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Review Article

Impact of 2013 ASCO/CAP guidelines on various HER2 reporting categories in breast cancer by fluorescent *in-situ* hybridization and Immunohistochemistry: A meta-analysis with systematic review

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ABSTRACT

Objective: The ASCO/CAP guidelines for reporting HER2 in breast cancer, first released in 2007, aimed to standardize the reporting protocol, and were updated in 2013 and 2018, to ensure right treatment. Several studies have analyzed the changes attributed to 2013 updated guidelines, and majority of them found increase in positive and equivocal cases. However, the precise implication of these updated guidelines is still contentious, in spite of the latest update (2018 guidelines) addressing some of the issues. We conducted systematic review and meta-analysis to see the impact of 2013 guidelines on various HER2 reporting categories by both FISH and IHC.

Materials and Methods: After extensively searching the pertinent literature, 16 studies were included for the systematic review. We divided our approach in three strategies: (1) Studies in which breast cancer cases were scored for HER2 by FISH or IHC as a primary test concurrently by both 2007 and 2013 guidelines, (2) Studies in which HER2 results were equivocal by IHC and were followed by reflex-FISH test by both 2007 and 2013 guidelines, and (3) Studies in which trends of HER2 reporting were compared in the two periods before and after implementation of updated 2013 guidelines. All the paired data in these respective categories was pooled and analyzed statistically to see the overall impact of the updated guidelines.

Results: In the first category, by pooled analysis of primary FISH testing there has been a significant increase in the equivocal cases ($P < 0.001$) and positive cases ($P = 0.037$). We also found 8.3% and 0.8% of all the negative cases from 2007 guidelines shifted to equivocal and positive categories, respectively. Similarly by primary IHC testing there has been a significant increase in both equivocal cases ($P < 0.001$) and positive cases ($P = 0.02$). In the second category of reflex-FISH testing there was a substantial increase in the equivocal cases ($P < 0.0001$); however there is insignificant decrease (10% to 9.7%; $P = 0.66$) in the amplified cases. In the third approach for evaluating the trend, with the implementation of 2013 guidelines, there was increase in the equivocal category ($P = 0.025$) and positive category ($P = 0.0088$) by IHC. By FISH test also there was significant increase in the equivocal category ($P < 0.001$) while the increase in the positive category was non-significant ($P = 0.159$).

Conclusions: The updated 2013 guidelines has significantly increased the positive and equivocal cases using primary FISH or IHC test and with further reflex testing, thereby increasing the double equivocal cases and increasing the cost and delaying the decision for definite management. However, whether the additional patients becoming eligible for HDT will derive treatment benefit needs to be answered by further large clinical trials.

Keywords: ASCO/CAP, Breast, FISH, HER2, Immunohistochemistry

INTRODUCTION

The Human epidermal growth factor receptor 2 (HER2) oncogene, also known as c-erbB-2 (v-erb-b2 avian erythroblast leukemic viral oncogene homolog 2), is located on chromosome 17 (17q12-21.32) and is a member of the HER family. Its tyrosine kinase activity signals cellular proliferation and indirectly affects programmed cell death. Amplification of HER2 gene or its protein overexpression is associated with rapid tumor growth, increased risk of recurrence, distant metastasis, and poor clinical outcome. Because of its location on the cell membrane and its role in the pathogenesis of breast cancer, HER2 has been considered an important molecular therapeutic target.[1-4]

Testing for HER2 is essential and critical in guiding the management of breast cancer patients. Patients with HER2 positive tumors receive HER2 directed therapy (HDT), and have a better prognosis than the patients not receiving HDT for equivalent stage. It is therefore important to determine the HER2 status accurately, so as to ensure that the drug be given only in indicated patients for effective results, and spare the HER2 negative patients who are unlikely to benefit from HDT. HER2 is amplified or overexpressed in 15-20% of invasive breast carcinoma (IBC), and has both prognostic and predictive value.[1,5-7]

The ASCO (American Society of Clinical Oncology) and CAP (College of American Pathologists) guidelines for HER2 reporting was first released in 2007 to standardize the HER2 test, which has significantly contributed in improving the laboratory performance for the accurate test results. Subsequently the updated ASCO/CAP guidelines in 2013 were released with an intention for detecting and eliminating any false negative cases with better precision.[8,9]

The 2013 ASCO/CAP guidelines have a lower threshold for reporting equivocal and positive categories as compared to 2007 guidelines, using both FISH (Fluorescent in-situ hybridization) and IHC (Immunohistochemistry) tests, which have apparently resulted in increase in both these categories. Several studies in the literature have analyzed the changes attributed to the 2013 updated guidelines by both FISH and IHC; however, the precise implications of these updated guidelines are still contentious. Retesting of equivocal cases by reflex method does resolve the HER2 status and yield a minor increase in the overall positive cases, but the significantly increased prevalence of equivocal (double equivocal) cases would increase the financial burden and thereby delay the decision for the final management of patient. Moreover, as by decreasing the threshold for the HER2 positive cases and increasing the additional patients eligible for HDT therapy, whether the clinical outcome is superior in these additional cases is an important research question which has been addressed to in some studies.[10,11]

This systematic review and meta-analysis has been undertaken to see the impact of these updated 2013 ASCO/CAP guidelines on various HER2 reporting categories by both FISH and IHC, and to assess the burden of additional reflex testing, which is more relevant in developing countries with financial constraints in the health sector. The paper also attends to see the impact of revised 2013 guidelines on the performance of reflex-FISH testing in equivocal IHC category on primary HER2 testing in comparison to 2007 guidelines.

At the time of this review and meta-analysis workup, the ASCO/CAP has released a 2018 focused update to address few clinical questions raised after 2013 updated guidelines, the implications of which are still debatable in evaluation by FISH, especially in categories where the result has to be adjudicated as per internal procedures for final category characterization.[12] This may ostensibly compromise the inter-laboratory standardization and decreasing the objectivity.

MATERIALS AND METHODS

The present systematic review collected primary-level studies by following a defined search strategy with inclusion and exclusion criteria on HER2 and ASCO/CAP guidelines.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Summary of 2007 ASCO/CAP guidelines[8] for quantification of HER2 status in invasive breast carcinoma is mentioned as under:

1. A positive HER2 test by IHC (score 3+) is defined as uniform intense circumferential membrane staining of >30% of tumor cells. A positive (amplified) HER2 by FISH is reported as an average HER2 copy number of ≥6.0 signals/cell on single probe while on dual probe, HER2/CEP17 ratio ≥2.2.
2. An equivocal IHC (score 2+) is defined as complete circumferential membrane staining with weak/moderate staining within >10% of tumor cells. Equivocal FISH cases are based on single probe average HER2 gene copy number 4–6 signals/cell, and on dual probe HER2/CEP17 ratio of 1.8–2.2.
3. A negative HER2 test is defines as IHC HER2 score 0 (no staining) or score1+ with weak, incomplete membrane staining in >10% of cells. FISH negative test based on single probe is average HER2 copy number <4 signals/cell, and on dual probe HER2/CEP17 ratio of <1.8

Summary of 2013 ASCO/CAP guidelines[9] for quantification of HER2 status in invasive breast carcinoma is as under:

1. A positive HER2 test by IHC (score 3+) is defined as complete, intense circumferential membrane staining in >10% tumor cells. A positive (amplified) HER2 by FISH is reported as an average HER2 copy number of ≥6.0 signals/cell on single probe while on dual probe, HER2/CEP17 ratio ≥2.0 with an average HER2 copy number ≥4.0/<4 signals/cell or HER2/CEP17 ratio < 2.0 with average HER2 copy number of ≥6.0 signals/cell.
2. An equivocal IHC (score 2+) is defined as incomplete/complete circumferential membrane staining with weak/moderate intensity staining within >10% of tumor cells; or complete and intense circumferential membrane staining within ≤10% of cells. Equivocal FISH cases are based on single-probe average HER2 copy number ≥4.0 and <6 signals/cell and on dual probe HER2/CEP17 ratio <2 with average HER2 copy numbers ≥4 and <6 signals/cell.
3. A negative HER2 test by IHC (score1+) is considered as incomplete faint membrane staining within >10% of tumor cells or score 0 with no staining or incomplete faint membrane staining within ≤10% of tumor cells. FISH negative test based on single probe is average HER2 copy number <4 signals/cell, and on dual probe is HER2/CEP17 ratio <2 with an average HER2 copy number <4 signals/cell.

Search strategy

After extensively searching the relevant literature by using pubmed, web of science and google scholar search engines, we have come across 663 citations. After evaluating all the retrieved citations, we have selected 52 journal article abstracts and 10 abstracts from other sources like meeting abstracts/posters/conference papers. On evaluating abstracts, full texts were obtained and after reading all the full text articles, subsequently 16 full text articles were included for the systematic review.

The following search method was adopted: HER2 OR Guidelines OR HER2 guidelines OR FISH test OR IHC test OR 2007 guidelines OR 2013 guidelines OR 2007 guidelines and 2013 guidelines OR HER2 test prognostic OR HER2 test diagnostic OR HER2 equivocal OR Human Epidermal Growth Factor Receptor 2 Testing OR HER2 reflex testing OR Breast cancer HER2 testing OR breast cancer equivocal report OR impact of guidelines OR ASCO/CAP guidelines OR ASCO/CAP 2007 guidelines OR ASCO/CAP 2013 guidelines. The above keywords OR search terms were used in variety of combinations for each outcome in each of the databases.

Inclusion criteria

This study included all primary research studies fulfilling the following criteria: (i) studies highlighting the comparison of 2007 v/s 2013 HER2 reporting guidelines by IHC and FISH test for primary HER2 reporting in consecutively diagnosed cases of invasive breast cancer, through paired data, highlighting the shift in the categories with updated 2013 guidelines, that were published in English, (ii) studies showing comparison of 2007 v/s 2013 HER2 reporting in breast cancer patients who underwent reflex-FISH testing (after initial IHC equivocal results) with paired data, highlighting the shift in the categories with updated 2013 guidelines (iii) Studies where the HER2 reporting trend was evaluated following 2007 and 2013 scoring guidelines, but in a different set of samples and period to observe the distribution trend of various HER2 categories (iv) either in hospital- or community-based setting, (v) a total study sample size of at least 100, and vi) Comparison studies done by IHC and FISH only.

Exclusion criteria

Case reports, case series, earlier reviews, HER2 testing by any other method than IHC/FISH, published in a language other than English were excluded. Qualitative studies and descriptive studies were also excluded.

Study records and data management

Selection process

This study has been conducted and reported in accordance with the PRISMA guidelines. Checklist on the search items has been included and the review was represented in a flowchart [Figure 1]. The analytical results of the study have been presented in the tables and figures as mentioned in index.

Two authors, SP and UK, independently carried out the literature search and identified 663 citations for HER2 guidelines. Full text articles were identified and assessed for eligibility after applying the inclusion and exclusion criteria. Critical appraisal of each study that found eligible was done by both investigators. Agreement of the requisite contents of the articles related to quality assessment and data extraction was performed. Any dispute in selection was resolved by third author (SA) after deliberation with SP and UK.

The studies thus collected were segregated as per the three categories mentioned in the inclusion criteria. A summary was prepared according to the WHO's regional classification of countries. The study location along with the period of study, study design, sample size, study setting and case ascertainment for each primary study is enlisted in Web Table 1. Most of the study settings based in a single hospital, with a few involving multiple-hospitals. Majority of the studies used FISH testing as primary method for HER2

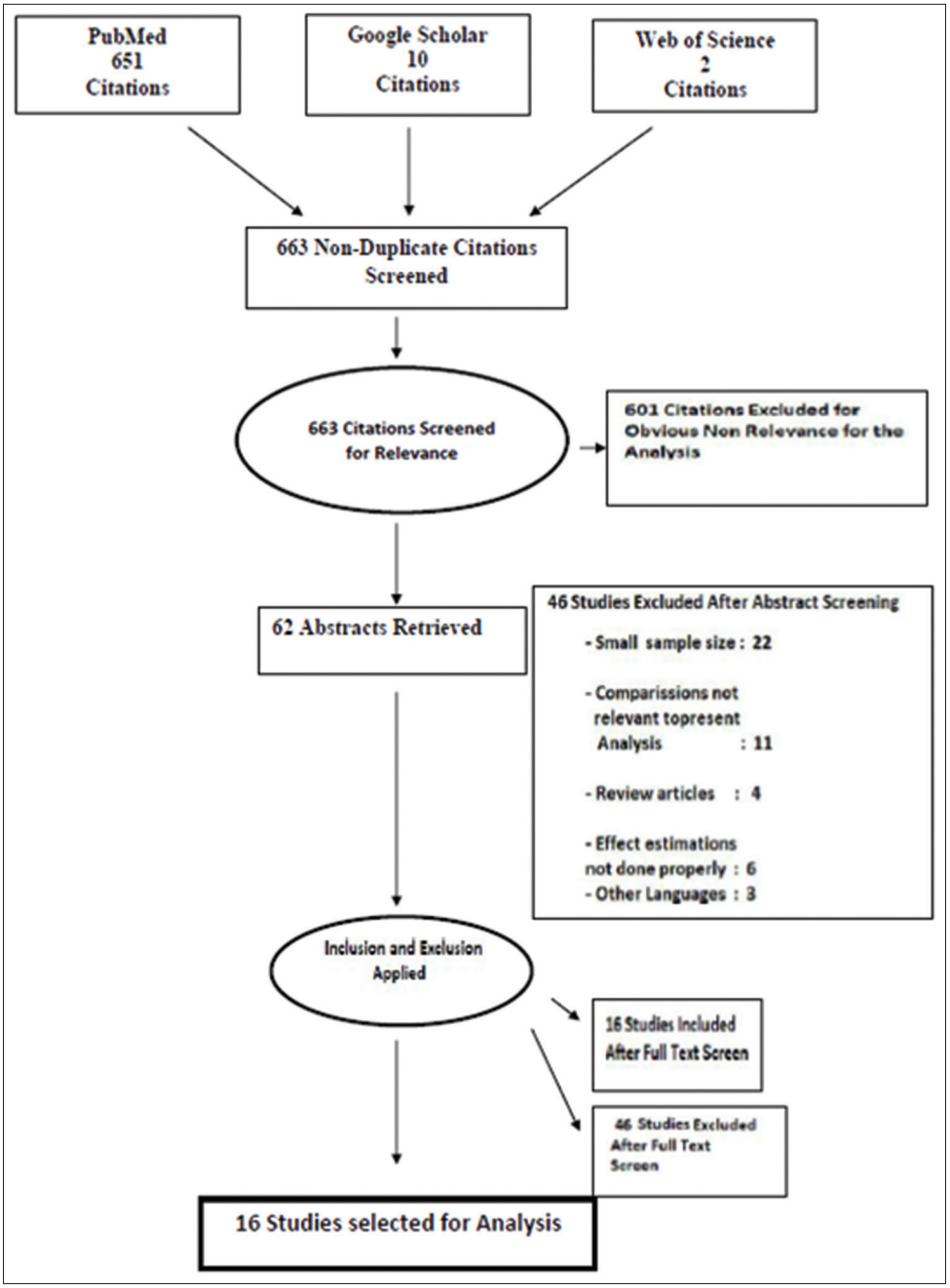

Figure 1: Prisma chart.

evaluation, while three studies have used IHC as primary testing modality.

We categorized the presentation of total studies in three different strategies/categories. The first strategy comprised of summarization of all studies in which the breast carcinoma cases were scored by FISH/IHC for primary HER2 reporting (paired responses) by both the guidelines (ASCO/CAP 2007 & 2013 guidelines), and have available data to evaluate shift in the trend. The second strategy comprised of all studies in which the breast carcinoma cases were scored for reflex-FISH (referred after equivocal IHC results) HER2 reporting by both the guidelines (ASCO/CAP 2007 & 2013 guidelines) and have results for comparison along with available data to evaluate the shifting trends in all the reporting categories as per the reclassification. The third strategy comprised of evaluating the impact on the trends and distribution of HER2 reporting categories before and after the implementation of updated ASCO/CAP 2013 guidelines in two periods both by FISH and IHC.

Data analysis

All the paired data in the respective 3 categories were pooled to evaluate the impact of the updated 2013 guidelines in HER2 reporting and analyzed statistically, to see the overall impact of the updated 2013 guidelines. Paired analysis of primary FISH/IHC testing (strategy I) and reflex-FISH testing (strategy II) was done by pooling of data from available studies. Shifting of cases from negative (as per 2007 guidelines) to equivocal and positive category (as per 2013 guidelines) is seen in paired data set. Similarly shifting of the cases from equivocal to negative or positive category is seen in paired data set. All statistical data was analysed using MedCalc Statistical Software version 16.4.3 (MedCalc Software bvba, Ostend, Belgium;https://www.medcalc.org; 2016).

RESULTS

Total 16 studies[11,13-27] were reviewed; of these there were 20 estimates, which has been summarized in web Table 1. This table highlights frequency and percentage of various HER2 reporting categories by FISH and IHC method according to both 2007 and 2013 guidelines.

In the first category we compared the results of pooled sample of 9074 cases from seven studies[11,13-18] in which the primary HER2 testing was performed by FISH and the cases were concurrently classified by both 2007 and 2013 ASCO/CAP guidelines [Table 1a-c]. As clearly evident, with updated 2013 guidelines, using primary FISH reporting, there has been a substantial increase (1.7 to 6.5%) in the equivocal cases (P = <0.001) [Table 1b] and significant increase (22.6 to 23.9%) in the HER2 amplified cases (P = 0.037) [Table 1c]. Hence 2013 guidelines selected more patients qualifying for HDT along with substantial increase in the reflex testing. There was good overall concordance between guidelines.

Table 1a: Concordance between HER2 reporting categories by Primary FISH testing as per 2007 and 2013 ASCO/CAP guidelines.

FISH	2007			
	Negative	Equivocal	Positive	Total
2013				
Negative	6271	39	0	6310 (69.6%)
Equivocal	547	44	0	591 (6.5%)
Positive	51	68	2054	2173 (23.9%)
Total	6869 (75.7%)	151 (1.7%)	2054 (22.6%)	9074

*Pooled analysis included six studies (11, 13-18)

We performed meta-analysis on proportions and found that 8.3% and 0.8% of all the negative cases as per 2007 guidelines shifted to equivocal category (95% C.I.:5.8 to 11.2) and positive category (95% C.I.: 0.4 to 1.5) respectively, as per 2013 guidelines on reclassification (Web Tables 2 and 3; Figures 2 and 3).

Of all the equivocal cases from 2007 guidelines, 70.5% shifted to negative or positive category [Figure 4] on reclassification

Table 1b: HER2 reporting by primary FISH; Equivocal cases versus others (Negative and Positive) according to 2007 & 2013 ASCO/CAP guidelines.

	Equivocal	Negative+Positive	Total
2007	151 (1.7%)	8923 (98.3%)	9074
2013	591 (6.5%)	8483 (93.5%)	9074

Equivocal cases shift: 1.7% to 6.5% increase; P value <0.001, *Pooled analysis included six studies (11, 13-18)

Table 1c: HER2 reporting by primary FISH; Positive versus others (Equivocal and Negative) according to 2007 & 2013 ASCO/CAP guidelines.

	Positive	Equivocal+Negative	Total
2007	2054 (22.6%)	7020 (77.4%)	9074
2013	2173 (23.9%)	6901 (76.1%)	9074

Positive cases shift: 22.6% to 23.9% increase; P value=0.037. *Pooled analysis included six studies (11,13-18).

Table 1d: Concordance between HER2 reporting categories by Primary IHC as per 2007 & 2013 ASSO/CAP guidelines.

Pooled IHC	2007			
	Negative	Equivocal	Positive	Total
2013				
Negative	203	0	0	203 (19.8%)
Equivocal	126	605	0	731 (71.2%)
Positive	1	27	65	93 (99.1%)
Total	330 (32.2%)	632 (61.5%)	65 (6.3%)	1027

*Analysis included three studies (14, 21, 27)

Table 1e: HER2 reporting by primary IHC: Equivocal versus others (Negative and Positive) according to 2007 & 2013 ASCO/CAP guidelines.

	Equivocal	Negative+Positive	Total
2007	632 (61.5%)	395 (38.5%)	1027
2013	731 (71.2%)	296 (28.8%)	1027

Equivocal cases shift: 61.5% to 71.2% increase; P value <0.001. *Analysis included three studies (14, 21 and 27)

(2013 guidelines) with 95% C.I. (62.4 to 78) (Web Table 4; Figure 4).

We also analyzed the results of pooled sample of 1027 cases from three studies[14,21,27] in which the primary HER2 testing was performed by IHC and the cases were concurrently classified by both 2007 and 2013 ASCO/CAP guidelines [Table 1d]. On reclassification as per 2013 guidelines, there has been a significant increase in the equivocal cases (61.5 to 71.2%; $P < 0.001$) and positive cases (6.3 to 9.1%; $P = 0.02$) [Table 1e and f].

Table 1f: HER2 reporting by primary IHC: Positive versus others (Equivocal and Negative) according to 2007 & 2013 ASCO/CAP guidelines.

	Positive	Equivocal+Negative	Total
2007	65 (6.3%)	962 (93.7%)	1027
2013	93 (9.1%)	934 (90.9%)	1027

Positive cases shift: 6.3% to 9.1% increase; *P*-value=0.02045. *Analysis included three studies (14, 21 and 27)

On analyzing the second strategy/approach on reflex-FISH testing (referred after equivocal IHC results), we compared the results of pooled paired sample of 2423 cases from four studies[19-22] in which reflex-FISH HER2 testing was performed and were concurrently classified by both 2007 and 2013 ASCO/CAP guidelines [Table 2a-2c]. As clearly evident, with updated 2013 guidelines there was a massive increase (3.5% to 22.6%) in the equivocal cases ($P < 0.0001$); however there is a mild decrease (10.1% to 9.7%) in the amplified cases ($P = 0.66$). Hence, updated 2013 guidelines result in substantial increase in equivocal cases and eventually double-equivocal cases, undergoing reflex-FISH testing.

We performed the meta-analysis on proportions and found that 24.7% and 0.9% of all the negative cases as per 2007 guidelines shifted to equivocal category (95% C.I.: 0.2 to 78.2) and positive category (95% C.I.: 0.4 to 1.6), respectively,

Table 2a: Concordance between HER2 reporting categories by Reflex-FISH test as per 2007 & 2013 ASCO/CAP guidelines.

Overall Reflex-FISH	Negative	Equivocal	Positive	Total
Negative	1559	21	61	1641 (67.7%)
Equivocal	518	29	0	547 (22.6%)
Positive	16	35	184	235 (9.7%)
Total	2093 (86.4%)	85 (3.5%)	245 (10.1%)	2423

*Analysis included 4 studies (19-22)

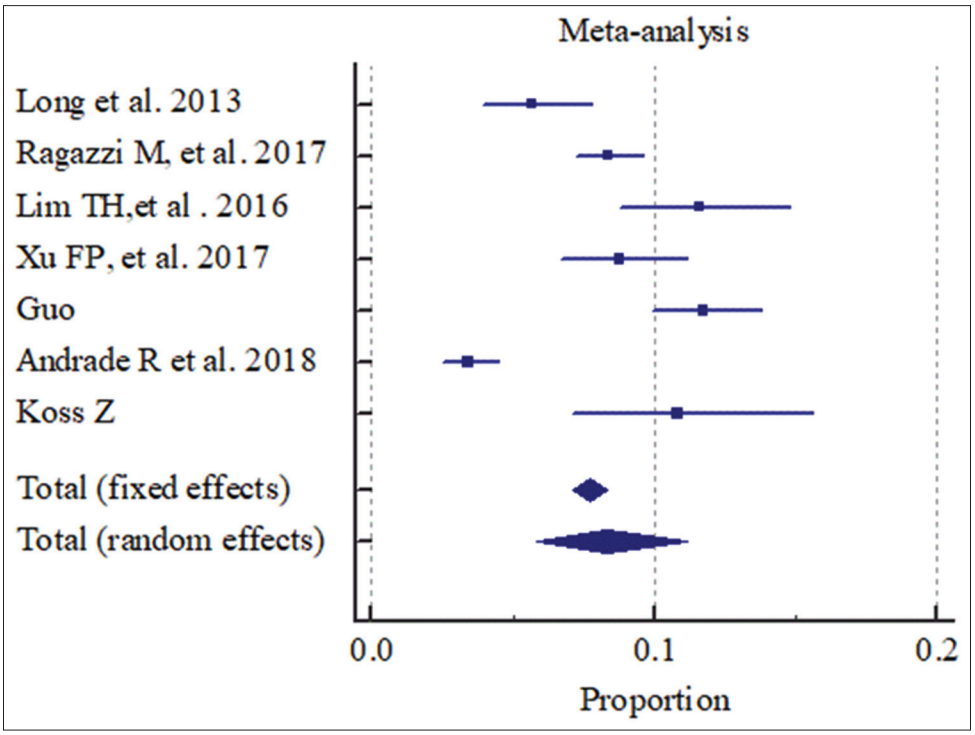

Figura 2: Forest plot for proportion of Negative cases in 2007 shifting to Equivocal in 2013 (Primary FISH).

Table 2b: HER2 reporting by Reflex-FISH: Equivocal versus others (Negative and Positive) according to 2007 & 2013 ASCO/CAP guidelines.

	Equivocal	Negative+Positive	Total
2007	85 (3.5%)	2338 (96.5%)	2423
2013	547 (22.6%)	1876 (77.4%)	2423

Equivocal cases shift: 3.5% to 22.6% increase; *P*-value <0.0001. *Analysis included 4 studies (19-22)

Table 2c: HER2 reporting by Reflex-FISH: Positive versus others (Equivocal and Negative) according to 2007 & 2013 ASCO/CAP guidelines.

	Positive	Equivocal+Negative	Total
2007	245 (10.1%)	2178 (89.9%)	2423
2013	235 (9.7%)	2188 (90.3%)	2423

Positive cases shift: 10.1% to 9.7% (decrease); *P*-value=0.66. *Analysis included 4 studies (19-22)

as per 2013 guidelines on reclassification (Web Table 5 and 6; Figure 5 and 6).

Of all the equivocal cases of reflex-FISH testing from 2007 guidelines, proportion of cases shifting to negative or positive category is 65.4% on reclassification (2013 guidelines) with 95% C.I.:55.3 to 74.9 (Web Table 7, Figure 7).

We evaluated as per the third approach and compared the trends of HER2 reporting categories in two periods before and after the implementation of updated ASCO/CAP 2013 guidelines both by FISH and IHC. The results are shown in Table 3a and b. In IHC category, data of 3 studies[23-25] were pooled with effective total sample size was 3056 (cases reported by 2007 guidelines) and 1554 (cases reported by 2013 guidelines). The increase in the equivocal category was from 23.7 to 26.7 % (*P* value: 0.025) while the increase in the positive category was from 12.9 to 15.8 % (*P* value: 0.0088), [Table 3a].

In FISH reporting category, data of 3 studies[23,25,26] were pooled with effective total sample size was 2811 (cases

Table 3a: Comparison of trends of HER2 reporting by IHC in the two periods (before and after implementation of 2013 ASCO/CAP guidelines).

Pooled IHC samples	Negative (0/1+score)	Equivocal (2+score)	Positive (3+score)	Total
2007	1937	723	396	3056
2013	893	415	246	1554
P-value	0.00010	0.025	0.0088	

*Analysis included 3 studies (23-25)

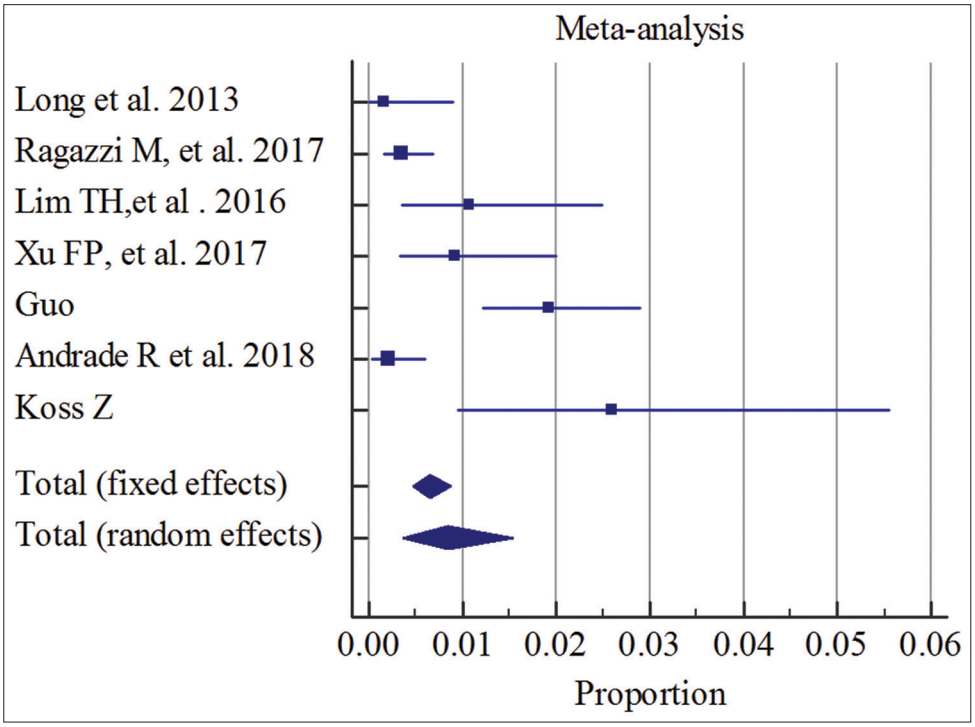

Figure 3: Forest plot for proportion of Negative cases in 2007 shifting to Positive in 2013 (Primary FISH).

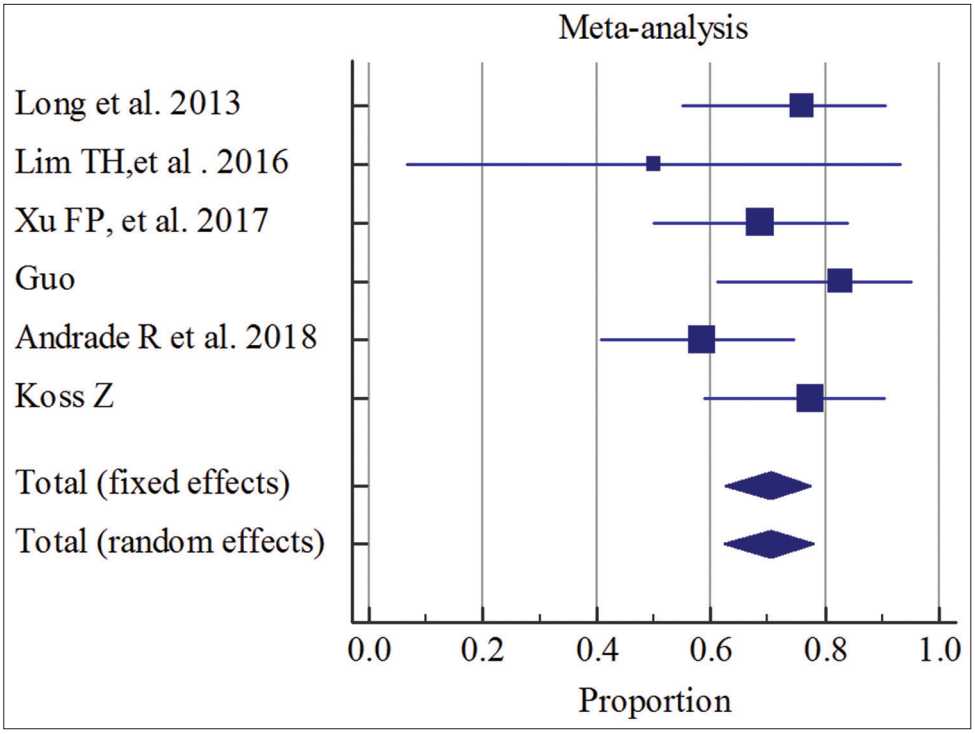

Figure 4: Forest plot for proportion of Equivocal cases in 2007 shifting to Negative or Positive in 2013 (Primary FISH).

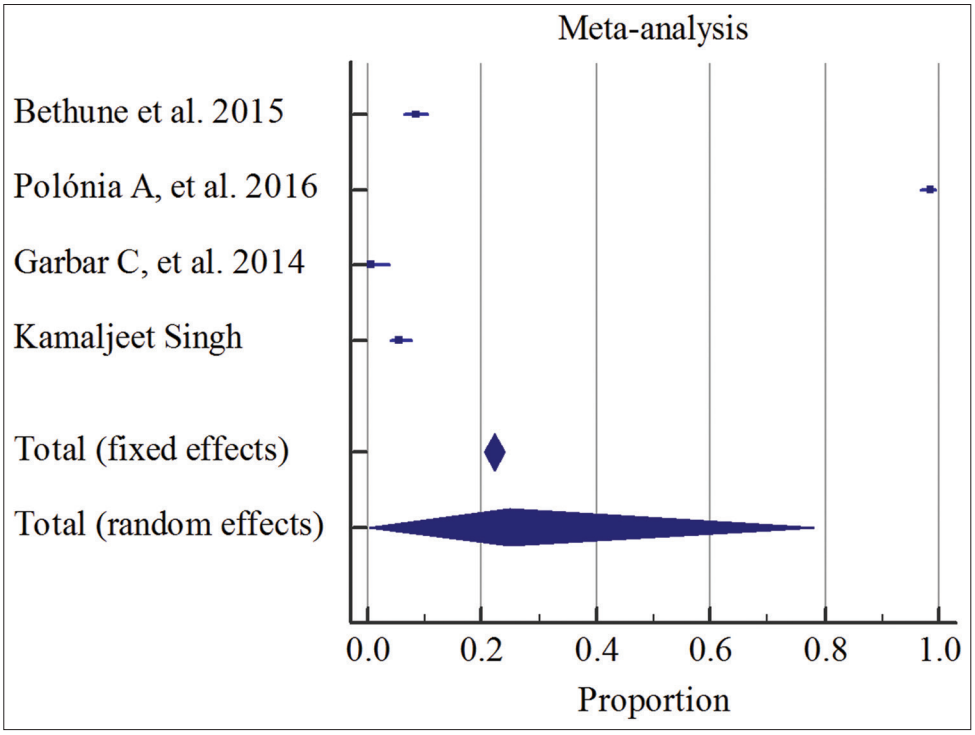

Figure 5: Forest plot for proportion of Negative cases in 2007 shifting to Equivocal in 2013 (Reflex-FISH).

reported by 2007 guidelines) and 1284 (cases reported by 2013 guidelines). The increase in the equivocal category was from 0.7 to 5.7 % ($P < 0.001$) while the increase in the positive category was from 12.5 to 14.2 % ($P = 0.159$), [Table 3b].

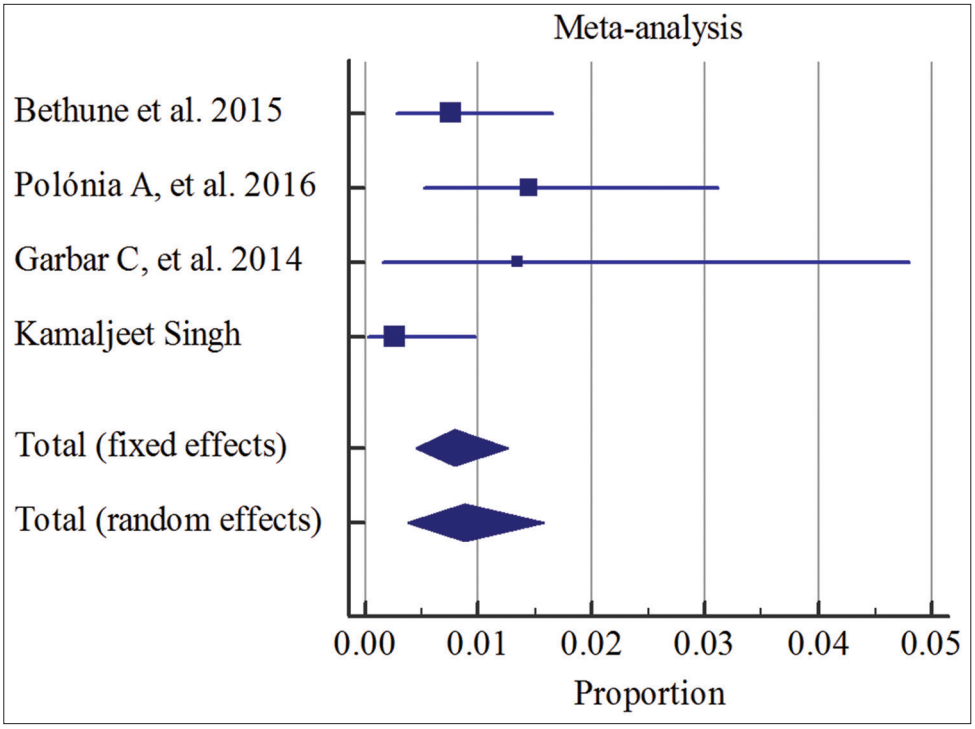

Figure 6: Forest plot for proportion of Negative cases in 2007 shifting to Positive in 2013 (Reflex-FISH).

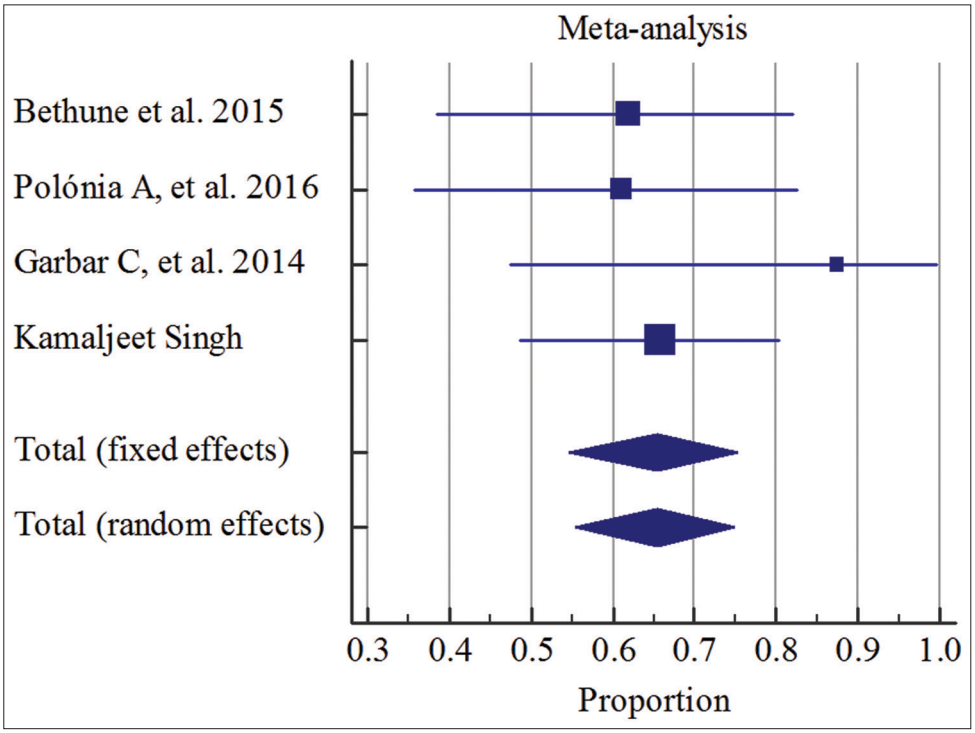

Figure 7 : Forest plot for proportion of Equivocal cases in 2007 shifting to Negative or Positive in 2013 (Reflex-FISH).

Therefore, on evaluating the trend of HER2 reporting categories, with updated 2013 guidelines there is a significant increase in equivocal cases by both IHC and FISH; while significant increase in the positive cases occurred by IHC and

Table 3b: Comparison of trends of HER2 reporting by FISH in the two periods (before and after implementation of 2013 ASCO/CAP guidelines by FISH.

Pooled FISH samples	Negative	Equivocal	Positive	Total
2007	2438	19	352	2811
2013	1015	73	182	1284
P-value	<0.001	<0.001	0.159	

*Analysis included 3 Studies (23,25,26)

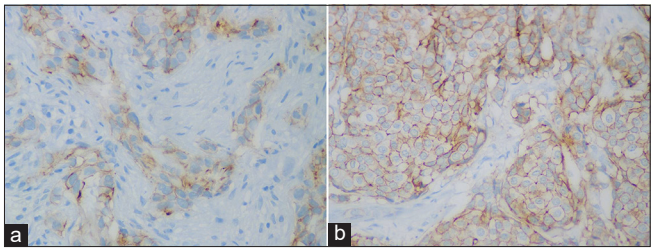

Figure 8: (a) HER2 immunostain shows incomplete partial membranous staining with weak to moderate intensity which is score 1+ (negative) as per 2007 ASCO/CAP guidelines and 2018 focused update. This staining pattern is reminiscent of score 2+ (equivocal) as per 2013 guidelines (DAB; x 200). (b) HER2 immunostain shows complete circumferential membranous staining, of weak to moderate intensity in more than 10% of cells, which is score 2+ (equivocal) as per 2018 ASCO/CAP focused update guidelines. (DAB; x 400).

not by FISH. Figures 8a and 8b depict HER2 IHC staining score 2+ as per 2013 and 2018 ASCO/CAP focused update guidelines, respectively.

DISCUSSION

The 2007 ASCO/CAP guidelines have appreciably standardized the HER2 testing which has resulted in selecting the patients for HDT in more reliable and accurate manner and has also increased the proficiency testing worldwide. The newer HER2 targeted drugs are more expensive, and have their own dose-related toxicities; therefore the 2013 updated guidelines for accurate HER2 testing which strives to ensure the right patient selection for HDT. The 2013 updated ASCO/CAP guidelines were released to select additional patients who could derive benefit from HDT.[3,13,28-33]

Many studies have assiduously evaluated the impact of 2013 ASCO/CAP guidelines as against 2007 guidelines for HER2 testing by FISH or IHC. The precise impact of these guidelines in clinical practice is still controversial. We performed a systematic review and meta-analysis of all primary studies highlighting the comparisons of HER2 reporting by FISH and IHC by 2007 & updated 2013 guidelines.

On evaluation of the pooled data of 7 studies,[11,13-18] which comprised of 9074 cases subjected to primary HER2 FISH testing, and concurrently classified by both 2007 and 2013 ASCO/CAP guidelines, a substantial increase in the equivocal and amplified cases has been seen with updated 2013 guidelines. Hence the updated (2013) guidelines selected more patients for HDT along with increase in the reflex testing and double equivocal cases, thereby increasing the cost and delaying the definite status.

All the seven pooled studies have shown an increase in overall positivity in HER2 testing by 2013 updated guidelines. Guo *et al*.[16] in his study reported that 90.6% (125 out of 138) of the equivocal cases by 2013 FISH guidelines were also IHC equivocal (2+). These double equivocal cases (n = 125) included 121 cases that were negative, and 4 cases that were equivocal by 2007 guidelines. Updated guidelines resulted in 1.7% increase in overall HER2 positivity while 6.1% increase in equivocal cases.

Ragazzi *et al*.[11] found that 58.9% (112 out of 190) of the equivocal cases by 2013 guidelines, were solved by reflex IHC and the rest remained double equivocal without any other additional testing.

Xu *et al*.[15] also found significant increase in FISH equivocal results with updated 2013 guidelines, and reflex testing could clarify the HER2 status in approximately 50% of the cases.

We came across few other primary FISH testing studies in the literature in which paired data was not available to highlight the shift in categories, and so were not included in meta-analysis. They also showed significant increase in the in the equivocal and amplified cases with updated 2013 guidelines.[3,10]

We also analyzed the pooled data of 3 studies,[14,21,27] comprising of total 1027 cases in which paired data was available for evaluating the HER2 status by primary IHC testing. They also demonstrated a significant increase in equivocal cases and positive cases, although with some contrast results among individual studies [Table 1d]. Garbar *et al*.[21] evaluated 186 patient samples and found substantial increase in equivocal (40/186 versus89/189; P = 0.001) and positive (09/186 versus 26/189; P = 0.001), and found a slight decrease in false negative cases (3/186 versus 1/186) with implementation of 2013 guidelines. Study by Lim *et al*.[14] showed the result at variance regarding the equivocal category. They evaluated 590 cases and found a slight decrease in equivocal cases (86.8% to 85.3%; P = 0.45) and increase in positive cases (4.9% to 6.4%; P = 0.26) with implementation of 2013 guidelines.

Few other studies which evaluated HER2 IHC have shown that a significant proportion of score1+ IHC by 2007 guidelines has shifted to IHC 2+ (equivocal) with implementation of updated 2013 guidelines, thereby significantly increasing reflex FISH testing with no significant additional case eligible for HDT.

Lambein *et al*.[34] showed that with implementation of 2013 guidelines about 18.7% (20/107) of cases changed from IHC 1+ to IHC 2+, and these additional 20 equivocal cases yielded only single FISH amplified (5%) and single (5%) equivocal case on reflex FISH test.

Pannacchia *et al*.[35] also found marked increase (11.4% to 18.9%) in IHC 2+ cases, with post 2013 updated guidelines. However on further analysis of IHC 2+ category (2013 guideline) they detected additional 10 cases eligible for treatment with anti-HER 2 therapy and this correspond to one of the major goals expected by updated 2013 ASCO/CAP guidelines.

Rakha *et al*.[36] stated that updated ASCO/CAP 2013 guidelines modified the criteria for equivocal (2+) IHC category by including weak/moderate incomplete membranous staining in more than 10% of tumor cells, which was considered negative in previous guidelines. The author states that no evidence has been provided for such a change, which would result in significant proportion of reflex testing with financial implications. The ASCO/CAP panel[37] responded to this issue, that 2013 guidelines were intended to reduce the false-negative results (by increasing the sensitivity), taking into consideration the longer term confirmation of the survival benefit achieved by anti-HER2 therapy.

On analyzing the second category, where the pooled data of 4 studies[19-22] comprising of 2423 cases [Table 2] which were subjected to reflex-FISH testing, there has been a prodigious increase (3.5 to 22.6%) in the equivocal cases ($P < 0.0001$); however there is a mild decrease (10% to 9.7%) in the amplified cases ($P = 0.66$), with the implementation of 2013 updated guidelines. Hence the updated guidelines results in substantial increase in equivocal cases and eventually double-equivocal cases undergoing reflex-FISH testing; thereby increasing the dilemma in targeted treatment decision and significantly increasing the cost involved with the reflex testing especially in financially and resource constrained countries.

Few studies have analyzed reflex-FISH testing concurrently by both guidelines in which paired data was not available to highlight the shift in categories, and not included in this meta-analysis, yet has shown significant increase in the in the equivocal cases and positive cases with updated 2013 guidelines.[38,39]

The ASCO/CAP 2013 guidelines also suggested the use of alternative ISH chromosome 17 probe as a reflex testing for the initial FISH equivocal cases and on calculating the HER2/alternate probe ratio. If the ratio is >2, the test should be considered positive.[9] However the precise implications of additional cases detected by using these alternate probes is still abstruse and contentious due to lack of consensus guidelines for reporting HER2 with alternate probes.[7,10,11,38,40,41]

The strength of the undertaken study was to highlight the shifting of the various reporting categories of HER2 with the implementation of the 2013 ASCO/CAP updated guidelines along with statistical evaluation in the pooled data. Regarding critic of our study, we could not evaluate and comment upon the therapeutic advantage of the additional patients who became eligible for the HDT with updated 2013 guidelines.

Although there has been a good concordance between IHC and FISH for testing, the preferred method still remains controversial. Few authors suggest FISH as a preferred primary modality as it is more reproducible, and standardization of IHC is problematic leading to some false negative results in HER2 0/1+ category.[42-46] Accurate determination of HER2 status is crucial for optimizing breast cancer outcomes for which standardization of guidelines and testing algorithm and regular participation in proficiency testing is essential.[47]

CONCLUSION

To summarize and conclude, the updated 2013 guidelines has significantly increased the equivocal cases by both primary FISH and IHC testing and eventually increased the double equivocal cases, and has proven to detect more patients eligible for anti-HER2 therapy and identifying patients with equivocal results who may potentially benefit from HDT. However further large prospective clinical trials are essential to address the treatment benefit in additional patients becoming eligible for anti-HER2 therapy so as to ensure that the right patient receives the right treatment and hence to optimize and balance the cost effectiveness and treatment outcomes. However at the time of doing this systematic review and meta-analysis the ASCO/CAP has released 2018 focused update recommendations as the need was felt since 2015. To address the issues from the previous guidelines, the 2018 ASCO/CAP focused update guidelines[12] have proposed new revised definition of IHC 2+ (equivocal): weak to moderate complete staining in >10% of the cell. In FISH reported cases with HER2/CEP17 ratio < 2 and HER2 signals/cell: 4-6 (equivocal as per 2013 guidelines), a definite diagnosis has to be rendered based on additional workup. The 2018 guidelines also modified the interpretation of FISH reported cases having HER2/CEP17 ratio >2 with less than 4 HER2 signals/cell (positive as per 2013 guidelines) to be considered as negative (if no change in result provided by additional workup). Hence, 2018 focused update will likely decrease the IHC 2+ (equivocal) cases, however the impact on FISH category is unpredictable especially in categories where the result has to be adjudicated as per internal procedures for final category characterization, will ostensibly compromise the inter-laboratory standardization and decreasing the objectivity. The precise impact of currently released 2018 focused update will take few years to evaluate.

Declaration of patient consent

A general informed consent was taken from the patient regarding sharing of clinical data for research purpose. All

the patients' information in the manuscript is anonymised and only de-identified data is used.

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Conflicts of interest

There are no conflicts of interest.

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