Laparoscopic ischemic conditioning of the stomach prior to esophagectomy induces gastric neo-angiogenesis

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Background: The risk of an anastomotic leakage (AL) following Ivor-Lewis esophagectomy is increased in patients with calcifications of the aorta or a stenosis of the coeliac trunc. Ischemic conditioning (ISCON) of the gastric conduit prior to esophagectomy is supposed to improve gastric vascularization at the anastomotic site. The prospective ISCON trial was conducted to proof the safety and feasibility of this strategy with partial gastric devascularization 14 days before esophagectomy in esophageal cancer patients with a compromised vascular status. This work reports the results from a translational project of the ISCON trial aimed to investigate variables of neo-angiogenesis.

Methods: Twenty esophageal cancer patients scheduled for esophagectomy were included in the ISCON trial. Serum samples (n=11) were collected for measurement of biomarkers and biopsies (n=12) of the gastric fundus were taken before and after ISCON of the gastric conduit. Serum samples were analyzed including 62 different cytokines. Vascularization of the gastric mucosa was assessed on paraffin-embedded sections stained against CD34 to detect the degree of microvascular density and vessel size.

Results: Between November 2019 and January 2022 patients were included in the ISCON Trial. While serum samples showed no differences regarding cytokine levels before and after ISCON biopsies of the gastric mucosa demonstrated a significant increase in microvascular density after ISCON as compared to the corresponding gastric sample before the intervention.

Conclusion: The data prove that ISCON of the gastric conduit as esophageal substitute induces significant neo-angiogenesis in the gastric fundus which is considered as surrogate of an improved vascularization at the anastomotic site.

Keywords: esophagectomy, gastric conduit, ischemic conditioning, angiogenesis

Introduction

Transthoracic esophagectomy with gastric reconstruction is generally accepted as surgical standard of multimodal treatment for esophageal cancer patients [1]. In this setting, anastomotic leakage remains the most common and serious complication considerably contributing to postoperative morbidity and mortality [2,3]. Despite a multifactorial etiology, a sufficient vascular perfusion of the gastric conduit is considered to be the most important factor for the development of AL. In addition to the general deterioration of gastric perfusion associated with the gastric tube formation, severe atherosclerosis of the thoracic aorta as well as a stenosing celiac axis have been recognised as significant risk factor of AL in recently published observational studies [4-9].

To improve gastric vascularisation and thereby to reduce the incidence of postoperative AL, ISCON of the gastric conduit has been investigated in several experimental human and animal studies. However, in recent meta-analyses summarizing the present evidence of clinical trials, ISCON could not prove to reduce the incidence of AL. This is mainly due to the fact that ISCON was applied to unselected cohorts not differentiating between patients with a normal or increased risk for the occurrence of AL [10]. Furthermore, the time interval between ISCON and esophagectomy was generally only one week, mostly too short as animal experiments show an optimal neovascularisation after 14 days [11].

Based on these findings, the ISCON trial was designed to investigate the feasibility and safety of ischemic conditioning in patients with a compromised vascular status defined by the amount of calcifications of the thoracic aorta and the degree of celiac trunc stenosis [8,9]. As part of this clinical feasibility trial[12], an accompanying translational program was set up to investigate serological and histopathological variables of neoangiogenesis aiming to demonstrate possible changes of microcirculation associated with ISCON.

Methods

The study protocol of this prospective, single-arm feasibility trial was approved by the Medical Ethical Committees of the University Medical Center Utrecht (reference number NL67819.041.18) and the University of Cologne (reference number 18-299). The study protocol has been previously published and was prospectively registered at clinicaltrials.gov [13]. The clinical results of the trial were recently published elsewhere [12].
Consecutive patients with a resectable esophageal carcinoma (cT1-4aN0-3M0) scheduled for an esophagectomy were eligible for this study. The main inclusion criteria were major calcifications in the thoracic aorta according to the Utrecht Calcification Score (UCS) and any degree of a celiac trunk stenosis according to the modified NASCET score [7,9]. ISCON includes a diagnostic laparoscopy to partially devascularise the stomach by transection of the left gastric artery and vein and the short gastric vessels. The right gastric artery and the right gastroepiploic artery along the greater curvature remained preserved. The ISCON procedure was followed by transthoracic esophagectomy after an interval of 12-18 days.

Blood samples (10 ml) were collected in the operation theatre before the ISCON procedure and 12-18 days later before the esophagectomy. The blood was stored in EDTA and immediately transferred to the lab for centrifugation at 2000 g for 10 minutes at + 4 ° C. Serum aliquots were stored at - 80 ° C. The cytokine array was performed *via* Luminex Discovery Assay (R&D Biotechne) with the indicated analytes according to the manufacturer's instructions. Frozen serum samples were thawed, centrifuged at 1000 g for 10 minutes and diluted 1:2 in Calibrator Diluent (R&D Biotechne) prior to analysis. Cytokines/Chemokines were measured with Luminex 200 xMAP system (Luminex) and quantified by comparison to a standard curve. xPONENT software was used for data collection and analysis.

Gastric biopsies of the fundus were endoscopically taken on the day before ISCON or intra-operatively immediately prior the ISCON procedure. The second set of endoscopic tissue samples were collected on the day before esophagectomy or alternatively half of the gastric stapling donut or the tip of the gastric conduit received after completion of esophagogastrostomy was sent in for further histopathological examination of the gastric mucosa. All tissue samples were stained against CD34 with mouse monoclonal anti-CD34 antibody (dilution 1:700, Cellmarque / CEQBEnd10) on Leica BOND-MAX stainer (Leica Biosystems, Germany) according to the manufacturers' protocol using a citrate-based antigen retrieval protocol. Slides were scanned using a slide scanner. At least three representative images (100x magnification) of the gastric mucosa were analysed via ImageJ using an experimentally determined threshold value. Mean values express the mean of these view fields per patient condition. We only analyzed gastric mucosal vessels as the fundus biopsies taken

prior to ISCON in a standardized fashion do not contain deeper tissue layers. Vessel density (expressed as area density %) and vessel average size were calculated by ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018) as previously described [14],[15]. Grading of vascularity was defined as follows: category 1 includes samples with a CD31 area density % < 2, category 2 includes samples with an area density between 2 and 3 and category 3 includes samples with an area density > 3.

Results obtained from pre- and post-ISCON serum and tissue samples were compared using an unpaired students t-test or a Chi-square test.

Results

In total, 11 serum samples were available prior and post ISCON for cytokine analysis. Serum samples prior and post ISCON procedure were analysed using a Luminex Assay (R&D Systems) including a set of 62 cytokines. No difference regarding cytokine levels in the serum of ISCON patients could be detected (p = 0.3956, 0.7439, 0.8571, for VEGF, TNF, IL-8) respectively). Figure 1 depicts a selection of 3 cytokines associated with neoangiogenesis. The total set is depicted in Supplementary file 1.

Figure 1

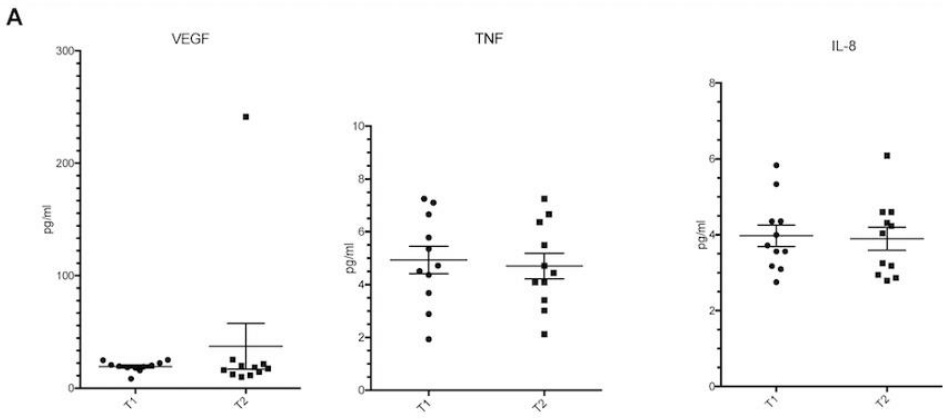

Figure 1. Comparison of selected proangiogenic cytokine (VEGF, TNF and IL-8) serum levels prior and post ISCON

In total, 12 sets of tissue samples before and after ISCON were available for histopathological analysis. For this cohort, there was a significant increase in the microvessel density (mean 1.970 ± 0.1619 SEM vs. mean 3.427 ± 0.3824 SEM, p=0.0020) as well as the vessel size (mean 68.47 ± 8.182 SEM vs. mean 95.80 ± 7.439 SEM, p=0.0217) comparing the tissue samples before and after the ISCON procedure. (Figure 2 A-C). This ISCON related histopathologic change could be demonstrated for each single patient included. We furthermore categorized the samples in three different grades of vascularization. We found that distribution of patients' samples over these categories significantly differs prior and post ISCON (p=0.008) (Figure 2 D). To explore whether the histopathologic changes upon ISCON might impact on anastomotic leakage we stratified the patients for the occurrence of an anastomotic leak and calculated the increase in vessel count before and after ISCON which can be seen as 'angiogenic' response. We found that the three patients in our collective that experienced an anastomotic leakage had a significantly less 'angiogenic response' upon

ISCON compared to patients' without anastomotic complications (mean -99.22 ± 68.73 SEM vs. mean 266.7 ± 80.28 SEM, p= 0.0329), (Figure 2 E).

Figure 2

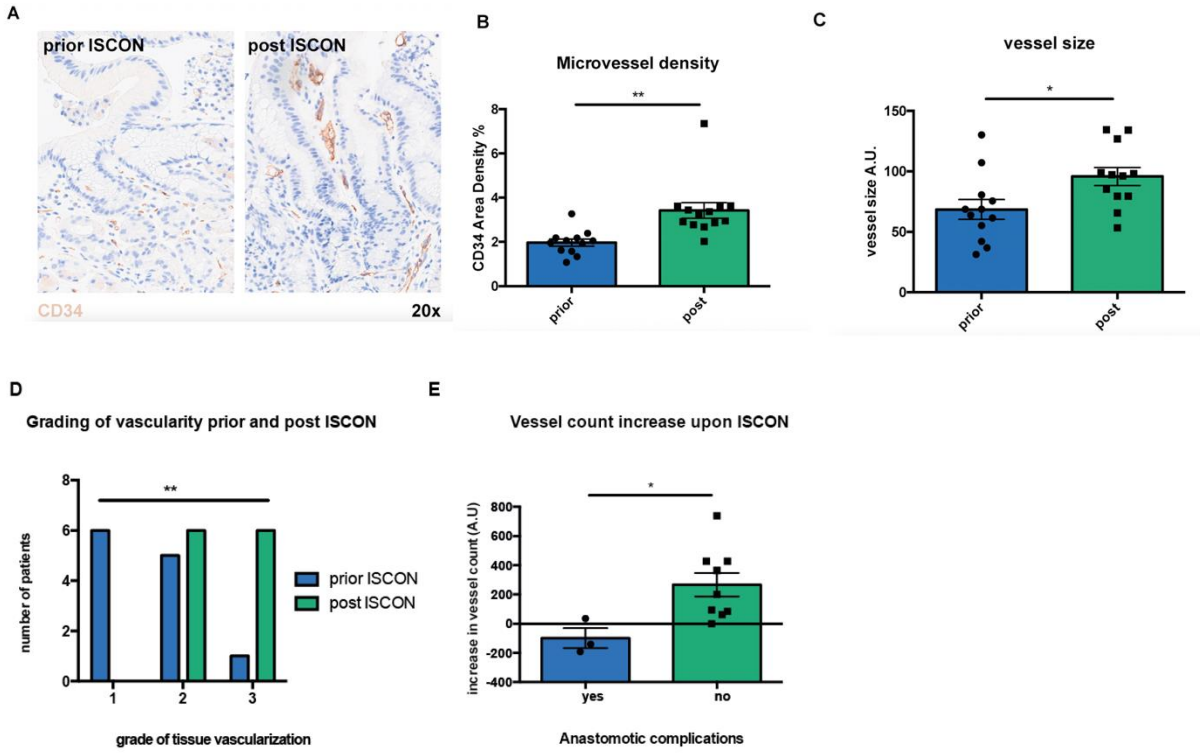

Figure 2. A Representative histopathologic sections of the gastric mucosa stained against CD34 (brown). **B** Quantification of mucosal microvessel density expressed as the proportion of CD34 positive area per image prior and post ISCON. **C** Quantification of vessel size prior and post ISCON. **D** Categorization of patients in 3 grades of gastric mucosal vascularization based on the distribution of patients in our study. **E** Angiogenic response in patients with or without anastomotic complications.

Discussion

Ischemic conditioning of the gastric conduit is considered to be a treatment option to improving perfusion at the anastomotic site. However, recent meta-analyses failed to demonstrate a significant reduction of the clinically most important variable AL [16,17]. The ISCON trial was designed as prospective observational study to demonstrate the clinical feasibility and safety in a selected cohort of esophageal cancer patients with an increased risk to develop an AL based on the presence of atherosclerotic disease.

To our knowledge, the data of this translational ISCON program demonstrate for the first time in humans that ischemic conditioning induces a strong pro-angiogenic effect in the gastric fundus proven by the CD34 histopathological increase of microvessel density and vessel size. This suggests that gastric vascularization improves at the critical site of the anastomotic region prior to esophagectomy. This is in line with other experimental data investigating neo-angiogenesis in rats as a response to ischemic conditioning of the stomach [18]. Our findings also demonstrate that an interval of 14 days between ISCON and esophagectomy is effective to induce a reproducible response at the level of gastric microvascularity. However, further investigations need to prove whether our histopathologic findings as surrogate of gastric vascularization are in fact associated with a measurable increase in gastric perfusion and finally reduce the incidence of anastomotic leakage in patients with an increased risk for this surgical complication. Currently applicable methods to detect this difference *in vivo* include the quantitative ICG measurement combined with the endoluminal measurement of the oxygen saturation [19,20].

The fact that gastric mucosa shows a distinct angiogenic response to ISCON on a histological level while serum samples were unaltered are not considered as conflicting results. The hypoxic stimulus following ISCON might induce the upregulation of proangiogenic genes resulting in increased cytokine levels. An altered serum profile with possibly increased serum concentrations is expected to happen immediately after the procedure, probably within minutes after dissection of the left gastric artery [21] reaching maximum levels after a few days at the latest [22]. Serum levels in this study were measured right before and 14 days after the ISCON procedure thereby possibly missing the expected angiogenic effect.

It is also important to note from a technical aspect that, theoretically, devascularization of the fundus can even more drastic by cutting the esophago-cardiac branch of the inferior phrenic artery. This would potentially result in severe adhesions at this anatomical region making the following esophagectomy more complex.

Another very important aspect is how short term altered serum cytokines might impact on the tumor micromilieu and accordingly to short-term tumor progression. It would be furthermore very interesting to see how blood vessel hierarchy (arterioles, venules, capillaries) is affected by ISCON which is technically challenging both in vivo (e.g.by ICG) or immunohistochemically.

Summarizing our clinical [12] and translational results from the ISCON Trial, together with reports from others [23], we can document the safety of the procedure and deliver evidence that ISCON produces a histologically measurable effect in the gastric conduit. Very interestingly, we can show that patients who experienced anastomotic leakage were those patients who responded poorly to the ISCON procedure in terms of neo-angiogenesis. Further experiments and trials should explore whether this poor response is patient intrinsic or related to extrinsic factors such as neoadjuvant treatment or medication.

Conclusion

In conclusion, the findings provide evidence that ISCON induces a significant proangiogenic effect at the level of gastric vascularization. Further studies need to prove that this histopathologic effect is truly associated with an improved microcirculation at the anastomotic site which results in the clinical benefit of reduced anastomotic leakage.

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Trial registration**:** Clinicaltrials.gov, NCT03896399. Registered 4 January 2019.

Disclosure

The authors declare no conflict of interest.

Data availability

Detailed data and methodology are available from the corresponding author upon request.

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