Dear Danish Working Environment Authority,

Thank you for the opportunity to comment on the evaluation from the Quality Committee of the report: Zinc Oxide. Scientific basis for setting a health-based occupational exposure limit. We are pleased to note that the Quality Committee agrees with the major decisions in our risk assessment, including selection of critical effect, selection of a threshold approach and the suggested long-term OEL.

Below, we respond to the points raised by the Quality Committee. Each rebuttal is provided in italics just below each comment from the Quality committee.

Yours sincerely Niels Hadrup, Anne Thoustrup Saber, Nicklas Raun Jacobsen, Pernille Danielsen, Sarah Søs Poulsen, Karin Sørig Hougaard and Ulla Vogel,

June 9, 2021.

NRCWE's response to the points raised by the Quality Committee

Each rebuttal is provided in italics just below each comment from the Quality committee.)

Short report from Danish Working Environment Authority´s (AT) Occupational exposure limit quality committee. Evaluation of the report: Zinc oxide: Scientific basis for setting a health-based occupational exposure limit.

Members of the quality committee: Nellie Anne Martin (Miljøstyrelsen); Anoop Kumar Sharma (DTU Fødevareinstituttet); Mette Lausten Hansen, (Arbejdsmedicin AUH); Jesper Bo Nielsen (Institut for Sundhedstjenesteforskning, SDU); Vivi Schlünssen (NFA)

This report is based on an online meeting 23th April 2021 headed by AT, where the results from the report were discussed after the authors presented the content of the report- The members of the quality committee had the chance to ask questions to the authors.

The Report: Niels Hadrup, Anne Thoustrup Saber, Nicklas Raun Jacobsen, Pernille Danielsen, Sarah Søs Poulsen, Karin Sørig Hougaard and Ulla Vogel. Zinc oxide: Scientific basis for setting a health-based occupational exposure limit. The National Research Centre for the Working Environment (NFA), Copenhagen 2021. ISBN 978-87-7904-378-7

Erratum: Page 18, Figure 1. No explanation for the star symbol – significance level? The resolution of Figure 1 figure (and also Figure 2 and 3) could be improved.

Page 17: Consider to remove "other metal oxides" from the subheading – the section is only dealing with papers on CU and Zn.

Response: Thank you for pointing out these inconsistencies. It is now stated that the stars indicate statistically significantly increased levels as compared to 0 mg/m^3 in fig 1. 'Other metal oxides' has been removed from the subheading. The quality of figures 1-3 has been improved.

Overall evaluation of the report

The report reviews data relevant to assessing the hazards of ZnO particles in humans and animals. Furthermore, toxico-kinetics and mechanisms of toxicity are reviewed, and previous risk assessments of

ZnO are summarized. The scientific basis for setting an occupational exposure limit (OEL) are presented and based on this, the authors suggest a health based OEL for ZnO.

In general, the report is well written with a clear structure and easy to follow. The Committee recommend more consistency with regards to evidence level. It is of importance to know whereas "no or limited evidence" is due to missing data or due to studies indicating no effect. As an example it is stated page 30, last line: "No evidence of mutagenicity was found".
Response: Thank you! In general, we have not commented on missing data, so no or conclusions regarding limited evidence should in general be understood as the available studies showing no effect or conflicting results. The only exception to this is for mutagenicity, where the current working group notes that we find evidence of genotoxicity, but no evidence of mutagenicity, where we were unable to identify studies addressing mutagenicity. Thus, concerning mutagenicity, the statement pertained to both to limited evidence and to a study with no effect. We now updated the text with additional knowledge from the EU report from 2004 that we already had included elsewhere in the report [1]. This means that we now pinpoint the number of negative and a few positive mutagenicity studies.

In the EU report, we identified 12 in vitro mutagenicity studies that were not included in the first version of the report. These studies were six studies in Salmonella typhimurium with different forms of Zn (oxide, sulphate, chloride, distearate) that were all negative. This was in line with one study by Gocke et al. that we already reported. In Escherichia coli, one study on Zn (chloride), described in the EU report, gave an ambiguous result.

Concerning eukaryotic cell studies, six additional mutagenicity studies described in the EU report provided the following results: three negative studies (sulphate, distearate, chloride), one weakly positive study (sulphate), and two positive studies (oxide, monoglycerate).

We have now inserted the following text into the report:

"In an EU report from 2004 (EU, 2004), 12 in vitro mutagenicity studies were described. These studies were six studies in Salmonella typhimurium with different forms of Zn (oxide, sulphate, chloride, distearate) that were all negative. In Escherichia coli, one study on Zn (chloride), gave an ambiguous result.

Concerning eukaryotic cell studies, six mutagenicity studies described in the EU report provided the following results: three negative studies (sulphate, distearate, chloride), one weakly positive study (sulphate), and two positive studies (oxide, monoglycerate)."

In addition, our previous overall conclusion on genotoxicity had the following wording

"

Overall conclusion on genotoxicity
After inhalation, one study was positive for genotoxicity while another was negative. After intratracheal instillation, one study was positive for genotoxicity and another was negative in the comet assay but showed effect on DNA damage checkpoint regulation. After oral dosage, three studies were positive and two were negative. One intraperitoneal injection study was positive. There are both in vitro studies suggesting presence and absence of genotoxicity.

Overall, the current working group finds evidence of ZnO-induced genotoxicity in vivo and in vitro. However, the current working group notes the absence of evidence of mutagenicity."

We now changed this wording into:

"Overall conclusion on genotoxicity
After inhalation, one study was positive for genotoxicity while another was negative. After intratracheal instillation, one study was positive for genotoxicity and another was negative in the comet assay but showed effect on DNA damage checkpoint regulation. After oral dosage, three studies were positive and two were negative. One intraperitoneal injection study was positive. There are both in vitro studies suggesting presence and absence of genotoxicity. Concerning mutagenicity, Zn was overall negative in bacterial cells (seven negative studies and one ambiguous study), while in eukaryotic cells there were three negative studies, one weakly positive and two positive studies.

Overall, the current working group finds evidence of ZnO-induced genotoxicity in vivo and in vitro. The current working group notes that, except for two positive studies, a majority of nine in vitro studies are negative for mutagenicity."

The literature search was performed by a research librarian, and details of searched databases and the search strings are included as an appendix in the report.
The vast majority of the included papers deals with welders, and barely any data for other working groups (e.g. workers producing ZnO containing products) is included. If this evidence is not available it would be helpful if this is explicitly stated.
*Response: Metal fume fever was first described in 1822 [2], but it has not been considered as a severe adverse outcome, as the short-term manifestation is transient discomfort. The fact that metal fume fever entails an acute phase response in humans was published in 2016 [3], and the implication that inhalation of ZnO may cause cardiovascular disease is therefore not really considered in the literature before this [4]. Therefore, very few occupational studies were found to report relevant endpoints and included in the report***.**

If there is any available literature on the origin of SAA and CRP (e.g. from the liver) it would be relevant information to include in the report.
Response: In humans, CRP and SAA levels are assessed in blood, and the origin of the proteins in blood cannot be established. Since acute phase protein synthesis is regulated at the level of transcription, increased mRNA levels should be considered as evidence of synthesis in the tissue.
In humans, liver, fat and the appendix have been identified as the major sites of Saa1 and Saa2 gene expression, whereas liver and gall bladder were identified as the major site of Crp expression [5]. However, differential expression of Crp and Saa1 and -2 mRNA levels has also been found in macrophages, fibroblasts and epithelial cells isolated from human lung tissue from COPD patients and smokers [6].
In mice, pulmonary exposure to many different kinds of particles leads to highly increased expression of Saa3, Saa2 and Saa1 in lung tissue, whereas the hepatic response is smaller and with much shorter duration [7, 8]. Saa3 is often identified as the most differentially expressed gene in murine lung following pulmonary exposure to nanomaterials [7]. Furthermore, pulmonary macrophages were identified as the major source of Saa3 gene expression following LPS exposure, whereas fibroblasts were the major source of Saa3 gene expression following exposure to carbon black nanoparticles [7]. Thus, in mice, the lung is a major site of Saa expression in response to pulmonary particle exposure, whereas the liver seems to contribute less. In humans, the available information is sparse, but there is evidence that several cell types in the lung are capable of SAA and CRP expression, but unfortunately, the organ of synthesis of SAA and CRP in humans has not been established for the many different stimuli that cause increased blood levels of acute phase proteins.

We anticipate a large within day and day – to day variability for ZnO (both for ZnO fume and dust), but information about this is not covered in the report. In order to be able to set an evidence based short-

term OEL, literature with this information is needed. Probably many of the papers included in the reference (EU, 2004) and summarised in Table 3 contain information about exposure variability. This information is not included in Table 3. Furthermore, it would be helpful if it was clear from Table 3 whether the documentation is based on measurements or other kinds of exposure information. With regard to existing OELs, Belgium and Finland have a factor 5 between the short term and the 8 hours OEL, and Switzerland and Germany a factor 4. For Denmark a factor 2 is present.
Response: In table 3, there is a column with reasonable worst-case exposure levels, and in addition, a column with typical exposure levels. For both columns, there is an additional column containing the source of this information (measured/analogy/expert opinion). Thus, table 3 provides information regarding typical exposure levels (and in addition, information on typical duration and frequency of exposure), as well as an estimate of high exposure levels (the worst-case exposure levels). Thus, we think that the requested information is to some extend already covered in table 3. The data given in table 3 are exposure levels without using personal protection equipment (PPE). The use of PPE would be expected to reduce the exposure 90% according to the EU report. However, we agree that it would be helpful with better information on current exposure levels.
The current working group is of the opinion that our recommendation for short-term exposure levels should be health-based. As the ZnO-induced acute phase response is dose-dependent with regards to the daily cumulative dose, we would recommend that the short-term OEL is twice the OEL, in order to avoid very high short term exposures leading to higher than expected cumulative deposited doses. Unfortunately, the information underlying national regulatory decisions regarding the factor between short term and 8-hour OEL are not publicly available.

On page 16 (Epidemiological studies of welders). It would be helpful with inclusion of more information on 1) exposure levels and 2) how smoking is taken into consideration (Ibfelt et al., 2010 and Mocevic et al., 2015).
Response: 1) in the Danish study, total accumulated exposure to welding fume particles was estimated using a job exposure matrix based on more than 1000 measurements of ambient air particles in the workplace between 1971 and 1985 made by the Danish Welding Institute and the National Institute of Occupational Health. In the meta-analysis, the athors state that 'the exposure of interest is welding exposure regardless of welded material (soft steel, mild steel, or stainless steel), welding processes (electrode welding or gas shielded welding),and welding intermittence'. Exposure was not addressed or discussed any further in the meta-analysis.
2) In the Danish study, risk estimates were adjusted for smoking. Self-reported information on smoking was collected in 1986. For the meta-analysis, five of nine included studies included information on smoking.

The authors regard acute phase response as the critical adverse effects and argue for a causal association to cardiac disease. The committee agree on this decision, but it is a new approach to use a normal physiological response reaction as the critical effect, as opposed to earlier settings where abnormal physiological responses or disease (e.g. carcinogenicity) has been the critical endpoint. The committee therefore recommend to include a few lines about this new approach in the report.
Response: the working group most respectfully disagrees and notes that many of the Danish OELs are based on airway irritation, which is a physiological and reversible response. In addition, inflammation was identified as the critical effect in several of the previous OEL reports evaluated by the Quality Committee in the recent years (Nanosized titanium dioxide, Carbon nanotubes and Nanosized carbon black). Lastly, we note that the German OEL for ZnO is based on metal fume fever, and we note that increased serum levels of CRP and SAA is now understood to be an integral aspect of metal fume fever.

On page 16 the authors summarise the epidemiological evidence for an association between acute phase response (especially CRP and SAA) and cardiovascular disease. The committee recommend to be less

firm in the conclusions on causality. The epidemiological studies with the strongest design using Mendelian Randomisation to adjust for confounding find that CRP is not causally related to cardiovascular disease. No Mendelian Randomisation studies are available for SAA and cardiovascular disease. The committee agree there is strong mechanistic evidence supporting that the underlying mechanism of action is a SAA-mediated effect on cholesterol transport, but until now this is not convincingly confirmed in the epidemiological literature.
Response: Thank you. The present working group has chosen to put weight on the very strong mechanistic evidence from animal studies showing that all isoforms of SAA are causally implicated in plaque progression [9, 10] by modifying the cholesterol efflux. SAA is highly conserved in mammals and human SAA1 is very homologous to Saa3 in mice.

Mechanistic evidence of these systemic effects are hard to find in humans. HDL-mediated efflux capacity was determined for 2468 participants of the Ludwigshafen Risk and Cardiovascular Health (LURIC) study who were referred to coronary angiography at baseline between 1997 and 2000 [11]. Cholesterol efflux was associated negatively with C-reactive protein (CRP), fibrinogen, interleukin-6 (IL-6), and serum amyloid A. Cardiovascular mortality was higher in patients in the lowest cholesterol efflux quartile. This association was weakened, but not fully abolished, after adjustment for HDL cholesterol.
With regard to studies on Mendelian Randomsation, the current working group was able to identify some relevant studies. Associations between SAA1 rs12218 and risk of CHD in a Chinese case-cohort study, such that C-allele carriers were at increased risk of CHD [12]. In another Chinese study, four single SAA SNPs (rs12218, rs4638289, rs7131332,and rs11603089) were assessed in two independent case–control studies, one of Han Chinese (1416 CAD patients and 1373 control subjects) and the other Uygur population (588 CAD patients and 529 control subjects)[13]. C-allele carriers of rs12218 had higher SAA levels and lower HDL levels in blood and homozygous CC carriers were at higher risk of coronary artery disease in both populations [13]. In another study, Chinese CC carriers of rs12218 had increased Carotid Intima Media Thickness as compared to homozygous wildtype carriers [14].

The authors chose to focus on studies dealing with occupational exposure by inhalation, and the committee support that decision, as inhalation is the major route of exposure for ZnO. We also agree with the notion that a joint OEL for all particle sizes is the adequate approach, as inhaled ZnO particles undergo rapid dissolution at the low pH in lysosomes after cellular uptake.
The authors based the suggested health-based OEL on data from a human experimental study (Monse et al 2018), and the Committee agree on this decision. The evidence from animal studies are quite limited, especially for CRP and SAA.
Response: Thank you! However, we most respectfully disagree with regards to evidence from animal studies, as we think that there is strong mechanistic evidence for the causal implication of SAA in atherosclerosis as described above, and in addition, strong evidence of particle-induced Saa expression at mRNA and protein levels in mice [7, 8, 15].

The authors state the risk assessment methodology follows the guidelines suggested by ECHA (ECHA 2019), and they adequately argue for a threshold level approach.
Setting an occupational exposure limit for ZnO
In setting an occupational exposure limit with acute phase response as the critical endpoint the authors used the NOAEC of 0.5 mg/m3 for increase in SAA and CRP after exposure (Monsé et l 2018, Figure 2 in the report). They corrected this value to an 8 hours working day = NOAEC *4 hour/8 hour = 0.25 mg/m3.
Due to a large inter-individual variation observed in the reviewed biomonitoring studies, the authors use the highest assessment factor for inter-individual variation suggested by ECHA, a factor of 5
This results in a suggested long-term threshold limit value of 0.25 mg/m3/5 = 0.05 mg/m3 ZnO

14. Xie X, Ma YT, Yang YN, Fu ZY, Li XM, Huang D, Ma X, Chen BD, Liu F: **Polymorphisms in the SAA1/2 gene are associated with carotid intima media thickness in healthy Han Chinese subjects: the Cardiovascular Risk Survey**. *PLoS One* 2010, **5**(11):e13997.
15. Poulsen SS, Knudsen KB, Jackson P, Weydahl IE, Saber AT, Wallin H, Vogel U: **Multi-walled carbon nanotube-physicochemical properties predict the systemic acute phase response following pulmonary exposure in mice**. *PLoS One* 2017, **12**(4):e0174167.