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ORIGINAL ARTICLE

Impact of delayed fixation and decalcification on PD-L1 expression: a comparison of two clones

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Abstract

The bone is a frequent localization for lung non-small cell cancer metastasis; decalcification is required to permit tissue section. Pre-analytical conditions can influence the detection of immunohistochemical markers. The aim of our work is to evaluate PD-L1 expression in samples with delayed fixation and in decalcified tissue with chelating agent or acid at different time. Tumor-expressing PD-L1 and placental tissue were fixed at different times or decalcified with an acid decalcifier or EDTA for different durations. For 22C3 antibody, when tissues were decalcified with DC3, there was a significant decrease in the percentage of tumor cells or placental villi stained which after 4 h ($p = 0.035$ at 4 h). When EDTA is used for 22C3 antibody, there was a slight decrease in the percentage of stained tumor cells or villi but although there was a trend ($p = 0.058$ at 20 h), this was never statistically significant. For E1L3N antibody, when tissues were decalcified either with DC3 or EDTA, there was no significant decrease for the proportion of stained tumor cells or placental villi, neither for staining intensity for the first 24 h. The proportion of placental villi and tumor stained or intensity of staining was not significantly lower for any sample after delayed fixation also at 24 h for both PD-L1 clones. Delayed fixation does not affect the proportion of stained cell and intensity with PD-L1 immunohistochemistry. Decalcification also performed with EDTA lower the proportion and intensity of stained cells with PD-L1 immunohistochemistry.

Keywords PD-L1 · Decalcification · Delayed fixation · EDTA · Acid decalcifier

Introduction

PD-L1 is an immunohistochemical marker used to determine response to checkpoint inhibitors in non-small cell lung carcinoma (NSCLC). It has been shown that a patient's response to checkpoint inhibitors such as anti-PD1/PD-L1 is better in patients with PD-L1 expression in tumor cells [1]. For NSCLC, patients with the highest tumor proportion score have the best benefits of checkpoint inhibitors therapy [1]. The greatest difference between checkpoint inhibitors plus chemotherapy versus chemotherapy with placebo was observed in patients with a tumor proportion score of 50% or greater with 22C3 antibody for pembrolizumab [1]. It has recently been shown that response to pembrolizumab improves overall survival compared with chemotherapy alone for stage IV NSCLC in the first line for patients with ≥ 50% PD-L1 expression in tumor cells [2].

Pre-analytical conditions can influence the detection of immunohistochemical markers. In breast carcinoma, delayed fixation can reduce HER2 immunohistochemical signal [3]. Furthermore, delayed fixation can decrease signal intensity for estrogen receptors and progesterone receptor [4]. In consequence, it is recommended to reduce as much as possible fixation delay for breast carcinoma.

The bone is a frequent localization for NSCLC metastasis; therefore, decalcification is required to permit tissue section. Two major decalcifiers are used in surgical

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pathology: calcium chelators such as EDTA and acid decalcifiers. EDTA has a neutral pH and is thought to better preserve proteins for immunohistochemistry and nucleic acid for DNA sequencing or FISH study than acid decalcifiers [5]. Again, in the well-studied breast carcinoma markers, decalcification influences the expression of hormone receptors and HER2 and might lead to false-negative, depending on the decalcifying agent used [5].

The use of EDTA decalcification allows molecular testing and immunohistochemistry in bone metastatic NSCLC [6]. Nevertheless, EDTA decalcification requires more time than decalcification with most acids. PD-L1 testing is mandatory for metastatic NSCLC without targetable mutation for therapies. For PD-L1, little is known about the effect of decalcification and how delayed fixation affects PD-L1 immunohistochemistry result [7]. The aim of our work is to evaluate PD-L1 expression in samples with delayed fixation and in decalcified tissue with chelating agent or acid at different times.

Material and methods

Tissue samples

Twelve samples were prospectively collected at the Department of Pathology of the University Medical Center of Saint Etienne, France. The local ethics committee approved the study (IRBN172018/CHUSTE). Tissue samples were collected from November 2017 to August 2018 from the leftover normal placenta or surgically resected tumor tissue. Placental tissue expresses PD-L1 constitutively in syncytiotrophoblast and intermediate trophoblastic cells. All experiments were performed at room temperature, without shaking or agitation. Tissue management is summarized in a flowchart (Fig. 1).

Decalcification

For decalcification, tissue samples with enough tumor material or placenta with short post-operative delay (cold ischemia time < 15 min) were cut into 6 pieces representing on cut-section 4 to 8 mm^2 then all immediately placed in 4% buffered formaldehyde. After 24 h of fixation, they were put into EDTA (MicroDec EDTA based, Diapath) or DC3 Qpath decalcifier (VWR International), an acid decalcifier containing chlorhydric acid for 4 h, 8 h, 12 h, 16 h, 20 h, and 24 h. Six different placental tissues and 2 lung cancer (two lung pleomorphic carcinoma) tissues were used.

We have also tested prolonged decalcification: tissue samples with enough tumor material or placenta with short post-operative delay (cold ischemia time < 15 min) were cut into 6 pieces representing on cut-section 4 to 8 mm^2 and then all immediately placed in formalin. After 24 to 72 h of fixation, each sample was placed either into EDTA or into DC3 for 1 day, 3 days, or 5 days. Five different placental tissues and 4 lung cancers (2 adenocarcinomas, 1 squamous cell carcinoma, and 1 pleomorphic carcinoma) were used.

After decalcification, samples were rinsed with water and placed in formalin for less than 24 h. Then, each sample was placed into a single cassette labeled with a different Indian ink derived from a previously described method [8]. One fragment of the same tumor or placenta for each patient was used as a control and was not put into a decalcifying solution; each tumor or placenta has its own control. Immunohistochemistry was performed on this tissue block.

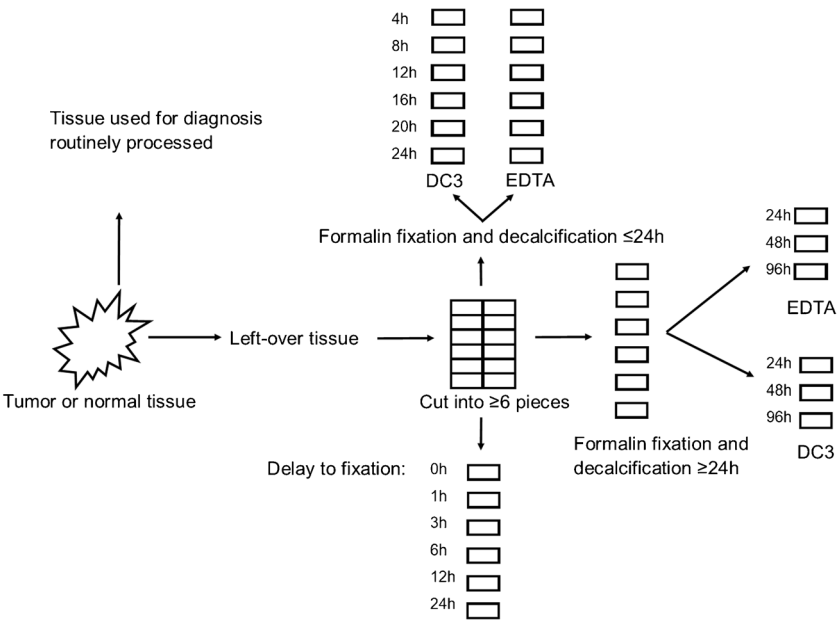


Fig. 1 Flowchart of tissue management for delayed fixation and decalcification

Delayed fixation

For delayed fixation, tissue samples with sufficient tumor material with short post-operative delay (cold ischemia time < 15 min) or placenta were cut into 6 pieces representing on cut-section 4 to 8 mm^2 then placed in formalin at different delays: immediately, at 1 h, 3 h, 6 h, 12 h, and 24 h and kept at room temperature (20–24 °C) in the dark. After cutting into 6 pieces and before fixation, tissues were stored in labeled sterile plastic tubes in humid atmosphere at room temperature in the dark, and tissues were not immerged in any liquids such as physiological serum or water. After at least 24 h to 48 h of fixation, each sample was placed into a single cassette labeled with a different Indian ink for each sample. Immunohistochemistry was performed on this tissue block. Five different placental tissues and 4 lung cancers (2 adenocarcinomas, 1 squamous cell carcinoma, and 1 pleomorphic carcinoma) were used. The fragment immediately fixed the same tumor or placenta for each patient was used as a control; each tumor or placenta has its own control.

Immunohistochemistry

Automated immunohistochemistry was performed on 4-μm sections with the Omnis platform (Agilent, CA) according to the manufacturer's instructions. PD-L1 with 22C3 clone (22C3, 1/40, Agilent, Santa Clara, CA) and E1L3N clone (E1L3N, 1/100, Santa Cruz Technology, TX) were used. 22C3 antibody and E1L3N in this work are laboratory-developed tests that were calibrated on 22C3 PharmDx test results. PD-L1 scoring was performed jointly by FF and GC. On each slide, external positive and negative controls were used (tonsil). For PD-L1 scoring, the percentage of stained tumor cells or placental villi was recorded. The intensity of staining for tumor cells and immune cells was also recorded with a semi-quantitative system: 0 (no staining), 1 (weak intensity), 2 (moderate intensity), and 3 (strong intensity).

Statistical analysis

Descriptive statistics such as mean and standard deviation for continuous variables are provided. Missing and failed data were not included for statistical analysis. Immunohistochemical results were compared by cross tables. Median percentages were compared using the Wilcoxon signed-rank test. P values < 0.05 were considered significant. Statistical analysis was performed with R software version 3.5.0 [9].

Results

Tissues

Seven placental tissues were included, and they all expressed PD-L1 on 100% of villi at an intensity of 3 on control tissue.

For tumor tissue, 12 cases were tested and excluded because PD-L1 expression was at 0% or because PD-L1 expression was heterogeneously seen in tumor tissue and could induce an allocation bias on tissue macro-array. A total of 5 tumor tissue could be included (2 adenocarcinomas, 2 pleomorphic carcinomas, 1 squamous cell carcinoma). On the control tumor tissue, PD-L1 expression of tumor tissue ranged from 30 to 100% with an intensity from 1 to 3.

Decalcification $\leq$ 24 h

Results are outlined in Fig. 2 and representative microphotographs are shown in Fig. 3.

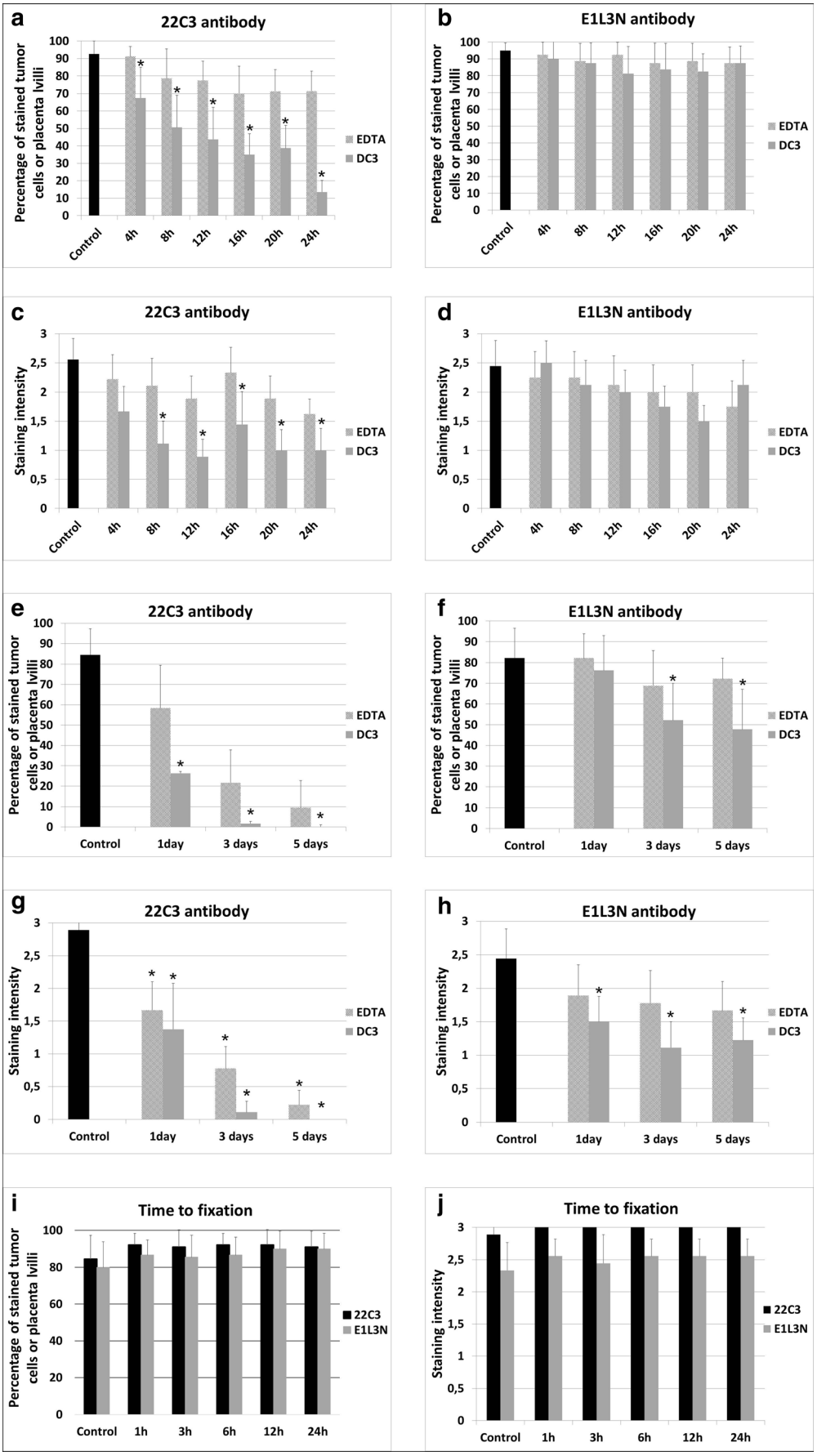
For 22C3 antibody, when tissues were decalcified with DC3, there was a significant decrease in the percentage of tumor cells or placental villi stained after 4 h ($p = 0.035$ at 4 h) (Fig. 2a, c). The mean percentage of tumor cells or placental villi stained was at 92.5%± 14.8 for the non-decalcified control tissue and was at 13.6%± 13.1 at 24 h. When EDTA is used for 22C3 antibody, there was a slight decrease in the percentage of stained tumor cells or villi overtime but although there was a trend ($p = 0.058$ at 20 h), this was never statistically significant. There was a significant decrease in staining intensity after 8 h ($p = 0.033$ at 8 h) for DC3 decalcification whereas the decrease in intensity of staining with EDTA appeared after 20 h ($p = 0.048$).

For E1L3N antibody, when tissues were decalcified either with DC3 or EDTA, there was no significant decrease for the proportion of stained tumor cells or placental villi, neither for staining intensity for the first 24 h (Fig. 2b, d).

Decalcification $\geq$ 24 h

For 22C3 antibody, with prolonged decalcification for 1 day, 3 days, and 5 days, there was a significant decrease in the percentage of stained tumor cells or placental villi with an acid decalcifier ($p = 0.014$ for 1 day). With EDTA decalcification, there was a statistically significant decrease after 48 h ($p = 0.006$). Nevertheless, the intensity of staining with both decalcifiers with 22C3 clone was significantly lower than in the control at each time (Fig. 2e, g).

For E1L3N clone and prolonged acid decalcification for 1 day, 3 days, and 5 days, there was a significant decrease in the percentage of stained tumor cells or placental villi after 3 days ($p = 0.020$) and a significant decrease in the intensity of staining at 1 day, 3 days, and 5 days ($p = 0.008$ at 1 day). With the use of EDTA, there was no decrease of staining intensity at 1 day,


a
22C3 antibody
Percentage of stained tumor cells or placenta lvilli
EDTA
DC3
Control
4h
8h
12h
16h
20h
24h
b
E1L3N antibody
c
22C3 antibody
Staining intensity
d
E1L3N antibody
e
22C3 antibody
1day
3 days
5 days
f
E1L3N antibody
g
22C3 antibody
h
E1L3N antibody
i
Time to fixation
22C3
E1L3N
1h
3h
6h
j
Time to fixation

◀ **Fig. 2** Histograms showing percentage of stained tumor cells or placental villi, staining intensity according to time, delayed fixation, type of decalcifier, and PD-L1 clone. Asterisk is for significant result compared with control (immediate fixation without decalcification). **a**, **b** and **e**, **f** Mean percentage of stained tumor cells or villi according to time of decalcification and decalcifying agent. **c**, **d** and **g**, **h** Mean percentage of intensity of stained tumor cells or villi according to time of decalcification and decalcifying agent. **i**, **j** Mean percentage of stained intensity and of stained tumor cells or villi according to delay to fixation

3 days, and 5 days; there was a trend to a decrease of intensity but at the limits of significance ($p = 0.053$) (Fig. 2f, h).

Delayed fixation

Results are outlined in Fig. 2i, j. The proportion of placental villi and tumor stained or intensity of staining was not significantly lower for any sample after delayed fixation also at 24 h for both PD-L1 clones.

Discussion

Bone metastasis is common in NSCLC at diagnosis or at disease progression and is present in one-third of cases [10]. For the diagnosis and choice of treatment options of metastatic NSCLC, molecular testing and immunohistochemistry can be performed on bone biopsy which is sometimes the only available tumor tissue [6]. PD-L1 immunohistochemistry recently gained interest because of the development of checkpoint inhibitor immunotherapy in lung cancer in patients with PD-L1-positive tumors [2]. An immune checkpoint inhibitor can be delivered in the first line or second line of metastatic NSCLC according to tumor proportion score [2].

In our study, we demonstrate that immunohistochemistry of PD-L1 is affected by decalcification with both decalcifying agents used. Both decalcifiers lower the percentage of PD-L1-positive cells for tumors and placental tissue. DC3, an acid decalcifier, affects more rapidly and profoundly PD-L1 immunohistochemistry results than EDTA, a chelating agent. Nevertheless, EDTA also lowers PD-L1 positivity. This result is similar to immunohistochemical markers and molecular techniques for breast cancer where EDTA seems to affect less these immunohistochemistry results than acid decalcifiers [5]. We have tested decalcification in a prolonged situation of several days that might not represent real-life diagnostic condition. Nevertheless, we have shown that also a few hours of EDTA decalcification decrease the percentage of PD-L1-stained structures and intensity with 22C3 antibody. Because EDTA decalcification requires more time than acid decalcification, this data might be of importance. PD-L1 expression is also decreased after decalcification in placental tissue that is known to strongly and diffusely express PD-L1. As far staining intensity is

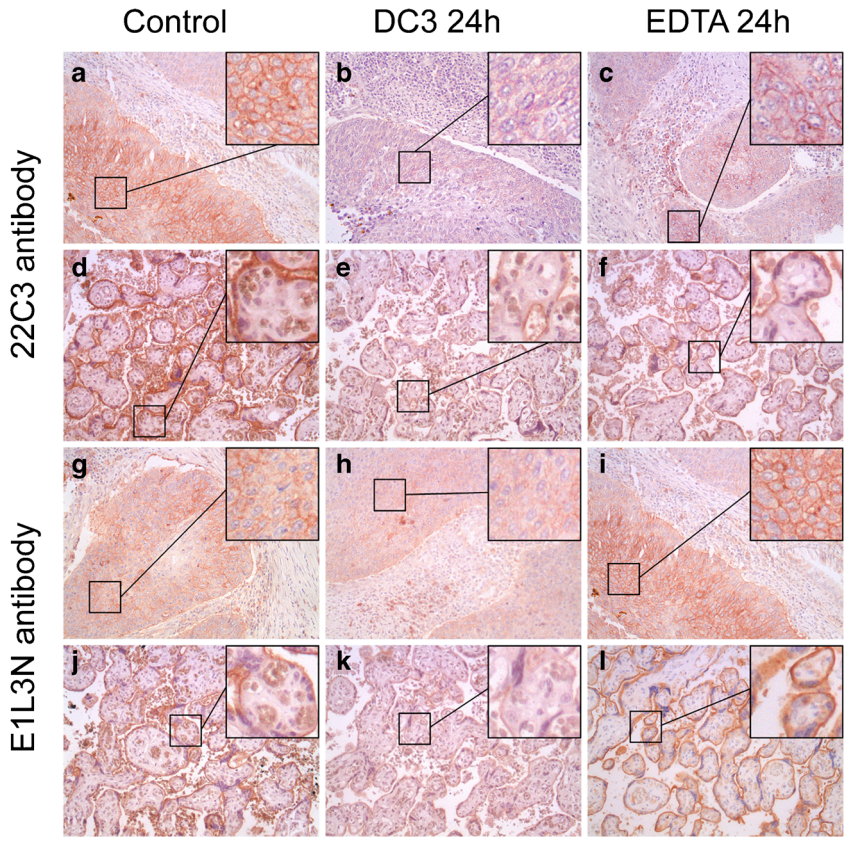


Fig. 3 Immunohistochemistry anti-PD-L1, × 200 magnification. **a**–**c** and **g**–**f** The same lung carcinoma. **d**–**f** and **j**–**l** Same placenta. After DC3 decalcification, there is a more pronounced decrease in staining intensity and percentage of stained structure with 22C3 clone than with E1L3N antibody. Right corner of each image: representative cellular detail of the staining

decreased especially with the use of DC3 and 22C3 antibody, we can raise the question of the effect of decalcification on samples with a low intensity of staining which might be "false-negative" of PD-L1 testing when decalcified.

Our work shows that the time of exposure to decalcifying agents affects PD-L1 results: the longer a tissue is exposed to a decalcifying agent, the higher the probability that PD-L1 immunohistochemistry might be affected.

Two clones were tested: 22C3 antibody and E1L3N on laboratory-developed tests that were calibrated on 22C3 PharmDx test results. We have chosen these clones because pembrolizumab 22C3 antibody was used in trials and because E1L3N has an intra-cellular binding site and has comparable results to other clones [2, 11, 12]. E1L3N seems to be less sensitive than 22C3 clone to both decalcifiers. We might hypothesize that E1L3N clone cross-react with intra-cellular protein or has an intra-cellular binding site or that is not present for 22C3 antibody and makes him more less sensitive to decalcifiers [11, 13]. The high reactivity of PD-L1 to decalcification might be related to the fact that 22C3 antibody binds only to extracellular domain of PD-L1 [14]. This extracellular domain might be more easily affected by decalcifying agent.

In contrast, PD-L1 immunohistochemistry results are not affected by delayed fixation. As far metastatic lung adenocarcinoma needs molecular testing, we do not recommend delayed fixation, but we show that PD-L1 E1L3N and 22C3 clone are robust markers that seem not to be sensitive to delayed fixation. For all cancer biopsies, delayed fixation must be avoided because it can lower DNA quality and affects other immunohistochemistry results [4, 15]. For breast cancer, several studies have shown that delay to formalin fixation has a negative effect on estrogen and progesterone receptor immunohistochemistry and on HER2 immunohistochemistry [16, 17]. PD-L1 seems a robust marker that can be performed reliably on archival material [18]. A recent study on more than 1000 patients showed that PD-L1 expression was adequately preserved following months of storage [19].

A limitation of our work is that PD-L1 does not stain every tumor and some of our tumors are negative for this marker and were not included. Nevertheless, staining of immune cells in this samples can be analyzed, but we have chosen not to include immune cell analysis as far immune cell staining does not seem to be as reproducible between pathologists as tumor proportion score [20]. Another limitation is that PD-L1 can stain heterogeneously a tumor with positive and negative areas within the same tumor, but also placental tissue with high level and homogeneous PD-L1 expression is affected by decalcification. We tried to lower the heterogeneity bias by taking large samples (4 to 6 mm^2) than biopsies used for tissue microarrays.

To our knowledge, this is the first study to describe the influence of different pre-analytical conditions such as delayed fixation and the use of two different decalcifiers on PD-L1 immunohistochemistry. For PD-L1, only the use of methanol-based fixative on cytology specimen has been shown to be less reliable than formalin fixation [21]. Our work might be relevant for clinical results: it could affect the treatment decision with patients changing of categories (0%, 1–50%, ≥ 50% of stained tumor cells).

In conclusion, delayed fixation does not affect the proportion of stained cell and intensity with PD-L1 immunohistochemistry with both clones. Decalcification also performed with EDTA lowers the proportion and intensity of stained cells with PD-L1 immunohistochemistry. Decalcification lowers more rapidly 22C3 clone intensity and proportion of stained structures than E1L3N clone. E1L3N clone seems more robust on decalcified tissue. EDTA should be preferred to acid decalcifiers when needed in case of PD-L1 testing. Whatever the decalcifying agent, decalcification should be shortened as much as possible when PD-L1 testing could be used. Several decalcifying agents exist with different protocols between laboratories so as many commercially available PD-L1 clones, so each laboratory should validate its PD-L1 test with its own decalcifying agent. Pathologists and physicians should be aware of our results when interpreting PD-L1 immunohistochemistry results on bone metastasis. A real-life study on decalcified bone tissue with matched primitive tumor sampled at the same time could bring further responses and a real-life counterpart to our work. A response to this question could be given by the nationwide prospective study on PD-L1 rate on different samples from different localizations in France.

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Authors' contributions FF and GC wrote the manuscript. FF and GC designed the study. FF, GC, DL, VDC, PDC, MM, AC, VY, and MP edited and reviewed the manuscript. FF and GC analyzed the data. FF, GC, DL, VDC, PDC, MM, AC, VY, and MP collected the data. All authors gave final approval for publication. FF takes full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

Compliance with ethical standards Local ethics committee approved the study (IRBN172018/CHUSTE). This study was performed according to the standards of French law.

Conflict of interest The authors declare that they have no conflict of interest.

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