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Can novel elastin stains improve  
pathologic assessment of microscopic  
vascular invasion in lung  
adenocarcinoma? A reproducibility  
study with comparative analysis of  
diverse histochemical stains for elastin

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**CAN NOVEL ELASTIN STAINS IMPROVE PATHOLOGIC ASSESSMENT OF  
MICROSCOPIC VASCULAR INVASION IN LUNG ADENOCARCINOMA? A  
REPRODUCIBILITY STUDY WITH COMPARATIVE ANALYSIS OF DIVERSE  
HISTOCHEMICAL STAINS FOR ELASTIN.**

by

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B.S., University of California-Riverside, Riverside, CA, 2013

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**ABSTRACT**

Visceral pleural invasion (VPI), tumor cell invasion into the elastic layer of the pleura, is a standard in staging lung adenocarcinomas. Many studies have found vascular invasion (VI), tumor cells within the lumen of veins or arteries, to be as significant a negative prognostic factor as VPI. In this study, we examined whether or not the use of the elastic trichrome (ET) stain to evaluate VPI and VI led to improved assessment. A cohort of attending pathologists (A) was assigned 50 cases of lung adenocarcinoma and asked to evaluate VPI and VI using 4 stains for each case (H&E, Verhoeff's Van Gieson, ET, and Movat Pentachrome) A second cohort of resident pathologists (B) was asked to do the same however the cases were split into 2 groups. The residents evaluated the first group of 25 cases and were then asked to watch an educational lecture regarding the stains and take an assessment. Afterwards, they evaluated the second group of 25 cases.

In cohort A, the ET did not improve accuracy or reproducibility in VI and VPI. This result led to the use of an educational video and assessment in cohort B. Overall accuracy improved in evaluation of VI and VPI in the second round (after

the lecture and assessment). Similarly, reproducibility improved from fair agreement to substantial agreement in VI and to moderate agreement for VPI.

In conclusion, the ET stain improved accuracy and reproducibility of evaluation of VI and VPI in lung adenocarcinomas with educational instruction. Since the results of VI are comparable to VPI, the current standard of care in staging lung adenocarcinomas, VI should also be implemented as a standard of care with the ET used as the standard for diagnosis.

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## **INTRODUCTION**

### **Structure and Anatomy of the Lung**

The lungs are the central organs of the respiratory system where they facilitate gas exchange between inhaled air and circulating red blood cells. The lungs are enveloped by visceral pleurae comprised of a thin mesothelial lining and underlying connective tissue containing a well-defined elastic layer which separates each lobe, creating a smooth surface which juxtaposes with the parietal pleura of the chest wall. The potential space between the two pleurae, the pleural cavity, contains serous fluid meant to create and maintain a pressure gradient and provide lubrication between the two layers. The serous fluid provides the only separation between the visceral and parietal pleura. The visceral pleura folds on itself (creating a double layer) and invaginates the lung tissue creating fissures which separate the lungs into lobes. The right lung has three lobes while the left has two lobes.<sup>1</sup>

Both lungs organize around the bronchial tree, beginning with a main bronchus originating from the trachea. The mainstem bronchus of each lung enters at its root and then subsequently divide into secondary (lobar) and tertiary (segmental) bronchi. At the tertiary bronchi, divisions continue into bronchopulmonary segments and then bronchioles. Each lung has ten bronchopulmonary segments. The bronchioles that branch off the

bronchopulmonary segments contain alveoli, which make up most of the lung tissue. It is in the alveolus that a complicated network of blood vessels allow for the exchange of blood and gases.<sup>2</sup>

### **Vasculature of the Lung**

The arterial supply of the lung comes from the pulmonary arteries which run alongside the bronchi with analogous branching divisions. These arteries ultimately supply deoxygenated blood to capillaries of the alveoli. After gas exchange in the alveolus, oxygenated blood returns to the heart via pulmonary veins that exit the lung through the septa of each bronchopulmonary segment. The pulmonary arteries that supply the capillary, consist of three layers: the tunica externa, tunica media and the tunica intima. The tunica externa is the outermost layer, the tunica media is within the tunica externa and finally, the tunica intima is the most inner layer. Pulmonary arteries and its branches are known as elastic arteries. The tunica externa and tunica media layers of these vessels are made of up elastic and collagen fibers, among other connective tissues. The tunica media layer contains smooth muscle as well, and in pulmonary arteries is very thick, allowing them stretchability. Pulmonary veins have the tunica externa, media and intima layers. However, the tunica media layer is thinner in veins. Capillaries are composed of only the tunica intima, which

also contains elastic and collagen fibers albeit not as much as the two outer layers. Capillary walls are thinner to allow for exchange of gases.<sup>3</sup>

## **Lung Adenocarcinoma**

In 2020, lung cancer was the most predominant form of cancer diagnosed in males and second most in females (after breast cancer) globally. Approximately 2,206,771 or 11.4% of all new cancer cases and 1,796,144 or 18% of all deaths caused by cancer were attributed to lung cancer, continuing to be the number one cause of death in males and females population<sup>4</sup>. In the United States, 228,820 new cases of lung cancer were diagnosed in 2020 and over 22% of all cancer deaths were attributed to lung cancer<sup>4</sup>. Lung cancer can be categorized into two main types: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). SCLC presents at an advance stage and is generally treated with chemotherapy and radiation therapy while early stage NSCLC can be managed by surgical resection alone. NSCLC can further be classified into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Of interest is adenocarcinoma, which constitutes the majority of NSCLC and approximately 40% of all lung cancers.<sup>5</sup>

Invasive adenocarcinomas are classified based on predominant growth pattern as: lepidic predominant, acinar predominant, papillary predominant, micropapillary predominant, and solid predominant<sup>6</sup>. The subtypes are correlated

to prognosis and range from poor to favorable. Lepidic is considered the most favorable, while acinar and papillary are intermediate. Poor prognosis is correlated to micropapillary and solid. The cell patterns, subtypes and staging of lung adenocarcinomas are determined by histologic analysis of the tissue in question.

### **Lung Adenocarcinoma and Staging**

The Tumor, Node, and Metastasis (TNM) staging system for lung cancer is widely recognized and accepted as the criteria used to characterize and classify lung cancer. Accurate staging is crucial in determining the prognosis and proper course of treatment. The TNM system defines specific characteristics of the primary tumor, regional lymph nodes, and distant metastasis. Factors such as tumor size and extent of anatomic invasion determine the “T” designation. Patients with lymph node metastasis (N) or distant metastasis (M) have advanced stage (III or IV) cancer and are generally treated with systemic chemotherapy whereas smaller ( $\leq 4$  cm) organ confined tumors without metastasis have stage I cancer, which can be treated by surgery alone. Approximately 15% of Stage I NSCLC will recur despite adequate surgical management and this has led to the need for further subclassification of Stage I into subcategories (Stage IA1, IA2, IA3, and IB), which can predict which individuals have a higher likelihood of recurrence and which might benefit from additional therapy beyond surgery.<sup>7</sup>

One of the two most important prognostic factors in stage I lung cancer is tumor size and visceral pleura invasion (VPI), presence of tumor cells invading through the elastic layer of the visceral pleura. The most recent TNM staging system designates any cancer > 3 cm or smaller tumors with VPI as stage IB<sup>8</sup>. Some have proposed that the TNM system should incorporate vascular invasion into the equation as it is associated with an outcome at least as poor as VPI<sup>9-12</sup>. Similar to VPI, vascular invasion (VI) is defined as the presence of tumor cells within the lumen of veins or arteries. However, the 8<sup>th</sup> edition of the TNM staging system fails to incorporate VI as a component of the T stage in lung cancer.

### **Vascular Invasion, Visceral Pleural Invasion and Special Staining**

The poor prognostic impact of VI has been widely studied and determined to be one of significance<sup>13</sup>. Multiple reviews and analyses have recognized VI as contributing to unfavorable outcomes for recurrence and overall survival as well as an increased risk for metastasis<sup>14,15</sup>. Several studies have also established VI as an independent prognostic factor from that of tumor size, lymphatic permeation, and VPI<sup>16-18</sup>. As a result, VI has once again been suggested as a staging factor in the next edition of the TNM system, after it was originally suggested for the 7<sup>th</sup> and 8<sup>th</sup> editions<sup>19-21</sup>.

As previously stated, staging is based upon histological analysis. The Hematoxylin and Eosin (H&E) stain has long been the standard stain for

histological evaluation of tissues. Hematoxylin is a dark purple stain and is used to visualize nuclei however it will also stain cell organelles, collagen, and elastic fibers. Hematoxylin is counterstained with eosin, which is a red stain used to visualize the cell cytoplasm and extracellular matrix. Other components of the cell that are stained will take on a mixture of the colors and possibly in different shades. While H&E staining is the standard, it is not ideal for all tissue types. In the case of lung tissue, the presence of elastic and collagen fibers in the pleura and blood vessels can be particularly difficult to distinguish (Figures 1,2,3 A).<sup>22</sup>

Special stains are utilized by pathologists to highlight specific components of tissue. The Verhoeff-van Gieson (VVG) stain is routinely used to highlight the elastic tissue of the visceral pleura in order to aid in the identification of VPI. The elastic tissue present in blood vessels also contains elastin, specifically in the tunica media layer. The elastin is arranged in concentric circles of fenestrated lamellae, between myocyte layers, giving it a unique appearance. The VVG stain has two stages: the Verhoeff component and the van Gieson counterstain. In the first part, iron (III) chloride and iodine solution are used as mordants to oxidize hematoxylin to hematein. The hematein is what stains the elastic tissue because of elastin's affinity for the oxidized complex, allowing it to retain the color. The counterstain produces contrast against the hematoxylin by staining the collagen and muscle fibers (if any) present. Differentiation is necessary to highlight specific structures that maintain the dye, while excess dye is removed from surrounding structures. Differentiation varies based on the elastin present in the tissue and

under-differentiation will allow for better visualization of the elastic fibers. The final result will have the nuclei and elastic fibers stained black, collagen will be a salmon hue, and the cytoplasmic elements will be a shade of yellow. In blood vessels, this will result in the tunica media to be stained black and a pink-red color, as it contains mostly elastic fibers and smooth muscle (Figure 1,3 B). The smooth muscle will be highlighted in the pink-red hue. The tunica intima would have some black staining because of its elastic membrane but would mostly be pink-red and yellow due to its endothelial cell and connective tissue composition. The tunica externa would have black staining in the elastic lamina while the connective fibers would be yellow.<sup>23</sup>

The elastic trichrome (ET) is a combination stain also used to stain elastic fibers. The first part of the stain is the Verhoeff stain while the second is the Masson trichrome. Bouin's fixative is used as a mordant to help the dye adhere to the tissue. Weigert's hematoxylin, a modified hematoxylin containing ferric chloride, stains the nuclei and elastic fibers blue-black. Biebrich scarlet acid highlights keratin and muscle fibers red. Finally, aniline blue stains collagen and bone either blue or green (if components present). This stain is most beneficial for vascular tissues, as elastic lamellae are easily visualized and distinguished (Figures 1,3 C). The tunica intima would have a thin dark blue-black lining while the tunica media would have dark blue-black staining because of the elastic along with red staining in the smooth muscle cells. The tunica externa would be blue with some blue-black staining owing to its connective tissue and elastic fiber composition. Identifying

veins and arteries would be particularly easier with this stain as the thicker tunica media in arteries would have significantly more red and blue-black while in veins, since the tunica media is thinner, more blue would be visible.<sup>24</sup>

The Movat Pentachrome is an elastin stain used principally for heart, lung, and connective tissue as well as blood vessels. It uses Weigert's hematoxylin, an iron hematoxylin, to stain elastin fibers and the nuclei. These components are negatively charged and therefore have high affinity for the hematoxylin, allowing them to retain the dye. Alcian blue binds with the mucopolysaccharides present at low acidity. Scarlet acid fuchsin and resorcin fuchsin stain acidophilic components (collagen and muscle). Phosphotungstic acid removes dye from both collagen and reticulin fibers, allowing them to be stained with alcoholic saffron, resulting in a yellow hue. The end result is a tissue section with five colors (Figures 1,2,3 D). Nuclei and elastic fibers are stained dark purple to black, collagen and reticulin yellow, mucin or ground substance blue to blue green. Muscle is highlighted in red and fibrin tissue in a bright red. Arteries would have a distinct middle with dark purple to blue linings and mostly red in between (some yellow), emphasizing the elastic and smooth muscle in the tunica media. Veins would have a prominent yellow outer layer, surrounded by some dark purple to black, showcasing the connective tissue and elastic lamina in the tunica externa.<sup>25</sup>

Since VPI is currently assessed as the standard of care in NSCLC staging and because VI has been established as an equally important prognostic measure, visualization and accurate identification of both is of utmost importance. Elastic

stains are preferred over H&E for proper assessment of the elastic layer within the visceral pleura<sup>26</sup> and the blood vessels<sup>27</sup>. Similar to the layers of blood vessels, the visceral pleura consists of multiple layers such as the mesothelial lining, superficial and deep elastic laminae, and loose connective tissue. All are highlighted in different colors, depending on the stain (Figure 2), making proper assessment less difficult. The VVG is used the most however, studies have found the VVG alone, though superior to H&E, still does not alleviate all the ambiguity associated with the visceral pleura and blood vessels<sup>28</sup>.

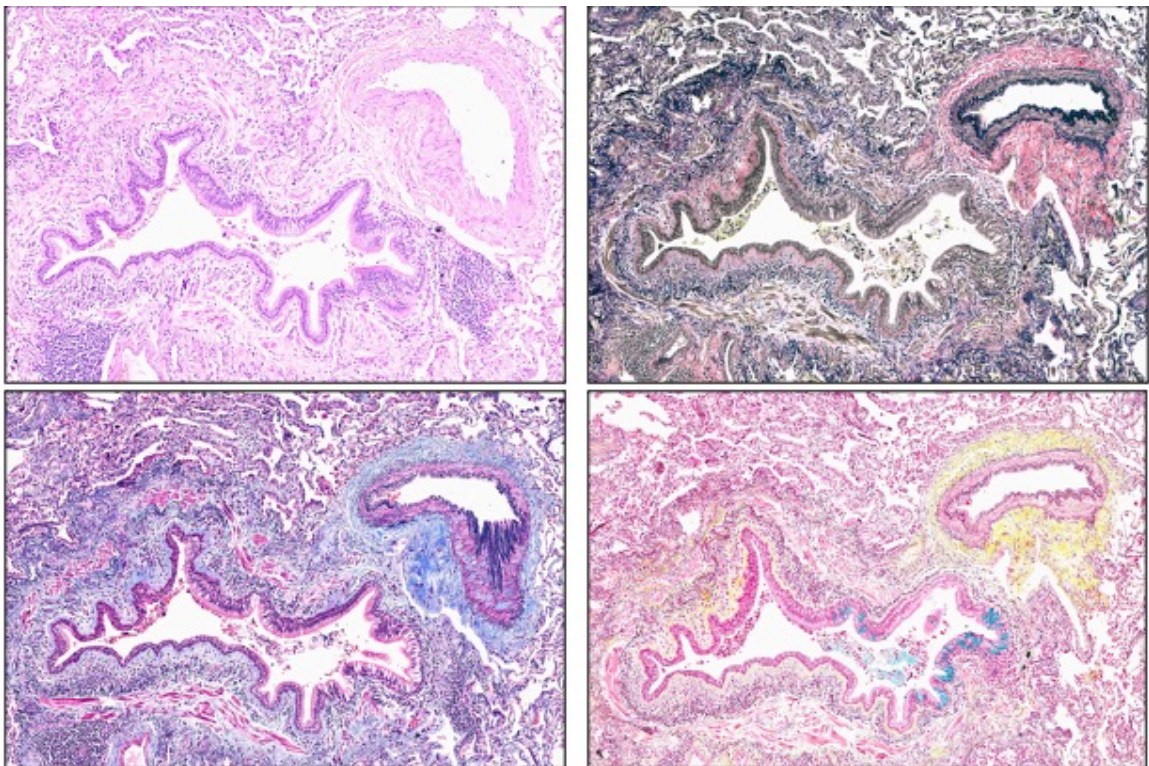


Figure 1. Representative histological sections of blood vessels with no VI or VPI stained with (clockwise) H&E, VVG, ET, and Movat Pentachrome.

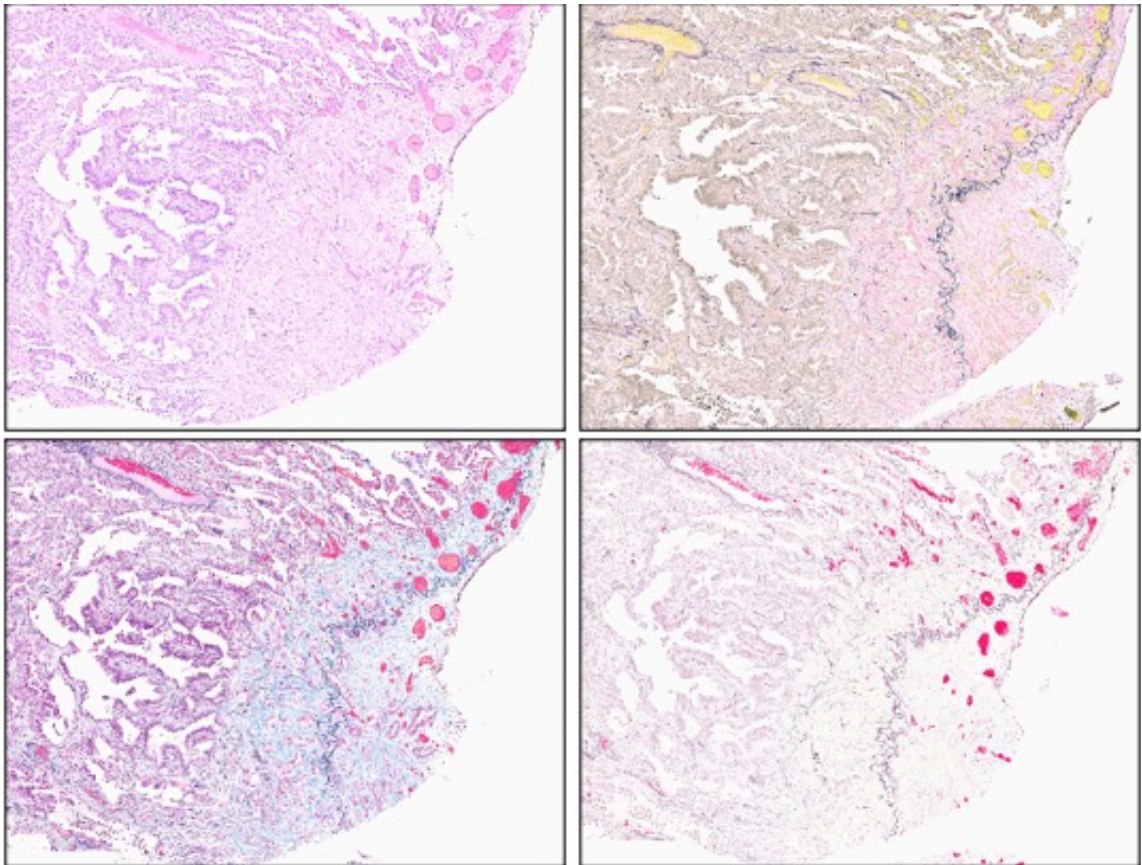


Figure 2. Representative histological sections of lung tissue with VPI stained with (clockwise) A.H&E, B.VVG, C.ET, and D.Movat Pentachrome.

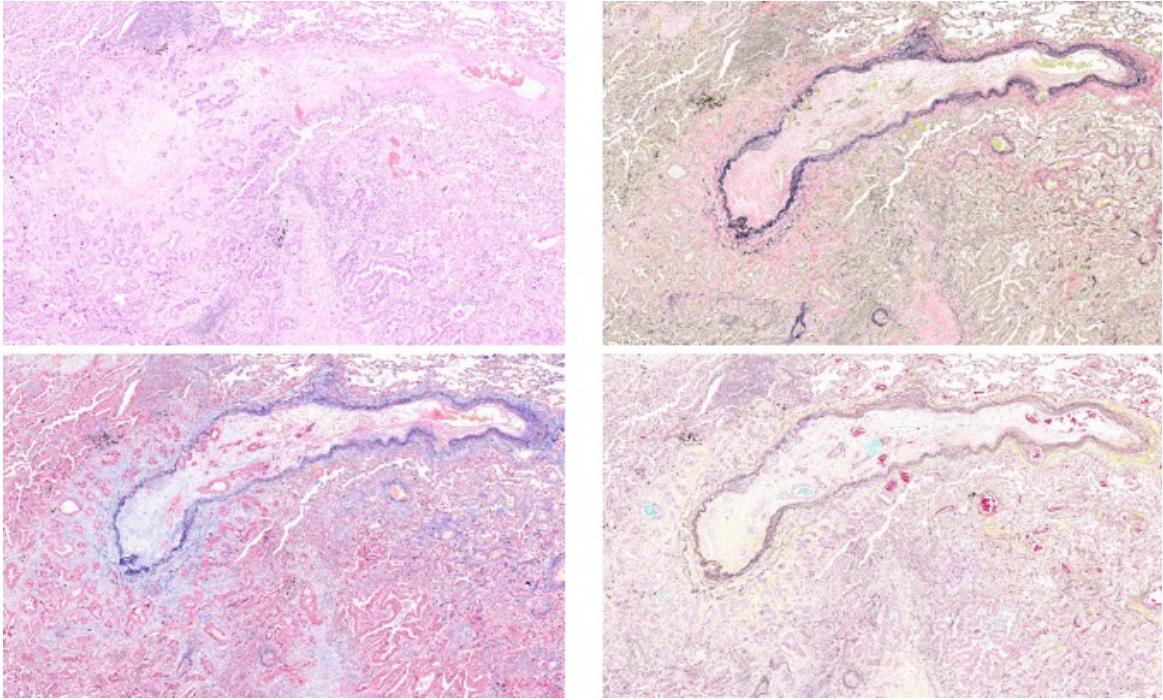


Figure 3. Representative histological sections of blood vessels with VI stained with (clockwise) A.H&E, B.VVG, C.ET, and D.Movat Pentachrome.

## OBJECTIVES

In this study, we sought to examine whether the use of the ET could improve assessment of VPI and VI in lung adenocarcinomas. Diagnoses using three elastin stains (ET, VVG, and the Movat pentachrome) will be compared with the standard H&E stain to determine if evaluation of VPI and VI is improved. Finally, reproducibility of the ET stain as the standard for diagnosis will be assessed among two cohorts (attending and resident pathologists).

## METHODS

Resected lung adenocarcinomas were identified from Boston Medical Center (BMC) and Lahey Hospital & Medical Center (LHMC). A cohort of approximately 230 lung adenocarcinoma tumor blocks, from between 2005 to 2015, were chosen. Slides from each block were produced and stained with H&E, VVG, ET and Movat pentachrome stains. The presence of VPI and VI was evaluated by a pulmonary pathologist (EJB) and specified as either present or not for each. A subset of 50 from this cohort was chosen. To ensure adequate representation and avoid possible bias, cases both positive and negative for VPI and VI were chosen. All slides were deidentified and given an identification number unique to this study.

All stains were done manually apart from the H&E, which was stained on an automated stainer. The stainer uses the standard H&E protocol, which after deparaffinization and rehydration, stains in Hematoxylin. The slides are rinsed in water and dipped in acid alcohol or a bluing reagent. After rinsing in water, they are stained in Eosin and dehydrated in 95% alcohol and absolute alcohol. Xylene is used to clear the slides and finally they are coverslipped. For the VVG, Verhoeff's working solution was prepared with alcoholic hematoxylin, ferric chloride 10% and iodine solution. Slides were stained for 20 minutes and then rinsed with distilled water. Ferric chloride 2% was used to differentiate the tissue until the tissue appeared gray. Excess chloride was rinsed off with tap water and the staining was

evaluated. The elastic fibers were expected to be a clear, sharp black stain to ensure under differentiation. The slides were then treated in sodium thiosulfate for 1 minute and rinsed in water. The Van Gieson solution was used to counterstain the tissue for 3-5 minutes. The slides were cleared in Xylene and coverslipped.

The ET stain required slides must be incubated in Bouin's fixative for an hour at 60° Celsius before being rinsed in distilled water. Similar to the VVG, Verhoeff's working solution was prepared, and slides were stained for 15 minutes before being rinsed in distilled water. Biebrich Scarlett Acid Fuchsin was utilized as an acid dye and slides were stained for 2 minutes before being rinsed in water and placed in Phosphotungstic Phosphomolybdic acid for 8 minutes. Next, they were placed in Aniline Blue Masson's Trichrome for 3 minutes and then rinsed with water. After 1 minute in acetic acid 1%, the slides were dehydrated in 95% alcohol, absolute alcohol, and cleared in xylene before being coverslipped.

The Movat pentachrome required the longest amount of time, as the Resorcin Fuchsin solution required overnight (minimum 16 hours staining). After deparaffinization and rehydration to water, the slides were stained in Alcian Blue 1% in 1% acetic acid for 20 minutes. They were rinsed in water and placed in Alkaline alcohol (pH over 8) for 2 hours. Before being placed in the Resorcin Fuchsin solution overnight, slides were rinsed in water followed by 70% alcohol. Slides were again rinsed in water for a minimum of 10 minutes. Afterwards, staining in Weigert's hematoxylin took place for 5 minutes followed by rinsing in running water. The slides stained in Woodstain Scarlet Acid Fuchsin for 5 minutes were

rinsed in 0.5% acetic acid. Next, the sections were rinsed in three changes of absolute alcohol before being placed in alcoholic saffron for 20 minutes. Dehydration in three changes of absolute alcohol took place before the collagen stain was evaluated for sufficient yellow. If not stained properly, incubation in the alcoholic saffron was repeated. If sufficiently stained, the slides were cleared in xylene and coverslipped.

A total of eleven pathologists participated, including five attendings and six pathology residents. The group of five attending pathologists (cohort A) were asked to evaluate VPI and VI on the subset of 50 cases using first, H&E and then one of the elastin stains. All three elastin stains were provided, and the pathologists used their preferred elastin stain to determine the presence of VPI and VI. Their diagnosis was compared to that determined by EJB.

The residents (cohort B) were provided 25 cases and all four stains upfront. After evaluating the cases for VPI and VI, they were asked to watch a short lecture providing training in how the elastin stains can be utilized. After viewing the lecture, the residents were asked to take an assessment. They were given 11 cases (separate from the total 50), 7 with all four stains and 4 with an H&E and ET. Disagreements on either VPI or VI was reviewed between EJB and each resident as part of the educational assessment. The residents were then given a second set of 25 cases to evaluate. With this second set, they were provided only an H&E and ET.

An Aperio AT2 digital slide scanner was used to scan each slide at 20x magnification and to manage assignments and workflow of each participant via imagescope software. Fleiss' k statistic was used to measure agreement among both cohorts. k statistic values ranging from 0.00 to 0.20 correspond to slight agreement, 0.21 to 0.40 to fair agreement, 0.41 to 0.60 to moderate agreement, 0.61 to 0.80 to substantial agreement, and 0.81 to 1.00 to near perfect agreement. The statistics were calculated using Microsoft Excel and R Studio.

## **RESULTS**

We initially assessed accuracy and reproducibility among the five attending pathologists without any education regarding the interpretation of the elastic stains regarding VPI or VI. Elastic trichrome was the preferred stain among all five attending pathologists. Both accuracy and reproducibility was higher in assessing VPI than VI (Table 1) in this cohort. However, elastic stains did not improve neither accuracy nor reproducibility for VPI or VI, yielding only fair agreement for both. The ET improved accuracy for VPI among 4 of the 5 pathologists (8-16%) when compared to H&E while one pathologist fared worse with ET (-16%). Accuracy improved in 3 of 5 pathologists for the interpretation of VI (14-34%) with ET but did not change the accuracy for the remaining 2 pathologists.

**Table 1. Percent Accuracy and k Statistic in Assessment of VI and VPI with H&E and Elastic Stains (Cohort A)**

	Attending 1	Attending 2	Attending 3	Attending 4	Attending 5	Overall Agreement (%)	k Statistic
H&E-VI	76%	54%	52%	50%	56%	64	0.21
Elastic-VI	76%	68%	76%	52%	90%	64	0.28
Difference	0%	14%	24%	2%	34%	0.4	
H&E-VPI	76%	82%	84%	72%	72%	74	0.31
Elastic-VPI	60%	92%	92%	80%	88%	73	0.38
Difference	-16%	10%	8%	8%	16%	-1.0	

Summary of each attendings' percent accuracy and improvement. The k statistics corresponded to fair agreement for all 4 categories.

Based on these results, we designed an instructional video and assessment to see if specific instruction might lead to uniform improvement of the interpretation of elastic stains with regards to VPI and VI in a second cohort (six residents). For this purpose, the 50 cases were divided into two groups of 25 cases (round 1 and round 2). Evaluation of elastic stains were performed as round 1 (25 cases prior to the lecture and assessment) and then round 2 (25 cases after lecture and assessment). Overall accuracy improved for both VPI (68% to 82%) and VI (71%

to 79%) after the instructional video and assessment (Table 2). The greatest improvement was seen among the residents with the poorest accuracy in round 1. Reproducibility improved from fair ( $k=0.30$ ) to moderate ( $k=0.58$ ) for VPI and fair ( $k=0.29$ ) to substantial ( $k=0.61$ ) agreement for VI comparing round 1 to round 2 (Table 3). Importantly, both accuracy and reproducibility for VI was similar to VPI, which is the current standard of care for staging purposes.

Representative images of the most common benign features that were inaccurately interpreted as VI are shown in Figure 4. These included inflammatory infiltration of vessels by either histiocytes or lymphocytes misinterpreted as tumor (Fig 4 A & B). In other cases, tumor stromal desmoplasia was misinterpreted as tunica media of the vessel wall (Fig 4 C). Finally, epithelioid endothelial cells were sometimes misinterpreted as tumor (Fig 4 D). These misinterpretations reflected the residents' unfamiliarity with the differences in nuclear staining in the ET compared to H&E stains.

**Table 2. Percent Accuracy in Assessment of VI and VPI with ET Stain  
(Cohort B)**

	Resident 1	Resident 2	Resident 3	Resident 4	Resident 5	Resident 6
Round 1 VI	75%	57%	79%	68%	64%	75%
Assessment Score for VI (%)	91	45	91	73	73	64
Round 2 VI	79%	79%	79%	86%	82%	75%
Improvement	4%	21%	0%	18%	18%	0%
Round 1 VPI	71%	68%	82%	43%	54%	68%
Assessment Score for VPI (%)	100	55	91	100	91	91
Round 2 VPI	82%	79%	89%	82%	71%	79%
Improvement	11%	11%	7%	39%	18%	11%

Summary of each residents' percent accuracy, assessment scores, and improvement.

**Table 3. Percent Overall Agreement and k Statistic for Agreement in Assessment of VI and VPI with ET Stain (Cohort B)**

	k Statistic	Interpretation	Overall Agreement(%)
Round 1 VI	0.29	Fair Agreement	69
Round 2 VI	0.61	Substantial Agreement	81
Round 1 VPI	0.30	Fair Agreement	65
Round 2 VPI	0.58	Moderate Agreement	84

Summary of Percent Overall Agreement, k Statistic, and Interpretation for Cohort B.

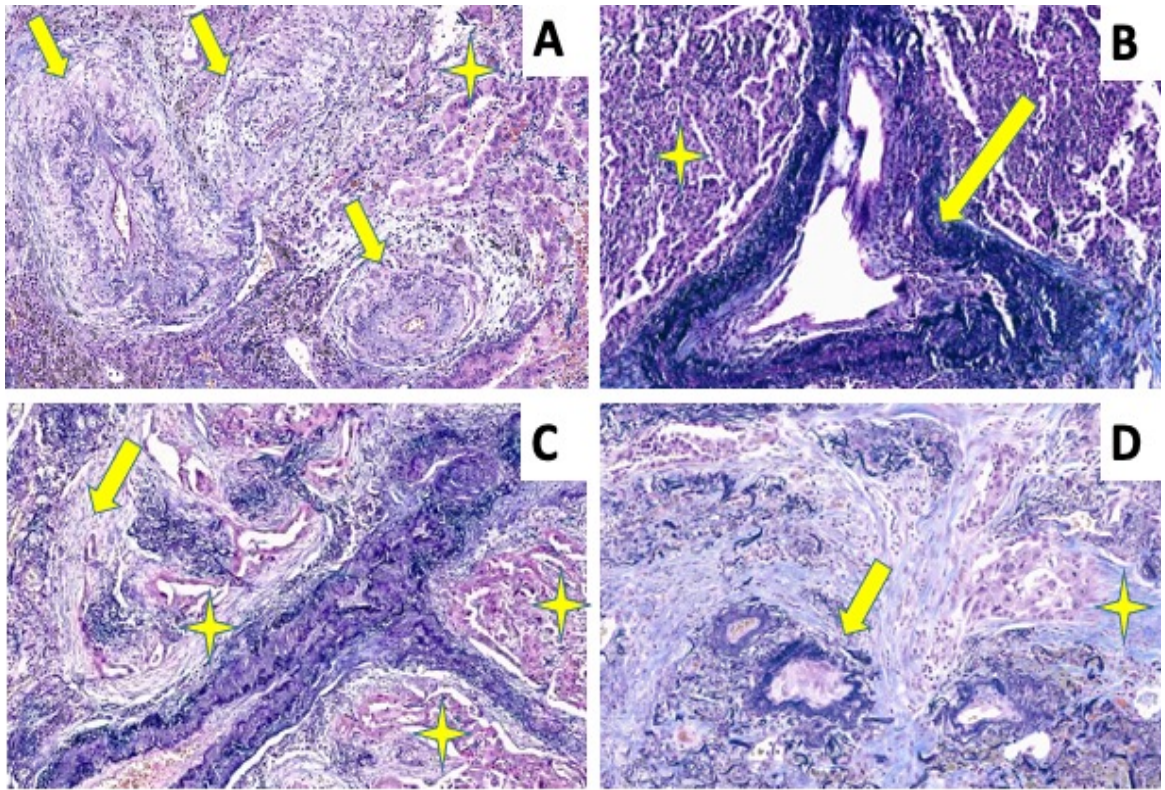


Figure 4. ET histologic sections of challenging cases leading to discrepancies among residents in the assessment slides. Stars designate tumor whereas arrows designate benign cell type misinterpreted as vascular invasion.

## DISCUSSION

The use of the ET stain to evaluate VI in lung adenocarcinomas was assessed. The elastin stains did not show improvement in determining presence of both VPI and VI among the attending pathologists. Reproducibility remained the same, with the use of H&E and ET both yielding only fair agreement. Therefore, an educational system was utilized to improve understanding of the ET in the second cohort. Accuracy and reproducibility improved from round 1 to round 2, with most improvement in residents 2, 4, and 5, who had scored the lowest in round 1 for both VPI and VI. The reproducibility of use of the ET for evaluation of VPI and VI in cohort B improved to substantial and moderate agreement, respectively.

In Shivji et al., the combination ET stain has been evaluated as the primary stain in determining VI in colorectal cancers. The study found ET superior to H&E because of improved assessment. The ET highlights elastic lamina in vessels regardless of the integrity of the structure, thereby resolving any concerns regarding undistinguishable architecture or structure. Relatedly, the ET provides a strong contrast in the elastin fibers, connective tissue, and tumor. The prominent contrast provides easy detection of VI as compared to the VVG, where the staining of different components may not be as apparent. Overall, the ET had superior accuracy and sensitivity to H&E, with most pathologists agreeing upon its use as a primary stain. Still, unfamiliarity with the stain and reduced comfort in evaluating ET stain alone resulted in adoption in only a few hospital labs.<sup>29</sup>

The ET and Movat pentachrome are both special histochemical stains that were essential to Pathology decades ago. Over time, with the emergence of immunohistochemical (IHC) staining, the use of special histochemical stains became scarce. However, routine use of certain IHC stains requires competency assessments assigned by the College of American Pathologists (CAP). The standard of care for breast cancer includes determining the presence of specific hormone receptors for estrogen (ER) and progesterone (PR) using IHC biomarker assays. The presence or lack of ER and PR in an individual will determine their course of treatment, prognosis, and risks of recurrence. Therefore, accurate testing and interpretation is essential.<sup>30</sup>

To ensure accuracy, CAP mandates that pathologists take and adequately pass a yearly assessment, evaluating their ability to accurately diagnose such cases. Similarly, Programmed death-ligand 1 (PDL-1) is a protein that downregulates an immune response, eventually inducing cell death in lymphocytes that are necessary for defense against cancer and pathogens. Tumors that upregulate PDL-1 successfully evade immune responses that would destroy them. Inhibitors of PDL-1 can be used as cancer therapeutics but first, the expression of PDL-1 on tumor cells would need to be determined. Pathologists are evaluated on their ability to correctly identify the presence or absence of PDL-1 using specific IHC biomarker assays.<sup>31-32</sup> Apart from evaluations and continuous monitoring, pathologists are required to participate in regular educational instruction as well.

Comparable to the ER, PR and PDL-1 IHC biomarker assays, the use of the ET for VI will require initial educational programming and assessments as well as continuous monitoring. The resident cohort showed improvement in assessing VPI and VI with the ET after a short training period. With the attending pathologists, lower reproducibility was most likely due to the newness and lack of understanding of the stain. This rationale is consistent with what was observed in evaluation of ET in colorectal cancer in Shivji et al: pathologists who did not agree with only the use of ET stated unfamiliarity as the reasoning behind their decision<sup>29</sup>.

VPI is already a prognostic factor in the TNM staging system and the prognostic importance of VI has been established as equivalent to that of VPI<sup>14-16</sup>. Its addition to the TNM staging system relies on multiple factors, one of which is reproducibility. Continuous, accurate evaluation of VI needs to be presented to ensure similar long-term results among pathologists. In cohort B, among the residents, we observed such results. The evaluation of VI with ET improved in percent overall agreement and reproducibility in round 2. These results represent the comparability of VI to VPI, the current standard of care. Because the accuracy and reproducibility of VI is similar to that of VPI, its evaluation with ET should also be considered a standard of care in the staging system.

Some factors that may have caused lower assessment scores or some disagreement include inaccurate interpretations. In order to properly measure the extent to which the educational programming improved knowledge of the stain, more complex and challenging cases were chosen as part of the assessment.

Characteristics such as histiocytic vasculitic and lymphocytic vasculitic inflammation reaction was misinterpreted as VI. Likewise, tumor and desmoplastic stroma with adjacent elastin fiber clumping and plump endothelial cells were misinterpreted as VI. Therefore if all types of lung cancer cases (routine, less complex) were included, reproducibility would increase additionally.

To further examine the accuracy and reproducibility of the ET in detecting VI, we continue to enlist pathologists from different institutes all over the world to take part in this study. This cohort will include general pathologists along with specialists to ensure universal relevance. We will be implementing the educational system used with the second cohort to also illustrate the necessity of educational programming and assessments for the use of this stain. Furthermore, the ET has been used to identify features in lung adenocarcinomas. These specific features were analyzed using spatial transcriptomics to aid in the identification of biomarkers specific to subcategories of lung adenocarcinomas. The data is currently being evaluated.

In conclusion, the ET stain improved evaluation of both VI and VPI in lung adenocarcinomas with educational instruction. In the first cohort of attending pathologists, reproducibility and accuracy of VI and VPI did not improve when compared to the H&E. Therefore, for the second cohort of resident pathologists, an educational lecture was used to improve understanding of the stain. Both accuracy and reproducibility improved. Since the results of VI are comparable to VPI, the current standard of care in staging lung adenocarcinomas, VI should also

be implemented as a standard of care with the ET used as the standard for diagnosis.

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## CURRICULUM VITAE

