as assessed using immunohistochemical and ISH methods. Specifically, we enrolled 10 TNBC patients who showed complete remission of the disease after both surgical intervention and chemo/radiotherapy (positive prognosis) and did not show any recurrence of the disease in a 10-year follow-up, and 10 TNBC patients who, besides the received treatments, experienced tumor recurrence and death within the follow-up (negative prognosis). Patients with known comorbidities or with multifactorial inflammatory pathology were excluded. The study was approved by the Local Ethics Committee, CE-AVEC (Comitato Etico Area Vasta Emilia Centro), with the project code 906-2020-Oss-UniFe, and followed the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from all individual participants included in the study.

Immunohistochemistry

All the reagents have been purchased from Merck Millipore (Burlington, USA) if not otherwise specified. Tumor specimens were collected and embedded in paraffin, then processed and analyzed for the routine

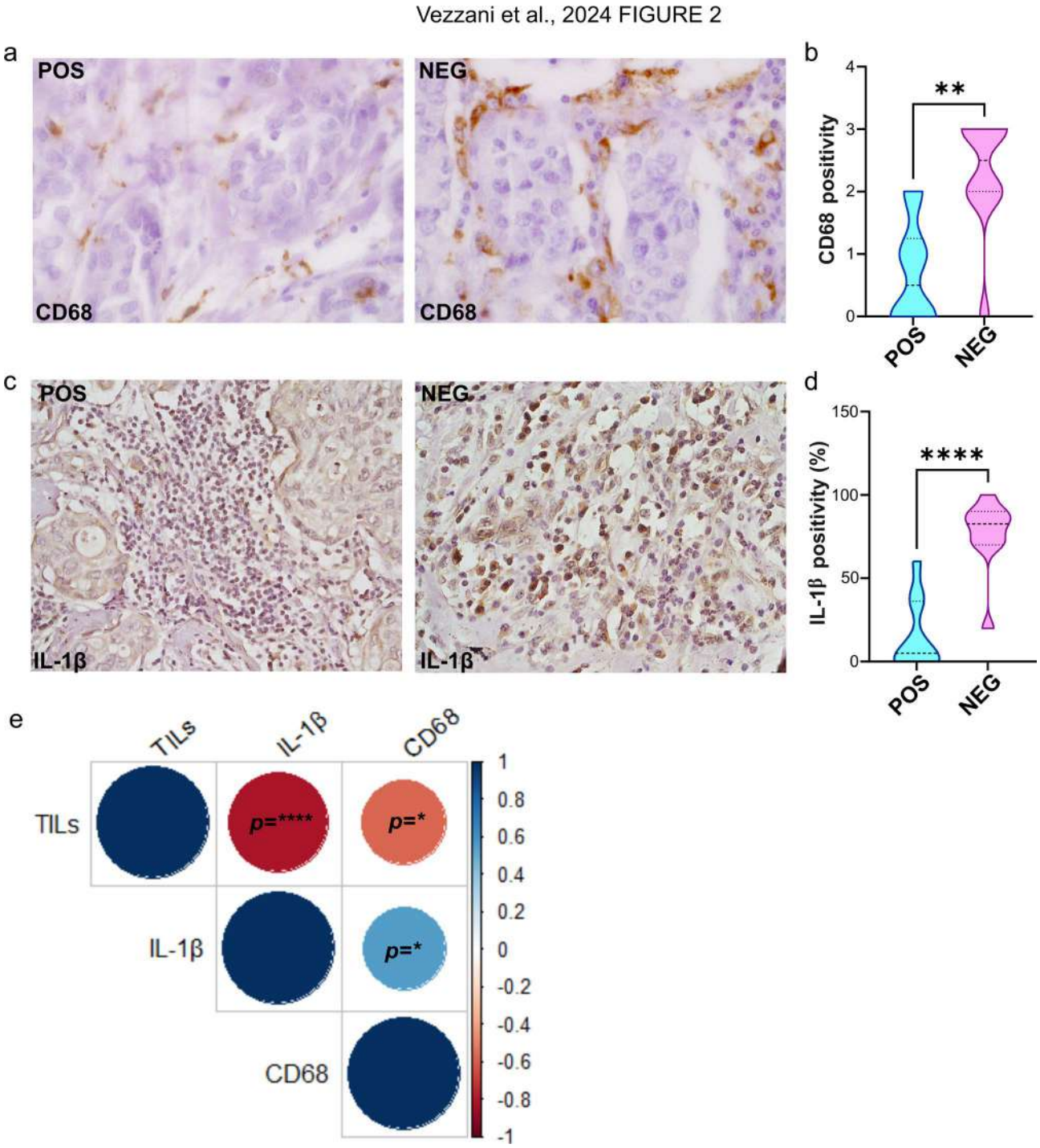


Fig. 2. TAMs and IL-1β expression in TNBC patients. (**a**) Representative images showing low (left) and high (right) CD68 + cell levels (TAMs) at the tumor margin, from patients with positive and negative prognoses, respectively. Images acquired at ×40, HPF. (**b**) Violin plots of CD68 + cell percentages (TAMs) in 20 TNBC patients, grouped by prognosis: POS (n = 10) vs. NEG (n = 10). Statistical analysis: two-tailed unpaired Mann–Whitney, $p < 0.0022$. Dashed line: median, dotted lines: quartiles. (**c**) Representative images of low (left) and high (right) IL-1β expression in patients from positive and negative prognosis groups, respectively. Images acquired at ×60. (**d**) Violin plots of IL-1β positivity percentages in 20 TNBC patients, grouped by prognosis: POS (n = 10) vs. NEG (n = 10). Statistical analysis: two-tailed unpaired Mann–Whitney, $p < 0.0001$. Dashed line: median, dotted lines: quartiles. (**e**) Correlation analyses using the Spearman coefficient between TILs and IL-1β levels (two-tailed, $p < 0.0001$), TILs and TAMs (two-tailed, $p = 0.0134$), and TAMs and IL-1β levels (two-tailed, $p = 0.0151$). A colorimetric scale indicates the correlation strength: + 1 (blue) denotes a direct correlation, and − 1 (red) denotes an inverse correlation.

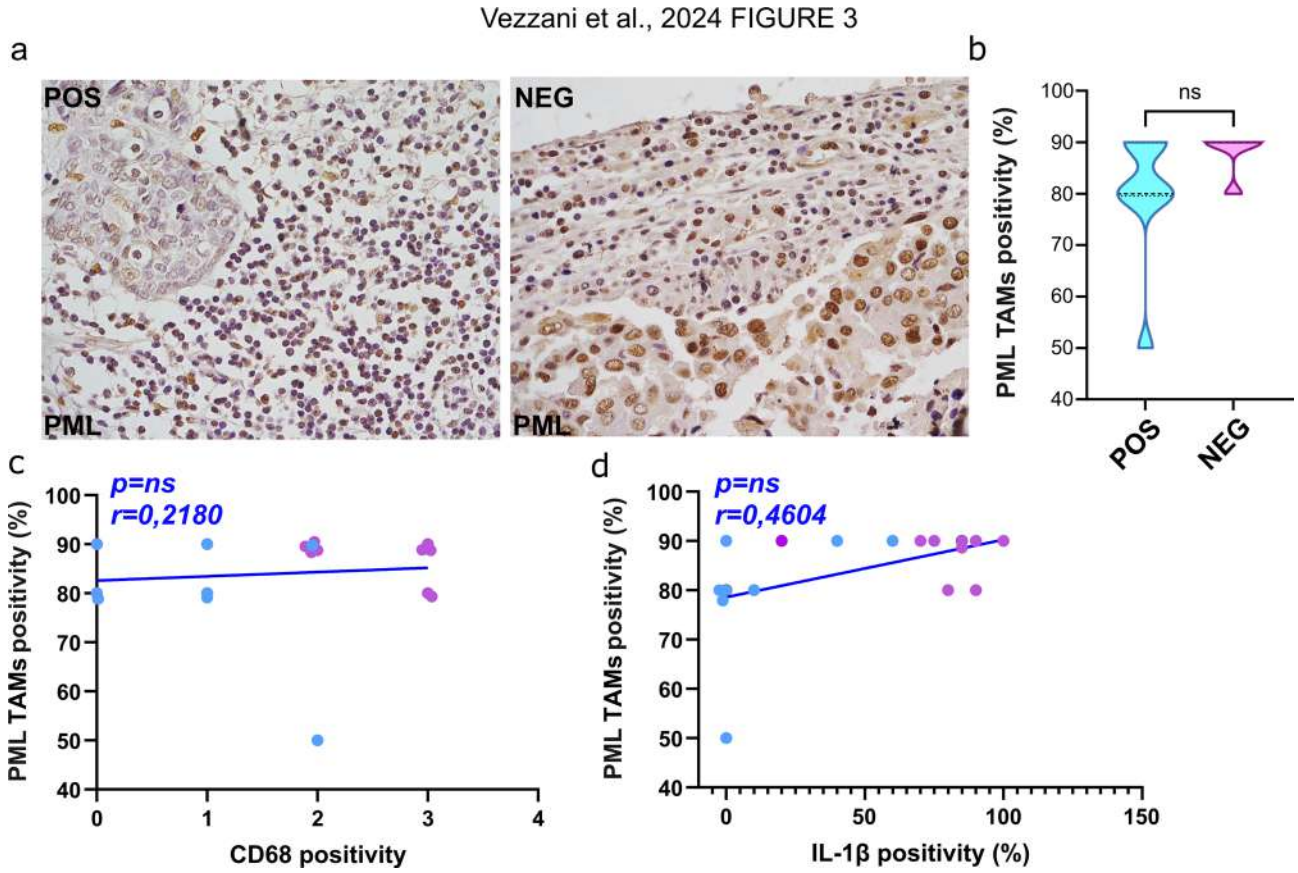
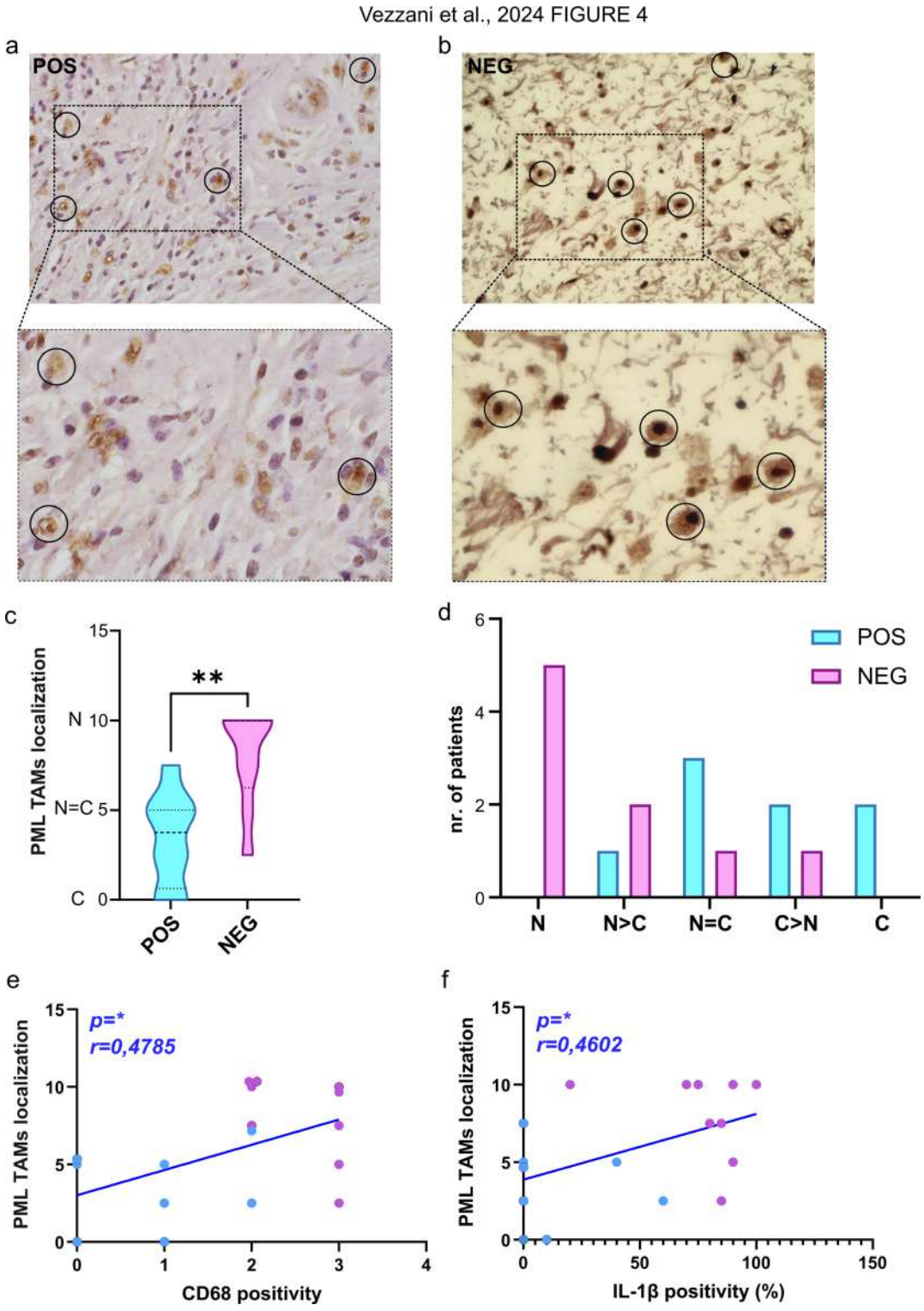


Fig. 3. PML expression in TAMs of TNBC patients. (**a**) Representative images showing PML expression levels in TAMs from patients with positive (left) and negative (right) outcomes. Images acquired at ×60 magnification. (**b**) Violin plots illustrate the distribution of qualitative percentages of PML positivity within TAMs across TNBC patients, grouped by prognosis: POS (n = 8) vs. NEG (n = 9). Statistical analysis: two-tailed unpaired Mann–Whitney, not significant (ns). Dashed line: median, dotted lines: quartiles. (**c**) Correlation analysis between the number of infiltrating TAMs (identified as CD68 + cells) and PML expression levels. The blue line represents simple linear regression. P-value was determined using a two-tailed Spearman correlation coefficient. Some data points (patients) may overlap, POS (n = 8), NEG (n = 9). (**d**) Correlation analysis between IL-1β positivity and PML expression levels within TAMs. The blue line represents simple linear regression. P-value was determined using a two-tailed Spearman correlation coefficient. Some data points (patients) may overlap, POS (n = 8), NEG (n = 9).

histopathological parameter analysis (H&E, IHC for CD8, CD68, ER, PR, and HER2) by the staff of the Pathology Unit at the University Hospital of Ferrara. Briefly, CD8 and CD68 immunodetection was performed on formalin-fixed paraffin-embedded tissue sections (4 µm thick) using the antibodies CD8 (SP58, ready to use, Roche Tissue Diagnostics, Basel, Switzerland) and CD68 (PG-M1, ready to use, Diagnostic Biosystems, Pleasanton, USA) and the Multimeric Detection Kit (Universal DAB Detection Kit Ultraview, Roche Tissue Diagnostics) on a Ventana Benchmark XT automated immunostainer (Roche Tissue Diagnostics). For the immunohistochemical evaluation of PML and IL-1β, 4 µm-thick tissue sections were first deparaffinated and rehydrated following 3 min incubation in xylene and then decreasing ethanol/water % solution (100%, 95%, 75%, 50%). Heat-induced antigen retrieval was performed with citrate buffer pH 6.00 for 20 min at 95 °C. The sections were then blocked with 10% goat serum in TBS and then immunostained with anti-PML (ab72137, 1:250 dilution, Abcam, Cambridge, UK), anti-IL-1β (ab9722, 1:500 dilution, Abcam) antibodies o.n. at 4 °C. Endogenous peroxidase activity was blocked by a 20 min incubation with 30% H_2O_2 solution in TBS, followed by incubation with an HRP-labeled polymer conjugated secondary antibody (1:500, Vector Lab, Newark, USA) for 1 h at RT. The signal was amplified using the ABC kit (Vector Lab) following manufacturer's instruction and the labeling was visualized by a 10 min incubation with the 3,3′ diaminobenzidine chromogen solution (Vector Lab), which resulted in a brown-colored precipitate at the antigen site. The sections were subsequently counterstained with hematoxylin.

Cell identification and scoring

Tumor cells were identified based on their nuclear and cytoplasmic characteristics and architectural features. For the evaluation of the stromal infiltrate, the peritumoral region and tumor margins were considered. Conversely, areas with detachment artifacts, necrotic areas, inflammation around the needle tract used for core



biopsy, and regression areas with central hyalinization were excluded. TILs were identified as mononuclear lymphoid cells (excluding granulocytes, dendritic cells, and macrophages), following H&E staining and IHC. From TILs evaluation was excluded any inflammatory infiltrate located outside the tumor borders (perilobular inflammation) or around any associated intraductal component, as well as areas of necrosis, fibrosis, sclerosis, and inflammation secondary to previous biopsy procedures. TILs presence was assessed as a continuous semi-quantitative parameter by assigning a percentage value (from 0 to 100%) to the proportion of stroma infiltrated by mononuclear cells relative to the total peritumoral stromal area. CD8 + TILs were evaluated by counting positive cells in 4 independent fields and represented as the average cell count. TAMs were identified using the pan-macrophagic CD68 marker, whose localization is primarily found in endosomal and lysosomal compartments, and at the plasma membrane level. CD68 positivity was evaluated in the peritumoral and intratumoral environment by observing its expression at the monocyte-macrophage cytoplasmic and membrane

◀**Fig. 4**. PML localization in TAMs and its impact on TNBC patient outcomes. (**a**,**b**) Representative images showing differential localization of PML within TAMs: cytoplasmic (**a**) for patients with a positive prognosis and nuclear (**b**) for those with a negative prognosis. Infiltrating macrophages and TAMs are highlighted with a black circle, and the dashed line marks the inset area. Images acquired at 40X magnification. The evaluation was performed on 4 independent fields for each patient. (**c**) Violin plots depict the distribution of PML localization within TAMs across 20 TNBC patients, categorized by prognosis: POS (n = 8) and NEG (n = 9). Statistical analysis: two-tailed unpaired Mann–Whitney, p = 0.0056. PML localization is quantified as indicated in Table 2 (0 C; 2,5 C > N; 5 C = N; 7,5 N > C; 10 N) with N for nuclear and C for cytoplasmic localization. Dashed line: median, dotted lines: quartiles. (**d**) Histograms display the count of patients featuring respective differential PML localization within TAMs, grouped by prognosis: POS (n = 8) vs. NEG (n = 9). (**e**) Correlation analysis between the presence of CD68 + cells (TAMs) and PML localization, with the blue line representing simple linear regression. The P-value was determined using a one-tailed Spearman correlation coefficient, p = 0.0269. Note: Some patient data points may overlap. (**f**) Correlation analysis between IL-1β positivity and PML localization within TAMs, with the blue line representing simple linear regression. The P-value was determined using a one-tailed Spearman correlation coefficient, p = 0.0333. Some patient data points may overlap. *N* nuclear.

Marker/cell types	Magnification	Scoring
TILs	20×	0–100%
CD8	20×	Average cell count in 4 independent fields
CD68	40× HPF	0, 1, 2, 3
IL-1β	20×	0–100%
PML expression	20×	0–100%
PML localization	40× HPF	0 C 2, 5 C > N 5 C = N 7, 5 N > C 10 N

Table 2. Magnification and qualitative scoring methods were used for IHC evaluation. *C* cytoplasmic, *N* nuclear.

levels at a 40X high power field (HPF). Based on the observed degree of positivity and, consequently, the extent of TAMs in the microenvironment, we applied the following classification: low or absent infiltration (0–1), moderate infiltration (2), and high infiltration (3). PML and IL-1β immunoreactivity were evaluated in the tumor and peritumoral stroma, depending on the research question. The positivity was evaluated on the entire slide excluding detachment artifacts, necrotic areas, inflammation around the needle tract used for core biopsy, and regression areas, and scored as a percentage, from 0 to 100. PML expression within tumor cells or TAMs was evaluated first morphologically, identifying tumor cells and TAMs, then proceeding with the evaluation of PML expression, scored as a percentage from 0 to 100. As for PML localization within TAMs, we identified 4 possible scenarios: prevalence of cytoplasmatic localization (score = 0), augmented cytoplasmic localization compared to the nuclear one (score = 2,5), equal cytoplasmatic and nuclear localization (score = 5), augmented nuclear localization compared to the cytoplasmic one (score = 7,5), predominant nuclear localization (score = 10). The scoring was performed in 4 independent fields (40X, HPF) for each patient.

All the described evaluations were conducted by two independent clinical pathologists (blinded to the patient outcomes) and scored as described and resumed in Table 2.

Statistical analysis

Statistical analysis was performed using the software Prism 9 and RStudio. The normality of data distribution was assessed by performing the Shapiro–Wilk test: if the distribution followed a Gaussian curve, datasets were compared by the two-tailed unpaired t-test, while if it did not, data were compared using the two-tailed unpaired Mann–Whitney test. Since the majority of the data sets analyzed showed a non-Gaussian distribution, the correlation analysis was performed by computing the one-tailed or two-tailed Spearman correlation coefficient, with a 95% confidence interval, depending on the directional hypothesis. Detailed information on the statistical methods used is reported in Supplementary Fig. 1. The correlation analysis shown in Fig. 2e was performed using TILs% shown in Fig. 1c, CD68 positivity shown in Fig. 2b, and IL-1β positivity shown in Fig. 2d.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

Conceptualization, C.G., P.Q., and PP; methodology, B.V., M.P., S.M.; formal analysis, B.V., M.P., P.Q., and R.G.; investigation, I.M. G.L., and P.C.; resources, P.Q., G.L., and P.C.; data curation, B.V.; writing—original draft preparation, B.V.; writing—review and editing, C.G., P.P and R.G.; visualization, B.V.; supervision, C.G., and P.P.; funding acquisition, C.G., and P.P. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to C.G.

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