Quantitative In Vivo Immunohistochemistry of Epidermal Growth Factor Receptor Using a Receptor Concentration Imaging Approach

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Abstract

As receptor-targeted therapies become increasingly used in clinical oncology, the ability to quantify protein expression and pharmacokinetics in vivo is imperative to ensure successful individualized treatment plans. Current standards for receptor analysis are performed on extracted tissues. These measurements are static and often physiologically irrelevant; therefore, only a partial picture of available receptors for drug targeting in vivo is provided. Until recently, in vivo measurements were limited by the inability to separate delivery, binding, and retention effects, but this can be circumvented by a dual-tracer approach for referencing the detected signal. We hypothesized that in vivo receptor concentration imaging (RCI) would be superior to ex vivo immunohistochemistry (IHC). Using multiple xenograft tumor models with varying EGFR expression, we determined the EGFR concentration in each model using a novel targeted agent (anti-EGFR affibody-IRDye800CW conjugate) along with a simultaneously delivered reference agent (control affibody-IRDye680RD conjugate). The RCI-calculated in vivo receptor concentration was strongly correlated with ex vivo pathologist-scored IHC and computer-quantified ex vivo immunofluorescence. In contrast, no correlation was observed with ex vivo Western blot analysis or in vitro flow-cytometry assays. Overall, our results argue that in vivo RCI provides a robust measure of receptor expression equivalent to ex vivo immunostaining, with implications for use in noninvasive monitoring of therapy or therapeutic guidance during surgery. Cancer Res; 74(24); 7465–74. ©2014 AACR.

Introduction

The ability to quantitatively measure the in vivo expression of extracellular proteins as well as the respective pharmacokinetics and receptor occupancy of any ligand-of-interest (i.e., therapeutics or imaging agents) in clinical oncology has long been recognized as an important key for personalized medicine, including tumor detection, treatment stratification, and therapeutic monitoring (1–5). The current practices for analyzing protein expression clinically and preclinically are performed on ex vivo tissues or in vitro cell culture; however, these tissue samples do not accurately represent the complexity of the in vivo environment and can grossly misinterpret the available receptor concentration for binding. Attempts at determining in vivo receptor concentration using a single targeted tracer have been studied, but are limited by the inability to distinguish actual binding events from vascular delivery and tumor retention. This article examines the hypothesis that in vivo receptor concentration imaging (RCI) shows the accessible concentration of extracellular receptors when complemented with an appropriate reference tracer to negate the dominant effects of drug transport in vivo (6); and this value directly correlates with standard ex vivo tissue processing methods. This hypothesis may seem obvious, yet the concept of applying immunohistochemistry (IHC) in vivo is largely undeveloped, and not validated in a panel of tumor lines. Moreover, the ability to dynamically quantify expression in vivo could be enormously important for evaluating the individual targeting of biologic therapies and changes in multiple receptors during the course of a therapy.

Clinically, the most widely used method of determining the molecular expression in a tumor is biopsy followed by IHC staining. Although IHC has been shown innumerable times to be correlative to the protein expression in a tumor, it suffers from a limited sampling of a potentially highly heterogeneous tissue, and is time consuming, and invasive to the patient. In addition, receptor staining in IHC is performed with all protein exposed on the cut surfaces, which is different than the in vivo situation in which drug access to extracellular proteins is via...
interstitial transport mechanisms. In vivo, the available receptors for binding can potentially be fewer than those available on an ex vivo slide. In vivo imaging with PET and single-photon emission computed tomography (SPECT) are two techniques that can be used to image the metabolic and molecular status of cancerous tissues; however, they cannot be interpreted as a direct receptor concentration image without accounting for the tumor and metastases detection but they are generally not saturating signals (21). RCI in PET and SPECT for applications in neurobiology has been successfully applied both clinically and preclinically for decades (3, 15, 17). However, these techniques do not simply transfer to oncology, even for brain tumors, because they rely on the simultaneous imaging of a single tracer in two structurally identical tissues where one tumor and metastases detection but they are generally not quantitative and like many other techniques can suffer from signal saturation (10, 11). Flow cytometry is an extremely sensitive quantitative technique (using calibration methods that use molecules of equivalent soluble fluorochrome, MESF, beads; ref. 12) but is most often applied to in vitro cells or intracellular protein expression measurements in ex vivo tissues, as extracellular proteins can be compromised during cellular retrieval from in vivo samples. In addition, techniques studying gene expression using techniques such as FISH can determine whether a mutated gene is present and in some cases (e.g., Her-2/neu mutations in breast cancer) provide a measure of gene amplification that correlates with patient prognosis (13). However, protein expression is not always correlated with gene expression, especially in moderately expressing tumors, because multiple copies of a single protein can be made from a single mRNA strand or the mutation of interest is not causing the overexpression (13, 14). It is important to note that all of these techniques have a varying degree of tissue integrity preservation and in vivo relevance, which can be essential for predicting the accessible and therapeutically relevant receptor population.

Molecular imaging and quantification of protein expression has had a large impact on preclinical drug design and development (4, 5, 15), validation and study of xenograft cancer models (16–18), and advancement of surgical resection techniques (19, 20). In vivo–targeted fluorescent imaging in both clinical and preclinical oncology settings has been limited in part by the extended times (24–48 hours) required to gain adequate contrast between tumor and normal tissues. Activatable fluorescent molecules are finding great success in tumor and metastases detection but they are generally not quantitative and like many other techniques can suffer from signal saturation (21). RCI in PET and SPECT for applications in neurobiology has been successfully applied both clinically and preclinically for decades (3, 15, 17). However, these techniques do not simply transfer to oncology, even for brain tumors, because they rely on the simultaneous imaging of a single tracer in two structurally identical tissues where one tissue (the "reference tissue") is devoid of any receptor (22). The hallmark of cancers is that they can be both physically and

![Figure 1](image_url)

**Figure 1.** The standard methods of quantifying protein expression in tissue and cell samples are compared in terms of type of sample preparation, detection technique, and sensitivity range. In each row, the relevance to in vivo tissues and tissue integrity decreases from left to right. IF, immunofluorescence; AR, amplification ratio. The sensitivity ranges for IHC and immunofluorescence (IF) are reported as pathologist score (top) and computer analyzed (bottom).
metabolically heterogeneous as compared with primary tissue, and so this two-tissue model fails to have value (23, 24). Multiple tracer imaging can be feasibly done with nuclear medicine methods, yet has been rarely attempted owing to the complexity of the signal and the complications of using multiple radiotracers in vivo.

The RCI method for fluorescence imaging of cancerous tissue overcomes the time delays and poor contrast of imaging a single fluorescent tracer and builds upon the RCI developed in the PET and SPECT neurobiology field (6). The RCI method enables in vivo quantification of receptor concentration within 1 hour of fluorescent tracer administration by “normalizing” the uptake of the targeted tracer by the uptake of a second, untargeted tracer—that has similar physical and pharmacokinetic characteristics to the targeted tracer—to account and correct for nonspecific uptake and delivery kinetics of the targeted tracer.

This study used clinically relevant targeted and untargeted affibody molecules (6 kDa antibody mimetics) labeled with near infrared fluorophores to determine the in vivo concentrations of five tumor models known to express varying levels of EGFR. The in vivo receptor concentrations determined by RCI were then compared with standard clinical and preclinical methods of protein concentration determination: pathologist scored IHC, computer-aided immunofluorescence, Western blot analysis, and flow cytometry. This study used EGFR as the model receptor system, but this technique is extendable to any extracellular protein receptor that has known and validated ligand or small-molecule–targeting agents.

Materials and Methods

Cell lines and culture methods

Four cell lines of varying origin and known EGFR expression were cultured for receptor quantification with in vitro flow cytometry and in vivo implantation: A431, a human epidermoid carcinoma (ATCC) expresses a very high level of EGFR; AsPC-1, a human pancreatic adenocarcinoma (from author T. Hasan, Massachusetts General Hospital, Boston, MA); and U251, a human neuronal glioblastoma (from Dr. Israel at Dartmouth College, Lebanon, NH) have moderate levels of EGFR expression; and 9L (from Dr. Wheeler at Wake Forest University, Winston-Salem, NC), a rat gliosarcoma known to be EGFR negative. Cell lines were cultured in either DMEM (A431, U251, and 9L) or RPMI (AsPC-1) with 10% fetal RPMI 10% (v/v) FBS and 100 IU/mL penicillin–streptomycin.

 Xenograft murine models

All animal procedures were conducted according to a protocol approved by the Dartmouth Institutional Animal Care and Use Committee (IACUC). A total of 30 female athymic nude mice were used with 6 mice per tumor group. The five tumor groups included subcutaneously implanted A431, AsPC-1, U251, and 9L, in addition to orthotopically implanted AsPC-1. For the subcutaneous tumor models, 1 × 10⁶ cells were implanted in the right flank of the mouse using 50 μL of a 1:1 mixture of Matrigel (BD Biosciences) and complete cell culture media. The orthotopic implantation of the AsPC-1 cells has been described elsewhere (25). Briefly, 1 × 10⁶ cells in 50 μL the 1:1 Matrigel to cell culture medium mixture is injected into the tail of the pancreas via a small incision in the mouse abdomen. All tumors were used for imaging and analysis when they reached 100 to 150 mm³ in volume.

Fluorescent labeling of anti-EGFR affibody proteins

The anti-EGFR affibody imaging agent (Affibody) was labeled with IRDye800CW maleimide (LI-COR Biosciences) and used as the targeted imaging agent. The negative control affibody imaging agent was labeled with IRDye680RS maleimide (LI-COR Biosciences) and used as the nontargeted reference imaging agent. The affibody proteins were prepared for labeling as described by the manufacturer’s instructions and using a Pierce Polyacrylamide Desalting Column (6K MWCO; Thermo Fisher Scientific) to purify fluorescent labeling. The prepared affibody proteins were subsequently labeled with the maleimide formulation of fluorescent dyes according to LI-COR Biosciences instructions with the modification that the free dye was separated from protein using the above described desalting column, and the fluorescently labeled affibody proteins were concentrated with using a spin column (Vivaspin 2, MWCO 3K; GE Healthcare Life Sciences) according to the manufacturer’s instructions.

Determination of EGFR expression levels

In vivo receptor concentration imaging. All animal procedures were conducted according to a protocol approved by the Dartmouth IACUC. The mice were prepared for imaging with an i.p. injection ketamine: xylazine (100:10 mg/kg) and when a surgical plane of anesthesia was obtained (confirmed by toe-pinch), superficial tissue was removed to expose the tumor and thigh muscle. Each mouse was placed with the tumor and muscle facing down onto a glass side, to which the mice were loosely secured with surgical tape to reduce movement during injection. The mice were positioned on an Odyssey Scanner (LI-COR Biosciences) and preinjection images were collected in both the 700 (excitation 685 nm and emission 700 nm LP) and 800 nm (excitation 785 nm and emission 800 nm LP) channels. The mixture of IRDye 800CW-AntiEGFR Affibody (0.2 nmol) and IRDye 680RD–control Affibody (0.2 nmol) was administered via tail vein injection and scanning was resumed with images taken every minute for the first 15 minutes and then every 2 to 5 minutes for a total of 40 minutes.

Estimates of EGFR-binding potential (BP) were determined on a pixel-by-pixel basis by applying a multiple-time-point dual-tracer kinetic model based on a simplified reference tissue model (22) to the temporal uptake curves of the targeted and untargeted tracers. The model, which has been described in depth elsewhere (6, 26), was fitted to the data using the optimization algorithm, lsqcurvefit() in MATLAB. Differences in the plasma pharmacokinetics were corrected using the deconvolution correction described previously (26). A region-of-interest was manually drawn around each tumor in the resulting BP parametric maps to calculate the mean tumor BP for each xenograft tumor, where BP is equal to the concentration of receptors available for binding multiplied by the binding affinity (kD) of the receptor–ligand pair (27). The mean receptor concentration of each xenograft tumor was

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determined by multiplying the BP by the dissociation constant \(k_{d} = 1/k_{a}\) determined by flow cytometry for each cell line (Supplementary Data).

**Immunohistochemistry (ex vivo).** All tissue was fixed in 10% buffered formalin (Biochemical Science Inc.). Tissue sections were cut at (4 µm), mounted on Leica Bond Plus Slides Cat # 00270, and air-dried at room temperature. Using the automated protocol of the Leica Bond Rx Automated Stainer (Leica Products/Equipment, Leica Microsystems, Inc.), the slides were baked for 30 minutes, and dewaxed with Leica Bond Dewax solution (Cat # AR9222). The antigen retrieval was Bond Epitope Retrieval 2 (Cat # ar9640), carried out in a pH 9.0 solution for 20 minutes. The EGFR primary antibody dilution was 1:300 for 15 minutes (Abcam Cat # ab52894; Abcam Inc.).

Primary antibody binding was visualized using the Leica Bond Refine Detection Kit (Cat # DS9800) with a diamino benzidine chromogen and a hematoxylin counterstain. The tissue sections were scored by a pathologist (author W. Wells, Department of Pathology, Geisel School of Medicine at Dartmouth College, Lebanon, NH) according to the following criteria: 0, no staining; 1, weaker membranous staining completely around cells; 2, weaker membranous staining completely around cells; and 3, strong membranous staining completely around cells.

**Immunofluorescence (ex vivo).** Half of the extracted tumor was covered in optimum cutting temperature compound (TissueTek) and flash-frozen in a mixture of methylbutane and dry ice. The tissues were prepared in 10-µm frozen sections, with three sections per slide, each 100 µm apart. Slides were fixed in precooled (−20 ºC) 1:1 acetonemethanol for 15 minutes at −20 ºC, air dried for 30 minutes, washed two times (5 min/wash) in PBS, and then blocked with prepared blocking solution (5% FBS, 1% BSA, 0.3% Triton X-100 in PBS) for 30 minutes at room temperature. Slides were then incubated overnight at 4 ºC with anti-EGFR (1:50; Cell Signaling Technology) in the blocking solution. Slides were washed three times for 5 minutes in PBS and incubated for 1 hour with Goat Anti-Rabbit Alexa Fluor 555 secondary (1:400; Invitrogen) in blocking solution and before a further three washes PBS. Slides were prepared for imaging by adding a coverslip with mounting medium (SlowFad Gold with DAPI, Invitrogen). Ten fluorescent images were obtained per tumor line using identical imaging agent control display similar uptake trends at short time periods after injection (Fig. 2, columns 1 and 2, respectively) and fluorescence intensity in the tumor region does not correlate with the previously determined concentration for each tumor group and control muscle tissue (Fig. 3) and correlates with the previously determined concentrations using the native human EGF ligand (Supplementary Data). The kinetic curves of the targeted and untargeted tracers (Fig. 2, column 4) display greater separation in tissues that have high EGFR concentrations and almost identical curves in the 9L model, which is an EGFR-negative tumor (6, 28). The calculated in vivo receptor concentration (Fig. 3) in A431, AsPC-1(Or), AsPC-1(SQ), and U251 tumor models
was statistically significant from the control muscle tissue ($P < 0.05$), known to lack endogenous EGFR, as well as the EGFR-negative 9L tumor ($P < 0.05$). In addition, the orthotopic and subcutaneous AsPC-1 tumor models were not significantly different from each other.

The anti-EGFR antibody in vivo receptor concentration was compared with standard techniques of receptor concentration determination, including ex vivo IHC, immunofluorescence, and Western blots in addition to in vitro flow cytometry (Fig. 4). The in vivo receptor concentration had a strong linear correlation to the EGFR concentration determined by ex vivo IHC ($r = 0.69, P = 2 \times 10^{-6}, m = 0.29 \pm 0.06, b = 0.8 \pm 0.2$) and immunofluorescence ($r = 0.62, P = 5 \times 10^{-4}, m = 1.0 \pm 0.2, b = 2.1 \pm 0.9$). There was no significant correlation between in vivo receptor concentration and ex vivo Western blot analysis ($r = 0.35, P = 0.08, m = 0.04 \pm 0.03, b = 0.06 \pm 0.08$), or in vivo receptor concentration and in vitro flow-cytometry ($r = 0.43, P = 0.017, m = 9 \times 10^8 \pm 3 \times 10^7, \text{intercept} = 4 \times 10^8 \pm 1.3 \times 10^9$) methods of receptor concentration determination. The IHC and immunofluorescence EGFR staining trends for each tumor model are shown in Fig. 5.

### Discussion

The successful delivery of molecular-targeted agents to tumors depends on many biologic and physiologic factors, including hemodynamics, vascular permeability, density, and diffusivity of the interstitial tissue, and nonspecific binding. These parameters can not only vary greatly from one region of a tumor to another but individual patients with tumors of the same origin can have vastly different biologic and physiologic parameters. In vivo single-tracer studies of receptor concentration have shown some success in correlating EGFR, or EGFR family receptors, concentrations (29–32); however, these studies look at target accumulation after a minimum of 24 hours where tumor to normal tissue contrast is high but the

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**Figure 2.** The concentration of in vivo EGFR is determined by RCI in five different tumor models (rows). The tumor lines are presented in the order of predicted EGFR concentration (fading purple triangle on far left). The first column, the fluorescence uptake of the EGFR-targeted tracer in the tumor (boundary designated by white dashed line) and surrounding tissues at 40 minutes after injection. The second column, the corresponding untargeted tracer uptake in the same tissue at the same time point. The third column, maps of the EGFR concentration (nmol/L), determined using the RCI method. The fourth column, the time trajectories of the average fluorescence within the tumor region of the targeted (red) and untargeted (green) tracers. The curves are normalized to the first time point.
The in vivo receptor concentration of each of the five tumor models [A431, AsPC-1(Or), AsPC-1(SQ), U251, and 9L] was determined and mapped using the RCI method and an anti-EGFR and control affibody imaging pair (Fig. 1). The in vivo receptor concentration for each tumor model was compared with standard methods of measuring receptor concentration in clinical and preclinical oncology using excised tissues or cells grown in culture (IHC, immunofluorescence, Western blot analysis, and flow cytometry; Fig. 4). The RCI-calculated receptor concentration had a high linear correlation and statistical significance with IHC scored by a trained pathologist and digitally analyzed immunofluorescence. These results are a key indication that in vivo RCI can determine receptor concentration with very high accuracy and is equivalent to ex vivo methods of IHC. In addition, the RCI method has the distinct advantage of imaging the tumor in its entirety, unlike IHC and immunofluorescence that require small biopsy samples or thinly sliced tissue sections, and also has the potential to be imaged repeatedly without the necessity of excising tissue multiple times.

Although there was a moderate linear correlation determined using Pearson Product Correlation Coefficient (defined as \(0.3 < r < 0.5\)) between the receptor concentrations determined by ex vivo Western blot analysis and in vitro flow cytometry with in vivo RCI, the correlation was not statistically significant. The largest discrepancy in both techniques can be attributed to the large overestimation of EGFR receptors in A431, a tumor line that expresses an order of magnitude more EGFR than the others tested. There are several possible explanations for this: (i) there was no interfering biologic physiology to limit delivery of molecular targets, such as tumor interstitium, restricted vascular flow or permeability, or varying cellular density; or (ii) both ex vivo Western blot analysis and in vitro flow cytometry use receptor-saturating concentrations for EGFR labeling, thus providing full and uninhibited access to all receptors (intracellular and extracellular). The inconsistencies between ex vivo Western blot analysis and the in vivo uptake of EGFR-targeted agents in A431 tumors have been observed before (6, 31). Both Tichauer and colleagues (6) and Aerts and colleagues (31) found that A431 tumors had significantly less delivery of an EGFR-targeted agent in vivo than was expected from ex vivo or in vitro quantification of receptor concentration. In fact, delivery of a single-targeted agent in A431 tumors was far lower in than tumor lines that were known to produce as much as an order of magnitude less EGFR.

Similarly, McLarty and colleagues (32) have demonstrated previously in breast cancer cell lines that highly overexpress HER2, that retention of a single agent is less than in cell lines that moderately overexpress HER2. Aerts and colleagues (31) attributes this discrepancy to physiologic parameters, such as vascular density and permeability, as well as cellular viability, which is likely true when considering the retention of an any single agent in a tumor (targeted or nontargeted). However, the RCI model accounts for these factors, suggesting that indeed there is a nonproportional discrepancy between in vivo receptor concentration (RCI, IHC, and immunofluorescence) and total protein content (Western
blot analysis and flow cytometry) with respect to the concentration of receptors physiologically available for targeted binding.

The in vivo receptor concentration determined for the five tumor models demonstrated expected trends when compared with the control tissues: muscle and 9L tumor model. The receptor concentration of the 9L tumor model was not significantly different from the muscle (Fig. 3), which was expected as the tumor line has been shown to have no apparent EGFR expression (6, 28) and was confirmed here (Figs. 3 and 5). The in vivo receptor concentrations of the EGFR-positive tumor models were all significantly different than the control muscle tissue and the 9L tumor line ($P < 0.05$, Fig. 3). It was hypothesized that EGFR expression would be different between the subcutaneous and orthotopic tumor models owing to the variations in physical environments, blood supply and distribution, and cellular content (34).

Interestingly, the receptor concentration of A431 was not significantly different from AsPC-1(Or), which has been determined previously using EGF as the targeted tracer (6). Although the available concentration determined by the RCI method of EGFR in the AsPC-1(SQ) model was lower than that of the AsPC-1(Or) model, the difference was not significant: Indeed the two tumor models both received a pathologist score of 2+ (Figs. 3 and 5). The staining difference is potentially attributable to a combination of tumor and normal pancreas tissue in the orthotopic tissues, and thus our calculated receptor concentration could also include benign EGFR-negative acinar cells. The significant contrast observed between the AsPC-1 tumor and the normal pancreas, illustrates the potential significance of this technique for surgical tissue resection or tumor detection in vivo.
Here, the experimentally determined \textit{in vivo} BP was converted to receptor concentration using the dissociation constants found by \textit{in vitro} flow-cytometry–binding studies. This requires the assumption that the \textit{in vivo} and \textit{in vitro} dissociation constants are the same. In neuroreceptor ligand studies, it has been demonstrated that \textit{in vivo} rate of dissociation is much slower than that \textit{in vitro}, but the dissociation constant itself stays relatively constant implying that ligand association is also decreased (27, 35, 36). However, this may be attributed to binding events occurring in the confined space of the synaptic barrier (37). Further studies on the apparent \textit{in vivo} dissociation constant need to be performed to confirm ligand–receptor affinity but is beyond the scope of the work presented here.

The results presented here highlight an important concept for therapeutic and imaging agent delivery to tumors: \textit{in vitro} or \textit{ex vivo} total protein measures are inaccurate methods of estimating the available receptor population for binding \textit{in vivo}. Although both IHC and immunofluorescence predicted the number of receptors \textit{in vivo}, they: (i) require invasive measures to acquire tissue; (ii) are not realistic techniques to monitor receptor expression changes over time; and (iii) do not account for dynamic biologic and physiologic parameters that influence vascular delivery. Here, the correlation of RCI determined \textit{in vivo} receptor concentration to \textit{ex vivo} IHC techniques was established for the first time with clinically relevant affibody molecules using minimally invasive techniques. Development of clinical noninvasive imaging techniques requires thorough analysis of native EGFR levels in the skin and other high EGFR-expressing tissues. In Supplementary Fig. S2, it is shown that mouse skin has far less EGFR than the A431 and AsPC-1 tumors but could significantly contribute to the signal in U251 or 9L and as such appropriate receptor concentration thresholds need to be determined. However, noninvasive RCI has recently been demonstrated in mice for lymph node imaging of breast tumor metastases using fluorescence alone (38) and glioma tumors using MRI- and CT-guided fluorescence tomography (39, 40). In addition, RCI has potential for imaging other primary cancers such as breast tumors (41–43) in which multiple measurements could be taken over a course antibody therapy to monitor treatment progress. Surface tumors such as skin cancers (melanomas, squamous, and basal cell carcinomas) and some head and neck cancers, as well as hollow organ tumors, such as esophageal and bladder cancers, would also be prime targets as optical or fluorescence imaging is often used preclinically in these instances. For instance, RCI of melanomas has recently been demonstrated using an intravascular compartment model similar to the one presented here and a vascular targeting agent (44). Currently, the use of RCI for fluorescence-guided surgical resection is being tested in our laboratories.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: K.S. Samkoe, K.M. Tichauer, J.R. Gunn, T. Hasan, B.W. Pogue


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.S. Samkoe, J.R. Gunn, W.A. Wells

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.S. Samkoe, K.M. Tichauer, J.R. Gunn, B.W. Pogue

Writing, review, and/or revision of the manuscript: K.S. Samkoe, K.M. Tichauer, W.A. Wells, T. Hasan, B.W. Pogue

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.W. Pogue

Study supervision: T. Hasan

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