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Short Communication

Synthetic Amorphous Silica in Food: Findings about "Liver Fibrosis" and Other Study-Related Findings in Van der Zande *et al*. (2014) are Questionable

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Abstract

Van der Zande., *et al.* [1] administered two synthetic amorphous silica to male Sprague-Dawley rats via food for 29 days (high dose groups up to 84 days). They concluded "an increased incidence of liver fibrosis after 84days of exposure", "increased height of jejunal villi" and silica accumulation in the spleen and kidneys.

The definition and determination of liver fibrosis is dubious. The silica uptake in spleen and kidneys has not been elucidated and even the term 'accumulation' is unclear. The published results of the statistical analysis cannot be reproduced. The study design did not follow OECD guidelines. The authors used an unusual range of 5 nm to 200 nm to define nano-particles. We recommend, first, repeating the tissue evaluation based on a reliable liver fibrosis definition and performed by an experienced pathologist and/or organizing an independent pathology working group; and, second, reanalyzing the findings with appropriate statistical procedures.

Keywords: *Synthetic Amorphous Silica; SAS; E551; Liver Fibrosis; Food Additive; Van der Zande., et al. 2014*

Introduction

Synthetic amorphous silica (SAS) is a form of silicon dioxide (SiO2) that is intentionally manufactured. SAS has been used in a wide variety of industrial and consumer applications including food, cosmetics and pharmaceutical products for many decades. Solid SAS are used as adsorbents, fillers, thickening agents, anti-caking agents, emulsion stabilisers, free-flow agents and carriers in a variety of industrial and consumer products, including pest control, pharmaceuticals, cosmetics, and food and feed products [2].

SAS is produced by thermal (pyrogenic) or wet (precipitated, incl. gel) processes. In the initial particle formation step, primary particles with dimensions below 100 nm are formed by nucleation, coagulation and coalescence. These primary particles form indivisible units by covalent binding, the aggregates that have external dimensions typically above 100 nm. They are fused together with no physical boundaries among them. The aggregates combine to form agglomerates in the micron size range by physical attraction forces (van der Waals and H-bridges). According to the EU Recommendation Definition [3] for "nanomaterial" 'particle' means a minute piece of matter with defined physical boundaries. 'Aggregate' is defined as a particle comprising of strongly bound or fused particles, 'agglomerate' means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components. An identifying feature of SAS powder is that it is on the market as micron-sized agglomerated aggregates. SAS has been produced and marketed for decades without significant changes in its physical-chemical properties [2,4].

Due to its morphology, SAS is affected by the evolving discussion about nanomaterials. No harmonized definition of nanomaterials, however, exists worldwide although many regulatory, industry and non-governmental organizations have their own definitions of nanomaterials. The International Organization for Standardization (ISO) developed ataxonomy of terms and definitions for nanomaterials [5-9]. ISO defines nanomaterials as a "*material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale*". Nanoscale is defined as a size range from approximately 1 nm to 100 nm. Nanomaterials comprise nano-objects and nanostructured materials. The identifying feature of nanostructured materials is that their internal or surface structure is in the nanoscale, but their external dimensions are greater than the nanoscale. The definition is covered by Working Draft document ISO/WD TS 80004-2 (former ISO/TS 12921) [7]. Pyrogenic and precipitated SAS are nanostructured materials according to the ISO definition with an internal structure in the nanoscale, the primary particles. These primary particles do not exist in the products in isolation, but as aggregates/agglomerates, with external dimensions above 100 nm [2,4]. Mean aerodynamic diameters of SAS were estimated to be about 200 µm based on analyses of the product as delivered see Table 12 in ECETOC [4]. Further descriptions of production processes and use of SAS are published [2,4].

The EU Commission has issued a recommendation on the definition of the term "nanomaterial" under regulatory aspects as a reference for determining whether a material should be considered as a nanomaterial. The Commission recommended to adopt this as an overarching classifier for all EU based regulations. The definition is based solely on the size of the constituent particles of the material, without regard to hazard or risk. A nanomaterial as defined in this recommendation should consist of 50% or more of particles by number size distribution having a size of 1 nm to 100 nm. The definition also includes particles in agglomerates or aggregates whenever the constituent particles, i.e., in the case of SAS the primary particles, are in the size range 1 nm to 100 nm. SAS is considered a nanomaterial under the current EU Commission Recommendation definition. Image analysis by transition electron microscopy confirms this conclusion [2,4].

SAS is widely applied in food products within the EU as a food additive (E551). SAS is permitted for application in food for decades. Triggered by the evolving discussion about nanomaterials new toxicological studies were launched to assess whether SAS in food is a health hazard to consumers. In the recent and potentially influential study of van der Zande., *et al*. [1], two SASs (identifiers: "SAS" and "NM-202") were administered to male Sprague-Dawley rats via food for 29 days, with additional administration of the high dose groups up to 84 days. The authors concluded that their study "showed an increased incidence of liver fibrosis after 84-days of exposure" and an "increased height of jejunal villi".

These findings reported by van der Zande., *et al*. [1] are questionable. In the following we comment on the characterization of the materials applied, the design of the study, the definition and determination of the endpoints, and the statistical analysis performed.

Material Characterization

Nanosize range

One major problem in the current "nano" discussion is a missing harmonized definition of "nano" [10]. The size range 1 nm to 100 nm has been issued by the ISO Technical Committee 229 "Nanotechnologies" [6] and is also used under regulatory aspects e.g. [3]. Therefore, the 5 to 200 nm size range described and used as the "nano-size range" in van der Zande., *et al.* [1] is not in accordance with these conventions and is misleading (cp. Section 2 in [10]). If applied, it needs a specific justification which was not given by the authors.

Terminology

Van der Zande., *et al.* [1] reported on two materials: NM-202 and "SAS". We use "SAS" to refer to the second specific substance tested by van der Zande., *et al.* [1] and distinguish this from SAS as the usual abbreviation for synthetic amorphous silica. Both materials are hydrophilic, pyrogenic synthetic amorphous silicas (SASs) and applied as food additives (E551). NM-202, supplied as a typical SAS to the OECD Sponsorship Programme for testing of manufactured nanomaterials, has been characterized by the manufacturer on relevant physical-chemical endpoints as requested under the OECD Sponsorship Programme [11].

Silanol groups of the test materials (IR Spectra)

Hydrophilic synthetic amorphous silicas, like pyrogenic SAS, bear process-related surface silanols (≡SiOH). The authors clearly indicated the absence of any band at a wave number of 3750 cm-1 (typical for isolated silanols) and they reported the absence of peaks at wavenumbers 3200 – 3500 cm-1 (typical forbridged silanols) in both spectra (NM-202, "SAS"). These data are incompatible with results from testing the original material NM-202 as summarized in Heinemann [11] and are also incompatible with the analysis of "SAS" [12]. We conclude that the material NM-202 has been either wrongly characterized or the material has been modified during storage and handling. Basic findings on silanol groups of synthetic amorphous silica are given inIler [13], Barthel [14], Mathias and Wannemacher [15].

Carbon content

Van der Zande *et al.* [1] Reported a prime facie surprisingly high carbon content of the "pristine" test substances (2.9% for NM-202; 2.1% for "SAS"). In addition, Table 2 shows that FTIR detected some C-H stretching vibrations around a wave number of 3000 cm-1. NM-202 and "SAS" are produced at temperatures above 1,000°C. Both substances are not "after-treated" with e.g. organic compounds. Therefore, they are no hydrophobic SASs.

Pyrogenic hydrophilic SASs typically contain ≥ 99.8% silica, with alkali and heavy metals in the low ppm range [4]. NM-202 has been analyzed concerning carbon content according to DIN ISO 10694 after different storage times [16]. The carbon content was always < 0.1% (Table 1). Barthel [14] characterized hydrophilic pyrogenic silica and mentioned a carbon content of 0.03% which confirms the data summarized in Table 1. The carbon content of "SAS" was also determined according to DIN ISO 10694and found to be< 0.1% [17]. We like to emphasize that the measurements discussed in this paragraph are defined on the mass scale which is the usual way to report content percentages.

Substance	Batch	Date of manufacture	Carbon content [%]
NM-202	VA76557	18.01.2013	< 0.1%
NM-202	VA77179	25.12.2013	< 0.1%
NM-202	VA77224	20.01.2014	< 0.1%
NM-202	VA77234	05.02.2014	< 0.1%

Table 1: *Carbon content of different batches of NM-202 (HDK® N20) with different period of storage [16].*

Van der Zande., *et al*. [1] cited Rasmussen., *et al*. [18] as having performed the carbon content measurements for NM-202. Rasmussen., *et al*. [18] described the measurement procedure on p.29: "The XPS analysis provides information on the surface composition (down to a depth of 10 nanometres) of the analysed material. The detection limit of the method is about 0.1% percent of the atoms (at%)." In brief, this means that van der Zande., *et al.* [1] reported on surface percentages but not on mass percentages. The difference is not made clear and discussed in van der Zande., *et al* [1]. This may mislead a reader to erroneously believe that the original materials are contaminated and the manufacturers' reports and publicly available Technical Data Sheets are not correct. We recommend that analytical results should always be reported in a transparent way to avoid potential misunderstandings. In addition, we note that XPS measurements performed by an accredited testing facility on "SAS" (as produced) resulted in a carbon content of 0.8% of the atoms (at %) [19]. The discrepancy between these measurements and the results reported by van der Zande., *et al.* [1] may only be explained by carbon contamination during sample preparation and/or material storage.

Analyses and report of particle size distributions

The authors report on size distributions in the feed matrix and intestinal content [1] (Table 2). The particles size findings determined with the cited method HDC-ICP-MS require to be cautiously interpreted, because the data deviate significantly from findings using AF-4, TEM or DLS techniques [11,18]. We note that the material was sonicated twice before the analyses were done [1, p.13]. Thus, the size

distribution of the material may have been changed by this procedure. The tables should be checked for errors; the authors reported in Table 2 that 106% of the material in the intestinal content showed a particle size between 5 nm to 200 nm in the medium exposure group. This cannot be true because the maximum percentage possible is 100%.

Material	Severity* of fibrosis	Length of bootstrap	Number of realizations	Two-sided p-value
"SAS"	≥ 1	11000	10185	8.05 %
"SAS"	≥ 2	22000	10368	10.17 %
NM-202	≥ 1	11000	10356	0.64 %
NM-202	≥ 2	22000	11058	7.74 %

Table 2: *Re-analysis of fibrosis incidence data given in Table S9 in [9]. Two-level non-parametric bootstrap of random effects logistic regression models. The percentile-t-method (asymptotic refinement) was used to calculate two-sided p-values based on a non-symmetrical, equal-tailed test.*

**Severity degrees: ≥ 1 means "at least very mild", ≥ 2 means "at least mild". There were no degrees observed > 2 (mild).*

Design of the repeated oral dose study

The described sub-chronic oral study does not meet the minimum requirements of the current OECD Test Guideline 408 "Repeated Dose 90-day Oral Toxicity Study in Rodents" [20] concerning duration of exposure (90 d) and number of animals (10 per group per sex). If toxicological effects are expected, a satellite group should be included in the test which was not considered. The limit test dose is defined as 1000 mg/kg/d in OECD 408 [20].

Group size

The number of animals of the test groups is too small in comparison to OECD Guideline 408 [20]. Van der Zande., *et al*. [1] studied only five rats per group. This means that results may be unstable, suffer from a sparse data bias [21] and are difficult to interpret.

Duration of exposure

No rationale is given for the shortened duration of the longest exposure period (completion after 84 days instead of 90 days requested by OECD) [20]. Again, this may complicate interpretations.

Chosen doses

The highest "SAS" dose was chosen 2.5 times higher than the limit test dose (1000 mg/kg/d) mentioned in OECD 408 [20]. In contrast, for NM-202 the highest dose was set at the maximum test dose of OECD 408[20]. The authors justified the excess dose of 2500 mg/kg/d by referring to a previously reported LOAEL at 1500 mg/kg/d in Dekkers., *et al*. [22] who referred to So., *et al* [23]. This means, however, that in this cited review and in the referenced study the maximum dose of the OECD Guideline (1000 mg/kg/d) was also not considered. We note that the maximum dose of 1000 mg/kg/d was adopted by OECD to exclude unspecific effects at extreme doses and to follow animal welfare guidelines. Furthermore, the application of extreme doses in toxicity studies, as applied in the study of van der Zande., *et al.* [1], may cause unrealistic overload phenomena that need to be considered.

Definition and determination of endpoints

Severity of fibrotic changes in the liver

In the INHAND publication for rodents, 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 [24]. In this recent INHAND publication, gradings were not discussed with the exception of cirrhotic changes representing a severe degree. The method section of the publication by van der Zande., *et al.* [1]

does not provide a reference or standard for the definition of the 6 fibrosis severity categories that have been applied by the authors. Thus, it is unclear how the decision of the observers may be reproduced when they graded the severity of fibrosis into the 6categories. In particular, the distinction between adjacent categories like "not remarkable" (0) and "very mild" (1) or "very mild" (1) and mild" (2) remain unclear. Does the term 'not remarkable' indicate that no change was seen at all, or does it indicate a subjective upper threshold assigned by the evaluating pathologist? Anyhow, a grading system based on a scale of fibrosis severity grades 1 to 5 should be based in the first instance on the recognition of normal, i.e., normal existing connective tissue need to be graded as 0.

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" [25]. This is even more important, when subtle changes are the focus of the investigation as in van der Zande., *et al.* [1], where the main findings about fibrosis were described as 'very slight'.

A possible evaluation scheme could be:

Fibrosis severity grade 1 – more than the normal contents of portal connective tissue up to doubling of the structures within n_1 (number) evaluated periportal fields in n_2 (number) evaluated sections per liver from lobes A, B, C, etc.

Fibrosis severity grade 2: connective tissue in defined fields 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) .

Fibrosis severity grade 3: in addition to features listed for Fibrosis severity grade 2, there is a fine connective tissue bridging between 2 or 3 lobules in the periportal region.

Fibrosis severity grade 4: connective tissue bridging between 4 to 6 lobules in the periportal region.

Fibrosis severity grade 5: larger bands of connective tissues bridging diffusely the liver lobules.

We like to emphasize that the presented evaluation scheme is our suggestion how a grading of the severity of liver fibrosis may be defined. Other examples for gradings are published [24]. Importantly, no definition was provided by van der Zande., *et al.* [1]. Thus, the evaluation and findings as reported on in Table S9 and Figure 6C in van der Zande., *et al*. [1] remain dubious.

Furthermore, the areas evaluated should be defined. In Figure 5F in van der Zande., *et al*. [1] a larger vein and portal arteries are shown associated with connective tissue. This, however, represents the normal condition. In Figure 5E, the anatomical situation seems to represent a periportal Glisson's triad with bile ducts and blood vessels surrounded by connective tissue. This also should be classified as normal.

Unfortunately, the staining method was not described by van der Zande and co-workers. The stain is obviously not hematoxylin and eosin but it could be Sirius red (Figures 5E and 5F in van der Zande., *et al*. [1]; Figure 1).

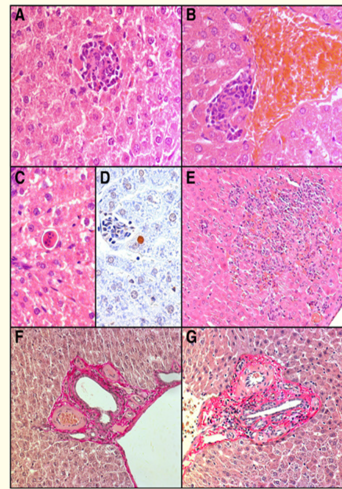

Figure 1: *Figure 5 van der Zande., et al. [1].*

(A) and (B): The inflammatory cells represent normal findings in most control rat livers.

(C) and (D): The single cell apoptosis is an indicator of hepatocellular turnover and represents a normal background finding in the rat liver.

(E): Small foci of hepatocellular necrosis are often observed in young adult control rats.

(F) and (G): The staining, although not indicated, is most likely Sirius Red. This is a method to stain collagen fibers. The amount of collagen fibers in the Glisson's triad as shown in both figures represents the normal anatomical situation.

Nevertheless, the amount of connective tissue shown in Figures 5E and 5F in van der Zande., et al. [1].

Original text (van der Zande., et al. [1]):

Histological images of livers from animals treated with SAS or NM-202 for 28 or 84 days. (A, B) Light microscopic images of an inflammatory granuloma after 84-days of exposure for (A) SAS high dose (magnification: 200x), and (Bi 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 (6) NM-202 high dose (magnification 100x).

Nevertheless, the amount of connective tissue shown in Figures 5E and 5F in van der Zande., *et al.* [1](Figure 1 in present paper) is unexceptional for typical 13-week studies of Hsd: SD rats (and other strains). Even a higher amount of connective tissue maybe noted. The latter observation is usually related to peribiliar fibrosis, a normal change related to ageing. Photographs at a lower magnification would allow the reader to compare and evaluate the findings on a larger area. Unfortunately, selected areas without presenting any overviews are no reliable illustrations. Photographs from control rats and slides categorized as "not remarkable" (grade 0) were not shown but would be helpful. Furthermore, in Table S9, five controls showed an incidence of fibrosis. It would be of value to include also photographs from the respective lesions from a control animal with fibrosis and without fibrosis.

A quantitative analysis of lymphocytic cells, inflammatory granulomatous foci, and apoptotic cells did not reveal differences between controls and treated animals. However, Figures 5 A-C in van der Zande., *et al.* [1] (Figure 1 in present paper) are titled "Histological images of livers from animals treated with "SAS" or NM-202 for 28 or 84 days". The title misleads the reader and makes him/her believe that the lesions presented and described as inflammatory granuloma and apoptosis are induced lesions. Inflammatory cell foci as shown in these pictures (Figures 5 A-Cin van der Zande., *et al.* [1]; Figure 1 in present paper) are, however, present in rat livers from any strain in 80-100% of control animals [26,27]. Similarly, a single apoptotic cell represents an indicator of normal turnover and may be found in any control animal too. The inflammatory cell foci are considered to be part of the clearance mechanism. Also, small foci of necrosis as shown in Figure 5E in van der Zande., *et al.* [1] (Figure 1 in present paper) are not unusual in the liver and are reported in up to 50% of

the control animals [26]. The absence of inflammatory or degenerative effects induced by the test items is supported by the data generated in Table S8 and S10 by van der Zande., *et al* [1]. In Table S8, even statistically significant differences for IL-2 are driven only by marginal numerical values. By its physiological function for differentiation of B- and T-cells, these values are deemed to be within the range of normal variation. IL-10 revealed higher values in control and NM-202 treated animals. However, IL-10 is deemed to be involved in processes of immune tolerance induction and hence, a supportive role for this interleukin in the process of liver fibrosis is unclear. In contrast, increased IL-10 values may be considered protective to liver fibrosis [28] Also, gene expression data in Table S10 do not support any pro-inflammatory effect by treatment.

It is no contradiction that in previous studies inflammatory and degenerative processes in the liver were described when using other routes of administration (i.v. and i.p.). An intravenous or intraperitoneal administration of nanomaterials is simply not comparable with an oral ingestion. These differences are well known since decades and have been described and explained for SAS in the scientific literature [29-35].

Analysis of silica uptake in the liver did not show significant differences except for the low dose. The authors explained this puzzling phenomenon by the gelating behavior of the silica.

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.

It was not reported which measures were taken to avoid a contamination of the liver tissue with intestinal contents. Based on our own experience such a contamination can even cause EDX analysis to show diatomeae in liver tissues [Weber, unpublished data]. Of note, EDX-SEM did not reveal any silica contents in the liver according to van der Zande., *et al* [1]. In Figure 7A in van der Zande., *et al.* [1] (Figure 2 in present paper), 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. The identity of these cells was not evaluated by any further histological method. Whatever the nature of this cell is, Figure 7C n van der Zande., *et al.* [1] (Figure 2 in present paper) does not show any peak for silica.

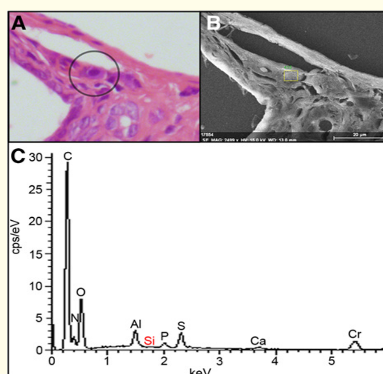

Figure 2*: Figure 7 van der Zande., et al. [1].*

In Figure 7A, a cell is shown that have been annotated as a macrophage. It is unclear why this cell has been termed '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.

Original text (van der Zande., et al. [1]):

Histological and electron microscopical images from the Ijver. (A) Light microscopic image of a macrophage (indicated by the circle) in liver tissue from an animal treated with the highest dose of SAS for 84 days, and (B) a corresponding SEM-EDX graph of the same macrophage (indicated by the rectangle) in which (C) the elemental composition was analyzed.

In summary, all facts described and shown from the liver sections in van der Zande., *et al*. [1] are not conclusive for any induced inflammatory and fibrotic change in the liver. No silica deposition was confirmed in the liver. The gene expression data showed significant differences, but van der Zande., *et al.* [1] stated that '"the observed differences ...were low". Therefore, the conclusion of an induced fibrosis in the liver by uptake of synthetic amorphous silica is deemed to be obsolete.

Villi of jejunum

Regarding the differences noted in the jejunal villi height, the authors did not describe their standardization of tissue sampling. This is in fact extremely difficult for this very long intestinal segment. It is known that the height of villi and the depths of crypts are changing in the course of the intestine. It is possible that plane-of-section artifacts changed the outcome of the image analysis. Similar circumstances have been reported under other conditions [36]. The suspicious cutting plane artifact is supported by the examples presented in Figure S3 in van der Zande., *et al*. [1] (Figure 3 in present paper) when comparing the areas in A, B, and C. Whereas 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 have been cut in an oblique position. It is therefore not comprehensible how these data have been generated. Furthermore, it need to made clear to the readership, if an increased proliferation could be proven to avoid a misinterpretation of normal tissue vs adaptive changes vs. proliferative changes.

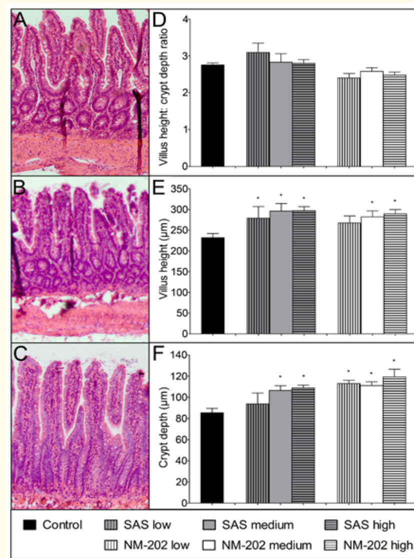

Figure 3: *Figure S3 van der Zande., et al. [1].*

Figures S3A and S3B represent oblique cuts through the mucosa. It is easily recognizable due to the transversal cuts through the crypts. Figure S3C represents well cut villi. The measurement was performed from the bottom of the crypts up to the of the villi.

Original text (van der Zande., et al. [1]):

Quantitative histopathological evaluation of jejunum from animals treated with SAS or NM-202 for 28 days. Cross-sections of jejunum (H&E stained) of (A) control, (B) SAS low, and (C) NM-202 low exposed animals, at 2.5x magnification, showing villi and crypts. Effect of the different treatments (mean ± SEM; n = 5) on (D) villus height: crypt depth ratio, (E) villus height, and (F) crypt depth. The villus height is significantly increased in all groups versus the control, except for the NM-202 low group. The crypt depths are significantly increased in all groups except for the SAS low group. Since both the villus height and the crypt depth increased in almost all groups there were no significantly differences in the ratio between the villus height and crypth depth. An increased villus: crypt ratio, in combination with an increased villus height suggests a well differentiated, active tissue. Abbreviations: SEM: standard error of the mean. * Significant difference vs. the control ($p < 0.05$)".

Silica in other organs

It was noted by van der Zande., *et al*. [1] that after 84 days of exposure with "SAS", there were clearly elevated silica levels in the spleen, whereas the exposure to the highest dose of NM-202 for 84 days did not result in accumulation of silica in any of the examined tissues. Histopathological assessment of the kidneys and spleen showed no differences between the treated animals and the controls; the detailed data had not been provided.

We believe, however, it would be fair to show these data as well. For example, after absorption from the intestine, the silica in the spleen could have been transported by macrophages. In such case, the pathologist should have evaluated the presence of histiocytes in the spleen. These cells could be increased in number or bearing particles in the cytoplasm or could be foamy type macrophages etc. Because of the suspected accumulation of silica in the tissues, the question should be answered why SEM-EDX was not applied to splenic tissues.

Similarly, it is of great interest to understand where the silica is present in renal tissues. It is important to know whether silica particles are excreted and hence are present in the primary urine but not being stored in the tissue, or if the silica is really present and stored in any renal cells. Unfortunately, urine analysis was not reported on.

Pathology review procedures

The authors counted all observations with "very mild degree of fibrosis" as positive in their analyses. Unfortunately, they dropped this important characterization in the abstract and described the finding simply as "increased incidence of liver fibrosis". This shortened endpoint description may mislead readers to believe that definite signs of fibrosis were documented and analyzed.

The authors did not report whether one observer evaluated all the slides or whether various observers made decisions on different slides or if a peer review was performed. How were the observer(s) trained to report the results reliably, i.e., was an experienced toxicologic pathologist present at evaluation? Is he/she experienced with the rat strain and its background alterations? We highly recommend undertaking an official retrospective peer review by an experienced toxicologic pathologist, according to current accepted standards [37]. In case of doubt on the study outcome, a pathology working group (PWG) could be organized by an independent group of pathologists, e.g., Experimental Pathology Laboratories Inc. who published the outcome of PWG results elsewhere [36].

Statistical Analysis

The main outcome of the study is described in the abstract as an increased incidence of fibrosis in the liver among the highest exposed animals ("SAS": 1000 mg/kg/d and NM-202: 2500 mg/kg/d; 84 days of exposure) in comparison to unexposed control rats. Table S9 in van der Zande., *et al*. [1] presents the individual data for all animals studied (5 rats per exposure group, 10 slides per rat). Fibrosis data "were analyzed by logistic regression using a binomial distribution between 0 and 10" [1, p.17]. Results are shown in Figure 6C in van der Zande., *et al*. [1] (Figure 1 in present paper), p. 10 on fibrosis of at least degree 1 (very mild) and the authors report:" ... the occurrence of periportal fibrosis in the liver ... was significantly increased in the NM-202 treated animals (p = 0.021), as compared with the control animals (Figure 6C in van der Zande., *et al*. [1] (Figure 1 in present paper); Additional file 1: Table S9). In the "SAS" treated animals the presence of fibrosis appeared to be increased too, but this was not significant (p = 0.073)." We tried to reproduce this statement with validated software [38] but failed. The reported p-values appear to be wrong or the data reported in Table S9 are wrong. The p-values can neither be reproduced with conventional logistic regression (which the authors probably used) nor with exact logistic regression to account for small numbers [39]. In addition, we note that the analysis strategy chosen is inappropriate: the study was designed and exercised as a two-level experiment with slides (level 1) clustered within rats (level 2). In contrast, the authors analyzed the data as if all slides were independent. One way to overcome this drawback is to collapse the information within rats. If we do so and analyze whether the rats show at least one positive slide (≥ degree 1 = "at least very mild", ≥ degree 2 = "at least mild") the differences between exposed and control rats were always far from being significant, even if exact logistic regression was applied. To analyze the data more appropriately in terms of a two-level experiment and to deal simultaneously with the small number of rats we additionally performed a hierarchical non-parametric bootstrap [40] on random effects logistic models two-level regression [41]. We summarize the main results in Table 2.

No significant effect of "SAS" on very mild or mild effects of fibrosis could be identified. NM-202 showed an association with very mild fibrosis but no clear effect on mild fibrosis (we note that about 50% of the models did not converge). In addition, the basic two-level analysis of "SAS" showed a pronounced variance of effect estimates across rats. i.e., the model showed that animals reacted differently (heterogeneity of effect).

Discussion

Unfortunately, it is no rare observation that p-values published in the peer-reviewed literature are wrong [42]. The paper of van der Zande., *et al.* [1] seems to be another example. This sheds doubt on the scientific reliability of this research. No effects could be seen in an exact analysis on the rat level, i.e., after collapsing the data within rats as usually done in toxicology. Two-level regression analyses, combined with bootstrap procedures indicated some associations with NM-202 exposure (assuming that Table S9 correctly reports the findings of the experiment which is in doubt).

The findings reported in Table S9 and Figure 6C in van der Zande., *et al.* [1] (Figure 1 in present paper) rely on a correct assessment of the endpoint studied. To assure reliability of the endpoint determination, randomized slides should be independently and blindly re-categorized by an experienced pathologist using a standardized reading procedure and a well-defined categorization of fibrosis severity. It is a major drawback of the van der Zande., et al. investigation [1] that no definition was presented how to categorize liver slides into the 6 grades of fibrosis severity they proposed. In addition, the published pictures and the accompanying data are not convincing because this material does not prove any fibrotic change related to the SAS ("SAS" and NM-202) exposure. We emphasize that rat studies should follow the instructions of OECD Guideline 408 to avoid sparse data problems and discussions about inappropriate dosing. This is of particular importance if the intention of such work is to re-evaluate or replace already existing official risk assessments.

We note that our observation appears to be no outlier. Our critical assessment of van der Zande., *et al.* [1] is in line with the overall finding of Prof. Krug who reviewed the literature of the last 10 to 15 years to establish whether an evaluation is possible for human toxicological end points of engineered nanomaterials: "Although only a couple of hundred papers had appeared on the topic of 'Nanomaterials: environmental and health effects' before 2000, this number has exploded to over 10,000 since 2001. Most of these studies, however, do not offer any kind of clear statement on the safety of nanomaterials. On the contrary, most of them are either self-contradictory or arrive at completely erroneous conclusions" [43]. We agree with Brock's statements who asked for a strengthening of the peer-review process [44].

Van der Zande., *et al.* [1] stated: "Since the original SAS exposure studies, performed in the 1980's, no oral *in vivo* study has been reported in the public domain." This statement is not correct as SAS has been investigated under the CEFIC Long-range Research Initiative (LRI) Nanotechnology Testing besides inhalation toxicity and mutagenicity experiments also in tests with oral application according to current OECD Testing Guidelines and under specific considerations of nano aspects applying the Principles of Good Laboratory Practice (Project N1-FRAU: Tiered Approach to Testing and Assessment of Nanomaterial Safety to Human Health: 28 d oral study (OECD Testing Guideline 412); N3-TNO: Testing and Assessment of Reproductive Toxicity of Nanomaterials: Prenatal developmental toxicity study in Wistar rats (OECD Testing Guideline 414); Two-generation reproduction toxicity study in Wistar rats (OECD Testing Guideline 416) [45,46]. In these tests, no systemic and no reproductive toxicity has been observed, respectively. Such data which can be made available after request should be considered before further testing is initiated in parallel [10].

It seems that very high doses, even above the maximum test dose mentioned in OECD 408 are necessary to see toxicological effects after oral SAS exposure- if any reliable and non-physiological changes exist at all. This is in line with results from previous studies with SAS [4] which clearly indicate the low order of toxicity of synthetic amorphous silica after oral exposure. One major outcome of an OECD 408 test is the definition of a No Observed Effect Level (NOAEL). If all the aspects mentioned above are considered we do not see that the study by van der Zande., *et al.* [1] generated any reliable evidence against the view that for both test substances the highest dose ("SAS": 2500 mg/kg/d; NM-202: 1000 mg/kg/d) can be considered as a NOAEL. We strongly recommend, first, repeating the tissue evaluation

based on a scientific standard, reliable liver fibrosis definition and performed by an experienced pathologist and/or organizing and independent PWG; and, second, reanalyzing the findings with appropriate and reliable statistical procedures before the study may be used in any risk assessment. This recommendation is in line with the more general statement made by Dr. Maynard when he discussed the study by van der Zande., *et al*. [1]: "Based on the available evidence, fumed silica in food is acceptably safe. It may be that future research leads to a re-evaluation of its safe use. But it would at this point be irresponsible to question its use" [1].

Conflict of Interest

Evonik Resource Efficiency GmbH and Wacker Chemie AG produce SAS.

Authors' Contributions

PM structured the paper and performed the statistical analysis. AB and MH drafted the passages about NM-202, NK about "SAS". KW drafted the section on the methods of pathology.

Appendix

The following five references may be received freely to the interested reader contacting the corresponding author:

AQura. Test report No. A140016150. 2014
AQura. Test report No. A140016199. 2014
AQura. Test report No. A140018861. 2014

Heinemann M. Technical report: OECD Sponsorship program for the testing of manufactured nanomaterials, nanomaterial: Silicon dioxide (SiO2); Co-sponsor: CEFIC ASASP sector group, BIAC; contribution by the Co-sponsor on physical-chemical properties and material characterization. 2010

WACKER. Internal test report, HDK® Carbon analysis according to DIN ISO 10694/internal test specification PV09522, 07.03.2014. 2014

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