

3D virtual histology demonstrates tumour connectivity in spread through air spaces (STAS) of non-small cell lung cancer

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Introduction: As the nature of spread through air spaces (STAS) in non-small cell lung cancers (NSCLC) remains a matter of debate, this paper presented the first application of 3D X-ray virtual histology to shed light on the origin of these elements.

Methods: Five adenocarcinomas and two squamous cell carcinomas were selected from a cohort of NSCLC cases to serve as representative examples of neoplasms in which the presence of STAS had already been assessed through conventional histology. Although available only for research purposes, synchrotron radiation X-ray phase-contrast micro-tomography (μ CT) allows virtual sectioning of whole paraffin blocks with spatial and contrast resolution similar to that of histology, thus enabling examination of STAS patterns (e.g., single and clustered tumour cells, micropapillary, solid nests).

Results: The 3D results demonstrated that free-floating STAS (i.e., micropapillary and solid patterns) were observed to be only the edges of tumour cell clusters connected to the primary tumour. In contrast, STAS located near alveolar walls or vascular structures suggested tumour cell migration along these surfaces away from the primary tumour.

Conclusion: These findings indicate that most STAS types are clusters of cells connected to the main tumour mass. 3D X-ray virtual histological investigation helps to understand the morphological composition and spatial evolution of the tumour, as well as the presence of a tumour larger than that visible in the histological slide. From a radiological and surgical perspective, these findings may influence the assessment of the extent of parenchymal involvement and help guide the surgical approach.

Keywords: 3D Pathology, histopathology, non-small cell lung cancer, spread through air spaces, X-ray computed tomography, X-ray phase-contrast micro-tomography, X-ray virtual histology

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Abbreviations: μ CT, micro-tomography; FFPE, formalin-fixed paraffin-embedded; H&E, Haematoxylin and Eosin; IHC, immunohistochemical; MIP, maximum-intensity projection; NSCLC, non-small cell lung cancers; PACS, picture archiving and communications system; STAS, spread through air spaces.

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Introduction

Spread through air spaces (STAS) refers to clusters or single tumour cells found within alveolar spaces beyond the edge of the main tumour, not attached to it. It reflects an invasive pattern in non-small cell lung cancers (NSCLC), and pathologists identify it by locating detached tumour cells in surrounding lung tissue.

STAS is linked to higher recurrence rates and poorer overall survival, particularly in early-stage lung adenocarcinoma.^{1,2} Although difficult to detect preoperatively, recognising STAS is critical for surgical planning. Several studies have explored imaging features that may predict STAS, with CT often showing solid nodules, spiculation, cavitation, clear boundaries, pleural indentation, vessel convergence, mediastinal lymphadenopathy, and pleural thickening in STAS-positive cases.^{3,4} Given its prognostic value also in risk stratification,^{5–7} STAS was included in the 2015 WHO Classification of lung adenocarcinoma as a key invasive pattern, defined as micropapillary clusters, solid nests, or single tumour cells beyond the tumour edge in air spaces.^{8–11} Subsequent studies proposed subclassifications based on morphology,¹² and spread quantity.¹³ The 2021 WHO update confirmed STAS as a prognostic histological feature.¹⁴ However, its interpretation remains debated. Onozato used 3D histology to suggest STAS-like patterns might actually be connected to the tumour.^{15,16} Others, including Blaauwgeers and Pelosi, argued STAS may be an artefact caused by tissue handling.^{17–19} Kadota and Yagi showed that many STAS cells are attached to alveolar walls, not freely floating.^{10,20} Travis proposed a dynamic process of detachment, migration, and reattachment to explain STAS behaviour.²¹

All these considerations were based on histology, which is the gold standard in clinical practice for its ability to visualise individual cells, subcellular structures, and selectively labelled tissue components with high accuracy and specificity. However, histology is limited by its two-dimensional nature and the introduction of artefacts during sample preparation. As a result, it cannot definitively determine whether STAS are free-floating cells, extensions of the primary tumour, or artefacts.

To address this limitation, X-ray phase-contrast Computed micro-Tomography (μ CT) offers a non-destructive technique capable of revealing the 3D microarchitecture of biological tissues. Unlike conventional CT, μ CT enhances visualisation of low-density tissues by detecting phase shifts in X-rays, rather

than relying solely on attenuation. This enables a more detailed and artefact-free view of tissue structure without the need for staining or sectioning. As such, μ CT bridges the gap between radiological imaging and histological analysis, providing complementary insights into tissue pathology. However, its current high-resolution implementation is limited to synchrotron radiation facilities, restricting widespread use.

This study employs synchrotron-based μ CT to reconstruct the high-resolution 3D microarchitecture of lung resection specimens and investigate the nature of STAS. Although μ CT has been used to examine lung biopsies,^{22–24} this is, to our knowledge, the first virtual histology study focused specifically on STAS. We identified STAS through conventional histology, categorised the patterns, and then analysed formalin-fixed paraffin-embedded (FFPE) blocks via μ CT to assess whether STAS are artefactual or connected to the main tumour. Preoperative CT scans were used to support and validate our findings.

Methods

Seven FFPE lung tumour specimens were selected from a cohort of 20 invasive NSCLC cases by the Department of Pathology, University Hospital of Trieste (Italy). These, representative of STAS-positive neoplasms, were analysed by synchrotron μ CT. True STAS were identified according to the 2021 WHO Classification,¹⁴ with distances ranging from 0.4 to 4 millimetres. The full cohort included 17 lung adenocarcinomas and 3 squamous cell carcinomas, seven of them presenting airspace invasion and 3 control tissues. Table 1 summarises the selected STAS-positive cases, including diagnosis, STAS pattern^{10,20} (single cells, micropapillary, solid nests), and cancer type. Each specimen was first histopathologically validated, then scanned using μ CT; therefore, an exact correspondence between the histology and a μ CT slice is not possible and not necessary. Diagnostic clinical CT scans were also reviewed.

CONVENTIONAL HISTOLOGY

FFPE blocks were prepared according to clinical practice standards. Tissue specimens were fixed in 10% buffered formalin and embedded in standard paraffin blocks, measuring $2.5 \times 3.5 \times 1.0$ cm³. Then, they were sectioned into 5 μ m slices and placed on glass slides. Tissue sections were stained by Haematoxylin and Eosin (H&E) for the histopathological

Table 1. Specimen information extracted from histological diagnosis

	NSCLC tumour	Grade	STAS pattern
1	Adenocarcinoma acinar, mucinous, and lepidic	G2	Micropapillary
2	Adenocarcinoma acinar and micropapillary	G2	Micropapillary
3	Adenocarcinoma acinar, papillar, micropapillary, solid	G2	Single cells, micropapillary, solid nests
4	Adenocarcinoma acinar, papillar, micropapillary, solid	G2	Single cells, micropapillary
5	Adenocarcinoma solid and micropapillary	G2	Single cells, micropapillary
6	Squamous cell carcinoma	G3	Solid nests
7	Squamous cell carcinoma	G3	Solid nests

examination. Immunohistochemical (IHC) staining was performed using a CK7 + TTF-1 antibody cocktail (Ventana, Roche). TTF-1 yielded brown nuclear staining and CK7 red cytoplasmic staining. Neoplastic cells showed co-expression of brown nuclei and red cytoplasm. Digital images were acquired using an Olympus VS200 slide scanner at a magnification of $\times 20$ with a pixel resolution of $0.274 \mu\text{m}/\text{pixel}$ and visualised using QuPath software.²⁵

IDENTIFICATION OF STAS VIA HISTOPATHOLOGICAL EXAMINATION

In the histopathological images, tumour STAS were mainly found within the air spaces of lung parenchyma in proximity of the main tumour. The three morphological patterns that characterised STAS are reported in Figure 1: sparse single cells scattered (Figure 1A), clusters and floret micropapillary (Figure 1B), and solid nests (Figure 1C) are visible free-floating in the air spaces. The representative cases were chosen due to their distance from the main tumour.

X-RAY PHASE-CONTRAST COMPUTED MICRO-TOMOGRAPHY (μCT)

The same FFPE blocks were considered for μCT investigation at the SYRMEP beamline of Elettra Synchrotron (Trieste, Italy).²⁶ The propagation-based imaging setup in white beam configuration (machine

operating voltage and current: 2.0 GeV, 308 mA) was exploited to achieve an optimal tissue contrast (200 mm sample-to-detector distance, $2.0 \mu\text{m}$ pixel size, 21 keV average energy spectrum). The projections were collected by the SCMOS detector (Orca-Flash 4.0, 2048×2048 pixels sensitive area) coupled with a $17 \mu\text{m}$ GGG scintillator. For each scan, 3600 projections were acquired over 360° with a 200 ms exposure time per projection. Employing Syrmep Tomo Project software,²⁷ projections were then filtered with a phase-retrieval algorithm²⁸ and reconstructed with the Filtered Back Projection algorithm. 3D volume renderings were retrieved employing the software Avizo 3D (Thermo Fischer Scientific, Massachusetts, United States).

CLINICAL COMPUTED TOMOGRAPHY

All CT scans were performed using a 256-slice multi-detector CT system (Brilliance iCT 256, Philips Healthcare, Best, The Netherlands). The patients were invited to hold their breath with tidal inspiration during scanning in the supine position. The technical parameters were: rotation time = 270 ms; beam collimation = $128 \times 2 \times 0.625 \text{ mm}$; normalised pitch = 0.975; z-axis coverage = 160 mm; reconstruction interval = 0.3 mm; section reconstruction thickness = 1 mm; tube voltage = 120 kV; tube current (effective mA) = 280–400 depending on patient size; and field of view = 40 cm. CT images were analysed by a thoracic radiologist with 15 years of experience, at standardised lung window settings (window level -600 HU and window width 1600 HU) on a picture archiving and communications system (PACS) integrated workstation and mediastinal window setting (window level $400\text{--}500 \text{ HU}$ and window width $20\text{--}40 \text{ HU}$).

Results

3D virtual histology provides a comprehensive view of lung tissue architecture in both healthy and diseased states, as demonstrated in Figure 2, with the potential of bridging high-resolution imaging with traditional microscopy. The control sample (Figure 2A) shows a well-aerated parenchyma with thin, uniform alveolar walls and clear airspaces. In contrast, the adenocarcinomas in Figure 2B,C display increased tissue density and loss of normal alveolar structure, with yellow boundaries marking the transition from healthy to malignant regions. The specimen in Figure 2C shows even more pronounced distortion,

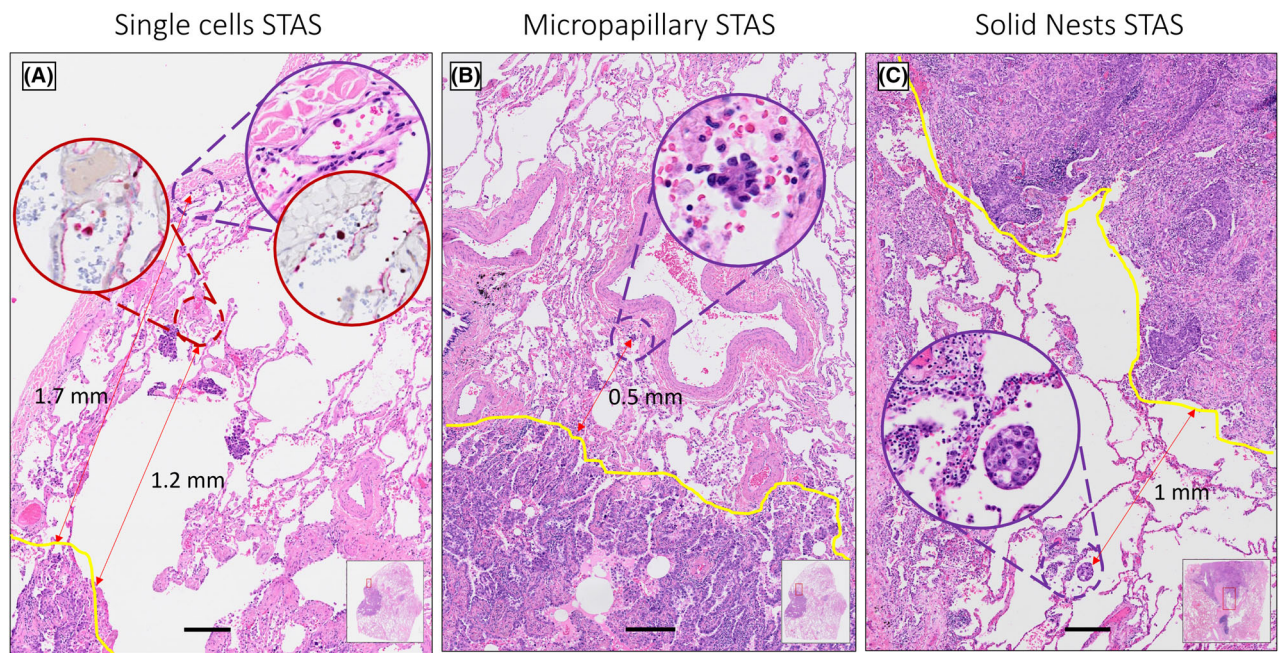


Figure 1. STAS patterns in non-small cell lung cancers histology. (A) Single cell pattern STAS consisting of scattered discohesive single cells; (B) Micropapillary pattern STAS floating in alveolar space; and (C) Solid pattern STAS in lung parenchyma beyond the edge of the main tumour. The tumour boundaries are outlined in yellow. H&E and IHC close-ups as well as representative distances (in red) from the main tumour boundary (in yellow) are included. Scalebars = 200 µm.

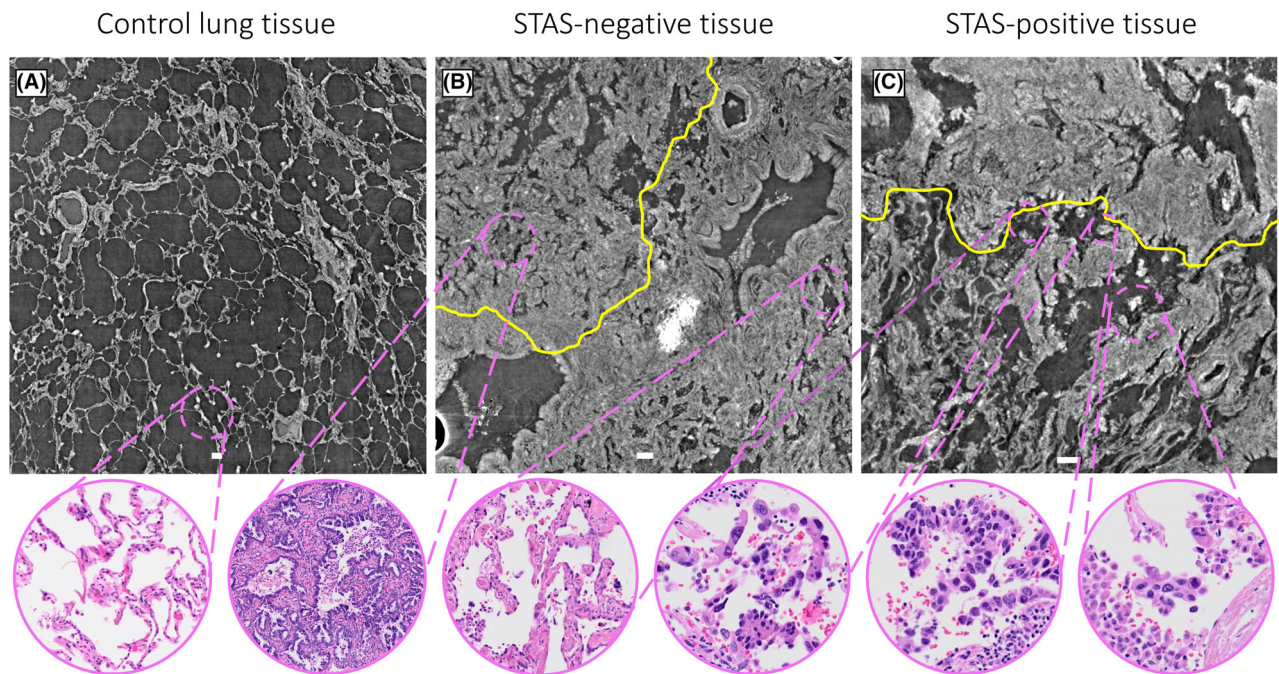


Figure 2. Specimen overview employing synchrotron-based µCT imaging. In the top panel, (A) a control lung tissue that shows normal lung parenchyma architecture and well-defined blood vessels, and (B) a STAS-negative adenocarcinoma case, exhibiting localised tumour growth without evidence of STAS beyond the borders of the tumour marked in yellow. In the bottom panel, (C) a STAS-positive adenocarcinoma case is shown, demonstrating evident STAS beyond tumour edges, with confirmation of STAS extension supported by the related corresponding histology. Scalebars = 100 µm.

with dense, irregular tumour extensions beyond the primary mass. Histology confirms atypical epithelial clusters within alveolar spaces—findings consistent with micropapillary STAS—and shows detached aggregates and cytologic atypia that underscore the tumour's invasive nature.

ATTACHMENT OF SINGLE CELLS AND MICROPAPILLARY STAS TO THE MAIN TUMOUR AND ALVEOLAR WALLS

Single cells and micropapillary clusters are typical STAS in histology. Figure 3 compares histology and

μ CT of the same adenocarcinoma area extending beyond the boundary of the main tumour, which is highlighted by the yellow contour. Floating single cells and clusters, which are indicated by red arrows in Figure 3A, occupy alveolar spaces, forming groups that attach to alveolar walls and vessels, ultimately suggesting a peritumoral spatial relationship with the primary tumour. Micropapillary clusters are more easily traceable (Figure 3C,D), while single cell STAS are also detectable but frequently aggregate into micropapillary groups while preserving spatial continuity within the peritumoral alveolar spaces. Consecutive μ CT images enable virtual 3D sectioning of the

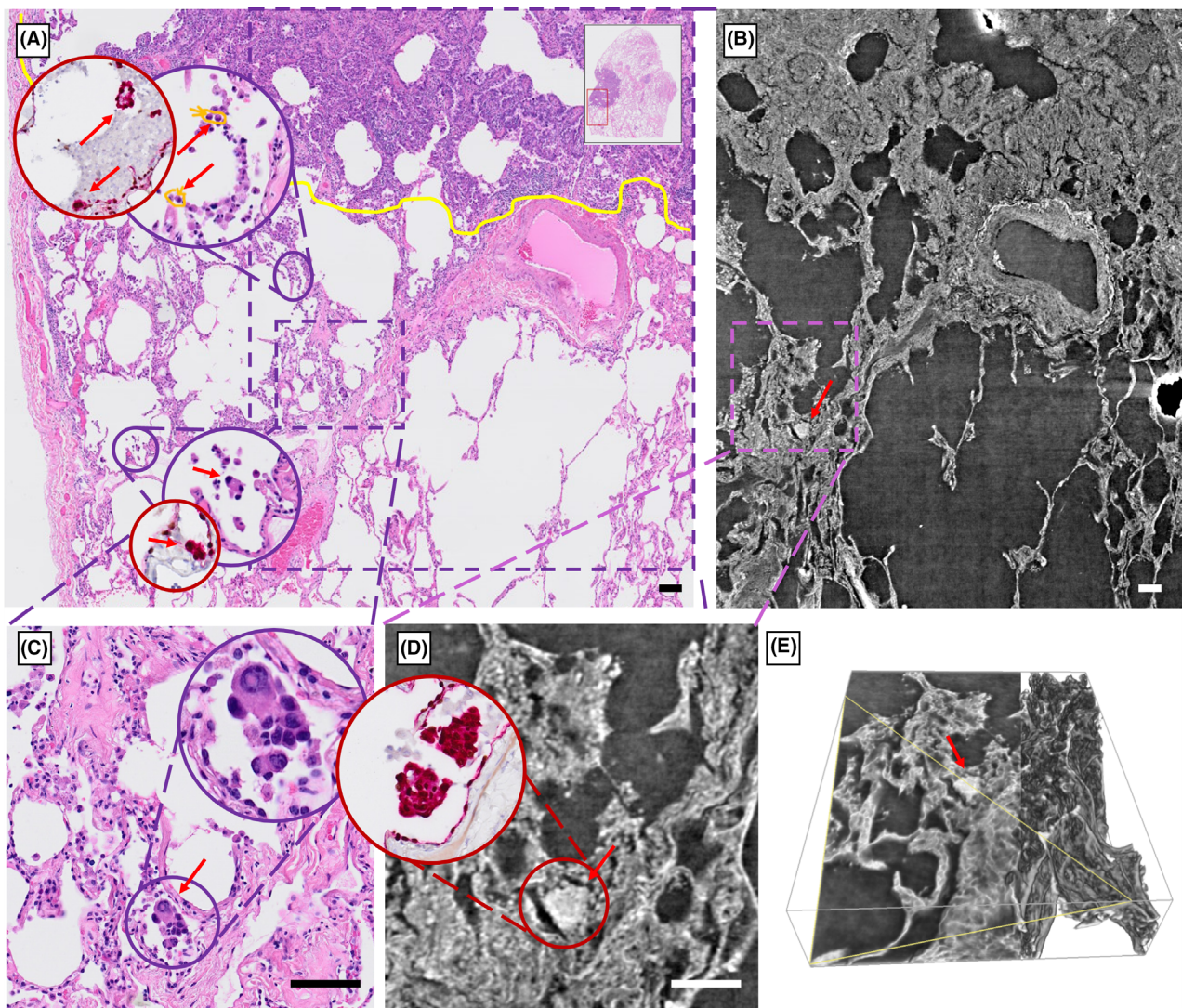


Figure 3. Comparison of single cells and micropapillary STAS of an adenocarcinoma neoplasm in histological and μ CT images. The same region of interest is shown in histology (A) and μ CT (B), with enlarged views containing STAS in histology (C) and μ CT (D). Single cells STAS, as well as floret micropapillary elements, are represented also via IHC and highlighted in the micropapillary environment by red arrows, including in 3D μ CT rendering (E). Refer to Video S1 for a visual illustration of the virtual sectioning of the μ CT volume. The tumour boundary is outlined in yellow in (A). Scalebars = 100 μ m. [Colour figure can be viewed at wileyonlinelibrary.com]

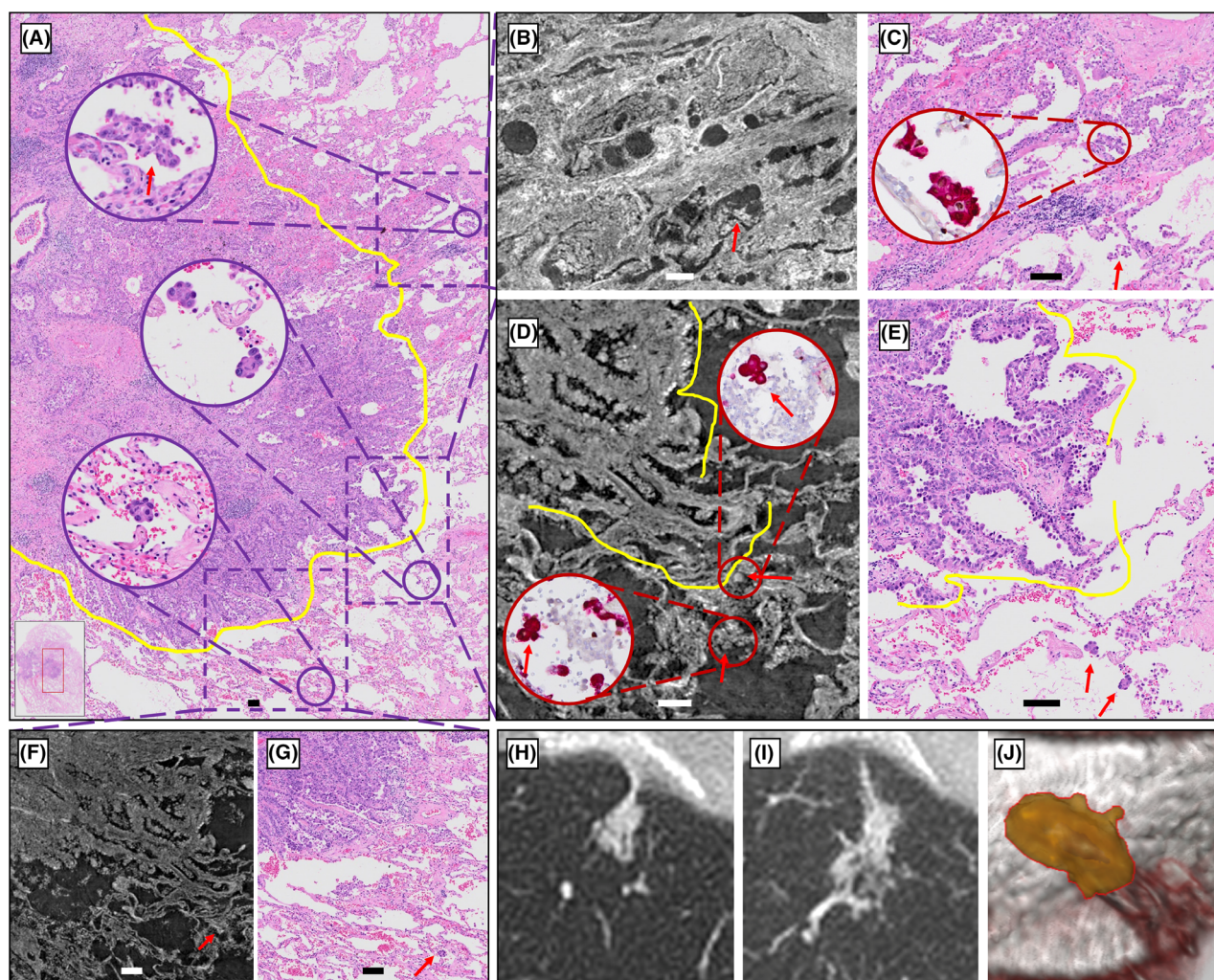


Figure 4. Histological, CT, and μ CT images of an adenocarcinoma neoplasm with marginal micropapillary growth. STAS are visible as micropapillary clusters highlighted by enlargements of the areas. (A) Histological image of a tumour mass growing adhesively to a blood vessel, with the tumour boundary outlined in yellow. Correlative imaging between histological (C, E, G) and μ CT (B, D, F) regions. (H) CT image and (I) MIP image representing the same lesion, with (J) its 3D reconstruction. Scalebars = 100 μ m. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1111/his.70130)]

volume, as shown in Figure 3E and Video S1, providing further insights into the spatial relationship between the STAS and the main tumour, highlighting micropapillary STAS near vessel and alveolar walls, seen as denser regions.

Figure 4A shows invasive lung adenocarcinoma with peripheral micropapillary STAS clusters. μ CT slices (Figure 4B,D,F) aligned to deeper histological sections (Figure 4C,E,G) demonstrate that μ CT preserves tissue compactness, whereas histologic processing stretches and slightly distorts tumour and lung architecture, artificially expanding STAS distribution. Thin-slice axial CT (Figure 4H,I) depicts a solid nodule with irregular margins and small peripheral branching extensions, likely correlating with

peritumoral ground-glass or tiny satellites, as evidenced by 3D CT rendering of Figure 4J. These features illustrate CT biomarkers associated with STAS-positive tumours.⁴

Figure 5A illustrates the distal branch of an extensive acinar-type adenocarcinoma with a micropapillary pattern at its margins. According to WHO 2021 guidelines, such STAS may represent a sectioning artefact; however, this case shows clear tumour connectivity. As detailed in Figure 5D, histology shows micropapillary clusters floating within alveoli and attaching to alveolar walls, while μ CT demonstrates the same microarchitecture a few micrometres deeper within the intact FFPE block in Figure 5E. The same micropapillary STAS clusters exhibit an attachment

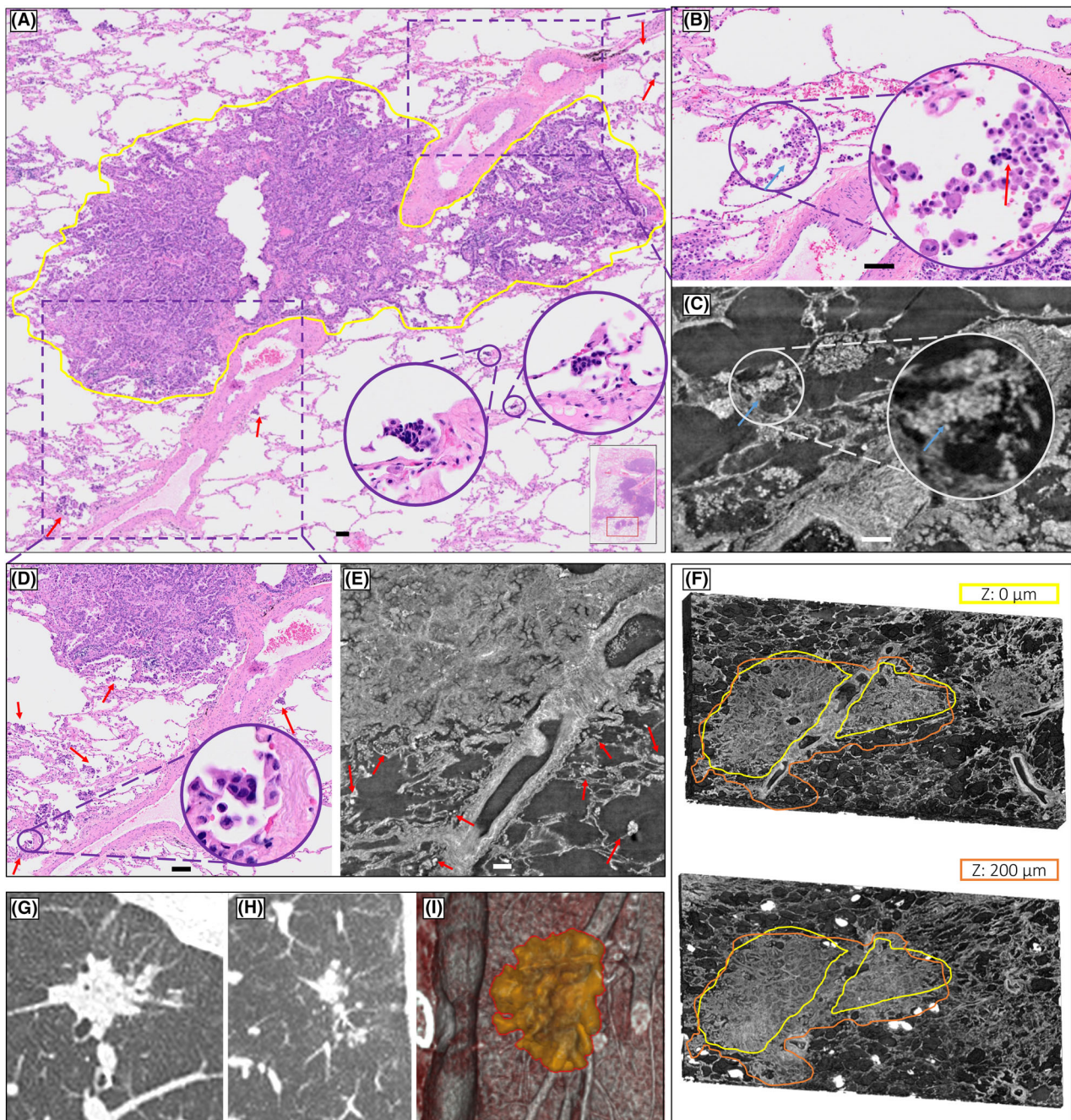


Figure 5. Histological, CT, and μ CT images of an adenocarcinoma neoplasm with marginal micropapillary growth. STAS are visible as micropapillary clusters pointed by red arrows, with some of them in proximity to a big vessel. (A) Histological image of a tumour mass growing adhesively to a blood vessel, with the tumour boundary outlined in yellow. Correlative imaging between histological (B, D) and μ CT (C, E) regions. Enlargement in (C) is a 6 μ m-depth maximum-intensity projection (MIP) image. (F) 3D reconstruction of the progression of the adenocarcinoma neoplasms (in yellow at $Z = 0 \mu\text{m}$ and in orange at $Z = 200 \mu\text{m}$) while virtually cutting the FFPE block. Consult Video S2 for the 3D virtual slicing animation of the volume. (G) CT image and (H) MIP image representing the same lesion, with (I) its 3D reconstruction. Scalebars = 100 μm . [Colour figure can be viewed at wileyonlinelibrary.com]

to the neoplasm in the μ CT images, as evidenced by the highlighted different tumour borders of the 3D volume rendering in Figure 5F at 200 μm depth

within the FFPE block. In addition, such tumour developed contiguously to an artery with a visible trait of invasiveness into lympho-vascular system. Of

particular interest is the 3D conformation of the tumour, which appears to expand visibly during the progressive μ CT virtual sectioning of Figure 5F and Video S2. Because μ CT lacks specific staining, definitive cellular identification cannot be confirmed, but morphology offers indirect clues: macrophages appear as smooth, rounded structures (Figure 5C), whereas micropapillary STAS exhibit irregular contours, reflecting the clustering of multiple neoplastic cells (Figure 5E). CT imaging evidences a solid spiculated nodule (Figure 5G), while it also depicts perilesional micronodules with a tree-in-bud distribution on MIP reconstruction (Figure 5H).²⁹ Figure 5I presents a three-dimensional representation of the NSCLC tumour.

SOLID TUMOURS AS COMPACT PAPULE THAT EXPAND FROM THE PRIMARY TUMOUR

Histology in Figure 6A shows solid STAS in squamous cell carcinoma, either connected by thin neck or fully detached as in Figure 1. These clusters resemble dense purple drops, while they result compact ball-shaped clusters extending beyond tumour edge into adjacent alveolar spaces via μ CT (Figure 6B,C). These clusters likely propagate toward alveolar walls for space and structural support while remaining connected to the main tumour, as shown in Video S3 and its 3D reconstruction (Figure 6G). CT images in Figure 6D,E, demonstrate a distinctive expansion of the lesion toward the pleura, mostly visible in the volume rendering (Figure 6F). Additionally, Figure 6E delineates primary nodules ramification into the surrounding lung parenchyma, while maintaining attachment to the vessel.

AN EXAMPLE OF ARTEFACT: INADVERTENT TUMOUR EMBEDDING

An interesting case of real artefact in an adenocarcinoma neoplasm is reported in Figure 7. In the histological section, such a cluster of tumour cells is located far away from the main tumour, even though it seems to be connected to the alveolar wall with an edge. With this observation, a pathologist would normally consider it as an artefact instead of true STAS due to its location and surroundings. The corresponding μ CT investigation confirms the artifactual nature of this cluster of tumour cells.

The presence of this structure within the intact paraffin block eliminates the possibility of an artefact induced by microtome sectioning. However, the most probable cause of the observed artefact is the

transport of neoplastic material due to the passage of the pathologist's knife (i.e., a knife-cut artefact).³⁰ Indeed, 3D μ CT analysis strongly supports this interpretation. First, the fragment is spatially isolated from the primary tumour and located close to the pleura, which raises the possibility that it originated from a region not included within the μ CT investigation volume. In addition, 3D μ CT revealed only small accumulations of blood and tumour cells within alveolar interstices (blue arrows) that remain completely disconnected from the primary tumour mass, without lepidic growth along alveolar fibrous septa. Even though the histology in Figure 7 demonstrates ruptured alveolar septa and communication between alveolar spaces, these features alone do not indicate airspace spread. Taken together, this interpretation aligns with the 2021 WHO criteria,¹⁴ which emphasise that isolated tumour fragments without continuity to the tumour edge are most consistent with knife-cut artifactual displacement of cellular cluster.

Discussion

Beyond previously reported CT findings associated with STAS, our research suggested a correlation between radiological “tree-in-bud” patterns and the histological STAS. This assumption requires validation through a prospective multicentric study of patients utilising radiological and histological examination methods.

Although some STAS appear as floating structures in 2D histology, μ CT showed most of them are simply tumour margins. Others, initially appearing detached, were seen via μ CT to adhere to vessels or alveolar walls. This insight may impact surgical strategies by informing decisions on resection extent to reduce local recurrence. Since assessing STAS can be challenging even for experienced pathologists, with studies showing notable interobserver variability in distinguishing true STAS from artefacts,³¹ this diagnostic uncertainty highlights the need for complementary approaches. In this context, while histology excels at revealing sub-cellular tumour morphology and cellular patterns, μ CT enables non-destructive virtual sectioning and bridges conventional histology with clinical CT through high-resolution 3D imaging across microscopic and macroscopic scales. Furthermore, μ CT further adds value by clarifying whether clusters represent true detached tumour elements, artefacts, or peripheral extensions.

Some limitations of this study should be considered. The small sample size naturally restricts the strength

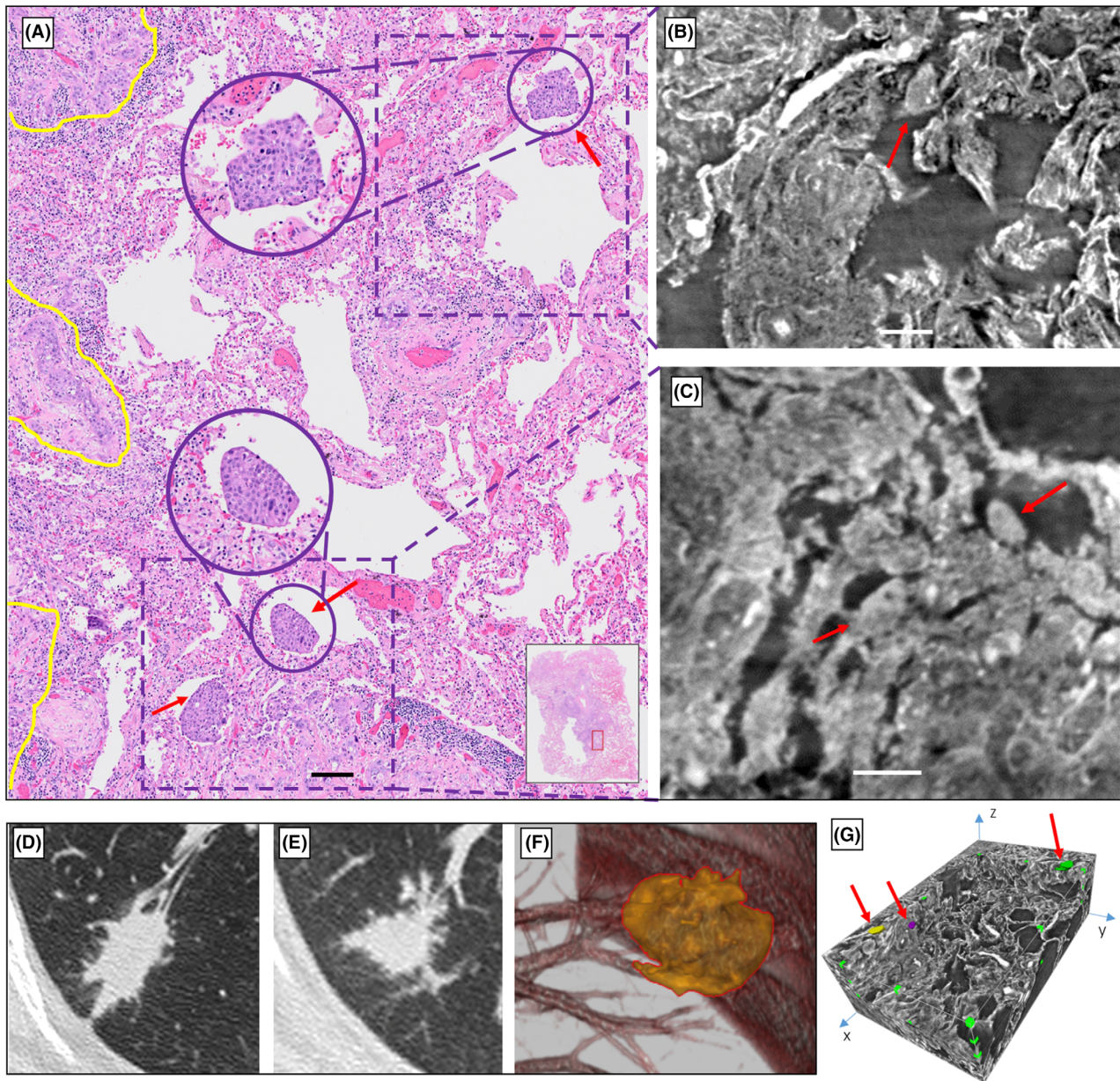


Figure 6. STAS represented as solid nests in a squamous cell carcinoma by means of histology, CT, and μ CT techniques. (A) Selected region from the histological section, with tumour boundary contoured in yellow, in which (B, C) two areas are investigated by means of μ CT due to the presence of solid tumour papule indicated by red arrows. (G) Three-dimensional rendering of the area previously represented in two dimensions, with some coloured solid clusters pointed by red arrows. For a visual representation of the virtual sectioning of the μ CT volume, refer to Video S3. (D, E) CT images representing the same lesion, with (F) its 3D reconstruction. Scalebars = 100 μ m. [Colour figure can be viewed at wileyonlinelibrary.com]

of conclusions and calls for validation in larger cohorts. In addition, synchrotron-based μ CT, while offering excellent resolution, is not yet widely accessible and involves non-negligible costs. Despite efforts to develop virtual histology with conventional X-ray sources,^{32–34} current state-of-the-art imaging still requires synchrotron light for adequate resolution.

Though access is limited, synchrotron facilities support advanced pulmonary imaging.^{35–37} Finally, the analysis of high-resolution 3D datasets requires dedicated time and expertise, which may currently limit its broader adoption. Nonetheless, these constraints are expected to lessen as technology becomes more available and streamlined.

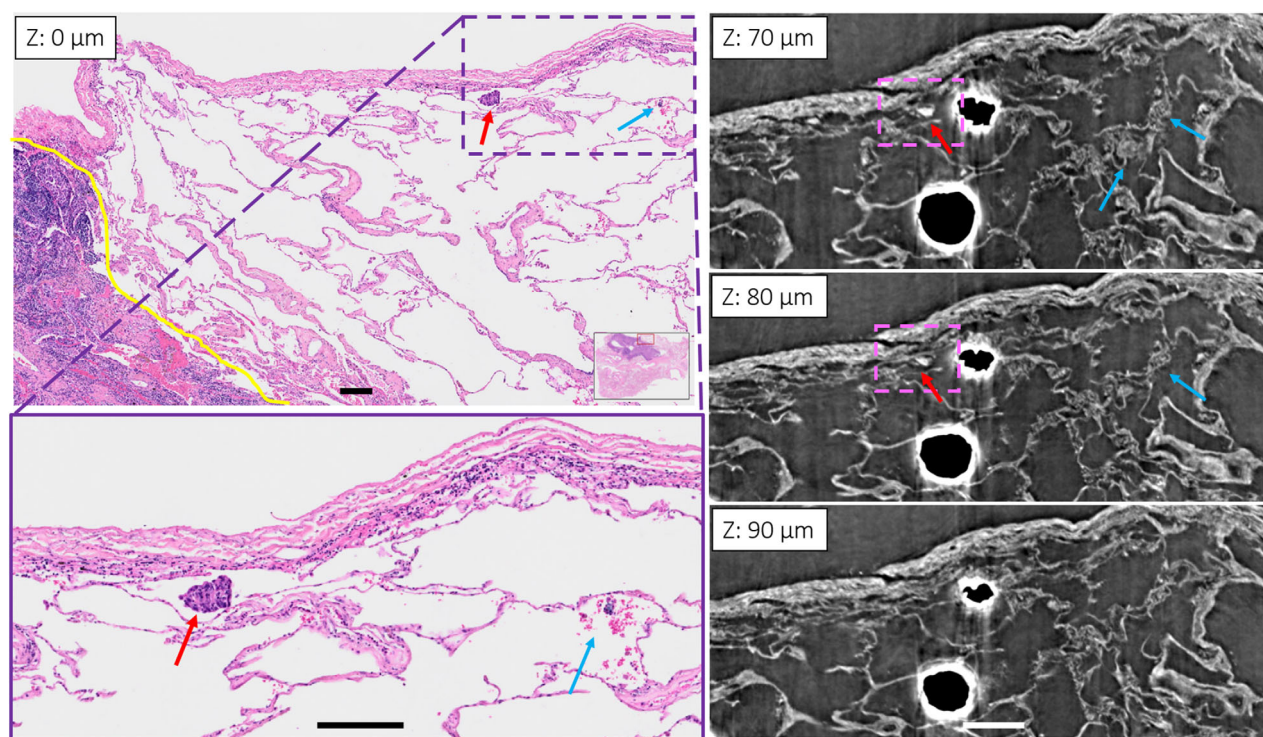


Figure 7. Solid STAS that appears as artefacts in an adenocarcinoma neoplasm by means of histology, CT, and μ CT techniques. Solid STAS that appears as artefacts in histological section (left) and in μ CT images (right). Considering the histological plane at reference plane ($Z = 0 \mu\text{m}$), μ CT images presented are taken at several depths into the paraffin block from the reference plane, thus $Z = 70 \mu\text{m}$, $80 \mu\text{m}$, $90 \mu\text{m}$. Red arrows point at the same solid STAS, while blue arrows point at blood particles accumulation. The tumour boundary is outlined in yellow. Scalebars = $200 \mu\text{m}$. [Colour figure can be viewed at wileyonlinelibrary.com]

In conclusion, X-ray virtual histology enables spatial analysis of tissue without physical sectioning and enhances our understanding of STAS origin and distribution, while histology remains essential for accurate interpretation. Our findings suggest that floating STAS are consistently connected to alveolar or vascular structures, often linked to the main tumour. Further studies with larger cohorts are necessary to confirm these preliminary observations and assess the prognostic role of STAS more precisely.

Author contributions

Giulia Saccomano: conceptualisation, methodology, validation, formal analysis, investigation, data curation, writing – original draft, visualisation. **Francesco Brun:** conceptualisation, methodology, investigation, writing – original draft, supervision, project administration, funding acquisition. **Fulvia Martellani:** conceptualisation, validation, formal analysis, resources, writing – review and editing, supervision. **Cristina Bottin:** resources, investigation, writing – review and

editing. **Stefano Lovadina:** conceptualisation, validation, writing – review and editing, supervision. **Marina Troian:** writing – review and editing, supervision. **Maria Assunta Cova:** writing – review and editing, supervision. **Elisa Baratella:** conceptualisation, methodology, validation, investigation, resources, writing – original draft, visualisation, supervision.

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Conflict of interests

All authors declare that they have no conflicts of interest.

Patient consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

The raw data directory is registered at <https://doi.org/10.34965/I11065>. Data is available upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1.