Journal of Current Medical Research and Opinion
CMRO 08 (10) 4275-4283 (2025)
DOI: https://doi.org/10.52845/CMRO/2025/8-10-1
www.cmro.in
Original Article
CMRO
ISSN (O) 2589-8779
(P) 2589-8760

Upregulation of Plasminogen and Fibronectin in Advanced Breast Carcinoma: An Immunohistochemical Study in Iraqi Women

Hazima Mossa Alabassi[1] | Mariam Qassim Al-Dulemey[2] | Farah M. Abdulsada[2] | Haneen Farooq Alaassi[3] | Adel Omran Salman[4]

[1]Department of Biology, College of Education ibn Al-Haitham, University of Baghdad, Baghdad, Iraq.; e-mail: Hazima.m.k@ihcoedu.uobaghdad.edu.iq
[2]Department of Bioinformatics, College of Biomedical Informatics, University of Information Technology and Communications, Baghdad, Iraq. e-mail: Maryam.qasim@uoitc.edu.iq
[3]Department of Medical Intelligence, College of Biomedical Informatics, University of Information Technology and Communications, Baghdad, Iraq. e-mail: haneen.farooq@uoitc.edu.iq
[4]Ministry of Environment, Technical Directorate, Baghdad, Iraq. e-mail: adelomransalman@gmail.com

Abstract:

Background: Breast cancer (BC) is by far the most prevalent form of cancer among Iraqi females. Both core extracellular matrix glycoprotein fibronectin (FN) and fibrinolytic system zymogen plasminogen (PLG) are considered to have a role in tumor invasion and metastasis. We investigated the relationship between the progression of a pathological process and the expression of FN and PLG in terms of immunohistochemistry (IHC).

Methods: FN and PLG were stained by a polymer-HRP detection method to stain twenty-five FFPE breast carcinomas. Semi-quantitative values were created with predetermined cut-offs as intensity and percentage positive were combined to produce semi-quantitative values (03). It is Wilcoxon signed-rank that was used to test the differences between pairs (FN vs. PLG).

Results: FN scores were higher than PLG, paired analysis(Wilcoxon two-sided $p = 0.0074$; one-sided p FN = PLG = 0.0037). PLG in Stage II and Stage III ($p = 0.03$ and $p = 0.04$ respectively) were statistically higher, but Stage IV was not significant ($p = 0.15$). FN had stage specific significant increase in Stage II ($p = 0.004$) and in Stage III-IV ($p = 0.04$). No correlation with the age of a patient was observed ($p > 0.05$).

Conclusions: FN and PLG are both up-regulated as the disease advances in this group, although FN is more important and more common in the Stages III and IV than PLG. These findings support the notion that they might be of use as aggressiveness biomarkers and potential targets in methods that address extracellular matrix (ECM) and fibrinolysis. In order to validate and elaborate on these, there is need to carry out larger, multi-center cohorts through digital image analysis.

Keywords: Breast cancer; Fibronectin; Plasminogen; Immunohistochemistry; Tumor progression; Biomarkers.

Corresponding Author: Hazima Mossa Alabassi, Department of Biology, College of Education ibn Al-Haitham, University of Baghdad, Baghdad, Iraq
E-mail: mohamed.hamza@alfarabiuc.edu.iq

Received: 15.08.2025 | **Revised:** 11.10.2025 | **Accepted:** 15.10.2025 | **Published:** 17.10.2025

Introduction:

In the world, approximately 2.3 million new cases of breast cancer and about 685,000 deaths due to this disease were observed in 2020, which makes it the most frequently diagnosed cancer in women and one of the leading causes of cancer-related deaths in general [1]. It must have context-specific biomarkers research to aid in prognosis and care plans in Iraq due to the population-based evaluations indicating a steady rise in incidence over the last two decades [2]. Another attribute of tumor development that simplifies invasion, metastatic spread and resistance to treatment is extracellular matrix (ECM) remodeling [3,4]. These syntheses identify the effect of the composition, cross-linking, and rigidity of the ECM on cell signaling and tumor behavior. Unregulated state in this axis in breast cancer is associated with aggression and worse, poor results [5]. In this environment, plasminogen activation system enhances fibrinolysis and pericellular proteolysis leading to cell migration and invasion. This system is called urokinase-type and tissue-type plasminogen activators or uPA and tPA, respectively.

Fibronectin (FN) is an important ECM glycoprotein with its roles in cell adhesion, migration, and angiogenesis, and accumulates in tumor and pre/metastatic sites, which can be used as a biomarker and a target of therapy in breast cancer [6,7].

A bit of cell-adhesion molecules (CAMs) background is required since adhesion signaling transforms extracellular matrix (ECM) signals into pro-invasive programs. FN increases motility, survival, and metastatic competence by initiating FAK/Src and tension-dependent signaling by canonical integrins, specifically, 5- and 3 [9,10]. This FN-integrin/adhesion framing of our markers, the FN and plasminogen axis, and invasion pathways can be realized to be much in line with the focus of the study on IHC. This study, therefore, employs immunohistochemistry to determine the levels of plasminogen (PLG) and fibronectin (FN) expression in formalin-fixed and paraffin-embedded breast cancer tissues of Iraqi women of different clinicopathologic grades and stages. Following title of the study and as per the focus of the abstract on prognostic effect and therapeutic potential in this situation, we suppose that paired analysis exhibits higher FN scores than PLG and that the two markers have an upregulated state which is dependent on stage/grade [11,12].

Materials and Methods:

Sample collection

The patients were aged between twenty two and seventy two years of age and were sampled in the pathology lab archive. All clinical diagnoses obtained through archival histology reports were obtained using the immunohistochemical staining approach.

Immunohistochemistry (IHC)

FFPE tissue sections (5 µm) were placed on positively charged slides and baked at 60 o C 60-120 minutes, deparaffinized using xylene and rehydrated with to water using graded ethanol. The heat-induced epitope retrieval (HIER) was done on Tris-EDTA, p.h. 9.0 on Fibronectin (FN) and EDTA, p.h. 8.0 on Plasminogen (PLG) at 95-98 o C in 20-30 minutes and thereafter, subjected to washes with buffer. The 3 percent H 2 O 2 was used to block endogenous peroxidase (10 minutes). [11]. Elaborations of the sections were incubated with the rabbit polyclonal primary antibodies as follows: Fibronectin -Elabscience. E-AB-67878 (IHC recommended dilution is 1:50 - 1:200, 45 mins, room temperature); Plasminogen - BT-LAB, Code BT-AP07254 (IHC recommended dilution 1:100-1:300, 45 mins, room temperature). Quality controls were positive controls (e.g., renal/testicular tissues suitable to be targeted) that were done in parallel and a negative control that was done without the primary antibody as part of the control.

Pathologic Assessment and Scoring

The extent of IHCs expression was determined semi-quantitatively by multiplying the staining intensity (0 to 3) and the percentage of positive tumor cells with a predetermined scheme: Score 0(negative; <10 percent), Score I (10 percent to 25

percent), Score II (26 percent to 50 percent) and Score III (51 percent to 75 percent/diffuse). The a priori definition of positivity was that intensity should be 1 or higher in at least 10% of tumor cells. All slides were rated by two identifying blinded observers and any discrepancies were sorted out by consensus, and inter-observer agreement was summarized using Cohens 6. Table 1 presents percentages of categories based on centage of stained tumor cells (category definitions of IHC scoring).

Table 1. Percentage of stained tumor cells

Score	Percentage of stained tumor cells
0	<10% (negative)
I	10–25%
II	26–50%
III	51–75% / diffuse

Statistical analysis

The data were analyzed using SPSS software 25 (IBM) and graph pad Prism 10. Continuous variables are shown in the form of the mean and standard deviation of minus or plus the mean (SD). We applied one-way ANOVA in case of group comparisons. Then, to manage Type I errors, we applied post hoc multiple comparisons by means of Bonferonni or False Discovery rate (FDR) correction. The Chi-square test was used to test the categorical variables. Where necessary, correlations were analyzed using the Pearson or Spearman correlation coefficients. A p-value less than 0.05 was considered statistically significant to us. In case of datasets with more than a few tests, a number of comparison corrections were applied so as to reduce the number of false positives [13].

Results and Discussion

Positive controls showed strong, diffuse cytoplasmic staining (Figures 3-4) following IHC procedure validation, whereas the negative controls of plasminogen and fibronectin had no apparent signal of DAB (Figures 1-2). The following interpretations of tumor samples are all based on the control results, which confirm the specificity of the antibodies and the lack of non-specific backgrounds [14]. Besides, this control performance is consistent with the revised CAP guidelines of analytic validation of IHC assays in 2024. According to these rules, when there is a change (in pre- or post-analytic conditions, i.e. decalcification, non-formalin fixation or scoring changes) then the assay should be re-validated to guarantee a consistent interpretation across tissues and indications [15].

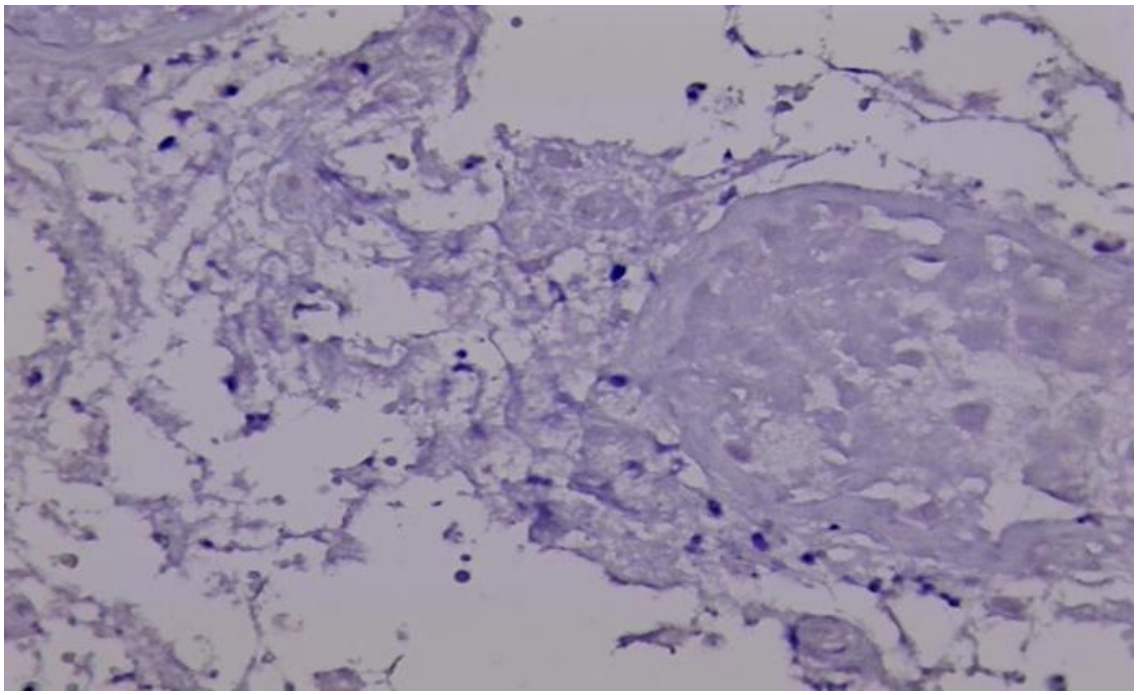

Figure 1. Negative control (no primary antibody added) of Plasminogen run on testicular tissue; no DAB signal (40×).

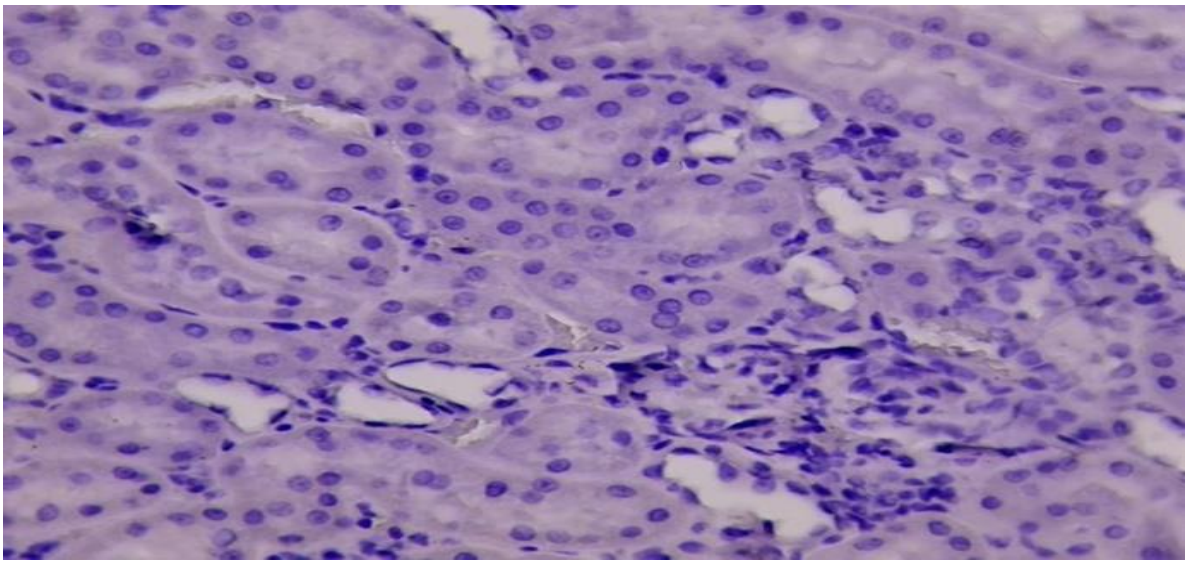

Figure 2. Negative control (primary antibody omitted) for Fibronectin run on renal tissue; no DAB signal (40×).

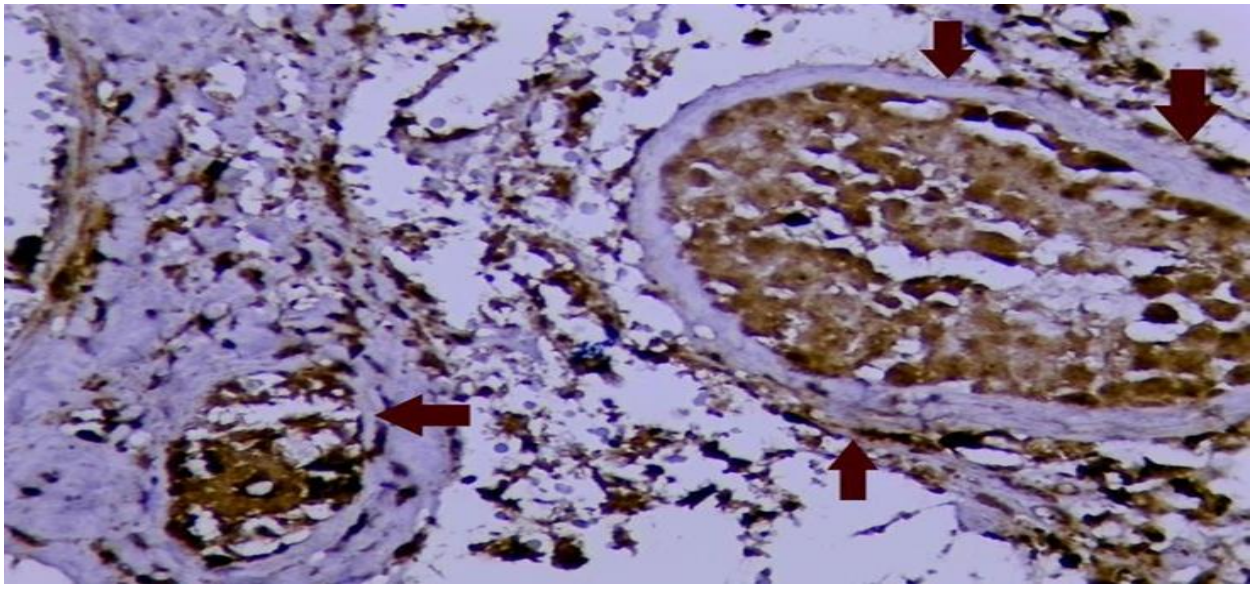

Figure 3. Positive control: testicular tumor (Plasminogen) showing cytoplasmic DAB staining (40×).

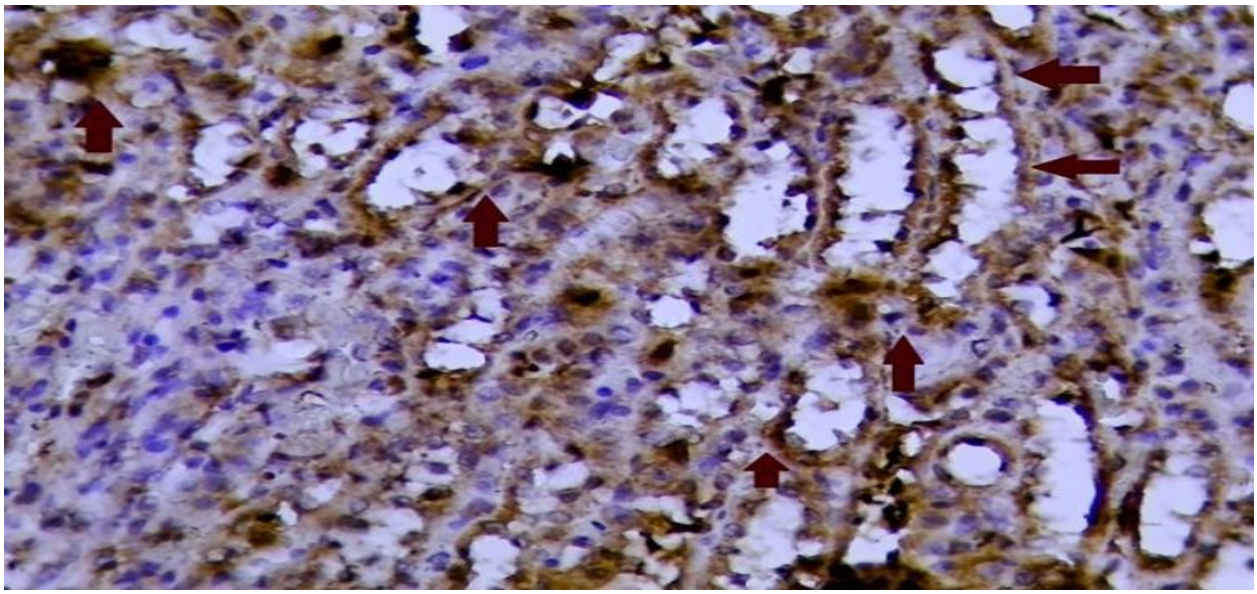

Figure 4. Positive control: renal tumor (Fibronectin) showing cytoplasmic DAB staining (40×).

The comparison on the plasminogen (PLG) and fibronectin (FN) scores were done, in a paired manner, using Wilcoxon signed-rank test, which is applicable in cases of non-parametric data that are paired. The p-value obtained was 0.0074 which was not adjusted. The result was not affected by multiplicity corrections (Bonferonni, FDR) (adjusted p = 0.0074 in both cases) as only a single paired comparison was analyzed. FN was more common than PLG in 14/25 pairs and vice versa in 1/25 with 10 ties and one-sided test FN the PLG showed a p-value of 0.0037. The paired difference was significant in favor of FN, which was estimated to be r = 0.70 (Table 2; Figures 5-6).

Table 2. Comparison of plasminogen (PLG) and fibronectin (FN) immunohistochemistry scores by the Wilcoxon signed-rank test.

Sample	Plasminogen	Fibronectin	Difference (Fibronectin − Plasminogen)	Absolute Difference	Rank	Sign
1	1	1	0	0	5.5	0
2	0	1		1	15.5	+
3	1	1	0	0	5.5	0
4	0	3	3	3	24.5	+
5	2	2	0	0	5.5	0
6	3	3	0	0	5.5	0
7	1	1	0	0	5.5	0
8	1	3	2	2	22.0	+
9	2	3	1	1	15.5	+
10	2	3	1	1	15.5	+
11	2	3	1	1	15.5	+
12	2	3	1	1	15.5	+
13	2	2	0	0	5.5	0
14	2	2	0	0	5.5	0
15	2	3	1	1	15.5	+
16	2	3	1	1	15.5	+
17	3	3	0	0	5.5	0
18	3	0	-3	3	24.5	-
19	2	2	0	0	5.5	0
20	3	3	0	0	5.5	0
21	2	3	1	1	15.5	+
22	1	3	2	2	22.0	+
23	2	3	1	1	15.5	+
24	1	2	1	1	15.5	+
25	1	3	2	2	22.0	+

- Difference column = Fibronectin score minus Plasminogen score.
- Absolute Difference = Difference for ranking.
- Rank = rank of absolute differences (used for Wilcoxon signed-rank test).
- Sign = direction of change (+ increase, − decrease, 0 no change).

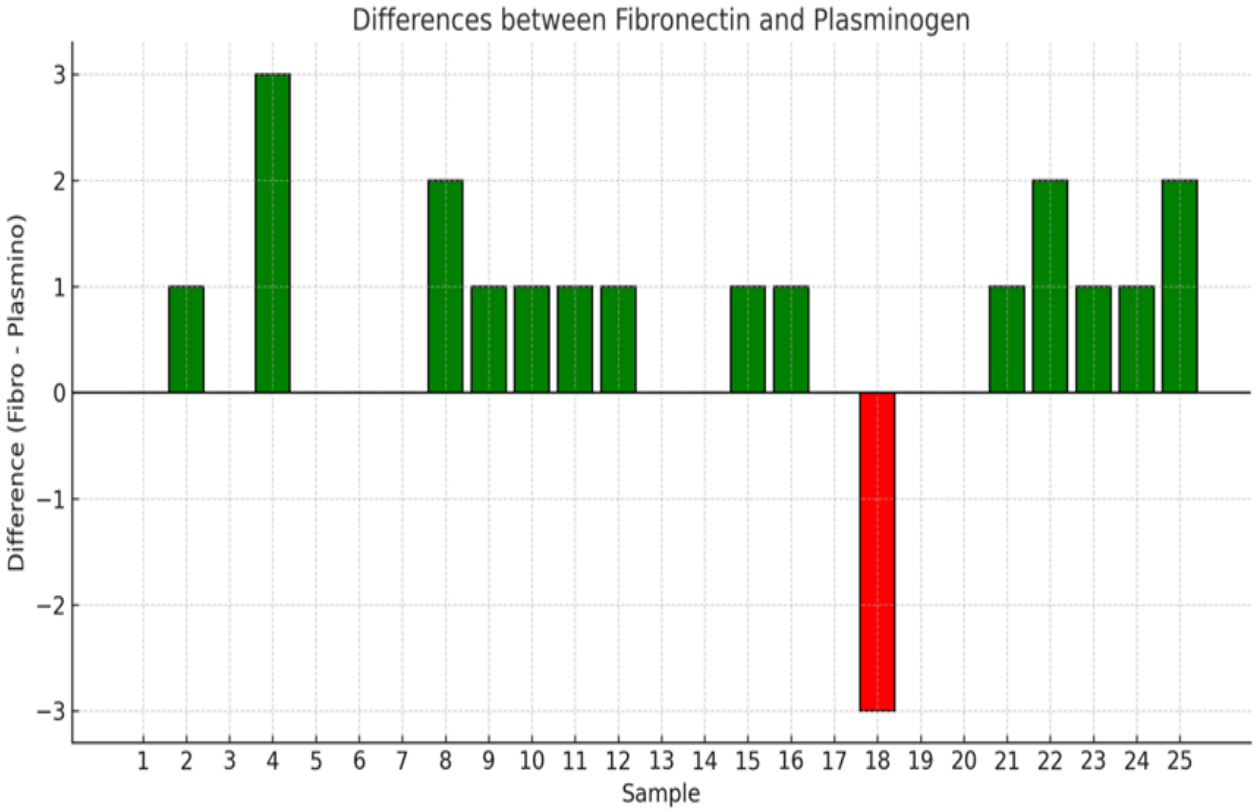

Figure 5. variation between Fibronectin and Plasminogen, with positives in green, negatives in red, and neutral values marked by the blue line.

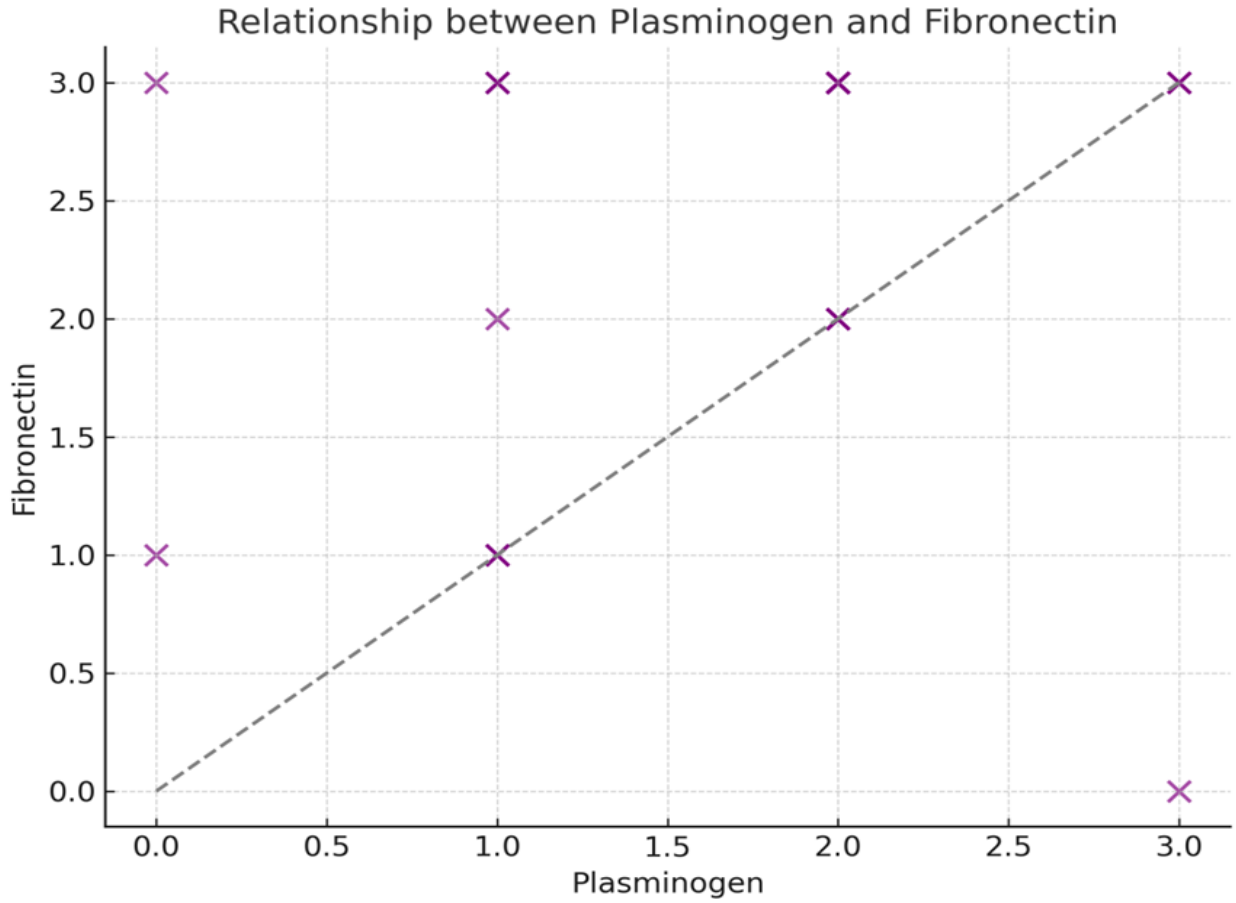

Figure 6. relationship between Plasminogen and Fibronectin, with the identity line (y = x) for comparison

In Stage III, Score I would show faint staining in early/low-grade lesions, Score II would show moderate staining (p = 0.03) and Score III would show high cytoplasmic staining (p = 0.04) and this would be observed at every stage of pathologic progression. The correlation was not found to be statistically significant, probably because the sample size was small, although Stage IV samples frequently appeared heavily stained. Figures 7-10 give representative IHC figures which show this gradient associated to stages. The findings are consistent with known information regarding the plasminogen-activation axis in biology where the urokinase-type plasminogen activator (uPA) converts plasminogen into plasmin that subsequently enables the degradation of the extracellular matrix (ECM) and invasion to occur [5,16].

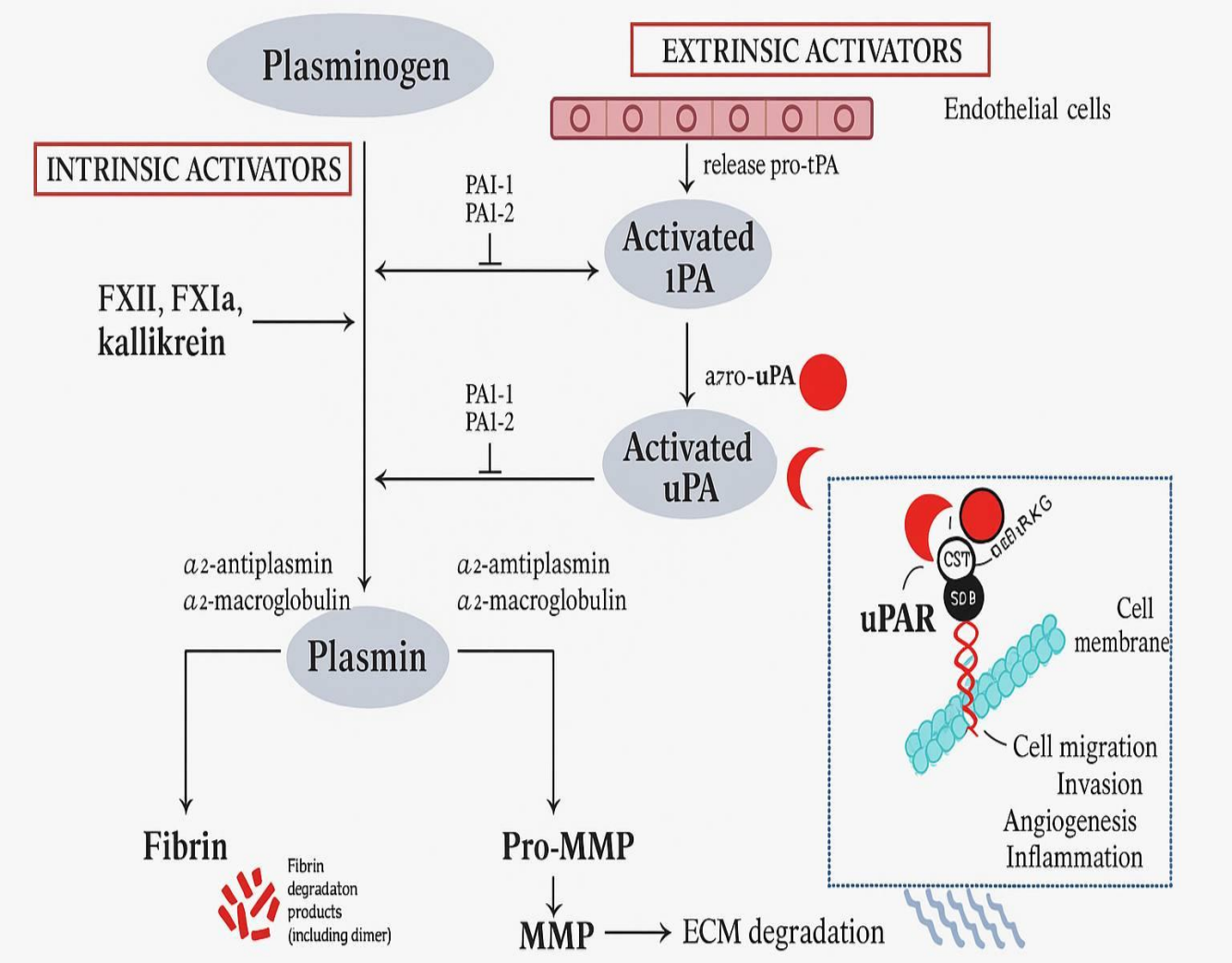

Figure 7. Schematic representation of the plasminogen activation system showing intrinsic (FXII, FXIa, kallikrein) and extrinsic (tPA, uPA) activators leading to plasmin formation, fibrin degradation, and extracellular matrix (ECM) remodeling involved in tumor invasion and metastasis [16].

New clinical and mechanistic data has also indicated the same trend: in validating analysis stromal uPA (IHC) was revealed to be an independent prognostic factor in endocrine - responsive early breast cancer, and uPA-PAI-1 heteromerization enhances pro-metastatic signaling by recruiting tumor-promoting neutrophils, as supplementary research indicated [17,18].

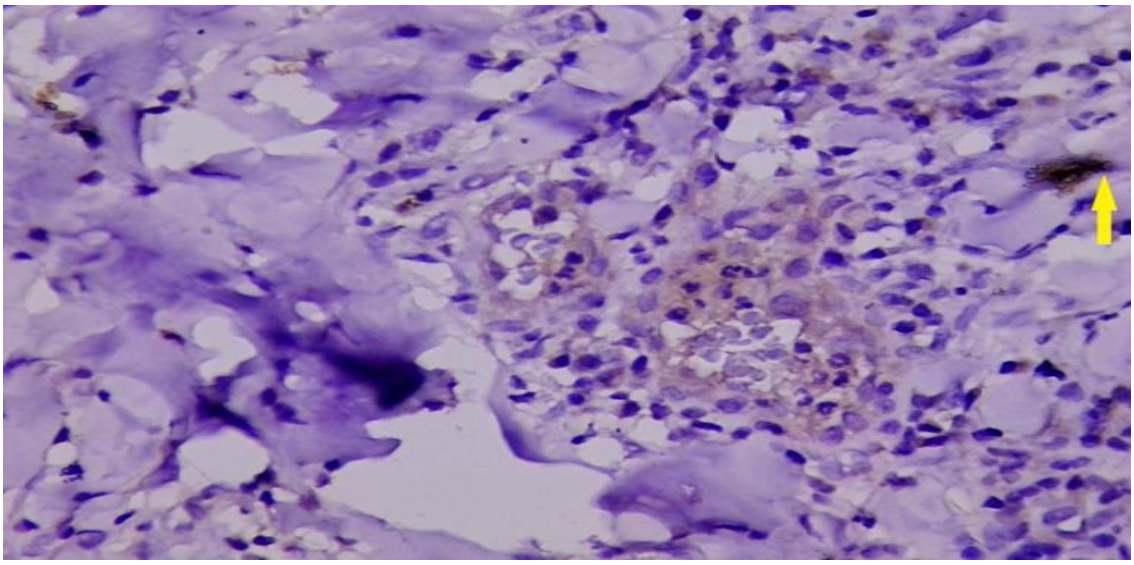

Figure 8. IHC of breast cancer showing positive Plasminogen (Score I), 40×. Arrows indicate positive cytoplasmic staining.

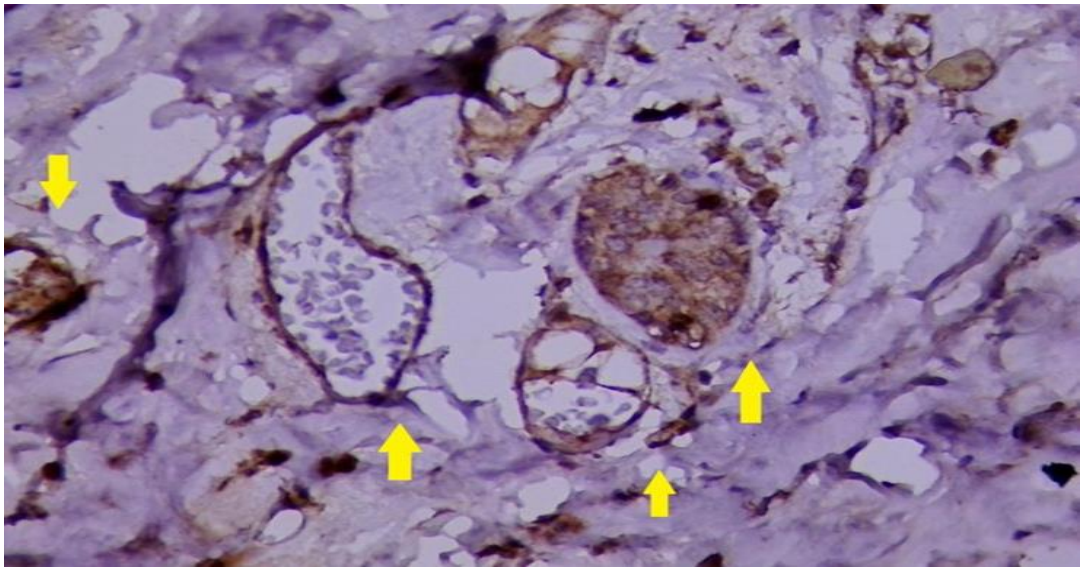

Figure 9. IHC of breast cancer showing positive Plasminogen (Score II), 40×. Arrows indicate positive cytoplasmic staining.

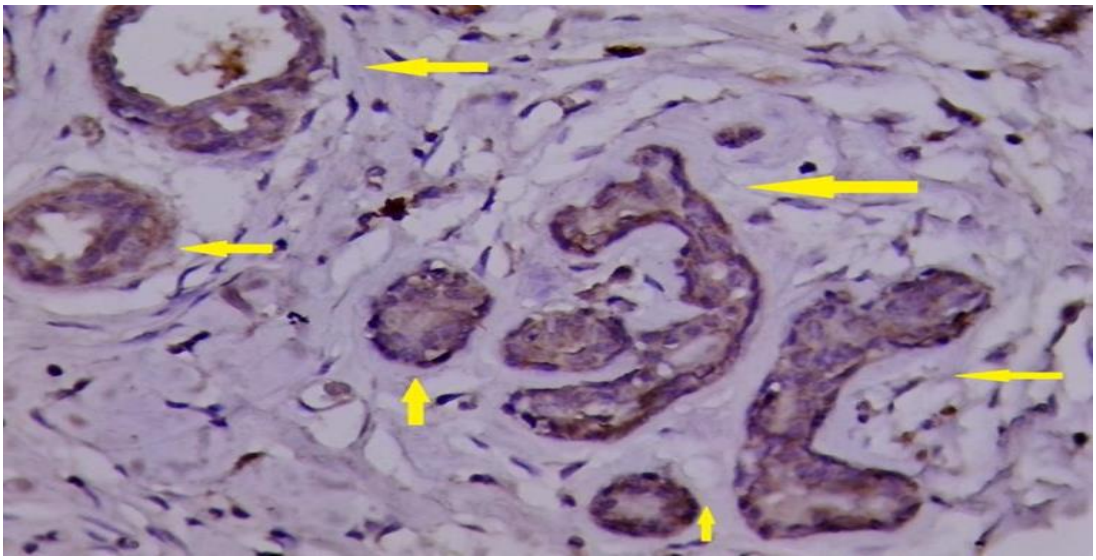

Figure 10. IHC of breast cancer showing positive Plasminogen (Score III), 40×. Arrows indicate positive cytoplasmic staining.

There were a minor accumulation in Score I lesions, a moderate accumulation in Score II tumors and a large and abundant accumulation of fibronectin (FN) in Score III tumors, which showed a definite progressive increase in fibronectin (FN) with tumor stage and grade. The first stage of our dataset where FN was statistically significant was Stage II (p=0.004); other significant stages of the dataset were Stages III and IV (p=0.04). Figure 11-14 representative IHC panels illustrating this gradient. Biologically, these trends are consistent with the existing knowledge: cancer-related fibroblasts establish FN-rich, stiffened matrices, which increase integrin (alpha5 beta1/ alpha v beta3) -FAK/SRC signaling, and promote the dissemination of cancer to other body regions [19,20,21].

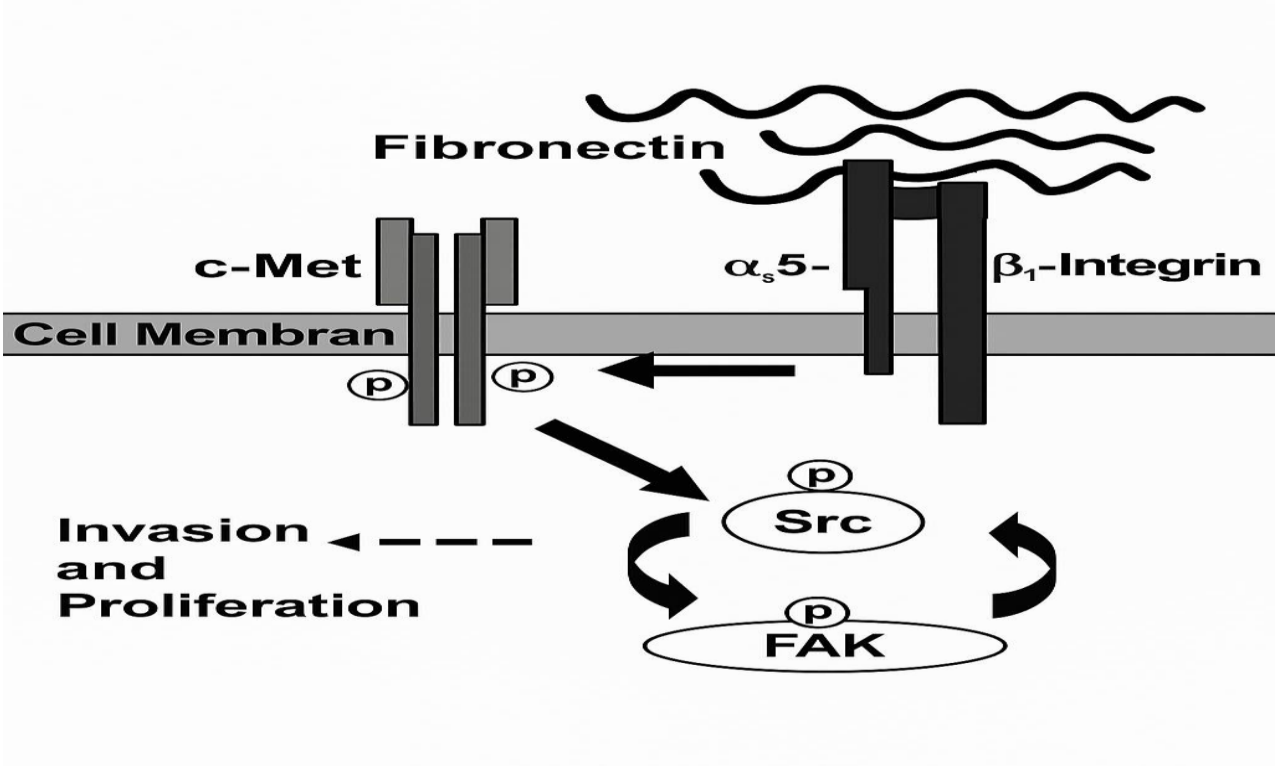

Figure 11. Fibronectin interaction with α5β1-integrin and c-Met receptors activates FAK/Src signaling, promoting cell invasion and proliferation in breast carcinoma. [6].

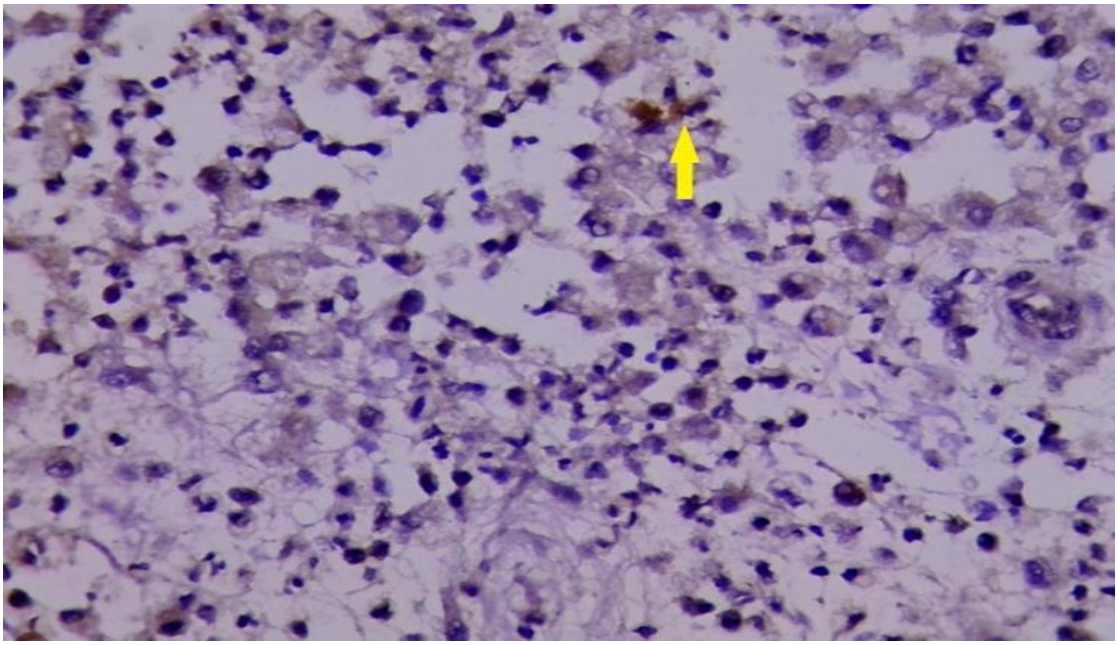

Figure 12. IHC of breast cancer showing positive Fibronectin (Score I), 40×. Arrows indicate positive cytoplasmic staining.

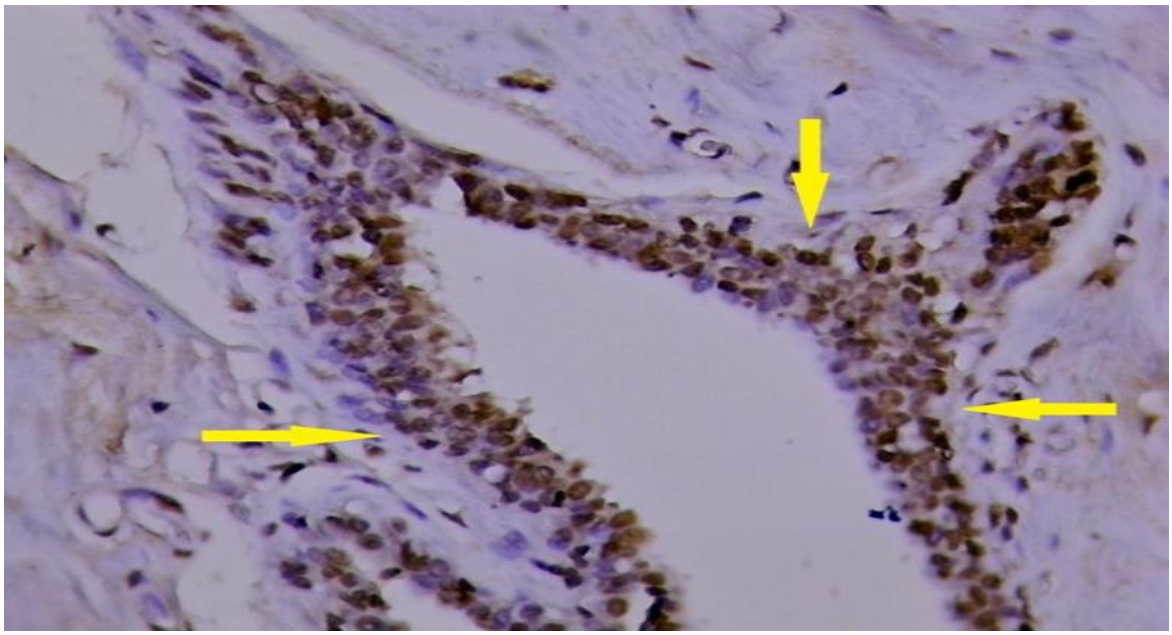

Figure 13. IHC of breast cancer showing positive Fibronectin (Score II), 40×. Arrows indicate positive cytoplasmic staining.

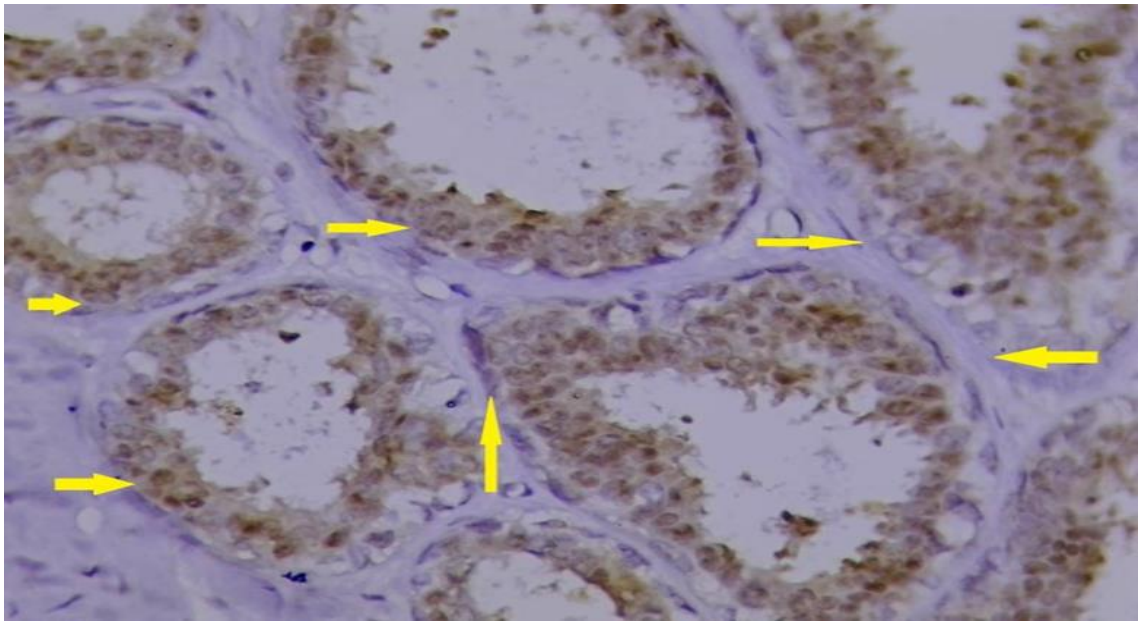

Figure 14. IHC of breast cancer showing positive Fibronectin (Score III), 40×. Arrows indicate positive cytoplasmic staining.

Table 1 indicates that the level of expression is primarily predetermined by tumor more than by age since the level of the two variables did not correlate statistically when chi-square testing was conducted. It implies that the elderly patients have higher chances of being positive. Mechano-responsive programs, including YAP/TAZ, activated by the stiffness of the stroma increase and the high level of fibronectin, can lead to the improvement of the properties of epithelial-mesenchymal transition, proliferation, survival, and therapy resistance [22,23]. Also, the constant production of plasmin increases proteolysis of ECM barriers due to uPA activity of which bioactive fragments are also released, potentially complementing the invasion process once again [15].

The other reason which might have led to the association between high fibronectin and bad results is that the changing of ECM interacts with antitumor immunity. This is backed up by open-access reviews which demonstrate that abnormal matrix composition and stiffness have the

potential to impede effective immune-cell infiltration and facilitate immune evasion [3,24,25]. Another reason is the pre-metastatic niche, which is conditioned by ECM reprogramming and helps distant organs to become receptive to incoming tumor cells, which also explains the intensities of staining are higher in the advanced stages [26].

The existing data and literature comparison suggest the situation where invasion, metastatic seeding, and immunological escape is stimulated by a joint effect of plasminogen activation and fibronectin-rich matrix remodeling. There is open-access evidence to support the use of these markers as pragmatic histopathologic adjuncts to risk stratify and serve as entry points to therapeutic intervention, including inactivating fibronectin-integrin signaling, preventing uPA-axis signaling or suppressing YAP/TAZ-dependent mechanotransduction. The observation is complemented by recent research: Biomechanical regulation of the extracellular matrix (ECM) can stimulate immune infiltration and treatment response [27,28]. On the one hand, breast-cancer-associated cancer-associated fibroblasts generate fibrillar matrices that increase the cancer stromal stiffness, elevate the integrin-FAK/Src and YAP/TAZ mechanosignaling, and restrict the entry of drugs and immune-cells.

Conclusions:

This study proves that both FN and PLG are up-regulated in Iraqi breast cancer. FN and PLG exhibit a consistent distribution of preference on semi-qualitative IHC with FN being more common than PLG and a rising prevalence with pathologic stage/grade. The results give support to the hypothesis that a combination of the plasmin-mediated proteolysis and the presence of FN-rich matrix remodeling facilitates the invasion and the progression of the disease. Interestingly, no correlation between the age of patients and FN or PLG expression was observed suggesting that it was tumor-intrinsic but not demographic regulation. In these parameters, FN and PLG may be applicable histopathologic tools in risk stratification and treatment targets (e.g., by inhibiting the uPA/PAI-1 axis, FN-integrin signaling, or matrix-mediated mechanotransduction). This study has a quasi-quantitative grading in FFPE tissue and a small, single-center study (n=25) which can limit the generalizability of the study. It would be wonderful had future research found ways to confirm these findings in larger prospective groups, combine biomechanical outputs with machine image analysis (such as H-score), and establish whether ECM-targeting or mechanotransduction-modulating approaches can be more effective on the FN-dominant cancers.

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