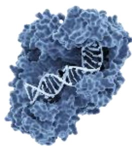

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Original Research Article

A Study Comparing Immunohistochemistry, Fluorescence in situ hybridization, GeneXpert® Breast Cancer STRAT4 Assay for Determining Hormone Receptor, Human Epidermal Growth Factor Receptor 2, Ki67 Status in Invasive Breast Carcinoma in Moroccan women

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Abstract: The accurate assessment of hormone receptors, HER2, and Ki-67 proliferative index provides meaningful information about breast cancer prognosis and prediction of therapy response. Immunohistochemistry, the most common method for evaluating these prognostic biomarkers, can be impacted by numerous variabilities due to pre-analytical/analytical factors and subjective interpretation by pathologists. The Xpert® Breast Cancer STRAT4, an RT-qPCR based system, can be used to classify invasive breast carcinomas based on the assessment of these four biomarkers; Methods: We evaluated *ESR1*, *PGR*, *ERBB2,* and *MKi67* mRNA expression by Xpert Breast Cancer STRAT4 and ER, PR, HER2 and Ki67 by IHC (FISH for HER2 IHC 2+) in 200 formalin-fixed paraffin-embedded (FFPE) tissue blocks with invasive breast cancer, collected from the Pathology Department of Casablanca Ibn Rochd University Hospital; Results: Concordance between Xpert ® Breast Cancer STRAT4 and IHC was 93.5% for ER, 83.51% for PR, 95% for HER2 (92% for IHC+FISH), and 81.20% for Ki67 (excluding intermediate IHC Staining $10 \leq$ %IHC <20). The simple Kappa coefficient was, for ER, 0.830 ($P < 0.0001$), 0.565 ($P < 0.0001$) for PR,

0.838 ($P < 0.0001$) for HER2-IHC, 0.771 ($P < 0.0001$) for HER2 IHC+FISH and, for, Ki67, 0.458 ($P < 0.0001$); Conclusions: We demonstrated globally a high concordance between centrally assessed IHC, IHC+FISH and mRNA measurements of ER/*ESR1* and HER2/*ERBB2*, and a moderate agreement between PR/*PGR* and Ki67/*MKi67*. These findings provide an additional, objective, and quantitative assessment of tumor receptor status in breast cancer.

Keywords: Breast cancer; hormone receptors; Human Epidermal Growth Factor Receptor 2; Ki-67; immunohistochemistry; Xpert STRAT4

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1. Introduction

Cancer poses a significant global health challenge, as evidenced by the staggering numbers of new cases and deaths recorded in 2020, which stood at 19.3 million and 10.0 million, respectively [1-4]. Individuals face a 20% chance of developing cancer at some point, with a 10% risk of dying. One in five people will develop cancer during their lifetime, and one in 10 will succumb to it [1].

In 2020, female breast cancer surpassed all other types of cancer as the most common cancer worldwide, with an estimated 2.26 million new cases, followed closely by lung cancer with 2.21 cases [1,3-4]. Approximately 494,000 new cases are diagnosed annually in Europe, and it is estimated that 143,000 women will succumb to the disease [5]. Morocco saw around 11,000 new breast cancer diagnoses in 2020 [6], making it the most common malignancy in the country and the leading cause of cancer-related deaths among Moroccan women [6].

Improved treatment and early detection have led to a 34% decrease in breast cancer mortality over the past 30 years [7]. Immunohistochemical assessment of estrogen and progesterone receptor protein expression has become a valuable tool for predicting patient outcomes and response to endocrine therapy in breast cancer [8-12]. Another alteration that has received significant attention in the last two decades is amplifying the HER-2/neu gene in human breast cancers. Measuring protein overexpression or gene amplification of the Human Epidermal Growth Factor Receptor 2 (HER2 or *ERBB2*) can serve as a useful prognostic marker and a predictor of response to trastuzumab or other HER2-targeted therapies [13,14].

Several retrospective studies of breast cancer patients have shown the marker of proliferation Ki67 (*MKi67*) to be an important prognostic factor. It has various potential uses, including prognosis, prediction of response to chemotherapy or endocrine therapy, estimation of residual risk in patients on standard therapy, and as a dynamic biomarker of treatment efficacy in samples taken before, during, and after neoadjuvant therapy [14,15].

The European Society for Medical Oncology (ESMO) recommends that all primary breast carcinomas be tested for estrogen receptor (ER), progesterone receptor (PR), HER2/*ERBB2*, and Ki-67 at the time of diagnosis, according to treatment guidelines [16-18].

Immunohistochemistry (IHC) on formalin-fixed paraffin-embedded (FFPE) tissues is the gold standard for assessing hormonal receptor status, HER2, and Ki67 in breast cancer [7,13,14]. When the HER2 score is equivocal (2+), fluorescence in situ hybridization (FISH) is typically used to clarify HER2 immunohistochemical results, and some institutions use FISH for initial HER2 evaluation status in all patients [13,19].

Despite being used for a long time, immunohistochemical assays have not been adequately standardized across laboratories. The accuracy and reproducibility of results for these four biomarkers can be impacted by pre-analytical or analytical limitations such as tissue fixation, choice of antibodies, use of manual versus computer-assisted scoring methods, and interpretation of results in assay performance, which can significantly affect IHC and FISH results [14,16,20-22].

The Xpert® Breast Cancer STRAT4 test is an *in vitro* diagnostic medical device (CE-IVD: *in vitro* diagnostic medical device. May not be available in all countries. Not available in the U.S.) that utilizes a semi-quantitative assay with qualitative cut-off values to detect the mRNA levels of Estrogen Receptor (*ESR1*), Progesterone Receptor (*PGR*), HER2/*ERBB2*, and Marker of Proliferation Ki-67 (*MKi67*) in FFPE invasive breast cancer samples [21,22]. This test measures the target mRNA levels of these four biomarkers and the mRNA levels of the reference gene CYFIP1 in FFPE breast cancer tissue using a self-contained cartridge. The GeneXpert® (GX) system is responsible for automating and integrating all aspects of the sample processing, including RNA isolation, amplification, and detection of the target sequences in FFPE samples through real-time reverse transcriptase, polymerase chain reaction assays (RT-PCR) [21,22].

The main goal of this first retrospective study in the North Africa region is to assess the clinical performance of the GeneXpert Instrument Systems compared to the currently used IHC and FISH methods in evaluating routine biomarkers (ER, PR, HER2 and Ki67) in Moroccan women. The study aims to verify the efficacy of this new approach. According to earlier research conducted in various other countries, Xpert® Breast Cancer STRAT4 is highly reproducible and shows a high level of agreement with IHC and HER2 FISH results [21-29].

2. Materials and Methods

Our study included two hundred blocks of FFPE tissue corresponding to 200 patients, aged ≤5 years and archived in the Pathology Department of Casablanca Ibn Rochd University Hospital. The histopathology of all samples remaining in the blocks was reviewed, and only specimens still containing invasive breast carcinoma cells were included in the study. The FFPE tissue sections were obtained from core biopsies (55 cases) and surgical specimens (145 cases), from a selection of patients with invasive breast cancer whose tumor samples were collected and routinely evaluated for breast cancer biomarkers (ER, PR, HER2 and Ki67) according to the standard of care (SOC) IHC and/or FISH assays at the Pathology laboratory. The immunohistochemical status (IHC) was sought on 4 µm tissue sections

treated and incubated with the antibodies ER: FLEX Monoclonal Rabbit Anti-Human Estrogen Receptor α Clone EP1 Ready-to-Use; PR: FLEX Monoclonal Mouse Anti-Human Progesterone Receptor Clone PR 636 Ready-to-Use and Ki67: FLEX Monoclonal Mouse Anti-Human Ki-67 Antigen Clone MIB-1 Ready-to-Use, according to the Dako protocol on the Autostainer Link 48 IHC platform. HER2 status is first assessed by IHC, using the antibody Ventana Pathway Anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody on a Ventana GX automated platform. Tumors were classified as ER positive or PR positive when ≥1 % of invasive tumor cells showed definite nuclear staining, irrespective of staining intensity. A tumor was considered HER2 positive if an IHC score equal to 3+ was found and HER2 negative if a score of 0 or 1+ was observed (ASCO/CAP guidelines) [30]. HER2 Equivocal (IHC 2+) results were subsequently tested by FISH with manual technique using the probes (HER2 IQFISH pharmDx) to confirm the final HER2 status.

The patient tumors selected for our study represent the various breast cancer subtypes as determined through surrogate IHC subtyping by the routine assays performed at our laboratory, as follows:

- 25 triple negatives (ER negative, PR negative and HER2 negative)
- 25 HER2+ (Hormone receptor (HR) negative / HER2 +)
- 100 Luminal A (HR positive / Ki67 < 20 %)
- 25 Luminal B (HR positive / Ki67 ≥ 20 %)
- 25 HER2 IHC 2+ (8 cases HER2 FISH positive and 17 HER2 FISH negative using current ASCO/CAP HER2 guidelines) [31].

The mRNA levels of *ESR1*, *PGR*, *ERBB2* (HER2), and *MKi67* were assessed by quantitative gene expression readouts using the Xpert® Breast Cancer STRAT4 assay. Breast Cancer FFPE tissue samples were prepared for the assay as tissue scrolls (10 µm thickness) and placed into a 1.5ml Eppendorf tube. For some surgical specimens (FFPE section contained <30% invasive tumor), macrodissection (tumor area defined by the pathologist) was carried out, and the FFPE section was scraped off the slide and placed at the bottom of the tube. FFPE samples were first treated with the recommended volumes of FFPE lysis reagent (1.2 ml) and proteinase K (20 µL) provided by the Xpert® FFPE Lysis Kit (CE-IVD*) before use in Xpert® Breast Cancer STRAT4. The solution was then incubated in a heat block at 80 °C for 30 minutes. Then 1.2 ml of ≥95% Ethanol was mixed with the sample.

Once the tissue lysate was prepared, a 520 µL aliquot was placed into the appropriate sample chamber in the Xpert® Breast Cancer STRAT4 cartridge. The testing cartridge was inserted into a module of a GeneXpert® System for processing, where the system fully automated and thoroughly integrated nucleic acid purification, amplification, and real-time

detection. The final results of STRAT4 testing are available approximately 70 minutes after starting the test.

2.1. Statistical analysis

Statistical analysis was done in GraphPad Prism Software. For each of the four biomarkers studied, agreement measurements between Xpert® Breast Cancer STRAT4 and IHC and/or FISH, which were considered as the reference methods, were based on contingency table analysis and included overall concordance (overall percent agreement), positive percent agreement (sensitivity) defined as the number of samples classified positive by both IHC and Xpert® Breast Cancer STRAT4 divided by the number of positive samples using immunohistochemistry, negative percent agreement (specificity), and Cohen's κ coefficient scores. The Kappa (κ) statistic numeric values are categorized into the slight agreement (≤0.2), fair agreement (between 0.21 and 0.40), moderate agreement (between 0.21 and 0.40), substantial agreement (between 0.61 and 0.80), and almost perfect agreement (between 0.81 and 1.00). All measurements were associated with 95% confidence intervals (95% CI), compared using Fisher's exact test, and considered significant for $P < 0.05$ [32].

3. Results

For each sample, we evaluated mRNA results by Xpert® Breast Cancer STRAT4 and compared them to the results obtained by the already routinely performed IHC+HER2 FISH.

3.1. Concordance between IHC/FISH and Xpert STRAT4

3.1.1. Estrogen receptor

The overall concordance rate between Xpert® Breast Cancer STRAT4 *ESR1* mRNA results and ER protein IHC results was 93.50% using either the IHC cut-off of ≥1% or ≥10% immunostaining level for positivity and using a pre-defined delta Ct cut-off (dCt ≥ −1) for *ESR1*-positivity by Xpert® Breast Cancer STRAT4 based on prior concordance studies [21,25]. The Cohen'κ coefficient score was equal to 0.830 (95% confidence interval: From 0.741 to 0.919) (Table 1).

Only 2% of immunohistochemistry-ER-positive samples were classified as negative using STRAT4, whereas 4.5% of immunohistochemistry-ER-negative samples showed a positive Xpert® Breast Cancer STRAT4 ER status (Figure 1). These results demonstrate perfect agreement between STRAT4 and IHC for the *ESR1*/ER biomarker and suggest a low discordance level exists between the two methods.

Table 1. Comparison of protein status for ER, PR, HER2, and Ki67 and mRNA expression for *ESR1*, *PGR*, *ERBB2,* and *MKi67* between Immunohistochemistry « IHC », Fluorescence in situ hybridization « FISH » and RT-qPCR « Xpert® Breast Cancer STRAT4 test*».

Analyte	Reference	Total	IHC+/ RTqPCR +	IHC+/ RTqPCR -	IHC-/ RTqPCR -	IHC-/ RTqPCR +	Sensitivity (PPA)	Specificity (NPA)	Concordance rate (OPA)	KAPPA Statistic
ER/*ESR1* (IHC+ 1%)	IHC	200	142	4	45	9	97.26%	83.33%	93.5%	0.830
ER/*ESR1* (IHC+ 10%)	IHC	200	142	4	45	9	97.26%	83.33%	93.5%	0.830
PR/*PGR* (IHC+ 1%)	IHC	194	130	5	32	27	96.3%	54.24%	83.51%	0.565
PR/*PGR* (IHC+ 10%)	IHC	194	114	1	36	43	99.13%	45.57%	77.32%	0.488
HER2/*ERBB2*	IHC	175	30	3	136	6	90.91%	95.77%	95%	0.838
HER2/*ERBB2*	FISH	25	7	1	10	7	87.50%	58.82%	68%	0.387
HER2/*ERBB2*	IHC/ FISH	200	37	3	147	13	92.5%	91.88%	92%	0.771
KI67/*MKI67* (IHC+ ≥20%)	IHC	184	92	3	32	57	96.84%	35.96%	67.39%	0.334
KI67/*MKI67* (IHC+ >10%)	IHC	184	94	6	29	55	94%	34.52%	66.85%	0.299
KI67/*MKI67* (excluding 10≤IHC%<20 range)	IHC	133	92	3	16	22	96.84%	42.11%	81.20%	0.458

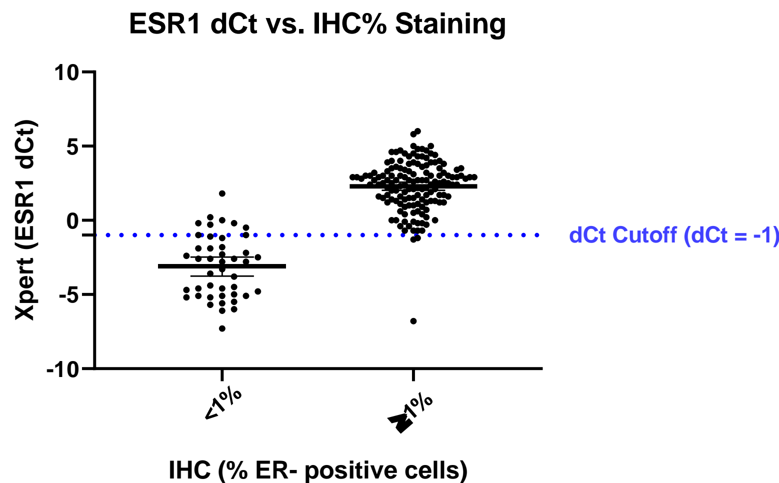

Figure 1. Comparison of estrogen receptor status determined by RT-qPCR and Immunohistochemistry. Graph of mRNA expression *ESR1* dCt determinated with Xpert® Breast Cancer STRAT4 test* by ER IHC result categorized as negative (<1%), or positive (≥1%).

3.1.2. Progesterone receptor

Regarding the PR data, six cases with "indeterminate" Xpert® Breast Cancer STRAT4 *PGR* results were excluded. Taking into account all the remaining cases, concordance between Xpert® Breast Cancer STRAT4 *PGR* and PR IHC results using an IHC cut-off of ≥1% as recommended by ASCO-CAP [16] was 83.51% using *PGR* dCt cutoff of -3.5. The statistical kappa value is around 0.565 (95% confidence interval: From 0.435 to 0.694) (Table 1).

Only 2.5% cases of IHC PR-positive samples were classified negative by RT-qPCR. In contrast, 14% of IHC PR-negative cases showed a positive Xpert® Breast Cancer STRAT4 *PGR* status based on current Xpert® Breast Cancer STRAT4 cutoffs (Figure 2). By using the IHC cut-off of ≥10% to determine PR-positive status, the overall concordance between both methods was 77.32%. In comparison, the Cohen's κ coefficient score was equal to 0.488 (95% confidence interval: From 0.372 to 0.603) (Table 1).

In this case, only 0.5% of the samples were classified PR-positive by IHC and negative by Xpert® Breast Cancer STRAT4, whereas 22% of the samples of IHC PR-negative became Xpert® Breast Cancer STRAT4 *PGR*-positive (Figure 2). Based on this data, we note a moderate agreement between STRAT4 and IHC for the *PGR*/PR biomarker with an acceptable discordance percentage between the two methods (Table 1).

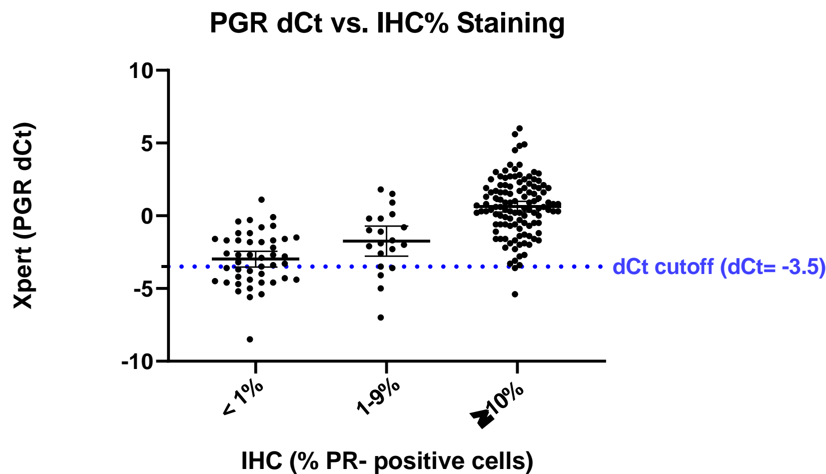

Figure 2. Comparison of progesterone receptor status determined by RT-qPCR and Immunohistochemistry. Graph of mRNA expression *PGR* dCt determined with Xpert® Breast Cancer STRAT4 test by PR IHC result categorized as negative (0%), low positive (1–9%), or positive (≥10%).

3.1.3. HER2

For HER2 status determination, we studied the concordance on three levels, first of all between IHC and STRAT4 excluding equivocal cases (HER2 score = 2+), secondly between FISH and STRAT4 exclusively for equivocal cases, and lastly between STRAT4 and the two reference methods (IHC and FISH), including all cases of the study.

The overall concordance rate between Xpert® Breast Cancer STRAT4 and IHC was approximately 95% (excluding equivocal cases) with a κ coefficient equal to 0.838 (95% confidence interval: From 0.735 to 0.940) (Table 1). The percentage of discordant cases classified by IHC as HER2- positive and ERBB2- negative on Xpert® Breast Cancer STRAT4 was only 1.7%, whereas 3.5% of HER2-negative cases on IHC were ERBB2-positive using Xpert® Breast Cancer STRAT4 (Figure 3).

The concordance between Xpert® Breast Cancer STRAT4 and FISH for equivocal cases was 68%, and the Cohen's coefficient was equal to 0.387 (95% confidence interval: From 0.073 to 0.700). Solely 4% of FISH positive samples were classified negative using Xpert® Breast Cancer STRAT4, while 28% of FISH negative samples were classified positive using Xpert® Breast Cancer STRAT4 (Figure 3).

Considering both reference methods (IHC and FISH) as ASCO/CAP Guidelines recommended in evaluating the HER2 status [33]. The concordance rate between Xpert® Breast Cancer STRAT4 and reference methods (IHC and FISH), including all samples, was 92%. The Kappa coefficient equals 0.771 (95% confidence interval: 0.666 to 0.877) (Table 1).

In this case, the cohort was divided into HER2- negative (Score 0, Score1, Score 2/FISH non-amplified) and HER2- positive (Score3, Score2/FISH amplified). Therefore, the

percentage of discordant between HER2- negative cases on IHC/FISH and HER2-positive cases using Xpert® Breast Cancer STRAT4 was 6.5%, while only 1.5% HER2– positive cases on IHC/FISH became HER2- negative using Xpert® Breast Cancer STRAT4 (Figure 3).

In general, the Cohen's kappa value and the overall percent agreement (OPA) showed that HER2 results ranged from good to very good agreement; however, results suggest a minimal percentage of disagreement between methods (Table 1).

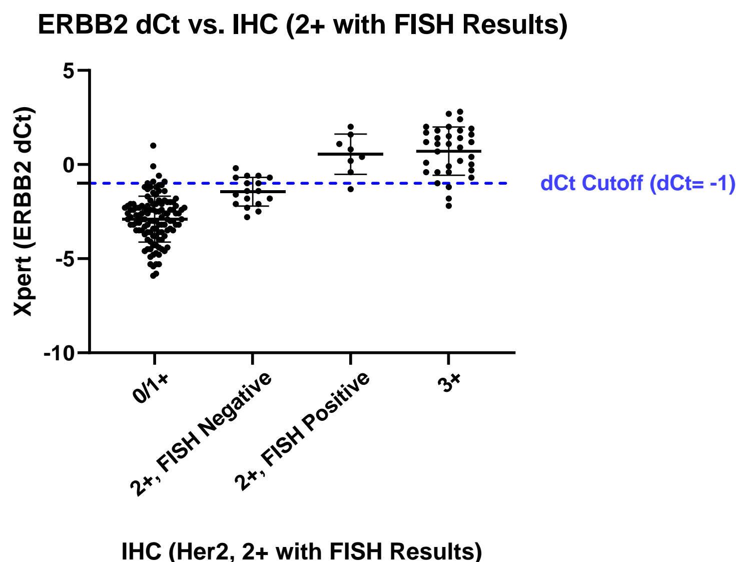

Figure 3. Comparison of HER2/*ERBB2* determined by either RT-qPCR or by immunohistochemistry with or without FISH assessment of IHC2+. Graph of mRNA expression *ERBB2* dCt determined with Xpert® Breast Cancer STRAT4 test by IHC/FISH Her2 results including all sample size.

3.1.4. Ki67

Last, we examined the Xpert® Breast Cancer STRAT4 *MKi67* dCt values and Ki67 results by using Ki67 IHC cutoff of 10% and 20% to discriminate "high proliferation rate" from "low proliferation rate". In comparison, we used an intermediate zone (equivocal results) between 10 and 20% for the *MKi67* dCt distribution. We excluded sixteen cases with « Indeterminate » Xpert® Breast Cancer STRAT4 *MKi67* status from this cohort.

The overall concordance between Xpert® Breast Cancer STRAT4 *MKi67* and Ki67 IHC, considering a high proliferation rate of≥20%, was 67.39% (Table 1). Only 1.6% of samples with high Ki67 IHC results were classified as low Ki67 using Xpert® Breast Cancer STRAT4, while 31% of cases with low Ki 67 showed a high ki67 in Xpert® Breast Cancer STRAT4 (Figure 4).

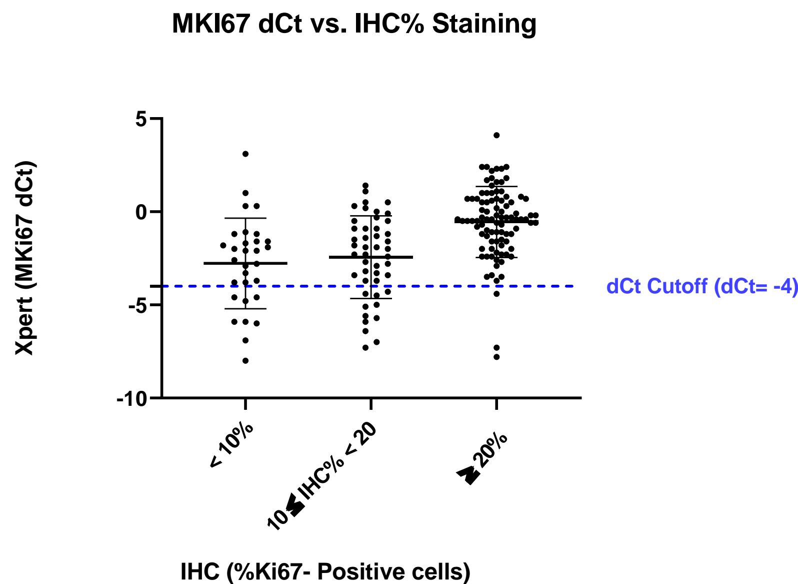

Figure 4. Comparison of Ki67 proliferation rate determined by either RT-qPCR or immunohistochemistry. Graph of Xpert® Breast Cancer STRAT4 *MKi67* dCt values by Ki67 IHC % staining where the IHC low proliferation rate cutoff is defined as <10%, IHC high proliferation rate cutoff is defined as ≥20%, and the intermediate proliferation rate (equivocal range) is defined as 10 %≤ IHC% < 20%.

When we considered >10% cutoff, the overall agreement was 66.85%. Discordant cases consisted of IHC high Ki67 and Xpert® Breast Cancer STRAT4 low Ki67 (3%) and IHC low Ki67 / Xpert® Breast Cancer STRAT4 high Ki67 (≈ 30%) (Table 1).

When we excluded samples with IHC staining in the 10 %≤ IHC % < 20% range, the overall agreement was 81.2% (Table 1). We noted only 2% of discordant cases with high Ki67 on IHC and low Ki67 using Xpert® Breast Cancer STRAT4. However, 16.5% cases showed a low ki67 on IHC and a high Ki67 by Xpert® Breast Cancer STRAT4 (Figure 4).

The κ coefficient equals 0.334 (95% confidence interval: 0.224 to 0.445) using 20% Ki67 IHC Cutoff, Kappa = 0.299 (95% confidence interval: 0.182 to 0.417) using 10% Ki67 IHC Cutoff. When we excluded equivocal cases, the Kappa equals 0.458 (95% confidence interval: From 0.289 to 0.628) (Table 1).

Comparison of *MKi67* STRAT4 results with IHC % immunostaining for Ki67 demonstrates that Ki67 results ranged from slight to moderate agreement between the methods and a significant discordance percentage.

For ER and PR, we used IHC cutoffs of 1% as recommended by the ASCO/CAP 2010 ER/PR testing guidelines [34] and 10% as described elsewhere [24,34]. Central IHC and central FISH resolve IHC HER2 2+ to either FISH-negative or FISH-positive, as recommended by the ASCO-CAP guidelines [33] for HER2 testing. For Ki67 we used IHC cutoff of ≥ 20% and >10% to discriminate "high proliferation rate" from "low proliferation rate"."INDETERMINATE" results for *PGR* or *MKi67* in the Xpert Breast Cancer STRAT4

indicate that the CYFIP1 reference gene included in the assay cartridge had a Ct that was not within the valid range or the endpoint was below the threshold setting required for *PGR* or *MKi67* status determination. For these cases, the assay needed to be redone using the concentrated lysate procedure provided by the test kit.

4. Discussion

Immunohistochemical assays are the gold standard for evaluating ER, PR, HER2, and Ki67 status in invasive breast carcinoma. The main advantages of IHC for assessing these markers are that it is rapid and simple, it can be performed in most pathology laboratories, and (compared with other assays) it is relatively inexpensive. However, IHC assay reliability has been questioned because alterations during tissue processing, manipulation, and fixation, as well as the antibody clone, internal controls, and scoring system, may affect the results' precision. In addition, inter-observer variability in interpretation may also play a role as IHC remains a semi-quantitative and non-standardized method [23-25].

The ambiguity encountered in interpreting HER2 IHC results, especially in cases with HER2 equivocal scores (HER2 IHC =2+), may represent an issue. Most laboratories in resource-constrained settings may be unable to overcome it, as the gold standard would be FISH for quantifying HER-2 gene amplification in these cases. Indeed, FISH has the advantage of being a quantitative method and is considered the gold standard method for confirming the HER-2 status, not only to resolve IHC 2+ cases but also for all other cases where it has an excellent correlation with the HER2 IHC results [36,37]. Major disadvantages are that FISH is technically complicated to execute, arduous to establish, has a long run time, and is costly, making it not routinely available in all pathology laboratories worldwide. Moreover, another limitation of this method is that it does not necessarily reflect target protein expression, and counting FISH spots is wearisome and can be biased by tumor heterogeneity [13,24,25]. Nevertheless, these causes of assay variability may explain the differences in ER, PR, HER2, and Ki67 IHC results in breast carcinomas reported previously.

Currently, treatment of invasive breast carcinoma relies essentially upon ER, PR, HER2, and Ki67 status [38,39], and the accuracy of assays is critical. Hence, to overcome the limitations of IHC and HER2 FISH, there have been efforts to establish alternative methods to assess the 4 biomarkers of interest as accurately as possible [40]. One of the options is to use RT-qPCR. Reverse transcription quantitative PCR (RT-qPCR) represents a sensitive, efficient, and reliable approach for analyzing RNA. The initial step in RT-PCR is the production of a single-strand complementary DNA copy (cDNA) of the RNA through the action of the retroviral enzyme reverse transcriptase, to amplify that part of this cDNA by PCR. RT-PCR is used to analyze differential gene expression or cloned cDNAs. RT-PCR is more sensitive and easier to perform than other RNA analysis techniques [41].

In this study, our statistical data demonstrated that the Xpert® Breast cancer STRAT4 closed-system RT-qPCR method shows a good concordance rate with IHC+HER2 FISH results. The concordance between Xpert® Breast cancer STRAT4 and HER2 IHC+HER2

FISH has been evaluated in other studies and varies between 91% and 98%. In the current analysis, our data demonstrate that the Xpert® Breast Cancer STRAT4 assay shows greater than 91% concordance with HER2 IHC+HER2 FISH, suggesting that our results are generally concordant with the previous studies [21-25,37,42,43].

For both *ESR1*/ER and *ERBB2*/HER2, data suggested almost perfect agreement between Xpert® Breast Cancer STRAT4 and central IHC (κ "ER"= 0.830; κ "HER2"= 0.838), with nearly all of the discordant cases with quantitative dCt values close to the *ESR1* and *ERBB2* dCt cutoffs, respectively. Our findings are in good agreement with previously reported results: we found overall concordance of 93.50% and 95% for ER and HER2, respectively, and previously published papers have reported values of 97% to 98% for ER and of 93% to 97% for HER2 [21-23,25].

The results showed a moderate Kappa correlation agreement for *PGR*/PR (using PR IHC+ 1%) and *MKi67*/Ki67 (excluding equivocal cases) between both assays (κ "PR"= 0.565; κ "Ki67"= 0.458). Xpert® Breast Cancer STRAT4 demonstrated a significant overall concordance with IHC for *PGR* (83.5%) and *MKi67* (81%). The concordance rates observed in other studies vary from 81% to 92% for PR and from 78% to 89% for Ki67, in accordance with agreement percentages obtained in our analysis [21-23,25,43].

Discordance between assay methods can be attributed to several factors, including tissue fixation, antibody clones used in IHC, and scoring methods. Preanalytical factors are essential to monitor, and a quality assessment scheme should be put in place in any laboratory routinely assessing the 4 biomarkers [44]. Particular attention should be paid to the impact of sample handling, time of fixation, duration of tissue fixation, antibody selection, control samples and interpretation of assay on Xpert® Breast Cancer STRAT4 results [11,14,15,18]. In spite of the systematic practice of immunohistochemistry methods, procedural inconsistency remains elevated in clinical settings, leading to interlaboratory and intralaboratory variations and high false-negative (for ER and PR) and false-positive (for HER2) [19]. This inconsistency emphasizes the importance of a standardized retrieval method in performing reliable IHC and/ or FISH for the four markers routinely screened in breast cancer diagnosis.

Xpert® Breast Cancer STRAT4 has already shown good agreement with automated semi-quantitative IHC [21-23,26,27,29]. *ESR1* and *ERBB2* assessments have the highest pertinence in all studies. Comparison between the Xpert® Breast Cancer STRAT4 *PGR* status and the PR IHC status resulted in more discrepancies. This discrepancy between the two methods could be explained by the fact that total mRNA does not necessarily reflect the total protein and vice versa [45]. Denkert *et al*. have demonstrated that Ki67 IHC results are greatly variable. Significant variability in concordance rate has been noted for this biomarker, although this is not unforeseen given the challenges associated with Ki67 IHC evaluation [23].

Major gaps in diagnostic availability exist in many low-income and middle-income countries (LMIC) [46]. IHC is not widely available and faces many challenges, such as lack of skilled human resources and adequate equipment, insufficient and unreliable funding to

run the facilities, and erratic supply chain management [47]. IHC would, therefore, either be unavailable at all or only available at a central level, in a centralized manner, resulting in very important delays in getting the results for breast cancer [13,15,21-25,43]. Since GenXpert technology is already widely available in LMIC, especially in Africa, as some countries have been largely equipped for the HIV and/or TB programs [48], existing instruments could be mutualised and used for breast cancer. Since GenXpert technology is very easy to use, implementing such technology would be very swift. In situations where delays exist in obtaining IHC results, such as heavy workloads or centralized IHC services, alternative diagnostic methods such as GenXpert can be utilized for timely preliminary results. This enables oncologists to initiate treatment promptly while awaiting IHC results. After IHC results become available, treatment plans can be adapted if necessary. Additionally, in cases where IHC is available but ineffective due to small specimen sizes or technical limitations, STRAT4 can be employed as a tiebreaker.

Considering our results and similar results found in other published papers, the Xpert® Breast Cancer STRAT4 assay is highly reproducible. It shows a high level of concordance with IHC (and HER2 FISH) results. Therefore, it may be a potential solution to overcome IHC/FISH limitations and improve the management of invasive breast cancer, especially in low-resource countries [13,15,21-25,43].

5. Conclusions

In conclusion, determining hormone receptors (ER/PR), HER2, and Ki67 status by immunohistochemistry (and in situ hybridization for HER2 IHC 2+ cases) is part of the standard management of invasive breast cancer. There are questions related to technical issues with the standard tests used, and all international recommendations insist on improving the quality of tissue samples analyzed (pre-analytical phase), analytical techniques, and interpretation of results to ensure quality immunohistochemistry and molecular biology tests. The new RT-qPCR assay « Xpert® Breast Cancer STRAT4 » gave promising results and a remarkable agreement with the reference techniques (IHC and FISH). Molecular diagnostics have become more and more essential in diagnosing and managing cancer in general, and further studies of Xpert® Breast Cancer STRAT4 are needed with a larger sample size to support and confirm the results already published.

Ethics approval and consent to participate: All experiments were performed retrospectively following the Moroccan Bioethics Law 28-13, and after the Casablanca biomedical research ethics committee (CERBC) approval.

Author Contributions: RE - Realised the technical part, analyzed and interpreted the data, and significantly contributed to the manuscript. AN - Study monitored and corrected the manuscript. AK - selected samples with the pathologist and Contributed to the technical analysis. YZ - contributed to the statistical study. MO & JW - corrected the manuscript. MK - study supervisor, selected eligible cases for the study, reviewed the H&E stained slide, interpreted the data, and corrected the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest: J.W. was an employee of Cepheid at the time of this study. The funders had no role in selecting samples used in the study, collecting data from participating sites, interpreting the final data analyses in the study, or deciding to publish the results. The other authors declare that they have no competing interests.

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