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ORIGINAL RESEARCH ARTICLE

Selecting Targets for Molecular Imaging of Gastric Cancer: An Immunohistochemical Evaluation

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Abstract

Purpose Tumor-targeted positron emission tomography (PET) and fluorescence-guided surgery (FGS) could address current challenges in pre- and intraoperative imaging of gastric cancer. Adequate selection of molecular imaging targets remains crucial for successful tumor visualization. This study evaluated the potential of integrin $\alpha_v\beta_6$, carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5), epidermal growth factor receptor (EGFR), epithelial cell adhesion molecule (EpCAM) and human epidermal growth factor receptor-2 (HER2) for molecular imaging of primary gastric cancer, as well as lymph node and distant metastases.

Methods Expression of $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 was determined using immunohistochemistry in human tissue specimens of primary gastric adenocarcinoma, healthy surrounding stomach, esophageal and duodenal tissue, tumor-positive and tumor-negative lymph nodes, and distant metastases, followed by quantification using the total immunostaining score (TIS).

Results Positive biomarker expression in primary gastric tumors was observed in 86% for $\alpha_v\beta_6$, 72% for CEACAM5, 77% for EGFR, 93% for EpCAM and 71% for HER2. Tumor expression of CEACAM5, EGFR and EpCAM was higher compared to healthy stomach tissue expression, while this was not the case for $\alpha_v\beta_6$ and HER2. Tumor-positive lymph nodes could be distinguished from tumor-negative lymph nodes, with accuracy ranging from 82 to 93% between biomarkers. CEACAM5, EGFR and EpCAM were abundantly expressed on distant metastases, with expression in 88–95% of tissue specimens.

Conclusion Our findings show that CEACAM5, EGFR and EpCAM are promising targets for molecular imaging of primary gastric cancer, as well as visualization of both lymph node and distant metastases. Further clinical evaluation of PET and FGS tracers targeting these antigens is warranted.

Key Points

Carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5), epidermal growth factor receptor (EGFR) and epithelial cell adhesion molecule (EpCAM) are promising targets for molecular imaging of primary gastric cancer, as well as lymph node and distant metastases.

This study forms the preclinical groundwork for further clinical evaluation of positron emission tomography (PET) imaging and fluorescence-guided surgery tracers directed against these targets, which could enhance pre- and intraoperative imaging of gastric cancer and, ultimately, patient outcomes.

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Graphical Abstract

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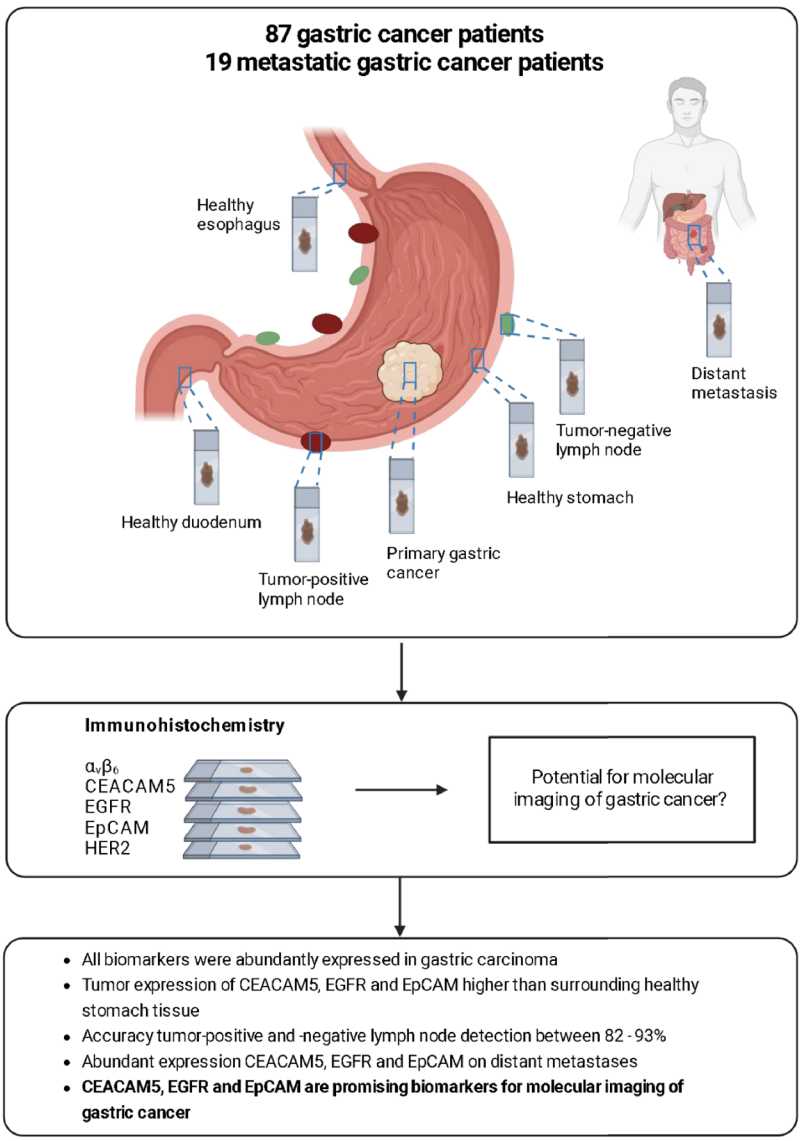

1 Introduction

Gastric cancer is the fifth most common malignancy, with a worldwide incidence of more than 1 million cases per year. Despite recent therapeutic advances, prognosis remains poor, with a 5-year survival of approximately 40%, resulting in more than 700,000 deaths worldwide annually [1, 2]. Achieving local control through subtotal or total gastrectomy combined with lymphadenectomy remains the cornerstone of multidisciplinary gastric cancer treatment [3]. Preoperatively, adequate disease staging is pivotal for patient-tailored treatment selection and maximizing its efficacy.

Preoperative work-up of gastric cancers is comprised of endoscopy, computed tomography (CT) imaging, ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG PET) and/or diagnostic laparoscopy in clinically curable locally advanced disease (>cT3 and/or N+, M0) [4, 5]. However, each modality has its limitations for accurate tumor detection, potentially leading to erroneous tumor staging and, consequently, unnecessary tumor resections, futile biopsies, extra imaging procedures and/or unnecessary administration of systemic therapy. For example, CT imaging provides accurate T-staging (sensitivity 83–100% for tumors with serosal involvement), while sensitivity for N-staging is lower at approximately 60% [4, 6, 7]. Importantly, sensitivity for small-sized distant metastases and peritoneal metastases is limited at 23–76%. Also, a significant proportion of gastric cancers have absent ^{18}F-FDG PET avidity (≈ 20%), and non-specific uptake in the stomach wall can also mask tumor presence [5, 8]. The use of ^{18}F-FDG PET for nodal and distant metastasis staging is also unsatisfactory, with sensitivity of 49% and 33–56%, respectively [5, 9].

To improve the accuracy of gastric cancer staging, diagnostic laparoscopy with or without peritoneal cytology is frequently performed [5, 10–12]. A systematic review highlighted that 9–60% of patients who were preoperatively staged as M0 had irresectable disease intraoperatively [10]. Recently, the PLASTIC trial reported the limited added value of ^{18}F-FDG PET and showed the superiority of diagnostic laparoscopy in accurate staging of locally advanced gastric cancer [5]. Limitations of laparoscopy, however, include its invasiveness, inability to accurately identify non-superficial liver metastases, lymph node metastases or extraperitoneal lesions, and the absence of tactile feedback for identifying malignant tissue [13, 14]. Besides tumor staging, an intraoperative challenge is encountered when radical resection is considered feasible. Studies showed that the presence of microscopically tumor-positive resection margins (i.e., R1 resection) is still observed in approximately 7% of gastric cancer patients, which has been associated with higher peritoneal recurrence rates and poorer survival [15, 16].

To address these challenges, tumor-targeted positron emission tomography (PET) and real-time fluorescence-guided surgery (FGS) using near-infrared light have emerged as valuable tools to enhance tumor imaging, respectively, by providing high-contrast visualization of malignant tissue [14, 17, 18]. These molecular imaging technologies could improve assessment of tumor localization, potentially avoiding resection for irresectable disease, as well as assisting surgeons in radical tumor resection. However, the success of molecular imaging hinges on the adequate selection of tumor-specific targets.

An ideal molecular imaging target is abundantly and homogenously expressed on the tumor cell membrane across all patients, while expression in healthy surrounding tissue is absent [17]. Preferably, the target-of-interest is also present on lymph node and distant metastases and its expression remains present in microscopic residual disease after neoadjuvant therapy (NAT). However, governed by tumor heterogeneity, among others, a universal molecular imaging target for gastric cancer has still not been identified.

Over the last years, several targets were recognized as promising for molecular imaging of gastrointestinal cancers, including integrin $\alpha_v\beta_6$, carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5), epidermal growth factor receptor (EGFR), epithelial cell adhesion molecule (EpCAM) and human epidermal growth factor receptor-2 (HER2) [19–24]. Consequently, tracers targeting some of these biomarkers were evaluated in clinical trials for various gastrointestinal tumor types [25–30]. However, their potential for molecular imaging of gastric cancer has been underexplored.

This study, therefore, provides the first crucial step towards application of these tracers in gastric cancer, by evaluating $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 as molecular imaging targets for gastric cancer and its metastases. To accomplish this, biomarker expression was evaluated within the full anatomical context of gastric cancer. Biomarker expression was, therefore, determined using immunohistochemistry on human tissue specimens of primary tumors, healthy surrounding stomach tissue, but also on esophageal and duodenal tissue, and LN^+ and LN^-, and distant metastases.

2 Materials and Methods

2.1 Patient and Tissue Specimen Selection

Pathology reports of patients who underwent resection for gastric adenocarcinoma at the Leiden University Medical Center (LUMC) from 2013 to 2020 were retrospectively reviewed. Representative formalin-fixed paraffin-embedded (FFPE) tissue blocks and hematoxylin and eosin (HE) slides

of 87 patients, containing primary gastric tumor, healthy stomach, esophageal, duodenal and/or (metastatic) lymph node tissue, were selected and obtained from the biobank at the LUMC. To allow proper subgroup analyses, the cohort was constituted to contain approximately a 1:1 ratio of patients with diffuse and intestinal type tumors according to the Laurén classification. Patients with mixed type Laurén classification were excluded. Selection of FFPE tissue blocks was performed by a gastrointestinal pathologist (ASLPC) based on the HE slides. Tissue specimens containing gastric adenocarcinoma metastases biopsy tissue were also obtained from 19 patients. Clinicopathological data were obtained from patients' medical records. Pathological tumor (pT) and pathological lymph node (pN) stages were defined according to the 8th edition of the American Joint Committee on Cancer and Union for International Cancer Control (AJCC/UICC) TNM staging system for gastric cancer. The study protocol was approved by both the Gastroenterology Biobank Review Committee (protocol reference: 2020-16) as well as the local medical ethical review committee (protocol reference: B20.052). This study was conducted in compliance with the Dutch code of conduct for responsible use of human tissue in medical research. Tissue specimens and clinicopathological data were handled in an anonymized manner and in compliance with the Declaration of Helsinki (1964).

2.2 Immunohistochemistry

Tissue sections 4 µm thick and cut from FFPE tissues were mounted on glass slides. Sections underwent deparaffinization in xylene for 15 min, followed by rehydration through sequential ethanol concentrations (100%, 50% and 25%). Subsequently, endogenous peroxidase was blocked using a 0.3% hydrogen peroxide solution. Antigen retrieval was tailored to the primary antibody employed, as outlined in Supplementary Table 1 in the electronic supplementary material (ESM). Post-antigen retrieval, slides were thoroughly rinsed in phosphate-buffered saline (PBS, pH 7.4). Primary antibodies (see Supplementary Table 1 in the ESM) targeting $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM or HER2 were applied to the tissue sections, which were subsequently left to incubate overnight at room temperature in a humid incubator. After overnight incubation, slides were rinsed in PBS to remove any residual primary antibodies. Anti-mouse horseradish peroxidase (HRP) or anti-rabbit HRP secondary antibodies (Envision, Dako, Glostrup, Denmark) were subsequently applied for 30 min at room temperature in a humid incubator for 30 min. Secondary antibodies were then removed by thorough PBS rinsing. Visualization of antibody binding was achieved using a 3,3-diaminobenzidine (DAB) tetrahydrochloride solution (K3468, Agilent Technologies, Inc., Santa Clara, CA, USA) for 10 min at room temperature. Finally, slides were counterstained with Mayer's hematoxylin (Klinipath B.V., Olen, Belgium), dehydrated in a dry incubator for 2 h and mounted using Pertex (Leica Microsystems, Wetzlar, Germany).

2.3 Evaluation of Immunohistochemical Staining

Whole slide images of the stained tissue slides were captured using the PANNORAMIC® 250 Flash III DX scanner (3DHISTECH Ltd, Budapest, Hungary). DAB staining was quantified using the total immunostaining score (TIS), which was computed by multiplying the staining proportion (0 = ≤ 9%, 1 = 10–25%, 2 = 26–50%, 3 = 51–75%, 4 = ≥ 76%) by the staining intensity (0 = none, 1 = weak, 2 = moderate, 3 = strong). Staining based on the TIS was categorized as follows: 0 = negative; 1, 2, 3, 4 = weak expression; 6, 8 = moderate expression; 9, 12 = strong expression. A panel of three independent observers (RDH, MvD, ASLPC) conducted the scoring. Instances of disagreement were discussed in a consensus meeting, during which the final score was conclusively determined.

2.4 Statistical Analysis

For categorial data, groups at baseline were compared using a Chi-square test. An independent samples *t* test was used to compare continuous variables of patient characteristics. TIS values between tumor, healthy surrounding stomach, esophageal and duodenal tissue were compared using Kruskal-Wallis test with Dunn's correction for multiple comparisons. Biomarker expression subgroup analyses were performed using a Mann-Whitney test. IBM SPSS statistics version 29 (IBM Corporation, Armonk, NY, USA) was used for all statistical analyses of patient characteristics. Graphs and statistical analyses for biomarker expression were created and performed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA, USA). Differences with a p value < 0.05 were considered significant.

3 Results

3.1 Patient Characteristics

Eighty-seven patients diagnosed with gastric adenocarcinoma were included, of which 45 (52%) had diffuse type disease and 42 (48%) had intestinal type disease. Clinicopathological characteristics are summarized in Table 1. In the intestinal type group, 17 patients (40%) had well–moderately differentiated tumors, compared to 0 (0%) in the diffuse type group ($p < 0.001$). NAT consisted of chemotherapy, while one patient received chemoradiotherapy. Albeit not statistically significant, there was a small

difference in the number of patients who received NAT in both groups (diffuse type: 32 [71%]; intestinal type: 22 [52%]; $p = 0.097$). Other baseline characteristics did not differ between both groups.

3.2 Biomarker Expression in Primary Gastric Cancer Tissue Specimens

Tissue slides were stained for $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 expression, and expression was quantified using the TIS. Representative examples of these stainings are shown in Fig. 1. Positive expression (TIS values ≥ 1) on primary gastric tumors was found in 86% of the tumors for $\alpha_v\beta_6$, 72% for CEACAM5, 77% for EGFR, 93% for EpCAM and 71% for HER2 (Table 2). Categorized staining intensities are depicted in Table 3. All biomarkers showed a membranous staining pattern, with $\alpha_v\beta_6$ and EpCAM showing a mostly homogenous staining pattern, while staining was slightly more heterogeneous for CEACAM5, EGFR and HER2 (Fig. 1). Additionally, biomarker co-expression in primary gastric tumors was analyzed (Table 4). The highest co-expressing biomarker combination was $\alpha_v\beta_6$ and EpCAM, which were simultaneously expressed in 84% for primary gastric tumors. The remaining biomarker combinations were always expressed in more than 55% of cases, indicating moderate co-expression. Additionally, for all biomarker

Table 1 Patient characteristics of the total gastric cancer cohort ($n = 87$) as well as diffuse type ($n = 45$) and intestinal type ($n = 42$) subgroups

Characteristic	Total ($n = 87$)	Diffuse type ($n = 45$)	Intestinal type ($n = 42$)	*P* value
Age, mean (SD)	67.2 (12.7)	64.3 (14.1)	70.3 (10.3)	0.073
Gender, *n* (%)				0.038
Male	28 (32)	26 (58)	33 (79)	
Female	59 (68)	19 (42)	9 (21)	
Surgery type, *n* (%)				0.407
Total gastrectomy	35 (40)	20 (44)	15 (36)	
Partial gastrectomy	52 (60)	25 (56)	27 (64)	
Tumor localization, *n* (%)				
Cardia/fundus	15 (17)	6 (13)	9 (21)	0.298
Corpus	24 (28)	11 (24)	13 (31)	
Antrum	34 (39)	20 (44)	14 (33)	
Pre-pyloric	8 (9)	3 (7)	5 (12)	
Other	6 (7)	5 (11)	1 (2)	
Tumor differentiation, *n* (%)				
Well–moderate	17 (20)	0 (0)	17 (40)	< 0.001
Poor	51 (59)	27 (60)	24 (57)	
Missing	19 (22)	18 (40)	1 (2)	
Primary tumor, *n* (%)				
pT1	15 (17)	5 (11)	10 (24)	0.210
pT2	9 (10)	7 (16)	2 (5)	
pT3	37 (43)	19 (42)	18 (43)	
pT4	26 (30)	14 (31)	12 (29)	
Regional lymph nodes, *n* (%)				
pN0	27 (31)	15 (33)	12 (29)	0.789
pN1	21 (24)	12 (27)	9 (21)	
pN2	16 (18)	8 (18)	8 (19)	
pN3	23 (26)	10 (22)	13 (31)	
Neoadjuvant therapy, *n* (%)				
Yes, chemotherapy	53 (61)	32 (71)	21 (50)	0.097
Yes, chemoradiotherapy	1 (1)	0 (0)	1 (2)	
No	33 (38)	13 (29)	20 (48)	
R-status, *n* (%)				
R0	76 (87)	38 (84)	38 (90)	0.234
R1	10 (11)	7 (16)	3 (7)	
Missing	1 (1)	0 (0)	1 (2)	

combinations, 88–98% of primary tumors were positive for at least one of the two biomarkers (≥ 1).

3.3 Subgroup Analyses of Biomarker Expression in Primary Gastric Cancer Tissue Specimens

Subgroup analyses revealed that median expression between diffuse and intestinal type tumors did not differ for all biomarkers except for HER2, which showed a lower median TIS on diffuse type tumors (median TIS 4.0 vs. 2.0, $p = 0.0004$; also see Supplementary Table 2 in the ESM). Moreover, subgroup analyses of biomarker expression in primary tumor tissues between patients who did not receive NAT and those who received NAT revealed that the median TIS for CEACAM5 and EGFR was lower on tumor specimens derived from patients who received NAT (CEACAM5: median TIS 9.0 vs. 3.5, $p = 0.0215$; EGFR: median TIS 6.0 vs. 3.0, $p = 0.0072$; also see Supplementary Table 3 in the ESM). For the remaining biomarkers, primary tumor expression was similar in patients who received NAT and patients who did not receive NAT.

3.4 Biomarker Expression in Primary Gastric Cancer Versus Healthy Surrounding Stomach, Esophageal and Duodenal Tissue Specimens

Images of sequential tumor sections showing biomarker expression in primary gastric cancer and healthy surrounding stomach, esophageal and duodenal tissue specimens are shown in Fig. 1. Quantified TIS values representing expression of $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 as well as the statistical comparison of biomarker expression is depicted in Fig. 2 and Supplementary Table 4 in the ESM. For $\alpha_v\beta_6$, median expression in primary gastric cancer tissue was lower compared to healthy surrounding stomach (median TIS 6.0 vs. 9.0, $p < 0.0001$) and duodenal tissue (median TIS 6.0 vs. 9.0, $p = 0.0427$), and similar to expression in esophageal tissue (median TIS 6.0 vs. 8.5, $p > 0.9999$). For CEACAM5, expression in primary tumor tissue was higher compared to healthy surrounding stomach (median TIS 4.0 vs. 0.0, $p < 0.0001$) and duodenal tissue (median TIS 4.0 vs. 0.0, $p = 0.0003$), but comparable to esophageal tissue (median TIS 4.0 vs. 4.0, $p > 0.9999$). EGFR expression in primary tumor tissue was higher compared to healthy surrounding stomach tissue (median TIS 4.0 vs. 2.0, $p = 0.0023$) but similar to esophageal (median TIS 4.0 vs. 6.0, $p = 0.2235$) and duodenal tissue (median TIS 4.0 vs. 3.0, $p > 0.9999$). EpCAM expression in tumor tissue was higher compared to healthy surround stomach (median TIS 9.0 vs. 0.0, $p < 0.0001$) and esophageal tissue (median TIS 9.0 vs. 0.0, $p < 0.0001$), but comparable to expression in duodenal tissue (median TIS 9.0 vs. 6.0, $p = 0.7003$). Lastly, HER2 expression in primary tumor tissue was not different from healthy surrounding stomach (median TIS 2.0 vs. 2.5, $p > 0.9999$) and esophageal tissue (median TIS 2.0 vs. 5.0, $p = 0.1454$) and lower than duodenal tissue (median TIS 2.0 vs. 8.0, $p = 0.0152$).

3.5 Expression of Biomarkers in Tumor-Positive and Tumor-Negative Lymph Node Tissue Specimens

Biomarker expression was evaluated on metastatic lymph nodes (LN^+) and tumor-negative lymph nodes (LN^-). Representative immunohistochemical (IHC) images showing expression of $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 on LN^+ tissue are shown in Fig. 3**.** Sensitivity, specificity, positive predictive value, negative predictive value and area under the curve were calculated based on dichotomous (positive/negative) biomarker expression and are depicted in Table 5**.** Although sensitivity for LN^+ versus LN^- differentiation was moderate for HER2 and CEACAM5 (both 56%), no false-positive staining was observed. For the remaining biomarkers, higher sensitivity (range 72–82%) and 100% specificity for differentiation between LN^+ and LN^- were observed, indicating their potential to serve as targets for imaging of metastatic lymph nodes. Accuracy for identifying LN^+ and LN^- ranged between 82% and 93% for all biomarkers.

3.6 Expression of Selected Biomarkers in Metastatic Gastric Cancer Tissue Specimens

Based on their tumor-specific expression pattern and accurate LN^+ detection potential, CEACAM5, EGFR and EpCAM were selected for further analysis of their expression in metastatic gastric cancer tissue specimens derived from 19 patients. Patient characteristics of this cohort are described in Supplementary Table 5 in the ESM. Tissue specimens were derived from various locations, with the most common locations including the abdominal wall (4/19, 21%), peritoneum (3/19, 16%) and large/small intestine (both 2/19, 11%). Representative IHC images of HE, CEACAM5, EGFR and EpCAM staining are depicted in Fig. 4**.** Positive biomarker expression in metastatic gastric cancer tissue specimens was observed in 94% for CEACAM5, 88% for EGFR and 95% for EpCAM (Table 6). As can be derived from Table 7, CEACAM5 and EpCAM staining was predominantly strong, while EGFR staining was somewhat weaker.

4 Discussion

Molecular imaging through tumor-targeted PET and FGS can address current limitations in pre- and intraoperative staging as well as resection margin assessment of gastric

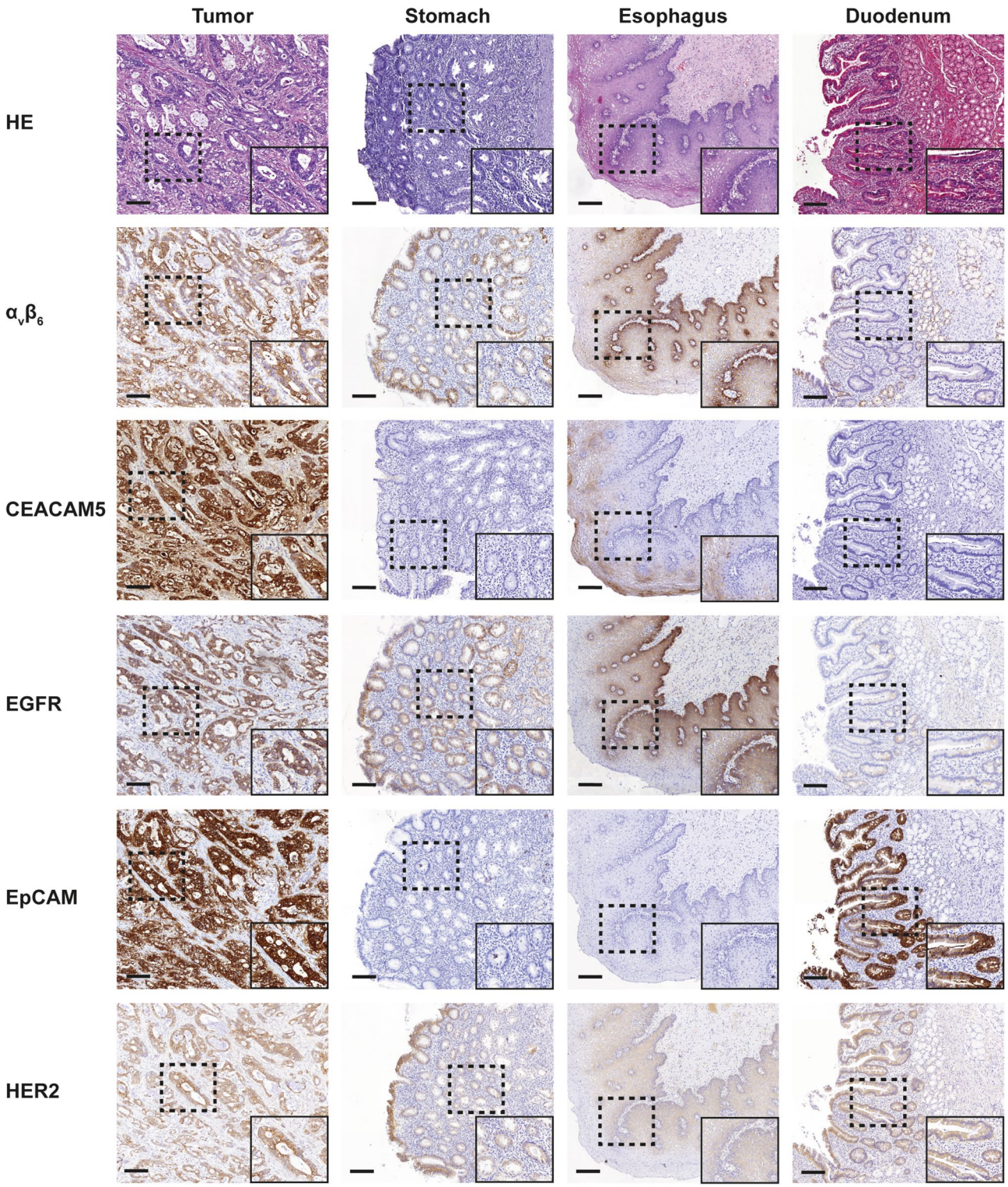

Fig. 1 Representative images of HE and immunohistochemical staining of $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 on primary gastric cancer, as well as healthy surrounding stomach, esophageal and duodenal tissue. Overview images and inserts are taken at 5× and 20× magnification, respectively. Scale bars represent 200 µM. *CEACAM5* carcinoembryonic antigen-related cell adhesion molecule 5, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *HE* hematoxylin and eosin, *HER2* human epidermal growth factor receptor-2

Table 2 Percentages of positive biomarker expression in primary gastric cancer tissue specimens (TIS ≥ 1)

Biomarker	Positive tumor expression (%)
$\alpha_v\beta_6$	86
CEACAM5	72
EGFR	77
EpCAM	93
HER2	71

CEACAM5 carcinoembryonic antigen-related cell adhesion molecule 5, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *HER2* human epidermal growth factor receptor-2, *TIS* total immunostaining score

cancer. Adequate selection and application of molecular imaging targets is the main prerequisite for adequate tumor visualization using these techniques. The current study showed that $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2, all promising tumor-specific targets for gastrointestinal cancers, were abundantly expressed in primary gastric tumor tissue specimens, with positive expression ranging from 71% to 93%. Regarding biomarker co-expression, 88–98% of primary gastric tumors showed positive expression of at least one of two biomarkers for all possible biomarker combinations, indicating the potential added value of bispecific tracers to increase the number of patients eligible for molecular imaging. Additionally, CEACAM5, EGFR and EpCAM showed higher expression in tumor tissue compared to healthy surrounding stomach tissue, classifying these targets as suitable for primary gastric cancer imaging. As $\alpha_v\beta_6$ and HER2 expression in healthy surrounding stomach tissue was higher or did not differ from primary tumor expression, respectively, we consider these targets not suitable for molecular imaging of primary gastric cancer. Despite moderate sensitivity for LN^+ detection observed for CEACAM5 and HER2 (both 56%), all biomarkers could distinguish LN^+ and LN^- with high accuracy, indicating the potential of these targets for pre- and intraoperative N-staging. Lastly, EGFR, EpCAM and CEACAM5 showed moderate to strong expression in virtually all distant (peritoneal) metastases, highlighting their potential as targets for M-staging. Our study therefore demonstrated the feasibility of EGFR, EpCAM and

Table 3 Distribution of $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 expression on primary gastric cancer as quantified by the TIS values categorized into negative (TIS = 0), weak (TIS = 1, 2, 3, 4) moderate (TIS = 6, 8) or strong expression (TIS = 9, 12)

Biomarker	No. of tissue	Negative *n* (%)	Weak *n* (%)	Moderate *n* (%)	Strong *n* (%)
$\alpha_v\beta_6$	*n* = 87	12 (14)	27 (31)	24 (28)	24 (28)
CEACAM5	*n* = 87	24 (28)	20 (23)	14 (16)	29 (33)
EGFR	*n* = 84	19 (23)	26 (31)	26 (31)	13 (15)
EpCAM	*n* = 86	6 (7)	7 (8)	18 (21)	55 (64)
HER2	*n* = 84	24 (29)	36 (43)	20 (24)	4 (5)

CEACAM5 carcinoembryonic antigen-related cell adhesion molecule 5, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *HER2* human epidermal growth factor receptor-2, *TIS* total immunostaining score

Table 4 Percentage of cases with positive $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 expression for at least one of two biomarker combinations (panel: ≥ 1) along with the percentage of cases with positive expression of both biomarkers (panel: both), as quantified by a dichotomized TIS (TIS = 0: negative expression; all other TIS values: positive expression)

Biomarker	Panel	$\alpha_v\beta_6$ (%)	CEACAM5 (%)	EGFR (%)	EpCAM (%)	HER2 (%)
$\alpha_v\beta_6$	≥ 1	–	93	94	95	94
	Both		66	77	84	71
CEACAM5	≥ 1	93	–	93	95	90
	Both	66		56	70	55
EGFR	≥ 1	94	93	–	98	88
	Both	77	56		74	59
EpCAM	≥ 1	95	95	98	–	94
	Both	84	70	74		71
HER2	≥ 1	94	90	88	94	–
	Both	71	55	59	71	

CEACAM5 carcinoembryonic antigen-related cell adhesion molecule 5, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *HER2* human epidermal growth factor receptor-2, *TIS* total immunostaining score

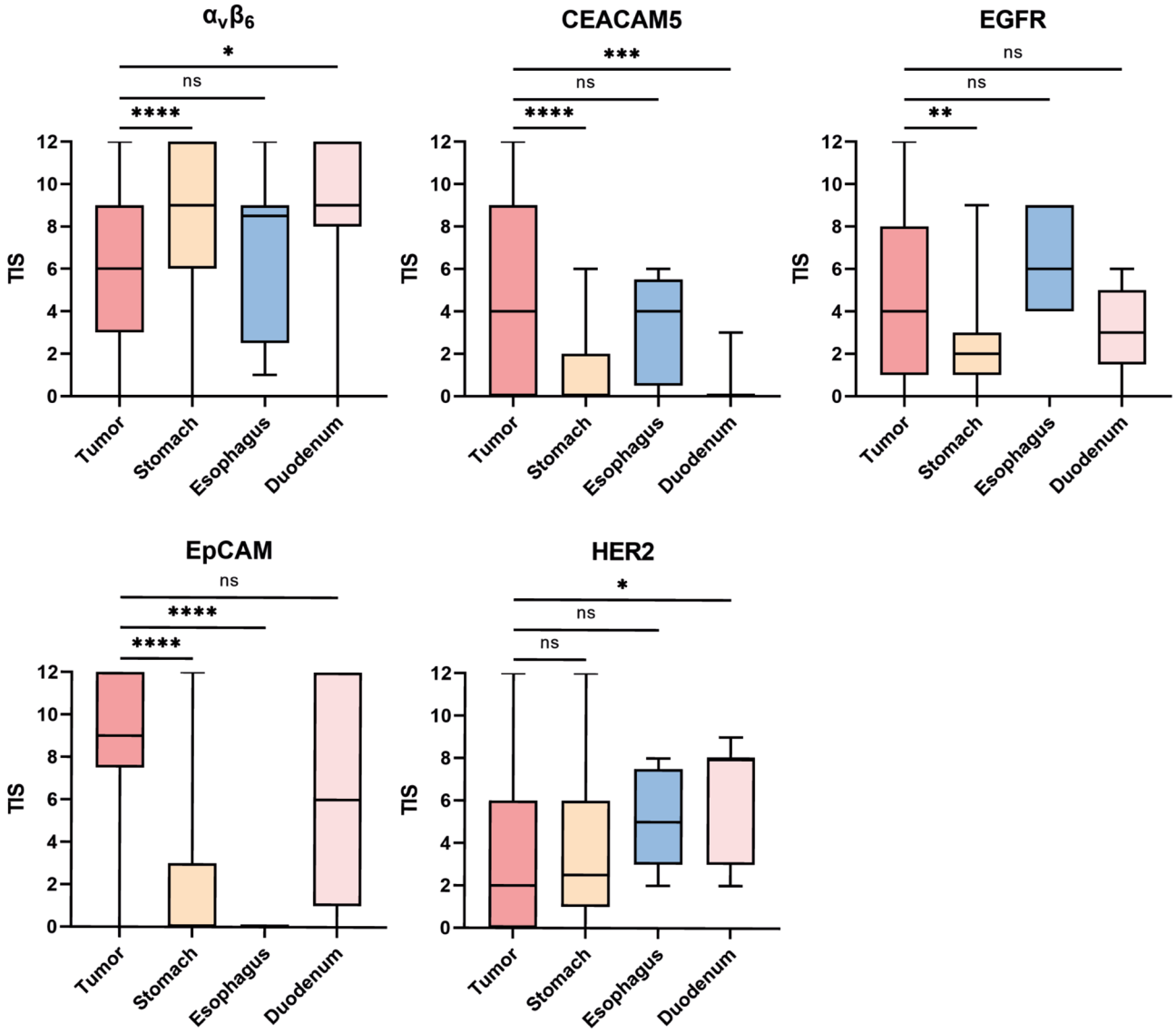

Fig. 2 Box plots representing TIS values of $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 staining on primary gastric cancer, as well as healthy surrounding stomach, esophageal and duodenal tissue. Horizontal lines represents the median TIS values, boxes represent interquartile range and brackets represent total TIS range. *CEACAM5* carcinoembryonic antigen-related cell adhesion molecule 5, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *HER2* human epidermal growth factor receptor-2, *ns* not significant, *TIS* total immunostaining score. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

CEACAM5 as molecular imaging targets for gastric cancer in a clinically relevant context.

The abundant tumor expression of the biomarkers reported herein is largely in line with previous studies, albeit we reported higher percentages of positive IHC staining compared to previous research, particularly for $\alpha_v\beta_6$, EGFR and HER2 [31–35]. This could, among others, have been caused by the use of different scoring systems, primary antibodies or antigen retrieval techniques during IHC staining, as well as inter- and intratumoral heterogeneity, and the relatively small sample sizes of previous IHC studies [36]. Confirmation of our results in a different or larger cohort of gastric cancer patients could verify validity of the results observed herein, as well as elucidate underlying mechanisms contributing to these discrepancies.

A strong methodological point of this study is the additional evaluation of biomarker expression in healthy surrounding esophageal and duodenal tissue specimens. Similar CEACAM5 and EpCAM expression levels were found on healthy esophageal and duodenal tissue compared to tumor expression, respectively, while EGFR expression in both tissue types did not differ from tumor expression. Consistent with our findings, expression of CEACAM5 and EGFR has been identified in healthy esophageal tissue,

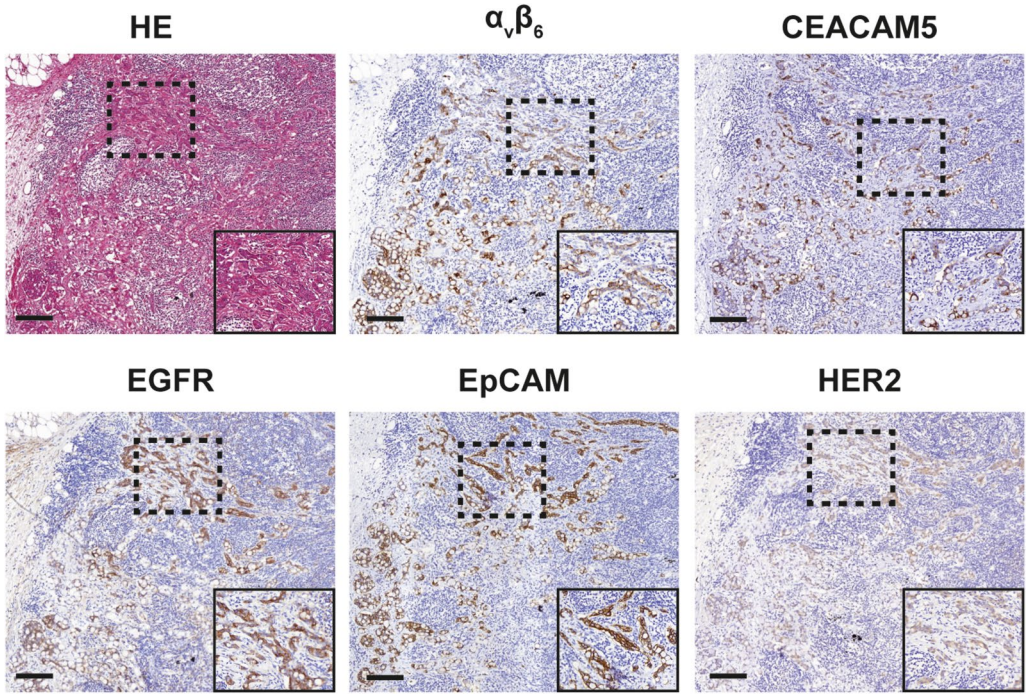

Fig. 3 Representative images of HE and immunohistochemical staining of $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 on lymph node metastases of gastric cancer. Overview images and inserts are taken at 5× and 20× magnification, respectively. Scale bars represent 200 µM. *CEACAM5* carcinoembryonic antigen-related cell adhesion molecule 5, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *HE* hematoxylin and eosin, *HER2* human epidermal growth factor receptor-2

Table 5 Sensitivity, specificity, PPV, NPV and accuracy along with the AUC and *p* value for identification of LN^+ tissue specimens based on $\alpha_v\beta_6$, CEACAM5, EGFR, EpCAM and HER2 expression

Biomarker	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)	Accuracy (%)	AUC (95% CI)	*P* value
$\alpha_v\beta_6$	72	100	100	85	89	0.860 (0.782–0.938)	< 0.0001
CEACAM5	56	100	100	78	83	0.780 (0.689–0.872)	< 0.0001
EGFR	74	100	100	86	90	0.872 (0.795–0.949)	< 0.0001
EpCAM	82	100	100	89	93	0.908 (0.843–0.974)	< 0.0001
HER2	56	100	100	77	82	0.780 (0.688–0.872)	< 0.0001

A dichotomized (positive/negative) TIS was used (TIS = 0: negative; all other TIS values: positive expression)

AUC area under the curve, *CEACAM5* carcinoembryonic antigen-related cell adhesion molecule 5, *CI* confidence interval, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *HER2* human epidermal growth factor receptor-2, LN^+ tumor-positive lymph node, *NPV* negative predictive value, *PPV* positive predictive value, *Sens.* sensitivity, *Spec.* specificity, *TIS* total immunostaining score

while EGFR and EpCAM expression in, respectively, duodenal mucosa and epithelia of both the small and large intestines was also reported [21, 37–40]. Although previous literature described that EpCAM is overexpressed in gastrointestinal tumors compared to healthy surrounding tissue, it should be noted that EpCAM's presence in the small and large intestine might impact the detection of peritoneal metastases of gastric cancer using EpCAM-targeted molecular imaging tracers [21]. Taken further, the absence of EpCAM in esophageal tissue makes EpCAM a more suitable target for delineation of proximal gastric cancers located near the esophageal-gastric junction (EGJ), while absence of CEACAM5 on duodenum epithelium makes this target appropriate for assessing resection margins of distal gastric cancers invading the duodenum. Of note, EGJ and duodenal invasion are frequently present in (sub)cardia (33–50%) and distal gastric cancer (14–33%), respectively [41–43]. Moreover, considering the increased R1 resection rate and reduced patient survival in these locally advanced cancers, adequate intraoperative tumor delineation may be a valuable tool to improve adequate

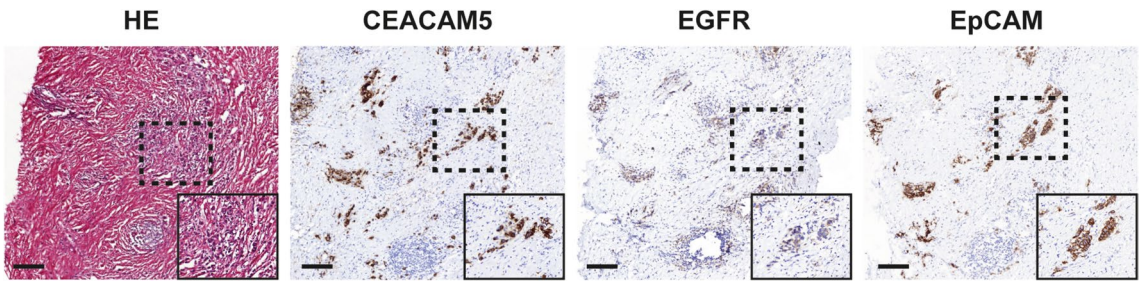

Fig. 4 Representative images of HE and immunohistochemical staining of CEACAM5, EGFR and EpCAM on distant metastases of gastric cancer. Overview images and inserts are taken at 5× and 20× magnification, respectively. Scale bars represent 200 µM. *CEACAM5* carcinoembryonic antigen-related cell adhesion molecule 5, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *HE* hematoxylin and eosin

Table 6 Percentages of positive biomarker expression in metastatic gastric cancer tissue specimens (TIS ≥1)

Biomarker	Positive tumor expression (%)
CEACAM5	94
EGFR	88
EpCAM	95

CEACAM5 carcinoembryonic antigen-related cell adhesion molecule 5, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *TIS* total immunostaining score

resection margin assessment and, potentially, patient outcomes [44, 45].

In addition, the inclusion of patients with diffuse and intestinal type adenocarcinomas, as well as patients who received NAT, allowed subgroup analyses to study potential effect of these clinicopathological factors on the biomarkers' expression level. Interestingly, we found similar biomarker expression in diffuse and intestinal type adenocarcinomas for all biomarkers, except for HER2, which showed lower TIS values on intestinal type tumor tissue specimens. This makes the remaining biomarkers broadly applicable as molecular imaging markers in gastric cancer patients. Moreover, this finding is particularly promising for molecular imaging of diffuse type gastric cancers, given the lower ^{18}F-FDG PET avidity, more frequent underestimation of the proximal margin length and increased irradical resection rate in this histological subtype [44, 46–49]. Additionally, subgroup analyses revealed that CEACAM5 and EGFR expression was lower in patients who received NAT. Consequently, care should be taken when targeting CEACAM5 and EGFR for molecular imaging of primary gastric tumors after NAT.

Preoperatively, several targeted PET tracers have aimed to address current limitations in staging of gastric cancer in both the preclinical and clinical setting, with a strong focus on fibroblast activation protein (FAP)-targeted agents [18]. FAP is expressed in 55–75% of gastric carcinomas and is associated with increased migration, invasion and reduced survival, while expression in healthy surrounding tissues is virtually absent [50–52]. A recent meta-analysis showed that FAPI PET outperformed conventional ^{18}F-FDG PET sensitivity for primary tumor, lymph node metastasis and peritoneal dissemination of gastric cancer, thereby indicating the potential of both FAPI PET and targeted PET in general [53, 54]. However, the overexpression of FAP in tissue during instances of tissue remodeling, such as wound healing or chronic inflammation, could pose a threat for its potential to delineate benign from malignant tissue [55]. Nevertheless, although our study intended to focus on tumor cell-specific molecular imaging targets, additional evaluation and comparison of FAP expression in our cohort would be an interesting continuation of this study.

Table 7 Distribution of CEACAM5, EGFR and EpCAM expression in metastatic gastric cancer tissue specimens as quantified by the TIS values categorized into negative (TIS = 0), weak (TIS = 1, 2, 3, 4) moderate (TIS = 6, 8) or strong expression (TIS = 9, 12)

Biomarker	No. of tissue	Negative *n* (%)	Weak *n* (%)	Moderate *n* (%)	Strong *n* (%)
CEACAM5	*n* = 18	1 (6)	6 (33)	4 (22)	7 (39)
EGFR	*n* = 16	2 (13)	10 (63)	2 (13)	2 (13)
EpCAM	*n* = 19	1 (5)	3 (16)	2 (11)	13 (68)

CEACAM5 carcinoembryonic antigen-related cell adhesion molecule 5, *EGFR* epidermal growth factor receptor, *EpCAM* epithelial cell adhesion molecule, *TIS* total immunostaining score

Of the targets evaluated herein, only HER2 has been clinically targeted for PET imaging in gastric cancer. Using ^{89}Zr-trastuzumab, O'Donoghue et al. observed tumor accumulation in 80% of patients with positive HER2 status; however, not all known lesions could be visualized in these patients [56]. Interestingly, the authors did not observe significant stomach uptake as one would expect based on our observation of similar HER2 expression in primary tumors and healthy surrounding stomach tissue. It should be noted that, although positive biomarker expression remains a fundamental criterion for successful molecular imaging, it does not invariably correlate with positive tumor uptake in the clinical setting, underscoring the importance of both tumor heterogeneity and extensive clinical validation of molecular imaging tracers. Noteworthy, significant stomach wall and intestine uptake is commonly reported for $\alpha_v\beta_6$-targeting PET tracers, thus reflecting our findings of high $\alpha_v\beta_6$ expression in these tissue types [57].

FGS-related research in gastric cancer has particularly focused on fluorescence-guided lymphadenectomy, as opposed to primary tumor imaging or intraoperative tumor staging. For instance, Chen et al. randomized gastric cancer patients between indocyanine green tracer-guided (ICG) lymphadenectomy using submucosal injection 1 day preoperatively and conventional laparoscopic gastrectomy [58]. The authors showed that ICG lymphadenectomy yielded more lymph nodes compared to the non-ICG group, leading to less unremoved lymph node stations, while complication rates between both groups were similar. However, sensitivity for metastatic lymph node detection was moderate at 56%. Considering these and previously outlined constraints in accurate intraoperative staging of gastric cancer staging, redirecting focus in FGS-related research towards tumor-targeted imaging could pave the way for novel tracers that address these limitations.

This study has some limitations. For instance, the relatively small sample size may have reduced the robustness of our subgroup analyses. Therefore, the findings of the subgroup analyses reported herein, although relevant for the assessment of a molecular imaging target's suitability, should be interpreted with caution. Secondly, due to the presence of staining artefacts, some slides were not suitable for scoring. Nevertheless, as the amount of excluded tissue slides per marker was limited (maximum 3/87 primary tumor specimens), we do not anticipate this influenced the findings of our study and the reproducibility thereof.

Future research into molecular imaging targets for gastric cancer could focus on their expression in premalignant tissue, such as chronic gastritis, intestinal metaplasia or dysplasia, thereby establishing the targets' potential for differentiation between malignant and benign tissue [59]. Also, as molecular targets are not expressed in all patients, preoperative screening for positive biomarker expression could be performed, followed by application of the most suitable molecular imaging tracer. When feasible, such a strategy would form a robust and efficient way of patient-tailored employment of molecular imaging tracers in gastric cancer, maximizing its potential to improve pre- and intraoperative staging as well as resection margin assessment. This may be performed using biopsy material of primary gastric tumors or metastases, which is routinely obtained for histological diagnosis. Moreover, the predictive value of biomarker expression in tumor biopsies for primary gastric tumor expression remains to be elucidated.

5 Conclusion

Our findings show that CEACAM5, EGFR and EpCAM are promising targets for molecular imaging of gastric cancer, as well as lymph node and distant metastases. By improving pre- and intraoperative identification of tumor tissue, targeted PET and FGS could enhance gastric cancer staging and resection, ultimately leading to improved patient outcomes. Further clinical evaluation of PET and FGS tracers targeting these antigens is warranted.

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Declarations

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Conflict of Interest RDH, MvD, KT, WdS, LFdGO, ASLPC, JB, ALV and PJKK declare no conflict of interest.

Availability of Data and Material The data presented in this study are available upon reasonable request from the corresponding author.

Ethics Approval The study protocol was approved by both the Gastroenterology Biobank Review Committee (protocol reference: 2020-16) and the local medical ethical review committee (protocol reference: B20.052). This study was conducted in compliance with the Dutch code of conduct for responsible use of human tissue in medical research. Tissue specimens and clinicopathological data were handled in anonymized manner and in compliance with the Declaration of Helsinki (1964).

Consent to Participate The need for informed consent was waived by the local medical ethical review committee.

Consent for Publication Not applicable.

Code Availability Not applicable.

Author Contributions RDH was responsible for conceptualization, methodology, formal analysis and visualization, and wrote the original draft of the manuscript. KT and RDH conducted the immunohistochemical experiments, and RDH, MvD and ASLPC contributed to scoring of the immunohistochemical stainings. All remaining authors contributed to reviewing and editing the manuscript. PJKK supervised the study. All authors read and approved the final version of the manuscript.

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