

# Intraoperative Molecular Imaging With Pafolacianine: Histologic Characteristics of Identified Nodules

Inderpal S. Sarkaria,<sup>1</sup> Timothy G. Biro,<sup>2</sup> Sunil Singhal,<sup>3</sup> Rishindra M. Reddy,<sup>4</sup> Linda W. Martin,<sup>5</sup> David C. Rice,<sup>6</sup> Alex S. Lopez,<sup>7</sup> Gary Stevens,<sup>8</sup> Tina Barret,<sup>9</sup> Sudish C. Murthy<sup>10</sup>

## Abstract

**A retrospective review of 273 resected malignant and nonmalignant lesions from 191 patients were analyzed to characterize the effect of pafolacianine identified lesions during its use in recent clinical trials. A surprisingly broad range of lesions were identified by pafolacianine that correlated with both FR $\alpha$  and FR $\beta$  expression on targeted lesions.**

**Background:** With increased early detection efforts, surgery for early-stage lung cancer is expected to rise. Pafolacianine is the first FDA approved targeted optical imaging agent indicated as an adjunct for intraoperative identification of malignant and nonmalignant pulmonary lesions in adult patients with known or suspected cancer in the lung. **Methods:** This is a retrospective review of the malignant and nonmalignant lesions identified by pafolacianine with intraoperative molecular imaging (IMI) in the multi-center Phase 2 and Phase 3 ELUCIDATE clinical trials. All lesions meeting the intent to treat criteria from the combined studies were included. Histopathology for malignant and nonmalignant lesions and immunohistochemistry (ICH) for folate receptor alpha (FR $\alpha$ ) and folate receptor beta (FR $\beta$ ), which pafolacianine binds to, were assessed. **Results:** A total of 273 lesions resected from 191 patients were analyzed. The identification of primary and occult malignant lesions with pafolacianine in combination with standard practice was improved ( $P < .001$ ) when compared to standard practice alone. A range of histologies were demonstrated including adenocarcinoma (primary and metastatic), squamous cell carcinoma, adenoid cystic carcinoma, chordoma, lymphoma, and papillary thyroid cancer. Ninety-two percent (205 of 223) of lesions tested for folate expression were positive for FR $\alpha$  or FR $\beta$  expression. **Conclusions:** While initially intended to identify adenocarcinoma, IMI with pafolacianine targets a broad histological cross-section of malignant and nonmalignant primary and metastatic lesions in the lung. As real-world use expands, additional insight will continue to inform utility of pafolacianine in clinical practice and may broaden clinical applicability.

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**Keywords:** Lung cancer, Surgery, Folate receptor, Targeted, Occult disease

**Abbreviations:** FR $\alpha$ , Folate receptor alpha; FR $\beta$ , Folate receptor beta; GCP, Good clinical practices; ICH, Immunohistochemistry; IMI, Intraoperative molecular imaging; IRBs, Institutional Review Boards; ITT, Intent to treat; IVD, In vitro diagnostic; NSCLC, Non-small cell lung cancer; RFC, Reduced folate carrier; SCLC, Small cell lung cancer; TAMs, Tumor-associated macrophages; TME, Tumor microenvironment.

<sup>1</sup>UT Southwestern Medical Center, Dallas, TX

<sup>2</sup>On Target Laboratories, West Lafayette, IN

<sup>3</sup>Penn Medicine Perelman Center for Advanced Medicine, Philadelphia, PA

<sup>4</sup>Department of Surgery, Michigan Medicine, Section of Thoracic Surgery, Ann Arbor, MI

<sup>5</sup>University of Virginia Medical School, Charlottesville, VA

<sup>6</sup>The University of Texas MD Anderson Cancer Center, Houston, TX

<sup>7</sup>Moffitt Cancer Center, Tampa, FL

<sup>8</sup>DynaStar Consulting, Inc., Bastrop, TX

<sup>9</sup>On Target Laboratories, Inc., West Lafayette, IN

<sup>10</sup>Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH

## Introduction

Lung cancer is the leading cause of cancer related deaths in the United States, with an estimated projection of 127,070 lung cancer related deaths for 2024. Among new cases of cancer, lung cancer is the third most frequent diagnosis, with an estimated projection of 234,580 newly diagnosed cases for 2024.<sup>1</sup> Surgery remains the best option for patients presenting with resectable disease; however,

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Address for correspondence: Sudish C. Murthy, MD, PhD, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Section Head of Thoracic Surgery, Cleveland Clinic Main Campus, Mail Code J4-1, 9500 Euclid Avenue, Cleveland, OH 44195

E-mail contact: [MURTHYS1@ccf.org](mailto:MURTHYS1@ccf.org)

# Intraoperative Molecular Imaging With Pafolacianine

the approximate 5-year survival rates for these patients remains low, 65% for localized disease and 35% for regionally advanced disease.<sup>2</sup>

Despite the widespread adoption of minimally invasive thoracoscopic and robotic surgery for lung cancer,<sup>3,4</sup> the ability to identify smaller targeted lesions, including ground glass nodules, is paradoxically more challenging. It is these smaller lesions that are being uncovered by the widespread use of CT scanning and introduction of lung cancer screening programs. In addition, thoracic surgeons have traditionally relied on visual inspection and manual palpation.<sup>5</sup>

Despite these efforts, the rates of local recurrence suggest that surgeons are unable to completely detect and remove primary tumor nodules using standard and white light visualization. Up to 24% will recur locally following lung cancer surgery, with up to 19% local recurrence in stage 1 patients.<sup>6</sup> In addition to localization of primary nodules, local recurrence can also be attributed to synchronous lesions (occult malignant lesions) that are not identified on preoperative imaging and not found during the initial surgery.<sup>7,8</sup> Positive surgical resection margins (the distance from the primary tumor to the closest resection margin) also contribute to the recurrence rate.<sup>9</sup> During video-assisted thoracoscopic surgery (VATS), smaller and deeper nodules are not always identified.<sup>10,11</sup> With the increasing trend towards robot-assisted thoracic surgery (RATS),<sup>3,4</sup> the ability of the surgeon to identify smaller malignant and nonmalignant lesions such as ground glass nodules is more challenging, as the ability to palpate the lesion is limited or lost.

With initiatives for earlier screening such as the American Lung Cancer Screening Initiative and the 2023 American Cancer Society guidance recommendations for annual lung cancer screening for individuals who meet the eligibility criteria,<sup>12</sup> there is expected to be an increase in the number of people screened, with the hope of finding cancer at an earlier stage. Localizing these smaller lesions, identifying occult malignant disease, and obtaining negative margins are important factors in achieving the goal of removing all cancer and minimizing removal of noncancerous tissue. Intraoperative Molecular Imaging (IMI) with imaging agents should help the surgeon in this goal, in real-time.

Pafolacianine is the first targeted drug approved by the FDA as an optical imaging agent indicated as an adjunct for intraoperative identification of malignant and nonmalignant pulmonary lesions in adult patients with known or suspected cancer in the lung. Pafolacianine identifies lesions by binding specifically to lesions overexpressing folate receptor alpha (FR $\alpha$ ) and folate receptor beta (FR $\beta$ ) receptors, and then endocytosed into these lesions. FR $\alpha$  and FR $\beta$  are overexpressed in both non-small cell lung cancer (NSCLC), including adenocarcinoma and squamous cell carcinoma, and in small cell lung cancer (SCLC). FR $\alpha$  and FR $\beta$  are also overexpressed in tumor-associated macrophages (TAMs), which account for the majority of leukocytes in the stroma of the tumor microenvironment (TME).<sup>13,14,15</sup> Labeling either FR $\alpha$  or FR $\beta$  could increase the spectrum of cancers identified with IMI and pafolacianine.<sup>16</sup>

Here we review the combined results from recently completed Phase 2 and Phase 3 clinical trials of pafolacianine. We sought to understand the spectrum of tumor histology (primary and metastatic) and FR expression on lesions identified by IMI with

pafolacianine. The goal is to anticipate the histopathologic correlates that will emerge as this technology is disseminated.

## Material and Methods

### Study Design

Table 1 below summarizes the trial design for both the Phase 2 and Phase 3 multicenter trials. Both trials evaluated the safety and efficacy of pafolacianine at a dose of 0.025 mg/kg, administered over 60 minutes, and completed at least 1 hour prior to imaging. The primary and secondary goals were reversed between the studies, as the ability to impact the surgery through the proportion of patients with one or more Clinically Significant Events (CSE) became the primary objective with concurrence from the FDA in a Special Protocol Assessment Agreement. CSEs are defined as (1) one or more primary lung nodules (cancerous or noncancerous, excluding normal lung parenchyma) not detected under normal light and/or palpation; or (2) one or more cancerous synchronous lesion not detected under normal light and/or palpation; or (3) the identification of a cancer-positive margin that fluoresces within (less than or equal to) 10 mm of the surgical resection staple line. The Phase 3 study was randomized in that all subjects were administered the imaging agent, and a subset (10%) were randomized to no imaging. The surgeon, patient and sponsor were blinded, with the surgeon becoming unblinded after the drug administration and the initial assessment with standard practice of visualization under white light and palpation. This was done to prevent relying on IMI imaging results rather than the surgeon identification prior to IMI.

Further details regarding the design and results for each trial can be found in the Phase 2 and Phase 3 publications.<sup>17,18</sup> Both trials were conducted in accordance with the Declaration of Helsinki and Good Clinical Practices (GCP) and were approved by the appropriate institutional review boards (IRBs). Ethics committee approval and IRB-approved informed consent were obtained for all patients enrolled in both trials.

### Histopathology of Resected Lesions

For the Phase 2 trial, all resected lesions were submitted to a central laboratory for identification of the histopathology of the lesion. If central laboratory results were not able to be provided as a result of inadequate tissue sample, the local pathology results were used for reporting of the histopathology results. Central and local pathologists were blinded to the imaging status for the lesion. For the Phase 3 trial, all histopathology results were obtained from the local pathology lab, and all pathologists were blinded to the imaging status.

### Immunohistochemistry (IHC) for Folate Receptor Alpha (FR $\alpha$ ) and Folate Receptor Beta (FR $\beta$ ) Expression of Resected Lesions

For both the Phase 2 and Phase 3 trials, all resected lesions (where an adequate tissue sample was available following other histological evaluation) were submitted to a central laboratory for an additional IHC assessment for measuring the expression of FR $\alpha$  and FR $\beta$ . If the lesion was FR $\alpha$  positive, then FR $\beta$  evaluation was not required to be assessed. If the lesion was FR $\alpha$  negative, then the lesion was tested for FR $\beta$  expression. All pathologists assessing folate receptor

**Table 1** Summary of Phase 2 and Phase 3 Trials

	<b>Surgical Criteria</b>	<b># Sites and # Subjects Full Analysis Set</b>	<b># Camera Imaging Systems</b>	<b>Study Drug Administration</b>	<b>Key Primary and Secondary Objectives:</b>
Phase 2	Confirmed diagnosis of adenocarcinoma of the lung, or primary diagnosis/high clinical suspicion of lung nodule(s) warranting surgery, based on CT and/or PET imaging, scheduled to undergo endoscopic or thoracic surgery	6 sites 92 subjects in the full analysis set	4	Same day as surgery, at least 1 hour prior to imaging	Primary Objective: Estimation of sensitivity and false positive rates of pafolacianine to identify lung cancer during intraoperative imaging. Secondary objective: the proportion of patients with at least 1 Clinically Significant Event (CSE) as a result of utilizing OTL38 and Near Infrared Imaging.
Phase 3	Primary diagnosis or a high clinical suspicion of cancer in the lung warranting surgery based on CT/PET or other imaging, scheduled to undergo surgical thoracoscopy for diagnostic wedge resection followed by anatomic lung resection	12 sites 111 subjects enrolled, 100 randomized to receive drug and imaging (Full Analysis Set) 11 randomized to drug only	1	Same day, or day before surgery, 1 to 24 hours prior to imaging	Primary Objective: To confirm the efficacy of pafolacianine used with Near Infrared (NIR) fluorescent imaging to detect at least 1 Clinically Significant Event in adult subjects scheduled to undergo surgical resection for known or suspected cancer in the lung. Secondary Objective: Sensitivity and False Positive Rate of pafolacianine for identification of cancer in the lung.

expression were also blinded to the fluorescent imaging status of the lesion. In the results and discussion sections, the percentage of lesions reported as folate receptor positive are for those lesions where folate receptor expression testing was completed.

### Immunohistochemistry Methods

Formalin-fixed, paraffin-embedded samples were immunohistochemically evaluated for the expression of FR $\alpha$  using the Folate Receptor antibody (Cat# API3005AA, Biocare Medical) for In Vitro Diagnostic (IVD) use, and for the expression of FR $\beta$  using the Folate Receptor antibody (Cat# NBP2-43654, Novus Biologicals).

### Scoring of Folate Receptor Expression

For comparison of folate receptor levels, expression scores were determined by the pathologist using the product of intensity of antibody expression / immunoreactivity staining and cellularity, where an intensity of 1 was considered weak, 2 moderate, and 3 strong and percent cells staining positive where a cellularity score assignment of 1 was  $\leq 33\%$ , 2 was 34% to 65%, and 3 was  $\geq 66\%$ . The values reported in the Phase 2 and Phase 3 studies are composite scores of the above, and characterized as 0, 1 (low), 2 (medium), and 3 (high). A reported value of 0 does not necessarily equate to the complete absence of folate receptor expression; samples with low antibody expression and/or low intensity could result in a low cellularity score  $< 1$ , but still be folate receptor positive.

### Imaging Agent: Pafolacianine

Pafolacianine is a molecule consisting of a folate receptor targeting agent combined with a fluorescent dye which absorbs light in the NIR region within a range of 760 nm to 785 nm with peak

absorption of 776 nm and emits fluorescence within a range of 790 nm to 815 nm with a peak emission of 796 nm. The molecular formula is  $C_{61}H_{63}N_9Na_4O_{17}S_4$  (Tetrasodium salt) with a molecular mass of 1414.42 for the Tetrasodium salt and 1326.4 for the Parent Ion: (LC-MS; free acid, sodium-free) 1326.4.

The mechanism of action for pafolacianine is to bind to FR $\alpha$  and FR $\beta$ , which are overexpressed on multiple cancers. Pafolacianine has a high affinity for both FR $\alpha$  (KD = 7.4 nM) and FR $\beta$  (KD = 7.4 nM) which compares well with the binding affinity of folic acid to the receptors (KD = 9.2 for FR $\alpha$  and KD = 8.2 for FR $\beta$ ). This high affinity for the folate receptor  $\alpha$  and  $\beta$  is critical to pafolacianine selectively binding to malignant tissue and sites of inflammation where expression is increased compared to normal tissues where there is very limited distribution and accessibility of these receptors.<sup>19</sup> (Salazar CTR). Pafolacianine does not bind to the reduced folate carrier (RFC) or SLC19A1, which is expressed in all healthy cells, and is recognized to be the major transport system for folates in mammalian cells and tissues.<sup>20</sup>

### Camera Imaging Systems

For the Phase 2 trial, a total of 4 different camera imaging systems were evaluated for safety and efficacy with pafolacianine. Table 2 provides a summary of the NIR excitation wavelength and the light source for each of the systems, as well as the mean estimations and variation for sensitivity and false positive rates for each system. For the Phase 3 pivotal trial, only 1 camera imaging system was allowed to be used for all patients for purposes of standardization.

### Statistical Analysis

The results for lesions meeting the Intent to Treat (ITT) criteria from both trials were combined for purposes of analysis. ITT

## Intraoperative Molecular Imaging With Pafolacianine

**Table 2** Phase 2 Camera Imaging Systems Specifications

Camera	System 1	System 2	System 3	System 4
NIR excitation	785 nm	755-765 nm	780 nm	700 - 750 nm
Light source	NIR laser diode	NIR laser diode	NIR laser diode	Xenon
Sensitivity	0.873 (0.76, 0.94)	0.913 (0.45, 0.99)	0.858 (0.45, 0.98)	NE
False positive rate	0.086 (0.04, 0.19)	0.205 (0.10, 0.37)	0.25 (NE)	NE

Abbreviation: NE = Not evaluable.

**Table 3** Histopathology of Lesions Resected in the Phase 2 and Phase 3 Studies

Histology Type	# Of Lesions	% IMI+ Pafolacianine	% FR $\alpha$ or $\beta$ positive
Adenocarcinoma	156	79%	96%
Adenosquamous	4	100%	100%
Carcinoid	2	50%	100%
Carcinoma	18	94%	82%
Chordoma	4	100%	100%
Granulosa cell tumor	2	100%	100%
Lymphoma	6	67%	75%
Melanoma	2	50%	100%
Neoplasm	3	33%	67%
Sarcoma	2	50%	100%
Squamous cell carcinoma	13	69%	100%
Adenomatous hyperplasia	1	100%	N/A
Hamartoma	1	100%	100%
Fibrosis	3	67%	67%
Granulomas	12	92%	100%
Inflammation	4	75%	100%
Lymph nodes	1	100%	N/A
Pneumonia	4	100%	100%
Unknown	2	100%	50%
No malignancy	33	100%	65%
<b>Total</b>	<b>273</b>		

criteria included localization of the primary target lesion (malignant or nonmalignant lesions) and identification of nontarget occult malignant disease (not identified preoperatively). Histopathology, folate receptor expression, and method of identification of the lesion were included in the analyses. Identification of the lesion included a) those lesions identified by IMI with pafolacianine only, and b) those lesions that were identified by both the surgeon and IMI with pafolacianine, and/or those lesions identified by the surgeon only, under standard practice of white light visualization and/or palpation. All statistical analyses for the combined data were via post hoc analysis. Lesion characteristics were assessed to identify if specific attributes were statistically significant variables in identifying lesions by IMI with pafolacianine only. The Fishers Exact test was used to analyze the 2-way tables, and the Cochran-Mantel-Haenszel tool was used to analyze multi-factor tables.

The impact of IMI with pafolacianine as an adjunct to the surgeon's standard lesion localization practices of visualization using white light and palpation was assessed by overall improvement in successful localization of malignant and nonmalignant primary

lesions and occult malignant lesions, when compared to standard palpation and visualization practices.

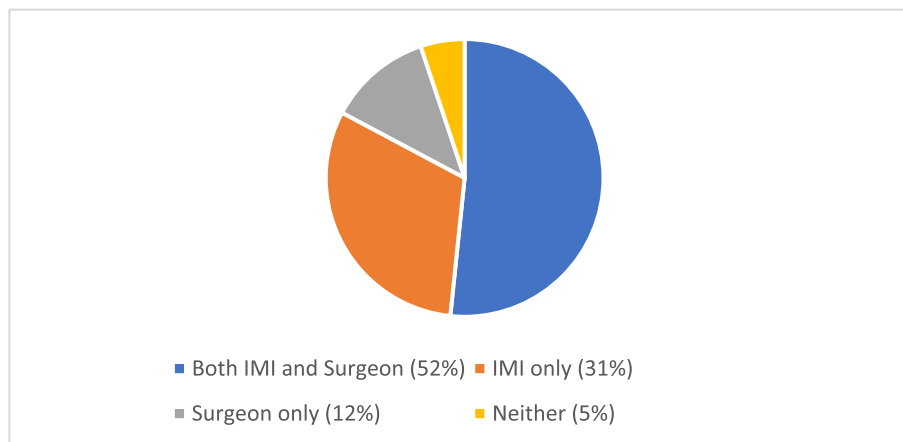
## Results

A total of 273 resected lesions in the Phase 2 and 3 trials were analyzed by histopathology. Figure 1 provides the proportion of lesions identified by localization method; 31% of lesions were identified by IMI alone.

Table 3 provides the lesion histopathologic results by type and number of lesions resected, the percentage of lesions identified by IMI with pafolacianine, and the folate receptor expression level for the lesions.

### Cancers Identified by IMI With Pafolacianine

Table 4 summarizes malignant lesions identified by IMI with pafolacianine resected in the studies, including those that were primary lung cancer, cancer that was metastatic to the lung, and occult malignant lesions. Also included are the percentage of those lesions by histological type that were folate receptor positive. One hundred thirty-five of the 142 malignant lesions identified by IMI

**Figure 1** Lesion identification method (total lesions: 273)**Table 4** Malignant Lesions Identified by IMI With Pafolacianine (Fluorescent Positive In Situ)

Histology Type	# Of Lesions	% FR Positive
Adenocarcinoma	124	95%
Adenosquamous	4	100%
Carcinoid	1	100%
Carcinoma	17	80%
Chordoma	4	100%
Granulosa cell tumor	2	100%
Lymphoma	4	100%
Melanoma	1	100%
Neoplasm	1	100%
Sarcoma	1	100%
Squamous cell carcinoma	9	100%
<b>Total</b>	<b>168</b>	<b>95% FR+</b>

with pafolacianine assessed for folate receptor expression (95%) were folate receptor positive.

#### Metastatic Cancers Identified by IMI With Pafolacianine

Thirty-nine metastatic lesions identified by IMI with pafolacianine were found in the Phase 2 and Phase 3 trials combined. Table 5 provides a summary of the cancers that were metastatic to the lung, with the organ of origin (when specified), the number of lesions, and the percentage of lesions that were folate receptor positive. Twenty-nine of the 30 metastatic cancer lesions (97%) that were assessed for folate receptor expressed FR $\alpha$  and/or FR $\beta$ .

#### Nonmalignant Primary Lesions Identified by IMI With Pafolacianine

A summary of the primary target nonmalignant lesions identified by IMI with pafolacianine is provided in Table 6. These lesions were identified in preoperative imaging as highly suspicious, leading the surgeon to perform the wedge resection for purposes of obtaining a

diagnostic wedge. Sixteen of the 17 nonmalignant lesions resected (94%) were FR positive in this Intent to Treat surgical diagnostic population.

#### Impact of FR $\beta$ Expression in Localization of Primary Lesions

The impact of pafolacianine targeting both FR $\alpha$  and FR $\beta$  is demonstrated in both malignant and nonmalignant lesions. All lesions in Table 7 and Table 8, were negative for expression of FR $\alpha$  but were positive for expression of FR $\beta$ . The lesions fluoresced with low (1) through high (2,3) levels of FR $\beta$  expression. Many of the primary malignant lesions that over-expressed FR $\beta$  were metastatic from other organs. Figure 2 is an example of cancer that expressed a high level of FR $\alpha$ ; Figure 3 is an example of cancer that expressed a high level of FR $\beta$  (Appendix 1).

#### Lesions Identified by the Surgeon Only

Table 9 provides a summary of lesions that were not identified by IMI with pafolacianine. Thirty-three of these lesions were identified by the surgeon only, under standard practices of localization techniques with white light/palpation. Fourteen lesions were not identified by the surgeon or by IMI with pafolacianine. The folate expression level for these lesions is also provided where available; 40 of the 43 lesions tested for folate receptor (93%) were folate receptor positive.

#### Statistical Analysis Results

*Pafolacianine as an Adjunct to the Surgeon.* We assessed the impact of pafolacianine as an adjunct for identifying malignant and nonmalignant primary lesions and occult malignant lesions. Table 10 lists by category the percentage of lesions identified by the surgeon using standard conditions of white light and palpation alone and the percentage of lesions identified by the surgeon with standard conditions and the addition of IMI with pafolacianine. The greatest increase by percentage is in the occult malignant lesions that were not identified by the preoperative imaging,

## Intraoperative Molecular Imaging With Pafolacianine

**Table 5** Metastatic Lesions Identified by IMI With Pafolacianine (Fluorescent Positive In Situ)

Metastatic lesion	# Of Lesions	% FR positive
Adenocarcinoma (metastatic origin not specified)	4	100%
Adenocarcinoma (colorectal)	8	100%
Adenocarcinoma (pancreatic)	2	100%
Adenocarcinoma (prostate)	1	100%
Adenoid cystic carcinoma	5	100%
Chordoma (spine)	4	100%
Lymphoma (not specified)	1	100%
Lymphoma (marginal zone)	2	100%
Lymphoma (diffuse Large B-cell)	1	100%
Papillary tumor (thyroid)	2	0%
Hepatocellular Carcinoma	2	100%
Granulosa cell tumor (ovarian)	1	100%
Granulosa cell tumor (not specified)	1	100%
Renal cell carcinoma	1	100%
Breast carcinoma	1	100%
Metastatic carcinoma (not specified)	1	100%
Melanoma (skin)	1	100%
Pleomorphic liposarcoma (not specified)	1	100%
<b>Total</b>	<b>39</b>	<b>97% FR+</b>

**Table 6** Nonmalignant Primary Lesions Identified by IMI With Pafolacianine

Histology type	Subtype	# of Lesions	% FR positive
Chondroid hamartoma	Not Applicable	1	100%
Chronic interstitial pneumonitis	Not Applicable	1	100%
Granulomas	Necrotizing	2	100%
	Non-necrotizing	3	100%
	Not specified	2	100%
Inflammation	Alveolated lung with reactive changes and inflammation negative for malignancy	1	100%
No malignancy	Not specified	2	100%
Pneumonia	Organizing granulomatous pneumonia Lipoid	2	100%
Inflammatory myofibroblastic tumor	Not Applicable	1	100%
Sclerosing pneumocytoma	Not Applicable	1	0%
Solitary fibrous tumor	Not specified	1	100%
<b>TOTAL</b>		<b>17</b>	<b>94% FR+</b>

or by the surgeon intraoperatively during the standard localization in absence of IMI with pafolacianine. The localization of primary malignant lesions with the addition of IMI with pafolacianine was statistically significant ( $P < .001$ ) when compared to identification of primary malignant lesions only by standard white light/palpation. Localization of primary nonmalignant lesions was not statistically significant. The identification of occult malignant lesions was also statistically significant ( $P < .001$ ). In further analysis of the occult malignant lesions, Table 11 captures the number of malignant occult lesions and the nonmalignant lesions and the method of identification for the lesions. Table 12 lists the contribution of IMI with pafolacianine alone in identifying lesions by a number of variables.

The sensitivity (True Positive)/(True Positive + False Negative) of IMI for identifying occult malignant lesions outside of what the surgeon identified by standard practices was 95% (19/(19+1)).

The positive predictive value (True Positive)/(True Positive + False Positive) for IMI outside of what the surgeon identified was 40.4%. Combining the performance of the surgeon's standard practice in combination with IMI, the sensitivity for identification of occult malignant lesions was 100%, and the positive predictive value was 46.4%. The major contributions of IMI with pafolacianine as an adjunct to the surgeon are in the identification of occult malignant disease, metastatic disease, and the localization of both malignant and nonmalignant primary lesions.

## Discussion

The key findings of the analyses:

A broad range of primary lesions are identified by IMI with pafolacianine including not only adenocarcinomas, but also

**Table 7** Fluorescent Positive, FRβ Positive Malignant Primary Lesions (FRα Negative)

Histology	Lesion Category	FRβ Score
Squamous cell carcinoma	Primary lung cancer	1
Squamous cell carcinoma	Primary lung cancer	2
Squamous cell carcinoma	Primary lung cancer	3
Squamous cell carcinoma	Primary lung cancer	3
Lymphoma	Metastatic B-cell	3
Lymphoma	Metastatic (not specified)	3
Adenocarcinoma	Metastatic prostate	1
Carcinoma	Not specified	3
Chordoma	Metastatic from spine	1
Melanoma	Metastatic	3
Neoplasm	Not specified	2
Sarcoma	Metastatic	3

**Table 8** Fluorescent Positive, FRβ Positive Nonmalignant Primary Lesions (FRα Negative)

Histology	Lesion Category	FRβ Score
Granuloma	Not specified	3
Granuloma	Not specified	3
Granuloma	Non-necrotizing	2
Granuloma	Necrotizing	2
Adenocarcinoma spectrum	Minimally invasive	2
Chondroid hamartoma	Not specified	3
Pneumonia	Lipoid	2
Solitary fibrous tumor	Not specified	2

other carcinomas (including squamous cell carcinoma of the lung), and additional malignancies such as lymphoma, chordoma, sarcoma, and adenosquamous.

Identification of metastatic lesions by IMI with pafolacianine included colorectal, adenoid cystic, papillary, hepatocellular, chordoma, and pancreatic.

Localization of targeted lesions from preoperative imaging that were suspicious for cancer but found to be nonmalignant included granulomas, pneumocytoma, myofibroblastic tumor, and hamartoma.

Using IMI with pafolacianine the surgeon identified an additional 19 occult malignant lesions that were not identified by standard practice.

Folate receptor expression (alpha and/or beta) in >95% of malignant lesions resected, including 12 fluorescent malignant lesions that were FRα negative, but were FRβ positive.

The false negative rate increases with deeper and smaller lesions but IMI with pafolacianine identifies additional lesions that would not have been found with standard practice. The greatest contributor to false negative lesions appears to be related to the imaging systems used at the sites.

*Cancers identified intraoperatively by IMI with pafolacianine:* The overexpression of FRα in lung adenocarcinomas is well established<sup>22</sup> and the Phase 2 and Phase 3 results confirmed pafolacianine targets these lesions because of high FR expression. In addition, IMI with pafolacianine identified a broad range of other cancer types as described in Table 3 and Table 4. As shown in Table 10, an additional 27 malignant lesions were localized only with IMI, improving upon the 138 lesions identified through standard practice ( $P < .001$ ). Figure 4 in Appendix 1 is an example of a primary lung cancer identified by IMI with pafolacianine only.

*Metastatic cancers identified intraoperatively by IMI with pafolacianine:* IMI with pafolacianine identified 39 metastatic lesions including 35 from cancers that originated in other organs. Of note, pafolacianine was very effective at identifying colorectal cancer metastases, likely due to the increased expression of FRα in colorectal cancers with distant metastases when compared to colorectal cancer without metastases at time of presentation ( $P = .043$ ).<sup>21</sup> Moreover, FRα expression might become a viable biomarker for diagnosis, progression, and prognosis for additional cancers including gastrointestinal, gynecological, breast, and lung cancers,<sup>22</sup> as all such lesions identified by pafolacianine (with the exception of papillary (thyroid) tumors) over-expressed folate receptor (Table 5).

**Table 9** Lesions Not Identified by IMI With Pafolacianine

Histopathology	# Of Lesions	% FR Positive
Adenocarcinoma	32	97%
Carcinoid	1	Not Available
Extranodal marginal zone lymphoma	1	0%
Fibrosis	1	100%
Granulomas	1	100%
Inflammation	1	100%
MALT Lymphoma	1	100%
Melanoma	1	100%
Metastatic leiomyosarcoma	1	100%
Metastatic metatypical basal cell carcinoma	1	100%
Spindle cell neoplasm	2	50%
Squamous cell carcinoma	4	100%
<b>Total</b>	<b>47</b>	<b>93% FR+</b>

## Intraoperative Molecular Imaging With Pafolacianine

**Table 10** Improvement in Localization of Primary and Occult Malignant Lesions Using IMI With Pafolacianine

Lesion Type	Total # of Lesions	Surgeon White Light and Palpation Only	Surgeon White Light, Palpation, and IMI With Pafolacianine
Primary malignant	178	78% (138)	93% (165) $P < .001$
Primary nonmalignant	19	69% (13)	95% (18) $P = .0625$
Occult malignant lesions	32	41% (13)	100% (32) $P < .001$

**Table 11** Sensitivity and Positive Predictive Value for Identification of Occult Malignant Lesions Using IMI With Pafolacianine

Method of Identification	Occult Malignant Lesions (N)	Occult Nonmalignant Lesions (N)
IMI pafolacianine only	19 (TP)	28 (FP)
Surgeon white light palpation only	1 (FN)	0
Identified by surgeon and IMI	12 (TP)	9 (FP)
Identified by neither	0	0
<b>Total</b>	<b>32</b>	<b>37</b>

**Table 12** Distribution of Lesion Types Identified by IMI With Pafolacianine Only

Lesion Type	% IMI Only	% IMI Only Malignant	% IMI Only Nonmalignant
All lesions	31.1%	22.2%	62.1%
Primary lesions	16.2%	15.2%	26.3%
Occult lesions	69.4%	59.4%	75.7%
Metastatic lesions	19.6%	19.6%	0%

*Localization of nonmalignant lesions by IMI with pafolacianine:* In addition to the identification and resection of metastatic malignant lesions, the localization and resection of primary malignant lesions, ground glass opacities (GGOs), and nonmalignant primary lesions was also demonstrated. Among the nonmalignant lesions identified by IMI with pafolacianine were multiple granulomas, pneumonias (organizing granulomatous, chronic interstitial pneumonitis, and lipoid), chondroid hamartoma, and inflammatory tissue. For the patients in the Phase 2 and Phase 3 trials, pafolacianine identified 18 of the 19 (95%) nonmalignant primary lesions that were ultimately resected, compared to 13/19 (69%) with standard methods ( $P = .0625$ ). While this  $P$ -value did not meet the level of statistical significance, IMI with pafolacianine aided the clinician in localizing 5 additional lesions that might have otherwise not been removed or might have required removal of additional lung parenchyma to localize and resect the primary lesion identified in the preoperative imaging.

*Identification of occult malignant lesions:* Using IMI with pafolacianine, the surgeon was able to identify an additional 19 occult malignant lesions that were not identified by the standard practice of white light and palpation. Figure 5 in Appendix 1 is an example of an occult malignant lesion identified by IMI with pafolacianine only. These lesions were not identified on preoperative imaging and likely would not have been found and resected. The surgeon was able to identify 13 occult malignant lesions, of which 12 fluoresced. In looking at the contribution of IMI to the identification of occult malignant lesions, the sensitivity of the surgeon improved from 41% (13/32) to 100%. The positive predictive value for identifying occult malignant lesions was reduced

as a result of the nonmalignant lesions that were identified by IMI alone. While the majority of these lesions (61%) were less than 6 mm in length, this resulted in removing nonmalignant tissue.

*Importance of FR $\alpha$  and FR $\beta$  overexpression on tumors and in the Tumor Microenvironment:* In the Phase 2 and Phase 3 combined trial results, 95% of all malignant lesions identified expressed either FR $\alpha$  or FR $\beta$ , and 94% of the nonmalignant primary lesions expressed folate receptor. The ability of pafolacianine to target both the alpha and beta receptor is demonstrated across multiple histologies. Twelve primary malignant lesions were FR $\alpha$  negative, but were FR $\beta$  positive and fluoresced in situ (Table 7). Consistent with previous studies which show FR $\alpha$  is highly expressed in adenocarcinoma but not in squamous cell carcinoma,<sup>23</sup> the expression of FR $\beta$  in squamous cell carcinoma adds to the benefit of pafolacianine in identifying malignant lesions. In addition to malignant primary lesions, 7 nonmalignant primary lesions (Table 8) were FR $\alpha$  negative but FR $\beta$  positive and fluoresced in situ. These results demonstrate the utility of pafolacianine targeting both FR $\alpha$  and FR $\beta$  overexpressed on the lesion or in the stroma of the TME. As demonstrated in the results, improvement in the sensitivity was observed in multiple cancer subtypes, metastatic and local disease, with folate receptor expression present in greater than 92% of the lesions.

There were false negatives in the Phase 2 and Phase 3 studies, defined as resected lesions that did not fluoresce intraoperatively but were confirmed to be malignant histologically. We evaluated several patient, tumor, and other variables to understand factors correlating with false negative lesions.

Age, sex, BMI, size, lesion depth and smoking history were not important (data not shown). Other studies have identified the depth of the lesion as a significant factor impacting in situ fluorescence of the lesion<sup>24,25</sup> however, our results did not identify depth as a significant factor differentiating identification. For lesions  $\leq 20$  mm in depth, the false negative rate for the surgeon and IMI with pafolacianine were similar. For lesions  $> 20$  mm in depth, the false negative rate for IMI was 37.5% in comparison to the false negative rate for the surgeon of 75%. In assessing the impact of the size of the lesion for false negatives, the false negative rate for IMI was highest for lesions 0-10 mm in size (24.2%) but compared favorably to the surgeon false negative rate of 39.4% for lesions  $\leq 10$  mm. The sensitivity for IMI with pafolacianine was also estimated based on the anatomical location of the lesion in the lung. The sensitivity was lowest for the right lower lobe (73.3%) while the sensitivity for the other lobes ranged from 80.3% to 92.3%. The lower sensitivity for lesions in the right lobe could be related to positioning of the scope to fluoresce the area of interest. We also assessed the sites for understanding the impact on false negative lesions. For the Phase 2 study, the false negative rates by site ranged from 5% to 40%. This translates to 40% of all false negative lesions in the study being at one site; this becomes more meaningful in that this site generated only 16.4% of all lesions assessed in the Phase 2 study. For the 5 remaining sites in the Phase 2 study, the percentage of false negative lesions was less than the percentage of all lesions across all sites. The likely explanation for the false negatives at this site is related to the imaging systems used. This site evaluated 4 camera imaging systems (Table 2), while other sites evaluated only 1 or 2 imaging systems. For the Phase 3 study, 2 sites had false negative rates that were disproportionate to the proportions of lesions across all sites. Site 1 generated 23.1% of all false negatives, but only 9% of all lesions in the Phase 3 study. Site 6 generated 31% of all false negative lesions, compared to 10.5% of all lesions. The high false negative rate at site 6 was likely related to the imaging system, which had performance issues that were not recognized until after completion of the Phase 3 study.

Based on the above assessments, it appears the imaging systems had the greatest impact on the generation of false negatives using IMI with pafolacianine. Therefore, the implementation of pafolacianine in clinical settings will require the use of camera imaging systems with FDA 510k clearance and extensive training on use of the device with the imaging agent. As imaging system development continues, the false negative rates should decrease.

The ability for IMI with pafolacianine to fluoresce a lesion can often be multifactorial and patient dependent. Because of the expression of folate receptors in the false negative lesions, other variables including imaging technology must be considered. As mentioned previously, the highest false negative rates were seen with smaller and deeper lesions, although IMI with pafolacianine was able to identify smaller and deeper lesions that were not found by standard practices. The imaging systems used in the clinical studies were also related to the generation of false negatives. As development in camera imaging systems continues to advance, there should be an improvement in identifying all malignant and benign lesions that express FR $\alpha$  or FR $\beta$ . Because FR $\beta$  is expressed in nonmalignant tissue, including inflammatory tissue, the surgeon's judgment will be

important in the resection of fluorescent tissue. As surgeons become more experienced with IMI imaging, results may also continue to improve.

There were several limitations associated with the data analysis. Because of the small size of a number of the resected lesions, not all lesions could be assessed for FR $\alpha$ , and not all FR $\alpha$  negative lesions were not tested for FR $\beta$  expression. The depth of the lesion was not measured in the Phase 2 study, and therefore only the Phase 3 lesion depths were measured. For the data analysis, we assessed only individual variables as independent. The results of the studies were generated in a controlled clinical trial environment, and therefore real-world data results could differ from results of the clinical studies. Recurrence and survival data were not part of the study goals, and therefore these outcomes data were not collected.

## Conclusion

Localization and resection of both primary malignant disease and cancer metastatic to the lung, to achieve R0 (no remaining visible disease), is the goal of oncologic lung surgery. Preoperative imaging will inform the surgeon of the presence of suspicious lesions but identification of primary and occult tumors with minimally invasive approaches can be challenging. Intraoperative molecular imaging has evolved as a potential tool for surgeons to successfully localize suspicious lesions identified through preoperative imaging, as well as for identification of additional previously undetected occult malignant lesions during the operative procedure.

Based on the results of the combined Phase 2 and Phase 3 studies, IMI with pafolacianine was capable of identifying a broad spectrum of cancer histologies. Both FR $\alpha$  and FR $\beta$  expression were important in identifying the lesions, and the ability of the surgeon to identify primary malignant and occult malignant lesions was statistically significant when compared to lesions they identified with standard conditions alone ( $P < .001$ ).

The applicability of the results from the clinical trials to the broader clinical arena will ultimately be determined by adoption in academic and community practices. These experiences are accruing, and real-world use and collection of metadata from additional institutions and surgeons will help to better define the role of IMI with pafolacianine in patients with known or suspected cancer in the lung.

## Clinical Practice Points

- What is already known about this subject?

It is estimated that 4-9% of patients with NSCLC will have additional occult tumors identified at time of surgery. With widespread use of minimally invasive approaches to early-stage lung cancer, localization of primary tumors and identification of occult tumors can be challenging.

- What are the new findings?

Intraoperative molecular imaging (IMI) with pafolacianine significantly improved the identification of a wide range of local primary and metastatic lung cancers, including occult malignant lesions not identified on preoperative imaging or routine white light inspection and palpation by the surgeon ( $P < .001$ ).

## Intraoperative Molecular Imaging With Pafolacianine

- How might it impact clinical practice in the foreseeable future?  
IMI with pafolacianine has been shown to identify additional occult tumors at the time of surgery, potentially improving accuracy of staging, complete resection rates, and cancer outcomes.

### Disclosure

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Doctors Murthy, Singhal, Reddy, Martin, Rice, and Sarkaria were investigators in the sponsored trials and report financial support, administrative support, equipment, drugs, or supplies, provided by On Target Laboratories during the course of the clinical trials. No compensation was paid directly to the investigators from On Target Laboratories. Timothy Biro and Tina Barrett report a relationship with On Target Laboratories that includes employment and equity or stock. Dr. Gary Stevens is with Dynastat which was paid by the sponsor On Target Laboratories for statistical analysis work related to the publication. Dr. Lopez is an employee of Moffitt Cancer Center which was paid by the sponsor On Target Laboratories for IHC and pathology related services. Dr. Murthy and Dr. Sarkaria report a relationship with On Target Laboratories. Dr. Reddy, Dr. Rice, Dr. Martin, and Dr. Singhal report no further relationship with On Target Laboratories.

This report includes data generated from a multi-center phase 2 trial and a randomized multi-center Phase 3 trial sponsored by On Target Laboratories.

### CRedit authorship contribution statement

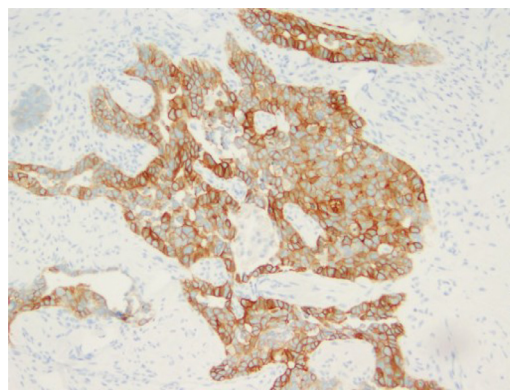
**Inderpal S. Sarkaria:** Writing – review & editing, Writing – original draft, Investigation. **Timothy G. Biro:** Writing – review & editing, Writing – original draft, Project administration, Data curation, Conceptualization. **Sunil Singhal:** Writing – review & editing, Investigation. **Rishindra M. Reddy:** Writing – review & editing, Investigation. **Linda W. Martin:** Writing – review & editing, Investigation. **David C. Rice:** Writing – review & editing, Investigation. **Alex S. Lopez:** Writing – review & editing, Methodology, Data curation. **Gary Stevens:** Writing – review & editing, Formal analysis. **Tina Barrett:** Writing – review & editing, Data curation, Conceptualization. **Sudish C. Murthy:** Writing – review & editing, Writing – original draft, Investigation.

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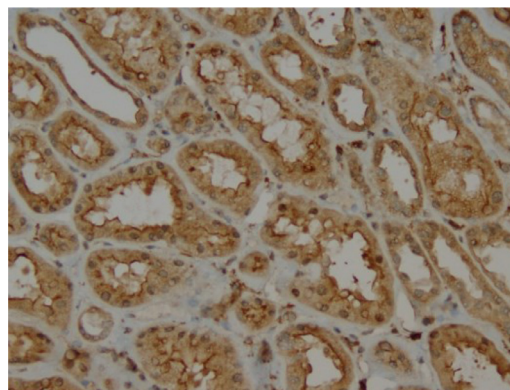
On Target Laboratories provided all the funding for the Phase 2 and Phase 3 clinical trials.

### Appendix 1

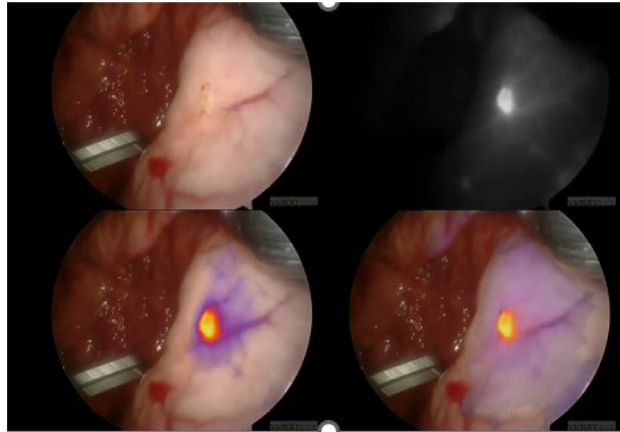
**Figure 2** Cancer overexpressing FR $\alpha$ .



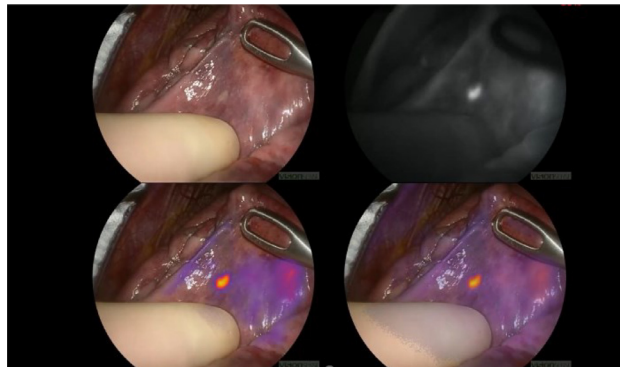
**Figure 3** Cancer overexpressing FR $\beta$ .



**Figure 4** Left Upper Lobe Lung Adenocarcinoma localized by IMI with pafolacianine; the lesion is 1.4 cm in size and was 2.2 cm deep. Upper left is white light, upper right is contrast, and lower images are fluorescent overlay.



**Figure 5** Left Upper Lobe Adenocarcinoma found by IMI with pafolacianine; the lesion is 1.5 mm in size. Upper left is white light, upper right is contrast, and lower images are overlay.



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## Intraoperative Molecular Imaging With Pafolacianine

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