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Chronic low-dose cadmium exposure accelerates the onset and metastasis of invasive mammary carcinoma and promotes immunosuppression in the tumor microenvironment in BALB–*neu*T mice

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ABSTRACT

Cadmium (Cd), a heavy metal known to act as an endocrine disruptor, has been implicated in breast cancer (BC) via mechanisms that involve both estrogen receptor (ER)-dependent and -independent pathways. We used BALB–*neu*T transgenic mice, a model of aggressive ErbB2-driven mammary carcinogenesis, to evaluate the effects of chronic exposure to an environmentally relevant, low-dose of Cd on tumor onset, multiplicity, progression, immune microenvironment, and metastasis. Cd exposure significantly accelerated tumor onset and increased tumor multiplicity and weight, reducing both tumor-free and overall survival. Cd-exposed mice displayed elevated serum estrogen levels, and increased expression of progesterone receptor and tumor progression markers, including CD31, Ki67, and phosphorylated Akt, in tumor tissues. Despite the increased recruitment of $CD4^+$ and $CD8^+$ T cells to the peritumoral stroma, Cd exposure fostered an immunosuppressive microenvironment with elevated Tregs and $PD\text{-}1^+$ exhausted T cells, both locally and systemically. Notably, lung metastases were threefold more frequent in Cd-exposed mice. Our results demonstrate that low-dose Cd exposure promotes the development and progression of ErbB2-driven breast tumors through hormonal, proliferative, and immune-modulating mechanisms. These findings reveal that exposure to environmentally relevant doses of Cd not only accelerates ErbB2-driven breast cancer but also reshapes the immune landscape toward dysfunction and immunosuppression, likely compromising both anti-tumor immunity and response to immunotherapies. These data advocate for tighter regulation of Cd exposure in populations at risk for breast cancer.

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1. Introduction

In recent years, growing consideration has been placed on the role of Cd in human health, in particular in tumor development and progression, and on its endocrine disrupting properties (Yang et al., 2025). Cd is widely diffused in the environment and, being highly soluble in water, it easily contaminates soil and aquatic system, posing significant risks not only to human health but also to flora, fauna, and ecosystem functioning through persistence, bioaccumulation, and trophic transfer (Cai et al., 2022; Li Z. et al., 2025; Qi et al., 2025; Zhang and Reynolds, 2019). Humans can assimilate Cd through food, air pollution and smoking. Cd has a very long half-life, is not biodegradable and is poorly excreted, so that it accumulates in the body over time (Yang et al., 2025). Women tend to be exposed to higher levels of Cd in comparison to men, a phenomenon that is partly explained by the evidence that intestinal absorption of Cd and iron occurs via the same transporter (DMT1); this transporter is in fact up-regulated as a result of increased iron demand, that in turn is often more pronounced in women due to menstruation and pregnancy (Kim et al., 2014).

As for its carcinogenic activity, Cd is designated as a Group I carcinogen by International Agency for Research on Cancer (IARC, 2012), and Cd exposure has been associated with development of different cancer types, including prostate, lung and breast cancer (Genchi et al., 2020). Its involvement in cancer development and progression appears to rely on multiple mechanisms, such as the induction of inflammation and oxidative stress, interference with signaling pathways involved in apoptosis and cell growth, DNA damage, inhibition of DNA repair and aberrant DNA methylation (Genchi et al., 2020; Qu and Zheng, 2024). In breast cancer (BC), the endocrine disrupting properties of Cd also play a key role in the carcinogenic process. In fact, Cd, as well as lead selenium, mercury and nickel, has been found to have estrogenic activity, which is why these elements are also known as metalloestrogens (Bimonte et al., 2024; du Plessis et al., 2023).

BC, one of the most common tumors in the world, is characterized by a multifactorial and complex etiology influenced by both genetic and environmental factors (Wan et al., 2022; Xiong et al., 2025). Molecular classification is based on the expression of prognostic and predictive biomarkers such as estrogen receptor (ER), progesterone receptor (PR), Human Epidermal growth factor Receptor 2 (HER2/ErbB2/neu) and Ki76, which allows a better understanding of tumor development, progression, and response to therapy (Allison et al., 2020; Testa et al., 2020). Among BCs subtypes, ErbB2-overexpressing tumors are highly aggressive subtypes usually associated with early recurrence and worse clinical outcomes (Testa et al., 2020).

Although genetic predisposition is a major risk factor for BC, the role of endocrine disrupting chemicals (EDCs) in breast carcinogenesis is increasingly recognized (Focaccetti et al., 2024; Song et al., 2025; Wan et al., 2022). Estrogens play a key role in the development of the mammary gland, and its exposure to estrogenic EDCs can deregulate hormone-related signaling pathways, leading to developmental and functional defects, and promote carcinogenesis (Singh, 2024; Wan et al., 2022). Cd, in particular, can mimic the action of estrogens by binding the αER and mediating receptor activation and nuclear translocation. Cd has also been found to replace zinc in the DNA-binding domain of αER and to increase the DNA binding affinity of the receptor. A possible interaction between this metalloestrogen and the membrane-bound form of αER and the non-classical estrogen receptor GPR30 (G-protein coupled receptor 30) has also been reported (du Plessis et al., 2023).

Genetically engineered mouse models of cancer are valuable tools to investigate how EDCs can interact with the genetic background to alter tumor development and progression. In this regard, we recently demonstrated that chronic administration of the EDC bisphenol-A (BPA) to BALB–*neu*T mice accelerates mammary carcinogenesis, stimulates tumor angiogenesis and favors an immunosuppressive tumor microenvironment (TME) (Focaccetti et al., 2024). BALB–*neu*T mice, transgenic for a mutated form of the rat ErbB2/neu receptor gene, represent one of the most aggressive breast cancer models, growing multifocal carcinomas in all mammary glands with full penetrance (Astolfi et al., 2005; Di Carlo et al., 1999). In this study, the BALB–*neu*T transgenic mouse model was employed to investigate the impact of chronic low-dose exposure to Cd, at 1 mg/l in drinking water, on ErbB2/neu-driven mammary carcinogenesis.

2. Materials and methods

2.1. Generation and maintenance of BALB–neuT transgenic mice

Male BALB–*neu*T transgenic mice were regularly bred with BALB/c females (H-2d; Charles River, Calco, Italy) in the animal facilities of the University of Rome Tor Vergata. Offspring were genotyped by PCR to detect the presence of the transgene (Focaccetti et al., 2024). Mice were maintained in pathogen-free conditions and all procedures were conducted in compliance with European Union regulations and institutional guidelines, following protocols approved by the Italian Ministry of Health (authorization no. 577–2022-PR).

2.2. Chronic Cd exposure of BALB-neuT mice

Cadmium chloride ($CdCl_2$, cat. no. 202908) was purchased from Merck-Italy–Sigma Aldrich (St. Louis, MO, USA). Starting from weaning (around 3 weeks old) until approximately 30 weeks of age, two separate groups of twelve virgin females, each individually identified, were maintained with free access to drinking water, either with or without Cd (1 mg/l). The water was supplied in bottles made of polycarbonate compliant with Title 21 FDA requirements, which ensure strict extractive limitations for the material intended for food contact (Tecniplast, Buguggiate, VA, Italy). The water, whether containing Cd or not, was replaced weekly, and the amount consumed in each cage was recorded. Body weight and mammary gland condition were monitored weekly. Tumors were recorded once they reached a minimum diameter of 3 mm, and their progression was tracked until either all ten mammary glands presented with palpable mass or a single tumor exceeded 10 mm in diameter. At that point, animals were euthanized and tissues, including tumors, organs, and blood, were gathered for further analysis. No animals were removed from the study due to adverse clinical signs, nor were any lost to follow-up for reasons other than those specified. The timing of initial tumor detection was recorded (Focaccetti et al., 2024). At weekly intervals, tumor multiplicity (i.e. the number of tumors per animal) was recorded for each individual mouse, and the mean tumor multiplicity across all animals was calculated. Additionally, two more mice groups (three mice per group) were treated under the same conditions but euthanized at 11 weeks of age to obtain mammary tissue during early-stage tumor development.

2.3. Histological and immunohistochemical evaluation of mammary and lung tissues

Three mice per experimental group were used to collect tissues for histological and immunohistochemical analyses. At the time of euthanasia, mammary tissue and internal organs were collected and fixed in 4 % paraformaldehyde for 24 h. Samples were then embedded in paraffin, sectioned, and prepared for both histological and immunohistochemical evaluations. Histological assessment of mammary samples was carried out on sections stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was conducted as previously described (Focaccetti et al., 2024). Briefly, antigen retrieval was carried out on 3-μm paraffin sections using either citrate buffer (pH 6.0) or EDTA–citrate buffer (pH 7.8) for 30 min at 95 °C. The sections were then incubated with the primary antibodies for 1 h at room temperature. Antibody–antigen complexes were visualized using the Horseradish Peroxidase/3,3′-diaminobenzidine (HRP-DAB) Detection Kit (cat. nos. AFN600 and ACH500, UCS Diagnostic, Rome, Italy).

Primary antibodies used are listed in Supplementary Table 1. Following immunostaining, hematoxylin was employed to counterstain tissue sections. Microscopic evaluation and imaging were carried out using an Olympus BX53 microscope. Immunostaining results were assessed independently by two investigators in blind fashion.

To maintain uniformity across samples, the percentages of cells positive for αER, PR, and Ki-67 were calculated relative to the total number of cells observed within each field (20x magnification). For markers such as CD4, CD8, F4/80, Foxp3, and PD-1, quantification was performed by counting positive cells across five high-power fields (HPFs) at 20x magnification. CD31 expression was evaluated by counting the number of stained blood vessels in five HPFs (20x).

The expression levels of ErbB2/neu, GPR30, total Akt, phospho-Akt, and PD-L1 were analyzed semi-quantitatively using a composite scoring approach, combining both the extent of positive staining and staining intensity as previously described (Focaccetti et al., 2024).

The histological analysis of lung tissues was performed on H/E-stained sections. ErbB2/neu was evaluated by immunohistochemistry (ErbB2/neu antibody C-18; cat. no. sc-284; 1:50). Images were acquired with the M8 Microscope and Scanner PreciPoint (Nikon Europe B.V., Amstelveen, Netherlands) and metastases were counted.

2.4. Preparation of murine tumor and spleen cell suspensions for flow cytometry

Six mice per experimental group were used to collect samples for flow cytometry studies. Tumor tissues and spleens were harvested and processed into single-cell suspensions as previously described (Benvenuto et al., 2021). Tumor-infiltrating leukocytes were isolated as previously reported (Pacella et al., 2018). Spleens were processed as previously described (Focaccetti et al., 2024). For flow cytometry, 5×10^5 cells per sample were labeled with Fixable Viability Dye eFluor780 (cat. no. 65086514, eBioscience, Thermo Fisher Scientific, Waltham, MA, USA). Surface markers were stained using fluorochrome-conjugated antibodies (Supplementary Table 1). Cells were then fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (cat. no. 00–5523–00, eBioscience, Thermo Fisher Scientific, Waltham, MA, USA). Intracellular staining included Foxp3 APC (clone FJK16s, cat. no. #17–5773–82, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and IFN-γ BV421 (clone XMG1.2, Sony Biotechnology Inc., San Jose, CA, USA). Data acquisition was performed on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA), collecting 50,000 events within the lymphocyte gate. Obtained data were analyzed using CytExpert software, version 2.5 (Beckman Coulter).

2.5. Evaluation of serum estrogen levels in mice

Serum samples were collected from the two groups of twelve female mice at weaning, before the beginning of Cd treatment, and at sacrifice (30 weeks of age on average), and stored at −80°C until analysis. Serum estrogen levels were measured using the Mouse Estrogen ELISA Kit (MBS 766177; MyBioSource, San Diego, CA, USA), following the provided kit instructions. The samples collected at the two time-points from control and Cd-exposed mice ($n = 12$/group) were run in duplicate.

2.6. Statistical analysis

Tumor weights were compared between groups using a two-tailed Student's *t*-test. Longitudinal data on tumor growth, evaluated in terms of tumor multiplicity, were analyzed with a Mixed-Effects Model (REML) with repeated measures (Forrest et al., 2020). Sphericity was not assumed, and p-values were corrected using the Geisser-Greenhouse method. The statistical significance of tumor multiplicity differences between groups at each individual time point was determined using Sidak's multiple comparisons test. Survival data were analyzed by the Kaplan–Meier approach, with differences between groups assessed via the log-rank (Mantel-Cox) test. Immunohistochemistry scores were compared using the non-parametric Mann-Whitney test. Flow cytometry data, and ELISA measurements were compared using a two-tailed Student's *t*-test. All statistical calculations were carried out using GraphPad Prism software (version X; GraphPad Software, San Diego, CA, USA), with statistical significance defined as p-values less than or equal to 0.05.

3. Results

3.1. Chronic Cd exposure promotes mammary carcinogenesis mediated by ErbB2/neu in the BALB–neuT murine model

BALB–*neu*T, a murine model transgenic for the activated rat neu oncogene, was used to investigate the impact of Cd intake on the mammary carcinogenesis mediated by ErbB2/neu. Female BALB–*neu*T mice spontaneously develop mammary carcinomas with high homology to human ErbB2-positive BC (Conti et al., 2014). Given that dietary intake is a primary source of exposure (Qu and Zheng, 2024), post-weaning (3 weeks of age) female BALB–*neu*T mice (12 mice/group) were given ad libitum access to drinking water either containing or not low Cd levels (1 mg/l) (Thijssen et al., 2007). This dose was chosen as it reflects environmentally relevant exposure levels and has been shown to induce biological effects in rodents (Thijssen et al., 2007).

Cd exposure had no observable impact on water consumption. Water intake was comparable between the experimental groups: control mice consumed an average of 21.9 ± 1.2 ml of water per week, while Cd-exposed mice consumed 22.2 ± 1.4 ml of Cd-containing water per week (Fig. 1**A**). This resulted in an average Cd intake of 0.022 mg/mouse/week, equivalent to 0.13 mg/kg body weight/day (Fig. 1**B**).

Given that female BALB–*neu*T mice spontaneously develop independent tumors in all ten mammary glands, tumor growth was assessed in terms of both tumor multiplicity and overall tumor weight. Mammary glands were examined weekly to monitor tumor development, and longitudinal data on tumor multiplicity were analyzed using a Mixed-Effects Model (Forrest et al., 2020). The analysis revealed that tumor multiplicity was significantly affected by time (week, $p < 0.0001$), treatment (Cd, $p = 0.0003$), and by the time × treatment interaction ($p < 0.0001$), indicating that the temporal pattern of tumor growth differs between groups. In Cd-exposed mice, palpable tumors appeared at 15 weeks of age (after 12 weeks of Cd exposure), whereas in the control group tumors were first detected at 18 weeks (Fig. 1**C**). The number of tumors per mouse was significantly higher in Cd-exposed mice starting at 18 weeks, and, by 21 weeks of age, Cd-exposed mice showed, on average, twice as many tumors as control mice (6.3 vs 3.3, $p \leq 0.01$) (Fig. 1**C**). This trend continued, with Cd-exposed mice consistently developing more tumors than controls up to week 23. Tumor weight from age-matched mice further revealed that tumors of Cd-exposed mice were significantly heavier compared to those of control mice (Fig. 1**D**).

Tumor-free survival was 18.5 weeks in the control group but shortened to 17 weeks in mice exposed chronically to Cd ($p \leq 0.0001$). By 18 weeks of age, all Cd-exposed mice had developed at least one mammary tumor, whereas in the control group this occurred at 22 weeks (Fig. 1**C, E**). Concurrently, a significant difference in survival was observed between Cd-exposed and control mice ($p = 0.002$) (Fig. 1**F**). In fact, the average survival was 28 weeks in the control group mice, while in Cd-exposed mice it was of 26 weeks. Due to tumor formation in all mammary glands, all Cd-exposed mice were euthanized between 24 and 30 weeks of age (Fig. 1**F**).

Overall, mice chronically exposed to Cd exhibited a higher tumor burden per animal, increased tumor mass, and a corresponding reduction in both tumor-free and overall survival. These findings collectively indicated that Cd intake accelerated ErbB2/neu-driven mammary carcinogenesis in the BALB–*neu*T mice.

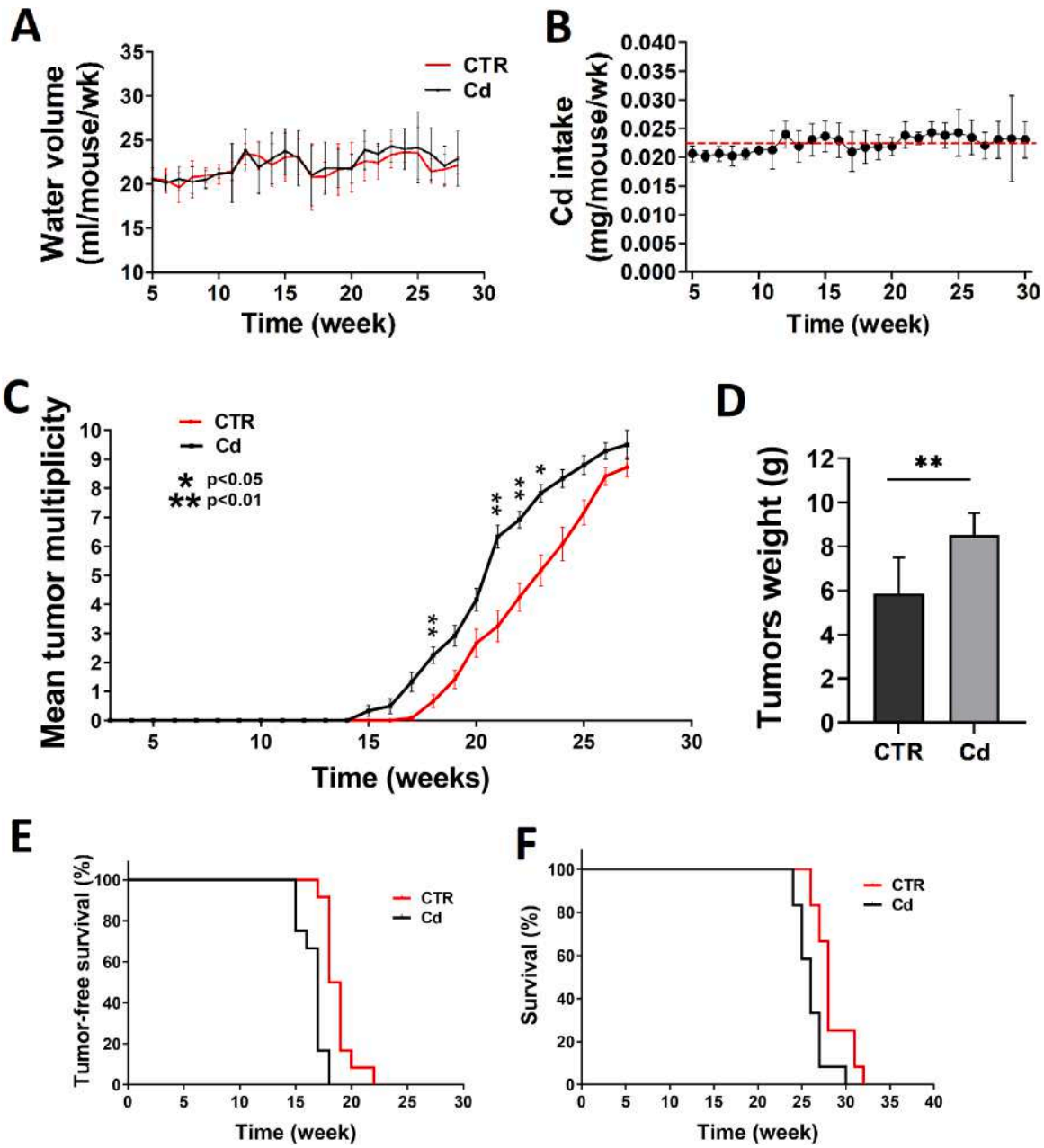

Fig. 1. Cadmium consumption and its impact on tumor growth and survival in BALB–*neu*T mice. Two groups of twelve female mice were maintained with free access to drinking water, either with or without Cd (1 mg/l). (A) Amount of water (mean ± SD) consumed by mice either untreated (control, CTR) or exposed to cadmium (Cd) during the study period. (B) Weekly average Cd intake (mean ± SD), with the red dashed line representing the mean Cd dose calculated over the entire experimental timeframe. (C) Mean tumor multiplicity (i.e. mean number of tumors per mouse, with a maximum of 10 tumors/mouse) in groups receiving water containing Cd or regular water, starting from 3 weeks of age (weaning) until sacrifice. Data are expressed as mean ± SD. Statistical significance of tumor multiplicity differences between groups at each individual time point was determined using Sidak's multiple comparisons test. (D) Total tumor mass measured per mouse (mean ± SD, 8 mice per group) at equivalent ages for both Cd-exposed and CTR groups. Data are presented as mean ± SD; **p ≤ 0.01 compared to CTR by Student's *t*-test. (E) Percentage of mice remaining tumor-free over time and (F) overall survival rates comparing Cd-exposed mice to CTR (12 mice per group).

3.2. Histopathological features and hormonal receptor profile of tumor tissues from BALB–neuT mice after chronic Cd exposure

BALB-*neu*T female mice develop, through a well-defined temporal sequence and reproducible histopathological changes, mammary carcinogenesis. In particular, preinvasive lesions, with features of atypical lobular hyperplasia and carcinoma *in situ*, are detected around the 11th week of age. These early lesions progress, around the 16th week, to invasive carcinomas, which become palpable in all mammary glands around the 30th week (Di Carlo et al., 1999; Focaccetti et al., 2024; Rovero et al., 2000). Based on this temporal sequence, mammary tissues of Cd-exposed and control mice were collected at both the preinvasive (11 weeks of age) and invasive stage of tumor development (30 weeks on average) for histological analysis and IHC.

Histological analysis of mammary tissues at the preinvasive stage confirmed the presence of atypical hyperplasia and carcinoma *in situ* in control animals, while at the same age the tissues of Cd-exposed animals showed extensive areas of neoplastic foci (Fig. 2**A**). No histological differences were instead observed in invasive stage lesions of mice chronically exposed to Cd compared with those of control mice (Fig. 2**A**).

The expression of ErbB2/neu, αER, PR and the non-canonical

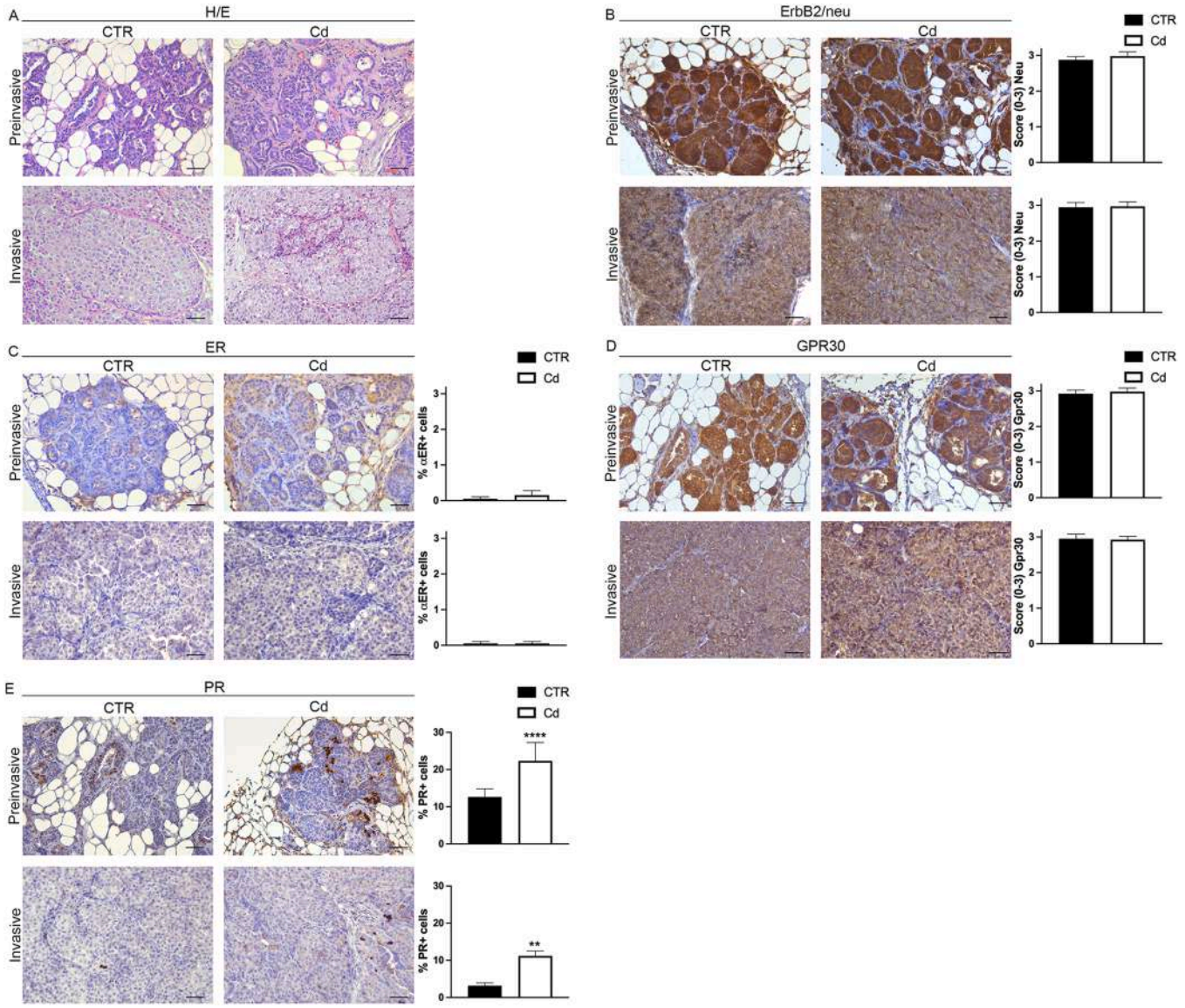

Fig. 2. Histopathological evaluation and hormonal receptor characterization of Mammary Tumor Progression in BALB-*neu*T Transgenic Mice Tumor tissues were collected from control (CTR) and Cd-exposed mice at both preinvasive and invasive stages of tumor progression (3 mice/group). (A) Hematoxylin and eosin (H/E) staining. IHC analysis was carried out for (B) ErbB2/neu, (C) αER, (D) GPR30 and (E) PR. Hematoxylin was used for nuclear counterstaining. Quantification of positive cells and IHC scoring were conducted as described in the Materials and Methods section. Data are presented as mean ± SD. Statistical significance: $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$ vs CTR, by Mann-Whitney test. Images were captured using an OLYMPUS BX53 microscope (Hachioji, Tokyo, Japan). Bar corresponds to 50 μm; original magnification 200x.

estrogen receptor GPR30 was then investigated by IHC. High expression levels of ErbB2/neu were detected in preinvasive and invasive lesions of Cd-exposed and control mice (Fig. 2**B**). At both stages of tumor development, the lesions of control and Cd-exposed mice had less than 1 % of αER positive cells (preinvasive lesions: 0.05 % ± 0.05 and 0.16 % ± 0.13, respectively, of αER positive cells; invasive lesions: 0.05 % ± 0.05 and 0.05 % ± 0.06, respectively, of αER positive cells), while all the analyzed animals showed a high expression level of the non-classical estrogen receptor GPR30 (Fig. 2**C,D**). On the other hand, significant differences in PR levels were noted between the two experimental animal groups. In particular, compared to control mice, an increased percentage of PR-positive cells was found in both preinvasive stage lesions ($p \leq 0.0001$) and invasive lesions ($p \leq 0.001$) of Cd-exposed mice (Fig. 2**E**). Worthy of note, in both groups of mice PR levels were strongly reduced during tumor progression but remained higher in tissues of Cd-exposed mice than in those of control mice.

3.3. Chronic Cd exposure induces tumor progression markers expression in BALB-neuT mice

IHC was used to study the expression of proliferation (Ki67) and the neo-angiogenesis (CD31) markers in mammary tumor tissues of Cd-exposed and control mice. Cd treatment increased the percentage of Ki67 positive cells in both preinvasive ($p \leq 0.001$ vs CTR) and invasive lesions ($p \leq 0.001$ vs CTR) (Fig. 3**A**). CD31-positive vessels were increased in tissues from Cd-exposed mice, regardless of tumor stage ($p \leq 0.0001$ vs CTR in preinvasive lesions; $p \leq 0.01$ vs CTR in invasive lesions) (Fig. 3**B**).

Akt kinase overactivation is correlated with BC progression and aggressiveness (Li X. et al., 2025). Therefore, the expression of Akt and its activated form (phospho-Akt) was also investigated by IHC. While Akt levels were similar in lesions of Cd-exposed and control mice at both the investigated time points (Fig. 3**C**), a significant elevation in phospho-Akt levels was detected in lesions from Cd-exposed mice at

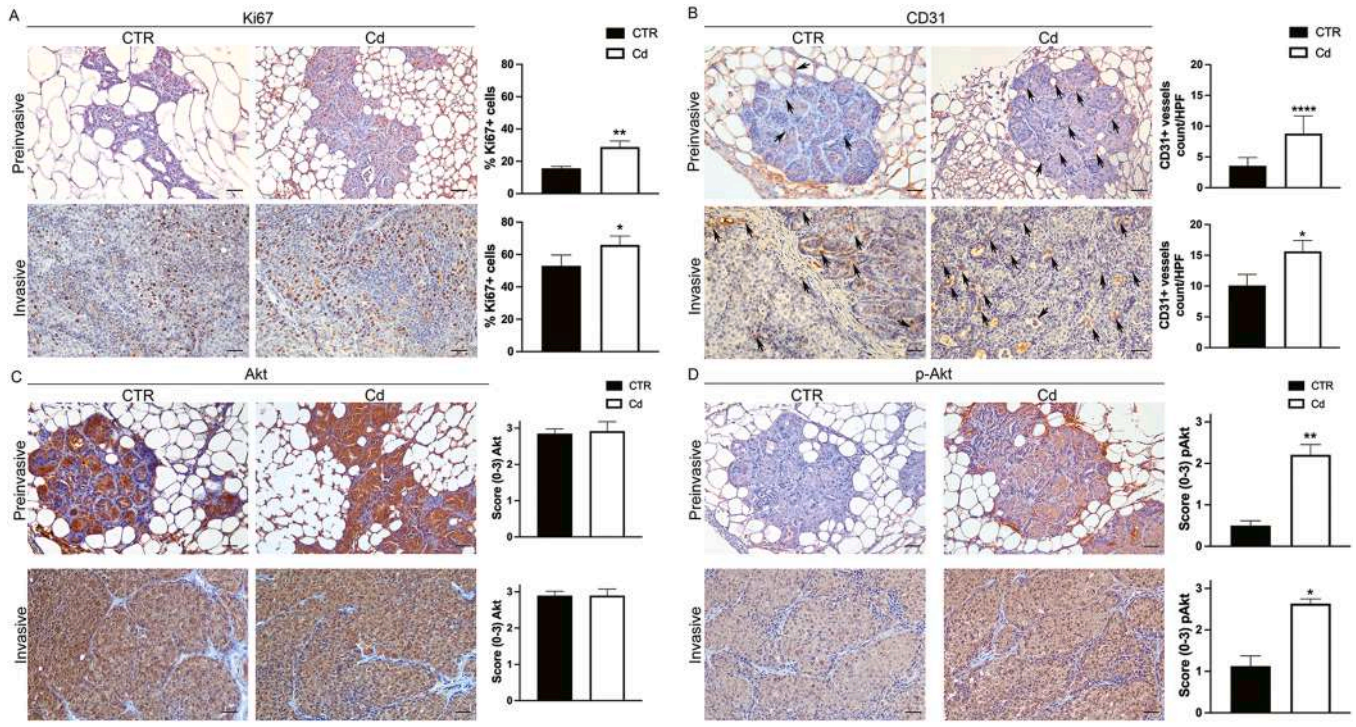

Fig. 3. Chronic Cd exposure enhances expression of tumor progression markers in mammary tumors of BALB-*neu*T mice. Tumor tissues collected at both the preinvasive and invasive stages from control (CTR) and Cd-exposed mice (3 animals per group) were analyzed by IHC for the expression of (A) Ki67 (proliferation marker), (B) CD31 (neoangiogenesis), (C) total Akt, and (D) phosphorylated Akt (p-Akt). Arrows in panel (B) indicate CD31-positive blood vessels. Hematoxylin was used for nuclear counterstaining. IHC quantification was carried out as described in the Materials and Methods section, and data are presented as the mean ± SD. Statistical significance: *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001, vs CTR, by Mann-Whitney test. Images were captured using an OLYMPUS BX53 microscope. Bar corresponds to 50 μm; original magnification 200x.

preinvasive as well as in invasive stage (p ≤ 0.001 vs CTR, at both time points) (Fig. 3**D**).

3.4. *Cd increases lung metastasis formation in BALB-neuT mice*

Lungs from Cd-exposed and control mice (3 mice/group) were collected at sacrifice (30 weeks of age), and processed for histological analysis and IHC. For histological analysis, whole lung sections were stained with H/E (Fig. 4). ErbB2/neu expression was investigated by IHC to confirm the presence and number of metastatic foci (Hüsemann et al., 2008) (Fig. 4**C,F**). The number of metastases detected in the lungs of Cd-exposed animals was increased about threefold as compared to that of the control group (p ≤ 0.01) (Fig. 4**G**).

3.5. *Effect of Cd on the tumor immune microenvironment of BALB-neuT mice*

The effect of Cd intake on the tumor immune microenvironment was first investigated by IHC. In both control and Cd-exposed mice, immune cells, rather than infiltrating the tumor, appeared mainly distributed in the peritumoral stroma (Fig. 5). The amount of cells expressing the macrophage marker F4/80 did not differ between the lesions of the two experimental groups at either stage of tumor development (Supplementary Figure 1 **A,** Fig. 5**A**). On the other hand, while no difference in $CD4^+$ and $CD8^+$ cell number was observed in preinvasive lesions of Cd-exposed as compared to control mice (Supplementary Figure 1**B,C**), in invasive tumors of Cd-exposed mice an increased number of both $CD4^+$ and $CD8^+$ T lymphocytes was recruited to the peritumoral stroma (≤ p 0.01 vs CTR) (Fig. 5**B,C**). At the same time, however, the invasive tumors of Cd-exposed animals showed an increased amount of cells expressing markers of immunosuppression and T cell exhaustion (Sato, 2025; Schütz et al., 2017), namely the regulatory T cells (Tregs) marker Foxp3 (p ≤ 0.001 vs CTR) and the immune inhibitory receptor Programmed Cell Death-1 (PD-1; p ≤ 0.01 vs CTR) (Fig. 5**D,E**). The ligand of PD-1, PD-L1 was instead uniformly expressed at high levels in invasive tumors of control mice, consistent with our previous report (Focaccetti et al., 2024), as well as in tumors of Cd-exposed mice (Fig. 5**F**).

To corroborate these IHC findings, flow cytometry was performed on immune cells extracted from invasive tumors (Fig. 6). Consistent with IHC results, a significant increase in the $CD4^+$ and $CD8^+$ T lymphocytes frequency (p ≤ 0.01 vs CTR) and of $CD4^+CD25^+Foxp3^+$ Tregs (p ≤ 0.05 vs CTR) was detected by flow cytometry in tumors of Cd-exposed mice (Fig. 6**A-C**). The proportion of B lymphocytes, identified by flow cytometry as $CD3^-B220^+$ cells was also significantly increased (p ≤ 0.00.1 vs CTR) in tumors of Cd-exposed mice (Fig. 6**D**). The increase in the amount of $CD8^+$ T and B lymphocytes recruited to the tumors of Cd-exposed animals appears remarkable, since the frequency of these immune cell subsets at the systemic level appeared instead reduced, as shown by the analysis of cells collected from the spleen. In comparison, the increase in the amount of Tregs recruited to the tumors of Cd-exposed mice was accompanied by a parallel increase of Tregs frequency in the spleen (Fig. 6).

On the other hand, as evidenced by the expression of the exhaustion marker PD-1 and the anticancer cytokine IFN-γ (Bhat et al., 2017), the functional profile of $CD4^+$ and $CD8^+$ T lymphocytes appeared to be variably affected by Cd exposure, both in the spleen and at the tumor site. More specifically, the percentage of $CD4^+$ and $CD8^+$ T lymphocytes expressing the inhibitory receptor PD-1 was significantly higher in both tumors and spleen of Cd-exposed mice as compared to control mice (Fig. 6**E,F**). At the same time, an increased expression of IFN-γ was detected in tumor-recruited and splenic $CD4^+$ T lymphocytes, while $CD8^+$ T lymphocytes in both tissue compartments showed a similar, though not statistically significant, increase (Fig. 6**G,H**).

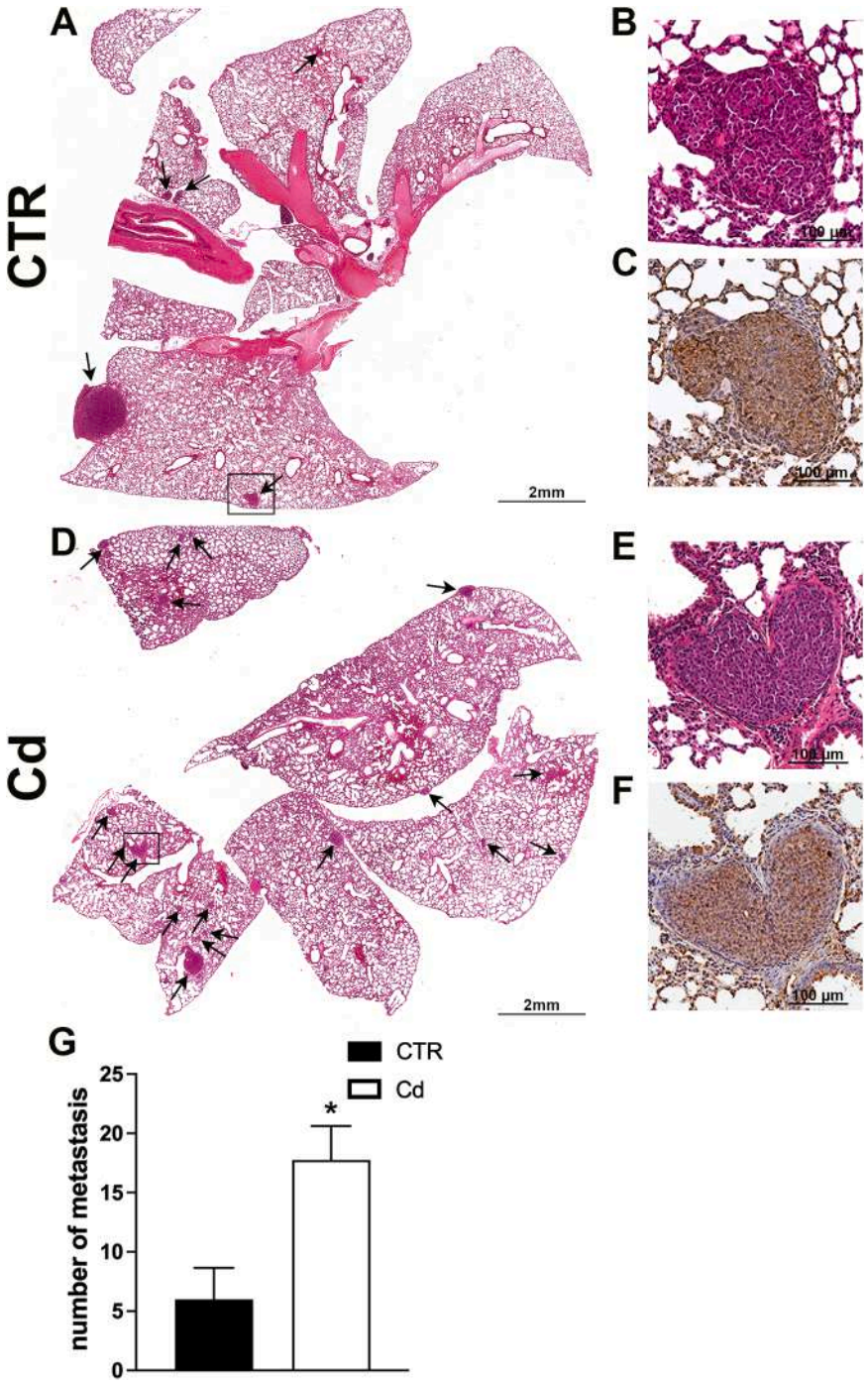

Fig. 4. Analysis of lung metastasis in Cd-exposed and control mice. (A,D) H/E staining of whole lung sections from (A) control (CTR) and (D) Cd-exposed mice (bar corresponds to 2 mm). Metastases are indicated by arrows. The rectangular inserts indicate metastases shown at higher magnification in (B) and (E) (bar corresponds to 100 μm). (C,F) immunostaining for ErbB2/neu. (G) Number of metastases in lungs of Cd-exposed and control (CTR) mice (3 mice/group). The results are expressed as the mean ± SD values of the number of metastases counted in three whole lung sections for each mouse. Statistical significance: *$p \leq 0.05$ vs CTR, by Mann-Whitney test. Images were captured with a M8 Microscope and Scanner PreciPoint (Nikon Europe B.V., Amstelveen, Netherlands).

3.6. Cd intake increases serum estrogen levels in female BALB-neuT mice

Serum estrogen levels were measured in female mice at weaning (t0), before the beginning of Cd administration, and at sacrifice (t1), i.e. at 30 weeks of age on average. While in control mice, serum estrogen levels were similar at the two investigated time points, in the Cd-exposed group estrogen levels at t1 were significantly higher than those measured at t0 ($p \leq 0.0001$) (Supplementary figure 2). Accordingly, at sacrifice Cd-exposed mice had significantly higher levels of serum estrogens as compared to control mice ($p \leq 0.05$). These findings indicate that Cd intake affects estrogen metabolism in female mice, leading to a significant increase in hormone levels over time.

4. Discussion

In this study, we provide evidence that chronic exposure to Cd, a heavy metal known for its endocrine-disrupting properties, significantly accelerates tumor development and progression in BALB–*neu*T female

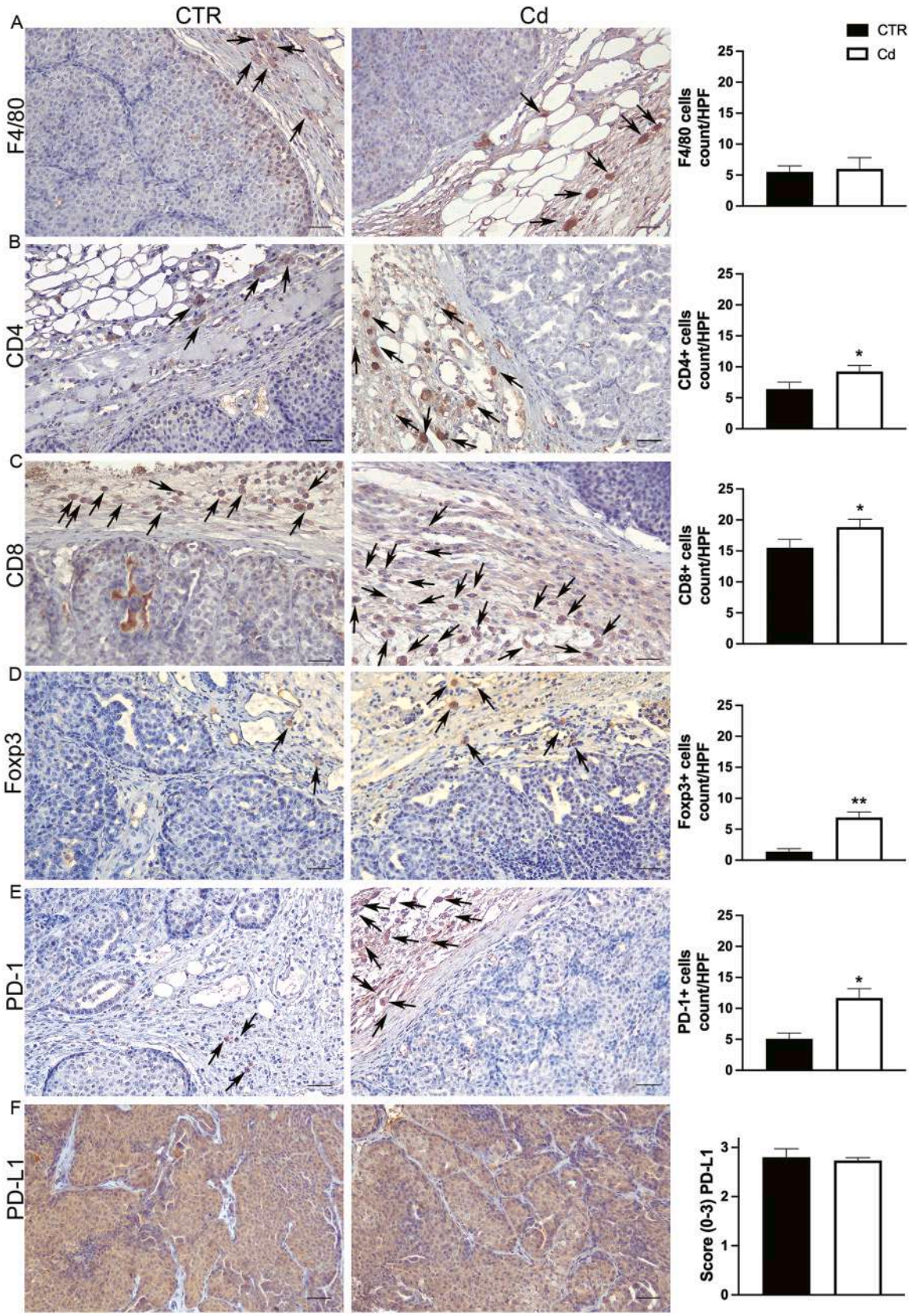

Fig. 5. Immunohistochemical detection of immune cell markers in tumors from control and cadmium-exposed mice. Tumor sections from control (CTR) and Cd-treated BALB–*neu*T mice at the invasive stage of tumor progression (3 mice per group) were subjected to IHC to assess the expression of (A) F4/80, (B) CD4, (C) CD8, (D) Foxp3, (E) PD-1, and (F) PD-L1. Positive cells are indicated by arrows. Hematoxylin was used to counterstain the tissue sections. IHC scoring was performed as described in Materials and Methods section, and results are presented as the mean ± SD. Statistical significance: *p ≤ 0.05, **p ≤ 0.01 vs. CTR, by Mann-Whitney test. Images were captured using an OLYMPUS BX53 microscope. Bar corresponds to 50 μm; original magnification 200x.

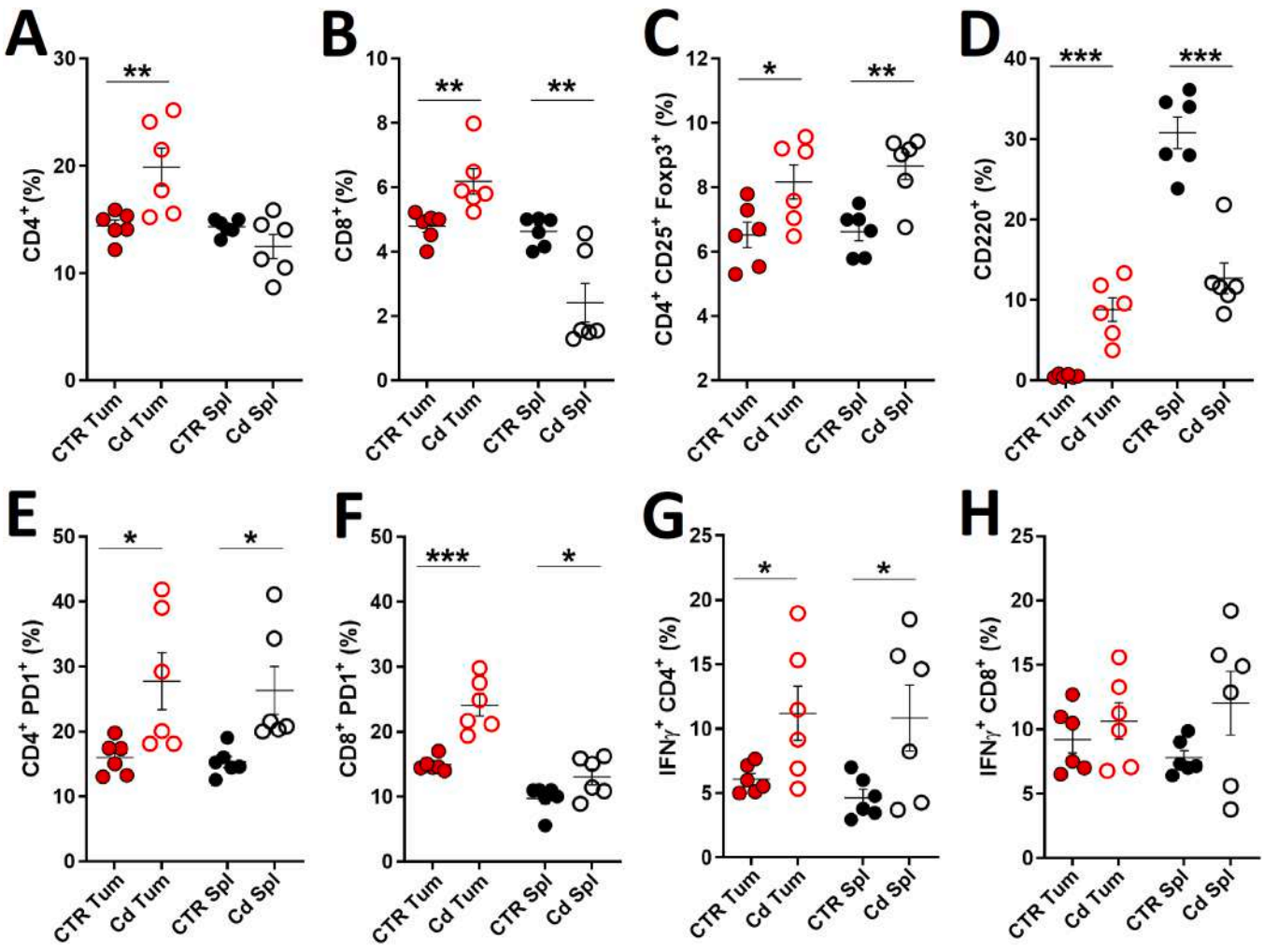

Fig. 6. Flow cytometric characterization of immune cell subsets and functional profile in tumors from invasive-stage BALB–*neu*T mice. Single-cell suspensions were prepared from spleens (Spl) and tumor tissues (Tum) of both control (CTR) and cadmium (Cd)-exposed animals (6 mice per group) and subjected to multiparametric analysis.The frequency of (A) $CD4^+$ cells, (B) $CD8^+$ cells, (C) $CD4^+CD25^+Foxp3^+$ cells (Tregs), (D) $CD220^+$ B cells, (E) $CD4^+PD\text{-}1^+$ and (F) $CD8^+PD\text{-}1^+$, (G) $CD4^+IFN\text{-}\gamma^+$ and (H) $CD8^+IFN\text{-}\gamma^+$, were quantified and presented as mean ± SD. Statistical significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, by Student's *t*-test.

mice, a model of ErbB2/neu-driven breast carcinogenesis that mimics ErbB2-positive human BC (Conti et al., 2014; Hüsemann et al., 2008). Even though the BALB–*neu*T model displays features that do not recapitulate the natural history of human breast cancer, such as the full penetrance and rapid onset of mammary tumors, it provides a sensitive platform to uncover how environmentally relevant Cd exposure may influence breast cancer development and tumor–immune interactions.

Our data demonstrate that mice drinking water containing a low dose of Cd, corresponding to environmental exposure levels reported in humans (Thijssen et al., 2007; Yue et al., 2023), exhibited an anticipated tumor onset and an increased tumor multiplicity, resulting in a shortened tumor-free and overall survival. Moreover, metastatic spread was significantly more pronounced in Cd-exposed mice, whose number of ErbB2-positive lung metastatic foci counted at the time of sacrifice was about three times that of control mice.

Similar to our results, it has been recently reported that chronic intake of water containing Cd, at a dose about four times higher than the one we used (3.6 mg/l for 23 weeks), accelerates mammary tumorigenesis in MMTV-ErbB2 mice (Yue et al., 2023). Compared with the tumors of MMTV-ErbB2 mice, a strain of transgenic mice in which mammary cancerogenesis is driven by the overexpression of wild-type ErbB2, those of BALB–*neu*T mice, which are transgenic for an activated form of ErbB2, are on the whole more aggressive and develop after a shorter latency (Focaccetti et al., 2024; Yue et al., 2023). Nonetheless, treatment with a lower dose of Cd was able to anticipate the onset of invasive mammary carcinoma and metastatization in the highly aggressive tumor model used in the present study.

Mammary tumors of BALB–*neu*T mice contained less than 1 % of αER positive cells, at both preinvasive and invasive stage of tumor development, consistent with our previous report (Focaccetti et al., 2024), and such expression level was not affected by Cd-treatment. Tumors from both groups of mice are thus classified as ER-negative, since a percentage of αER positive cells ≥ 1 % is required to define a ER-positive status (Reinert et al., 2022).

The carcinogenic activity of Cd can rely on both αER-dependent and -independent mechanisms (du Plessis et al., 2023; Psaltis et al., 2024; Qu and Zheng, 2024). Despite the absence of αER expression in BALB-*neu*T lesions from the 11th week onward, Cd may exert estrogenic effects in the early stages of tumorigenesis, when ER expression is higher before being progressively downregulated during tumor progression (Zattarin et al., 2020). Acting as a metalloestrogen, Cd can bind αER and promote receptor activation, nuclear translocation, and DNA-binding affinity (du Plessis et al., 2023; Psaltis et al., 2024). Thus, before the 11th week, these ER-dependent effects may facilitate the onset of early-stage hyperplasia. In parallel, Cd is known to trigger ER-independent signaling through GPR30, a receptor abundantly expressed in BALB–*neu*T tumors,

whose activation triggers ERK and PI3K/Akt pathways (Liu et al., 2008; Yu et al., 2010). These pathways are known to cooperate with ErbB2 signaling to promote proliferation, survival, and tumor progression (Li et al., 2025, 2025). Therefore, the earlier onset and accelerated tumor growth observed in Cd-exposed mice can be supported by both ER-dependent and ER-independent mechanisms. In addition to the estrogenic activity of Cd itself, its effect on circulating estrogen levels may also contribute to the acceleration of carcinogenesis in our model. Indeed, Cd-exposed animals had a significant increase in serum estrogen levels, both over time and in comparison to control mice, a finding that aligns with recent human data showing a positive association between blood Cd and circulating estrogens (Gerald et al., 2023). These increased estrogen levels may derive from Cd interference with steroidogenic enzymes, hormone metabolism, or endocrine feedback pathways (Qu and Zheng, 2024; Yang et al., 2025). In turn, elevated systemic estrogens can synergize with Cd in activating GPR30 signaling, thus amplifying the activation of proliferative and survival pathways.

As we previously reported (Focaccetti et al., 2024), PR is expressed in BALB–*neu*T mice mammary lesions at both the preinvasive and invasive stage, with lower levels detected in the latter case (Focaccetti et al., 2024). In the lesions of Cd-exposed mice, increased PR levels were observed at both stages, and this may have further contributed to the acceleration of carcinogenesis, as PR is increasingly regarded as an important player in BC (Focaccetti et al., 2024; Scabia et al., 2022). These changes in PR levels mirror the findings of our recent study on the effect of the EDC Bisphenol-A (BPA) on BALB–*neu*T mice (Focaccetti et al., 2024). Although PR levels are primarily regulated by αER transcriptional activity, PR expression and up-regulation in ER-negative tumors (like those of BALB–*neu*T mice) can be explained by alternative transcriptional and post-transcriptional mechanisms (Focaccetti et al., 2024; Pu et al., 2023). Intriguingly, a positive transcriptional regulator of the PR gene is cyclin D1, whose protein levels have been found to be increased in both Cd-exposed cells and mammary tumors with overexpression or activating mutations of ErbB2/neu (Lee et al., 2000; Topisirovic et al., 2002; Yang et al., 2010). This convergence suggests a mechanistic link through which Cd and ErbB2/neu could cooperate to enhance PR expression.

The levels of the tumor progression markers Ki67, CD31 and phosphorylated Akt, were markedly increased in the lesions of Cd-exposed mice compared to control mice (Li X. et al., 2025). In this regard, Cd-induced engagement of GPR30 can synergize with ErbB2-dependent pathways in inducing tumor cell proliferation and resistance to apoptosis via Akt activation as well as in promoting angiogenesis (Li X. et al., 2025; Liu et al., 2008; Yu et al., 2010).

The results of the present study further demonstrate that the tumor immune microenvironment was also significantly altered by Cd exposure, as assessed by both IHC and flow cytometry. In fact, invasive lesions of Cd-exposed mice showed an increased recruitment of $CD4^+$ and $CD8^+$ T lymphocytes to the peritumoral stroma; however, this rise was associated with a concomitant increase in the number of immunosuppressive regulatory T cells (Tregs, $Foxp3^+$) and T cells expressing the exhaustion marker PD-1 (Masuelli et al., 2021; Turley et al., 2019). These findings suggest that in this tumor model, where immune cells are restricted to the peritumoral area, Cd exposure could impact the metastatic process by facilitating the immune evasion of metastatic cells.

It is of note that although the effects of Cd on some immune cell subsets appeared specific for the TME, in many cases parallel changes were observed both in the tumor milieu and systemically. For instance, in Cd-exposed mice, compared to controls, the amount of $CD8^+$ T cells was increased in tumor tissues and decreased at the systemic level, according to the analysis of cells collected from the spleen, the latter finding being consistent with previous studies performed in different models (Mirkov et al., 2021). This increase of tumor-recruited $CD8^+$ T lymphocytes may be explained by the earlier tumor onset induced by Cd-treatment. The elevated presence of tumor-recruited Tregs and $CD4^+$ and $CD8^+$ T lymphocytes expressing the inhibitory receptor PD-1 was instead accompanied by a parallel increase of the frequency of these subsets in the spleen of Cd-exposed mice. This finding, coupled with the increase of both tumor-recruited and splenic $CD4^+$ IFN-γ^+ T lymphocytes, and possibly $CD8^+$ IFN-γ^+ T lymphocytes, observed in Cd-exposed mice, suggest that Cd may cause a persistent and systemic stimulation of T cell activation, which ultimately is known to drive T cell exhaustion (Chow et al., 2022; Zuniga and Harker, 2012). In conclusion, according to these results, the effects of Cd on the immune TME seem to mainly mirror those caused by this heavy metal at the systemic level (Mirkov et al., 2021). Interestingly, this is at variance with the results obtained treating BALB–*neu*T mice with BPA. In fact, similar to the effect of Cd, the chronic intake of BPA accelerated mammary cancerogenesis in BALB–*neu*T mice and favoured an immunosuppressive TME; still, the changes in the frequency of immune cell subsets recruited to the tumors of BPA-treated mice were specific for the tumor milieu and not paralleled by systemic changes. In particular, exposure to BPA was associated with a higher recruitment of regulatory T cells and functionally exhausted CD8+ T lymphocytes within the TME, while it had no effect on their frequency in the spleen (Focaccetti et al., 2024). Nonetheless, the results presented herein and in our previous study demonstrate that Cd and BPA exert comparable tumor-promoting effects in the BALB–*neu*T model, where these compounds act through pathways which converge on the upregulation of PR expression in tumor tissues, activation of pro-survival and angiogenic signaling, and immunosuppressive reprogramming. These findings highlight that setting safe exposure limits for these compounds should take into account their possible combined and cumulative effects, which may lower the threshold for breast cancer initiation and progression.

The translational implications of the present study are particularly relevant for women living in Cd-polluted areas or working in Cd-related industries, and warrant the implementation of enhanced health surveillance protocols to facilitate early tumor detection in these specific populations.

CRediT authorship contribution statement

Agnese Po: Writing – review & editing, Validation. **Elisabetta Ferretti:** Writing – review & editing, Conceptualization. **Eleonora Leti Maggio:** Investigation, Formal analysis. **Nicla Cristina:** Visualization, Investigation. **Silvia Migliaccio:** Writing – review & editing, Conceptualization. **Michele Milella:** Writing – review & editing, Conceptualization. **Valeria Lucarini:** Validation. **Alessandra Fabi:** Writing – review & editing, Conceptualization. **Monica Benvenuto:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Roberto Bei:** Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Francesca Servadei:** Writing – original draft, Methodology. **Laura Masuelli:** Writing – review & editing, Supervision, Resources, Conceptualization. **Manuel Scimeca:** Writing – review & editing, Methodology. **Loredana Cifaldi:** Writing – review & editing, Resources. **Martino Tony Miele:** Writing – review & editing. **Camilla Palumbo:** Writing – original draft. **Valentina Angiolini:** Validation. **Daniela Nardozi:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Chiara Focaccetti:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Zein Mersini Besharat:** Validation, Investigation. **Alessandro Mauriello:** Writing – review & editing, Methodology.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.119757.

Data availability

Data will be made available on request.

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