Bevacizumab-Induced Normalization of Blood Vessels in Tumors Hampers Antibody Uptake

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Abstract
In solid tumors, angiogenesis occurs in the setting of a defective vasculature and impaired lymphatic drainage that is associated with increased vascular permeability and enhanced tumor permeability. These universal aspects of the tumor microenvironment can have a marked influence on intratumoral drug delivery that may often be underappreciated. In this study, we investigated the effect of blood vessel normalization in tumors by the antiangiogenic drug bevacizumab on antibody uptake by tumors. In mouse xenograft models of human ovarian and esophageal cancer (SKOV-3 and OE19), we evaluated antibody uptake in tumors by positron emission tomographic imaging 24 and 144 hours after injection of89Zr-trastuzumab (SKOV-3 and OE19),89Zr-bevacizumab (SKOV-3), or89Zr-IgG (SKOV-3) before or after treatment with bevacizumab. Intratumor distribution was assessed by fluorescence microscopy along with mean vessel density (MVD) and vessel normalization. Notably, bevacizumab treatment decreased tumor uptake and intratumoral accumulation compared with baseline in the tumor models relative to controls. Bevacizumab treatment also reduced MVD in tumors and increased vessel pericyte coverage. These findings are clinically important, suggesting caution in designing combinatorial trials with therapeutic antibodies due to a possible reduction in tumoral accumulation that may be caused by bevacizumab cotreatment. Cancer Res; 73(11); 1-9. ©2013 AACR.

Introduction
Angiogenesis, the formation of new blood vessels, is one of the hallmarks of cancer and is therefore an anticancer drug target (1, 2). In solid tumors, extensive angiogenesis is accompanied by defective vascular architecture, leading to increased vascular permeability, hypoxia, low pH, and high interstitial fluid pressure. Several preclinical and clinical studies indicate that antiangiogenic drugs, including the anti-VEGF-A antibody bevacizumab, lead to vessel normalization in addition to their antivascular effect. Major characteristics of vessel normalization include reduced number and size of immature vessels, increased vessel pericyte coverage, and reduced interstitial fluid pressure (3, 4). Normal (or normalized) blood vessels are lined with pericytes. This lining is absent in the abnormal, immature vessels created during tumor angiogenesis, which can cause increased leakiness (5).

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We have developed zirconium-89 (\(^{89}\text{Zr}\))-labeled bevacizumab and \(^{89}\text{Zr}\)-trastuzumab as tracers for positron emission tomographic (PET) scanning to visualize and quantify bevacizumab and trastuzumab biodistribution for both preclinical and clinical purposes (22–24). These tracers can provide insight into how bevacizumab affects uptake of other antibodies.

We evaluated the effect of bevacizumab treatment on the tumor uptake of \(^{89}\text{Zr}\)-trastuzumab, \(^{89}\text{Zr}\)-bevacizumab, and \(^{89}\text{Zr}\)-immunoglobulin G (IgG). In addition, we tracked the localization of IgG in the tumors with \textit{ex vivo} fluorescent labeling and related the findings to effects of bevacizumab treatment on tumor vessel density and vessel normalization.

Materials and Methods

Antibody labeling

Trastuzumab (Roche), bevacizumab (Roche), and human IgG (Sanquin) were conjugated and labeled with \(^{89}\text{Zr}\) as described previously (22, 24). IgG served as control because it is biochemically comparable to trastuzumab and bevacizumab but has no affinity for a specific antigen. In short, purified trastuzumab, bevacizumab and IgG were conjugated with the chelator tetra-fluorophenyl-N-succinimidylserylter (TPF-N-sucDf; kindly provided by Dr. G.A. van Dongen, VUMC). Radiolabeling of all conjugates (N-sucDf-trastuzumab, -bevacizumab, and -IgG) was conducted with clinical grade \(^{89}\text{Zr}\)-oxalate (IBA). All \(^{89}\text{Zr}\)-tracers had a purity of >95% before administration to the animals.

IgG was also labeled with IRDye 800CW (LI-COR Biosciences) as described previously (25). Purified IgG was labeled with IRDye 800CW and the solution was purified with a PD-10 desalting column (GE Healthcare). Labeling efficiency and purity of IgG-800CW as determined by SE-HPLC were, respectively, 85% to 90% and >95%.

Animals and tumor inoculation

The human ovarian cancer SKOV-3 cell line was selected for xenograft experiments because of its high expression of VEGF and HER2 (24, 26). In addition, the HER2-overexpressing esophageal adenocarcinoma cell line OE19 (27) was used for xenograft experiments. Both tumor models had comparable tumor growth kinetics \textit{in vivo}, which allowed the evaluation of both before- and during-treatment scans at a comparable tumor size. SKOV-3 was obtained from American Type Culture Collection (ATCC) and OE19 from Sigma-Aldrich. Both cell lines were authenticated by short tandem repeat profiling (Baseclear). ATCC HTB-77 was used as the reference profile for SKOV-3. The evaluation value (EV) obtained for SKOV-3 was 1 and for OE19 was 1.06. SKOV-3 cells were cultured in Dulbecco’s Modified Eagle Medium (Invitrogen) and OE19 cells in Roswell Park Memorial Institute medium (Invitrogen), both with 4.5 g/mL glucose and supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO\(_2\). For tumor inoculation, the cells were harvested by trypsinization and resuspended in culture medium supplemented with fetal calf serum. \textit{In vivo} imaging and \textit{ex vivo} biodistribution experiments were conducted in male nude mice (HSD, athymic nude-nu, average weight 30 g) obtained from Harlan. To avoid a pharmacologic effect of the \(^{89}\text{Zr}\)-bevacizumab tracer, in the \(^{89}\text{Zr}\)-bevacizumab group, a lower bevacizumab tracer dosage was essential to evaluate pre- and posttreatment tumor uptake. To enable imaging of low tracer doses, male nude mice (BALB/cOlaHDS-foxn\(^{nu}\), average weight 20 g) from Harlan were used in the \(^{89}\text{Zr}\)-bevacizumab group because of slower clearance of human IgG in these animals (28). At 6 to 8 weeks of age, mice were subcutaneously injected with 1 × 10\(^6\) SKOV-3 cells or 3 × 10\(^6\) OE19 cells in 0.3 mL Matrigel (BD Biosciences) and culture medium (1:1). Tumor growth was assessed by caliper measurements. When tumors measured 6 to 8 mm in diameter, about 2 to 3 weeks after inoculation, \textit{in vivo} studies were started. All animal experiments were carried out with isoflurane inhalation anesthesia (induction 3%, maintenance 1.5%). The animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Groningen, the Netherlands).

PET scan imaging and bevacizumab treatment

Clear tumor uptake of \(^{89}\text{Zr}\)-trastuzumab and \(^{89}\text{Zr}\)-bevacizumab is known to occur 24 hours after tracer injection with an optimal time point 144 hours after tracer injection (22, 24). Therefore, PET imaging was conducted 24 or 144 hours after tracer injection. In the SKOV-3 model, on day 0 of the study schedule, animals received the first tracer gift of \(^{89}\text{Zr}\)-trastuzumab (100 μg, ±5 MBq), \(^{89}\text{Zr}\)-bevacizumab (10 μg, ±5 MBq), or \(^{89}\text{Zr}\)-IgG (100 μg, ±5 MBq). In the OE19 model, animals received \(^{89}\text{Zr}\)-trastuzumab (100 μg, ±5 MBq) on day 0 of the study schedule. The tracers were injected intravenously in the penile vein. PET imaging was conducted on days 1 and 6 (respectively, 24 and 144 hours after first tracer injection). From day 7 until 12, we allowed the tracer to decay and the antibody to remove form the tumor. On days 13, 16, and 19, the animals received a 5 mg/kg nonlabeled treatment dose bevacizumab according to earlier studies or 0.9% NaCl as placebo (29). The second tracer injection was administered on day 14, and PET imaging was conducted on days 15 and 20 (respectively, 24 and 144 hours post second radioactive tracer injection). Animals were sacrificed after the last scan on day 20. For the second tracer injection, both IgG groups received a co-injection of \(^{89}\text{Zr}\)-(5 MBq) and IRDye 800CW–labeled IgG to study the intratumor distribution of IgG with fluorescence microscopy. The total protein dose was the same as in the first tracer injection, with half of the dose labeled fluorescently. In total 5 groups were scanned with different tracers and interventions: (i) \(^{89}\text{Zr}\)-trastuzumab (n = 7), (ii) \(^{89}\text{Zr}\)-bevacizumab (n = 6), (iii) \(^{89}\text{Zr}\)-IgG (n = 6) all treated with bevacizumab, (iv) \(^{89}\text{Zr}\)-IgG (n = 6) treated with placebo all in the SKOV-3 model, and (v) \(^{89}\text{Zr}\)-trastuzumab (n = 6) in the OE19 model treated with bevacizumab. During each scan sequence, static images of 15 minutes (24 hours postinjection) and 45 minutes (144 hours postinjection) acquisition time were obtained with a microPET Focus 220 rodent scanner (CTI Siemens) followed by an 8-minute transmission scan. \textit{In vivo} quantification was conducted after image reconstruction with AMIDE Medical Image Data Examiner software (version 0.9.1). The data are presented as the mean standardized uptake value (SUV\(_{\text{mean}}\)) and percentage injected dose per gram tissue (%ID/g).
**Ex vivo biodistribution and tumor tissue analysis**

After the last PET scan on day 20, animals were sacrificed and organs were excised and weighted for biodistribution. Organs and primed standards were then counted for radioactivity in a calibrated well-type LKB-1282 Compu-gamma system (LKB Wallac) and corrected for physical decay. Hematoxylin and eosin staining was conducted to assess tumor viability for SKOV-3 and OE19. For immunohistochemical and fluorescence imaging, harvested tumors were formalin-fixed and paraffin-embedded. For SKOV-3, slides (5 μm) were stained with antibodies against Ki67 (1:350, Dako) and von Willebrand Factor (vWF, 1:250, Dako) to calculate the proliferation index and the mean vessel density (MVD), respectively. The proliferation index was calculated as the percentage of Ki67-positive nuclei in 3 fields at ×400 magnification using a calibrated grid. The MVD was analyzed as described earlier (22). Angiogenic hot spot areas were determined in 3 predefined hot spot areas by counting the number of positive vessels at ×200 magnification using a calibrated grid. Images were acquired by the NanoZoomer 2.0-HT slide scanner.

Figure 1. SKOV-3 representative transversal and coronal microPET images of ⁸⁹Zr-trastuzumab, ⁸⁹Zr-bevacizumab, and ⁸⁹Zr-IgG tumor uptake at 24 hours (day 1 vs. 15; A) and at 144 hours (day 6 vs. 20; B) after tracer injection. Bevacizumab (5 mg/kg) or placebo treatment was given on days 13, 16, and 19. In the graphs, quantification of ⁸⁹Zr-trastuzumab, ⁸⁹Zr-bevacizumab, and ⁸⁹Zr-IgG tumor uptake is shown for every individual animal (black) and the mean tumor uptake (dashed red line) expressed in SUV<sub>mean</sub>. When the difference in tumor uptake before and after bevacizumab treatment was significant, the decrease (%) is given with corresponding P value.
Tissue viability was expressed as the total percentage of necrosis in the tumor within fixed size squares overlaying the tumor sections. Analysis was conducted using NanoZoomer Digital Pathology viewer software (Hamamatsu). To visualize endothelial cells, a fluorescent double staining with an antibody against Meca32 (1:40, BD Biosciences) was conducted, whereas to visualize pericytes an antibody, α-smooth muscle actin was used (SMA: 1:100, Sigma). Nuclei were stained with Hoechst 33528 (1:5,000, Molecular Probes, Invitrogen). Tumor sections were analyzed using a Leica DM6000 microscope, and images shown were captured with a Leica DFC360FX camera and processed with LAS-AF2 software (Leica). In 3 predefined angiogenic hot spot areas, the degree of pericyte coverage was examined at a ×200 magnification and scored for no, partial, or full coverage.

Fluorescence microscopy analysis of IgG-800CW together with Hoechst staining was determined in tumor sections as described before (25), and overview images were obtained with the Odyssey infrared imaging system (LI-COR Biosciences). Image analysis was conducted using FV10-ASW (version 1.6).

**Statistical analysis**

Data are presented as mean ± SD. PET data were analyzed with the paired Student’s t test. Ex vivo analysis was executed with the unpaired t test. All statistical analyses were conducted with Prism 5 (GraphPad). P ≤ 0.05 (2-tailed) was considered significant.

**Results**

**Bevacizumab treatment decreases tumor uptake of 89Zr-trastuzumab, 89Zr-bevacizumab, and 89Zr-IgG**

PET images obtained at 24 and 144 hours after tracer injection visibly showed a lower tumor uptake of 89Zr-trastuzumab, 89Zr-bevacizumab, and 89Zr-IgG during bevacizumab treatment. Already on the 24-hour PET images after tracer injection (day 1 vs. 15), SUVmean tumor uptake values decreased from 3.5 ± 0.8 to 2.2 ± 1.0 (−38% ± 20%, P = 0.004), 1.5 ± 0.1 to 1.3 ± 0.3 (−16% ± 16%, NS), and 1.5 ± 0.3 to 1.0 ± 0.2 (−27% ± 21%, P = 0.04) for, respectively, 89Zr-trastuzumab, 89Zr-bevacizumab, and 89Zr-IgG (Fig. 1A). This effect was even more pronounced at 144 hours (day 6 vs. 20), with a reduction in 89Zr-trastuzumab tumor uptake from a SUVmean of 5.2 ± 1.2 before to 3.0 ± 1.0 after bevacizumab treatment (−41% ± 5%, P < 0.0001) and a similar reduction in 89Zr-bevacizumab tumor uptake with an SUVmean of 2.8 ± 0.3 to 1.6 ± 0.2 after bevacizumab treatment (−43% ± 4%, P < 0.0001; Fig. 1B).

Tumor uptake of 89Zr-IgG was also lowered, although to a lesser extent. SUVmean decreased from 1.7 ± 0.2 before to 1.2 ± 0.1 after bevacizumab treatment (−28% ± 6%, P = 0.0004). Placebo treatment did not affect 89Zr-IgG uptake in the tumor with SUVmean tumor uptake of 1.6 ± 0.3 before and 1.6 ± 0.1 after placebo at 24 hours and 1.8 ± 0.4 before and 1.8 ± 0.2 after placebo at 144-hour PET images (Fig. 1).

In the OE19 model, bevacizumab treatment also decreased the tumor uptake of 89Zr-trastuzumab. Already on the 24-hour PET images (day 1 vs. 15), the SUVmean tumor uptake values decreased from 2.7 ± 0.7 to 1.8 ± 0.7 (−34% ± 15%, P = 0.004). At 144 hours (day 6 vs. 20), the SUVmean tumor uptake decreased even more from 3.9 ± 1.2 to 2.5 ± 1.4 (−39% ± 16%, P = 0.003; Fig. 2).

*Ex vivo* biodistribution of the tracer in nontumor tissues and the tumor, for all 4 groups in the SKOV-3 model, are shown in Fig. 3A. *Ex vivo* biodistribution for both 89Zr-trastuzumab and 89Zr-bevacizumab were comparable with earlier described data, with high uptake of the tracer in the tumor, moderate uptake in the blood pool and highly vascularized organs, and low uptake in the remaining organs. (22, 30). The *ex vivo* biodistribution data of 89Zr-trastuzumab in the OE19 model were similar to the pattern in the SKOV-3 model (Supplementary Fig. S1A) For 89Zr-IgG, tumor uptake was lower when the animals had received bevacizumab than the placebo-treated 89Zr-IgG group for both the *ex vivo* biodistribution tumor uptake (P = 0.02) and the *in vivo* PET tumor uptake (P = 0.0001; Fig. 3B). Tumor uptake of the *in vivo* PET data nicely
correlated with ex vivo tumor uptake as measured during biodistribution analysis for both the SKOV-3 ($R^2 = 0.81$; Fig. 3C) and the OE19 model ($R^2 = 0.71$; Supplementary Fig. S1B).

Tumor growth and tumor size were comparable between all groups throughout the experiment, with no significant differences. For the SKOV-3 model, the average tumor size of all 4 tracer groups was 288 ± 66 mm³ on day 1 and 494 ± 123 mm³ on day 15. On day 6, this was 327 ± 74 mm³ and 543 ± 86 mm³ on day 20. For the OE19 model, on day 1, the average tumor size was 238 ± 71 mm³ and 334 ± 117 mm³ on day 15. On day 6, this was 298 ± 114 mm³ and 404 ± 158 mm³ on day 20.

**Figure 3.** A, day 20 ex vivo biodistribution data of the target-specific tracers $^{89}$Zr-trastuzumab, $^{89}$Zr-bevacizumab, and the aviscific tracer $^{89}$Zr-IgG in mice bearing SKOV-3 treated with bevacizumab (5 mg/kg) or placebo on days 13, 16, and 19. The %ID/g ± SD is shown for nontumor tissue and tumor for $^{89}$Zr-trastuzumab, $^{89}$Zr-bevacizumab, and both $^{89}$Zr-IgG groups. B, day 20 ex vivo biodistribution (%ID/g ± SD) and in vivo PET (SU recovery) data of tumor uptake for the $^{89}$Zr-IgG groups, treated with bevacizumab or placebo in the SKOV-3 model. Tumor uptake was lower in the $^{89}$Zr-IgG group treated with bevacizumab compared with the placebo-treated $^{89}$Zr-IgG group for biodistribution and PET data. C, the day 20 ex vivo biodistribution tumor uptake (%ID/g) in the SKOV-3 model nicely correlated with the day 20 in vivo PET tumor uptake (%ID/g); $R^2 = 0.81$ for SKOV-3.

**Bevacizumab treatment normalizes the tumor vasculature and reduces uptake of fluorescent-labeled IgG**

Bevacizumab treatment reduced the tumor vessel density. In the placebo-treated group, the MVD was 9.0 ± 1.3 vessels/high-power field, whereas the MVD in bevacizumab-treated tumors was 5.8 ± 1.2 ($P < 0.0003$), 5.9 ± 1.1 ($P = 0.0001$), and 5.4 ± 0.8 ($P < 0.0001$) for the $^{89}$Zr-trastuzumab, $^{89}$Zr-bevacizumab, and $^{89}$Zr-IgG group, respectively (Fig. 4A and C). Bevacizumab treatment also induced vessel normalization. In placebo-treated tumors, 68% ± 21% of the tumor vessels had no pericyte
coverage and only 7% ± 11% of the vessels were fully covered with pericytes. After bevacizumab treatment, pericyte coverage was absent in only 10% ± 14% of the tumor vessels (P < 0.0001), whereas 75% ± 21% of the vessels were fully covered (P < 0.0001; Fig. 5). Ex vivo, we observed that IgG-800CW was mainly present in the extracellular matrix, and bevacizumab treatment reduced the accumulation of IgG-800CW relative to the placebo (Fig. 4B). This matches the PET results. Bevacizumab treatment did not affect tumor morphology and viability in both the SKOV-3 and OE19 model. In addition, bevacizumab did not affect the proliferation index in the SKOV-3 model.

Discussion

This study shows that bevacizumab treatment substantially reduces the tumor uptake of the antibodies trastuzumab, bevacizumab, and IgG. In addition, we show an induction of vessel normalization and decrease in MVD in these tumors by bevacizumab treatment. As bevacizumab also lowered the uptake of IgG in the tumor, the results for the antibodies cannot solely be explained by a therapeutic effect or tumor saturation by bevacizumab itself. Therefore, these results show that bevacizumab treatment can impair penetration of large molecules such as antibodies into the...
tumor, resulting in a reduced tumor uptake of these antibodies.

In several preclinical settings such as murine mammary carcinoma, human small cell lung carcinoma, human glioblastoma multiforme, and human colon adenocarcinoma, blocking VEGF signaling not only led to a depletion of the vasculature but also created a morphologically and functionally normalized vascular network of the remaining vessels (3, 4, 8, 31, 32). There are also additional preclinical data supporting the effect of vessel normalization on drug uptake by tumors. In an orthotopic mammary model, the effect of vessel normalization by antiangiogenic treatment was shown for uptake of nanomedicines ranging from 12 to 125 nm in size. The mice received 5 mg/kg of the mouse anti-VEGF antibody DC101 every 3 days. Intravital imaging showed that DC101 treatment led to vessel normalization in the tumor and thereby influenced the penetration of nanomedicines in the tumor. Small 12-nm particles took advantage of the induced vessel normalization, leading to better penetration of these particles into the tumor. Conversely, tumor uptake of 125-nm particles was reduced (33). We observed a reduction in tumor antibody uptake within 24 hours of tracer injection. This reduction was even more pronounced after 6 days, which coincided with a normalized tumor vasculature illustrated by increased pericyte coverage of the remaining vessels in the tumor.

Another recent study described the effect of a single administration of the species cross-reactive anti-VEGF-A antibody B20-4.1 24 hours before tracer injection on the biodistribution and pharmacokinetics of trastuzumab (34). Tumor-bearing mice received trastuzumab labeled with the SPECT tracers indium-111 (111In) or iodine-125 (125I) and were treated with or without B20-4.1. Biodistribution data showed a 25% to 30% decrease for both 111In- and 125I-trastuzumab tumor uptake for up to 7 days. SPECT-CT images made 2 days after anti-VEGF therapy also showed a decrease of 111In-trastuzumab uptake in the tumor. The advantage of PET scanning, which we used over SPECT scanning, is that it is easier to quantify uptake. We observed a 41% decrease in vivo of the PET tracer 89Zr-trastuzumab tumor uptake after bevacizumab treatment. This decrease already occurred within 1 day after tracer injection and the start of bevacizumab treatment. Moreover, we observed a decrease in uptake of 89Zr-bevacizumab and 89Zr-IgG after bevacizumab treatment.

We observed no effect on tumor size of bevacizumab treatment and 89Zr-trastuzumab compared with placebo and 89Zr-IgG in the SKOV-3 model. This might be due to the fact that we treated the animals over a relatively short period of time (1 week) with bevacizumab (5 mg/kg) and one single gift of trastuzumab (100 μg, corresponding with a dose of 4 mg/kg). However, there are only very limited preclinical data on the biologic effect of combining bevacizumab with trastuzumab (35, 36) This will likely be due to the fact that testing antitumor efficacy of humanized antibodies in an animal model has its limitation, as trastuzumab has inherent immunomodulatory activity (37–40). More importantly, findings in the clinic show that bevacizumab treatment does not contribute favorably to the effect of trastuzumab and support the idea that vessel normalization by bevacizumab has the same effects in patients.
(20, 21). Combination studies of bevacizumab plus another antibody have shown a detrimental or only modestly beneficial effect. In colorectal cancer, bevacizumab and the EGF receptor 1 (EGFR1) antibody cetuximab are both active drugs when combined with chemotherapy. In addition, cetuximab has some activity as monotherapy (41, 42). But the combination of chemotherapy, bevacizumab and cetuximab even negatively affected the progression-free survival of patients with colorectal cancer compared with chemotherapy and bevacizumab alone in the CAIRO2 trial (18). A possible explanation of this reduced therapeutic efficacy was considered to be the fact that tumor-promoting M2 macrophages are activated by cetuximab in the local tumor microenvironment. This would lead to increased local VEGF production (43), resulting in insufficient depletion by bevacizumab. In addition, on the basis of the present study, one could argue that the cetuximab levels in the tumors of these patients were likely lower because of the simultaneous treatment with bevacizumab. Analogously, in the PACCE trial, the addition of the EGFR1 antibody panitumumab to bevacizumab and chemotherapy in patients with colorectal cancer also led to a decrease in progression-free survival (19). That vessel normalization by bevacizumab affects antibody uptake in the clinic is supported by a preliminary report in which we showed a 47% lower tumor uptake of $^{89}$Zr-bevacizumab after bevacizumab treatment in 11 patients with renal cell cancer (44). Overall these clinical data are in line with a considerable biologic effect of reduced antibody uptake after bevacizumab treatment.

PET scanning with $^{89}$Zr-labeled antibodies can be used in future clinical studies as a supportive tool for more rationally designed treatment regimens containing bevacizumab combined with other antibodies. This can give additional insight whether choosing a treatment schedule affects tumor antibody distribution in patients and in this way PET imaging may contribute to improved and rational use of bevacizumab.

Disclosure of Potential Conflicts of Interest
J.A. Gietema has a commercial research grant from Roche to support a clinical study with bevacizumab. E.G.E. de Vries has a commercial research grant from Roche to the University Medical Center for imaging study in patients and is a consultant/advisory board member of Genentech. No potential conflicts of interest were disclosed by the other authors.

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