Effects of endothelial defects and venous interposition grafts on the acute incidence of thrombus formation within microvascular procedures

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Endothelial defects (ED) and the usage of interposition vein grafts (IVG) are known risk factors for free flap failure. This experimental study aimed to compare both situations of thrombus formation and fluorescence angiographic behavior. Indocyanine green videoangiography (ICGVA) with the FLOW 800 tool was systematically performed in groups I = ED, II = IVG, and III = ED and IVG (each n = 11). ICGVA was able to detect thrombosis in five animals and safely ruled it out in 26 with two false-positive cases (sensitivity, specificity, and positive and negative predictive values were 100%, 90%, 62%, and 100%, respectively). The difference between visually and ICGVA-assisted ED measurements was significant (p = 0.04). The areas of thrombosis showed no significant difference. Moreover, ICGVA detected a decrease of all parameters at the ED area and/or within the IVG section in all groups. The presence of an endothelial defect had a higher impact on thrombus formation than the IVG usage. ICGVA is qualitatively able to detect endothelial defects and clinically evident thrombosis. However, the quantitative values are not yet attributable to one of the clinical scenarios that may jeopardize free flap transfer.

Microvascular techniques have been established and developed within different surgical fields in the last decades. Free microvascular tissue transfer represents the gold standard for complex reconstructions in the head and neck area following ablative tumor surgery, excessive trauma, or other indications (e.g., because of side effects such as medication-related osteonecrosis of the jaw or osteoradionecrosis) in most specialized centers1–3. On the one hand, this patient cohort is challenging because of multiple organic or vascular comorbidities with dissolved and altered vascular architecture resulting from arteriosclerosis, radiation therapy, or previous surgical interventions4–7. These patients need to be handled with a distinct approach in the pre-, intra-, and postoperative settings. Missed salvage operations of compromised free flaps, especially in this high-risk group, may result in increased rates of flap necrosis and consecutive prolonged hospital stay and costs8–10.

On the other hand, thrombosis still represents one of the most common reasons for flap loss despite the progress in microvascular surgery on the refinement of surgical techniques, instruments, and different monitoring devices. Borderline microsurgical situations, such as the irradiated and vessel depleted neck, are often associated with a hypercoagulable vascular state or may demand the use of an interposition vein graft (IVG), which is described by many authors as a high-risk factor for thrombosis3,11,12.

Previous studies revealed that the majority of thrombotic events appear within the first hours after performing an anastomosis1. Therefore, a high level of interest exists in an early, reproducible, and objective assessment of patency and blood flow13. However, indocyanine green (ICG), an injectable dye that binds to plasma proteins and remains intravascular, has been introduced to free flap surgery in the last two decades14,15. The intraoperative use of ICG videoangiography (ICGVA) in combination has increased, allowing quantitative and qualitative

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evaluation of blood flow and the detection of endogenous (e.g., endothelial defects) and exogenous (e.g., vascular compression due to hematoma) complications\(^{13,16–21}\).

This study aims to critically evaluate the diagnostic accuracy of ICGVA combined with the FLOW 800 tool in detecting microvascular thrombosis in three different procoagulant situations in a sensitive rat model\(^{22}\) by creating different potentially thrombogenic microvascular situations. Furthermore, this study also aims to compare thrombosis rates between endothelial defects and IVGs in an attempt to determine which combination creates a higher risk of thrombosis.

### Results

This study operated on 33 rats, and the infrarenal aorta had a median diameter of 1.72 mm (1.11–2.53). Moreover, no side effects attributable to ICG application were observed.

#### Detection and determination of endothelial defect and thrombosis (Tables 1, 2).

A significant difference \((p=0.05)\) was noted between groups I and III in terms of the probability of thrombosis with an increased risk of thrombosis after endothelial defect only in contrast to the combined intervention. Further comparisons of the defect group (I) with the IVG alone group (group II) and between groups II and III showed no significant differences in terms of thrombus formation \((p=0.14\) and \(p=0.32\), respectively).

Changes in ICGVA fluorescence behavior identified four, one, and two thromboses in groups I, II, and III, respectively. Visually (with a tenfold magnification), which served as reference, thrombus formation was however detected in four and one rats in groups I and II, respectively. However, no clinical evidence of thrombus in any vessel was noted in group III. Furthermore, ICGVA was able to reliably detect thrombosis in five animals and safely rule it out in 26 animals, with two false-positive cases. This resulted in sensitivity, specificity, and positive and negative predictive values of 100%, 90%, 62%, and 100%, respectively (Table 1).

#### Impact of the endothelial defect and thrombus formation on ICGVA (Tables 3, 4).

All four defined parameters (first maximum, maximal increase and decrease, and area under the curve (AUC\(^{ICG}\)) of the detected ICG fluorescence curve were calculated for each region of interest (ROI) to compare them within and between the groups (Table 3). Additionally, the differences between ROIs 1 and 2, 1 and 3, and 1 and 4 were calculated and are displayed in Table 4. All three groups (I–III) showed a decrease in all parameters within the ROIs at the defect area and/or within the area of the IVG (Table 3 and Fig. 1). The value of the first maximum significantly changed between ROI 1 vs. 2 in group I \((p<0.01)\); between ROI 1 vs. 3 in group II \((p<0.01)\); and between ROI 1 vs. 2 \((p<0.01)\), ROI 1 vs. 3 \((p<0.01)\), and ROI 1 vs. 4 \((p=0.05)\) in group III. The maximal increase between the following ROIs was significantly different: ROI 1 vs. 2 \((p<0.01)\) in group I and between ROI 1 vs. 2 \((p<0.01)\) and ROI 1 vs. 3 \((p=0.01)\) in group III. The values of maximal decrease showed no significant changes within the different ROIs for each group. The values of AUC\(^{ICG}\) significantly changed between ROI 1 vs. 2 in.

|                | Clinical/visual assessment | ICGVA-assisted assessment |
|----------------|---------------------------|---------------------------|
|                | Positive | Negative | Total |
| ICGVA-assisted assessment |          |          |       |
| Positive | 5       | 3        | 8     |
| Negative | 0       | 25       | 25    |
| Total    | 5       | 28       | 33    |

**Table 1.** Cross table for ICGVA-assisted thrombus detection.

| Group | Visual area of thrombosis (mm\(^2\)) | ICGVA area of thrombosis (mm\(^2\)) | \(p\) value | Visual area of ED (mm\(^2\)) | ICGVA area of ED (mm\(^2\)) | \(p\) value |
|-------|--------------------------------------|--------------------------------------|-------------|--------------------------------|--------------------------------|-------------|
| I     | 0.56 (0.36–1.06)                     | 0.96 (0.42–1.14)                     | 0.11        | 0.72 (0.25–2.30)               | 0.65 (0.35–1.59)               | 0.93        |
| II    | 0.44                                | 0.34                                |             | –                               | –                              |             |
| III   | –                                   | 0.36 (0.15–0.56)                     | 0.19        | 0.19 (0.02–0.30)               | 0.30 (0.05–0.74)               | <0.01       |
| Total | 0.50 (0.36–1.06)                     | 0.69 (0.15–1.14)                     | 0.47        | 0.26 (0.02–2.30)               | 0.39 (0.05–1.59)               | 0.04*       |

**Table 2.** Descriptive results of clinical ED area and thrombosis in visual and ICGVA analysis using NIH Image software (ImageJ 1.41o, National Institutes of Health, Bethesda, MD, USA). *IGVA Indocyanine green videoangiography, ED Endothelial defect, group I Endothelial defect, group II Interposition vein graft (IVG), group III Endothelial defect with interposition vein graft. \(^a\)Wilcoxon test.
The comparison of registered corresponding ICG values of each ROI (2, 3, and 4) between groups I–III showed a significant difference for first maximum \((p < 0.01)\), maximal increase \((p = 0.02)\), and \(AUC_{ICG}\) \((p < 0.01)\) within the corresponding ROI 2 between groups I and III. A comparison of registered ICG values within group I \((p < 0.01)\), between ROI 1 vs. 3 in group II \((p < 0.01)\), and in group III between ROI 1 vs. 2 \((p < 0.01)\), ROI 1 vs. 3 \((p < 0.01)\), and ROI 1 vs. 4 \((p = 0.01)\).

| Parameter           | Group   | I       | II      | III     |
|---------------------|---------|---------|---------|---------|
| First maximum       |         | 345.07  | 378.28  | 566.49  |
| Maximal increase    |         | 0.59 (0.27–0.75) | 0.64 (0.18–1.84) | 0.69 (0.28–3.79) |
| Maximal decrease    |         | −0.10 (−0.38–0.05) | −0.28 (−2.71–0.03) | −0.19 (−0.57–0.08) |
| \(AUC_{ICG}\)       |         | 327,850.14 (144,045.89–483,015.33) | 404,530.41 (147,937.59–656,325.44) | 498,672.58 (202,527.95–586,814.66) |
| First maximum       |         | 140.63 (102.39–224.89)* | –       | 410.88 (124.67–594.93)*# |
| Maximal increase    |         | 0.20 (0.12–0.75)* | –       | 0.52 (0.19–2.69)*# |
| Maximal decrease    |         | −0.10 (−0.89–0.04)) | –       | −0.16 (−0.51–0.04)) |
| \(AUC_{ICG}\)       |         | 158,318.35 (78,380.48–279,790.88)* | –       | 295,815.71 (152,913.84–398,368.67)*# |
| First maximum       |         | 318.66 (209.17–536.32) | 333.03 (118.44–644.13) | 508.60 (187.70–626.00)## |
| Maximal increase    |         | 0.53 (0.33–0.98) | 0.58 (0.16–1.05) | 0.73 (0.16–2.63) |
| Maximal decrease    |         | −0.14 (−0.59–0.06) | −0.06 (−0.17–0.09) | −0.13 (−0.69–0.07) |
| \(AUC_{ICG}\)       |         | 327,119.88 (141,131.65–441,150.91) | 368,974.45 (185,822.91–464,508.97) | 380,680.60 (180,753.86–520,915.12)# |
| First maximum       |         | 76.44 (−0.91–276.86) | 185.97 (23.98–290.91) | – |
| Maximal increase    |         | 0.09 (−0.33–0.46) | 0.09 (−0.06–1.34) | – |
| Maximal decrease    |         | −0.00 (−1.87–0.20) | 0.02 (−0.17–0.07) | – |
| \(AUC_{ICG}\)       |         | 82,172.10 (−1,286.26–359,731.52) | 154,991.18 (52,849.27–201,659.19) | – |
| First maximum       |         | 39.51 (−174.00–107.49) | −15.54 (−171.90–172.06) | 90.07 (−165.91–271.81) |
| Maximal increase    |         | 0.02 (−0.37–0.18) | −0.05 (−0.40–0.83) | 0.08 (−0.60–1.17) |
| Maximal decrease    |         | 0.03 (−0.04–0.21) | 0.04 (−2.30–0.23) | 0.01 (−0.16–0.19) |
| \(AUC_{ICG}\)       |         | 35,304.97 (−11,221.14–110,432.71) | −3,990.70 (−113,622.70–314,222.50) | 119,735.07 (−82,066.72–174,410.91) |

Table 3. Absolute values (arbitrary units, AU) and descriptive statistics of ICGV A for the three experimental groups: endothelial defect (ED group I), interposition vein graft (IVG, group II), and ED with IVG (group III). I defect, II interposition vein graft, III defect and interposition vein graft, ROI region of interest, \(AUC_{ICG}\) area under the curve. Median (range). Wilcoxon test: significant \((p < 0.05)\) change in ROI 1 vs. 2, 3, and 4 \(\ast, \ast, \ast\) within groups I–III and compared values. Mann–Whitney U test: significant \((p < 0.05)\) change between groups I vs. II, I vs. III, and II vs. III \(\%, \& , \?\) for the compared values.

| Parameter           | Groups | I       | II      | III     |
|---------------------|--------|---------|---------|---------|
| 

\(\Delta ROI \) 1 and 2
| First maximum       | 122.38 (9.99–377.92) | –       | 200.30 (0.00–329.93) |
| Maximal increase    | 0.23 (−0.02–0.49) | –       | 0.10 (0.02–1.11) |
| Maximal decrease    | 0.02 (0.20–0.73) | –       | 0.01 (−0.20–3.45) |
| \(AUC_{ICG}\)       | 169,534.79 (−2062.07–344,419.32) | –       | 119,513.21 (28,090.56–277,780.77) |
| 

\(\Delta ROI \) 1 and 3
| First maximum       | –       | 76.44 (−0.91–276.86) | 185.97 (23.98–290.91) |
| Maximal increase    | –       | 0.09 (−0.33–0.46) | 0.09 (−0.06–1.34) |
| Maximal decrease    | –       | 0.00 (−1.87–0.20) | 0.02 (−0.17–0.07) |
| \(AUC_{ICG}\)       | –       | 82,172.10 (−1,286.26–359,731.52) | 154,991.18 (52,849.27–201,659.19) |
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Table 4. Differences of fluorescence behavior (AU) in ICGV A between the defined regions of interest (ROI, 1–4) for the three experimental groups: endothelial defect (ED group I), interposition vein graft (IVG, group II), and ED with IVG (group III). I defect, II interposition vein graft, III defect and interposition vein graft, ROI region of interest, \(AUC_{ICG}\) area under the curve. Median (range). Wilcoxon test: significant \((p < 0.05)\) change in ROI 1 vs. 2, 3, and 4 \(\ast, \ast, \ast\) within groups I–III and compared values. Mann–Whitney U test: significant \((p < 0.05)\) change between groups I vs. II, I vs. III, and II vs. III \(\%, \& , \?\) for the compared values.
corresponding ROI 3 between groups II and III revealed no significant differences. A comparison of the ICG values within corresponding ROI 4 between groups I vs. II and II vs. III revealed no significant differences. Only the first maximum was significantly different between groups I and III ($p = 0.03$) within ROI 4.

**Uni- and multivariate regression analyses (Table 5).** Univariate regression analysis for thrombosis formation and visual (reference) detection showed a significant association with the size of endothelial defect ($\text{mm}^2; p < 0.01; 95\% \text{ confidence interval (CI)}, 0.42–0.78$) and usage of an IVG ($p = 0.02; 95\% \text{ CI}, −0.57–(−0.07))$. Multivariate regression analysis only showed a significant association with the size of endothelial defect ($\text{mm}^2; p < 0.01; 95\% \text{ CI}, 0.40–0.89$).

**Discussion**

Technically meticulous flap raising, correct and gentle handling of vessels in microvascular preparation and technically flawless microsurgical anastomosis are essential and mandatory requirements in reconstructive microsurgery. Nevertheless, flap failure rates for head and neck reconstruction have ranged between 0 and 7.7% in the literature over the decades\(^{23,24}\). These generally good results also include cases that were surgically reexplored (set back) in the operation room because of an imminent loss of free flap\(^{25}\). However, success can only be assumed if the salvage operation was performed within transplant-specific congestion or ischemic time interval in the case of a successful revision of the anastomosis or elimination of the decisive cause\(^{26,27}\). Although the sensitivity and specificity of ICGVA have already been investigated in a few clinical and animal studies, the simulation of complicated microsurgical situations requiring an IVG has not yet been investigated. A more realistic vascular analog model to a demanding clinical situation was created and evaluated by setting endothelial defects and/or using an IVG.

The first 72 postoperative hours remain the main interval for free flap loss\(^{1,24}\). Sweeney et al. and others described that flap loss attributable to venous congestion occurs earlier than flap loss due to arterial insufficiency or infection\(^{28}\). Clinically, persisting arterial vasospasm, endothelial cell damage, or inadequate microsurgical sutures (e.g., intraluminal adventitia) subsequently lead to early thrombus formation and intraoperative occlusion. Thus, a clinical observation period of at least 45 min is recommended, during which, for example, reconstruction with the free flap can be performed\(^1\). Late free flap failure may have different reasons, which can be classified into surgical (including intra- and extravascular) and nonsurgical (including alcohol withdrawal) causes. Based on this knowledge, this study has chosen the acute observation period of 60 min similar to Mordick et al.\(^{28}\) to analyze the possibly altered perfusion changes after a reestablished blood flow after approximator clamp removal. An earlier timepoint would only reflect immediate changes. Scientifically, earlier and later timepoints
Incidence of thrombosis 0.35 (−267.17–97.23) 0.19 (−1.05–0.21) 0.28 (−0.23–0.77) 0.68 (−101,600.43–698,051.60)

Area of thrombosis 0.34 (−937.43–463.27) 0.96 (−0.93–0.96) 0.66 (−0.21–0.15) 0.72 (−653,469.09–510,601.89)

Area of ED 0.01 (−323.11–(−44.23)) 0.25 (−0.94–0.26) 0.20 (−0.04–0.17) 0.92 (−517,867.41–191,121.70)

ROI 1 First maximum (95% CI) Maximal increase (95% CI) Maximal decrease (95% CI) AUCICG (95% CI)

Incidence of thrombosis 0.16 (−317.83–56.82) 0.30 (−0.96–0.31) 0.52 (−1.29–0.68) 0.20 (−190,852.74–43,431.62)

Area of thrombosis 0.60 (−310.80–414.97) 0.38 (−0.54–0.93) 0.69 (−2.91–2.34) 0.12 (−99,464.44–422,500.89)

Area of ED 0.04 (−260.72–(−8.12)) 0.19 (−0.74–0.16) 0.55 (−0.91–0.49) 0.83 (−165,791.55–10,858.94)

ROI 2 First maximum (95% CI) Maximal increase (95% CI) Maximal decrease (95% CI) AUCICG (95% CI)

Incidence of thrombosis 0.67 (−240.35–366.56) 0.35 (−1.71–0.64) 0.59 (−0.56–0.95) 0.87 (−169,413.58–200,025.36)

Area of thrombosis – 0.59 (−0.93–0.96) 0.69 (−2.91–2.34) 0.12 (−99,464.44–422,500.89)

Area of ED 0.04 (−260.72–(−8.12)) 0.19 (−0.74–0.16) 0.55 (−0.91–0.49) 0.83 (−165,791.55–10,858.94)

ROI 3 First maximum (95% CI) Maximal increase (95% CI) Maximal decrease (95% CI) AUCICG (95% CI)

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ROI 4 First maximum (95% CI) Maximal increase (95% CI) Maximal decrease (95% CI) AUCICG (95% CI)

Incidence of thrombosis 0.91 (−136.73–153.81) 0.42 (−0.64–0.28) 0.15 (−0.07–0.41) 0.72 (−78,677.48–112,855.86)

Area of thrombosis 0.49 (−583.95–357.12) 0.71 (−1.15–1.49) 0.26 (−0.10–0.26) 0.55 (−311,891.11–479,577.77)

Area of ED 0.11 (−183.62–19.72) 0.32 (−0.64–0.21) 0.63 (−0.11–0.18) 0.19 (132,104.38–29,150.71)

Table 5. Univariate regression analysis of incidence of thrombosis, visual area of thrombosis, and visual area of endothelial defect of the analyzed ICGVA parameters. 95% CI 95% Confidence interval, ROI Region of interest, AUCICG Area under the curve.
and negative predictive value each 100%). However, an exact assessment of the presence of a thrombus can be generally difficult due to overlapping phenomena, e.g., vascular sutures. Furthermore, the exact delineation of a thrombus is sometimes difficult and depends on the subjective assessment of the surgeon although fluorescence reduction in thrombosed vessels could be visualized in this study. In difficult situations, it may therefore be useful not to rely solely on ICGVA but to use a combination of different methods such as Doppler or clinical inspection.

The fluorescence behavior showed a significant reduction of the first maximum, maximal increase, and AUC<sub>ICG</sub> (Tables 3, 4). However, no significant correlation could be described in univariate regression analysis (Table 5). The reason for this may be the small number of animals as well as the low count of evident thrombus formations. This study failed to associate the incidence of thrombosis with a certain numerical or quantitative value in ICGVA, but this failure could also be a result of the lack of statistical power for this precise question.

Mücke et al. have previously analyzed the feasibility of ICGVA to detect immediate arterial thrombus formation. The authors observed that macroscopical analysis correlated well with ICGVA<sup>22</sup>. Thus, a histological value in ICGVA, but this failure could also be a result of the lack of statistical power for this precise question.

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and the procedure is performed by an experienced microsurgeon even though the underlying indication of an IVG reflects the clinical and microsurgical complexity.

The nature of the study (animal) does not allow direct transfer to the clinical situation because it represents a maximal standardized situation in an animal model that will not approach the microsurgical complexity when IVGs are used. Nevertheless, this study tried to generate a more realistic vascular model analogous to the demanding clinical situation that necessitates interposition vein grafting by setting endothelial defects and/or using an IVG. However, the rat model remains the gold standard experimental model for microvascular procedures because it offers comparable physiology, rheology, and coagulative behavior as the human being.

**Methods**

**Ethical statement and animal welfare.** The study conforms with current German regulations, guidelines for animal welfare, and the international principles of laboratory animal care. The study was carried out following the ARRIVE guidelines and the local government approved the animal experiments (Regierung Oberbayern; AZ.: 03-35-08). The animals were housed in filter-top cages under hygienic conditions according to the Federation of Laboratory Animal Science Associations (FELASA) guidelines. Water and standard rodent diet (Altromin; Altromin Spezialfutter GmbH & Co. KG; Lage, Germany) were provided ad libitum. All animals were sacrificed following a standardized protocol at the end of the measurements and observation time.

**Workflow, model generation, and experimental groups.** The anesthesia was induced with a weight-dependent intraperitoneal injection of ketamine–xylazine (1 and 0.25 mL/kg/weight, respectively). The anesthesia was then continued intravenously using femoral vein access after insertion of a microcatheter (Premicath; VYGON GmbH & Co. KG; Aachen, Germany). Maintenance dosage was a one-eighth dose of 10% ketamine when needed, as previously described in detail.

The abdomen was shaved, and an approximately 5 cm midline laparotomy was performed. The prolapsing intestine was carefully retracted with moist swabs andatraumatic, blunt hooks. The retroperitoneal space was opened, the abdominal aorta was defined, the perivascular sheet was incised, and the infrarenal aorta was carefully freed from the surrounding tissue and separated from the vena cava inferior. All branching arterial vessels were ligated and cut. The preparation was randomized for the subsequent procedures according to three experimental groups (each n = 11): endothelial defect (ED, group I), interposition vein graft (IVG, group II), and ED with IVG (group III; Fig. 1). The EDs were generated with microforces by removing the endothelial layer within a distinct area opposite to a longitudinal incision (Fig. 2A). A 1/10-mm paper was positioned adjacent to the defect on the aorta to enable an orientated and standardized defect size preparation. The femoral vein served as an IVG donor site. It was harvested with a standardized length of 10 mm distal of the inserted and fixed microcatheter. Before microsurgical grafting, the IVG was rinsed with heparin-diluted highly purified water and gently dilated with a dilator (D-5a, S&T AG; Neuhausen, Switzerland).

After all group-related surgical procedures, microvascular anastomosis and longitudinal incision closure were performed with interrupted sutures using 10–0 Ethilon (Ethilon; Ethicon Division of Johnson & Johnson; Livingston, Scotland). The temporary Acland approximator (ABB-22 V, S&T AG; Neuhausen, Switzerland) was then removed and the blood flow was reestablished.

**Indocyanine green fluorescent videoangiography and analysis.** Intraoperative ICGVA was previously performed as described in detail. 60 min after blood flow reestablishment following the aforementioned surgical procedures. Indocyanine green dye (ICG-PULSION; Pulsion Medical System AG; Munich, Germany) was weight-dependently (0.03 mg/kg body weight) injected intravenously to assess the fluorescence behavior using the operating microscope type OPMI Pentero with an integrated near-infrared videoangiography detection system and the FLOW 800 tool (INFRARED 800; Carl Zeiss Meditec AG; Oberkochen, Germany). Fluorescence angiography was conducted at a fixed working distance of 300 mm and with a tenfold magnification. The ICG dye-specific emission was detected and color encoded concerning fluorescence intensity over time (AU). The immediate analysis of three or four regions of interest (ROI) was systematically positioned. The number of ROIs depends on the group. Group I had three ROIs (proximal, inside, and distal to the ED), group II had three ROIs (proximal, inside, and distal to the IVG), and group III had four ROIs (proximal to the ED and IVG, inside the ED, inside the IVG, and distal to the ED and IVG). The values of the first maximum, maximal increase to the first maximum, maximal decrease after first maximum, and the AUC of the resulting fluorescence curve over time were captured for 120 s with 25 images per second and analyzed within all ROIs (Fig. 2B,C).

**Analyses and correlation of incidence of thrombosis and defect area.** The incidence of thrombosis was dichotomously analyzed. Visually, a macroscopic clot at a tenfold magnification and a spearing of the fluorescent signal in ICGVA were rated positively for thrombosis formation.

The defect size of the endothelium (in square millimeter) was planimetrically measured using NIH Image software (ImageJ 1.41o, National Institutes of Health; Bethesda, MD, USA) both for clinical macroscopic and ICGVA analyses.

**Statistical analyses.** Fisher’s exact test was used to determine the thrombosis probability of groups I–III. The Wilcoxon test was used to analyze the measurements of two related samples (development of ICG fluorescence behavior within groups I, II, or III). Moreover, the Mann–Whitney U test was used to compare the registered corresponding ICG (first maximum, maximal increase and decrease, and AUC) values of each ROI (2, 3, and 4) between groups I–III. For the incidence of clinically proven thrombosis, uni- and multivariate regression
analyses were performed with the clinically measured ED size and the IVG usage. P values are two-sided and subject to a global significance level of 0.05. The data were analyzed using the Statistical Package for the Social Sciences, version 23.0, for Windows software (IBM Corp; Armonk, NY, USA).

Conclusions
The presence of endothelial defects had a higher impact on thrombus formation than the usage of an IVG in the abdominal aorta model of the rat. Moreover, indocyanine green videoangiography using the FLOW 800 tool is qualitatively able to detect endothelial defects and clinically evident thromboses. Significant quantitative values are not yet attributable to one of the clinical scenarios that may jeopardize successful free flap transfer. Thus, further research is needed to translate these observations into the clinical setting.

Data availability
All data generated or analyzed during this study are included in this published article.

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Figure 2. Endothelial defect (group I). (A) Prepared defect area (Δ indicating the borders of the defect), (B) three regions of interest (ROI, 1 = proximal, 2 = in, and 3 = distal to the ED), and (C) corresponding fluorescence intensity curve (in AU) for capturing time of 120 s.
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Author contributions
L.M.R.: Study design/conduction, operations, data acquisition and interpretation, and major contribution in manuscript writing. M.-K.H.: Study design/conduction, data acquisition and interpretation, contribution in manuscript writing, and manuscript revision. C.T.W.: Data interpretation and manuscript revision. L.H.S.: Data acquisition and interpretation and manuscript revision. K.-D.W.: Study conception and manuscript revision. A.M.F.: Study design, contribution in manuscript writing, and figure designing. T.M.: Study design, analyses, and contribution in manuscript writing.

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The authors declare no competing interests.

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