

Belgian guidelines for HER2 testing in gastric cancer

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As HER2 testing in gastric cancer is different from that in breast cancer, the Working Group Molecular Pathology, a section of the Belgian Society of Pathology in collaboration with the Belgian Club of Digestive Pathology, the Belgium Group of Digestive Oncology and the Belgium Society of Medical Oncology, formed a committee to establish Belgian guidelines for HER2 testing in gastric cancer. These guidelines are based on the international recommendations and take the Belgian criteria for reimbursement of trastuzumab into account. The aim of these guidelines is to report the consensus of when and how HER2 testing should be performed in gastric cancer in order to establish strategies for ensuring optimal performance, interpretation and reporting assays.

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Introduction

Human epidermal growth factor receptor 2 (HER2, ErbB2, Neu) is a 185-kDa transmembrane tyrosine kinase receptor. It is a member of the EGFR family and plays a central role in growth factor signal transduction. HER2 overexpression/amplification is implicated in the development of various solid tumours and plays a pivotal role in oncogenic transformation and tumorigenesis.¹ The HER2 signaling pathway represents a major therapeutic target.

It is well known that, in breast cancer, HER2 overexpression/amplification is associated with aggressive tumour growth, poor prognosis and an increased risk of disease recurrence.^{2,3} Moreover, in breast cancer, HER2 overexpression/amplification is a predictive marker for targeted therapy with the monoclonal

antibody trastuzumab, a fully humanised anti-HER2 monoclonal antibody.^{4,5}

HER2 overexpression, however, has not only been reported in breast cancer, but also in ovarian, lung, endometrium, salivary duct, colon and gastric cancer.⁶⁻⁸ In gastric cancer overexpression/amplification of HER2 were first described already in 1986.⁹ In conjunction with the knowledge obtained from breast cancer, this leads to experiments to investigate an antitumor effect of anti-HER2 antibodies in gastric cancer. A growth inhibitory effect of trastuzumab was demonstrated in vitro in cultured human gastric tumour cell lines overexpressing HER2 and in vivo in these cell lines growing as xenografts.¹⁰ As long ago as 1992, combined chemotherapy (capecitabine/cisplatin) in a xenograft model with trastuzumab

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was reported to have a remarkable tumour growth inhibitory effect compared to chemotherapy alone.⁹ Subsequently, a few case studies showing successful treatments with trastuzumab in gastric cancer, as well as abstracts on ongoing pilot/phase II studies suggesting an inhibitory effect of trastuzumab on gastric cancer were presented.¹¹ Most information concerning the effect of trastuzumab on gastric cancer growth, however, came from the Trastuzumab for Gastric cancer (ToGA) trial, an open-label randomized multicenter phase III study conducted in 24 countries (Asia, Australia, Europe, South and Central America, South Africa). In this trial trastuzumab was added to chemotherapy (fluoropyrimidine, either 5-FU or capecitabine/cisplatin) in patients with advanced gastric or gastro-oesophageal junction cancer (n=584) with overexpression /amplification of HER2. The primary endpoint was overall survival (OS). The combination of chemotherapy plus trastuzumab was shown to be statistically superior to chemotherapy alone, with an increased median overall survival of nearly 3 months (OS 13.8 versus 11.1 months without trastuzumab). Moreover, increased benefit from trastuzumab was seen in patients who had higher levels of HER2 protein expression, including subgroups with IHC score 2+/FISH + and IHC score 3+. In these patients median overall survival increased from 11.8 months for the chemotherapy treatment arm to 16.0 months for the chemotherapy with trastuzumab arm. The combination of chemotherapy plus trastuzumab prolonged also the progression-free survival and increased the response rate.¹²

In addition to showing the efficacy of trastuzumab in gastric cancer patients, analysis of tumour samples of patients enrolled in the ToGA trial confirmed the previous observation that HER2 overexpression is found in approximately 15-25% of cases of advanced gastric and gastro-oesophageal junction cancer, and that there is a strong correlation with tumour type and location in the stomach.^{9,13-15} Indeed, HER2 overexpression/amplification is described in 33% of gastro-oesophageal adenocarcinoma and in 21% of gastric cancers, in 32.2% of adenocarcinomas of intestinal type, in 20.4% of mixed type and in only 6.1% of diffuse type.¹⁶ The virtual absence of HER2 overexpression in the diffuse type of gastric cancer is support for the idea of substantial molecular differences between the different histological tumour

types which may develop through different molecular alterations. For example, reduced or abnormal E-Cadherin expression is a molecular feature strongly linked to the diffuse type of gastric cancer.¹⁷

Following the presentation of the ToGA trial results, the EMA (European Medicines Agency) reported on December 17th 2009 the approval of trastuzumab (Herceptin[®]) for the treatment of advanced/metastatic adenocarcinoma of the stomach or gastro-oesophageal junction, in combination with capecitabine or 5-fluorouracil (5-FU) and cisplatin, for patients who have not received prior anti-cancer treatment for their metastatic disease. The EMA limited the approval to patients whose tumours have HER2 overexpression as defined by HER2 IHC score 2+, confirmed by a positive FISH, or by HER2 IHC score 3+, as shown by an accurate and validated assay.¹⁸ This is different from the Belgian reimbursement criteria published in the *Moniteur Belge/Belgisch Staatsblad* on September 20th 2010 which stated that trastuzumab can be given in combination with capecitabine/5-FU and cisplatin to not previously treated patients with metastatic carcinoma of the stomach or gastro-oesophageal junction. For reimbursement of trastuzumab in Belgium, amplification of HER2 should be demonstrated by positive ISH test (FISH/SISH/CISH). The test should be performed in a laboratory that can prove validation of the test and can prove that the result is considered positive if HER2/CEP17 ratio ≥ 2 .

Accurate assessment of HER2 status is essential to determine which patients are eligible for trastuzumab treatment. This requires optimal tissue handling and standardized immunohistochemical staining (IHC) and in situ hybridisation (ISH) for amplification testing. As HER2 testing in gastric cancer is different from breast cancer, the scientific societies and the Working Group Molecular Pathology formed a committee to establish guidelines for HER2 testing for gastric cancer in Belgium. The aim of these guidelines is to report the consensus of when and how HER2 testing should be performed in gastric cancer in order to establish strategies for ensuring optimal performance, interpretation and reporting assays.

When to perform HER2 testing?

HER2 testing should be performed on request of the oncologist.

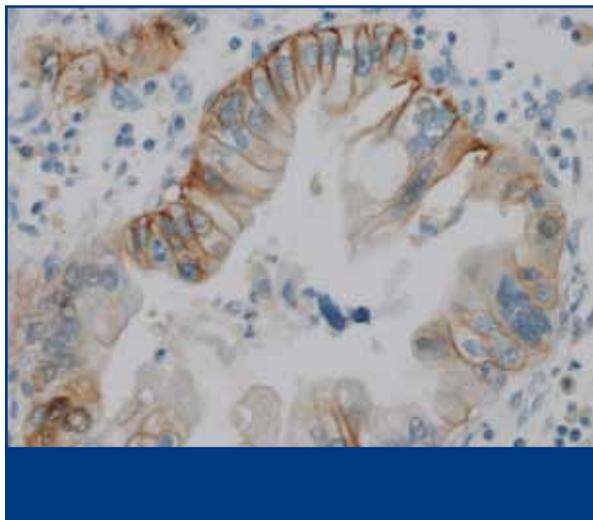


Figure 1. Incomplete membrane positivity in HER2 IHC in gastric cancer (lateral and basolateral staining) (x20).



Figure 2. Heterogeneity for HER2 IHC in gastric cancer (x5).

How to perform HER2 testing?

In the ToGA trial (registration trial) to be considered eligible for trastuzumab treatment IHC score 3+ and/or amplification with HER2/CEP17 ratio ≥ 2 as determined by FISH was required.¹² Criteria for HER2 scoring within the phase III ToGA trial were essentially based on a separate validation study (so-called pre-ToGA) in which protein expression as determined by immunohistochemistry (IHC) was correlated with gene amplification as determined by fluorescence in situ hybridisation (FISH) in a series of 168 gastric cancer resection specimens.¹⁴ In all cases IHC and FISH were performed in parallel. All cases with discordant IHC/FISH results were re-evaluated. This analysis showed that HER2 overexpression in gastric cancer is different from breast cancer and a consensus was established to modify the breast scoring system for HER2 IHC, taking the differences between HER2 overexpression in gastric cancer and breast cancer into account. By applying the rules for scoring HER2 IHC in breast cancer to gastric cancer, we would deny therapy to a considerable number of gastric cancer patients appearing to be responders to trastuzumab therapy.^{14,15,19} The most important differences between breast and gastric cancer HER2 testing are the incomplete membrane positivity (lateral or basolateral membrane staining) and the heterogeneity of the overexpression/amplification of HER2 in gastric cancer.

Incomplete membrane positivity

Breast cancer most often consists of solid nests of tumour cells and HER2 IHC positivity requires complete membranous staining creating a chicken wire pattern.^{20,21}

In gastric cancer, however, lateral membranous staining with linear staining at contact sites between 2 cells or basolateral membranous staining creating a U-shaped staining pattern is observed (Figure 1). This is due to the higher frequency of glandular formations with luminal in gastric cancer (intestinal type) wherein basolateral (not luminal) membranes are stained. The basolateral staining pattern is probably secondary to the absence of growth factor receptors at the luminal part of the cell. This incomplete membrane positivity would be considered negative in IHC HER2 scoring in breast cancer, but should be considered positive in gastric cancer. The requirement for complete membranous staining thus is omitted in gastric cancer.^{14,19}

Heterogeneity

In contrast to breast cancer where IHC for HER2 is usually homogeneous, tumour heterogeneity is more common in gastric cancer (Figure 2).^{14,19,22} Heterogeneous staining can sometimes be seen within one gland. The most important reason for heterogeneous staining, however, is that up to one third of gastric cancers are of mixed intestinal/diffuse type.

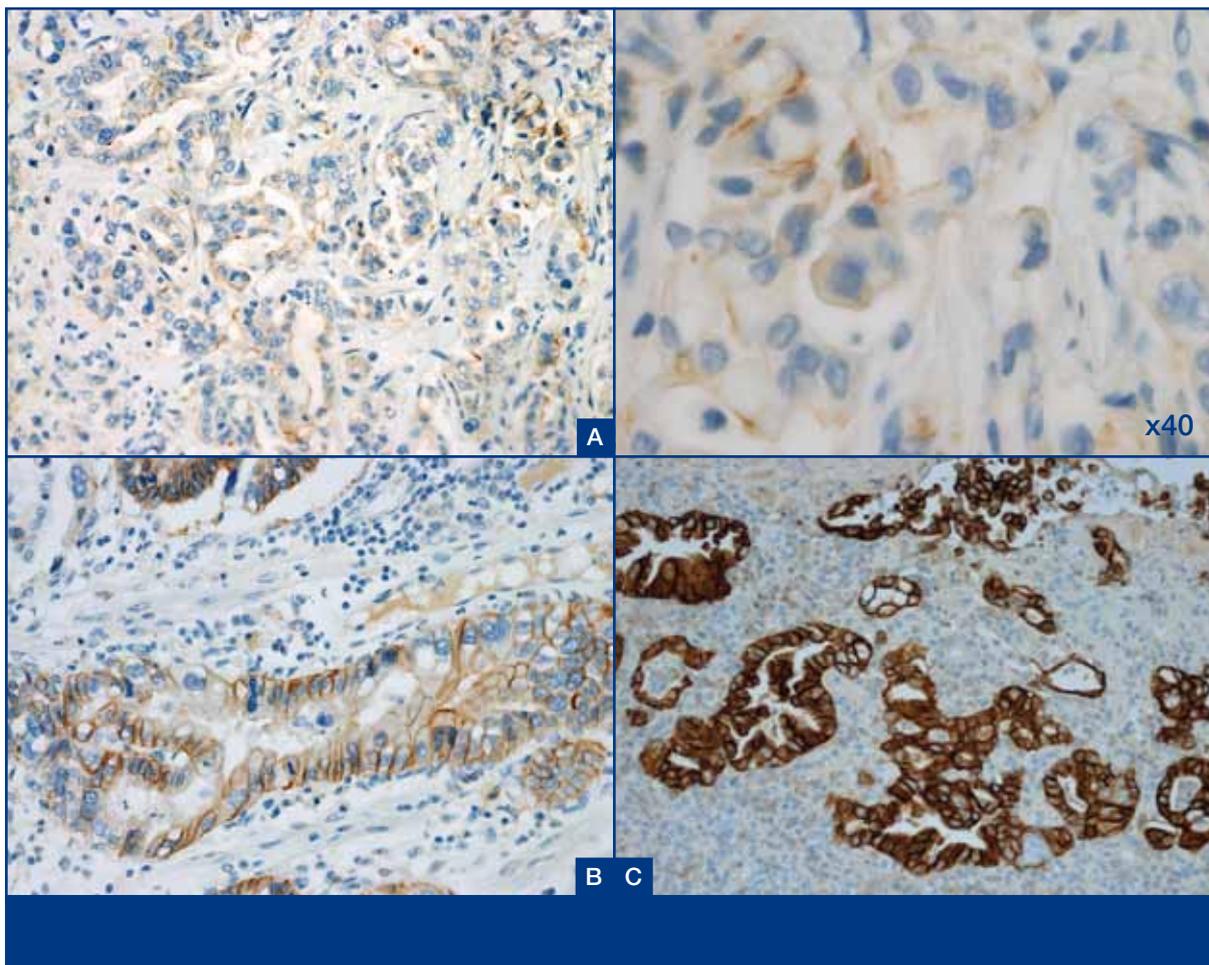


Figure 3. A. Gastric cancer with HER2 IHC score 1+ (x5, x40). B. IHC score 2+ (x20). C. IHC score 3+ (x5).

Strong staining is often seen in areas of an intestinal type of differentiation, while areas of diffuse types of differentiation are negative. Signet ring cell carcinomas are almost always negative. An identical heterogeneous pattern of staining is identified at the DNA level (amplification). Because of heterogeneity, the 10% cut-off level for positivity, which is required in breast cancer, is omitted in gastric cancer. Positivity in gastric cancer specimens thus is independent of the percentage of stained cells and it is sufficient to have a cohesive group of cells displaying HER2 positivity. The first papers on HER2 scoring in gastric cancer it stated that a least 5 cohesive HER2-positive cells were required.¹⁹ However, clearly it is more appropriate to require at least 20 cohesive cells for positivity, as a number of 20 cells showing amplification for HER2 is required to conclude for positivity in amplification studies with ISH (J. Rüschoff, personal communication). The 10% cut-off level for positivity was in the first instance only omitted for biopsy specimens, while for

surgical resection specimens a 10% cut-off level was required instead of the 30% cut-off for breast cancer resection specimens.^{14,19} However, the 10% cut-off level is now also considered inappropriate for testing in surgical resection specimens, as often these cases with less than 10% positive IHC score 2+ or IHC score 3+ cells as determined by IHC contain a cluster of 20 cells with amplification of HER2 as detected by ISH (J. Rüschoff, personal communication). For this reason the Belgian working group for HER2 testing in gastric cancer advises to apply the same rules to resection specimens as to biopsies, and to require in both cases for positive IHC at least 5 (or better 20) cohesive HER-2 positive cells.

Assessment of HER2 status

Accurate assessment of HER2 status is essential to determine which patients might benefit from therapy. This requires optimal tissue handling and stan-

Table 1. Algorithm for reproducible intensity scoring in HER2 IHC in gastric cancer (magnification rule).

Score 3+: Tumour cell clones with a strong basolateral or lateral membranous reactivity irrespective of percentage of tumour stained	Positivity directly visible by eye or at low magnification (5X)
Score 2+: Tumour cell clones with a weak to moderate basolateral or lateral membranous reactivity irrespective of percentage of tumour cells stained	Requires a more detailed magnification with a 10X or 20X objective for demonstration of membranous staining
Score 1+: Tumour cell clones with a faint/barely perceptible membranous reactivity irrespective of percentage of tumour cells stained	If high magnification, a 40X objective, is required for demonstration of membranous staining

standardized immunohistochemical staining and in situ hybridisation for amplification testing.

Optimal tissue sampling and handling requirements.

During endoscopy, as many biopsies as possible must be taken from the area suspicious for cancer. It is recommended to take at least 6 biopsies to increase the results (J. Rüschoff, personal communication). A joint effort by gastroenterologists and pathologists is needed to achieve this aim. There is no rule for the size of tumour specimens. However, reports should mention that results could be false negative because the amount of tumour tissue is too small.

Today it is the pathologist's responsibility to pay careful attention to fixation in anticipation of potential molecular testing, to use an appropriate fixative and to aim for an optimal fixation time. The interval between tissue sampling and fixation should be as short as possible; ideally the tissue should be fixed within 20-30 min, but at least best within 1 hour. The best fixative is 10% neutral buffered formalin. Bouin's fixative is proscribed for ISH. Fixation time should be at least 6 hours and should not be more than 48 hours. Predominantly underfixation is a problem as this can lead to false positive IHC results for HER2. Overfixation is less a problem for IHC, but should be avoided for ISH. The surgical specimen should be fixed after slicing of the specimen, to allow adequate fixation.²³

Biopsies as well as surgical specimens can be used for HER2 testing with similar success rates. Indeed, the percentage of positivity is not statistically different for biopsies (22.8%) and surgical samples (20.0%) in the ToGA trial.¹⁶ In addition, there is no statistical difference between the overexpression/amplification of HER2 in metastatic tissue versus primary tumours,

and both were used in the ToGA trial.

On resection specimens, because of the strong correlation between HER2 overexpression/ amplification and tumour type, blocks with areas of gland formation (intestinal type) should be selected if present.

Optimal IHC testing requirements and correct interpretation of the results

Specificity and sensitivity of the currently available anti-HER2 antibodies (e.g. HercepTest – Dako, 4B5 clone - Ventana, CB11 clone - Novocastra) used in IHC testing differ. The antibody clone used should be mentioned in the report.

IHC positivity for HER2 should be determined by an accurate and validated assay. The use of controls with known HER2 levels is mandatory.²³ Moreover, it is advised to participate in proficiency testing programs, e.g. as organised by NordiQC. Tissue sections should not be used for HER2 testing if cut more than 6 weeks before.

For reproducible intensity scoring it is advised to apply the algorithm, the so-called magnification rule, proposed by J. Rüschoff (*Table 1* and *Figure 3*).¹⁹ Real positivity requires a linear intercellular staining pattern. A granular staining pattern, cytoplasmic staining, nuclear staining or staining occurring at the luminal surface or only basal staining should be considered as false positivity. If staining only occurs at the edge of the tissue sample or in crashed areas this should be considered as an artefact. It should be noted that with the 4B5 HER2 antibody clone positivity is also observed in areas of intestinal metaplasia and in normal foveolar cells. However, usually these cells display nuclear and cytoplasmic positivity. Nuclear and cytoplasmic positivity with this antibody is also sometimes observed in signet ring cells. These features must be interpreted as falsely-positive.¹⁹

Optimal ISH testing requirements and correct interpretation of the results

Tissue should be well fixed (see above) as inadequate fixation may contribute to background auto-fluorescence. Pre-treatment is very important. Underdigested tissue needs a longer protease incubation time. This appears more often in overfixed tissue. Overfixation could cause loss of signal or weak signal, strong non-specific background staining and strong DAPI staining. Overdigested tissue needs a shorter protease incubation period. Typical signs of overdigestion are absent signals and nuclei with irregular outline and central holes. Evaluation of HER2 in situ hybridization (ISH) in gastric cancer is similar to that in breast cancer except for HER2/CEP17 ratio ≥ 2.0 indicating HER2 gene amplification. In case of a ratio between 1.8 and 2.2 an additional count of 40 cells in a different area is required. The final cut-off is then a ratio of ≥ 2.0 (19). Interpretation must include the counting of at least 20 non-overlapping, adjacent tumour cells in the chosen cancer area, only leaving out those cells that do not meet the quality criteria (overlapping nuclei). Counting can be done by a trained technologist, but must be confirmed by a pathologist.

Polysomy, defined as 3 or more chromosome 17-signals per cell, is a factor to consider when interpreting ISH-test results, although polysomy appears to be rare in gastric cancer compared to breast cancer. From a clinicopathological point of view, recent studies have shown that unamplified polysomal breast carcinomas might be considered for therapy with trastuzumab.^{24,25} In gastric cancer, non-amplified polysomal carcinomas are actually considered equivalent to HER2-negative carcinomas. Definite recommendations, however, can be made only after randomised clinical trials testing the efficacy of trastuzumab therapy in patients with unamplified polysomy have taken place in breast cancer as well as in gastric cancer.

Due to heterogeneity, a precise screening of the whole tumour area is very important. It is highly recommended to start by performing IHC to facilitate identification of an amplified region in case of heterogeneity. When HER2 IHC is performed in the primary pathology laboratory and HER2 ISH in a reference laboratory, it is indispensable to send the HER2 IHC slide to the reference laboratory together with the tissue block.

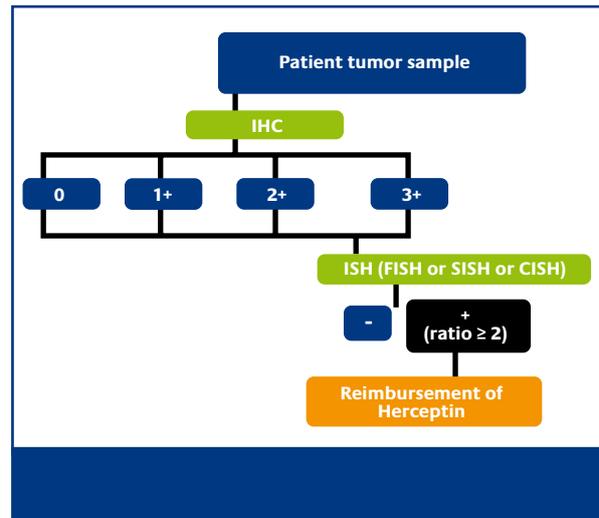


Figure 4. Belgian algorithm for HER2 testing in gastric cancer.

Concordance between IHC and ISH

In most studies a high concordance between IHC and ISH for IHC score 3+ is observed, but this seems quite different for IHC score 2+ and score 1+. In the study of Rüschoff et al. 32% of cases with IHC score 2+ and 5% of IHC score 1+ were amplified. Most IHC score 1+ and 2+, however, showed low level amplification, which was only the case in 16% of IHC score 3+ tumours.¹⁹ Also Bilous et al. showed a significant number of gastric cancers with HER2 IHC score 0/1+ and gene amplification.²² These patients with amplification but low level HER2 expression might respond less well to trastuzumab therapy.¹² These observations, however, are based on a small number of patients, and larger studies are needed to confirm these findings.

Belgian reimbursement criteria and testing algorithm

On September 20th 2010, Belgian reimbursement criteria for trastuzumab in the adjuvant treatment of metastasised gastric/gastro-oesophageal junction cancer were published in the Moniteur Belge/Belgisch Staatsblad. For reimbursement of trastuzumab amplification of HER2 should be demonstrated by positive ISH test (FISH/SISH/CISH). The test should be performed in a laboratory that can prove validation of the test and can prove that the result is considered positive if HER2/CEP17 ratio ≥ 2 .

The Belgian Working Group Molecular Pathology

Table 2. Pathological report for HER2 testing in gastric cancer.

Patient identification:	
Requesting physician identification:	
Date specimen received :	Date results available:
Tissue block number (including name of primary pathology department) :	
Specimen type:	Specimen site :
Histological type :	
Fixation method and time of fixation (if available) :	
<u>HER2 IHC</u>	
Antibody clone used :	
IHC score:	
Estimated number of tumour cells on which the scoring was performed and percentage of positive cells :	
<u>HER2 ISH</u>	
Technique used :	
HER2/CEP17 ratio :	
Interpretation of the results	

supports the decision to require HER2 ISH as the sole criterion to determine which patients are eligible for trastuzumab treatment and thus for reimbursement of therapy and not to rely solely on IHC, in case of IHC score 3+. It has been shown that reproducibility between laboratories is less good for IHC than for ISH, predominantly because clinical samples have non-standardized fixation methods and fixation times and ISH is relatively independent of tissue fixation, compared to IHC, where staining intensity is highly dependent on fixation time. Moreover, in ISH we have to count the number of signals, while IHC includes an interpretation step. In IHC we have to score the intensity of the staining, and our eyes cannot adequately distinguish small differences in staining intensity. However, as overexpression/amplification of HER2 in gastric cancer is often heterogeneous, the Belgian working group for HER2 testing in gastric cancer recommends to start by performing IHC for HER2, in order not to miss a small focus of HER2 overexpression/ amplification (Figure 4, page 19). This IHC positive focus can subsequently be selected for further ISH analysis.

The Belgian committee for HER2 testing in gastric cancer in addition supports the decision to analyse all tumours by ISH, thus including those with IHC score 0/1+, to avoid false negatives. Moreover, about 7.5% of IHC score 0/1+ cases appear to be amplified and it has not yet been unequivocally proven that these patients with IHC score 0/1+, but with amplification of HER2, do not respond to trastuzumab treatment,

as the number of patients with IHC score 0/1+ and FISH+ was small.^{12,22}

The Belgian committee for HER2 testing in gastric cancer thus recommends using both IHC and ISH in clinical testing. HER2 IHC as well as ISH should be standardised, using written procedures and should be regularly validated, using the quality control and quality assurance measures as described in the Belgian guidelines for HER2/neu testing in breast cancer.²⁰ The pathological report should be standardised as described in *Table 2*. The primary pathologist should include the results into the original or a complementary pathology report and transmit the results to the requesting oncologist. The report should include interpretation of the results.

Conclusion

The Belgian committee for HER2 testing in gastric cancer recommends to perform ISH preceded by IHC in all cases in clinical testing in gastric cancer, in order not to miss the amplified region in cases of heterogeneity. Amplification as demonstrated by HER2 ISH (HER2/CEP17 ratio ≥ 2) is however the sole criterion for the decision to treat with trastuzumab and thus for reimbursement of therapy in Belgium. Moreover, all patients considered for trastuzumab treatment, as well as all patients with IHC score 0/1+, should be analysed by ISH, as they are also eligible for trastuzumab therapy if they show amplification.

Concerning surgical samples, the pathologist

Key messages for clinical practice

- 1.** Accurate HER2 testing of metastatic gastric cancer is required for optimal disease management. It must be performed on request of the oncologist.
- 2.** Important differences exist between gastric cancer and breast cancer concerning HER2 testing: The most important differences are the incomplete membrane positivity (lateral or basolateral membrane staining) and the heterogeneity of the over-expression/amplification of HER2 in gastric cancer. Application of a IHC scoring system specific for gastric cancer is necessary as the requirement for complete membranous staining as well as the 10% cut-off level for breast cancer is omitted in gastric cancer. The Belgian working group for HER2 testing in gastric cancer advises to apply the same rules for resection specimens as for biopsies, i.e. to require in both cases for positive IHC at least 5 (or better 20) cohesive HER-2 positive cells.
- 3.** Tumour tissues should be fixed in 10% buffered formalin for 6 to 48 hours and the interval between tissue acquisition and fixation should be as short as possible (< 1 hour).
- 4.** Concerning surgical samples, the pathologist should select the tissue blocks with the largest area of intestinal differentiation (glandular structures).
- 5.** Amplification as demonstrated by HER2 ISH (HER2/CEP17 ratio ≥ 2) is the sole criterion for decision to treat with trastuzumab and thus for reimbursement of therapy in Belgium. ISH preceded by IHC should however be performed in all patients in clinical testing in gastric cancer, in order not to miss the amplified region in cases of heterogeneity.
- 6.** In case HER2 IHC is performed in the primary pathology laboratory and HER2 ISH in a reference laboratory, the HER2 IHC slide should be send together with the tissue block to the reference laboratory.
- 7.** Quality assurance and optimal internal validation procedures are requested as well for IHC as for ISH.

Committee for HER2 testing in gastric cancer:

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should select the tissue blocks with the largest area of intestinal differentiation (glandular structures). The pathologist should clearly identify the strongest IHC-positive area in order to perform ISH con-

firmation on that particular area. For IHC modified HER2 scoring as described in *Table 1* on page 19, should be applied. A key issue for the scoring of positive HER2 expression is a membranous stain-

ing that can be unequivocally assessed as linear staining at cell-cell contact sites. Importantly, in contrast to breast cancer, incomplete lateral or basolateral membrane positivity should be considered positive in gastric cancer. Because of heterogeneity the 10% cut-off was omitted for biopsy specimens, and, it is now also becoming considered inappropriate for testing in surgical resection specimens, as some of these cases with less than 10% positive cells in IHC contain a cluster of 20 cells with amplification of HER2 as detected by ISH (J. Rüschoff, personal communication).

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