

MASTERARBEIT | MASTER'S THESIS

Titel | Title

Interaction of human omental fibroblasts with apoptotic cells in peritoneal metastasis

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of
Master of Science (MSc)

Wien | Vienna, 2026

Studienkennzahl lt. Studienblatt |
Degree programme code as it appears on the
student record sheet:

UA 066 606

Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:

Masterstudium Drug Discovery and Development

Betreut von | Supervisor:

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Abstract in German

Kolorektale Peritonealmetastasen sind durch ein ausgeprägtes stromadominiertes Tumormikromilieu gekennzeichnet, welches von Fibroblasten gebildet wird. Metastasenassoziierte Fibroblasten spielen bei der Remodellierung der extrazellulären Matrix und der Immunsuppression eine zentrale Rolle. Ob diese Fibroblasten auch an der Beseitigung apoptotischer Zellen beteiligt sind, wurde bislang nicht vollständig untersucht. Die Efferozytose stellt dabei mehr als einen passiven „Housekeeping“-Prozess dar, vielmehr kann dieser Prozess die Immunantwort sowie das Verhalten stromaler Zellen aktiv beeinflussen und dadurch bei der Tumorprogression mitwirken. Ziel dieser Arbeit war es daher zu untersuchen, ob MAFs in peritonealen Metastasen sowohl funktionell zur Efferozytose befähigt als auch anatomisch vorteilhaft positioniert sind, dass sie an diesem Prozess beteiligt sein können.

Die Efferozytosekapazität patientenabgeleiteter Fibroblasten wurde in vitro mithilfe eines durchflusszytometriebasierten Kokultur-Assays mit fluoreszenzmarkierten apoptotischen Tumorzellen untersucht. Ergänzend wurden histologische Analysen von CPM-Gewebeschnitten aus zwei unabhängigen Patientenkohorten durchgeführt. Hierzu wurden immunhistochemische Färbungen für cleaved Caspase-3, M30, FAP und α -SMA sowie TUNEL-Färbungen und immunfluoreszente Kofärbungen verwendet, um die räumliche Verteilung apoptotischer Zellen und ihre Beziehung zu aktivierten Fibroblasten zu charakterisieren.

Es konnte gezeigt werden, dass diese MAFs apoptotische Tumorzellen effizient und dosisabhängig aufnehmen, damit bestätigt, dass ihre bereits zuvor in diesem Patienten gemachten Modellsystem beschriebene Efferozytosefähigkeit. Die histologischen Untersuchungen zeigten in beiden Patientenkohorten zwei reproduzierbare räumliche Verteilungsmuster: Zum einen fanden sich apoptotische Zellen gehäuft in mizingefüllten, fibroblastenarmen luminalen Strukturen, die räumlich vom stromalen Kompartiment getrennt waren. Zum anderen waren apoptotische Zellen diffus im Stroma verteilt und lagen regelmäßig in unmittelbarer Nähe zu FAP- und α -SMA-positiven Fibroblasten. Die apoptotische Identität dieser Zellen konnte

mithilfe komplementärer Marker über verschiedene Stadien der Apoptose hinweg bestätigt werden.

Zusammenfassend zeigen diese Ergebnisse, dass MAFs in kolorektalen Peritonealmetastasen sowohl funktionell zur Efferozytose befähigt als auch anatomisch so positioniert sind, um diesen Prozess in vivo auszuführen. Zwar reichen diese Beobachtungen allein nicht aus, um eine aktive Aufnahme apoptotischer Zellen nachzuweisen. Jedoch liefert die Übereinstimmung funktioneller und anatomischer Befunde eine belastbare Grundlage für zukünftige Untersuchungen der Frage, ob die fibroblastenvermittelte Efferozytose zum immunsuppressiven Charakter des peritonealen metastatischen Tumormikromilieus beiträgt.

Abstract in English

Colorectal peritoneal metastasis (CPM) is characterised by a stromal-dominant tumour microenvironment in which metastasis-associated fibroblasts (MAFs) act as key regulators of extracellular matrix remodelling and immune suppression. Whether these fibroblasts also participate in the clearance of apoptotic cells has not been investigated. Efferocytosis is not a passive housekeeping process but can actively shape immune tone and stromal behaviour, with direct consequences for tumour progression. This study asked whether MAFs within the peritoneal metastatic niche are both functionally capable and anatomically positioned to contribute to this process.

Patient-derived fibroblasts were assessed for efferocytic capacity in vitro using a flow cytometry-based co-culture assay with fluorescently labelled apoptotic tumour cells. Histological analyses of CPM tissue sections from two independent patient cohorts were performed using immunohistochemistry for cleaved caspase-3, M30, FAP and α -SMA, combined with TUNEL and immunofluorescence co-labelling, to characterise the spatial distribution of apoptotic cells and their relationship to activated fibroblasts. MAFs engulfed apoptotic tumour cells efficiently and in a dose-dependent manner, confirming the efferocytic capacity previously described for peritoneal MAFs in this patient-matched context. Histological analysis revealed two reproducible spatial patterns across both cohorts: apoptotic cells clustered within mucin-filled, fibroblast-poor luminal structures, physically separated from stromal contact, and apoptotic cells dispersed throughout the stromal compartment in consistent proximity to FAP- and α -SMA-positive fibroblasts. Apoptotic identity was confirmed across multiple stages using complementary markers.

Together, these findings establish that MAFs in CPM are both functionally capable of efferocytosis and anatomically positioned to engage in it in vivo. Spatial proximity alone cannot demonstrate active engulfment, but the convergence of functional and anatomical evidence provides a grounded basis for investigating whether fibroblast-mediated efferocytosis contributes to the immunosuppressive character of the peritoneal metastatic microenvironment.

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List of Abbreviations

ACs	Apoptotic Cells
CRC	Colorectal Cancer
α -SMA	α -smooth muscle actin
Apaf-1	Apoptosis protease activating factor 1
BSA	Bovine Serum Albumin
CAFs	Cancer associated fibroblasts
DAB	3,3'-Diaminobenzidine
DAMPs	damage-associated molecular patterns
DED	Death Effector Domain
DISC	death-inducing signaling complex
DPBS	Dulbecco's phosphate-buffered saline
EDTA	ethylenediaminetetraacetic acid
FAP	fibroblast activation protein
FasL	Fas Ligand
FCS	Fetal Calf Serum
FFPE	Formalin-Fixed Paraffin-Embedded
HIPEC	hyperthermic intraperitoneal chemotherapy
IHC	Immunohistochemistry
IF	Immunofluorescence
MAFs	metastasis-associated fibroblasts
MMT	mesothelial-to-mesenchymal transition
MOMP	Mitochondrial Outer Membrane Permeabilization
OCT	optimal cutting temperature
PBMCs	peripheral blood mononuclear cells
PFA	paraformaldehyde
PSMs	Peritoneal surface malignancies
TdT	terminal deoxynucleotidyl transferase
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand

TUNEL

Terminal deoxynucleotidyl transferase dUTP Nick
End Labeling

Introduction

Colorectal Cancer and Peritoneal Surface Malignancies

Colorectal cancer (CRC) is the third most prevalent cancer worldwide.¹ Approximately 20% of patients present with metastases at diagnosis, with a five-year survival rate of roughly 15%. Common metastatic sites include the liver, lungs, and peritoneum.² Among these, peritoneal metastases are associated with a particularly poor prognosis, partly due to limited therapeutic options. Combined treatment strategies involving cytoreductive surgery to remove macroscopic lesions and hyperthermic intraperitoneal chemotherapy (HIPEC) to target microscopic disease have improved outcomes. However, recurrence remains frequent, and experts continue to investigate and debate the most effective drug choices and treatment protocols for HIPEC.^{2,3}

The peritoneum (Fig. 1), the largest serous membrane in the human body,⁴ consists of a monolayer of mesothelial cells supported by a basement membrane and an underlying submesothelial stroma rich in fibroblasts, extracellular matrix components, and immune cells.⁵ Beyond its structural role, the peritoneum functions as a biologically active microenvironment in which mesothelial and stromal cells secrete cytokines, chemokines, and adhesion molecules, mediating immune regulation and intercellular interactions within the peritoneal cavity.⁶ Mesothelial cells exhibit considerable plasticity and can undergo mesothelial-to-mesenchymal transition (MMT) in response to injury or tumour-derived signals, acquiring fibroblast-like features that promote extracellular matrix deposition and fibrosis.⁷ Together with the rich stromal cell content and active cytokine milieu of the submesothelial compartment, these features collectively render the peritoneum a dynamic and tumour-supportive microenvironment that is particularly susceptible to metastatic colonization.

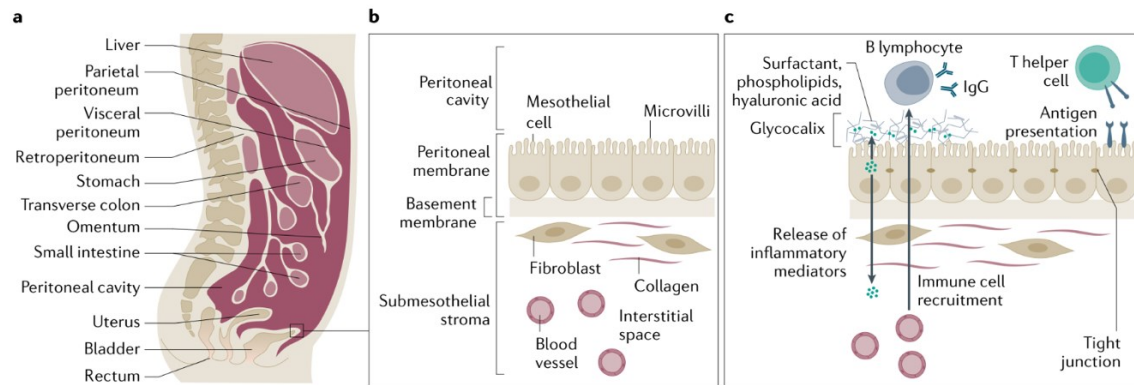


Figure 1 Anatomy and structure of the peritoneum. (A) Anatomical location of the peritoneum within the abdominal cavity, showing the parietal and visceral peritoneum in relation to surrounding organs. (B) Layered structure of the peritoneal membrane, consisting of a surface monolayer of mesothelial cells, an underlying basement membrane, and the submesothelial stroma containing fibroblasts, collagen, blood vessels, and interstitial space. (C) Biological activity of the peritoneal surface, including release of inflammatory mediators, immune cell recruitment, and antigen presentation. Adapted from Cortés-Guiral et al., 2021.

Peritoneal surface malignancies (PSMs) comprise tumours confined to the peritoneal lining, regardless of their primary origin, and involve the visceral and parietal peritoneum, omentum, and mesentery.⁸ They are broadly classified into primary tumours, such as malignant peritoneal mesothelioma or primary peritoneal carcinoma, and secondary tumours arising from intra-abdominal malignancies, most commonly colorectal cancer at 22%, ovarian cancer at 16%, and gastric cancer at 13%.⁷ Despite their diverse origins, PSMs share key biological characteristics, including diffuse intraperitoneal spread and a strong dependence on the peritoneal microenvironment, both of which contribute to poor clinical outcomes.⁹ Secondary peritoneal metastasis develops through a well-defined, multistep process (Fig. 2).³ Tumour cells initially detach from the primary lesion, penetrate the serosal surface, and are released into the peritoneal cavity. Once in this environment, they are transported by peritoneal fluid and tend to accumulate in regions of reduced flow, such as the pelvis, greater omentum.^{8,10} Within the peritoneal fluid, tumour cells frequently form multicellular aggregates, a configuration that enhances their survival and increases their capacity to adhere to the mesothelial lining. Following attachment, the cells invade the underlying submesothelial stroma, where they interact with and activate resident stromal cells.^{10,11} This leads to the differentiation of fibroblasts into cancer-associated fibroblasts (CAFs), which contribute to extracellular matrix remodeling, immune regulation, and tumour progression. As the metastatic niche evolves, distinct populations of metastasis-associated fibroblasts (MAFs) emerge, further shaping the

local microenvironment and supporting the establishment and growth of peritoneal lesions.⁷

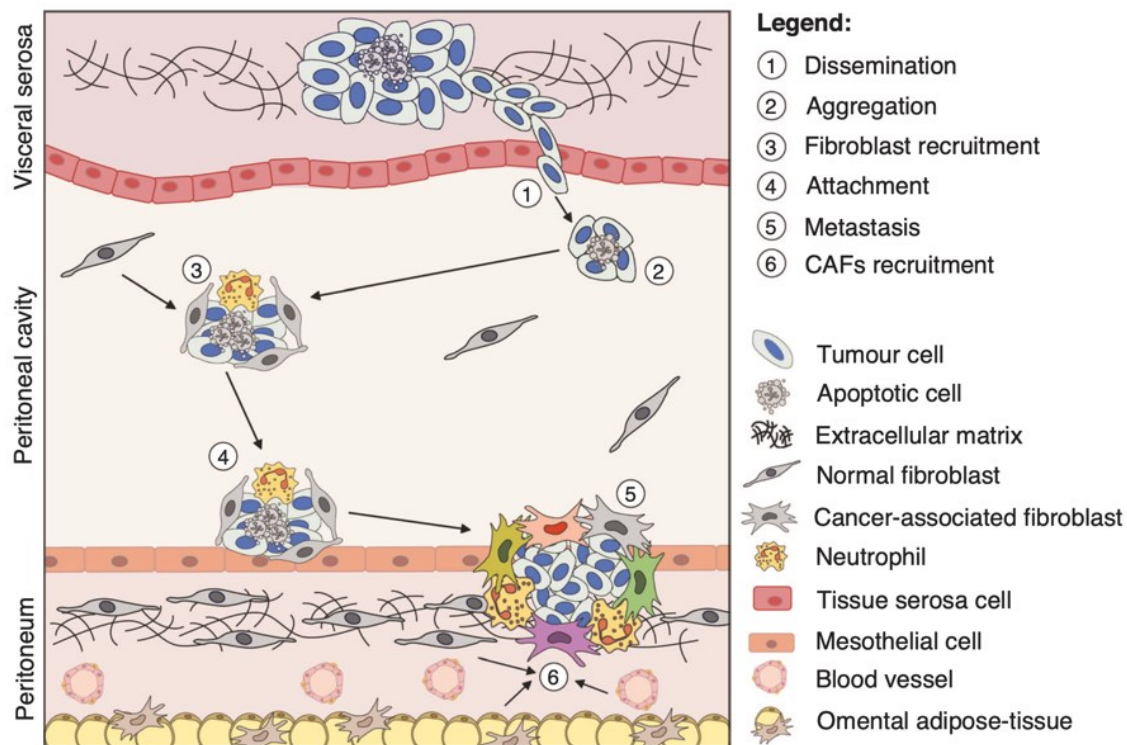


Figure 2 Schematic overview of the multistep process of peritoneal metastasis. Including tumour cell detachment, dissemination within the peritoneal cavity, aggregation into multicellular clusters, adhesion to the mesothelium, and invasion into the submesothelial. Adapted from Ramos et al., 2024.

Fibroblasts in the peritoneal tumour microenvironment

Fibroblasts are a major stromal component of the tumour microenvironment and play a central role in shaping the peritoneal metastatic niche.^{3,12} Ongoing tumour growth and invasion disrupt tissue structure, leading to the release of damage-associated molecular patterns (DAMPs), which are further increased by spontaneous tumour cell death. Fibroblasts sense these signals through pattern recognition receptors, such as Toll-like receptors, and migrate to sites of damage, where they become persistently

activated.⁷ These activated fibroblasts, known as CAFs, drive tumour progression and therapy resistance by releasing growth factors and chemokines, providing metabolic support, and remodeling the extracellular matrix, thereby promoting invasion and fibrosis.¹³ In PSMs, CAFs are particularly abundant and associated with high expression of fibroblast activation protein (FAP) and pronounced fibrotic remodeling.¹⁴ However, no universally specific marker exists for CAFs. Commonly used proteins such as FAP, α -smooth muscle actin (α -SMA), and PDGFR α are not consistently expressed across all subpopulations, which complicates their classification and reflects their considerable functional heterogeneity.¹⁵

Fibroblast heterogeneity within the peritoneal tumour microenvironment is largely driven by diverse cellular origins. In contrast to earlier assumptions that stromal cells are recruited from the circulation, most fibroblasts in peritoneal metastases arise from local precursor cells, including resident submesothelial fibroblasts, adipose-derived stromal cells from the omentum, and mesothelial cells undergoing MMT under the influence of tumour-derived factors such as TGF- β .⁷ This diverse cellular origin gives rise to a heterogeneous population of MAFs with site-specific phenotypes, as further highlighted by recent single-cell RNA sequencing studies.⁷ These MAF subpopulations exhibit substantial functional diversity, encompassing roles in matrix remodeling, fibrosis, and immune regulation. Notably, peritoneal MAFs have been shown to display distinct immunomodulatory properties compared to fibroblasts in primary tumours, including the capacity to suppress immune cell activity and contribute to an immunosuppressive microenvironment.^{3,16} Together, these findings underscore that fibroblasts in the peritoneal tumour microenvironment are not merely structural components but active regulators of tumour progression, whose phenotype and function are strongly shaped by the local niche.

Given their abundance, plasticity, and close spatial interaction with tumour cells, particularly MAFs are key regulators of the metastatic niche.¹⁷ Their central role in tissue remodeling, immune modulation, and tumour progression suggests that they may also participate in additional microenvironmental processes, including the clearance of apoptotic cells, although this function remains insufficiently understood in peritoneal metastases.

Apoptosis

Apoptosis is a tightly regulated form of programmed cell death essential for eliminating damaged or unwanted cells and maintaining tissue homeostasis.¹⁸ In normal physiology, its role is as significant as that of mitosis and cell proliferation, together forming the opposing forces that regulate cell population size across tissues. Throughout development and aging, apoptosis acts as a homeostatic mechanism, but also serves a protective function during immune responses and in reaction to cellular injury caused by disease or harmful agents.¹⁸ Despite the wide range of physiological and pathological stimuli capable of triggering apoptosis, cellular responses are not uniform, the same stimulus does not necessarily induce cell death in all cell types.^{18,19}

Apoptosis produces a characteristic sequence of morphological changes. The earliest features include cell shrinkage and pyknosis, the most defining hallmark of apoptotic cells, accompanied by increased cytoplasmic density and tightly packed organelles. Chromatin condensation leads to pyknosis, visible on hematoxylin and eosin stained sections as round or oval structures with dense eosinophilic cytoplasm and darkly stained nuclear chromatin fragments, appearing as isolated cells or small clusters within the tissue (Figure 3). As apoptosis progresses, extensive plasma membrane blebbing occurs, followed by nuclear fragmentation through karyorrhexis and separation of cellular contents into apoptotic bodies through a process termed budding.¹⁸ Apoptotic bodies consist of cytoplasm with tightly packed organelles, with or without a nuclear fragment, enclosed within an intact plasma membrane, without any lysis or damage to neighboring cells.¹³ They are subsequently recognized and engulfed by neutrophils, macrophages and dendritic cells and degraded within phagolysosomes.²⁰ Caspases are central to the mechanism of apoptosis, acting as both initiators and executioners of cell death.²¹ Of the 18 identified caspases in mammals, the apoptotic subfamily is composed of initiator caspases (caspase-2, -8, -9, and -10) and effector caspases (caspase-3, -6, and -7). Apoptotic stimuli trigger activation of initiator caspases, which leads to cleavage and activation of effector caspases, ultimately executing cell death.²² Both pathways converge on effector caspases, particularly caspase-3, which drives the controlled dismantling of the cell through membrane remodeling, nuclear fragmentation, and formation of apoptotic bodies.²² Importantly, apoptotic cells are not inert remnants of cell death. Rather, their

clearance can mediate not only immunologically silent processes but also specialized functions such as cellular differentiation and organ development.²³

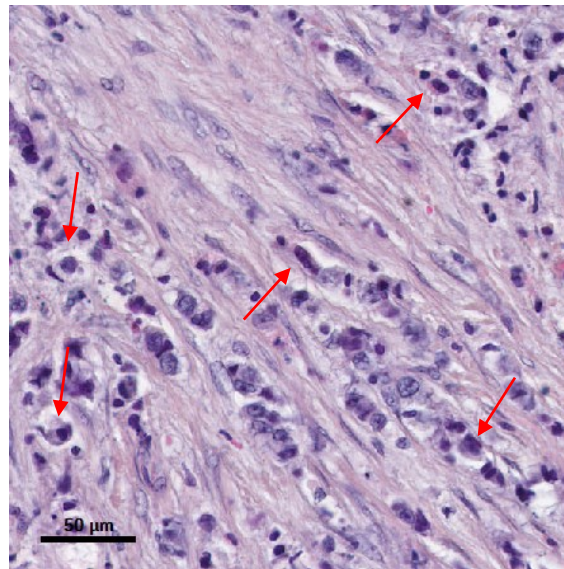


Figure 3 Morphological features of apoptosis in colorectal peritoneal metastasis tissue. Hematoxylin and eosin-stained section showing examples of apoptotic tumour cells (red arrows) characterized by nuclear pyknosis, chromatin condensation, and dense eosinophilic cytoplasm. Apoptotic cells appear as isolated round to oval structure within the tumour stroma, consistent with the classical morphological hallmarks of apoptosis. Scale bar: 50 μ m

Apoptosis can be triggered via two main pathways. The extrinsic pathway is initiated by the binding of extracellular death ligands, including tumour necrosis factor (TNF), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL), to transmembrane death receptors on the cell surface. Upon ligand binding, the intracellular death domain of these receptors recruits adapter proteins FADD and TRADD, which interact with pro-caspase-8 through their shared Death Effector Domain (DED), leading to formation of the death-inducing signaling complex (DISC) and autocatalytic activation of caspase-8. Active caspase-8 subsequently activates downstream effector caspases, which dismantle the nucleus and other intracellular structures, executing cell death.^{20,24} The intrinsic pathway is activated by intracellular stress signals including DNA damage, hypoxia, oxidative stress, viral infections, and chemotherapeutic agents. These stimuli converge on the mitochondria, triggering

Mitochondrial Outer Membrane Permeabilization (MOMP), regulated by the BCL-2 family of proteins comprising approximately 25 pro- and anti-apoptotic members whose balance determines cell fate. Anti-apoptotic members including BCL-2, BCL-xL and MCL-1 suppress apoptosis by inhibiting their pro-apoptotic counterparts, while pro-apoptotic members are divided into effector proteins, BAX, BAK and BOK, and BH3-only proteins including BID, BIM, PUMA and NOXA. Following apoptotic stimuli, BAX and BAK insert into the outer mitochondrial membrane, releasing cytochrome c and other apoptogenic factors into the cytosol. Cytochrome c then binds apoptosis protease-activating factor 1 (Apaf-1) to form the apoptosome, a multi-protein complex that activates initiator caspase-9, which in turn activates effector caspase-3, initiating the caspase cascade that ultimately dismantles the cell. Additionally, mitochondrial proteins Smac/DIABLO and Omi/HtrA2 are released into the cytosol where they antagonize Inhibitor of Apoptosis proteins, further promoting caspase activation and ensuring progression of apoptosis.^{24,25} Beyond drug- or radiation-induced cell death, spontaneous apoptosis is a recurrent feature of CRC and is thought to reflect the genomic instability and elevated cellular turnover characteristic of the disease.²⁶ Apoptotic tumour cells are commonly detected within CRC tissue using markers such as caspase-cleaved cytokeratin-18 (M30), which distinguishes epithelial and tumour cell apoptosis from stromal cell death. Notably, these apoptotic cells tend to accumulate within so-called pseudolumina, irregular glandular structures formed by neoplastic crypts, a pattern histopathologically described as "dirty necrosis" despite an underlying apoptotic rather than necrotic mechanism.²⁶ Rather than acting purely as a tumour-suppressive mechanism, spontaneous apoptosis in CRC appears to shape the surrounding tissue microenvironment. Apoptotic tumour cells release soluble factors, most notably interleukin-8, that recruit neutrophils to sites of cell death; these neutrophils in turn promote an anti-inflammatory, M2-like polarisation of neighbouring macrophages, potentially fostering an immunologically permissive niche that favours tumour progression.²⁶ This is consistent with a broader body of evidence indicating that apoptosis can exert paradoxical, pro-oncogenic effects. Apoptotic cells can stimulate proliferation of surviving neighbours, alter local immune tone, and correlate with a higher mitotic index and shorter overall survival in several cancers.²⁷ In CRC specifically, an elevated apoptotic index in tumour tissue relative to adjacent mucosa has been linked to an independent increase in metastatic risk and shorter survival, with dying cells frequently found within hollow spheroid nests, structures in

which cells progress from an apoptotic to a secondary necrotic state before their remnants are shed.²⁸ This tissue-level observation is mirrored in the blood, where patients with higher pre-treatment CK18 levels tend to have a poorer prognosis, further supporting a link between apoptotic burden and disease outcome.²⁹ More generally, colorectal cancer is characterised by a disturbed balance between proliferation and apoptosis.³⁰

Taken together, these findings suggest that spontaneous apoptosis in CRC should not be regarded solely as a marker of tumour cell attrition, but rather as an active process that shapes the tumour microenvironment by influencing immune cell recruitment and polarisation. Since the biological consequences of apoptosis depend not only on cell death itself but also on the efficient recognition and removal of apoptotic cells, identifying the cellular mediators of apoptotic cell clearance becomes particularly relevant.²⁸

This concept is especially relevant in CPM, where rapid tumour cell turnover, intratumoural hypoxia, and therapeutic interventions such as chemotherapy and HIPEC generate abundant apoptotic cells within the metastatic niche.^{26,31} Efficient clearance of these cells through efferocytosis is therefore essential not only to prevent secondary necrosis and local inflammation but also because the clearance process itself may actively influence the stromal and immune landscape of peritoneal lesions.^{32,33} Consequently, understanding which cell populations participate in efferocytosis may provide important insights into the mechanisms governing the CPM microenvironment.

Efferocytosis

Although large numbers of cells undergo apoptosis on a routine basis, apoptotic cells are rarely observed *in vivo*. This is due to the close coupling between cell death and the highly efficient phagocytic clearance of dying cells, a process referred to as efferocytosis.³² The uptake of apoptotic cells by both professional and non-professional phagocytes is essential for maintaining tissue homeostasis.³⁴ In most tissues, efferocytosis is carried out either by professional phagocytes with high phagocytic capacity, such as macrophages and dendritic cells, or by non-professional phagocytes with lower phagocytic activity, including neighbouring cells like epithelial

cells and fibroblasts.³⁵ Professional phagocytes originate from the bone marrow and express a range of scavenger receptors that facilitate the recognition and uptake of dying cells. Although non-professional phagocytes are not primarily specialized for this function, they have also been shown to contribute to apoptotic cell clearance, particularly during tissue repair processes.³⁶ Efferocytosis fulfills two key functions in tissue homeostasis. First, the rapid clearance of apoptotic cells prevents their progression to secondary necrosis and formation of necrotic cores, thereby avoiding the release of pro-inflammatory mediators that would otherwise amplify inflammatory cascades and compromise tissue repair.³² Second, phagocytes that engulf apoptotic cells shift toward a pro-resolving phenotype, dampening pro-inflammatory signaling while promoting the production of anti-inflammatory mediators to resolve inflammation.³⁴ By upregulating pro-repair transcriptional programmes, this allows the continuous turnover of billions of dying cells to occur in a non-immunogenic manner.³² Following engulfment, macrophages and neutrophils downregulate pro-inflammatory cytokines such as TNF- α , while macrophages may further polarize into diverse anti-inflammatory and pro-healing phenotypes that support the resolution of inflammation and tissue repair.³⁷

Efferocytosis is a coordinated multistep process (Fig. 4) comprising four sequential phases: apoptotic cell finding, binding, internalization, and degradation. In the first phase, apoptotic cells release soluble „find-me“ signals including chemokines such as CX3CL1, bioactive lipids such as sphingosine-1-phosphate and lysophosphatidylcholine, and nucleotides including ATP and UTP, which attract phagocytes to the site of cell death. In the second phase, efferocytes engage apoptotic cells through two types of cell surface receptors. Some receptors bind apoptotic cell surface molecules directly, including stabilin 1, stabilin 2, BAI1, TIM family receptors and LRP1. Others function indirectly through bridging molecules, including GAS6, protein S and MFG-E8, which simultaneously bind phosphatidylserine on the apoptotic cell surface and receptors on the efferocyte, including TAM family receptors TYRO3, AXL and MERTK, integrins α V β 3 and α V β 5, and CD36.³⁴ Phosphatidylserine externalized on the apoptotic cell membrane represents the most important „eat-me“ signal, though additional signals including calreticulin and ICAM3 also contribute.³⁸ Receptor engagement activates RAC1, a member of the RHO family of small GTPases, which drives actin polymerization and formation of the phagocytic cup,

enabling internalization of the apoptotic cell. Following internalization, phagolysosomal assembly and acidification are facilitated through LC3-associated phagocytosis, a process involving PI3K complex assembly and NOX2-dependent reactive oxygen species production, which together promote efficient degradation of the internalized apoptotic material.³⁴

In the tumour microenvironment, where elevated levels of apoptosis result from rapid cellular turnover and therapeutic stress, efferocytosis can function as a double-edged process. Although wound-healing cytokines released during efferocytosis are beneficial in normal tissue repair, within the tumour microenvironment these same mediators can enhance tumour cell motility, facilitate immune evasion, and actively exaggerate the immunosuppressive character of the tumour microenvironment, thereby contributing to metastatic progression.³⁹ Furthermore, many of the receptors and bridging molecules involved in efferocytosis are overexpressed in cancer, suggesting that tumours may exploit these pathways to support their own survival and escape immune surveillance.³⁹

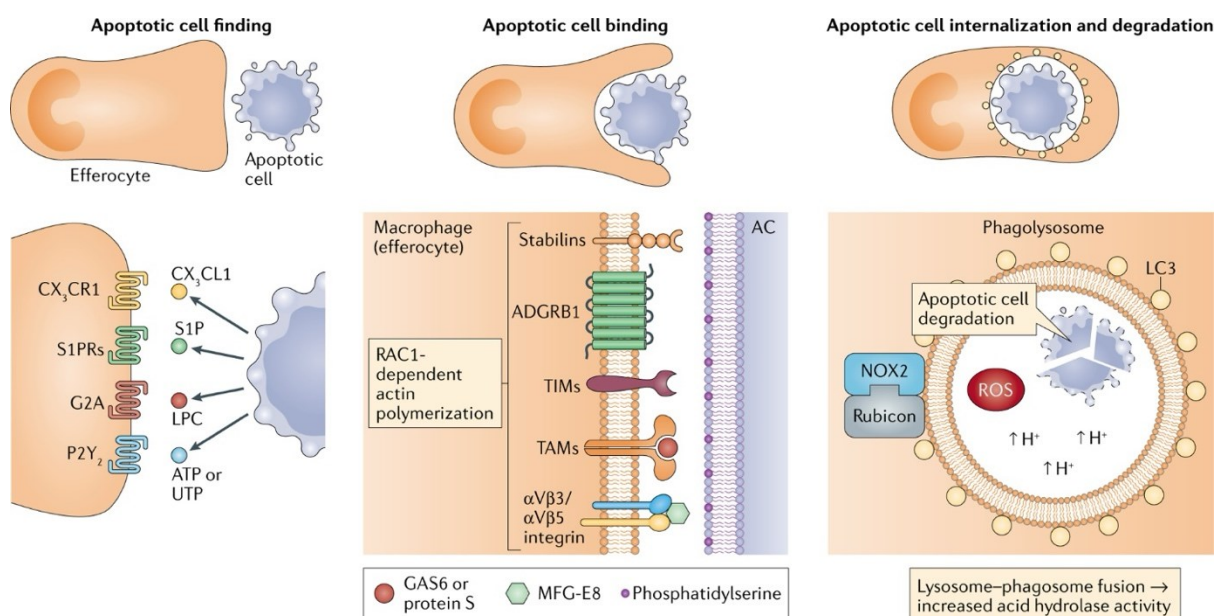


Figure 4 The multistep process of efferocytosis. Dying cells release soluble "find-me" signals (CX₃CL1, S1P, LPC, ATP/UTP) that recruit efferocytes. Binding is mediated by direct receptors (stabilins, ADGRB1, TIMs, TAMs) or bridging molecules (GAS6/Protein S, MFG-E8) recognising phosphatidylserine on the apoptotic cell surface, triggering RAC1-dependent actin polymerisation and engulfment. The internalised cell is subsequently degraded within the phagolysosome via NOX2-dependent ROS production and LC3-associated phagocytosis. AC, apoptotic cell; LPC, lysophosphatidylcholine; ROS, reactive oxygen species; S1P, sphingosine-1-phosphate. Adapted from Doran, Yurdagul & Tabas, *Nature Reviews Immunology*, 2020.

Detection of Apoptosis

The sequential biochemical and structural changes that characterise apoptosis provide the basis for its experimental detection. Detection methods can be broadly categorised according to the apoptotic feature they target: morphological approaches visualise characteristic nuclear and cytoplasmic changes directly, molecular biology techniques detect the biochemical hallmarks of apoptosis, most notably internucleosomal DNA fragmentation, and immunological methods identify specific proteins whose activation or cleavage is exclusive to the apoptotic process.^{40,41} Morphological assessment on HE stained sections allows identification of cells displaying pyknosis, chromatin condensation, and apoptotic body formation, but relies on subjective criteria and is limited to later apoptotic stages. Molecular approaches such as the TUNEL assay localise DNA strand breaks directly within tissue sections, while immunological detection of apoptosis-specific proteins enables both spatial localisation and stage-specific identification of apoptotic cells within complex tissue.⁴⁰

In this study, three complementary immunological and molecular markers were applied, targeting apoptosis at successive stages of the cell death process. The M30 antibody detects an early apoptotic event specific to epithelial cells. Cytokeratin 18 is an intermediate filament protein constitutively expressed in epithelial cells. During the early execution phase of apoptosis⁴², effector caspases cleave cytokeratin 18 at Asp396,⁴³ exposing a neoepitope that is absent in the intact, uncleaved protein. The M30 antibody recognises exclusively this cleavage-generated neoepitope and therefore labels only apoptotic epithelial cells, without reacting with viable cells expressing intact cytokeratin 18.⁴² In the context of CPM tissue, this specificity makes M30 particularly useful for identifying apoptotic cells of epithelial origin against the stromal background.

At a subsequent stage, cleaved caspase-3 immunostaining provides a molecular readout of active caspase-3, the central executioner of apoptosis.²⁴ Caspase-3 is constitutively expressed in all nucleated cells as an inactive proenzyme, procaspase-

3, of approximately 32 kDa.⁴⁴ In its inactive form, the protein is held in a catalytically incompetent conformation. Upon apoptotic signalling, upstream initiator caspases, primarily caspase-8 via the extrinsic pathway and caspase-9 via the intrinsic pathway, cleave procaspase-3 at defined aspartate residues within the interdomain linker, generating subunits of approximately 17 and 12 kDa that assemble into the catalytically active heterotetramer.⁴⁴ This cleavage exposes a neoepitope absent in the intact precursor, and antibodies directed against this neoepitope react exclusively with cells in which caspase-3 has been irreversibly activated, without labelling viable cells expressing procaspase-3.⁴⁵ Cleaved caspase-3 immunostaining thus identifies cells at early-to-mid apoptotic stages, prior to extensive cellular disintegration.

The TUNEL assay (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) detects the internucleosomal fragmentation of genomic DNA, a later apoptotic event.⁴⁰ During the execution phase of apoptosis, caspase-activated endonucleases cleave chromatin into oligonucleosomal fragments of approximately 180–200 base pairs, generating free 3'-hydroxyl termini at strand break sites. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyses the template-independent addition of modified nucleotides (in this study EdUTP) to these 3'-OH ends, which are subsequently visualised via „click chemistry“, enabling in situ detection of apoptotic nuclei in tissue sections.^{40,41} The molecular basis for this fragmentation pattern lies in caspase-activated DNase, which cleaves DNA at internucleosomal linker regions, generating fragments whose size corresponds to nucleosome-protected DNA of approximately 167 base pairs. ⁴⁶ Because DNA fragmentation occurs downstream of caspase-3 activation, TUNEL-positive cells represent more advanced apoptotic stages than cleaved caspase-3-positive cells, making the two markers temporally complementary. ⁴¹

Together, M30, cleaved caspase-3, and TUNEL detect apoptosis at successive molecular stages and provide a temporally layered characterisation of apoptotic cell death within tissue, as applied across consecutive sections in this study.

Pilot Experiments

Initial evidence that peritoneal MAFs possess efferocytic capacity was provided by Chiara Zotter (2023)⁴⁷, who characterised the ability of patient-derived CAFs and

MAFs to engulf apoptotic CRC cells in vitro. Both fibroblast populations were shown to take up apoptotic material in a metabolically active, serum-dependent manner, with uptake detectable as early as four hours after co-culture. Critically, MAFs exhibited an approximately two-thirds higher efferocytosis capacity compared to CAFs, a difference confirmed in matched CAF/MAF pairs isolated from the same patients, indicating that it reflects a consistent and reproducible functional distinction between the two populations rather than inter-individual variation.

These findings raised the question of whether fibroblast-mediated efferocytosis also occurs in the context of peritoneal metastasis in vivo. To address this, the present study examined the spatial distribution of apoptotic cells relative to activated fibroblasts in CPM tissue sections, reasoning that anatomical co-localisation of MAFs and apoptotic cells in situ would be a necessary prerequisite for efferocytic engagement in the peritoneal metastatic niche. The efferocytosis assay using MAF49, derived from the same patient whose tissue sections were analysed histologically, confirmed that this patient's MAFs possess the efferocytic capacity previously established for peritoneal MAFs in vitro,⁴⁷ thereby directly linking functional competence to the in vivo tissue context examined in this study.

Objective and Aims

Given the abundance of activated fibroblasts and the continuous generation of apoptotic cells within the peritoneal metastatic niche, we hypothesized that MAFs in CPM are spatially positioned to interact with apoptotic tumour cells in vivo, suggesting a potential role in their clearance. To address this, two complementary approaches were employed. First, the efferocytic capacity of patient-derived MAFs was assessed in vitro to confirm, in a patient-matched design, that MAFs from the same patient examined histologically retain the capacity for apoptotic cell engulfment previously established for peritoneal MAFs. Second, immunostaining-based analyses of CPM tissue sections were performed to characterize the spatial distribution of apoptotic cells and their relationship to activated fibroblasts within the tumour microenvironment, providing histological evidence for their potential efferocytic engagement in vivo.

Materials and Methods

The following section outlines the patient cohort, sample collection, and analytical methods applied in this study.

Human patient samples

Patient material used in this study was obtained from individual patients diagnosed with peritoneal metastatic colorectal cancer who underwent HIPEC surgery at the General Hospital of Vienna. All material was provided voluntarily and with written informed consent in accordance with the European General Data Protection Regulation. Ethical approval for the acquisition and use of these human samples was granted by the Ethics Committee of the Medical University of Vienna. Samples were collected at the Department of General Surgery, Division of Visceral Surgery. A complete case consisted of peripheral blood, peritoneal lavage, and tumour as well as adjacent non-tumourous tissue. However, not all components could be obtained from every patient. Peripheral blood samples were processed for the isolation of peripheral blood mononuclear cells (PBMCs), peritoneal lavage was used to obtain fibroblasts, and whole tissue samples were either or both utilized for the establishment of primary fibroblast cultures or preserved as Formalin-Fixed Paraffin-Embedded (FFPE) sections and frozen tissue sections (cryosections) for histological analyses.

A list of samples used in this study and the corresponding patient information is shown in table 1. Sample selection was carried out independently of patient characteristics such as age, sex, or treatment history. Samples included in the final analysis were selected based on tissue quality and completeness of available FFPE material, following preliminary experiments to establish staining protocols.

Table 1 List of patient samples; n.e. = not evaluated

Type of Tissue	Age	Gender	Tumour Type	Localization
Carc 24	n.e.	m	Peritoneal carcinomatosis from Colon adenocarcinoma	parietal peritoneum
Carc 29	n.e.	n.e.	Sigma, CRC	Peritoneum
Carc 38	54		invasive tubular adenocarcinoma (origin: rectum)	Omentum
Carc 39	58	f	Ovarian metastasis and intestine metastasis; (Origin: right sided colon carcinoma)	Parietal peritoneum
Carc 41	26	m	n.e.	Peritoneum
Carc 49	68	f	Colon Ascendent Carcinoma	Omentum
Carc 52	71	m	Small bowel cancer	Omentum
Carc 57	51	m	Appendicial carcinosis	n.e.
Carc 64	54	f	Appendix adenocarcinoma with peritoneal metastasis	Parietal peritoneum
Carc 72	40	m	mucocele of the appendix and pseudomyxoma peritonei	Visceral peritoneum
Carc 73	46	m	Kolonkarzinom (TripeCa - Rektum, Transversum, Sigma)	n.e.
Carc 77	57	f	mucocele of the appendix	Omentum
Carc 86	58	f	Left sided colon carcinoma	peritoneum
Carc 97	n.e.	n.e.	n.e.	Visceral peritoneum
Carc 97	n.e.	n.e.	n.e.	Omentum
Carc 101	n.e.	n.e.	n.e.	Parietal peritoneum
Carc 101	n.e.	n.e.	n.e.	Omentum

CAFs were isolated from primary tumour tissue, whereas MAFs were isolated from peritoneal metastases or from the peritoneal lavage of patients diagnosed with peritoneal metastatic CRC. For the purpose of this study, only MAFs were used for further experiments. Freshly obtained tissue samples were washed twice with sterile 1× Dulbecco's phosphate-buffered saline (DPBS) to remove blood and debris. The tissue was then cut into small fragments using sterile scissors to facilitate enzymatic digestion. The minced tissue was incubated in a dissociation solution consisting of 1× DPBS supplemented with 2% fetal calf serum (FCS), 1 mM ethylenediaminetetraacetic acid (EDTA), 100 U/mL collagenase IV, and 50 U/mL DNase I. Samples were placed on a MACSmix™ Tube Rotator and incubated at 37 °C for 90 minutes to allow efficient tissue dissociation. Following digestion, the cell suspension was passed through a 100 µm cell strainer to remove remaining tissue fragments. The filtrate was centrifuged at 300 × g for 5 minutes at room temperature, and the cell pellet was treated with VersaLyse solution for 5 minutes to lyse residual red blood cells. The cells were then washed with RPMI 1640 medium supplemented with 10% FCS and 10 mM HEPES and centrifuged again under the same conditions. After the final wash, the cells were resuspended and seeded into culture dishes containing EGM™ MV2 medium supplemented with penicillin and streptomycin. Cells were cultured under standard conditions, and the medium was replaced every 2–3 days to remove non-adherent cells. Once the cultures reached approximately 80–90% confluence, the adherent fibroblast-like cells were expanded and used for subsequent in vitro experiments.

Tumour fixation for FFPE and cryo sectioning

Fresh human tissue samples were placed in sterile 10 cm Petri dishes and dissected into smaller fragments using a scalpel. For paraffin embedding, tissue pieces were immediately fixed in 4.5% paraformaldehyde (PFA) for 24 hours to preserve tissue architecture and protein integrity. After fixation, samples were transferred into histology cassettes and stored in 70% ethanol at 4 °C until further processing. Tissues were subsequently dehydrated using a KOS Microwave Histostation (Milestone) according to the manufacturer's instructions and embedded in paraffin with the TEC2900 Embedding Center (Histo-Line Laboratories). The resulting FFPE blocks were sectioned into 3–4 µm thick slices using a rotary microtome (Leica RM2255). Cutted

sections were stored at room temperature until used for immunohistochemistry (IHC), immunofluorescence (IF), and TUNEL analyses.

For cryosection preparation, freshly dissected tissue fragments were placed into cryomolds and completely covered with optimal cutting temperature (OCT) compound. The samples were immediately shock-frozen in liquid nitrogen to preserve cellular morphology and antigenicity and stored at -80°C until further use. Cryosections were cut using a cryostat and mounted onto microscope slides. Cutted were stored at -20°C , until used for IHC, IF, and TUNEL analyses.

CG08 cell line and culture conditions

In addition to patient-derived MAFs, the in-house established CG08 cell line was used in this study. CG08 cells were originally isolated from ascitic fluid obtained from a patient diagnosed with peritoneal CRC at the General Hospital of Vienna. Following isolation, CG08 cells were maintained in RPMI 1640 medium supplemented with GlutaMAX™, 10% FCSm and 10 mM HEPES. MAFs were isolated from peritoneal metastatic tumour tissue of CRC patients, as described above, and cultured in EGM™. All cell cultures were maintained at 37°C in a humidified incubator with 5% CO_2 under standard culture conditions.

Efferocytosis Assay

The preparations for the assessment of the efferocytosis capacity of MAFs were performed as described in the following subsections.

Induction of apoptosis in CG08 cells

Apoptosis was induced by replacing the medium with RPMI supplemented with $100\text{ }\mu\text{g/mL}$ TNF α . After incubation for 1h at 37°C the TNF α -mix was removed, washed 2 times with 1x PBS and 1 ml of RPMI was added. The cells were irradiated with UV-C at 250 mJ/cm^2 (without the lid) and another 1 ml of RPMI medium was added. The cells were then incubated at 37°C for 24h.

Preparation of CFSE-labeled apoptotic CG08 cells

Apoptotic cells were harvested and transferred into 2 mL reaction tubes. Cells were centrifuged at $10,000 \times g$ for 10 minutes at 4°C and resuspended in $1\times$ PBS. For labeling of apoptotic bodies, a $2\ \mu\text{M}$ CFSE solution was added, followed by incubation for 5 minutes in the dark. The staining reaction was terminated by adding 1 mL FCS. Cells were then centrifuged at $10,000 \times g$ for 5 minutes at 4°C and washed twice with RPMI medium. After the final wash, apoptotic bodies were resuspended in RPMI and added to PKH26-labeled fibroblasts at the indicated ratios (1:0, 1:1, 1:5 fibroblasts to ACs). The co-culture was incubated for 18 hours at 37°C .

Preparation of PKH26-labeled fibroblasts

Fibroblasts were incubated overnight at 37°C , harvested, and resuspended in $1\times$ PBS. The cell number was determined prior to centrifugation at $400 \times g$ for 5 minutes. During centrifugation, a working solution of PKH26 dye was prepared according to the manufacturer's instructions. For staining, the cell pellet was resuspended in Diluent C, and the PKH26 dye solution was added immediately. The cell suspension was gently mixed by pipetting for 1 minute, followed by an incubation period of 2 minutes at room temperature. The staining reaction was terminated by adding 1 mL FCS, after which the suspension was diluted with RPMI medium and centrifuged at $400 \times g$ for 5 minutes. The cell pellet was resuspended in RPMI medium, and cells were counted again. Subsequently, 1×10^6 cells in 1 mL were seeded per well in a 24-well plate.

Flow cytometry sample preparation and measuring

For flow cytometry analysis, cells from the different experimental conditions were harvested using TrypLE, washed twice with PBS, and centrifuged at $400 \times g$ for 5 minutes. The cell pellet was resuspended in RPMI medium and transferred to FACS tubes. For viability staining, cells were centrifuged again ($400 \times g$, 5 minutes) and washed twice with PBS. The pellet was then resuspended in Zombie Yellow solution (1% in PBS) and incubated for 15 minutes at 4°C in the dark. The staining reaction was stopped by adding FACS buffer. Cells were subsequently washed twice with PBS to remove excess dye and finally resuspended in FACS buffer. Samples were

measured with a Galios Flow Cytometer. The Zombie Yellow viability and active caspase-3 staining were analyzed using Kaluza software.

Histology

This section describes the detailed procedures used for tissue preparation, staining, and immunohistochemical analysis to investigate the cellular and molecular characteristics of the samples.

Hematoxylin and Eosin stain

The tissue samples were fixed in formalin overnight and then embedded in paraffin. To prepare the sections, the paraffin was removed using xylene, followed by rehydration through a decreasing ethanol gradient (100%, 80%, 70%, and 50%) and a final rinse in deionized water. For nuclear staining, the slides were immersed in Harris hematoxylin for one minute, then placed in Scott's solution for ten minutes at room temperature. Cytoplasm and extracellular matrix staining were achieved by incubating the slides in eosin solution for 30 seconds. Finally, the stained sections were dehydrated through an ethanol gradient (80%, 96%, and 100%), cleared in xylene, and mounted using coverslips and Entellan®.

Immunohistochemistry

For IHC staining of formalin-fixed, paraffin-embedded tissues, followed the processes of deparaffinization and rehydration as previously described. To block endogenous peroxidase activity, the slides undergo a 10 min in dark-incubation treated with a 0.3% hydrogen peroxide solution in 1x DPBS for 10 minutes. For Antigen retrieval the samples were heated up to 120°C in a 10 mM sodium citrate buffer (pH 6.0). Then the tissue section on the microscope slide was circled with a pap pen to create a hydrophobic barrier keep incubation reagents contained. The 2.5% normal goat serum for 20 minutes in a humid chamber. Protein blocking was performed by treating the slides with 2.5% normal goat serum for 20 minutes in a humid chamber. The slides then followed an overnight, 4°C incubation with the primary antibody, again in a humid chamber. The primary antibodies used included FAP, Sirius Red, cleaved Caspase-3, and α -SMA. After incubation, excess primary antibody was washed off using a buffer solution containing 1x DPBS and 0.1% Tween®20. The slides were then incubated for

45 minutes with a secondary antibody (goat-anti-mouse/goat-anti-rabbit IgG) for polymeric labeling, followed by another wash with the buffer solution. A two-component system comprising a liquid stable DAB chromogen and DAB substrate buffer was used for polymeric labeling and 3,3'-Diaminobenzidine (DAB) staining of the tissue sections. The staining reaction was halted with deionized water. Nuclei were counterstained using Hematoxylin Gill III, and the reaction was stopped with fresh water before intensifying the staining with Scott's solution. Finally, the tissue sections were dehydrated using a graded ethanol series (70%, 80%, 96%, and 100%), cleared in butyl acetate, and mounted with coverslips and Entellan®.

TUNEL and Immunfluorescence co-labeling

Before the start of the experimental procedure the FFPE tissue slides were incubated overnight (or min. 1h) at 60° before. For deparaffinization the samples followed the procedure in table 1. The tissue samples were fixed in formalin overnight and then embedded in paraffin. To prepare the sections, the paraffin was removed using xylene substitute, followed by rehydration through a decreasing ethanol gradient (table 2), an incubation in NaCl, and a final rinse in DPBS. For Antigen retrieval the samples were heated up to 120°C in a 10 mM sodium citrate buffer (pH 6.0). After that the tissue samples were three times washed in washing buffer for three minutes. Then the tissue section on the microscope slide was circled with a pap pen to create a hydrophobic barrier keep incubation reagents contained.

Table 2 Rehydration procedure through a decreasing ethanol gradient

Solution	Incubation time
Xylene substitute	5 minutes
Xylene substitute	5 minutes
50%:50% Xylene sub.: EtOH	3 minutes
100% EtOH	5 minutes
100% EtOH	3 minutes
96% EtOH	3 minutes
80% EtOH	3 minutes
70% EtOH	3 minutes

50% EtOH	3 minutes
0,85% NaCl	5 minutes
1x PBS	5 minutes

For fixation, the tissue sections were immersed in 4.5% paraformaldehyde for 15 minutes at 37 °C, followed by two washes in DPBS for 5 minutes each. Permeabilization was performed by treating each tissue section with Proteinase K solution for 15 minutes in a humidified chamber. The slides were then washed in DPBS for 5 minutes. This was followed by a second fixation step in 4.5% paraformaldehyde for 5 minutes at 37 °C, two additional washes in DPBS for 5 minutes each, and a final rinse in deionized water. To prepare a positive control, strand breaks got induced by incubating the fixed and permeabilized tissue sections with DNase II (1:50 in DPBS) for 30 minutes at room temperature, followed by a wash in deionized water. To perform the TdT reaction, 25 µL of the TdT reaction mix was added to the tissue samples and the positive control, while the negative controls were incubated in deionized water. The TdT reaction mix was freshly prepared according to Table 3. Each tissue section was covered with a piece of Parafilm to ensure uniform distribution and to prevent evaporation, then incubated for 60 minutes at 37 °C. After incubation, all slides were rinsed in deionized water, followed by a 5-minute wash with 3% BSA and 0.1% Triton™ X-100 in PBS, and finally a rinse in DPBS.

Table 3 TdT reaction mix preparation

Reaction components	Number of slides			
	1	2	5	10
TdT Reaction Buffer (Component A)	23,5 µL	47 µL	119 µL	235 µL
EdUTP (Component B)	0,5 µL	1 µL	2,5 µL	5 µL

TdT enzyme (Component C)	1 μ L	2 μ L	5 μ L	10 μ L
Total volume	25 μ L	50 μ L	125 μ L	250 μ L

The Click-iT™ Plus reaction cocktail was freshly prepared according to Table 4. therefore, a 10× Click-iT™ Plus TUNEL Reaction Buffer Additive was prepared by diluting the original 100× solution 1:10 in deionized water. The reaction cocktail must be used within 15 minutes of preparation.

Table 4 Click-iT™ Plus reaction cocktail preparation

Reaction components	Number of slides			
	1	2	5	10
Click-iTTM Plus TUNEL Supermix	22,5 μ L	45 μ L	112,5 μ L	225 μ L
10X Click-iTTM Plus TUNEL Reaction Buffer Additive	2,5 μ L	5 μ L	12,5 μ L	25 μ L
Total volume	25 μ L	50 μ L	125 μ L	250 μ L

Each tissue section was covered with 25 μ L of reaction cocktail and a piece of Parafilm, including the negative controls. The slides were incubated for 30 minutes at 37 °C in a humidified chamber, protected from light. After incubation, the Click-iT™ Plus reaction cocktail was removed, and each slide was washed with 3% BSA in PBS

for 5 minutes, followed by a final rinse in 1× DPBS. To proceed with the immunofluorescence labeling, each tissue section was blocked with 3% BSA in 1× DPBS for 30 minutes at room temperature in a humidified chamber, protected from light. Then, 50 µL of the primary antibody solution (1:100 in BSA-T) was applied to each slide, except for the negative controls, and the slides were incubated overnight at 4 °C in a humidified chamber, protected from light. The next day, the slides were washed twice in BSA. Then, 50 µL per slide of the secondary antibody solution (1:200 in BSA-T) was applied and incubated for 1 hour at room temperature in a humidified chamber, protected from light, followed by two washes in BSA-T. For nuclear staining, 50 µL per section of DAPI solution (1:1000 in BSA-T) was applied and incubated for 15 minutes at room temperature, protected from light, followed by two washes in 1× PBS. Finally, each slide was mounted with Fluoromount-G and a cover slip. The slides were stored at 4 °C, protected from light.

Lists of reagents and consumables, buffers and solutions, and Antibodies

Reagents, consumables, buffers, solutions, and antibodies used in this study are listed in the Tables below.

Table 5 List of reagents and consumables

List of reagents and consumables		
Product	Supplier	Catalogue Number
Albumin bovine Fraction V, Protease-free	SERVA	11926.03
Bloxall™ Blocking Solution SP-6000	Vector	VECSP-600
Bovine serum albumin (BSA)	Sigma Aldrich	A7906-100G
CellTrace™ CFSE	Invitrogen	C34554
Citric acid	Sigma	C-8532
Click-iT™ Plus TUNEL Assay	Invitrogen	C10618
Collagenase IV	Gibco	17104019

DAPI (1 mg/mL)	Thermo Fisher Scientific	62248
DMEM (1x), high glucose	Gibco	21969035
DNase I	Sigma Aldrich	D5307
Dulbecco's phosphate-buffered saline 1x (DPBS)	Gibco	14190-094
EDTA 0.5 M pH 8	Invitrogen	15576-038
Endothelial Cell Growth Medium MV 2	PromoCell	C-22022
Entellan™	Merck	1.07961
Eosin	Dako	CS701
Ethanol absolute	VWR Chemicals	20821.330
Fetal Calf Serum (FCS)	Linaris GmbH	SBF3111KUA
Fluoromount	Southern Biotech	No. 0100-01
GlutaMAX (100x)	Gibco™	3505-038
Goat Serum 1%	Dako	X0907
Harris Hematoxylin solution	Merck	HHS32
Hematoxylin solution modified acc. To Gill III	Merck	HX99385674
HEPES, 1 M pH 7.3 UltraPure	Thermo Scientific	15624198
Hydrogen peroxide 30%	Merck	1.07209
ImmPRESS Goat Anti-Mouse IgG Polymer Kit	Vector	MP-7452
ImmPRESS Goat Anti-Rabbit IgG Polymer Kit	Vector	MP-7451
Liquid DAB+ Substrate Chromogen System	Dako	K3468
n-Butyl acetate	Merck	1.09.652.2500
PAP Pen	Thermo Fisher Scientific	VECH-4000
Paraformaldehyde (PFA)	SAV Liquid Production GmbH	29401
Paraformaldehyde 4% in PBS	SAV Liquid Production GmbH	29401
Penicillin-Streptomycin (10,000 U/mL)	Gibco™	15140122
Permealisation buffer 10X	eBioscience	00-8333-56

PKH26 Fluorescent Cell Linker Dye	Sigma Aldrich	PKH26GL
RPMI 1640 Media, GlutaMAX Supplement	Gibco	61870010
Scott's solution	Morphisto	11192.01000
Supplement Mix Endothelial Cell Growth Medium MV2	PromoCell	C-39226
Triton X-100	Sigma	036K00851
TrypLE	ThermoFisher	A1217701
Trypsin	Sigma	161069
Tween-20	Merck	8.22184.0500
VersaLyse Lysing Solution	Beckman Coulter	A09777
Xylo	Fisher Chemicals	X/0250/15
Zombie-yellow	BioLegend	423103

Table 6 List of buffers and solutions

List of Buffers and Solutions	
Buffer	Components
FACS Buffer	1x DPBS 2% BSA
Citrate Buffer pH 6	10 mM Sodium Citrate
TUNEL Wash Buffer	5 mg/mL Bovine serum albumin and 0.1% Triton TM -X in PBS pH 7.4
TUNEL Block Buffer	3% BSA in DPBS pH 7.4
TUNEL Permeabilization Reagent	0.25% Triton TM X in DPBS
IF Wash Buffer	1X DPBS (-/-) 1% BSA 0.1% Tween20
IF Block Buffer	1X DPBS (-/-)

	0.1% Tween20
Freezing Medium	10% DMSO 90% FCS

Table 7 List of Antibodies

Antobodies	Dilution	Group Intern Nomenclature	Provider	Catalogue Number
Cleaved-caspase-3, rabbit	1:200	P250	Cell Signaling	9664S
M30, mouse	1:60	P242	Abcam	ab183200
Smooth muscle actin, rabbit	1:100	P235	Proteintech	14395-1-AP
anti-human Fap, mouse	1:100	P164	Invitrogen	BMS168
Secondary Anti-rabbit IgG, goat	1:200	S005	Invitrogen	A21072
Secondary anti- Mouse IgG, donkey	1:500	S008	Thermo Fisher	A-21202

Results

This study aimed to determine whether fibroblasts in colorectal peritoneal metastases are spatially associated with apoptotic cells, thereby suggesting a potential role in their clearance in vivo. To address this, a combined experimental approach was applied. First, the efferocytotic capacity of patient-derived fibroblasts was assessed in vitro using flow cytometry-based co-culture assays. Second, histological analyses of CPM tissue sections, including immunohistochemical analysis, TUNEL assay and Immunofluorescence analysis, were performed to examine the spatial distribution of apoptotic cells and their relationship to activated fibroblasts within the tumour microenvironment. In a subsequent histological analysis, the apoptotic nature of these cells was further confirmed using complementary apoptosis markers. Together, these approaches were designed to evaluate both the functional capability of fibroblasts to engulf apoptotic cells and their anatomical positioning within metastatic lesions.

Efferocytosis Assay

To evaluate the efferocytic ability of patient-derived MAF49 fibroblasts, flow cytometry analysis was employed. MAF49 was selected because it derives from the same patient whose tissue was subsequently examined histologically, allowing functional and anatomical evidence to be linked within a single patient. This experiment aimed to examine the engulfment capacity of MAF49 fibroblasts towards ACs derived from CG08 tumour cells treated with TNF α and UV irradiation. Fibroblasts were labeled with PKH26 and co-cultured with CFSE-labeled ACs at different ratios, 1:0, 1:1, and 1:5 (fibroblasts to ACs), for 18 hours.

MAF 49 fibroblasts engulf apoptotic cells in vitro

The flow cytometric gating strategy for the identification of viable PKH26⁺ fibroblast singlets, defined as Zombie Yellow⁻, is presented in Figure 5 top panels, while the PKH26⁺ and CFSE⁺ populations are shown in the bottom panels. Viable PKH26⁺ and fibroblasts singlets were identified by sequential gating on FCS/SSC, exclusion doublets, and removal of Zombie Yellow⁺ events, as shown in the upper panels of Figure 5.

The analysis revealed that MAF 49 fibroblasts efficiently engulf CFSE-labeled apoptotic bodies after 18 hours, indicated by a clear double-positive PKH26⁺ CFSE⁺ population, shown in Figure 5 D-E. In the absence of ACs, used as a baseline control, nearly all events were PKH26⁺ CFSE⁻, confirming specific labeling with minimal background fluorescence, with only 1.14 % double-positive events. At a 1:1 co-culture ratio, approximately 49.0 % of MAF 49 fibroblasts became double-positive, demonstrating substantial uptake capacity. This difference became even more pronounced at a 1:5 ratio, where 93.5 % of fibroblasts exhibited CFSE positivity, indicating near-complete engulfment under conditions of AC excess.

Quantitative analysis in Figure 6 shows that MAF 49 fibroblasts actively perform efferocytosis, with the percentage of PKH26⁺ CFSE⁺ double-positive cells rising in a clear ratio-dependent manner, from 1.14% (no ACs) to 49.0% (1:1) and 93.5% (1:5), across co-culture conditions. These results confirm that MAF49 retains the efferocytic phenotype previously described for peritoneal MAFs,⁴⁷ thereby directly linking functional competence to the patient tissue examined histologically in this study.

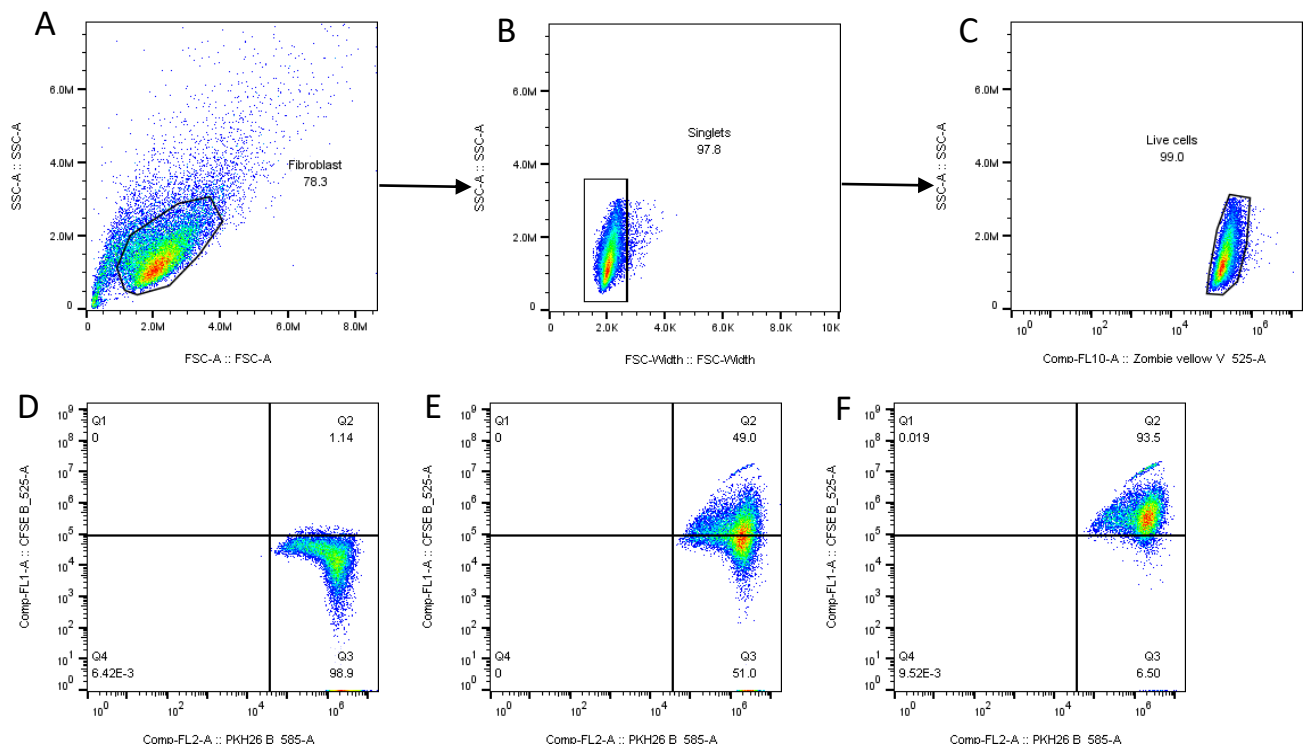


Figure 5 Figure Flow cytometric analysis of efferocytosis by MAF49 fibroblasts using Kaluza software. Flow cytometry plots generated with Kaluza software depict the gating strategy (top row) and PKH26/CFSE expression in viable MAF49 fibroblast singlets (bottom row). Top row: (A) FSC-A vs. SSC-A gating identifies the main fibroblast population (78.3%). (B) FSC-A vs. FSC-W selects singlets (97.8%). (C) Zombie Yellow staining excludes dead cells (99% viable). Bottom row: Overlay of three conditions: (D) MAF49 without ACs with 1.14% double-positive. (E) MAF49 + ACs 1:1 with 49.0% PKH26+ CFSE+. (F) MAF49 + ACs 1:5 with 93.5% double-positive, demonstrating dose-dependent uptake of CFSE-labeled apoptotic bodies by PKH26-labeled fibroblasts.

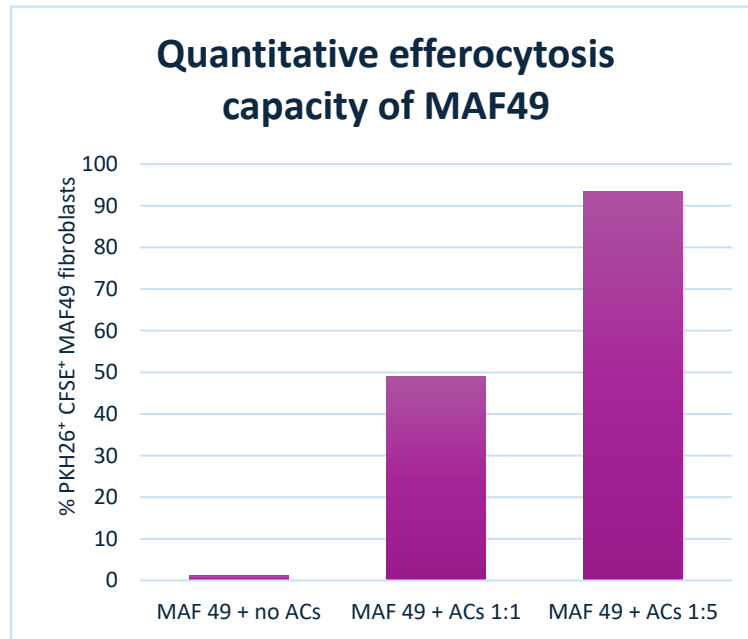


Figure 6 Quantitative efferocytosis capacity of MAF49 fibroblasts. Bar graph illustrating the percentage of PKH26+ CFSE+ double positive MAF49 fibroblasts across conditions: baseline (MAF49 no ACs: 1.14%), 1:1 co-culture with apoptotic bodies (49.0%), and 1:5 ratio (93.5%). Data derived from flow cytometric analysis (Fig.1) highlight dose-dependent uptake efficiency

Histological analysis of CPM tissue

To investigate the spatial relationship between fibroblasts and apoptotic cells in vivo, selected FFPE tissue samples, originated from patients with PSMs, were stained with IHC, IF and TUNEL. Two series of stainings were performed to achieve a different perspective. The first series was used primarily for comprehensive histological characterization of the presence of fibroblasts next to apoptotic cells. For this, 5 tissue samples per CPM patient of a cohort of 10 patients was stained. The initial staining was H&E, to visualise overall tissue morphology and architecture, Sirius Red highlighting collagen fibres, as fibroblasts are the main producers of collagens, IHC for FAP identified activated fibroblasts, while IHC for α -SMA marked myofibroblasts. This series was completed with an IHC stain for cleaved caspase 3, which recognises apoptosis. This combination of stains allowed simultaneous evaluation of tissue structure, fibroblast presence, and the spatial relationship between fibroblasts and apoptotic cells.

The second series was used for observing whether activated fibroblasts are positioned to interact with dispersed apoptotic cells in vivo. For this, 5 tissue samples per CPM

patient of a cohort of 14 patients was stained. The stains included HE, Caspase3, IHC M30 which identifies Cytokeratin18 a marker of early apoptosis epithelial cells, TUNEL assay was used to detect DNA fragmentation during later apoptosis stages, and IF α -SMA on the same tissue section as the TUNEL assay to visualize the spatial organization and detect potential co-localizations.

ACs are organized with two distinct patterns of spatial distribution

IHC analysis revealed the presence of caspase positive cells within tumour tissue in all patients examined. Based on their dark, condensed morphology, these cells were identified as ACs. Cleaved caspase-3–positive cells were detected both within tumour cell populations and the surrounding stroma and were found organized in two distinct spatial distribution patterns. In some regions, caspase-3–positive cells could be identified forming clearly defined clusters, inside of the tumour lumina, boarded by a one line epithelial cell wall filled with mucus. In contrast, in other areas caspase-3–positive cells were distributed throughout the stromal compartment, referred to as dispersed apoptotic regions.

These two spatial distribution patterns are illustrated in Figure 7 A and B. In A, a representative example of a clustered apoptotic region is represented, where cleaved caspase-3–positive cells are concentrated within a well-defined area of the tumour tissue (outlined in red), forming a localised cluster of apoptotic cells. In B, the dispersed apoptotic pattern is demonstrated, characterised by caspase-3–positive cells distributed more evenly and uniformly throughout the stromal compartment. Arrows indicate individual caspase-3–positive apoptotic cells in both panels.

In the clustered apoptotic regions, caspase-3–positive cells were predominantly concentrated within a well-demarcated central area of the tumour tissue. While additional apoptotic cells were also present in the surrounding stroma, the defining feature of this pattern was the dense accumulation of apoptotic cells within the central cluster. Indicating that, the apoptotic clusters are localized in the tumour stroma.

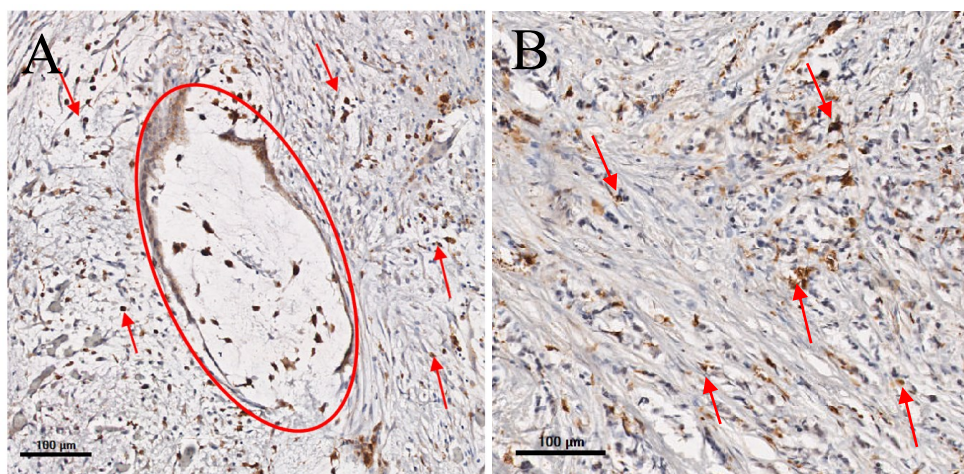


Figure 7 Spatial distribution patterns of apoptotic tumour cells in CPM tissue.

(A) Representative example of a clustered apoptotic region identified by cleaved caspase-3 immunohistochemistry. Caspase-3–positive apoptotic cells are concentrated within a well-defined area of the tumour tissue (outlined in red), forming a localized cluster. **(B)** Representative example of a dispersed apoptotic pattern, characterized by caspase-3–positive cells distributed more evenly throughout the stromal compartment. Arrows indicate individual caspase-3–positive apoptotic cells in both panels.

As

shown in Figure 8, diffuse brown staining was occasionally observed along the luminal tumour cell border. This signal was morphologically distinct from cleaved caspase-3–positive apoptotic cells, which appeared as discrete, darkly stained cells with condensed and shrunken morphology. Within this marginal area, individual caspase-3–positive apoptotic cells could be identified as isolated, strongly stained cells (red arrow), clearly distinguishable from the surrounding non-specific staining. Accordingly, only cells displaying both strong caspase-3 staining and characteristic apoptotic morphology were classified as apoptotic cells.

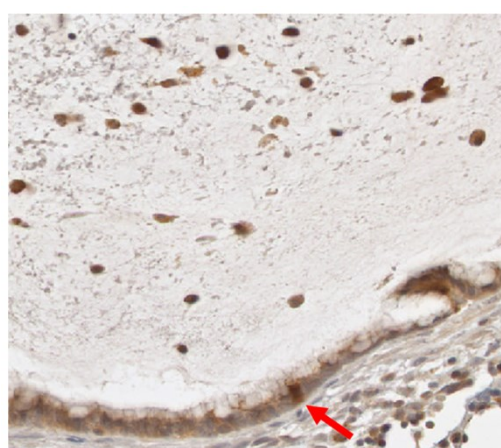


Figure 8 Morphological discrimination of cleaved caspase-3–positive apoptotic cells.

Diffuse brown staining was occasionally observed along the luminal border of tumour cells following cleaved caspase-3 immunohistochemistry. This signal was morphologically distinct from true apoptotic cells, which appeared as discrete, strongly stained cells with condensed and shrunken morphology. Within this marginal region, individual cleaved caspase-3–positive apoptotic cells could be identified as isolated, intensely stained cells (arrow), clearly distinguishable from the surrounding diffuse staining. Only cells displaying both strong cleaved caspase-3 staining and characteristic apoptotic morphology were classified as apoptotic cells.

Dispersed apoptotic areas

In dispersed apoptotic regions, cleaved caspase-3–positive cells appeared as isolated, scattered cells within a matrix-dense stromal background, as seen on H&E overview (Figure 9). Sirius Red staining demonstrated dense collagen deposition in the same areas, while FAP and α -SMA immunostaining confirmed the presence of activated fibroblasts and myofibroblasts in the regions of interest. Caspase-3–positive cells were distributed among these stromal cell populations rather than forming compact aggregates. ACs were located in close spatial proximity to FAP- and α -SMA-positive stromal cells, indicating frequent direct cell–cell contact between apoptotic tumour cells and activated fibroblasts/myofibroblasts. This arrangement placed fibroblasts in an optimal position to potentially engage in efferocytic clearance of apoptotic cells, although functional efferocytosis was not directly evaluated in this study.

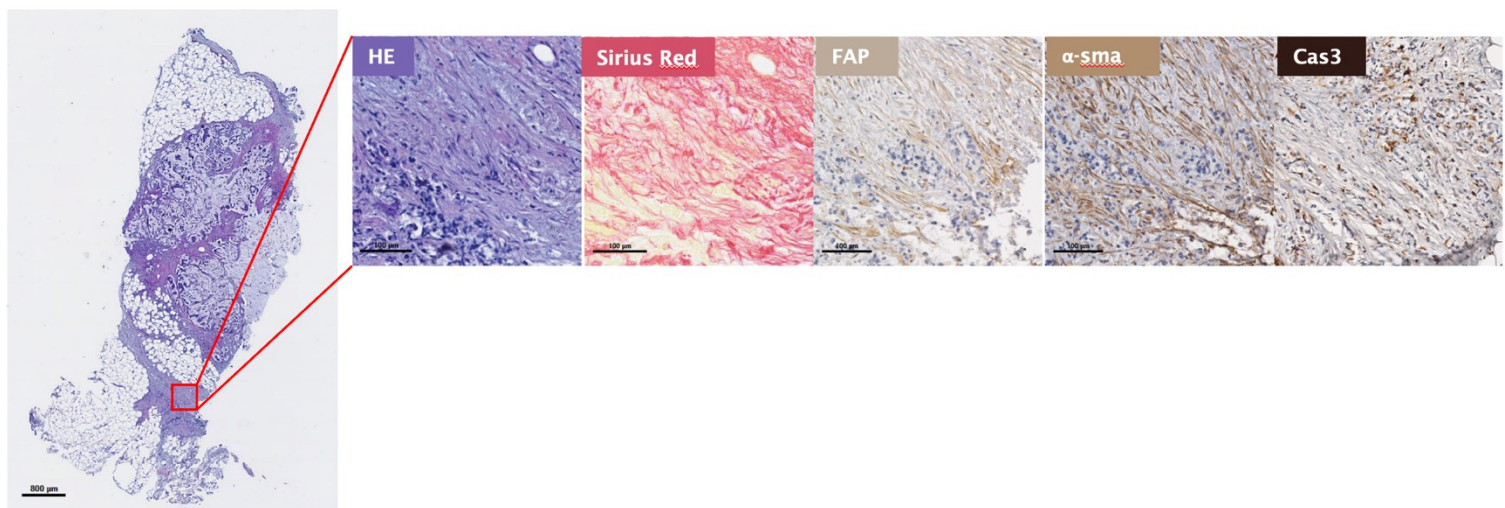


Figure 9 Representative dispersed apoptotic regions in CPM tissue.

H&E overview shows apoptotic cells distributed as isolated, scattered cells within a matrix-dense stromal background. Cleaved caspase-3 immunohistochemistry identifies individual apoptotic tumour cells dispersed throughout the stroma rather than forming compact aggregates. Sirius Red staining demonstrates dense collagen deposition in these regions. Immunohistochemical staining for fibroblast activation protein (FAP) and α -smooth muscle actin (α SMA) confirms the presence of activated fibroblasts and myofibroblasts within the same stromal areas. Caspase-3–positive apoptotic cells are located in close spatial proximity to FAP- and α SMA-positive stromal cells.

Clustered apoptotic areas

Different than in the dispersed areas, caspase-3–positive cells were organized in separate clusters in specific areas within the tumour tissue referring to the clustered apoptotic areas. Figure 10 shows a representative clustered apoptotic area. The H&E

overview represents a typical tissue morphology. Typically, a cluster is located within stromal tissue and is prominently circled with a boarder of mucin producing tumour cells forming a pseudo lumina filled with mucus. The negative staining for FAP and α SMA clearly indicates minimal fibroblast activity within the clusters, corroborated by the low collagen levels. Dark brown, concentrated apoptotic cells are observed within the circled regions. Caspase-3-positive cells inside these enclosed regions showed characteristic apoptotic morphology and could be clearly distinguished from surrounding viable tumour cells. Here, caspase-3-positive cells are also detected outside of the cluster, as they represent the typical tumour microenvironment. With the clusters it is indicated that the apoptotic cells inside the clusters don't have a direct contact to fibroblasts other than the ACs outside of the clusters or located in the stroma have a high contact with fibroblasts.

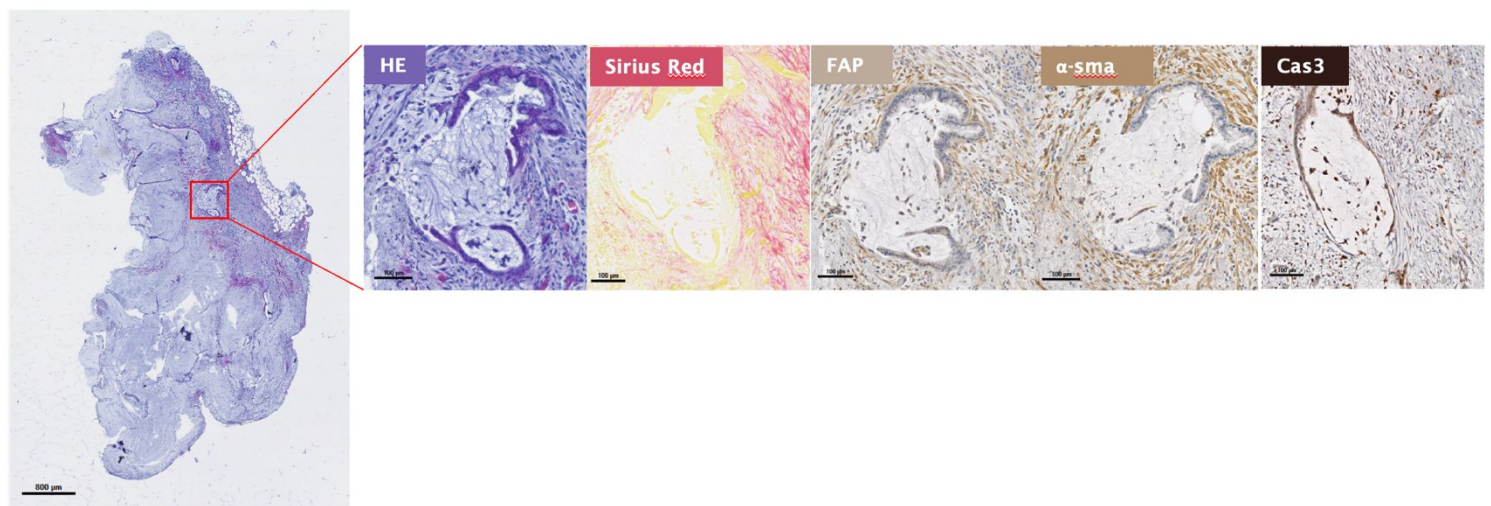


Figure 10 Representative clustered apoptotic region in CPM tissue.

H&E overview showing a typical clustered apoptotic area within the tumour tissue. The cluster is located within stromal tissue and is surrounded by a layer of mucin-producing tumour cells forming a pseudo-luminal structure filled with mucus. Cleaved caspase-3 immunohistochemistry identifies a dense accumulation of apoptotic cells within the luminal cluster, visible as dark brown-stained cells with characteristic condensed morphology. Caspase-3-positive apoptotic cells are concentrated within the clustered region, while additional apoptotic cells can also be detected in the surrounding tumour stroma

Apoptotic tumour cell identity and stage

Initial histological analysis of the first patient cohort (n=10) established two distinct spatial distribution patterns of apoptotic cells and suggested a close proximity between apoptotic cells and activated fibroblasts. However, as these findings relied primarily on caspase-3 immunostaining and morphological criteria, a second independent

patient cohort (n=12) was analyzed to address three specific aims: first, to validate the spatial distribution patterns observed in the first cohort; second, to confirm apoptotic cell identity more rigorously using complementary markers detecting distinct apoptotic stages; and third, to directly visualize the spatial relationship between apoptotic cells and activated fibroblasts on the same tissue section using TUNEL and α -SMA co-labeling.

Caspase-3 staining in the second cohort confirmed the presence of both clustered and dispersed apoptotic patterns in every patient examined, validating the observations from the first cohort. M30, detecting caspase-cleaved cytokeratin 18 as a marker of early apoptosis, and TUNEL, marking DNA fragmentation at later apoptotic stages, showed staining patterns consistent with caspase-3, confirming that the observed cells represented authentic apoptotic cells across multiple stages rather than artefactual staining. Together, these three markers provide complementary evidence for genuine ongoing apoptosis within the tumour tissue.

To enable reliable cross-marker comparison, each patient was analyzed using a series of 20 consecutive 3 μ m sections, ensuring any two analyzed sections were at most 60 μ m apart. As the three markers detect distinct apoptotic features at different stages, labeling the exact same cell with all three was not technically feasible. Instead, consistent staining patterns across consecutive sections were used to confirm apoptotic identity. TUNEL and α -SMA co-labeling was performed on the same tissue section to allow direct visualization of spatial proximity between apoptotic cells and activated fibroblasts. Within the tumour tissue, α -SMA-positive cells were observed adjacent to apoptotic cells across both cohorts, confirming the consistent presence of fibroblasts in close proximity to apoptotic cells. Figure 11 provides an overview of the staining series for each patient, showing consecutive H&E, Caspase-3, M30, and TUNEL + α -SMA sections, while the complete series for all patients is included in the appendix.

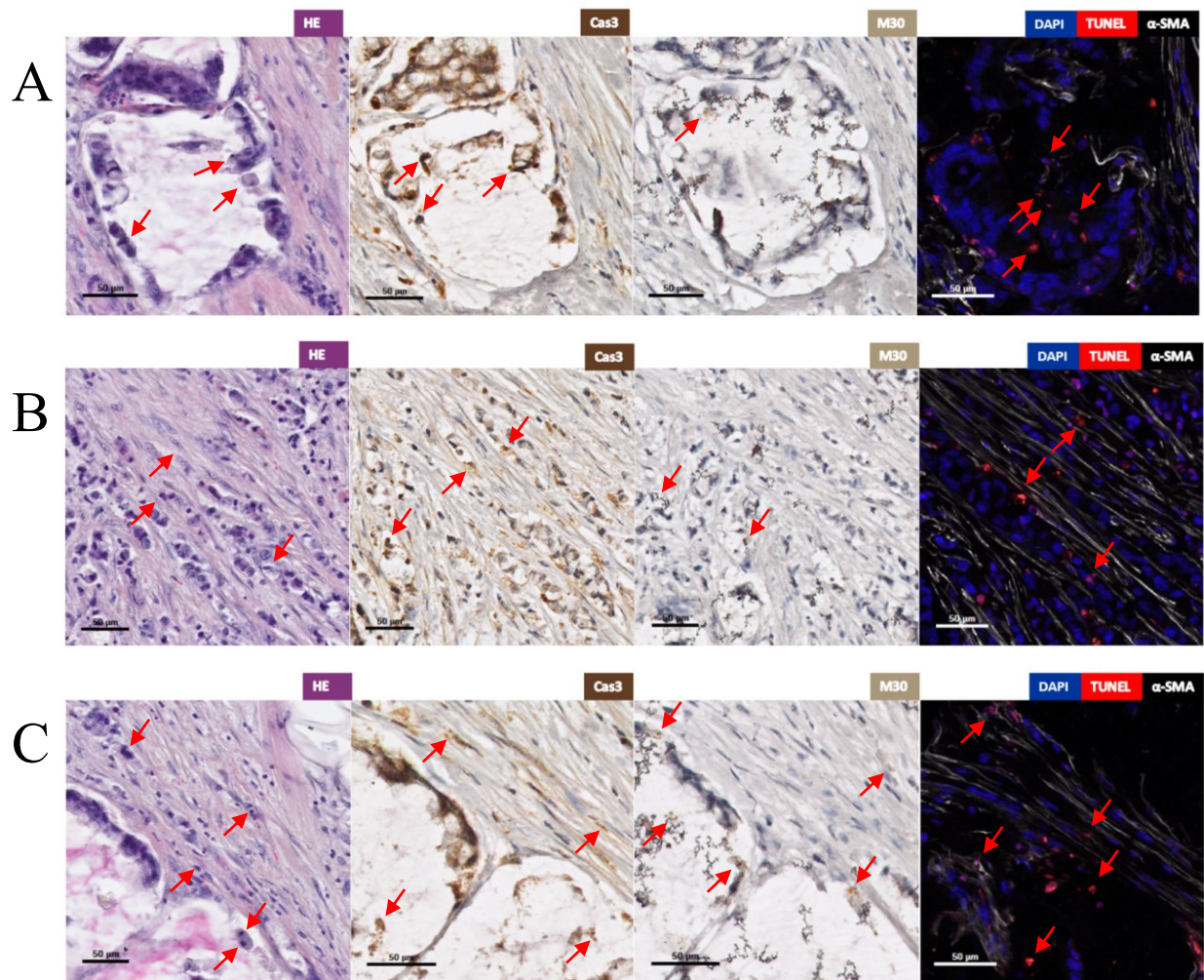


Figure 11 Representative images of apoptotic cells and fibroblasts in tumour tissue from CPM patients. Cas3 staining identified apoptotic cells, with additional M30 IHC and TUNEL assay confirming apoptosis at different stages. Co-labeling with α -SMA IF staining revealed fibroblasts adjacent to apoptotic cells. Consecutive tissue sections were analyzed to compare apoptosis markers across consecutive samples. Apoptotic cells indicated with red arrows. **(A)** Clustered apoptotic areas, **(B)** Dispersed apoptotic areas, **(C)** Apoptotic cluster within fibroblast-rich tumour stroma.

Spatial proximity with activated fibroblasts

Taken together, the two spatial distribution patterns identified across both cohorts reveal a striking difference in fibroblast accessibility to apoptotic cells. In clustered apoptotic regions, apoptotic cells are physically enclosed within tumour-bordered structures lacking FAP and α -SMA expression, effectively isolating them from fibroblast contact. In contrast, dispersed apoptotic cells within the stromal compartment were consistently found in direct proximity to α -SMA-positive myofibroblasts, including along the submesothelial layer, an anatomical niche known to be enriched in activated fibroblasts in peritoneal metastases. This spatial arrangement is particularly relevant in light of the *in vitro* efferocytosis data, which demonstrated that patient-derived fibroblasts are capable of engulfing apoptotic cells efficiently and in a dose-dependent manner. The close positioning of dispersed apoptotic cells to activated fibroblasts *in vivo* indicates that the anatomical prerequisite for efferocytic interaction exists within the tumour microenvironment.

Taken together, the *in vitro* and *in vivo* findings converge on a consistent picture. Patient-derived fibroblasts demonstrated robust efferocytotic capacity *in vitro*, efficiently engulfing apoptotic cells in a dose-dependent manner. Within CPM tissue, ACs segregated into two spatially distinct populations: clustered aggregates physically isolated from fibroblast contact, and dispersed stromal cells consistently localized adjacent to activated fibroblasts. The latter population, confirmed across both patient cohorts using Caspase-3, M30, and TUNEL, was found in direct proximity to α -SMA-positive myofibroblasts within collagen-dense stroma and along the submesothelial interface. Together, these observations establish both the functional capacity and the anatomical prerequisite for fibroblast-mediated efferocytosis within the peritoneal tumour microenvironment.

Discussion

This study investigated the spatial distribution of apoptotic tumour cells in CPM and the potential *in vivo* role of MAFs in their clearance. Building on previously demonstrated *in vitro* efferocytic capacity of peritoneal MAFs,⁴⁷ this study extends

these findings to their anatomical context by providing histological evidence that fibroblast activation status in CPM tissue correlates with the spatial distribution of apoptotic cells. Histological analyses revealed two reproducible spatial patterns of apoptosis: clustered apoptotic cells within mucin-rich luminal structures and dispersed apoptotic cells embedded in fibroblast-rich stroma. Complementary in vitro experiments demonstrated that patient-derived MAFs possess the capacity to engulf apoptotic cells in a ratio-dependent manner. Together, these findings suggest that fibroblasts within the peritoneal metastatic niche are both spatially positioned and functionally capable of participating in apoptotic cell clearance, potentially influencing stromal remodeling and immune regulation.

Fibroblasts exhibit efferocytosis capacity towards apoptotic cells in vitro

The first aim of this study was to determine whether fibroblasts identified in CPM tissues are functionally capable of apoptotic cell uptake. Using MAF49, derived from the same patient examined histologically, this study confirmed, in a patient-matched design, the ratio-dependent efferocytic capacity previously established for peritoneal MAFs.⁴⁷ This patient-matched design is a particular strength of the study, as it allows functional observations to be directly related to the tumour microenvironment from which the fibroblasts originated, rather than relying on unrelated cell lines. Uptake increased markedly as the ratio of apoptotic material increased, while non-specific uptake remained negligible, confirming the functional competence of these cells.

These findings are consistent with previous work by Zotter (2023),⁴⁷ who demonstrated dose-dependent efferocytosis as a characteristic phenotype of CAFs and MAFs across a cohort of patient-derived samples. The present in vitro experiment was not intended to re-establish this capacity at a population level, but to confirm it specifically in MAF49, the fibroblast line derived from the same patient whose tissue was analysed histologically, thereby linking functional and anatomical evidence within a single patient.

Although efferocytosis is traditionally associated with professional phagocytes such as macrophages and neutrophils, growing evidence indicates that non-professional phagocytes, including fibroblasts, can also contribute to apoptotic cell clearance. While

fibroblasts generally exhibit a lower engulfment capacity and slower kinetics in vitro, they possess key advantages in vivo, including their numerical abundance and close proximity to dying cells within tissues. These features likely enable fibroblasts to contribute substantially to apoptotic cell clearance under physiological and pathological conditions.⁴⁸

Evidence from other disease contexts demonstrates that efferocytosis alters fibroblast behavior in terms of activation state and cytokine secretion,^{49–51} although whether comparable consequences occur in CPM-associated fibroblasts remains unknown, and could prove central to understanding how apoptotic cell clearance shapes the metastatic niche.

Distinct spatial organization of apoptotic cells in CPM

Histological examination of CPM specimens revealed two reproducible spatial patterns of ACs. In the first pattern, ACs accumulated in dense clusters within luminal structures rich in mucin. In the second pattern, ACs appeared as dispersed individual cells embedded within the tumour stroma. These patterns were consistently observed across the analyzed patient cohort, suggesting that they represent reproducible features of CPM tissue architecture.

Clustered apoptotic cells were largely confined to intratumoural luminal compartments and appeared physically separated from the surrounding stroma. This pattern is consistent with observations in other solid cancers, where dying tumour cells have been reported to accumulate within the central cavities of glandular tumour structures, enclosed by a surrounding layer of viable tumour cells.²⁶

FAP and α -SMA staining was absent within these clusters, suggesting that fibroblasts are either not activated or not present within this compartment. The epithelial tumour cell layer forming the glandular wall, together with the dense mucin matrix, may act as a structural barrier that limits fibroblast access or suppresses fibroblast activation within the luminal space. However, this barrier does not appear to exclude all immune cells, as neutrophils have been shown to infiltrate clustered apoptotic regions in similar tissue contexts, likely due to their small size and high migratory capacity compared to stromal fibroblasts.²⁶ Whether fibroblasts are physically excluded or simply not activated within these luminal compartments therefore remains an open question that cannot be resolved from the markers used in the present study.

The physical enclosure of apoptotic cells within these mucin-filled structures raises the question of whether and how clearance occurs within these regions. In the absence of direct fibroblast contact, professional phagocytes such as neutrophils or macrophages represent the most likely alternative efferocytic cell type.⁵²

Evidence from similar tissue contexts suggests that clustered apoptotic regions are highly infiltrated by neutrophils, recruited through apoptotic cell-derived IL-8/CXCL8 signaling.²⁶ Rather than simply clearing apoptotic debris, these neutrophils have been shown to drive anti-inflammatory polarization of tumour-associated macrophages, thereby actively shaping the local immune microenvironment in a tumour-supportive direction.²⁶ This suggests that apoptotic cell clearance within clustered regions may not be absent, but rather carried out by immune cells rather than fibroblasts, with distinct functional consequences. However in the absence of efficient clearance, uncleared apoptotic cells may undergo secondary necrosis, releasing intracellular components that contribute to local inflammation and tissue damage, further influencing stromal activation and immune cell recruitment within the local tumour microenvironment.⁵³

In contrast, dispersed apoptotic cells were frequently observed within fibroblast-rich stromal regions, including areas with dense collagen deposition. These cells often resided in close proximity to activated fibroblasts, suggesting that stromal cells may participate in the recognition and clearance of apoptotic material.⁵⁴ Fibroblast-rich stromal niches are known to support metastatic persistence and therapy resistance through extracellular matrix remodeling, tumour cell survival signaling, and modulation of immune responses.^{7,55} The presence of apoptotic cells within these same niches raises the possibility that efferocytosis-induced signaling in fibroblasts further reinforces these tumour-supportive properties,⁵⁶ representing a potential mechanistic link between apoptotic cell clearance and stromal activation in CPM.

Apoptotic cells were identified at both early and late stages across both patient cohorts, suggesting that apoptosis within CPM lesions is not restricted to a single synchronized event but may represent a continuous process. Beyond their role as markers of cell death, apoptotic cells are increasingly recognized as active modulators of their microenvironment through the release of soluble mediators that shape immune cell recruitment and influence the cellular composition of surrounding tissue.^{26,56}

Activated Fibroblasts are positioned to interact with apoptotic cells in vivo

To further characterize the microenvironment surrounding apoptotic cells, the expression of fibroblast activation markers, including α -SMA, FAP, and collagen deposition, was examined in CPM tissue sections. Regions containing dispersed apoptotic cells frequently exhibited strong staining for activated fibroblast markers as well as a dense, collagen-rich extracellular matrix, indicative of fibroblast activation and stromal remodeling. These observations are consistent with the concept that MAFs are key organizers of the metastatic niche in CPM, where activated fibroblasts are known to produce extracellular matrix components, secrete cytokines and growth factors, and regulate immune cell recruitment.^{7,57}

FAP expression warrants specific consideration among the markers examined. FAP is a serine protease selectively expressed on activated stromal fibroblasts, associated with extracellular matrix remodeling, tumour invasion, and immunosuppressive signaling within the tumour microenvironment. Unlike α -SMA, which marks myofibroblastic differentiation more broadly and can also be expressed by smooth muscle cells and pericytes, FAP expression is more selective for activated cancer-associated fibroblasts, although its expression may vary by CAF subtype, providing greater specificity in identifying the stromal population of interest.⁵⁸ The consistent detection of FAP-positive fibroblasts in regions containing dispersed apoptotic cells therefore suggests that the fibroblasts positioned adjacent to apoptotic cells represent a functionally activated stromal population capable of active microenvironmental remodeling, rather than non-activated resident fibroblasts.

A notable finding was the frequent co-localisation of apoptotic cells and FAP-positive fibroblasts within the peripheral stromal regions of CPM tissue sections. The presence of both cell populations within the same stromal compartment raises the possibility that direct interactions between fibroblasts and apoptotic cells occur within the peritoneal metastatic niche, a finding that aligns with the efferocytotic capacity observed in vitro.⁴⁷ In this context, it is worth noting that apoptotic cells and their membrane-bound vesicular remnants are no longer considered passive byproducts of

cell death, but rather active participants in intercellular signaling, capable of transmitting bioactive molecules including damage-associated molecular patterns, growth factors, and profibrotic microRNAs that have been shown to promote fibroblast activation and extracellular matrix remodeling.⁵⁹ In wound healing contexts, efferocytosis of apoptotic cells has been shown to promote TGF- β release and myofibroblast differentiation, suggesting a direct link between apoptotic cell clearance and stromal activation.⁵¹

Fibroblast-mediated efferocytosis as a potential mechanism in colorectal peritoneal metastasis

Taken together, the findings of the present study support a model in which fibroblasts are anatomically and functionally positioned to contribute to apoptotic cell clearance within the CPM microenvironment. It should be emphasised that the two lines of evidence address distinct questions. The in vitro assay establishes that MAF49 fibroblasts are functionally capable of engulfment, while the histological analysis establishes that activated fibroblasts and apoptotic cells co-occur spatially in vivo. Neither observation, individually or in combination, demonstrates that engulfment actually occurs within CPM tissue. The two datasets converge on a consistent and plausible model without constituting direct proof of it. Histological analyses demonstrated that apoptotic cells are frequently located within collagen-rich stromal regions populated by activated fibroblasts, placing these cells in an anatomical position compatible with efferocytic engagement. Complementing these observations, patient-derived MAF49 fibroblasts efficiently engulfed apoptotic tumour cells in vitro in a ratio-dependent manner, demonstrating that peritoneal MAFs possess the intrinsic capacity to function as non-professional efferocytes. Although these experiments cannot fully reproduce the complexity of the tumour microenvironment, they provide functional support for the hypothesis that fibroblasts participate in apoptotic cell clearance in vivo.

An important question arising from these findings is whether fibroblast-mediated efferocytosis represents merely a mechanism for removal of apoptotic cells or whether apoptotic cell uptake actively alters fibroblast behaviour. Increasing evidence suggests

that efferocytosis is not a passive clearance process but can induce profound phenotypic changes in non-professional phagocytes, including fibroblasts.^{49,50,60}

Studies in primary human fibroblasts have shown that apoptotic tumour cells are recognised through dedicated phosphatidylserine-dependent mechanisms involving the receptor BAI1, with preferential uptake of late apoptotic cells.⁵⁰ These observations indicate that fibroblast-mediated efferocytosis is a regulated biological process rather than unspecific phagocytosis, supporting the interpretation that the efferocytic capacity observed in peritoneal MAFs reflects an intrinsic property of activated fibroblasts. The observation that MAF49 fibroblasts in the present study efficiently engulfed apoptotic material in a dose-dependent manner is consistent with this interpretation, suggesting that their efferocytic activity reflects an actively regulated capacity rather than non-specific uptake.

Beyond apoptotic cell recognition, efferocytosis appears capable of reshaping fibroblast function. In models of myocardial injury, activated α -SMA-positive myofibroblasts acquire efferocytic competence during differentiation and undergo marked functional reprogramming following apoptotic cell uptake. This response is characterised by reduced IL-6 production, increased TGF- β 1 secretion and the acquisition of a pro-repair phenotype, suggesting that efferocytosis reinforces rather than attenuates fibroblast activation.⁴⁹ Interestingly, enhanced extracellular matrix deposition has also been proposed as a downstream consequence of this response.⁴⁹ This concept is particularly relevant in the context of the present findings, where apoptotic cells were predominantly detected within collagen-rich stromal areas containing activated α -SMA-positive fibroblasts. Rather than representing unrelated observations, the spatial association between apoptotic cells, activated fibroblasts and dense collagen deposition raises the possibility that apoptotic cell clearance contributes to maintenance of the highly desmoplastic architecture characteristic of colorectal peritoneal metastases.

At the same time, available evidence indicates that fibroblast responses to efferocytosis are highly context dependent. In tumour-associated fibroblasts derived from lung cancer, apoptotic cell uptake activated Notch1 signalling and induced secretion of WISP-1 while suppressing fibroblast activation markers including α -SMA and fibronectin, and reducing invasive behaviour.⁶⁰ These findings demonstrate that

efferocytosis does not trigger a universal fibroblast programme but instead modulates fibroblast phenotype according to the surrounding tissue environment and disease context.

Collectively, these observations provide an important framework for interpreting the findings of the present study. While the current data do not demonstrate that efferocytosis directly alters fibroblast phenotype in CPM, they support the hypothesis that apoptotic cell uptake may represent more than a simple housekeeping mechanism. Given the activated, collagen-associated phenotype of the MAFs identified in this study, it is plausible that fibroblast-mediated efferocytosis contributes to stromal remodelling and maintenance of the metastatic niche. However, whether efferocytosis reinforces fibroblast activation, modifies cytokine secretion or influences extracellular matrix production in colorectal peritoneal metastasis remains unknown and should be addressed in future mechanistic studies.

Indeed, efferocytosis is recognized as an active biological process that can induce immunomodulatory signaling in the phagocytic cell, thereby contributing to the resolution of inflammation and tissue remodeling.⁶¹ In physiological contexts such as wound healing or tissue homeostasis, this typically supports restoration of tissue integrity and dampening of pro-inflammatory responses.⁶² Within the tumour microenvironment, however, similar pathways may be subverted to support tumour progression,⁶³ for example by promoting immunosuppressive niches and stromal adaptations that favor tumour cell survival and escape from immune control.⁵⁹

This is particularly relevant in CPM, where the metastatic niche is characterized by pronounced stromal dominance,⁷ limited immune cell infiltration, and an overall immunosuppressive microenvironment that contributes to therapy resistance.⁶⁰ Fibroblast-mediated efferocytosis could therefore represent one mechanism by which the stromal compartment contributes to maintaining this tumour-supportive niche. Whether this process influences local immune responses or represents a therapeutically targetable component of the peritoneal tumour microenvironment remains an important question for future investigation.

Fibroblast-mediated clearance of apoptotic cells may not simply serve as a neutral housekeeping process, but could represent one additional mechanism by which

activated fibroblasts contribute to the tumour-supportive character of the peritoneal metastatic niche. Future work will therefore be needed to determine whether fibroblast efferocytosis modulates local immune responses, stromal remodeling, or tumour cell behavior in CPM, and whether this process represents a therapeutically relevant node in the metastatic microenvironment.

Limitations and future perspectives

Several limitations of this study should be considered when interpreting its findings.

The most fundamental constraint of this study is that efferocytosis could not be directly demonstrated *in vivo*. The histological tissue sections used are fixed and static by nature, meaning that any active engulfment of apoptotic cells by fibroblasts can only be inferred from spatial proximity and co-localization patterns, not directly observed. Capturing efferocytosis as a live, sequential process requires purpose-built tools, as illustrated by CharON, a dual-fluorescence reporter system that enabled real-time tracking of apoptotic cell death and subsequent macrophage-mediated engulfment and digestion in living *Drosophila* embryos.⁶⁴ Equivalent live imaging or lineage-tracing approaches in human peritoneal metastasis tissue are currently not feasible, and direct *in vivo* confirmation of fibroblast-mediated efferocytosis in CPM therefore remains an important goal for future work.

A further limitation relates to the multi-marker apoptosis analysis. The three detection methods employed, namely H&E morphology, cleaved caspase-3 immunohistochemistry, and TUNEL, each capture apoptosis at a distinct stage of the cell death process. It is therefore not biologically realistic to expect all three to label the same cell simultaneously. In addition, the chemical requirements of each staining protocol are mutually incompatible, precluding their combination on a single tissue section. Each staining was consequently performed on separate serial sections, meaning that a true co-localization of all apoptosis markers within the same individual cell was not achievable.

Patient-level clinical variables such as age, sex, and prior treatment including neoadjuvant chemotherapy or HIPEC regimens were not systematically accounted for in the analyses. The cohort was clinically heterogeneous and complete data were not available for every patient, which made it difficult to assess how these factors may have influenced the distribution of apoptotic cells or the activation state of fibroblasts within the tissue.

Conclusion

That apoptosis occurs frequently within CPM is already understood. What remained unknown was what happens to the ACs that accumulate within the metastatic niche and whether fibroblasts might play an active role in their clearance. This distinction matters as efferocytosis does not act as a neutral clearance mechanism. The outcome of this process depends on which cell type performs it and in what tissue context with direct consequences for immune tone, stromal behaviour, and ultimately disease progression.

This study approached that question through two complementary lines of evidence. First, patient-derived MAF49 fibroblasts demonstrated a robust and dose-dependent capacity to engulf apoptotic tumour cells in vitro, confirming that peritoneal fibroblasts are functionally equipped to act as efferocytes. Second, histological analysis of CPM tissue revealed that apoptotic cells adopt two spatially distinct patterns within peritoneal lesions: clustered within mucin-filled, fibroblast-poor luminal structures where stromal contact is limited, and dispersed throughout the stromal compartment where they are consistently found in direct proximity to activated FAP- and α -SMA-positive fibroblasts. This spatial distinction, together with the underlying apoptotic cell identity, was independently validated in a second patient cohort using complementary markers detecting distinct stages of apoptosis, strengthening confidence that the observed cells represent genuine ongoing apoptosis rather than artefactual staining. Together, these findings establish both the functional capacity and the anatomical opportunity for fibroblast-mediated efferocytosis to occur within the peritoneal metastatic niche. What the functional consequences of this engagement might be remains an open question. In other tissue contexts, efferocytosis has been shown to drive fibroblast activation, promote myofibroblast differentiation, and stimulate the

secretion of pro-fibrotic mediators including TGF- β . Whether peritoneal MAFs respond similarly after engulfing apoptotic tumour cells is unknown, but it is a question worth asking, particularly given the already immunosuppressive and stromal-dominant character of the CPM microenvironment.

These findings remain observational. Direct observation of efferocytosis in living peritoneal tissue and functional characterisation of its downstream consequences in fibroblasts will be essential to test this model. Nevertheless, the work presented here offers a starting point for investigating whether fibroblast-mediated efferocytosis contributes to the immunosuppressive character of the peritoneal metastatic niche, supported by patient-matched in vitro data and consistent histological evidence. Whether fibroblast-driven efferocytosis represents a therapeutically relevant process in CPM is a question that this work now places within reach.

References

1. Klimeck, L., Heisser, T., Hoffmeister, M. & Brenner, H. Colorectal cancer: A health and economic problem. *Best Practice & Research Clinical Gastroenterology* **66**, 101839 (2023).
2. Ren, Q. & Vermeulen, L. Shaping the microenvironment in peritoneal metastases. *Nat Cancer* **6**, 412–414 (2025).
3. Ramos, C. *et al.* Colorectal cancer peritoneal metastasis is promoted by tissue-specific fibroblasts that can arise in response to various local disorders. *Cancer Letters* **633**, 217686 (2025).
4. Healy, J. C. & Reznick, R. H. The peritoneum, mesenteries and omenta: normal anatomy and pathological processes. *European Radiology* **8**, 886–900 (1998).
5. Van Baal, J. O. A. M. *et al.* The histophysiology and pathophysiology of the peritoneum. *Tissue and Cell* **49**, 95–105 (2017).
6. Cortés-Guiral, D. *et al.* Primary and metastatic peritoneal surface malignancies. *Nat Rev Dis Primers* **7**, 91 (2021).
7. Ramos, C., Gerakopoulos, V. & Oehler, R. Metastasis-associated fibroblasts in peritoneal surface malignancies. *Br J Cancer* **131**, 407–419 (2024).
8. Miguez González, J. *et al.* Primary and secondary tumors of the peritoneum: key imaging features and differential diagnosis with surgical and pathological correlation. *Insights Imaging* **14**, 115 (2023).
9. Gerber, S. A. *et al.* Preferential Attachment of Peritoneal Tumor Metastases to Omental Immune Aggregates and Possible Role of a Unique Vascular Microenvironment in Metastatic Survival and Growth. *The American Journal of Pathology* **169**, 1739–1752 (2006).

10. Bootsma, S., Bijlsma, M. F. & Vermeulen, L. The molecular biology of peritoneal metastatic disease. *EMBO Mol Med* **15**, e15914 (2023).
11. Yamaguchi, H. & Miyazaki, M. Cell Biology of Cancer Peritoneal Metastasis: Multiclonal Seeding and Peritoneal Tumor Microenvironment. *Cancer Science* **116**, 1171–1180 (2025).
12. Tao, L., Huang, G., Song, H., Chen, Y. & Chen, L. Cancer associated fibroblasts: An essential role in the tumor microenvironment. *Oncology Letters* **14**, 2611–2620 (2017).
13. El-Khattouti, A., Selimovic, D., Haikel, Y. & Hassan, M. Crosstalk between Apoptosis and Autophagy: Molecular Mechanisms and Therapeutic Strategies in Cancer. *J Cell Death* **6**, JCD.S11034 (2013).
14. Lee, H. H. & Al-Ogaili, Z. Fibroblast activation protein and the tumour microenvironment: challenges and therapeutic opportunities. *Oncol. Rev.* **19**, 1617487 (2025).
15. Li, Y. *et al.* Cancer-Associated Fibroblasts: Heterogeneity, Cancer Pathogenesis, and Therapeutic Targets. *MedComm* **6**, e70292 (2025).
16. Walterskirchen, N. *et al.* Metastatic colorectal carcinoma-associated fibroblasts have immunosuppressive properties related to increased IGFBP2 expression. *Cancer Letters* **540**, 215737 (2022).
17. Wang, Z. *et al.* Metastasis-associated fibroblasts: an emerging target for metastatic cancer. *Biomark Res* **9**, 47 (2021).
18. Elmore, S. Apoptosis: A Review of Programmed Cell Death. *Toxicol Pathol* **35**, 495–516 (2007).
19. Wong, R. S. Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res* **30**, 87 (2011).

20. Xu, X., Lai, Y. & Hua, Z.-C. Apoptosis and apoptotic body: disease message and therapeutic target potentials. *Bioscience Reports* **39**, BSR20180992 (2019).
21. Tang, H. L. *et al.* Cell survival, DNA damage, and oncogenic transformation after a transient and reversible apoptotic response. *MBoC* **23**, 2240–2252 (2012).
22. Eskandari, E. & Eaves, C. J. Paradoxical roles of caspase-3 in regulating cell survival, proliferation, and tumorigenesis. *Journal of Cell Biology* **221**, e202201159 (2022).
23. Han, M. *et al.* Physiological Roles of Apoptotic Cell Clearance: Beyond Immune Functions. *Life* **11**, 1141 (2021).
24. Goldar, S., Khaniani, M. S., Derakhshan, S. M. & Baradaran, B. Molecular Mechanisms of Apoptosis and Roles in Cancer Development and Treatment. *Asian Pacific Journal of Cancer Prevention* **16**, 2129–2144 (2015).
25. Khatun, J., Gelles, J. D. & Chipuk, J. E. Dynamic death decisions: How mitochondrial dynamics shape cellular commitment to apoptosis and ferroptosis. *Developmental Cell* **59**, 2549–2565 (2024).
26. Schimek, V. *et al.* Tumour cell apoptosis modulates the colorectal cancer immune microenvironment via interleukin-8-dependent neutrophil recruitment. *Cell Death Dis* **13**, 113 (2022).
27. Castillo Ferrer, C., Berthenet, K. & Ichim, G. Apoptosis – Fueling the oncogenic fire. *The FEBS Journal* **288**, 4445–4463 (2021).
28. Wimmer, K., Sachet, M. & Oehler, R. Circulating biomarkers of cell death. *Clinica Chimica Acta* **500**, 87–97 (2020).
29. Greystoke, A. *et al.* Multi-level evidence that circulating CK18 is a biomarker of tumour burden in colorectal cancer. *Br J Cancer* **107**, 1518–1524 (2012).

30. Ismail, N. I., Othman, I., Abas, F., H. Lajis, N. & Naidu, R. Mechanism of Apoptosis Induced by Curcumin in Colorectal Cancer. *IJMS* **20**, 2454 (2019).
31. Kim, Y. J. & Kim, C. H. Treatment for Peritoneal Metastasis of Patients With Colorectal Cancer. *Ann Coloproctol* **37**, 425–433 (2021).
32. Mehrotra, P. & Ravichandran, K. S. Drugging the efferocytosis process: concepts and opportunities. *Nat Rev Drug Discov* **21**, 601–620 (2022).
33. Schilperoort, M. *et al.* The role of efferocytosis-fueled macrophage metabolism in the resolution of inflammation. *Immunological Reviews* **319**, 65–80 (2023).
34. Doran, A. C., Yurdagul, A. & Tabas, I. Efferocytosis in health and disease. *Nat Rev Immunol* **20**, 254–267 (2020).
35. Werfel, T. A. & Cook, R. S. Efferocytosis in the tumor microenvironment. *Semin Immunopathol* **40**, 545–554 (2018).
36. Romana-Souza, B., Chen, L., Leonardo, T. R., Chen, Z. & DiPietro, L. A. Dermal fibroblast phagocytosis of apoptotic cells: A novel pathway for wound resolution. *The FASEB Journal* **35**, e21443 (2021).
37. Lam, A. L. & Heit, B. Having an Old Friend for Dinner: The Interplay between Apoptotic Cells and Efferocytes. *Cells* **10**, 1265 (2021).
38. Zhang, Y.-Q. *et al.* Efferocytosis in tissue engineering: A comprehensive review of emerging therapeutic strategies for enhanced tissue repair and regeneration. *Bioactive Materials* **52**, 155–181 (2025).
39. Efferocytosis Creates a Tumor Microenvironment Supportive of Tumor Survival and Metastasis. *Can Cell Microenviron* <https://doi.org/10.14800/ccm.666> (2015)
doi:10.14800/ccm.666.

40. Guo, M. *et al.* Apoptosis detection: a purpose-dependent approach selection. *Cell Cycle* **20**, 1033–1040 (2021).
41. Kari, S. *et al.* Programmed cell death detection methods: a systematic review and a categorical comparison. *Apoptosis* **27**, 482–508 (2022).
42. Sol, M. Y. Assessment of Apoptosis by M30 Immunoreactivity and the Relationship with the MSI status and the Clinicopathological Characteristics of Colorectal Carcinomas.
43. Eren, F. *et al.* Caspase-cleaved fragments of cytokeratin 18 in patients with chronic hepatitis B. *Clinica Chimica Acta* **411**, 2029–2032 (2010).
44. Maurya, S. K., Tewari, M., Sharma, B. & Shukla, H. S. Expression of procaspase 3 and activated caspase 3 and its relevance in hormone-responsive gallbladder carcinoma chemotherapy. *Korean J Intern Med* **28**, 573 (2013).
45. Ai, X. *et al.* Generation and characterization of antibodies specific for caspase-cleaved neo-epitopes: a novel approach. *Cell Death Dis* **2**, e205–e205 (2011).
46. Heitzer, E., Auinger, L. & Speicher, M. R. Cell-Free DNA and Apoptosis: How Dead Cells Inform About the Living. *Trends in Molecular Medicine* **26**, 519–528 (2020).
47. Zotter, C. Functional Characterization of Cancer- and Metastasis-Associated Fibroblasts in the Context of Efferocytosis. [unpublished Master thesis]. FH Campus Wien - University of Applied Sciences. (2023).
48. Fond, A. M. & Ravichandran, K. S. Clearance of Dying Cells by Phagocytes: Mechanisms and Implications for Disease Pathogenesis. in *Apoptosis in Cancer Pathogenesis and Anti-cancer Therapy* (ed. Gregory, C. D.) vol. 930 25–49 (Springer International Publishing, Cham, 2016).
49. Nakaya, M. *et al.* Cardiac myofibroblast engulfment of dead cells facilitates recovery after myocardial infarction. *Journal of Clinical Investigation* **127**, 383–401 (2016).

50. Hermetet, F. *et al.* Efferocytosis of apoptotic human papillomavirus-positive cervical cancer cells by human primary fibroblasts. *Biology of the Cell* **108**, 189–204 (2016).
51. Kim, Y.-B., Yoon, Y.-S., Choi, Y.-H., Park, E.-M. & Kang, J. L. Interaction of macrophages with apoptotic cells inhibits transdifferentiation and invasion of lung fibroblasts. *Oncotarget* **8**, 112297–112312 (2017).
52. Zhang, M. *et al.* The efferocytosis process in aging: Supporting evidence, mechanisms, and therapeutic prospects for age-related diseases. *Journal of Advanced Research* **69**, 31–49 (2025).
53. Szondy, Z., Garabuczi, Á., Jo³s, G., Tsay, G. J. & Sarang, Z. Impaired Clearance of Apoptotic Cells in Chronic Inflammatory Diseases: Therapeutic Implications. *Front. Immunol.* **5**, (2014).
54. Dogusan, Z., Montecino-Rodriguez, E. & Dorshkind, K. Macrophages and Stromal Cells Phagocytose Apoptotic Bone Marrow-Derived B Lineage Cells. *The Journal of Immunology* **172**, 4717–4723 (2004).
55. Wang, W., Cheng, B. & Yu, Q. Cancer-associated fibroblasts as accomplices to confer therapeutic resistance in cancer. *Cancer Drug Resist* **5**, 889–901 (2022).
56. Morana, O., Wood, W. & Gregory, C. D. The Apoptosis Paradox in Cancer. *IJMS* **23**, 1328 (2022).
57. Wang, Z. *et al.* Metastasis-associated fibroblasts: an emerging target for metastatic cancer. *Biomark Res* **9**, 47 (2021).
58. Nilforoushan, N. *et al.* Cancer-Associated Fibroblasts: Clinical Applications in Imaging and Therapy. *Tomography* **11**, 143 (2025).
59. Ciceri, Paola, C., Mario. Apoptotic Bodies in Kidney Disease: Orchestrators of Inflammation, Fibrosis, and Repair. (2026) doi:10.1681/ASN.0000001073.

60. Kim, H. J. *et al.* Reprogramming of cancer-associated fibroblasts by apoptotic cancer cells inhibits lung metastasis via Notch1-WISP-1 signaling. *Cell Mol Immunol* **19**, 1373–1391 (2022).
61. Pandit, R., Hillman, H., Williams, J. W. & Yurdagul, A. More Than a Cleanup Crew: The Expanding Biology of Efferocytosis. *ATVB* **46**, (2026).
62. Sukka, S. R. *et al.* Efferocytosis drives a tryptophan metabolism pathway in macrophages to promote tissue resolution. *Nat Metab* **6**, 1736–1755 (2024).
63. Li, G. *et al.* Efferocytosis: the art of cellular clearance and novel perspectives in disease therapy. *Mol Cancer* **24**, 268 (2025).
64. Raymond, M. H. *et al.* Live cell tracking of macrophage efferocytosis during *Drosophila* embryo development in vivo. *Science* **375**, 1182–1187 (2022).

Role of Artificial Intelligence in this project

AI tools have been aiding in various stages of this project. Significant contributions include:

1. Anthropic. (2026). *Claude Sonnet 4.6* [Large language model]. <https://claude.ai> was used to improve grammar and readability with the prompt: “Please correct the grammar and improve the organization of the information to improve readability: [Paragraph written by the author.]”. Subsequently, the content was reviewed and edited as required, assuming full responsibility for the content.

2. Perplexity. (2025; 2026) Perplexity AI [Large language model]. <https://www.perplexity.ai> was used to retrieve up-to-date information and gain insights into the current state of knowledge on the research topic.

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Acknowledgment

I would like to express my deepest thanks and appreciation to my supervisor Rudolf Oehler, for giving me the opportunity to carry out this project and for his continuous guidance, support, and the valuable discussions we shared throughout.

I would also like to warmly thank my colleague Chiara Zotter, whose support during my master's thesis was invaluable to me. I am likewise thankful to Stefania Vintila and Sonja Kappel-Latif for their assistance with the immunohistochemical staining procedures. My thanks also goes to Cristiano Ramos, who was always ready to help whenever I had questions.

My appreciation also goes to the entire Department of General Surgery, whose thoughtful advice contributed greatly to a pleasant and productive working environment.

Appendix A: IHC Analysis

Figures 12-19 IHC analysis of CPM tissue (A) *H&E overview showing a typical clustered apoptotic area within the tumour tissue. The cluster is located within stromal tissue and is surrounded by a layer of mucin-producing tumour cells forming a pseudo-luminal structure filled with mucus. Cleaved caspase-3 immunohistochemistry identifies a dense accumulation of apoptotic cells within the luminal cluster, visible as dark brown-stained cells with characteristic condensed morphology. Caspase-3-positive apoptotic cells are concentrated within the clustered region, while additional apoptotic cells can also be detected in the surrounding tumour stroma. Arrows indicate apoptotic cells*

(B) *H&E overview shows apoptotic cells distributed as isolated, scattered cells within a matrix-dense stromal background. Cleaved caspase-3 immunohistochemistry identifies individual apoptotic tumour cells dispersed throughout the stroma rather than forming compact aggregates. Sirius Red staining demonstrates dense collagen deposition in these regions. Immunohistochemical staining for fibroblast activation protein (FAP) and α -smooth muscle actin (α SMA) confirms the presence of activated fibroblasts and myofibroblasts within the same stromal areas. Caspase-3-positive apoptotic cells are located in close spatial proximity to FAP- and α SMA-positive stromal cells. Arrows indicate apoptotic cells.*

Figures 20,21 IHC analysis of CPM tissue In these two patients, a clustered apoptotic pattern was present but could not be reliably captured photographically due to insufficient histological detectability, only the dispersed pattern is therefore shown. *H&E overview shows apoptotic cells distributed as isolated, scattered cells within a matrix-dense stromal background. Cleaved caspase-3 immunohistochemistry identifies individual apoptotic tumour cells dispersed throughout the stroma rather than forming compact aggregates. Sirius Red staining demonstrates dense collagen deposition in these regions. Immunohistochemical staining for fibroblast activation protein (FAP) and α -smooth muscle actin (α SMA) confirms the presence of activated fibroblasts and myofibroblasts within the same stromal areas. Caspase-3-positive apoptotic cells are located in close spatial proximity to FAP- and α SMA-positive stromal cells. Arrows indicate apoptotic cells.*

Patient 29

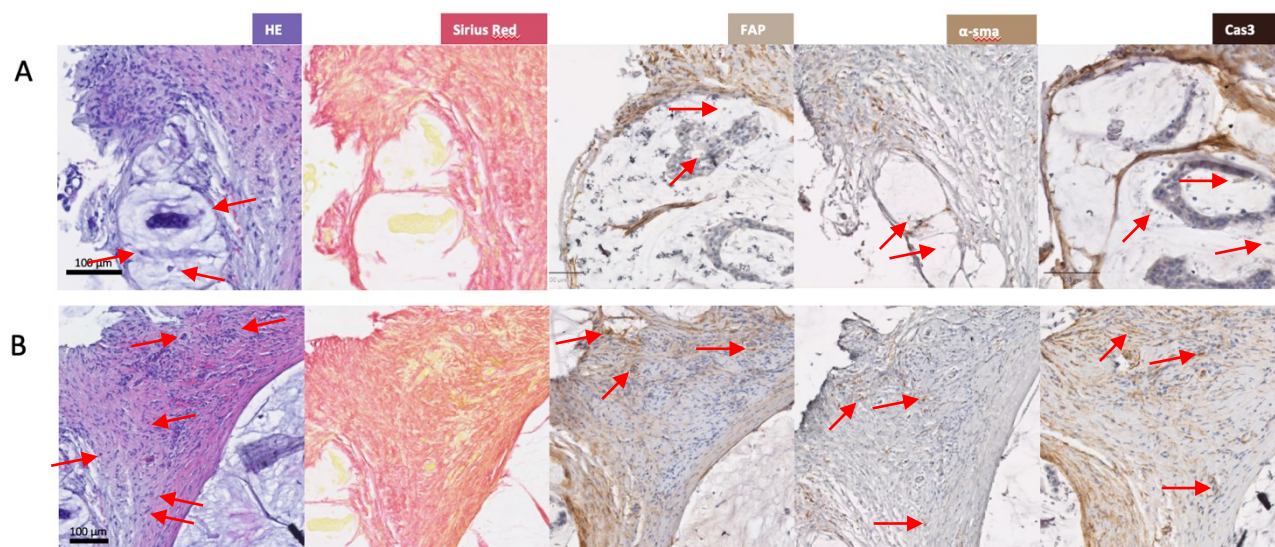


Figure 13 IHC analysis of CPM tissue of patient 29

Patient 38

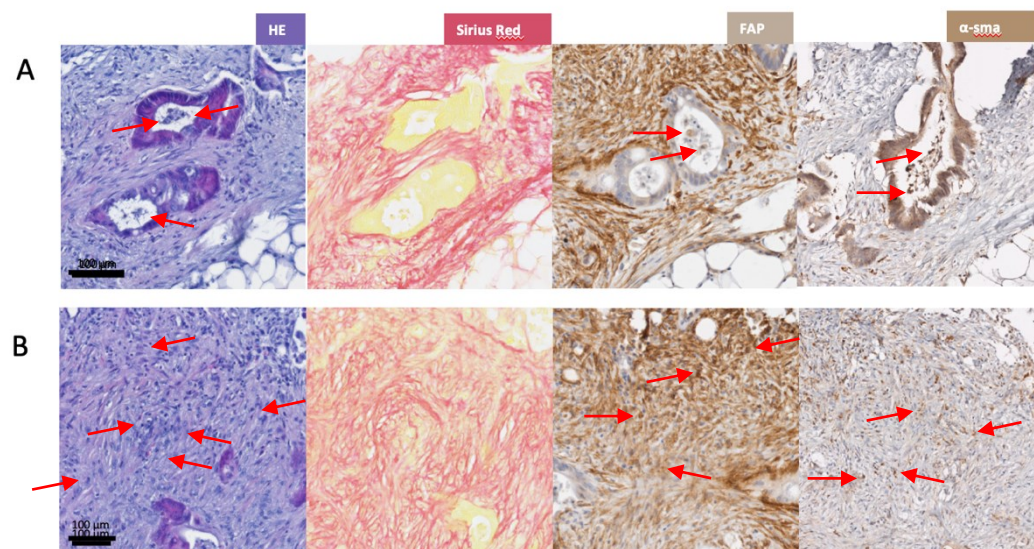


Figure 12 IHC analysis of CPM tissue of patient 38

Patient 39

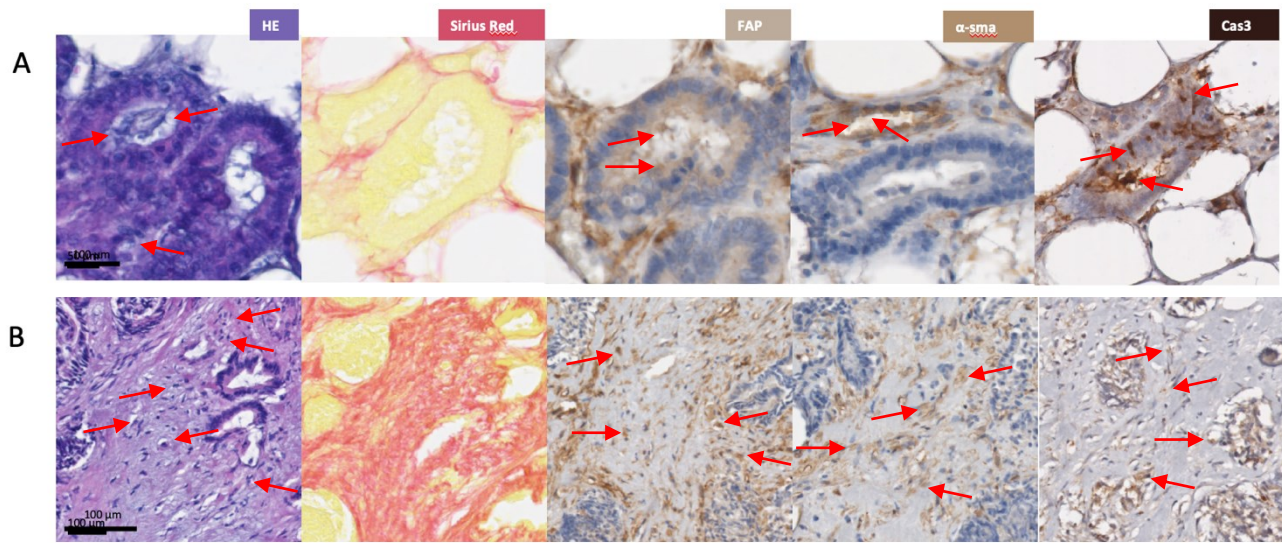


Figure 15 IHC analysis of CPM tissue of patient 39

Patient 41

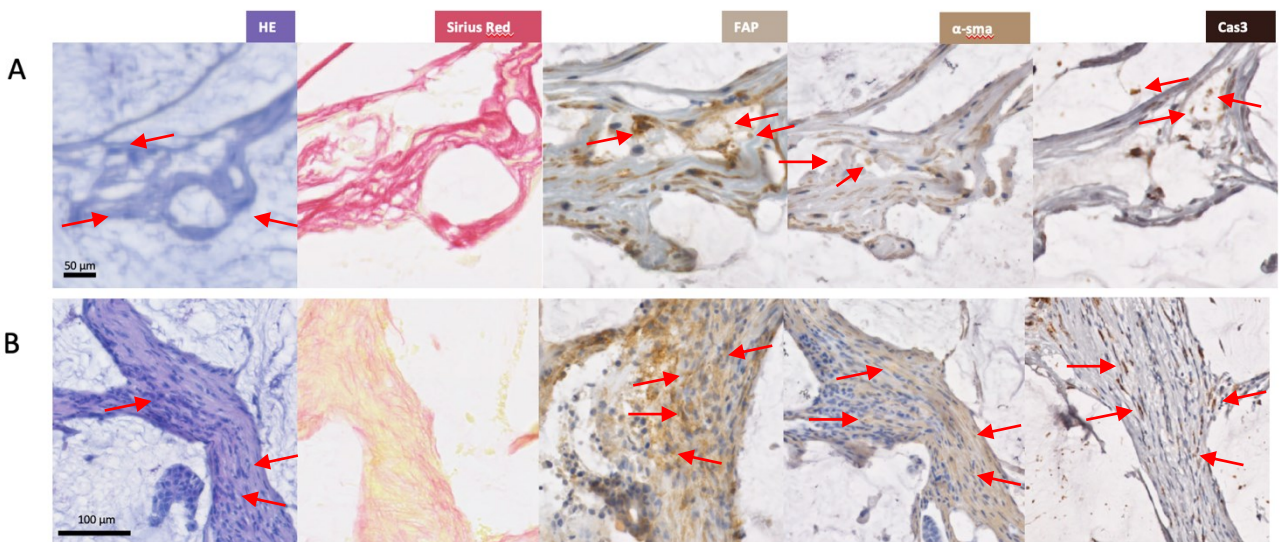


Figure 14 IHC analysis of CPM tissue of patient 41

Patient 49

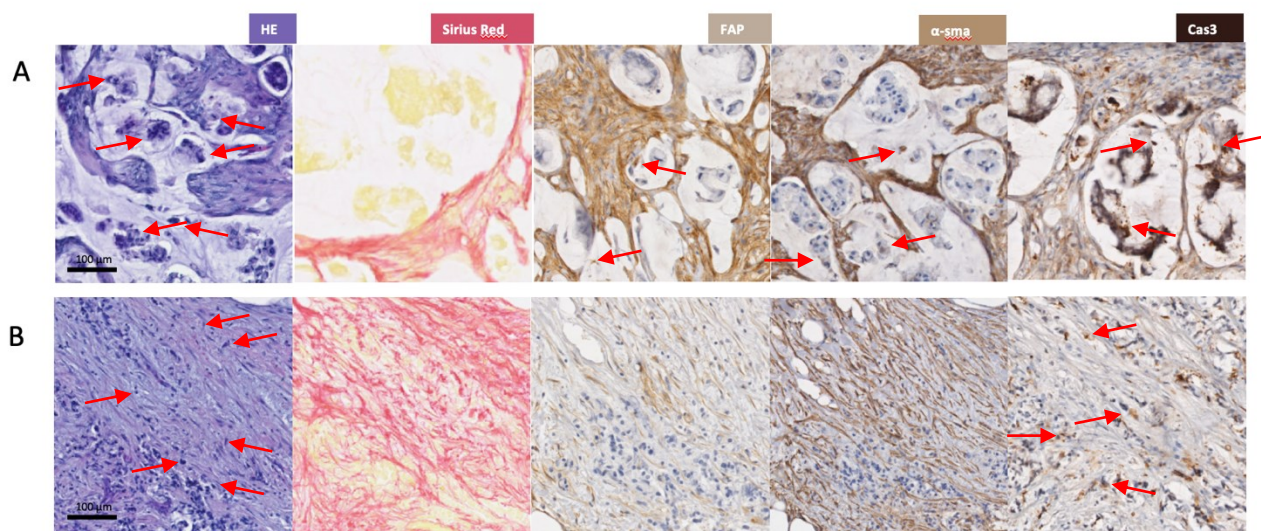


Figure 16 IHC analysis of CPM tissue of patient 49

Patient 72

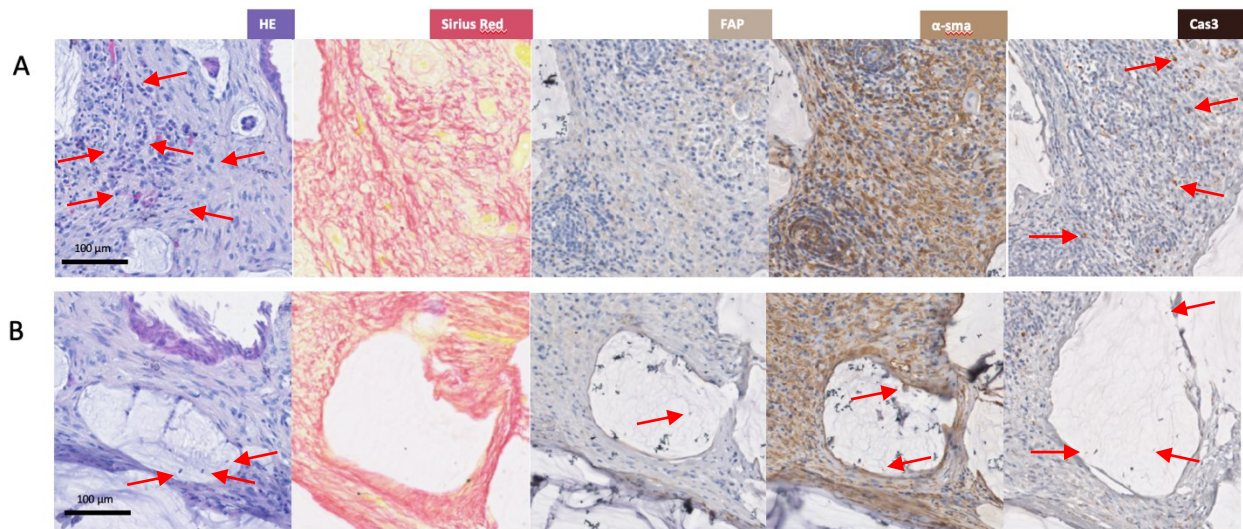


Figure 17 IHC analysis of CPM tissue of patient 72

Patient 73

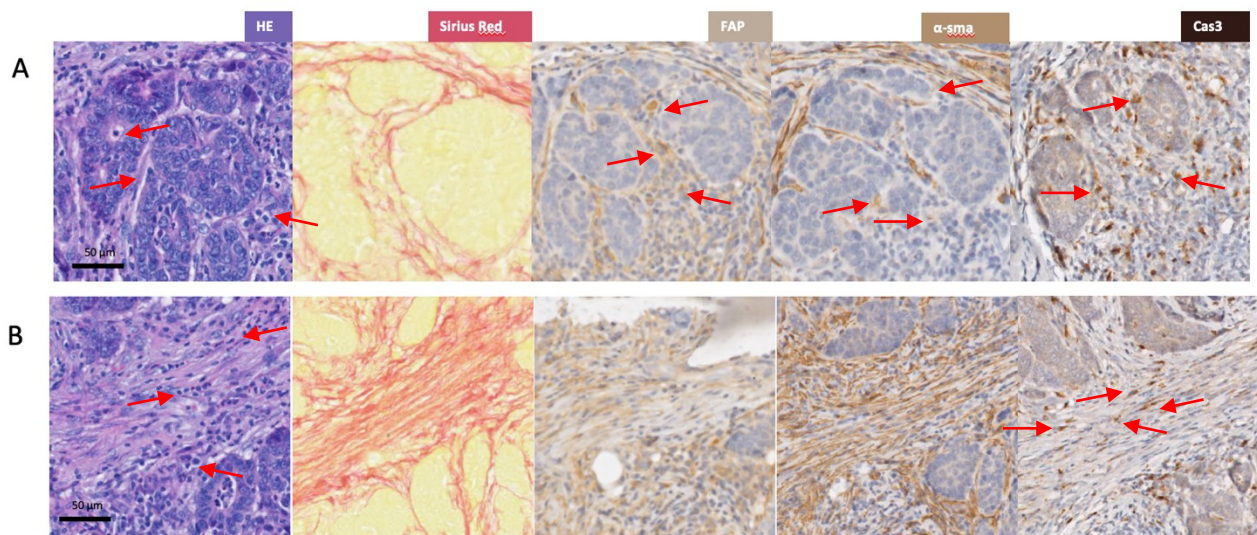


Figure 18 IHC analysis of CPM tissue of patient 73

Patient 77

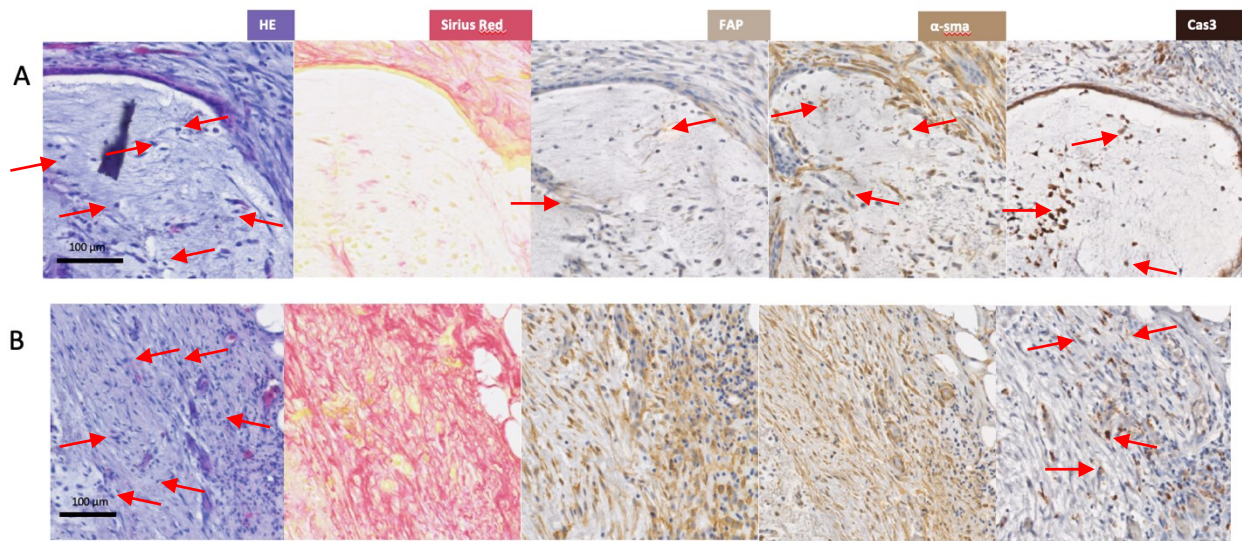


Figure 19 IHC analysis of CPM tissue of patient 77

Patient 74

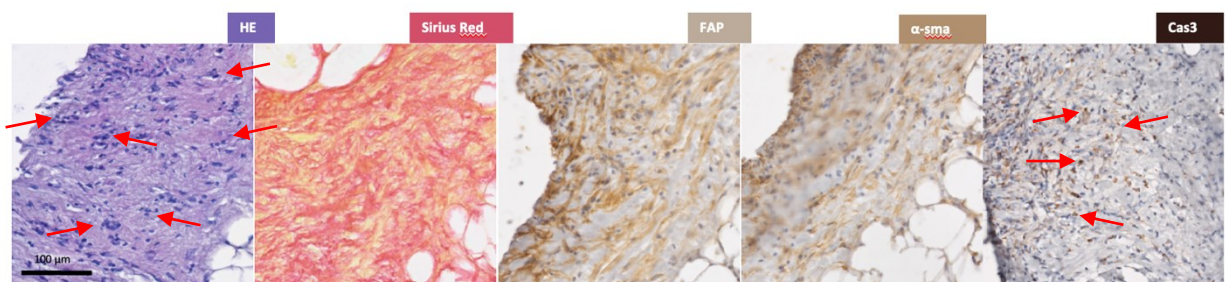


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Patient 57

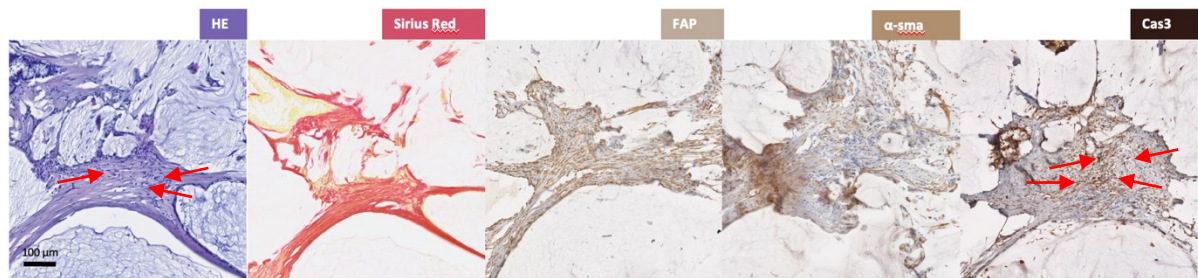


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Appendix B: TUNEL and Immunofluorescence Analysis

Figures 22-28: Representative apoptotic patterns per patient. For each patient, a whole-slide H&E overview of the same tissue section is shown, with the respective region of interest indicated (red box) and magnified alongside serial sections stained for cleaved caspase-3 and M30, and immunofluorescence for DAPI, TUNEL and α -SMA. **(A)** shows the clustered apoptotic pattern: apoptotic cells accumulate within mucus-filled pseudolumina, shielded from the surrounding stroma and largely devoid of direct fibroblast contact, as indicated by the absence or scarcity of α -SMA-positive cells within the cluster. **(B)** shows the dispersed apoptotic pattern within the same section: apoptotic cells are located directly within the stroma and are in close, direct contact with α -SMA-positive fibroblasts. Cleaved caspase-3, M30 and TUNEL staining confirm apoptotic cell identity in both patterns. Arrows indicate apoptotic cells.

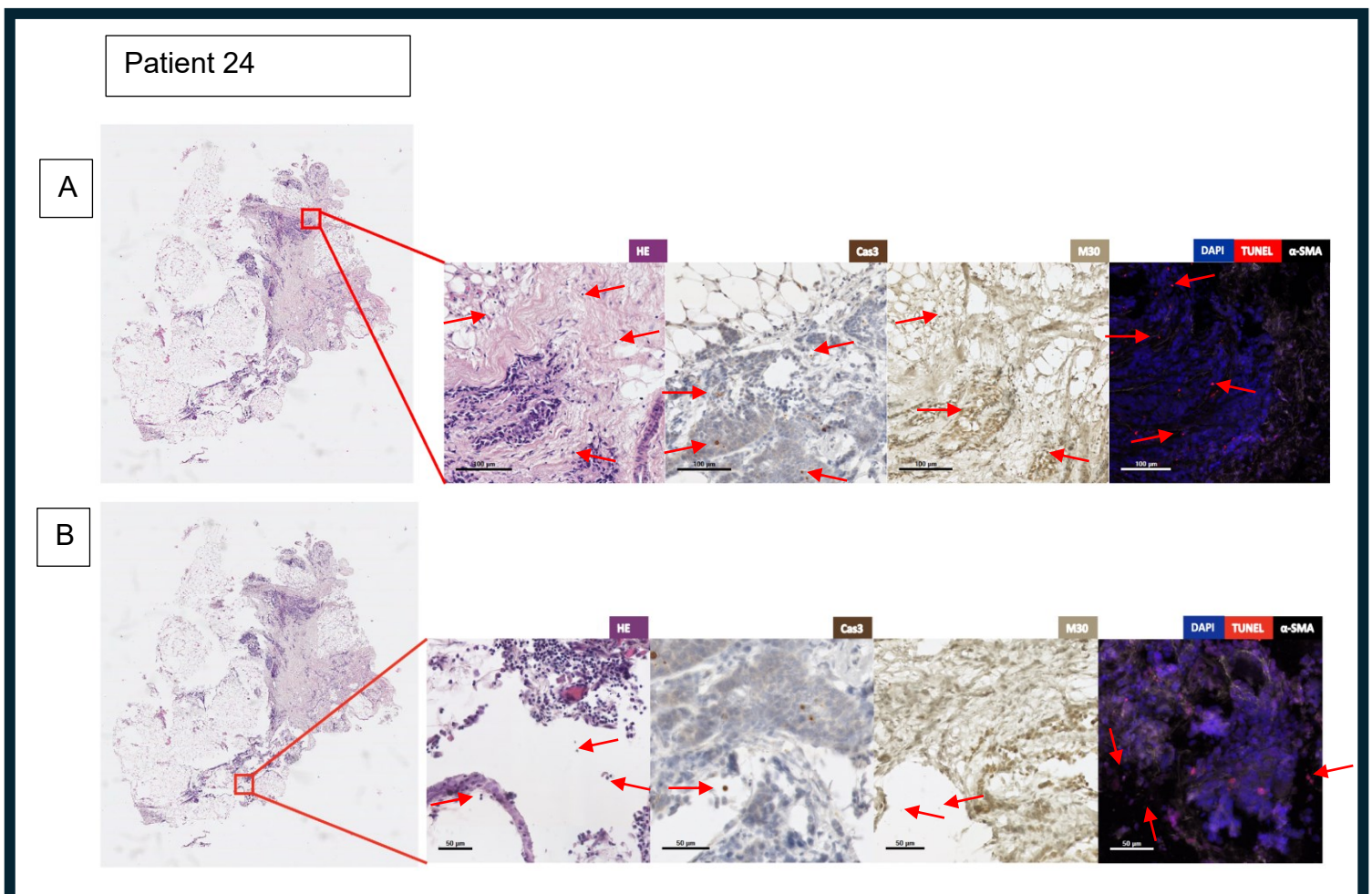


Figure 22 Histological and Immunofluorescence analysis of CPM tissue of patient 24

Patient 41

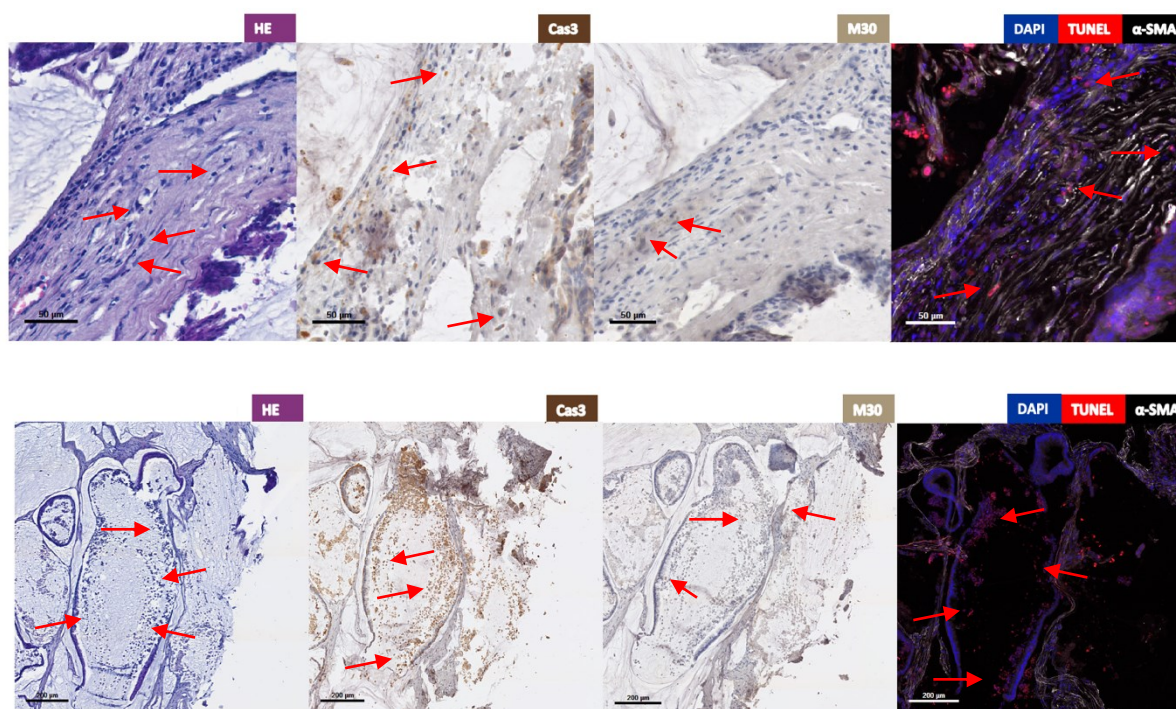


Figure 23 Histological and Immunofluorescence analysis of CPM tissue of patient 41

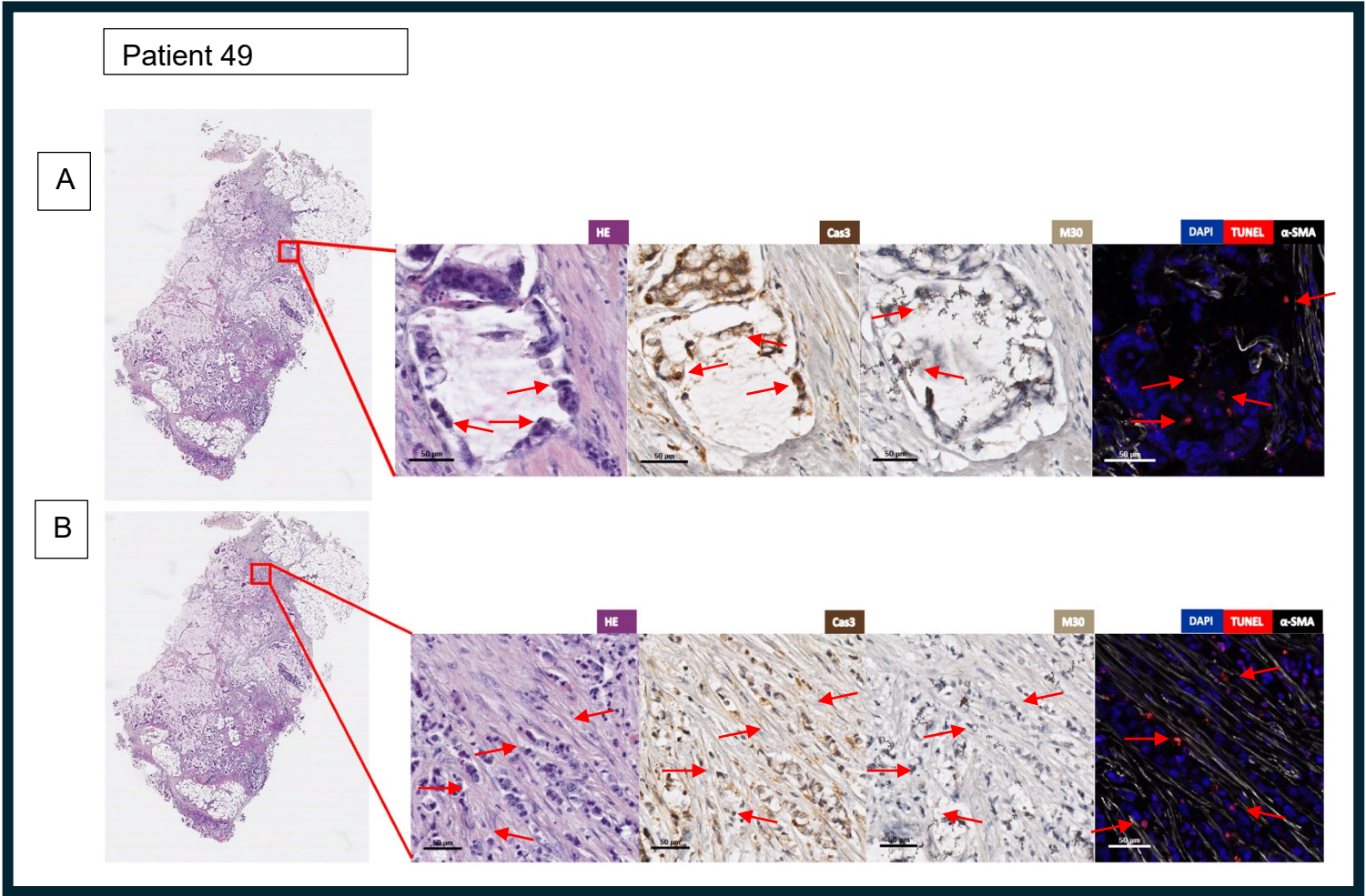
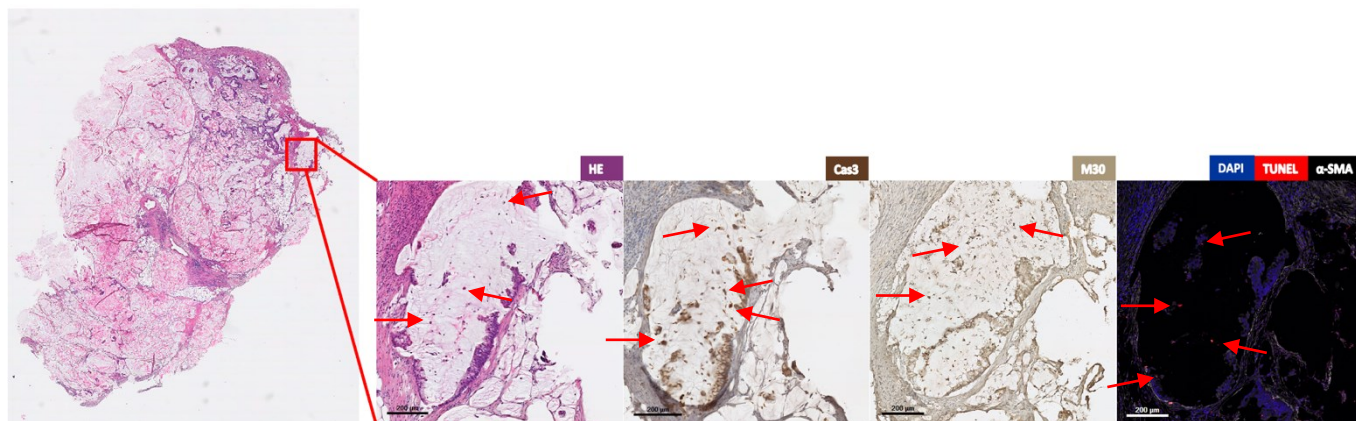


Figure 24 Figure 24 Histological and Immunofluorescence analysis of CPM tissue of patient 49

Patient 52

A



B

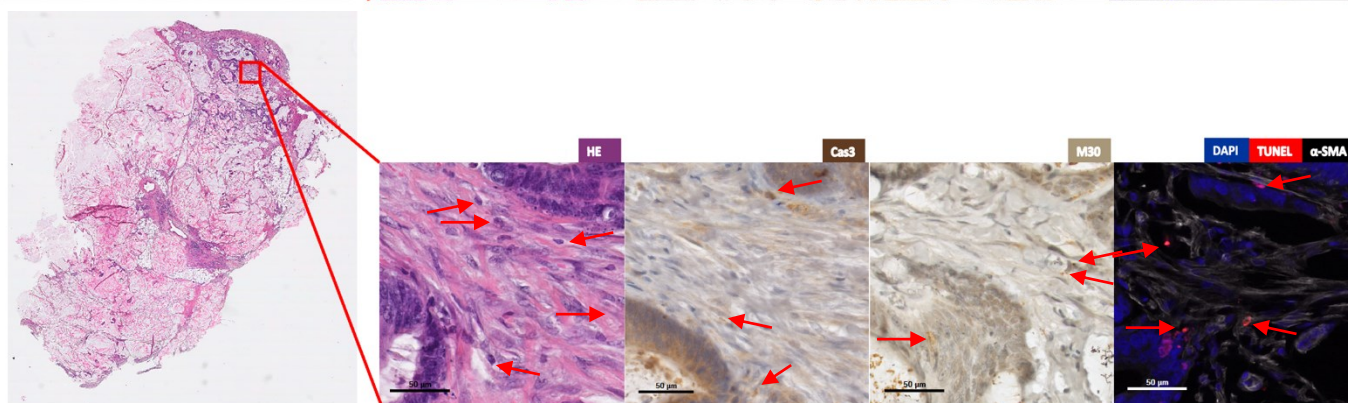


Figure 25 Figure 24 Histological and Immunofluorescence analysis of CPM tissue of patient 52

Patient 72

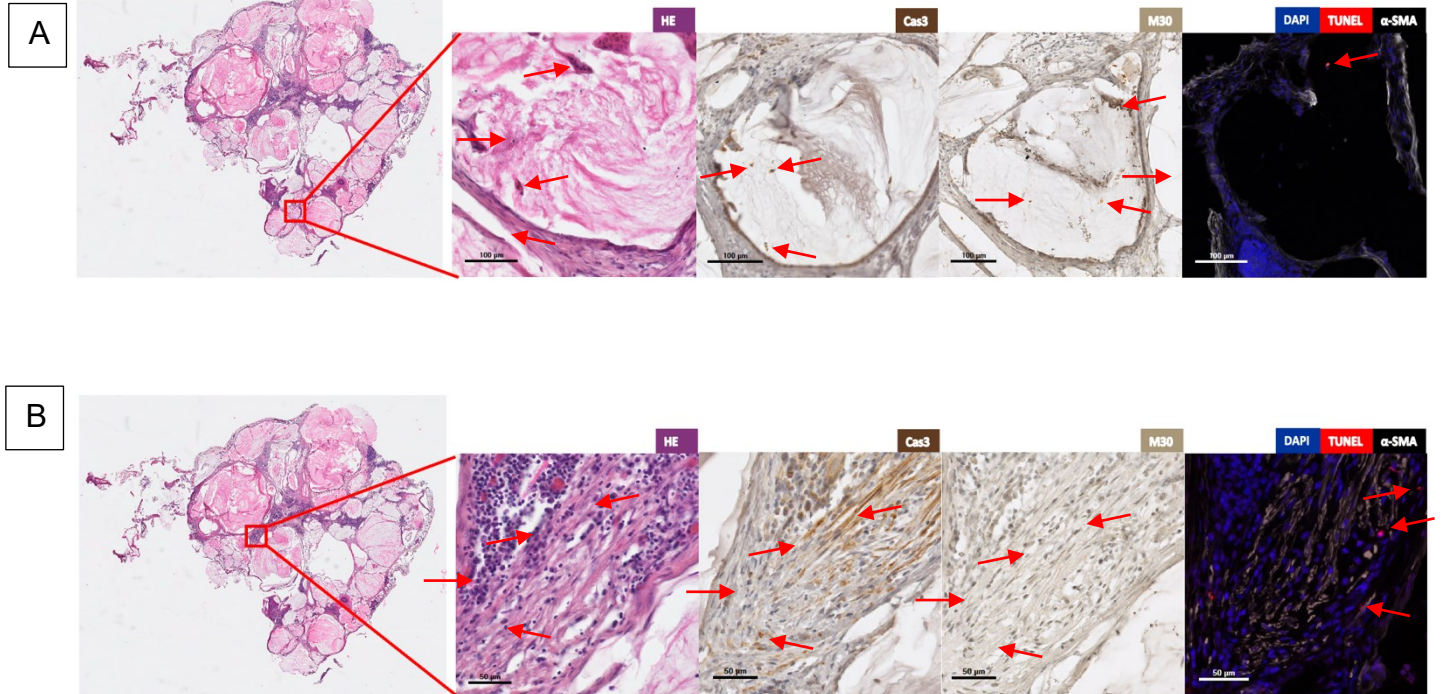


Figure 26 Histological and Immunofluorescence analysis of CPM tissue of patient 72

Patient 97 omentum

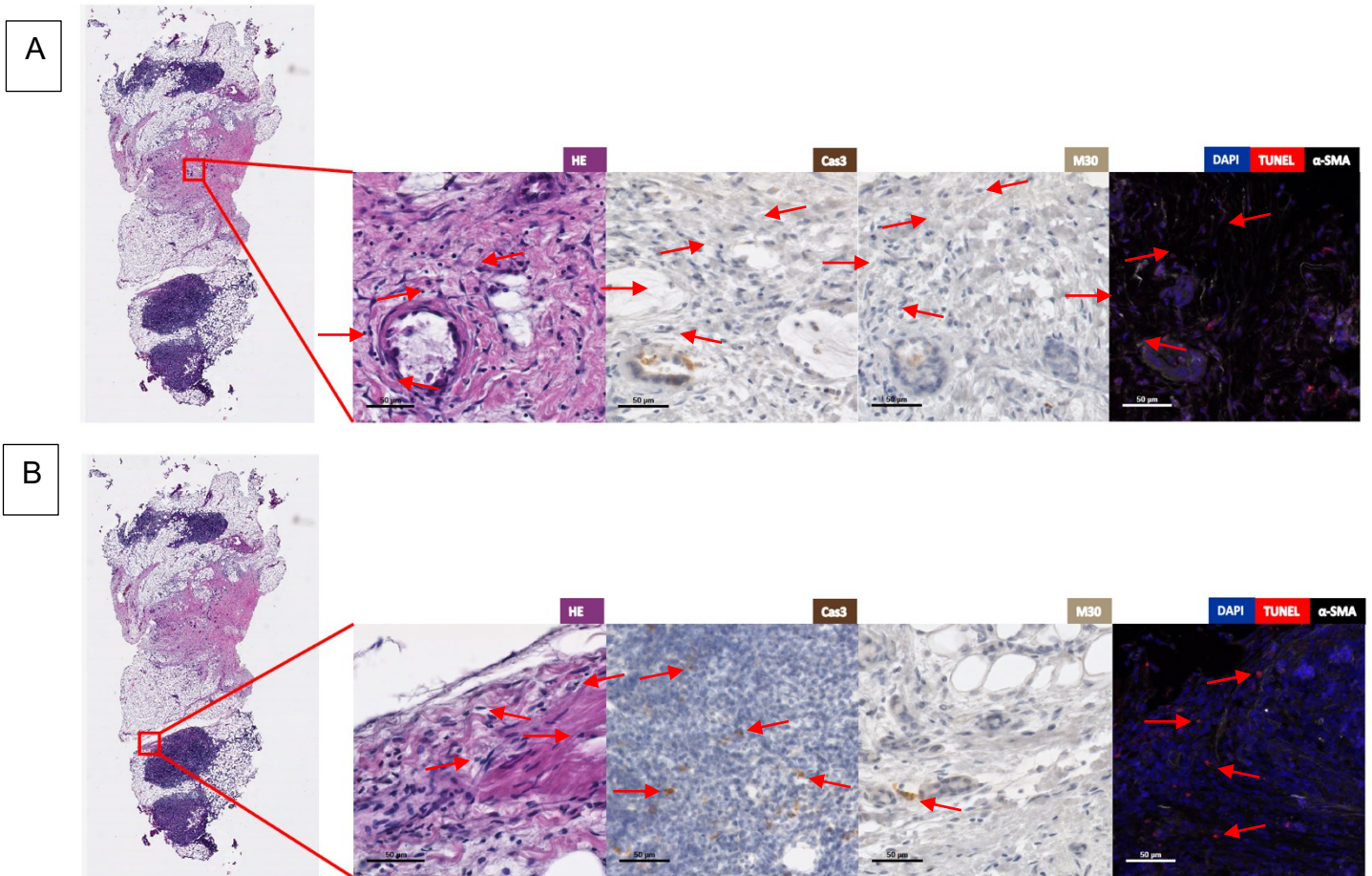
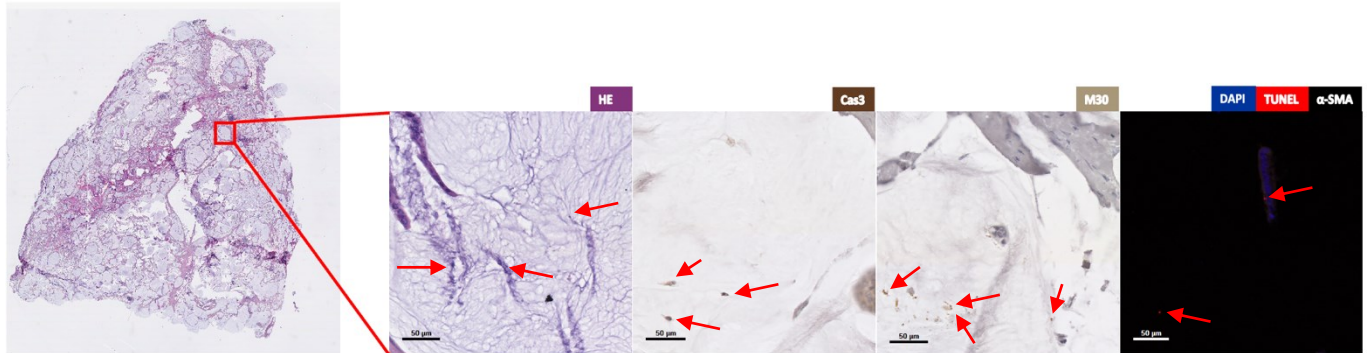


Figure 27 Histological and Immunofluorescence analysis of CPM tissue of patient 97 omentum

Patient 101

A



B

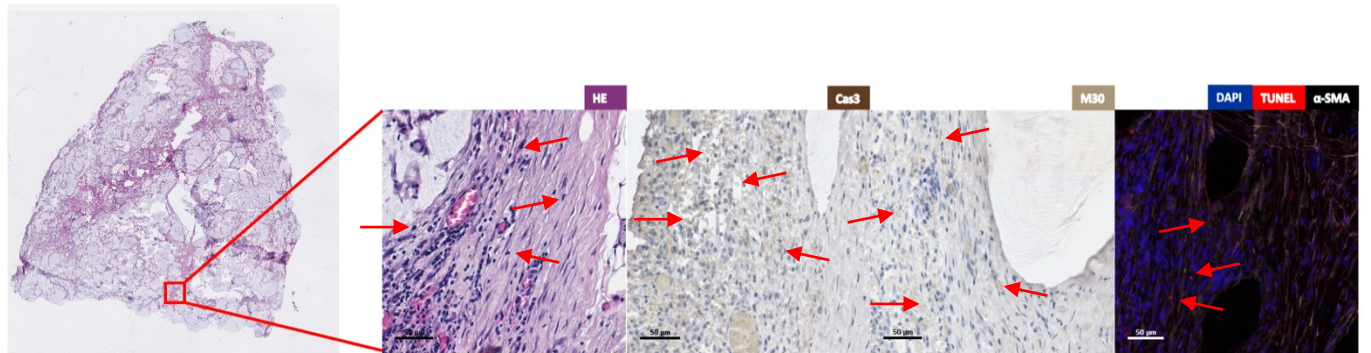


Figure 28 Histological and Immunofluorescence analysis of CPM tissue of patient 101

Figures 29-30: Representative apoptotic patterns per patient. A whole-slide H&E overview of the tissue section is shown, with the region of interest indicated (red box) and magnified alongside serial sections stained for cleaved caspase-3 and M30, and immunofluorescence for DAPI, TUNEL and α -SMA. In these patients, both the clustered and the dispersed apoptotic pattern are visible within the same field of view: apoptotic cells accumulate within a mucus-filled pseudolumen, shielded from the surrounding stroma and largely devoid of direct fibroblast contact, while adjacent apoptotic cells are located directly within the stroma in close, direct contact with α -SMA-positive fibroblasts. Cleaved caspase-3, M30 and TUNEL staining confirm apoptotic cell identity in both areas. Arrows indicate apoptotic cells.

Patient 77

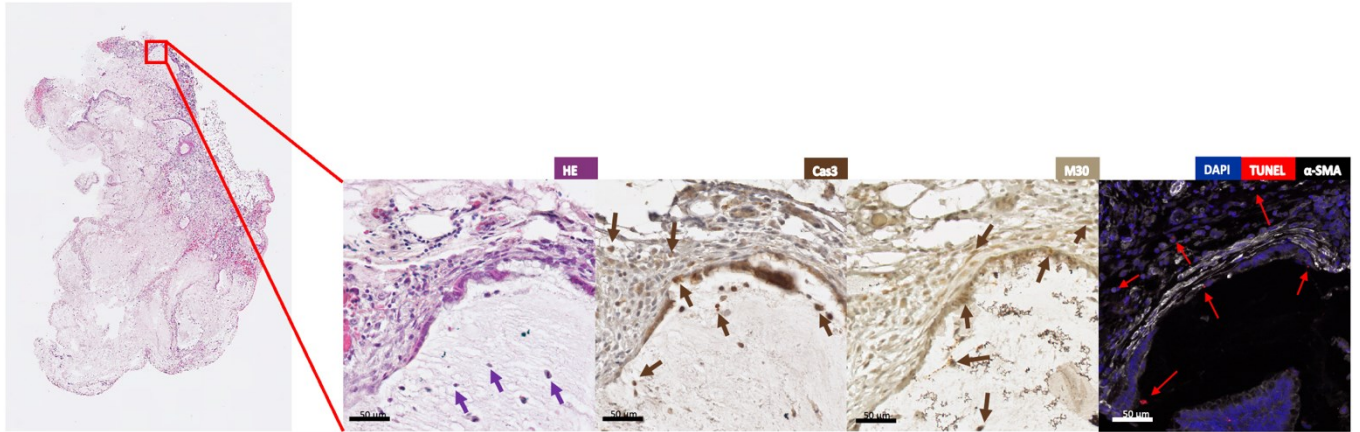


Figure 29 Histological and Immunofluorescence analysis of CPM tissue of patient 77

Patient 64

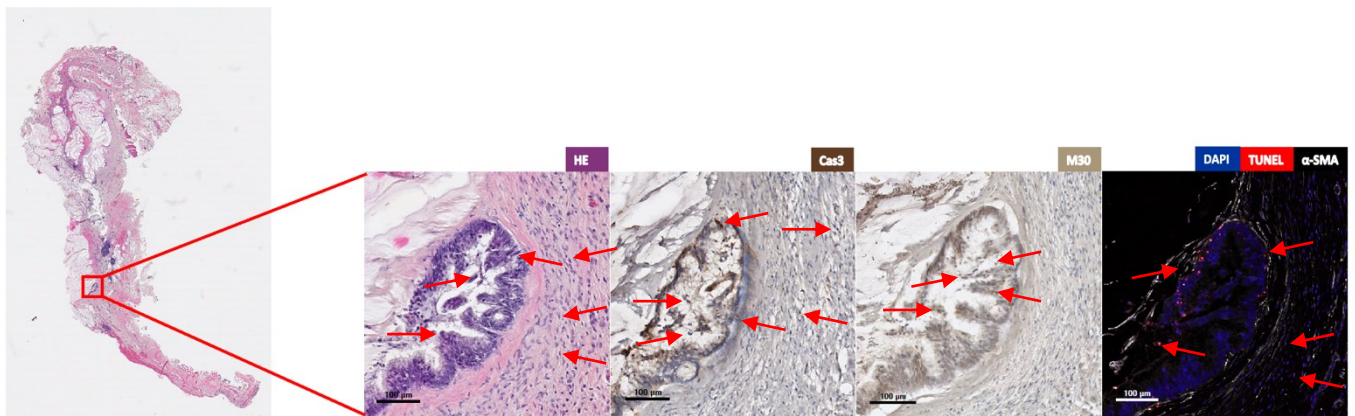


Figure 30 Histological and Immunofluorescence analysis of CPM tissue of patient 64

Figure 31 Representative apoptotic pattern of patient 29. A whole-slide H&E overview of the same tissue section is shown, with the respective region of interest indicated (red box) and magnified alongside serial sections stained for cleaved caspase-3 and M30. (A) shows the clustered apoptotic pattern: apoptotic cells accumulate within mucus-filled pseudolumina, shielded from the surrounding stroma and largely devoid of direct fibroblast contact. (B) shows the dispersed apoptotic pattern within the same section: apoptotic cells are located directly within the stroma. Cleaved caspase-3 and M30 staining confirm apoptotic cell identity in both patterns. TUNEL and immunofluorescence staining are not shown for this patient due to unsuccessful staining. Arrows indicate apoptotic cells.

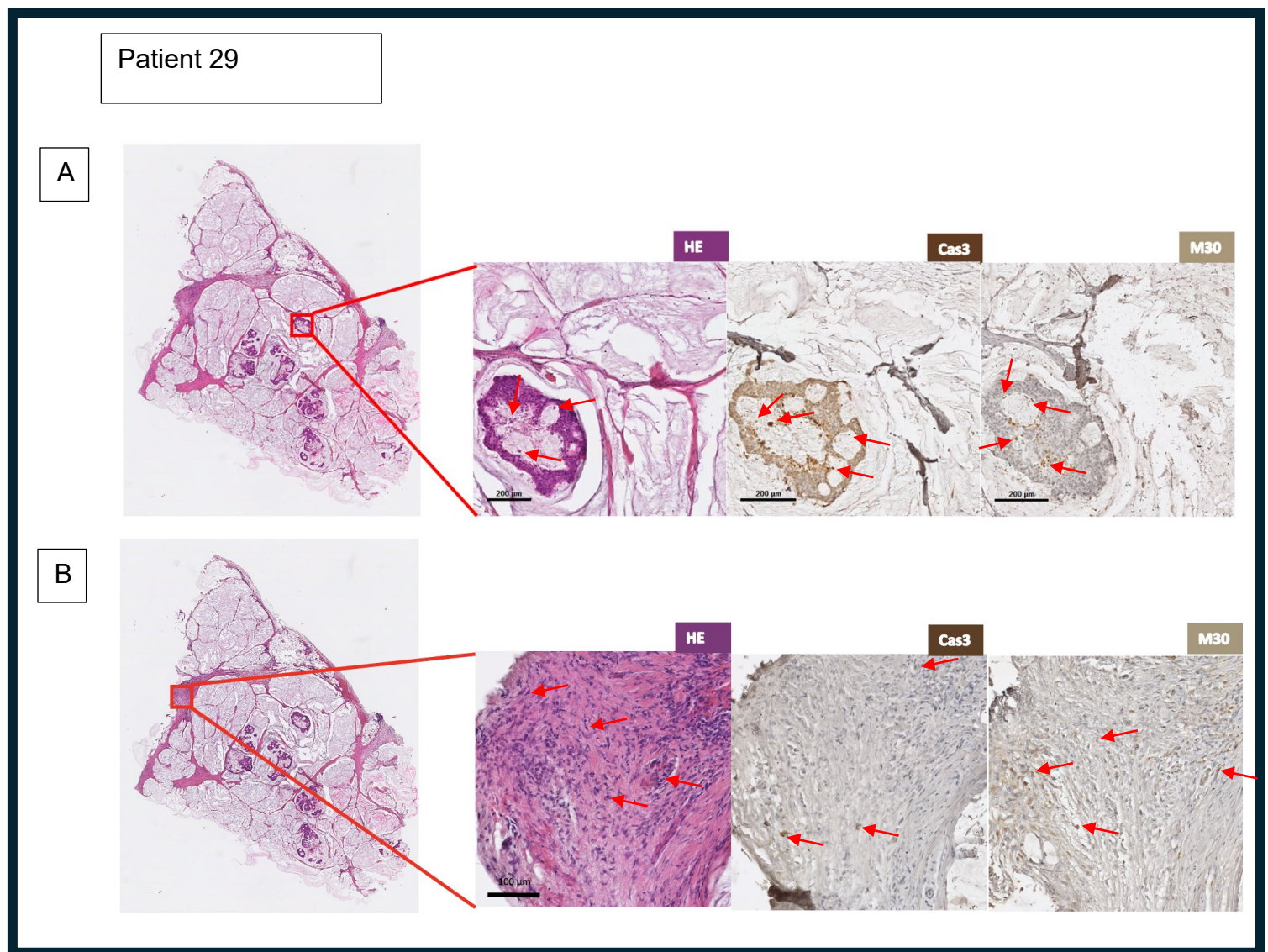


Figure 31 Histological and Immunofluorescence analysis of CPM tissue of patient 29.

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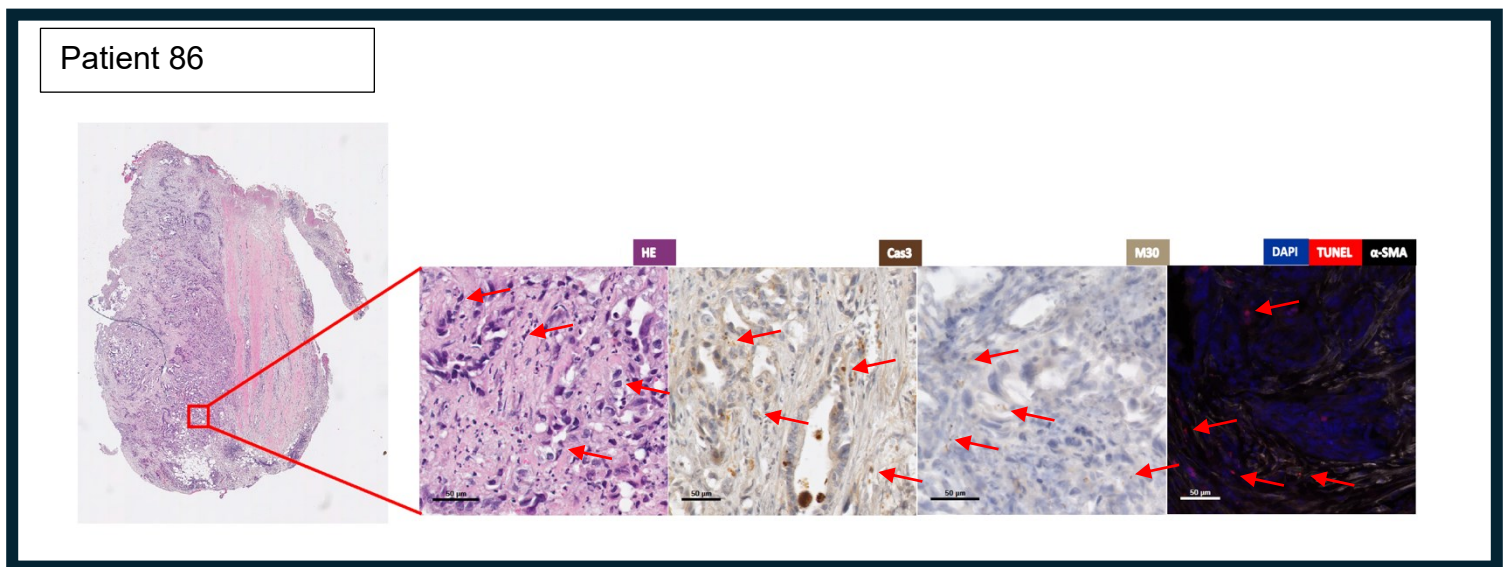


Figure 32 Representative apoptotic pattern of patient 86.

Figure 33 Representative apoptotic pattern of patient 97. A whole-slide H&E overview of the same tissue section is shown, with the respective region of interest indicated (red box) and magnified alongside serial sections stained for cleaved caspase-3, and, where available, M30 and immunofluorescence for DAPI, TUNEL and α -SMA. (A) shows the clustered apoptotic pattern: apoptotic cells accumulate within mucus-filled pseudolumina, shielded from the surrounding stroma and largely devoid of direct fibroblast contact; M30 and TUNEL/immunofluorescence staining are not shown for this area due to unsuccessful staining. (B) shows the dispersed apoptotic pattern within the same section: apoptotic cells are located directly within the stroma and are in close, direct contact with α -SMA-positive fibroblasts, confirmed by cleaved caspase-3 and TUNEL staining; M30 staining is not shown for this area due to unsuccessful staining. Arrows indicate apoptotic cells.

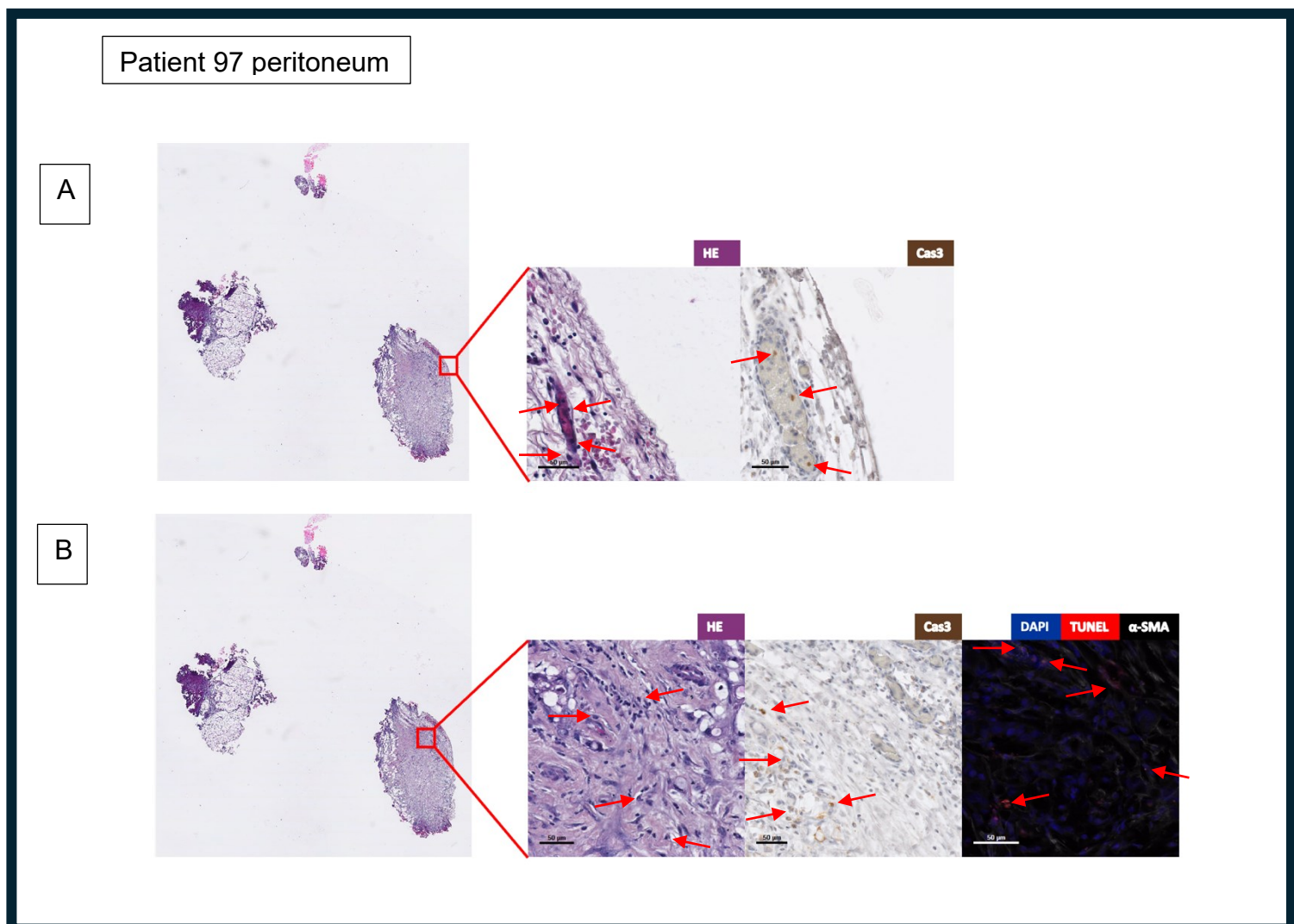


Figure 33 Representative apoptotic patterns of patient 97.

