INTRAOPERATIVE NEAR-INFRARED FLUORESCENCE IMAGING FOR SENTINEL LYMPH NODE DETECTION IN VULVAR CANCER: FIRST CLINICAL RESULTS

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ABSTRACT

OBJECTIVE
Disadvantages of the combined sentinel lymph node (SLN) procedure with radiocolloid and blue dye in vulvar cancer are the preoperative injections of radioactive tracer in the vulva, posing a painful burden on the patient. Intraoperative transcutaneous imaging of a peritumorally injected fluorescent tracer may lead to a one-step procedure, while maintaining high sensitivity. Aim of this pilot study was to investigate the applicability of intraoperative fluorescence imaging for SLN detection and transcutaneous lymphatic mapping in vulvar cancer.

METHODS
Ten patients with early stage squamous cell carcinoma of the vulva underwent the standard SLN procedure. Additionally, a mixture of 1ml patent blue and 1ml indocyanine green (ICG; 0.5 mg/ml) was injected immediately prior to surgery, with the patient under anesthesia. Color and fluorescence images and videos of lymph flow were acquired using a custom-made intraoperative fluorescence camera system. The distance between skin and femoral artery was determined on preoperative CT-scan as a measure for subcutaneous adipose tissue.

RESULTS
In 10 patients, SLNs were detected in 16 groins (4 unilateral; 6 midline tumors). Transcutaneous lymphatic mapping was possible in five patients (5 of 16 groins), and was limited to lean patients, with a maximal distance between femoral artery and skin of 24 mm, as determined on CT. In total, 29 SLNs were detected by radiocolloid, of which 26 were also detected by fluorescence and 21 were blue.

CONCLUSIONS
These first clinical results indicate that intraoperative transcutaneous lymphatic mapping using fluorescence is technically feasible in a subgroup of lean vulvar cancer patients.
INTRODUCTION

The sentinel lymph node (SLN) procedure using a combination of radiocolloid and blue dye has been proven safe in early-stage vulvar cancer, breast cancer and melanoma, and is associated with a significant decrease in short-term and long-term morbidity. In vulvar cancer, however, important disadvantages are the painful peritumoral injections of radiocolloid in the vulva several hours to one day prior to surgery.

Recently, intraoperative near-infrared fluorescence (NIRF) imaging was introduced as a new technique for SLN detection. This approach is based on the intraoperative injection of a fluorescent agent around the primary tumor, which will flow through the lymphatic vessels and accumulate in the SLN(s). Upon excitation with a laser beam, the agent emits light of a longer wavelength which is captured and processed by a fluorescence camera. Real-time images can be displayed on monitors in the operating theatre. Currently, the fluorescent agent of choice is indocyanine green (ICG), an FDA-approved agent with little toxicity that has been used for decades for ophthalmic angiography and evaluation of liver perfusion. Recent studies on the feasibility of SLN detection using ICG and NIRF imaging in breast cancer, early gastric cancer and skin cancer, describe detection rates comparable to the standard procedure. Transcutaneous lymphatic mapping was possible in 46-97% of breast cancer patients. Reported advantages of intraoperative NIRF imaging are the high signal-to-background ratio (SBR), the real-time feature for lymphatic mapping and the avoidance of radioactivity. In contrast with radiocolloid, the fluorescent agent is injected during surgery, when the patient is already under anesthesia, thus reducing patient discomfort associated with the peritumoral injections prior to surgery. Furthermore, theoretically, the superficial localization of most inguinal lymph nodes may enable transcutaneous lymphatic mapping in the groin, making NIRF imaging an interesting modality in vulvar cancer. A significant proportion of the tumors is located near or in the midline, therefore, transcutaneous lymphatic mapping will be useful for determining both the side and the number of SLNs, comparable to a lymphoscintigram when using a radioactive tracer.

This pilot study was initiated to prospectively assess the technical feasibility of intraoperative NIRF imaging and the possibility of transcutaneous lymphatic mapping using the fluorescent agent ICG for detection of the SLN in patients with early stage vulvar cancer.

PATIENTS AND METHODS

PATIENT SELECTION

In this single-center technical feasibility pilot study, 10 consecutive patients older than 21 diagnosed with T1/2 (< 4 cm) squamous cell cancer of the vulva eligible for SLN biopsy were included. Exclusion criteria included significant renal, cardiac or pulmonary disease (ASA III-IV), previous severe allergic or anaphylactic reactions against medication or
insect bites, and past or present hyperthyroidism. A preoperative CT-scan was obtained to exclude bulky metastatic inguinofemoral lymph node metastases. The pilot study was conducted in accordance with the ethical standards of the Helsinki Declaration of 1975 (Dutch Trial Register number NTR1983; EudraCT number 2009-010561-23), approved by the Institutional Review Board (IRB) of the University Medical Center Groningen, and all patients provided written informed consent.

STUDY DESIGN AND SURGICAL PROCEDURE

In this pilot study, NIRF imaging was performed in addition to the standard SLN procedure using the combined technique of a radioactive tracer (99mTc-nanocolloid, GE Healthcare BV, Eindhoven, the Netherlands) and patent blue (Bleu patenté, Guerbet, France). In this procedure, approximately 100 MBq 99mTc-nanocolloid was injected peritumorally one day prior to the surgical procedure.10 With the patient in lithotomy position under general or regional anesthesia, the fluorescent agent (for preparation, see below) was injected immediately prior to the incision in the groin, in four quadrants around the primary tumor, together with patent blue to ensure synchronous propulsion through the lymphatics. Upon injection, the camera system was placed above the patient to detect fluorescence transcutaneously. Subsequently, the SLN was localized based on radioactivity, blue discoloration, and/or fluorescence intensity. Throughout the procedure of opening the groin(s), identification and excision of the SLN, the handheld gamma probe for detection of radioactivity and the intraoperative camera system for detection of fluorescence were used simultaneously. Fixed moments for image capture were: I) detection of transcutaneous fluorescence prior to incision; II) after superficial opening of the groin based on the maximal radioactivity count, and III) after exposing the lymph node. Detection of the SLN using fluorescence was compared to detection by radiocolloid and patent blue in terms of detection ease for the surgeon. After excision, the groin was imaged for residual fluorescence. Additionally, all excised SLNs were imaged ex vivo for the presence of a fluorescent signal, and blue staining and radioactivity were again evaluated. SLNs were sent to the pathologist as individual specimens, and processed according to the standard SLN protocol.10 During and after surgery, patients were monitored for possible side effects of the injection.

The distance between the femoral artery and the skin was determined at the level of the pubic tubercle on the preoperative CT-scan,11 as a standardized measure for the amount of subcutaneous adipose tissue. This distance was used to relate transcutaneous lymphatic mapping to the penetration depth of ICG.

FLUORESCENT IMAGING AGENT PREPARATION

The imaging agent, indocyanine green (ICG) was prepared by dissolving a vial of 25 mg ICG green (Pulsion Medical Systems AG, Munich, Germany) in 50 ml of water for injection (B. Braun Medical), yielding a concentration of 0.5 mg/ml. For each patient, a new vial of ICG was used because of degradation of the fluorescence signal of ICG.
after dissolving. For injection around the primary tumor, 1 ml of undiluted patent blue was mixed with 1 ml of ICG solution in a syringe, and protected from light to prevent bleaching.

**INTRAOPERATIVE IMAGING SYSTEM**

The custom-made prototype intraoperative multispectral fluorescence camera system used in this study was developed at the Helmholtz Zentrum of the Technical University Munich, Germany in close collaboration with SurgOptix (SurgOptix Inc, Redwood Shores, CA, USA). The system and its components were recently described in detail by Themelis et al. and will be described here briefly. The operating field is illuminated by a white light source; a laser diode provides light of the desired wavelength for excitation of the fluorescent agent, in this study ICG. Light emitted by ICG passes through a system of optics and is separated into visible light (color), light at the emission (fluorescence) wavelength band and light at the excitation wavelength band (intrinsic). These bundles are detected by sensitive charge-coupled device (CCD) cameras. Multispectral signals from all three cameras are processed and combined in order to correct for artifacts. Color and fluorescence signals can be displayed as separate images on external monitors or superimposed in one image, allowing for accurate anatomical positioning of the fluorescence signal. Still images and real-time video images up to 12 frames per second are generated using customized software. During surgery, both the color image and the fluorescence signal are presented on external monitors, providing the surgeon with an unhindered view. Sterile drapes are used in the operating room to cover the camera system, making it applicable and safe for intraoperative use. The technical service of the University Medical Center Groningen tested the system and approved it for intraoperative application in its current form.

**RESULTS**

**PATIENT CHARACTERISTICS**

Demographic and clinical characteristics of the 10 patients included in the pilot study are shown in Table 1. Median age was 67.5 (range, 46 to 82); median BMI was 26.5 (range, 21 to 41). Four patients presented with a lateralized tumor, the other six patients had a tumor close to or on the midline. Median tumor size was 14.5 mm (range, 2.5 to 40).

**INTRAOPERATIVE SLN DETECTION**

A total of 29 SLNs (range, 1 to 7) were detected with the gamma probe, of which 26 (89.7%) were also detected with fluorescence, and 21 (72.4%) were blue (see Table 2). In 10 patients, SLNs were detected in 16 groins. Transcutaneous tracing of the lymph flow was observed in five of ten patients, all unilaterally (5 of 16 groins); the average BMI of these patients was 25.6. An example of transcutaneous lymphatic mapping is shown in
Figure 1. Overlying adipose tissue impeded transcutaneous fluorescence detection in the other five patients, who had an average BMI of 28.8. However, in 10 of 11 groins with no transcutaneous signal, fluorescence was detected after a superficial incision in the groin. The position of the incision was determined with the handheld gamma probe. The median distance between the femoral artery and the skin as determined on the pre-operative CT scan was 23.7 mm (range, 19-28.7) for the SLNs that were detected by transcutaneous fluorescence, versus 36.5 mm (range, 21.1-132) for SLNs that were not detected with transcutaneous fluorescence (see Table 3).

An interesting finding in a patient with two SLNs (patient 6), was that detection of the second SLN was facilitated by fluorescence, as this signal was more explicit than the radiocolloid signal. An example of in vivo fluorescence is shown in Figure 2, in which a bright fluorescent SLN is seen prior to excision and absence of fluorescence after excision. Continuous real-time imaging was performed during SLN dissection, shown in the Supplemental.

After dissection, all SLNs were imaged ex vivo. We observed bright fluorescence in all but one (28 of 29) SLNs. Of three SLNs that were identified solely by radioactivity in vivo, two showed fluorescence ex vivo. One SLN showed neither fluorescence nor blue discoloration (patient 10).

None of the patients experienced side effects related to intraoperative injection of ICG. Sterility of the camera system throughout the procedure was warranted by covering the system in sterile drapes. Operation time was prolonged with a maximum of 15 minutes; use of the intraoperative camera system did not cause major hindrance or problems during the dissection.

HISTOPATHOLOGIC OUTCOME
All SLNs were sent to the pathologist and examined according to the standard protocol for SLN biopsy. Two of 29 SLNs turned out to contain metastases; both of these SLNs were detected by fluorescence as well as radiocolloid; furthermore, both lymph nodes were blue.

DISCUSSION
This pilot study is, to our knowledge, the first clinical study evaluating the technical feasibility of intraoperative near-infrared fluorescence (NIRF) imaging for detection of the sentinel lymph node (SLN) in vulvar cancer. The greatest advantage of this technique is that it can be performed in a one-step, all intraoperative procedure, thus reducing patient discomfort that is associated with the current two-step procedure including pre-operative injections of radiocolloid in the vulva under local anesthesia. Furthermore, transcutaneous lymphatic mapping has the same advantages as preoperative lymphoscintigraphy, i.e. non-invasive identification of the number and location of the SLNs. Radiocolloid may also be injected intraoperatively, but this method does not allow for lymphoscintigraphy.
However, a combined intraoperative injection with ICG and radiocolloid could yield high detection rates while avoiding patient discomfort. We observed that an intraoperative injection of indocyanine green (ICG) around the primary tumor yields a bright fluorescent signal in the SLNs. In vivo, 26 of 29 radioactive SLNs could be detected with fluorescence alone, whereas three deeper seated SLNs were detected only with radioactivity. No SLNs were detected solely by fluorescence. All but one excised radioactive SLNs showed a clear fluorescent signal ex vivo, indicating accumulation of both ICG and radiocolloid in the SLNs, whereas only 21 SLNs were blue. One SLN (patient no. 10) was neither fluorescent nor blue, indicating that the mixture was most likely not adequately transported to the SLN.

Fluorescence was easier to detect intraoperatively than blue discoloration of lymph nodes. Unambiguous transcutaneous lymphatic mapping, however, was observed in only four of 10 patients, while a weak transcutaneous signal was observed in one patient. These findings are in contrast with previous studies on NIRF imaging of the SLN in breast cancer, reporting transcutaneous detection rates of 46-97%. The difference may be partly explained by the fact that most of these studies were performed in Asian countries, where obesity is much less prevalent. The patients in the current pilot study in whom transcutaneous lymphatic mapping was possible had an average body mass index of 25.6. In more obese patients (average BMI 28.8), the SLN was not detected with fluorescence until after the first, radioactivity guided, incision in the groin, thus exposing the lymph basin more. In a pilot study on SLN detection with ICG in cervical cancer performed by our group, we observed similar problems in obese patients, in whom fluorescence detection was impeded by adipose tissue.

The distance between the femoral artery and the skin was determined on the preoperative CT-scan and was considered a standardized measure for the degree of subcutaneous adipose tissue. We found a median distance of 23.7 mm in patients in whom transcutaneous fluorescence mapping was observed, versus 36.5 mm in those patients in whom transcutaneous SLN detection was not possible. These findings implicate that fluorescence imaging may be limited to lean patients, while in more obese patients radiocolloid cannot be safely omitted. Our next step will be to evaluate the technique in a selected patient population with a BMI <25 or a distance between femoral artery and skin <24 mm in order to obtain the maximum benefit of the procedure. In this follow-up study, the surgeon will at first be blinded for the lymphoscintigraphy results in order to determine whether the fluorescence signal alone can be used for adequate SLN localization before making the first incision.

Currently, ICG is the only FDA-approved NIR fluorescent agent. Future research on intraoperative fluorescence imaging for SLN detection may benefit from fluorescent agents with stronger penetration properties, like IR-dye CW800, which has a >50 times higher brightness compared to ICG. Detection of deeper seated SLNs may also be improved with photoacoustic imaging. This technique combines optical and acoustic waves to visualize subsurface structures. After injection of a fluorescent agent, the tissue is illuminated...
with laser light of the desired wavelength. Accumulated contrast agent in the lymph node absorbs the light and as a result, the tissue expands slightly, creating mechanical waves. These can be detected using an ultrasonic (US) device, which needs to be placed directly on the skin for adequate localization of the signal. This technique was shown to detect SLNs at a depth of 2.5 cm in rats when using methylene blue as contrast agent. ICG has favorable optical characteristics due to its near-infrared wavelength and may yield even better results. Kim et al demonstrated the feasibility of photoacoustic and fluorescence imaging using ICG for lymphatic mapping and SLN detection in rats. In vulvar cancer, the same concept could be applied in a dual approach in which first the SLN is noninvasively localized using US. Subsequently, after superficially opening the groin based on highest signal intensity, NIRF imaging of accumulated ICG serves as guide to identify the SLN. Photoacoustic imaging for SLN detection has not yet been applied in humans, but may be advantageous and is therefore worth exploring for clinical application.

CONCLUSION
These first clinical results indicate the technical feasibility of intraoperative transcutaneous lymphatic mapping using fluorescence in a subgroup of lean vulvar cancer patients. Given the limited penetration depth of ICG, application of this technique in its current form is restricted to patients with a BMI <25. A follow-up study in a selected group of lean patients is planned to determine the definitive value of fluorescence imaging for SLN detection in vulvar cancer.

ACKNOWLEDGEMENTS
The authors wish to thank A. Motekallemi for assistance in data collection and R.G. Pleijhuis for assistance in preparing illustrations.
Table 1 – Patient characteristics

<table>
<thead>
<tr>
<th>N=10</th>
<th>Median</th>
<th>Min-Max</th>
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<tbody>
<tr>
<td>Age</td>
<td>67.5</td>
<td>46-82</td>
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<tr>
<td>BMI</td>
<td>26.5</td>
<td>21-41</td>
</tr>
<tr>
<td>Linear extension (mm)</td>
<td>14.5</td>
<td>2.5-40</td>
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</table>

a. BMI = Body Mass Index
b. Maximal linear extension of the tumor, in millimeters

Table 3 - Physical characteristics of patients in whom transcutaneous fluorescence was detected (middle column) versus patients in whom no fluorescence was detected transcutaneously (right column).

<table>
<thead>
<tr>
<th>Transcutaneous fluorescence:</th>
<th>Yes</th>
<th>No</th>
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<tbody>
<tr>
<td>No. of groins</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>BMI (median, min-max)</td>
<td>25.6</td>
<td>28.8</td>
</tr>
<tr>
<td>Distance SLN-skin (median, min-max)</td>
<td>23.7</td>
<td>36.5</td>
</tr>
<tr>
<td>(22-30)</td>
<td>(21-41)</td>
<td>(21.1-132)</td>
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Table 2 – *In vivo* results of SLN detection

<table>
<thead>
<tr>
<th>Study nr.</th>
<th>Localization primary tumor</th>
<th>No. of SLNs <em>a</em></th>
<th>SLNs showing fluorescence <em>in vivo</em> <em>b</em></th>
<th>SLNs detected with patent blue <em>c</em></th>
<th>SLNs with metastases <em>d</em></th>
<th>Transcutaneous lymphatic mapping <em>e</em></th>
<th>Signal after superficial incision <em>f</em></th>
<th>Distance femoral artery – skin (mm) <em>g</em></th>
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<tbody>
<tr>
<td>1</td>
<td>Midline right</td>
<td>3 right 1 left</td>
<td>3 right 1 left</td>
<td>3 right 0 left</td>
<td>0</td>
<td>No</td>
<td>Yes</td>
<td>31.4 right 36.5 left</td>
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<tr>
<td>2</td>
<td>Right</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
<td>23.7</td>
</tr>
<tr>
<td>3</td>
<td>Left</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>Yes</td>
<td>Yes</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>Left</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>No</td>
<td>Yes</td>
<td>44.7</td>
</tr>
<tr>
<td>5</td>
<td>Midline</td>
<td>1 right 1 left</td>
<td>1 right 1 left</td>
<td>1 right 1 left</td>
<td>0</td>
<td>Only on left side</td>
<td>Yes</td>
<td>40.1 right 26.6 left</td>
</tr>
<tr>
<td>6</td>
<td>Right</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>No</td>
<td>Yes</td>
<td>132</td>
</tr>
<tr>
<td>7</td>
<td>Midline</td>
<td>2 right 5 left</td>
<td>2 right 4 left</td>
<td>2 right 3 left</td>
<td>0</td>
<td>No</td>
<td>Yes</td>
<td>23.7 right 27.1 left</td>
</tr>
<tr>
<td>8</td>
<td>Midline</td>
<td>1 right 2 left</td>
<td>1 right 1 left</td>
<td>1 right 1 left</td>
<td>0</td>
<td>No</td>
<td>Yes</td>
<td>32.4 right 39.4 left</td>
</tr>
<tr>
<td>9</td>
<td>Midline left</td>
<td>1 right 2 left</td>
<td>1 right 2 left</td>
<td>0 right 1 left</td>
<td>0</td>
<td>Only on left side</td>
<td>Yes</td>
<td>21.1 right 22.2 left</td>
</tr>
<tr>
<td>10</td>
<td>Midline left</td>
<td>1 right 3 left</td>
<td>0 right* 3 left</td>
<td>0 right* 2 left</td>
<td>0</td>
<td>Weak signal on left side</td>
<td>Only on left side</td>
<td>43.4 right 28.7 left</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>29</td>
<td>26</td>
<td>21</td>
<td>2</td>
<td>5 of 16 groins</td>
<td>15 of 16 groins</td>
<td></td>
</tr>
</tbody>
</table>

a. Total number of SLNs as detected by radiocolloid. b. Number of SLNs that could be detected *in vivo* based on fluorescence. c. Number of SLNs that showed blue discoloration. All of these were fluorescent. d. Number of SLNs containing metastatic disease. 

e. Transcutaneous detection of fluorescence. In patients 5, 9, 10, transcutaneous fluorescence was only seen on the left side, while SLNs were found bilaterally. f. Detection of fluorescence after a superficial incision was made in the groin. g. Distance between the femoral artery and skin, determined on preoperative CT-scan.

* In patient no. 10, weak transcutaneous fluorescence was seen on the left side. Both SLNs were fluorescent. No fluorescence was detected in the right groin, neither transcutaneously nor in the SLN.
REFERENCE LIST
