Synthetic Amorphous Silica – No Adverse Effects in Lung, Liver and Intestine

Klaus Weber[1], Axel Bosch[2]; Chirukandath Gopinath[3]; Jerry Hardisty[4]; Nils Krueger[5]; Ernest McConnell[6]; Günther Oberdörster[7], Tobias Schuster[5]

[1]AnaPath GmbH, Oberbuchsiten, Switzerland

[2] Wacker Chemie AG, Burghausen, Germany

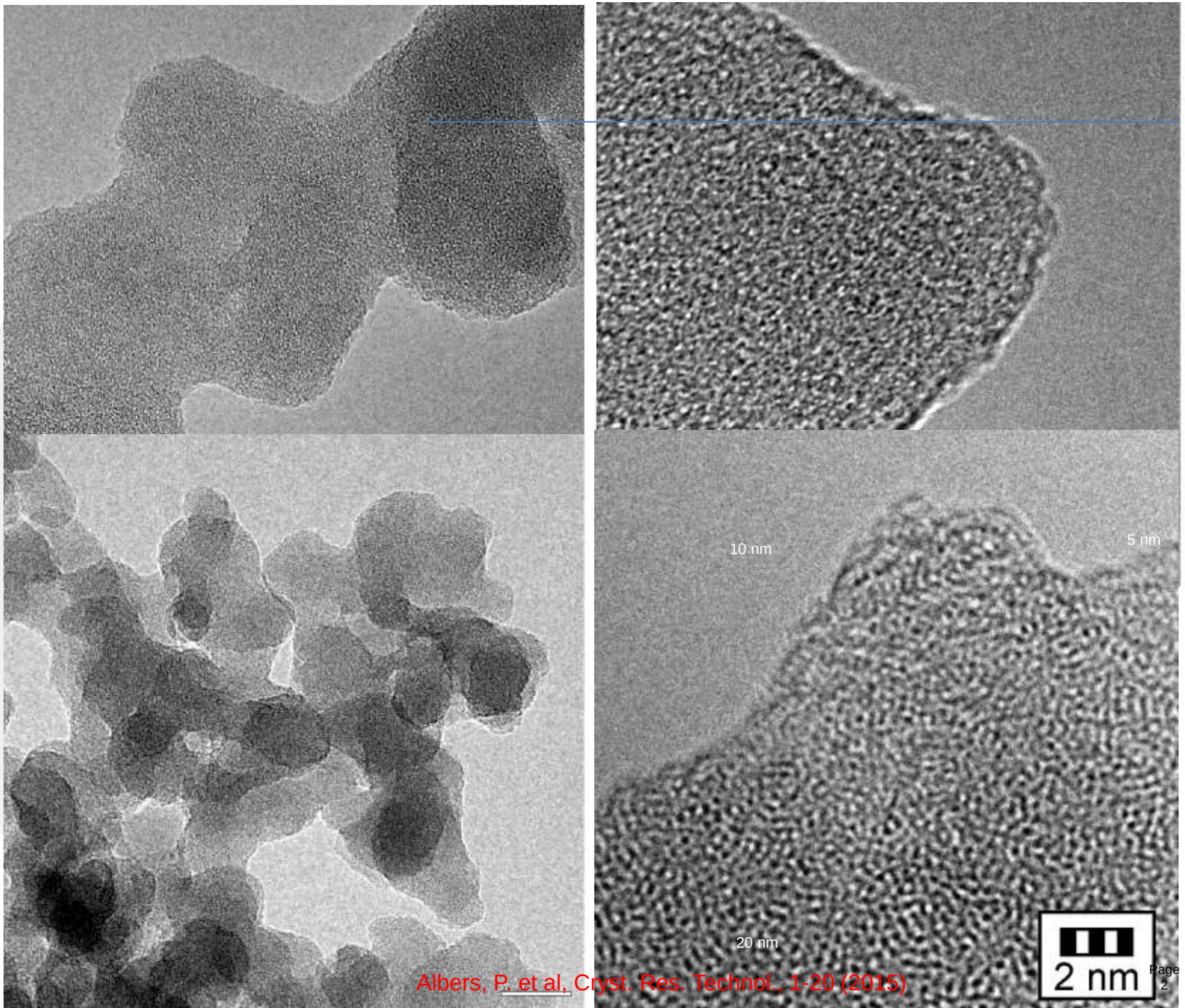
[3] Independent Consultant, Peterborough, UK

[4] EPL Inc., Research Triangle Park, NC, USA

[5]Evonik Resource Efficiency GmbH, Hanau-Wolfgang, Germany

[6] ToxPath, Inc. Raleigh, NC, USA

[7] University of Rochester, Rochester, NY, USA



Albers, P. et al, Cryst. Res. Technol., 1-20 (2015)

Is there currently a hype? Example: TiO_2

- **Human body contains approx. 15 mg Ti principally in the lungs. Urine levels are normally below the detection limit of 1.5 ug/l**
  (Whitehead J; pp. 1261-7 in Metals and their Compounds in the Environment., Merian E, ed. Weinheim, Germany: VCH (1991))
- **Liver and spleen level? References?**
- **15 subjects: '…All …the Netherlands their entire life …followed a Dutch diet…' What is this???**
- **'…TiO_2 particles are present in human liver and spleen, with ≥24% of nanosize (< 100 nm). The levels are below the doses regarded as safe in animals, but half are above the dose that is deemed safe for liver damage in humans when taking into account several commonly applied uncertainty factors. …'**

Hype? Example.

- TiO_2: probably the most common accessory mineral in magmatites, and hence in sediments
- Two human subjects .. Titanium implant, the total-Ti content … comparable to those observed in the liver and spleen in other subjects.
- Conclusion:

  '…With these new and unique human data, we remain with the conclusion that health risks due to oral exposure to TiO_2 cannot be excluded….'

Heringa MB, Peters RJB, Bleys RLAW, van der Lee MK, Tromp PC, van Kesteren PCE, van Eijkeren JCH, Undas AK, Oomen AG, Bouwmeester H. Detection of titanium particles in human liver and spleen and possible health implications. Part Fibre Toxicol. 2018 Apr 11;15(1):15.

Synthetic Amorphous Silica (SAS): Toxic or Not? Weight of Evidence.

- **Overview of toxicology data: for SAS no hazard has been identified**
  - **Skin irritation**
  - **Eye irritation**
  - **Skin sensitisation**
  - **Sub-chronic/chronic toxicity (oral)**
  - **Mutagenicity / Genotoxicity**
  - **Reproductive / developmental toxicity**
  - **Carcinogenicity (oral)**

Inhalation Toxicity Data

- **Long history of inhalation toxicity testing on various SAS types since 1950s (mostly available in open literature - Klosterkötter et al., ECETOC JACC 51, others)**
- **Subacute/subchronic/chronic studies (see next slide)**
- **Specific morphology of SAS (primary particle, aggregate, agglomerate) known and considered by the different study authors**
- **Toxicokinetics measurements (Si-contents)**

Tested SAS - Inhalation Toxicity (Repeated Dose)

SAS TYPE	STUDIES
Pyrogenic	5d 6w 90d 90d 6w, 18w, 12m
Precipitated	3m, 6m, 12m, 13m, 18m 3d 5d 2w 90d
Gel	2w 90d 3m, 6m, 12m, 13m, 18m
Colloidal	5d 3m, 6m, 12m, 13m, 18m 2w, 4w 4w

All Negative, except:

Reuzel PG, Bruijntjes JP, Feron VJ, Woutersen RA.

‘Subchronic inhalation toxicity of amorphous silicas and quartz dust in rats.’

Food Chem Toxicol. 1991 May;29(5):341-54.

Inhalation Study Design: Reuzel et al., 1991

The treatment schedule was as follows:

- **Group A: Sham**
- **Group B: AEROSIL® 200, low dose (1.3 mg/m^3)**
- **Group C: AEROSIL® 200, mid dose (5.9 mg/m^3)**
- **Group D: AEROSIL® 200,high dose (31 mg/m^3))**
- **Group E: AEROSIL® R 974, high dose (34,7 mg/m^3))**
- **Group F: SIPERNAT® 22S, high dose (34.9 mg/m^3))**
- **Group G: Quartz (58.5 mg/m^3).**

- **AEROSIL® 200: pyrogenic SAS**
- **AEROSIL® R 974: surface treated pyrogenic SAS**
- **SIPERNAT® 22S: precipitated SAS**

Inhalation Study Design: Reuzel et al., 1991

In this study, there were several scheduled sacrifices, including:

- **After 13 weeks (end of treatment)**
- **13 week recovery**
- **26 week recovery**
- **39 week recovery**
- **52 week recovery**

Peer Review and Pathology Working Group

The treatment schedule was as follows:

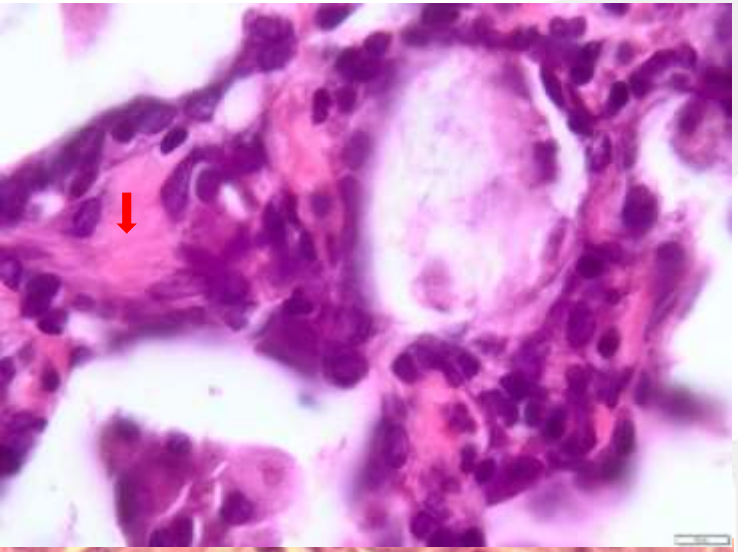
- **Group A: Sham**
- **Group B: AEROSIL® 200, low dose (1.3 mg/m3)**
- **Group C: AEROSIL®, mid dose (5.9 mg/m3)**
- **Group D: AEROSIL® 200,high dose (31 mg/m3)**
- **Group E: AEROSIL® R 974, high dose (34,7 mg/m3)**
- **Group F: SIPERNAT® 22S, high dose (34.9 mg/m3)**
- **Group G: Quartz (58.5 mg/m3).**

In this study, there were several scheduled sacrifices, including:

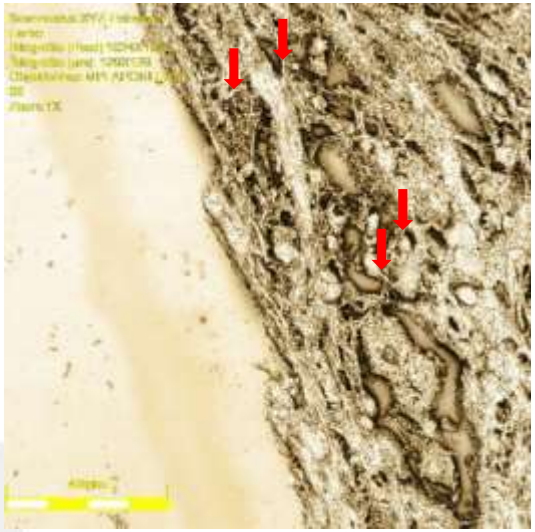
- **After 13 weeks (end of treatment) (males only*)**
- **13 week recovery***
- **26 week recovery**
- **39 week recovery**
- **52 week recovery***

*available for re-evaluation

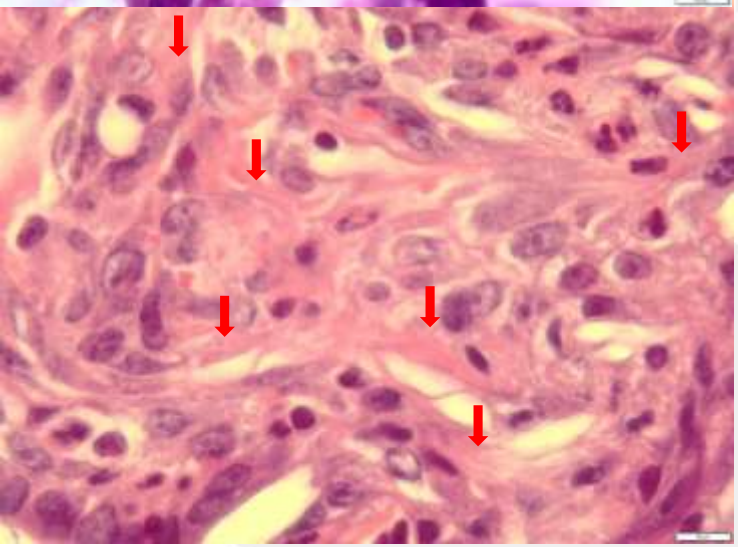
Fibrogenesis vs Fibrosis



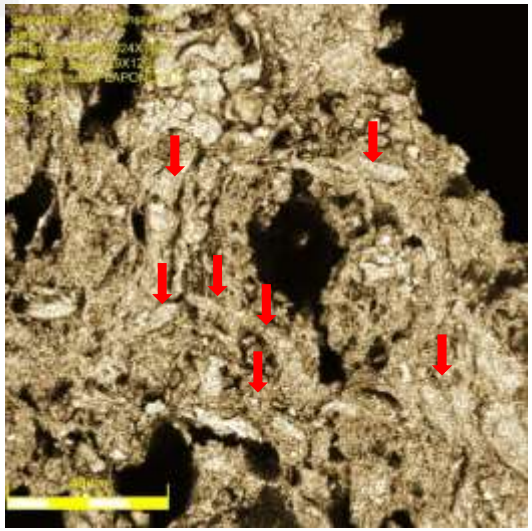
Fibrogenesis (fibroblastic response) - potentially reversible fibroblast proliferation with minimal cross-linking



Laserscan (LEXT OLS4000) at x 2160
Single fibers.



Fibrosis – irreversible crosslinking of collagen fibers. Note thick collagen bands.



Laserscan (LEXT OLS4000) at x 2160
Bands of collagen Fibers.

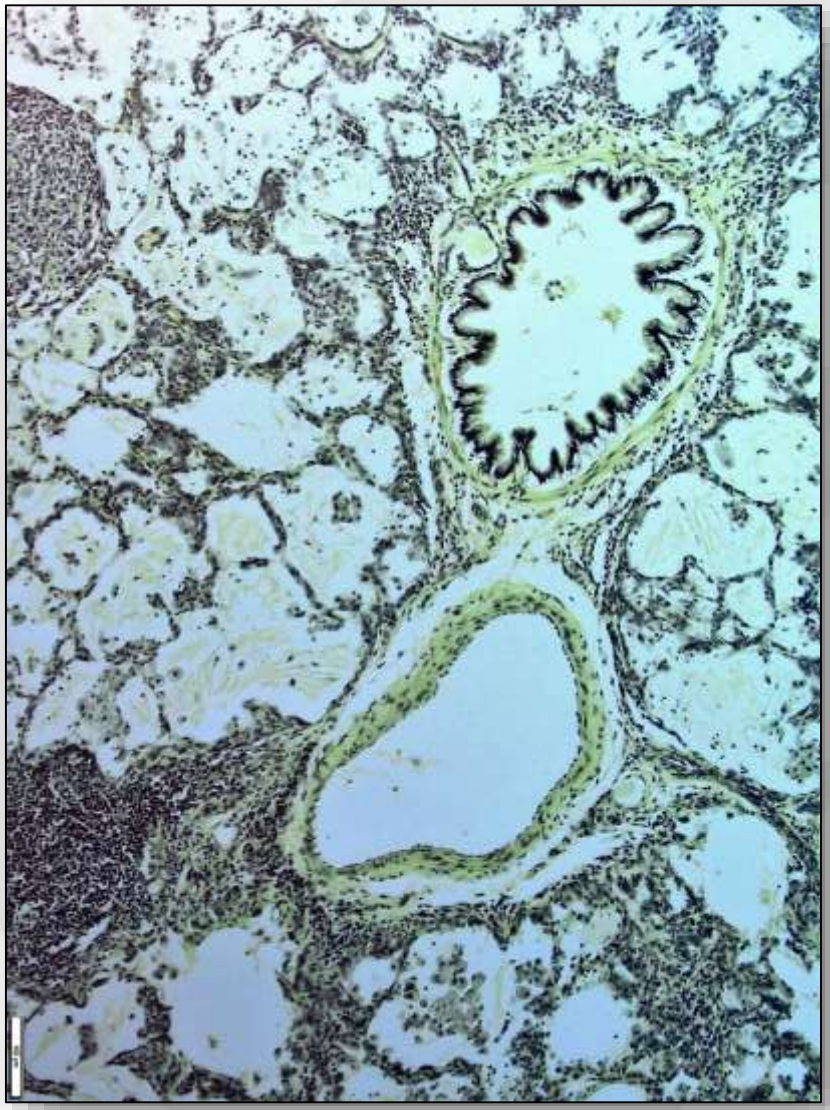
And a Special Stain?

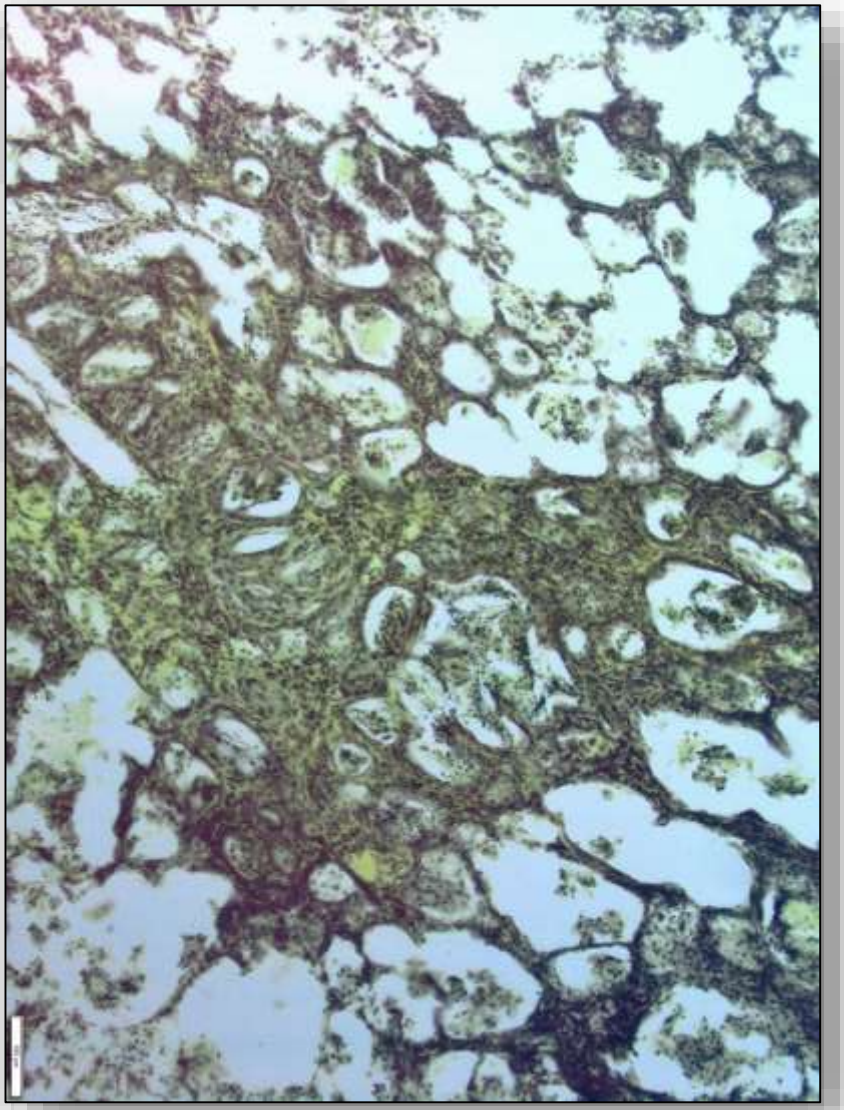
- **Weigert van Gieson was applied:**
- **'…interstitial fibrosis, seen as an amorphous eosinophilic thickening if the septa, which appeared to be positive for collagen (Weigert van Gieson) stain…..**

- **Weigert's elastic stain: combination of basic fuchsin and hematoxylin (cell nuclei will be counterstained)**
- **Van Gieson stain: mixture of picric acid and acid fuchsin as simple method for differential staining of collagen and other connective tissue.**

- **However, Elastic fibers are present in every alveolar wall!**

And the fibers? Examples: Quartz Group

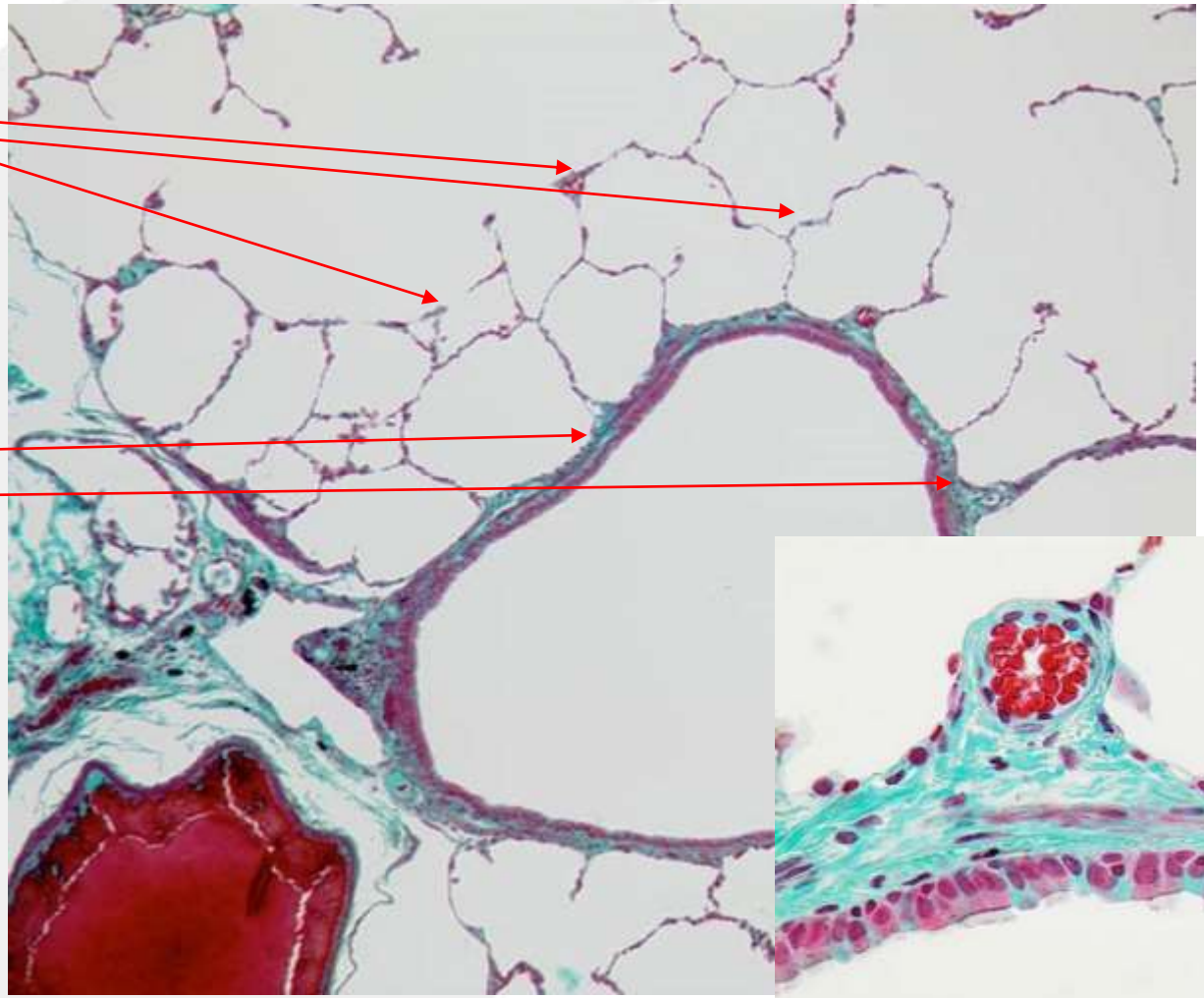




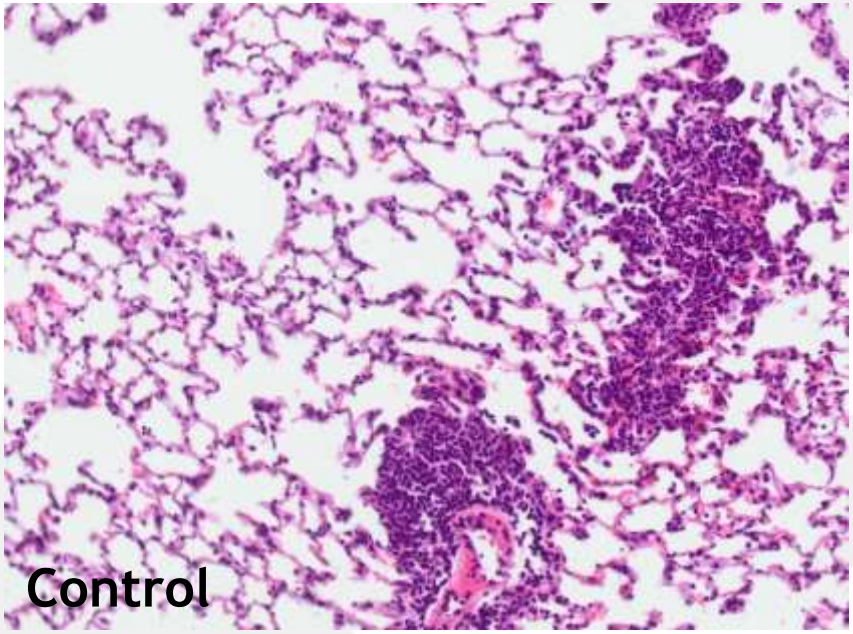
Normal Histology

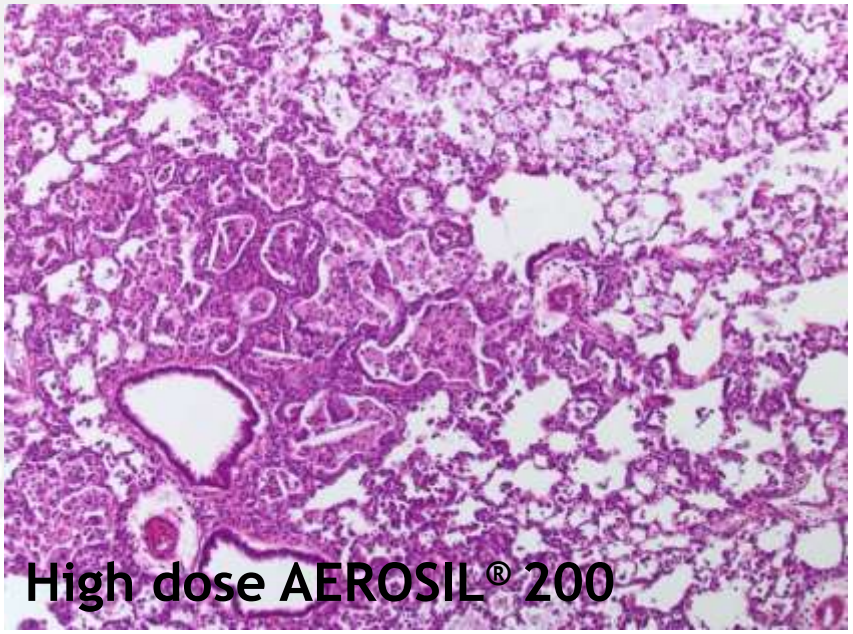
Normal collagen in blood vessels crossing alveolar walls

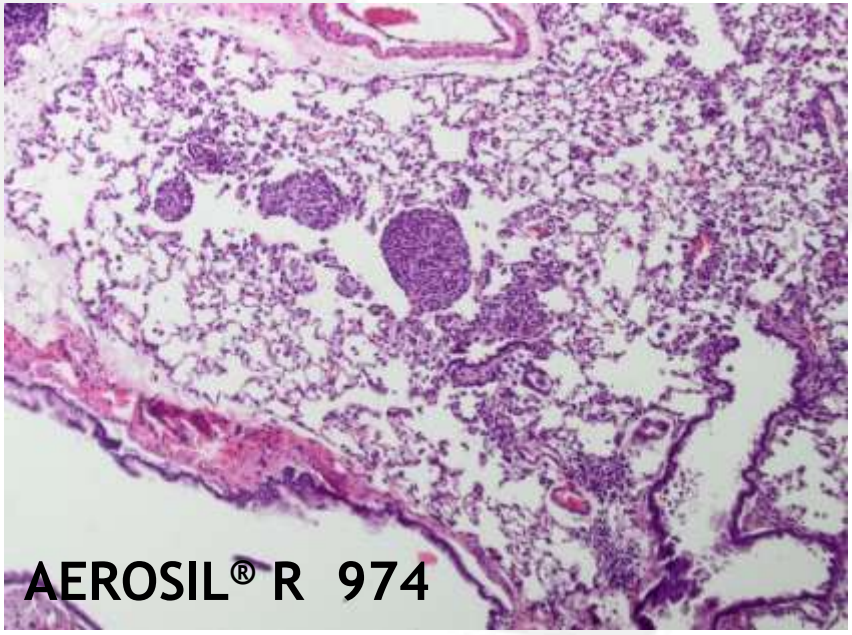
Normal collagen in blood vessels for archiec-tureal support of bronchioli

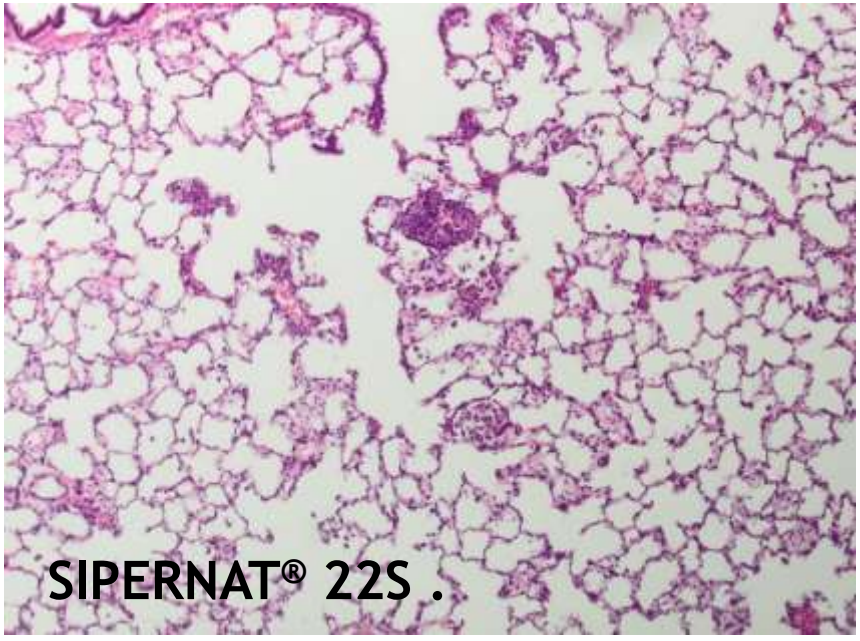


End of Treatment

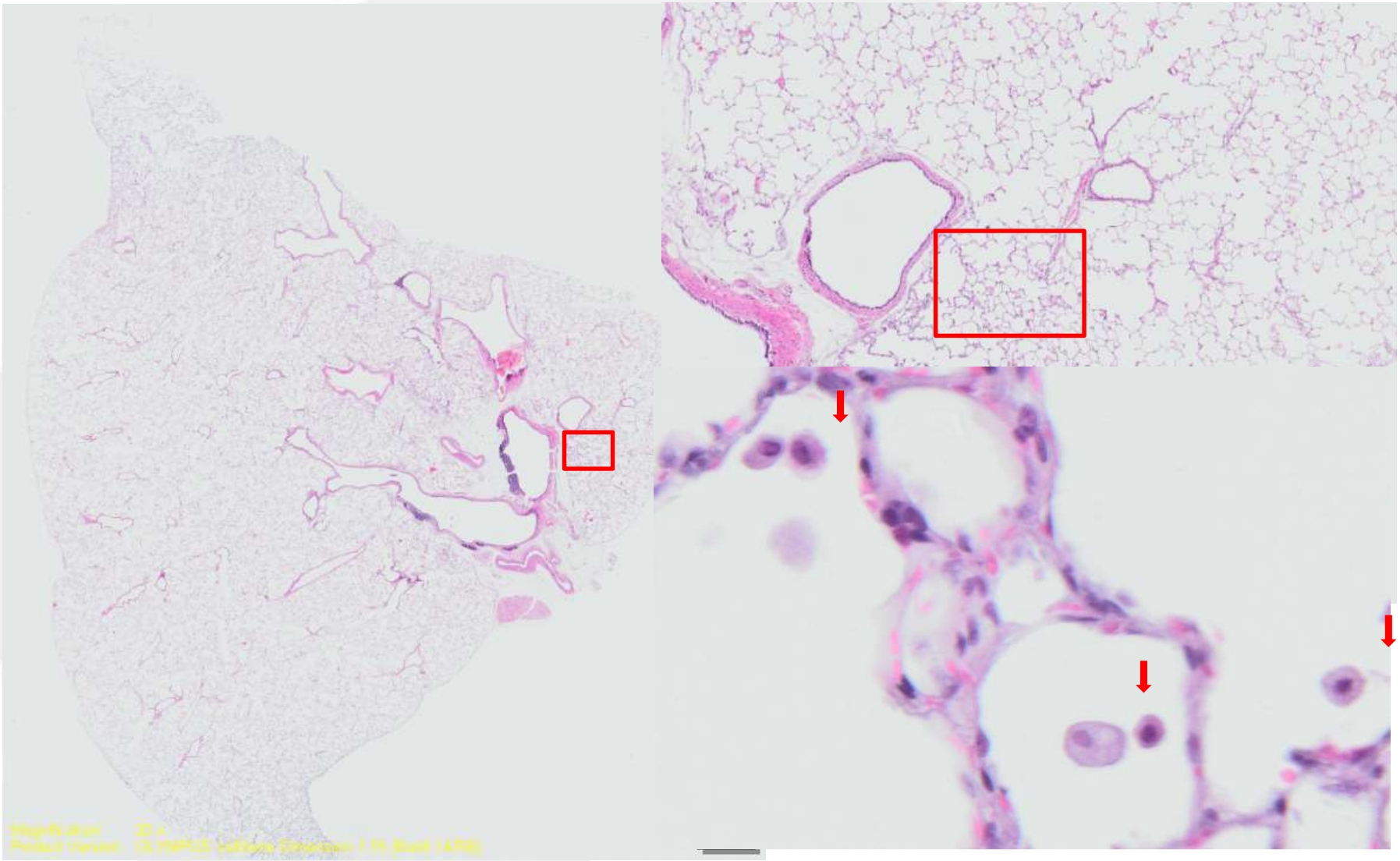






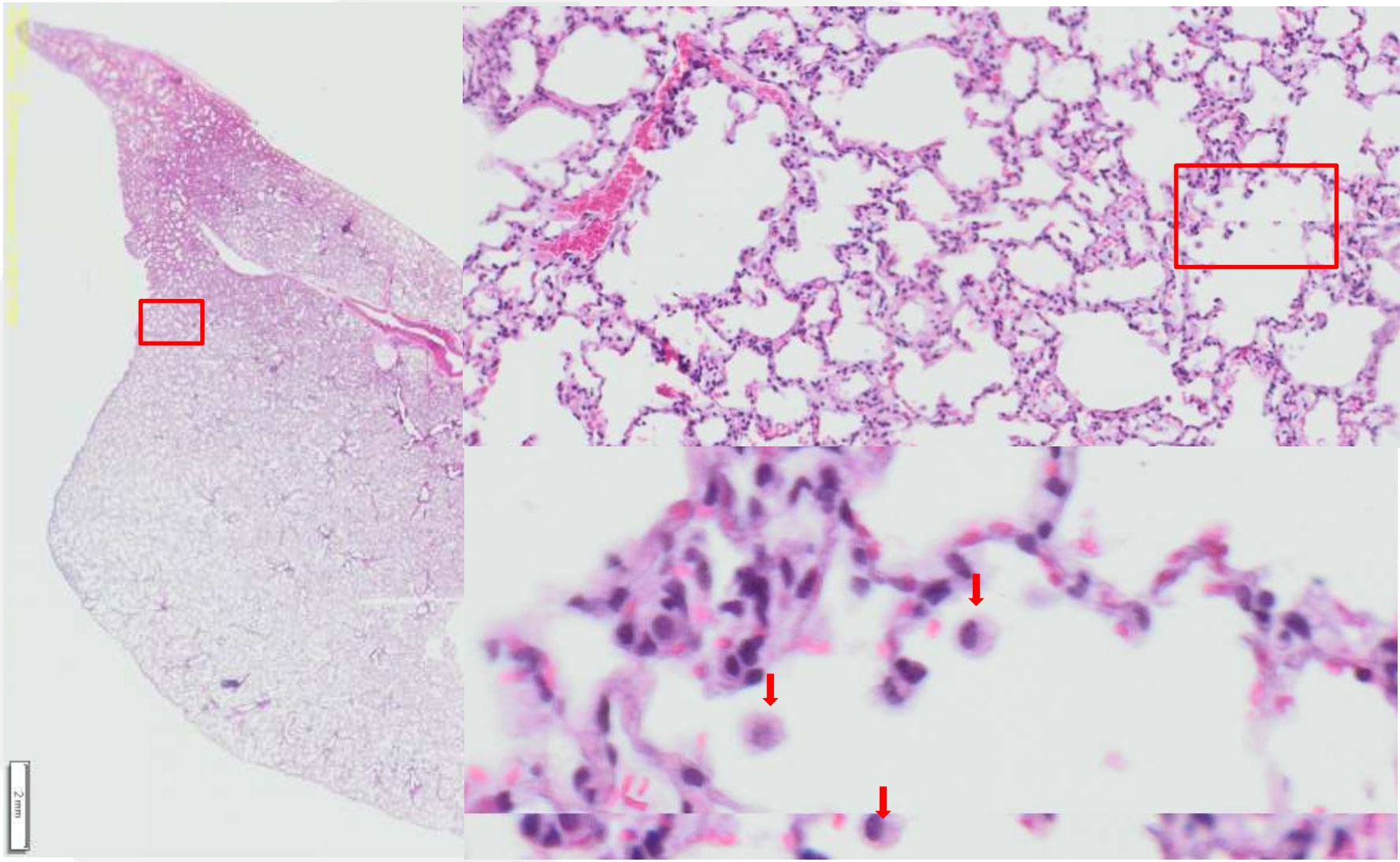


52-Week Recovery. Control



52-week recovery. Control A101. Normal lung. Focally single reactive alveolar macrophages.

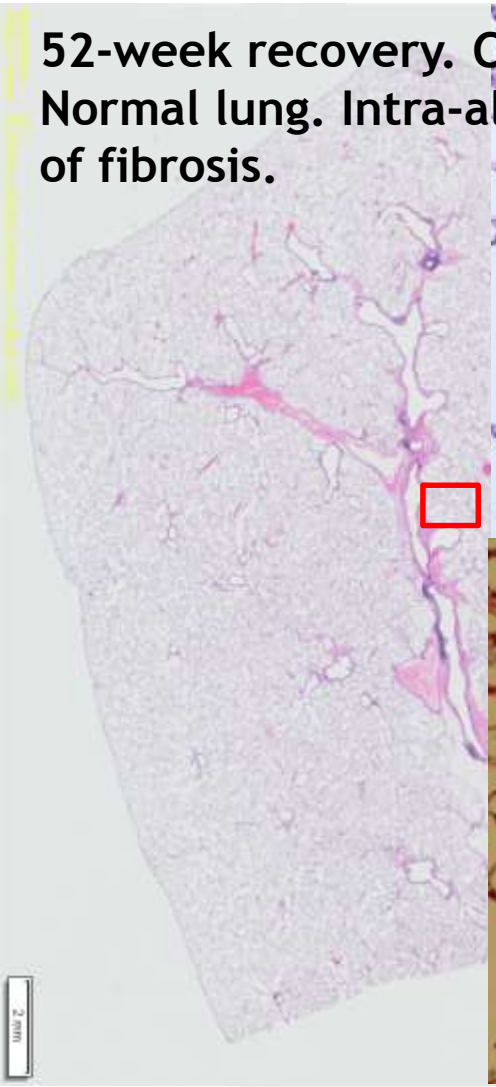
52-Week Recovery. AEROSIL® 200, high dose

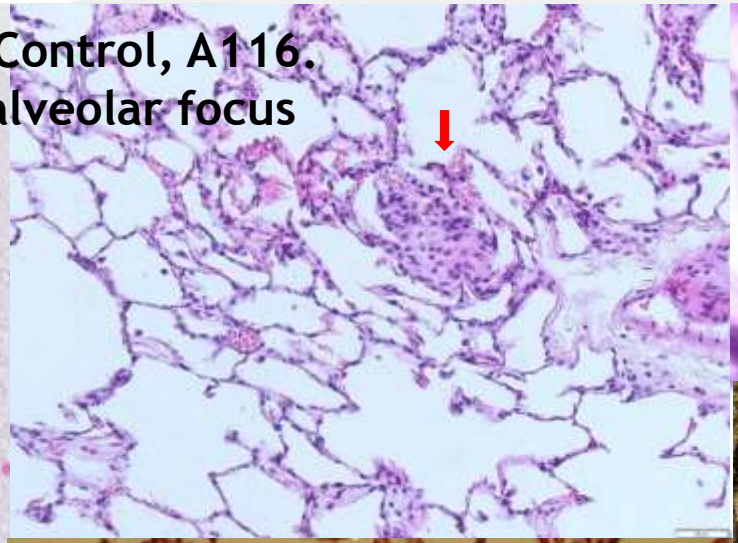


52-week recovery. Aerosil 200,high dose, D132. Normal lung. Focally single reactive alveolar macrophages.

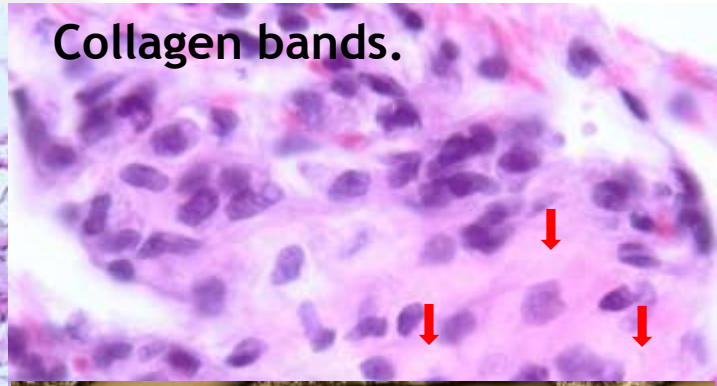
52-Week Recovery. Control

52-week recovery. Control, A116.
Normal lung. Intra-alveolar focus of fibrosis.

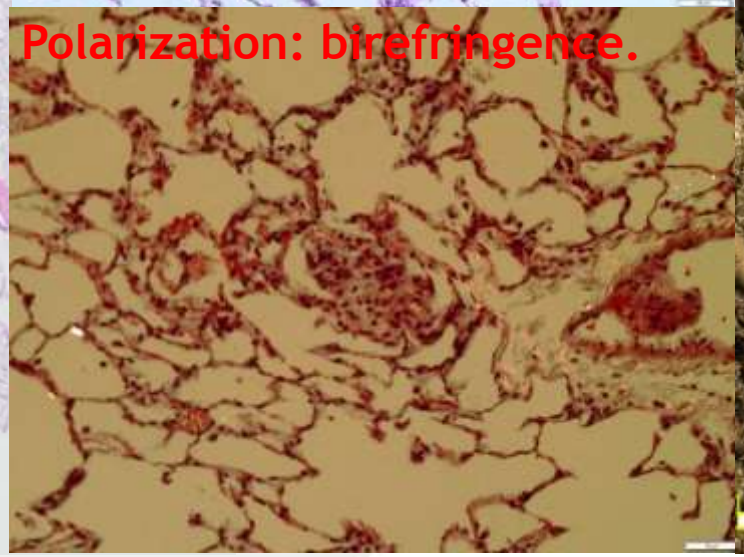




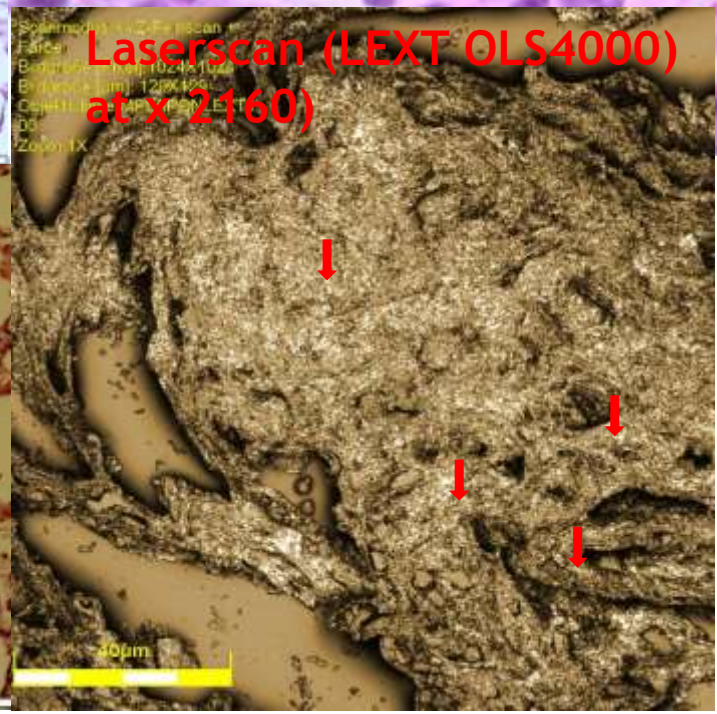
Collagen bands.



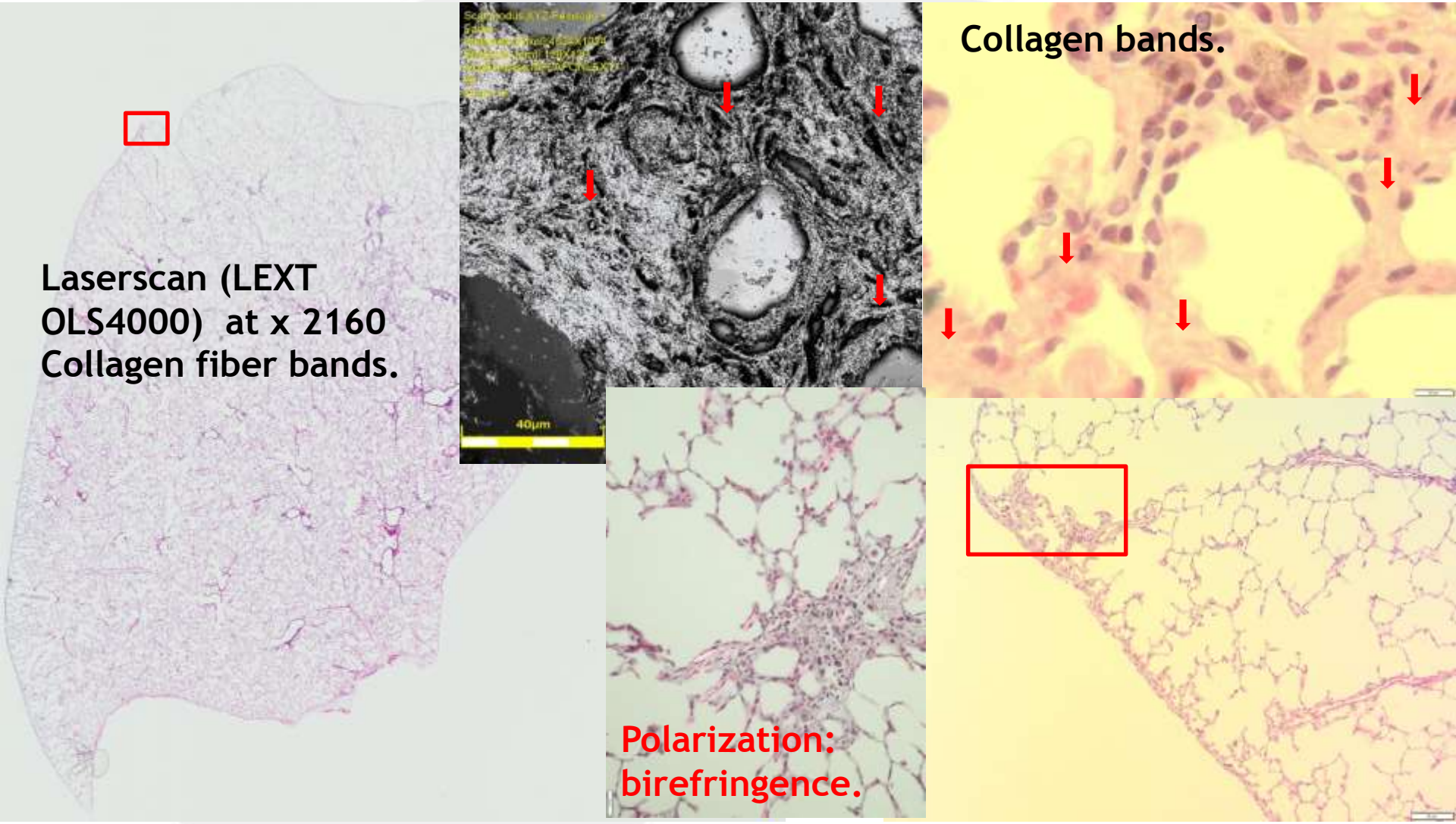
Polarization: birefringence.



Laserscan (LEXT OLS4000) at x 2160)



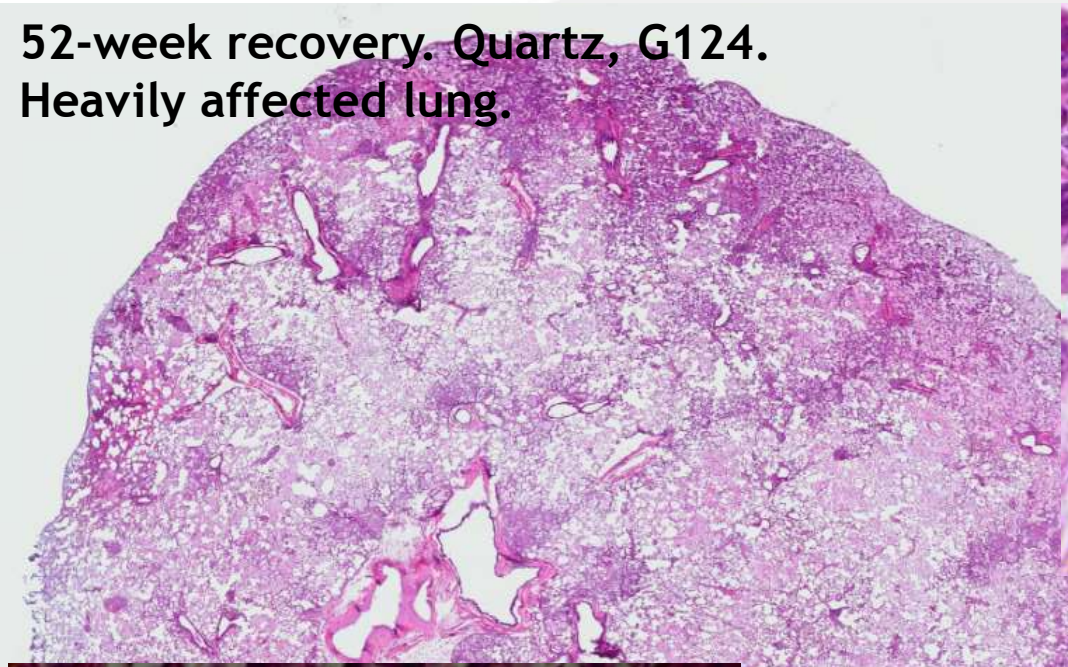
52-Week Recovery. AEROSIL® 200, high dose



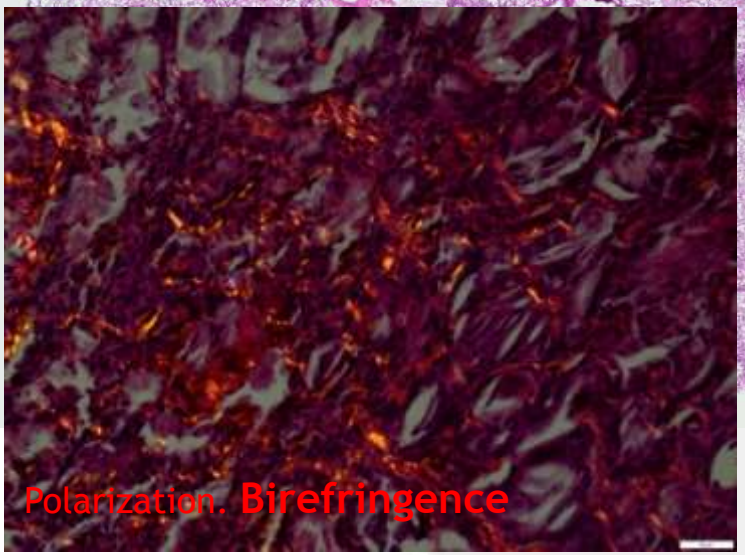
52-week recovery. AEROSIL® 200,high dose, D113. Normal lung. Subpleural focus of fibrosis.

52-Week Recovery. Quartz

52-week recovery. Quartz, G124.
Heavily affected lung.

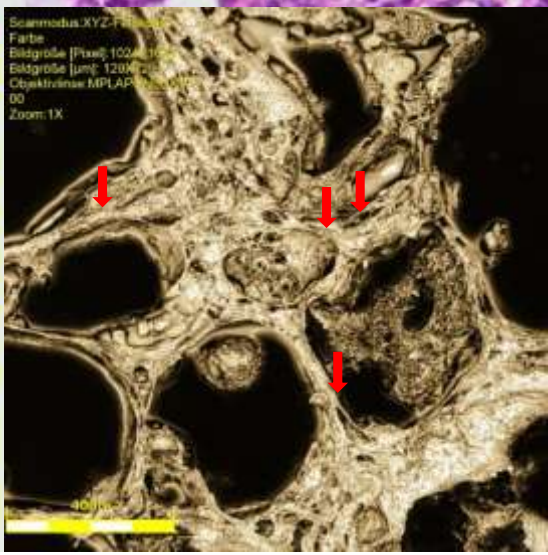


Inflammation, crystal clefts, collagen bands (arrows).



Polarization. Birefringence

Laserscan (LEXT OLS4000)
at x 2160
Bands of collagen fibers



After the PR and PWG: 52-Week Recovery

	Original		PR/PWG	
Group	Fibrogen.	Fibrosis	Fibrogen.	Fibrosis
Sham	0	0	0	1
AEROSIL® 200 Low	0	1	0	0
AEROSIL® 200 Mid	0	3	1	1
AEROSIL® 200 High	0	20	3	3
AEROSIL® R 974	0	0	0	0
SIPERNAT® 22S	0	0	0	0
Quartz	0	20	0	20

- Limited inflammatory changes, i.e., findings that are not normal in the lungs of aged animals
- All considered to be within the range of spontaneous background lesions (macrophage accumulation, macrophage type II hyperplasia, cleft granulomas)

Example for Age-Related Increase of Lung Fibrosis RccHan™: WIST dose

28-Day Studies: Male	Total n	Total %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	1979					
Interstitial fibrosis	1	0.05	0.06	1.13	0.00	20.00
28-Day Studies: Female	Total n	Total %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	2008					
Interstitial fibrosis	1	0.05	0.06	1.14	0.00	20.00
26-Week Studies: Male	Total n	Total %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	425					
Fibrosis	4	0.94	0.53	1.58	0.00	5.00
26 Week Studies: Female	Total n	Total %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	389					
Fibrosis	1	0.26	0.14	0.59	0.00	2.50
78-Week Studies: Males	Total n	Total n %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	438					
Fibrosis	16	3.65	3.80	6.63	0.00	20.00
78-Week Studies Females	Total n	Total n %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	438					
Fibrosis	7	1.60	0.96	2.15	0.00	7.69
104-Week Studies: Males	Total n	Total n %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	3865					
Fibrosis	128	3.31	3.23	7.87	0.00	33.00
104-Week Studies: Females	Total n	Total n %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	3806					
Fibrosis	137	3.60	3.49	9.09	0.00	40.00

http://www.envigo.com/products-services/research-models-services/models/rcchanwist-background-data/

Re-Evaluation and PWG: Results

- No `lung overload´ phenomenon (irreversible chronic persistent inflammation)
- No substance induced fibrosis
- SAS types show comparable effects – similar behaviour in the lung
- In association with inflammation `Fibrogenesis´ (reversible effect in all SAS dose groups)
- Expected minimal age related fibrosis (not substance related) was observed at the end of recovery

Re-Evaluation and PWG: Results

Overall conclusion:

- **Comparable toxicity of all tested SAS types, reversibility of effects, no substance induced fibrosis**

Toxicology Research and Application. In publication.

Weber K, Bosch A, Bühler M, Gopinath C, Hardisty JF, Krueger N, McConnell EE, Oberdörster G.

Aerosols of synthetic amorphous silica do not induce fibrosis in lungs after inhalation - Pathology working group review of histopathological specimens from a subchronic 13-Week inhalation toxicity study in Rats.

And, liver and intestine:

Morfeld P, Bosch A, Weber K, Heinemann M, Krueger N.:

Synthetic amorphous silica in food: Findings about "liver fibrosis" and other study-related findings in van der Zande et al. (2014) are questionable.

EC Pharmacology and Toxicology 3(2): 49-61 (2017).

Study Outcome (Zande et al., 2014)

- two SASs (identifiers: “SAS” and “NM-202”) were administered to male Sprague-Dawley rats via food for 29 days
- additional administration of the high dose groups up to 84 days
- Group size: 5 animals per sex

Conclusion:

- the study “…showed an increased incidence of liver fibrosis after 84-days of exposure…”
  and
  “…increased height of jejunal villi…”

van der Zande M, Vandebriel R, Groot M, Kramer E, Herrera Rivera Z, Rasmussen K, Ossenkoppele J, Tromp P, Gremmer E, Peters R, Hendriksen P, Marvin H, Hoogenboom R, Peijnenburg A and Bouwmeester H. Sub-chronic toxicity study in rats orally exposed to nanostructured silica. Particle and Fibre Toxicology. 11: 8. 2014.

Endpoint liver fibrosis

- **Liver fibrosis is defined by the presence of connective tissue in the liver (above the normal low rate seen in portal areas) as a reaction to acute or prolonged toxicity.**
- **The recent INHAND publication did not discuss gradings with the exception of cirrhotic changes representing a severe degree.**
- **The method section of the publication by van der Zande et al. does not provide a reference or standard for the definition of the 6 fibrosis severity categories that have been applied by the authors**

- **distinction between adjacent categories, e.g., "not remarkable" (0) and "very mild" (1) or "very mild" (1) and mild" (2) remain unclear**

Endpoint Liver Fibrosis: Proposal

- **The STP Best Practice Paper on pathology report writing recommends: "…When severity grading is important to the understanding of major study findings, it may be useful to provide a description of the distinguishing features of each severity grade…"**

- **This is even more important, when subtle changes are the focus of the investigation as in van der Zande et al., where the main findings about fibrosis were described as 'very slight'**

Proposal: Gradings

- Grade 0: no finding present
- Grade 1: doubling of the normal structures within n evaluated periportal fields in
- Grade 2: increased from double of normal up to 3 times of normal in more than two from all evaluated areas (to avoid counting focal changes of peribiliar fibrosis which should be considered as background lesions and not as a result representing exposure effects)
- Grade 3: in addition, a fine connective tissue bridging between 2 or 3 lobules in the periportal region
- Grade 4: bridging between 4 to 6 lobules in the periportal region
- Grade 5: larger bands of connective tissues bridging diffusely the liver lobules.

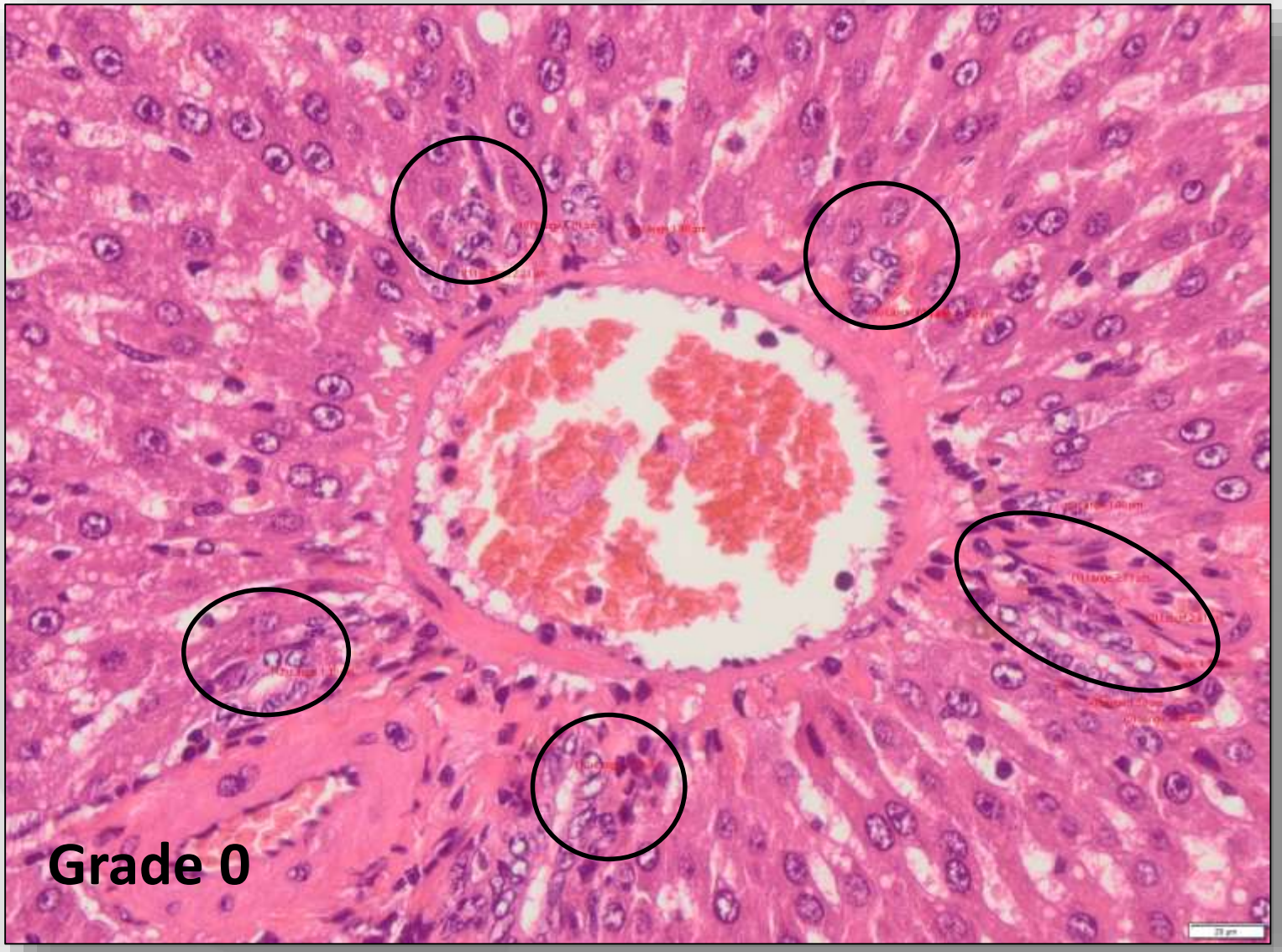
Proposal: by Measurement

- **Measuring the normal existing fibrous tissue and application of numerical gradings (Control Animals): Example: taken by normal background lesions in animals from 13- and 26-week studies (Sprague-Dawley rats)**

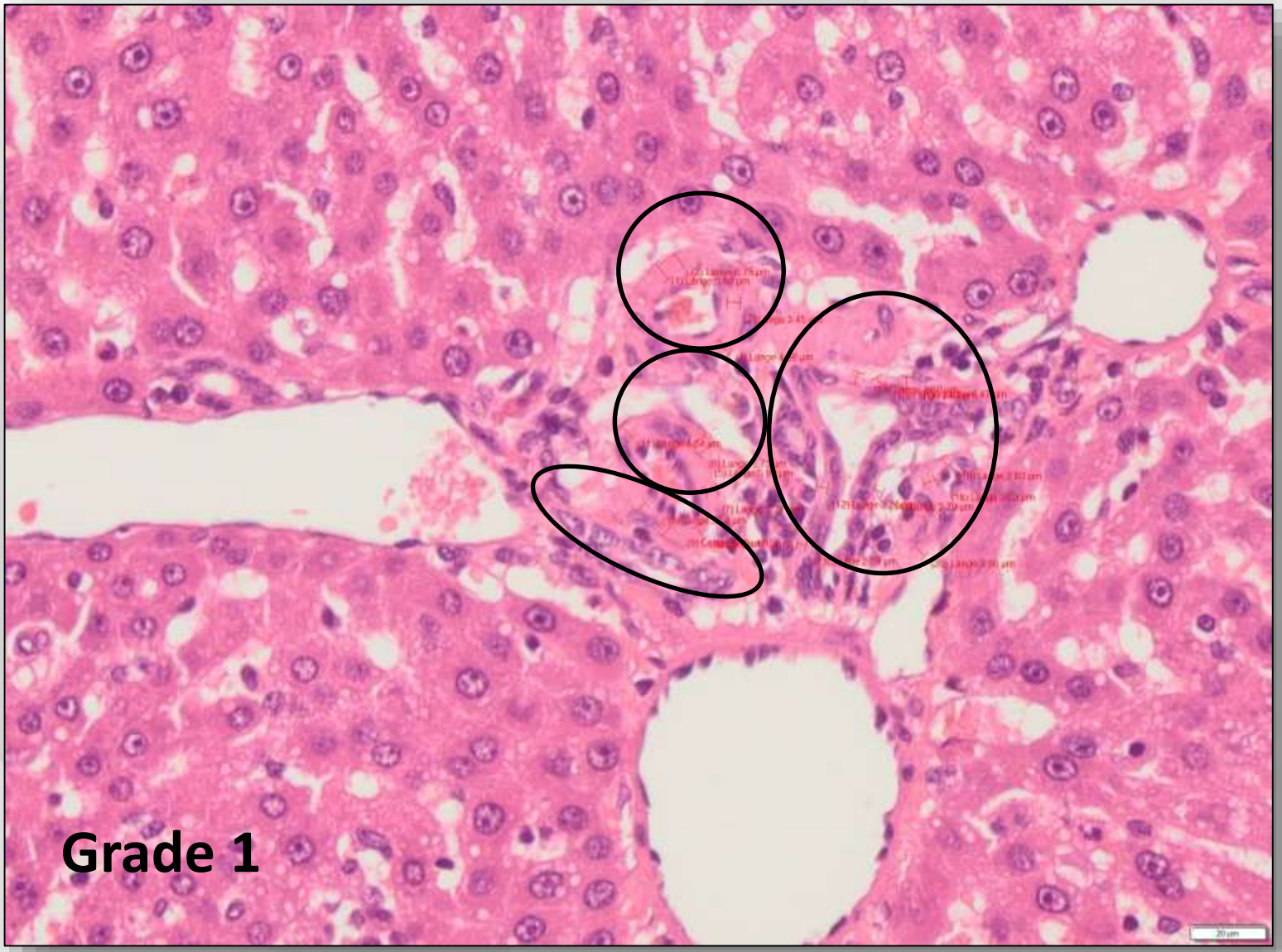
Number of measurements	20	20	20	20	20	20
Minimum	1.006	2.593	2.588	6.361	8.142	3.282
Maximum	4.023	6.747	9.971	22.614	35.595	52.554
Mean	1.979	4.058	5.735	14.809	17.576	20.289

- **Distribution for Gradings by means:**
  0 = up to 2 µm, 1= up to 4 µm, 2= up to 10 µm,
  3 = up to 15 µm, 4 = up to 20 µm, 5 = > 20 µm

Proposal: Gradings, Example by Measurement:
Control Animal

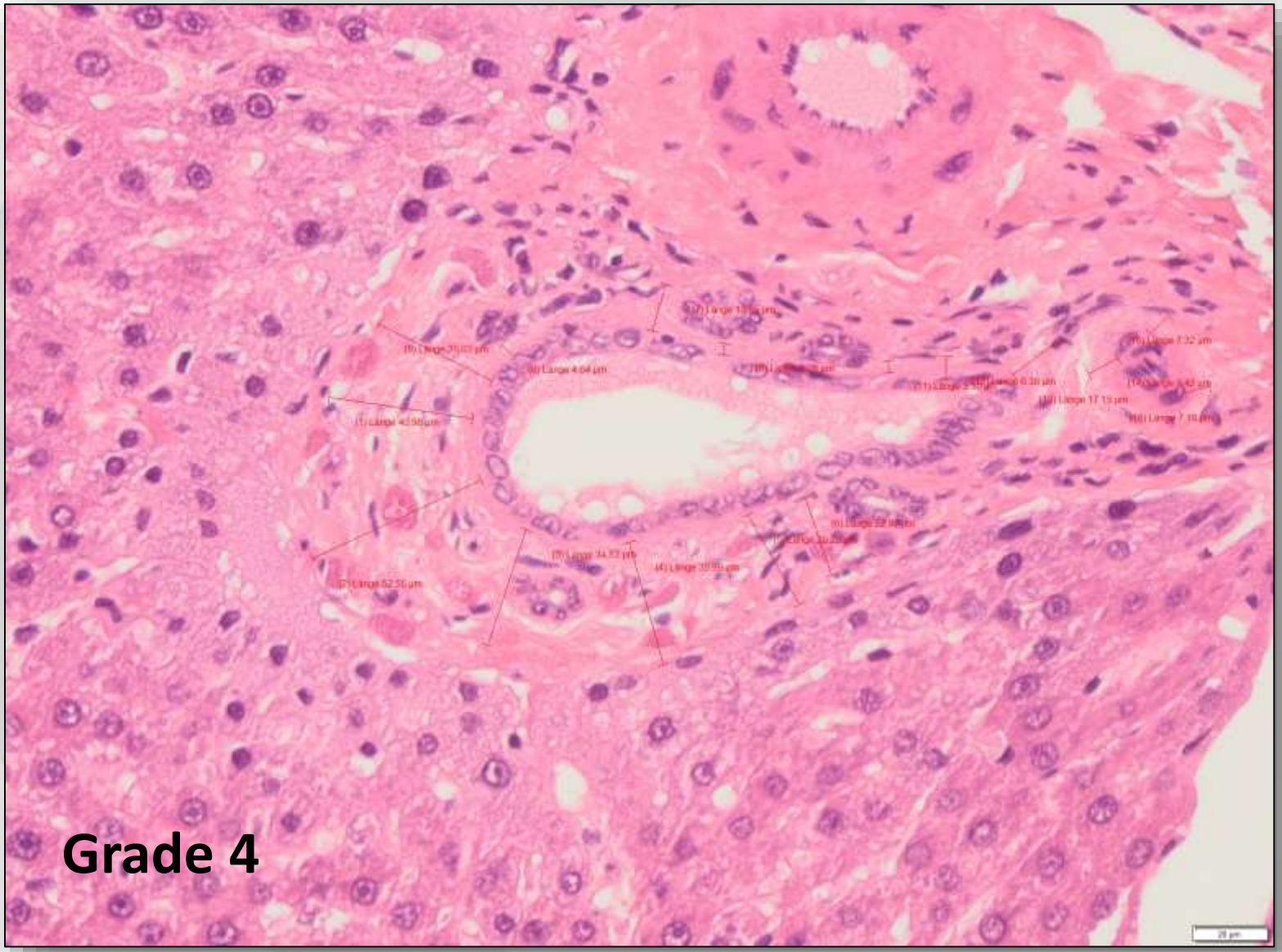


Proposal: Gradings, Example by Measurement:
Control Animal

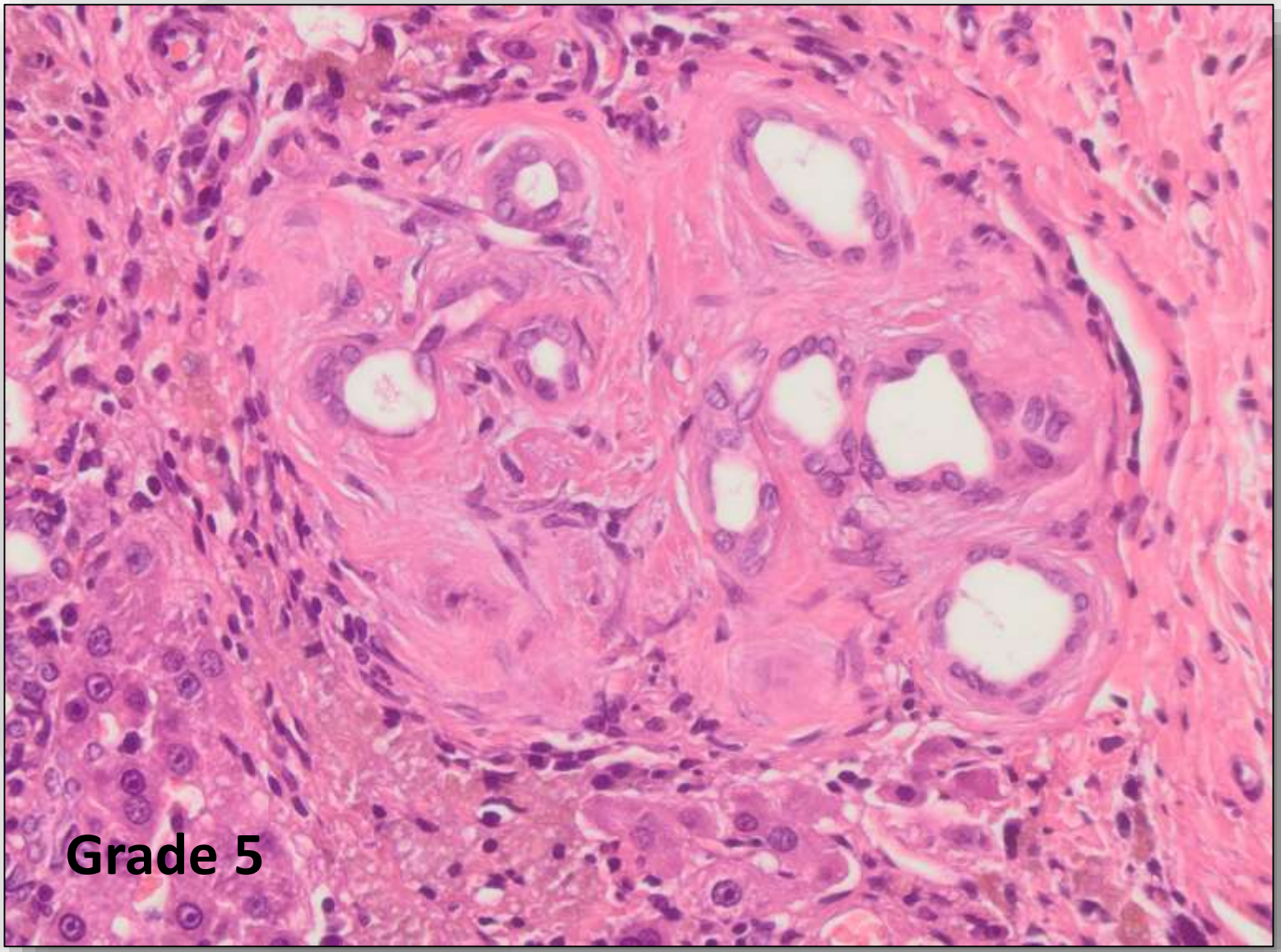


Proposal: Gradings, Example by Measurement:

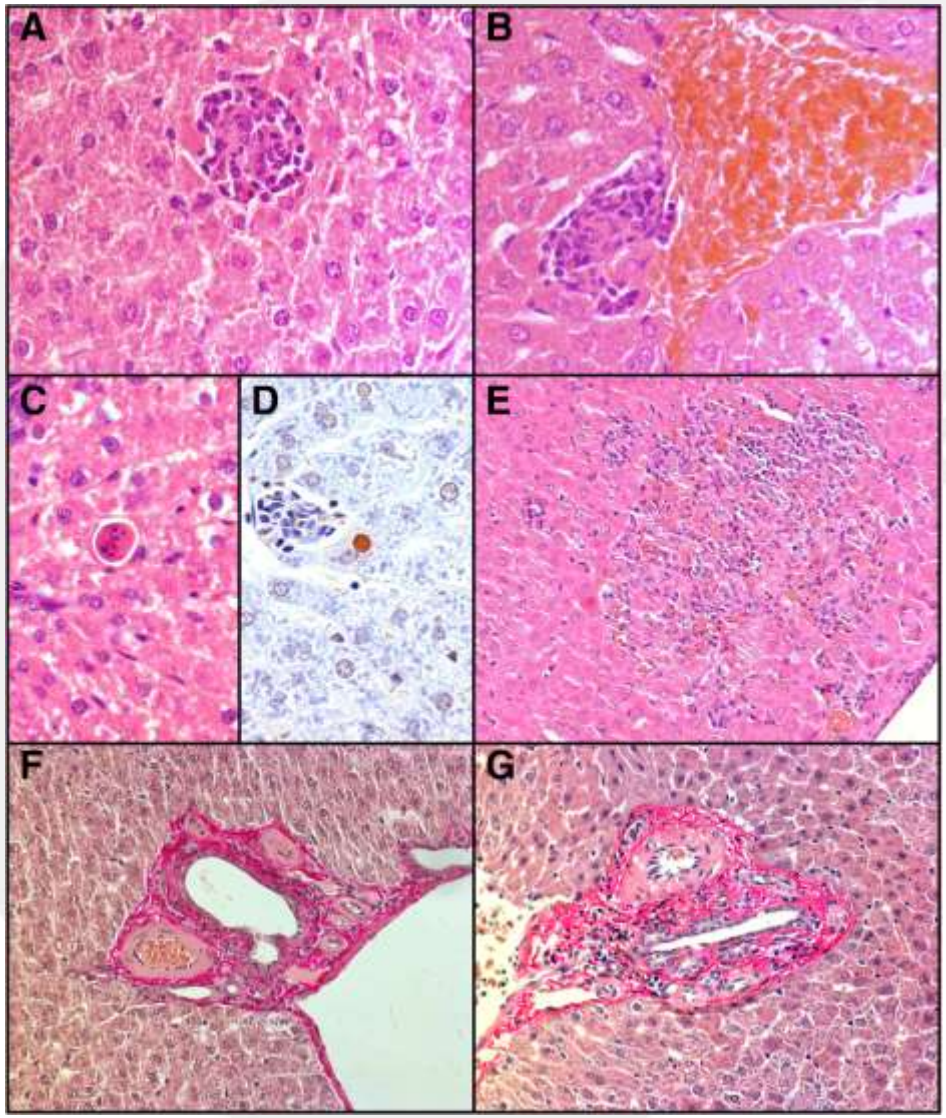
Control Animal



Proposal: Gradings, Example by Measurement: Control Animal

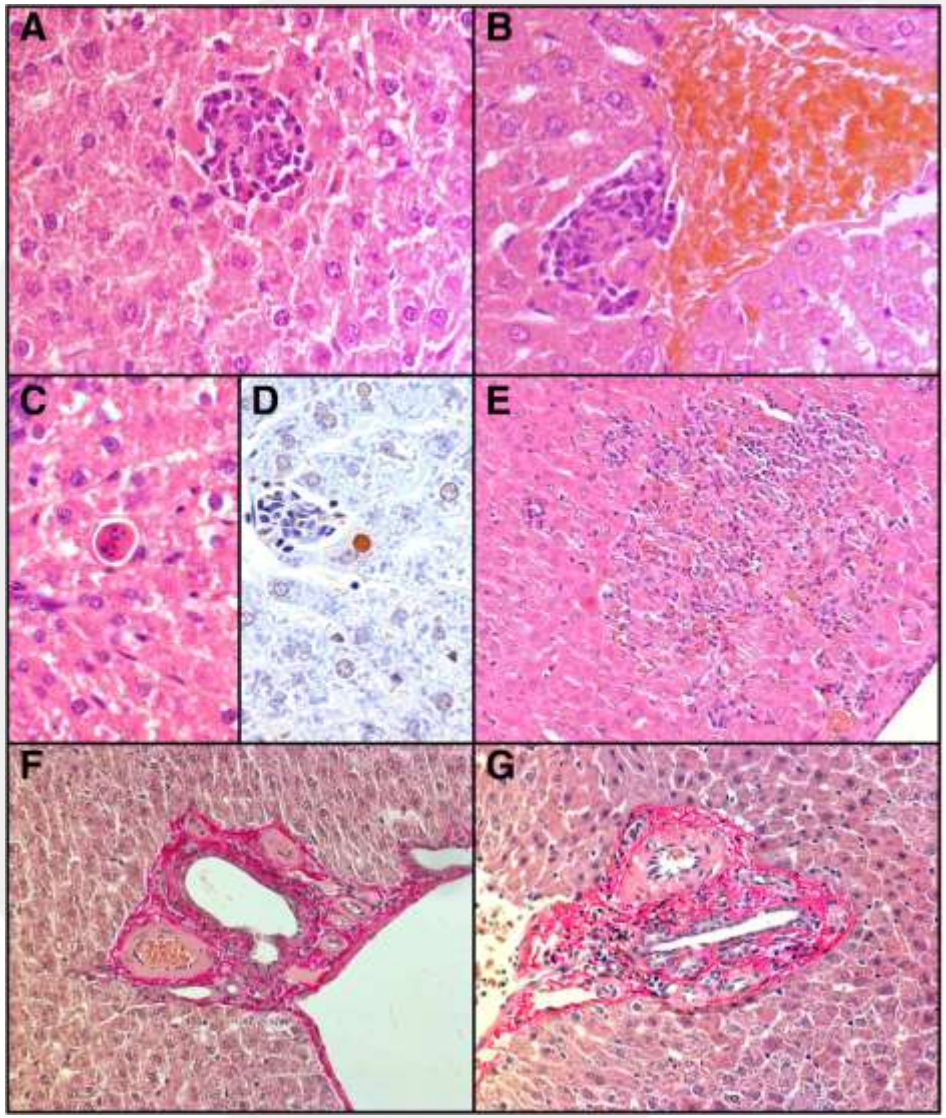


Comparison to Zande et al.



(A, B) ..inflammatory granuloma after 84-days of exposure for **(A)** SAS high dose (magnification: 200x), and **(B)** NM-202 high dose (magnification: 200x). **(C)** Apoptosis after 28-days of exposure (SAS low dose, H&E staining; magnification: 200x), and **(D)** apoptosis after 28-days of exposure (NM-202 high dose; immunohistochemically stained apoptosis; magnification: 200x). **(E)** Necrosis after 28-days of exposure (NM-202 medium dose; magnification: 25x), and **(F, G)** fibrosis after 84-days of exposure to the **(F)** SAS high dose (magnification 100x), and **(G)** NM-202 high dose (magnification 100x).

Real Interpretation

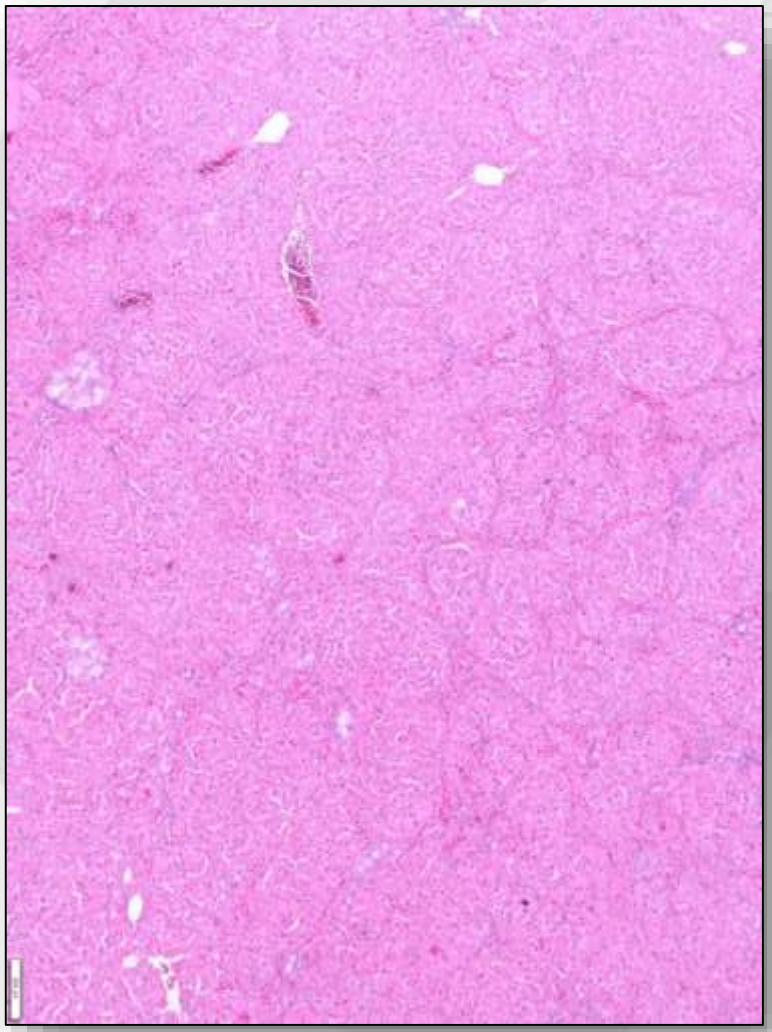


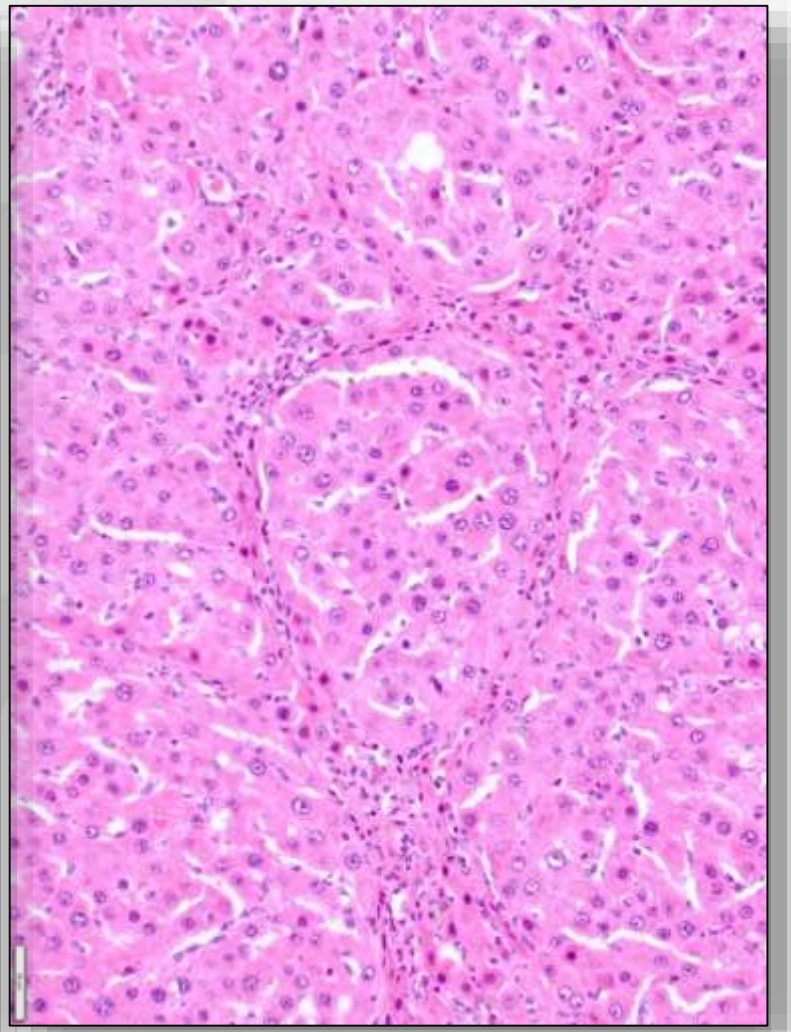
(A, B) ..inflammatory cell infiltrates as normal turnover of rat lives. Normal control lesion (up to 80-100%)
(C, D) Apoptosis yes, but is normal in rat livers, also in control animals.
(E) Necrosis yes. In control data e.g., RccHanTM rats 14-50%.
(F, G) peribiliar fibrosis after 84-days of exposure. Normal background finding in 13-week studies. Usually related to bile duct proliferation. Compare to previous shown pictures.

The staining for F and G was not indicated. It is Sirius Red.

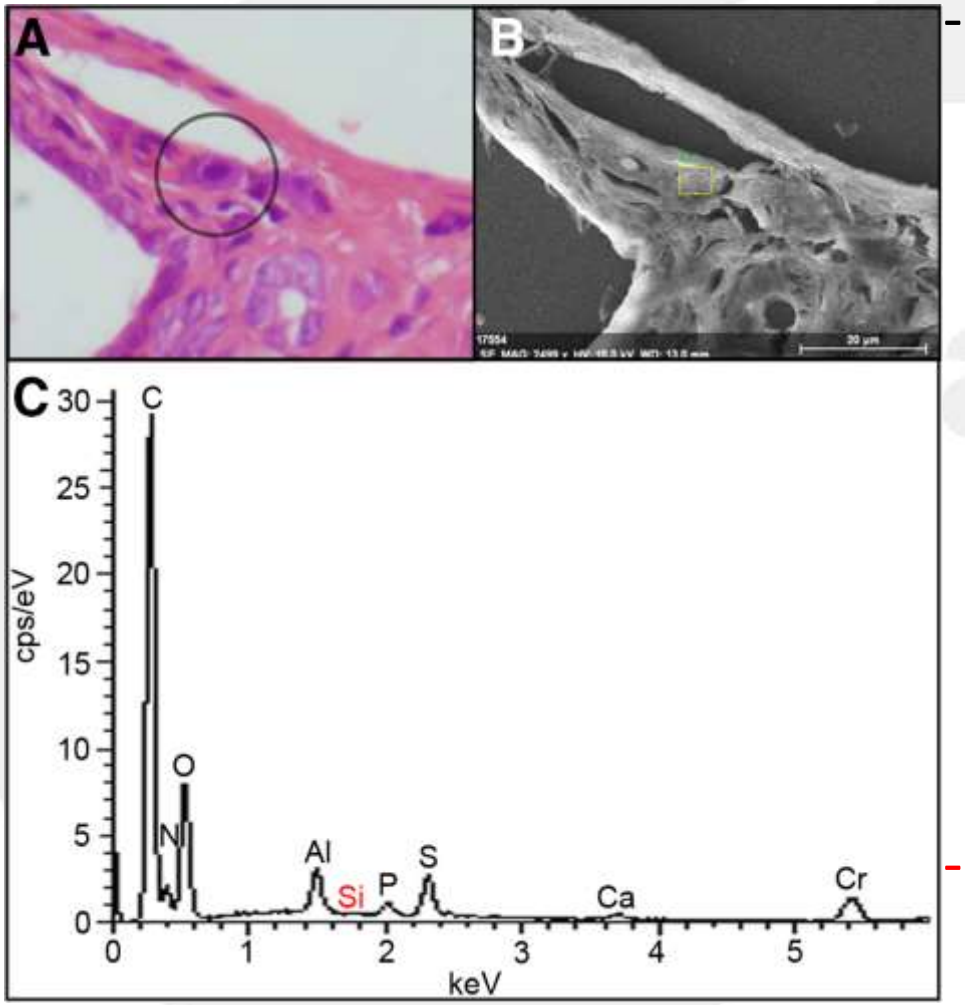
This is Liver Fibrosis!

- **Peribiliar fibrosis is normal background lesion.**
- **Induced liver fibrosis takes form of cirrhotic changes.**





Silica Uptake and Organ Weights: Where is the peak?



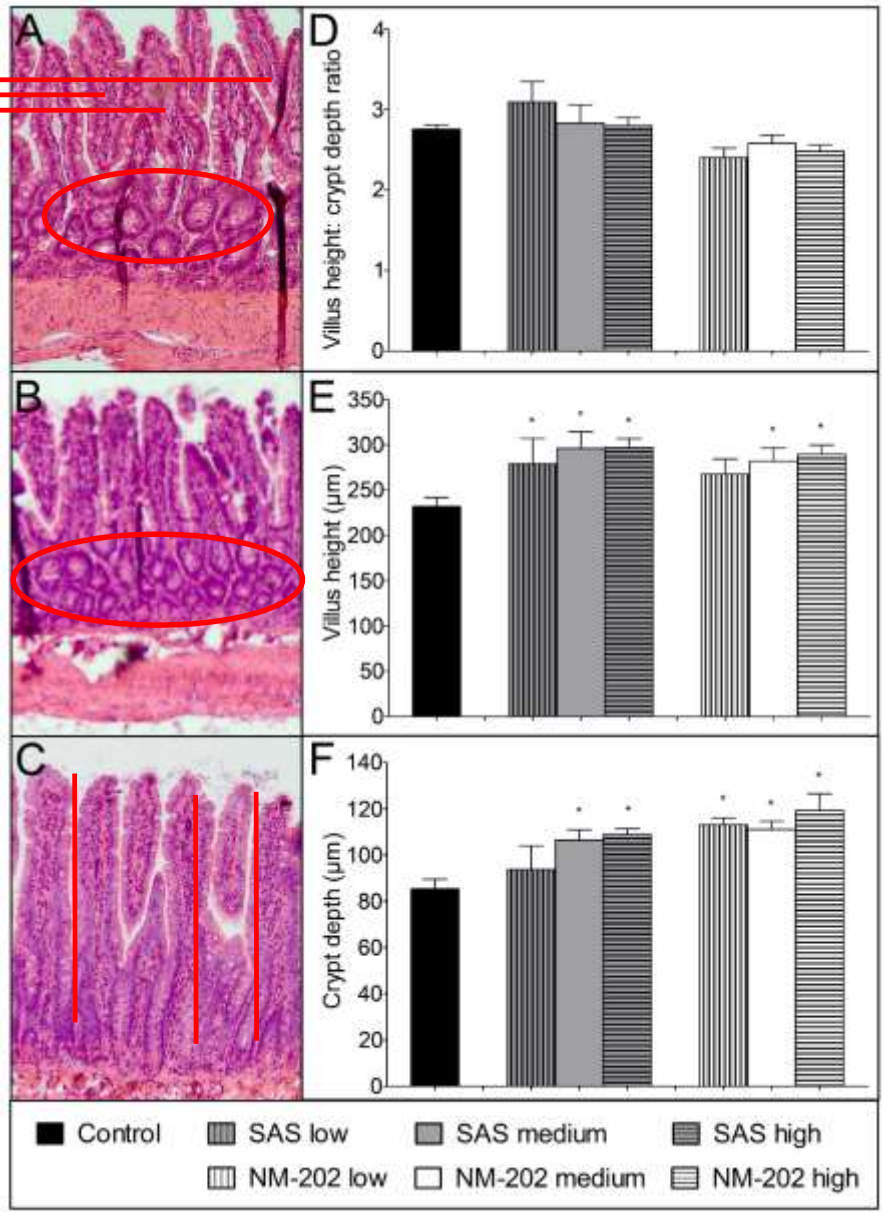
- Figure 7A, a cell is shown that have been annotated as a macrophage. It is also possible that this cell represents an oval cell together with a few more cells shown in the same picture at the right, the underlying small bile duct and a few lymphocytes can be recognized.
- Figure 7C does not show any peak for silica.

Intestinal Villi Height: Plane of Section Artifacts

Oblique section. Note: Several villi are cut in upper thirds only.
Crypts are visible by transversal section planes.

Again:
Crypts are visible by transversal section planes.

Note: Villi are cut longitudinally until the depths of crypts.

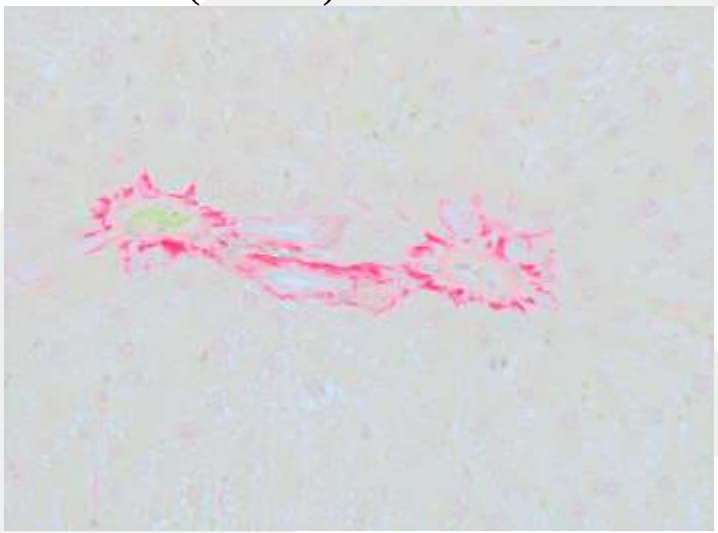


Current Investigation

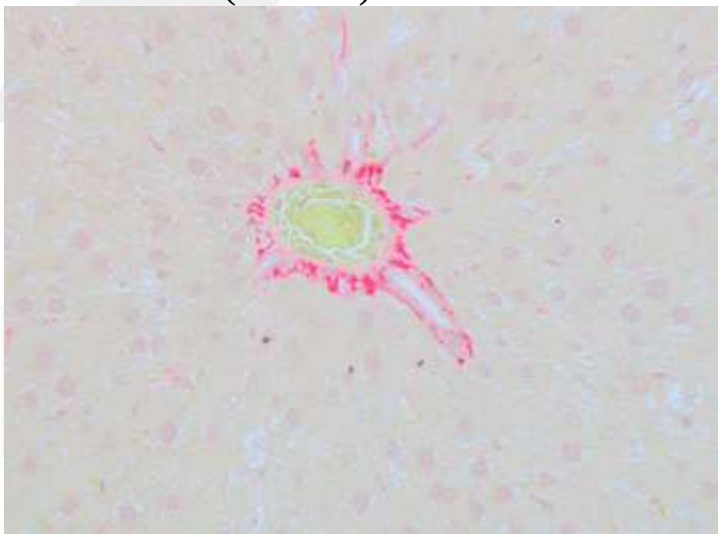
Evaluation of Si-distribution in organs and deposition-related tissue alterations on materials obtained from TNO Triskelion study V9127 (Oral two-generation reproduction study with NM-200 synthetic amorphous silica in Wistar rats)

Current Investigation: Liver – Collagen I-III, Sirius Red (Example: Sirius Red)

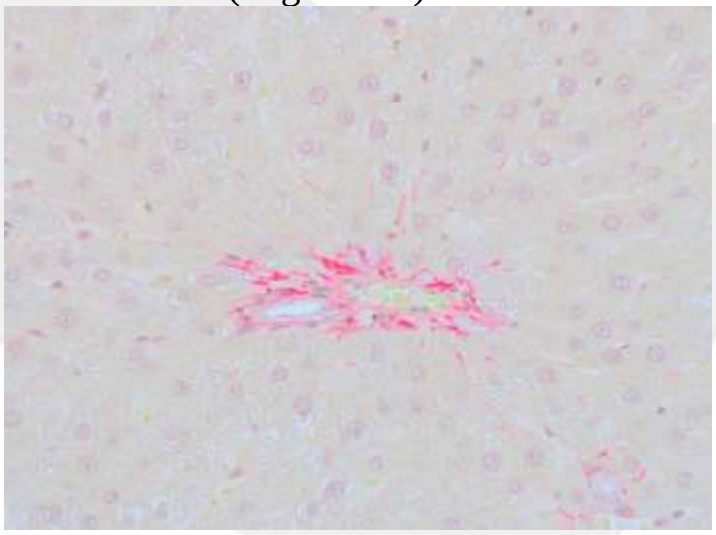
Male no. 1 (Control). ROI 1.



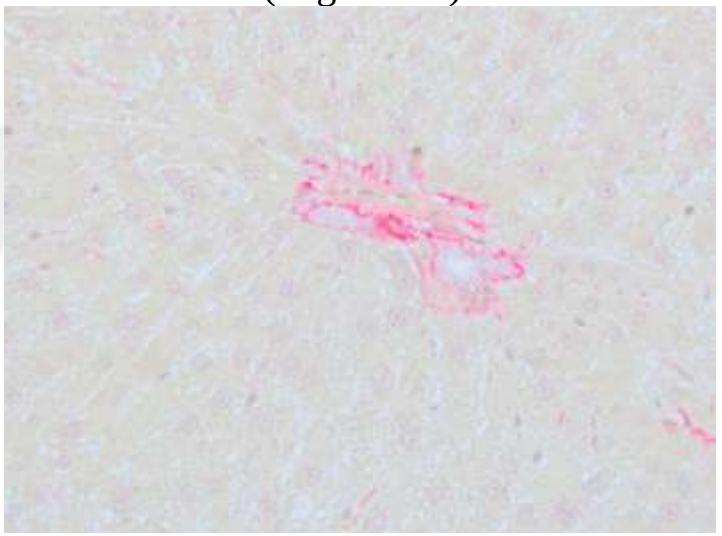
Female no. 2 (Control). ROI 1.



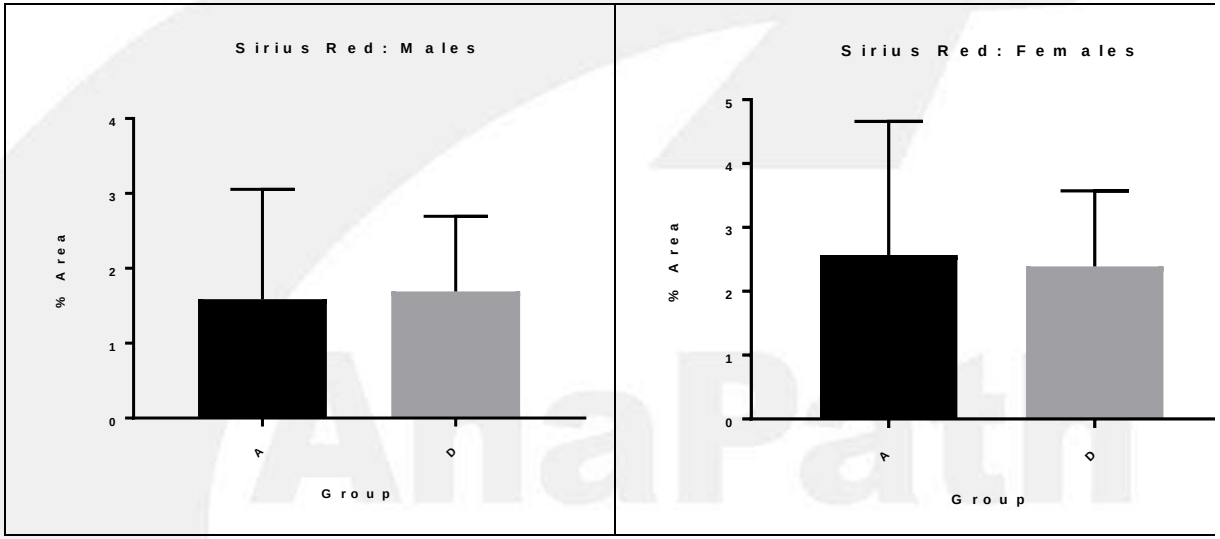
Male no. 169 (High Dose). ROI 1.

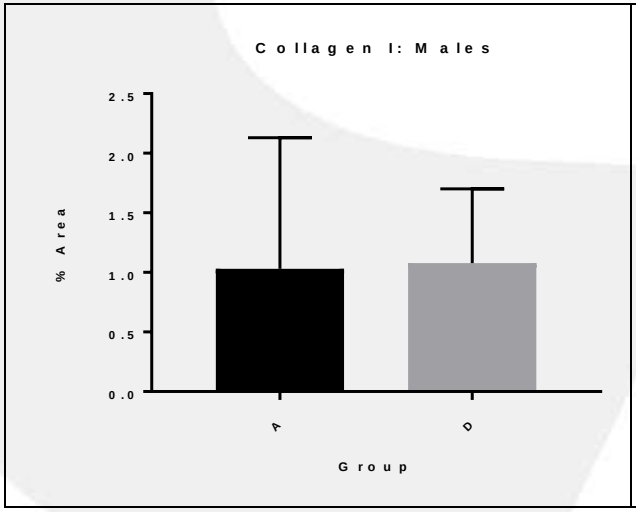


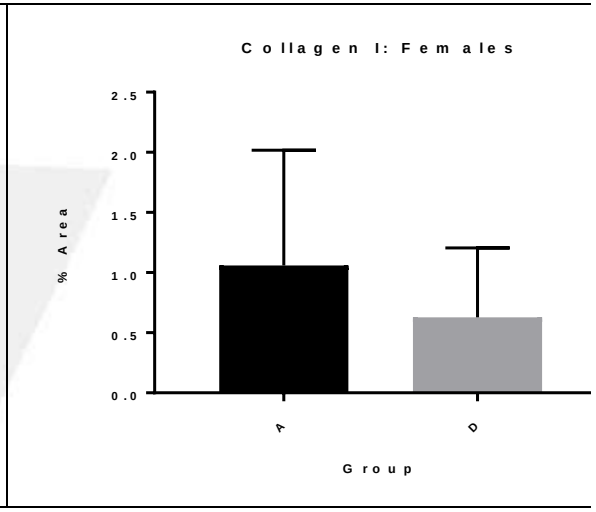
Female no. 170 (High Dose). ROI 1.



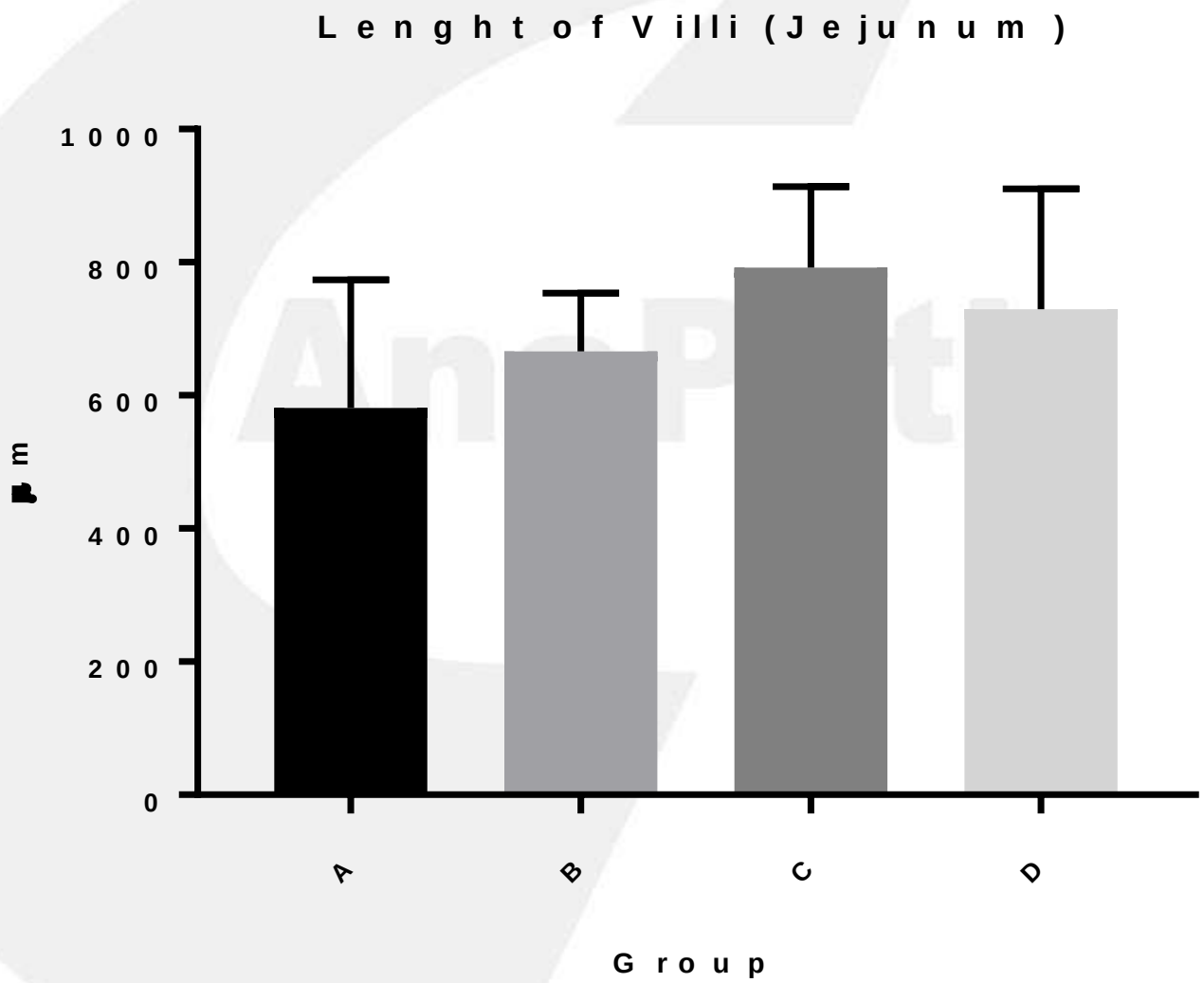
Current Investigation: Liver Sirius Red and Collagen 1: Example Graphs





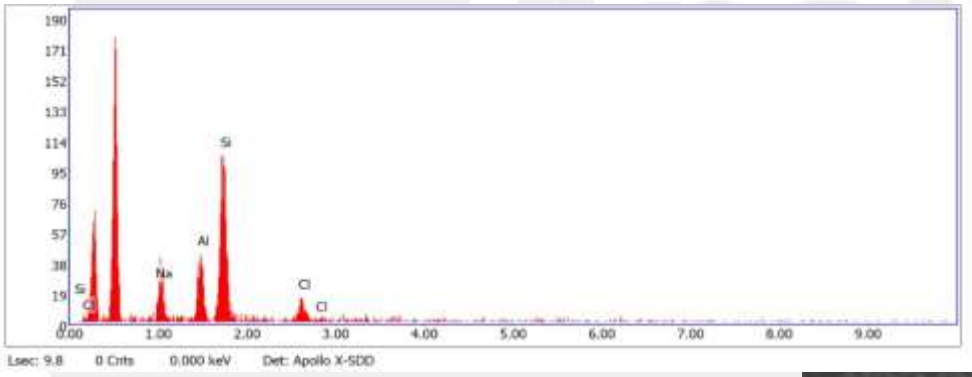


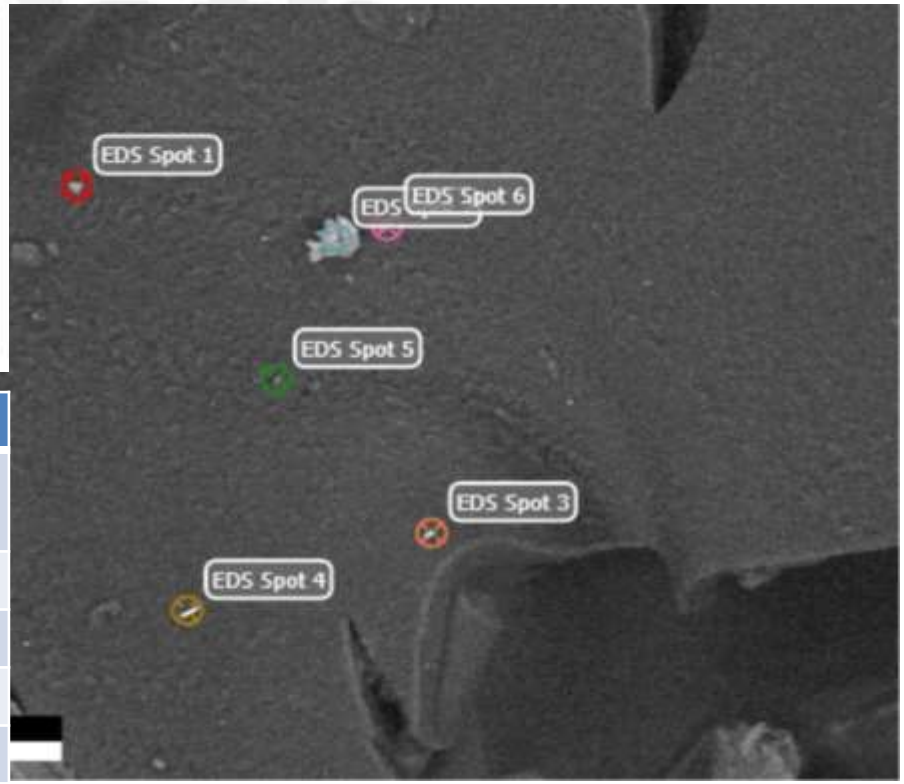
Current Investigation: Examples - Intestine



Current Investigation: Examples – Si-Contents

- **Liver**
- **EDS Spot 1**
- **This is no the test item. This is the feldspar Albit: $Na[AlSi_3O_8]$**





eZAF Smart Quant Results

Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	A	F
NaK	13.99	16.86	19.58	16.60	0.1127	1.0140	0.7920	1.0036
AlK	17.47	17.94	30.42	11.71	0.1478	0.9910	0.8465	1.0086
SiK	56.68	55.92	87.81	8.41	0.4531	1.0117	0.7888	1.0018
ClK	11.87	9.28	11.58	25.53	0.0858	0.9398	0.7680	1.0019

Summary

- Inflammatory lesions in lungs after treatment, but recovery
- No lung fibrosis
- Confirmation in current inhalation study

- No findings in liver and intestine
- Confirmed in recent evaluation
- No increased Si-Contents in organs different from esophagus and GIT