National Research Centre
for the Working Environment

Zinc oxide:

Scientific basis for setting a health-based occupational exposure limit

(Zinkoxid:

Videnskabelig dokumentation for en helbredsbaseret grænseværdi)

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Det Nationale Forskningscenter for Arbejdsmiljø, København 2021

Report

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Foreword

A research group at The National Research Centre for the Working Environment has as part of the Nanosafety effort, proposed that the acute phase response is a mechanism for particle-induced cardiovascular disease, underscoring cardiovascular disease as an occupational disease caused by particle exposure (Hadrup et al., 2020; Saber et al., 2014, 2013; Vogel and Cassee, 2018). In 2018, a controlled human study demonstrated dose-dependent acute phase response in healthy human volunteers following inhalation exposure to zinc oxide (ZnO) fumes at air concentrations well below the current occupational exposure limits (OELs) (Monsé et al., 2018; Vogel and Cassee, 2018).

Based on this new evidence, the Danish Working Environment Authority asked the National Research Centre for the Working Environment (NFA) to evaluate ZnO and the possibility to establish a health-based occupational exposure limit for ZnO particles. NFA has made a health-based risk assessment of ZnO and derived a health-based OEL. The health-based OEL will, together with socio-economic considerations, form the basis for the negotiation of an occupational exposure limit for ZnO.

The working group wishes to thank Chief Toxicologist Poul Bo Larsen, DHI, Denmark, for reviewing the report.

Copenhagen, December 2020

Contents

Executive summary

In this report, a working group from the National Research Centre for the Working Environment reviewed data relevant to assessing the hazard of zinc oxide (ZnO). The following chapters were included: human studies (Chapter 2), toxicokinetics (Chapter 3), animal studies (Chapter 4), mechanisms of toxicity (Chapter 5), previous risk assessments of ZnO (Chapter 6), scientific basis for setting an occupational exposure limit (OEL) (Chapter 7), and finally we summarise and suggest a health-based OEL for ZnO (Chapter 8). The focus of this report is only occupational exposure by inhalation. Both ZnO on the nanoform and as larger entities were evaluated in the current document.

Industrially manufactured ZnO is used in sunscreens, cosmetics, food additives, pigments, rubber manufacturing, electronics, agriculture, and antimicrobial products (Burnett and Wang, 2011). ZnO is a powder when in dry form.

Inhalation exposure to ZnO as fumes e.g. from welding induces metal fume fever (Bodar et al., 2005) and acute phase response (Krabbe et al., 2018) in a dose dependent manner, where the acute phase response is induced at lower air concentrations than metal fume fever (Krabbe et al., 2018).

Acute phase response is causally related to ischemic heart disease (Gabay and Kushner, 1999; Hadrup et al., 2020; Thompson et al., 2018; Vogel and Cassee, 2018). There is moderate evidence that welders, who are occupationally exposed to metal oxide fumes, are at increased risk of ischemic heart disease and acute myocardial infarction in a Danish cohort study and in a systematic meta-analysis (which included the Danish study) (Ibfelt et al., 2010; Mocevic et al., 2015).

ZnO has not been evaluated by The International Agency for Research on Cancer (IARC). Zinc is often a constituent of welding fumes, which are classified as Group 1 carcinogen by IARC (IARC, 2018), but welding fumes contain other metal oxides, including metal oxides that are known carcinogens.

The current working group finds evidence of ZnO-induced genotoxicity *in vivo* in animal studies and *in vitro*. However, the current working group notes the absence of evidence of mutagenicity, and absence of chronic inhalation studies or epidemiological studies with cancer as endpoint, where the effect can be ascribed to ZnO. Based on this, the current working group does not consider cancer as a critical endpoint for ZnO.

The current working group therefore considers ZnO-induced increased levels of the two acute phase proteins Serum Amyloid A (SAA) and/or C-reactive Protein (CRP) as the critical effect based on the causal link to coronary heart disease.

Controlled exposure studies with healthy volunteers show that inhalation exposure induces increased blood levels of SAA and CRP 24 hours after exposure. Following repeated exposures, signs of adaptation were observed for interleukin (IL-6), but not for CRP and SAA (Krabbe et al., 2018).

The mechanism of action is considered to be: inhalation of ZnO induces acute phase response that in relation to prolonged and repeated exposure may lead to formation of atherosclerosis and myocardial infarction. Specifically, increased levels of SAA have been causally linked to atherosclerosis in animal studies (Dong et al., 2011; Thompson et al., 2018) and to increased risk of coronary heart disease in epidemiological studies (Ridker et al., 2000).

Inhaled ZnO particles undergo rapid dissolution at the low pH in lysosomes after cellular uptake. Consequently, inhaled ZnO particles do not accumulate in lung tissue after inhalation exposure. ZnO-mediated acute phase response is likely mediated by dissolution-mediated tissue injury. ZnO-induced increased blood levels of acute phase response proteins during a 45-year work-life would provide a causal link to increased risk of atherosclerosis and myocardial infarction.

The current working group notes that the toxicity of ZnO is driven by rapid dissolution of ZnO occurring at the low pH in lysosomes. The rapid dissolution minimises the size-dependence of the observed toxicity (Kim et al., 2016). Consequently, the current working group suggests using the same exposure limit for all ZnO particle sizes.

The study by Monsé et al. was identified as the only study with dose-response relationship for ZnO exposure, but many other studies have studied single dose ZnO exposures or exposures to mixed metal oxides (ZnO/CuO) with similar results. In the study by Monsé et al. (Monsé et al., 2018), 16 healthy, non-smoking volunteers (mean age 26 years) were exposed to nanosized ZnO generated by pyrolysis. SAA and CRP levels were increased in a dose-dependent manner 24 hours post-exposure. SAA and CRP were highly correlated (correlation coefficient (R) = 0.78). Exposure to 0.5 mg/m^3 for 4 hours corresponding to 0.25 mg/m^3 during an 8-hour working day was identified as the No Observed Adverse Effect Concentration (NOAEC).

Due to the very large inter-individual variation (>20 fold) observed in all the reviewed controlled exposure studies and since only healthy volunteers have been studied, the assessment factor 5 (as recommended by ECHA), was used for inter-individual variation.

Thus, the suggested threshold is 0.25 mg/m^3/5 = 0.05 mg/m^3 ZnO, corresponding to 0.04 mg/m^3 Zn, for occupational exposure to ZnO and ZnO fumes.

Dansk sammenfatning

Ved fastsættelse af grænseværdier i arbejdsmiljøet indgår en række hensyn. Det drejer sig om helbredsrisikoen, men også tekniske og samfundsmæssige hensyn.

I NFA's arbejde med grænseværdidokumentation anvendes risikoestimater, som er et teoretisk mål for hvor mange, der ved dagligt udsættelse for stoffet ved grænseværdien efter et helt arbejdsliv (typisk efter 40-45 år) vil blive syge. I disse beregninger, er der *ikke* taget hensyn til personlige værnemidler eller andre kendte foranstaltninger til beskyttelse mod eksponering.

NFA udarbejder dokumentation for helbredsbaserede grænseværdier. Der tages udgangspunkt i publiceret systematisk litteraturgennemgang af epidemiologiske studier, dyrestudier og cellestudier af sammenhængen mellem udsættelse og risiko for forskellige helbredsudfald og de biologiske virkningsmekanismer. På baggrund af dette videnskabelige arbejde beregnes risikoestimaterne.

Dokumentation for helbredsbaserede grænseværdier vil sammen med de tekniske og samfundsmæssige betragtninger ligge til grund for forhandlinger mellem arbejdsmarkedets parter om endelig fastsættelse af grænseværdierne.

I denne rapport har en arbejdsgruppe fra Det Nationale Forskningscenter for Arbejdsmiljø gennemgået den relevante videnskabelige litteratur for at vurdere faren ved udsættelse for zinkoxid (ZnO). Dette er gjort med henblik på at give et sundhedsmæssigt grundlag for at kunne fastsætte en arbejdsmiljømæssig grænseværdi. En grænseværdi er ikke alene fastsat ud fra helbredsmæssige aspekter. Det kan også være en afvejning af sundhedsaspektet i forhold til de tekniske/økonomiske aspekter eller kontroltekniske muligheder.

Rapporten omfatter en gennemgang af humane studier (Kapitel 2), toksiko-kinetik (Kapitel 3), dyrestudier (Kapitel 4), virkningsmekanismer (Kapitel 5), tidligere risikovurderinger af ZnO (Kapitel 6), den videnskabelige basis for fastsættelse af helbredsbaseret risikovurdering til brug for fastsættelse af en helbredsbaseret grænseværdi (Kapitel 7), samt opsummering og forslag til helbredsbaseret grænseværdi for ZnO (Kapitel 8).

Denne rapport fokuserer alene på erhvervsmæssig eksponering ved indånding. Både ZnO på nanoform og i form af større partikler er blevet evalueret i dette dokument.

Industrielt fremstillet ZnO anvendes i solcreme, kosmetik, fødevarer, pigmenter, gummifremstilling, elektronik, landbruget og i antibakterielle produkter (Burnett and Wang, 2011). ZnO fremstår som et hvidt pulver.

Indånding af ZnO røg, e.g. i form af svejserøg, kan inducere metalfeber (Bodar et al., 2005) og akutfase respons (Krabbe et al., 2018), begge dele med en dosis-afhængig sammenhæng. Akutfase respons bliver induceret ved lavere koncentrationer end det der skal til for at inducere metalfeber (Krabbe et al., 2018).

Akutfase respons er kausalt relateret til iskæmisk hjertesygdom (Gabay and Kushner, 1999; Hadrup et al., 2020; Thompson et al., 2018; Vogel and Cassee, 2018). Der findes moderat evidens for at svejsere, som er erhvervsmæssigt udsat for indånding af metaloxid røg, har øget risiko for iskæmisk hjertesygdom og akut myokardieinfarkt. Dette ses i et dansk kohortestudie samt en systematisk meta-analyse (som også inkluderede det danske studie) (Ibfelt et al., 2010; Mocevic et al., 2015).

ZnO har ikke været evalueret af IARC. Zink (Zn) indgår dog ofte i svejserøg, som er klassificeret som kræftfremkaldende for mennesker (Gruppe 1 karcinogen) af IARC (IARC, 2018), men svejserøg indeholder ud over ZnO oftest andre metaloxider, heriblandt kendte karcinogener.

Arbejdsgruppen finder evidens for ZnO-induceret genotoksicitet in vivo i dyrestudier og in vitro. Arbejdsgruppen noterer imidlertid fravær af evidens for mutagenicitet og fravær af kroniske inhalationsstudier og epidemiologiske studier med kræft som endepunkt, hvor effekten kan tilskrives ZnO. Baseret på dette anser arbejdsgruppen ikke kræft som værende et kritisk endepunkt for ZnO.

Arbejdsgruppen anser ZnO-inducerede stigninger i blod-niveauer af de to akutfase proteiner Serum Amyloid A (SAA) og/eller C-reactive Protein (CRP) som værende den kritiske effekt. Dette er baseret på den kausale sammenhæng mellem forøgede blodniveauer af akutfase proteiner og hjertekarsygdom.

Kontrollerede biomoniteringsstudier med raske frivillige forsøgspersoner viser, at indånding af ZnO partikler inducerer forøgede blodniveauer af SAA og CRP 24 timer efter eksponering. Ved gentagne eksponeringer sås tegn på adaptation for interleukin-6 (IL-6), men ikke for CRP og SAA (Krabbe et al., 2018).

Den biologiske virkningsmekanisme er sandsynligvis, at indånding af ZnO inducerer akutfaserespons som ved gentagne eksponeringer øger risikoen for åreforkalkning og myokardieinfarkt. Øgede blodniveauer af SAA forårsager åreforkalkning i dyrestudier (Dong et al., 2011; Thompson et al., 2018) og er forbundet med øget risiko for hjertekarsygdomme i epidemiologiske studier (Ridker et al., 2000).

Efter indånding går de lungedeponerede ZnO partikler hurtigt i opløsning pga. den lave pH i cellernes lysosomer, når partiklerne bliver taget op af makrofager og andre celler i lungen. De indåndede ZnO partikler ophobes derfor ikke i lungevæv ved lungeeksponering. ZnO-medieret akutfaserespons opstår sandsynligvis på grund af vævsskader forårsaget af den kemiske reaktion når ZnO opløses. ZnO-induceret akutfaserespons i form af forhøjede niveauer af akutfase proteiner i blodbanen gennem et 45 års arbejdsliv udgør en kausal årsagssammenhæng til øget risiko for åreforkalkning og myokardieinfarkt.

Arbejdsgruppen bemærker, at ZnO-toksicitet er drevet af høj opløsning ved lav pH. Den relativt store opløselighed ved lavt pH mindsker toksicitetens afhængighed af de

indåndede partiklers størrelse (Kim et al., 2016). Arbejdsgruppen foreslår derfor, at der anvendes den samme grænseværdi for alle størrelser af ZnO partikler.

Et studie af Monsé et al. blev identificeret som det eneste studie som undersøgte dosis-respons sammenhængen for ZnO eksponering, men mange andre studier af enkelt doser eller med eksponering for blandede metaloxider (ZnO og CuO) gav lignende resultater. I det kontrollerede biomoniteringsstudie af Monsé et al. (Monsé et al., 2018), blev seksten raske ikke-rygende frivillige forsøgspersoner (gennemsnitsalder 26 år) udsat for ZnO nanopartikler genereret ved pyrolyse. SAA og CRP niveauer i blod var forøget og udviste dosis-respons sammenhæng 24 timer efter eksponering. SAA og CRP niveauer var tæt korrelerede (R=0.78). Udsættelse for 0,5 mg/m^3 i 4 timer svarende til 0,25 mg/m^3 for en 8-timers arbejdsdag blev identificeret som No Observed Adverse Effect Concentration (NOAEC).

På grund af stor inter-individuel variation (>20-fold) observeret i alle kontrollerede studier, og fordi kun raske forsøgspersoner har været inkluderet, blev en "assessment factor" på 5 (som anbefalet af ECHA) brugt for inter-individuel variation.

Den foreslåede tærskelværdi for ZnO er derfor 0,25 mg/m^3/5 = 0,05 mg/m^3 ZnO, svarende til 0,04 mg/m^3 Zn for erhvervsmæssig udsættelse for ZnO og ZnO røg.

Abbreviations

8-oxo-dG	8-Oxo-2'-deoxyguanosine
BAL	Broncho alveolar lavage
Bw	Body weight
CI	Confidence interval
CRP	C-reactive Protein
Cu	Copper
CuO	Copper oxide
ECHA	European Chemicals Agency
EU	European Union
HDL	High density lipoproteins
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
IARC	The International Agency for Research on Cancer
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IL-6	Interleukin 6
LOAEC	Lowest Observed Adverse Effect Concentration
LOAEL	Lowest Observed Adverse Effect Level
NOAEC	No Observed Adverse Effect Concentration
NOAEL	No Observed Adverse Effect Level
NFA	National Research Centre for the Working Environment
OEL	Occupational exposure limit
PM 2.5	Particulate matter <2.5 μm in diameter
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
R	Correlation coefficient
RR	Relative risk
SAA	Serum Amyloid A
TiO_2	Titanium dioxide
Zn	Zinc
ZnO	Zinc oxide

Introduction

Zinc (Zn) is an essential element that serves as a co-factor in various enzymes in the body. Humans ingest Zn in food supplements and via food. The intake via food, is approximately 10 mg/day (Bodar et al., 2005). Industrially manufactured zinc oxide (ZnO) particles are used in sunscreens, cosmetics, food additives, pigments, rubber manufacturing, electronics, agriculture, and antimicrobial products (Burnett and Wang, 2011). Inhalation exposure to ZnO fumes is seen at the workplace, e.g. during welding, Zn die casting, and brass casting (Bodar et al., 2005).

Forms of Zn includes metallic Zn, ZnO as powders or nanoparticles, and various Zn containing salts. The current report is focussed on ZnO. Selected physico-chemical properties of ZnO are provided in Table 1.

Table 1: Selected physico-chemical properties of ZnO.

ZnO ID and physico-chemical properties		**References**
CAS number	1314-13-2	(ECHA, 2020)
EC number	215-222-5	(ECHA, 2020)
Physical state and colour	The physical state of ZnO is solid powder. Its colour is white. This description is valid for both standard and nano forms	(ECHA, 2020)
Density	The density of ZnO is 5.68 g/cm^3 The density of nano ZnO (uncoated) is reported to be 5.47 g/cm^3	(ECHA, 2020)
Water solubility	2.9 mg/L at 20 °C	(ECHA, 2020)
pH value	6.7 of 2w% suspensions of nano ZnO uncoated prepared in demineralised water	(ECHA, 2020)
Particle size distribution (Granulo-metry) according to ECHA	The D50 of ZnO is 1.05 µm, the D80 is <20 µm	(ECHA, 2020)
Particle sizes in the inhalation studies included in the current report	*Human studies:* 40 nm and 291 nm 60 nm 48-86 nm *Animal studies:* 35 nm 61 nm and >100 nm 50 nm 50-70 nm 48-51 nm 35 and 250 nm 13 nm and 36 nm 50 nm 25 nm	*Human studies:* (Beckett et al., 2005) (Gordon et al., 1992) (Monsé et al., 2018) *Animal studies:* (Morimoto et al., 2016) (Landsiedel et al., 2014) (Conner et al., 1988) (Warheit et al., 2009) (Chien et al., 2017) (Ho et al., 2011). (Larsen et al., 2016) (Lam et al., 1985) (Kao et al., 2012)
Particle sizes (of Zn) in welding fumes in the inhalation studies included in the current report	141-142 nm	(Markert et al., 2016)

Abbreviations: D50: The portions of particles with diameters smaller and larger than this value are 50%; and similarly for D80. W% is weight percentage.

ZnO has not been evaluated by IARC. Yet Zn is often a constituent of welding fumes, and welding fumes are classified as Group 1 carcinogen by IARC (IARC, 2018).

The current Danish OEL is set for "ZnO and ZnO fumes, calculated as Zn" and is at present 4 mg/m^3. Notably, the current assessment focusses on total ZnO, and not specifically nanosized ZnO. The current working group notes that ZnO fumes consist of nanosized particles. OELs for ZnO from different countries are presented in Table 2.

Table 2: Occupational exposure limits (8-hour TWAs) for ZnO in different countries included in the GESTIS database (DGUV/IFA, 2018), and for Denmark (Arbejdstilsynet, 2019).

Country	**Limit value ZnO – eight hours (mg/m^3)**	**Limit value ZnO – Short term (mg/m^3)**
Denmark	4 (ZnO and ZnO fumes calculated as Zn)	8
Belgium	2 (respirable fraction)	10 (15 minutes average value, respirable fraction
Finland	2	10 (15 minutes average value)
Switzerland	0.1 (zinc and its compounds, inorganic respirable fraction)	0.4 (respirable aerosol)
Germany (DFG)	0.1 (zinc and its compounds, inorganic respirable fraction) 2 inhalable fraction	0.4 (15 min average value)

The aim of the present report is to review the data and investigate if the present knowledge allows for a suggestion of a health-based OEL for ZnO. In this document, we review the relevant literature on the adverse effects of ZnO with the scientific peer-reviewed literature as central sources of information.

The risk assessment methodology of this report will follow the guidelines suggested by ECHA (ECHA, 2019). First, threshold or non-threshold effects are determined. For an OEL based on threshold effects, the following traditional approach is utilised: 1) identification of critical effect, 2) identification of the No Observed Adverse Effect Concentration (NOAEC), 3) calculation of OEL using assessment factors adjusting for inter and intra species differences. Conclusively, the calculated OELs will be compared; and lastly, a recommended OEL for ZnO exposure will be proposed.

Human studies

Human exposure

ZnO is synthesised as powders, and besides dermal exposure, inhalation as fine dust is therefore considered an important route of occupational exposure. Exposure through inhalation may occur during the entire ZnO lifecycle: Manufacturing, storage, transporttation, product application, and end-of-life processes.

Moreover, ZnO is a component of welding fumes and other related process fumes (e.g. brass casting), and this poses a source of inhalation exposure in workers. This exposure occurs simultaneous with exposure to other metal oxides, which may also have similar and/or additional toxic effects.

Thus, overall, the greatest potential for human exposure is in the working environment, especially during production and handling of large quantities of ZnO and during welding and other related techniques.

In 2004, The European Union (EU) made a risk assessment of Zn in general and estimated the exposure levels given in Table 3 (EU, 2004). In the report, it was noted that these estimates were considered conservative values and would probably overestimate real exposure levels to an unknown extent. The typical exposure levels were reported to be in the range of 0.1-0.9 mg/m^3 ZnO for various industrial processes including ZnO production, paint production and welding. Reasonable worst-case scenarios were reported to be up to the current Danish OEL of 5 mg/m^3 ZnO corresponding to 4 mg/m^3 Zn with short term exposure scenarios of 10 mg/m^3 ZnO.

Table 3. Inhalation exposure levels of ZnO in various work situations, estimated by EU in 2004 (EU, 2004).

Scenario / sub-scenario	Activity	Frequency (days/year)	Duration (hours/day)	Reasonable worst case (mg ZnO/m³) ‡	Method	Typical exposure (mg ZnO/m³) ‡	Method
1. Production of ZnO	full shift production recycling workplace 1 workplace 2 workplace 3 workplace 4 short term	100-200 100-200	6-8 0.25	 4.8 (3.9) 4.8 (3.9) 2.1 (1.7) 2.0 (1.6) 2.0 (1.6) 5.3 (4.3) 10 (8)	 measured expert	 0.85 (1.1) 0.9 (1.1) 0.4 (0.5) 0.6 (0.7) 0.6 (0.7) 0.8 (1.0)	 measured
2. Production of paints containing ZnO	dumping full shift short term	100-200 100-200 100-200	2-4 6-8 0.25	5 (4) 2.5 (2) 10 (8)	analogy measured /analogy analogy	0.5 (0.4)	measured
3. Production of rubber products containing ZnO	dumping full shift short term	100-200 100-200 100-200	0-2 6-8 0.25	1.5 (1.2) 0.4 (0.3) 5 (4)	analogy analogy analogy	0.1 (0.08)	expert
4. Use of paint containing ZnO	spraying full shift short term	100-200 100-200 100-200	2-4 4-6 0.1-0.3	4 (3.2) 2 (1.6) 8 (6.4)	analogy calculated expert	0.5 (0.4)	expert
5. Zn die casting	full shift short term	100-200 100-200	6-8 0.25	1.0 (0.8) 2.0 (1.6)	measured /expert expert	0.1 (0.08)	measured
6. Brass casting	full shift full shift, very fine particles (< 0.52 µm) short term short term, very fine particles (< 0.52 µm)	100-200 100-200 100-200 100-200	6-8 6-8 0.25 0.25	2.0 (1.6) 0.2 (0.16) 4.0 (3.2) 0.4 (0.32)	measured /expert measured /calculated	0.5 (0.4)	-
7. Welding of Zn coated steel	full shift short term	100-200 100-200	6-8 0.25	0.8 (0.6) 1.6 (1.3)	measured expert	0.1 (0.8)	measured

‡ Data without parenthesis are expressed in mg ZnO/m³, data within parenthesis are expressed in mg Zn/m³

Metal fume fever: Description of an endpoint addressed in epidemiological studies and experimental studies in humans

Inhalation exposure to ZnO fumes induces metal fume fever (Bodar et al., 2005). Metal fume fever is induced by inhalation of many different metal oxides including aluminium, antimony, beryllium, boron, cadmium, chromium, cobalt, copper (Cu), iron, lead, manganese, magnesium, nickel, silver, selenium, tin, vanadium and Zn (Greenberg and Vearrier, 2015).

Metal fume fever is characterised by fatigue, chills, fever, myalgias, cough, dyspnoea, leucocytosis, thirst, metallic taste, and salivation (Barceloux, 1999). The flu-like symptoms disappear with continued exposure to ZnO and other metal oxides, but are induced again if exposure is continued after a short break such as a weekend (hence this condition is also termed Monday Morning Fever) (Barceloux, 1999). Welders are exposed to welding fumes containing various metal oxides. The composition of metal oxides depends on the type of welding.

In a study by Krabbe et al. (Krabbe et al., 2018), 15 healthy male volunteers were exposed to 2.5 mg/m^3 Zn- and Cu-containing welding fumes for 6 hours on 4 consecutive days. Serum levels of CRP, IL-6, and SAA were determined before and after the exposure on each day and 24 hours after the last exposure began. IL-6 levels were increased 6 hours after onset of exposure, but had returned to baseline after the end of exposure on the second exposure day. IL-6 levels were increased before onset of exposure on day 4 and 5. None of the subjects reported fever or any other symptoms related to metal fume fever. Lung function was unaffected for all exposures. CRP and SAA blood levels were statistically significantly increased 24 hours after the first exposure, and remained elevated at all post-exposure time points assessed (1-5 days after first exposure corresponding to 1 day after beginning of the last exposure). This suggests that there was no adaptation of the metal oxide-induced acute phase response over a period of 4 days. Conversely, signs of adaptation were observed for IL-6 levels. Notably, IL-6 is a pyrogen (Luheshi and Rothwell, 1996), causing fever, and an adaptation of IL-6 would parallel the Monday Morning Sickness characteristic of metal fume fever.

Thus, inhalation of metal oxides induces inflammation and acute phase response below the air concentrations that induce metal fume fever. The acute phase response is a risk factor for coronary heart disease (Gabay and Kushner, 1999; Hadrup et al., 2020; Saber et al., 2014). This suggests that occupational exposure to various metal oxide fumes is related to increased risk of cardiovascular disease, and mechanistically to atherosclerosis and ischemic heart disease (Hadrup et al., 2020; Saber et al., 2014; Vogel and Cassee, 2018). Rupture of an atherosclerotic plaque can lead to acute myocardial infarction or stroke, depending on where the blood stream is blocked.

Acute phase response proteins CRP and SAA as biomarkers for increased risk of atherosclerosis and increased risk of coronary heart disease

The acute phase response is induced in humans in response to inflammatory states caused by e.g. infection, infarction, and trauma, and it is defined by increases in acute phase response proteins with the most predominant being C-reactive protein (CRP), Serum amyloid A (SAA), and fibrinogen. During an acute phase response, these proteins can increase several thousand fold (Gabay and Kushner, 1999). CRP and SAA levels are highly correlated (Jylhävä et al., 2009). Elevated plasma levels of CRP and SAA have been reported as a risk factor for cardiovascular disease in prospective epidemiological studies in humans (Johnson et al., 2004; Lowe, 2001; Mezaki et al., 2003; Ridker et al., 2000).

Epidemiological studies using Mendelian Randomisation suggest that CRP is not causally related to risk of cardiovascular disease. Genetic variation in the promoter region of the human *CRP* gene is associated with blood levels of CRP but not with risk of cardiovascular disease (Elliott et al., 2009; Pai et al., 2008; Vogel, 2013), suggesting that the association between CRP levels and risk of cardiovascular disease is caused by co-variation with the causal factor, which could be SAA.

In the prospective epidemiological study of Nurses Health Cohort, a 5-fold increase in SAA levels was associated with 3-fold increased risk for cardiovascular events defined as death from coronary heart disease, nonfatal myocardial infarction or stroke, or the need for coronary-revascularisation procedures (Ridker et al., 2000). This shows that relatively modest increases in SAA levels are associated with increased risk of cardiovascular disease.

Based on this evidence, the current working group considers work-related increased blood levels of the acute phase proteins CRP and/or SAA following occupational exposure as biomarker of an occupational exposure that causes increased risk of atherosclerosis and increased risk of coronary heart disease. The effect is considered a threshold effect, dose-dependency is observed, and therefore NOAEL levels can be determined.

Epidemiological studies of welders

In a Danish study of 5,866 welders, the incidence of each of nine cardiovascular outcomes among welders was compared with 5-year age- and calendar year-specific male national rates (Ibfelt et al., 2010). The relative risks of disease were increased for acute myocardial infarction (standardised incidence ratio, 95% confidence interval (CI)) (1.12, 1.01 to 1.24), angina pectoris (1.11, 1.01 to 1.22), coronary heart disease (1.17, 1.05 to 1.31) and cerebral infarct (1.24, 1.06 to 1.44). Dose-response relationship was found in relation to cumulative exposure except for the highest dose group (> 100 mg/m^3*years).

A systematic meta-analysis (Mocevic et al., 2015) identified 18 epidemiological studies with at least one risk estimate of ischemic heart disease morbidity or mortality among workers exposed to welding fumes. Nine studies, including a total of 49,864 welders and

persons with welding-related work, were eligible for meta-analysis. The weighted relative risk (RR) for ischemic heart disease following exposure to welding fumes was 1.09 (95 % CI 1.00, 1.19). An increased risk was observed for acute myocardial infarction, relative risk (RR) = 1.69 (95 % CI 1.18, 2.42) and for other ischemic heart diseases RR = 1.06 (95 % CI 0.98, 1.14).

A recent report by the Nordic Expert Group (NEG, 2020) concluded: "There is insufficient evidence for an association between exposure to Zn and CVD [cardiovascular disease]."

The current working group is of the opinion that there is moderate evidence that welders, who are occupationally exposed to metal oxide fumes, are at increased risk of ischemic heart disease and acute myocardial infarction in a Danish cohort study and in a systematic meta-analysis (which included the Danish study) (Ibfelt et al., 2010)(Mocevic et al., 2015).

Based on this, the current working group considers ZnO-induced increased SAA and/or CRP levels as the critical effect based on the causal link to coronary heart disease.

Short-term controlled exposure studies with humans

Controlled mixed exposure to other metal oxides and to Cu and Zn

Baumann et al. performed a controlled exposure study with 15 non-smoking healthy male volunteers (mean age 26 years; mean weight 85.4 kg; healthy lung function data). The subjects were exposed in a randomised threefold cross-over study to welding fumes containing at 1 day "Zn only" (target Zn concentration (tc): 1.5 mg/m^3), at another day "Cu only" (target Cu concentration tc: 0.4 mg/m^3) and on the third day both "Zn and Cu" (Zn tc: 1.5 mg/m^3; Cu tc: 0.4 mg/m^3). This study showed that 6 hours of inhalation exposure to Zn (1.5 mg/m^3), or Cu (0.4 mg/m^3), or both (1.5 mg/m^3 Zn and 0.4 mg/m^3 Cu) induced significantly increased blood levels of SAA and CRP 24 hours after onset of exposure (Baumann et al., 2017). The SAA and CRP levels were highly correlated ($r=0.751$, $P<0.0001$).

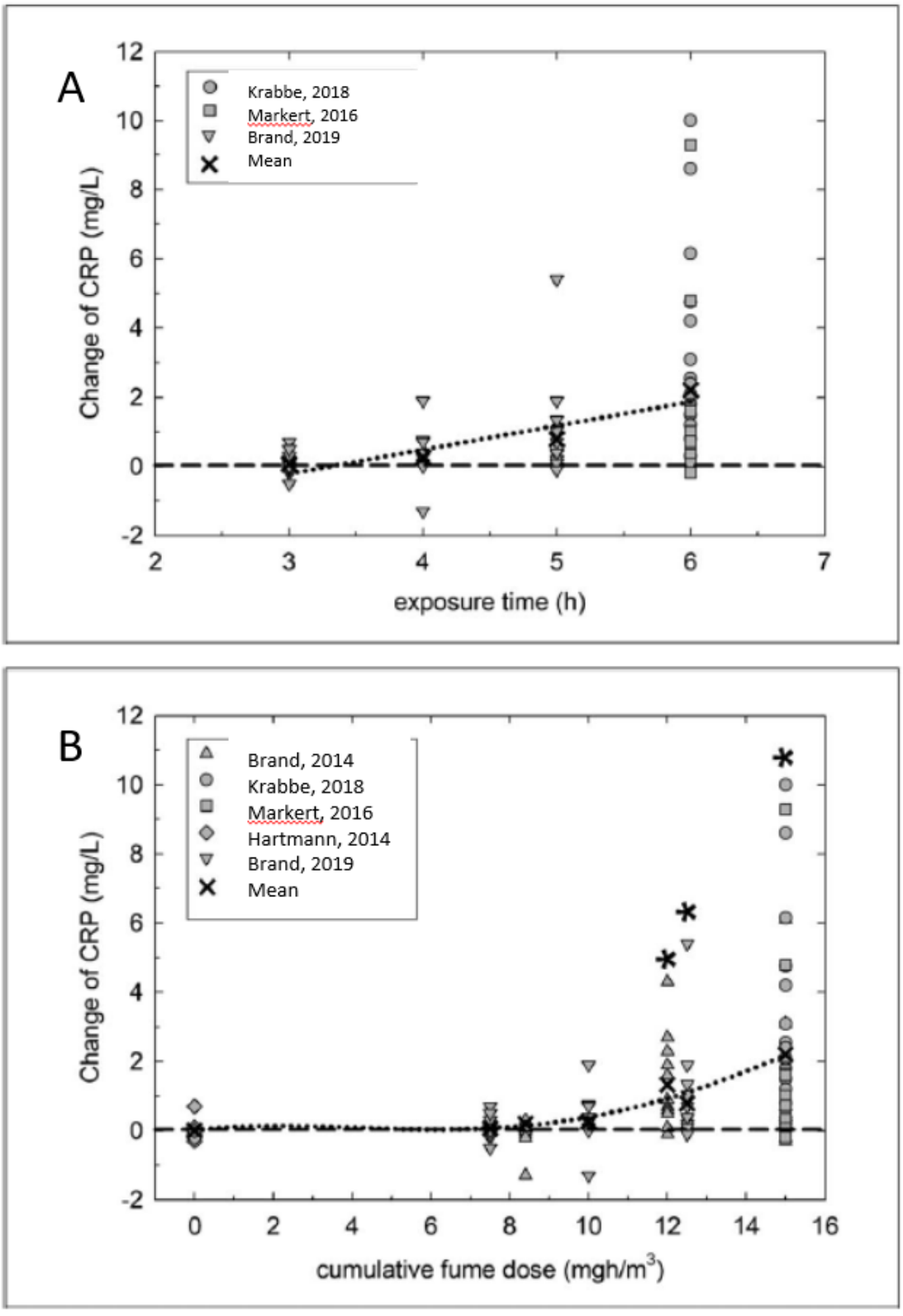

Figure 1: Summary of five different biomonitoring studies, where healthy volunteers are exposed to a mix of ZnO and CuO at exposure durations.
Top panel. Changes in blood levels of CRP 24 hours after exposure as function of exposure duration, Bottom panel: Changes in blood levels of CRP at 24 hours after exposure as function of the cumulative dose. Adapted from (Brand et al., 2019). Data from (Brand et al., 2014; Hartmann et al., 2014; Krabbe et al., 2018; Markert et al., 2016) were also included.

Brand et al. in addition to their own Brand et al. (Brand et al., 2019) summarised a number of biomonitoring studies of volunteers exposed to welding fumes containing a mixture of ZnO and CuO with 59.9% Zn and 22.2% Cu at different exposure durations (Peter Brand et al., 2014; Hartmann et al., 2014; Krabbe et al., 2018; Markert et al., 2016). The outcome was changes in CRP levels 24 hours post-exposure. The study reported dose-response relationship across different studies between exposure to metal oxides when exposure was calculated as the cumulative dose in mg*hour/m^3.

Unfortunately, data were only presented as graphical representations as shown in Figure 1. The current working group adapts the approach to calculate the cumulative exposure and convert the cumulative exposure to 8-hour averages.

Exposure to ZnO

Beckett et al. (Beckett et al., 2005) performed an exposure study of twelve healthy volunteers exposed to 0.5 mg/m^3 fine or ultrafine ZnO for two hours. ZnO particles were generated by an electric arc discharge system, between two consumable Zn electrodes (Zn, 99.99% pure; ESPI, Inc., Ashland, OR), in an argon gas environment with added oxygen, and then mixed with air before inhalation. For the ultrafine particle exposures, the count median diameter was 40.4 +/- 2.7 nm geometric standard deviation (GSD) 1.7, whereas for the fine particle exposures, the count median diameter was 291.2 +/- 20.2 nm GSD 1.7. CRP and SAA were not assessed, but IL-6 levels were unaffected by exposure. The volunteers had no symptoms of metal fume fever. Thus, 1 mg/m^3*h, corresponding to 0.125 mg/m^3 for an 8-hour working day, did not induce inflammation in terms of increased IL-6 levels in blood and did not induce symptoms of metal fume fever. Furthermore, there was no difference in the response to fine and ultrafine ZnO.

In a study by Gordon et al. (Gordon et al., 1992), four human volunteers were exposed to 5 mg/m^3 freshly formed ZnO (median count particle diameter 60 nm) for 2 hours. The volunteers were reported to develop classical symptoms of metal fume fever beginning 4 to 8 hours after exposure. Thus, exposure to 10 mg/m^3*h, corresponding to 1.25 mg/m^3 ZnO during an 8-hour working day, induced symptoms of metal fume fever. CRP and SAA were not assessed.

In a study by Fine et al. (Fine et al., 2000), nine volunteers were exposed to 5 mg/m^3 ZnO for 2 hours. Briefly, Zn shavings were heated to approximately 550° C in a furnace ventilated with inert argon gas. The released Zn vapours were carried downstream to react with oxygen, yielding a supersaturated atmosphere of ZnO vapour that condenses to ultrafine particles. These primary particles aggregate in chains to form secondary particles. Eighty percent of the volunteers developed symptoms of metal fume fever. Thus, a cumulative daily dose of 10 mg/m^3*h ZnO, corresponding to 1.25 mg/m^3 ZnO during an 8-hour working day, induced symptoms of metal fume fever. CRP and SAA were not assessed.

A controlled exposure study (Baumann et al., 2017) showed that 6 hours of inhalation exposure to welding fumes containing ZnO (1.5 mg/m^3 Zn) induced significantly increased blood levels of SAA and CRP 24 hours after onset of exposure. The cumulative

daily dose of ZnO was 9 mg/m^3*h corresponding to 1.125 mg/m^3 for an 8-hour working day.

In the controlled study by Markert et al. (Markert et al., 2016), 15 healthy male subjects were exposed to welding fumes containing 1.9 mg/m^3 ZnO (medium count diameter 141-142 nm) for 6 hours. CRP levels in blood were statistically significantly increased 24 hours after exposure. Thus, a cumulative exposure of 11.4 mg/m^3*h, corresponding to 1.425 mg/m^3 for an 8-hour working day increased CRP levels in blood.

In the study by Monsé et al. (Monsé et al., 2018), 16 healthy, non-smoking volunteers (mean age 26 years) were exposed to nanosized ZnO generated by pyrolysis. The mean diameter of the inhaled ZnO agglomerates was 48-86 nm. The volunteers were exposed to 0, 0.5, 1 and 2 mg/m^3 ZnO for 4 hours including 2 hours of cycling to mimic light work on different exposure days separated by 2 weeks. Biomarkers of effect were assessed at study entry (baseline), before and after exposure, after 24 hours and two weeks after last exposure. Blood levels of IL-6 were unaffected by exposure, while SAA and CRP levels were increased 24 hours post-exposure. SAA and CRP were highly correlated (R=0.78). When compared to the levels before exposure (Figure 2), blood CRP levels were signify-cantly increased at 24 hours after exposure for all ZnO concentrations. SAA levels were increased 24 hours after exposure to 1.0 and 2.0 mg/m^3 ZnO. Compared to the sham exposure, ZnO exposures yielded significantly higher CRP values 24 hours after exposure to 2.0 mg/m^3 ZnO, and higher SAA values after 1.0 and 2.0 mg/m^3 ZnO. ZnO exposures also yielded significantly higher CRP values 24 hours after exposure to 1.0 mg/m^3 ZnO, but this did not withstand correction for multiple testing. The cumulative doses were 2, 4 and 8 mg/m^3*h ZnO. The current working group notes that no increase in IL-6 levels were observed even though SAA and CRP levels were highly increased, suggesting that IL-6 levels cannot be used as proxy for SAA and CRP levels. The current working group notes that large inter-personal variation was seen in the response. Furthermore, for 3 out of 16 subjects, a dose-response dependent increase in SAA levels was observed already at 0.5 mg/m^3 (corresponding to 0.25 mg/m^3 for an 8-hour working day). In addition, CRP levels were increased at 0.5 mg/m^3 as compared to CRP levels before exposure (on the same day). This was also seen for sham exposure, but to a lesser extent, and no statistically significant difference was seen for sham exposure.

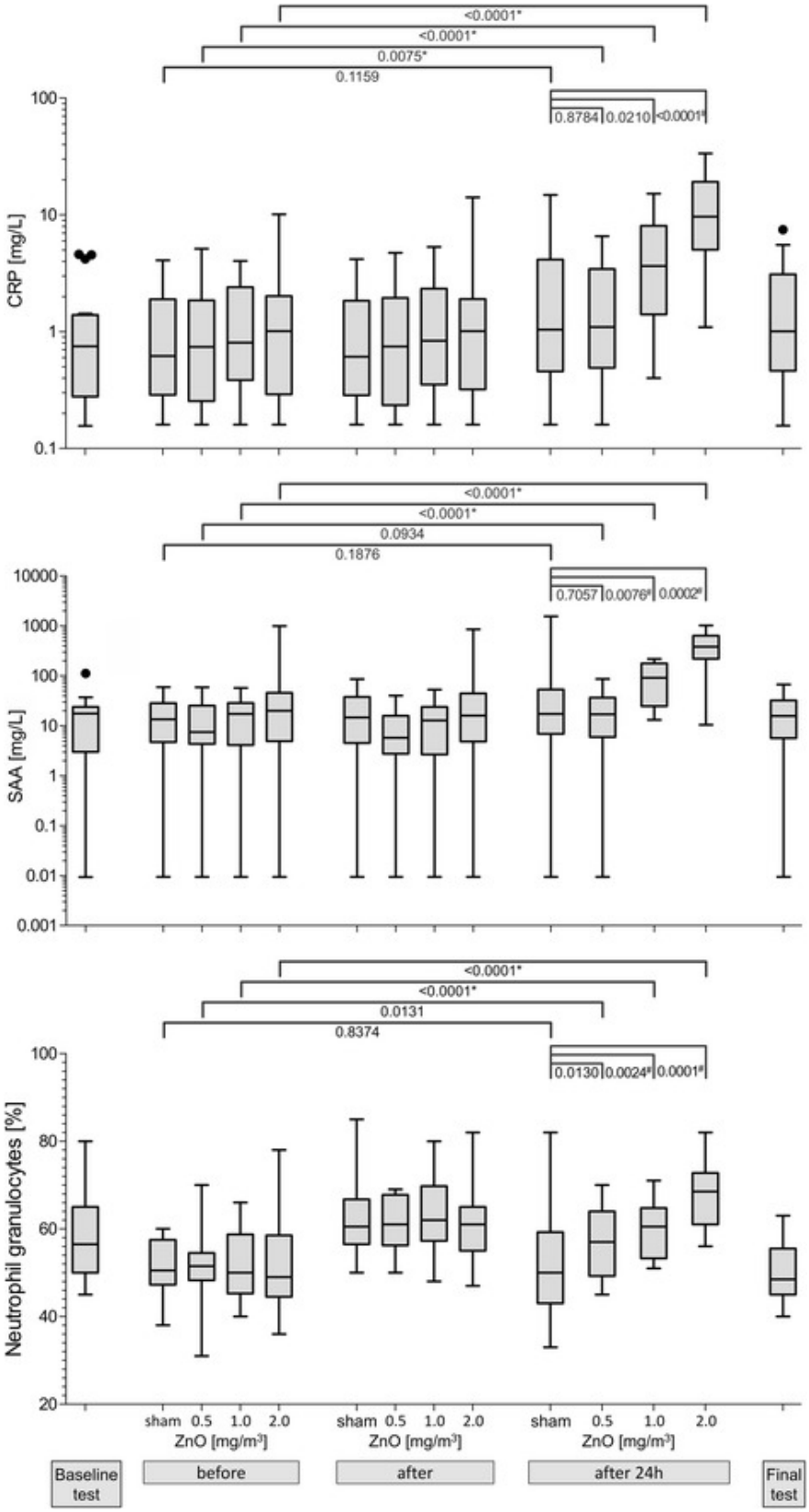

Figure 2: Median values of selected blood parameters according to ZnO concentrations and time points.
*significant values with significance level α = 0.0125 (after Bonferroni correction). #significant values with significance level α = 0.0167 (after Bonferroni correction). Outliers are defined as values above median + 1.5 x interquartile range or values below median – 1.5 x interquartile range (reproduced from (Monsé et al., 2018) with permission).

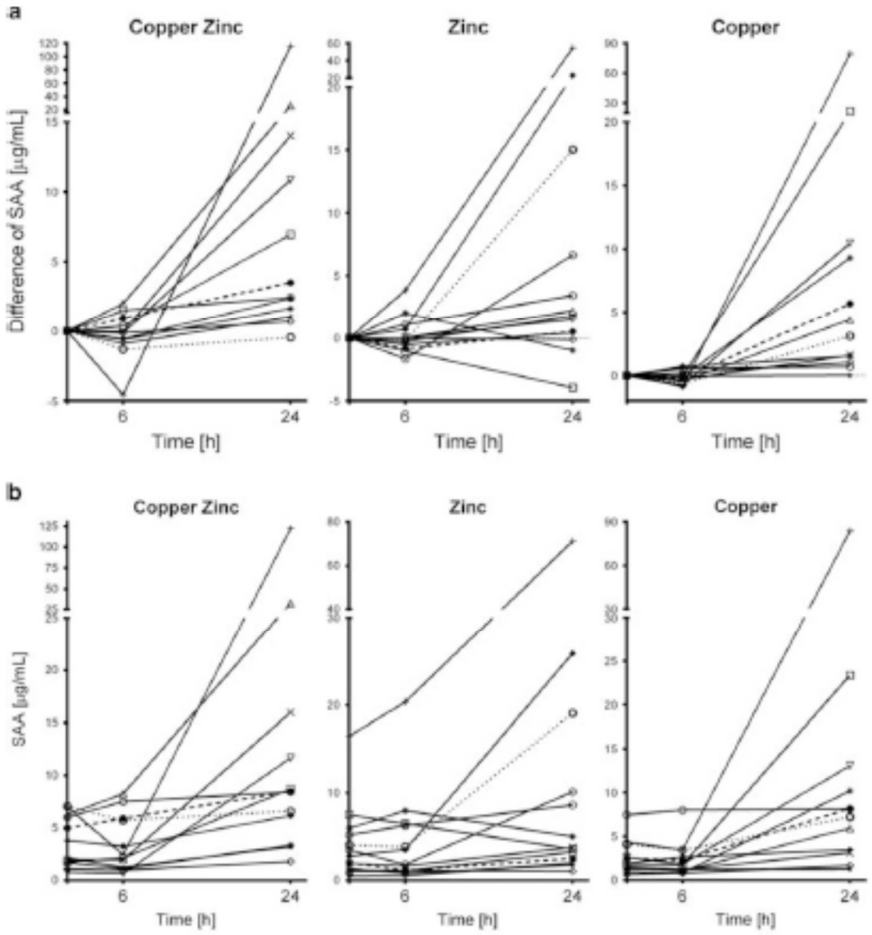

Figure 3: Blood levels of CRP and SAA before and after 6 hours exposure to ZnO, CuO or a combination of ZnO and CuO for 15 healthy non-smoking volunteers. Each curve represents one person, while the dotted lines represent the mean (adapted from (Baumann et al., 2017)).

Inter-individual variation and susceptible subpopulations:

The current working group notes that extensive inter-individual variation is observed in all the controlled studies (see Figures 1-3). The current working group estimates from the figures that more than 20 fold inter-individual variation in the magnitude of the response at the same exposure level is observed among young, healthy male volunteers.

Krabbe and co-workers (Krabbe et al., 2018) discuss susceptible populations: '*The subjects in this study were all healthy male students without chronic diseases, smoking habits, or occupational exposure to welding fumes. Thus, no conclusions can be drawn about the influence of these variables. Particularly since smoking, overweight, or diseases like arterial hypertension and diabetes can also contribute to chronic slight elevations of CRP and increased cardiovascular risk, the influence and interference of those variables on the effects of Zn- and Cu-containing welding fumes should be addressed in future studies.*' (Figure 1).

Other (occupational) exposures with similar effects

A number of other work-related exposures are known to induce acute phase response. Data from biomonitoring studies (summarised in (Hadrup et al., 2020)) have shown that

this includes organic dust, and paper mill dust, and for workers at steel production, iron foundry workers, high way maintenance workers, and wildland fire fighters.

A study from Taiwan reported correlation between air pollution levels (particulate matter <2.5 μm in diameter (PM 2.5)) and CRP levels among 30,034 participants with 39,096 CRP measurements. Every 5 μg/m^3 PM2.5 increment was associated with a 1.31% increase in CRP (95% confidence interval (CI): 1.00%, 1.63%) after adjusting for confounders (Zhang et al., 2017). Two-year averaged air pollution levels were 26 μg/m^3 PM 2.5. There was no significant effect modification by sex, age, educational level, smoking, hypertension, diabetes or body mass index on the association between PM2.5 and CRP (all *p*-values were greater than 0.10). The current working group notes that in this large study with more than 30,000 participants, correlation between PM 2.5 and CRP levels were found at very low PM concentrations (PM 2.5: 10-40 μg/m^3) compared to air concentrations used in biomonitoring studies, with no indications of threshold effects. Furthermore, no effect modification by lifestyle factors was seen, suggesting that there was no interaction between air pollution and lifestyle factors. This suggests that air pollution and lifestyle factors act as additive effects.

Lifestyle factors that influence acute phase response

A number of lifestyle factors influence the baseline blood level of acute phase response proteins. These lifestyle factors include physical activity (Pitsavos et al., 2005) which lowers acute phase protein levels, and body mass index and waist circumference (Jylhävä et al., 2009; Thorand et al., 2006), metabolic syndrome (Liu et al., 2006), age, and smoking (Pierce et al., 2009) which are associated with increased levels of acute phase proteins.

The current working group notes that this could mean that older persons and persons with high body mass index could represent susceptible sub-populations, who have higher baseline blood levels of acute phase response proteins.

Toxicokinetics

Exposures to ZnO particles and nanoparticles occur in many occupational settings; primarily via inhalation. Focus in this section will be on inhalation, the most critical occupational exposure pathway.

There are some inhalation studies with animals describing the toxicokinetics of ZnO nanoparticles. Rossner et al. exposed mice to ZnO nanoparticles at 0.02 mg/m^3 continuously for three days (72 h) and detected Zn by flame and electrothermal atomic absorption spectrometry (ET-AAS). They found 20.6 mg/kg in lung whereas controls contained 13.1 mg/kg lung. Surprisingly, after 3 months of exposure to the same nanoparticles at a higher mass concentration, 0.625 mg/m^3, resulted in no difference to controls with some 13 mg/kg lung (Rossner et al., 2019), indicating that elimination mechanisms had been upregulated over this long duration.

In another study, mice were exposed to ZnO nanoparticles (10 nm in diameter) at 3.5 mg/m^3, 4 hours/day for 2 or 13 weeks. Each at recovery times of 0 or 500 h. Zn levels were increased on lungs only in the subacute study at the end of exposure. There were no changes in Zn levels in any of the other tested organs (heart, liver, spleen, kidney or brain) and also there were no changes in blood (Adamcakova-Dodd et al., 2014).

These studies show that ZnO does not accumulate in tissues. ZnO undergoes dissolution at low pH (Adam et al., 2014; Eixenberger et al., 2017; Reed et al., 2012; Xia et al., 2008). A likely elimination pathway of (nano)particles could therefore be that ZnO undergoes dissolution in lysosomes in macrophages and lung epithelial cells (Cho et al., 2011). The resulting Zn^+ ion is soluble.

Animal studies

Selection of studies and endpoints

Since data on effects following inhalation of ZnO in humans are available, data from animal studies are primarily included to strengthen the conclusions drawn upon the human data and to allow comparison of health effects caused by other types of particles. In the present report, inhalation studies will be prioritised. Yet we also included studies using pulmonary deposition such as intratracheal instillation.

Pulmonary inflammation

Animal inhalation studies

Rats inhaled 35 nm ZnO nanoparticles at 2 or 10 mg/m^3, 6hours/day, 5 days/week for 4 weeks. Neutrophil numbers were increased in bronchoalveolar (BAL) fluid at the highest dose at 3 days of recovery. ($NOAEC_{neutrophils}$: 2 mg/m^3)(Morimoto et al., 2016).

Rats were exposed to ZnO of nano- (NM-111, 61 nm) or microsize (>100 nm). Mass concentrations of the nanoparticle were 0.5, 2.5 or 12.5 mg/m^3. The inhalation period was 6hours/day for 5 days, followed by 21 days of recovery. Both particles increased neutrophil numbers in BAL fluid and necrosis of the olfactory epithelium – the nanosized at the two highest doses, and the microsized one at its only tested dose, 12.5 mg/m^3. ($NOAEC_{ZnO\ nanoparticle}$: 0.5 mg/m^3) (Landsiedel et al., 2014).

Rats were exposed to either nanoparticulate (50-70 nm) or fine ZnO (<1000 nm) by inhalation at 25 or 50 mg/m^3 (1 or 3 h) resulting in a "metal fume fever" response with transient short-term lung inflammation (increased polymorphonuclear cells in BAL fluid), and cytotoxicity. (The current working group has set no NOAEC for this high dose study) (Warheit et al., 2009).

Guinea pigs inhaled 50 nm ZnO nanoparticles (3 hours/day for 1, 2, or 3 days) (2.3, 5.9, or 12.1 mg/m^3). The two highest levels were associated with increased neutrophils and protein in BAL fluid. Other changes included histologic evidence of inflammation. ($NOAEC_{neutrophils}$: 2.3 mg/m^3) (Conner et al., 1988).

Rats inhaled ZnO nanoparticles (48-51 nm) at 1.1 or 4.9 mg/m^3 for 5hours/day 5 days/week for 2 weeks. Recovery periods were 24 hours, 7 days, and 1 month. This regimen resulted in increased inflammation involving lymphocytic infiltration (both evaluated by histopathology) (Chien et al., 2017).

Concerning ZnO at sizes not necessarily in the nano-range, mice were exposed to 1 mg/m^3 ZnO– described as ultrafine particles, 3 hours/day for 1, 3, or 5 days. On day 5, an increase in BAL fluid neutrophils had almost returned to normal. Yet re-exposure after an additional 5 days to ZnO increased the neutrophil number again. Metallothionein was also increased. (Lowest Observed Adverse Effect Concentration $(LOAEC)_{neutrophils}$: 1 mg/m^3) (Wesselkamper et al., 2001).

Rats inhaled ZnO (not reported specifically to be on the nanoform) at 6.9 mg/m^3 6 hours/day for 7 days followed by recovery periods of 1, 3 or 15 days. The body weight was unaffected, while the number of alveolar macrophages were elevated at all time-points as determined by histopathology. Other effects, seen only at the two first time-points, included increased lung weight, BAL fluid protein and lactate dehydrogenase levels; while BAL fluid neutrophil numbers were only increased on day 1. ($LOAEC_{neutrophils}$: 6.9 mg/m^3) (Pauluhn et al., 2003).

Animal intratracheal and intranasal instillation studies

Rats were dosed 0.2 or 1 mg ZnO nanoparticles (35 nm) by intratracheal instillation. Recovery periods were 3 days, 1 week, 1, 3 or 6 months. Transient increases in neutrophil numbers in BAL fluid were observed (Morimoto et al., 2016). Rats were administered ZnO nanoparticles by intratracheal instillation. Recovery periods were 1 day or 1 week. Acute oxidative stress was found by ZnO nanoparticles (Fukui et al., 2015). Intranasal instillation of ZnO nanoparticles at 5 mg/kg body weight (bw) induced pulmonary inflammation seen by histopathology and as altered mRNA levels of various inflamematory markers (Saptarshi et al., 2015). After intratracheal instillation into mice, ZnO nanoparticles were found to aggravate lung inflammation occurring after lipopolysaccharide treatment. In addition, the combination of these two substances increased 8-Hydroxyguanosine in the lungs (Wang et al., 2020). ZnO nanoparticles and fine ZnO produced inflammation following intratracheal instillation into rats (Sayes et al., 2007). After intratracheal instillation into rats, ZnO nanoparticles at 1 mg/kg bw, but not 0.2 mg/kg bw, induced increased neutrophil numbers in BAL fluid (Konduru et al., 2014). After instillation into rats, ZnO nanoparticles were inflammogenic (Cho et al., 2010). Rats were dosed with ZnO nanoparticles by intratracheal instillation leading to disturbed cytokine regulation and increased oxidative injury (Liu et al., 2013). Zn but not a range of other metals instilled as salts into mouse lungs increased protein content and inflamematory cells in lung lavage fluid (Adamson et al., 2000). In mice, a coated ZnO nanoparticle (NM-111) but not an uncoated one (NM-110) increased neutrophil numbers in BAL fluid at 0.1 mg/kg bw, but not at the next lowest dose – 0.03 mg/kg bw (Hadrup et al., 2019).

Summary pulmonary inflammation

Several studies demonstrated pulmonary inflammation measured as increased neutronphil numbers in BAL fluid following inhalation or pulmonary exposure to ZnO nanoparticles. This was seen after inhalation and intratracheal instillation. NOAEC values for increased neutrophil numbers in BAL fluid following exposure to ZnO nanoparticles were as low as 0.5 mg/m^3 in one inhalation study; and a LOAEC in another study was 1 mg/m^3.

Pulmonary function

Animal inhalation studies

Guinea pigs inhaled 50 nm ZnO nanoparticles at 5 mg/m^3, 3 hours/day for 6 days; followed by recovery periods of 1, 24, 48, or 72 hours. Pulmonary function parameters including vital capacity and functional residual capacity were reduced at all time-points.

Initial increases in flow resistance and decreases in total lung capacity and compliance had normalised at 72 hours of recovery. Inflammation of the lungs persisted throughout the 72 hours. ($LOAEC_{pulmonary\ function}$: 5 mg/m^3)(Lam et al., 1985). Guinea pigs inhaled ZnO particles, submicron in size, at 1 mg/m^3 for 1 hour. Lung compliance was decreased, while minute volume, tidal volume, resistance, and frequency were not affected (NOAEC not set by the current authors) (Amdur et al., 1982).

Allergic reactions

Data from animal studies
Nanoparticles of ZnO, TiO_2 or SiO_2 were administered to mice by pharyngeal aspiration at 50 μg/mouse. Subsequently the mice were challenged with inhalation of ovalbumin. ZnO aggravated the expected ovalbumin reaction in that the serum concentrations of total IgE and ovalbumin specific IgE and IgG1 were increased. Pharyngeal aspiration of $ZnCl_2$ formulated in solution did not have this effect. This made the authors suggest that a continuous release of Zn^{2+}, rather than a bolus exposure, is needed for the aggravation of allergic reactions (Horie et al., 2015).

Other toxicities

Decreased body weight

Decreases in body weight have, to our knowledge, only been observed after intratracheal instillation. Jacobsen *et al.* found severe pulmonary toxicity of ZnO nanoparticles in mice after intratracheal instillation at 25 to 100 μg/mouse, which induced mortality; while ≥6 μg (~0.3 mg/kg bw) reduced weight gain and caused epithelial cell desquamation resulting in increased barrier permeability (Jacobsen et al., 2015).

Mice were given ZnO nanoparticles by intratracheal instillation at 200, 400, or 800 μg/kg bw. Body weight was decreased at the two highest doses and a range of other endpoints were affected already at the lowest dose (Wang et al., 2017).

BAL fluid and blood biochemical parameters

Rats were exposed to 25 nm ZnO nanoparticles at 23.2 mg/m^3 for 10 hours; or 34 nm at 6.6 mg/m^3 for 5 hours. Both treatments increased lactate dehydrogenase levels in BAL fluid (Kao et al., 2012).

One study tested ZnO, which was not specifically mentioned to be on the nanoform, in guinea pigs, rats and rabbits – all exposed to 2.5 or 5.0 mg/m^3 ZnO for up to 3 hours. In guinea pigs and rats, effects included changes in BAL fluid lactate dehydrogenase, beta-glucuronidase, and protein content, seen at both doses. ($LOAEC_{biochemistry}$: 2.5 mg/m^3). In contrast, no changes were observed in biochemical or cellular parameters in rabbits (Gordon et al., 1992).

Rats were exposed to ZnO 10 mg/m^3 for 2 hours/day, 5 days/week for 28 days inducing increased lactate dehydrogenase, total protein, and albumin in BAL fluid (Jain et al., 2013).

Histopathologic changes

Olfactory epithelium necrosis was seen in rats after inhalation of ZnO micro- or nanoparticles. ($NOAEC_{ZnO\ nanoparticle}$: 0.5 mg/m^3) (Landsiedel et al., 2014).

Rats were exposed to 20 nm ZnO nanoparticles at 2.5 mg/kg bw sprayed directly into the nasal passages. Zn increased in liver, while a range of biochemical markers were decreesed in serum, and severe tissue damage was observed in liver and lung (by histopathological evaluation) (Wang et al., 2010).

In a study by Jacobsen et al., histopathological changes were investigated in lungs and livers from mice, two days after a single exposure of ZnO nanoparticles by intratracheal instillation (0, 2, 6, and 18 μg ZnO nanoparticles/mouse). The effects included excessive desquamation of epithelial cells of bronchioles as well as lung oedema in treated animals. In addition, some effects were seen in liver, the most noteworthy change being increased presence of binucleate hepatocytes and enlargement of single hepatocytes (Jacobsen et al., 2015).

Cardiovascular effects

Acute phase response

Inhalation and intratracheal instillation studies in animals

To our knowledge, there are no inhalation studies in animals investigating the effect of ZnO nanoparticles on the acute phase response. However, we recently published an intratracheal instillation study in which we found the acute phase response gene *Saa3* mRNA level to be increased in lungs of mice after exposure to uncoated ZnO nanoparticles (NM-110) at 0.100 mg/kg bw. After exposure to the coated ZnO nanoparticle, NM-111, this effect was seen both at 0.033 and 0.100 mg/kg bw but not 0.011 mg/kg bw (Hadrup et al., 2019).

In mice, the SAA isoforms are the main acute phase response proteins, while CRP is only moderately induced by inflammatory stimuli (Pepys and Hirschfield, 2003; Whitehead et al., 1990). SAA (SAA1-4) is a highly conserved family of apolipoproteins associated with high density lipoproteins (HDL).

Other cardiovascular effects

ZnO was a pro-coagulant in Bmal1 (brain and muscle ARNT-like protein-1) knockout (Bmal1(-/-)) mice – a strain with disturbed circadian rhythm and at the same time described as a "pro-coagulant phenotype". This was observed after oropharyngeal aspiration (once a week for 5 weeks) at cumulative doses of 32 or 64 μg (~1.6 and 3.2 mg/kg bw) (Luyts et al., 2014).

Genotoxicity and cancer

Cancer

ZnO has not been evaluated by IARC. Zn is often a constituent of welding fumes and welding fumes are classified as Group 1 carcinogen by IARC (IARC, 2018), but welding fumes also contain other metal oxides with known carcinogenic effects.

Inhalation studies
We identified no studies of carcinogenicity after dosage with ZnO. We identified one study investigating a complex mixture in which Zn was present: The exposure to smoke produced by "ignition of a Zn oxide/hexachloroethane pyrotechnic composition" was investigated in 3 animal species at 3 dose levels 1 hour/day, 5 days a week at up to a total of 100 exposures. The investigated species were mice, rats, and guinea pigs (only 15 exposures due to high mortality). Mortality was increased at the highest dose in mice, and alveologenic carcinoma was seen in this animal species at the highest dose. Organ specific toxicity was seen in the respiratory system of all species and involved inflammation. The authors of that study discuss that apart from Zn, the carcinogenicity could also be induced by hexachloroethane or carbon tetrachloride, both are likely present in the smoke, and carbon tetrachloride has previously been shown to be a carcinogen in humans (Marrs et al., 1988).

Conclusion
The current working group is of the opinion that at present there is insufficient evidence of cancer as a critical endpoint for ZnO exposure.

In vivo genotoxicity studies
Inhalation
In rats, increased oxidative DNA damage in terms of 8-Oxo-2'-deoxyguanosine (8-oxo-dG) in lung was seen after 6 hours of inhalation of ZnO nanoparticles at 3.7 and 12 mg/m^3 (35 nm particle) and 45 mg/m^3 (250 nm particle). No effect was seen on this endpoint at 2.4 mg/m^3 (35 nm particle) and 7.2 and 11.5 mg/m^3 of the 250 nm particle (Ho et al., 2011).

No effect was observed in mice lung in the comet assay after 1 hour of inhalation of 58 or 53 mg/m^3 of ZnO nanoparticles (13 and 36 nm, respectively; Geometric Mean Diameters: 323 - 384 nm and 270 – 403 nm, respectively) (Larsen et al., 2016).

Intratracheal instillation
After intratracheal instillation into mice, genotoxicity was measured in BAL fluid cells and lung in the comet assay. Genotoxicity was observed only in single dose groups, with no dose-response relationship. But changes in cell cycle G2 to M phase DNA damage checkpoint regulation was seen in lung in global transcriptional response ZnO nanoparticles (Hadrup et al., 2019).

ZnO nanoparticles given by intratracheal instillation increased the 8-oxo-dG level in serum din rats at high 33 mg/kg bw dose of 50 nm ZnO nanoparticles (Chuang et al., 2014).

Oral exposure studies
Mice were orally dosed with ZnO nanoparticles at 300 mg/kg (bw) each day for 14 days. Zn was found to be accumulated in the liver, which also exhibited pathological lesions. And this was accompanied by increased DNA damage as measured in liver in the comet assay along with an induction of apoptosis (Sharma et al., 2012).

Mice were orally dosed with ZnO nanoparticles (200 or 500 mg/kg bw) causing DNA damage in peripheral blood and bone marrow cells, as measured by comet assay, micronuclei formation, chromosomal fragmentation, and increased phosphorylation of γH2AX (Pati et al., 2016).

ZnO nanoparticles were orally dosed to mice at 300 and 2000 mg/kg bw causing an increased number of chromosome aberrations in bone marrow cells and elevated levels of 8-oxo-dG in liver. In addition, a positive effect was seen at the highest dose in the randomly amplified polymorphic DNA (RAPD) assay in blood (Srivastav et al., 2017).

In two studies, no genotoxicity was observed after the dosage of ZnO nanoparticles: Four types of ZnO nanoparticles (20nm and 70nm size, positively or negatively charged) after oral administration by gavage at doses of 500, 1000 or 2000 mg/kg bw (dosed by oral gavage given on three occasions, it is not specified whether these were the cumulative doses). In rats, genotoxicity was measured in the comet assay in liver and stomach, whereas in mice it was tested in the micronucleus test in erythrocytes. Also in this study, *in vitro* genotoxicity was found to be negative as presented in the next sections (Kwon et al., 2014).

No changes were found in micronuclei formation in erythrocytes from mice dosed ZnO nanoparticles at the extremely high doses of 1.25, 2.5, or 5 g/kg bw (Li et al., 2012).

Concerning Zn dosed as a salt, Zn chloride orally dosed induced a synergistic effect on chromosome aberrations together with a low-calcium diet in bone marrow cells of mice. No effect was seen when the diet calcium content was normal (Deknudt and Gerber, 1979).

Intraperitoneal injection
Concerning intraperitoneal injection, chromosome aberrations and micronuclei formation in bone marrow cells were observed at all doses in mice intraperitoneally injected ZnO nanoparticles at 25, 50 or 100 mg/kg bw followed by 18 hours of recovery. Comet assay was only positive at the lowest dose in liver, and not positive at any dose in bone marrow or blood (Ghosh et al., 2016).

Concerning other Zn formulations, $ZnSO_4$ was negative in micronucleus test in bone marrow from mice treated with two doses of either 28.8, 57.5, or 86.3 mg/kg bw (Gocke et al., 1981).

Conclusion on in vivo genotoxicity findings
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. The current working group concludes that there is evidence of ZnO-induced genotoxicity following pulmonary exposure in animal studies. No evidence of mutagenicity was found.

In vitro genotoxicity studies of ZnO

Studies comparing ZnO nanoparticles to other nanomaterials

A number of studies have benchmarked ZnO nanoparticles to other nanomaterials in in vitro studies. We consider these studies relevant as the comparisons give an idea on how potent ZnO nanoparticles are. ZnO nanoparticles but not TiO_2 nanoparticles induced micronuclei (effect at 3.5 and 5 but not 2 μg/cm^2) and DNA damage in the comet assay (already at 1 μg/cm^2) in human colon carcinoma cells (Caco-2 cells) (both increased 8-oxo-dG) (Zijno et al., 2015).

A 3D human liver micro-tissue model was used to test the genotoxicity of ZnO nanoparticles and in this model the ZnO nanoparticles were found to be more genotoxic than TiO_2 (and to be at a similar level to silver nanoparticles) (Kermanizadeh et al., 2014).

In contrast to TiO_2 nanoparticles, ZnO nanoparticles induced genotoxicity using a battery of cell culture test systems (Hackenberg et al., 2017).

Human renal proximal tubule epithelial cells (HK-2) were incubated with ZnO nanoparticles (NM 110 and uncoated – NM-111), and compared to MWCNT, silver and five different TiO_2 nanoparticles. DNA damage was observed for NM-110, but not NM111. Silver nanoparticles and one of the TiO_2 particles were the most genotoxic particles (Kermanizadeh et al., 2013).

A549 human lung epithelial carcinoma cells were incubated with ZnO nanoparticles and these were found to induce increased micronuclei frequency at 50 μg/mL – at this concentration, strong cytotoxicity was observed. In comparison, no genotoxicity was seen for Lys-SiO_2 nanoparticles at 5 μg/mL, and TiO_2 nanoparticles or MWCNTs at up to 250 μg/mL (Corradi et al., 2012).

Genotoxicity profiles of ZnO exhibited genotoxicity to a higher extent than nanoparticles of Ag, Fe_2O_3, CeO_2 and SiO_2 in both TK6 and H9T3 cells (Watson et al., 2014).

ZnO (NM-110) was compared to CeO_2, TiO_2 and silver nanoparticles. ZnO, CeO_2, and TiO_2 were genotoxic in the comet assay in TK6 cells at 0.42 μg/mL and above, while silver nanoparticles had effect already at 0.14 μg/mL. In A549 cells the concentrations were given as μg/cm^2 and in these cells ZnO nanoparticles were considerably more toxic than the other materials (El Yamani et al., 2017).

ZnO nanoparticles were compared to carbon black, single wall carbon nanotube, and SiO_2 nanoparticles at 5 and 10 μg/mL in primary mouse embryo fibroblasts (PMEF) cells. CNT produced more DNA damage than ZnO in the comet assay while SiO_2 displayed weaker effects (Yang et al., 2009).

Human peripheral blood lymphocytes were incubated with ZnO nanoparticles or Al_2O_3 nanoparticles and both caused a concentration-dependent increase of DNA single-strand breaks in the comet assay, starting at 0.01 mM (Sliwinska et al., 2015).

ZnO nanoparticles (20 nm) and Al_2O_3 nanoparticles (4 nm) were added to human peripheral blood lymphocytes at 1, 12.5, 25, 50, 100, 250 μg/mL; the number of chromosome aberrations and micronuclei were increased at 12.5 and above for ZnO, whereas Al_2O_3 nanoparticles had no effect (Akbaba and Türkez, 2018).

Taken together, ZnO nanoparticles seem to be more genotoxic *in vitro* than TiO_2 and SiO_2 nanoparticles, whereas carbon nanotubes seem to be more potent.

Studies investigating only Zn
In some studies, genotoxicity was observed at concentrations at or below 10 μg/mL: ZnO nanoparticles in lung fibroblast cells (V-79) exhibited genotoxicity by Comet assay at 5 μg/mL and above, but not at 1 μg/mL. Moreover, ZnO nanoparticles increased Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene mutant frequency at 10 μg/mL and above, but not at 1 or 5 μg/mL (Jain et al., 2019).

ZnO nanoparticles were added to human epidermal cells (A431) and found to be genotoxic in the comet assay at 0.8 and 5 μg/mL, but not at 0.08 μg/mL and below (Sharma et al., 2009).

ZnO nanoparticles induced genotoxicity at 1, 2 and 4 μg/mL in the comet assay in wild type (effect at 4 μg level only) and 8-oxo-guanine DNA glycosylase-1 knock-out mouse embryonic fibroblast (Annangi et al., 2016).

Human hepatocyte (L02) and human embryonic kidney (HEK293) cells displayed oxidative DNA damage in the comet assay at 5 μg/mL ZnO nanoparticles and above in HEK293 cells, whereas the effect was only seen at 25 μ/mL in L02 cells (Guan et al., 2012).

Human bronchial epithelial BEAS-2B cells were incubated with ZnO nanoparticles and so-called fine ZnO. In the comet assay, only the nanoparticles exhibited effect and that was seen at 6.8 and 7.5 μg/mL while no effect was seen at 3.8 μg/mL. Both particle sizes induced micronuclei formation at two highest concentrations. The genotoxic effects were observed at doses overlapping with cytotoxicity (Roszak et al., 2016).

ZnO nanoparticles were found to induce DNA damage in mini organ cultures (MOCs) of human nasal mucosa at 5 μg/mL, no effect was observed at 0.1 μg/mL (Hackenberg et al., 2011).

ZnO nanoparticles were added to human peripheral lymphocytes and chromosomal aberrations were increased at 5 and 10 μg/mL. Micronuclei induction occurred at 10 and 15 μg/mL (Gümüş et al., 2014).

In human mesenchymal stem cells (hMSC), genotoxicity was observed in the comet assay at 1 and 10 μg/mL ZnO nanoparticles (Ickrath et al., 2017).

Human squamous cell carcinoma-derived FaDu cells were incubated with ZnO nanoparticles and DNA damage was induced in the comet assay at 5, 10 and 15 μg/mL (Moratin et al., 2018).

Three-dimensional mini organ cultures (MOCs) were exposed to ZnO nanoparticles or ZnO fine powder. DNA damage was detected in the comet assay at 10 μg/mL (and dose-dependently above) of ZnO nanoparticles but not at 5 μg/mL. ZnO powder had no effect in this assay (Hackenberg et al., 2011).

And genotoxic effects of ZnO nanoparticles were observed *in vitro* in mouse macrophage RAW 264.7 cells (Pati et al., 2016). In WIL2-NS, human lymphoblastoid cells ZnO particles (26 nm, 78 nm, or 147 nm) were tested at 10 μg/mL for micronucleus formation in the CBMN cytome assay. Increases were seen for the two largest particles. Dissolution and cellular uptake were also determined and although there were fewer particles per cell at the two largest particles, the amount of Zn mass wise was unchanged at the mid size and increased at the large one, suggesting that it is the total amount of Zn that matters in regard to genotoxic potency (Yin et al., 2015).

Some studies compare the effect of ZnO nanoparticles to those of Zn ions. These studies provide a picture of the roles of the particle form and released ions, and whether both entities contribute to the toxicity of ZnO nanoparticles. A549 cells were incubated with ZnO nanoparticles 15-18 nm in diameter (0.1, 10 and 100 μg/mL). DNA double strand breaks were induced at ZnO nanoparticles at 0.1 and 10 μg/mL at 24 hours. Similar doses of $ZnCl_2$ had no statistical effect on DNA damage although some increase at 0.1 μg/mL, no effect was observed at 10 μg/mL. The amount of intracellular and nuclear fraction of Zn was determined and Zn entered the nucleus suggesting Zn can interact directly with DNA here (Heim et al., 2015).

In other studies, the effect was observed only at higher concentrations (>10 μg/mL): In human liver cells (HepG2), ZnO nanoparticles were found to cause DNA damage as measured in the comet assay after 14 or 20 μg/mL, but not after 8 μg/mL (Sharma et al., 2011).

ZnO nanoparticles added to MRC5 human lung fibroblasts at 25 μg/mL induced genotoxicity measured by comet, and on 8-hydroxydeoxyguanosine (8-OHdG) assays (Ng et al., 2017).

ZnO nanoparticles at 12.5-50.0 μg/mL in rat kidney epithelial cells (NRK-52E) caused DNA damage in the comet assay at 50 μg/mL. No effect was seen at 32.5 μg/mL and below (Uzar et al., 2015).

ZnO nanoparticles of either ≤ 35 nm or 50-80 nm were added to human lymphoblastoid cell line TK6. The highest concentration 100 μg/mL exhibited effect in the comet assay (Demir et al., 2014).

In Chinese hamster ovary (CHO) cells, ZnO nanoparticles increased chromosome aberrations at high concentrations of 105 μg/mL – and above (Dufour et al., 2006).

ZnO nanoparticles were added to human SHSY5Y neuronal cells. The nanoparticles did not enter the cells but still induced micronuclei formation and DNA damage in the comet assay at 20, 30, and 40 μg/mL, perhaps reflecting that released ions entered the cells (Valdiglesias et al., 2013).

In some studies, the nanoparticle concentration was only reported in μg/cm^2. In one such study, the uptake of Zn into cells was confirmed by ICP-MS: In the comet assay, ZnO (NM-110) displayed a dose-dependent genotoxicity between 10 and 25 μg/cm^2 in the EpiAirway™ 3D human bronchial model. Uptake of the metal into the cell was confirmed by ICP-MS (Haase et al., 2017).

Human colon carcinoma (LoVo) cells were incubated with ZnO nanoparticles at 5 μg/cm^2 resulting in increased 8-oxodG and γ-H2AX (Condello et al., 2016).

Studies with ZnO (not specifically on nano-form)
Zn acetate had a dose-related positive effect in the L5178Y mouse lymphoma assay as well as in Chinese hamster ovary cells. In contrast, this salt did not affect unscheduled DNA synthesis measured in cultured rat hepatocytes and no effect was seen in the *Salmonella typhimurium* mutation assay. Another formulation, Zn-2,4-pentanedione, also had no effect on unscheduled DNA synthesis but induced frameshift mutations in the *Salmonella typhimurium* TA1538 and TA98 strains (Thompson et al., 1989).

ZnO (not reported to be on the nanoform), but not ZnCl2, induced chromosome aberrations in human dental pulp cells (Someya et al., 2008).

In vitro studies with no effects
ZnO nanoparticles 20 nm including one amorphous particle were tested in the Cytokinesis Block Micronucleus Assay (CBMN) in V79 cells. At 120 μM, the ZnO nanoparticles were genotoxic (Reis et al., 2015).

ZnO induced conflicting results in two different genotoxicity assays - the GreenScreen and the comet assay (Bayat et al., 2014). ZnO nanoparticles showed no effect at 20 μg/mL in the comet assay in mouse embryonic stem (mES) cells (Karlsson et al., 2014).

In one study, ZnO nanoparticles were not found to be genotoxic in the chromosomal aberration test when tested at 3.75, 7.5, and 15 μg/mL (Kwon et al., 2014).

Concerning bacterial assays, ZnO nanoparticles nor Zn were genotoxic in the SOS chromotest in *Escherichia coli* at up to 100 μg/mL (Nam et al., 2013).

ZnO nanoparticles were not found to be mutagenic in the bacterial mutagenicity assay at up to 5000 μg/plate (Kwon et al., 2014).

Concerning other Zn formulations, $ZnSO_4$ did not have strong genotoxic effects in the Basc test (one positive test and two negative ones) and did not exhibit effects in Ames test (*Salmonella typhimurium* test) (Gocke et al., 1981).

$ZnCl_2$ was compared to 6 other inorganic metal salts in their ability to induce mutations in L5178Y/TK+/- mouse lymphoma cell. $CdCl_2$, $NiCl_2$, and trans-platinum(II) diaminedichloride, but not $ZnCl_2$ (1.2 to 12.3 μg/mL), induced dose-related elevated number of mutants (Amacher and Paillet, 1980).

Conclusion in vitro gentoxicity
There are both *in vitro* studies suggesting presence and absence of genotoxicity.

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.

Mechanisms of toxicity

Metal fume fever, acute phase response and cardiovascular disease

There is evidence that inhaled ZnO undergoes relatively rapid dissolution at the low pH in the lysosomes of macrophages and other pulmonary cells following pulmonary exposure to nanomaterials. This is based on the rapid *in vitro* dissolution in various fluids mimicking those present in the body (Adam et al., 2014; Eixenberger et al., 2017; Reed et al., 2012; Xia et al., 2008), and direct evidence for dissolution of ZnO inside lysosomes (Cho et al., 2011). Furthermore, ZnO does not accumulate in lung tissue following inhalation exposure (Adam et al., 2014; Rossner et al., 2019). The current working group considers a likely mechanism of action for ZnO-induced pulmonary toxicity to be dissolution induced dose-dependent oxidative stress, inflammation, and tissue injury causing induction of acute phase response in lung tissue (Cho et al., 2011; Halappanavar et al., 2020; Jacobsen et al., 2015).

In humans, inhalation exposure to ZnO is accompanied by increased blood levels of the acute phase response proteins CRP and SAA (Monse 2018, Baumann 2018, Markert 2016). CRP and SAA levels are highly correlated.

The acute phase response is an established risk factor for atherosclerosis and coronary heart disease (Gabay and Kushner, 1999; Hadrup et al., 2020; Shridas and Tannock, 2019). Serum Amyloid A has been shown to be causally implicated in atherosclerosis in murine models of atherosclerosis (mostly ApoE knock-out mice). Inactivation of all three inducible SAA isoforms in ApoE -/- mice reduces plaque formation (Thompson et al., 2018), while virus-mediated overexpression of SAA1 (Dong et al., 2011) or SAA3 (Thompson et al., 2018) increased plaque formation. Epidemiological studies using Mendelian Randomisation suggest that CRP is not causally related to risk of cardiovascular disease (Elliott et al., 2009; Pai et al., 2008).

The underlying mechanism of action seems to be an SAA-mediated effect on cholesterol transport. Under homeostasis, HDL (high density lipoprotein) facilities cholesterol transport from peripheral macrophages to the liver. During the acute phase response, SAA becomes incorporated into HDL lipoproteins, causing reversal of the cholesterol flow, in turn causing peripheral macrophages to accumulate cholesterol and turn into foam cells, thus causing plaque progression and atherosclerosis (Feingold and Grunfeld, 2016; Lee et al., 2013; Vogel and Cassee, 2018). Rupture of an atherosclerotic plaque may cause myocardial infarction. The mechanism of action for particle-induced acute phase response leading to atherosclerosis has been described in Adverse Outcome Pathway 273 (https://aopwiki.org/aops/237)(Halappanavar et al., 2020).

Thus, the mechanism of action is likely that inhalation of ZnO induces acute phase response leading to formation of atherosclerosis and myocardial infarction. The inhaled ZnO does not accumulate due to rapid dissolution occurring at low pH inside the lysosomes. The contribution to risk of cardiovascular disease would therefore be mediated by ZnO-induced increased blood levels of acute phase response during a 45-

year work life. The study by Krabbe et al. showed that even though repeated inhalation exposure to ZnO induced adaptation of IL-6 levels, no such adaptation was observed for acute phase proteins (Krabbe et al., 2018). Modest increases in CRP and SAA levels are associated with increased risk of coronary heart disease in epidemiological studies.

The ZnO-induced level of acute phase response in a controlled study was high enough to be considered of clinical relevance. In a study of repeated exposure to 2.5 mg/m^3 welding fumes containing 60% Zn and 19.6% Cu for 6 hours on 4 consecutive days, increased CRP and SAA levels ($p<0.001$) were observed in 15 male volunteers from 24 hours after onset of first exposure to 24 hours after onset of the last exposure (Krabbe et al., 2018). All subjects were healthy male non-smokers, including never smokers or ex-smokers. Following exposure, CRP and SAA levels remained elevated 24 hours after onset of the last exposure (last time point assessed). The authors stated : '*Individuals with a chronic CRP level more than 3 mg/L have an increased risk for the occurrence of cardiovascular events like myocardial infarction or stroke compared with individuals with a CRP level less than 1 mg/L. In the current study, approximately 50% of the subjects had a CRP level more than 3 mg/L for the entire exposure week. If this would also be true for repeated exposure over a longer time course, for example, a work life, the increased cardiovascular risk would be an imminent threat for welders exposed to zinc- and copper-containing welding fume. The mass concentration of zinc and copper used in this study corresponds to the occupational exposure limits (2.5 mg/m^3 assuming a density of 5 g/cm^3) in Germany. Therefore, the present study contains a realistic work week scenario with four consecutive welding shifts of 6 and 18 hours of non-exposure*' (Krabbe et al., 2018).

In animal studies, particle-induced inflammation and pulmonary acute phase response are highly correlated (Poulsen et al., 2017; Saber et al., 2014). Inflammation is considered a threshold effect. Similarly, the current working group decided to consider ZnO-induced acute phase response a threshold effect.

Dose-response relationships

Inflammation and acute phase response

Human data

In the study by Monsé et al. (Monsé et al., 2018), 16 healthy, non-smoking volunteers (mean age 26 years) were exposed to nanosized ZnO generated by pyrolysis. SAA and CRP levels were increased in a dose-dependent manner 24 hours post-exposure (fig 2). SAA and CRP were highly correlated (R=0.78). The NOAEC was identified as 0.5 mg/m^3 for 4h.

Animal data

Dose-response relationships: dose-dependent pulmonary inflammation and acute phase response was found following pulmonary exposure to ZnO nanoparticles in mice (Hadrup et al., 2019; Jacobsen et al., 2015).

Previous evaluations of ZnO

ZnO has not been evaluated by IARC. Yet Zn is often a constituent of welding fumes and welding fumes are classified as Group 1 carcinogen by IARC (IARC, 2018). However, welding fumes often contain various other metal oxides, which are known carcinogens.

EU's Scientific Committee on Occupational Exposure Limits (SCOEL) has not published a report on Zn. The EU commission published in 2005 a risk assessment of Zn and Zn compounds including ZnO (Bodar et al., 2005). The review found for ultrafine particles in fumes a LOAEC of 5 mg/m^3 for ZnO based on metal fume fever. Notably, new studies published have since then enabled the setting of NOAEC values as presented in the current report.

There are to our knowledge no reports on hazard assessment of Zn from the European Chemical Agency's (ECHA's) Committee for Risk Assessment (RAC), nor from the Dutch Expert Committee on Occupational Safety (DECOS). The Nordic Expert Group (NEG) has evaluated "zinc" in 1981 (Tola, 1981). This was deemed to be too long ago to be relevant for the current report.

According to Brand (P Brand et al., 2014) (please see 'Short-term controlled studies'), the German MAK Commission used the study of Beckett et al. (Beckett et al., 2005) as a basis for the determination of the workplace threshold concentration. They extrapolated the data showing no symptoms of metal fume fever and no effect on IL-6 levels for twelve healthy volunteers following a two-hour exposure to 0.5 mg/m^3 corresponding to 0.125 mg/m^3 for a work shift duration of 8 hours, and concluded that 0.1 mg/m^3 Zn should be safe. The current working group notes that Beckett et al. did not assess CRP or SAA levels and that only one dose level was assessed.

Scientific basis for setting an occupational exposure limit

Different methods exist for calculating OELs. The choice of method depends on the mode of action of the substance, and can fundamentally be divided into two approaches: Threshold effects or non-threshold effects. The threshold effect approach relies on the assumption that the organism can withstand a certain dose before adverse effects occur, whereas for non-threshold effects it is assumed that any exposure to the substance can result in adverse effects.

The current working group concluded that there is no evidence of ZnO-induced carcinogenicity or mutagenicity, but found evidence of ZnO-induced genotoxicity. The current working group considers the available evidence of genotoxicity to be insufficient to regard cancer as a critical effect.

The current working group considers acute phase response-mediated risk of cardiovascular disease as the most severe critical effect. Furthermore, the current working group considers ZnO-induced acute phase response to be a threshold-based mechanism. Therefore, in this report, we will calculate a proposed OEL based on a threshold effect for induction of acute phase response based on data from human studies.

Health-based exposure limit based on human biomonitoring study

Exposure to ZnO induces metal fume fever and acute phase response, which is a risk factor for cardiovascular disease. There is some epidemiological evidence that welders are at increased risk of cardiovascular disease. The current working group considers the critical effect to be acute phase response-mediated risk of cardiovascular disease. The critical effect is considered induction of acute phase proteins CRP and SAA. The current working group furthermore notes that IL-6 levels in blood show signs of adaptation in a study of repeated exposure (Krabbe et al., 2018), and that ZnO-induced increased CRP and SAA levels were observed in absence of changes in IL-6 (Monsé et al., 2018). Thus, there is substantial evidence that IL-6 cannot be used as biomarker of ZnO-induced acute phase response.

Controlled exposure studies have shown that daily cumulative exposures can be used for determining a dose-response relationship.

The study by Monsé et al. (Monsé et al., 2018) is identified as the only study of the dose-response relationship between exposure to ZnO and induction of acute phase response.

In the study by Monsé et al. (Monsé et al., 2018), 16 healthy, non-smoking volunteers (mean age 26 years) were exposed to nanosized ZnO generated by pyrolysis. The mean diameter of the inhaled ZnO agglomerates was 48-86 nm. The volunteers were exposed to 0, 0.5, 1 and 2 mg/m^3 ZnO for 4 hours including 2 hours of cycling to mimic light work on different exposure days separated by 2 weeks.

Biomarkers of effect were assessed at study entry (baseline) and two weeks after last exposure. In addition, biomarkers of effect were assessed for each exposure, before and

after exposure, and after 24 hours. SAA and CRP levels were increased 24 hours post-exposure. SAA and CRP were highly correlated (R=0.78).

When compared to the levels before exposure, blood CRP levels were significantly increased 24 hours after exposure for all ZnO concentrations. SAA levels were increased 24 hours after exposure to 1.0 and 2.0 mg/m^3 ZnO. Compared to the sham exposure, ZnO exposures yielded significantly higher CRP values 24 hours after exposure to 2.0 mg/m^3 ZnO, and higher SAA values after 1.0 and 2.0 mg/m^3 ZnO. The no-effect level for SAA levels in blood was exposure to 0.5 mg/m^3 for 4 hours, corresponding to 0.25 mg/m^3 over an 8-hour working day.

Concerning assessment factors, all the identified biomonitoring studies were performed using young, healthy and non-smoking volunteers. Nevertheless, substantial variation (more than 20 fold) in SAA and CRP levels were observed in all studies.

Due to the large inter-individual variation observed in all the reviewed biomonitoring studies of healthy volunteers and the life-style factors known to increase the baseline blood levels of the acute phase proteins, the current working group has chosen to use the highest assessment factor for inter-individual variation suggested by ECHA, a factor of 5 (REACH, 2018).

Thus, the suggested threshold is 0.25 mg/m^3/5 = 0.05 mg/m^3 ZnO corresponding to 0.04 mg/m^3 Zn for occupational exposure to ZnO and ZnO fumes.

In summary, the critical effect of ZnO exposure was identified as induction of acute phase response in a threshold-dependent mode of action. The controlled exposure study by Monsé et al. reporting dose-response relationship between ZnO exposure and blood levels of acute phase proteins CRP and SAA were identified as suitable for risk assessment.

Exposure to 0.5 mg/m^3 for 4 hours corresponding to 0.25 mg/m^3 during an 8-hour working day was identified as the NOAEC.

Due to the very large inter-individual variation and since only healthy volunteers have been studied, the highest assessment factor suggested by ECHA, 5 was used for inter-individual variation.

Thus, the suggested threshold is 0.25 mg/m^3/5 = 0.05 mg/m^3 ZnO corresponding to 0.04 mg/m^3 Zn for occupational exposure to ZnO and ZnO fumes.

Conclusion

The current working group evaluated the relevant literature on ZnO from both epidemiological and animal inhalation studies.

The current assessment covers ZnO particles and fumes.

Exposure to ZnO fumes induces metal fume fever and acute phase response, which is a risk factor for cardiovascular disease. There is modest epidemiological evidence that welders are at increased risk of cardiovascular disease.

The current working group concluded that there is no evidence of ZnO-induced carcinogenicity or mutagenicity, but found evidence of ZnO-induced genotoxicity. The current working group considers the available evidence of genotoxicity to be insufficient to regard cancer as a critical effect.

The current working group considers the critical effect to be acute phase response-mediated risk of cardiovascular disease, and thus ZnO-induction of acute phase proteins CRP and SAA, measured as blood levels of CRP and SAA. The acute phase response is likely caused by dissolysis of ZnO at the low pH in lysosomes after cellular uptake. The rapid dissolution minimises the size-dependence of the observed toxicity. Consequently, the current working group suggests using one exposure limit for all ZnO particle sizes.

The current working group found that the mechanism of action of ZnO-induced pulmonary acute phase response was not fully clarified, whereas the mechanism of action of SAA-induced atherosclerosis was clear. Particle-induced inflammation and acute phase response are closely linked in animal studies (Saber 2014) and consequently, the current working group decided to perform the risk assessment based on this threshold-based mechanism of action.

The study by Monsé et al. (Monsé et al., 2018) is identified as the only study of the dose-response relationship between exposure to ZnO and induction of acute phase response. The no-effect level for SAA levels in blood was exposure to 0.5 mg/m^3 for 4 hours, corresponding to 0.25 mg/m^3 over an 8-hour working day.

Due to the large inter-individual variation observed in all the reviewed biomonitoring studies, the current working group has chosen to use the highest assessment factor for inter-individual variation suggested by ECHA, a factor of 5 (REACH, 2018).

Thus, the suggested threshold is 0.25 mg/m^3/5 = 0.05 mg/m^3 ZnO corresponding to 0.04 mg/m^3 Zn for occupational exposure to ZnO and ZnO fumes.

The current working group notes that the toxicity of ZnO is driven by rapid dissolution of ZnO occurring at the low pH in the lysosomes following cellular uptake. The dissolution is rapid, thus minimizing the particle size-dependence of the observed toxicity (Kim et al., 2016). In addition, no difference between exposure to ultrafine or fine ZnO was

reported in a controlled human exposure (Beckett et al., 2005). Consequently, the current working group suggests to use the same exposure limit for all ZnO particle sizes.

The current working group notes that many other metal oxides (especially CuO) also potently induce acute phase response following inhalation exposure (Baumann et al., 2017) and that co-exposures to CuO, ZnO and other metal oxides are common, e.g. during welding (Brand et al., 2019), and shooting (Sikkeland et al., 2017).

In conclusion, a health-based occupational exposure limit for ZnO and ZnO fumes, calculated as Zn, at 0.04 mg/m^3 is suggested.

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Appendix 1

Literature search for the document: ZINC OXIDE: SCIENTIFIC BASIS FOR SETTING A HEALTH-BASED OCCUPATIONAL EXPOSURE LIMIT

To compile this document, we retrieved relevant articles from the PubMed database (Pubmed, 2020) by using the search terms: 1) "zinc oxide AND inhalation" yielding 177 hits; 2) "zinc oxide AND risk assessment" yielding 182 hits; 3) "zinc oxide AND genotoxicity" yielding 169 hits; and 4) "zinc oxide AND carcinogenicity" yielding 169 hits; which were all assessed manually. We supplemented this search strategy with the reading of reference lists of the retrieved articles to identify additional literature with an older date. We identified a total of 136 references were relevant for inclusion in the current document.

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