



Article

Concordance of HER2 Expression in Paired Primary and Metastatic Sites of Endometrial Serous Carcinoma and the Effect of Intratumoral Heterogeneity

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Abstract: Primary endometrial serous carcinoma, known for its aggressive nature and poor prognosis, shares similarities with breast and gastric cancers in terms of potential HER2 overexpression as a therapeutic target. Assessing HER expression is complicated by tumor heterogeneity and discrepancies between primary and metastatic sites. In this study, we retrospectively analyzed HER amplification and expression in 16 pairs of primary endometrial serous carcinoma resections and corresponding metastases. HER2 status was determined using immunohistochemistry (IHC), with criteria based on the percentage and intensity of tumor cell staining. Confirmatory techniques, such as dual in situ hybridization (DISH) and fluorescence in situ hybridization (FISH), were also employed. This study reports on the concordance rates and the presence and pattern of HER2 heterogeneity. Our results showed an 87.5% concordance rate in HER2 amplification status between primary and metastatic sites, with 33% of cases scored as 2+ being amplified. Heterogeneity was observed in 100% of amplified cases and 95% of non-amplified cases on in situ testing, with variations in heterogeneity patterns between techniques. In conclusion, our findings emphasize the importance of testing both primary and metastatic sites or recurrences, with a concordance rate of 87.5%. In addition, a review of the literature and combining the results showed a concordance rate of up to 68%. The presence and pattern of heterogeneity, particularly in cases of mosaic or clustered heterogeneity in the primary tumor, may serve as reliable indicators of concordance, predicting a non-amplified HER2 status in corresponding metastases.

Keywords: endometrial serous carcinoma; HER2; metastasis; uterine serous carcinoma



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1. Introduction

Primary endometrial serous carcinoma represents an aggressive variant of endometrial carcinoma with a bleak prognosis, with a mere 55% estimated survival rate over a 5-year period [1–3]. Previous research indicated that human epidermal growth factor 2 (HER2) overexpression and/or amplification has been observed in 25–30% of endometrial serous carcinomas [4,5].

Studies conducted by Togami et al. through multivariate analysis and a comprehensive multi-institutional cohort study by Erickson et al. have consistently revealed an association between HER2 gene amplification and increased recurrence rates, leading to poorer outcomes [6,7]. However, the promising aspect lies in HER2 proving to be an effective therapeutic target [8]. In a phase II study, the use of single-agent trastuzumab in combination with carboplatin–paclitaxel exhibited both good tolerability and improved progression-free survival for advanced and recurrent HER2-positive endometrial cancers when compared to the standard platinum-based chemotherapy [9,10].

The present HER2 scoring guideline for endometrial serous carcinoma along with its testing algorithm are rooted in the findings of the clinical trial by Fader et al. (Figure 1). These findings have been advocated by Buza [11,12] and have been integrated into the endometrial biomarker testing template by the College of American Pathologists (CAP) [13]. Recent research encompassing 2192 cases of endometrial serous carcinomas of which 1686 (76.9%) were primary tumors and 506 (23.1%) were metastatic tumors highlighted that the incidence of HER2 status is significantly influenced by the diverse testing methods and interpretation guidelines employed [14]. The reported incidence varies from 10.5% when utilizing next generation sequencing to 19.6% following the 2018 breast in situ (ISH) criteria and to 18.6% with the 2007 breast ISH criteria [14]. Buza and Hui's recent evaluation of HER2 expression in ninety-four endometrial serous carcinomas using various evaluation methods, such as IHC and FISH, based on the CAP 2016 gastric criteria and CAP 2013 and CAP 2018 breast criteria showed a substantial 99% concordance with the 2018 gastric and breast criteria [15].

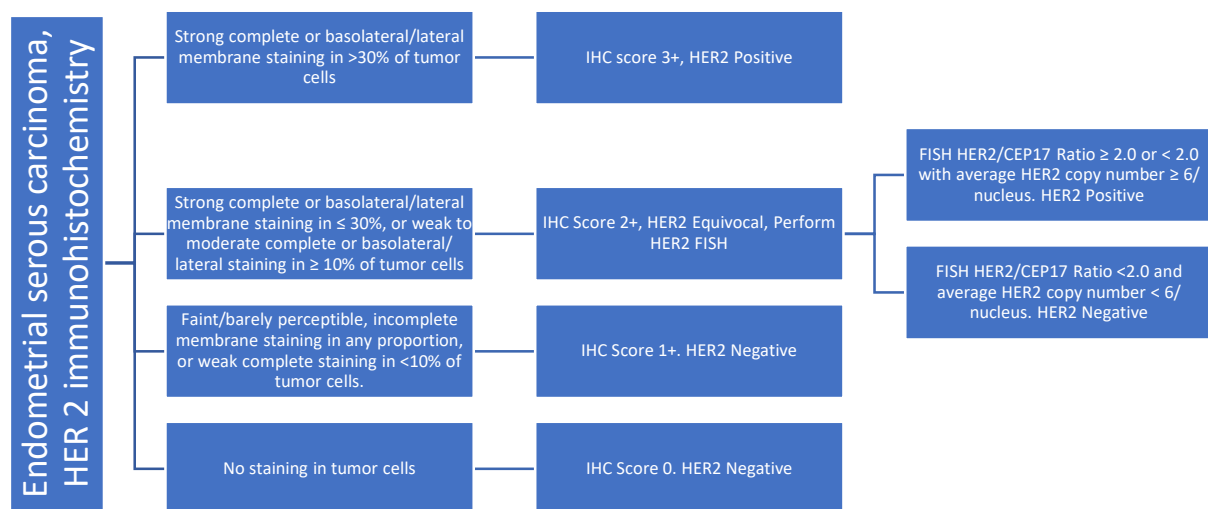


Figure 1. HER2 testing algorithm in endometrial serous carcinoma.

Moreover, the specimen types can be influenced by factors like tumor volume, intratumoral heterogeneity, and cold ischemic and fixation times. Although a universally accepted definition of HER2 heterogeneity is lacking, Albarello et al. defined HER2 heterogeneity in gastric cancers as a two-point difference in HER2 expression in at least 5% of the tumor [16]. Buza et al. further categorized heterogeneity, defining clustered heterogeneity as involving groups of tumor cells (20 cells or more) and mosaic heterogeneity as isolated amplified tumor cells within a background of unamplified tumor cells [17]. Buza et al. revealed that up to 72% (10 out of 14) of endometrial serous carcinomas exhibited HER2 clustered heterogeneity at the gene copy level [17]. In a separate report, she also demonstrated that up to 53% displayed significant heterogeneity in HER2 protein expression by IHC, with patchy IHC expression [5]. More recently, Shen et al. demonstrated that *HER2* genetic intratumoral heterogeneity defined as HER2 amplification in 5% to 50% of tumor cells examined by FISH was identified in 19/53 cases of high-grade endometrial carcinomas [18].

Despite the high rates of recurrence and metastatic spread, there are limited studies examining the concordance of HER2 status between primary and metastatic sites in endometrial serous carcinoma. It is unclear whether the HER2 status is maintained during metastasis. Establishing a high concordance rate between primary and metastatic sites would justify the testing of metastatic sites, as these are often more readily accessible for assessment prior to hysterectomy. Seven years ago, Halle et al. looked at HER2 expression in paired primary and metastatic endometrial cancers of which they had 35 paired cases of endometrial serous carcinomas [19]. In particular, in endometrial serous carcinoma, they found that 45% (9/20) of patients with HER2 high primary tumors had concordant

HER2 levels in metastasis [19], and 20% (3/15) with 0–2+ staining had metastases with 3+ staining [19]. This study compares HER2 status between primary and metastatic sites of endometrial serous carcinoma using the most recent HER2 assessment criteria for endometrial serous carcinoma with the multimodal analysis of HER2 status.

2. Materials and Methods

This study was approved by the King Edward Memorial Hospital Ethics committee, comprising endometrial serous carcinoma diagnosed from 2010 onwards selected from departmental archives. Out of fifty-two cases, there were sixteen hysterectomies and sixteen corresponding metastases; a total of 32 samples were selected. Relevant H&E and diagnostic slides from both hysterectomy and metastasis were reviewed to confirm the diagnosis. Thirteen cases were taken from the concurrent site of the disease, and three cases were recurrences.

Fresh tissue was fixed in 10% neutral buffered formalin (NBF) for at least 12 h before being processed. The chosen paraffin block had the largest volume of tumor on the slide to account for intratumoral heterogeneity and to increase the likelihood of identifying a HER2-overexpressed clone. We performed HER2 IHC, DISH, and FISH on both hysterectomies and corresponding metastases.

2.1. HER2 Immunohistochemical (IHC) Testing

Immunohistochemical testing was performed on formalin-fixed, paraffin-embedded tumor blocks (FFPE) cut at a thickness of 4 µm using 4B5 antibody (Ventana Medical Systems, Oro Valley, AZ, USA). The Ventana Benchmark XT autoimmunostainer uses the indirect polymer method after heat-induced epitope retrieval. The polyclonal ERBB2 antibody (DAKO, Glostrup, Denmark) was applied at a 1:4000 dilution. The IHC was scored independently by two pathologists (FY, YW) based on the criteria of Fader et al. (Figure 1).

2.2. HER2 Dual In Situ Hybridization (DISH)

DISH was performed on FFPE cut at a thickness of 4 µm using the automated Ventana INFORM HER2 Genomic probe platform, VENTANA HER2 Dual ISH DNA Probe Cocktail.

2.3. Fluorescence In Situ Hybridization (FISH)

FISH was performed using the ZytoLight in vitro (IVD) ERBB2/CEN17 dual-color probe (ZY Z-2077-200, Zytovision, Bremerhaven, Germany) to detect numerical changes in HER2. The specific procedures had been described in our validation of HER2 FISH probe paper [20].

2.4. Scoring of DISH and FISH

In order to account for tumor heterogeneity, we used the HER2 IHC as a guide, focusing on the areas where there was the highest expression in tumor cells. If there was heterogeneous amplification in clusters, 20 contiguous tumor cells with the highest amplification were counted to obtain the HER2/CEP17 ratio and average HER2 copy number per cell [17]. In mosaic heterogeneity, isolated tumor cells with a HER2/CEP17 ratio of more than or equal to 2.0 in a background that was unamplified, 20 contiguous cells were counted, and if the ratio was close to 2, up to 50 contiguous cells were counted in FISH and up to 40 contiguous cells were counted in DISH, recommended by the manufacturer, based on the breast protocol [17,21].

3. Results

A total of 16 paired primary tumors and metastases were analyzed in this series (Table 1). A total of 87.5% showed concordant HER2 amplification status (Table 2).

Table 1. IHC, DISH, and FISH results comparing primary tumor and metastasis (met).

Case No.	HER2 IHC YW	HER2/CEP17 YW	Avg. HER2/Nuclei YW	HER2 Amplified YW	HER2 IHC FY	HER2/CHR17 FY	Avg. HER2/Nuclei FY	HER2 Amplified FY	Heterogeneity of DISH FY	HER2/CEP17 Ratio	HER2 Per Nuclei	FISH Amplified	Heterogeneity of FISH
1	0	1.07	3.6	no	+1	1.32	3.5	no	mosaic	1.13	3	no	mosaic
1 met	0	1.05	3.4	no	0	1.16	2.85	no	mosaic	1.09	1.9	no	mosaic
2	+1	0.97	1.55	no	+1	0.97	1.65	no	mosaic	0.94	1.6	no	none
2 met	0	0.82	2.05	no	+1	0.77	1.85	no	none	1.2	2.4	no	mosaic
3	+1	1.7	3.75	no	+1	1.22	2.25	no	mosaic	1	2.3	no	none
3 met	+1	1.02	2.85	no	+1	1.82	4.1	no	mosaic	1.42	2.55	no	mosaic
4	+1	0.98	2.7	no	+1	1.17	2.05	no	mosaic	1	2	no	none
4 met	0	0.96	2.5	no	0	1.14	2.1	no	mosaic	0.87	2.6	no	none
5	+3	8.13	9.35	yes	+3	6.1	9.45	yes	cluster	>10	20	yes	cluster
5 met	+3	9.88	12.85	yes	+3	5.67	7.65	yes	cluster	12	24	yes	cluster
6	+1	1.18	2.65	no	+1	1.46	2.7	no	mosaic	1.04	2.5	no	none
6 met	+1	1.12	2.4	no	+1	1.5	2.7	no	mosaic	1.1	2.2	no	mosaic
7	+2	4.52	6.55	yes	+2	4	8.2	yes	cluster	2.977	6.4	yes	cluster
7 met	+3	2.93	6.75	yes	+2	3.68	9.75	yes	cluster	>7.3	14.6	yes	cluster
8	+1	1.15	2.35	no	+1	1.22	2.25	no	mosaic	0.98	2.8	no	none
8 met	+2	1.36	3.6	no	+2	1.31	4.65	no	mosaic	1	3.1	no	none
9	+3	4.69	9.85	yes	+2	4.07	9.35	yes	cluster	4.2	8.4	yes	cluster
9 met	+1	1.77	3.45	no	+1	1.68	3.45	no	mosaic	1.15	2.3	no	none
10	+2	1.62	3.4	no	+2	1.49	2.6	no	mosaic	1.33	2.6	no	mosaic
10 met	+1	1.29	2.2	no	+1	1.44	2.8	no	mosaic	1.21	1.75	no	mosaic
11	+2	4.44	8.65	yes	+2	5.18	8.8	yes	cluster	4.3	8.7	yes	cluster
11 met	0	1.23	2.4	no	0	0.94	1.6	no	none	1.1	1.9	no	mosaic
12	+3	5.8	13.05	yes	+3	3.36	6.55	yes	cluster	>5	10	yes	cluster
12 met	+3	5.2	10.65	yes	+3	3.63	7.8	yes	cluster	6	14.4	yes	cluster
13	+3	8.15	13.85	yes	+3	4.59	8.95	yes	cluster	4.45	8.9	yes	cluster
13 met	+3	7.26	9.8	yes	+3	6.63	7.95	yes	cluster	>10	20	yes	cluster

Table 1. Cont.

Case No.	HER2 IHC YW	HER2/CEP17 YW	Avg. HER2/Nuclei YW	HER2 Amplified YW	HER2 IHC FY	HER2/CHR17 FY	Avg. HER2/Nuclei FY	HER2 Amplified FY	Heterogeneity of DISH FY	HER2/CEP17 Ratio	HER2 Per Nuclei	FISH Amplified	Heterogeneity of FISH
14	+2	1.07	2.2	no	+1	1.49	2.6	no	mosaic	1.45	3.2	no	mosaic
14 met	0	1.22	3	no	0	1.12	1.85	no	mosaic	1.64	3.6	no	mosaic
15	+1	0.85	2.75	no	+1	0.93	1.85	no	mosaic	0.949	1.85	no	mosaic
15 met	+2	1.02	2.6	no	+2	1	2.5	no	mosaic	1	2.95	no	none
16	+1	1.26	1.95	no	+1	1.18	2	no	mosaic	1.73	2.6	no	mosaic
16 met	+1	1.4	3.65	no	+1	1.05	2.15	no	mosaic	1.29	2.45	no	mosaic

HER2/CEP 17—human epidermal growth factor receptor 2/chromosome enumeration probe 17. IHC—immunohistochemistry. DISH—dual in situ hybridization. FISH—fluorescent in situ hybridization. FY—Pathologist 1. YW—Pathologist 2.

Table 2. A comparison of *HER2* status in paired hysterectomy and metastasis samples.

Cases by <i>HER2</i> Status (<i>n</i>)	Metastasis Positive	Metastasis Negative	Total
Hysterectomy positive	4	2	6
Hysterectomy negative	0	10	10
Total	4	12	16

HER2—human epidermal growth factor receptor 2.

A total of 10/16 (62%) primary hysterectomies were *HER2* non-amplified by FISH and DISH, and 6/16 primary hysterectomies were *HER2* amplified by FISH and DISH (Table 3). Of those that were *HER2* non-amplified by FISH and DISH, 8/10 cases had a *HER2* IHC score of 0 or 1+, and 2/10 cases had an equivocal *HER2* IHC score of 2+ (case 10 and 14) (Table 1). Amongst the six *HER2* amplified cases, 4/6 cases had a *HER2* IHC score of 3+, and 2/6 cases had an equivocal *HER2* IHC score of 2+ (case 7 and case 11) (Table 1).

Table 3. Summary of *HER2* status in 16 cases of paired primary and metastatic sites.

	Primary	Metastases
IHC 0/1+ and DISH/FISH negative (<i>HER2</i> negative)	8	10
IHC 0/1+ and DISH/FISH positive (<i>HER2</i> positive)	0	0
IHC 2+ and DISH/FISH negative (<i>HER2</i> negative)	2	2
IHC 2+ and DISH/FISH positive (<i>HER2</i> positive)	2	0
IHC 3+ and DISH/FISH positive (<i>HER2</i> positive)	4	4
Total	16	16

HER2/CEP 17—human epidermal growth factor receptor 2/chromosome enumeration probe 17. IHC—immunohistochemistry. DISH—dual in situ hybridization. FISH—fluorescent in situ hybridization.

Amongst the 16 corresponding metastases, 12/16 (75%) were *HER2* non-amplified by FISH and DISH, and 4/16 cases were *HER2* amplified by FISH and DISH (Table 2). Of those that were *HER2* non-amplified by FISH and DISH, 10/12 cases had a *HER2* IHC score of 0 or 1+, and 2/12 cases had an equivocal *HER2* IHC score (case 8 met and case 15 met) (Table 1). Amongst the four *HER2*-amplified cases, all had a *HER2* IHC score of 3+ (Table 1).

Furthermore, 13% of primary hysterectomies were *HER2* amplified, but their corresponding metastases were *HER2* non-amplified (case 9 and case 11) (Table 1). Of all primary hysterectomies that were *HER2* non-amplified, none of the corresponding metastases were *HER2* amplified (Table 2).

3.1. IHC Compared with DISH and FISH

Of all cases that had a *HER2* IHC score of 0 or 1+ by both pathologists, none of them were *HER2* amplified by DISH. Of all cases that had a *HER2* IHC score of 3+ by both pathologists, all of them were *HER2* amplified by FISH and DISH, thus showing 100% specificity and 100% positive predictive value (Table 1).

Of the nine cases that had a *HER2* IHC score of 2+ by the first pathologist (YW), two were positive by DISH and FISH. Of the seven cases that had a *HER2* IHC score of 2+ by the second pathologist (FY), four were positive by DISH and FISH.

3.2. Intratumoral Heterogeneity

A total of 100% of amplified cases and 95% of non-amplified cases showed heterogeneity on in situ testing (ISH). The pattern of heterogeneity (clusters, mosaic) differed between the in situ techniques, but the overall amplification scores concurred.

In terms of intratumoral heterogeneity, we found that, in both sample types, there was a significant association between the type of heterogeneity and *HER2* amplification. All

cases with mosaic heterogeneity or none were HER2 non-amplified, and only those with cluster heterogeneity were HER2 amplified. This was crucial, and the authors emphasize counting 20 contiguous tumor cells to overcome mosaic intratumoral heterogeneity to provide an accurate assessment of HER2 status.

Interestingly, different levels of heterogeneity were present, whether mosaic heterogeneity or no heterogeneity (none) were present, even between DISH and FISH techniques for the same case on the same paraffin block. This is most likely attributed to endometrial serous carcinoma being a heterogeneous tumor, and each level highlights different tumor cells of which some show isolated HER2 amplification. All 10 primary tumors and metastases with cluster heterogeneity showed the same heterogeneity between DISH and FISH scoring.

Importantly, cluster heterogeneity was also associated with discordance between primary and metastatic sites, as 2/6 primary tumors with cluster heterogeneity, amplified on FISH and DISH, showed discordance, with the metastasis showing mosaic or no heterogeneity. As a result, HER2 was not amplified in the corresponding metastasis (case 9 and 11). These two cases were taken at the same time, and thus, intratumoral heterogeneity is more likely a factor as compared to tumor evolution at the metastatic site. Therefore, it is important to test both the primary hysterectomies and the metastatic deposits as they might have different HER2 amplification due to intratumoral heterogeneity.

4. Discussion

The incidence of HER2 amplification can be influenced by numerous pre-analytical and analytical factors, including a cold ischemic time of less than 1 h and fixation in 10% neutral buffered formalin for 6 to 72 h [22]. Metastatic deposits were placed directly into formalin at the time of the procedure, and all hysterectomies were opened and fixed within the same day, although the data on time to fixation were not routinely collected at our institution. Regarding the selection of the appropriate block, in our case, we targeted the largest volume of tumor to account for intratumoral heterogeneity.

Intratumoral heterogeneity, while rare in breast cancer, was observed in endometrial serous carcinoma, even in targeted sequencing and multiplex ligation [5,23]. Gastric/GOJ Ca also shows higher intra/intertumoral heterogeneity, and the VARIANZ trial shows that more stringent criteria for calling amplification may optimize testing to predict response [24]. Currently, the data on the heterogeneity between primary hysterectomies and corresponding metastases in endometrial serous carcinoma are limited.

Rottmann et al. examined 40 paired endometrial biopsy and hysterectomy specimens [25]. Their findings revealed a concordance rate of 84% for HER2 status, indicating a moderate level of agreement [25]. However, relying solely on HER2 testing performed on the hysterectomy specimens resulted in a false-negative rate of 26.7% [25]. Alternatively, if only the endometrial biopsy was tested for HER2, the false-negative rate was 15.4% [25]. We have shown in 14/16 (87.5%) cases a concordance with paired hysterectomy specimens to the corresponding metastasis. If only the hysterectomies were tested for HER2 amplification, two out of six cases would have been falsely positive concerning the metastasis, and none would have been falsely negative. Similarly, if only the metastasis were tested, two of the twelve cases would be falsely negative compared to the hysterectomies, and none would have been falsely positive.

With respect to the results from Halle et al., they examined 35 cases of paired endometrial serous carcinomas with their corresponding metastases [19]. The scoring criteria used for HER IHC were based on the 2013 breast criteria; therefore, basolateral/lateral membranous staining was not taken into account [19]. Amplified DISH was based on an absolute number of the *ERBB2* gene of >6, borderline was 4–6, and normal was less than 4, different to current CAP recommendations [19]. All cases had IHC performed; however, only 12/35 of the metastatic cases had confirmatory DISH performed [19].

They had 20 patients that were HER2 IHC 3+ in the original tumor; nine (45%) of their corresponding metastases also demonstrated HER2 IHC 3+, of which four had additional

DISH testing [19]. Interestingly, only three out of four were amplified and concordant, and one was negative by DISH [19]. It is not known if this discordance was due to sampling or other factors, including whether it was an interpretation error in the HER2 IHC or DISH results or preanalytical factors. In our sample, all cases that were HER2 IHC3+ were HER2 amplified by DISH and FISH. The remaining 11 patients' metastases showed a range of HER2 IHC expression between 0 and 2+; three patients had DISH performed on their metastasis [19]. Of note, two cases were HER2 IHC 2+ but negative for DISH [19].

Eleven patients demonstrated HER2 IHC 2+, and four patients had HER2 IHC 1+ in their original tumor [19]. Assuming all HER2 IHC 2+ were positive, except in cases where DISH showed negative amplification, the concordance rate between primary tumor and metastasis is 19/35 (54%) [19]. On the other hand, assuming HER2 IHC 2+ were negative, the concordance rate is 21/35 (60%) [19]. A combination of the results of the current study and that of Halle et al. shows the overall concordance between paired primary tumor and metastatic samples in endometrial serous carcinoma is between 33/51 (65%) and 35/51 (68%).

From our study, we noted that all our HER2-amplified cases were either HER2 3+ or HER2 2+, and all HER2 1+ or 0 cases were not HER2 amplified.

However, Halle et al. had three metastatic samples that were HER2 IHC 3+ but negative for DISH [19]. There were no cases that were HER2 IHC negative but positive by DISH [19]. Although the authors did not explain the discrepancy in the results, many factors, including sampling and intratumoral heterogeneity, could have played a role [19]. Therefore, the combined data support DISH/FISH testing for only HER2 IHC 2+ or 3+ cases in both primary and metastatic sites, which is in line with current screening recommendations in Australia for breast cancer. However, the CAP only recommends ISH testing for IHC-equivocal cases. This might mean that there might be some false positive cases. Further studies need to be conducted to determine if reflex ISH testing should be performed only for HER2 IHC-equivocal cases in endometrial serous carcinoma, particularly if tumoral heterogeneity is a significant factor.

Our study had certain limitations, including the limited matched cases, which precluded us from conducting an extensive statistical analysis. Overall, endometrial serous carcinoma is an uncommon tumor, and we have included all suitable cases from 2010.

5. Conclusions

In conclusion, the combined results show only up to 68% concordance between HER2 status of primary hysterectomies and matched metastatic deposits. The authors recommend that both the primary and metastatic sites with HER2 IHC 2+ and 3+ be considered for HER2 ISH testing. Differences in the presence and distribution of heterogeneity between primary and metastatic sites are significant, indicating that this factor could serve as a reliable indicator of concordance. Specifically, minimal or absent heterogeneity in the primary tumor is likely to indicate a non-amplified HER2 status in the corresponding metastasis.

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Informed Consent Statement: Patient consent was waived as this study is using archival tissue and there is no treatment; there is no projected risk to patients and no ethical issues of note.

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