Digital image analysis of HER2 immunohistochemistry in gastric- and oesophageal adenocarcinoma

Koopman, Timco; de Bock, Geertruida H.; Buikema, Henk J.; Smits, Maria M.; Louwen, Maarten; Hage, Mariska; Imholz, Alex L. T.; van der Vegt, Bert

Published in:
Histopathology

DOI:
10.1111/his.13322

Publication date:
2018

Histopathology

Histopathology 2018, **72**, 191–200. DOI: 10.1111/his.13322

Digital image analysis of HER2 immunohistochemistry in gastric- and oesophageal adenocarcinoma: a validation study on biopsies and surgical specimens

Timco Koopman,[1] Geertruida H de Bock,[2] Henk J Buikema,[1] Maria M Smits,[3] Maarten Louwen,[3] Mariska Hage,[3] Alex L T Imholz[4] & Bert van der Vegt[1]

[1]*Department of Pathology,* [2]*Department of Epidemiology, University of Groningen, University Medical Center Groningen, Groningen,* [3]*Department of Pathology and* [4]*Department of Medical Oncology, Deventer Hospital, Deventer, the Netherlands*

Date of submission 18 May 2017
Accepted for publication 23 July 2017
Published online *Article Accepted* 26 July 2017

Koopman T, de Bock G H, Buikema H J, Smits M M, Louwen M, Hage M, Imholz A L T & van der Vegt B (2018) *Histopathology* **72**, 191–200. https://doi.org/10.1111/his.13322

Digital image analysis of HER2 immunohistochemistry in gastric- and oesophageal adenocarcinoma: a validation study on biopsies and surgical specimens

Aims: To test the validity of diagnostics incorporating digital image analysis (DIA) for human epidermal growth factor 2 (HER2) immunohistochemistry (IHC) in gastro-oesophageal adenocarcinomas, as an alternative to current standard diagnostics using manual scoring.

Methods and results: We included 319 consecutive gastro-oesophageal adenocarcinomas (232 biopsies and 87 surgical specimens). DIA was applied to determine HER2 IHC classification, using both standard breast cancer (BC) and modified gastro-oesophageal cancer (GEC) cut-offs. Consensus manual scores were established by four independent observers. Chromogenic *in-situ* hybridization (CISH) was performed on all 2+ cases by manual scoring, DIA or both. HER2 status was considered positive in 3+ and CISH-positive 2+ cases. Overall agreement between DIA and consensus manual scores was 76.5% (weighted $\kappa = 0.66$, BC cut-offs) and 85.6% (weighted $\kappa = 0.80$, GEC cut-offs). Agreement was similar for biopsies and surgical specimens. All disagreement occurred in the manual IHC equivocal cases. DIA resulted in a reduction of 2+ cases: 75.8% with BC cut-offs and 46.5% with GEC cut-offs. HER2 status was positive in 48 cases (15%) with standard diagnostics and DIA using GEC cut-offs, and 46 cases (14.4%) using BC cut-offs (all with CISH in 2+ cases). Considering standard diagnostics as a reference, DIA showed 93.8% sensitivity and 99.6% specificity (BC cut-offs) or 97.9% sensitivity and 99.6% specificity (GEC cut-offs).

Conclusions: DIA is a reliable and feasible alternative to manual HER2 IHC scoring in gastro-oesophageal adenocarcinoma, both in biopsies and surgical specimens, leading to a reduction of 2+ cases for which subsequent ISH testing is required.

Keywords: digital image analysis (DIA), gastric cancer, human epidermal growth factor 2 (HER2), immunohistochemistry (IHC), oesophageal cancer

Introduction

Gastro-oesophageal cancers are among the most commonly diagnosed cancers worldwide, with 5-year survival rates of 19–32%.[1,2] Adenocarcinoma is the most common type of both gastric and distal

Address for correspondence: B van der Vegt, Department of Pathology and Medical Biology, University Medical Center Groningen, Groningen, the Netherlands, PO Box 30001, 9700 RB Groningen. e-mail: b.van.der.vegt@umcg.nl

oesophageal cancer.[3–5] Overexpression of human epidermal growth factor receptor 2 (HER2) occurs in 15–30% of gastro-oesophageal adenocarcinomas.[5–9] In this subgroup of patients, targeted anti-HER2 therapy offers an additional treatment modality, which was shown to improve survival rates in advanced stages.[10,11]

HER2 status in gastro-oesophageal cancer is determined using immunohistochemistry (IHC) and *in-situ* hybridization (ISH). IHC membrane staining is scored semiquantitatively negative (0 or 1+), equivocal (2+) or positive (3+) using a modified version of the breast cancer scoring system.[12–14] In equivocal cases, additional ISH is performed to determine HER2 gene amplification.

Digital image analysis (DIA) has emerged as an alternative method to classify HER2 IHC. In breast cancer, a variety of DIA tools in different platforms is able to determine HER2 status accurately.[15–19] DIA provides an objective and reproducible HER2 classification method to support pathologists in daily practice. DIA is recognized in the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines for HER2 in breast cancer as a diagnostic modality.[20] Potentially, DIA can reduce IHC equivocal (2+) cases, decreasing the number of cases requiring subsequent ISH.[18,19,21] DIA could offer these same advantages in gastro-oesophageal cancer. However, present data are limited, with contradictory results in the literature on this subject to date. Studies have focused predominantly on surgical specimens, while in clinical practice HER2 status is often determined on biopsies, as anti-HER2 therapy is currently used for unresectable (locally advanced or metastasised) carcinomas.[10]

The aim of this study was to validate DIA of HER2 IHC in a large cohort of biopsies and surgical specimens of gastro-oesophageal adenocarcinomas. To this end, DIA results were compared to consensus manual IHC scores by four independent observers. Furthermore, HER2 status using DIA with ISH on 2+ cases was compared to HER2 status by standard diagnostics, consisting of consensus manual scoring with ISH on 2+ cases.

Materials and methods

CASES

A total of 321 consecutive gastric and oesophageal adenocarcinomas, diagnosed from January 2004 to December 2011 in the Deventer Hospital (the Netherlands), were included. Two cases were excluded, as ISH failed in repeated tests, resulting in a study population of 319 cases (Table 1). All patient material was handled according to the 'Code of conduct for health research' of the Dutch Federation of Biomedical Scientific Societies.[22] Therefore, no additional permission from our Ethics Committee was needed.

Table 1. Clinicopathological characteristics (n = 319)

	n	%
Gender		
Male	218	68.3
Female	101	31.7
Age at diagnosis (years)		
<65	112	35.1
≥65	207	64.9
Disease status		
Advanced*	165	51.7
Not advanced	130	40.8
Status unknown	24	7.5
Specimens used for HER2 testing		
Biopsy	232	72.7
Surgical specimens	87	27.3
Tumour type (Laurén[35])		
Intestinal	188	58.9
Diffuse	85	26.6
Mixed	38	11.9
Indeterminate	8	2.5
Primary tumour location†		
Oesophagus	151	47.3
Distal oesophagus	54	16.9
Gastro-oesophageal junction	97	30.4
Stomach	161	50.5
Cardia	28	8.8
Non-cardia	133	41.7
Unknown	7	2.2

HER2, Human epidermal growth factor 2.
*Metastasised or inoperable locally advanced cancer.
†Primary tumour location according to TNM7 guidelines.

IMMUNOHISTOCHEMISTRY AND MANUAL SCORING

HER2 IHC was performed on whole tissue samples of biopsies or surgical specimens using the PATHWAY HER2/*neu* 4B5 monoclonal antibody (Ventana Medical Systems, Illkirch, France), following the manufacturer's protocol. HER2 was scored using the modified scoring system for gastro-oesophageal adenocarcinoma by Hofmann *et al.*,[12] with additional guidelines by Rüschoff *et al.*[13,23] (Table 2). Three clinical pathologists (M.M.S., M.L. and M.H.) and one resident (T.K.) received training on this scoring system, as described previously.[14] All observers scored all cases independently on glass slides (M.M.S., M.L. and M.H.) or digital images (T.K). Manual consensus score was defined as IHC-positive for a 3+ score by at least two observers (with minimum 2+ by other observers), IHC-negative if all observers scored 0 or 1+ and IHC-equivocal (2+) in all remaining cases.

DIGITAL IMAGE ANALYSIS

Digital images were acquired by scanning the glass slides in a NanoZoomer 2.0 HT (Hamamatsu Photonics, Hamamatsu City, Shizuoka, Japan) with a ×40 magnification lens, using a single focus layer without Z-stacking. Tissue detection with focus points was applied automatically. Digitized slides were stored on a hard disk and loaded into the DIA software module Visiopharm Integrator System (VIS) platform version 6.5.02303 (Visiopharm, Hørsholm, Denmark).

The HER2-CONNECT algorithm was used to classify immunohistochemical HER2 staining. This algorithm analyses membrane staining in a user-selected region of interest (ROI) by calculating a connectivity value based on diaminobenzidine (DAB) staining of linear structures corresponding to membrane fragments, as described in detail by Brügmann *et al.*[19] This connectivity value can vary continuously from 0 to 1, and is converted to a HER2 classification (0, 1+, 2+ or 3+) with specific cut-offs. The standard breast cancer (BC) cut-offs,[21] as well as modified gastro-oesophageal cancer (GEC) cut-offs, are displayed in Table 2. These GEC cut-offs were established with 12 randomly selected cases, three for each consensus manual score (two biopsies and one surgical specimen).

ROIs with the most pronounced membrane staining containing a minimum of 30 tumour cells were selected; maximum size was 0.5 mm^2. Multiple ROIs were selected: five to 35 ROIs in each case, depending on tissue size and staining heterogeneity, to ensure that a representative sample of HER2 expression was included. In biopsies, ROIs were selected in multiple sections on multiple levels. Artefacts and non-tumour tissue staining were carefully avoided, if possible.

The algorithm determines connectivity value for each individual ROI. In biopsies, the highest connectivity value of a single ROI among all analysed ROIs was interpreted as representative for the case, in accordance with the manual scoring guidelines of requiring a single cluster of ≥5 tumour cells.[12,23] In

Table 2. HER2 by manual scoring and digital image analysis classification in gastric and oesophageal adenocarcinoma

	HER2	Manual scoring of IHC staining*	DIA algorithm connectivity value (breast cancer cut-offs)	DIA algorithm connectivity value (gastro-oesophageal cancer cut-offs)
0	Negative	No reactivity or no membranous reactivity (visible at ×40)	Connectivity = 0	Connectivity = 0
1+	Negative	Faint or barely visible membranous reactivity (visible at ×40)	0 < connectivity ≤0.40	0 < connectivity ≤0.20
2+	Equivocal (requires subsequent ISH)	Weak to moderate complete, basolateral or lateral membranous reactivity (visible at ×10–20)	0.40 < connectivity ≤0.64	0.20 < connectivity ≤0.64
3+	Positive	Strong complete, basolateral or lateral membranous reactivity (visible at ×2.5–5)	Connectivity >0.64	Connectivity >0.64

HER2, Human epidermal growth factor 2; IHC, Immunohistochemistry; DIA, Digital image analysis; ISH, *In-situ* hybridization.
*According to the modified scoring system for gastro-oesophageal adenocarcinoma by Hofmann *et al.*[12] with additional guidelines by Rüschoff *et al.*[13,23] Sufficient well-preserved tumour tissue should be present, staining should be membranous and there should be a cluster of ≥5 stained tumour cells in biopsies or staining in ≥10% of tumour cells in surgical specimens.

surgical specimens, positive staining should include ≥10% of all tumour cells, and as such the highest connectivity score given by the algorithm was verified visually to be representative of ≥10% of the tumour.

IN-SITU HYBRIDIZATION

ISH was performed on all samples scored or classified 2+ by manual scoring or DIA. Chromogenic *in-situ* hybridization (CISH) was performed using ZytoDot SPEC HER2 Probe kit (ZytoVision, Bremerhaven, Germany), following the manufacturer's protocol. Negative CISH was defined as diploidy (two dots per nucleus) or polysomia (three to five dots per nucleus). Positive CISH was defined as low amplification (six to 10 dots per nucleus or small clusters) or high amplification (>10 dots per nucleus or large clusters) in >50% of tumour cells in at least 20 cells.

COMPARISON OF DIA WITH MANUAL SCORING

DIA classification of IHC was compared to consensus manual scores (negative, equivocal or positive) in the total study population as well as stratified between biopsies and surgical specimens. The clinically relevant outcome is HER2 status after ISH in 2+ cases. As such, HER2 status outcome when using DIA with subsequent CISH on 2+ cases was compared to HER2 status by standard diagnostics, which consisted of consensus manual scoring with subsequent CISH on 2+ cases. HER2 status was considered positive in IHC 3+ cases or IHC 2+ cases with positive CISH.

STATISTICAL ANALYSIS

To establish agreement between DIA classification and manual consensus scores of HER2 IHC, linear weighted kappa (κ) statistics were performed in R for Windows version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria), using the 'irr' package for κ statistics. κ values were interpreted as <0.2, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial; and 0.81–1.00, almost perfect agreement.[24]

Results

A flowchart of HER2 status as determined by standard diagnostics and DIA is displayed in Figure 1. DIA images are shown in Figure 2. Distribution of connectivity values in 1+, 2+ and 3+ cases is displayed in Figure 3. Standard diagnostics resulted in HER2-positive status in 48 of 319 cases (15%). DIA with CISH in 2+ cases resulted in HER2-positive status in 46 cases (14.4%) with BC cut-offs and 48 cases (15%) with GEC cut-offs.

DIA CLASSIFICATION COMPARED TO CONSENSUS MANUAL SCORES

Comparison of consensus manual scores and DIA classification of IHC in the total study population and stratified between biopsies and surgical specimens is outlined in Table 3. In the total study population, overall agreement was 76.5% (95% confidence interval (CI): 71.5–80.8%, 244 of 319 cases) with BC cut-offs and 85.6% (95% CI: 81.3–89.0%, 273 of 319 cases) with GEC cut-offs. Using BC cut-offs, kappa value was $\kappa = 0.66$ ('substantial' agreement). Using GEC cut-offs, this rose to $\kappa = 0.80$ ('substantial', nearly 'almost perfect' agreement). Kappa values were similar in biopsies and surgical specimens. Among biopsies, overall agreement was 73.3% (95% CI: 67.2–78.6%, 170 of 232 cases) with BC cut-offs and 83.6% (95% CI: 78.3–87.8%, 194 of 232 cases) with GEC cut-offs. For surgical specimens, overall agreement was 85.1% (95% CI: 76.1–91.1%, 74 of 87 cases) and 90.8% (95% CI: 82.9–95.3%, 79 of 87 cases) with BC and GEC cut-offs, respectively.

In the total study population, all 180 cases with a consensus manual IHC-negative score were classified as negative by DIA with both cut-offs. Similarly, all 40 cases with a consensus manual 3+ score were classified as 3+ by DIA with both cut-offs.

DIA IN MANUAL EQUIVOCAL CASES AND CONCORDANCE WITH CISH

From the total of 99 cases with a manual equivocal (2+) IHC score, 2+ cases were reduced by 46 (46.5%) using DIA with GEC cut-offs. Two cases were discordant with CISH: one case was false-positive 3+ (CISH-negative) and one case was false-negative 1+ (CISH-positive). Using BC cut-offs, 2+ cases were reduced by 75 (75.8%), which is 29 more than with GEC cut-offs, but at the cost of two additional false-negative cases (both classified 1+). Compared to manual scoring, there were no additional 2+ cases by DIA.

HER2 STATUS USING DIA VERSUS STANDARD DIAGNOSTICS

Sensitivity, specificity, positive and negative predictive value of HER2 status by DIA compared to standard diagnostics are displayed in Table 4.

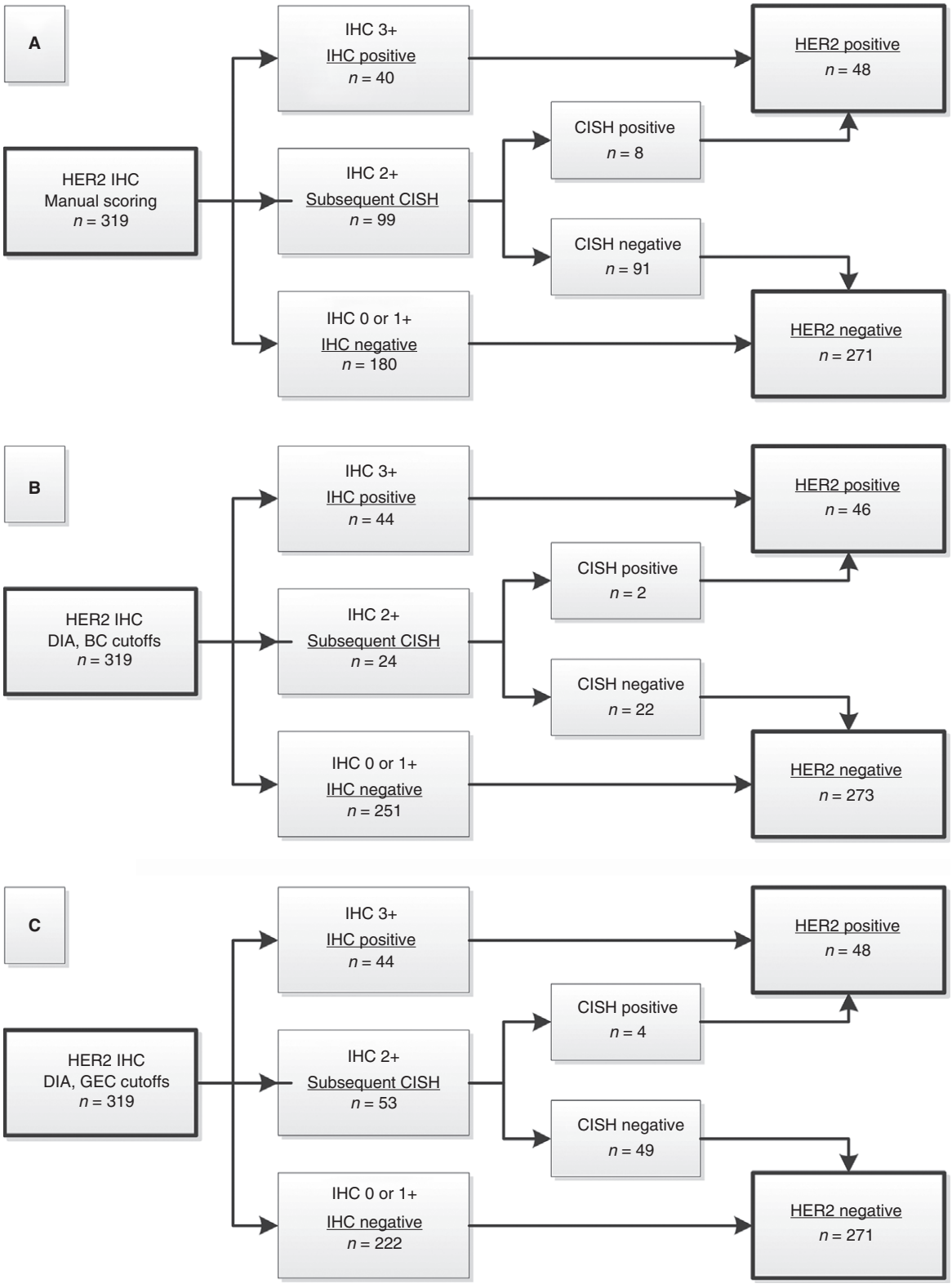

Figure 1. Flowchart of HER2 status as determined by standard diagnostics with consensus manual scoring (**A**) and by digital image analysis classification (**B**,**C**), with subsequent CISH on 2+ cases. HER2, Human epidermal growth factor 2; IHC, Immunohistochemistry; CISH, Chromogenic *in-situ* hybridization; DIA, Digital image analysis; BC, Breast cancer; GEC, Gastro-oesophageal cancer.

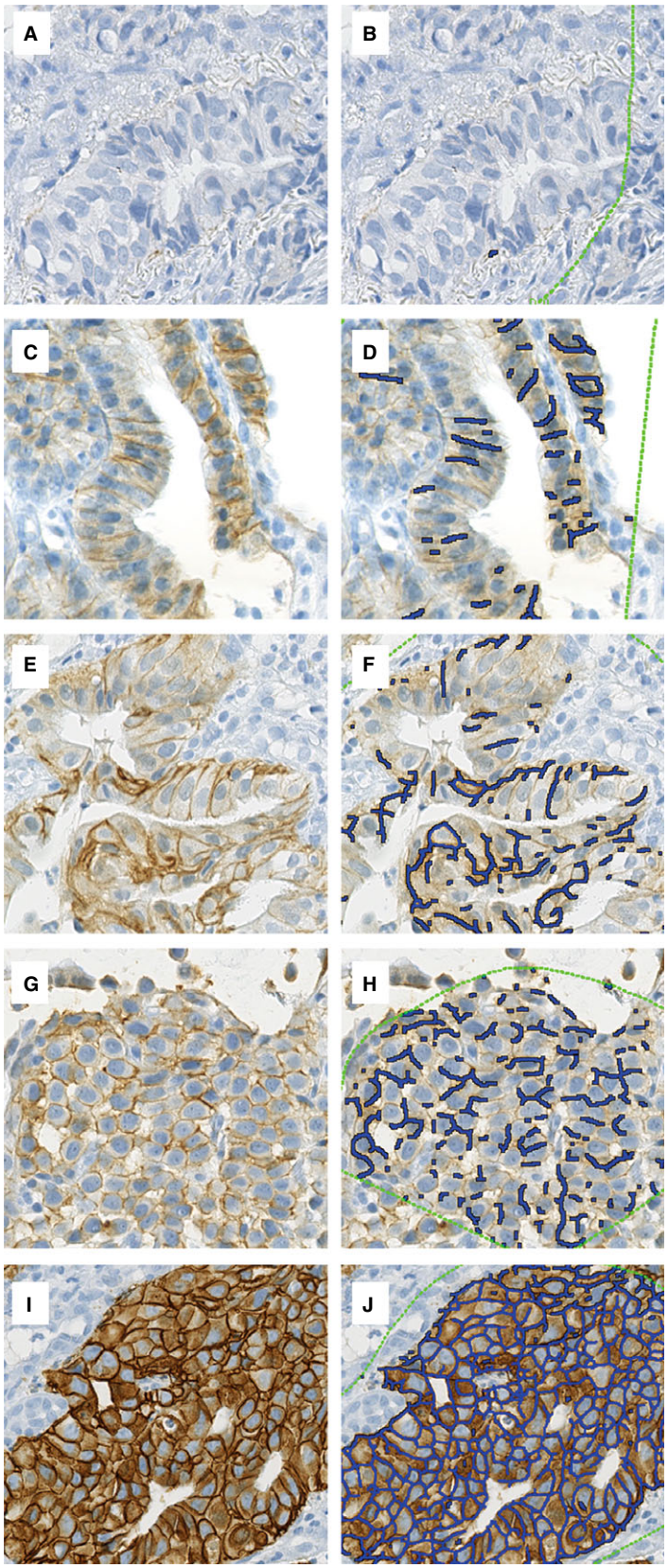

Figure 2. Digital image analysis of HER2 immunohistochemistry in gastro-oesophageal adenocarcinoma; examples with and without membrane connectivity mark-up. Classification of 0 (**A**,**B**), 1+ (**C**,**D**), 2+ in intestinal tumour type (**E**,**F**), 2+ in diffuse tumour type (**G**,**H**) and 3+ (**I**,**J**). Connectivity values were 0 (**B**), 0.171 (**D**), 0.256 (**F**), 0.386 (**H**) and 0.983 (**J**). CISH was negative in the 1+ case and in both 2+ cases, and positive in the 3+ case. HER2: human epidermal growth factor 2; CISH: chromogenic *in-situ* hybridization.

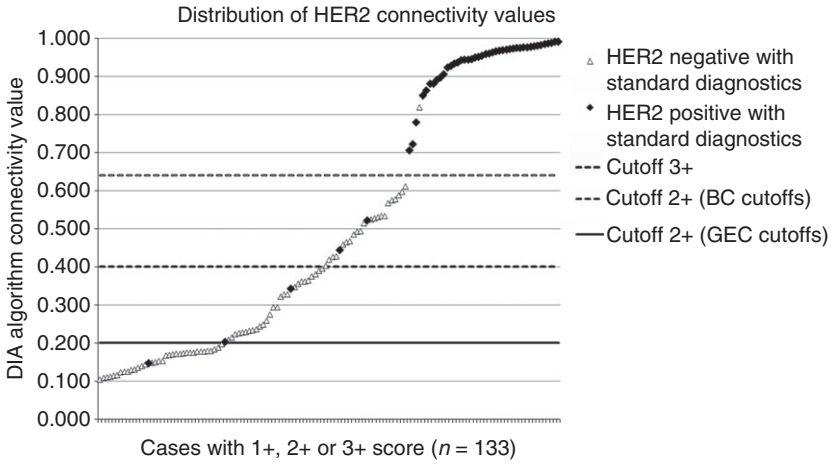

Figure 3. Distribution of HER2 connectivity values in 1+, 2+ and 3+ cases by DIA (n = 133). HER2, Human epidermal growth factor 2; DIA, Digital image analysis; BC, Breast cancer; GEC, Gastro-oesophageal cancer.

Table 3. Comparison of HER2 immunohistochemistry manual scores and digital image analysis classification in the total study population, biopsies and surgical specimens

	Consensus manual score											
	Total study population				Biopsies				Surgical specimens			
Digital image analysis	0/1+	2+	3+	Total	0/1+	2+	3+	Total	0/1+	2+	3+	Total
BC cut-offs												
0/1+	180	71	0	251	117	59	0	176	63	12	0	75
2+	0	24	0	24	0	19	0	19	0	5	0	5
3+	0	4	40	44	0	3	34	37	0	1	6	7
Total	180	99	40	319	117	81	34	232	63	18	6	87
Kappa*				κ = 0.66				κ = 0.65				κ = 0.69
GEC cut-offs												
0/1+	180	42	0	222	117	35	0	152	63	7	0	70
2+	0	53	0	53	0	43	0	43	0	10	0	10
3+	0	4	40	44	0	3	34	37	0	1	6	7
Total	180	99	40	319	117	81	34	232	63	18	6	87
Kappa*				κ = 0.80				κ = 0.78				κ = 0.82

HER2, Human epidermal growth factor 2; BC, Breast cancer; GEC, Gastro-oesophageal cancer.
*Linear weighted kappa (κ) score.

Discussion

We aimed to validate DIA of HER2 IHC in gastro-oesophageal tumours, predominantly biopsies. Agreement between DIA classification and manual scores was high, and similar in surgical specimens and biopsies. DIA led to a reduction of 2+ cases. Additionally, DIA (with ISH on 2+ cases) resulted in high sensitivity and specificity to establish HER2 status when compared to standard diagnostics (manual scoring with ISH on 2+ cases).

To the best of our knowledge, six studies implementing DIA of HER2 in gastro-oesophageal cancer have been published to date,[25–30] four studies of which compared directly DIA with manual scoring.[27–30] Our results were comparable to three studies.[27–29] The first[27] and second[29] found overall agreement between DIA and manual scores in 92%

Table 4. HER2 status using digital image analysis compared to standard diagnostics

Digital image analysis	Standard diagnostics (consensus manual scoring with ISH in 2+ cases)			
	Negative	Positive	Total	
BC cut-offs (with ISH in 2+ cases)				
Negative	270	3	273	NPV: 98.9%
Positive	1	45	46	PPV: 97.8%
Total	271	48	319	
	Spec: 99.6%	Sens: 93.8%		
GEC cut-offs (with ISH in 2+ cases)				
Negative	270	1	271	NPV: 99.6%
Positive	1	47	48	PPV: 97.9%
Total	271	48	319	
	Spec: 99.6%	Sens: 97.9%		

HER2, Human epidermal growth factor 2; ISH, *In-situ* hybridization; BC, Breast cancer; GEC, Gastro-oesophageal cancer; Spec, Specificity; Sens, Sensitivity; PPV, Positive predictive value; NPV, Negative predictive value.

of 103 cases (95% CI: 85.4–96.0%) and 97% of 68 cases (95% CI: 90.0–99.2%), respectively, which is comparable to the 85.6% (95% CI: 81.3–89.0%) overall agreement we found (using GEC cut-offs). A third study[28] on 110 cases reports 76% sensitivity of DIA with BC cut-offs, 100% with their GEC cut-offs and 100% specificity with both cut-offs when compared to ISH. We found similar results: 93.8% sensitivity with BC cut-offs, 97.9% with GEC cut-offs and specificity 99.6% with both cut-offs when compared to standard diagnostics. They performed ISH on all cases and we performed ISH only when clinically applicable (2+ cases). The fourth study by Jeung *et al.*[30] on 116 cases found 100% agreement between DIA and manual IHC-negative cases (also 100% in our study), 20–50% agreement in 3+ cases (100% in our study) and 0% agreement in 2+ cases, the latter being lower than, but in line with, the reduction of 2+ cases in our study. ISH was not performed in their study. As addressed by the authors, their algorithm was optimized for breast cancer and consequently unable to classify membrane staining adequately in gastro-oesophageal cancer. Although the algorithm in the current study was also developed for breast cancer, agreement of DIA with manual scores was high nonetheless.

As anti-HER2 therapy is used currently for unresectable gastro-oesophageal cancers, in clinical practice HER2 status is often determined on biopsies.[10] However, only two studies included biopsies besides surgical specimens.[29,30] Ormenisan *et al.*[29] found

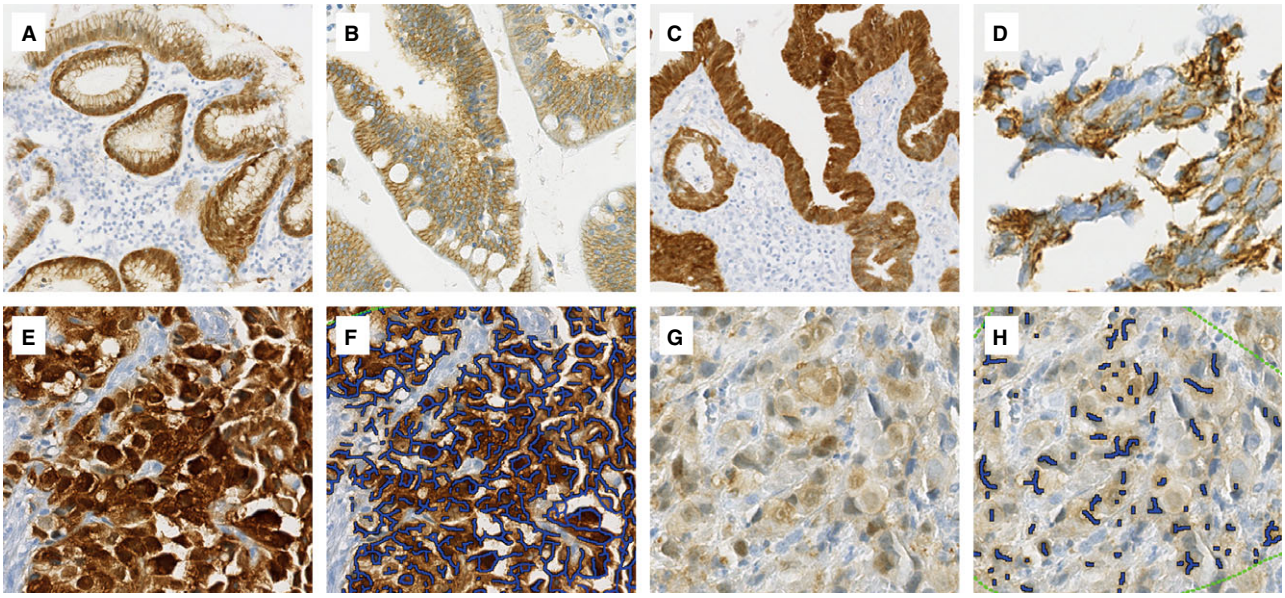

Figure 4. Artefacts and staining of non-tumour tissue with HER2 immunohistochemistry. There is immunoreactivity with normal gastric epithelium (**A**), intestinal metaplasia (**B**) and dysplastic epithelium (**C**). Aberrant staining can occur in edge artefacts (**D**). The false-positive (**E**,**F**) and false-negative (**G**,**H**) cases of this study illustrate artefactual nuclear and cytoplasmic staining, complicating manual and digital evaluation (images with and without membrane connectivity mark-up). Connectivity values of the discordant cases were 0.819 (**F**) and 0.147 (**H**). HER2: human epidermal growth factor 2.

high agreement in both biopsies and surgical specimens, but in a substantially smaller cohort (68 cases) than our study. Jeung *et al.*[30] found most disagreement among biopsies, while in the current study agreement rates in biopsies and surgical specimens were similar. The authors attribute this to the fact that in biopsies only a small number of HER2-positive cells are required,[12,23] which did not reach the threshold for a positive result in their DIA algorithm. As we used ROIs centred on positive clusters, this issue did not arise in our study.

In breast cancer, DIA can reduce the amount of IHC-equivocal (2+) cases.[18,19,21] In gastro-oesophageal cancer, Nielsen *et al.*[28] reported 36.4% reduction of 2+ cases using HercepTest and 50% reduction using the 4B5 antibody. In the current study, using 4B5 and applying GEC cut-offs, a 46.5% reduction of 2+ cases was achieved, which confirms these results. The reduction of 2+ cases decreases the need for subsequent ISH testing, potentially lowering diagnostic costs and reducing turnaround time in daily practice.

Three CISH-positive cases were classified false-negatively as 1+ when using BC cut-offs. In two of these cases, GEC cut-offs resulted in a 2+ classification, which would have triggered subsequent CISH. The third case was also a false-negative 1+ with GEC cut-offs. Upon review, manual scoring was complicated due to aberrant staining in nuclei and cytoplasm. Membrane staining was faint, and only one observer scored 2+; the other three scored negative. Additionally, the tumour was a diffuse type, on which HER2 scoring is known to be difficult.[5] One CISH-negative case was classified false-positively as 3+ due to strong aberrant cytoplasmic staining, which could not be avoided when selecting ROIs as all tumour tissue expressed aberrant staining. This case was scored manually as 0 by three and 3+ by one observer, who also interpreted the aberrant staining as positive membrane staining. As such, when using DIA with GEC cut-offs to determine HER2 status in our study population, only two of 319 cases (0.6%) were classified discordantly. In both cases manual scoring was troublesome, due partly or entirely to flawed staining. Aberrant staining can occur as nuclear or cytoplasmic staining, edge artefacts or crushing artefacts.[30–32] Cytoplasmic staining with the 4B5 antibody could be related to cross-reactivity with HER4.[33] Additionally, immunoreactivity can occur in pre-existent epithelium and pre-neoplastic tissue (intestinal metaplasia and dysplasia).[25] Interestingly, this occurred in all our cases if such tissue was present. Figure 4 displays examples of aberrant staining and images of the discordant cases. When conducting DIA, artefacts and aberrant staining should be avoided carefully.

HER2 membrane staining does not have to be circumferential in gastro-oesophageal cancer, as in breast cancer.[20] Although the algorithm we used evaluates membrane connectivity, both Nielsen *et al.*[28] and our team found that it can be applied successfully to gastro-oesophageal cancer specimens. We established GEC cut-offs at connectivity values of 0.20 (1+ to 2+) and 0.64 (2+ to 3+), but GEC cut-offs by Nielsen *et al.* were notably lower (0.09 and 0.30). This could be related to ROI size, as they selected entire tissue microarray images and HER2 classification is based on the membrane connectivity within the complete ROI. We used relatively small ROIs containing the strongest HER2 expression, as only five clustered positive tumour cells are required.[12,23]

The adjusted GEC cut-offs in this study were established on samples processed and stained in one laboratory. Further studies should be performed to validate these cut-offs, including stains from other laboratories and different HER2 antibodies. Although appropriate staining and training protocols have led to acceptable interobserver and interlaboratory concordance, manual scoring remains a subjective method with interobserver variability.[14,34] DIA could provide an objective and reproducible alternative, but no data are available on interplatform variability between different DIA platforms on identical cases.

In conclusion, our data suggest that DIA is a reliable and feasible alternative to manual scoring of HER2 immunohistochemistry in gastro-oesophageal adenocarcinoma, which can reduce equivocal cases requiring subsequent ISH testing and can be applied on both biopsies and surgical specimens.

Conflicts of interest

None to declare.

References

1. American Cancer Society. *Global cancer facts and figures.* 3rd ed. Atlanta, GA: American Cancer Society, 2015.
2. Howlader N, Noone AM, Krapcho M *et al.* SEER cancer statistics reviews 1975–2013, Bethesda, MD: National Cancer Institute based on November 2015 SEER data submission, posted to the SEER web site, April 2016 [internet]. Available at: http://seer.cancer.gov/csr/1975_2013/ (accessed 18 May 2017).
3. Napier KJ, Scheerer M, Misra S. Esophageal cancer: a review of epidemiology, pathogenesis, staging workup and treatment modalities. *World J. Gastrointest. Oncol* 2014; **6**; 112–120.

4. Nagini S. Carcinoma of the stomach: a review of epidemiology, pathogenesis, molecular genetics and chemoprevention. *World J. Gastrointest. Oncol.* 2012; **4**; 156–169.
5. Koopman T, Smits MM, Louwen M, Hage M, Boot H, Imholz ALT. HER2 positivity in gastric and esophageal adenocarcinoma: clinicopathological analysis and comparison. *J. Cancer Res. Clin. Oncol.* 2015; **141**; 1343–1351.
6. Janjigian YY, Werner D, Pauligk C *et al.* Prognosis of metastatic gastric and gastroesophageal junction cancer by *HER2* status: a European and USA international collaborative analysis. *Ann. Oncol.* 2012; **23**; 2656–2662.
7. Gravalos C, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. *Ann. Oncol.* 2008; **19**; 1523–1529.
8. Yoon HH, Shi Q, Sukov WR *et al.* Association of HER2/ErbB2 expression and gene amplification with pathological features and prognosis in esophageal adenocarcinomas. *Clin. Cancer Res.* 2012; **18**; 546–554.
9. Hu Y, Bandla S, Godfrey TE *et al.* HER2 amplification, overexpression and score criteria in esophageal adenocarcinoma. *Mod. Pathol.* 2011; **24**; 899–907.
10. Bang YJ, van Cutsem E, Feyereislova A *et al.* Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of *HER2* positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; **376**; 687–697.
11. Meulendijks D, Beerepoot LV, Boot H *et al.* Trastuzumab and bevacizumab combined with docetaxel, oxaliplatin and capecitabine as first-line treatment of advanced HER2-positive gastric cancer: a multicenter phase II study. *Invest. New Drugs* 2016; **34**; 119–128.
12. Hofmann M, Stoss O, Shi D *et al.* Assessment of a HER2 scoring system for gastric cancer: results from a validation study. *Histopathology* 2008; **52**; 797–805.
13. Rüschoff J, Dietel M, Baretton G *et al.* HER2 diagnostics in gastric cancer – guideline validation and development of standardized immunohistochemical testing. *Virchows Arch.* 2010; **457**; 299–307.
14. Koopman T, Louwen M, Hage M, Smits MM, Imholz ALT. Pathologic diagnostics of HER2 positivity in gastroesophageal adenocarcinoma. *Am. J. Clin. Pathol.* 2015; **143**; 257–264.
15. Skaland I, Ovestad I, Janssen EAM *et al.* Comparing subjective and digital image analysis HER2/neu expression scores with conventional and modified FISH scores in breast cancer. *J. Clin. Pathol.* 2008; **61**; 68–71.
16. Dobson L, Conway C, Hanley A *et al.* Image analysis as an adjunct to manual HER-2 immunohistochemical review: a diagnostic tool to standardize interpretation. *Histopathology* 2010; **57**; 27–38.
17. Laurinaviciene A, Dasevicius D, Ostapenko V, Jarmalaite S, Lazutka J, Laurinavicius A. Membrane connectivity estimated by digital image analysis of HER2 immunohistochemistry is concordant with visual scoring and fluorescence *in situ* hybridization results: algorithm evaluation on breast cancer tissue microarrays. *Diagn. Pathol.* 2011; **6**; 87.
18. Helin HO, Tuominen VJ, Ylinen O, Helin HJ, Isola J. Free digital image analysis software helps to resolve equivocal scores in HER2 immunohistochemistry. *Virchows Arch.* 2016; **468**; 191–198.
19. Brugmann A, Eld M, Lelkaitis G *et al.* Digital image analysis of membrane connectivity is a robust measure of HER2 immunostains. *Breast Cancer Res. Treat.* 2012; **132**; 41–49.
20. Wolff AC, Hammond ME, Hicks DG *et al.* Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *J. Clin. Oncol.* 2013; **31**; 3997–4013.
21. Holten-Rossing H, Møller Talman ML, Kristensson M, Vainer B. Optimizing HER2 assessment in breast cancer: application of automated image analysis. *Breast Cancer Res. Treat.* 2015; **152**; 367–375.
22. FMWV Code of Conduct for Health Research, 2011 [internet]. Available at: https://www.federa.org/sites/default/files/bijlagen/coreon/code_of_conduct_for_medical_research_1.pdf (accessed 17 May 2017).
23. Rüschoff J, Hanna W, Bilous M *et al.* HER2 testing in gastric cancer: a practical approach. *Mod. Pathol.* 2012; **25**; 637–650.
24. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; **33**; 159–174.
25. Fusco N, Rocco EG, Del Conte C *et al.* HER2 in gastric cancer: a digital image analysis in pre-neoplastic, primary and metastatic lesions. *Mod. Pathol.* 2013; **26**; 816–824.
26. Feuchtinger A, Stiehler T, Jütting U *et al.* Image analysis of immunohistochemistry is superior to visual scoring as shown for patient outcome of esophageal adenocarcinoma. *Histochem. Cell Biol.* 2015; **143**; 1–9.
27. Radu OM, Foxwell T, Cieply K *et al.* HER2 amplification in gastroesophageal adenocarcinoma: correlation of two antibodies using gastric cancer scoring criteria, H score, and digital image analysis with fluorescence *in situ* hybridization. *Am. J. Clin. Pathol.* 2012; **137**; 583–594.
28. Nielsen SL, Nielsen S, Vyberg M. Digital image analysis of HER2 immunostained gastric and gastroesophageal junction adenocarcinomas. *Appl. Immunohistochem. Mol. Morphol.* 2017; **25**; 610–617.
29. Ormenisan C, Wang J, Lawson D, Cohen C. Image cytometric HER2 in gastric carcinoma: is a new algorithm needed? *Appl. Immunohistochem. Mol. Morphol.* 2013; **21**; 414–419.
30. Jeung J, Patel R, Vila L, Wakefield D, Liu C. Quantitation of HER2/*neu* expression in primary gastroesophageal adenocarcinomas using conventional light microscopy and quantitative image analysis. *Arch. Pathol. Lab. Med.* 2012; **136**; 610–617.
31. Rakha EA, Pinder SE, Bartlett JMS *et al.* Updated UK recommendations for HER2 assessment in breast cancer. *J. Clin. Pathol.* 2015; **68**; 93–99.
32. HER2 testing site: a comprehensive resource for healthcare professionals outside the US [internet]. HER2 testing on gastric cancer. 2014. Available at: her2testing.org (accessed 18 May 2017).
33. Schrohl AS, Pederson HC, Jensen SS, Nielsen SL, Brünner N. Human epidermal growth factor receptor 2 (HER2) immunoreactivity: specificity of three pharmacodiagnostic antibodies. *Histopathology* 2011; **59**; 975–983.
34. NordiQC Immunohistochemical Quality Control [internet]. Assessment Run B21 2016 HER2 IHC. Available at: http://www.nordiqc.org/downloads/assessments/80_11.pdf (accessed 18 May 2017).
35. Laurén P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol. Microbiol. Scand.* 1965; **64**; 31–49.