In situ hybridization for the assessment of urokinase plasminogen activator and plasminogen activator inhibitor type-1 in formalin-fixed paraffin-embedded breast cancer specimens

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Abstract. Urokinase plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), have been reported as prognostic and predictive biomarkers in breast cancer, particularly in patients with node-negative tumors. uPA and PAI-1 expression levels classify patients into a poor-prognosis subgroup, requiring adjuvant chemotherapy and a favorable-prognosis subgroup, which can be considered for de-escalation. However, the clinical use of these two biomarkers remains limited, since fresh-frozen/fresh tumor samples are currently required for their quantification. The aim of the present study was to compare *PLAU* and *SERPINE1* mRNA expression levels (corresponding to uPA and PAI-1 proteins, respectively), assessed using *in situ* hybridization in 83 formalin-fixed paraffin-embedded (FFPE) breast tumor samples, with uPA and PAI-1 protein expression assessed using immunometric assay with paired fresh-frozen breast cancer samples. The results from the two methods significantly correlated as regards uPA quantification; however, >30% of the samples were discordant, according to the clinically validated threshold. Concordance between the two analytical methods was less prominent for PAI-1 protein and SERPINE1 mRNA. Taken together, the results of the present study indicate that although *PLAU* and *SERPINE1* mRNA may be reliably detected in FFPE samples using *in situ* hybridization, this technology cannot be used as a substitute for the replacement of the immunometric assay-derived quantification on fresh-frozen samples.

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Introduction

Urokinase-type plasminogen activator (uPA) and its main inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are proteolytic factors, which have been reported to be involved in extracellular matrix degradation and cell migration (1). These factors have been characterized by the American Society of Clinical Oncology (ASCO) and the French National Cancer Institute (INCa) as biomarkers for therapeutic de-escalation (chemotherapy withdrawal) in localized estrogen receptor (ER)-positive/human epidermal growth factor receptor (HER2)-negative ($ER^+/HER2^-$) breast cancer (highest level of evidence: A1) (2-4). This may indicate that they can be considered as more direct and affordable alternatives to multigenic signatures such as the Oncotype DX Recurrence Score (5), EndoPredict (EP/EPclin) (6) and Prosigna® Risk Of Recurrence score (7). uPA and PAI-1 expression levels improve the management of $ER^+/HER2^-$ early-stage breast cancer when used in conjunction with other clinicopathological data. Despite a high level of evidence, their use remains limited as they can be reliably quantified only using ELISA, by using a cytosolic preparation obtained from a freshly collected, rapidly frozen and pathologically controlled tumor sample of at least 125 mm^3 in size (~100 mg). In clinical routine practice, tumors are formalin-fixed and paraffin-embedded (FFPE) for diagnostic purposes and for investigating classical prognostic and predictive biomarkers (hormone receptors, HER2, cell proliferation) (8). Tumor sample freezing is limited to expert centers with specific facilities. Moreover, small tumors (<1 cm in diameter) are generally excluded from this analysis, since the whole sample is then required for FFPE fixation, in order to be adequate for anatomopathological analysis. As a result, the use of uPA/PAI-1 testing by ELISA remains exclusively in few expert centers, in which only tumors that are of adequate

size are used, in order to permit FFPE fixation and fresh-frozen tissue storage. Two prospective studies have underlined the limited feasibility of uPA/PAI-1 testing in routine practice (2,9) by reporting that only 48-57% of breast tumor samples could be assessed with validated ELISA. A method compatible with FFPE specimens could facilitate uPA/PAI-1 testing in all breast cancers (even small tumors), thus contributing to the selection of the most appropriate treatment for each patient. Several studies have tried to use immunohistochemistry as an alternative method to ELISA for the quantification of uPA and PAI-1 (10-13). However, although immunohistochemistry may be inexpensive and universally implemented in pathology laboratories, it has not been validated for uPA and PAI-1, as discrepant results have been reported. These conflicting results are probably related to the absence of consensus regarding the cellular compartment designated for analysis (stromal vs. epithelial cells) and the lack of validated antibodies, signal quantification methods and discriminating thresholds (14).

Compelling evidence has suggested that mRNA expression may be considered as an alternative for protein expression. Actually, the majority of studies in which uPA/PAI-1 mRNA and protein expression levels were compared have revealed satisfying concordance (15-18). However, until recently, mRNA quantification was mainly performed in frozen samples and therefore, presented with the same drawbacks as ELISA. Transcriptomic investigations based on FFPE samples have been limited due to the fact that sample handling for FFPE has been reported to damage RNA (19).

In the present study, RNAscope®, an *in situ* hybridization (ISH) technology was used, allowing the assessment of gene expression in FFPE tissue sections using a series of double-Z target probes and branched DNA (20) for the quantification of *PLAU* and *SERPINE1* gene mRNA expression levels (corresponding to uPA and PAI-1 proteins, respectively) in FFPE breast cancer samples. The objective was to determine the concordance between uPA/PAI-1 protein quantification in fresh tumor samples by using the ELISA reference test, and *PLAU*/*SERPINE1* mRNA levels in paired FFPE breast tumor samples by applying RNAscope® technology. It was hypothesized that in case the protein and mRNA levels were concordant, mRNA testing in FFPE breast tumor samples could be easily integrated in the clinical routine, in order to improve breast cancer management, particularly for patients with early $ER^+/HER2^-$ breast cancer.

Patients and methods

Patients and tumor samples. For the present study, breast tumor samples in which uPA and PAI-1 levels had been quantified using the FEMTELLE® uPA/PAI-1 ELISA kit (cat. no. 11-899; Cryopep) for routine diagnosis (15,21), were retrieved from a retrospective monocentric tumor biobank, which included samples and data on 520 patients with hormone-sensitive HER2-negative breast cancer, admitted to the Montpellier Cancer Institute between 2006 and 2011. According to the determined optimal sample size (as described below in the *Statistical analysis* paragraph), 83 patients were randomly selected to represent the dynamic ranges of uPA and PAI-1 concentrations (from 0.3 to 15 ng/mg and from 3.7 to 104.3 ng/mg proteins for uPA and PAI-1, respectively) and to obtain four equilibrated groups of patients (low/high uPA and low/high PAI-1), by using the established cut-off values of 3 and 14 ng/mg total protein for uPA and PAI-1, respectively (22,23) (Table SI).

The corresponding FFPE tissue blocks were identified, de-archived by the Clinical Resources Center of the Montpellier Cancer Institute (CRB-ICM, declaration number BB-033-00059) and used for the present study. The main clinical and pathological characteristics of the patients are presented in Table I.

The Institutional Review Board of the Montpellier Cancer Institute provided the ethical approval for the use of patient samples for the present study (Reference no. ICM-CORT2019-20). According to the French national ethics and legal provisions, all patients were informed prior to surgery and provided consent for the use their surgical specimens and the associated clinicopathological data for the present study.

ISH. ISH analyses were performed using 5-μm-thick sections from FFPE breast tumor tissue blocks and the RNAscope® 2.5 HD detection kit (cat. no. 322350; Advanced Cell Diagnostics, Inc.; Bio-Techne) according to the manufacturer's instructions. Briefly, following an initial drying step at 60˚C for 1 h, slides were deparaffinized in xylene (2x10 min at room temperature) and absolute ethanol (2x2 min at room temperature). To grant target RNA site accessibility, the slides were dipped in boiling Target Retrieval Solution (cat. no. 322000; Advanced Cell Diagnostics, Inc.; Bio-Techne) for 15 min, dried, dehydrated in absolute ethanol, and then incubated with protease solution (cat. no. 322331, RNAscope® Protease Plus; Advanced Cell Diagnostics, Inc.; Bio-Techne) at 40˚C for 30 min. Hybridization with the specific mRNA probes was performed in a humid chamber, inserted in a hybridization oven at 40˚C for 2 h. Signal amplification, based on branched DNA, was performed according to the manufacturer's protocol (30, 15, 30 and 15 min at 40˚C for Amp1, Amp2, Amp3 and Amp4 steps, respectively, followed by 2 steps of 30 and 15 min at room temperature for Amp5 and Amp6, respectively), and signals were detected using the Fast Red solution (cat. no. 322360; Advanced Cell Diagnostics, Inc.; Bio-Techne). The slides were then counterstained for 2 min at room temperature with hematoxylin (cat. no. K8008; Dako; Agilent Technologies, Inc.), dehydrated and mounted with permanent mounting medium (EcoMount; Biocare Medical LLC).

The double-Z probes that target human *PLAU* (uPA) and *SERPINE1* (PAI-1) were custom-designed by the supplier (cat. nos. 575931 and 569281, respectively; Advanced Cell Diagnostics, Inc.; Bio-Techne). A probe targeting the human *PolR2A* housekeeping gene was used as positive control (cat. no. 310451; Advanced Cell Diagnostics, Inc.; Bio-Techne). Experiments were done in batches and the three probes were used in parallel on three consecutive sections from the same block. A probe targeting the bacterial gene *dapB* was used as a negative control.

Following ISH, the sections were digitalized with a NanoZoomer slide scanner system (Hamamatsu Photonics K.K.) with a x40 objective. For each patient, five non-overlapping regions of interest (ROI) of 0.5 mm^2 in size were selected within the tumor (IT) and at its periphery (PT), using NDP.

Table I. Clinicopathological characteristics of the study population (n=83).

Characteristics	No. of patients	%
Age (years); range, 36-69		
≤54	41	49.4
>54	42	50.6
Tumor size		
pT1	66	79.5
pT2	17	20.5
Lymph node status		
pN0	83	100
pN1	0	0
Histological grade[a]		
SBR I	15	18.1
SBR II	58	69.9
SBR III	10	12.0
Radiotherapy		
No	5	6.0
Yes	78	94.0
Adjuvant chemotherapy		
No	52	62.7
Yes	31	37.3

[a]SBR, Scarff-Bloom-Richardson histological grade (33).

view2 software (Hamamatsu Photonics K.K.). The same ROIs were selected in the three consecutive sections hybridized with the probes targeting *PLAU*, *SERPINE1* and *PolR2A*, respectively (Fig. 1).

Image analysis. ROIs were exported at the highest available resolution using ImageJ 1.53c software (24) (National Institutes of Health) macro-toolset NDPI Export Regions Tool (https://github.com/MontpellierRessourcesImagerie/imagej_macros_and_scripts/wiki/ndpi_export_regions_tool). In the exported images, the ISH signal density was measured using the MRI Fibrosis Tool (https://github.com/MontpellierRessourcesImagerie/imagej_macros_and_scripts/wiki/mri_fibrosis_tool), which applies color-deconvolution to segment the signal. The vectors for the color-deconvolution were calculated from manually drawn ROIs using the ImageJ-plugin 'Colour Deconvolution' (25). The quantification was performed twice, using different vectors each time. For one vector and a specific probe, the values obtained for the five IT areas, the five PT areas, and the 10 areas (5 IT + 5 PT) were added and expressed as a percentage of the stained area/analyzed area. Data are expressed as absolute levels (% of *PLAU*- or *SERPINE1*-stained area/studied area) or as relative levels to *PolR2A* mRNA expression (% of *PLAU*- or *SERPINE1*-stained area/% of *PolR2A*-stained area).

Statistical analysis. The optimal sample size was calculated to be adequate for the detection of statistical significance. According to the primary objective, 83 samples were required (sample size estimated with the 'Large sample normal approximation' section of the nQuery software; Statsols), in order to detect any expected concordance of 95% between methods with a two-sided 95% confidence interval (CI) range from 0.903 to 0.997.

Categorical variables were described as the number of observations (N) and frequency (%) of each modality. Continuous variables are presented as the median, minimum and maximum values, and compared using the Wilcoxon rank sum test. Correlations between measurements were evaluated using Spearman's correlation coefficient. The RNAscope® test performance was assessed using Receiver Operating Characteristic (ROC) curve analysis. The ROC curves, defined as the plot of the true positive rate (=sensitivity) vs. false positive rate (=1-specificity) for all possible thresholds, are represented. The area under the ROC curve (AUC) and its 95% CI were calculated (non-parametric estimator). The AUC is the most commonly used index to provide an estimate of the predictive accuracy. Its value can vary from 0.5 (if the marker is not informative), to 1 (if the marker is perfectly discriminating). An optimal threshold was determined by maximizing the Youden index, defined by J=sensitivity + specificity-1. Sensitivity, specificity, positive predictive value and negative predictive value were also calculated. Statistical analyses were performed using STATA 16.0 software (StatCorp LP).

Results

RNAscope® technology implementation in routine clinical practice. The RNAscope® technology was used on 83 breast tumor samples that had been surgically removed and were FFPE-fixed 8 to 12 years prior to the ISH analysis. First, the pre-treatment steps were optimized (15 min of target retrieval and 30 min of permeabilization with protease) using a single sample and control probes, to obtain a high positive control signal with the *PolR2A* probe, no background with the *dapB* probe, and a tissue morphology compatible with morphological analysis. The same conditions were then applied along with the *PLAU* (uPA), *SERPINE1* (PAI-1), and *PolR2A* probes, in order to assess all selected breast tumor samples (n=83; n=249 slides). Overall, at the end of the ISH protocol, tissue detachment was observed in 11 slides. This issue was resolved by analyzing the breast tumor samples for a second time by increasing the slide drying time. The *PolR2A* probe yielded a punctuate signal, of variable intensity, in all 83 samples analyzed, permitting the validation of the mRNA quality in all samples. No signal was detected using the *PLAU* probe, in one sample only. As the *SERPINE1* probe yielded a good signal in the same sample, the absence of the *PLAU* signal suggested a technical issue, or an expression level below the test sensitivity threshold. Since the re-analysis of the same sample confirmed the absence of the *PLAU* signal, this sample was excluded from the statistical analyses. Taken together, the data suggested that the RNAscope® technology may be a robust and reliable method for ISH of FFPE specimens in clinical settings.

Since *in situ* techniques allow spatial and morphological cell characterization, it was revealed that *PLAU* and *SERPINE1* were mainly expressed in the stroma (Fig. 2A and B), whereas *PolR2A* was present both in tumor and stromal cells, as was expected (Fig. 2C).

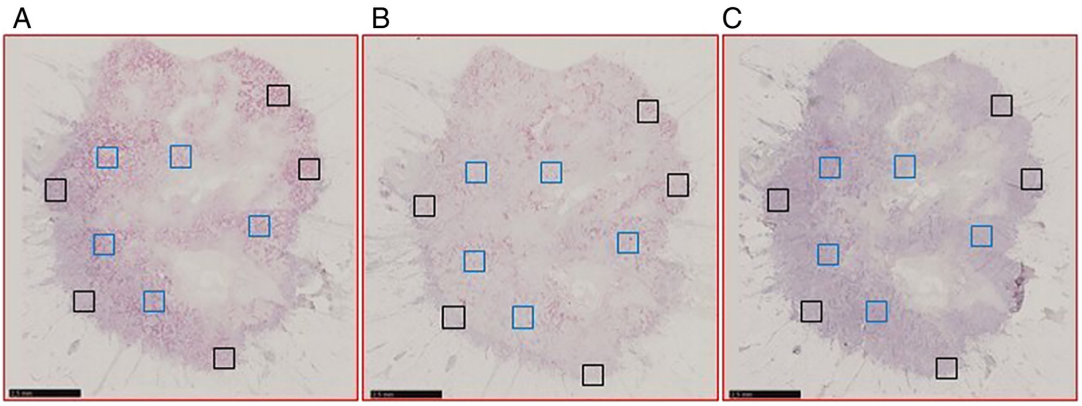

Figure 1. Regions of interest in three consecutive formalin-fixed paraffin-embedded tissue sections of a breast carcinoma sample hybridized with probes targeting (A) *PLAU* (uPA), (B) *SERPINE1* (PAI-1) and (C) *PolR2A*. Blue boxes indicate intra-tumor areas, and black boxes peripheral zones. Scale bar, 2.5 mm. uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; *PLAU*, uPA gene; *SERPINE1*, PAI-1 gene; *PolR2A*, RNA polymerase II subunit A gene.

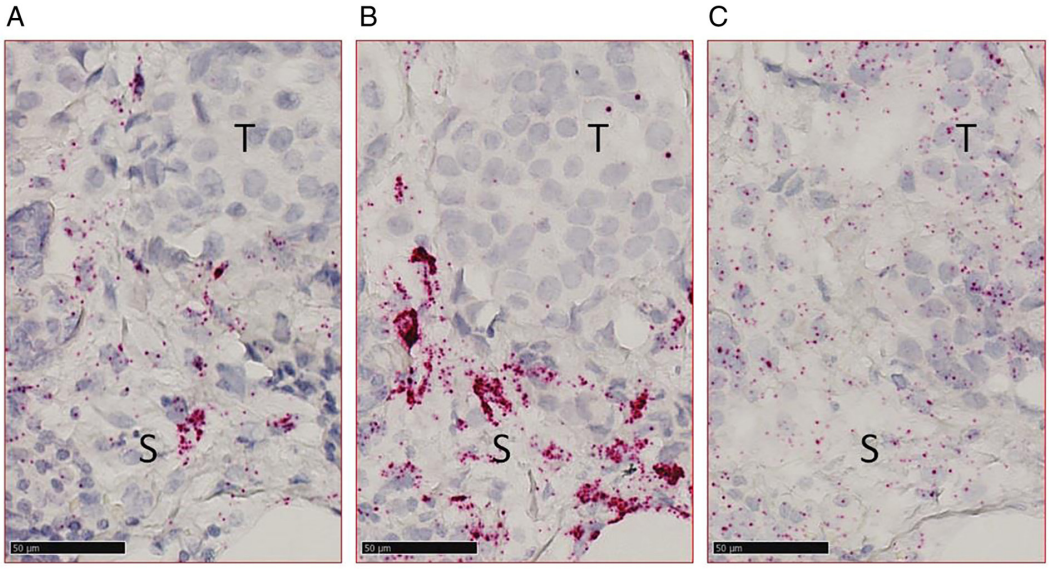

Figure 2. Detection of (A) *PLAU* (uPA), (B) *SERPINE1* (PAI-1), and (C) *PolR2A* in a formalin-fixed paraffin-embedded breast carcinoma sample using *in situ* hybridization. Scale bar, 50 μm. T, tumor area; S, stromal area; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; *PLAU*, uPA gene; *SERPINE1*, PAI-1 gene; *PolR2A*, RNA polymerase II subunit A gene.

An ImageJ tool was then developed by one of the authors (VB) to quantify the ISH signals in the five ROIs delineated within the tumor (IT) and at its periphery (PT), and also in both regions (IT + PT). To validate the quantification, the analysis was performed twice, using independent vectors (vectors A and B) and revealing a strong correlation (ρ=0.893 to 0.901, in function of the mRNA and area; Table II). In view of high correlation levels, the results of all analyses were subsequently presented only for vector B.

Strong correlations were also revealed between the IT and PT signals (ρ=0.834 and 0.875 for *PLAU* and *SERPINE1*, respectively; data not shown). Therefore, the expression values obtained in the IT and PT zones were combined, in order to obtain a single expression value for each target (IT + PT).

Evaluation of PLAU and SERPINE1 mRNA expression levels in FFPE breast tumor samples. It was hypothesized that in FFPE samples analyzed using the RNAscope® technology, each dot represented a single RNA molecule, provided that the RNA target is intact and properly unmasked to allow probe access. Unlike *PolR2A,* which demonstrated a punctuate expression pattern (Fig. 2C), the signals corresponding to the *PLAU* and *SERPINE1* probes were clustered (Fig. 2A and B), precluding their individual quantification. Therefore, by using an in-house ImageJ tool, the percentage of the stained area relative to the surface analyzed was quantified, for each target and each sample. In both the IT and PT areas, *PLAU* and *SERPINE1* mRNA expression levels were determined between 1 and 7% of the analyzed area, with only few specimens outside this range (Fig. 3A). Similar expression levels were also observed for the housekeeping gene, *PolR2A*. As no significant correlations were observed between the *PLAU*/*SERPINE1* and *PolR2A* expression levels, it was assumed that the variations observed for *PLAU* and *SERPINE* mRNA were not related to mRNA degradation (data not shown).

Table II. Correlations (Spearman's coefficients) between independent analyses to quantify target gene intensity (using ImageJ software).

Biomarker	IT (Vector B)	PT (Vector B)	IT + PT (Vector B)
uPA			
IT (Vector A)	0.893	-	-
PT (Vector A)	-	0.897	-
IT + PT (Vector A)	-	-	0.908
PAI-1			
IT (Vector A)	0.895	-	-
PT (Vector A)	-	0.893	-
IT + PT (Vector A)	-	-	0.901

uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; IT, intra-tumor area; PT, peripheral zone.

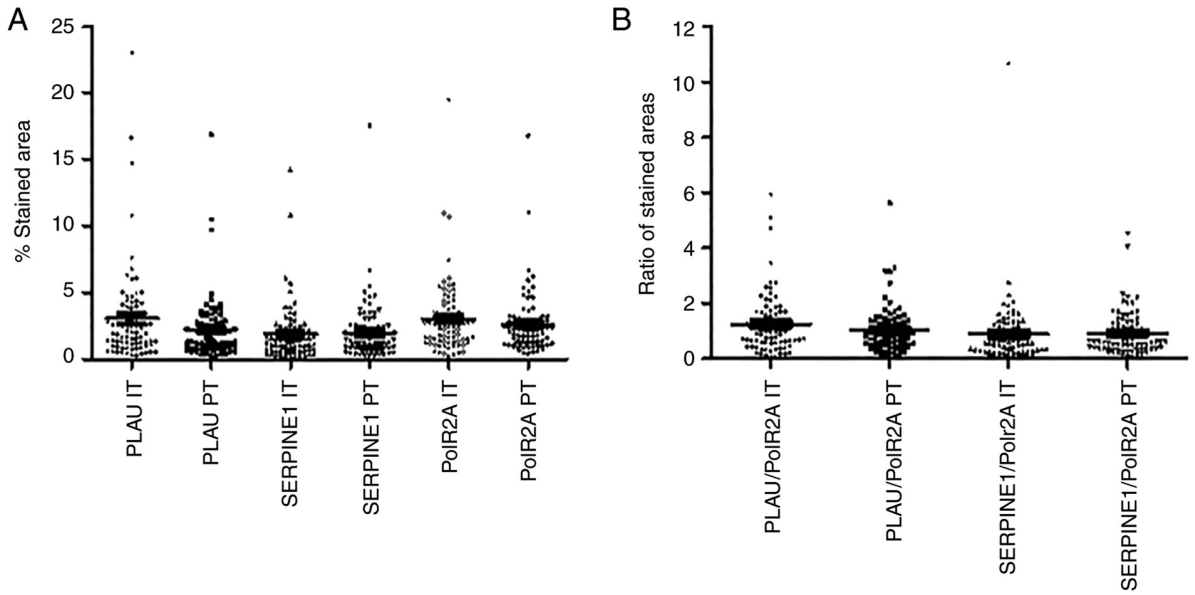

Figure 3. (A) Absolute and (B) relative (normalized to *PolR2A*) *PLAU* (uPA) and *SERPINE1* (PAI-1) gene expression analyzed using *in situ* hybridization in 83 formalin-fixed paraffin-embedded breast cancer samples. Gene expression was calculated as the surface of the stained area x100/surface of assessed area. IT, intra-tumor area; PT, peripheral zone; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; *PLAU*, uPA gene; *SERPINE1*, PAI-1 gene; *PolR2A*, RNA polymerase II subunit A gene.

PolR2A is commonly used as a reference gene for accurate gene expression normalization, since its mRNA expression is considered to be stable among samples (26,27). Therefore, it was hypothesized that the expression variations observed amongst the samples were mostly related to variations in RNA integrity. Considering RNA degradation in FFPE samples, *PLAU* and *SERPINE1* expression was normalized by using the *PolR2A* expression level for the same sample. Following this normalization, the *PLAU* expression level ranged between 0.06 and 5.95 in the IT areas, and between 0.05 and 5.61 in the PT areas (Fig. 3B). The normalized *SERPINE1* expression levels ranged between 0.01 and 10.67 in the IT areas, and 0.06 and 4.53 in the PT zones (Fig. 3B).

Comparison of the results obtained from RNAscope® and the ELISA reference test. Breast cancer samples of 83 patients were selected based on their u-PA and PAI-1 expression levels (obtained using the ELISA reference test) for the present study, and were classified into four balanced populations according to the established clinical thresholds (3 and 14 ng/mg for uPA and PAI-1, respectively; Table SI).

The normalized *PLAU* expression level (RNAscope®) was significantly higher in all analyzed areas in uPA-positive samples as compared to the uPA-negative samples (using ELISA), (according to ELISA) (P<0.001, P=0.002 and P<0.001 for the IT, PT and IT + PT areas, respectively; Fig. 4A). Similar results were also observed, concerning the normalized *SERPINE1* expression levels, although the differences were less significant between the PAI-1-negative and -positive specimens (P=0.032, P=0.039 and P=0.035 for the IT, PT and IT + PT areas, respectively; Fig. 4B). Moderate, yet significant correlations were also found between the normalized *PLAU* and *SERPINE1* expression levels (ISH) and the uPA and PAI-1 concentrations (ELISA) using continuous variables (Fig. S1).

Table III. Sensitivity, specificity, agreement rate, positive and negative predictive values of uPA expression analysis using *in situ* hybridization.

Area/zone analyzed	Optimal threshold using the Youden index	Agreement rate	Sensitivity	Specificity	Positive predictive value	Negative predictive value
IT	0.6537	0.68	0.88	0.49	0.63	0.80
PT	0.9142	0.73	0.68	0.78	0.76	0.71
IT + PT	0.6088	0.68	0.90	0.46	0.63	0.83

uPA, urokinase plasminogen activator; IT, intra-tumor area; PT, peripheral zone.

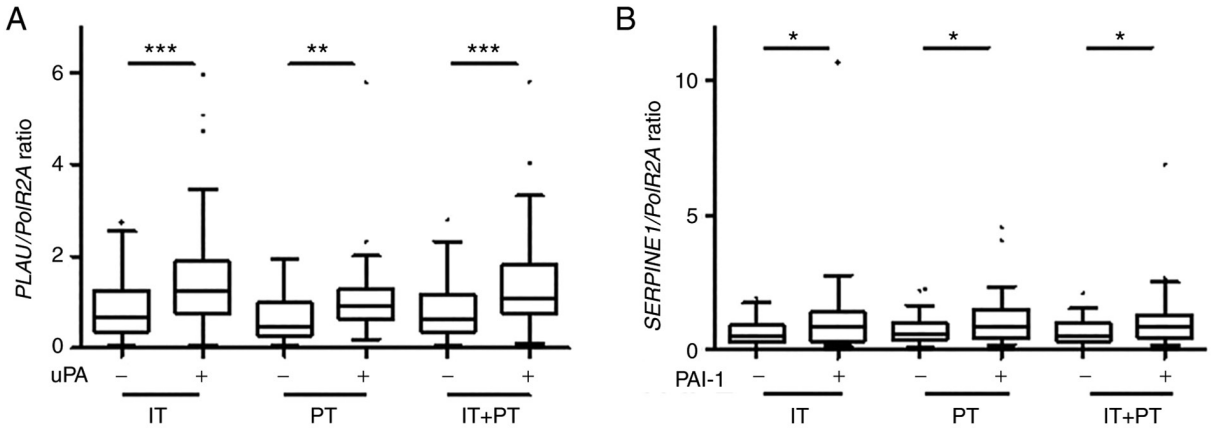

Figure 4. Evaluation of (A) *PLAU* (uPA) and (B) *SERPINE1* (PAI-1) mRNA expression in breast tumor samples according to the corresponding protein expression in positive (+) and negative (-) samples (uPA and PAI-1) determined using ELISA as the reference assay. *P<0.05; **P<0.01; ***P<0.001. IT, intra-tumor area; PT, peripheral zone; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; *PLAU*, uPA gene; *SERPINE1*, PAI-1 gene.

To better evaluate the correlations between the ISH and ELISA results, ROC curves and the Youden index were applied, in order to define the optimal thresholds for the discrimination of uPA- positive (or PAI-1- positive) from uPA-negative (or PAI-1- negative) samples. As regards uPA, the AUC values were 0.716 (95% CI, 0.604-0.827), 0.744 (95% CI, 0.635-0.852) and 0.717 (95% CI, 0.606-0.829) for the IT, PT and IT + PT areas, respectively, demonstrating significant concordance between the techniques (Fig. S2A, C and E). According to the optimal Youden indexes, the ISH test was considered positive when the normalized *PLAU* expression was higher than 0.6537, 0.9142 and 0.6088 in the IT, PT and IT + PT areas, respectively. By using the aforementioned cut-off values, a significant association between the ISH and ELISA methods (P<0.001, P=0.042 and P<0.001 for the IT, PT and IT + PT areas, respectively) was observed; however, 33% of the discordant results in IT and IT + PT, and 39% in the PT areas were also found. The discordant cases were distributed throughout the dynamic range of uPA expression determined using ELISA and not only around the clinical established cut-off value. The sensitivity, specificity, positive and negative predictive values obtained from the optimal Youden index are presented in Table III.

As regards PAI-1, the AUC values were <0.7, regardless of the analyzed areas (Fig. S2B, D and F). Therefore, the optimal thresholds were not determined and the concordance between analytical methods was not assessed.

The same statistical analysis using the values obtained with vector A were also performed, and similar results concerning the concordance between the results obtained with RNAscope® and the reference method were obtained (data not shown).

Discussion

Despite the development of molecular signatures, the pathological examination remains the cornerstone of breast cancer clinical management. Currently, the analysis of three biomarkers is mandatory (estrogen receptor, progesterone receptor and HER2), and the determination of the proliferative marker, Ki-67, is also recommended (8). In early $ER^+/HER2^-$ pN0 early-stage breast tumors, high uPA and PAI-1 levels have been found to be associated with a poor patient outcome (level of evidence: 1A) (2-4). However, their use in clinical management remains limited, mainly due to the lack of reliable alternative testing methods to the validated ELISA. A method that would permit the analysis of these established prognostic markers in the FFPE tissue samples routinely used by pathologists may expand the clinical use of these two biomarkers.

Over the past years, a number of studies have focused on the analysis of possible correlations between the uPA

and PAI-1 mRNA and protein levels (15-18,28,29). Several studies have reported significant correlations for both biomarkers (17,18) or only for PAI-1 (16), whereas other failed to identify any correlation for either (28). Witzel *et al* (29) revealed discrepant prognostic values of uPA and PAI-1 mRNA levels (using Affymetrix microarray) and protein levels, in a cohort of 200 patients with breast cancer without systemic treatment. These conflicting results, combined with the fact that they were obtained using frozen material, have limited the dissemination of these alternative methods in clinical practice.

In the present study, *PLAU* and *SERPINE1* mRNA expression in FFPE sections was analyzed using ISH, which could be easily implemented in routine diagnostic histopathology settings. This assay may detect RNA molecules down to single-copy sensitivity, due to the use of a branched DNA technology for signal amplification. For each biomarker, 20 probe pairs were designed, each spanning less than 50 nucleotides along the target mRNA. This design increases the sensitivity and specificity of mRNA detection and allows their use in samples with partially degraded RNA, which is one of the characteristics of FFPE samples (20). This technology has been previously evaluated in routine settings for PD-L1 quantification in non-small cell lung cancer, head and neck squamous cell carcinoma, and urothelial carcinoma tissue specimens (30), and for human papillomavirus testing in oropharyngeal squamous cell carcinoma (31). In the 83 FFPE breast cancer-sample cohort, 96.2% of the samples were successfully tested in a single run apart from a limited number of failures related to tissue detachment during the retrieval phase. This issue was solved by increasing the drying time before the retrieval step. Finally, among 249 sections (83 samples x 3 probes), only one sample was excluded due to lack of *PLAU* signal. Altogether, these results suggested that if concordant with protein levels, *PLAU* and *SERPINE1* mRNA detection in FFPE tumor samples is feasible and could be translated to clinical settings.

Similar to conventional immunohistochemistry assay, the quantification of the ISH signal is a crucial step for biomarker analysis. In routine clinical practice, even though tremendous efforts have been made to implement unbiased quantitative methods using image analysis, biomarkers are often assessed by pathologists by visual evaluation (percentage of immunoreactive cells and/or intensity of staining). By using the RNAscope® technology, each individual dot should represent a single RNA molecule, provided that the RNA target is intact and properly unmasked. Thus, counting the dots in a given area is the most reliable way to assess the number of mRNA copies. However, in the majority of the samples in the present study, clustered dots were observed, located within the same area, preventing the individual counting of the dots. As an alternative, a macro-toolset was used, in order to analyze the surface occupied by the dots. Since large ranges of *PolR2A* expression without significant correlation with *PLAU* and *SERPINE1* expression levels were observed, the results were normalized to *PolR2A* expression level, to eliminate the bias due to mRNA degradation variations, by analogy with what is done for RT-PCR data and by assuming that *PolR2A* expression is constant in a sample (26,27). Moreover, with the aim of using this test routinely, a reference gene for normalization allows the exploitation of clinical samples with variable mRNA quality, inherent to the pre-analytical conditions. Following normalization, significantly higher *PLAU* and *SERPINE1* levels in uPA- and PAI-1-positive (i.e., above the established clinical cut-off values) clinical samples were detected. Although significantly correlated, the *PLAU* expression status by ISH analysis (i.e., positive vs negative according to the optimal thresholds determined using the Youden index) was concordant with uPA ELISA status in only 68-73% of cases, according to the studied area (IT, PT and IT + PT). This was below the threshold of 95% which was set for clinical significance. Moreover, no discriminant cutoff for PAI-1 quantification was detected. It was hypothesized that the lack of correlation is mainly related to the fact that mRNA level incompletely predicts protein expression in breast tumors (32), particularly for uPA/PAI-1 (16,18,28).

In conclusion, while technically feasible in clinical practice and despite the possibility to spatially analyze RNA transcripts in FFPE samples in a routine setting, the RNAscope® technology to quantify *PLAU* and *SERPINE1* mRNA levels cannot be used as a substitute to uPA and PAI-1 protein quantification using ELISA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FBM planned the present study, designed and conducted the experiments, analyzed the data, and drafted and edited the manuscript. CM participated in the study conception and design, performed the statistical analysis and edited the manuscript. VB developed the new ImageJ macro toolset and provided advice on how to complete the image analysis, and also edited the manuscript. EC participated in the study conception and design and edited the manuscript. WJ participated in the study conception and design, provided study patients and material, and helped to draft the manuscript. FBM and CM confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Institut du Cancer de Montpellier, France (approval no. ICM-CORT2019-20). All patients were informed before surgery and provided consent for the use their surgical specimens and the associated clinicopathological data for the present research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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