Intestinal Uptake of Particles

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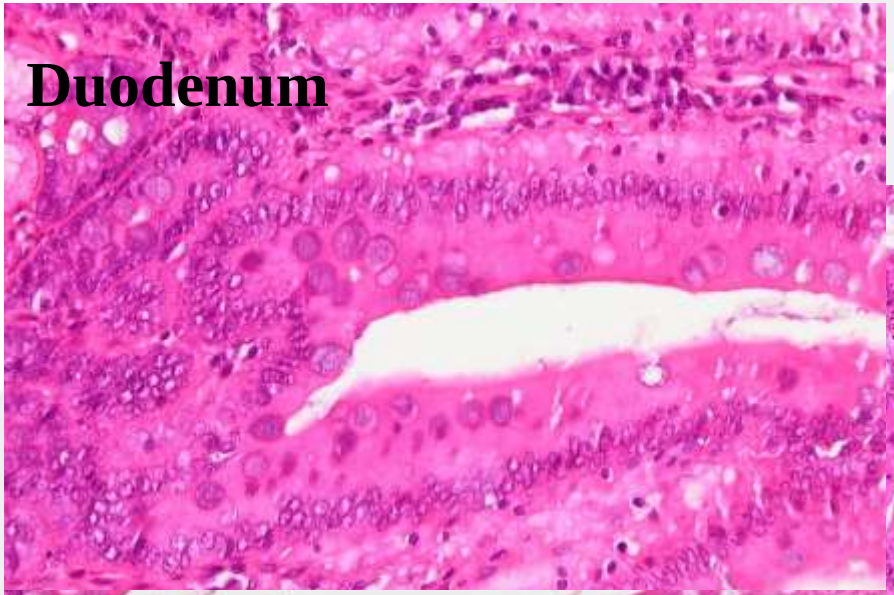
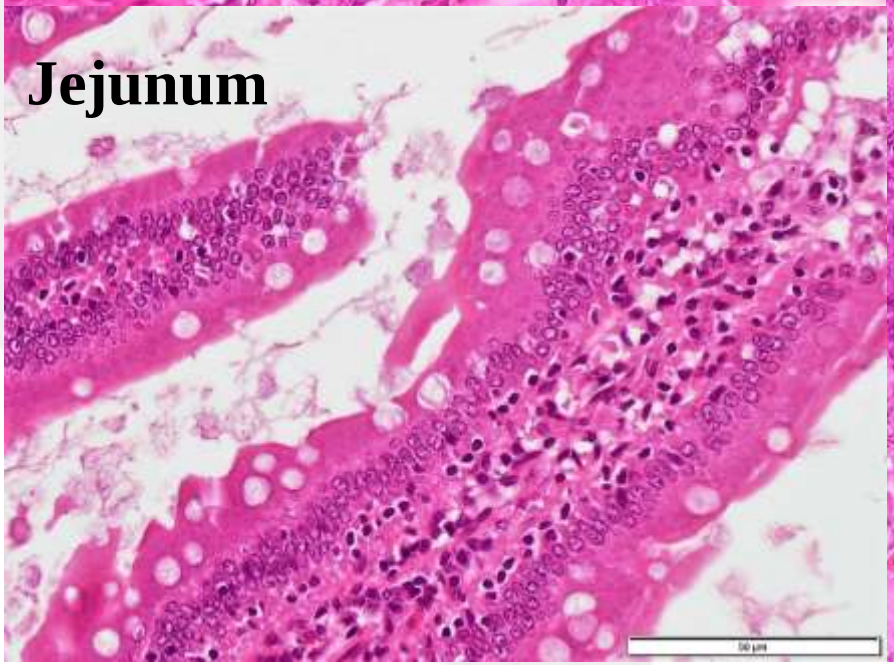
Intestinal Resorption, Functional Anatomy

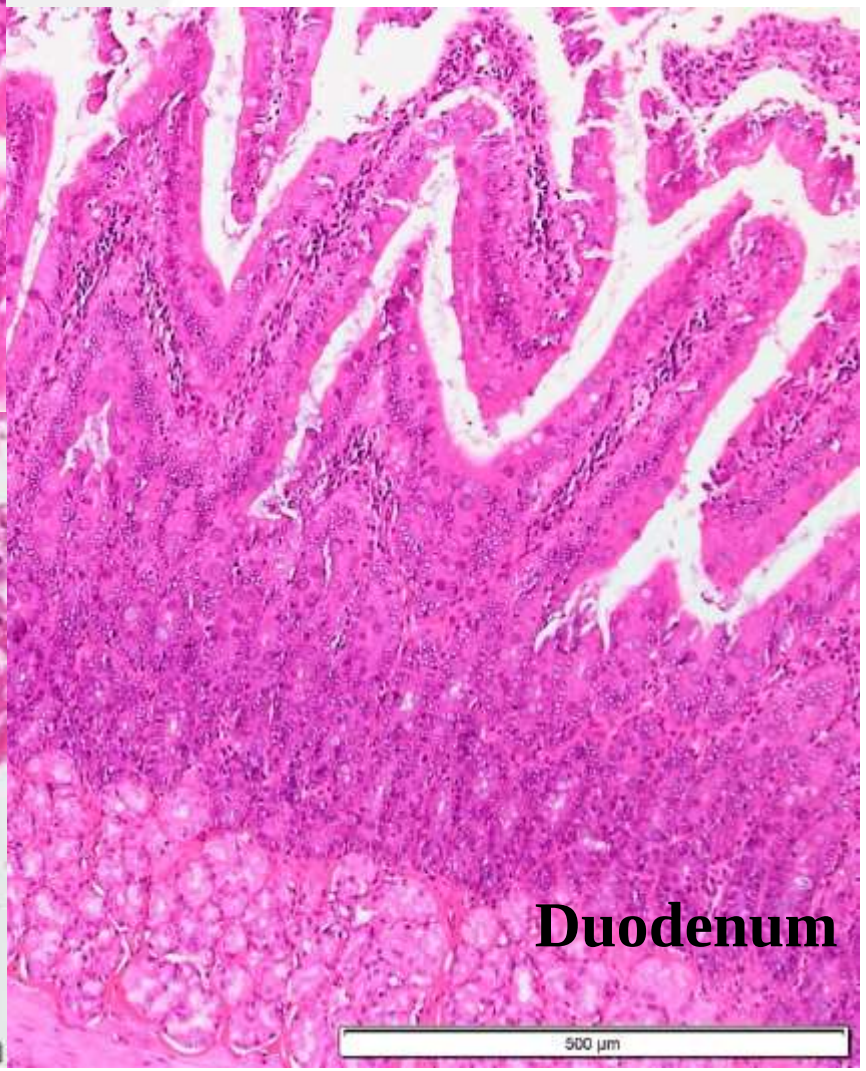
- Small intestine: length approx. 6 m, diameter approx. 2.5 cm
- Villi 0.5–1.5 mm in length cover the surface of epithelial cells at the tip
- Surface area enhanced by villi and microvilli for 30–600 fold,
- Absorptive cells at villi and secretory cells in crypts
- Colon: length approx. 1.5 m, diameter approx. 7 cm
- Most nutrients are absorbed in the small intestine
- Water and electrolytes primarily absorbed by colon

- Summary:
  Kiela PR, Ghishan FK (2016). Physiology of Intestinal Absorption and Secretion. Best Pract Res Clin Gastroenterol. 30:145-59.

Duodenum, Jejunum: Example







Intestinal Resorption, Physiology

- All nutrients can be absorbed into blood across polarized epithelial cell layer of intestinal mucosa
- Passive and active mechanisms
- Passage per day:
  - approx. 8–10 L fluid containing approx.:
    800 mmol of Na^+
    700 mmol of Cl^-
    100 mmol of K^+

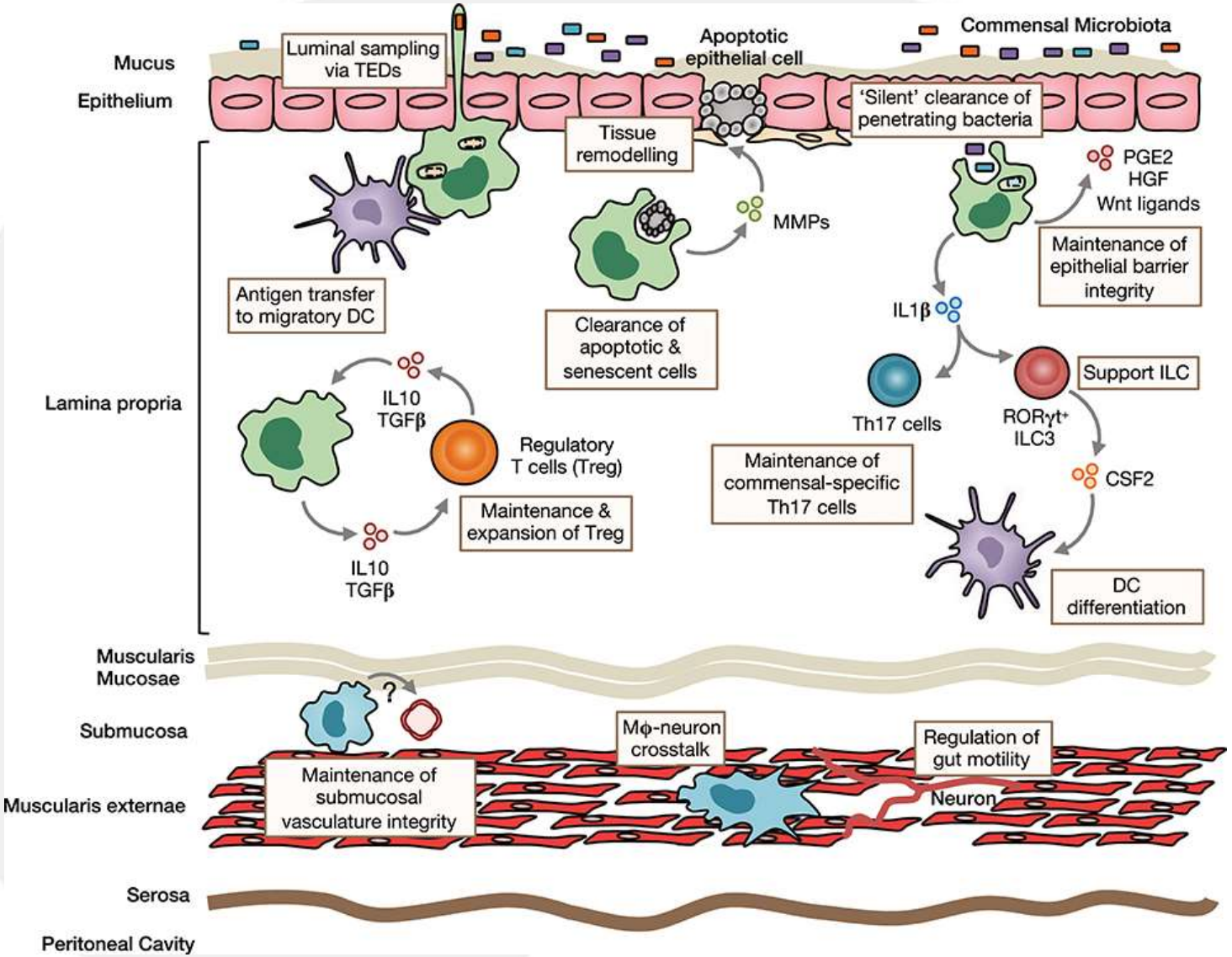
- Mechanisms for solute transport are secondary to several transport proteins located at the brush border membranes

Intestinal Macrophages: Functions

- Intestinal lamina propria macrophages (LPM) under epithelial monolayer are highly phagocytic and responsible for clearing apoptotic and senescent epithelial cells, and bactericidal activity
- LPM promote epithelial integrity (tissue-remodeling metalloproteinases, secretion of stem cell renewal factors (PGE2, HGF, Wnt ligands))
- LPM transfer antigens to migratory dendritic cells (DCs) for presentation to T cells in mesenteric lymph nodes
- Production of IL10 and TGFβ to maintain and facilitate secondary expansion of regulatory T cells (Tregs) locally in the LPM
- Support of Th17 cells and ILC3s through their production of IL1β, which is induced by exposure to the microbiota or its derivatives.
- Macrophages also present in deeper layers of intestinal wall (submucosa/muscularis: other functions)
- No macrophages in intestinal lumen

- *Bain CC, Schridde A (2018). Origin, Differentiation, and Function of Intestinal Macrophages. Front Immunol. 27;9:2733*

Intestinal macrophages



https://www.frontiersin.org/files/Articles/408505/fimmu-09-02733-HTML/image_m/fimmu-09-02733-g001.jpg

Particle Uptake

Depends from:

- Particle size
- Particle surface (e.g. hydrophobicity/charge)
- Dose of particles administered
- Administration vehicle
- Use of targeted delivery to M* cells
- Fed state of the animal
- Age of the animal
- Species under investigation
- Method used to quantify uptake

*Microfold cells

Particle Uptake

Site/Mechanism	Particle Size
Villus tips - resorption	5-150 nm
Intestinal macrophages - phagocytosis	1 µm
Enterocytes – endocytosis	<200 nm
Peyer's patches - transparacellular	<10 µm

(evaluated for Poly-styrene, -methyl methacrylate, -lactide, -lactide co-glycolide, and Ethyl cellulose)

O'Hagan DT (1996). The intestinal uptake of particles and the implications for drug and antigen delivery. J Anat. 189 (Pt 3):477-82.

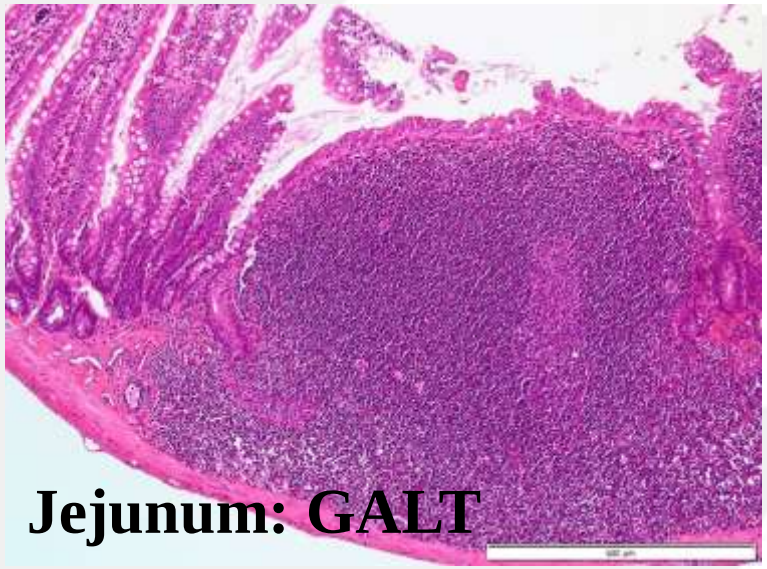
Intestinal Uptake, Polymeric Microspheres (MSs)

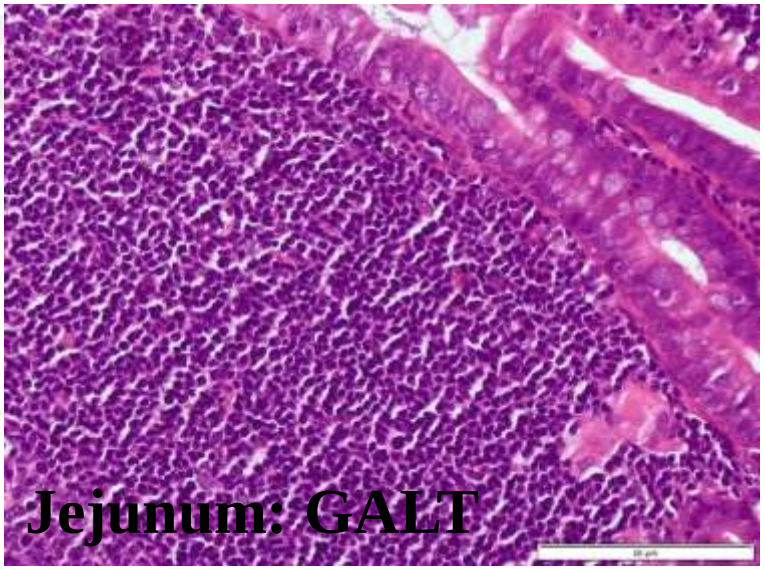
- nonbiodegradable polystyrene MSs (500 nm to 5 µm) delivered locally to the jejunum or ileum or by oral administration to young male rats
- Rapid uptake (≤ 5 min)
- Detected by TEM and confocal laser SM
- Gel permeation chromatography: MSs in all tissue samples
- High concentrations: in liver, kidneys, lungs.
- Pharmacologic inhibitors chlorpromazine, phorbol 12-myristate 13-acetate, and cytochalasin D caused reduction in total number of MSs absorbed
- Nonphagocytic processes (including endocytosis) direct the uptake of MSs (Microfold cells in GALT).

Reineke JJ, Cho DY, Dingle YT, Morello AP 3rd, Jacob J, Thanos CG, Mathiowitz E (2013). Unique insights into the intestinal absorption, transit, and subsequent biodistribution of polymer-derived microspheres. Proc Natl Acad Sci U S A. 110: 13803-8.

Intestinal Uptake, Microfold Cells in GALT

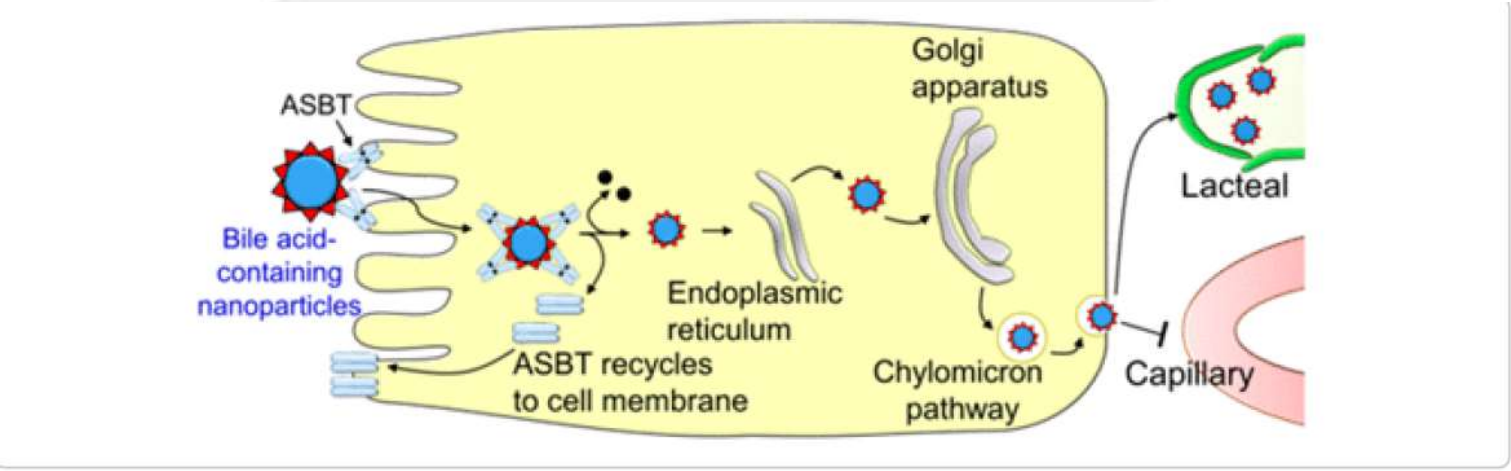
- known to initiate mucosal immunity responses on the apical membrane of the M cells and allow for transport of microbes and particles across the epithelial cell layer from the gut lumen to the lamina propria where interactions with immune cells can take place





Reineke JJ, Cho DY, Dingle YT, Morello AP 3rd, Jacob J, Thanos CG, Mathiowitz E (2013). Unique insights into the intestinal absorption, transit, and subsequent biodistribution of polymer-derived microspheres. Proc Natl Acad Sci U S A. 110: 13803-8.

Intestinal Resorption by GALT



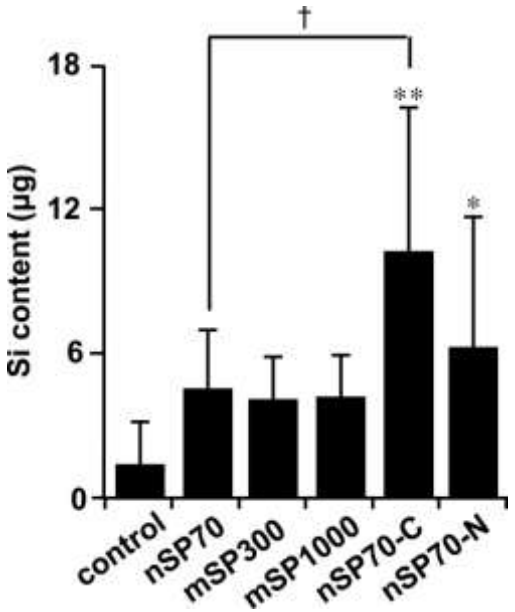
- Nanoparticle transport: combination of apical sodium-dependent bile acid transporter-mediated cellular uptake and chylomicron transport pathways.
- Particle-size- and dose-dependent oral bioavailability was observed for oral nanoparticle dosing up to 20 mg/kg.
- Probe nanoparticles appeared to be transported to systemic circulation via the gut lymphatic system.

Kim KS, Suzuki K, Cho H, Youn YS, Bae YH (2018). Oral Nanoparticles Exhibit Specific High-Efficiency Intestinal Uptake and Lymphatic Transport. ACS Nano. 12(9):8893-8900.

Example: Amorphous Silica Particles

- Evaluation: in vitro intestinal absorption and in vivo biological effects in mice of orally administered amorphous silica particles with diameters of 70, 300, and 1,000 nm (nSP70, mSP300, and mSP1000, respectively) and surface-modified nSP70 (carboxyl or amine groups: nSP70-C and nSP70-N, respectively).
- Everted gut sac method:
  Absorption through the intestine depends on particle diameter and surface properties,
  However: solution of 12.5 mg/mL!
- Hematological, histopathological, and biochemical analyses showed no significant differences between control mice and mice treated with the silica particles

Yoshida T, Yoshioka Y, Takahashi H, Misato K, Mori T, Hirai T, Nagano K, Abe Y, Mukai Y, Kamada H, Tsunoda S, Nabeshi H, Yoshikawa T, Higashisaka K, Tsutsumi Y (2014). Intestinal absorption and biological effects of orally administered amorphous silica particles. Nanoscale Res Lett. 9:532



Interim Summary

- Particle uptake via intestine is possible, however…
- Uptake depends on mechanism
- ‘Pinocytotic’ processes allows uptake of particles by enterocytes (endocytosis) if <200 nm (not possible for aggregates)
- Possibility for uptake via chylomicrons possible if soluble in fat
- Uptake by macrophages not possible, since no macrophages in intestinal lumen
- Uptake by M-cells into GALT possible for particle <10 µm, however, high concentration of particles necessary to trespass intestinal wall
- Uptake by M-cells from aggregates larger than 10 µm needs aggregate breakdown

Small Intestinal Transit Time

- In human, the median SITT: 219 min for females and 191 min for males.
- In rats: 1–2 h for transit of contents to reach the cecum, and 4–6 h to transit from the stomach to the colon.
- Longer retention period in rats compared to human

Fischer M, Fadda HM (2016). The Effect of Sex and Age on Small Intestinal Transit Times in Humans. J Pharm Sci. 105:682-686.

Horiuchi A, Tanaka N, Sakai R, Kawamata Y (2014). Effect of age and elemental diets on gastric emptying in rats. J Gastroenterol Hepatol Res. 3: 1340–3.

How quick work M-cells?

'…The formation of these "pockets" greatly reduces the intracellular distance that antigens have to travel and allows M cells to rapidly transport (within 10 to 15 min) antigenic materials to the basolateral membrane…'

Miller H, Zhang J, Kuolee R, Patel GB, Chen W (2007). Intestinal M cells: the fallible sentinels? World J Gastroenterol. 13(10):1477-86.

Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health: Page 35: Step 0 In vitro digestion

- consider the time for degradation
- consider the degraded amount
- consider the consitutents after degradation

Expected Pathology with High Uptake

1. Accumulation of reactive macrophages in Peyer's patches
2. Accumulation of reactive macrophages in mesenteric lymph nodes and related inflammatory lesions (e.g., latex, carbon)
3. Presence of particles in different organs with not predictable pathological lesions.

Ikomi F, Kawai Y, Ohhashi T (2015). Recent advance in lymph dynamic analysis in lymphatics and lymph nodes. Ann Vasc Dis. 5:258-68.
LeFevre ME, Olivo R, Vanderhoff JW, Joel DD (1978). Accumulation of latex in Peyer's patches and its subsequent appearance in villi and mesenteric lymph nodes. Proc Soc Exp Biol Med. 159:298-302.

Guidance on risk assessment …1a/1b, 2a

'…Review all existing physicochemical and toxicological information as well as information relevant to grouping/read-across…'

or

'…Including dissolution under lysosomal conditions…'

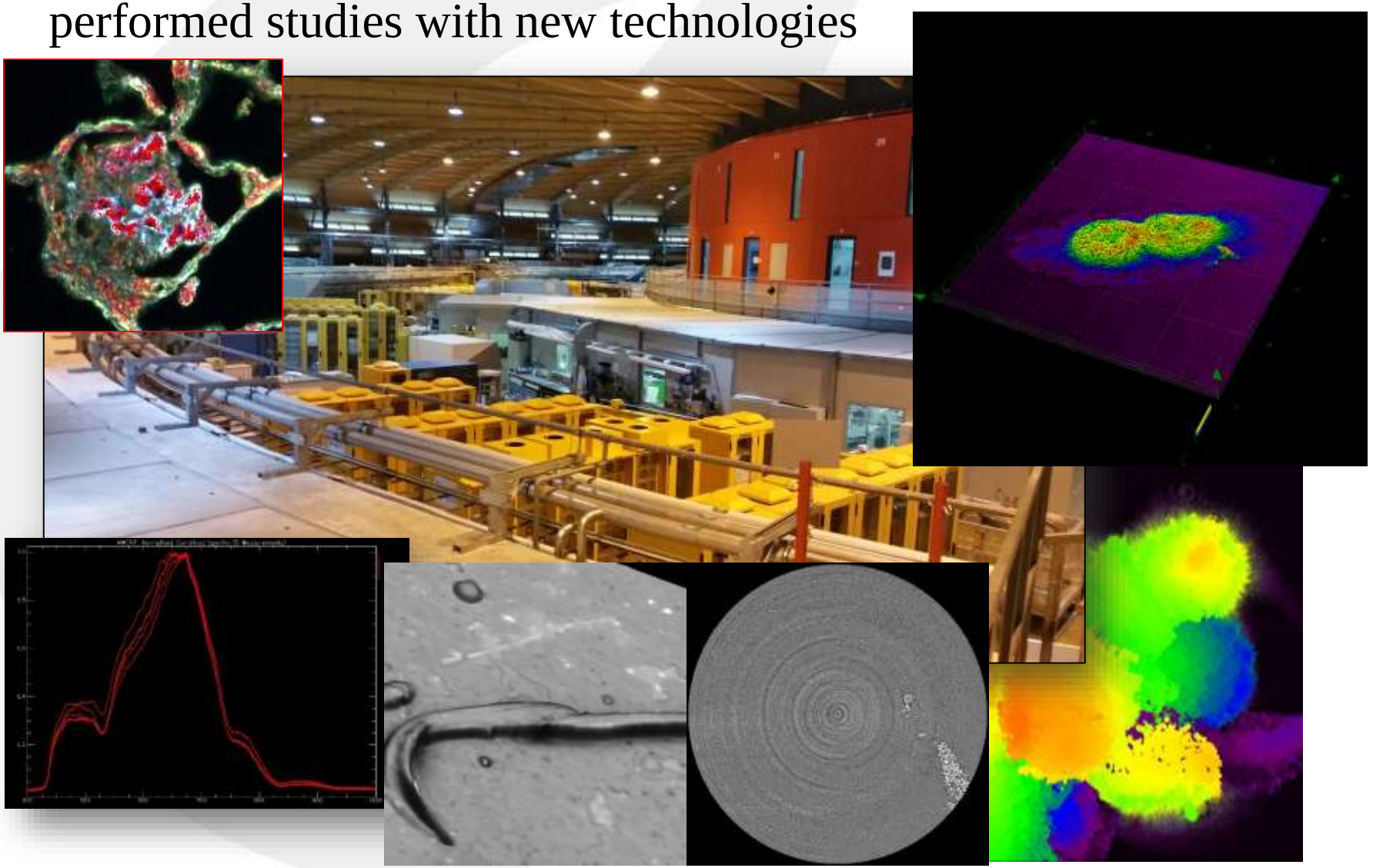
or

Is the nanomaterial non-persistent AND no indication of potential toxicity is observed

'…2a) A pilot study for dose finding and assessment of absorption, tissue distribution and accumulation and elimination phases…'

Or:

Exploit, corrected and enhance the evaluation of previously performed studies with new technologies



Example for Evaluation of Published Data and Use of Material from Previously Performed Studies

- Data contradictory to present knowledge might be published in peer-reviewed journals
- Critical view on surprising data is necessary
- Previously performed studies might be ‘exploited’ for additional data in order to follow such new findings

‘…Step 1a Review existing information(b). See Sections 3, 4, 6.3: Review all existing physicochemical and toxicological information as well as information relevant to grouping/read-across…’

- See example

Evidence?

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.

Morfeld P, Bosch A, Weber K, Heinemann M, Krueger N (2017). 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

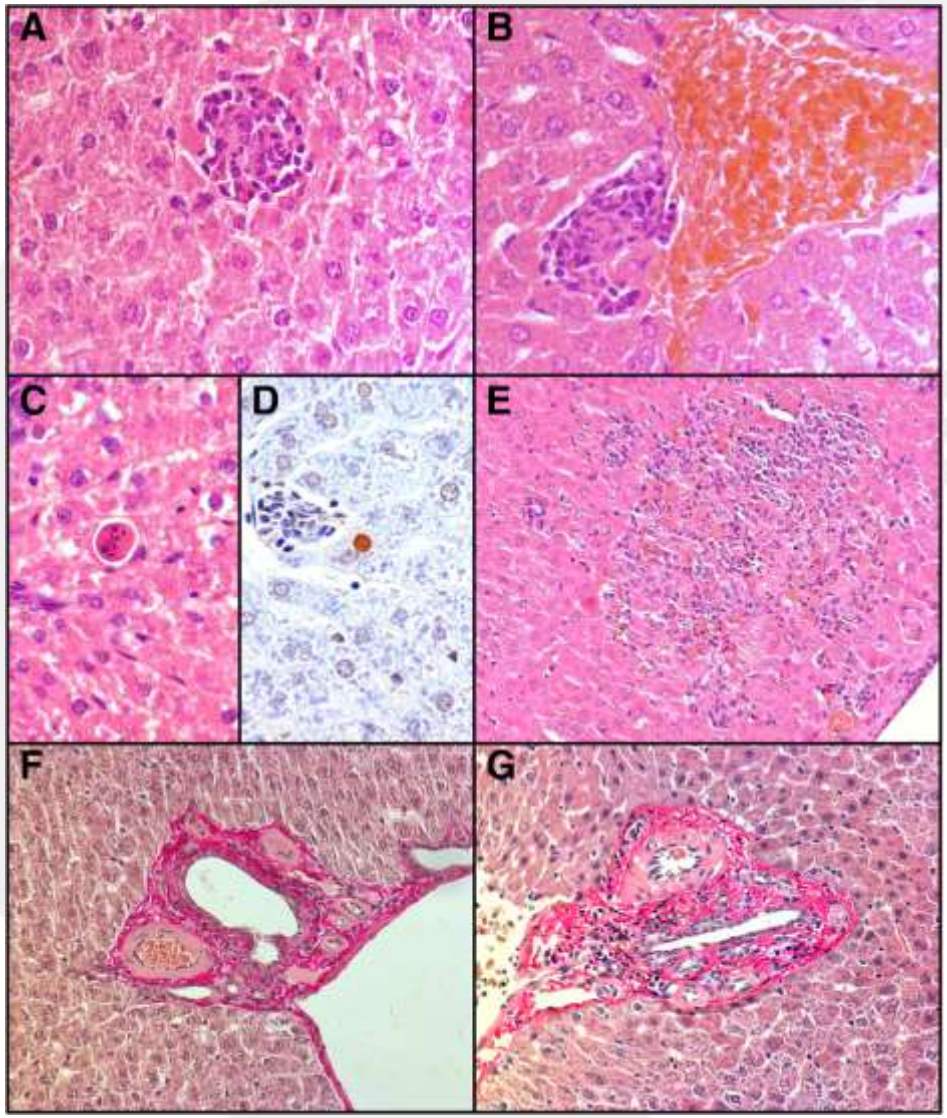
Study Outcome

- 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:

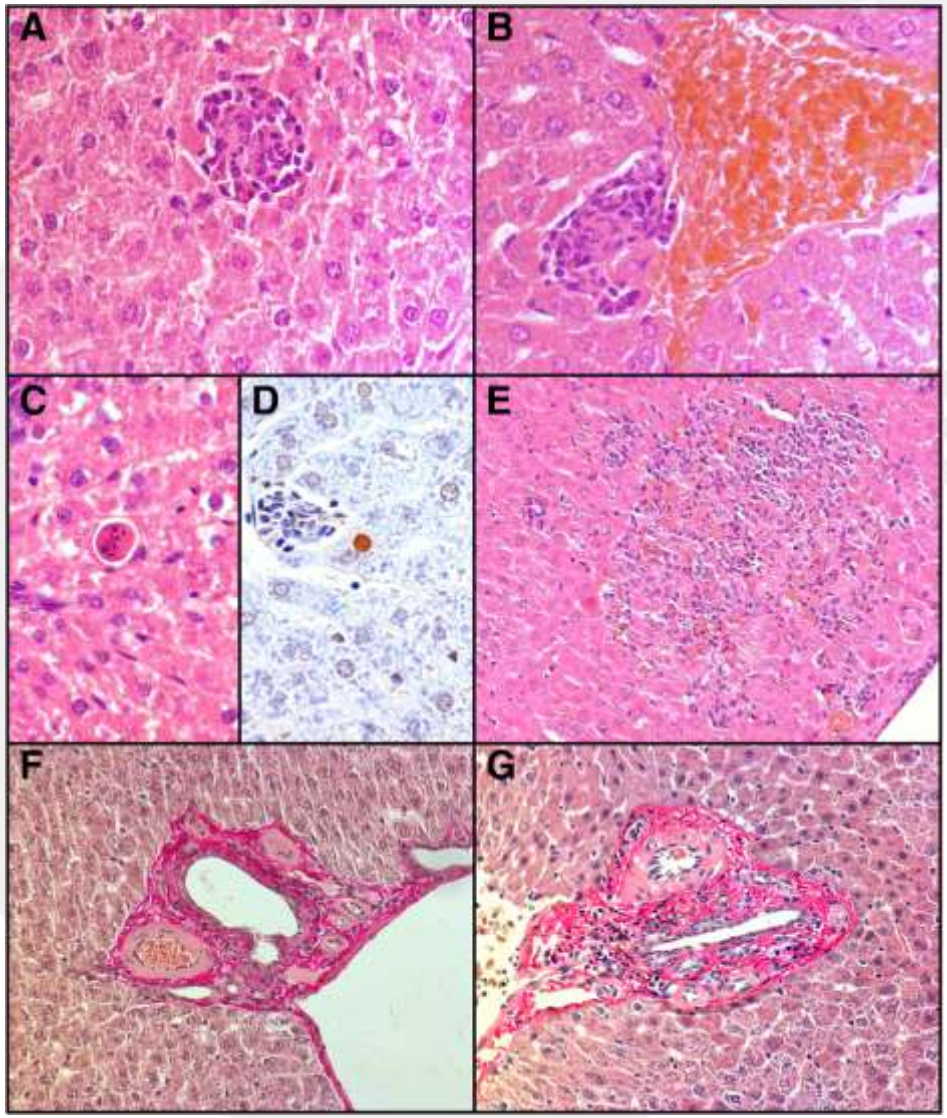
- the study “…showed an increased incidence of liver fibrosis after 84-days of exposure…”
  and
  “…increased height of jejunal villi…”

Interpretation by 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).

Interpretation by an Experienced Pathologist



(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., RccHan™ rats 14-50%.
(F, G) minimal and expected peribiliar fibrosis after 84-days of exposure. Normal background finding in 13-week studies. Usually related to bile duct proliferation. Compare to pictures shown below.

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

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

Proposal: by Measurement

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

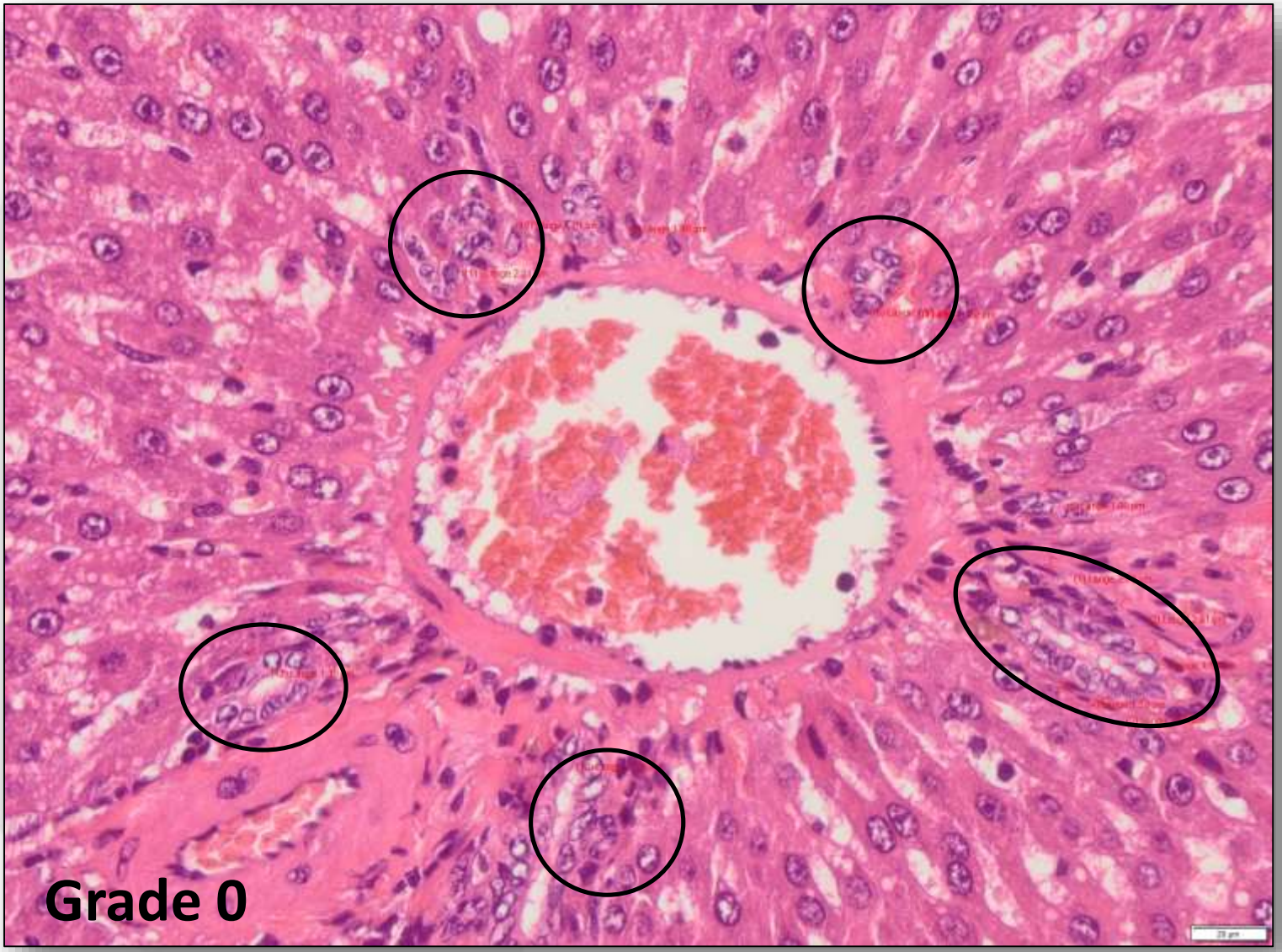
Number of measurements	20	20	20	20	20	20
Minimum	1.006	2.593	2.588	6.361	8.142	3.282
Maximum	4.023	6.747	9.971	22.614	35.595	52.554
Mean	1.979	4.058	5.735	14.809	17.576	20.289

Distribution for Gradings by means:

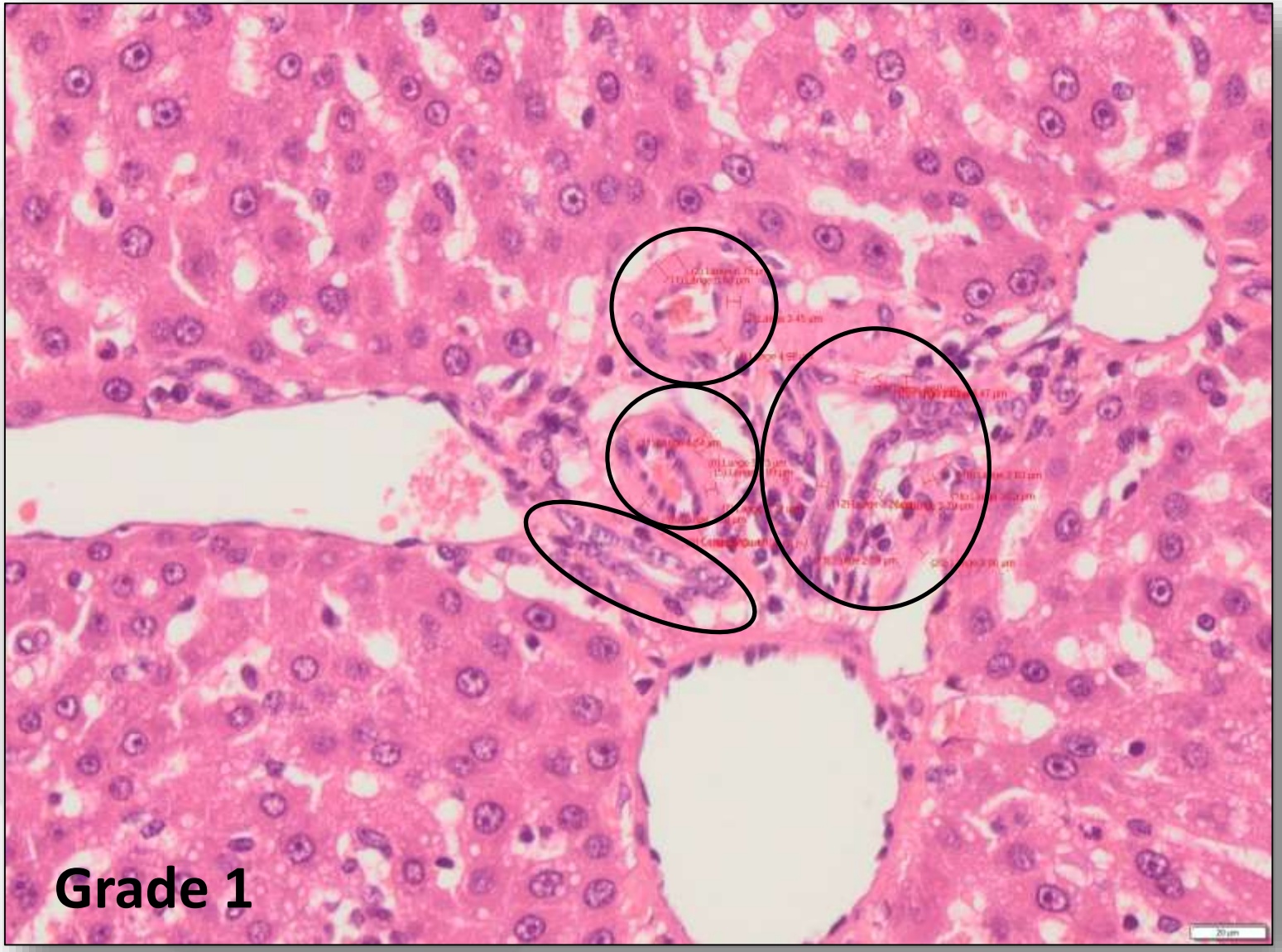
0 = up to 2 µm, 1= up to 4 µm, 2= up to 10 µm,

3 = up to 15 µm, 4 = up to 20 µm, 5 = > 20 µm

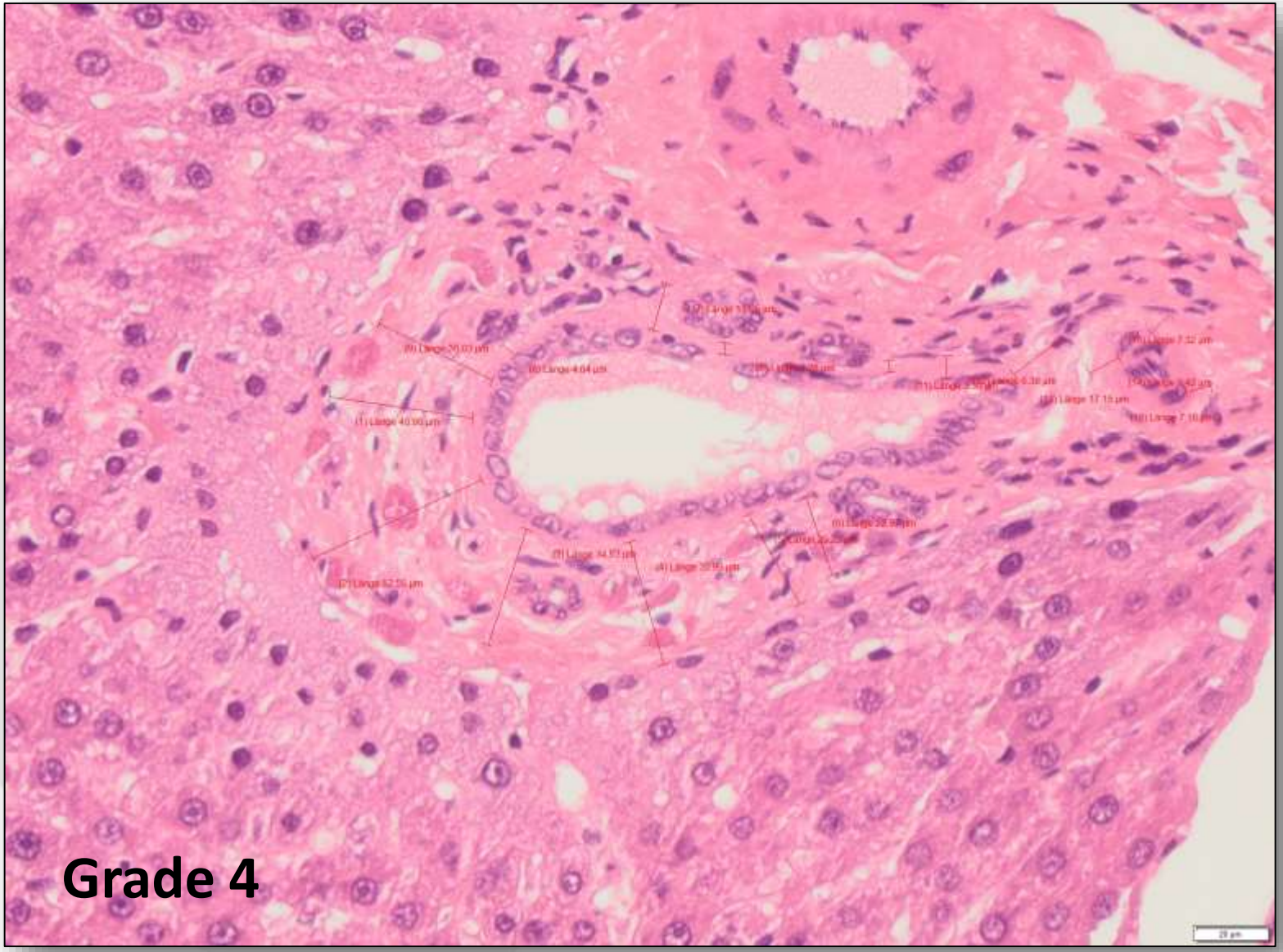
Proposal: Gradings, Example by Measurement



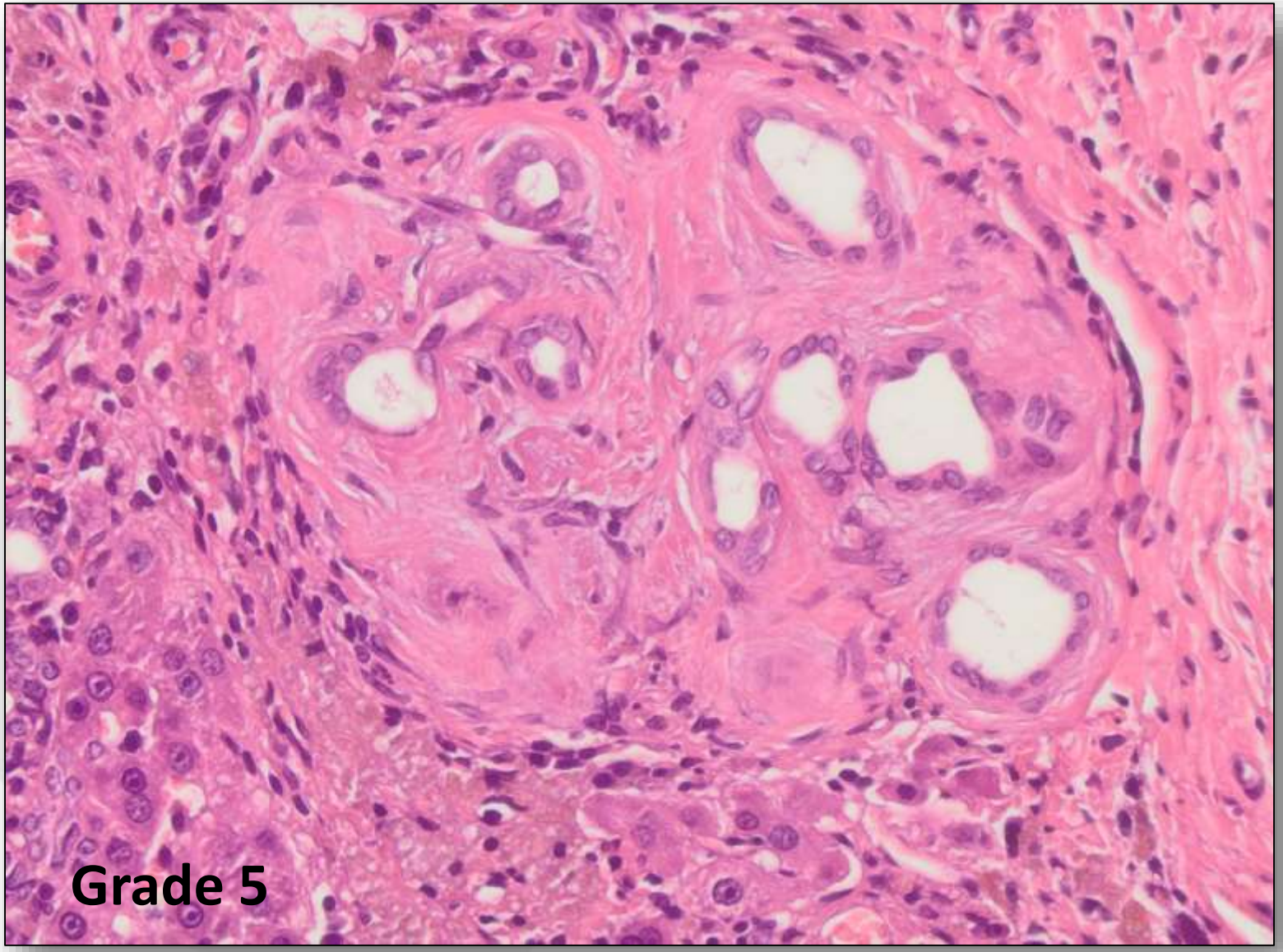
Proposal: Gradings, Example by Measurement



Proposal: Gradings, Example by Measurement



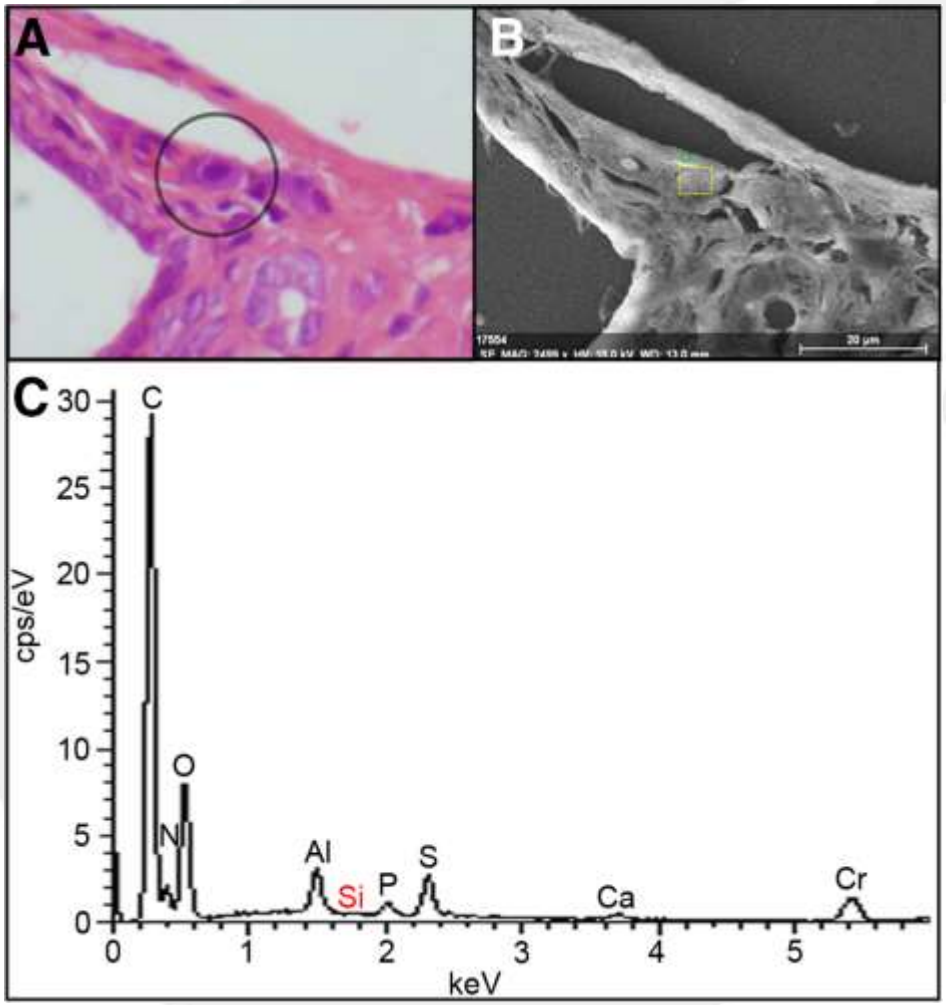
Proposal: Gradings, Example by Measurement



Silica Uptake and Organ Weights

- Analysis of silica uptake in the liver did not show significant differences except for the low dose.
- It may be expected that organ weight would be changed if silicon accumulates. However, absolute organ weights have been reported only in Table S4. When calculating relative organ weights, no difference can be established for liver, kidney and spleen. In contrast, this calculation reveals even lower organ/body weight ratios in several cases.

Silica Uptake and Organ Weights



- In 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 Villous Height

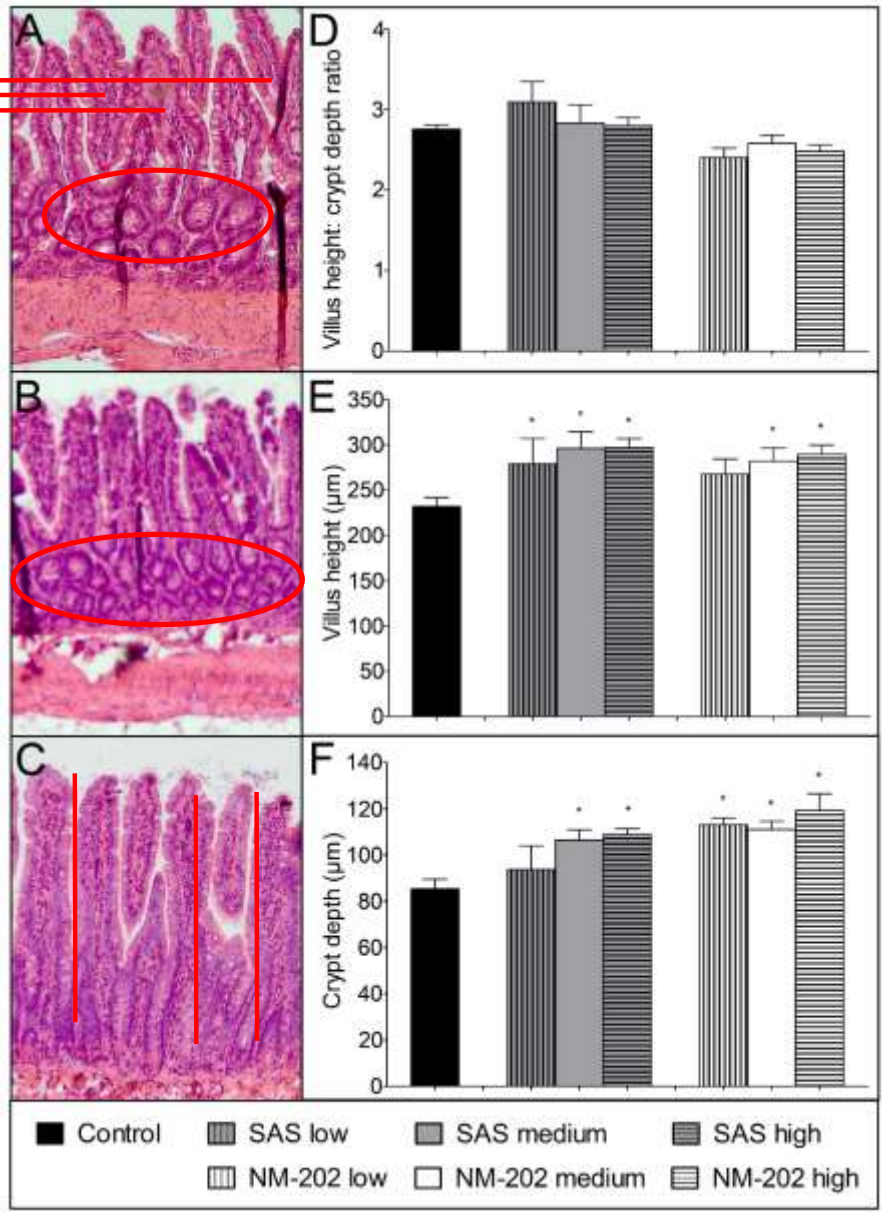
- Authors did not describe their standardization of tissue sampling.
- Height of villi and the depths of crypts are changing in the course of the intestine.
- Plane-of-section artifacts changed the outcome of the image analysis: Figure S3 - compare the areas in A, B, and C.
- In C a sharp demarcated longitudinal section shows the borderline between submucosa and overlying villi, in A and B crypts have been met in a transversal section.
- Comparing A and B, it is obvious that in A several villi been cut in a oblique position.

Villi Height. No Comparison Possible!

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.



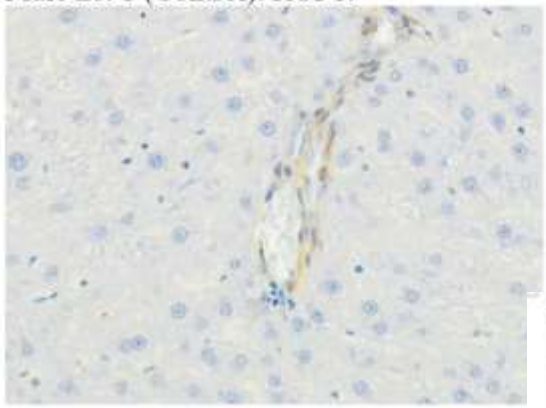
Proof

Previously performed reprotoxicity study with NM-200 underwent additional evaluation:

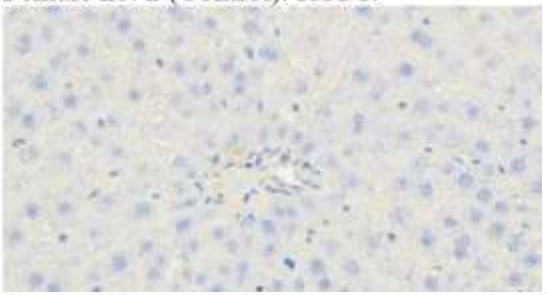
- Check on liver and intestine
- Liver: Sirius Red, Col I-III
- Intestine: Measurements of villus lenght
- Several organs: Si load (EDX)

Of course not: Liver

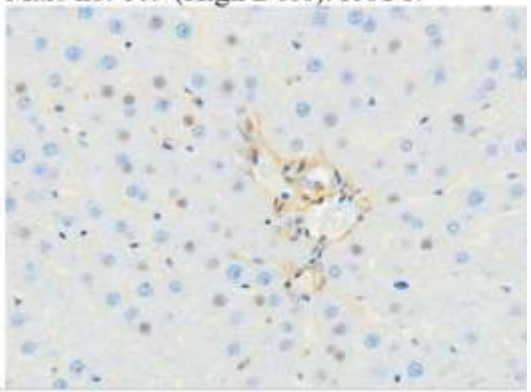
Male no. 1 (Control). ROI 1.



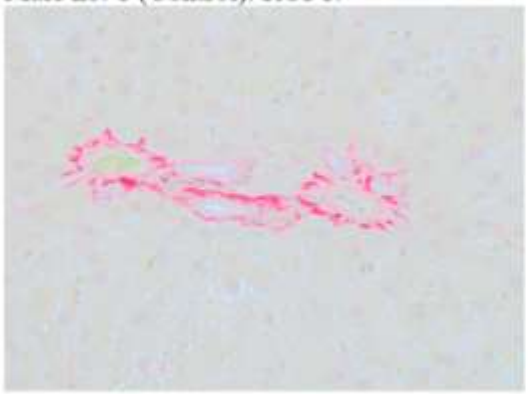
Female no. 2 (Control). ROI 1.



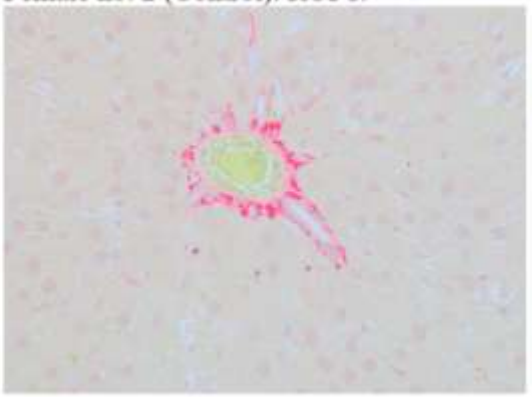
Male no. 169 (High Dose). ROI 1.



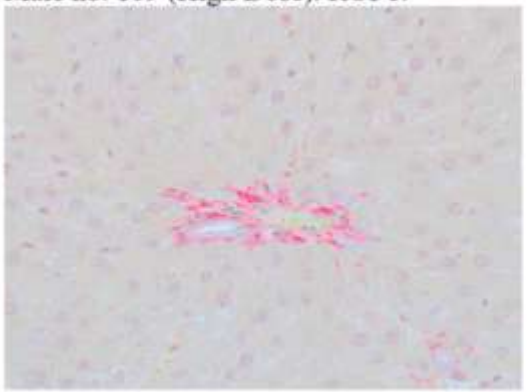
Male no. 1 (Control). ROI 1.



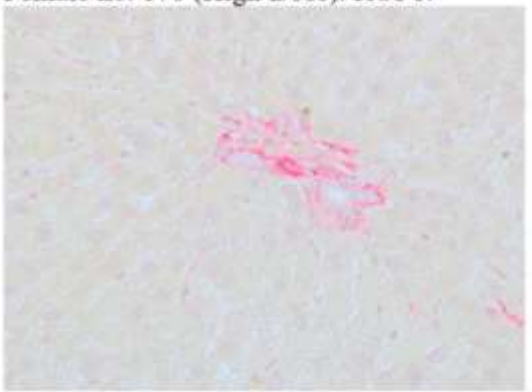
Female no. 2 (Control). ROI 1.



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

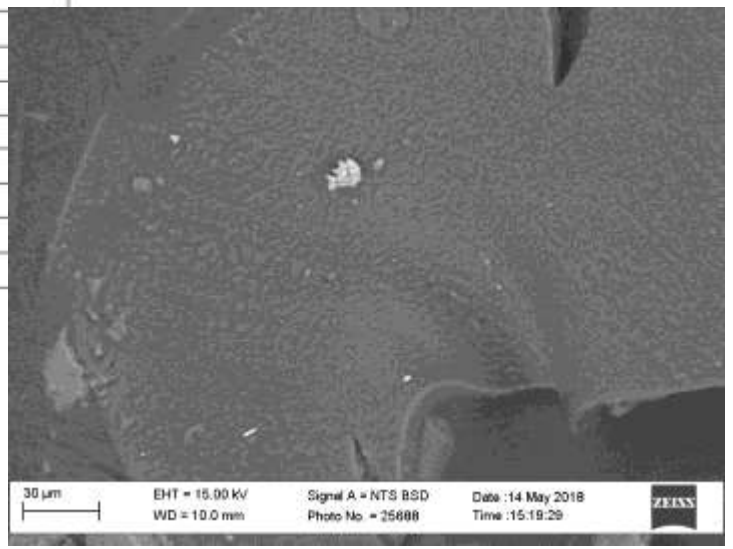


Female no. 170 (High Dose). ROI 1.

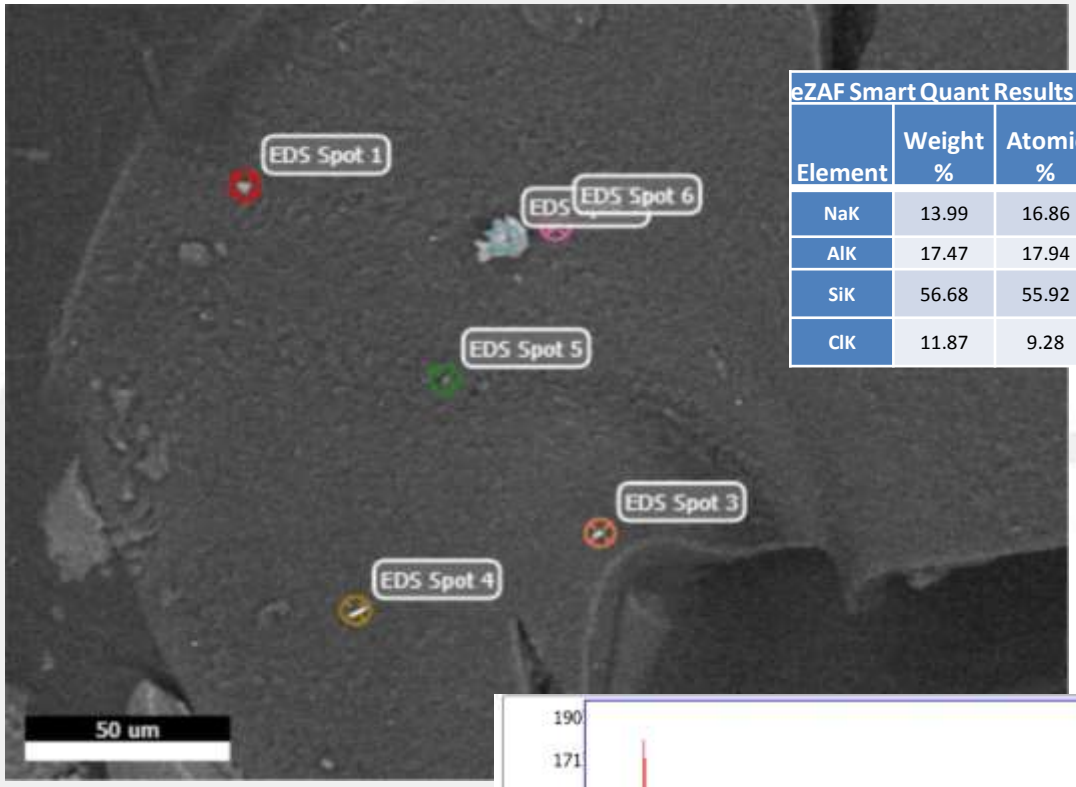


Of course not: Test Item load

Ileum	503_13_02	-		
	503_13_03	503_13_03	N	
	503_13_04	-		
	503_13_05	503_13_05	Y	Feldspar
Ceacum	503_14_01	-		
	503_14_02	-		
	503_14_03	503_14_03	N	
	503_14_04			
	503_14_05	503_14_05	N	
Colon	503_15_01	-		
	503_15_02	-		
	503_15_03	503_15_03	N	
	503_15_04	503_15_04	N	
Stomach, cardia	503_16_01	-		
	503_16_02	-		
	503_16_03	503_16_03	Y	Feldspar
Stomach, fundus	503_17_01	-		
	503_17_02	-		
	503_17_03	503_17_03	N	
	503_17_04	-		
	503_17_05	503_17_05	N	
Stomach, pylorus	503_18_01	-		
	503_18_02	-		
	503_18_03	503_18_03	N	
	503_18_04			

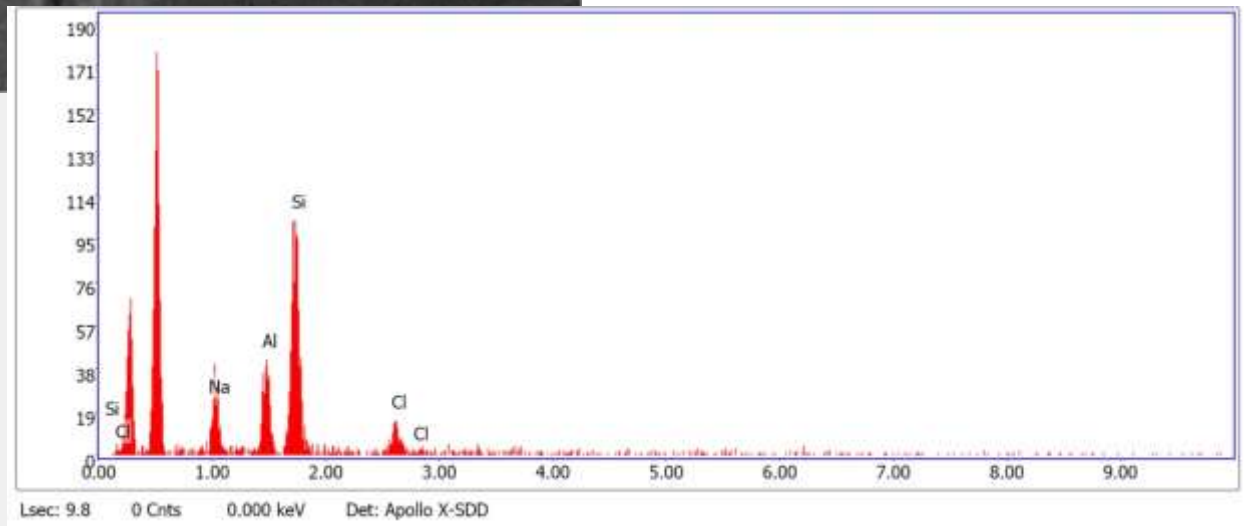


Of course not: Test Item load



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

$Na[AlSi_3O_8]$



Other Examples…

- Use of material from previously performed studies, e.g. material from reprotoxicity studies:
  F1-generation tissue material can be used in to order avoid new OECD 443 studies etc., e.g.,
  - application of measurements according to Garman et al. (2013) and special staining to avoid additional neurodevelopmental studies
  - enhanced histopathology of immune organs according to Elmore (2012)

Garman RH, Li AA, Kaufmann W, Auer RN, Bolon B (2016). Recommended Methods for Brain Processing and Quantitative Analysis in Rodent Developmental Neurotoxicity Studies. Toxicol Pathol. 44:14-42. Toxicol Pathol. 2012; 40(2): 148–156.

Elmore SA (2011). Enhanced Histopathology of the Immune System: A Review and Update. Toxicol Pathol, 40: 148-156.

Guidance on Nanotechnologies…

- If there is no quick degradation, follow from steps 2a onwards.

Questions:

- Is a 90-day study necessary? Or is a carfully designed 28-day study sufficient?
- Recovery groups are not recommaned but should be optional to the manufacturer
- Detection option for absorption might be an issue (e.g., carbonanotubes consist of approx. 98-99% Carbon only. Currently no well defined detection method is established.)

Guidance on Nanotechnologies…

- If there is no quick degradation, follow from steps 2a onwards.

Recommandations:

- Sampling and preservation of a full organ list
- Histological evaluation might be limited to selected organs, but local lymph nodes (e.g., mesenteric lymph nodes) and Peyer's patches should be included.
- For immune organs (including GALT and MALT), the STP described enhanced immune system evaluation might be considered as a tool on HE-stained sections
- Special stains (e.g., Masson's Trichrome) for intestinal mucosa and lympathic organs in order to detecd fibrosis
- Pathology evaluation is a key point. A Peer Review should be obligatory.

Guidance on Nanotechnologies…

- Step 3

Recommandations:

- An orderly performed 28 or 90-Day study might be a waiver for follow up studies (reproductive toxicology, cancerogenicity)
- Several in-vitro or ex-vivo models might elucidate further questions

Summary

- Particle uptake via intestine is possible mainly by M-cells into GALT possible for particles <10 µm (nanoparticles up to nanoparticle aggregtaes) (otherwise by endocytosis)
- Uptaken amount very limited:
  depending on concentration, diet, chemical surface, and prolonged transit times
- Solubility of particles causes resolution before inflammatory processes can start (no reports for Peyer' patches or mesenteric lymph nodes)
- Some publications very doubtful or wrong
- Own studies did not confirm previously reported results