

Amorphous Silica Producers (ASASP). JF (PQ Corporation), MK (Wacker Chemie AG), NK and TBS (Evonik Resource Efficiency GmbH), JN (Grace Europe Holding GmbH), J-AS (Solvay), and DS (Pittsburgh Plate Glass Company) produce synthetic amorphous silica.

## SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary References.

1. Boudard D, Aureli F, Laurent B, et al. Chronic oral exposure to synthetic amorphous silica (NM-200) results in renal and liver lesions in mice. *Kidney Int Rep.* 2019;4:1463–1471.
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**The Authors Reply:** In this response, we reply to the concerns raised by Weber *et al.*<sup>1</sup> on our recently published article (Boudard *et al.* 2019)<sup>2</sup> that provided evidence that chronic oral exposure to a form of synthetic amorphous silica (SAS) used as the food additive E551 (i.e., precipitated silica as NM-200) results in renal and liver lesions in mice. The letter criticizing our article, authored by an



industry consultant (pathologist) and co-authored by representatives of several SAS manufacturers, claims deficiencies in the study design and other methodological issues that, according to their judgment, would jeopardize the overall conclusions of our study. An itemized reply to their criticism is given hereunder.

## STUDY DESIGN

### Selection of Strains

Weber *et al.* argue that the C57BL/6 mouse line may not be the first choice for long-term studies because male individuals have been found to develop, at least to a certain extent, liver tumors and renal diseases as their age increases.<sup>1</sup> Of course, functional losses and appearance of neoplastic or non-neoplastic lesions in aging animals are inevitable in all mouse lines, similar to what is observed in humans. The pattern of disease susceptibility with age differs mainly because mice are more prone to cancer<sup>S1</sup>; however, neoplastic lesions were not the focus and have not been observed in our study. Our selection of C57BL/6 mice, *females* rather than males, was based on more than 20 years of experience in long-term studies using this mouse line. For specialized investigations aiming at comparing normal aging with slow neurodegenerative process, we have developed an ability to deal with aging animals, optimizing and standardizing experimental conditions in which groups of female mice could be followed for 1 to 3 years.<sup>S2,S3</sup> In our experience, abnormal occurrence of liver or renal lesions in these aging female mice, even in very elderly individuals, can be definitely ruled out. The same holds true for the other mouse line introduced more recently and directly derived from the C57BL/6 line, the C57BL/6S line solely differing for the absence of alpha-synuclein expression.

Our study<sup>2</sup> confirmed that it is possible to breed these types of animals for 18 months without apparent morbidity and spontaneous deaths in the control groups. The key point that Weber *et al.* fail to appreciate is that the kidney and liver lesions documented in our study were observed solely in exposed animals. This rules out that selection of the mouse line played any role in the finding. That having been said, it would be interesting to reproduce the experiment with other rodent lines and we hope that the scientific community will be encouraged to do so by our study.

### General Study Design

#### *Silica Content of Water and Diet*

Weber *et al.* complain about the lack of information on the silica content in the drinking water and animal diet

and the associated background silicon exposure.<sup>1</sup> However, as discussed hereunder (see the section “Silica Content in Different Organs”), the deposition resulting from this background exposure was characterized, because tissue silicon concentrations of control animals were analytically determined in the study. Therefore, there is no reason for concern here. On the other hand, the key point is that whatever the silica content in the water and diet was, the very same conditions were applied to both control and exposed animals, the only difference being for the latter addition of SAS to the drinking water. Therefore, exposed and control animals experienced the same background exposure. We reiterate here that histopathological findings were seen solely in exposed animals (irrespective of the mouse line).

### Design of the Study

Our study was designed following high-level standards and according to the principles of good laboratory practice, with the aim to investigate long-term exposure to a dose relevant to the estimated dietary intake of SAS in humans and identify potential hazards focusing on key target organs. The impetus for such a study was the fact that existing regulatory toxicological studies used high dose levels that make them questionable for human risk assessment of SAS exposure because of the physicochemical changes SAS undergoes at these levels and associated decreased bioavailability.<sup>3</sup> The necessity to test lower doses and long exposure times guided the study design, along with the reduction of the number of animals used to a minimum on the basis of our expertise in successfully dealing with studies of long duration without excessive expansion of the number of animals tested.

Weber *et al.* complain about lack of clarity in daily SAS intake estimation.<sup>1</sup> However, this is clearly explained in the [Supplementary Methods](#) (“Estimation of mouse daily intake”) and, as stated there, is based on the average daily water intake of 1.5 ml/10 g body weight in mice.<sup>2</sup> The mean administered dose over the entire experiment duration was then estimated to be 4.8 mg SiO<sub>2</sub>/kg body weight per day based on the actual weight of individual mice.

## PATHOLOGY EVALUATION

### Missing Animal Data

As stated in the article, no mortality was observed in the C57BL/6 mouse lines until mice were killed at 18 months of exposure. On the contrary, mortality was observed in the TgHuA53T mice as described in detail in the [Supplementary Material](#), affecting males more than females. In absence of mortality for the C57BL/6 mouse lines, Weber *et al.* complain about the lack of

results for organs other than kidney and liver.<sup>1</sup> In fact, necropsy was performed on all mice under strict clean room conditions and several organs were collected, including brain, spinal cord, gut, and spleen, and fixed in 10% buffered formalin solution for potential subsequent analyses. However, the primary objective of our study was to deal with kidney and liver as key target organs involved in detoxification and elimination and closely associated with potential adverse effects resulting from oral exposure.

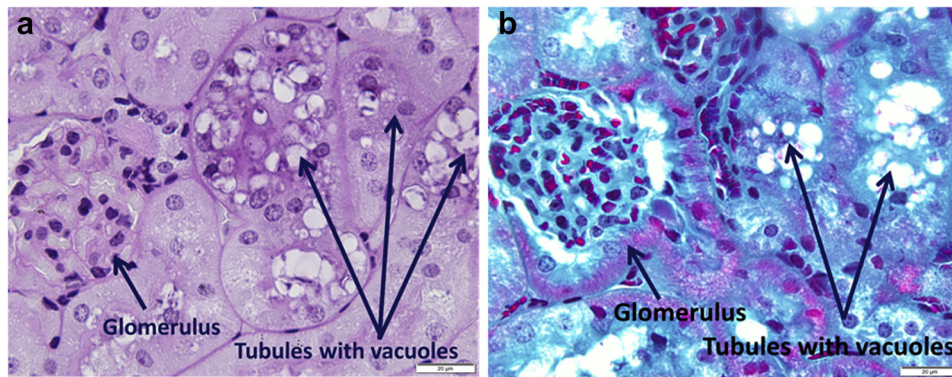
Weber *et al.* also complained about an apparent discrepancy in the number of mice analyzed as reported in different figures and tables.<sup>1</sup> Because we kept the number of animals in the study as low as possible, whenever feasible, tissues from the same animal were shared among the laboratories performing different analyses. In other words, at necropsy, the organs were divided to allow a maximum of analyses (e.g., 1 kidney was cut into 2 parts to allow different preparations from fresh materials compared with fixed embedded tissues). Some kidneys and livers were devoted to the determination of silica content. The selection of the organs/their portions for different uses was random. This explains why the number of samples is variable among different analyses.

About the controls, as clearly stated in the Methods section of the main article, they consisted of groups of females of each mouse line ( $n = 7$  and  $n = 8$ ) housed at the same time and in the same room as the exposed groups. They were called age-matched controls. On the other hand, at the histopathological level, we thought it was interesting to add material from younger individuals of control mice (unexposed to NM-200), namely 6-month old females ( $n = 7$  and  $n = 9$ ). There is nothing obscure in that, and in the Results it is stated “No morphological abnormalities were noted on young adult or age-matched control mice. . . .”<sup>2</sup>

Weber *et al.* additionally complain about the different age of mice of the transgenic line at the start of the experiment (2 months old compared with 3 months old in C57BL/6 mouse lines).<sup>1</sup> The difference depends on the specificities of this mouse line, which expresses the human mutated (A53T) alpha-synuclein protein triggering a programmed neurodegeneration leading to death at approximately the age of 12 to 14 months. Therefore, in this transgenic mouse line, the impact of 3-, 6-, and 9-month exposure to silica was studied on young animals (8 weeks old,  $n = 13$ , male and female) compared with the age-matched controls ( $n = 10$ , male and female unexposed transgenic mice).

### Evaluation

Weber *et al.* complain about the 3-grade level lesion score used and express their preference for a 5-grade



**Figure 1.** Kidney from a TgHuA53T mouse after 6 months of exposure to NM-200, view at 1/100 in oil. (a) Periodic acid–Schiff staining. (b) Trichrome staining. Arrows indicate glomerulus and tubules. The presence of vacuoles of different sizes is seen solely at the level of focal proximal tubes. The glomeruli are not damaged. Bars = 20 µm.

system.<sup>1</sup> We applied the scores commonly used in the classification of kidney diseases (LED, Oxford, Banff) combining accurate qualitative analysis and semi-quantitative analysis to score lesions from 0 to 3 (grade 1 for 0%–25% lesions, grade 2 for 25%–50% lesions, and grade 3 for >50% lesions). We agree that different choices are possible on this specific aspect and recognize the validity of other alternatives.

### Renal Changes

An experienced pathologist carried out the analysis and found no specific lesions in the controls, neither in the aged nor in the youngest, in any of the 2 C57BL/6 lines as well as in the transgenic mouse line. That was not the case of the exposed groups, in which lesions could be observed. We are sorry that this outcome disappoints Weber *et al.*

### Vacuolar Changes

Weber *et al.* are right indeed in noticing that in Figure 2h there are no visible vacuoles.<sup>1</sup> However, the silver staining of Figure 2h allows analyzing the basal membranes to eliminate the atrophic or necrotized tubes (no thickened membrane, no rupture), and it is not the appropriate coloration to observe with a suitable definition vacuoles into tubular epithelial cells. In addition, vacuoles are present on all kidneys but with focal presence.

On the other hand, [Supplementary Figure S4](#) clearly shows in the transgenic mouse line, with the use of the periodic acid–Schiff staining, the presence of vacuoles of different size at the level of focal proximal tubes, which is suggestive of toxic damage. The glomeruli are, however, not damaged. We also made this observation using a trichrome staining that shows the same and a more accurate view at 1/100 in oil, which is shown here in [Figure 1](#).<sup>2</sup>

### Glomerular Changes

Weber *et al.* admit that C57BL/6 mice are relatively resistant toward developing glomerulosclerosis,

proteinuria, and hypertension, but argue that a number of age-related glomerular changes are still possible.<sup>1</sup>

Indeed we are very aware that the number of permeable glomeruli decreases with age. A segmental and focal glomerulosclerosis appears, resulting in the destruction of glomeruli, which takes a typical appearance of fibrohyaline glomeruli associated with some interstitial fibrosis lesions and atrophic tubes. This physiological impairment was considered as the basal state of the kidney in aged individuals, for both controls and exposed mice. However, we did not identify fibrohyaline glomeruli in the aged control mice analyzed in our study.

### Amyloidosis

Weber *et al.* argue that amyloidosis is an aged-related background lesion that some studies were able to quantify at >80% in C57BL/6 males, although such evidence was not accompanied with the demonstration that the amyloidosis was of the serum amyloid A (SAA) type. In our study using solely female mice, all the aged controls were negative; the only amyloidosis case was observed in an exposed mouse and could be typed as an SAA case.

Weber *et al.* claim that the urine test used for transgenic mice was useless, as control animals were not monitored. As clearly shown in [Supplementary Table S4](#), all 10 control animals were monitored in the same way as the exposed animals until the end of the study (9 months). In addition, the differences observed between exposed and control mice could be statistically analyzed and turned out to be significant. As stated in the [Supplementary Material](#): “The rate of 1 g/L revealing a clear proteinuria was more frequent in exposed mice (38 to 75%) compared to controls (0 to 43%) for the available data collected between 3rd and 6th month of exposure ( $P < 0.001$ ).”<sup>2</sup> Therefore, criticism about this complementary analysis is not understood.



## Liver Changes

Weber *et al.* complain about absence of liver results in the main document.<sup>1</sup> Because renal lesions were considered as more serious and more marked than liver lesions, and because renal lesions following SAS exposure are less documented, our study was submitted to a scientific journal dedicated to kidney. The journal enforces a strict policy in terms of word limit requirements, and it is ultimately the editor (and the reviewers) who decides what goes in the main text and in the [supplementary file](#).

The experienced pathologist, specialized in liver examination, who carried out the analyses reported all the lesions observable in liver with hematoxylin and eosin staining. Further analysis using specific markers could not be performed, except SAA staining that confirmed the type of amyloidosis observed in exposed mice. There was no overinterpretation about other lesions, despite the outstanding observation in a C57BL/6S mouse that displayed steatotic-like cytoplasmic vacuolization (that Weber *et al.* also found notable).

## Changes in Transgenic Mice

Weber *et al.* focus on vacuolar changes observed in the transgenic mouse line and share their interpretation as lipid inclusions in mesangial cells.<sup>1</sup> [Supplementary Figure S4D](#) illustrates a mouse of this group (number 12 in [Supplementary Table S4](#)): as illustrated at high magnification in [Figure 1](#) of this response, vacuoles are present in only a few tubes, glomeruli are normal; there are no lipid inclusions within the mesangial cells, erythrocytes are seen within the glomerular capillaries, which is not pathological. The same is found for kidney of mouse number 13 of [Supplementary Table S4](#), as illustrated by [Supplementary Figure S4C](#).

Weber *et al.* complain about the low number of animals tested<sup>1</sup>; however, the number of animals evaluated is not unusual compared with the vast majority of *in vivo* studies, and we have already explained our choice to avoid overuse of experimental animals. They complain also about the alleged lack of clarity of the status (i.e., control or exposed) of the animals found dead. However, all the details are given in the [supplementary data](#), in [Supplementary Table S4](#), as well as in the text: “Several cases of unexpected death were observed (6 males and *only in the exposed group* [emphasis added]). These animals died before the 6th month of exposure except one that died at 7 months and one week of exposure with a 1 g/L proteinuria.”<sup>2</sup> These premature deaths were seen in animals exhibiting high proteinuria (regularly detected >1 g/L), which suggests a possible link to kidney alteration, but without conclusive evidence.

We, however, wish to thank Weber *et al.*, because further examination of [Supplementary Table S4](#) led us

to identify an incorrect value for the proteinuria of the mouse number 12, which remarkably reached 3 g/L during the sixth month of exposure. The correction in [Table S4](#) has been published separately.<sup>2</sup>

## Silica Content in Different Organs

Similar to the criticism on the other sections of the article, in their letter Weber *et al.* raise a number of questions that easily find their answer in the article itself and in the associated [Supplementary Material](#). Once these questions are addressed one by one, the issues themselves dissolve and the same happens for the overall criticism.

The first question they pose is “How much Si is normally present in the organs of control animals?”<sup>1</sup> This background silicon concentration depends on a number of factors, the most important being the silicon level and speciation in the diet and in the drinking water given to animals and the associated silicon intake. This point is actually meaningless because the true question is “Was the silicon content in kidneys and livers of the specific animals used in the study as controls (i.e., not exposed to NM-200) characterized?” This silicon background level of nonexposed animals was analytically determined in the study and, whatever the magnitude was, kidney and liver silicon concentrations of NM-200-exposed animals were compared with those of control, nonexposed animals (“Silica Deposition in NM-200-Exposed Mice” and “[Figure 1](#)”).<sup>2</sup>

The second question Weber *et al.* pose is “What could be the source of silicon in organs?”<sup>1</sup> and argue that inductively coupled plasma mass spectrometry (ICP-MS) analytical determination, being “nonsubstance-specific,” cannot clarify the source of silicon found in animal tissues. First, Weber *et al.* do not mention that ICP-MS/MS detection as used in the study is a highly sensitive, state-of-the-art element-specific analytical approach, which enabled, along with other experimental conditions (clean room laboratory, use of ultrapure reagents, analytical quality control), accurate measurement of silicon levels in organs of both NM-200 exposed and control animals. What ICP-MS/MS cannot do, is to discriminate soluble silicon from particulate silica (including manufactured silica, i.e., SAS). Based on available data,<sup>S4</sup> background dietary silicon from animal feed and drinking water comprises naturally occurring soluble silicon (orthosilicic acid and associated silicon-containing species with high bioavailability) and, mainly in solid food, some polymeric and particulate silica originating from natural sources or, in some cases, also from SAS occasionally used as an additive in animal feed. On the top of this dietary silicon background, treated animals were exposed to SAS as NM-200 through drinking water for 18 months. SAS exposure resulted in

higher tissue levels, measured as total silicon, detected in the livers of C57BL/6 and C57BL/6S mice (not statistically significant) and in kidneys of C57BL/6 mice (statistically significant) (Figure 1).<sup>1</sup> Irrespective of the statistical significance of the observed differences with controls, the biological significance of the finding is clear: SAS is known to be absorbed in the gastrointestinal tract (although to a low degree)<sup>3,4</sup> and, being slowly eliminated, accumulates in tissues following long-term exposure.<sup>3</sup> Being that SAS is negligibly soluble to insoluble material,<sup>S5,S6</sup> with a marked biopersistence, it is expected that silicon deposition in NM-200-exposed animals occurred in the form of silica particles.

The doubts and concerns of Weber *et al.* on the controls of the tissue distribution study are difficult to understand. The number of kidney and liver samples analyzed for the 2 NM-200-administered mouse lines and the controls are clearly stated (Figure 1).<sup>2</sup> It was dependent on the biological material available, considering that tissues from the same animal were shared among the laboratories performing the different analyses to keep the number of animals used as low as possible, and not all tissues from all animals happened to be available for all analyses. Silicon background levels in unexposed mice of the 2 lines did not show statistically significant differences, and thus these specimens were gathered to form the control group that was compared with the NM-200-exposed groups of the 2 lines. Although recognizing that this detailed explanation did not survive the harsh word limit requirements of the journal, we do not see here room for complaining about “limitations of the data,” as do Weber *et al.*

## CONCLUSIONS

In this reply, we demonstrated that the concerns of Weber *et al.* about the methodology of our research are unjustified and their criticism of the outcome of the study has no sound scientific foundation.

They maintain that the applied dose was “very low” and fail to appreciate evidence from previous nanotoxicology studies on SAS,<sup>3</sup> which highlighted that the use of “low,” realistic dose levels (i.e., close to exposures associated with actual use levels of SAS as food additive) is indeed key for assessing the risks associated with E551 long-term oral exposure. Existing evidence demonstrates that the use of unrealistically high doses leads to changes in the physicochemical properties of the material altering its toxicological behavior.

It is indeed worth noting that a recent guideline 90-day oral toxicity study on pyrogenic SAS (NM-203) identified adverse effects on the same target organs of the present study (i.e., liver and kidney) and at the same exposure range (Tassinari *et al.* 2019).<sup>5</sup>

We conclude that long-term studies at relevant doses are critical to address the potential risks for human health arising from daily lifelong dietary exposure to SAS. The design of our study was fit for this purpose and the results warrant further, targeted studies to characterize the dose-response relationship for the observed adverse effects.

## SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Table S4.

Supplementary References.

1. Weber K, Debraise N, Franklin J, et al. Letter regarding “chronic oral exposure to synthetic amorphous silica (NM-200) results in renal and liver lesions in mice”. *Kidney Int Rep.* 2020;5:550–554.
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