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Mechanisms of esophageal stricture after extensive endoscopic resection: a transcriptomic analysis

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ABSTRACT

Background and study aims Esophageal stricture is the most frequent adverse event after endoscopic resection for early esophageal neoplasia. Currently available treatments for the prevention of esophageal stricture are poorly effective and associated with major adverse events. Our aim was to identify transcripts specifically overexpressed or repressed in patients who have developed a post-endoscopic esophageal stricture, as potential targets for stricture prevention.

Patients and methods We conducted a prospective single-center study in a tertiary endoscopy center. Patients scheduled for an endoscopic resection and considered at risk of esophageal stricture were offered inclusion in the study. The healthy mucosa and resection bed were biopsied on Days 0, 14, and 90. A transcriptomic analysis by microarray was performed, and the differences in transcriptomic profile compared between patients with and without esophageal strictures.

Results Eight patients, four with esophageal stricture and four without, were analyzed. The mean ± SD circumferential extension of the mucosal defect was 85 ± 11 %. The transcriptomic analysis in the resection bed at day 14 found an activation of the interleukin (IL)-1 group (Z score = 2.159, P = 0.0137), while interferon-gamma (INFγ) and NUPR1 were inhibited (Z score = –2.375, P = 0.0022 and Z score = –2.333, P = 0.00131) in the stricture group. None of the activated or inhibited transcripts were still significantly so in any of the groups on Day 90.

Conclusions Our data suggest that IL-1 inhibition or INFγ supplementation could constitute promising targets for post-endoscopic esophageal stricture prevention.

Introduction

Endoscopic resection, with endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD), is the mainstay of management of early esophageal neoplasia [1]. While early adverse events (AEs) such as bleeding or perforations occur infrequently (<2%), post-endoscopic esophageal strictures remains a concern in 10% to 20% of patients [2, 3]. Post-endoscopic esophageal strictures occur in up to 90% of the patients when the resected circumference exceeds 75% of the esophageal lumen [4–6]; however, the pathophysiology of these strictures is poorly understood. As a result, stricture prevention strategies, based on mechanical interventions such as esophageal stent or preemptive dilatations, wound covering agents or cell sheets, stem cell therapy, or pharmacological interventions such as antimitotic, anti-fibrotic or anti-inflammatory agents, have not proven effective [7]. Topical steroid administration, either oral budesonide slurry or submucosal triamcinolone injection has been proposed with variable efficacy, especially on circumferential mucosal defects [8–10]. The most promising candidate for stricture prevention, oral corticosteroids [10, 11], has never demonstrated its efficacy in a randomized study, but has been associated with major adverse events [12, 13].

Our aim was to perform a mechanistic study of the esophageal wound healing process after extensive mucosal resection, using a transcriptomic analysis of esophageal biopsies, in order to identify overexpressed or underexpressed transcripts that could constitute targets for post-endoscopic esophageal stricture prevention.

Patients and methods

Patient selection

Consecutive patients with early esophageal neoplasia scheduled for endoscopic resection at high risk of esophageal stricture (i. e. involving at least three-quarters of the esophageal circumference) were offered to take part in the study before performing the resection. Patients were excluded if the endoscopic and transcriptomic assessment of the esophagus was impossible (e. g. in case of an esophagectomy following a non-curative endoscopic resection).

Endoscopic procedures and data collection

Endoscopic resections consisted in multiband mucosectomy or ESD, and were conducted under general anesthesia with endotracheal intubation and CO_2 insufflation by three expert endoscopists. Antiplatelet agents other than aspirin and anticoagulant therapy were discontinued before the procedure. High-definition upper gastrointestinal endoscopes with narrow-band imaging (GIF-H180J or GIF-HQ190, Olympus, Japan) or blue light imaging (EG-L590ZW, Fujifilm, Japan) were used. Procedures were carried out with a soft distal attachment cap. ESD knives included 1.5-mm Dual knife (Olympus, Japan) and straight or ball tip 1.5 mm Flush knife (Fujifilm, Japan), using a VIO 200 or 300 D electrosurgery unit (Erbe Medizin, Germany) with standard settings (Endocut I for incision, swift coagulation for submucosal dissection and soft coagulation for vessel coagulation). The ESD procedure was carried out as previously described [14], using an indigo-carmine-stained lifting solution made of 5% fructose and 10% glycerol mixed with saline [15]. Hemostasis of submucosal vessels was achieved with the ESD knife or a coagulation forceps (Coagrasper, Olympus, Japan). After completing the resection, four mucosal biopsies were taken in the healthy esophageal mucosa, 2 cm proximal to the resection bed. A liquid dye was allowed during the first 24 hours following the resection, and double dose proton pump inhibitors were administered to all patients during the month following the resection. At day one, soft diet was resumed and the patient discharged from the hospital.

On Days 14 and 90, a clinical consultation and an esophagogastroduodenoscopy were performed to check for signs of an early esophageal stricture, obtain mucosal biopsies in the healthy esophageal mucosa, 2 cm proximal to the resection bed (n = 4 biopsies), and on the resection bed itself (n = 4 biopsies). A hydrostatic balloon dilatation was performed in case of an esophageal stricture defined by the association of dysphagia and inability to pass a 10 mm gastroscope through the esophagus. Additional endoscopic balloon dilatations were performed whenever necessary, every 2 to 4 weeks [16], until stricture resolution.

Demographic, clinical, and procedure data were recorded on the day of the endoscopic resection, and at each follow-up endoscopy on Days 14 and 90. All esophageal biopsies taken on Days 0, 14, and 90 were immediately dispatched between 10% formol for histological analysis and Trizol reagent for –80 °C storage and further RNA extraction and transcriptomic analysis.

Histological and transcriptomic analysis

Mucosal biopsies were fixed in 10% formalin for 24 hours, embedded in paraffin, and blocks were sliced at 4 µm and stained with hematoxylin-eosin-saffron. Histological slides were assessed by two pathologists with expertise in digestive pathology (Pr B. Terris and Dr. F. Beuvon).

Total RNA from biopsies was extracted using Trizol (Invitrogen) according to the manufacturer's instructions and Diethyl Pyrocarbonate (DEPC). The quantity of RNA was measured using a Nanodrop ND-1000 spectrophotometer (Nyxor Biotech, Paris, France), and quality of the RNA was assessed using the Agilent 2100 Bioanalyzer (and the Agilent RNA6000 nano chip kit). All samples had an RNA Integrity number of more than 7 (between 7 and 9). After validating the quality of the RNA with Bioanalyzer 2100, 5 ng of total RNA was reverse transcribed in accordance with the Ovation PicoSL WTA System (Nugen). After controlling fragmentation using the Bioanalyzer 2100, cDNA was then hybridized to the genechip Human genome U133 Plus 2.0 array (Affymetryx) covering 47,000 transcripts and variants, to identify differentially expressed genes between the samples of cicatricial tissue in patients developing esophageal stricture and those who did not. Microarray reaction was obtained according to the manufacturer's instructions. After overnight hybridization, the chips were washed on the fluidic station FS450 following specific protocols (Affymetrix) and scanned using the GCS3000 7G. The scanned images were

▶ **Table 1** Patient and lesion characteristics.

	Post-endoscopic esophageal stricture N = 4	No post-endoscopic esophageal stricture N = 4	Overall
Sex ratio M/F	4/0	3/1	7/1
Mean ± SD age, years	64 ± 8	70 ± 9	67 ± 9
Indication for endoscopic resection, EAC/ SCC	2/2	3/1	5/3
Prior endoscopic therapy, n (%)	1 (25 %)	1 (25 %)	2/8 (25 %)
Site of the resection in the esophagus, n (%)			
▪ Middle third	2 (50 %)	1 (25 %)	3 (38 %)
▪ Lower third	2 (50 %)	3 (75 %)	5 (62 %)
Final histology, n (%)			
▪ T1am1	3 (75 %)	–	3 (38 %)
▪ T1am2	1 (25 %)	1 (25 %)	2 (25 %)
▪ T1am3	–	2 (50 %)	2 (25 %)
▪ T1b	–	1 (25 %)	1 (13 %)
Mean ± SD mucosal defect after ER, (%)	95 ± 6	75 ± 0	85 ± 11

EAC, early adenocarcinoma; N, number; SCC, squamous cell carcinoma; SD, standard deviation; ER, endoscopic resection.

then analyzed with Expression Console software (Affymetrix) to obtain raw data and metrics for Quality Control.

Bioinformatics

The raw data were normalized using the Robust Multichip Algorithm (RMA) in the Bioconductor R. All quality controls and statistics were then carried out using Partek GSsoftware. First, we established hierarchical clustering (Pearson's dissimilarity and average linkage) and Principal Component Analysis to control the data. To find differentially expressed genes, we used a classic variance analysis for each gene and made paired Tukey's post hoc tests between groups. We then used $P<0.05$ and fold changes >1.2 or <–1.2, further computed into Z-scores, to filter and select differentially expressed genes. Data were analyzed through the use of IPA (QIAGEN Inc., https://wwwquiagenbioinformatics.com/products/ingenuity-pathway-analysis).

Ethical aspects

An independent ethics committee (Comité de Protection des Personnes Ile de France 3, n° 2014-A01323-44) approved the study. All patients provided written informed consent for their participation to the study.

Descriptive statistics and comparisons between the groups

Continuous data were expressed as median values and interquartile range (IQR) and compared with an unpaired *t*-test. Categorical data were expressed as percentages and compared with a Fisher's exact test. $P<0.05$ was considered to indicate statistical significance.

Results

Patients

Between October 2014 and August 2016, 15 patients with a planned esophageal endoscopic resection at high risk of stricture agreed to take part in the study. Six patients were excluded because of absence of endoscopic follow-up: in five patients because of poor histoprognostic factors resulting in an esophagectomy or a chemoradiotherapy, and one because of a concomitant diagnostic metastatic rectal carcinoma, declined endoscopic follow-up. In a last patient, RNA was found to be degraded on Day 14 and 90 biopsy samples (RNA Integrity number of 2.6 and 1.4, respectively). Finally, eight patients, four with esophageal stricture and four without, were analyzed. Patients' characteristics are presented in ▶ **Table 1**.

One patient in each group had undergone prior endoscopic therapy, one with EMR and radiofrequency ablation, and the second with EMR and photodynamic therapy, both for dysplastic Barrett's esophagus. The resection type was ESD in seven of eight cases, and the only EMR was performed in a patient with dysplastic nodular high-grade dysplasia on Barrett's esophagus resected over 90 % of the esophageal circumference, resulting in a post-endoscopic stricture.

Long-term follow-up

The mean ± SD follow-up was 52 ± 29 months. All post-endoscopic strictures were successfully managed by a median (range) of one (1–4) endoscopic balloon dilatation. In the post-endoscopic stricture group, one patient with squamous

cell neoplasia and one patient with Barrett's neoplasia remained in complete remission of dysplasia and intestinal metaplasia, one patient had residual non-dysplastic intestinal metaplasia that was not eradicated owing to the risk of stricture recurrence, and one patient with squamous cell neoplasia underwent an esophagectomy for recurrent squamous cell carcinoma. In the non-stricture group, one patient underwent four radiofrequency ablation procedures with successful eradication of intestinal metaplasia, one patient had residual non-dysplastic intestinal metaplasia and declined endoscopic eradication therapy, and the two other patients remained in complete remission of intestinal metaplasia and dysplasia.

Histological analysis

The histological analysis of the resection specimens showed T1a lesions without poor histoprognostic factors and complete resection in all patients of the stricture group, and in two patients of the non-stricture group; another patient of this group had a T1am3 adenocarcinoma with moderate differentiation and lymphovascular involvement, and the last one had a deep submucosal infiltrating (1900 µm) T1b squamous cell carcinoma. Of these two patients, one declined further treatment, and one was unfit for surgery. While histological analysis of the normal appearing esophageal mucosa proximal to the resection was always normal on Days 0, 14, and 90, the biopsies of the scarred area revealed nonspecific granulation tissue with neovascularization, polymorph inflammatory cells, edema, and absence of epithelial cells in both groups. On Day 90, the endoscopic biopsies were normal in one patient of the stricture group, and showed moderate fibrosis of the lamina propria associated with moderate lympho-plasmocytic infiltration in the two other patients of this group. Meanwhile, no fibrotic changes were evidenced in the non-stricture group, two biopsies being strictly normal, and two revealing dysplastic Barrett's esophagus (low-grade dysplasia). The histological results are presented in the ▶ **Table 2**.

Transcriptomic analysis

The comparison of the levels of transcript expression in the resection bed at day 14 between the stricture and the non-stricture groups revealed statistically significant differences. The transcripts with Z scores >1 or <–1 (indicating activation or inhibition, respectively) and $P<0.05$ (indicating enriched pathways) are presented in the ▶ **Table 3** and ▶ **Table 4**. Mainly, interleukin-1 (IL-1) was activated (Z score = 2.159, $P=0.0137$), while interferon-gamma (INFγ) and NUPR1 were inhibited (Z score = –2.375, $P=0.0022$ and Z score = –2.333, $P=0.00131$) in the stricture group.

On Day 14, none of the activated transcripts were still significantly differentially expressed between the stricture and the non-stricture group in the resection bed on Day 90. No transcript was significantly activated at day 90. The significantly inhibited transcripts on Day 90 are presented in ▶ **Table 5**. However, NUPR1 and vascular endothelial growth factor were inhibited in the stricture group at day 90 (Z score = –1.342, $P=0.0301$ and Z score = –1.067, $P=0.0178$, respectively). In addition, Oncostatin M was moderately inhibited in the stricture group at day 90 (Z score = –1.069, $P=0.00806$).

Finally, we did not observe any statistically significant difference between the stricture and non-stricture group Days 0, 14 or 90 in terms of transcript expression in normal esophageal mucosa above the endoscopic resection bed.

Discussion

The expanding indications of esophageal ESD result in larger resection specimens and an increased frequency of post-endoscopic esophageal strictures [17, 18]. There is no currently recommended preventive treatment for these strictures, and their management relies on iterative endoscopic dilatation. These strictures result from inflammatory and fibrogenetic processes taking place in the first month following the mucosal resection [7], and which seem to be extinct seem after two to three months. We identified IL-1 and estrogen receptor 1 (ESR1) to be significantly upregulated at day 14 in the resection wound of patients who developed an esophageal stricture, compared to those who did not. Conversely, IFNγ, Nuclear Protein 1 (NUPR1), GLI2, and CD40 L were significantly downregulated in patients with post-endoscopic esophageal stricture. Importantly, these modifications were only expressed in the resection bed and during the early phase of the wound healing process.

The pathophysiology of esophageal stricture formation is poorly understood. The combined effects of decreased extracellular matrix degradation by metalloproteinases, increased synthesis of collagen and fibronectin, and wound contraction by myofibroblasts are thought to be the key mechanisms at stake. This inflammatory and fibrogenic cascade is obviously triggered by the epithelial damage [7]. However, the origin of the myelofibroblasts is still debated [19], and these cells could originate from epithelial cells undergoing epithelial to mesenchymal transition, or from a differentiation of local fibroblasts or muscle cells.

IL-1 is a proinflammatory cytokine, produced by monocytes and macrophages, involved in innate immunity mechanisms. Il-1 β, in particular, has been shown in vitro to drive epithelial to mesenchymal transition (toward myofibroblasts) of esophageal epithelial cells and contribute to esophageal inflammation and fibrogenesis [20]. Il-1β is also overexpressed at the early stages of laryngeal stricture formation after mucosal injury [21] and radiation induced esophageal stricture [22] in animal models. Considering the availability and excellent tolerability of IL-1 inhibitors and IL-1 receptor antagonists inhibitors [23], IL-1 pathway blockade could constitute a promising target to prevent post-endoscopic esophageal stricture. ESR1or ESRα and more generally the estrogen-signaling pathway have not been specifically studied in esophageal or digestive fibrosis. In animal models of dermal fibrosis however, estrogens rather seem to exhibit antifibrotic properties, estrogen inhibition by tamoxifen leading to increased dermal fibrosis [24]. Similarly, a recent database analysis found ESR1 to play a role in the pathogenesis of the chronic diabetic wound by limiting the survival of the resident fibroblasts [25]. Therefore, ESR1 might not be an optimal

▶ **Table 2** Histological analysis of esophageal biopsies.

Patient	Post-endoscopic esophageal stricture	Endoscopic resection specimen (D0)	Proximal squamous epithelium (D0)	Wound healing zone (D14)	Proximal squamous epithelium (D14)	Endoscopic resection scar (D90)	Proximal squamous epithelium (D90)
1	Yes	T1am1 adenocarcinoma	Normal squamous mucosa	Fibrinous exsudate, granulation tissue with predominantly polynuclear leukocytes	Normal squamous mucosa	Normal squamous mucosa	Normal squamous mucosa
2	Yes	T1am2 squamous cell carcinoma	Normal squamous mucosa	Fibrinous exsudate, granulation tissue with predominantly polynuclear leukocytes	Normal squamous mucosa	Normal squamous mucosa	Normal squamous mucosa
3	Yes	T1am1 adenocarcinoma	Normal squamous mucosa	Fibrinous exsudate, granulation tissue with predominantly polynuclear leukocytes	Normal squamous mucosa	Columnar cardial mucosa without intestinal metaplasia or dysplasia, fibrosis of the lamina propria	Normal squamous mucosa
4	Yes	T1am1 squamous cell carcinoma	Normal squamous mucosa	Fibrinous exsudate, granulation tissue with predominantly polynuclear leukocytes	Normal squamous mucosa	Squamous mucosa with slight dystrophic changes and congestion of the papillae	Normal squamous mucosa
5	No	T1am2 adenocarcinoma	Normal squamous mucosa	Fibrinous exsudate, granulation tissue with predominantly polynuclear leukocytes	Normal squamous mucosa	Barrett's esophagus with low-grade dysplasia	Normal squamous mucosa
6	No	T1am3 adenocarcinoma	Normal squamous mucosa	Fibrinous exsudate, granulation tissue with predominantly polynuclear leukocytes	Normal squamous mucosa	Normal squamous mucosa	Normal squamous mucosa
7	No	T1b squamous cell carcinoma	Normal squamous mucosa	Fibrinous exsudate, granulation tissue with predominantly polynuclear leukocytes	Normal squamous mucosa	Normal squamous mucosa	Normal squamous mucosa
8	No	T1am3 adenocarcinoma	Normal squamous mucosa	Fibrinous exsudate, granulation tissue with predominantly polynuclear leukocytes	Normal squamous mucosa	Normal squamous mucosa	Normal squamous mucosa

target to prevent esophageal stricture, and its upregulation in the stricture group could reflect a compensatory mechanism to limit stricture development. IFNγ is a soluble cytokine with immunomodulatory activity, reducing fibroblast activation and differentiation, resulting in an antifibrotic activity in animal and in vitro models of lung, kidney, and liver fibrosis [26–28]. In addition, IFNγ reduced the rate of arterial stenosis after intimal lesions in the rat [29]. Finally, IFNγ reduced the rate of esophageal stricture and collagen production after caustic burns in the esophagus in an animal model [30]. Considering our results, IFNγ administration during the early phase of the wound healing process could be a feasible and valid option to limit the rate of esophageal stricture. Nuclear Protein 1 (NUPR1) is a stress-inducible protein, involved in many fibrogenic processes in the kidney, heart, and pancreas [31]. To the best of our knowledge, NUPR1 has not been studied in the esophagus or in stricture mechanisms of the digestive tract. Its downregulation in the esophagus of patients with post-endoscopic esophageal strictures is unexpected. GLI 2 is a zinc finger transcription factor that is activated in the canonical, SMAD3-mediated, transforming growth factor-β pathway. As such, GLI2 belongs to profibrogenic proteins [32], and its downregulation in the

▶ **Table 3** Significantly activated transcripts on Day 14 in the stricture and non-stricture **groups.**

Transcript	Activation Z-score	*P* value
Interleukin 1 (IL-1)	2.159	0.0137
Estrogen Receptor 1 (ESR 1)	1.554	0.0443
TP53	1.277	0.00416
Vascular endothelial growth factor (VEGF)	0.987	0.000496
TP63	0.832	0.0292

▶ **Table 4** Significantly inhibited transcripts on Day 14 in the stricture and non-stricture groups.

Transcript	Activation Z-score	*P* value
Interferon-gamma (IFNγ)	−2.375	0.0022
Nuclear Protein 1 (NUPR1)	−2.333	0.00131
GLI2	−1.922	0.000174
CD154 (CD40 L)	−1.457	0.0109
Interleukin 5 (Il-5)	−1.4	0.000536
CG	−1.342	0.000222
Hypoxia-induced factor 1 A (HIF1A)	−1.311	0.00595
Transforming growth factor β1 (TGFβ 1)	−1.09	0.0000181
Epidermal growth factor receptor (EGFR)	−1.073	0.0116
Retinoblastoma protein (RB 1)	−1	0.000605

▶ **Table 5** Significantly inhibited transcripts on Day 90 in the stricture and non-stricture groups.

Transcript	Activation Z-score	*P* value
Nuclear Protein 1 (NUPR1)	−1.342	0.0301
Oncostatin M	−1.069	0.00806
Vascular endothelial growth factor (VEGF)	−1.067	0.0178

esophageal biopsies of patients with strictures is unexpected. Again, it might constitute a compensatory mechanism to limit stricture development. CD40 L (CD154), the natural ligand of CD40, is located at the surface of B and T cells, as well as macrophages, dendritic cells, endothelial cells, fibroblasts, and epithelial cell. Its activation results in the triggering of the PI3K, NF-κB, and p38/ERK cascades, resulting in inflammatory and fibrogenic activity [33, 34]. Here again, the downregulation of CD40 L is surprising, but could be associated to the underexpression of IFNγ.

Our study is the first to study the mechanistic aspects of post-endoscopic esophageal stricture formation in man, identifying potential targets for a pharmacological prevention of esophageal stricture. Importantly, we identified specific factors to the fibrogenesis in the human esophagus that are not predictable from works on pathological wound healing in other organs. Despite a large number of patients that were ultimately excluded from the analysis, the two study groups were well balanced in terms of patient number and interventions. As expected however, the stricture group had endoscopic resections involving larger proportions of the esophageal circumference, with more squamous cell neoplasia, located in the middle third of the esophagus. Contrary to previous reports [35], the invasion depth of the resected neoplasia had no impact on the occurrence of post-endoscopic stricture.

The main limitations of our work include the absence of validation of our microarray findings by RT-PCR and the small sample size (considering the costs involved in the transcriptomic analysis). However, the multiplicity of the control groups (normal squamous mucosa proximal to the resection vs wound healing mucosa, wound healing mucosa on Day 14 vs. Day 90, and stricture vs. non-stricture groups) increases the robustness of our findings. Our data can only point out an association between IL-1 activation or IFNγ and NUPR1 inhibition and post-endoscopic stricture formation, and no causal relationship can be concluded from our work. Therefore, we can only conclude that IL-1 inhibition (using readily available drugs such as anakinra) or INFγ are targets for post-endoscopic esophageal stricture prevention. We believe that these hypotheses should be tested in animal models of post- endoscopic esophageal stricture, such as the porcine model [36], before considering a phase I study in Man.

Because we used Human Genome U133 Plus 2.0 array chips (Affymetryx), we actually repeated the statistic testing for each transcript of the chip (n = 47 000). However, unlike RNA sequencing, for micro-arrays, as for proteomic analysis, we generally do not consider adjusted p-values, as we often process few samples, which implies low statistical power. Applying a *P* value adjustment would further increase the risk of erroneously excluding true positive results.

The inclusion of patients with squamous cell and Barrett's neoplasia, requested by the relatively small number of patient eligible for inclusion, is questionable. Indeed, the distal esophagus is wider, possibly more flexible, and exposed to a different chemical stress (gastric reflux) than the proximal esophagus. Therefore, the risk of esophageal stricture could be different in the proximal or distal esophagus. However, the risk of stricture following circumferential endoscopic resection in the lower esophagus reached 88 % in a prospective multicenter study of stepwise complete endoscopic resection of early Barrett's neoplasia [5], and 100 % in a smaller series involving ESD in a single treatment session for Barrett's neoplasia [6]. In addition, our team recently reported that for a given circumferential mucosal defect, the stricture rate was similar between squamous cell carcinoma and Barrett's neoplasia [16]. Although we cannot exclude that different molecular mechanisms lead to post-endoscopic esophageal stricture are different in the proximal and distal esophagus, we did observe similar stricture rates at both sites.

Conclusions

In conclusion, we identified IL-1 and INFγ as key players in the constitution of post-endoscopic esophageal stricture. This suggests that the administration of IL-1 inhibitors or INFγ during the early phases of the wound healing process following an endoscopic resection at high risk of esophageal strictures should be considered in preclinical models.

Competing interests

M Barret: Medtronic, olympus (medical training), Dr Falk pharma (oral presentation), Norgine, Ambu (board participation);
The other authors declared that they do not have any conflict of interest.

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