Ramazzini Institute

Cesare Maltoni Cancer Research Center

"Results of rodent carcinogenicity studies on aspartame"

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21 January, 2011
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Aspartame (APM): production and use

- 18,000 tons produced as of 2007
- second artificial intense sweetening agent after saccharin
- 62% of the intense sweetening agents market
- present in more than 6,000 products
- hundreds of millions of consumers worldwide
- Average daily intake in US and Europe
  - Projected maximum consumption: 22-34 mg/Kg b.w.
  - General population: 2-3 mg/Kg b.w.
  - Children/women of childbearing age: 2.5-5 mg/Kg b.w.

APM: hypothetical daily intake

Average Daily Intake of Aspartame		
Substance	Quantity/day	Concentration of aspartame consumed
Diet soda (200 mg/can)	2 cans	400 mg
Yogurt (125 mg/yogurt)	2 yogurts	250 mg
Diet custard/pudding (75mg/mousse)	1 serving	75 mg
Coffee with sweetener (40/mg packet)	4 cups	160 mg
Candy/chewing gum (2,5/candy)	10 candies	25 mg
Totals		**910 mg**

Equivalent to:

Woman 60 kg = 15,1 mg/kg body weight

Woman 50 kg = 18,2 mg/kg body weight

Child 30 kg = 30,3 mg/kg body weight

Child 20 kg = 45,5 mg/kg body weight

APM: regulatory approval

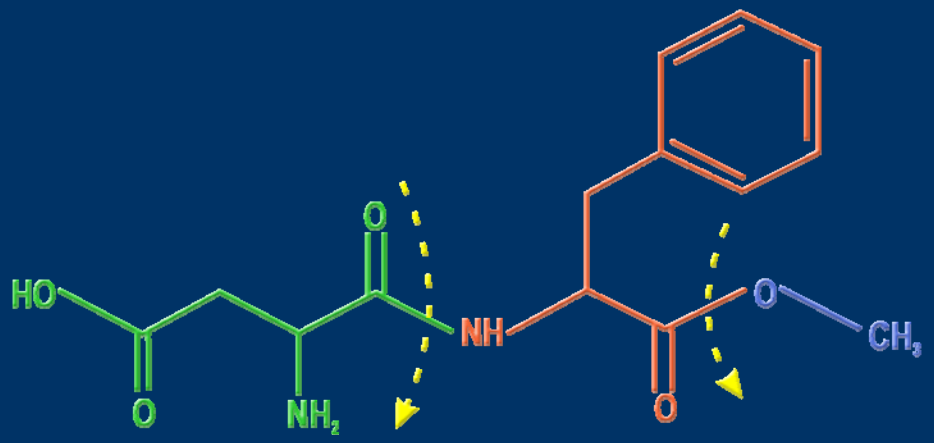
US FDA

- 1981 approved for solid food
- 1983 approved also for gaseous beverages
- 1996 all foods
- Acceptable Daily Intake (ADI) = 50mg/Kg bw
  (≅ 4000mg/day for a person of 80 Kg)

European Union

- 1994 determined safe for all use
- ADI = 40mg/Kg bw
  (≅ 3200mg/day for a person of 80 Kg)

APM: metabolism and genotoxicity



Aspartate Phenylalanine Methanol

- metabolized in the GI tract as aspartic acid, phenylalanine and methanol, both in humans and animals
- the metabolites, once absorbed, enter the blood circulation
- Methanol is not metabolized within the enterocite and rapidly enters the portal circulation and is oxidized in the liver to formaldehyde, which strongly binds to proteins and nucleic acids forming adducts

APM: genotoxicity

➔ Genotoxicity: APM has been shown to be non genotoxic in various tests in vitro and in vivo

APM: carcinogenicity

1973. Groups of 40 M and 40 F post-weaning Sprague-Dawley rats treated with APM in feed at doses of 0, 1, 2, 4, 6, 8 g/Kg b.w./day for 104 weeks. 60 M and 60 F served as controls.

No dose-related increase of brain tumors in treated groups.

1974. Groups of 40 M and 40 F Sprague-Dawley rats treated from fetal life and, after weaning, for 104 weeks with APM in feed at doses of 0, 2, 4 g/Kg b.w./day. 60 M and 60 F served as control.

Decrease of brain tumors incidence in treated groups.

APM: carcinogenicity

1981. Groups of 86 M and 86 F, 6 week-old Wistar rats treated with APM in feed at doses of 0, 1, 2, 4 g/Kg b.w./day from 6 to 104 weeks of age.
No increase of brain tumors observed.

1981. Groups of 36 M and 36 F CD-1 mice treated with APM in feed at doses of 1, 2, 4 g/Kg b.w./day from 6 to 110 weeks of age. 72 males and 72 females served as controls.
No carcinogenic effects observed.

Motivation to study APM

Given the limited sensitivity of these studies (small number of animals per group, duration of observation 110 weeks), and the ever-growing use of aspartame throughout the years, the ERF **(now RI)** decided in the late 1990s to plan an integrated project that would finally provide an adequate evaluation of the potential carcinogenic effects of aspartame based on:

> total number of animals used
> number of dose levels studied
> conduct of the experiment according to GLP

The integrated experimental project of the Ramazzini Institute on Aspartame started in 1997

RI integrated project on APM

	Animals		
Experiment	Species	No.	Status
first	S-D rats	1800	published (2005)
second	S-D rats	470	published (2007)
third	S-D rats	429	ongoing (biophase ended)
fourth	S-D rats	430	ongoing (biophase ended)
fifth	Swiss mice	852	ongoing (pre-pubblication)
Total		**3981**	

Materials and conduct (1/2)

APM chemical analysis:

- ➔ Purity: > 98%
- ➔ Diketopiperazine: < 1.5%
- ➔ Other impurities: < 1.0%

Route of administration: ingestion by feed

Start of the experiments: 8 weeks/fetal life

Materials and conduct (2/2)

Experimental conduct:

- ➔ Water and food consumption
- ➔ Body weight
- ➔ Clinical control
- ➔ Complete necropsy
- ➔ Histopathology
  - Full evaluation of all tissues and organs of each animal of each group
- ➔ Statistical evaluation
  - Cochran – Armitage; poly-K test; Cox proportional hazard model

First Aspartame experiment

(Sprague-Dawley rats)

First experiment: the plan

Age at start	Animals	dose/group ppm (mg/kg b.w.) [a,b]							
		100,000 (5,000)	50,000 (2,500)	10,000 (500)	2,000 (100)	400 (20)	80 (4)	0 (control)	**TOTAL**
6 weeks	n. males	100	100	100	150	150	150	150	900
8	n. females	100	100	100	150	150	150	150	900
Total		200	200	200	300	300	300	300	1800

[a] Considering the average weight of a rat as 400g, and average food consumption as 20g per day
[b] The treatment lasts for the entire life span

APM First Experiment: Results (part I)

INCIDENCE OF MALIGNANT SCHWANNOMAS OF PERIPHERAL NERVES IN MALES [a]

Tumor-bearing animals	ppm in feed (mg/kg b.w.) [b]						
	100,000 (5,000)	50,000 (2,500)	10,000 (500)	2,000 (100)	400 (20)	80 (4)	0 (control)
Incidence (%)	**4.0**	**3.0**	**2.0**	**1.3**	**2.0**	**0.7**	**0.7 *#**

[a] Historical control incidence of malignant schwannomas in males (2,265): 0.4% (range: 0-2.0%)

[b] p-values associated with the trend test are near the control incidence

* Statistically significant ($p<0.05$) using Cochran-Armitage test.

Statistically significant ($p<0.05$) using poly-k test ($k = 3$)

APM First Experiment: Results (part II)

INCIDENCE OF PRENEOPLASTIC LESIONS WITH ATYPIA (PLA) AND CARCINOMAS (CA) OF THE RENAL PELVIS AND URETER IN FEMALES

Tumor-bearing animals	ppm in feed (mg/kg b.w.) [a, b]						
	100,000 (5,000)	50,000 (2,500)	10,000 (500)	2,000 (100)	400 (20)	80 (4)	0 (control)
PLA Incidence (%)	11	7.1	7.0	4.7	4.0	3.3	1.3**
CA Incidence (%) [c]	**4.0**#	3.0	3.0	2.0	2.0	0.7	-
Total Incidence (%)	**15.0**##	**10.1**##	**10.0**##	**6.7**#	**6.0**#	4.0	1.3 **##

[a] p-values corresponding to pairwise comparison between the controls and the dosed group are near the dosed group incidence.

[b] p-values associated with the trend test are near the control incidence

[c] Historical control incidence of renal pelvis CA in females (2,274): 0.04% (range: 0-1.0%)

** Statistically significant ($p<0.01$) using Cochran-Armitage test

Statistically significant ($p<0.05$) using poly-k test (k = 3)

Statistically significant ($p<0.01$) using poly-k test (k = 3)

APM First Experiment: Results (part III)

INCIDENCE OF LYMPHOMAS AND LEUKEMIAS IN FEMALES [a]

Tumor-bearing animals	ppm in feed (mg/kg b.w.) [b, c]						
	100,000 (5,000)	50,000 (2,500)	10,000 (500)	2,000 (100)	400 (20)	80 (4)	0 (control)
Incidence (%)	**25.0**##	**25.0**##	**19.0**#	**18.7**#	**20.0**##	14.7	8.7**#

[a] Historical control incidence of lymphomas and leukemias in females (2,274): 13.3% (range: 4.0-25.0%)

[b] p-values corresponding to pairwise comparison between the controls and the dosed group are near the dosed group incidence.

[c] p-values associated with the trend test are near the control incidence

** Statistically significant ($p<0.01$) using Cochran-Armitage test

Statistically significant ($p<0.05$) using poly-k test (k = 3)

Statistically significant ($p<0.01$) using poly-k test (k = 3)

APM: reactions to the results: EFSA

➔ Lymphomas and leukemias

"[…] In detail the Panel concluded the following:

The slight increase in incidence of cancers known as lymphomas and leukemias in treated rats was considered to be unrelated to aspartame treatment and most likely attributed to the high background incidence of inflammatory changes in the lung. In addition, there was no dose-response relationship with respect to increasing doses of aspartame.

APM: reactions to the results: EFSA

➔ Kidney tumors

The findings in the kidney, ureter and bladder, observed mainly in female rats, are not specific to aspartame and have been observed with a number of chemicals administered to rats at high dose levels. Such changes are normally the result of irritation or imbalances in calcium metabolism specific to rats and are of no relevance for humans.

APM: reactions to the results: EFSA

➔ Peripheral nerves tumors

Concerning the malignant tumors of the peripheral nerves, the numbers of tumours were low with no clear dose- response relationship over a wide dose range. There is also uncertainty about the diagnosis of these tumours. The Panel indicated that this finding can only be fully evaluated by an independent peer-review of the relevant tissues. […]"

Second Aspartame experiment

(Sprague-Dawley rats)

Started: January 2004

Second experiment: the plan

Age at start	Animals	Dose/group,ppm (mg/kg b.w.) [a,b]			
		2,000 (100)	400 (20)	0 (control)	**TOTAL**
Fetal life	n. males	70	70	95	**235**
Fetal life	n. females	70	70	95	**235**
Total		140	140	190	**470**

[a] Considering the average weight of a rat as 400g, and average food consumption as 20g per day
[b] The treatment lasted for the entire life span

Second experiment: malignant tumors (%)

Animals	dose/group, ppm (mg/kg b.w.) [a]		
	2,000 (100)	400 (20)	0 (control)
Males (%)	**40.0 ****	**25.7**	**24.2****
Females (%)	**52.9**	**44.3**	**44.2**

[a] p-values associated with the trend test are near the control incidence

** significant ($p<0.01$) using Cox Regression Model.

Second experiment: mammary cancers (%)

Animals	dose/group, ppm (mg/kg b.w.) [a]		
	2,000 (100)	400 (20)	0 (control)
Males (%)	**2.9**	**-**	**-**
Females (%)	**15.7***	**7.1**	**5.3***

[a] p-values associated with the trend test are near the control incidence

* significant ($p<0.05$) using Cox Regression Model

Second experiment: lymphomas and leukemias (%)

Animals	dose/group ppm (mg/kg b.w.) [a]		
	2,000 (100)	400 (20)	0 (control)
Males (%)	**17.1***	**15.7**	**9.5**
Females (%)	**31.4****	**17.1**	**12.6****

[a] p-values associated with the trend test are near the control incidence

* significant ($p<0.05$) using Cox Regression Model

** significant ($p<0.01$) using Cox Regression Model

Comparison of lymphomas/leukemias in females: prenatal vs. postnatal exposure

Dose, ppm (mg/kg b.w.)	Females with lymphomas/leukemias (%) [a, b, c]	
	Prenatal exposure	Postnatal exposure
2,000 (100)	**31.4°°**	**18.7#**
400 (20)	**17.1**	**20.0##**
0 (control)	12.6°°	8.7(**#)[c]

[a] p-values corresponding to pairwise comparison between the controls and the dosed group are near the dosed group incidence.

[B] p-values associated with the trend test are near the control incidence

[c] The p-values associated with the trend test is referred to the 7 groups of first APM experiment

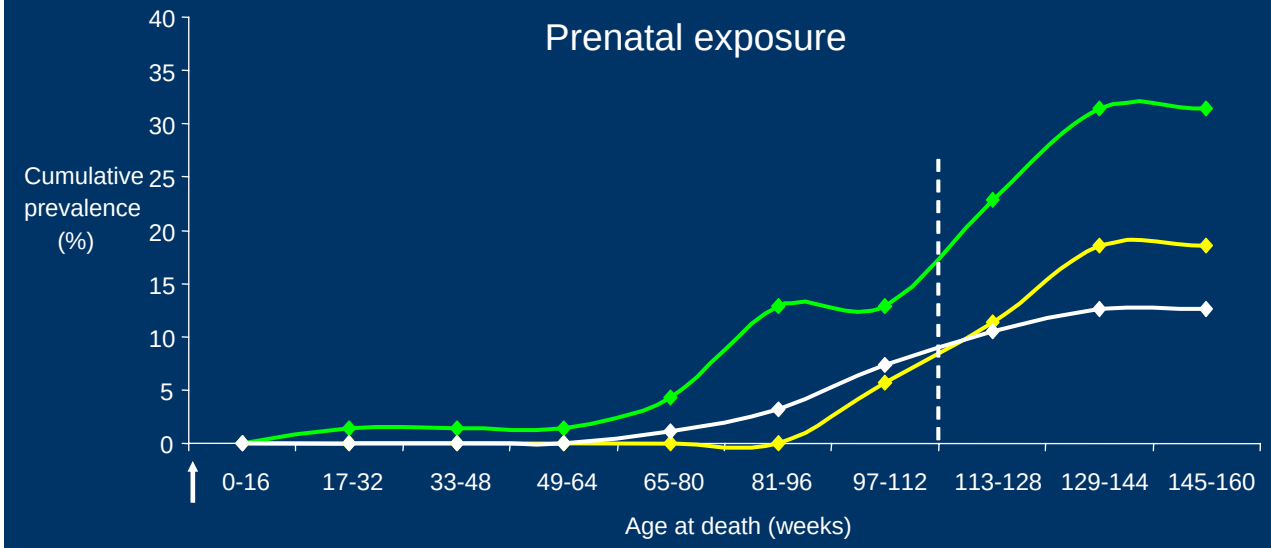
** Statistically significant ($p<0.01$) using Cochran-Armitage test

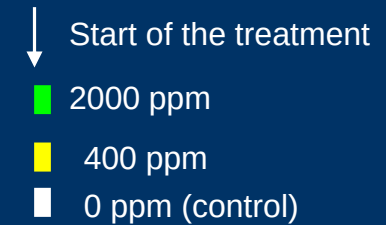
Statistically significant ($p<0.05$) using poly-k test ($k = 3$)

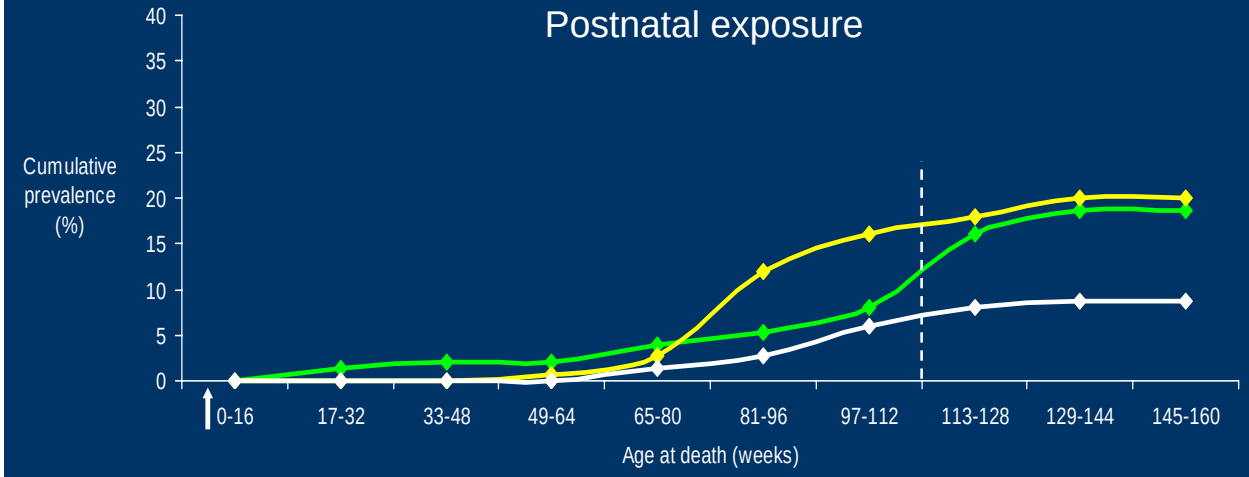
Statistically significant ($p<0.01$) using poly-k test ($k = 3$)

°° Statistically significant ($p<0.01$) using Cox Regression Model

Comparison of the cumulative prevalence of hemolymphoreticular neoplasia by age of death







Swiss mice Aspartame experiment

Swiss mice experiment: the plan

Age at start	Animals	Dose/group, ppm [a] (mg/Kg b.w.)					
		32,000 (3909)	16,000 (1919)	8,000 (987)	2,000 (242)	0 (Control)	**TOTAL**
fetal	n. males	83	64	73	122	102	444
fetal	n. females	62	64	62	103	117	408
Total		145	128	135	225	219	852

[a] **The treatment lasts for the entire life span**

Swiss mice experiment: malignant tumors (%)

Animals	Dose/group, ppm (mg/Kg b.w.)				
	32,000 (3909)	16,000 (1919)	8,000 (987)	2,000 (242)	0
Males (%)	68.7	60.9	72.6	56.3	56.4
Females (%)	64.5	68.8	64.4	73.8	67.6

Swiss mice experiment: Alveolar Bronchiolar Adenomas (A/BA) and Carcinomas (A/BC) in males[a] (%)

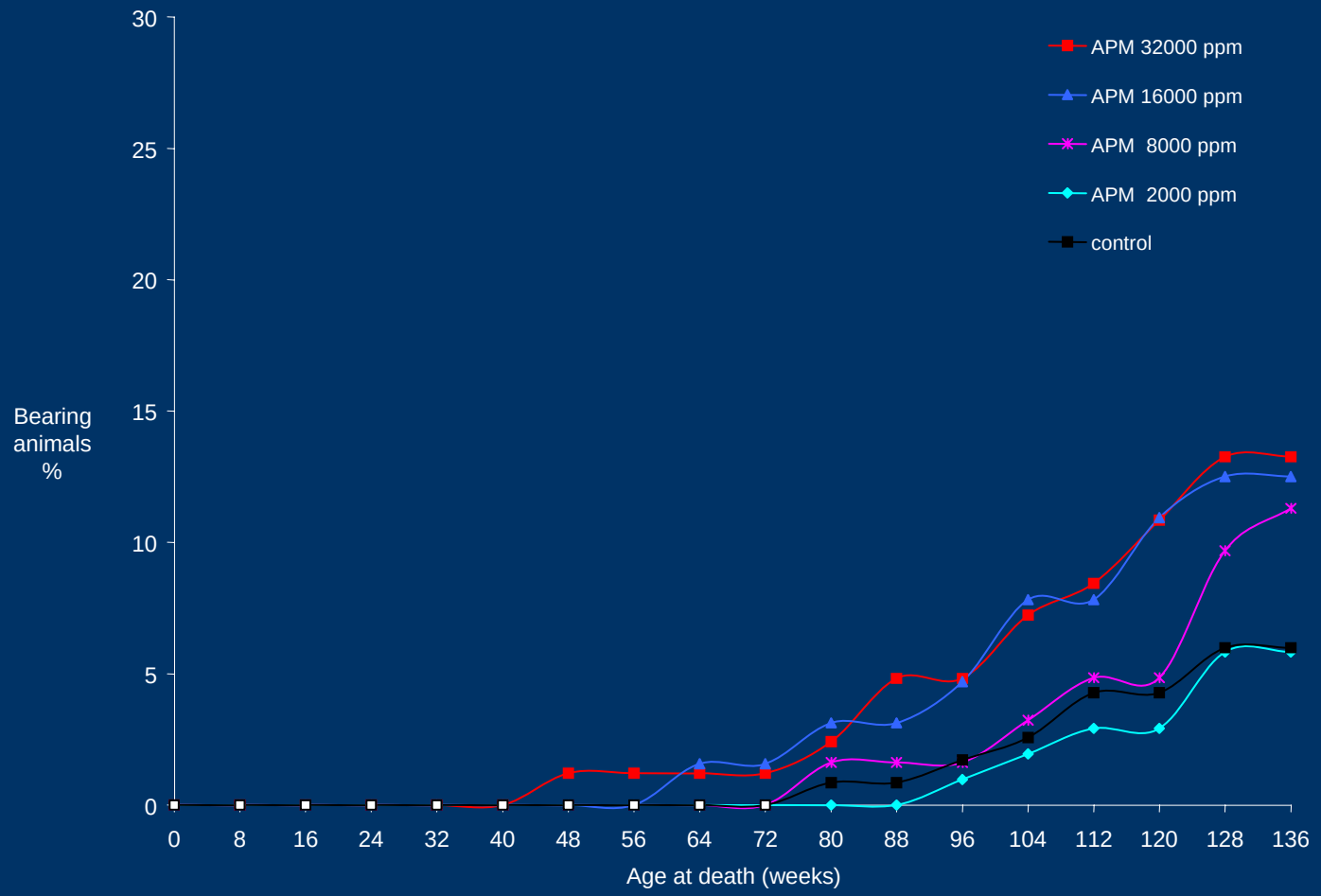
Animals	Tumors	Dose/group, ppm (mg/Kg b.w.)				
		32,000 (3909)	16,000 (1919)	8,000 (987)	2,000 (242)	0
Males	A/BA	7.2	10.9	11.3	8.7	6.8
Males	**A/BC**	**13.3***	**12.5**	**11.3**	**5.8**	**6.0***
Total		**20.5#**	23.4	22.6	14.6	**12.8#**

[a] p-values associated with the trend test are near the control incidence

* significant ($p<0.05$) using Cox Regression Model

significant ($p<0.05$) using Logistic analysis

Cumulative prevalence of lung A/BC by age of death



Swiss mice experiment: Hepatocellular Adenomas (HA)/ Carcinomas (HCC) in males[a], %

Animals	Tumors	Dose/group, ppm (mg/Kg b.w.)				
		32,000 (3909)	16,000 (1919)	8,000 (987)	2,000 (242)	0
Males	HA	2.4	9.4	6.5	9.7	7.7
Males	**HCC**	**18.1****	**15.6***	**14.5**	**11.7**	**5.1****
	Total	20.5	**25.0**[#]	21.0	21.4	12.8

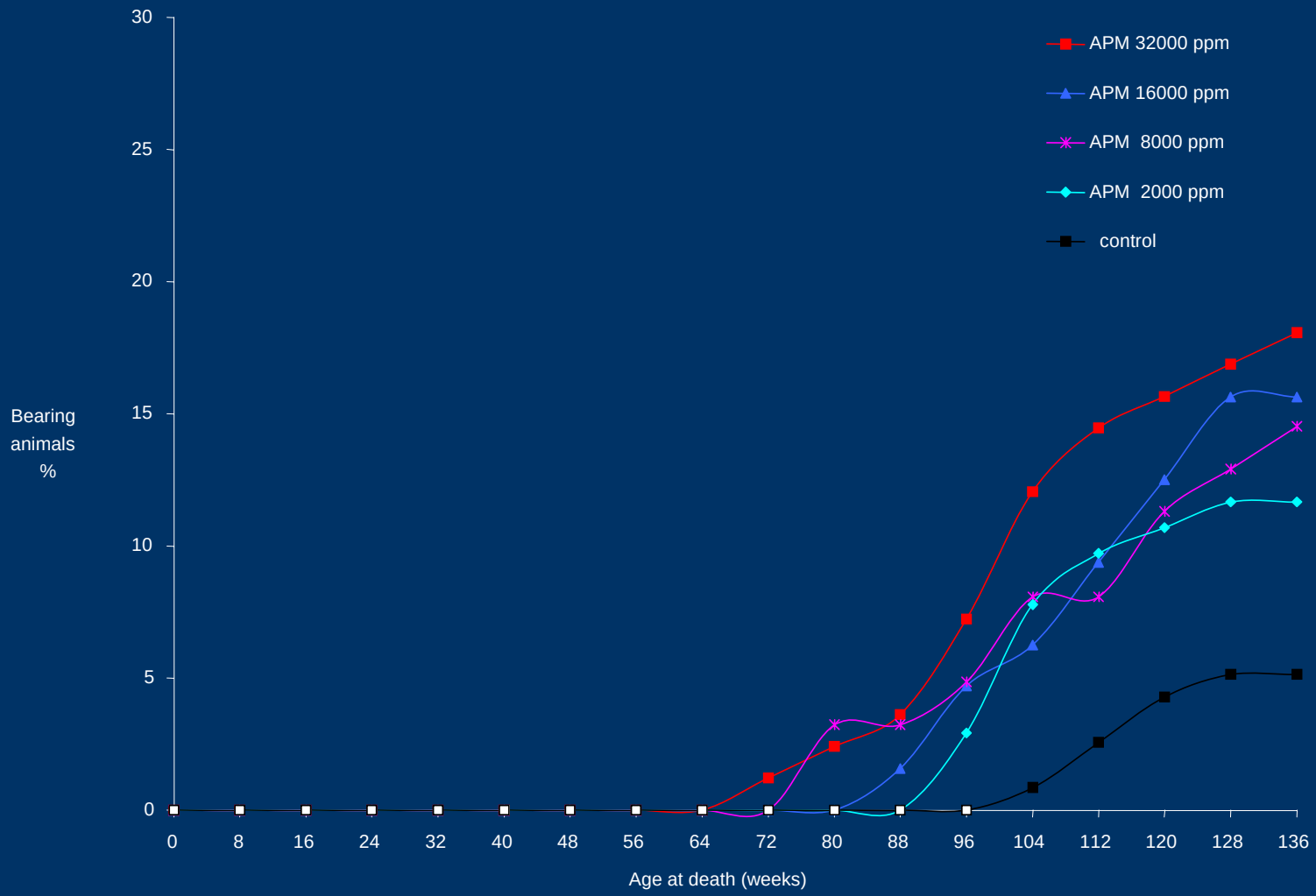
[a] p-values associated with the trend test are near the control incidence

* significant ($p<0.05$) using Cox Regression Model

** significant ($p<0.01$) using Cox Regression Model

[#] significant ($p<0.05$) using Logistic analysis

Cumulative prevalence of HCC by age of death



Swiss mice experiment: liver hemangioma and hemangiosarcomas (%)

Animals	Tumors	Dose/group, ppm (mg/Kg b.w.)				
		32,000 (3909)	16,000 (1919)	8,000 (987)	2,000 (242)	0
Males	Hemangiomas	-	1.6	1.6	1.9	1.7
Males	**Hemangiosarcomas**	**9.6**	**7.8**	**8.1**	**4.9**	**4.3**
	Total	9.6	9.4	9.7	6.8	6.0

Summary of the carcinogenic effects of APM in rodents

Significantly increased malignant tumors

Species	Age at start	Lymph/ leuk		Kidneys CP		Nervous sys. MS		Mammary ADC		Lung ADC		Liver HCC	
		M	F	M	F	M	F	M	F	M	F	M	F
S-D rats	8 weeks		+ DR		+ DR		+ DR						
S-D rats	fetal	+	+ DR						+ DR				
S-mice	fetal										+ DR		+ DR

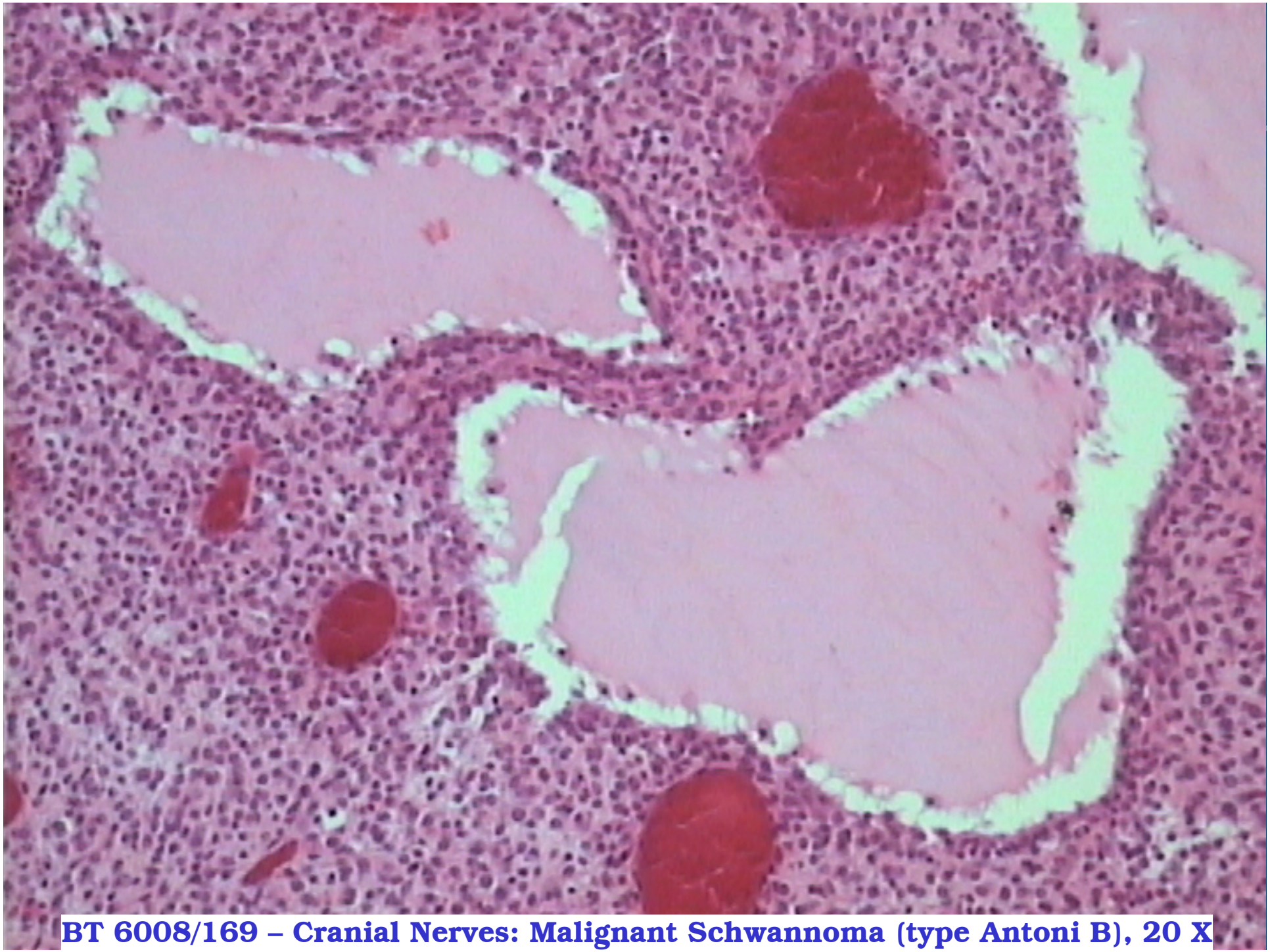
+= significantly increased;
DR= Dose-related;
CP= Carcinomas of the pelvis & ureter;

MS= Malignant Schwannomas;
ADC= Adenocarcinomas;
HCC= Hepatocellular carcinomas;

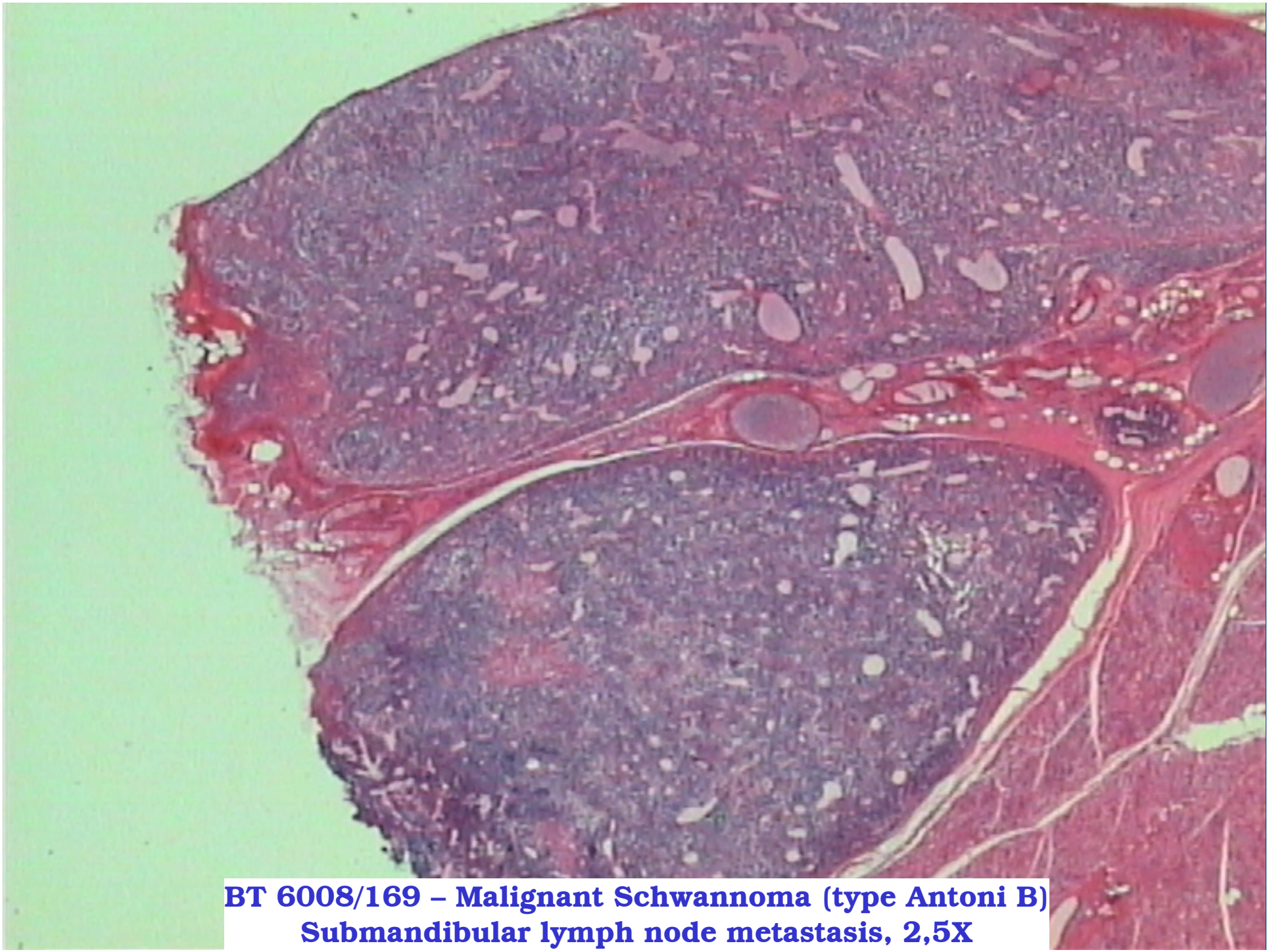
Histopatological lesions

Peripheral Nerves: Malignant Schwannomas

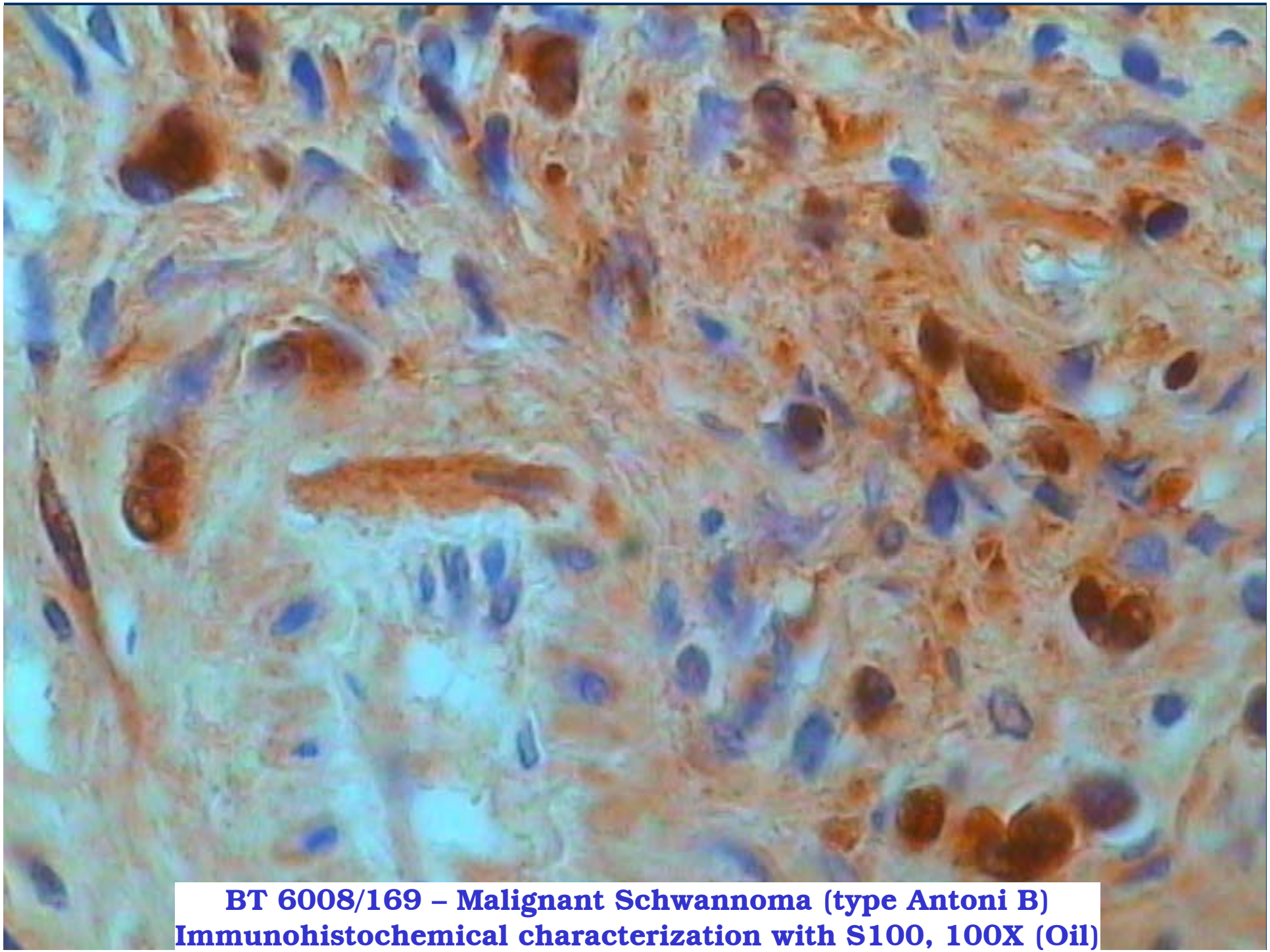
BT 6008/169 – Cranial Nerves: Malignant Schwannoma (type Antoni B), 2,5 X



BT 6008/169 – Cranial Nerves: Malignant Schwannoma (type Antoni B), 20 X



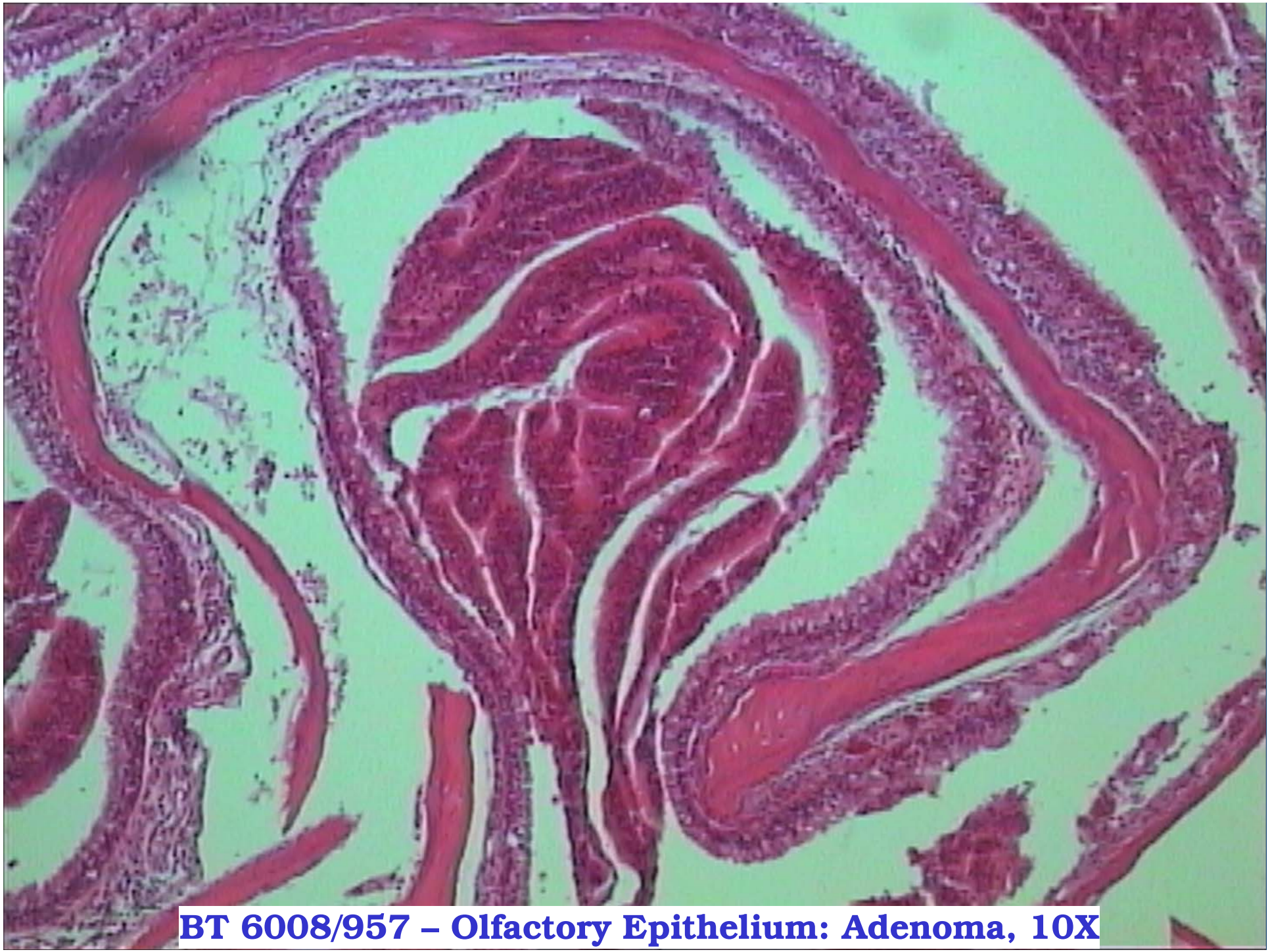
BT 6008/169 – Malignant Schwannoma (type Antoni B)
Submandibular lymph node metastasis, 2,5X



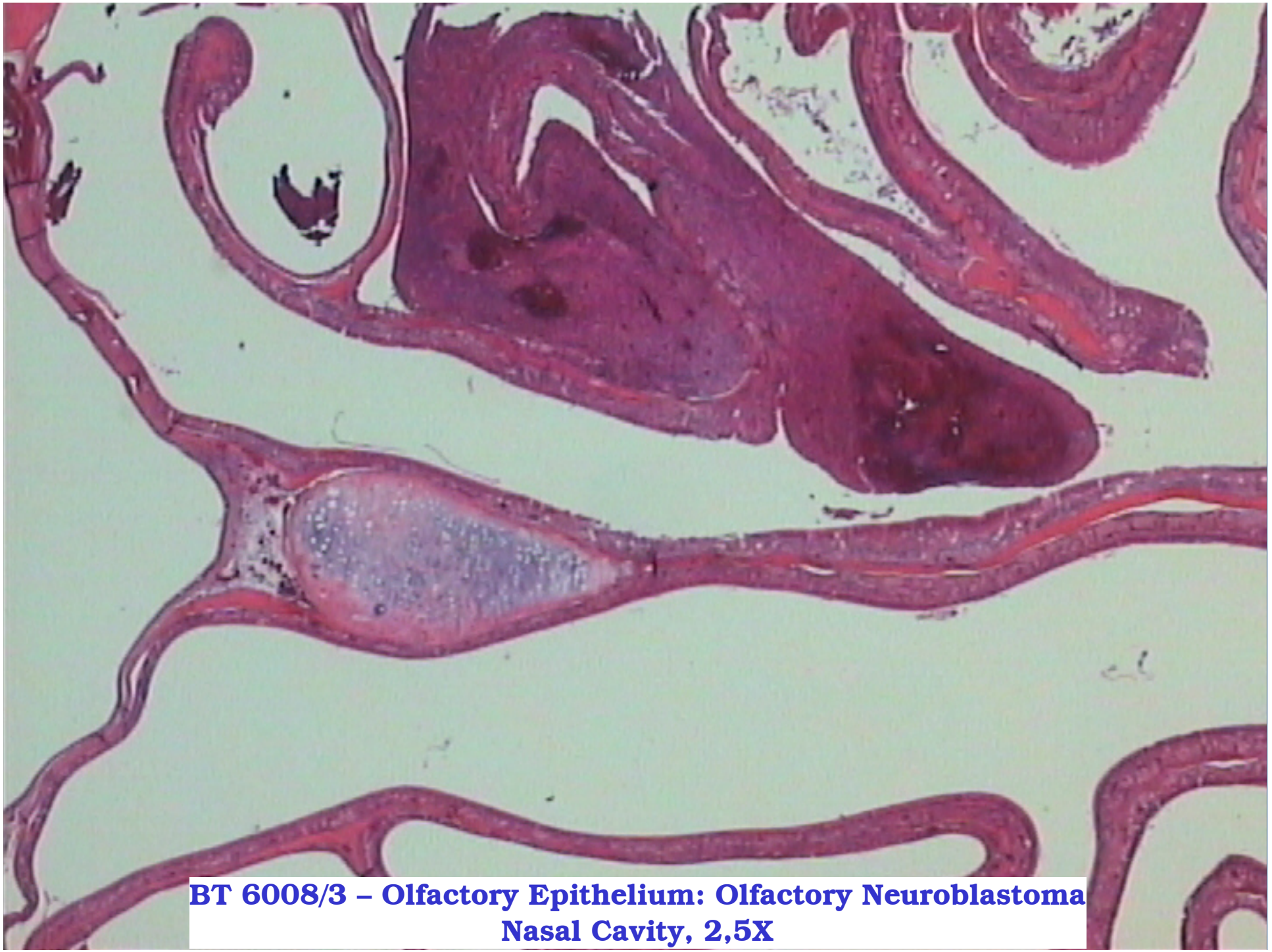
BT 6008/169 – Malignant Schwannoma (type Antoni B)
Immunohistochemical characterization with S100, 100X (Oil)

Preneoplastic and Neoplastic lesions of Olfactory Epithelium

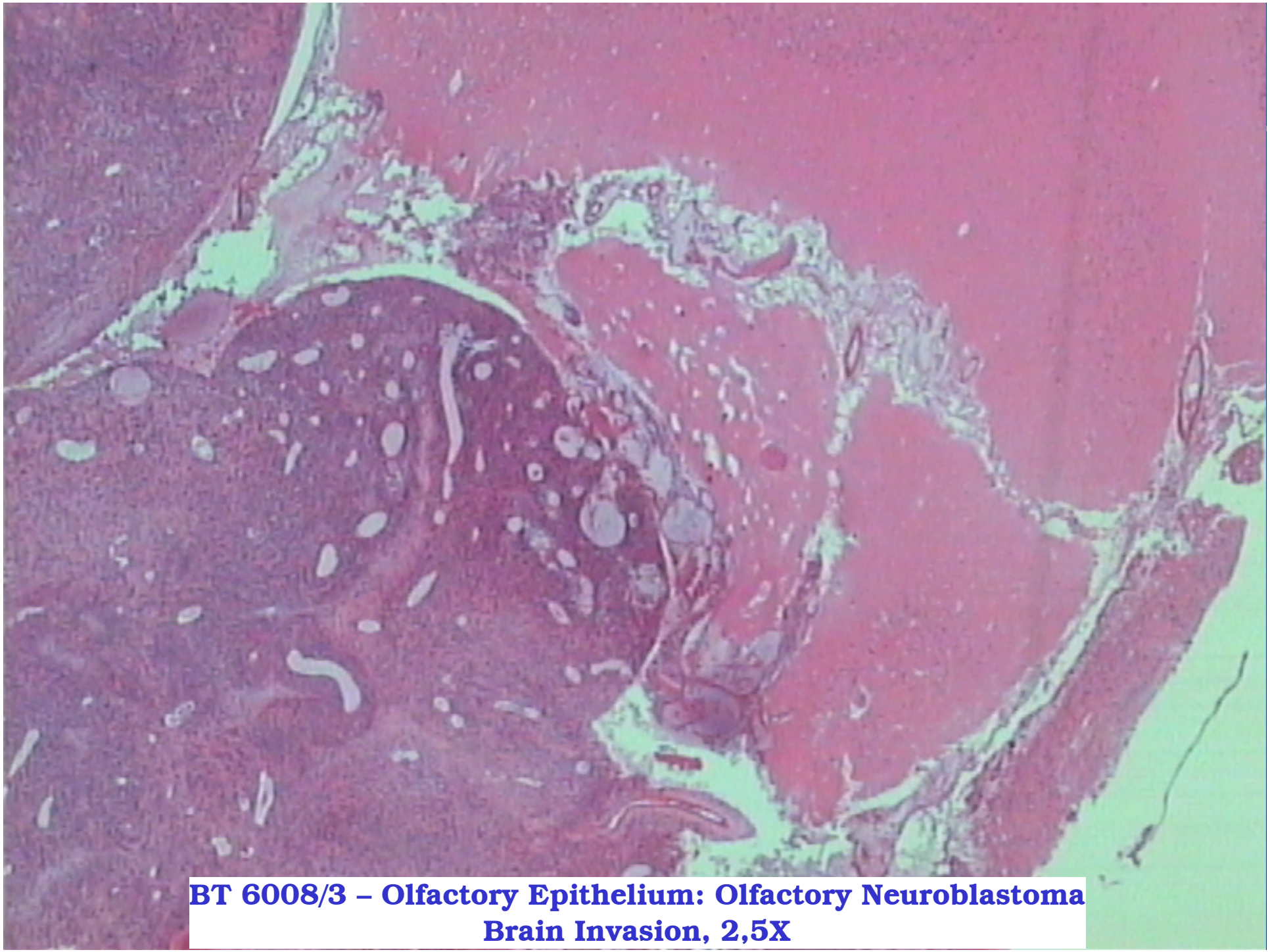
BT 6008/957 – Olfactory Epithelium: Adenomas, 2,5X



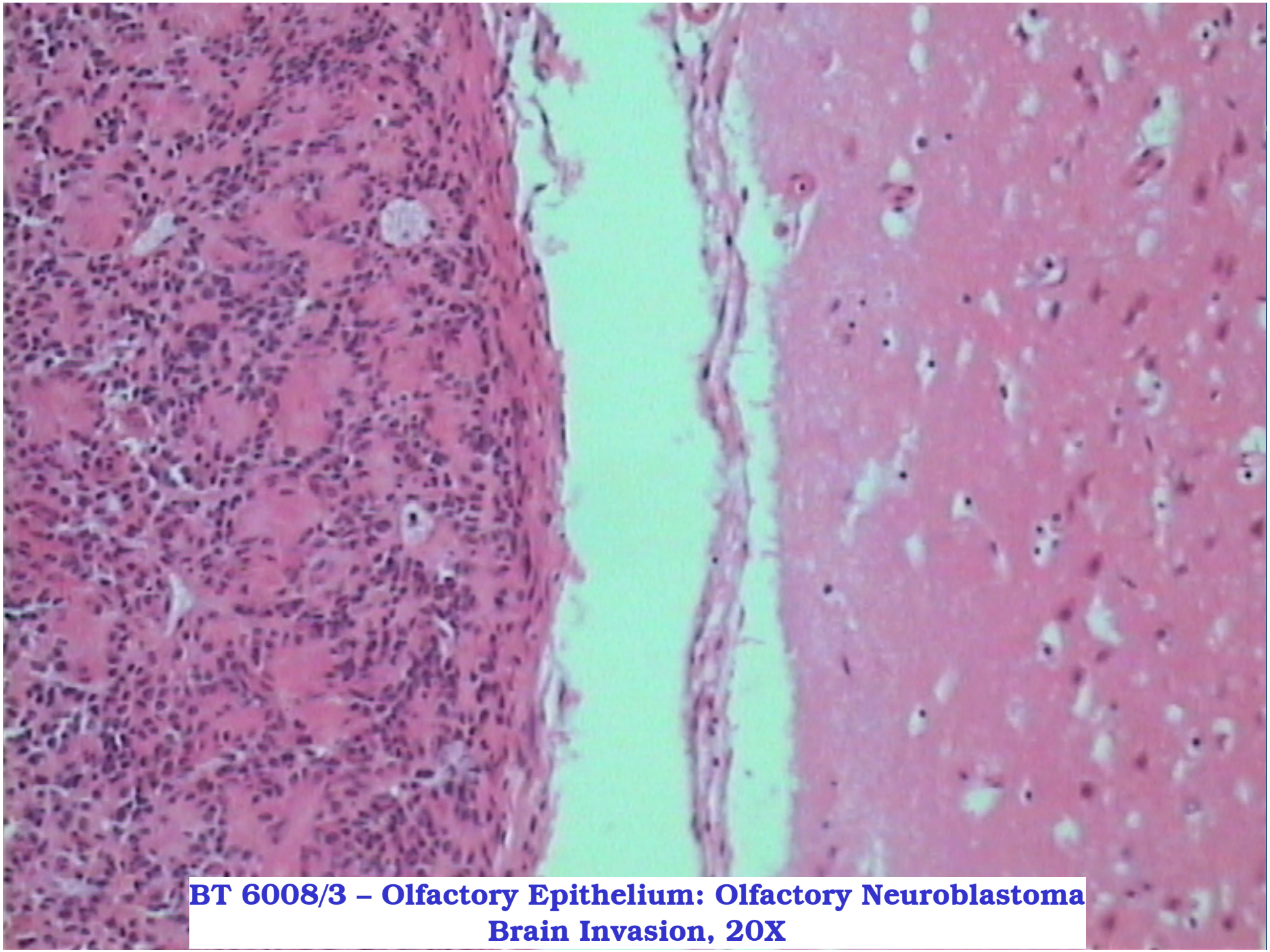
BT 6008/957 – Olfactory Epithelium: Adenoma, 10X



BT 6008/3 – Olfactory Epithelium: Olfactory Neuroblastoma
Nasal Cavity, 2,5X



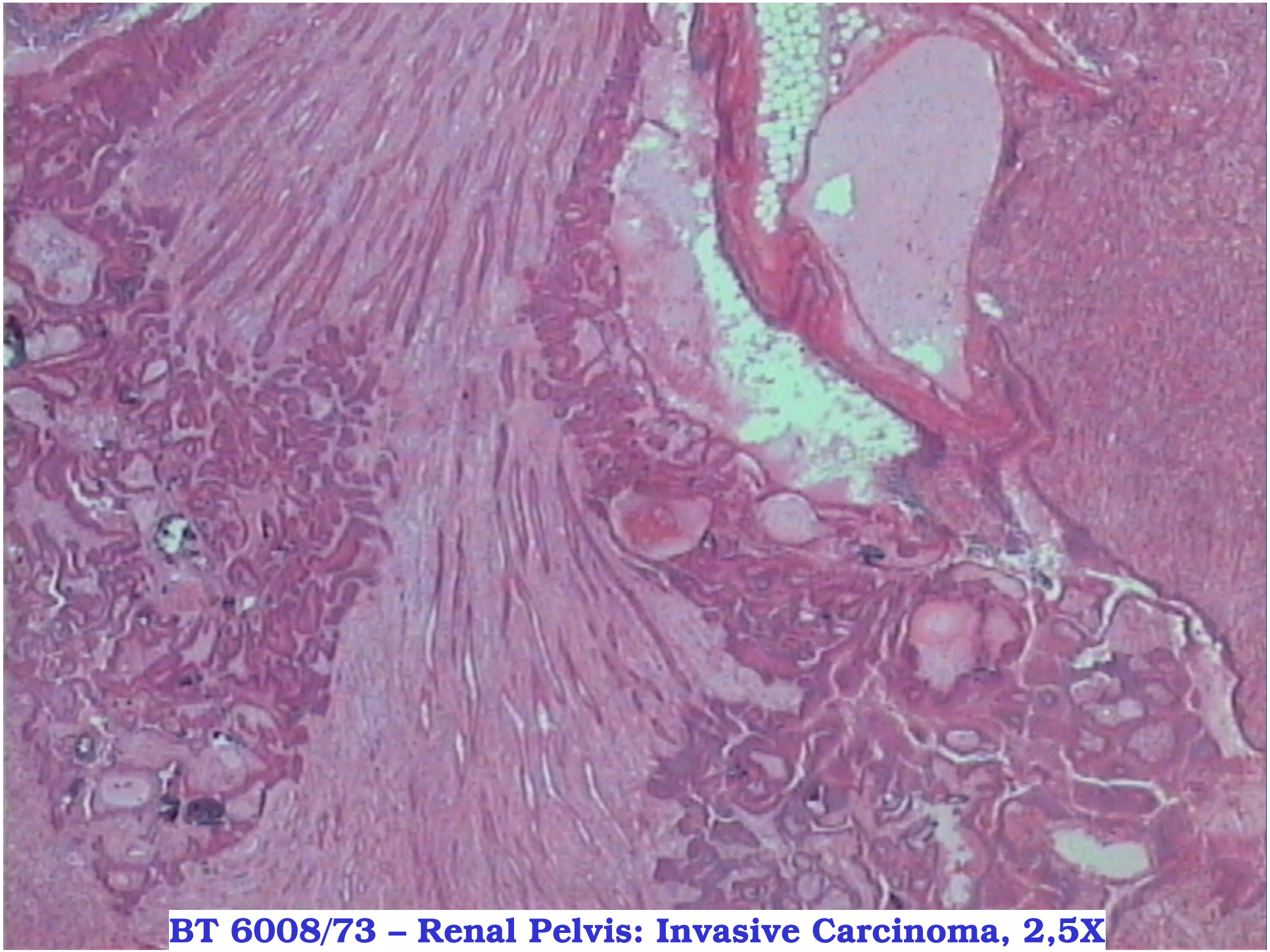
BT 6008/3 – Olfactory Epithelium: Olfactory Neuroblastoma
Brain Invasion, 2,5X



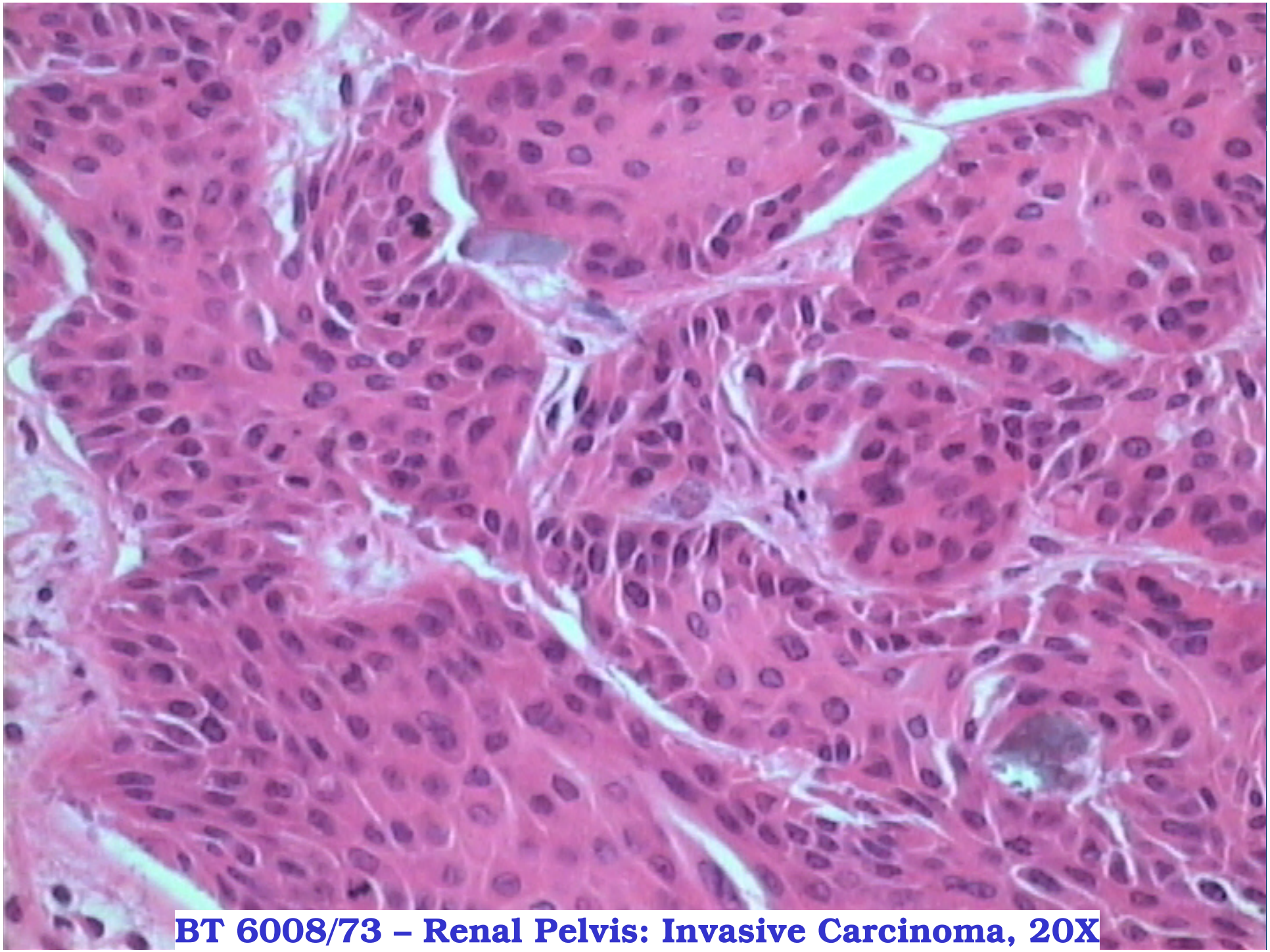
BT 6008/3 – Olfactory Epithelium: Olfactory Neuroblastoma Brain Invasion, 20X

BT 6008/3 – Olfactory Epithelium: Olfactory Neuroblastoma
Immunohistochemical characterization with Chromogranin A, 100X (Oil)

Renal Pelvis and Ureter lesions of oncological interest

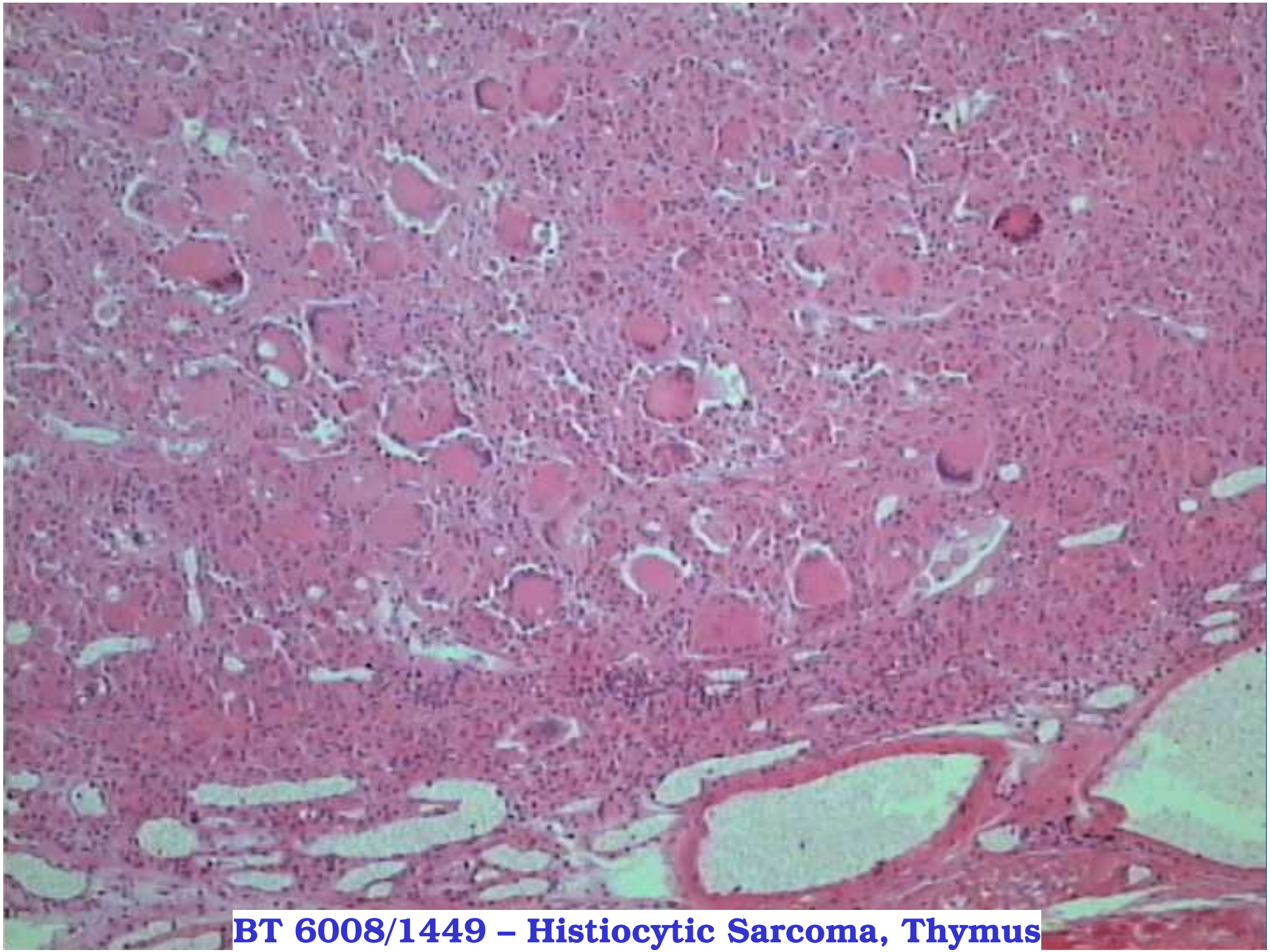


BT 6008/73 – Renal Pelvis: Invasive Carcinoma, 2,5X

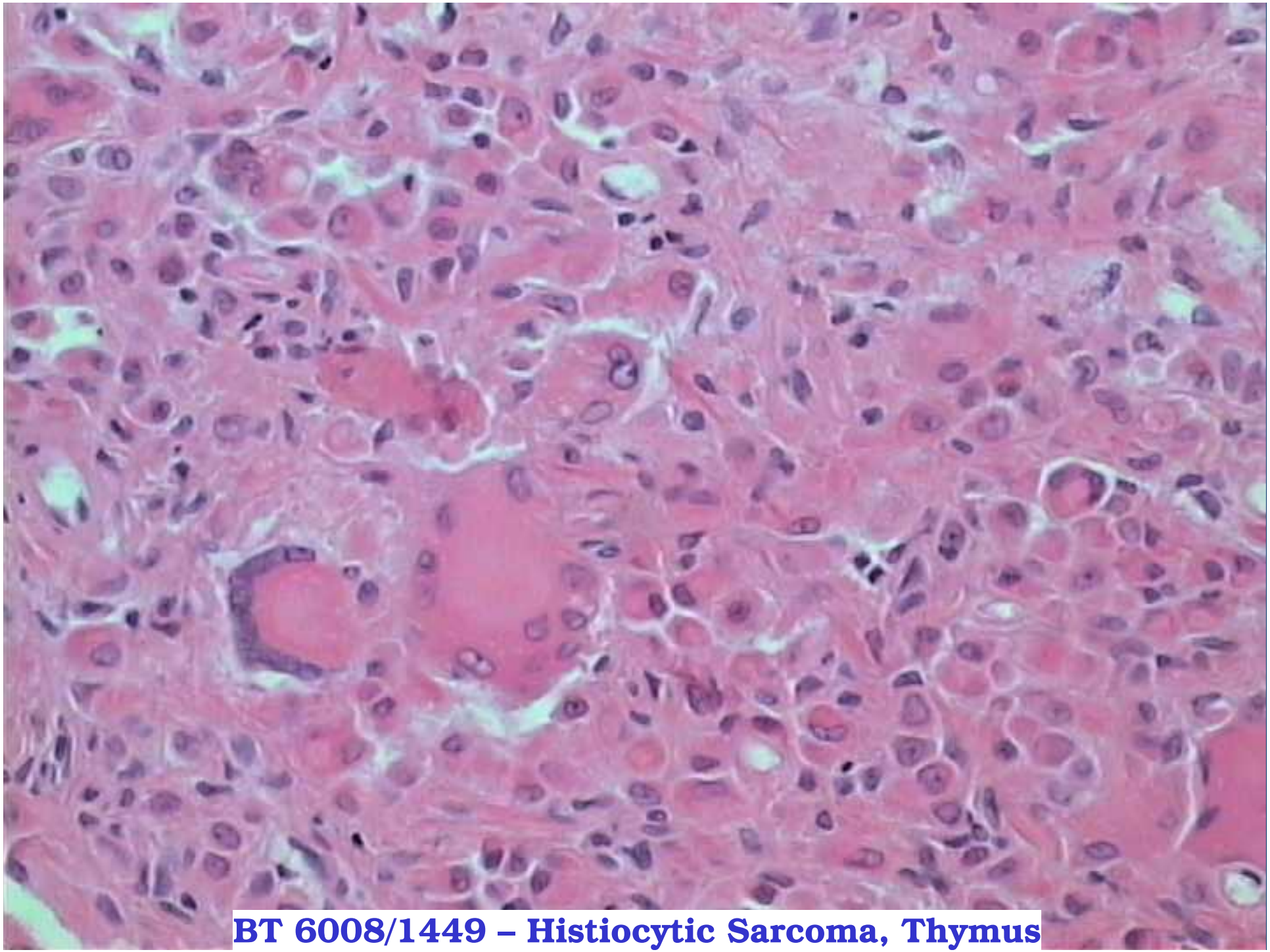


BT 6008/73 – Renal Pelvis: Invasive Carcinoma, 20X

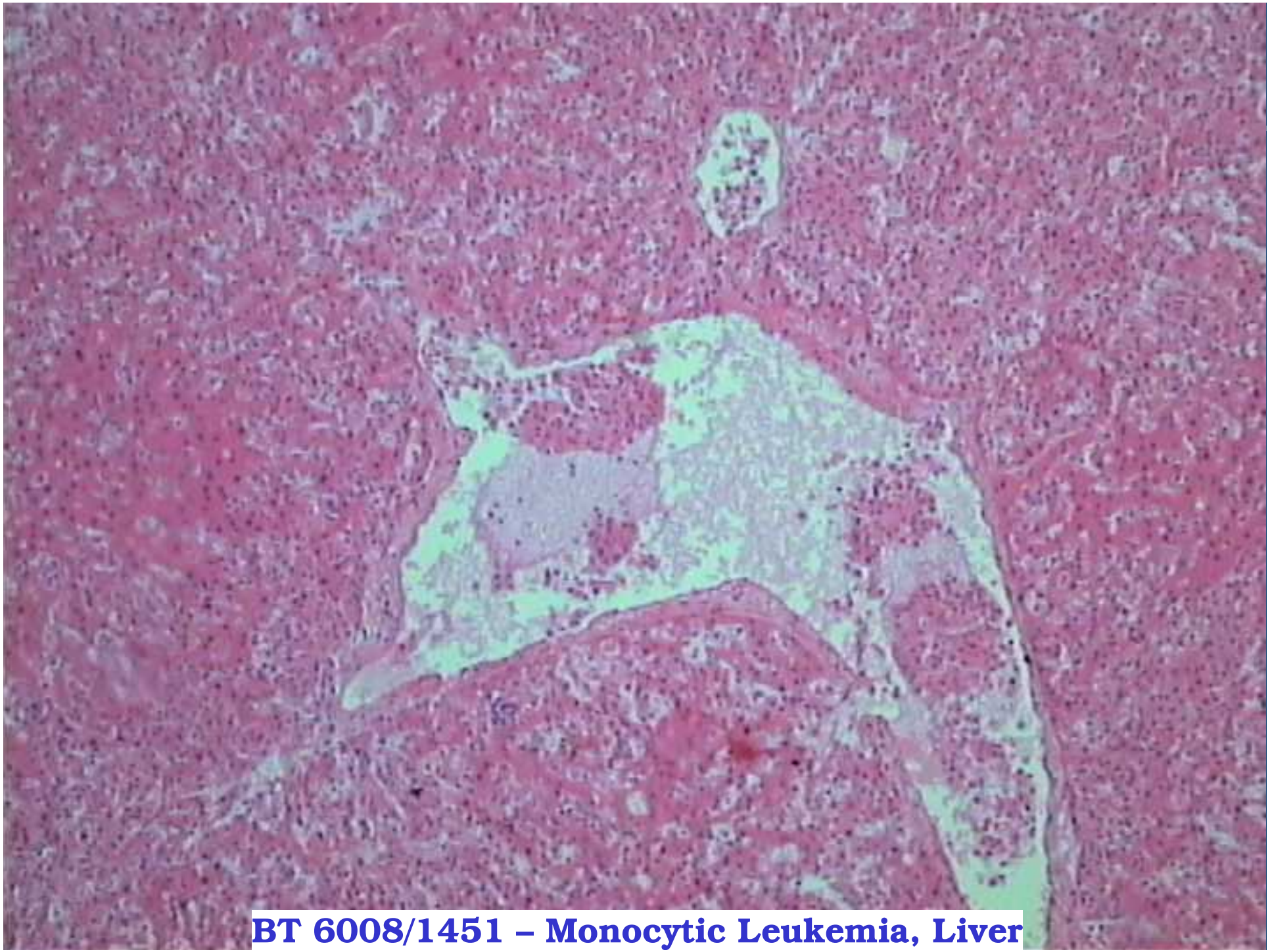
Haemolymphoreticular Neoplasias



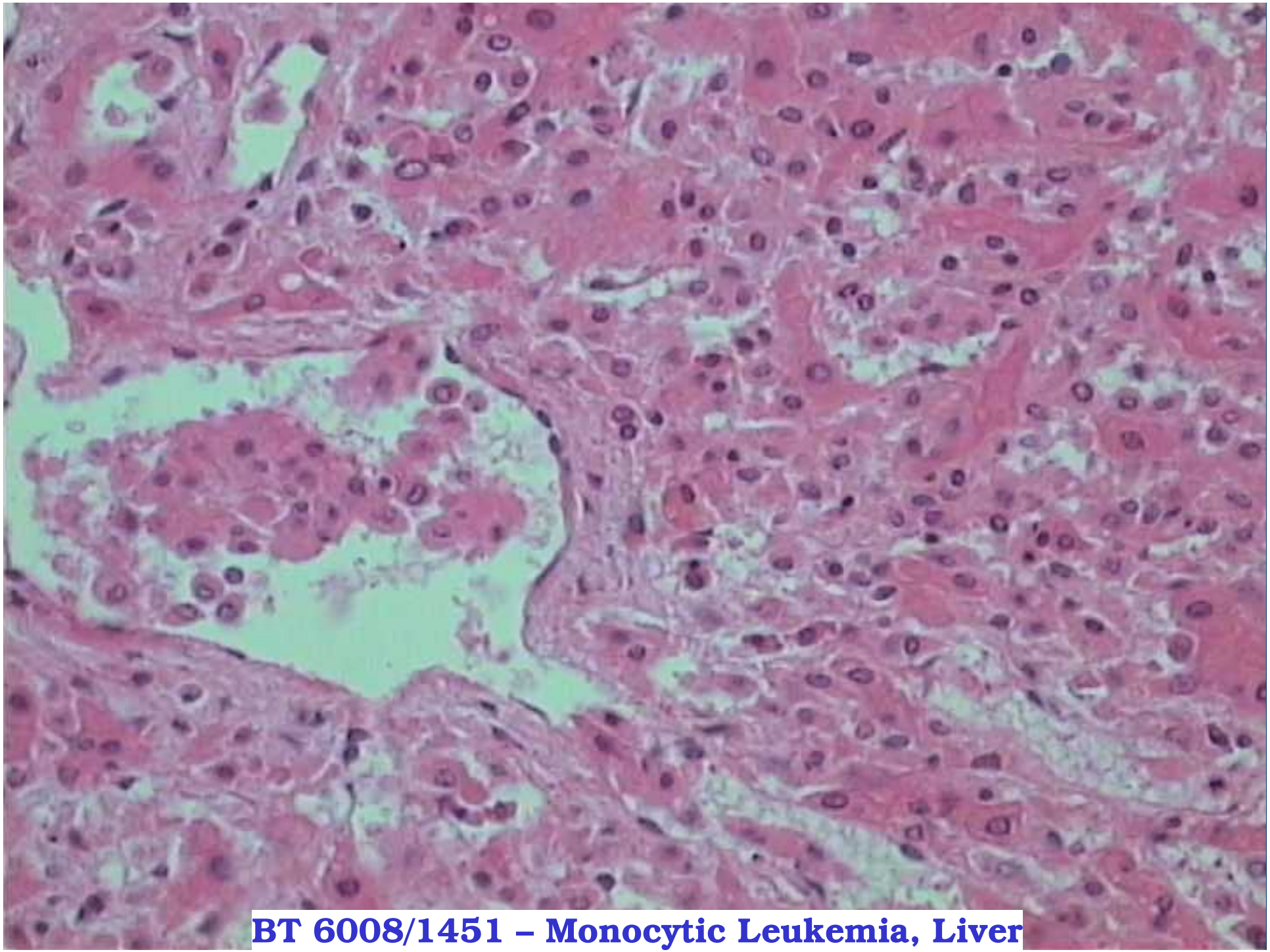
BT 6008/1449 – Histiocytic Sarcoma, Thymus



BT 6008/1449 – Histiocytic Sarcoma, Thymus

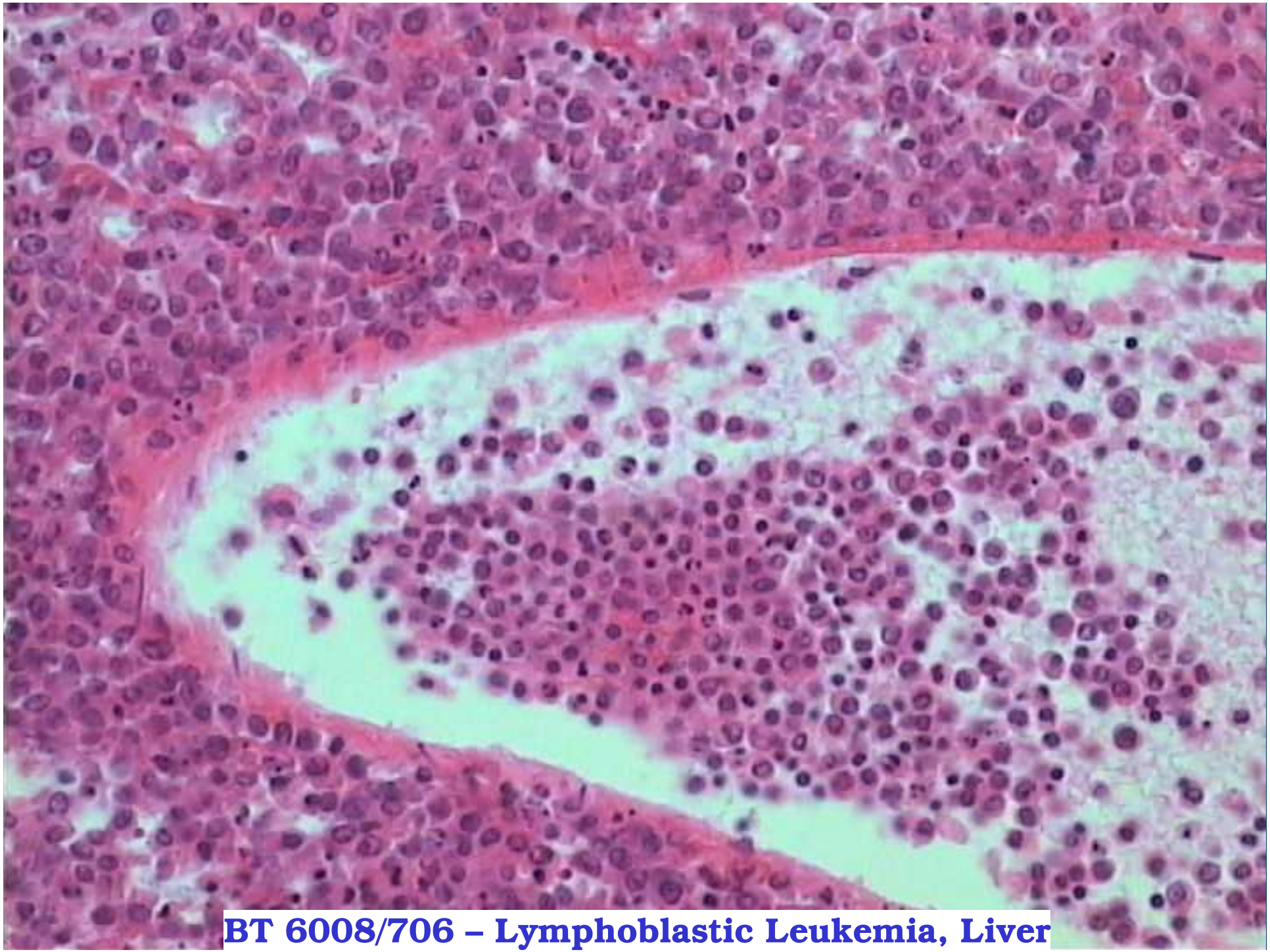


BT 6008/1451 – Monocytic Leukemia, Liver



BT 6008/1451 – Monocytic Leukemia, Liver

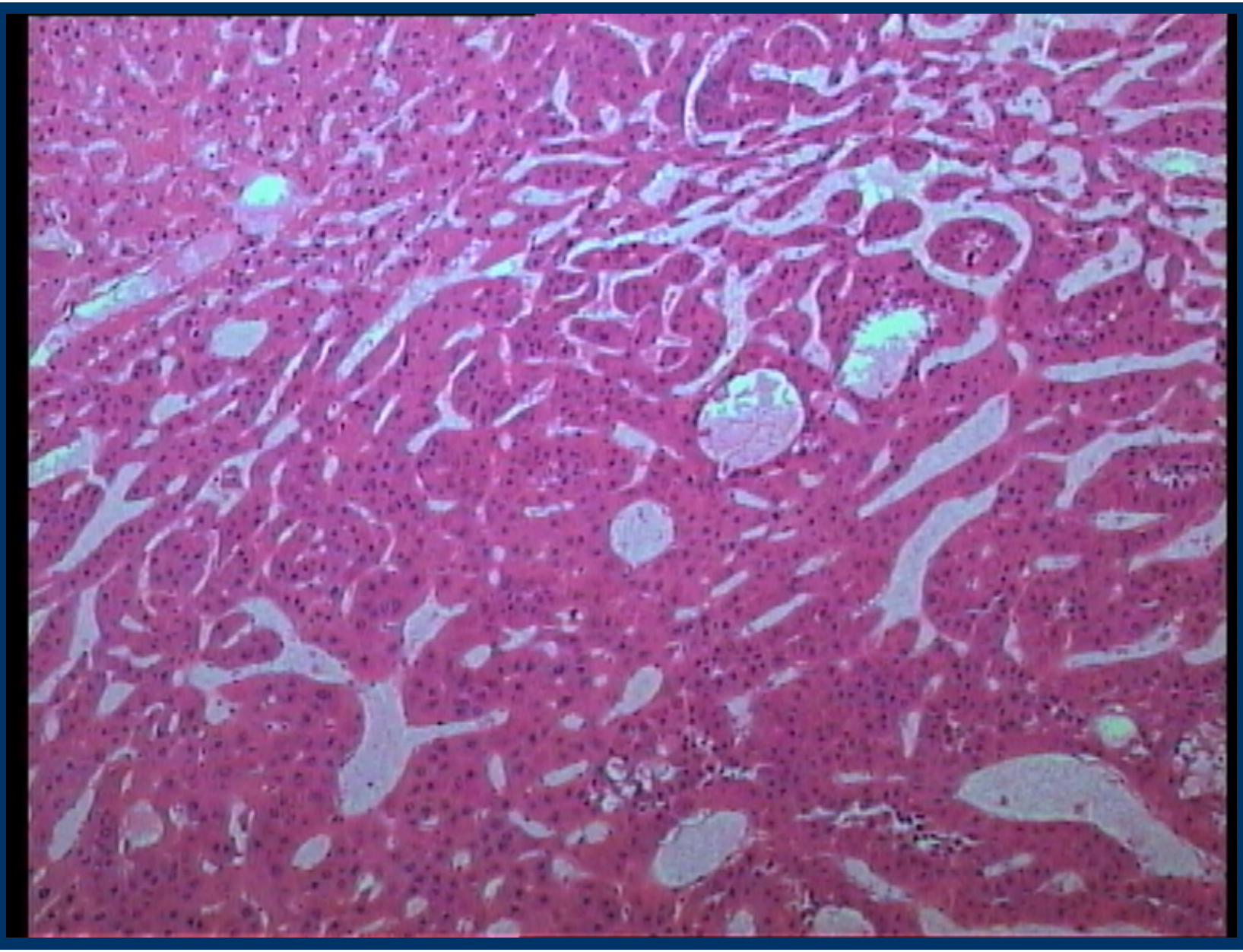
BT 6008/706 – Lymphoblastic Leukemia, Liver



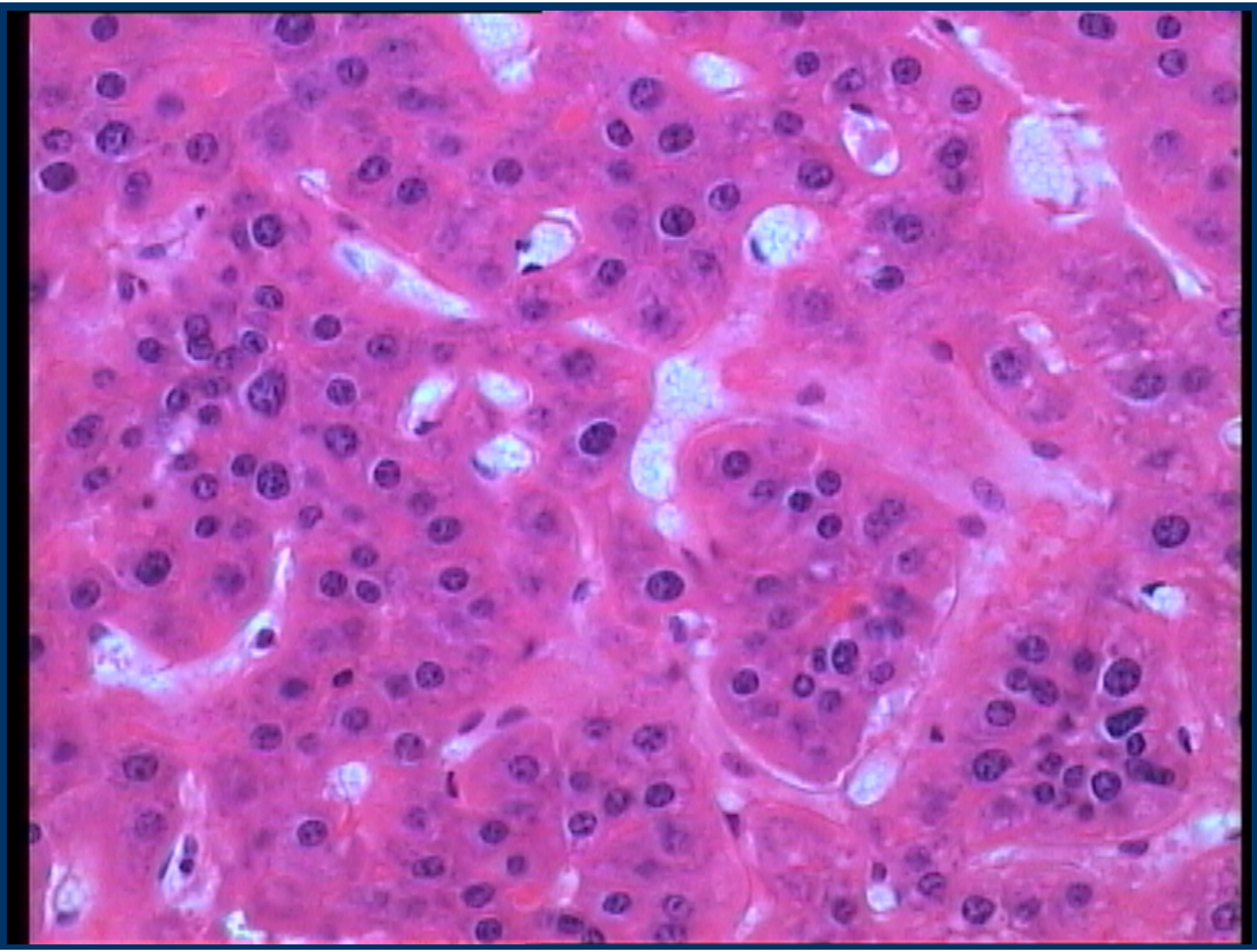
BT 6008/706 – Lymphoblastic Leukemia, Liver

Swiss mice: hepatocellular carcinomas

Swiss mice experiment: hepatocellular carcinoma (10X)

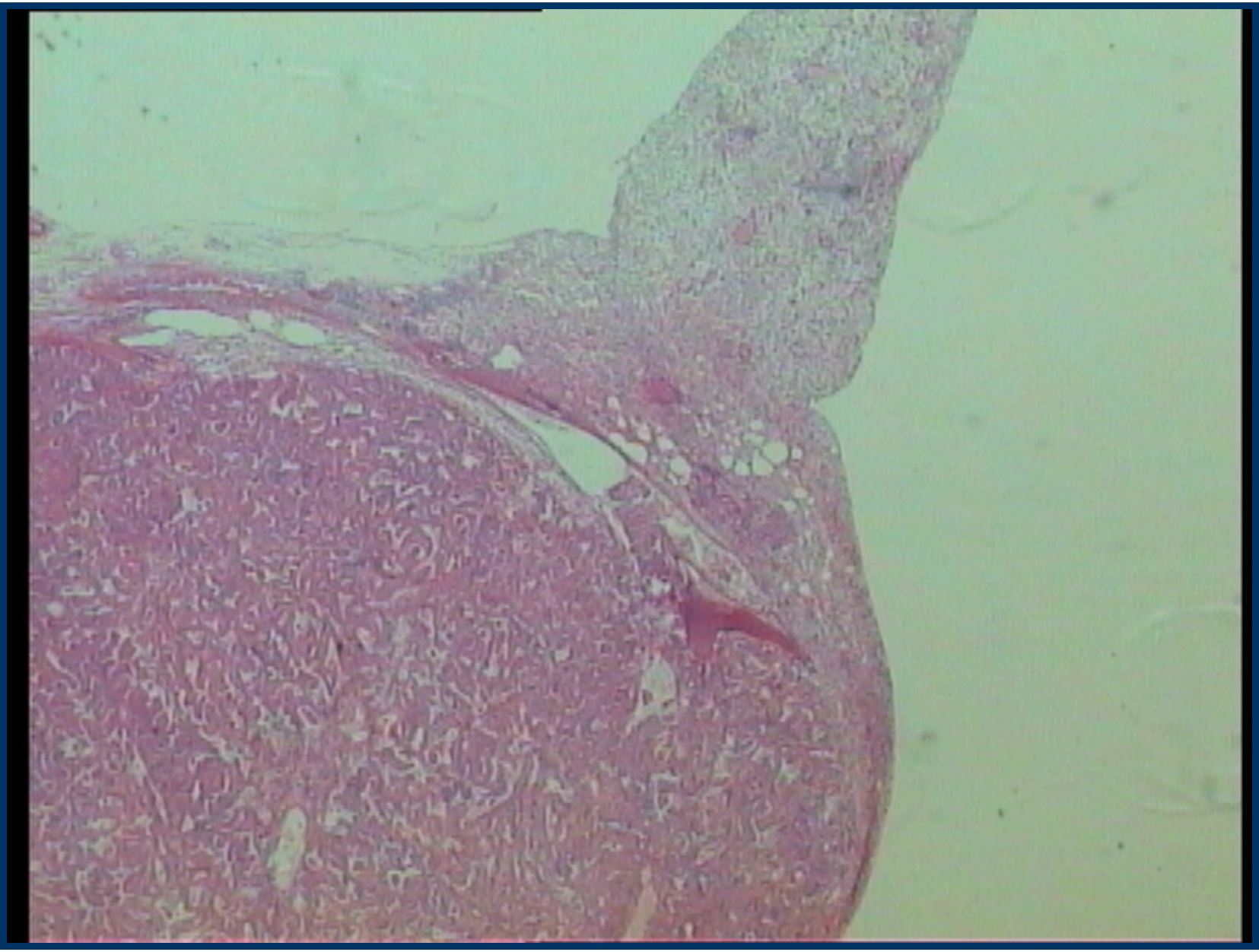


Swiss mice experiment: hepatocellular carcinoma (40X)

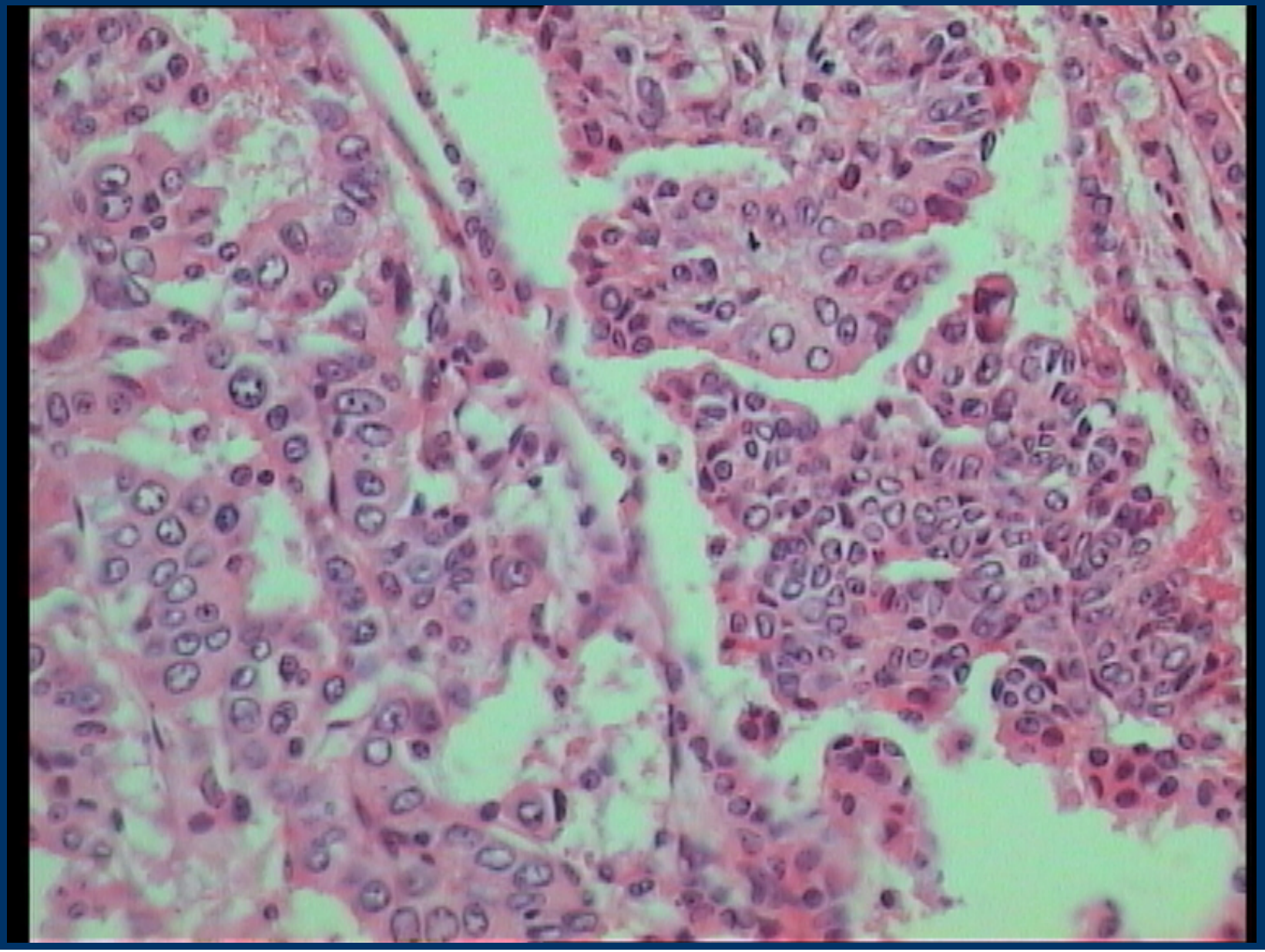


Swiss mice: Lung adenocarcinomas

Swiss mice experiment: lung adenocarcinoma (2.5X)



Swiss mice experiment: lung adenocarcinoma (40X)



Further reactions to the Ramazzini results (1/3)

- More recently, some industry consultants stated:

  “The most frequently reported hematopoietic neoplasm was lympho-immunoblastic lymphoma, the most affected organ was the lung and, in almost half of the rats with diagnosis, the lung was the only affected organ”

Reactions to the Ramazzini results (2/3)

- Industry consultants continued:

  "Because lymphocyte and plasma cell accumulation in the lung is characteristic of *M. pulmonis* disease, and because *M. pulmonis* disease can be exacerbated by experimental manipulations, including chemical treatment, we suggest that a plausible alternative explanation for the reported results of these bioassays is that the study was confounded by *M. pulmonis* disease and that lesions of the disease were interpreted as lymphoma"

Reactions to the Ramazzini results (3/3)

- Industry consultants continued:

  "The weight of available evidence favors the hypothesis that lesions of *Mycoplasma pulmonis* disease were interpreted as lymphomas in APM bioassay "

Ramazzini Institute responses (1/7)

- Infections and lymphoimmunoblastic lymphomas of the lungs +/- other organs

  1. Animals that die naturally are subject to infectious pathologies, both in rodents and in humans
  2. Among the animals bearing lymphoimmunoblastic lymphomas, the diffusion of neoplastic tissue involved sometime the lung and concurrently various other organs (liver, spleen, mediastinal and other lymph nodes)

Ramazzini Institute responses (2/7)

3. Even excluding from the census of the Hematopoietic Neoplasms (HPN) the animals with lung inflammation or lymphoimmunoblastic lymphomas there is a dose-related increased incidence of Lymphomas/leukemias

Ramazzini Institute responses (3/7)

4. Of 49 agents reported by the Ramazzini Institute to be carcinogenic in rats, only 8 induced hematopoietic neoplasms (VCM and benzene were not among them)

Ramazzini Institute responses (4/7)

- Pathology misinterpretation

  From the Pathology Working Group NTP Report, November 30, 2004

  "The diagnosis of lymphoblastic and histocytic neoplasms in the cases reviewed (10) were generally confirmed.

Ramazzini Institute responses (5/7)

- Pathology peer review

1. Because APM is a widely used intense artificial sweetener, peer review of the raw data, in particular peer review pathology, have been requested by several stakeholders as EFSA, FDA, NTP.
2. Ramazzini Institute has been always open to external collaborations, national and international, public and private.

Ramazzini reflections and responses (7/7)

3. As soon we started the first experiment, we contacted ILSI (International Life Science Institute) asking for collaboration and some support. They answered that were not interested, because the safety of APM was very well documented.

4. When we started the following experiments, we asked to EFSA to be involved in our research program on APM and other intense sweeteners. This could accelerate the conclusion of the studies. For their own reasons EFSA declined to accept our proposal.

Concluding remarks on the APM project

Concluding remarks on the APM project (1/3)

In our experimental conditions APM has been shown to induce a significantly increased incidence of malignant tumors in:

- multiple tissues in male and female rats
- multiple tissues in male mice
- an earlier occurrence in treated animals
- an higher incidence and an anticipated onset of cancers when the treatment starts from fetal life

The carcinogenic effects of APM were shown also at dose levels to which humans could be exposed

Concluding remarks on the APM project (2/3)

- Given that APM is completely metabolized in the GIT, it may be concluded that the carcinogenic effects were caused not by itself but rather by its metabolites

- The role of methanol inducing malignant neoplasms is reinforced by the following considerations based on experiments performed in our laboratory under the same experimental conditions:

➢ Methanol, which is metabolized to formaldehyde in humans and rats, induces:

- In female Sprague-Dawley rats a significant increase of hematopoietic neoplasms
- in male mice it cannot be disregarded that the conversion of APM methanol into formaldehyde adducts in liver cells may results in a progressive accumulation of adducts, which could explain the plausibility of the hepatocarcinogenic effects of APM

Concluding remarks on the APM project (3/3)

- MTBE, which is metabolized to methanol, induces, in female Sprague-Dawley rats, a significant increase of HLRN

- Formaldehyde induces, in both male and female Sprague-Dawley rats a significant increase in hematopoietic neoplasms

APM and childhood leukaemias

Childhood leukaemia: incidence and etiology (1/2)

- In USA leukaemia accounts for > 30% of new cancer cases; from 1975 to 2002 incidence rates of leukaemia increased 0.7% per year (total 19%)

  All accounts for ~ 80% leukaemia cases

- Ionizing radiation is the sole environmental risk established to date
- Other potential risk factors include: parental smoking and alcohol consumption, EMF exposure, hydrocarbons, immunity and infection, pesticides basis of our studies

From EHP, January 2010

Childhood leukaemia: incidence and etiology (2/2)

- On the basis of the results of our studies, APM should be added to the list of the potential risk agents of childhood leukaemia
- In particular should be recommended an epidemiological study among children

Final general comments and recommendation 1/2

- Absolute certainty in science is rarely an option; uncertainty is the norm, not the exception and the scientists base their judgement on the weight of evidence
- Uncertainty does not mean the science is flawed
- Disagreement does not mean that one of the parties is wrong or practicing junk science
- Checklists of criteria, while appealing in their convenience, are inadequate tools for assessing many scientific issues, certainly including causation

From: David Michaels, Doubt is their product

Final general comments and recommendation 2/2

On the basis of the weight of evidence of our results, APM should be considered a multiple site, trans-species carcinogenic agent in rodents.

A re-avaluation of the current regulations on APM remains, in our opinion, urgent.

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