VEGF-targeted multispectral optoacoustic tomography and fluorescence molecular imaging in human carotid atherosclerotic plaques

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Research Article

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Abstract

Background: Vulnerable atherosclerotic carotid plaques are prone to rupture resulting in ischemic strokes. Molecular imaging techniques have the potential to assess plaque vulnerability by visualizing molecular markers. Bevacizumab-800CW is a near-infrared fluorescent contrast agent antibody targeting vascular endothelial growth factor-A (VEGF-A). Here, we study if administration of bevacizumab-800CW is safe and can be visualized using multispectral optoacoustic tomography (MSOT) to evaluate atherosclerotic carotid plaques in vivo by visualizing intra-plaque neovascularization.

Methods: Healthy volunteers were imaged with MSOT to determine the technical feasibility of human carotid imaging with MSOT. Patients with symptomatic carotid artery stenosis scheduled for carotid artery endarterectomy were intravenously administered with a bolus injection of 4.5 mg bevacizumab-800CW. Before and two days after tracer administration, in vivo non-invasive MSOT was performed. For validation, ex vivo fluorescence molecular imaging of the surgically removed plaque specimen was performed and correlated with histopathology.

Results: Administration of 4.5 mg bevacizumab-800CW was safe in five patients. MSOT achieved accurate visualization of the carotid bifurcation area and assessment of the plaque in all five patients. Bevacizumab-800CW-resolved signal could not be detected with MSOT prior to surgery. However, ex vivo analysis of the carotid plaque showed accumulation of bevacizumab-800CW.

Conclusions: These first-in-human MSOT and fluorescence molecular imaging results in carotid artery plaques suggest that bevacizumab is a potential tracer for imaging of vulnerable plaques. However, the microdose used here cannot be detected with MSOT. A subsequent phase I dose-finding study is needed to evaluate bevacizumab-800CW in higher doses as a useful optoacoustic imaging agent. Moreover, the development of dedicated optoacoustic contrast agents for signal attenuation of the targeting moiety is advisable for carotid atherosclerotic plaque assessment using MSOT.

Background

Carotid atherosclerotic plaque rupture is a major cause of ischemic stroke, accounting for 18–25% of all stroke events. (1) Currently, carotid endarterectomy (CEA) is recommended if the degree of extracranial internal carotid artery (ICA) stenosis is > 70% in symptomatic patients to prevent a second ischemic stroke event or death. (2) However, some ischemic strokes do not correlate with stenosis severity but with plaque rupture. (3)

Identification of these ‘vulnerable plaques’ by obtaining information on plaque composition could be a valuable tool to select symptomatic and eventually asymptomatic patients with increased risk for ischemic stroke that would benefit from a CEA. (4, 5) There is strong evidence that vulnerable plaques show increased levels of inflammation, characterized by a thin fibrous cap, a necrotic core and increased macrophage infiltration. (6–8) Particularly, vulnerable plaques show intra-plaque neovascularization, induced by the intra-plaque release of vascular endothelial growth factor (VEGF-A). Therefore, the
overexpression of VEGF-A seems to have an influence on plaque instability and could be used as a target to identify vulnerable, unstable plaques, as has been shown through Positron Emission Tomography. (9, 10)

Optoacoustic imaging combines the favorable characteristics of optical imaging and ultrasound (US) for high-resolution imaging at depths of several centimeters.\(^{(11)}\) Specifically, multispectral optoacoustic tomography (MSOT) can detect multiple photoabsorbers in tissue, including both intrinsic tissue chromophores (e.g. hemoglobin, deoxyhemoglobin) and exogenous contrast agents. Here, we study if intravenous injection of bevacizumab-800CW, a near-infrared fluorescent tracer that has been used in pilot studies in surgical oncology, is safe in patients undergoing CEA, a vulnerable patient population with multiple risk factors of cardiovascular disease.\(^{(12, 13)}\) We aim to determine if in vivo MSOT of bevacizumab-800CW is feasible in symptomatic patients with carotid atherosclerotic plaques.

**Methods**

**Clinical trial design**

This microdosing safety and proof-of-concept study was performed at the University Medical Center Groningen. Healthy volunteers were recruited and underwent MSOT without prior tracer administration to study technical feasibility. Subsequently, symptomatic patients (i.e. after a cerebrovascular accident or a transient ischemic attack) scheduled for CEA were included. Patients received an intravenous microdose of 4.5 mg bevacizumab-800CW three days before surgery. In this vulnerable patient cohort, an FDA-approved microdosing strategy with no direct pharmaceutical effect which gives insight in the pharmacokinetics was used to grant the safest environment for patients while still being able to study the binding of the tracer.\(^{(14)}\) The used time interval (3 days prior to surgery) is used in a variety of oncological studies using bevacizumab-800CW based on the optimal imaging period due to the antibody half life. MSOT was performed before and two days after tracer administration. Two carotid artery specimens from consenting symptomatic patients without tracer administration were used as a negative control. The primary endpoints were tracer safety and feasibility of bevacizumab-800CW visualization with in vivo MSOT and ex vivo fluorescence molecular imaging. The study workflow is summarized in Fig. 1.

Approval for this study was obtained at the Institutional Review Board (IRB) of the UMCG (METc 2018/477). The study was performed in accordance with the Helsinki Declaration (adapted version 2013, Fortaleza, Brazil). Written informed consent was obtained from all individual participants included in the study. The trial was registered at the Clinical Trials Register (NCT03757507).

**Safety data**

Safety for this vulnerable patient group was a primary endpoint of this clinical study, both regarding MSOT and bevacizumab-800CW administration. Bevacizumab-800CW was in-house produced in the Good Manufacturing Practice facility of the UMCG, as previously described in detail.\(^{(15)}\) Vital signs and
physical examinations were obtained before and after MSOT and bevacizumab-800CW administration. Two weeks after tracer administration, patients were contacted by phone to identify adverse events, according to the National Cancer Institute CTCAE version 5.0.(16)

**Optoacoustic imaging**

All MSOT procedures were performed using a clinical research hybrid ultrasound (US) - MSOT system (MSOT Acuity Echo prototype; iThera Medical GmbH, Germany). This system uses a 25 Hz pulsed Nd:YAG laser for emission of 25 mJ pulses. The two-dimensional (2D) concave detector (4-MHz center frequency, 256 transducer elements) provides cross-sectional imaging with an in-plane spatial resolution of ~180 µm and field of view of 40x40 mm. Simultaneously, US signal is detected for anatomical guidance of the imaging procedure.

Imaging of the common carotid artery (CCA), the carotid bifurcation and the internal and external carotid artery was performed. Subjects were in a supine position with the neck in hyperextension and the chin rotated to the contralateral side. MSOT signals were acquired using a preset with six wavelengths (700nm, 730nm, 760nm, 780nm, 800nm and 850nm) to sample the absorption spectrum profiles of hemoglobin (HbO2) deoxyhemoglobin (HbR) and IRdye800CW.

MSOT data were analyzed using cLabs (iThera Medical GmbH) and reconstructed using a back-projection algorithm. The lumen of the CCA and the plaque in patient’s data were manually segmented in the US image. The lumen and plaque ROIs were duplicated at the same depth in the background region to serve as a reference. Optoacoustic signals in arbitrary units (a.u.) were extracted from the ROIs on three consecutive frames and average optoacoustic signals were registered. For image reconstruction, single wavelength analyses were performed at 800nm to detect both HbO2 and HbR, 850nm to detect HbO2 and 780 nm to detect IRDye800CW.(17) In addition, spectral unmixing was performed to study HbO2, HbR and IRDye800CW signal.

For quantitative analysis, the mean intensity ratios between the ROI and the background ROI were calculated to allow for data comparison of different patients, as reported earlier.(18) As such, the effects of imaging depth and light fluence are minimized.

**Ex vivo analyses of surgical specimen**

Directly after CEA, the atherosclerotic plaque was imaged using a closed-field fluorescence camera system (PEARL Trilogy, LI-COR Biosciences, Lincoln, NE, USA). Subsequently, the specimen was formalin-fixed for 24 hours and serially sliced in ~1 mm thick tissue slices. Fluorescence flatbed scanning of tissue slices was performed using the Odyssey® CLx (LI-COR Biosciences) and embedded in paraffin blocks (formalin-fixed paraffin-embedded [FFPE]-blocks).

ROIs were drawn of the plaque within the surgical specimen using white light images with plaque anatomy as a reference. Mean fluorescence intensities (MFI) of the bevacizumab-800CW group and the negative control from all tissue slices (2–3 tissue slices per patient) were calculated. 4 µm tissue sections
were obtained of all FFPE-blocks and hematoxylin and eosin (H&E) staining and VEGF immunohistochemistry were performed to correlate imaging results with histology. A pathologist (GFHD) blinded for imaging results analyzed all H&E tissue sections.

To assess the presence of intact bevacizumab-800CW in the atherosclerotic plaque, we performed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page) on one sample of a fresh frozen surgical carotid artery specimen, as previously described. Bevacizumab-800CW was yielded in concentrations of 10 to 80 µg total protein on a biorad mini protein TGX precast gel (7.5%). Gels were stained with imperial protein stain (Thermo Scientific). Unlabeled bevacizumab was used as a reference. Subsequently, we scanned the gel using the 800 nm channel of the Odyssey CLX ® flatbed scanning system.

Statistics

Due to this proof-of-concept study’s explorative character and the subsequent small sample size, only descriptive statistics were used. MFI was defined as total counts per ROI pixel area (signal/pixel). Descriptive statistics and graph design were conducted using Graphpad Prism version 8.

Results

Five healthy volunteers and five patients with symptomatic carotid artery stenosis were included in this study. Since we could not detect bevacizumab-800CW with MSOT in vivo, the trial was stopped before completing the target patient sample size (n = 10). In total, four patients (all males, mean age 76) underwent the complete in- and ex vivo imaging protocol. For one patient who received bevacizumab-800CW, the surgical plan was converted to carotid artery stenting resulting in no surgical specimen obtainment. Patient and healthy volunteer characteristics and safety data are shown in Table 1. No drug-related or imaging related (serious) adverse events were observed.

In vivo MSOT

Healthy volunteer imaging showed the technical feasibility of in vivo optoacoustic visualization of the carotid artery and detection of intrinsic tissue chromophores such as HbR and HbO2. In all patients, handheld MSOT enabled real-time visualization of the CCA, internal carotid artery and external carotid artery. In all patients, the atherosclerotic plaque could be identified. In all patients, the plaque was localized in the internal carotid artery and CCA. Yet, as the light fluence in the internal carotid artery was limited by absorption of the external carotid artery, we used the CCA for further analyses. Strong absorption of hemoglobin restricted further penetration light, resulting in a “cap” of optoacoustic signal in the CCA. In all images, the optoacoustic intensity in the CCA was higher than that of surrounding tissue, both when using single wavelength analyses and spectral unmixing. (Fig. 2A).

Subsequently, we assessed the ability of MSOT to identify a vulnerable plaque by detecting bevacizumab-800CW signal within the atherosclerotic plaque. Pre-injection and post-injection data of
single wavelength analysis at 780nd and IRDye800CW spectral unmixing were analyzed per patient. In one patient, no pre-injection MSOT was performed due to the unavailability of the dedicated MSOT room, resulting in a total of seven data points. Upon spectral unmixing, no optoacoustic signal of bevacizumab-800CW could be detected with MSOT (Fig. 2A). Also, no increase in single wavelength analysis of 780 nm was observed after tracer administration (1.13 (IQR 1.00-1.27) a.u. post-injection vs 1.26 (IQR 0.79–1.17) a.u. pre-injection(Fig. 2)).

**Ex vivo analyses**

To further evaluate specific bevacizumab-800CW tracer uptake in the atherosclerotic plaque, we performed closed-field fluorescence imaging of the freshly excised surgical specimen. Macroscopically, fluorescence signal correlated well with areas within the plaque containing calcifications (Fig. 2B). To evaluate whether the fluorescence signal was derived from the tracer, we included two carotid plaque specimen of consenting negative controls. Tissue slices of the experimental group showed an MFI of 2.37 (IQR 1.90–3.99) x 10^{-4} compared to 1.86 (0.79–2.26) x 10^{-4} in negative controls (Fig. 2B).

Analysis of 10 µm sections showed increased MFI compared to the vascular wall (Fig. 3A). In the atherosclerotic plaques, mainly large fibrotic and necrotic tissue was observed upon H&E histopathological examination (Fig. 3B). A representative image of high VEGF-A expression in the carotid artery is demonstrated in correlation with a fluorescence signal (Fig. 3B). The presence and integrity of bevacizumab-800CW within the excised vulnerable plaque was demonstrated using SDS-page on a fresh frozen excised specimen (Supplemental Fig. 1).

**Discussion**

Currently, no imaging method can differentiate vulnerable from non-vulnerable atherosclerotic plaques for risk stratification and patient selection for surgery. To our knowledge, this is the first proof-of-concept study that aimed to visualize vulnerable atherosclerotic plaques by MSOT using bevacizumab-800CW for VEGF expression as a biomarker of intra-plaque neovascularization. Our data demonstrate that microdosing 4.5 mg bevacizumab-800CW is safe in a vulnerable patient population undergoing CEA. Although we could not detect bevacizumab-800CW-specific signal through *in vivo* MSOT, accumulation of intact bevacizumab-800CW in the carotid plaque specimen was objectified with SDS-page.

In all patients, we could visualize the entire carotid artery bifurcation area and identify the atherosclerotic plaques. Using both single wavelength analyses and spectral unmixing we could differentiate several intrinsic tissue chromophores, regardless of different imaging depths affecting light fluence. However, 4.5 mg bevacizumab-800CW could not be detected with MSOT to visualize intra-plaque neovascularization within plaques. Further *ex vivo* analysis with fluorescence imaging systems neither proved bevacizumab-800CW-specific signal. Nevertheless, the accumulation of bevacizumab-800CW was demonstrated through SDS-page (Supplemental Fig. 1). We surmise that a microdose bevacizumab-800CW does not result in a detectable increase in optoacoustic signal, or fluorescence signal surpassing plaque autofluorescence.
The findings of this study are in line with previous studies that showed accumulation of bevacizumab in carotid atherosclerotic plaques expressing VEGF-A. Recently, our group has shown that the conjugate bevacizumab-800CW can discriminate between vulnerable and non-vulnerable plaques, and identify intraplaque angiogenesis by targeting VEGF-A. (19) Although the accumulation in atherosclerotic plaques was detected with $^{89}\text{Zr}$-bevacizumab micro-PET in ex vivo CEA specimens, which in fact correlated with VEGF immunohistochemistry scores, we could not detect bevacizumab-800CW in the current dose with neither MSOT or fluorescence imaging. (10)

We hypothesize that the inability to detect bevacizumab-800CW with MSOT or fluorescence imaging is due to two causes. First, the current microdose might be too low to be detected or surpass the auto fluorescence in the atherosclerotic plaque. Secondly, the photophysical properties of the tracer are not optimal for optoacoustic signal generation. Therefore, we propose two strategies for future clinical studies that pursue MSOT for identification of vulnerable plaques by targeting VEGF-A, in which is used as targeting moiety since it has shown specific discrimination between vulnerable and non-vulnerable plaques.

The first and most straightforward approach could be a dose-escalation study employing an increasing dose of bevacizumab-800CW to approximate the detection limit of optoacoustic imaging, as has been carried out in multiple clinical trials in fluorescence-guided surgery and fluorescence-guided endoscopy. (12, 20) An alternative approach is to conjugate bevacizumab to a dedicated optoacoustic signaling compound, currently not clinically available but has gained increasing attention last decade. (21, 22) Optoacoustic contrast agents have been reviewed extensively and ideally exhibit a high molar extinction coefficient in the NIR, a characteristic absorption spectrum with sharp peaks, high photostability, high photothermal conversion efficiency, low quantum yield and favourable biocompatibility. (21)

Our data processing framework is a step-up towards the standardization of dual modality optoacoustic imaging together with fluorescence imaging for visualizing plaque biology and atherosclerosis. Further studies for optimizing optoacoustic contrast agents are needed to enable non-invasive transcutaneous visualization of vulnerable plaques in vivo.

**Declarations**

*Data availability*

All data obtained during the current study are available from the corresponding author on reasonable request.

*Conflict of interest*

GMvD is CEO, founder and shareholder of TRACER Europe BV. No other potential conflicts of interest relevant to this article exist
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Author contributions

PJS, JV, HHB and GMvD designed the study, PJS and JV performed data acquisition, analyzed and interpreted data and drafted the manuscript, PJS and JV contributed equally to this work, GFHD and JH were involved in histopathological analyses and reviewing of the manuscript, GM assisted in processing and analysis of the surgical specimens, LAH, GFHD, JH, WBN, CJZ, RHJAS, HHB and GMvD supervised the study, interpreted data and supervised writing of the manuscript.

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Table 1 | Clinical characteristics of healthy volunteers and patients who underwent bevacizumab-800CW administration and finished the study

| Characteristics | 5 volunteers | 4 patients |
|-----------------|--------------|------------|
| Age (years), *mean (range)* | 28 (23-38) | 76 (58-82) |
| Sex: males, *n (%)* | 5 (100) | 4 (100) |
| Weight (kg), *mean (range)* | 82 (74-86) | 71.5 (65-80) |
| Height (cm), *mean (range)* | 186 (172-189) | 172.5 (170-178) |
| Imaging time (min), *mean (range)* | 11 (5-12) | 9 (5-12) |
| MSOT related adverse events | 0 | 0 |

Data are presented in numbers with percentages (%) or means with range.

Figures

A. **MSOT validation**
   - Optoacoustic imaging

B. **Imaging procedures**
   - Day 0
     - Optoacoustic imaging
     - Intravenous injection 4.5 mg **bevacizumab-800CW**
   - Day 1
     - Optoacoustic imaging
   - Day 2
     - Carotid endarterectomy
     - Fluorescence imaging **Whole carotid specimen**

**Tissue processing**
- Day 3-10
  - Fluorescence imaging
  - Carotid Tissue Slices
  - FFPE blocks
  - Immunohistochemistry
    - VEGF-A
  - Fluorescence microscopy

*Figure 1*
Imaging workflow MSOT was performed on healthy volunteers for technical feasibility (A). On day 0, optoacoustic imaging and tracer administration were performed, followed by bevacizumab-800CW-targeted optoacoustic imaging one day prior to carotid endarterectomy (B). Afterward, tissue processing using fluorescence imaging of the carotid specimen was performed (C). Abbreviations: MSOT, multispectral optoacoustic tomography; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery.

**In vivo multispectral optoacoustic tomography**

![Image A](image1.jpg)

**Ex vivo closed-field fluorescence imaging**

![Image B](image2.jpg)

Figure 2
Macroscopic optoacoustic and fluorescence imaging

Representative hybrid optoacoustic image of carotid atherosclerosis pre- and postinjection (A), the region of interest resembling the carotid plaque used for measurements is delineated with a red line. Pre- and postinjection measurements of 780 nm optoacoustic imaging of all carotid arteries showed no difference on 780 nm and IRDye800CW. Fluorescence imaging of a whole surgical carotid specimen and a formalin-fixed tissue slice with standard histopathology (B). Mean fluorescence intensities (PEARL) of the plaque in all surgical tissue slices from negative patients and bevacizumab-800CW patients. Abbreviations: MSOT, multispectral optoacoustic tomography; HbR, deoxygenated hemoglobin; HbO2, oxygenated hemoglobin; HbT, total hemoglobin.

Figure 3

Microscopic tracer distribution

Fluorescence imaging of a formalin-fixed paraffin-embedded carotid tissue section of a vulnerable atherosclerotic plaque (A, B). Both regions with VEGF-A overexpression and calcifications due to autofluorescence show high fluorescence signal, which cannot be attributed to VEGF-specific signal (B). Abbreviations: VEGF, vascular endothelial growth factor A.

Supplementary Files

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- SupplementalFigure1.SDPage.png