Workshop in Diagnostic Immunohistochemistry
Oud St. Jan/ Old St. John – Brugge (Bruges), Belgium June 13th – 15th 2018

The unkown primary tumour: IHC Classification, antibody selection, protocol optimization, controls and EQA (part I)

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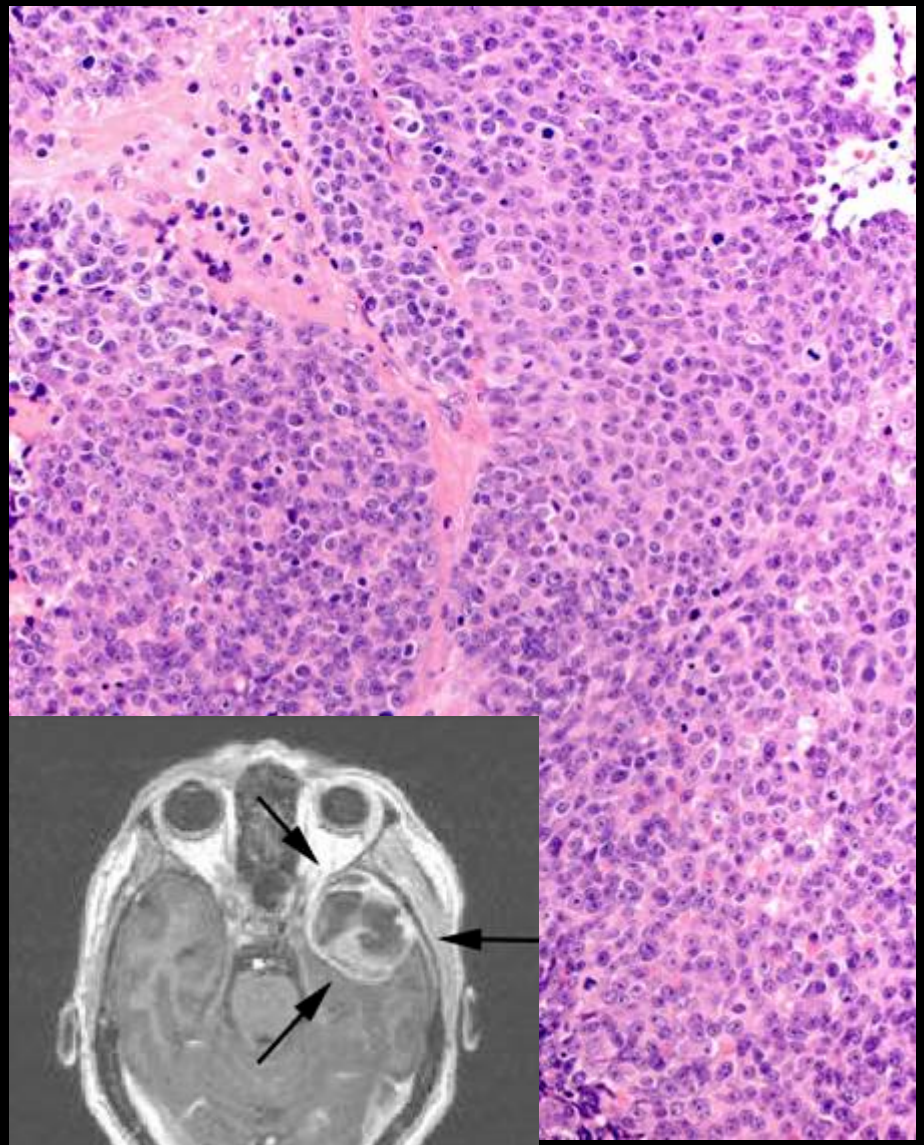
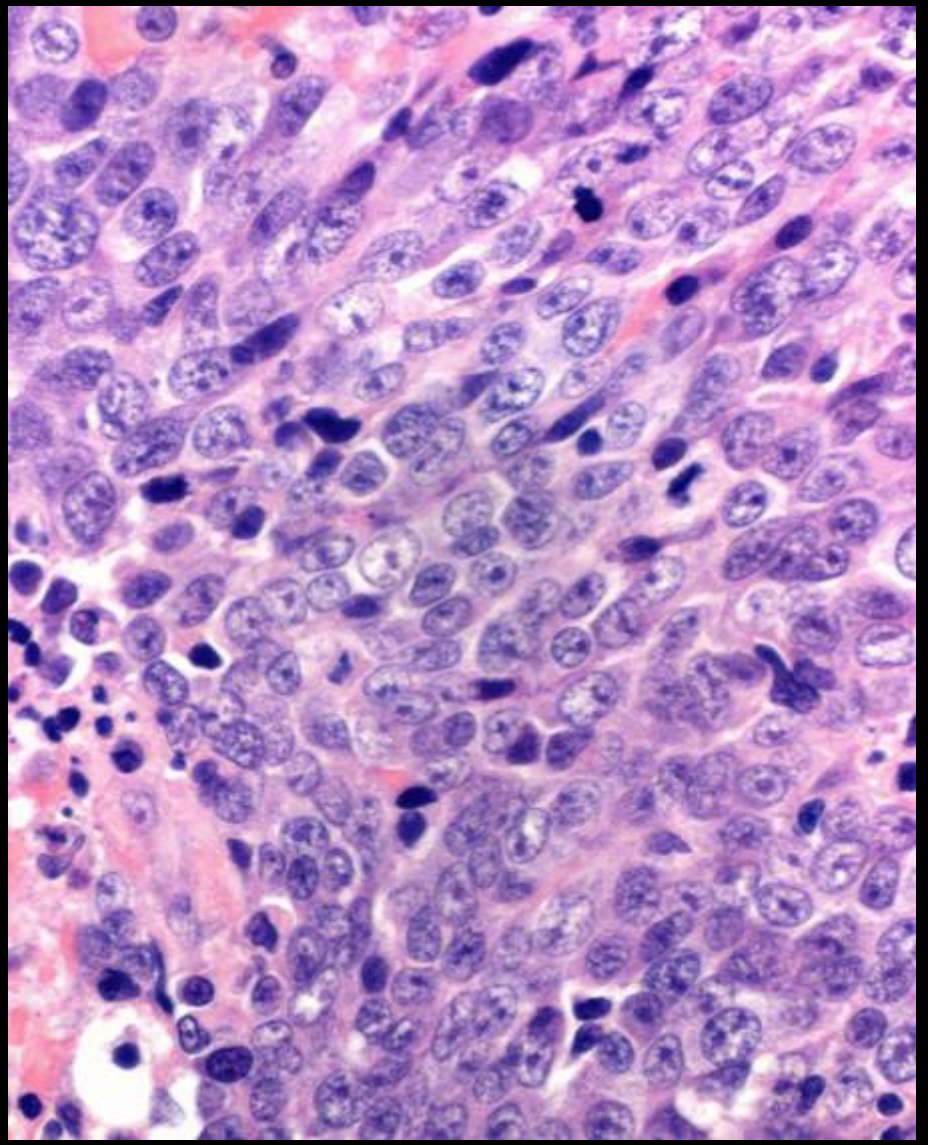
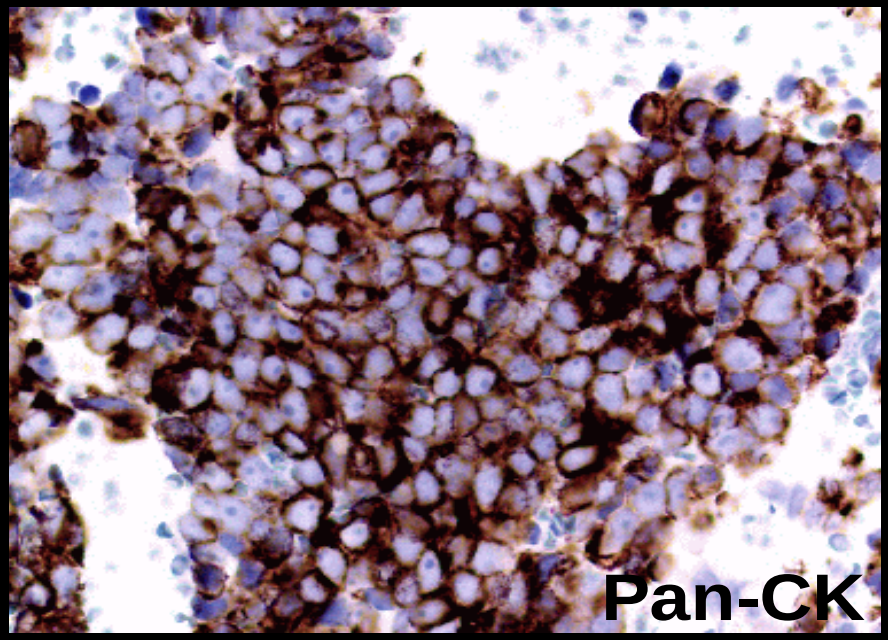
Tumours of unknown origin: Histology

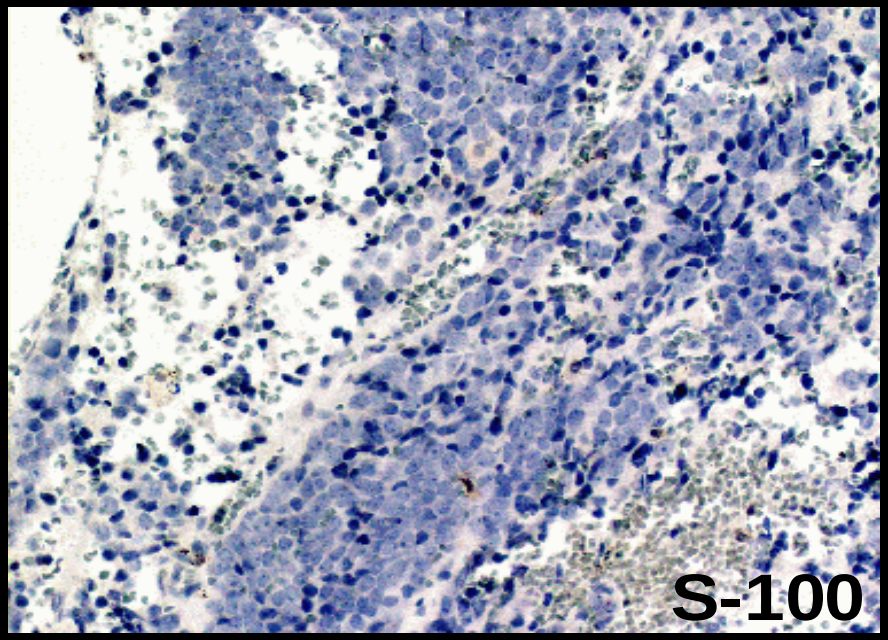


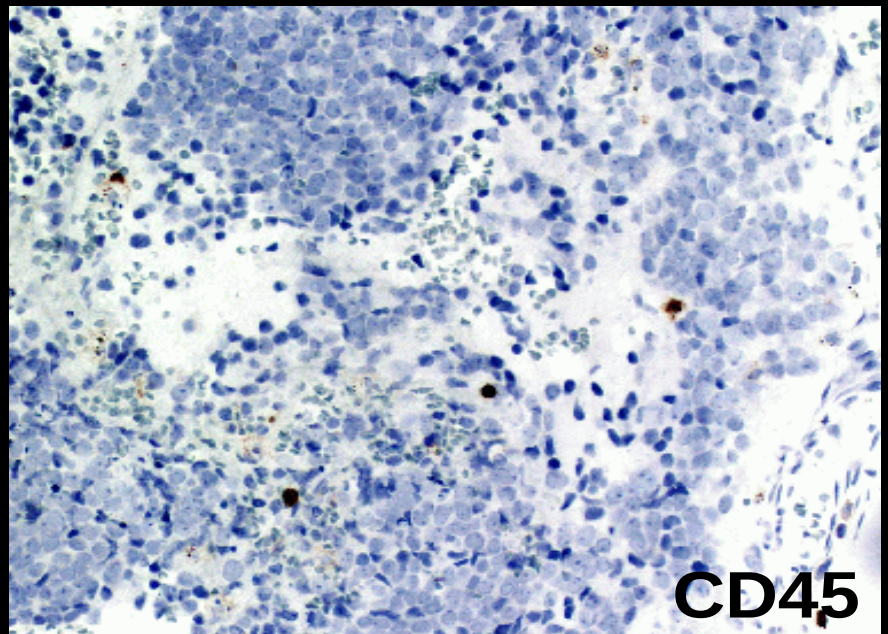


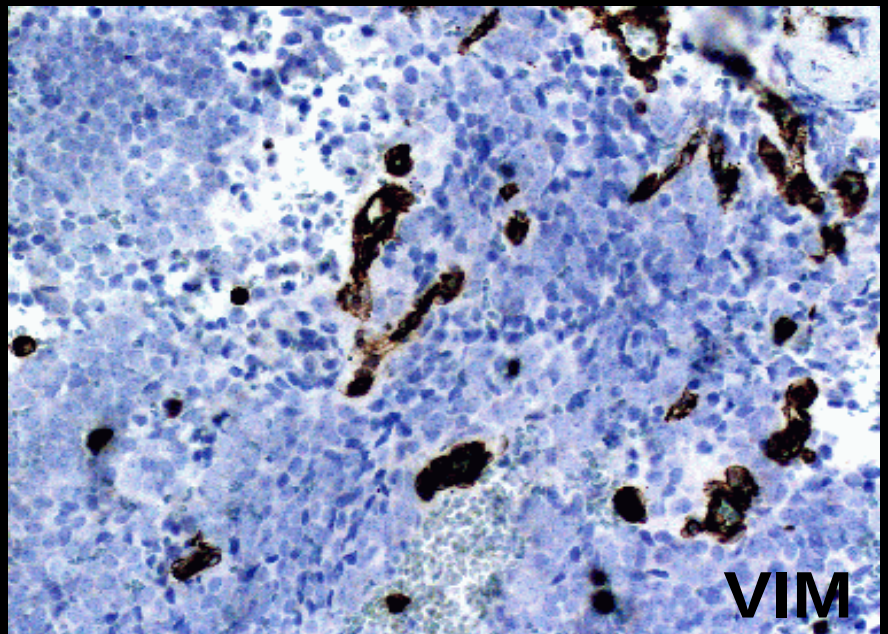
Brain tumour - biopsy

Tumours of unknown origin: Immunohistochemistry









IHC classification of the Unknown Primary Tumour

UPT: A tumour appearing in metastatic setting without a histologically proven primary tumour.

UPT pose an increasing challenge for the pathologist - due to the progress in surgical and oncological treatment possibilities.

IHC classification of the Unknown Primary Tumour

New, relatively specific antibodies give the pathologist more and better diagnostic tools.

But the diagnostic work also become more complex in terms of planning, optimization of protocols, interpretation of reaction patterns and error trapping.

IHC classification of the Unknown Primary Tumour

10 - 15% of cancers remained UPTs

+ ??% uncertain if primary or metastatic

- liver, lung, bone, lymph nodes, brain, peritoneum . . .

‘Undifferentiated’ neoplasms (5-10%)

- carcinomas, sarcomas, melanomas, germ cell tumours
- malignant lymphomas

- Adenocarcinomas (80-90%)
  - lung, breast, prostate, colorectum, ovary, pancreas …
- Squamous cell carcinomas (5-10%)
  - lung, esophagus, uterine cervix …

IHC classification of the Unknown Primary Tumour

Differences in prognosis

Differences in treatment regimes

- malignant lymphomas
- carcinomas (breast, prostate, ovary . . .)
- sarcomas (GIST, synovial sarcoma . . .)
- germ cell tumours

Pathology tests cost effective

Pathology tests save patient discomfort

The patient's 'right to know'

The risk of hereditary cancer

IHC classification of the Unknown Primary Tumour

- Most likely diagnoses
- Relevant differential diagnoses

↓

- Optimal selection of antibodies for a diagnostic algorithm
  - Primary and secondary antibody panels
  - Turn-around-time
  - Laboratory expenses

IHC classification of the Unknown Primary Tumour

Pathologist

- knowledge, acceptance, skill

Tumour material

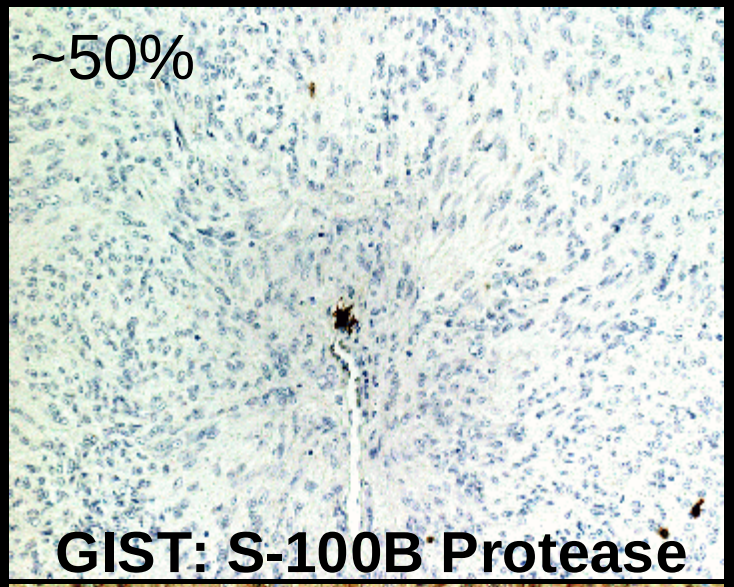
- diagnostic markers

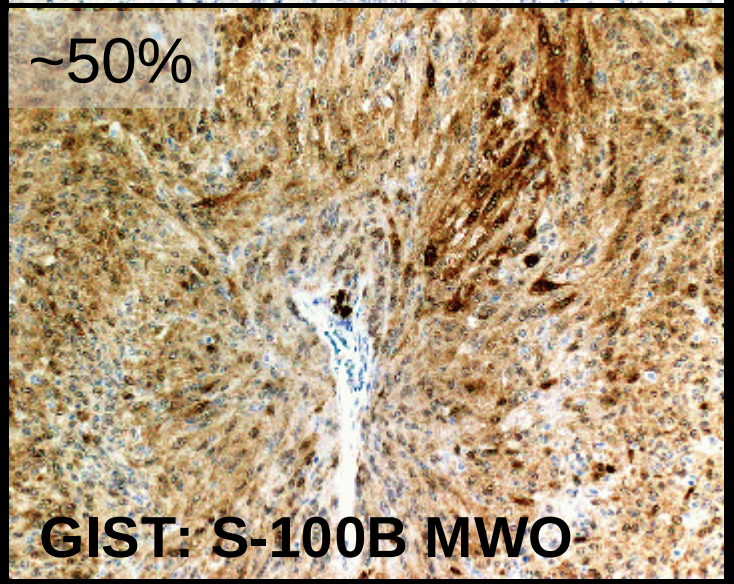
Antibodies available

- applic. in diagnostic algorithms

Methods

- protocol:
  sensitivity, specificity, reliability
- interpretation:
  cut-off level for positivity
  clinical relevance





IHC classification of the Unknown Primary Tumour

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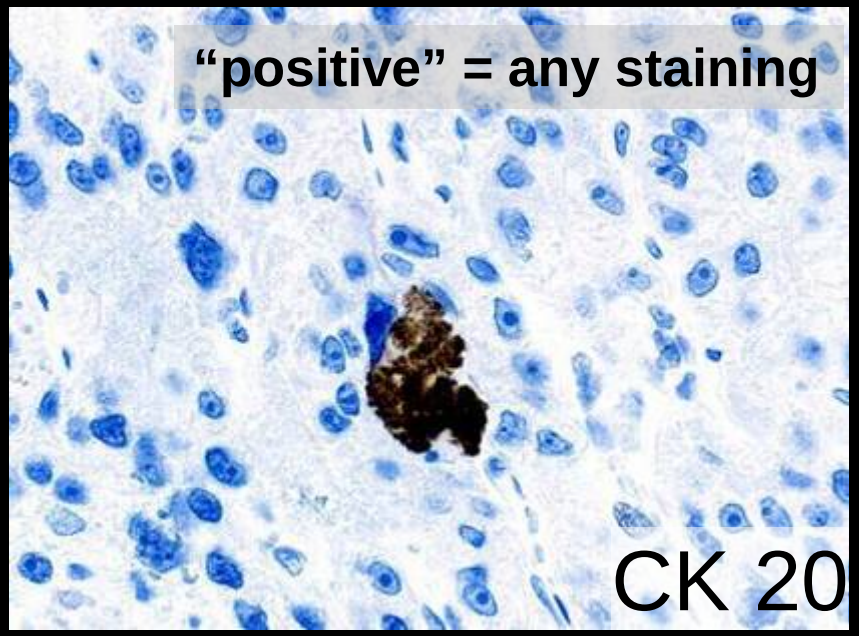
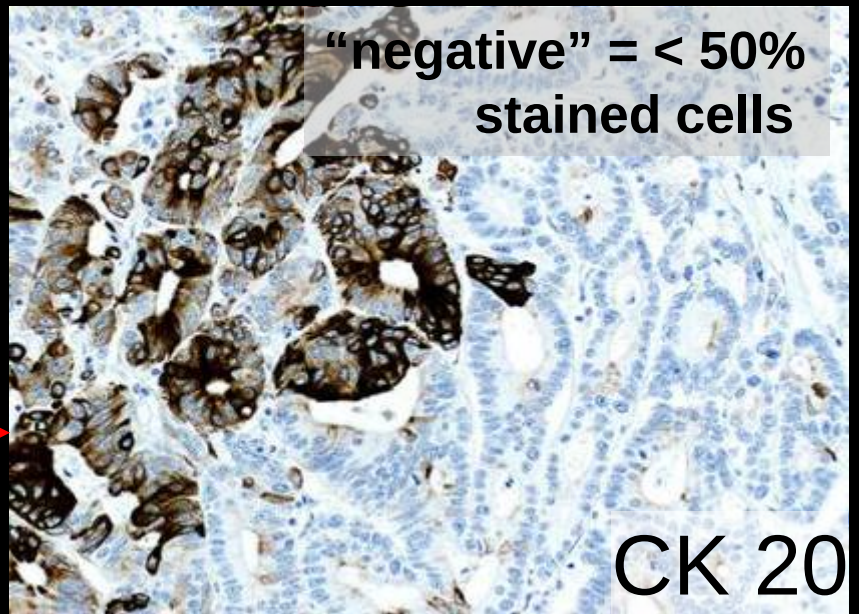


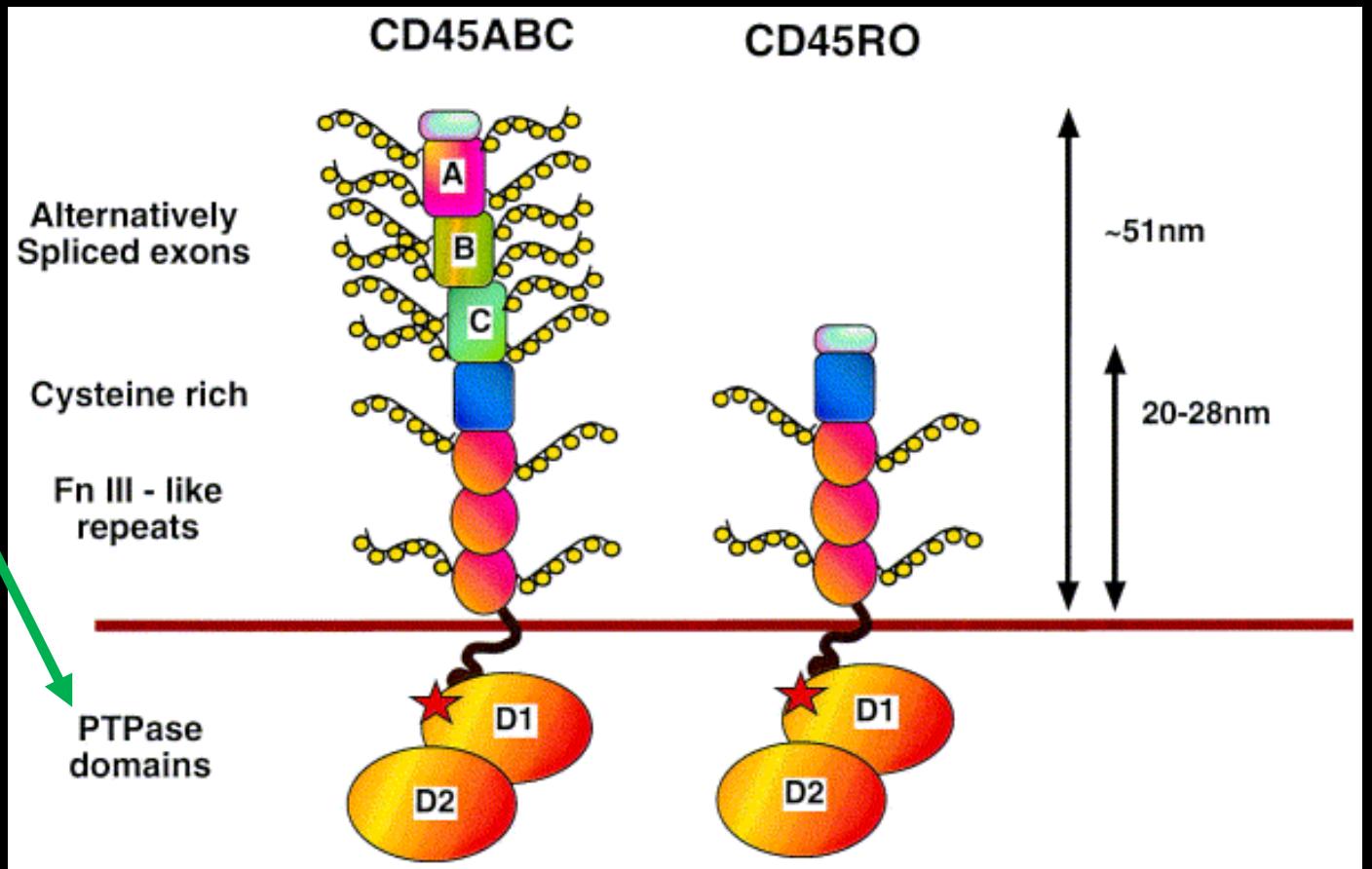


Primary panel for the unknown primary tumour

	CD45	Pan-CK	S-100	VIM
Haemato-lymphoid neoplasms	+/(-)	-/(+)	-/(+)	+/(-)
Epithelial neoplasms	-	+/(-)	-/+	-/+
Mesothelial neoplasms	-	+	-	+
Mesenchymal and neuronal neoplasms	-	-/(+)	-/+	+
Non-neuronal neuroepithelial neoplasms	-	-/(+)	+	+
Germ cell neoplasms	-	-/+	-/+	+

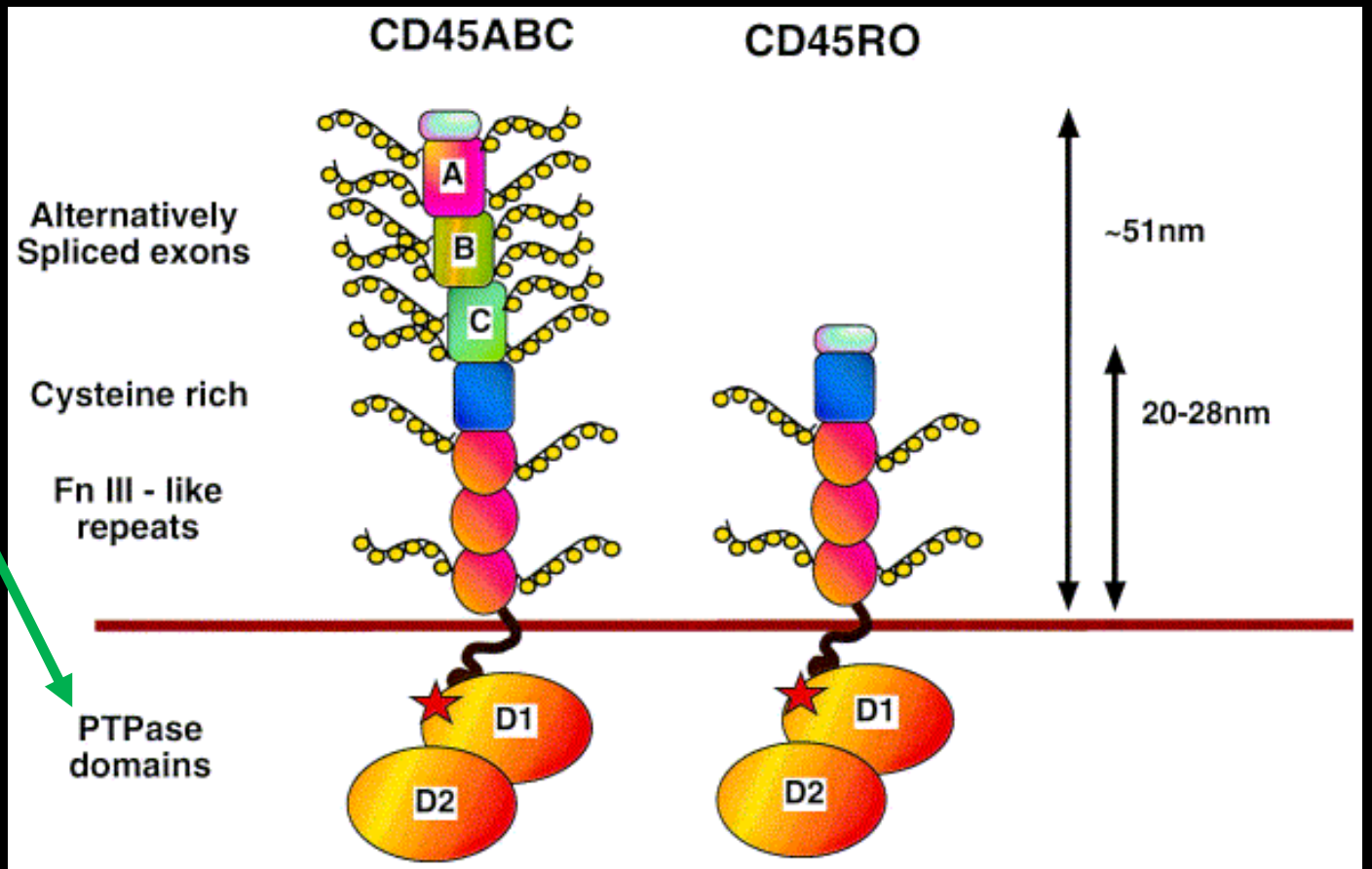
CD45 - Leucocyte common antigen (LCA)

- Transmembrane protein tyrosin phosphatase essential for **haematopoietic signal transduction and cell activation**
- Membrane associated component: 5 isotypes
- Intracellular component: one common type

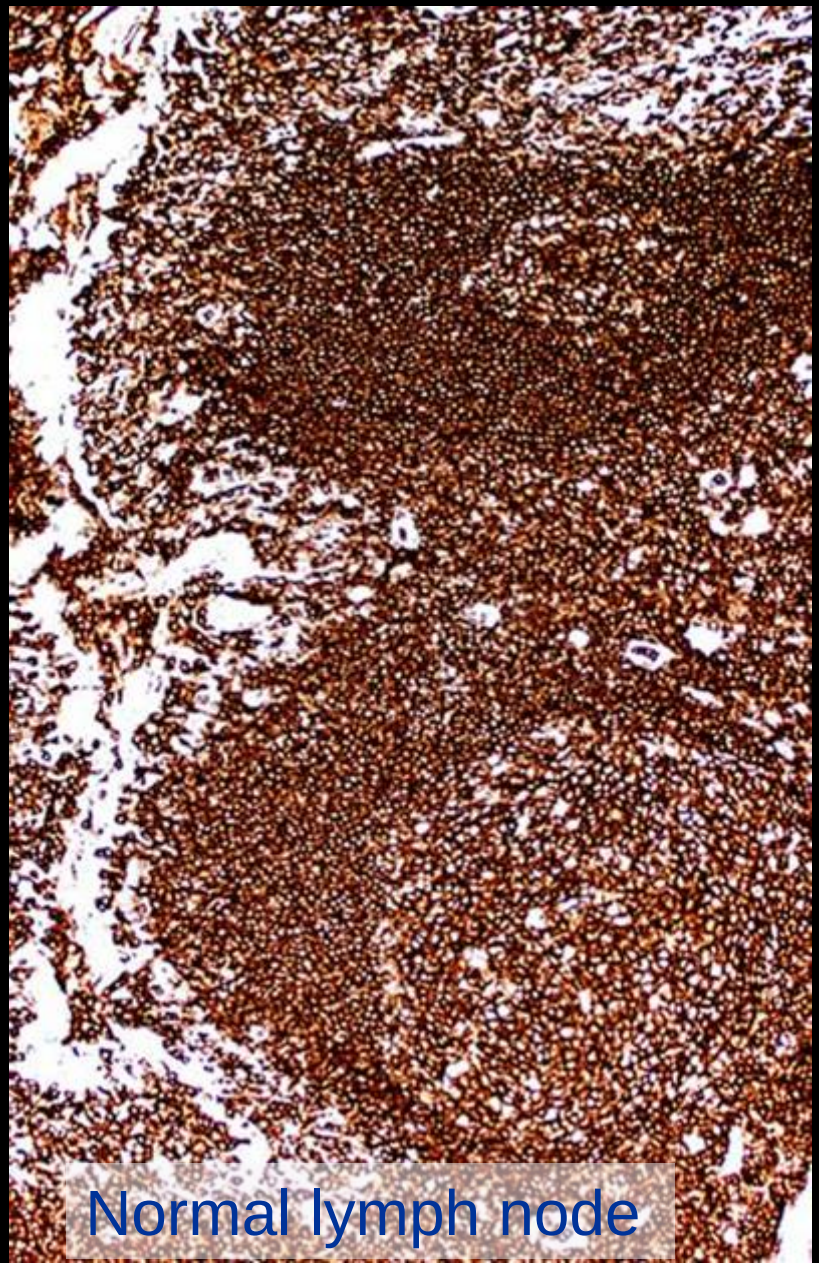


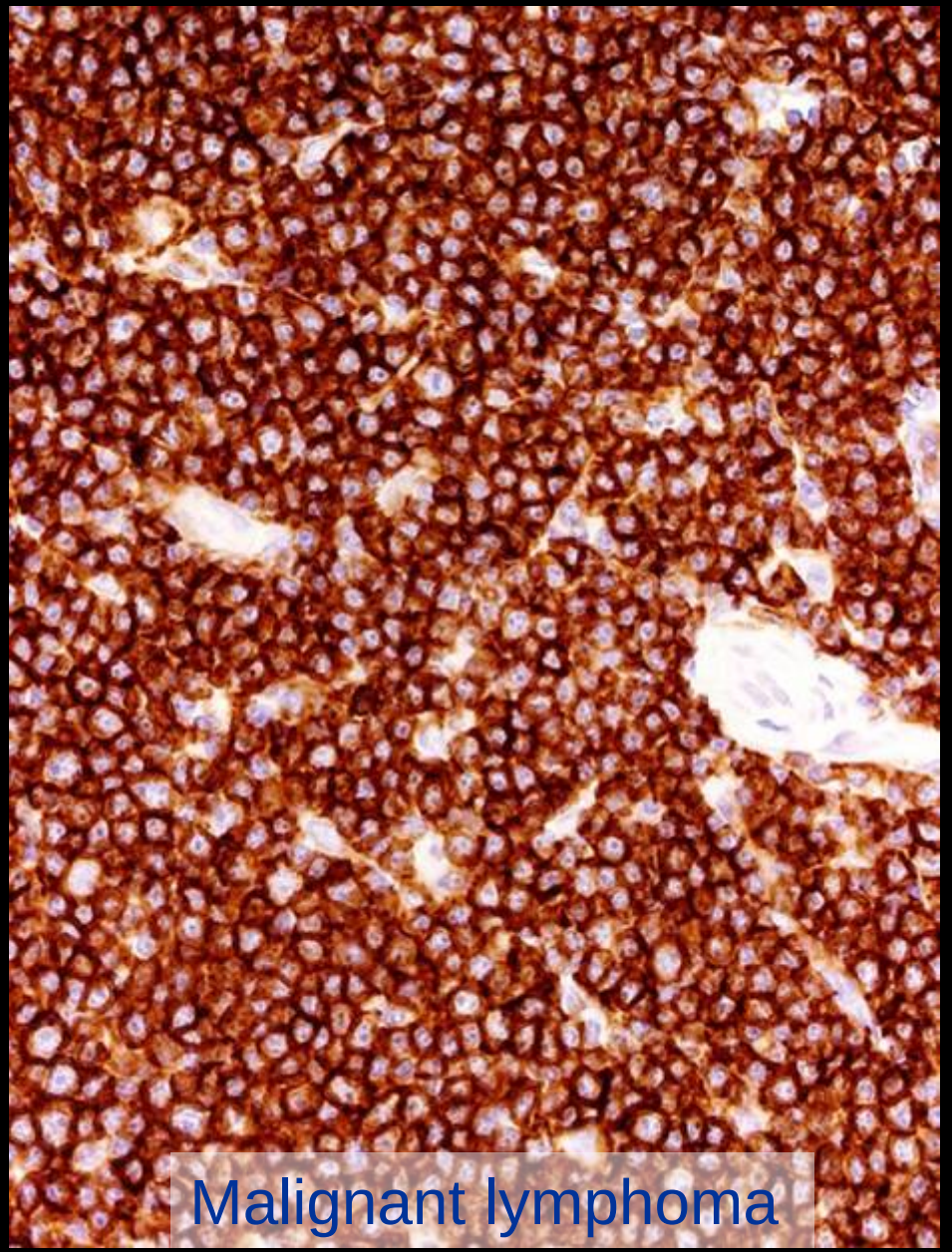
CD45 - Leucocyte common antigen (LCA)

- Large majority of haematolymphoid cells and neoplasms
- Lost in *maturing erythocytes, megakaryocytes and plasmacells*
- ”Never” found in non-haematolymphoid cells and neoplasms



CD45 - Leucocyte common antigen (LCA)





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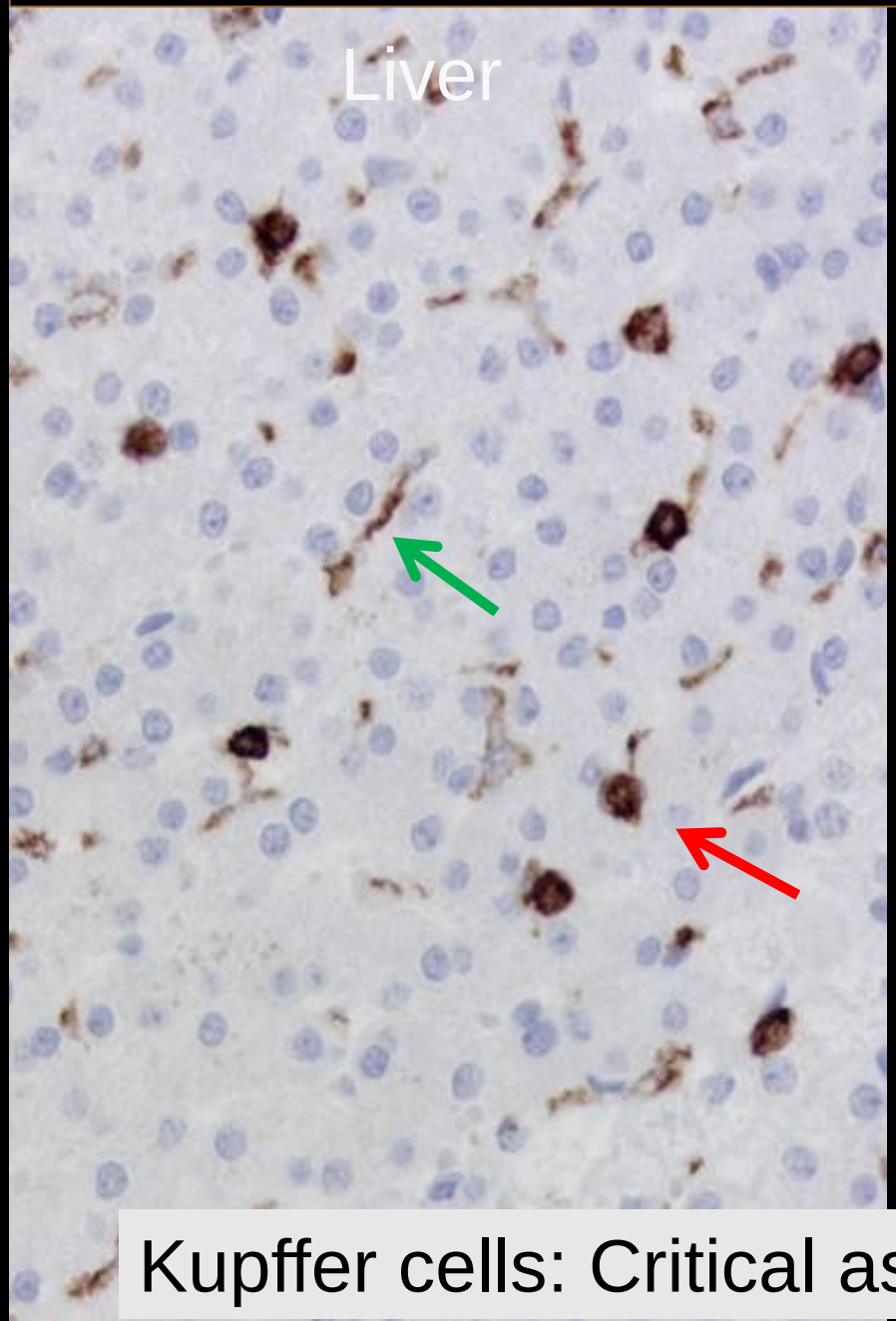
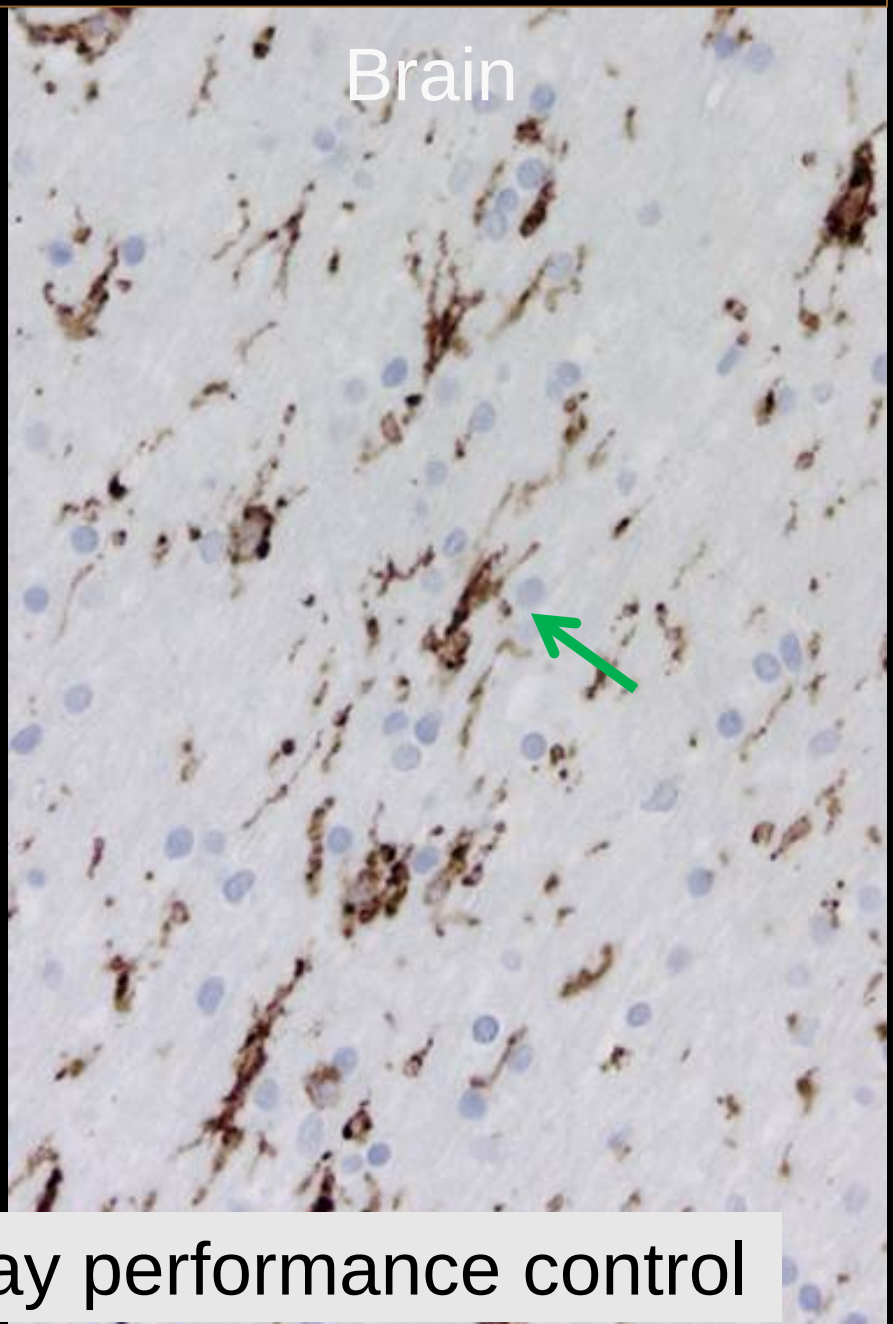
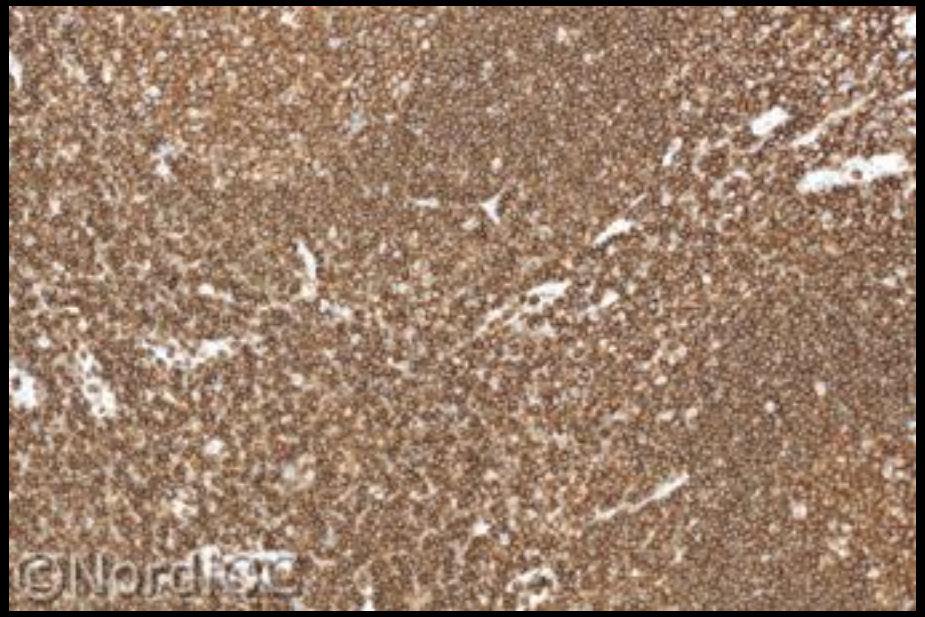
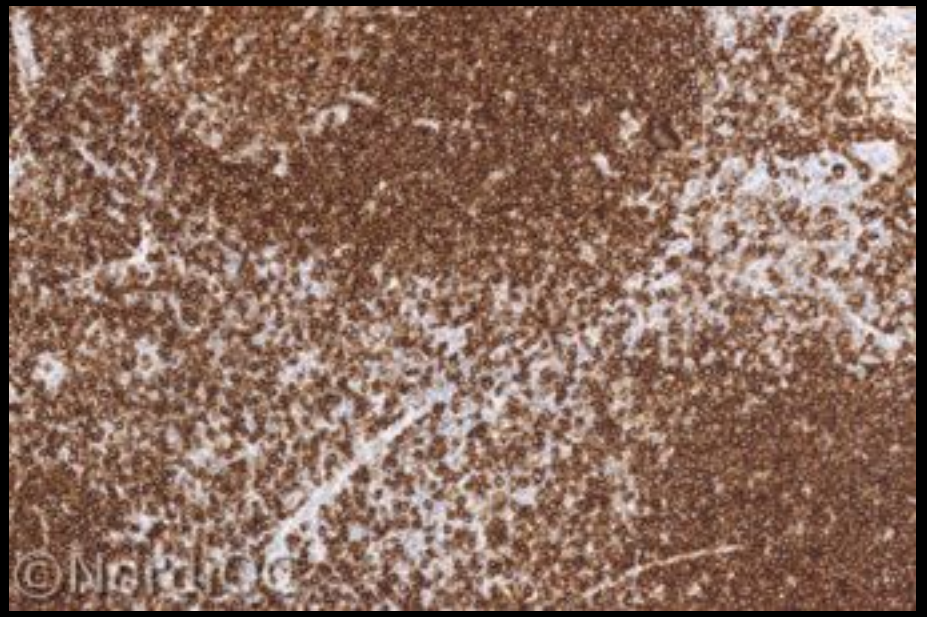




Kupffer cells: Critical assay performance control

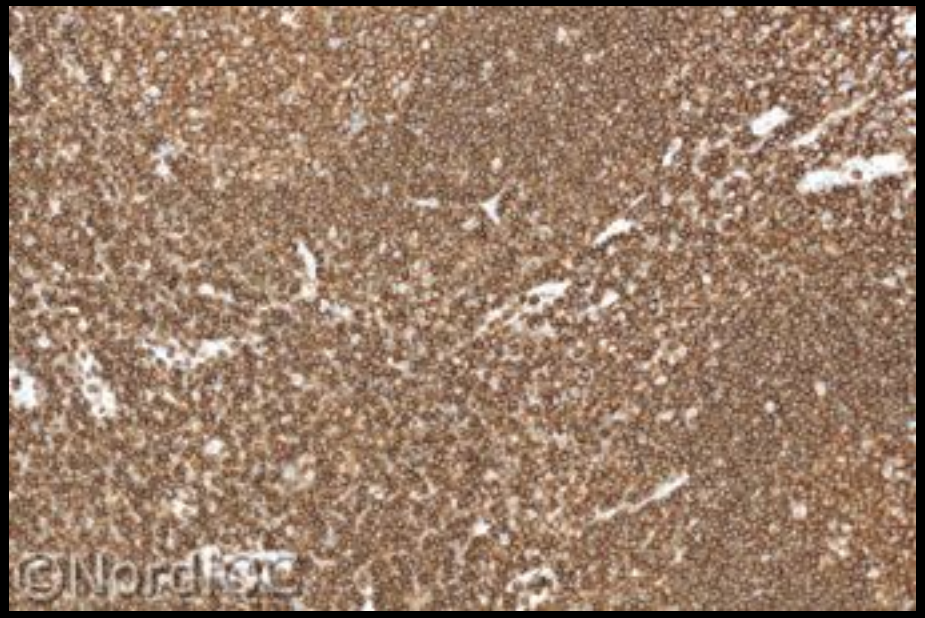
CD45 – NordiQC run 37 2013

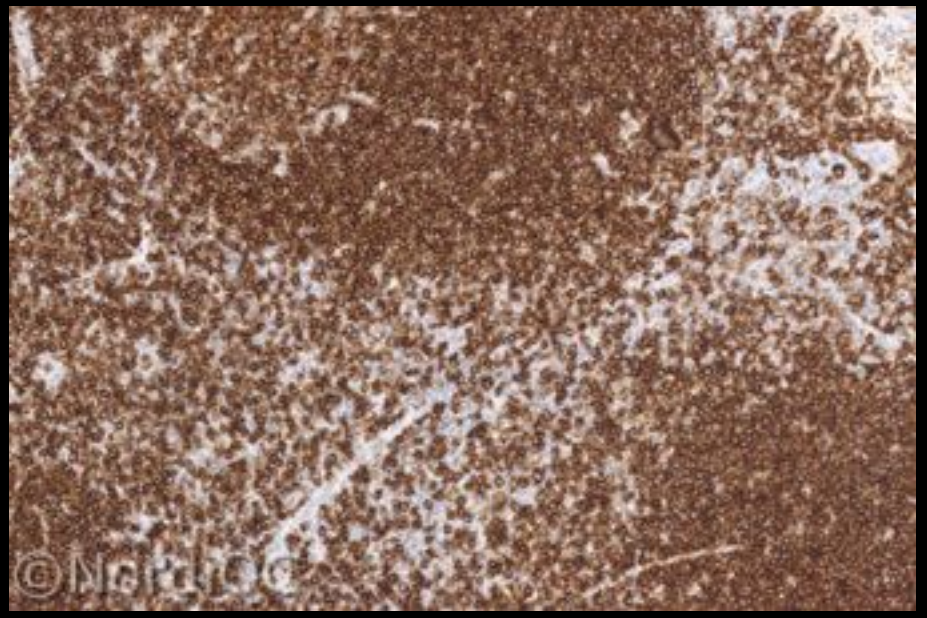


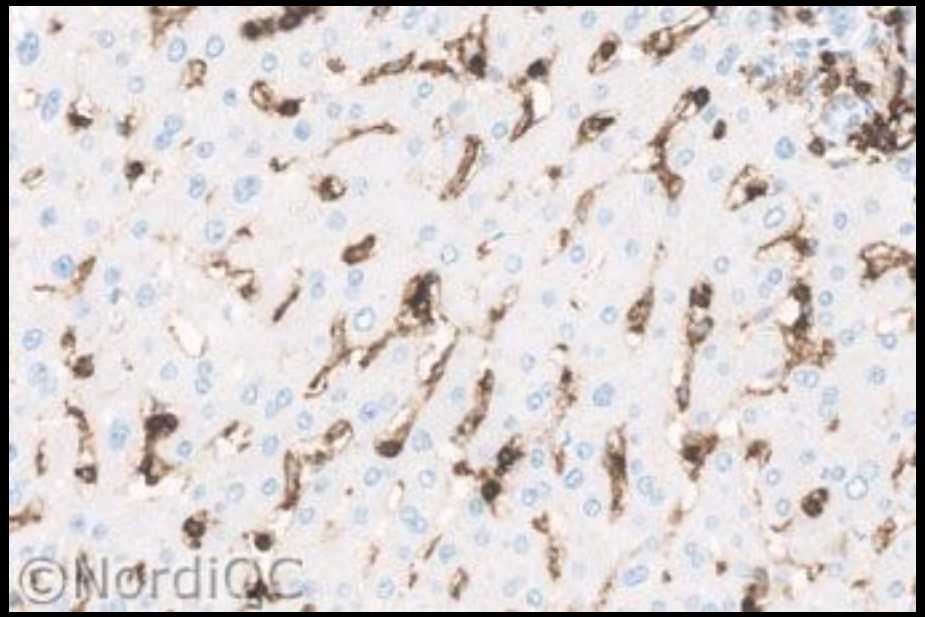


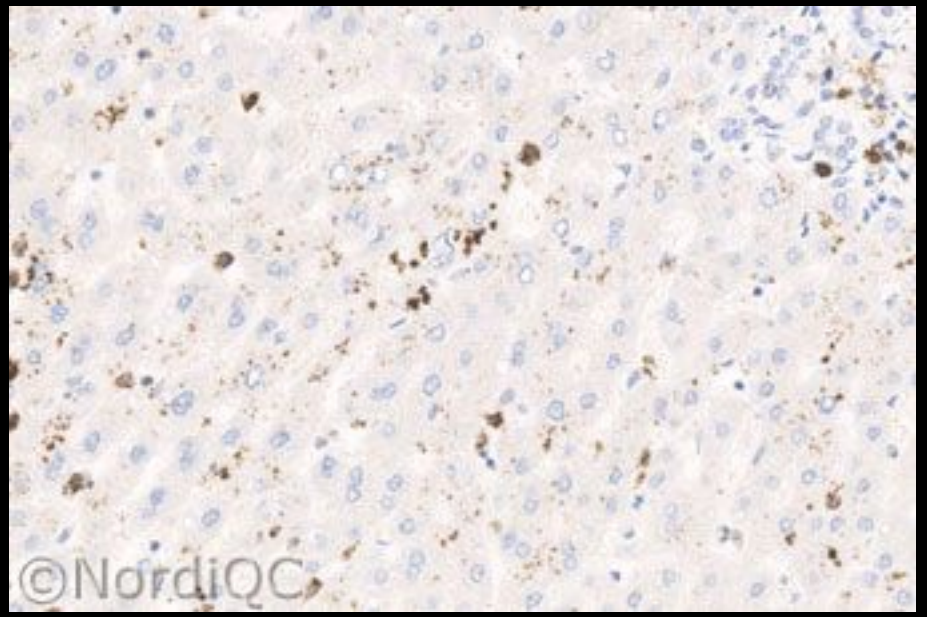
Which is best?

CD45 – NordiQC run 37 2013





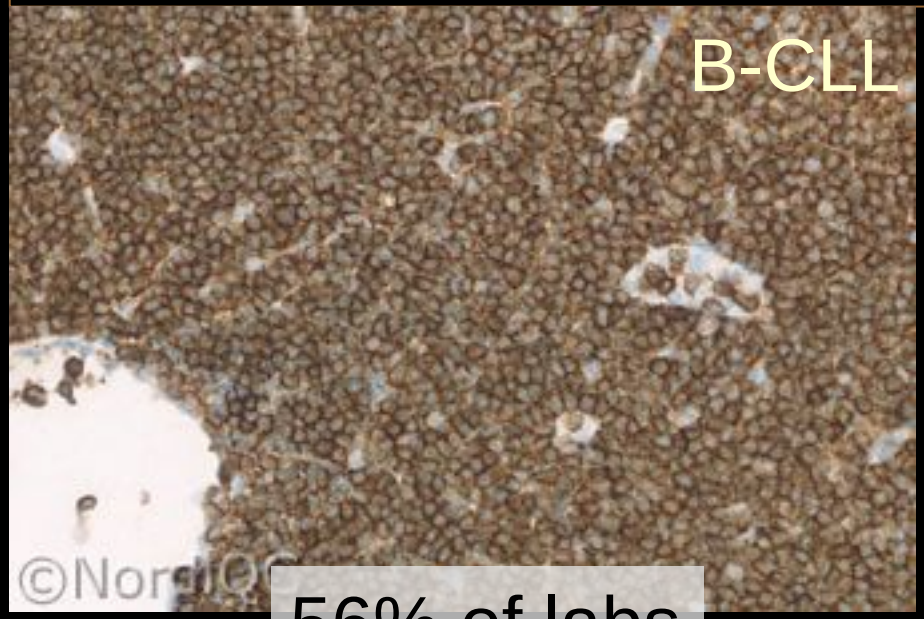


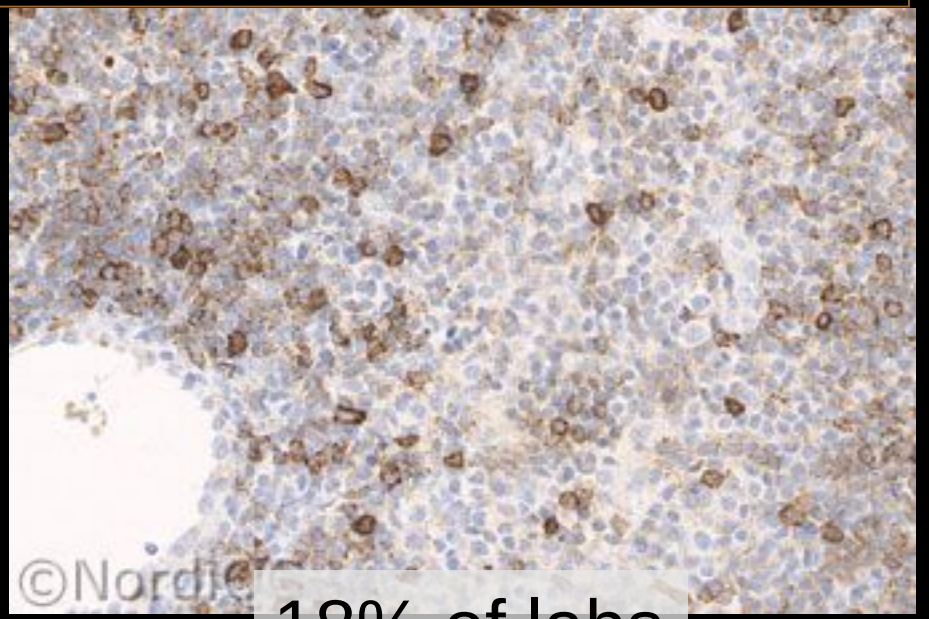


Optimal

Insufficient

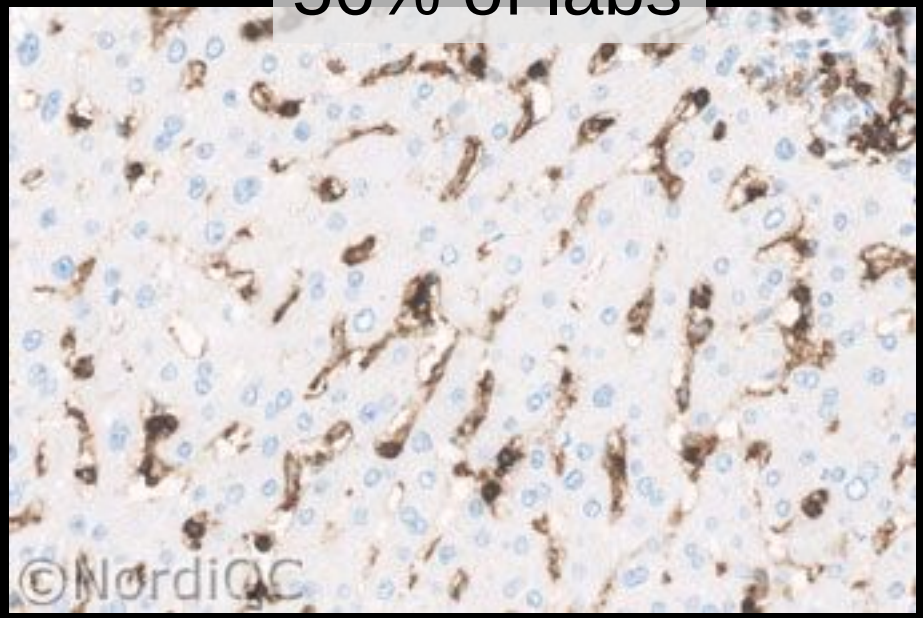
CD45 – NordiQC run 37

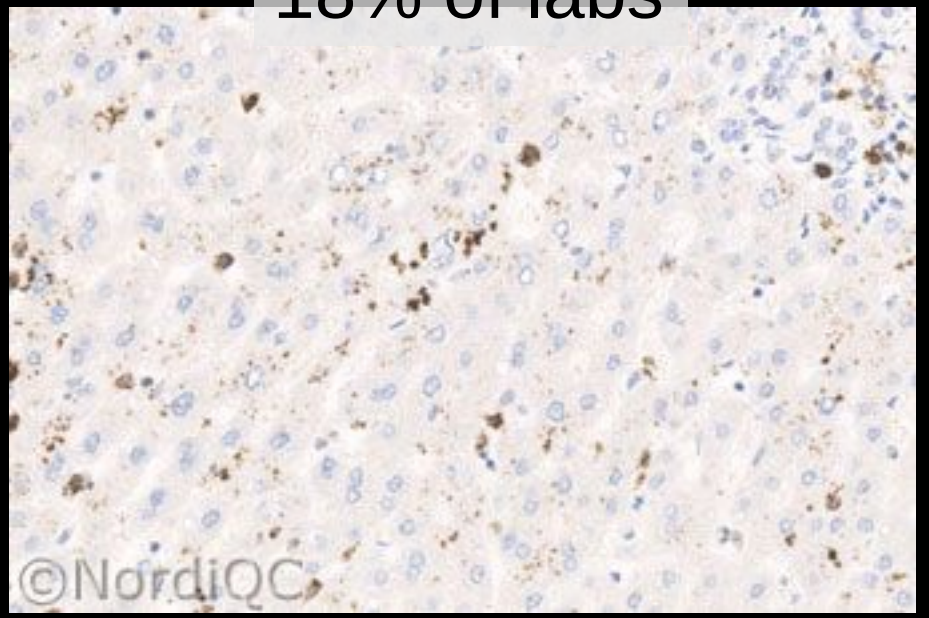




56% of labs

18% of labs

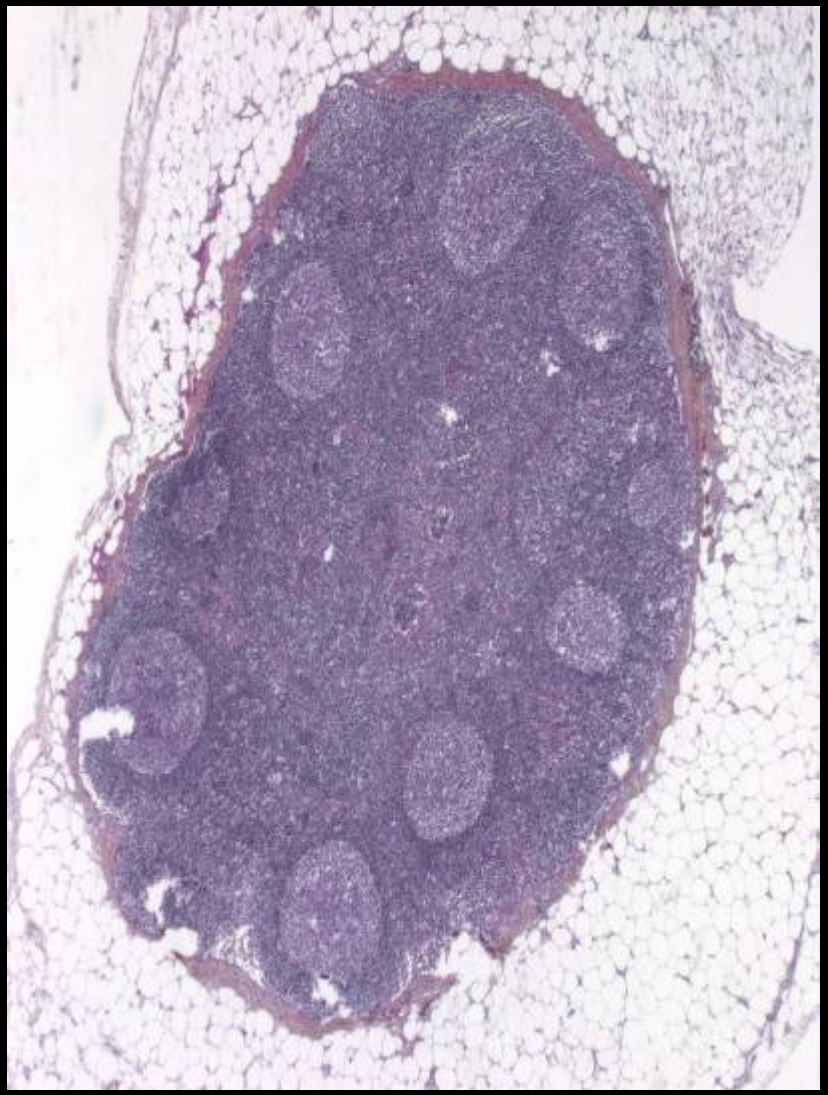




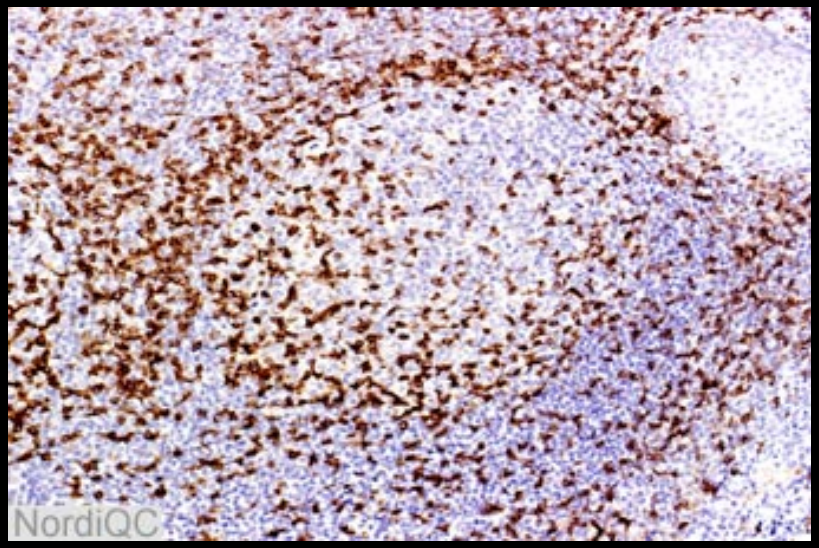
Optimal

Insufficient

CD45 - Leucocyte common antigen (LCA)



Lymph node/Tonsil



- CD45 RO ~ T-cells

- CD45 RA ~ B-cells

CD45 - Leucocyte common antigen (LCA)

Cytokeratin-Positive, CD45-Negative Primary Centroblastic Lymphoma of the Adrenal Gland

A Potential for a Diagnostic Pitfall

Ludvik R. Donner, MD, PhD; Frank E. Mott, MD; Isaac Tafur, MD

● We report a case of cytokeratin-positive, CD45-negative primary polymorphic centroblastic lymphoma of the adrenal gland. Additional immunostaining, which demonstrated positivity for CD20 and κ light chain, as well as detection of the monoclonal rearrangement of the immunoglobulin heavy chain gene, helped to establish the diagnosis of lymphoma and to rule out an initially favored diagnosis of poorly differentiated carcinoma.

(*Arch Pathol Lab Med.* 2001;125:1104–1106)

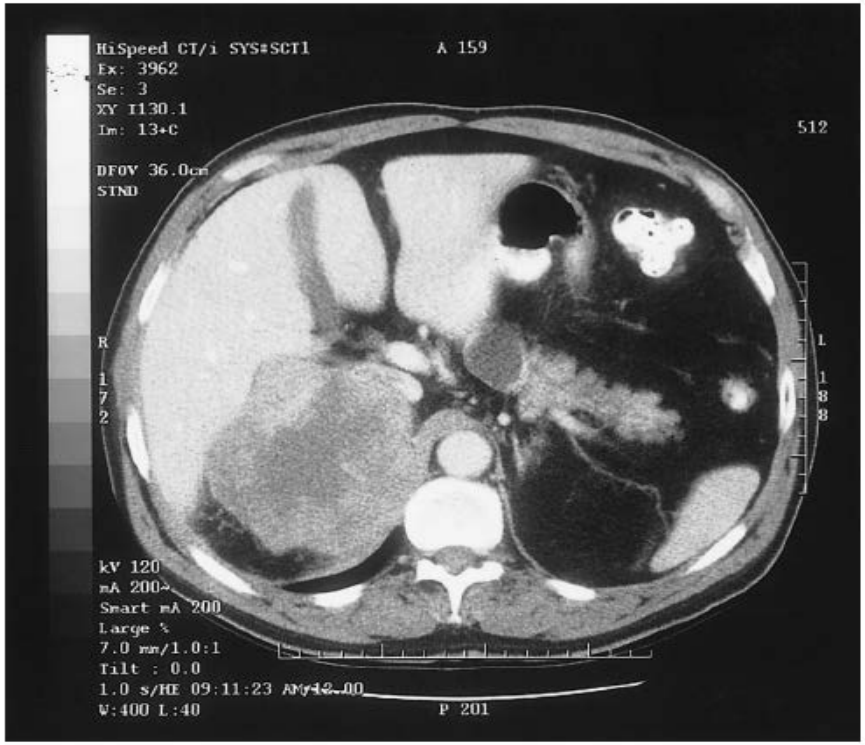


Figure 1. *Computed tomography of a large right suprarenal mass involving the liver.*

CD45 - Leucocyte common antigen (LCA)

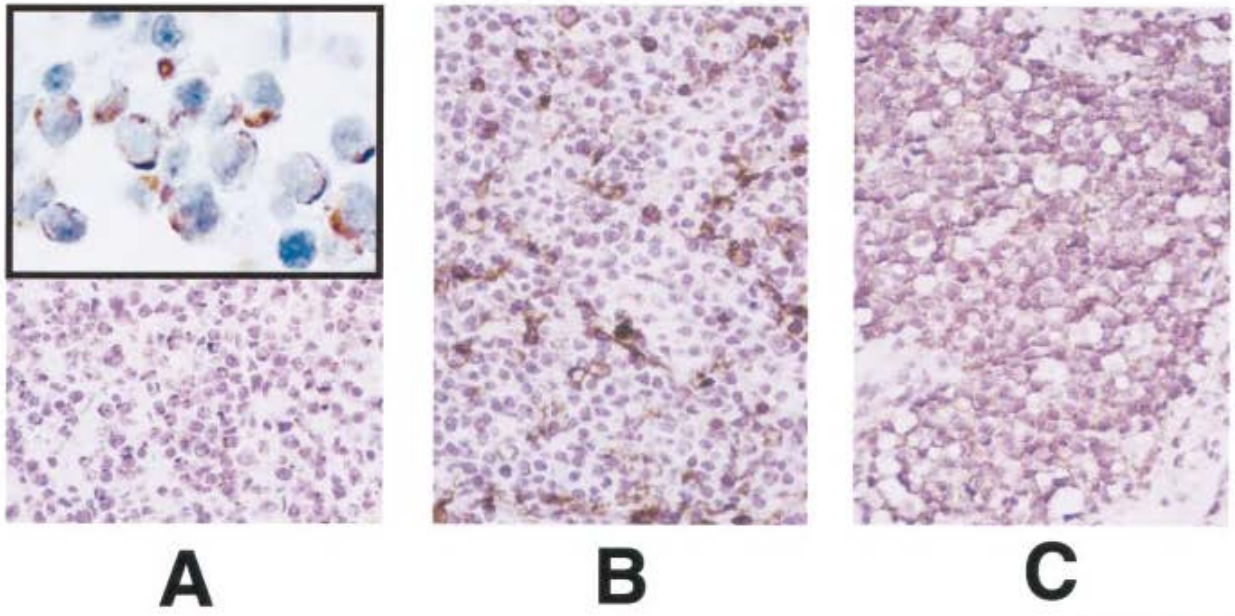


Figure 3. *Note immunoreactivity of the lymphoma cells for cytokeratin (A) and CD20 (C) but not CD45 (B) (original magnification ×100, inset ×250).*

Molecular Biologic Findings

Monoclonal rearrangement of the immunoglobulin heavy chain gene was identified by polymerase chain reaction (data not shown).

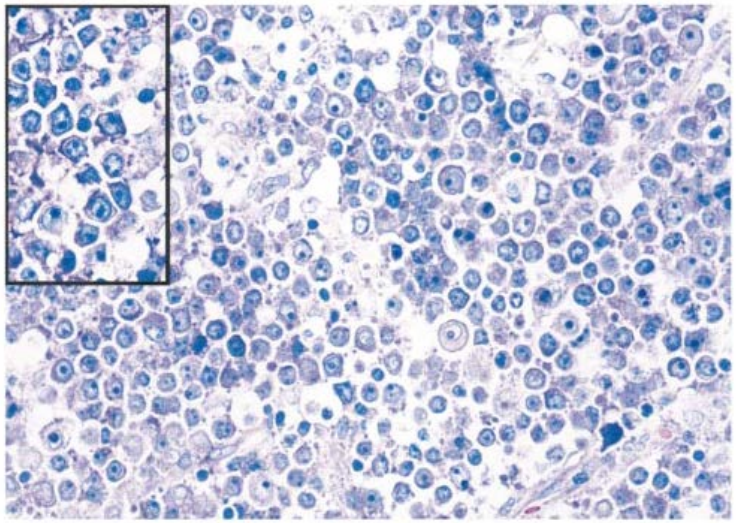


Figure 2. *Light microscopic appearance of the tumor (Giemsa stain, original magnification ×100, inset ×250).*

CD45 - Leucocyte common antigen (LCA)

MATERIALS AND METHODS

We performed immunohistochemical stains for cytokeratin (AE1/AE3. Cell Marque. Austin. Tex: CAM5.2. Becton Dickinson. San Jose, Calif; cytokeratins 5/6, Zymed, San Francisco, Calif; cytokeratin 7, Dako Corporation, Carpinteria, Calif; cytokeratin 20, Dako; 34βE12, Enzo, New York, NY), CD3, CD20, CD30, CD45RO, CD68, κ light chain, λ light chain, myeloperoxidase, epithelial membrane antigen, neuron-specific enolase, synaptophysin, S100 protein, HMB-45 (Dako), and chromogranin A (Cell Marque) on a TechMate 500 with a ChemMate Secondary Detection Kit–Peroxidase/DAB (Ventana Medical Systems, Tucson, Ariz). The histologic sections were pretreated by steaming in citrate buffer solution (Target Retrieval Solution, Dako) for 30 minutes at 99°C.

The monoclonal antibodies AE1/AE3 (working concentration, 0.4 μg of protein/mL) were applied for 25 minutes at room temperature. The immunostaining was repeated twice, each time with identical results.

Primary panel for the unknown primary tumour

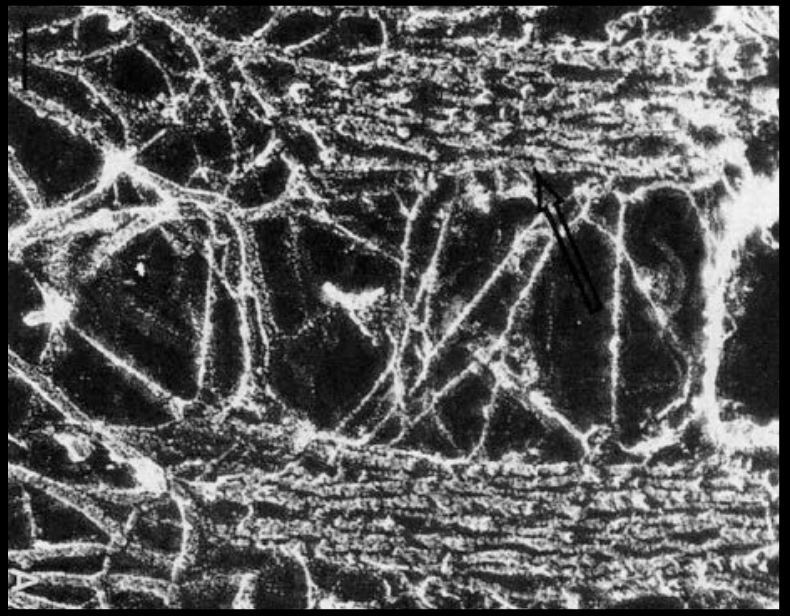
	CD45	Pan-CK	S-100	VIM
Haemato-lymphoid neoplasms	+/(-)	-/(+)	-/(+)	+/(-)
Epithelial neoplasms	-	+/(-)	-/+	-/+
Mesothelial neoplasms	-	+	-	+
Mesenchymal and neuronal neoplasms	-	-/(+)	-/+	+
Non-neuronal neuroepithelial neoplasms	-	-/(+)	+	+
Germ cell neoplasms	-	-/+	-/+	+

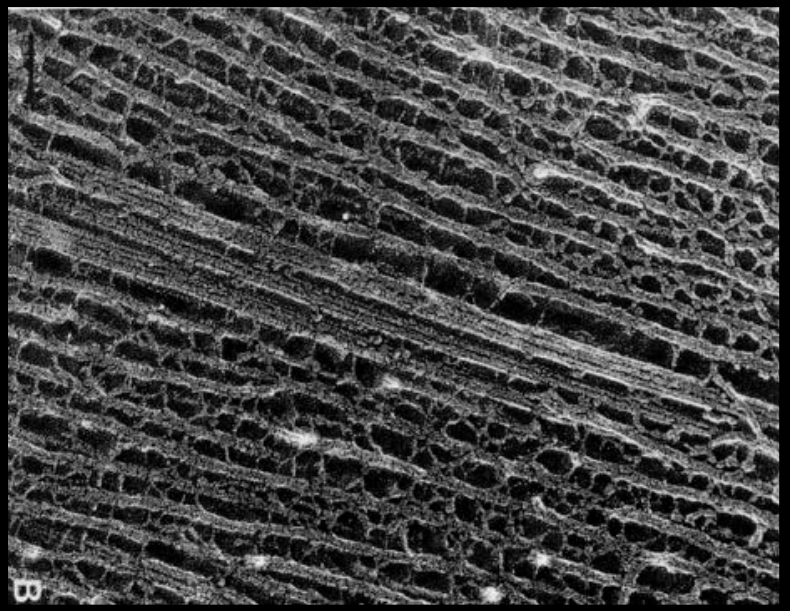
Cellular filaments

Microfilaments: (6 nm)

Intermediate filaments (7- 11 nm)

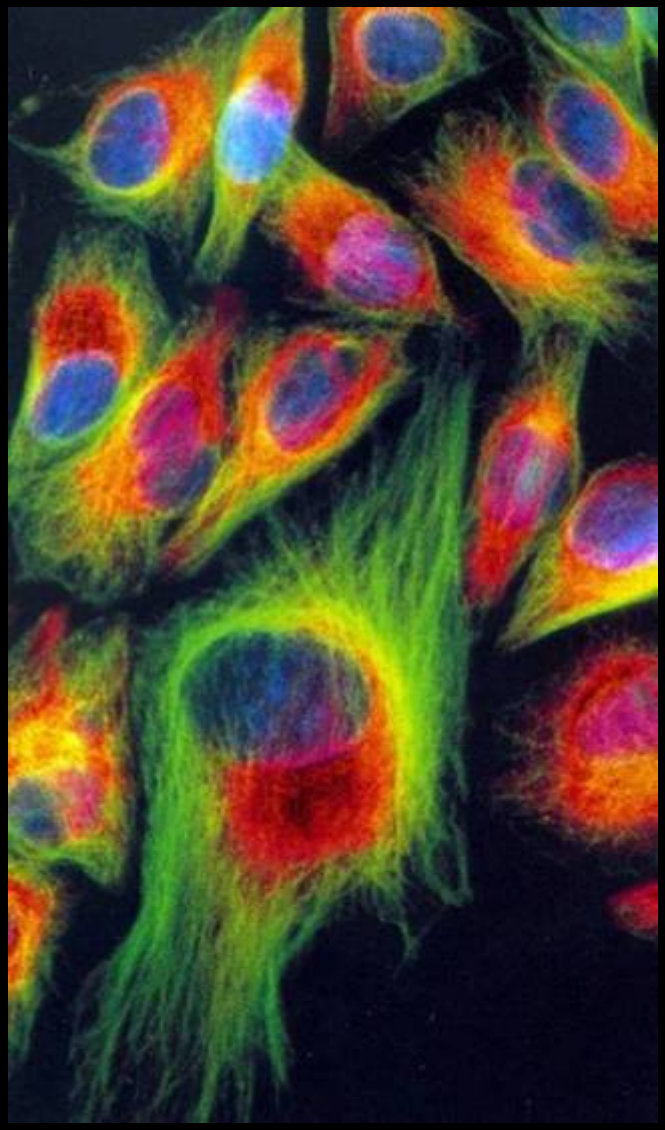
Microtubuli (23 nm)



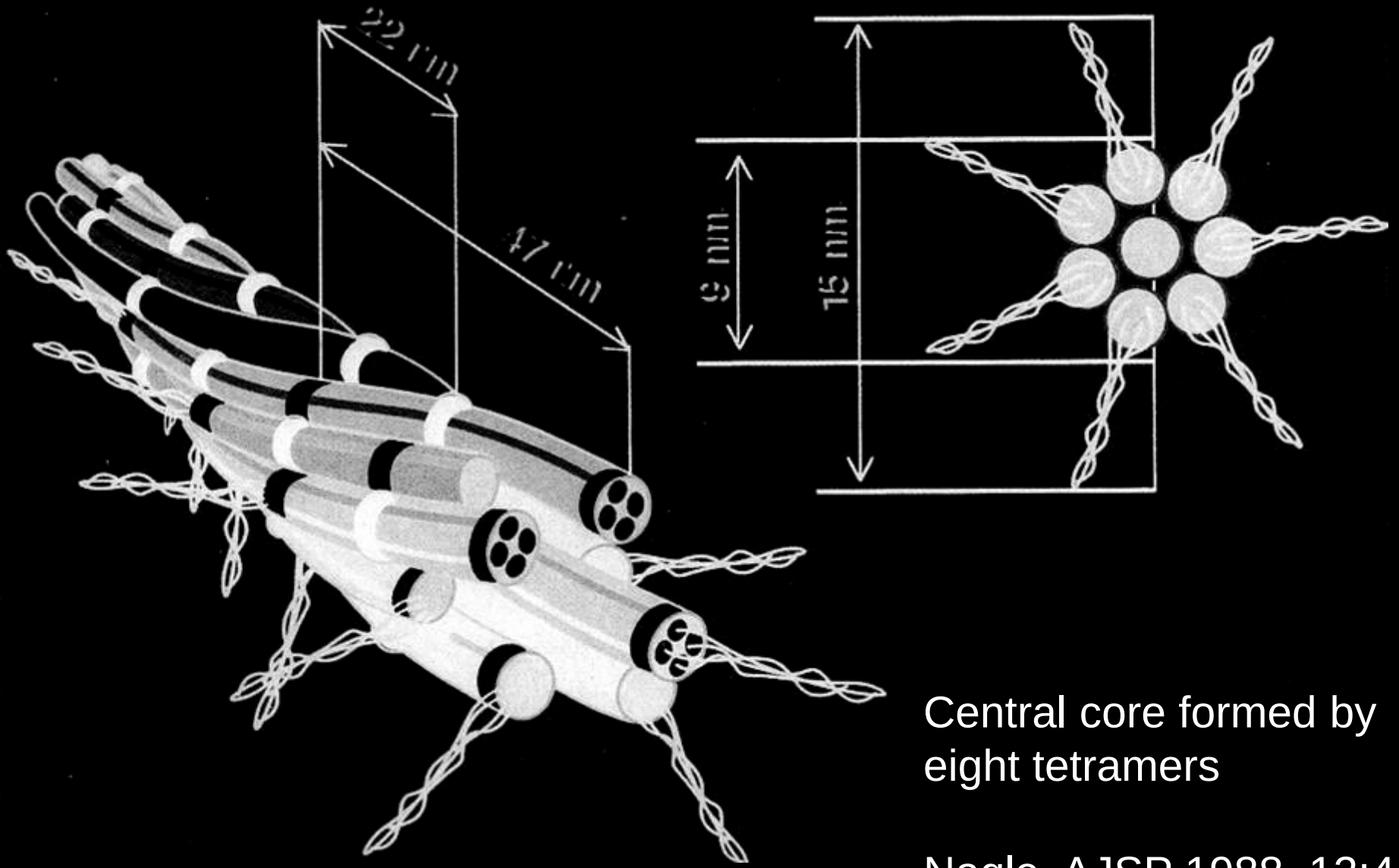


Intermediate filaments

- Group of mainly cytoplasmic filaments 7 – 11 nm in diameter
- Part of the cytoskeleton in virtually all cells, creating as meshwork and connecting nuclear membrane with cell membrane
- Often associated with microfilaments (6 nm) and microtubules (23 nm)
- Important for mechanical strength and cellular functions



Intermediate filaments – tetrameric units

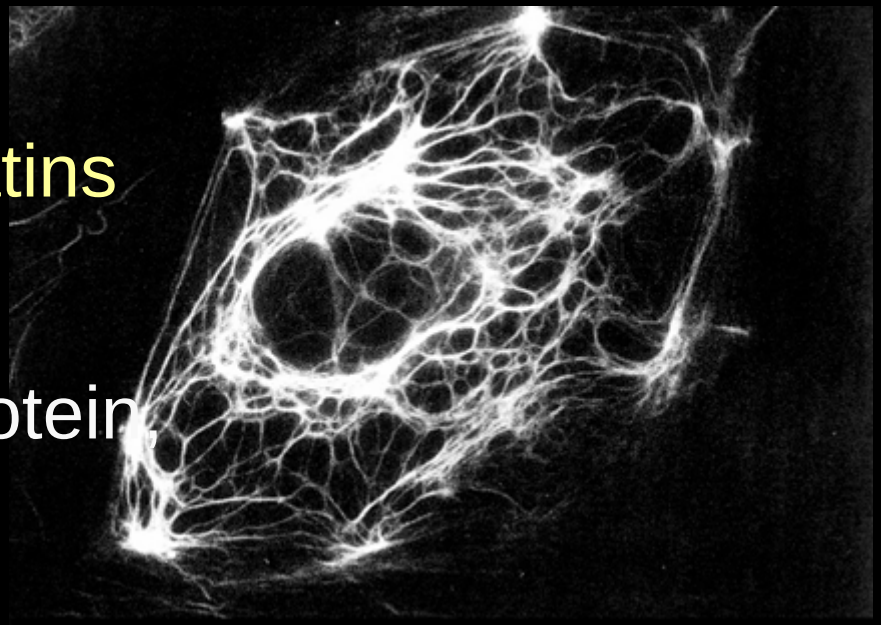


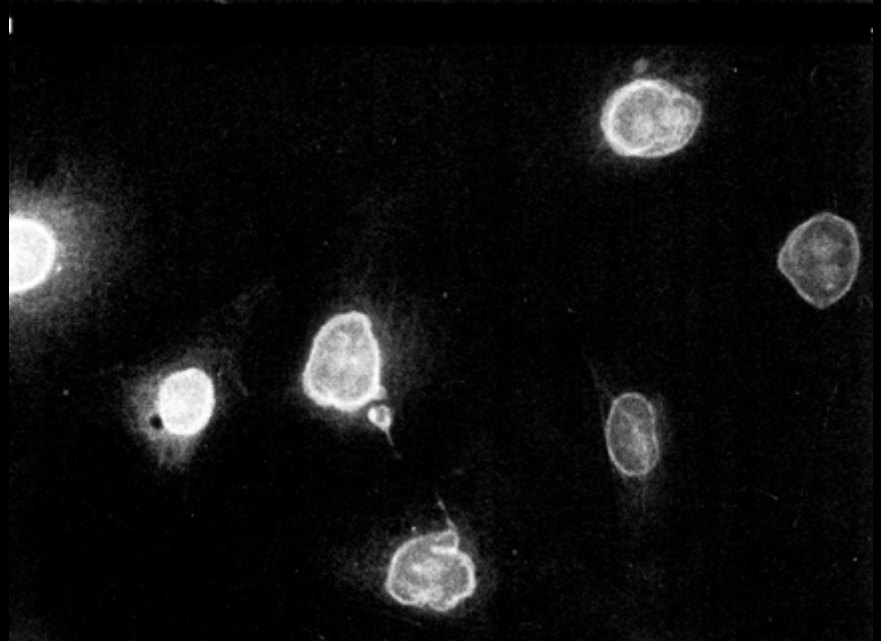
Central core formed by eight tetramers

Nagle, AJSP 1988, 12:4

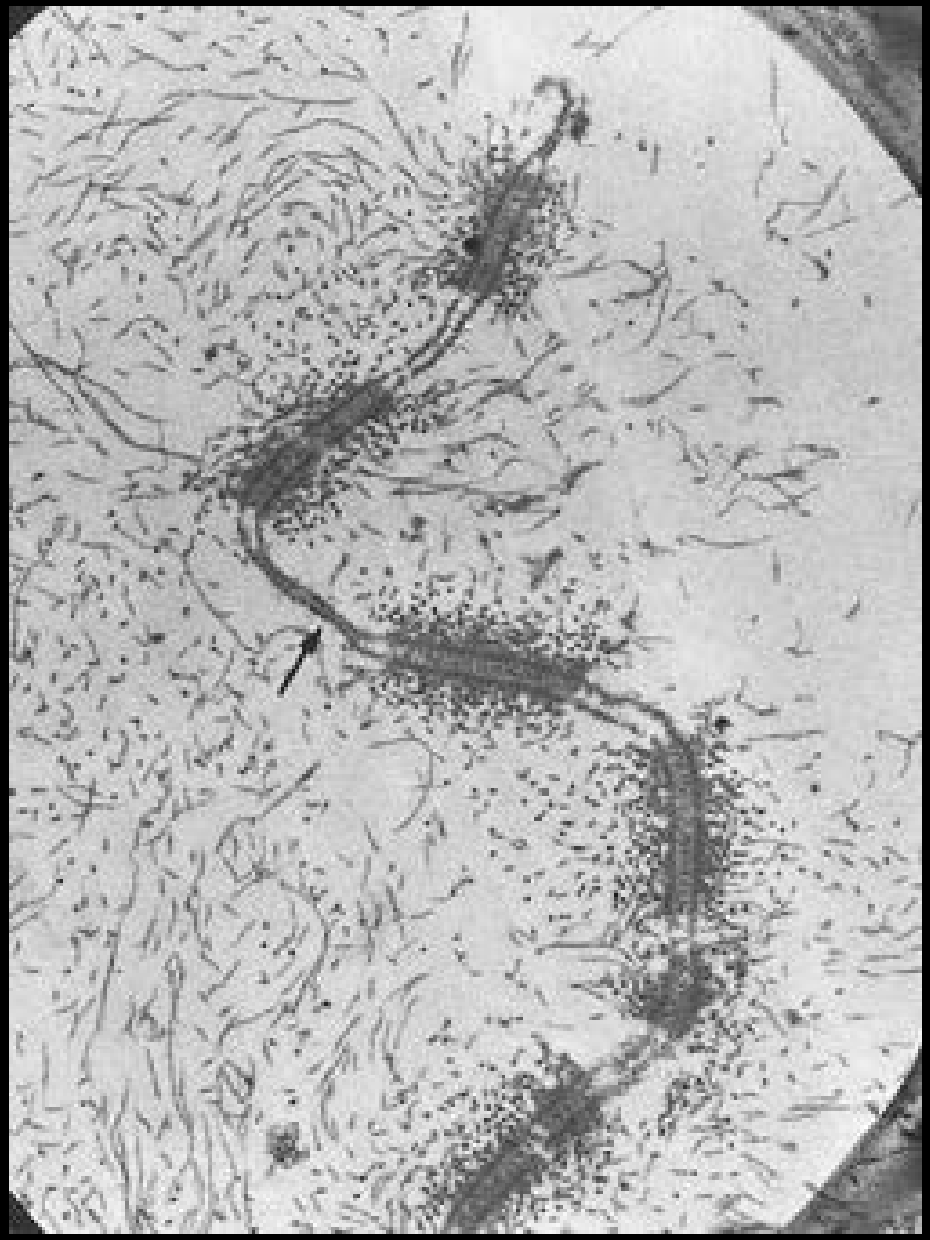
Intermediate filaments - 5 classes

- **I** acidic cytokeratins
- **II** basic-neutral cytokeratins
- III vimentin, desmin,
- glial fibrillary acidic protein,
- peripherin
- IV neurofilament protein,
- α-internexin, nestin
- V lamins





Cytokeratins as tonofilaments



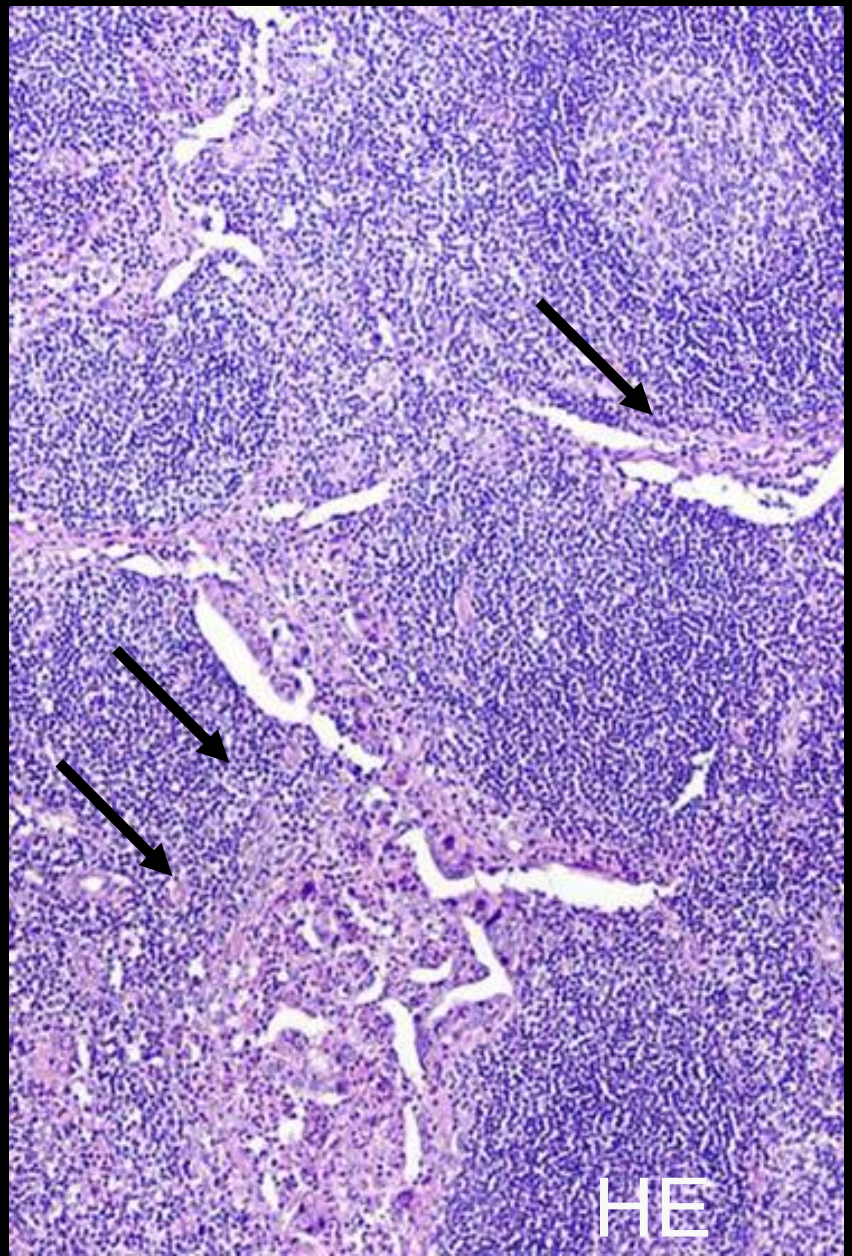
Cytokeratin intermediate filaments attached to desmosomes

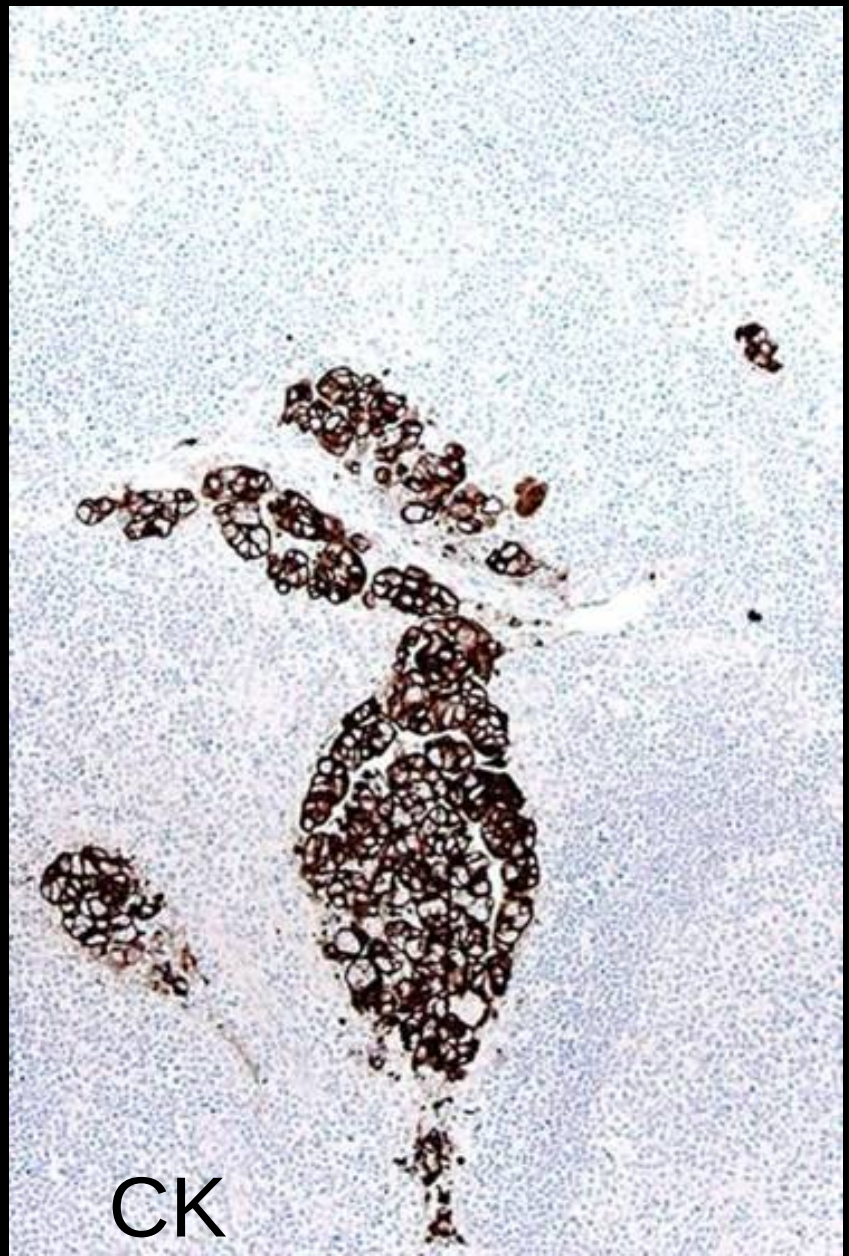
Drochmans et al.
J Cell Biol. 1978, 79:427

Cytokeratins in diagnostic pathology

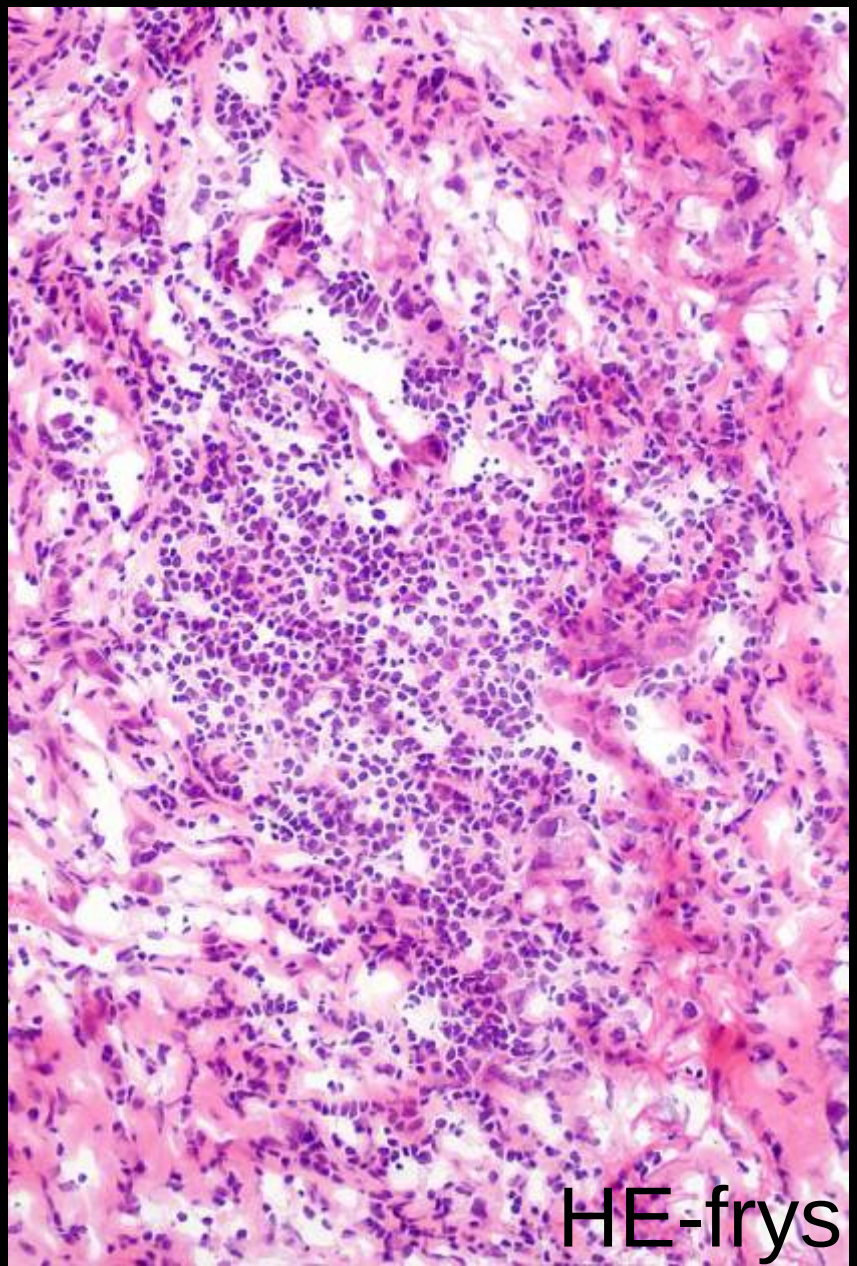
- Cytokeratins (CKs) belong to the most fundamental markers of epithelial differentiation
- CKs comprise a large family of subtypes. Different cell types express different patterns of CK subtypes
- Cancers generally express CK patterns that at least in part represent the pattern of the putative cell of origin
- Metastases express CK patterns fairly concordant with those of the primary tumours

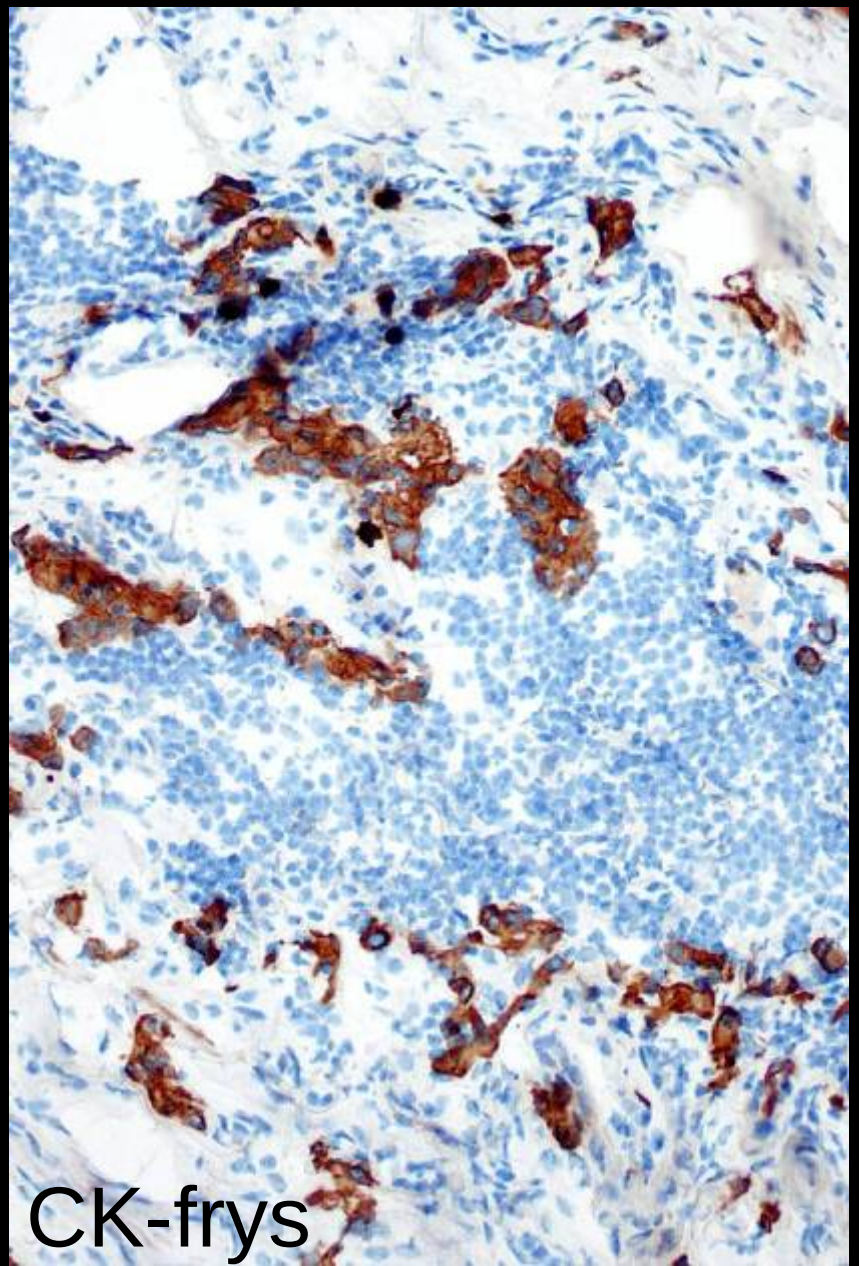
Micrometastases identified by cytokeratin





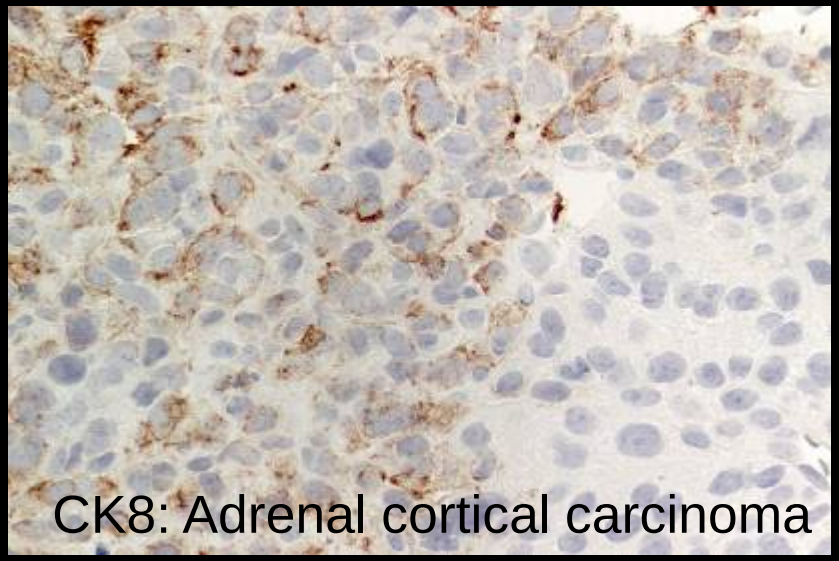
Carcinoma in frosen section identified by cytokeratin



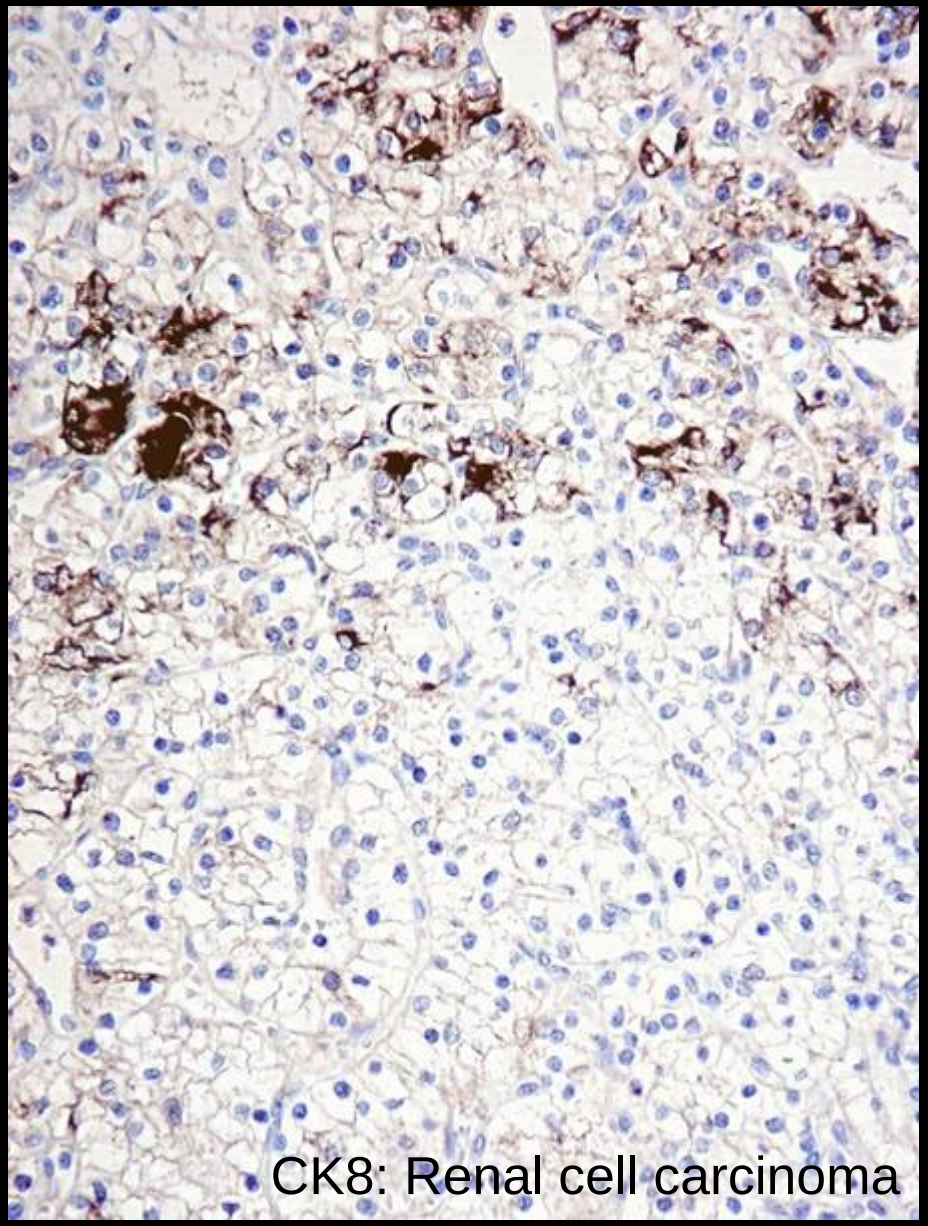


Low molecular weight cytokeratins in carcinomas

- Carcinomas “always” LMW-CK-positive, except some cases of
  - Renal cell carcinoma
  - Adrenal cortical carcinoma
  - Small cell carcinoma



CK8: Adrenal cortical carcinoma



CK8: Renal cell carcinoma

Primary panel for the unknown primary tumour

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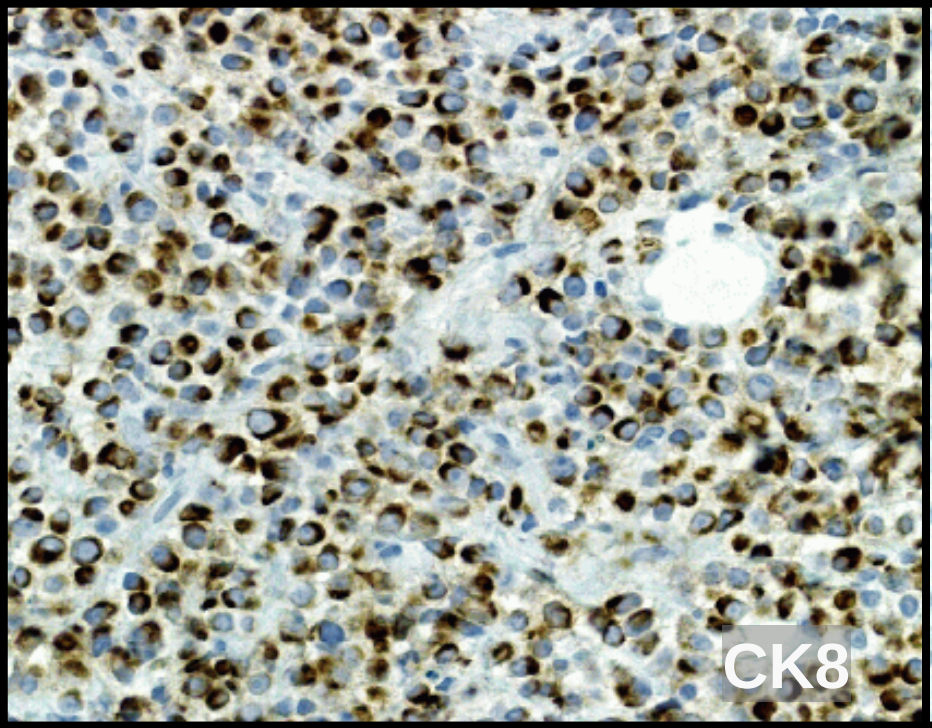
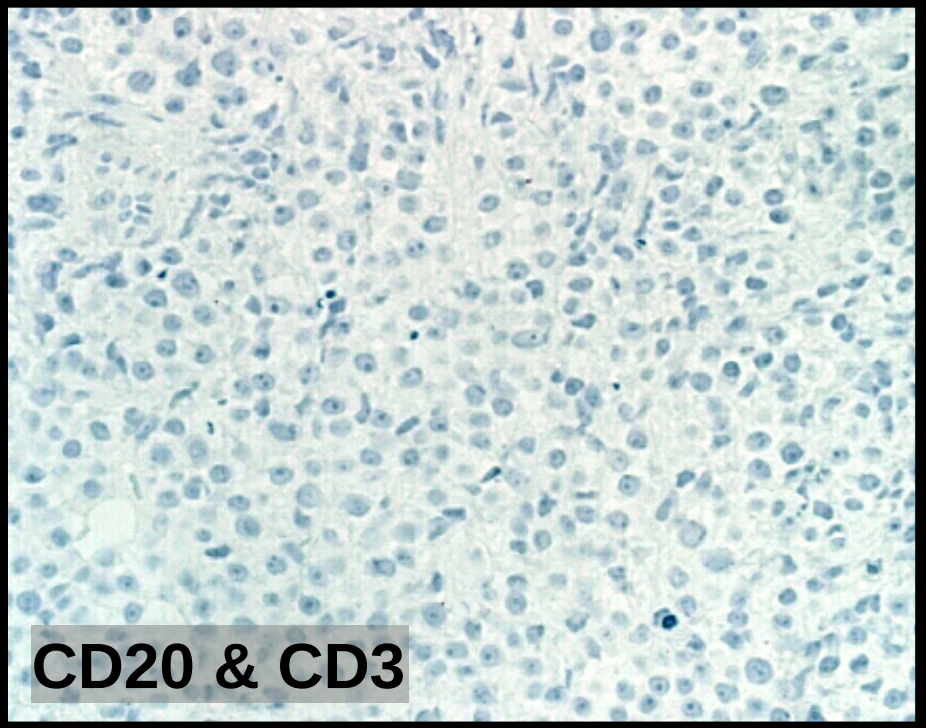
Epithelial neoplasms	-	+/(-)	-/+	-/+
Mesothelial neoplasms	-	+	-	+
Mesenchymal and neuronal neoplasms	-	-/(+)	-/+	+
Non-neuronal neuroepithelial neoplasms	-	-/(+)	+	+
Germ cell neoplasms	-	-/+	-/+	+

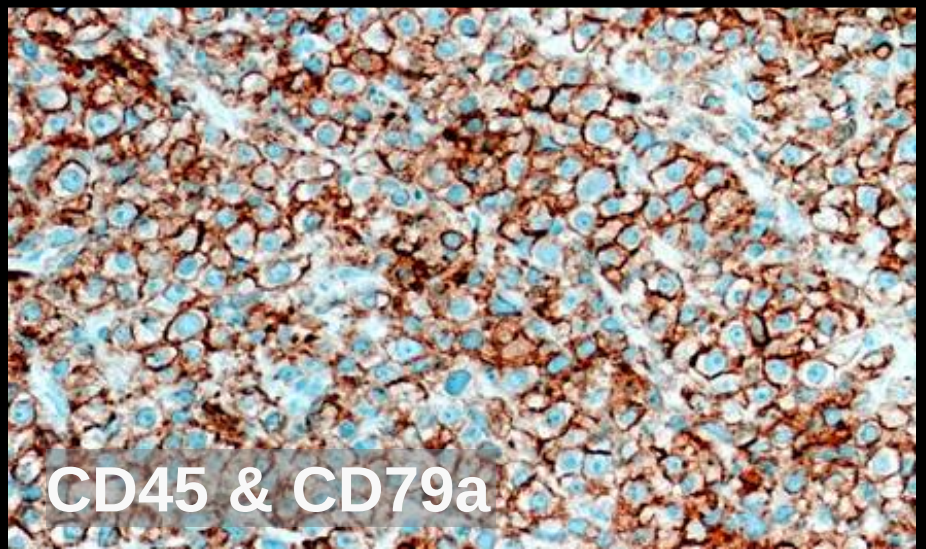
Cytokeratins in non-epithelial tumours





♀ 42 y, tumour infiltrating retroperitoneum

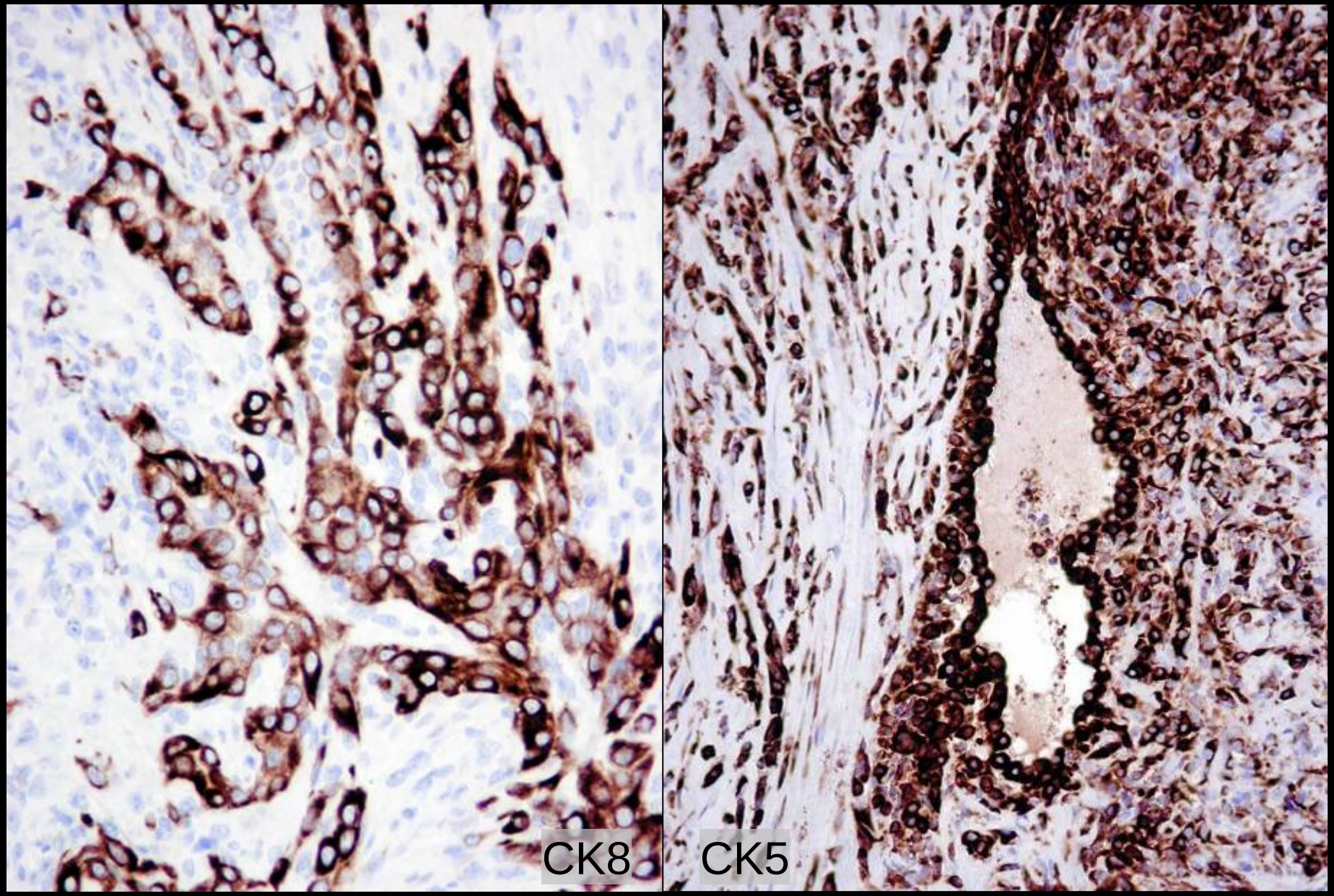
Malignant lymphoma !



Primary panel for the unknown primary tumour

	CD45	Pan-CK	S-100	VIM
Haemato-lymphoid neoplasms	+/(-)	-/(+)	-/(+)	+/(-)
Epithelial neoplasms	-	+/(-)	-/+	-/+
Mesothelial neoplasms	-	+	-	+
Mesenchymal and neuronal neoplasms	-	-/(+)	-/+	+
Non-neuronal neuroepithelial neoplasms	-	-/(+)	+	+
Germ cell neoplasms	-	-/+	-/+	+

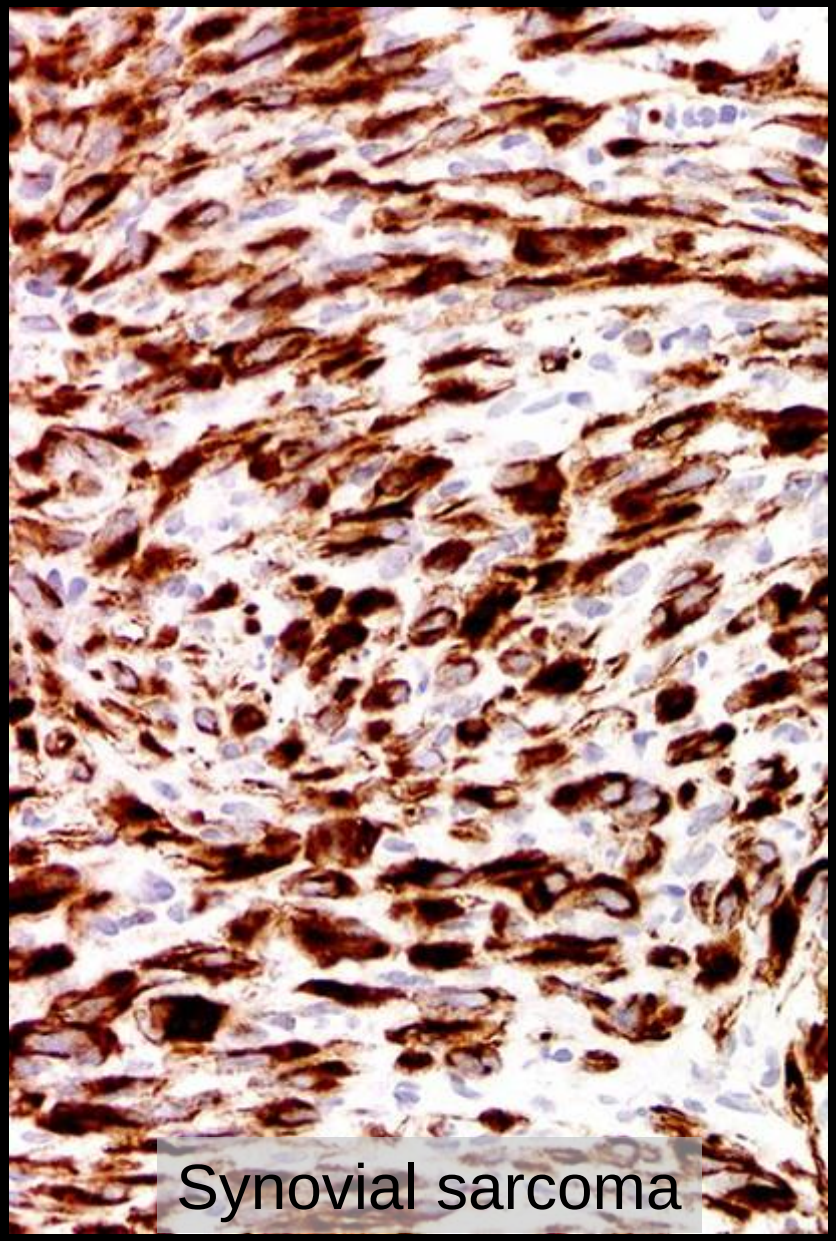
Cytokeratins in malignant mesothelioma



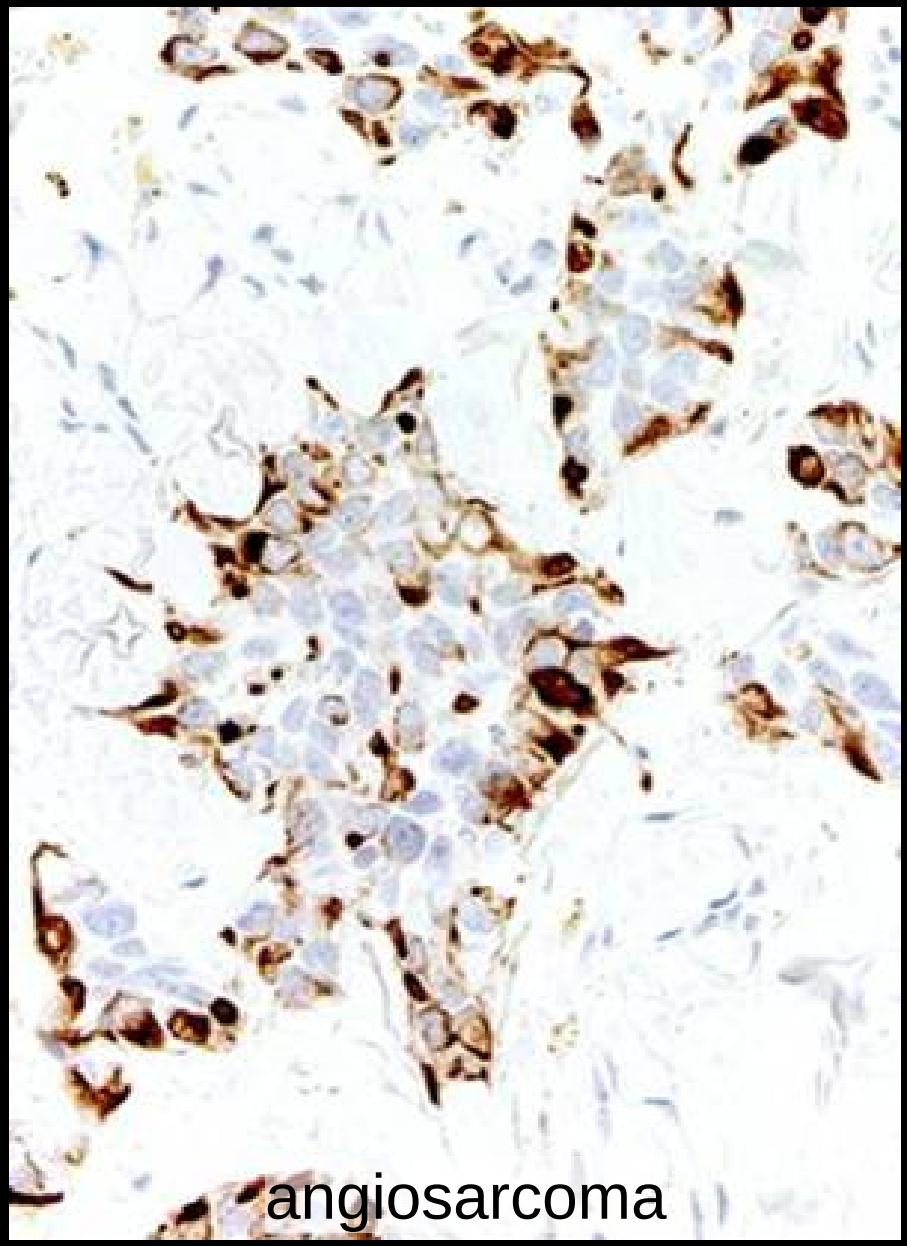
Primary panel for the unknown primary tumour

	CD45	Pan-CK	S-100	VIM
Haemato-lymphoid neoplasms	+/(-)	-/(+)	-/(+)	+/(-)
Epithelial neoplasms	-	+/(-)	-/+	-/+
Mesothelial neoplasms	-	+	-	+
Mesenchymal and neuronal neoplasms	-	-/(+)	-/+	+
Non-neuronal neuroepithelial neoplasms	-	-/(+)	+	+
Germ cell neoplasms	-	-/+	-/+	+

Cytokeratins in sarcomas

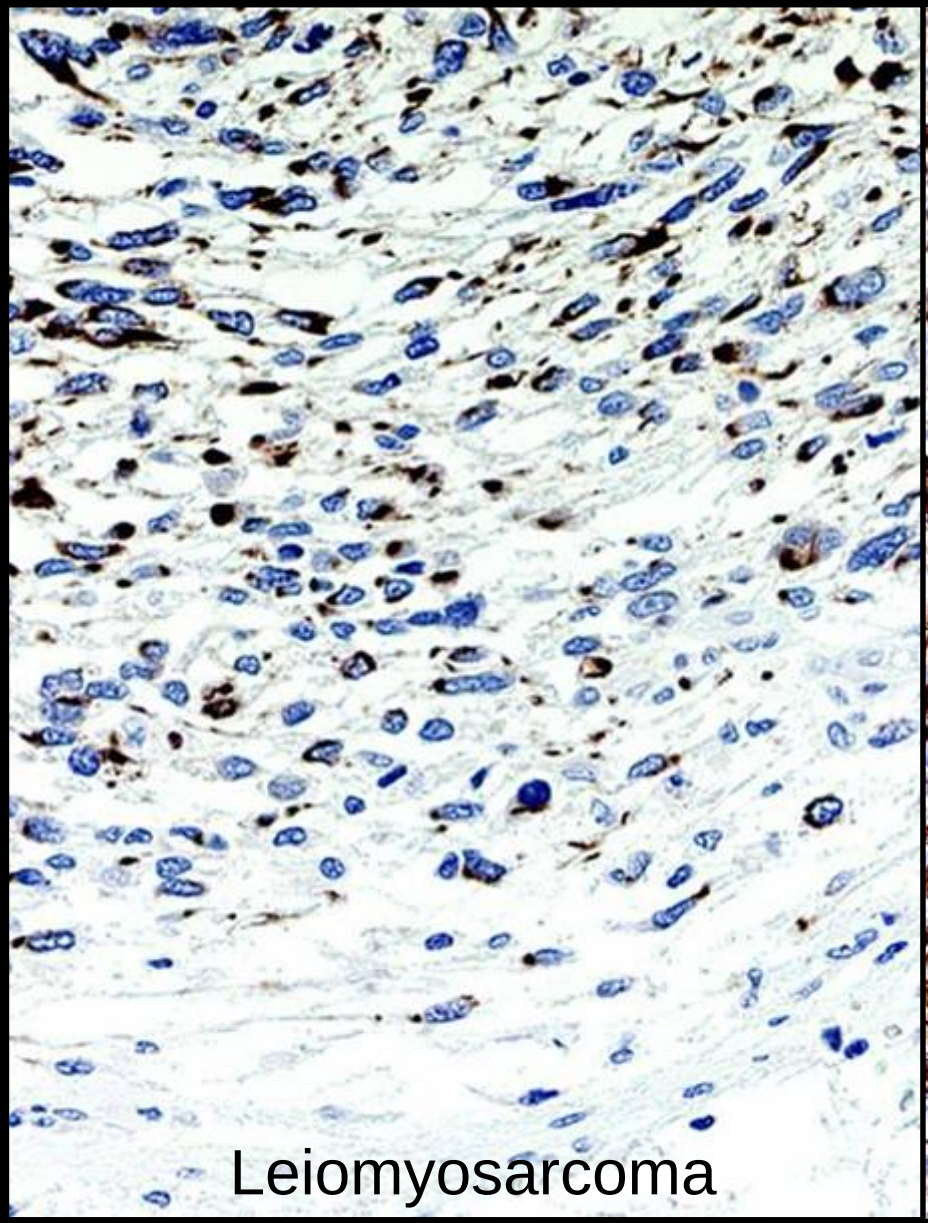


Synovial sarcoma

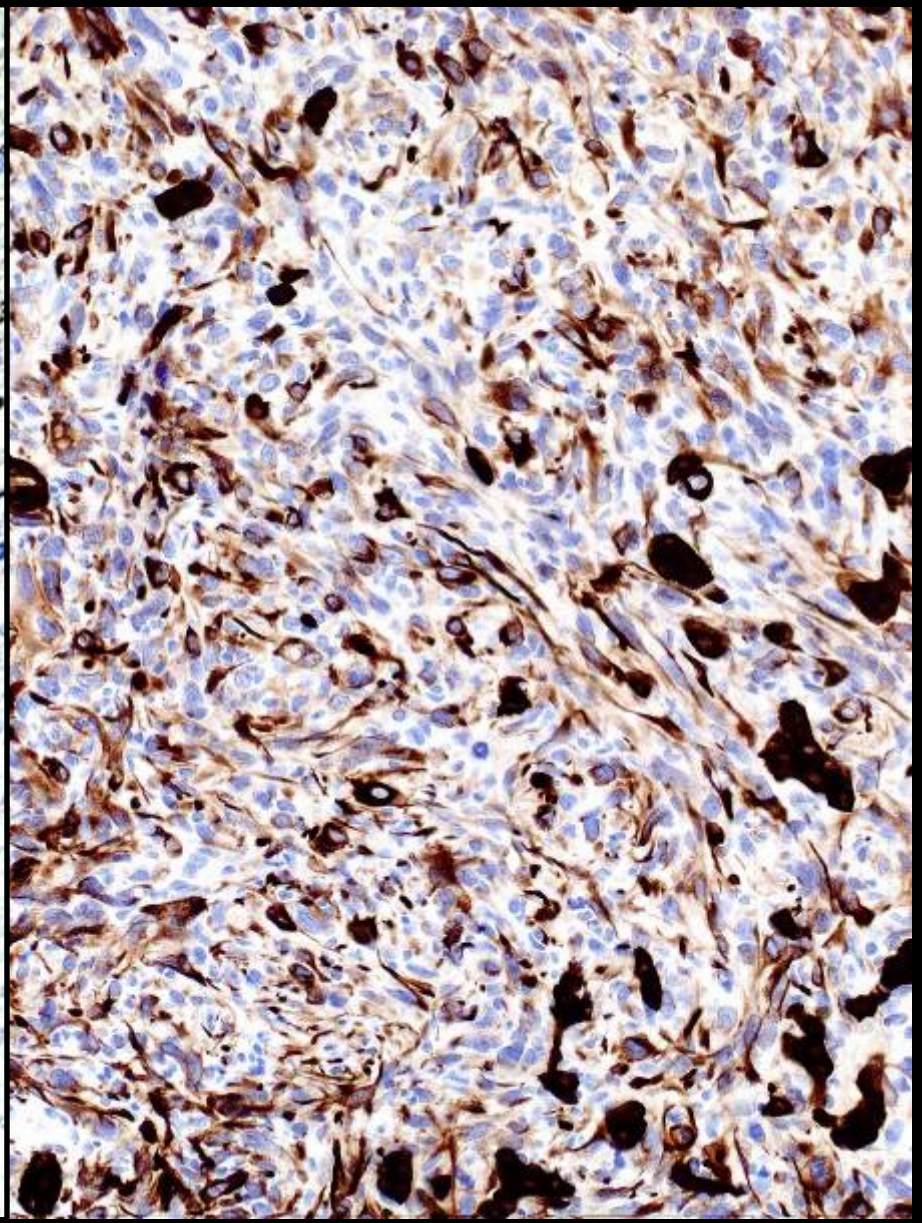


angiosarcoma

Cytokeratins in non-epithelial tumours



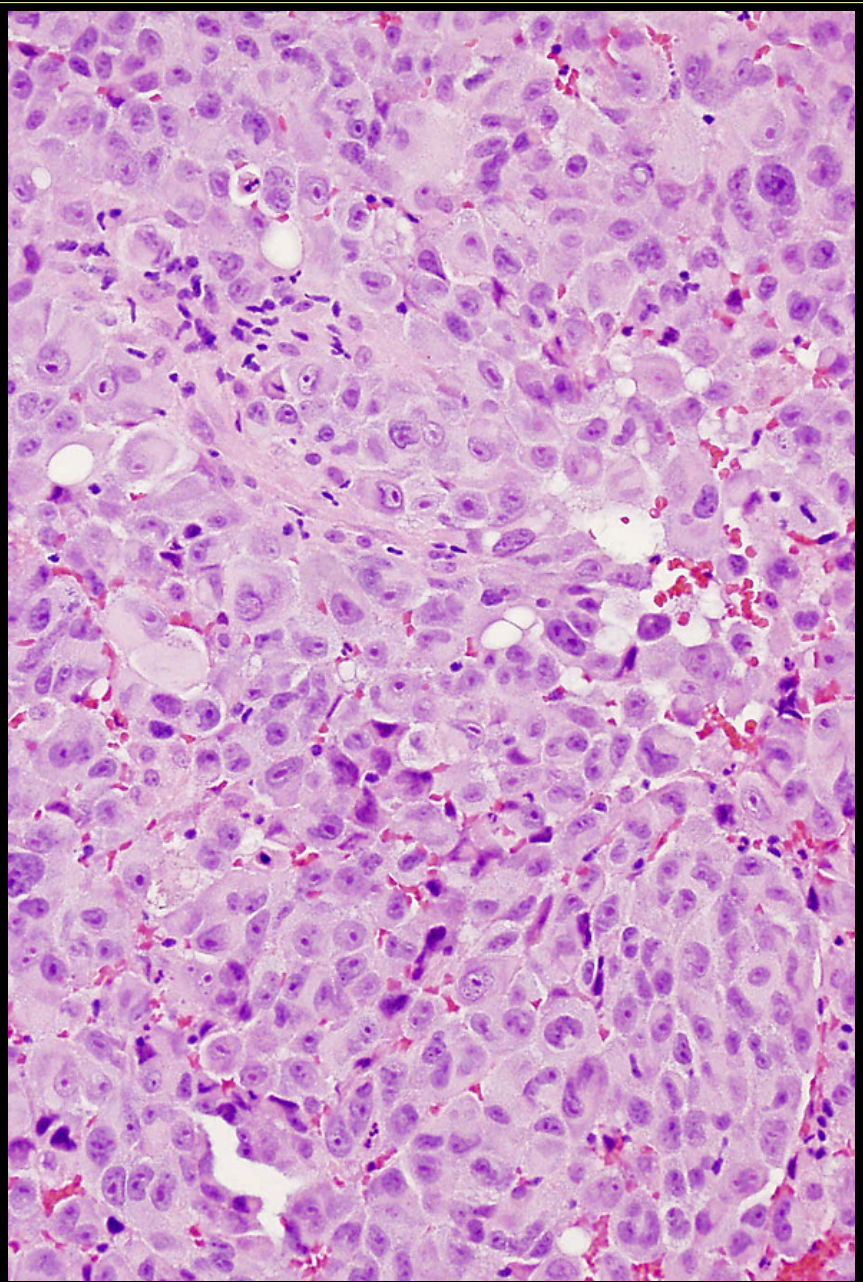
Leiomyosarcoma

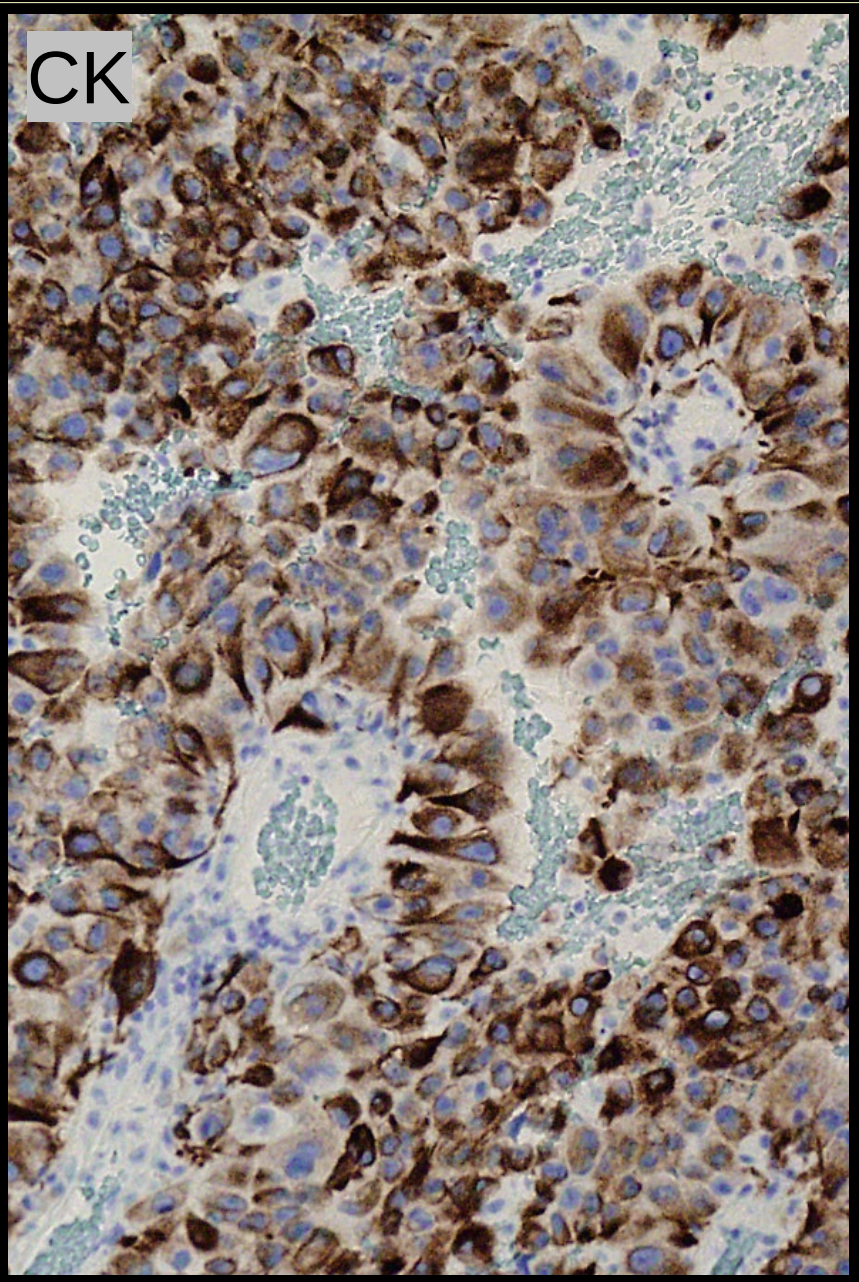


Primary panel for the unknown primary tumour

	CD45	Pan-CK	S-100	VIM
Haemato-lymphoid neoplasms	+/(-)	-/(+)	-/(+)	+/(-)
Epithelial neoplasms	-	+/(-)	-/+	-/+
Mesothelial neoplasms	-	+	-	+
Mesenchymal and neuronal neoplasms	-	-/(+)	-/+	+
Non-neuronal neuroepithelial neoplasms	-	-/(+)	+	+
Germ cell neoplasms	-	-/+	-/+	+

Cytokeratins in malignant melanoma

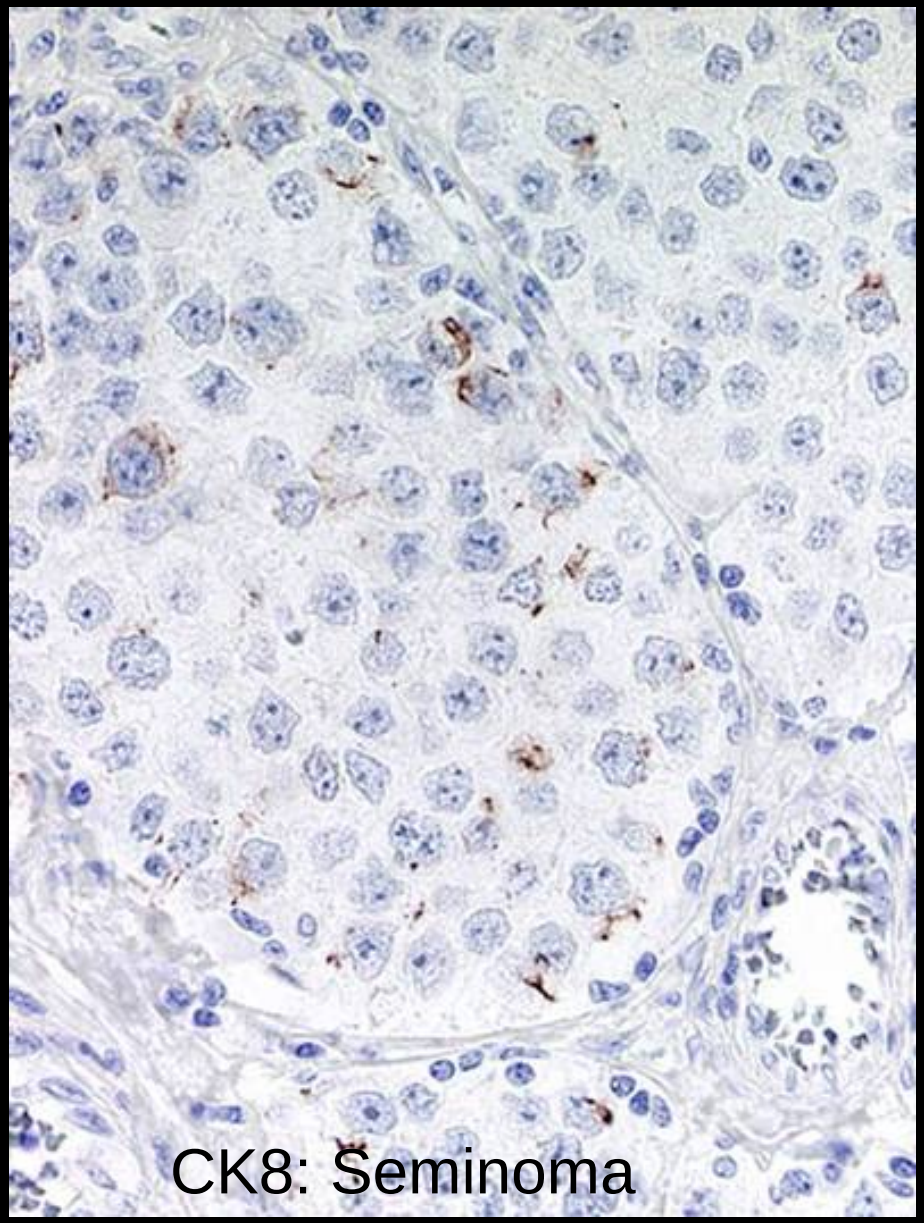




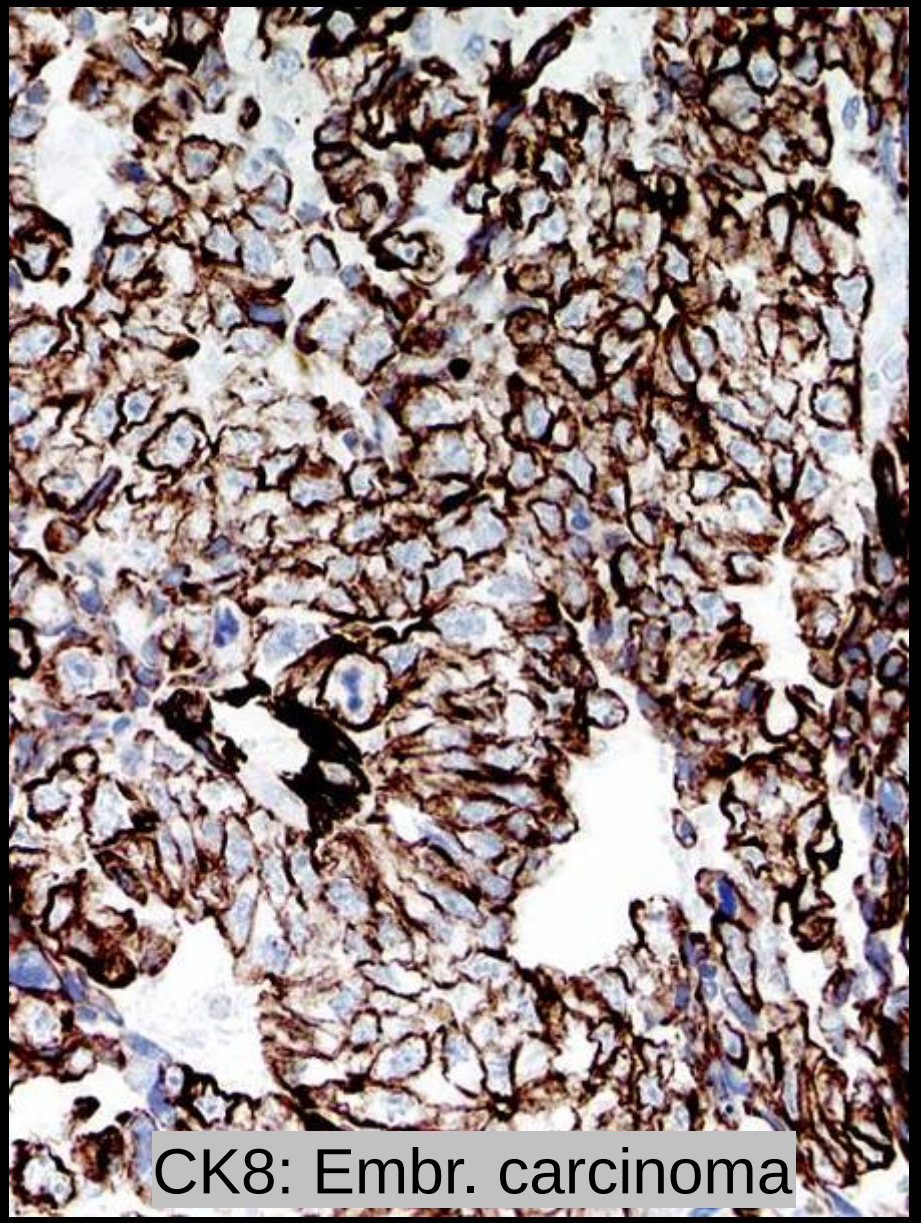
Primary panel for the unknown primary tumour

	CD45	Pan-CK	S-100	VIM
Haemato-lymphoid neoplasms	+/(-)	-/(+)	-/(+)	+/(-)
Epithelial neoplasms	-	+/(-)	-/+	-/+
Mesothelial neoplasms	-	+	-	+
Mesenchymal and neuronal neoplasms	-	-/(+)	-/+	+
Non-neuronal neuroepithelial neoplasms	-	-/(+)	+	+
Germ cell neoplasms	-	-/+	-/+	+

Cytokeratins in germ cell tumours

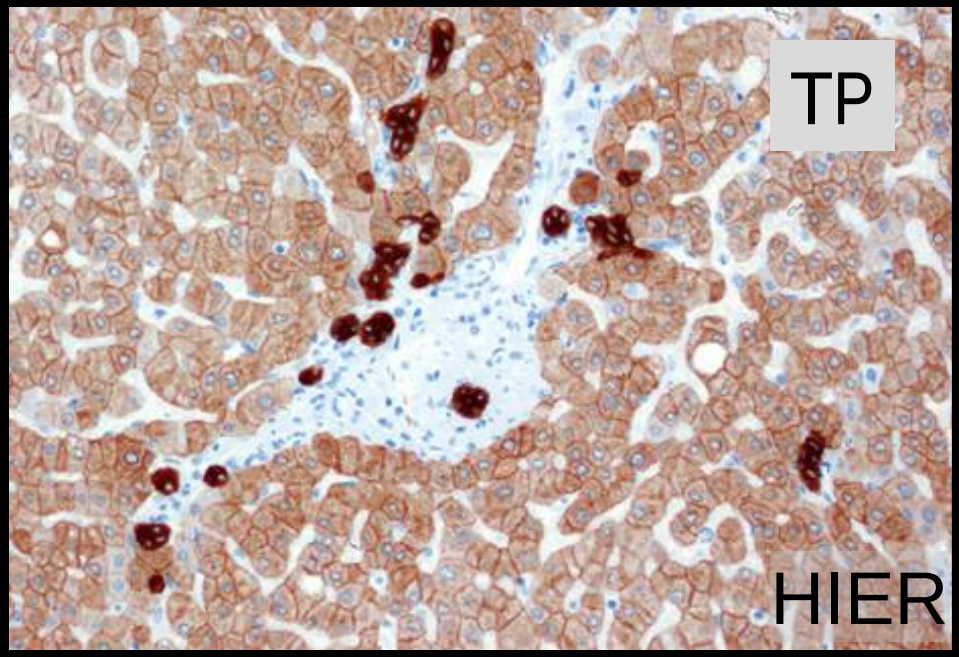


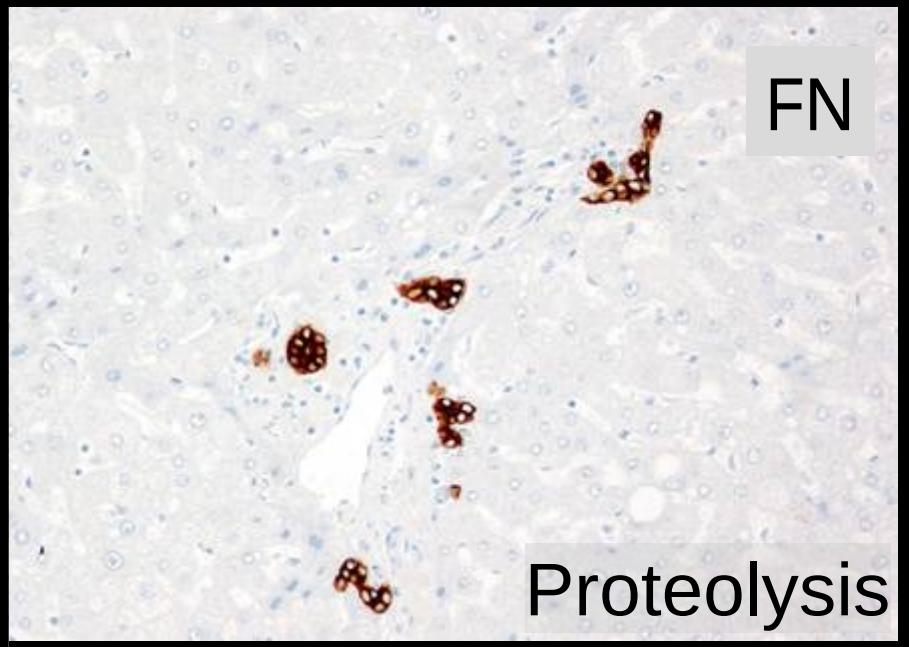
CK8: Seminoma



CK8: Embr. carcinoma

Cytokeratins: proteolysis causes false negativity

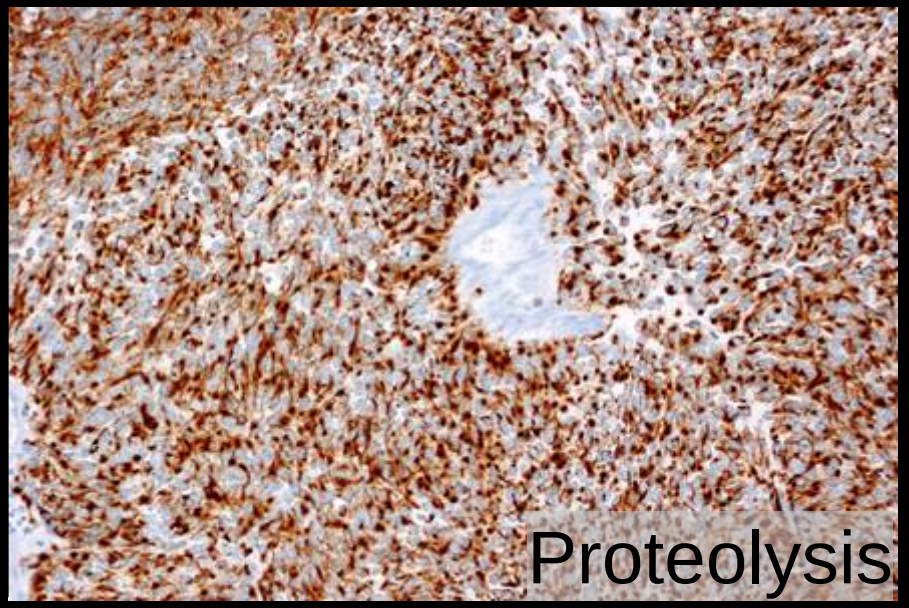




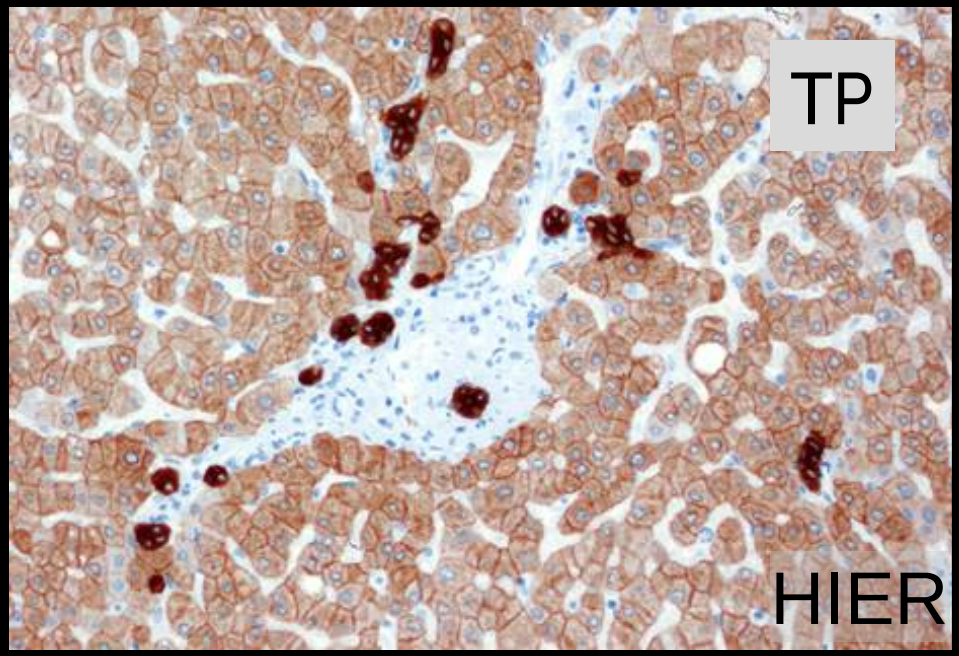
PAN-CK - AE1/AE3 clone cocktail:

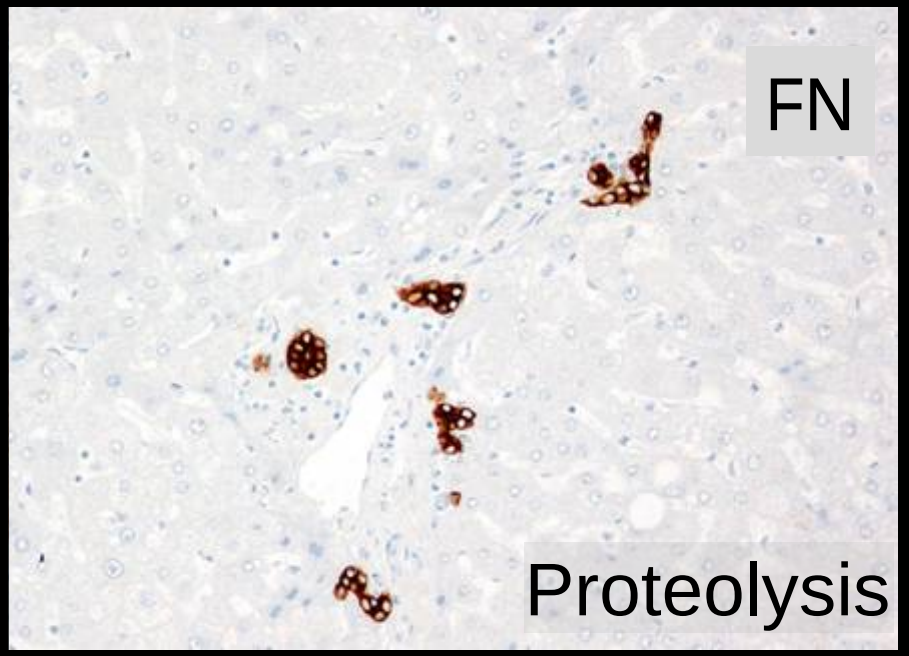
- AE1 detects CK8 after HIER only
- AE1 does not detect CK18
- AE3 neither detects CK8 or CK18

SCLC

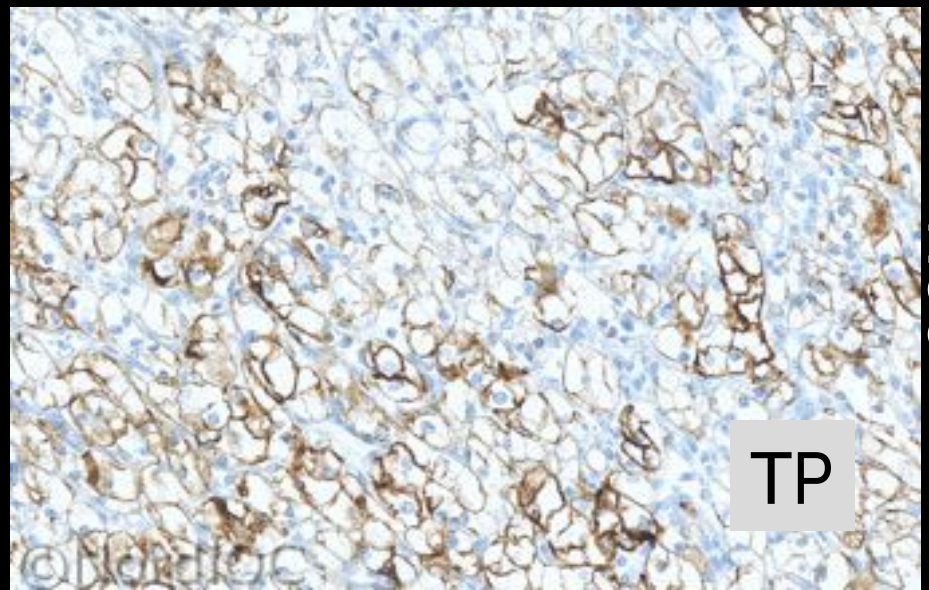


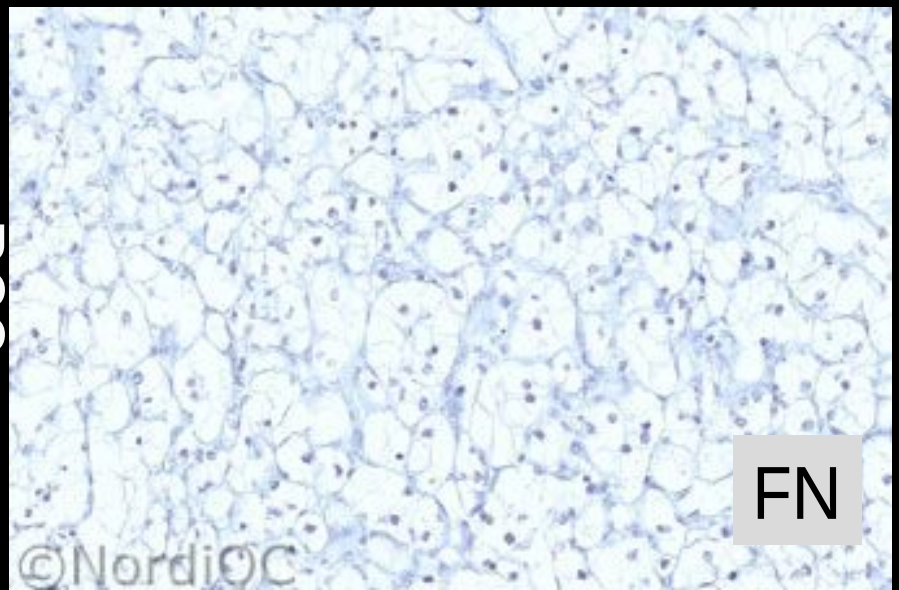
Cytokeratins: retrieval causing false negativity





RCC





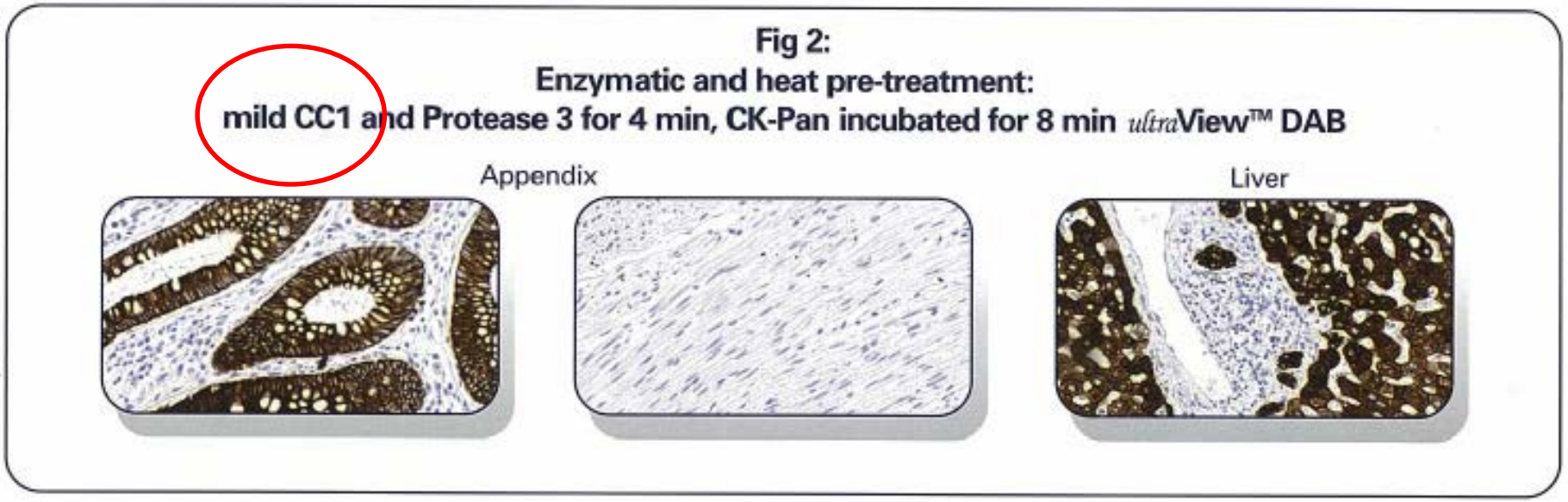
Misleading datasheets

Table 1. Recommended Staining Protocols for Anti-Pan Keratin (AE1/AE3/PCK26)

Procedure Type	Platform/Method	
	ES or NexES IHC	BenchMark or BenchMark XT
Deparaffinization	Off Line	Selected
Cell Conditioning (Antigen Unmasking)	None Required	None Required
Enzyme (Protease)	Protease 1, 4 minutes	Protease 1, 4 minutes
Antibody (Primary)	Pan Keratin, approximately 16 minutes	Pan Keratin, approximately 16 minutes
A/B Block (Biotin Blocking)	Optional	Optional
Amplify (Amplification)	Optional	Optional
Counterstain (Hematoxylin)	Hematoxylin, 2 to 4 minutes	Hematoxylin, 2 to 4 minutes
Post Counterstain	Bluing, 2 to 4 minutes	Bluing, 2 to 4 minutes

Giving false negative results when only LMW-CKs are present

Misleading datasheets



IHC - NordiQC 2016

Table 2. **Proportion of sufficient results for CK-PAN in the eight NordiQC runs performed**

	Run 8 2003	Run 15 2005	Run 20 2008	Run 24 2008	Run 30 2010	Run 36 2012	Run 41 2014	Run 47 2016
Participants, n=	72	85	103	123	168	202	233	275
Sufficient results	53%	58%	62%	60%	65%	65%	67%	72%

AE1/AE3 : Optimal results only obtained by **HIER** in NordiQC runs

Dako: RTU – HIER
Leica: RTU – Proteolysis
Thermo:

Conc: Proteolysis or HIER
Conc: HIER
Conc: HIER Quanto – Proteolysis UltraVision

.....

AE1/AE3/PCK26: Optimal results mainly obtained by HIER+protelysis in NordiQC runs

VMS: RTU - Proteolysis

Misleading data sheets + Wrong control material used

Improved datasheets

By 17th October 2014

Table 1. Recommended Staining Protocol for anti-Pan Keratin (AE1/AE3/PCK26) with ultraView Universal DAB Detection Kit on a BenchMark XT instrument.

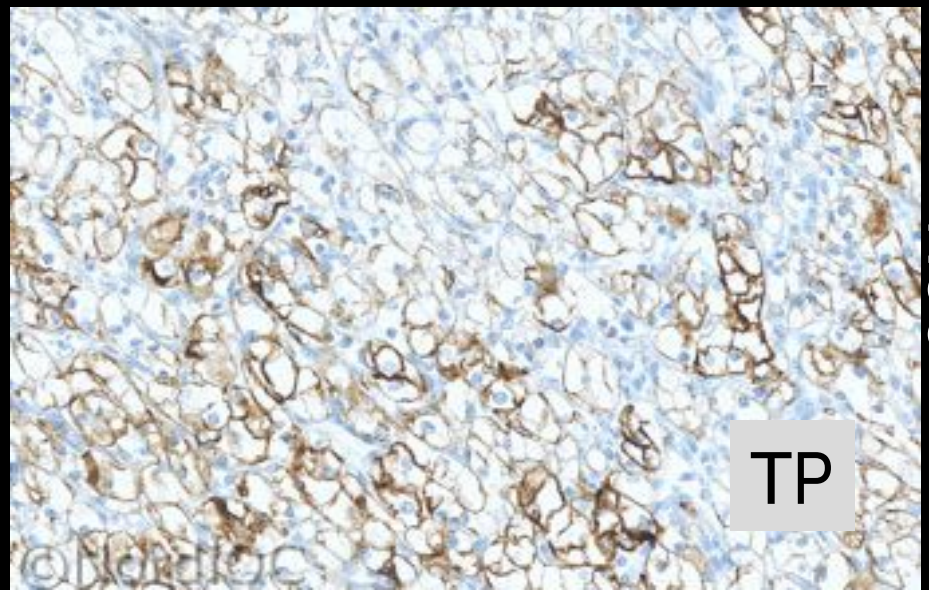
Procedure Type	Method
Deparaffinization	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, Mild
Enzyme (Protease)	Protease 3, 4 minutes
Antibody (Primary)	BenchMark XT instrument 8 minutes, 37° C
ultraBlock	*VENTANA Antibody Diluent with Casein (760-219), 4 minutes
Counterstain	Hematoxylin II, 4 minutes
Post Counterstain	Bluing, 4 minutes

*Use of VENTANA Antibody Diluent with Casein (760-219) at the ultraBlock step is recommended to reduce staining on smooth muscle

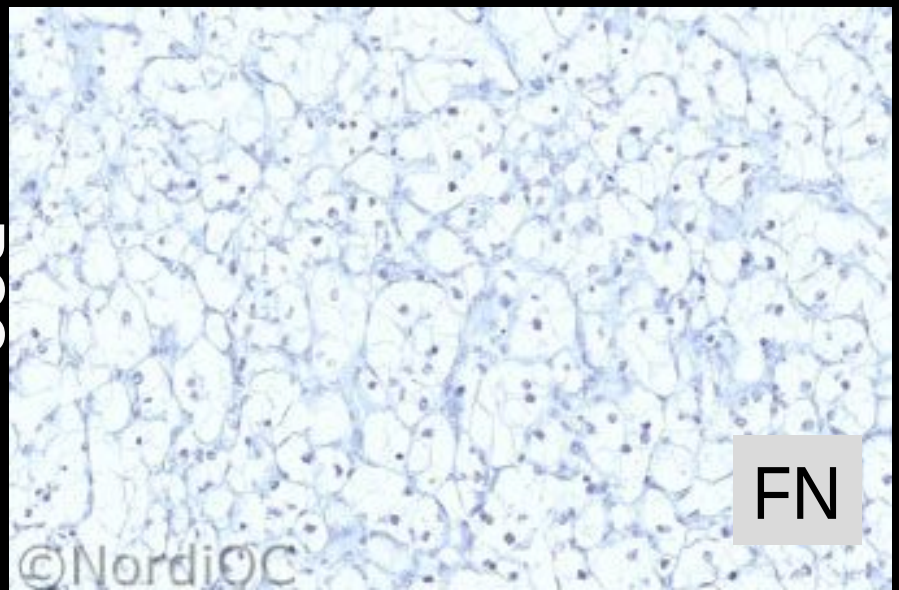
NordiQC run 41 2014

Fra: Galloway, Mary [mailto:Mary.Galloway@fda.hhs.gov]
Sendt: 13. november 2014 01:14
Til: Søren Nielsen / Region Nordjylland
Emne: RE: Changes Made to Package Inserts

Sören,
*Thanks for identifying and alerting us to the issues with **...anti-Pan Keratin**. The package inserts are now changed (see links below).*
I hope we can continue to learn of any future staining problems you may uncover.
Much appreciated!
Mary



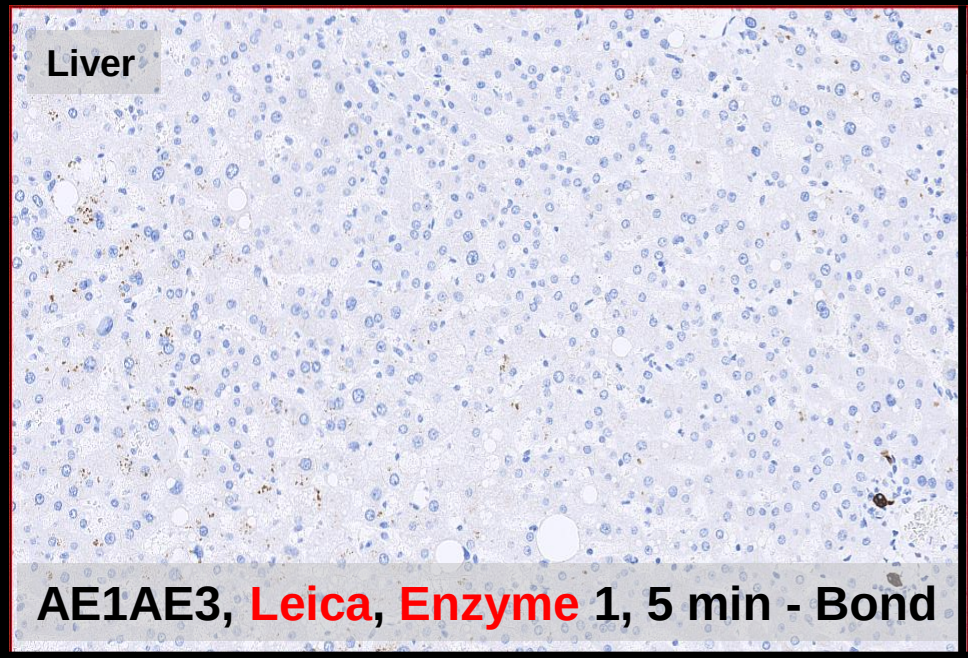
RCC



IHC - NordiQC 2016

Ready-To-Use antibodies								
mAb clone cocktail **AE1/AE3 IR053**	36	Dako/Agilent	28	5	2	1	92%	95%
mAb clone cocktail **AE1/AE3 GA053**	19	Dako/Agilent	18	0	1	0	95%	100%
mAb clone cocktail **AE1/AE3 313M-18**	3	Cell Marque	0	1	0	2	-	-
mAb clone cocktail **AE1/AE3 MAD 001000QD**	1	Master Diagnostica	1	0	0	0	-	-
mAb clone cocktail **AE1/AE3 Kit-0009**	1	Maixin	1	0	0	0	-	-
mAb clone cocktail **AE1/AE3 PA0909**	5	Leica/Novocastra	0	1	3	1	20%	-
mAb clone cocktail **AE1/AE3 RTU-AE1/AE3**	2	Leica/Novocastra	0	0	2	0	-	-
mAb clone cocktail **AE1/AE3/5D3 IP162**	2	Biocare	1	1	0	0	-	-
mAb clone cocktail **AE1/AE3/PCK26 760-2135/2595**	62	Ventana/Roche	37	8	5	12	73%	96%
rmAb clone cocktail **EP24/EP67/B22.1/B23.1 MAD-000680QD**	2	Master Diagnostica	0	2	0	0	-	-
Total	275		132	65	43	35	-	
Proportion			48%	24%	16%	12%	72%	

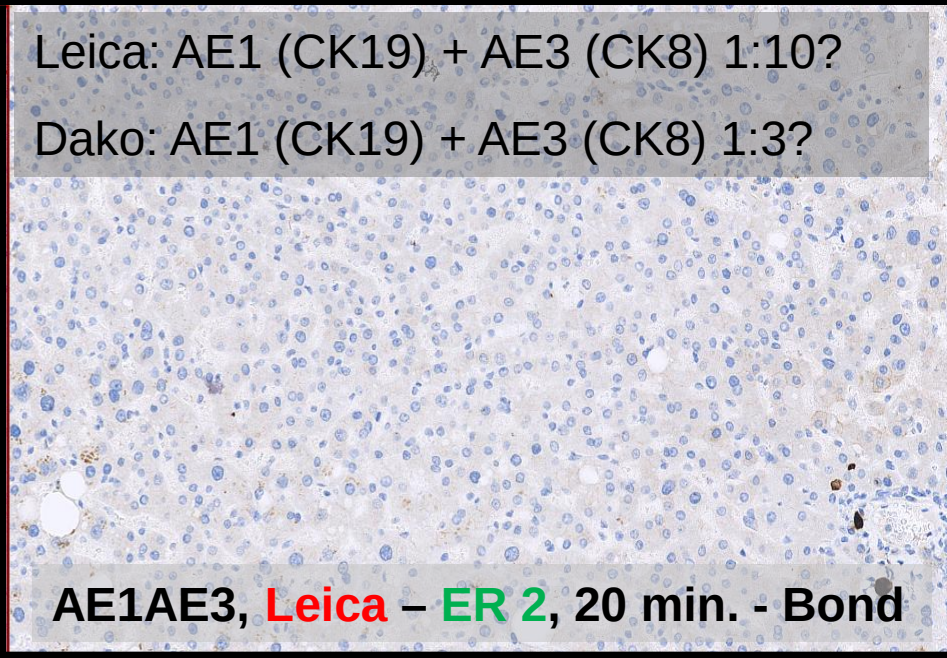
IHC – Controls and CSQI for the primary panel



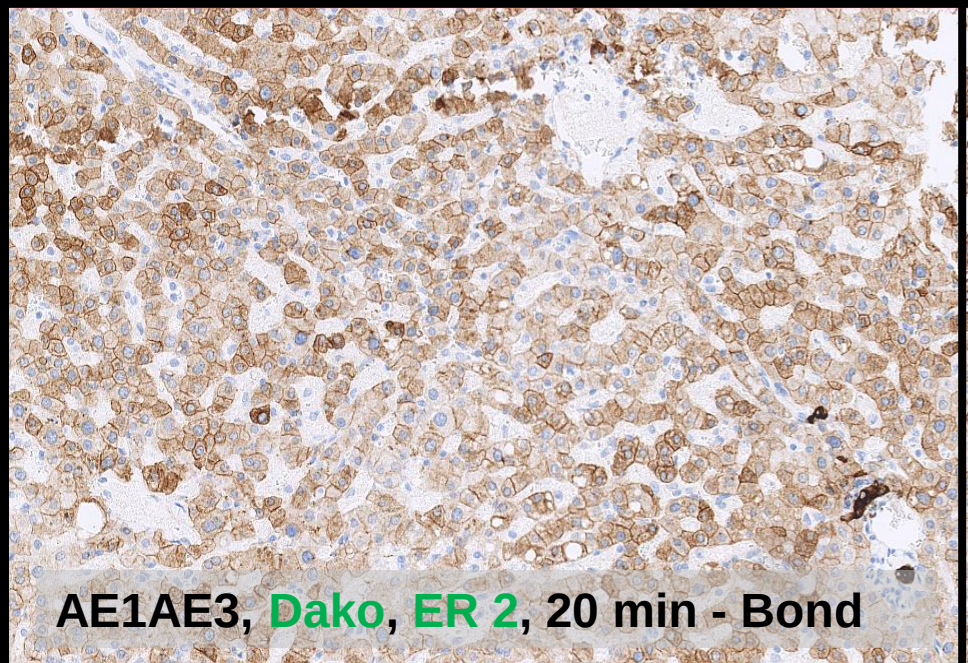
AE1AE3, Leica, Enzyme 1, 5 min - Bond

Leica: AE1 (CK19) + AE3 (CK8) 1:10?

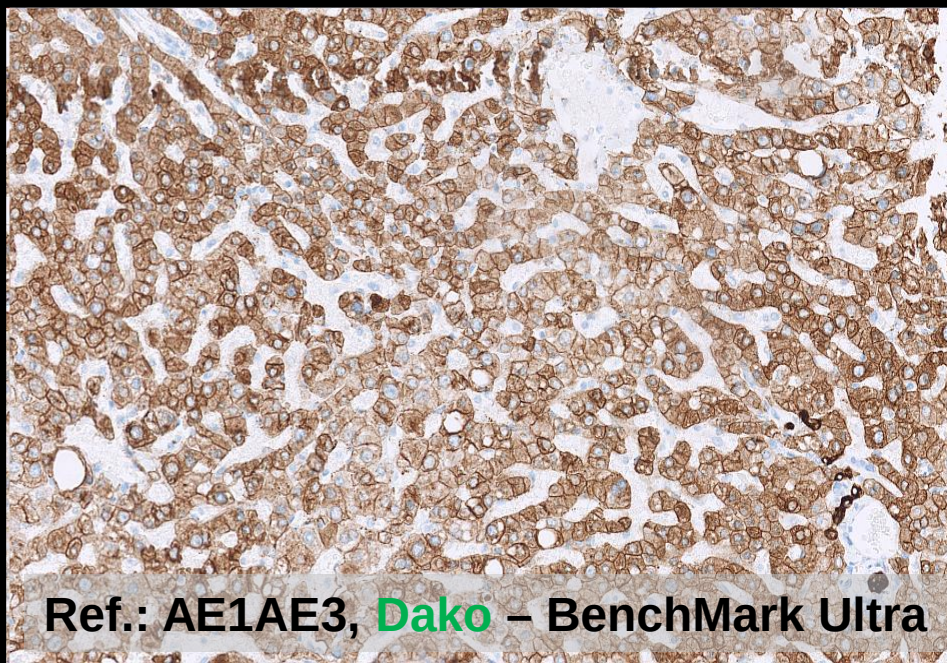
Dako: AE1 (CK19) + AE3 (CK8) 1:3?



AE1AE3, Leica – ER 2, 20 min. - Bond



AE1AE3, Dako, ER 2, 20 min - Bond



Ref.: AE1AE3, Dako – BenchMark Ultra

Primary panel for the unknown primary tumour

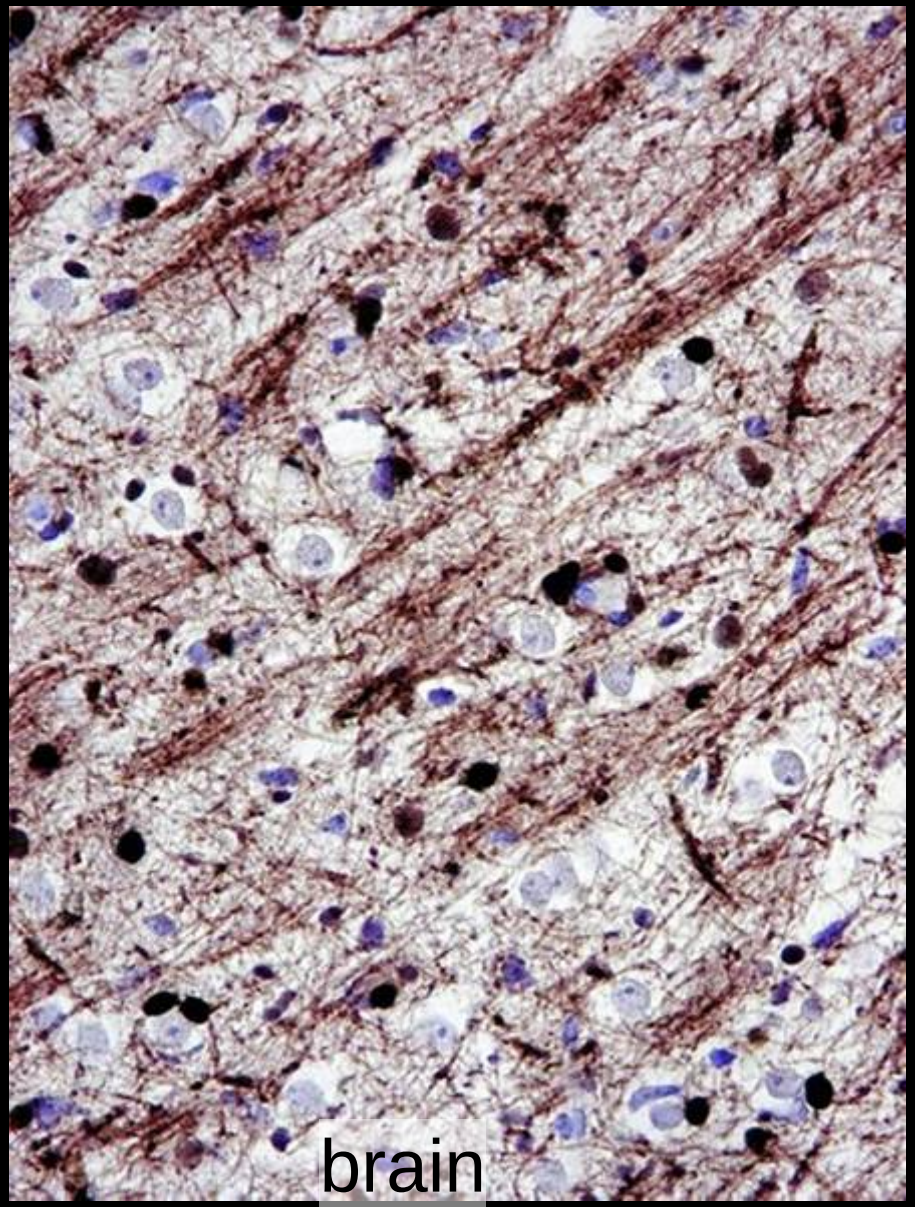
	CD45	CK	S-100	VIM
Haemato-lymphoid neoplasms	+/(-)	-/(+)	-/(+)	+/(-)
Epithelial neoplasms	-	+/(-)	-/+	-/+
Mesothelial neoplasms	-	+	-	+
Mesenchymal and neuronal neoplasms	-	-/(+)	-/+	+
Non-neuronal neuroepithelial neoplasms	-	-/(+)	+	+
Germ cell neoplasms	-	-/+	-/+	+

S-100 protein

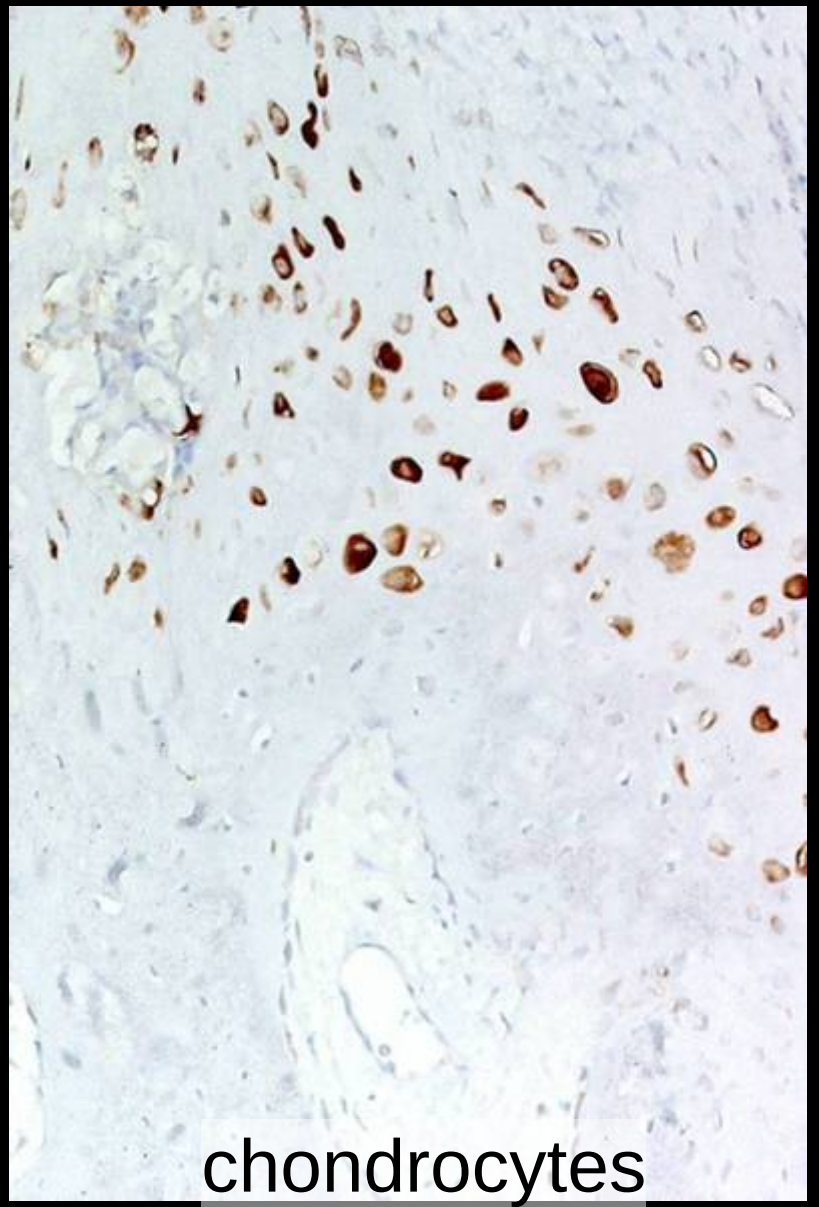
- Family of acid calcium binding proteins 9/13 kDa
- Located in nuclei, cytoplasm and cell membranes
- at least 10 α-chains and one β-chain creating homo- and heterodimers

- S-100 β-chain mainly found in
  - Melanocytes
  - Glial cells
  - Langerhans' cells / interdigitating reticulum cells
  - Fat cells
  - Myoepithelial cells
- Polyclonal antibodies primarily detects the β-chain

S-100 protein

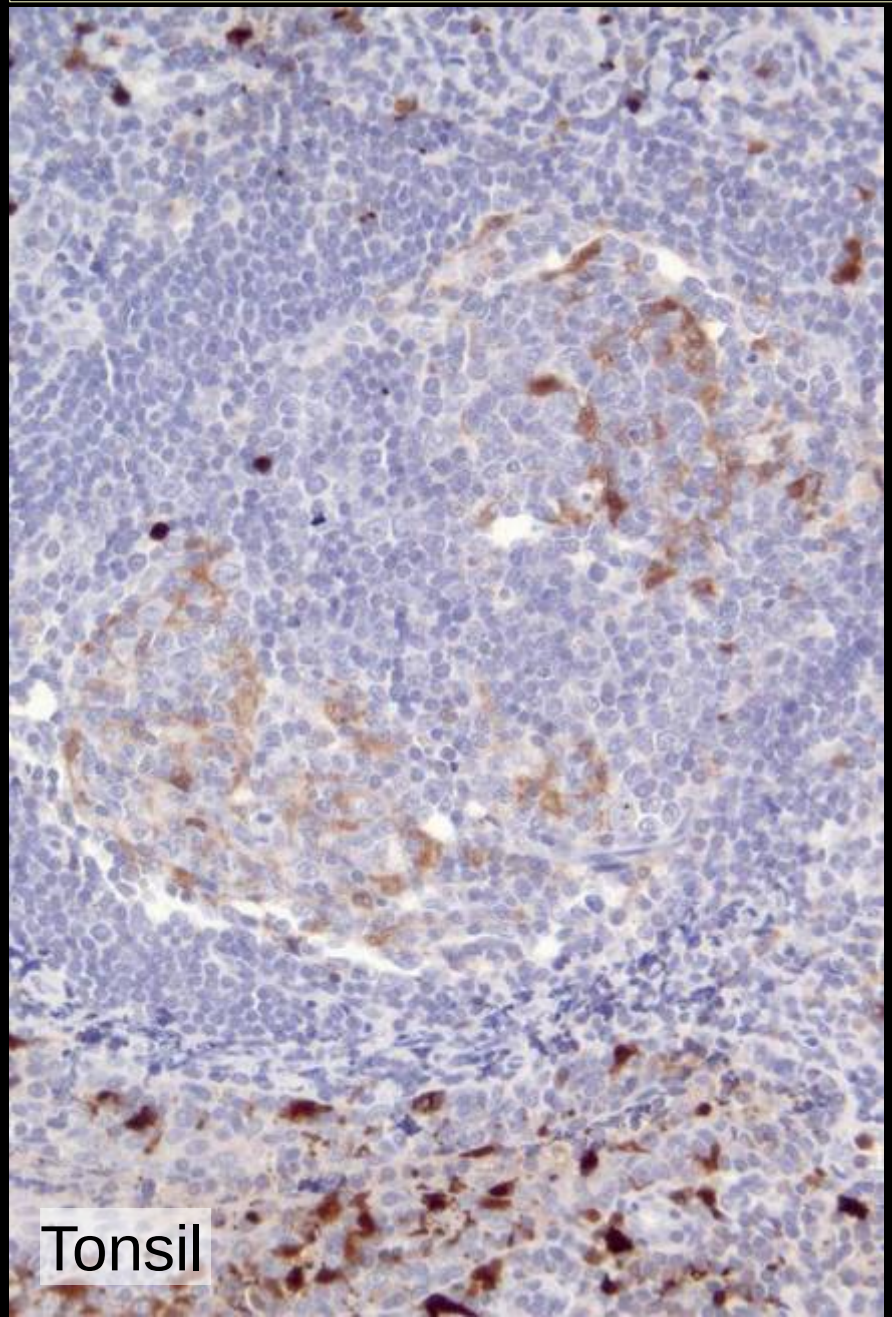


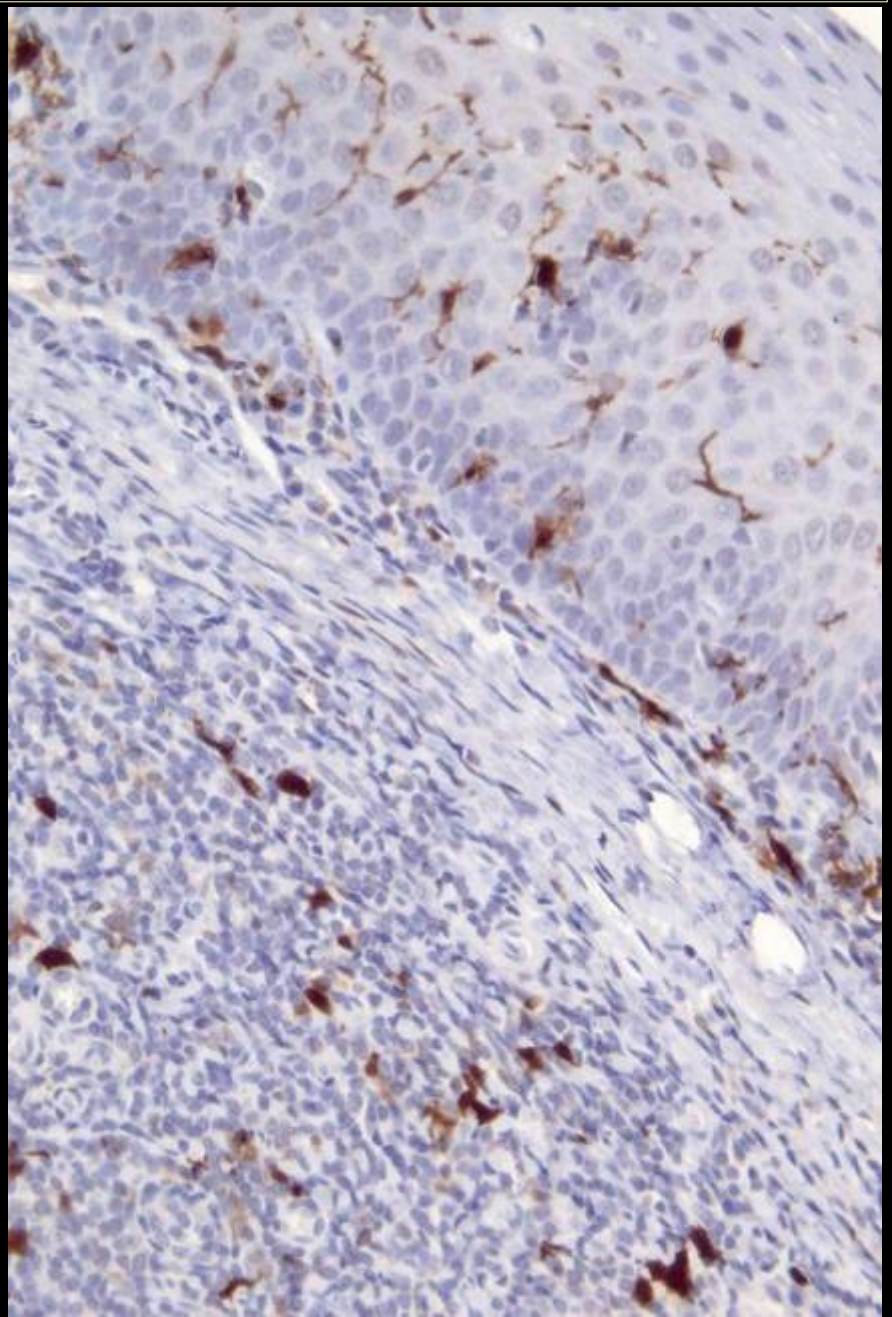
brain



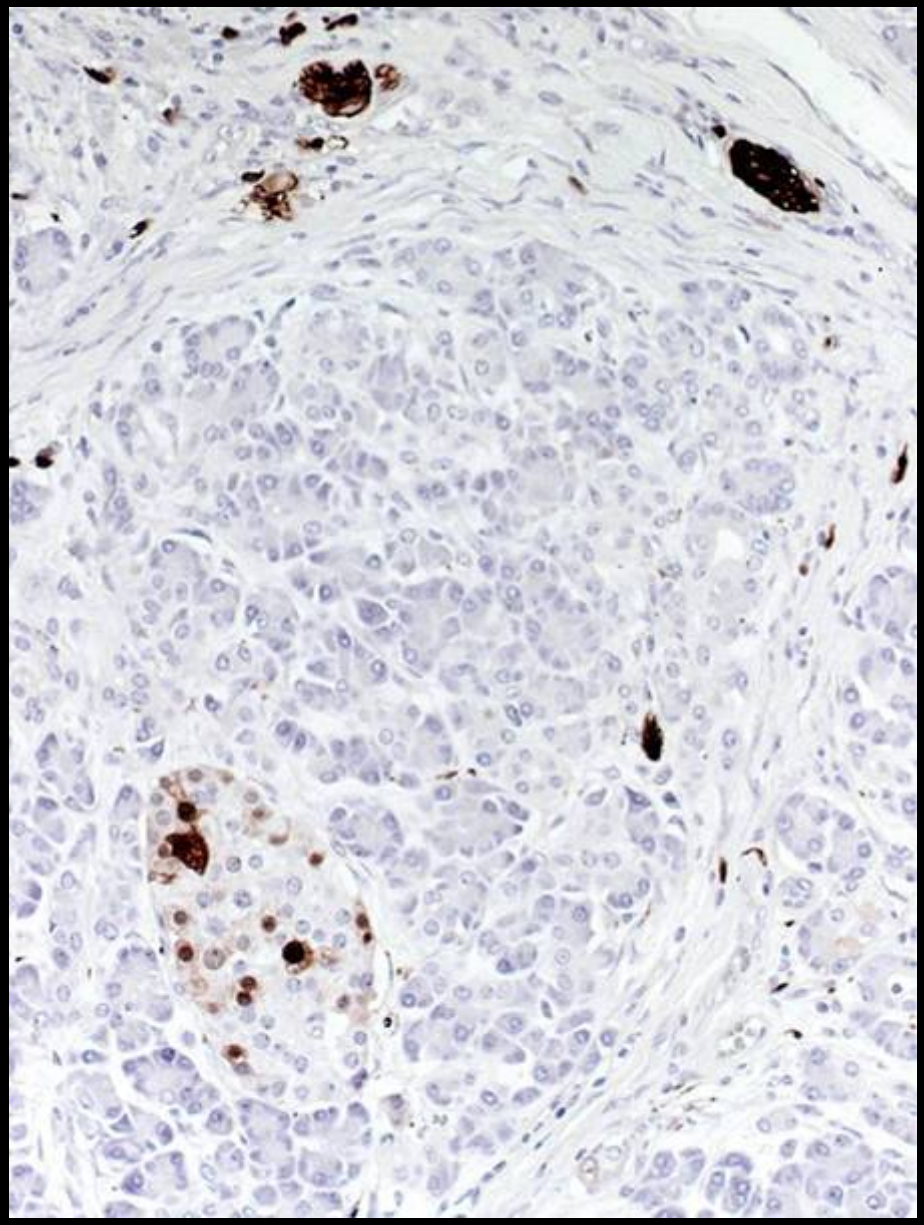
chondrocytes

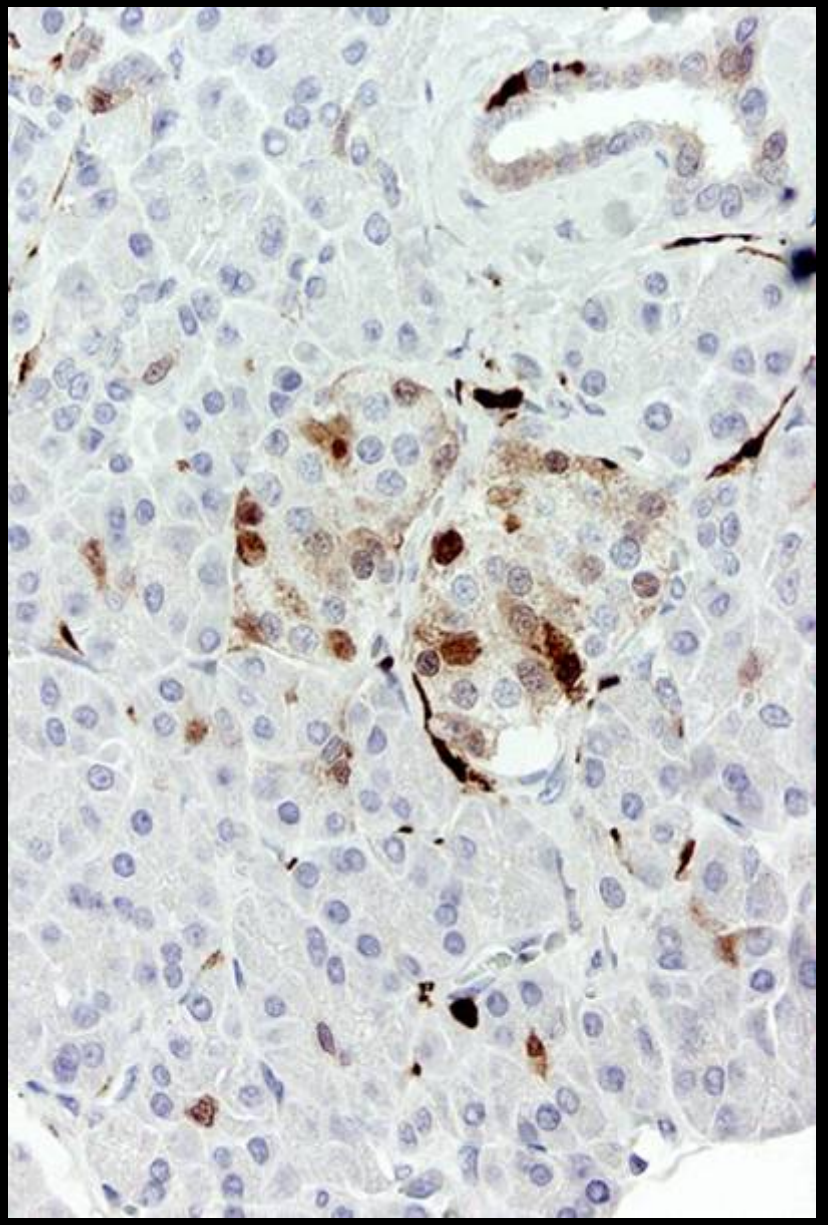
S-100 protein



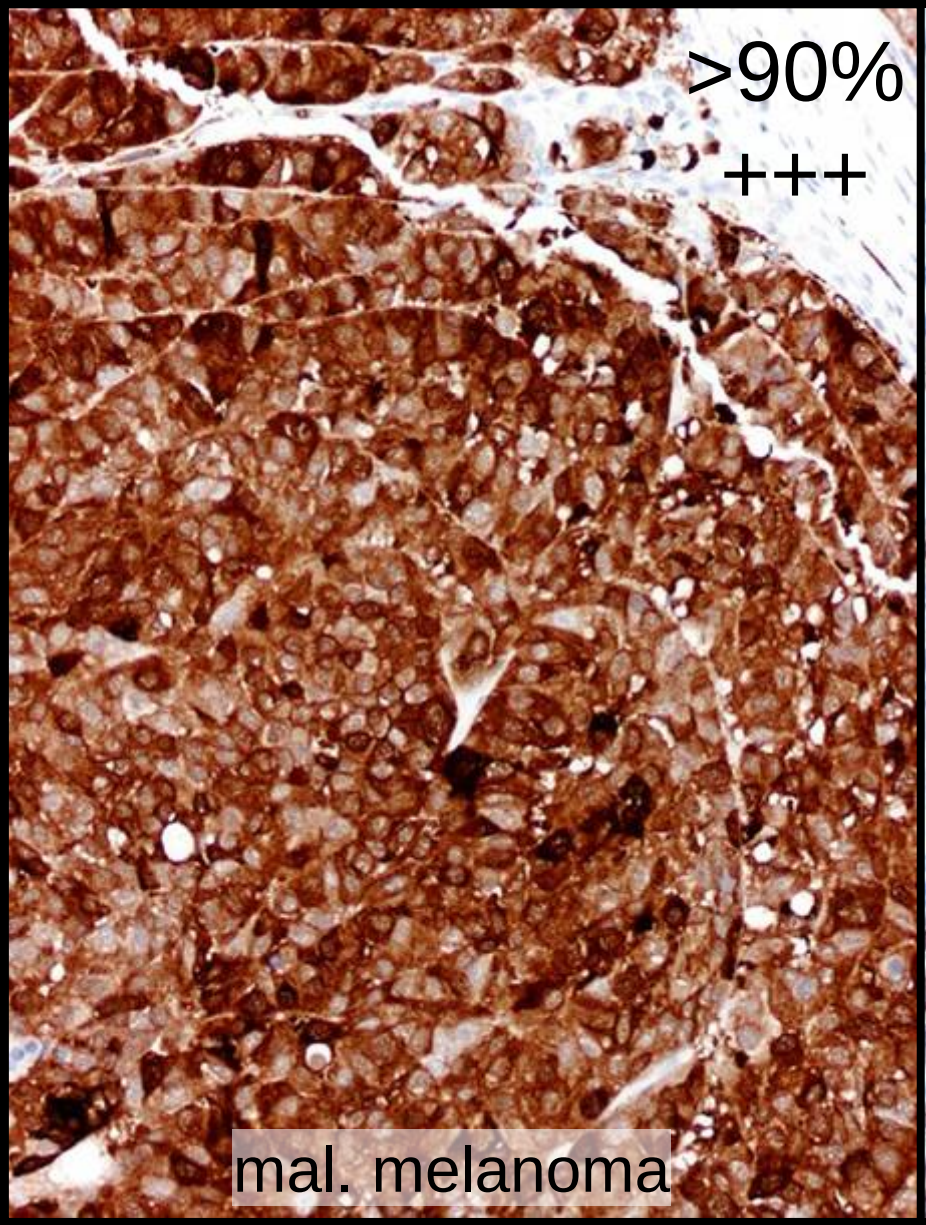


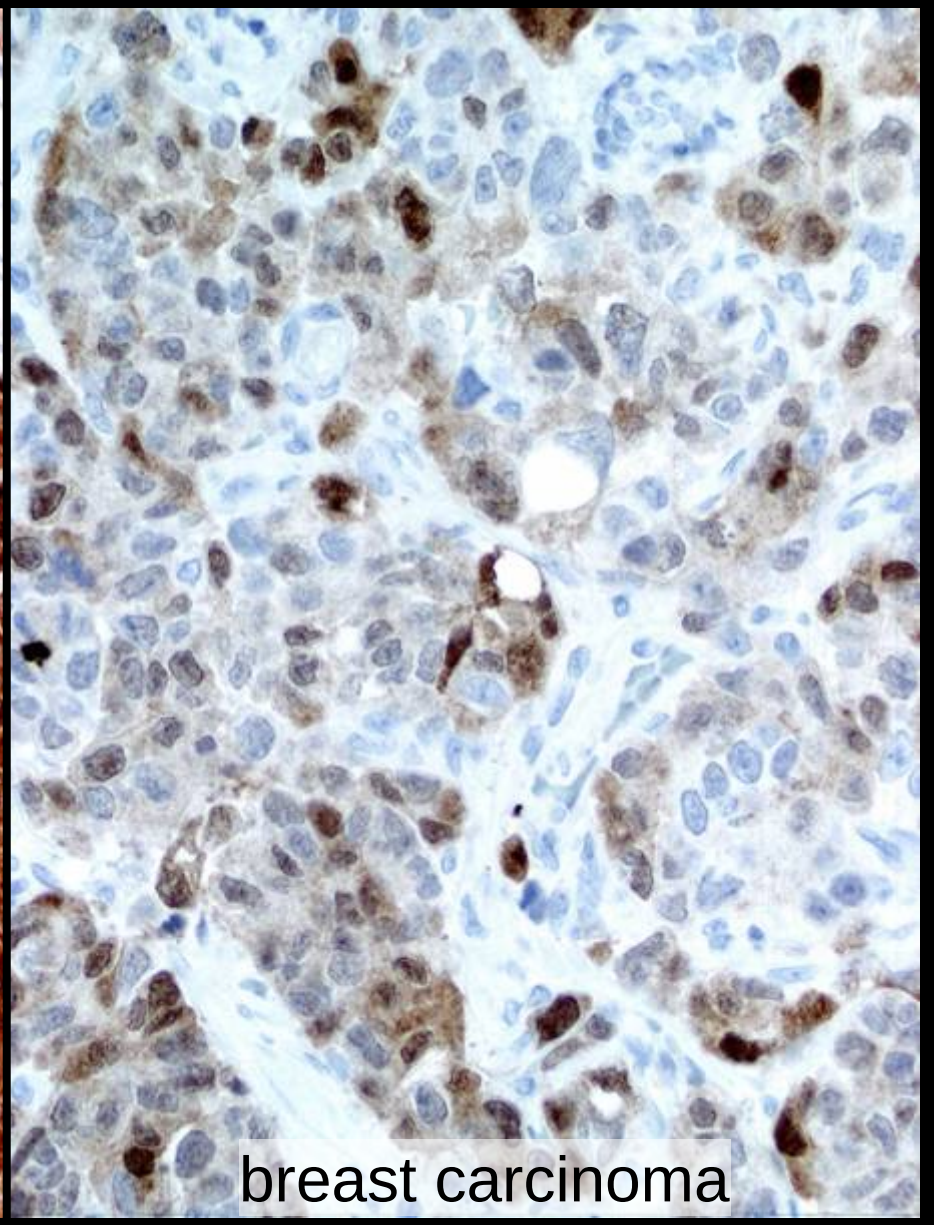
S-100 protein – pancreas





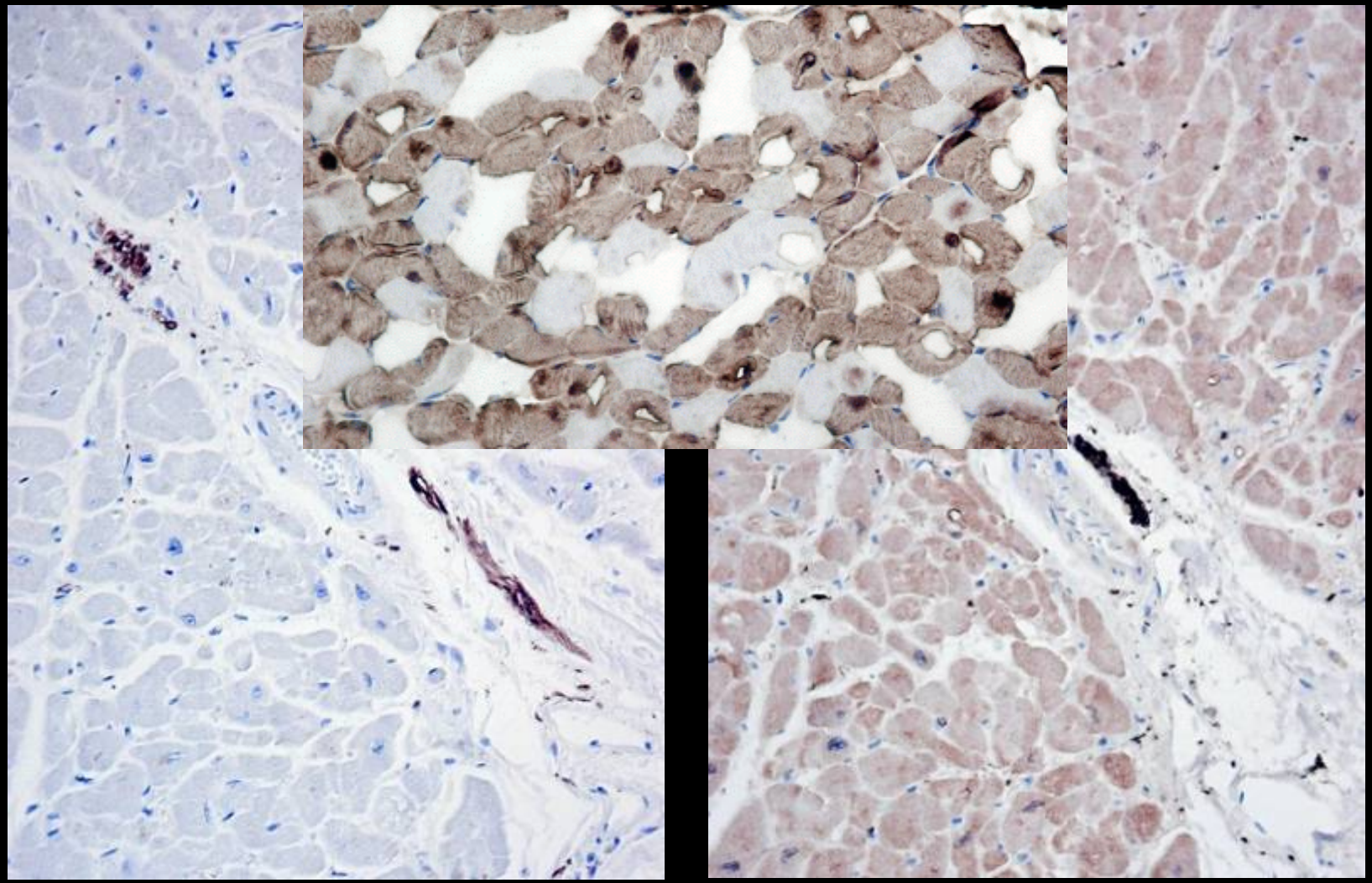
S-100 in malignant tumours





S-100 protein

To HIER or not..



Proteolytic

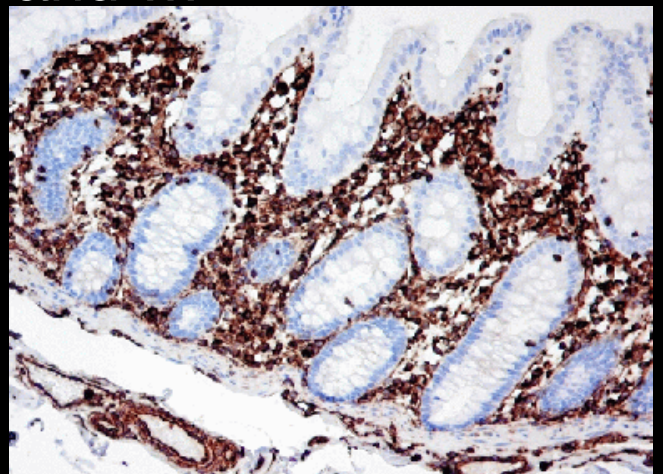
HIER

Primary panel for the unknown primary tumour

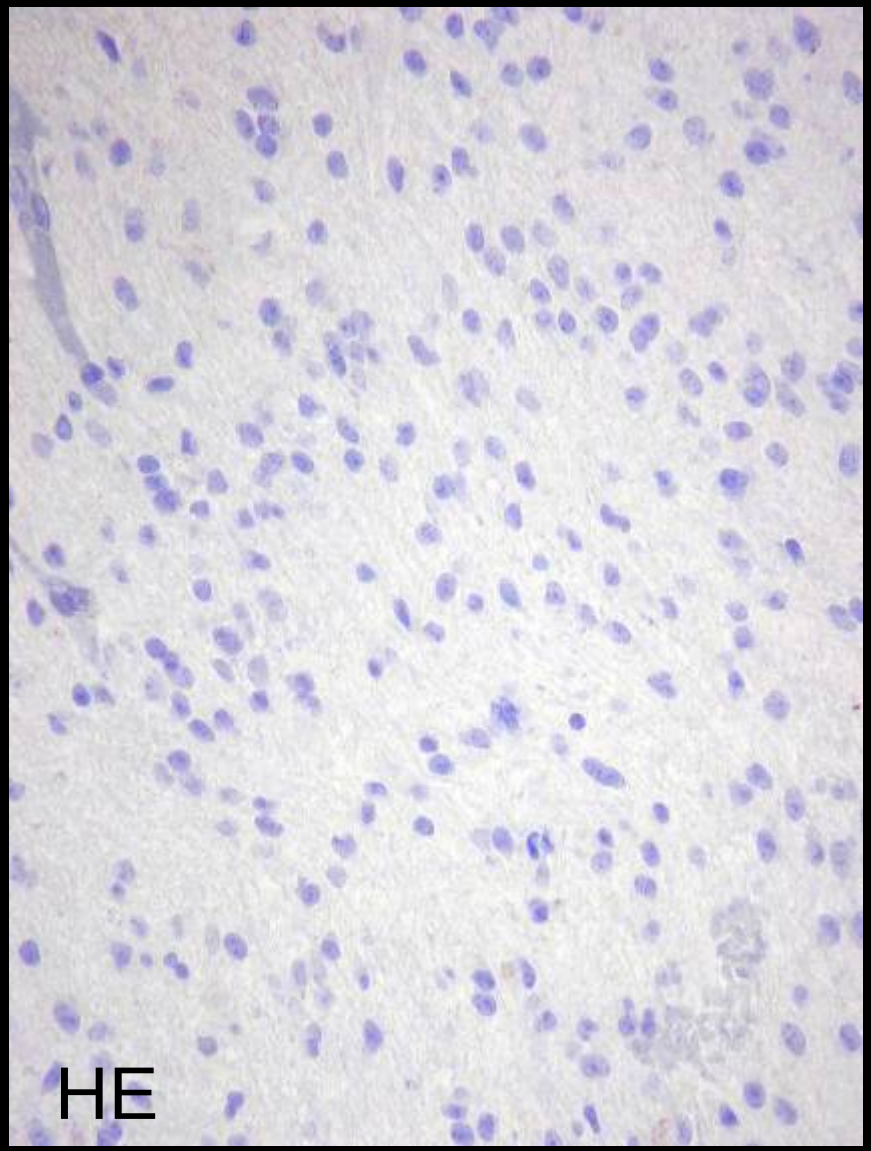
”Real”	CD45	CK	S-100	VIM
Haemato-lymphoid neoplasms	+/(-)	-/(+)	-/(+)	+/(-)
Epithelial neoplasms	-	+/(-)	-/+	-/+
Mesothelial neoplasms	-	+	-	+
Mesenchymal and neuronal neoplasms	-	-/(+)	-/+	+
Non-neuronal neuroepithelial neoplasms	-	-/(+)	+	+
Germ cell neoplasms	-	-/+	-/+	+

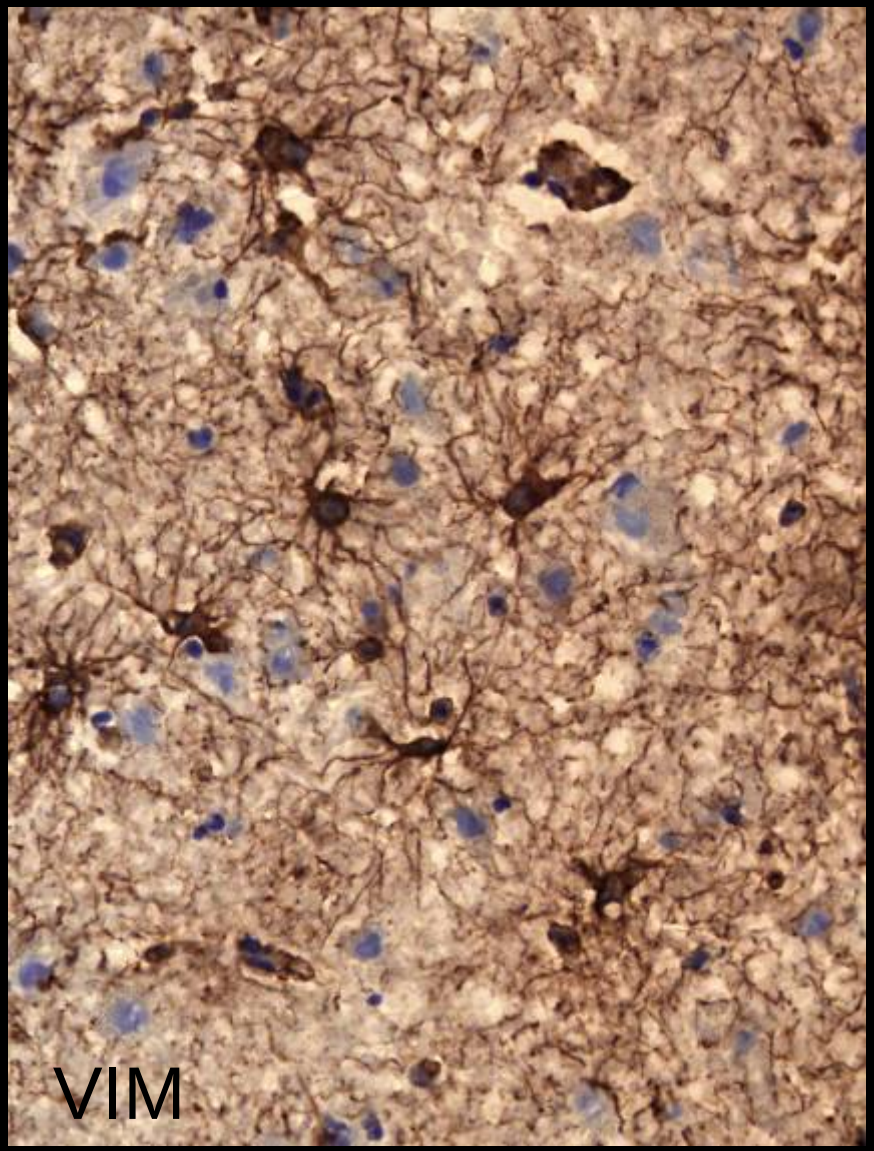
Vimentin

- Cytoplasmic intermediate filament, 57 kDa
- Present in all mesenchymal cells
- Present in early stages of all cells, replaced by other intermediate filaments in most non-mesenchymal cells
- Coexpressed with cytokeratin in some epithelia
  - Endometrium, renal tubules, thyroid gland …
- Coexpressed with cytokeratin in some non-epithelial cells
  - Mesothelium



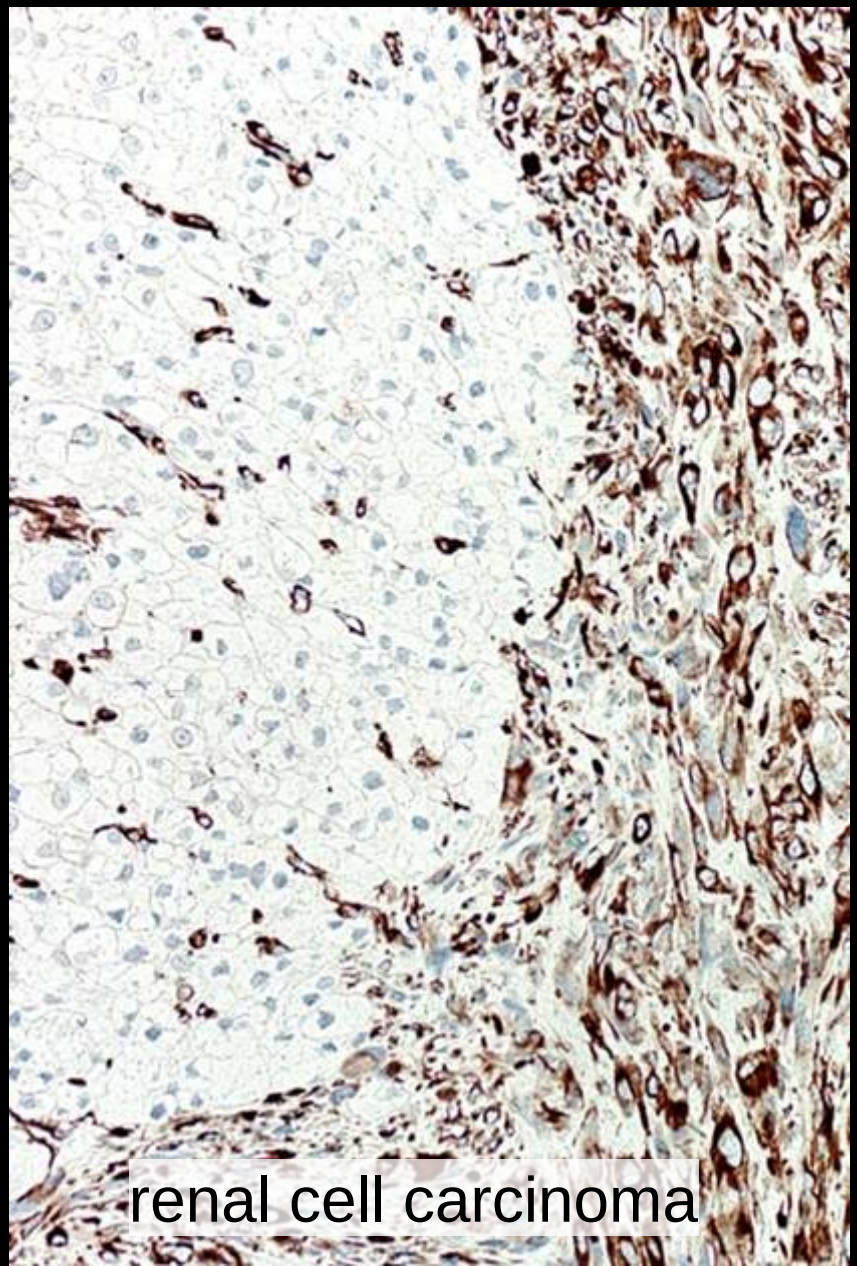
Vimentin in normal tissue



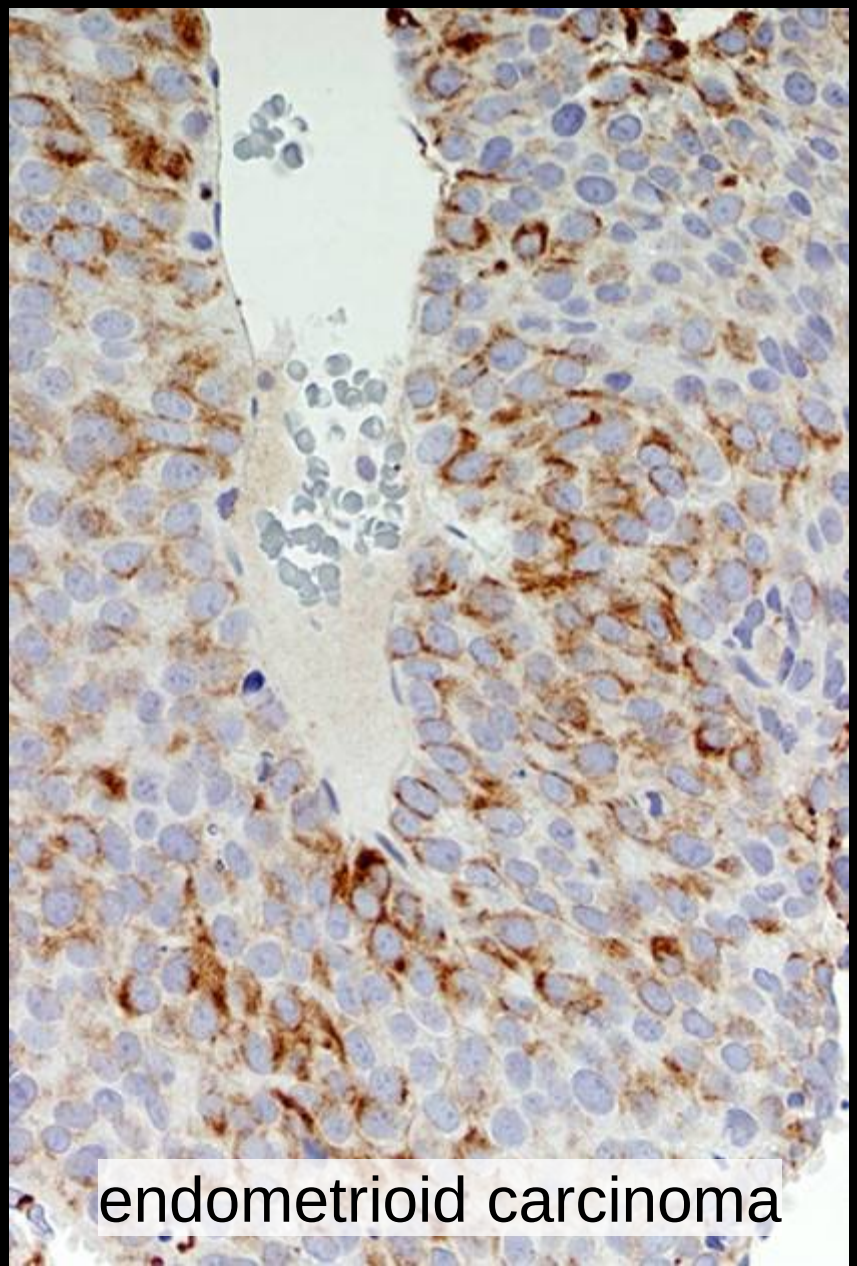


Normal brain

Vimentin in carcinomas

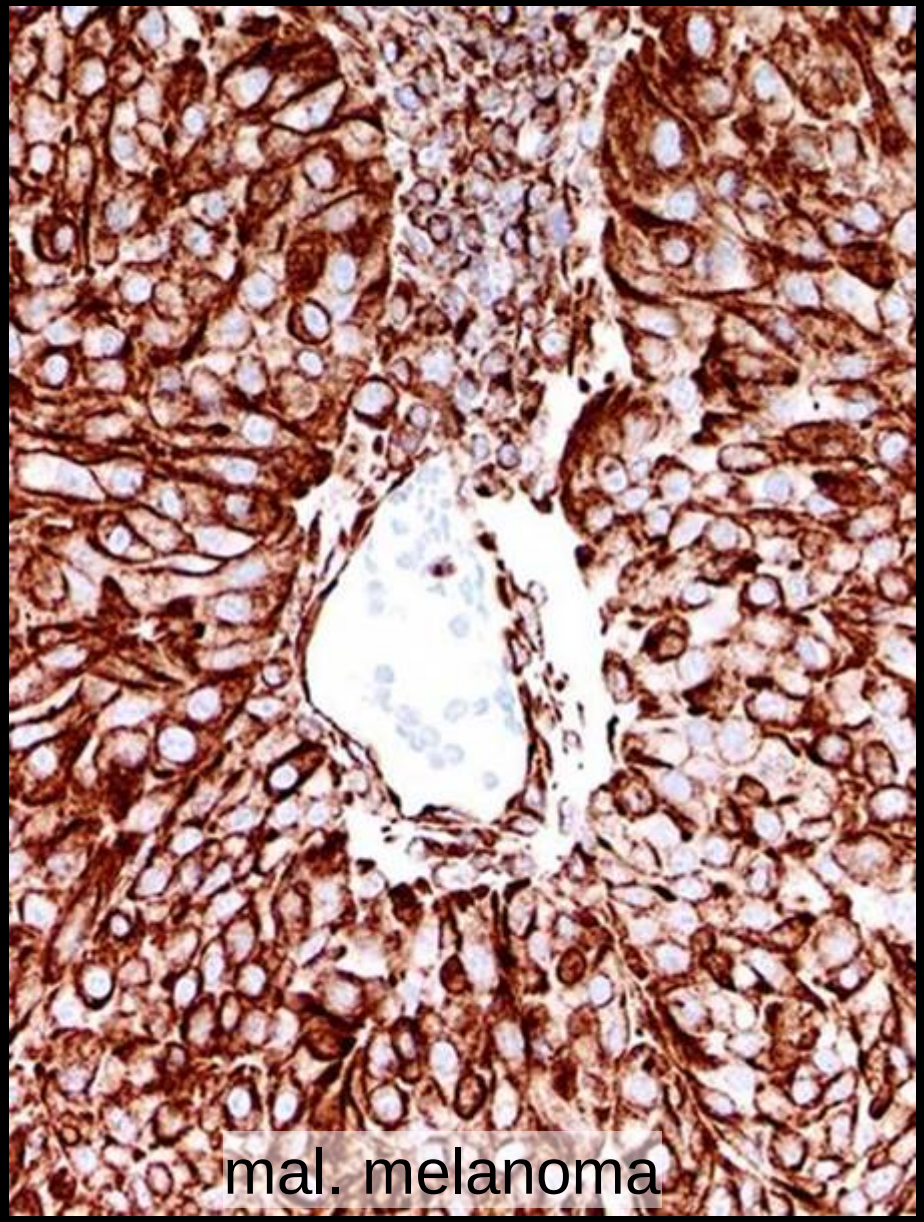


renal cell carcinoma

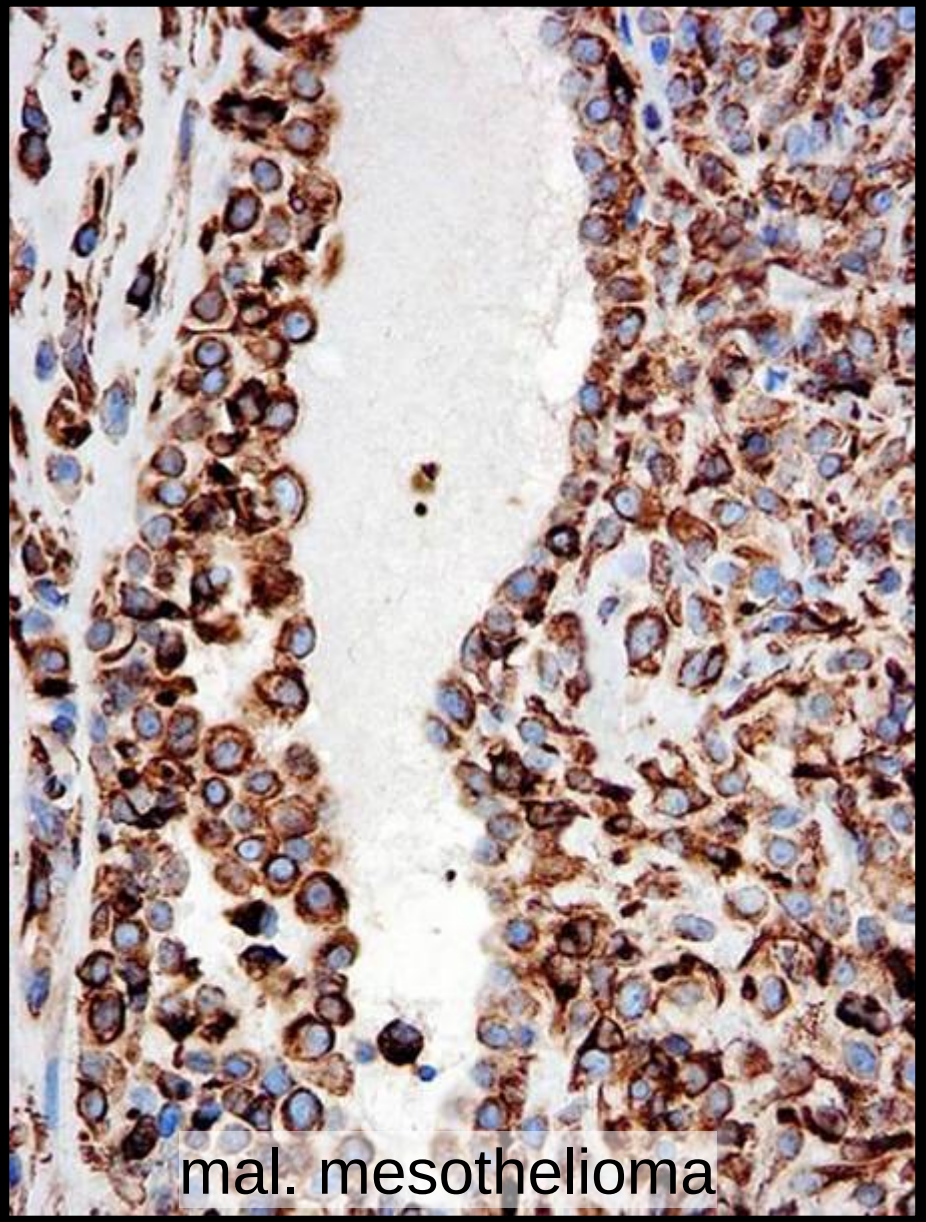


endometrioid carcinoma

Vimentin in non-epithelial tumours



mal. melanoma

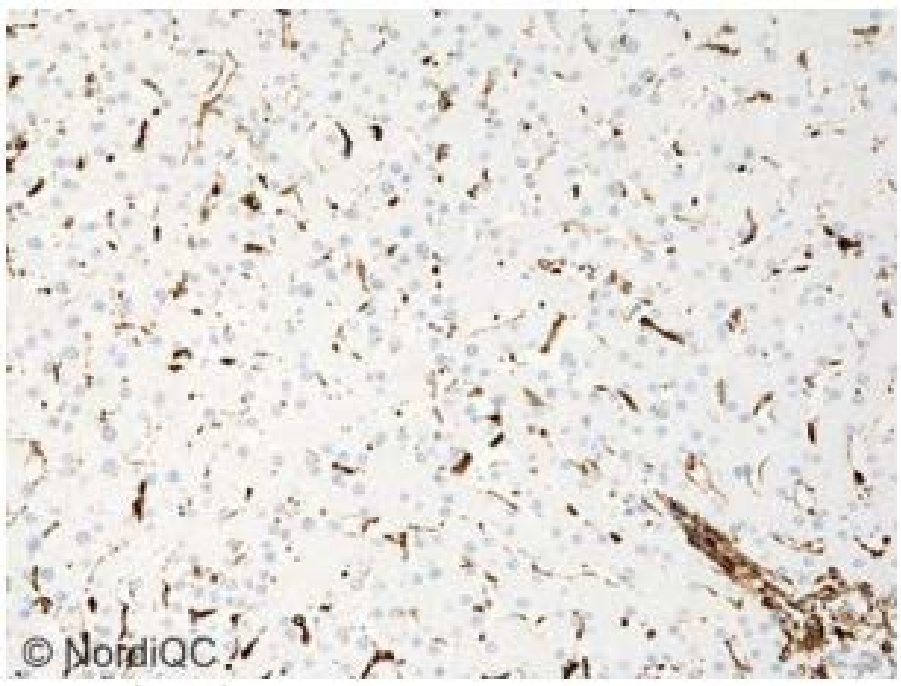


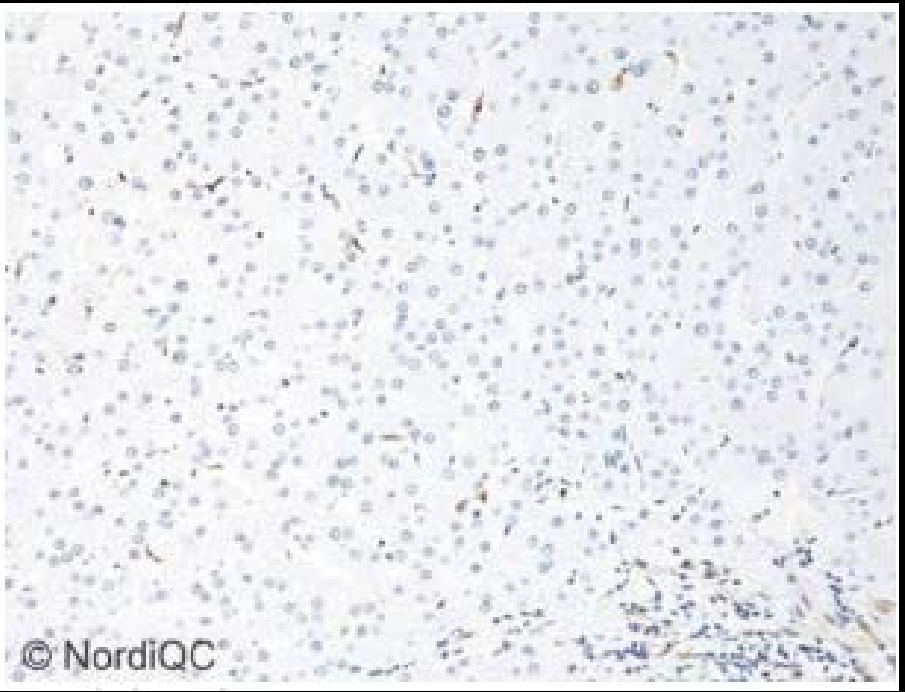
mal. mesothelioma

NordiQC runs 12 (2004) – 52 (2018)

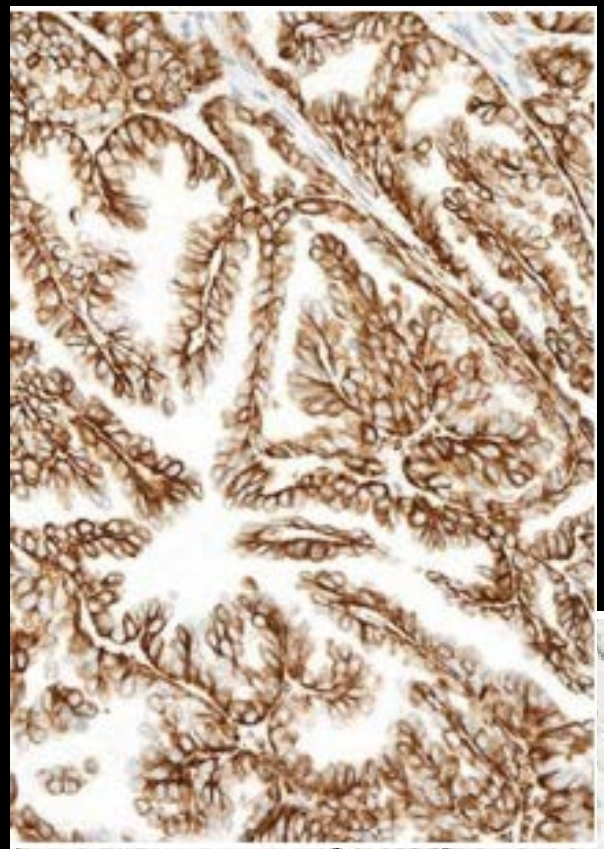
Table 2. **Proportion of sufficient results for VIM in the three NordiQC runs performed**

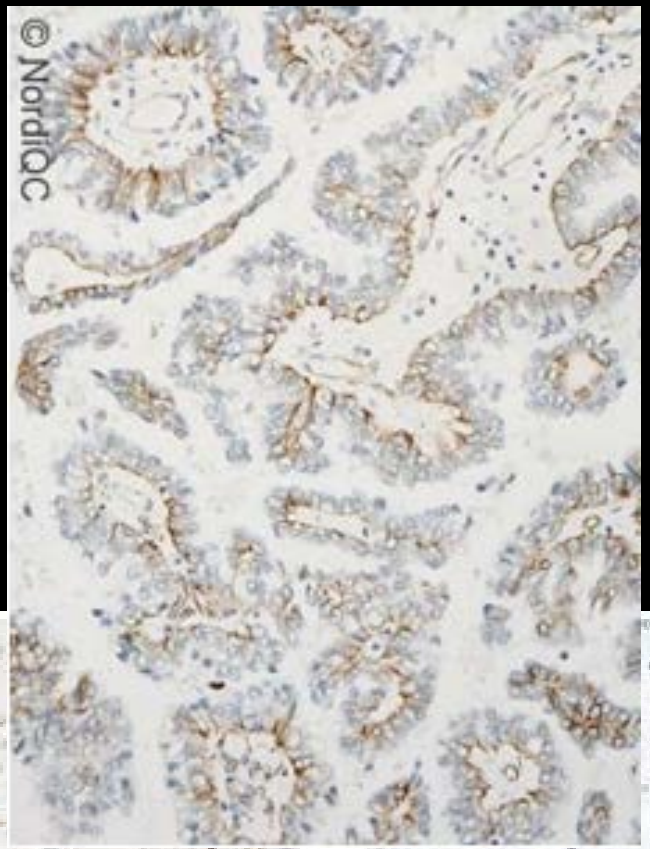
	Run 12 2004	Run 30 2010	Run 52 2018
Participants, n=	79	164	308
Sufficient results	94%	83%	74%

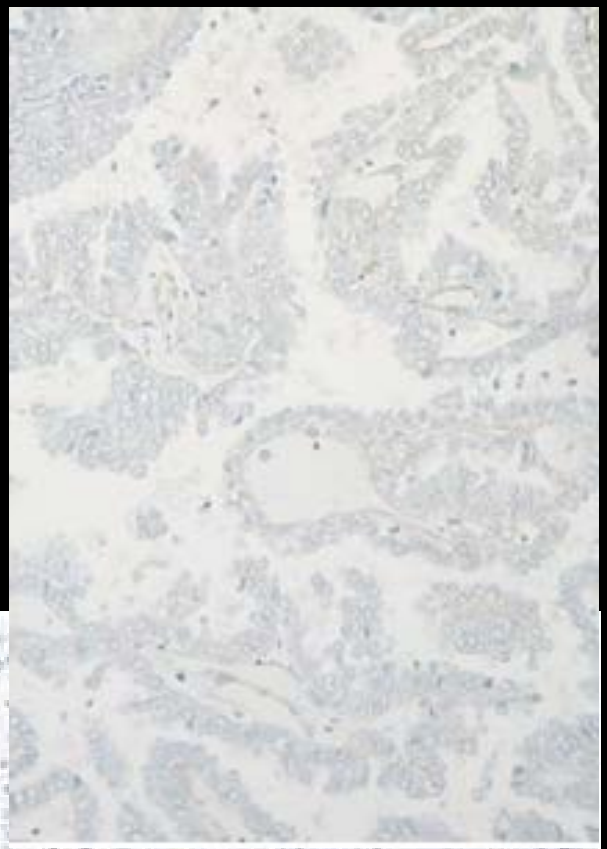


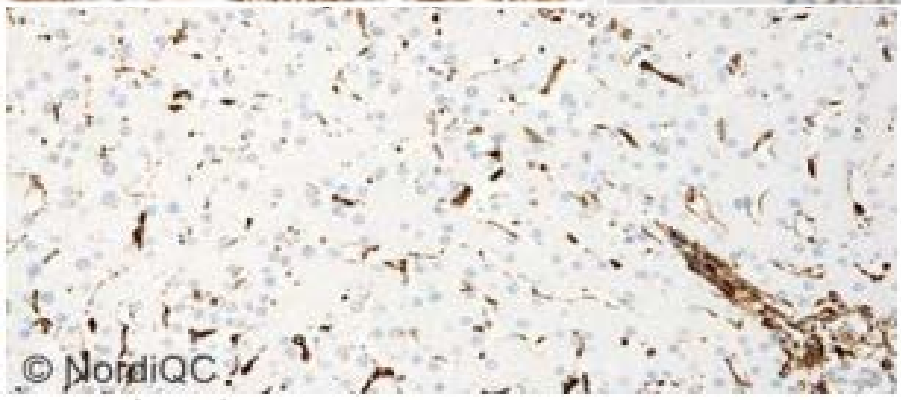


NordiQC runs 12 (2004) – 52 (2018)









Too dilute Ab
Too little or no HIER

Workshop in Diagnostic Immunohistochemistry
Oud St. Jan/ Old St. John – Brugge (Bruges), Belgium June 13th – 15th 2018

The unkown primary tumour: IHC Classification antibody selection, protocol optimization, controls and EQA (part I)

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NORTH DENMARK REGION