- ECHA project SR 23 -

Support to the assessment of remaining cancer risks related to the industrial use of cobalt salts in the context of chemical risk management procedures under REACH.

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EXECUTIVE SUMMARY

The objective of this project is to support the assessment of remaining cancer risks related to the industrial use of cobalt(II) sulphate (EC#:233-334-2), cobalt dichloride (EC#: 231-589-4), cobalt(II) dinitrate (EC#:233-402-1), cobalt(II) carbonate (EC#:208-169-4) and cobalt(II) diacetate (EC#:200-755-8) in the context of chemical risk management procedures under REACH.

In the project, specific attention is given to expert assessments performed by international or national bodies (published during the period 2004 – 2014) and also newer literature not included in these assessments. Thus, a top-down procedure is used for this project to evaluate the conclusions of the most recent expert assessments. Based on the data provided herein, supplemented with the data from REACH registrations and recent data from the cobalt industry, conclusions are made regarding the mode of action of the cobalt salts and dose-reponse relationships are established for their carcinogenic effects.

Based on the review of the expert asssessments, it is concluded that the five cobalt salts should be considered as carcinogens by inhalation. This conclusion is based on two NTP carcinogenicity studies performed with mice and rats in which increased incidences of local alveolar/bronchiolar neoplasms were observed at exposure levels of 0.3 mg/m³ of cobalt sulphate heptahydrate and above (in both sexes of both species). From these data OECD in 2014 derived a BMDL10 value of 0.414 mg cobalt sulfate heptahydrate /m³ (corresponding to 0.093 Co/m³) with respect to the development of lung tumours in female rats.

Concerning mutagenic properties, the cobalt(II)-ion is considered genotoxic *in vitro* due to the induction of chromosome damage observed in several assays using mammalian cells. *In vivo* data indicate that cobalt salts may induce a variety of genotoxic alterations (DNA damage, gene mutations and chromosomal aberrations) in connection with intraperitoneal administration. Only very limited and non-conclusive human data are available with respect to the assessment of genotoxic effects from cobalt/ cobalt salt exposure. Based on this it is concluded that genotoxicity as a mode of action behind lung tumours cannot to be ruled out.

The underlying mechanisms for the genotoxic and carcinogenic effects of the cobalt salts have not been fully elucidated, but it is a general view that key mechanisms involved are:

- oxidative DNA damages due to cobalt(II) induced ROS generation as Co(II) catalyses the generation of reative oxygen species through a Fenton like mechanism;
- cobalt(II) induced impairment of DNA-repair mechanisms due to cobalt (II) binding to DNA-repair enzymes.

Overall, it has to be noted that specific thresholds remains to be identified for the Co(II)-ion with respect to tumour formation. Mechanistically, uncertainties pertain to whether the initial event of a catalytic effect of the cobalt(II) ions leading to oxidative DNA damages through a Fenton-like mechanism can be considered a threshold or a non-threshold effect. Further it is not clear whether the induction of alveolar proteinosis, chronic inflammation, hyperplasia (all of which may be considered as thresholded events) are prerequisite for the development of a carcinogenic response of Co(II).

So, although the suggested mechanisms may have thresholds, the current data does not allow identification of this. Overall, it can be concluded:

- carcinogenicity data are only available for local tumours in the respiratory tract in relation to

inhalation exposure, thus dose response estimations can only be made for inhalation exposure.

- the current scientific findings and mode of action considerations support the notion that water soluble cobalt substances may be threshold carcinogens although there are some uncertainties related to initiation by catalytic ROS generation and direct oxidative DNA damage. In addition, the genotoxicity data may indicate a non-threshold mechanism.

- thresholds have, however, not been identified for the cobalt salts in relation to the carcinogenicity and genotoxicity in the respiratory tract

Therefore at present, due to lack of identified thresholds and due to remaining uncertainties regarding the mechanisms involved, the water soluble cobalt salts are considered as genotoxic carcinogens and are to be assessed using a non-threshold approach.

Dose-response relationship

As a starting point for the dose-response estimations, the BMDL10 of 0.093 mg Co/m^3 with respect to development of bronchoalveolar tumours in female rats was identified as the most appropriate dose metric. As the animals were exposed to cobalt sulphate particle with a MMAD (Mass Median Aerodynamic Diameter) in the range of 1 µm – 3 µm, and as the tumours were located in the deeper part of the lung, the dose-response relationship pertains to the *respirable fraction* of the particles. Inhalable particles would - for the particle fraction above the size of the respirable range – to a great extent be deposited in the upper part of the respiratory tract. Data from the NTP (1998) indicate that both rats and mice develop hyperplasia, metaplasia and atrophy in epithelial cells of the nose, and metaplasia of the squamous epithelium of the larynx. Although inhalable particles should also be considered as carcinogenic the dose-response related to this metric is far more uncertain as this will very much depend of the content of respirable particles.

It should be noted that the dose response relationships were derived by linear extrapolation, which is to be considered as a very conservative approach, especially at very low exposure levels. It is acknowledged therefore that excess risks in the lower exposure range might be overestimated following this approach.

Using a linearised non-threshold approach the following dose response relationships were derived.

Worker exposure, dose-response:
The following dose-response relationship was dervied for worker exposure (8 h/d) with respect to the respirable fraction of the particles:

$$\text{Excess risk} = \text{exposure level} \times 1.05\ (\text{mg Co/m}^3)^{-1}$$

General population exposure, dose-response:
The following dose-response relationship was dervied for general population exposure (24 h/d) with respect to the respirable fraction of the particles:

$$\text{Excess risk} = \text{exposure level} \times 5.88\ (\text{mg Co/m}^3)^{-1}$$

1 INTRODUCTION

1.1 Objective

The objective of this project is to support the assessment of remaining cancer risks related to the industrial use of cobalt(II) sulphate (EC#:233-334-2), cobalt dichloride (EC#: 231-589-4), cobalt(II) dinitrate (EC#:233-402-1), cobalt(II) carbonate (EC#:208-169-4) and cobalt(II) diacetate (EC#:200-755-8) in the context of chemical risk management procedures under REACH.

A key issue is to evaluate the mode of action of the cobalt salts to assist in the identification of the most appropriate risk management action for these substances. Furthermore, justifications should be given for the selection of the most relevant studies and exposure-related parameters for establishing a dose-response model in relation to human exposure to cobalt salts and cancer risk.

In the project, specific attention is given to assessments performed by international or national bodies and newer literature not included in these assessments. Thus, a top-down procedure is used for this project to evaluate the conclusions of the most recent expert assessments and based on the data provided herein to conclude on mode of action and the dose-reponse for carcinogenicty of the cobalt substances.

1.2 Key data on cobalt salts

In connection with web based literature search and in consultation with ECHA, the following key expert evaluations were identified (in chronological order) and these evaluations will be examined in this report:

ATSDR (2004). Toxicological Profile for Cobalt. U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry, 486 pp.

SWH (2005a). Cobalt and Cobalt Compounds. Criteria Document for Swedish Occupational Standards. National Institute for Working life. Swedish Work and Health 2005:12. http://www.inchem.org/documents/kemi/kemi/ah2005_12.pdf

SWH (2005b). Scientific Basis for Swedish Occupational Standards xxv. Cobalt and cobalt compound. National Institute for Working life. Swedish Work and Health 2005:7,16-43. http://www.inchem.org/documents/kemi/kemi/ah2005_07.pdf

WHO/CICAD (2006). Cobalt and inorganic cobalt compounds. Concise International Chemical Assessment Document 69, WHO, 85 pp.

IARC (2006). IARC monographs on the evaluation of carcinogenic risks to humans: Cobalt in hard metals and cobalt sulfate, gallium arsenide, indium phosphide and vanadium pentoxide. Vol 86, 37-158.

MAK (2007). Cobalt and its compounds (as inhalable dusts or aerosols). Evaluation from 2004. http://onlinelibrary.wiley.com/doi/10.1002/3527600418.mb744048e0023/pdf

MAK (2009). Cobalt und Cobaltverbindungen. http://onlinelibrary.wiley.com/doi/10.1002/3527600418.mb744048verd0046/pdf

EFSA (2009). Scientific Opinion on the use of cobalt compounds as additives in animal nutrition. EFSA Journal 2009;7(12):1383.

ECHC (2011). Screening Assessment for the Challenge: Cobalt, Cobalt chloride, Cobalt sulphate. Environment Canada, Health Canada. https://www.ec.gc.ca/ese-ees/8E18277B-457E-4073-8F27-EF5878648820/batch10_4substances(1)_en.pdf

EFSA (2012). Scientific Opinion on safety and efficacy of cobalt compounds (E3) as feed additives for all animal species: Cobaltous acetate tetrahydrate, basic cobaltous carbonate monohydrate and cobaltous sulphate heptahydrate, based on a dossier submitted by TREAC EEIG. EFSA Journal 2012;10(7):2791.

Danish EPA (2013). Cobalt(II), inorganic and soluble salts. Evaluation of health hazards and proposal of a health based quality criterion for drinking water. Environmental Project No. 1520, 2013. Danish Environment Protection Agency.

NTP (2013). NTP TECHNICAL REPORT ON THE TOXICOLOGY STUDIES OF COBALT METAL (CAS NO. 7440-48-4) IN F344/N RATS AND B6C3F1/N MICE AND TOXICOLOGY AND CARCINOGENESIS STUDIES OF COBALT METAL IN F344/NTac RATS AND B6C3F1/N MICE (INHALATION STUDIES). NTP TR 581.NIH Publication No. 14-5923. National Toxicology Programme

NTP (2014). Cobalt Sulfate CAS No. 10124-43-3. Report on Carcinogens, Thirteenth Edition. Research Triangle Park, NC: U.S. Department of Health and Human Services, Public Health Service.
http://ntp.niehs.nih.gov/pubhealth/roc/roc13/

OECD (2014a). SIDS Initial Assessment Report on soluble cobalt salts. For CoCAM 6 Paris, 30 September – 3 October 2014.

OECD (2014b). SIDS Initial Assessment Profile on soluble cobalt salts. For CoCAM 6 Paris, 30 September – 3 October 2014.
http://webnet.oecd.org/HPV/UI/handler.axd?id=b789fd1c-bab3-433c-9f47-3cbd49042976

ANSES (2014). Valeurs limites d'exposition en milieu professionnel Évaluation des effets sur la santé et des methods de mesure des niveaux d'exposition sur le lieu de travail pour le cobalt et ses composes à l'exception du cobalt associé au carbure de tungstène. Rapport d'expertise collective. Septembre 2014.

In addition, data from the CSR reports in REACH registrations of the cobalt salts will be examined with respect to the evaluation and description of the carcinogenicity and the mode of action of the cobalt salts. Additional data provided for this project by the Cobalt Development Institute (CDI/CoRC) will also be considered in this project.

1.3 Outline of the report

Chapter 2 provides an introduction to the cobalt salts by describing the physico-chemical properties of the substances.

Chapter 3 provides the harmonised classification of the five cobalt salts and an overview of the data in relation to their toxicokinetic properties and their carcinogenicity and mutagenicity. This chapter is based on the most updated in-depth expert evaluations of the substances among the references shown above.

Chapter 4 gives an overview of the discussions/conclusions made by international and national expert groups specifically with regard to the carcinogenic mode of action of the substances. Differences in the interpretation of key data are discussed and the most important references for mode of action and

use of threshold/non-threshold approach are identified. Based on this discussion, the use of a threshold/non-threshold approach for the cobalt salts in the context of the REACH Regulation is concluded.

Chapter 5 goes into detail with respect to dose-response relationship for the carcinogenic effects. The relevant exposure route for the carcinogenic risk is identified and the most relevant point of departure (POD) for establishing dose-response estimations is identified. Appropriate modifications of the POD metric are made and explained and considerations regarding interspecies differences are presented. In accordance with the methodology described in the ECHA Guidance R.8[1], the carcinogenic dose-response relationship is presented for worker exposure as well as for exposure to the general population.

[1] Guidance on information requirements and chemical safety assessment, Chapter R.8: Characterisation of dose [concentration]-response for human health (version 2.1)

2 IDENTITY AND PHYSICO-CHEMICAL PROPERTIES

The five cobalt salts are described and evaluated as a category for the purposes of this report, as the divalent cobalt cation (Co^{2+}) moiety is considered to constitute the critical entity of the cobalt salt substances that has been evaluated related to their carcinogenicity. The counter ions of the cobalt salts (i.e. sulphate, nitrate, chloride, acetate, and carbonate) are not considered to contribute to any toxicity of the cobalt salts and are therefore not evaluated further. Thus, the cobalt salts are considered to have very similar toxicological properties. Such a grouping/read-across approach has been used by several expert group evaluations that often even used a broader category approach covering both metallic cobalt and less water soluble cobalt compounds.

The following description of the physical and chemical properties of the cobalt salts are referenced from OECD (2014a). Further details are given in the table below.

"Cobalt sulphate is typically marketed as the heptahydrate, which is a rose, odourless, crystalline, inorganic solid. The relative density of cobalt sulphate is 3.71. Upon heating of the hydrated form, water of crystallisation is lost and the anhydrous form is formed. The melting point for the anhydrous cobalt sulphate is reported to be > 700°C. The water solubility of cobalt sulphate monohydrate at 20°C and 37°C is 376.7 g/L and 391.5 g/L (measured), respectively."

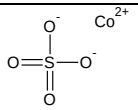
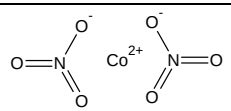
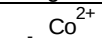
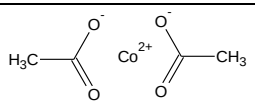
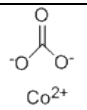
"Cobalt dinitrate is typically marketed as the hexahydrate, which is a red purple, flaked, inorganic solid. The relative density of cobalt dinitrate is 2.49. Cobalt dinitrate decomposes at ca. 100 °C before melting. The water solubility of cobalt dinitrate hexahydrate at 20 °C is > 669.6 g/L (measured)."

"Cobalt dichloride is typically marketed as the hexahydrate, which is a purple, odourless, crystalline, inorganic solid. The relative density of cobalt dichloride is 3.36 - 3.37. Upon heating of the hydrated form, water of crystallisation is lost and the anhydrous form is formed. The melting point for the anhydrous cobalt dichloride is reported to between 735°C - 737°C. The water solubility of cobalt dichloride hexahydrate at room temperature is 585.9 g/L (measured)."

"Cobalt diacetate is typically marketed as the tetrahydrate, which is a red, crystalline inorganic solid with a relative density of 1.76 (measured at 21.4 °C). A decomposition temperature of cobalt diacetate tetrahydrate was determined at 370°C. Distinct melting or boiling points are not available. The water solubility of cobalt diacetate tetrahydrate at 20 °C is 348.04 g/L and 360 g/L (measured)."

For cobalt carbonate the commercial form is cobalt(II) carbonate (CAS number 7542-09-8), a material of indeterminate stoichiometry, (CoCO3)x _ (CO(OH)2)y _ zH2O, that contains 45 – 47 % cobalt (ECHA 2010). Cobalt carbonate decomposes at 280 °C and is only sparingly soluble in water with a water solubility of up to 0.022 g/L depending on the conditions for measuring the solubility (e.g. load of the substance and duration) (REACH-registration data of the substance, public domain).

Table 2.1 Physico-chemical properties of the 5 cobalt substances (OECD 2014a + REACH registration).

Common name	Cobalt (II) sulphate	Cobalt (II) dinitrate	Cobalt (II) dichloride	Cobalt (II) diacetate	Cobalt(II)carbonate
Molecular Formula	$CoSO_4$	$Co(NO_{3)2}$	$CoCl_2$	$Co(C_2H_3O_2)_2$	$CoCo_3$
Physical state	Cobalt sulphate heptahydrate is a rose, odourless, crystalline, inorganic solid	Cobalt dinitrate hexahydrate is a red purple, flaked, inorganic solid	Cobalt dichloride hexahydrate is a purple, odourless, crystalline, inorganic solid	Cobalt diacetate tetrahydrate is a red, crystalline, inorganic solid	Cobalt carbonate is a red powder or rhombohedral crystals
Structural formula					
Molecular weight	154.99 (anhydrous) 281.10 (heptahydrate)	182.94 (anhydrous) 291.03 (hexahydrate)	129.84 (anhydrous) 237.93 (hexahydrate)	177.02 (anhydrous) 249.08 (tetrahydrate)	118.94 (anhydrous)
CAS number	10124-43-3 10026-24-1 (heptahydrate)	10141-05-6 10026-22-9 (hexahydrate)	7646-79-9 7791-13-1 (hexahydrate)	71-48-7 6147-53-1 (tetrahydrate)	513-79-1 (Anhydrous) 57454-67-8 (Hydrate)
EINECS number	233-334-2	233-402-1	231-589-4	200-755-8	208-169-4
Melting point	>700°C	Decomposes at about 100 °C. No melting point can be determined	735°C and 737°C	Decomposes around 370 °C. No melting point can be determined	Decomposes at 280 °C.
Density	Cobalt sulphate has a relative density of 3.71.	Cobalt dinitrate has a relative density of 2.49.	Cobalt dichloride has a relative density of about 3.36 - 3.37.	Cobalt diacetate tetrahydrate has a relative density of 1.76.	Cobalt(II)carbonate has a relative density of 4.2.
Vapour pressure	Negligible, i.e. below the level of significance (10^{-5} Pa).	Negligible, i.e. below the level of significance (10^{-5} Pa).	Negligible, i.e. below the level of significance (10^{-5} Pa).	Negligible, i.e. below the level of significance (10^{-5} Pa).	Negligible, i.e. below the level of significance (10-5 Pa).
Water solubility	376.7 g/L at 20°C and 391.5 g/L at 37°C	> 669.6g/L at 20°C	585.9 g/L at RT	348.04 g/L and 360 g/L at 20°C	< 0.022 g/L
Log octanol/water partition coefficient	Not relevant for cobalt salts. (inorganic ions with negligible transfer to organic phase)	Not relevant for cobalt salts. (inorganic ions with negligible transfer to organic phase)	Not relevant for cobalt salts. (inorganic ions with negligible transfer to organic phase)	Not relevant for cobalt salts. (inorganic ions with negligible transfer to organic phase)	Not relevant for cobalt salts. (inorganic ions with negligible transfer to organic phase)

It can be seen that cobalt carbonate differs from the other, highly water soluble cobalt salts by the fact that it is only sparingly water soluble. However, in terms of toxicological relevance and for pragmatic reasons, the five cobalt salts will be referred to in this study as soluble salts and considered as a group. A similar approach was taken by MAK (2007), which termed all cobalt compounds with a water solubility above 0.1 g/L as “water soluble”.

It should be noted that the properties of the possible nanoforms of these salts are not considered in this report.

3 HUMAN HEALTH HAZARD WITH FOCUS ON CANCER

3.1 Human health classification

The cobalt salts are subjected to the following harmonised CLP-classifications:

Table 3.1 Harmonised human health classification of cobalt salts

Substance	Harmonised classification
Cobalt (II) diacetate CAS 71-48-7	Skin Sens. 1 (H317); Resp. Sens. 1 (H334); **Carc. 1B (H350i); Muta. 2 (H341)**; Repr. 1B (H360F)
Cobalt (II) dichloride CAS 7646-79-9	Acute Tox. 4 (H302); Skin Sens. 1 (H317); Resp. Sens. 1 (H334); **Carc. 1B (H350i); Muta. 2 (H341)**; Repr. 1B (H360F)
Cobalt (II) carbonate CAS 513-79-1	Skin Sens. 1 (H317); Resp. Sens. 1 (H334); **Carc. 1B (H350i); Muta. 2 (H341)**; Repr. 1B (H360F)
Cobalt (II) dinitrate CAS 10141-05-6	Skin Sens. 1 (H317); Resp. Sens. 1 (H334); **Carc. 1B (H350i); Muta.2 (H341)**; Repr. 1B (H360F)
Cobalt (II) sulphate CAS 10124-43-3	Acute Tox. 4 (H302); Skin Sens. 1 (H317); Resp. Sens. 1 (H334); **Carc. 1B (H350i); Muta. 2 (H341)**; Repr. 1B (H360F)

From this it can be seen that identical classification applies to all cobalt salts covered by this project with the exception of cobalt (II) sulphate and cobalt (II) dichloride that are furthermore classified as Acute Tox 4 (H302). The identical hazard profile as indicated by the classification supports the category approach for the substances. In that respect it is interesting to notice that classification as Muta.2 applies to all the cobalt salts and that the classification as Carc 1B only pertains to the inhalation route of exposure.

It may be noted that other non-water soluble (or less water soluble) substances such as metallic cobalt, cobalt oxide and cobalt sulphide are classified with neither Carc 1B nor Muta. 2 in the entries of the harmonised classification of the substances.

IARC (2006) has concluded that cobalt (II) sulphate and other soluble cobalt (II) salts are possibly carcinogenic to humans (Group 2B). Also metallic cobalt metal (without tungsten carbide) was concluded as possibly carcinogenic to humans (Group 2B), due to sufficient evidence in animals.

3.2 Toxicokinetics

3.2.1 Absorption and distribution

Inhalation exposure

Absorption data from inhalation are sparse; however, an approximately absorption rate of 30 % of inhaled cobalt oxide particles has been found in a study using hamsters. After inhalation of cobalt and cobalt substances in experimental animals, marked increases of cobalt have been found in the lungs, but increased cobalt levels were also found in the liver, kidney, trachea, spleen, bones and heart with the highest levels in the liver and kidneys (WHO/CICAD 2006).

Increased levels of cobalt in urine from humans exposed by inhalation to cobalt substances indicate

that absorption takes place after inhalation; however, absorption may also occur from the gastrointestinal tract due to deposition of particles in the upper respiratory tract and swallowing of the particles. The distribution of cobalt in humans is similar to that described for experimental animals (WHO/CICAD 2006).

Oral exposure

In experimental animal rat studies gastrointestinal absorption of 13-14 % has been found for cobalt chloride compared to only 1-3 % for the unsoluble cobalt oxide. Cobalt chloride administered together with milk had a significant higher absorption rate of approximately 40 %.

In experimental animals after oral absorption, cobalt is distributed from the blood to other tissues: liver (primarily), kidneys, heart, stomach and intestines. After long-term oral exposure in rats, increased cobalt levels were found in liver, kidneys, muscle, brain and testes. In pregnant rats increased cobalt levels were found in fetal blood and amniotic fluid after oral exposure (WHO/CICAD 2006).

In humans, absorption rates of 18 to 97 % of an oral dose have been found depending on type and dose of the cobalt compound and the nutritional status of the individual. The absorption is increased among individuals with iron deficiency as rates of 31-71 % were found compared to individuals without iron deficiency (18-44 %) (WHO/CICAD 2006).

Dermal exposure

With respect to dermal exposure, hamsters exposed to 100 µl of a solution containing 2 µg cobalt chloride/µl on shaved skin eliminated 33 µg of this dose during 48 hours indicating a significant degree of absorption (MAK 2009).

Increased levels of cobalt in urine has also been determined after dermal exposure of humans to lubricating oil containing cobalt substances (MAK 2009).

An *in vitro* experiment using human skin determined the dermal absorption using applications of cobalt dichloride of approx. 100 µg/cm² and approx. 1000 µg/cm² for an exposure duration of 8h. For the two exposure concentrations, the corresponding absorbable doses (sum of absorbed dose + skin and stratum corneum) were calcultated to 0.38% and 1.08%, respectively (OECD 2014a).

3.2.2 Elimination

Inhalation exposure

In experimental animals, soluble cobalt compounds are absorbed into the blood at a faster rate than less soluble compounds and excreted in the urine and faeces. Urinary excretion rates seem to correlate with the translocation rate of cobalt from the lungs to blood, whereas faecal excretion rates seem to correlate with mechanical clearance rates of cobalt from the lungs to the gastrointestinal tract. Following an initial high rate of faecal clearance, urinary excretion is the primary route of cobalt elimination after a single inhalation exposure or 3 months of exposure (WHO/CICAD 2006).

No human data are available regarding elimination after inhalational exposure.

Oral exposure

In experimental animals, faecal elimination is the primary route of excretion following oral exposure. Faecal clearance has been noted to decrease as cobalt particle solubility increases. In several species, oral exposure to cobalt(II,III) oxide (with ^{57}Co tracer) resulted in little gastrointestinal absorption and a rapid elimination in faeces (>96 %). Cobalt(II) chloride was excreted primarily via faeces (70–83 % of the administered dose) in rats, with urinary excretion accounting for the remainder of the dose. Single exposures in beagle dogs demonstrated that insoluble cobalt(II,III) oxide was eliminated in the faeces and urine at 90 % and 5 %, respectively, while the more soluble cobalt nitrate was eliminated at 70 % in the faeces and 25 % in the urine (WHO/CICAD 2006).

In humans, faecal elimination is also the primary route of excretion following oral exposure. Faecal elimination has been found to vary (3–99 % of the dose) depending on the amount and type of cobalt administered and the nutritional status of the subject. Several days after an oral exposure, ten times more cobalt was excreted in faeces than in urine. In subjects with an iron deficiency, less cobalt was eliminated in the faeces, and more was absorbed (WHO/CICAD 2006).

Dermal exposure

No data available.

3.3 Carcinogenicity

3.3.1 Experimental animal data

Only carcinogenicity studies in relation to the inhalational route are available. Thus, two 2-year carcinogenicity studies with inhalational exposure to cobalt sulphate heptahydrate in rats and mice are available (NTP 1998 (full study report) and publication by Bucher et al. 1999).

In these studies, increased incidences of alveolar/bronchiolar neoplasms in both sexes of both species at concentrations ≥ 0.3 mg/m³ cobalt sulphate heptahydrate (equivalent to ≥ 0.067 mg Co/m³) were seen (see Tables 3.2 and 3.3 below with detailed information). No NOAEC for formation of lung tumours could be derived from these studies.

Table 3.2 Results of the NTP study on the carcinogenicity of cobalt sulfate in rats from the NTP (1998) study. (Table from MAK 2007)

Species:		F344/N rats, 50 ♂, ♀ per group			
Application:		inhalation, whole animal exposure chamber			
Concentration:		0, 0.3, 1.0, 3.0 mg cobalt sulfate heptahydrate/m^3			
Duration:		6 hours/day, 5 days/week, 105 weeks			
Toxicity:		0.3 mg/m^3 and above: ♂, ♀: interstitial fibrosis			
		Cobalt sulfate-heptahydrate concentration [mg/m^3]			
		0	0.3	1.0	3.0
Surviving animals after 24 months	♂	17/50 (34%)	15/50 (30%)	21/50 (42%)	15/50 (30%)
	♀	28/50 (56%)	25/49 (51%)	26/50 (52%)	30/50 (60%)
Lungs					
hyperplasia of the alveolar epithelium	♂	9/50 (18%)	20/50 (40%)*	20/48 (42%)*	23/50 (46%)**
	♀	15/50 (30%)	7/49 (14%)	20/50 (40%)	33/50 (66%)
atypical hyperplasia of the alveolar epithelium	♀	0/50 (0%)	0/49 (0%)	3/50 (6%)	5/50 (10%)*
metaplasia of the squamous epithelium	♀	0/50 (0%)	1/49 (2%)	8/50 (16%)*	3/50 (6%)
metaplasia of the alveolar epithelium	♂	0/50 (0%)	50/50 (100%)**	48/48 (100%)**	49/50 (98%)**
	♀	2/50 (4%)	47/49 (96%)**	50/50 (100%)**	49/50 (98%)**
bronchoalveolar adenomas	♂	1/50 (2%)	4/50 (8%)	1/48 (2%)	6/50 (12%)
	♀	0/50 (0%)	1/49 (2%)	10/50 (20%)**	9/50 (18%)*
bronchoalveolar carcinomas	♂	0/50 (0%)	0/50 (0%)	3/48 (6%)	1/50 (2%)
	♀	0/50 (0%)	2/49 (4%)	6/50 (12%)*	6/50 (12%)*
bronchoalveolar adenomas or carcinomas	♂	1/50 (2%)	4/50 (8%)	4/48 (8%)	7/50 (14%)*
	♀	0/50 (0%)	3/49 (6%)	16/50 (32%)**	16/50 (32%)**
Nose					
hyperplasia of the lateral wall epithelium	♂	2/50 (4%)	14/50 (28%)	21/49 (42%)	20/50 (40%)
	♀	1/50 (2%)	8/49 (16%)	26/50 (52%)	38/50 (76%)
metaplasia of the lateral wall squamous epithelium	♂	1/50 (2%)	3/50 (6%)	5/49 (10%)	8/50 (16%)
	♀	1/50 (2%)	1/49 (2%)	4/50 (8%)	10/50 (20%)
olfactory epithelial atrophy	♂	8/50 (16%)	24/50 (48%)**	42/49 (86%)**	48/50 (96%)**
	♀	5/50 (10%)	29/49 (59%)**	46/50 (92%)**	47/50 (94%)**
olfactory epithelial metaplasia	♂	5/50 (10%)	1/50 (2%)	5/49 (10%)	30/50 (60%)**
	♀	2/50 (4%)	2/49 (4%)	3/50 (6%)**	40/50 (80%)**
Larynx					
metaplasia of the squamous epithelium of the epiglottis	♂	0/50 (0%)	10/49 (20%)**	37/48 (77%)**	50/50 (100%)**
	♀	1/50 (2%)	22/49 (45%)**	39/50 (78%)**	48/50 (96%)**
Adrenals					
benign, complex or malignant phaeochromocytomas	♂	15/50 (30%)	19/50 (38%)	25/49 (51%)*	20/50 (40%)
	♀	2/48 (4%)	1/49 (2%)	4/50 (8%)	10/48 (21%)*

* $p \leq 0.05$ (Fisher's exact test)
** $p \leq 0.01$ (Fisher's exact test)

Table 3.3 Results of the NTP study on the carcinogenicity of cobalt sulfate in mice from the NTP (1998) study. (Table from MAK 2007)

Species:		B6C3F1 mice, 50 ♂, ♀ per group			
Administration:		inhalation, whole animal exposure chamber			
Concentration:		0, 0.3, 1.0, 3.0 mg cobalt sulfate heptahydrate/m^3			
Duration:		6 hours/day, 5 days/week, 105 weeks			
		Cobalt sulfate heptahydrate concentration [mg/m^3]			
		0	0.3	1.0	3.0
Surviving animals after 24 months	♂	22/50 (44%)	31/50 (62%)	24/50 (48%)	2050 (40%)
	♀	34/50 (68%)	37/50 (74%)	32/50 (64%)	28/50 (56%)
Lungs					
Bronchoalveolar adenomas	♂	9/50 (18%)	12/50 (24%)	13/50 (26%)	18/50 (36%)*
	♀	3/50 (6%)	6/50 (12%)	9/50 (18%)	10/50 (20%)*
Bronchoalveolar carcinomas	♂	4/50 (8%)	5/50 (10%)	7/50 (14%)	11/50 (22%)*
	♀	1/50 (2%)	1/50 (2%)	4/50 (8%)	9/50 (18%)**
Bronchoalveolar adenomas or carcinomas	♂	11/50 (22%)	14/50 (28%)	19/50 (38%)	28/50 (56%)**
	♀	4/50 (8%)	7/50 (14%)	13/50 (26%)*	18/50 (36%)**
Nose					
olfactory epithelial atrophy	♂	0/50 (0%)	0/50 (0%)	29/48 (60%)**	48/49/98%)**
	♀	0/50 (0%)	2/50 (4%)	12/49 (24%)**	46/48 (96%)**
olfactory epithelial hyperplasia	♂	0/50 (0%)	0/50 (0%)	0/48 (0%)	10/50 /20%)**
	♀	0/50 (0%)	0/50 (0%)	0/49 (0%)	30/48 (63%)**
Larynx					
metaplasia of the squamous epithelium	♂	0/48 (0%)	37/49 (76%)**	48/48 (100%)**	44/49 (90%)**
	♀	0/50 (0%)	45/49 (92%)**	40/47 (85%)**	50/50 (100%)**

* $p \leq 0.05$ (Fisher's exact test)
** $p \leq 0.01$ (Fisher's exact test)

Based on the findings from these studies, OECD (2014a) calculated benchmark doses (BMD) using the US EPA BMD software (Version 2.0) with the Gamma Model (Version 2.13). The numbers of alveolar/bronchiolar adenoma or carcinoma in the lungs of rats and mice were selected as benchmark response. The 95 % lower confidence limit of the BMD for a treatment-related increase in response of 10 % was calculated (BMDL10). The lowest BMDL10 value of 0.414 mg/m³ cobalt sulphate heptahydrate was found for female rat tumours.

It should be noted that in the NTP (1998) study, increased incidences of liver hemangiosarcomas occurred in male mice, which may indicate concern for carcinogenic effects after systemic uptake as well (which then also could be relevant for systemic uptake from other routes of exposure). However, the male mice were infected with Heliobacter Hepaticus and in earlier NTP studies, infections caused by Heliobacter Hepaticus had led to increased incidences of hemangiosarcoma in the livers of the mice. Therefore, this finding could not be associated to the cobalt sulphate exposure (NTP 1998; Bucher 1999).

Furthermore, adrenal pheochromocytomas were increased in female rats, and to some extent in male rats (NTP 1998; Buchner 1999). However, the induction of pheochromocytoma is known to occur in rats in connection with inhalation exposure to particles that lead to inflammation, fibrosis and chronic

pulmonary lesions, which then further reduces the gas exchange and lead to hypoxemia of the animals (Ozaki 2002). Therefore, in the current CLP guidance document, the induction of this type of tumours in connection with inhalation of particles in rats is not considered relevant for cancer classification (ECHA 2015).

Based on this, it may be concluded that the local induction of alveolar/ bronchiolar tumours in the lungs can be considered the only carcinogenic relevant response from inhalation exposure to water soluble cobalt salts.

3.3.2 Human data

Very limited human data are available with regard to carcinogenicity.

Thus, only two epidemiological studies concerning occupational exposure to cobalt salts have been identified by OECD (2014) as well as by IARC (2006).

Mur et al. (1987) studied the mortality in a cohort of 1 143 workers at an electrochemical plant in France that produced cobalt, cobalt oxides, cobalt salts and sodium. The cohort included all men who had worked one year or more between 1950 and 1980 and was split in several subgroups related to their tasks in the production. However, the exposure levels of cobalt were not reported. Among cobalt production workers, there was a relative increase in deaths from cancers of the trachea, lungs and bronchus (SMR 4.66; 95 % CI 1.46-10.64 based on four cases). No corrections for cigarette smoking were possible. The relationship between cobalt production and lung cancer mortality seemed to be supported by a case-control analysis nested in the cohort study. Among cases of deaths from lung cancer, there were 44 % of the workers who had ever been employed at the cobalt production (all for more than 10 years); there were only 17 % among the controls. However, the difference was not statistically significant. The interpretation of this study should be done with caution due to the low number of cases and because the role of smoking and of simultaneous exposure to arsenic and nickel could not be taken into account.

Moulin et al. (1993) did a follow-up study of the same population, extending the observation period from 1980 to 1988. The total cohort comprised 1 148 subjects. The SMR for all causes of death was 0.85 (95 % CI 0.76-0.95) for the whole cohort, and 0.95 (95 % CI 0.78-1.26) for the sub-cohort of workers born in France. With regard to lung cancer mortality among cobalt production workers, the SMRs were 0.85 (95 % CI 0.18-2.50, 3 cases) for the whole cohort and 1.16 (95 % CI 0.24-3.40, 3 cases) for the sub-cohort. Any excess of mortality from diseases of the circulatory and of the respiratory systems did not appear among cobalt production workers. Maintenance workers, however, exhibited a non-significantly elevated SMR for lung cancer (1.80, 95 % CI 0.78-3.55), reaching statistical significance for duration of exposure and time since first exposure ≥ 30 years. This finding could not be clearly explained apart from the fact that asbestos exposure may have occurred. Again, it may be noted that the study is limited by the very small number of cases.

Thus, all in all the human data are too limited to draw any conclusions regarding the carcinogenicity of the cobalt salts.

3.4 Mutagenicity

3.4.1 *In vitro data*

From the IARC (2006) evaluation on the water soluble cobalt salts, it can be seen that generally there was a lack of mutagenic activity in bacteria, although isolated positive finds occurred and a comutagenic potential was noted in connection with co-exposure to known mutagens e.g. benzo(a) pyrene and napthtylamine.

In saccharomyces cerevisiae gene conversion and petite *ρ*-mutation in mitochondrial DNA was seen, but no other types of mutation occurred.

IARC (2006) noted several positive results in mammalian cells cultured *in vitro* with respect to induction of DNA–protein cross-linkage, DNA strand breakage and sister chromatid exchange in most of the studies.

Further, cobalt (II) chloride induced mutations at the Hprt locus in Chinese hamster V79 cells, but not at the 8AG and the Gpt loci. At the same Gpt locus in a transgenic Chinese hamster V79 G12 cell line, lower concentrations of cobalt (II) chloride did induce gene mutations. In a single study, at the Tk locus in mouse lymphoma L5178Y cells, the results were negative.

Also, cell transformation of Syrian hamster embryo cells was found to be induced by the cobalt salts (IARC 2006).

In cultured human cells *in vitro*, positive results were noted for inhibition of protein-DNA binding activities and inhibition of p53 binding to DNA and for induction of gene expression (in Cap43 in human lung cells), induction of DNA strand breakage and sister chromatid exchange. In cultured human lymphocytes, induction of aneuploidy was noted (IARC 2006).

When looking through the different expert evaluations, there are no substantial differences in the interpretation of the *in vitro* mutagenicity data, and it is overall acknowledged that cobalt metal particles and soluble cobalt (II) salts have the capacity to cause DNA damage and chromosomal damage in mammalian cells *in vitro*.

It should, however, be noted that the negative results in bacteria may be probably due to the test conditions of the assays performed. The difficulty of detecting cobalt and other metals and their salts in bacteria was shown by Pagano and Zeiger (1992) to be due to interaction with test media (precipitation in the PO_4-buffer, which is used as standard in the Ames test). If HEPES buffer or water was used instead, cobalt chloride was much more potent in inducing gene mutations. A clear concentration response effect was observed from 12.5 to 800 µM and a more than threefold increase in revertants was observed already at 50 µM $CoCl_2$.

For further details see Appendix A, which presents the mutagenicity data in a tabulated form and the conclusions made from the data by expert groups.

3.4.2 *In vivo* data

Rather limited *in vivo* genotoxicity data are available on the cobalt salts.

An overview of the experimental animal *in vivo* genotoxicity test results is shown below in Table 3.4.

Table 3.4. *In vivo* genotoxicity data on water soluble and sparingly soluble cobalt salts. (Compiled from, IARC 2006, MAK 2007, ECHC 2011, OECD 2014a, Kirkland et al. 2015).

Substance and reference	Assay	Exposure	Result
Intraperitoneal exposure			
Cobalt(II) chloride, Farah 1983	Aneuploidy, Male hamsters - bone marrow - germ cells	400 mg/kg bw i.p. dosed over 9 days	 Positive Positive
Cobalt(II) chloride, Suzuki et al. 1993	MN, Mice -bone marrow	25-90 mg/kg bw i.p.	Positive dose related
Cobalt(II) chloride, Rasgele et al. 2013	MN, Mice -bone marrow	11.2, 22.5, 45 mg/kg bw i.p.	Positive
Cobalt(II)acetate Kasprzak et al. 1994	Oxidative DNA base damage, rats - kidney, liver, lung	50 µM/kg bw i.p. (~2.9 mg Co/ kg bw)	Positive
Oral exposure			
Cobalt(II) chloride, Palit et al. 1991	Chrom. abb. Mice -bone marrow	0, 20, 40, 80 mg/kg bw oral	Positive at all exposure levels and dose-related
Cobalt(II) chloride, Gudi 1998	Chrom. Abb. Rats -bone marrow	50,200,600 mg/kg bw oral	Negative
Cobalt(II)sulfate Legault 2009	Chrom abb. Rats – bone marrow	80, 160, 320 mg/kg/d single dose oral, and during 5 days oral	Negative
Cobalt(II) chloride, Kirkland et al. 2015	Chrom abb. Rats – sperm cells	0, 3, 10, 30 mg/kg bw/d oral during 28 days	Negative, no signs of toxicity noted apart from a small reduction in body weight
Inhalation exposure			
Cobalt (II)sulphate, heptahydrate NTP 1998	K-*ras* mutation, mice -in lung neoplasms	0, 0.3, 1, 3 mg/m^3 inhalation 2 years	Positive

Intraperitoneal exposure

IARC (2006) highlighted three studies using i.p. administration. In these studies, cobalt(II) chloride induced aneuploidy (pseudodiploidy and hyperploidy) in bone marrow and testes of Syrian hamsters (Farah 1983) and micronuclei in bone marrow in male BALB/c mice (Suzuki et al.,1993). Cobalt(II) acetate was shown to induce DNA base damage in female and male Fischer 344/NCr rats (Kasprzak et al. 1994).

Overall, the data indicate that the water cobalt salts are genotoxic in vivo in connection with i.p. administration (Farah 1983, Suzuki et al. 1993, Rasgale et al. 2013, Kasprzak et al. 1994). Although these studies were acknowledged by the other expert assessments, the relevance of the studies by Farah 1983, Suzuki et al. 1993, Rasgale et al. 2013 were questioned by OECD (2014) and Kirkland et al. (2015) as the exposure route was not considered relevant for human exposure. Furthermore, shortcomings of the studies were argued (i.e. poor reporting, too high dose level used) and the increase in micronuclei found by Rasgele et al. (2013) and Suzuki et al. (1993) was suggested to be a follow from increased erythropoiesis. Although, different interpretations apply for these studies the data cannot be dismissed or neglected as indications for a genotoxic potential of water soluble cobalt salts *in vivo*.

Also, i.p. micronucleus test data cannot be said to be irrelevant for the assessment of mutagenicity when it comes to a soluble in vitro genotoxic substance that is a potential lung carcinogen. When assessing i.p. micronucleus test data, there are two different issues:

(i) testing for inherent potential to be mutagenic in whole animals, the hazards of concern being anything in any tissue that could be caused by chemically-induced mutagenic lesions in DNA;

and

(ii) testing specifically for the ability of a chemical to produce heritable mutations in the germ cells.

The *in vivo* micronucleus test (or a comparable chromosome aberration test) has been the key study to investigate (i) for many years for substances that have been found to be genotoxic in *in vitro* systems. For all types of chemical, internationally, the i.p. route has been considered valid for this test since the early 1990es. Its use was routine in new substance dossiers, for example. The OECD test guideline No. 474, does not exclude the use of this exposure route, if justified, and such a justification could be that the target cells are to be regarded as a surrogate for any tissue in the body. In contrast, in tests such as COMET, UDS and transgenic mice gene mutations, the targets are in specific tissues and the i.p. route may not be justified.

Oral exposure

Palit et al. (1991) dosed groups of five male Swiss albino mice orally with cobalt dichloride at 0, 20, 40 and 80 mg/kg, the top dose being 10 % of a lethal dose. Bone marrow preparations were made 6, 12, 18 and 24 hr later. Fifty metaphases were scored from each animal per sampling time. For all sample periods, a significant dose-related trend was observed for chromosome aberrations compared to the control group. The authors concluded that the chromosome damage was directly related to the exposure concentration of cobalt(II) chloride and also to the increased period after administration.

This study was referred to and cited in several of the expert assessments, e.g. WHO/CICAD (2006), MAK (2007), EFSA (2009) (but not IARC 2006). In the OECD (2014a) assessment, the validity of the study was questioned as the clear dose-response and time-response relationship found in the study was considered to be a very unusual finding and thus the study was found to be of limited relevance.

Instead, OECD (2014a) focussed on two unpublished oral studies by Gudi (1998) and Legault (2009), which are studies not covered by the other expert group assessments:

Gudi (1998) studied bone marrow chromosome aberration in groups of male and female Sprague-Dawley rats at oral dose levels of 50, 200 and 600 mg/kg of cobalt dichloride hexahydrate. The substance was dissolved in water and administered once by oral gavage. Some animals died at the

highest dose, and also at the next lower dose (200 mg/kg), and so both of these dose levels were higher than the MTD. Clinical signs, including lethargy and piloerection, indicated systemic exposure. Animals were administered colchicine 2-4 hrs before sacrifice in order to arrest dividing cells of the bone marrow in metaphase. Most animals were sacrificed 18 hrs after dosing, while others 42 hrs after dosing (not clearly indicated). Bone marrow was aspirated from 1 femur per animal. Where possible, 100 cells per animal were analysed microscopically for presence of chromosome aberrations. There were some reductions in mitotic index in the bone marrow preparations of treated animals (up to 34% reduction compared to control), which may be taken as indicative of bone marrow toxicity. In addition, severe reductions in the percentage of polychromatic erythrocytes gave clear indications of bone marrow toxicity. Frequencies of chromosomal aberrations in treated groups were low and similar to control, and there were no significant increases. Thus, cobalt dichloride hexahydrate did not induce chromosome aberrations in bone marrow of rats at lethal doses that induced bone marrow toxicity (OECD 2014a).

Legault (2009) studied bone marrow chromosomal aberration in rats following single oral administration to cobalt sulphate at dose levels of 80, 160 and 320 mg/kg cobalt sulphate (two rats per dose group). Further, in a multi-dose study, rats (5 rats per group) were exposed orally to 100, 300 and 1000 mg/kg cobalt sulphate daily for 5 days; however, only the low-dose animals survived the five days of dosing. Fifteen hours after the last dosing, the animals were given 4 µg/kg colchicine to accumulate bone marrow cells in metaphase, and one hour later they were sacrificed and bone marrow smears were made and stained. Chromosome aberrations were scored from 100 cells/animal. Chromosome aberrations were within normal ranges at 160 and 320 mg/kg whereas chromosome aberration frequencies in the range of 5 % were noted at the dose-level of 80 mg/kg. These were considered outside historical controls but were not different from the vehicle control values without colchicine (OECD 2014a).

Very recently, Kirkland et al. (2015) further described the data from this study as the study also covered results from repeated oral exposure of five male and five female rats to 100 mg/kg/d, 300 mg/kg/d and 1000 mg/kg/d (these data were not clearly reported in OECD (2014)). Due to high mortality at the two highest dose levels, results only became available from the low dose group (after five days of exposure) and from the high dose group (after two days of exposure). The frequency of chromosome aberration in the control group was very low (0.2 %) so although a level of 1.2 % was found in the high dose group, this was not considered an effect attributed to the exposure.

In addition to this, Kirkland et al. (2015) reported data from a study where chromosome aberrations in sperm cells were studied after 28 days of oral exposure of rats to 0, 3, 10 and 30 mg/kg/d of cobalt chloride. Higher dose levels of 100 mg/kg/d and 300 mg/kg/d were not tolerated by the animals. The frequencies of chromosome aberrations were determined from 200 metaphases per animal. No signs of toxicity were noted in the study apart from a small reduction in body weight. At none of the dose levels, increased frequencies of chromosome aberrations or of polyploidy were observed. Data on mitotic index did not indicate toxicity towards the bone marrow cells.

It is acknowledged that the data, as tabulated in the original reference by Palit et al. (1998), can be considered rather unusual as clear stepwise dose-responses can be seen for chromosome aberrations in relation to the three dose levels used (in the interval from 20-80 mg/kg), and a clear time-response relationship was seen for the four timing intervals for the sampling of bone marrow cells. Therefore, it may be difficult to make any firm conclusion based on this study that indicates a clear positive response.

In opposition to this, the study by Gudi (1998) did not find induction of chromosome aberrations in the bone marrow in rats exposed to cobalt(II) chloride at higher and cytotoxic doses. The study has not been published and has therefore not undergone a peer-review.

The study by Legault (2009) is less informative, as the increase in chromosome aberrations at the lowest dose level after single exposure compared to the higher dose levels is difficult to interpret. In addition, the findings in relation to repeated exposure were considered negative, but only data from one dose level were obtained. The study has not been peer-reviewed.

Thus, to some extent weight of evidence suggests a lack of genotoxic effects after oral exposure. However, no firm conclusion can be made based on these data. Although some doubt pertain to the Palit et al. (1998) study, differences in species sensitivity towards the genotoxicity in bone marrow of the cobalt(II)-ion cannot be ruled out.

Inhalation exposure

In relation to inhalation exposure, NTP (1998) examined tissues from lung neoplasms in mice obtained from the 2-year inhalational carcinogenicity study for genetic alterations in the K-ras gene. A dose response relationship in the frequency of K-ras mutations was observed in cobalt sulphate heptahydrate-induced lung neoplasms: 14 %, 38 %, and 45 % at the dose levels of 0.3, 1.0, and 3.0 mg/m3 doses, respectively. There were generally no differences in the mutation frequency or spectra between benign and malignant lung neoplasms. NTP (1998) noted that the higher number of k-ras mutations (G to T transversions at codon 12) is supportive evidence that cobalt sulphate heptahydrate may indirectly damage DNA by oxidative stress. According to NTP (1998), the observation of similar frequencies and spectra of mutations in cobalt sulphate heptahydrate-induced alveolar/bronchiolar adenomas and carcinomas is consistent with other studies showing that K-ras activation occurs as an early and initiating event. If mutations in the K-ras gene occurred later in the carcinogenic process, an increased frequency of K-ras mutations would have been expected in the carcinomas.

3.4.3 Human mutagenicity data

OECD (2014) and IARC (2006) referred to a study by De Bock et al. (2000) in which comet assays and micronuclei detection were performed on lymphocytes from 35 workers exposed to cobalt and inorganic cobalt salts (average exposure level estimated to be 0.020 mg Co/m^3 based on 5 weeks measurement of urinary concentration of 0.020 mg Co/g creatinine). Micronuclei were scored both as binucleates (MNCB) and as mononucleates (MNMC) to discriminate between micronuclei accumulated during chronic exposure *in vivo* (mononucleates) and additional micronuclei expressed during the culture period *in vitro* (binucleates). Also biomarkers of DNA damage: 8-hydroxy-deoxyguanosine (8-OHdG) in urine, DNA single-strand breaks and formamido-pyrimidine DNA glycosylase (FPG)-sensitive sites with the alkaline Comet assay in mononuclear leukocytes were determined. No significant increase in genotoxic effects was detected in workers exposed to cobalt-containing dust compared with controls. No difference in any genotoxicity biomarker was found between workers exposed to cobalt and to hard-metal dusts (a further group included). It was found that smoking status affected the levels of micronucleated binucleates (MNCB) in lymphocytes; however, the exposure to cobalt was not found to induce an increased frequency as compared to a control group.

The Swedish Work and Health report (SWH 2005a) referred to a study by Hengstler et al. (2003) that studied DNA-damage in mononuclear blood cells from 78 workers subject to a combined exposure to cobalt, lead and cadmium. A strong correlation between DNA single strand breaks and cobalt exposure was found among workers exposed to mean levels in the range of 4-10 µg Co/m^3 (species not indicated). An inhibition of repair activity of DNA adducts (8-oxoguanine) in blood from these workers was also reported (referred to as unpublished data). The authors concluded that cobalt was the strongest determinant for DNA-damage, but that interactions with cadmium and/or lead seemed likely.

MAK (2007) referred to a study by Oesch et al. (1999), in which increased numbers of DNA single strand breaks and reduced repair capacity for oxidative DNA damage in lymphocytes were found in a subgroup of 11 workers from a groups of 78 metal workers, who were exposed to > 4 µg/m^3 of cobalt (species not indicated) at the work site. Although the workers were exposed to considerably higher cadmium levels as well, statistical analysis revealed that the cobalt exposure had the dominant impact on the occurrence of strand breaks.

3.4.4 Conclusion mutagenicity

Only very limited and non-conclusive human data are available with respect to the assessment of genotoxic effects from cobalt/ cobalt salt exposure.

Most consistently the cobalt(II)-ion is considered genotoxic *in vitro* due to the induction of chromosome damage in mammalian cells.

In addition:

- Several i.p. studies on water soluble cobalt salts have been positive for genotoxic effects after systemic uptake.
- Oral studies are non-conclusive i.e. no clear evidence on systemic genotoxicity after oral exposure.
- There may be local genotoxic effects, but these have not been really studied in appropriate studies (e.g. by *in vivo* comet assay in respiratory epithelial cells). NTP results on k-*ras* mutations in lung tumours suggest oxidative damage in lung tissue. In addition, i.p. data indicate oxidative damage on DNA.

Based on this it is concluded that genotoxicity as a mode of action behind lung tumours cannot to be ruled out. This is also supported by the positive findings from the *in vitro* studies.

4 OVERVIEW AND CONCLUSIONS REGARDING CARCINOGENIC MODE OF ACTION AND THRESHOLD/ NON-THRESHOLD

4.1 Overview of expert conclusions

From the description in Chapter 3 and after having a close look of the expert assessment identified in Section 1.2, the following overview of the expert group assessments can be given regarding:

- *Carcinogenicity in relation to exposure routes*
- *Mutagenicity in vitro and in vivo (for specific exposure routes)*
- *Mode of action considered relevant*
- *Carcinogenic threshold/ non-threshold mode of action*
- *Point of departure (POD) for carcinogenic effect*
- *POD for most critical effect*

Table 4.1 Overview regarding conclusions of the various expert groups

Expert evaluation	**Carc.**	**Muta.** ***In vitro/ in vivo***	***Mode of action****	**Carc. threshold/ non-threshold**	**Cancer POD; Reference**	**Critical effect; POD; (Reference)**
ATSDR (2004)	+inhalation	+ / + oral; +i.p.	*ROS*	No discussion	-	Reduced lung function, humans NOAEL: 0.0058 mg Co/ m^3 Occupational exposure, metallic cobalt (Nemery et al. 1992)
Swedish Work and Health SWH (2005a+b)	+ inhalation	+ / + oral; +i.p.	*ROS* *(DNA repair)*	No discussion	-	Respiratory tract irritation, humans LOAEL: 0.003 mg Co/ m^3 Occupational exposure, hard metal (Alexanderson 1979)
IARC (2006)	+ inhalation; + i.p.	+ / + i.p.	*ROS* *DNA repair*	No discussion	-	Not assessed
WHO/CICAD (2006)	+ inhalation	+ / + oral; +i.p.	*ROS* *DNA repair*	No discussion but attempt was made regarding low-dose risk	BMDL10 (male mice): 0.358 mg Co/m3 (NTP 1998)	Reduced lung function, humans NOAEL: 0.0058 mg Co/ m^3 Occupational exposure, metallic cobalt (Nemery et al. 1992)

Expert evaluation	Carc.	Muta. *In vitro/ in vivo*	*Mode of action**	Carc. threshold/ non-threshold	Cancer POD; Reference	Critical effect; POD; (Reference)
				estimation		
MAK (2007) + MAK (2009)	+ inhalation, also relevant for dermal exposure route	+ / + oral; +i.p.	*ROS* *(DNA repair)*	No threshold could be derived in relation to genotox and cancer	-	Various effects on the respiratory tract: various LOAELs presented No specific POD
EFSA (2009) + EFSA (2012)	+ inhalation	+ / + oral; +i.p.	*ROS;* *DNA repair*	No discussion	-	Polycythaemia LOAEL (oral): 1 mg Co/kg (ATSDR 2004)
Environment Canada, Health Canada (2011)	+ inhalation	+ / + oral; +i.p.	*ROS* *DNA repair*	No direct interaction between Co(II) and genetic material. MoE approach to be used	-	Reduced lung function, humans NOAEL: 0.0058 mg Co/ m^3 Occupational exposure, metallic cobalt (Nemery et al. (1992)) Cardiomyopathy, humans, LOAEL (oral): 0.04 mg/kg-bw/day (ATSDR 2004); (WHO/CICAD 2006)
Danish EPA (2013)	+ inhalation, other exposure routes not excluded	+ / + oral; +i.p.	*ROS;* *DNA repair*	No discussion	-	Polycythemia, humans LOAEL (oral): 1 mg/kg/d (Davis and Fields 1958)
NTP (2013)	+ inhalation (cobalt metal)	+/+ inhalation	*ROS* *K-ras mutations*	No discussion	-	-
NTP 2014	+ inhalation (cobalt sulphate)	+/ not addressed	*ROS* *DNA repair*	No discussion	-	-
OECD (2014a+b)	+ inhalation	+/- oral	*ROS*	Threshold approach as not genotoxic *in vivo*	BMDL10 (female rats): 0.414 mg/m^3 as cobalt sulfate heptahydrate (NTP 1998)	Cobalt asthma, humans NOAEC: 0.12 mg Co/m^3 (Sauni et al., 2010)

Expert evaluation	Carc.	Muta. *In vitro/ in vivo*	*Mode of action**	Carc. threshold/ non-threshold	Cancer POD; Reference	Critical effect; POD; (Reference)
ANSES 2014	+ inhalation	Metallic cobalt concluded as a weak genotoxic substance	*(ROS* *DNA repair)*	Non-threshold	Uncertain	Cancer/ inflammation. *Pragmatic* 8-h occupational limit value of 2.5 µg Co/m3 based on a BMDL10 (inflammation, rats) of 0.07 mg Co/m3
REACH CSR (2014)	+; inhalation	+/- oral (inhalation metallic Co)	*ROS* *Non-DNA damage*	Threshold approach as not genotoxic *in vivo*	BMDL10 (female rats): 0.414 mg/m^3 as cobalt sulphate heptahydrate (NTP 1998)	DNEL (workers, long-term): 0,105 mg/m^3 based on repeated dose toxicity DNEL (general population, long-term): 0.0166 mg/m^3 based on cancer - both as cobalt sulphate (DNEL values as reported in public version of REACH registration of cobalt sulphate)

+/- indicate positive/ negative conclusion regarding genotoxicity
Mode of action set in ( ) indicate that the mode of action was only briefly mentioned

The various expert evaluations have been considered, and details of our considerations can be found in Appendix B.

4.2 Key findings and discussion

Especially a publication by Beyersmann and Hartwig (2008) as referred to by EFSA (2009) and ECHC (2011) seems important, as this publication makes a state-of-science review regarding possible mode of actions of cobalt and cobalt substances in relation to their genotoxicity and carcinogenicity.

Induction of ROS and oxidative stress:

It was noted by Beyersmann and Hartwig (2008) that cobalt ions are able to induce the formation of reactive oxygen species (ROS) both *in vitro* and *in vivo* and that cobalt(II)-ions catalyse the generation of hydroxyl radicals from hydrogen peroxide in a Fenton type reaction. Such a mechanism was supported by the i.p. study by Kasprzak et al. (1994) in which cobalt(II) resulted in the formation of oxidative DNA base damage in kidney, liver and lungs. In addition, the analysis of mutations in tumour tissues in a carcinogenicity study with cobalt sulphate in mice (NTP 1998) revealed that five of nine mutations were G-T transversions in codon 12 of the K-*ras* oncogene, which might be due to oxidative DNA damage.

As shown in Table 4.1, there is a general consensus among the expert group assessments that ROS generation is a relevant mode of action for the genotoxic effects of the Co(II)-ion (see also Appendix B for a detailed description of each expert assessment).

Inhibition of DNA repair:

Beyersmann and Hartwig (1998) found that the genotoxicity of other mutagenic agents was augmented by soluble cobalt salts as well as by cobalt metal dust. Further, cobalt(II) inhibited the nucleotide excision repair of DNA damage caused by UV-C radiation in human fibro- blasts. Both the incision and polymerisation steps were inhibited. In particular, cobalt inhibited the Xeroderma

pigmentosum group A (XPA) protein, a zinc finger protein involved in nucleotide excision repair where it substituted for the zinc ion.

As support for this, it was mentioned that the co-mutagenicity of cobalt observed *in vitro* corresponds to its co-carcinogenic effect in an animal study, where cobalt(II) oxide enhanced the carcinogenicity of benzo[a]pyrene (a study by Steinhoff and Mohr (1991) using intratracheally administration of the substances).

Upregulation of hypoxia-inducible factor HIF-1α:

Beyersmann and Hartwig (2008) also referred to data indicating a cobalt(II) induced upregulation of hypoxia-inducible factor HIF-1α. Such an upregulation will result in hypoxia in the tissue, which is well-known to promote the growth of tumours.

4.2.1 Assessment of expert groups of mode of action

The above described mode of actions (especially induction of ROS and impairment of DNA-repair) were recognised and addressed by most of the expert group assessments (see overview Table 4.2). IARC (2006) specifically discussed:

- a direct effect of cobalt(II) ions causing damage to DNA through a Fenton-like mechanism; and
- an indirect effect of cobalt(II) ions through inhibition of repair of DNA damage caused by endogenous events or induced by other agents.

Also, data submitted from CDI/CoRC provide evidence for ROS generation and inhibition of DNA-repair as relevant modes of action for the genotoxic responses in relation to exposure to the cobalt(II)-ion (CDI/CoRC(2015), see Appendix C).

Thus, the overall weight of evidence points towards cobalt(II) induced ROS generation and inhibition of DNA-repair as key events that to a great extent can explain the genotoxic effects of the cobalt-ion. None of the expert assessments specifically addresses a direct interaction between the cobalt(II)-ion and the genetic material as a mode of action.

However, it cannot be said that the mechanisms regarding mutagenicity and carcinogenicity have been fully elucidated or to what extent the various mechanisms may interact with each other.

With respect to interaction of the proposed key mechanisms, a synergistic effect of the genotoxic mode of actions may be postulated, as the cobalt-(II)-ion both leads to increased ROS generation inducing oxidative DNA-mutations and at the same time impairs the DNA repair mechanisms of the cell.

4.2.2 Assessment of expert groups on threshold/ non-threshold approach

Few of the expert assessments have specifically addressed the issue whether the carcinogenic effects from inhalational exposure should be considered a threshold or non-threshold phenomenon.

From the discussion above concerning mode of action, the question would be whether ROS mediated DNA damage and inhibition of DNA-repair may be considered as threshold mechanisms.

Arguments for *threshold mechanisms* have been put forward by **ECHC (2011); OECD (2014a+b); the REACH CSRs (2014); Kirkland et al. (2015)** and **CDI/CORC (2015)**.

In these assessments, the genotoxic potential observed in *in vitro* and *in vivo* studies (using i.p. administration) was not considered to be expressed in relation to relevant human exposure routes, as

effective *in vivo* defence mechanisms were considered to overcome the indirect genotoxic effects of the cobalt((II)-ion. Emphasis was put on the negative results from the most recent *in vivo* oral exposure studies by Gudi (1998) and by Legault (2009), in which no increase in chromosome aberrations was found. Consequently, as no genotoxic concern could be documented for the relevant route of exposure *in vivo,* the substances were evaluated as non-genotoxic and having a threshold.

It should however be noted that the NTP (1998) data regarding K-*ras* mutations from neoplasm in mice exposed by inhalation to cobalt(II) sulfate heptahydrate were not considered or even mentioned by any of these assessments.

Data submitted by CDI/CORC (2015) provided the most detailed analysis regarding whether the substances should be considered as threshold/non-threshold (see Appendix C). CDI/CoRC (2015) acknowledged that specific data demonstrating a threshold for carcinogenic effects were lacking. Nevertheless, and based on a weight of evidence approach in which genotoxicity data and mechanistic data concerning mode of action were combined with data on histopathological findings from the carcinogenicity studies, a threshold mode of action was concluded.

The arguments can be summarised as follows:

- the histopathological picture from the repeated dose toxicity studies and the cancer studies fit into the pattern of a well-known mode of action for development of lung tumours where cytotoxicity and chronic inflammation (threshold effects) are necessary events for inducing hyperplasia that further progresses into tumours.

- the cells of the body are subjected to spontaneous endogenous ROS generation that leads to several hundred oxidative DNA damages each day in a cell. These damages are repaired by existing homeostatic DNA repair mechanisms. Thus, the increased ROS generation induced by cobalt(II) has to exceed an upper threshold for the capacity of the DNA repair system in order to elicit further toxic responses. Therefore, the initiation event of DNA damage due to the cobalt(II) induced ROS generation can be considered as a threshold mechanism.

- the inhibition of DNA repair is considered to be a result of competitive Co(II) binding to the DNA repair enzymes. Such binding has been shown to follow sigmoidal dose-response curves and thresholds for the enzyme binding have been established in several in vitro systems.

On the other hand, non-threshold mechanisms were referred to by **MAK (2007)** and **ANSES (2014)**. MAK (2007) very shortly stated that the available epidemiological and genotoxicity data did not allow for the derivation of a threshold that would protect against carcinogenic effects. ANSES (2014) also very shortly stated that exposure levels protecting against inflammatory effects in the lung would not necessarily protect against the carcinogenic effect as this effect should be considered as a stochastic (i.e. non-threshold) effect (no further details were given).

4.3 Conclusions

The five cobalt salts covered by this project should be considered carcinogens in relation to inhalational exposure, the only exposure route considered relevant for a carcinogenic response. Furthermore, although not clarified, a genotoxic mode of action cannot be ruled out.

The underlying mechanisms for the potential genotoxic and carcinogenic effects of the water-soluble cobalt salts have not been fully elucidated, but it is a general view that key mechanisms for initiation of DNA-damage are cobalt(II) induced ROS generation in combination with impairment of DNA-repair due to cobalt (II) binding to DNA-repair enzymes. For further progression into cancer, the current data support a cytotoxic mode of action where chronic cobalt(II) exposure by inhalation may trigger a sequence of events going from cytotoxicity, chronic inflammation, proteinosis, hyperplasia and into the

development of tumours in the lung tissue. Also, upregulation of HIF-1α and induction of hypoxia may enhance the process of tumour progression.

In the REACH Guidance R.7a[2], it is stated that impairment of DNA repair *may* lead to genotoxicity via a non-linear or threshold dose-response. In addition, it is stated that thresholds *may* be present for certain carcinogens that cause genetic alterations via indirect effects on DNA as a result of interaction with other cellular processes, e.g. cellular processes where the compensatory capacity or physiological or homeostatic control is exceeded. Also, it is recognised that for certain genotoxic carcinogens causing genetic alterations, a practical threshold *may* exist for the underlying genotoxic effect. For example, this has been shown to be the case for aneugens (agents that induce aneuploidy – the gain or loss of entire chromosomes to result in changes in chromosome number), or for chemicals that cause indirect effects on DNA that are secondary to another effect (e.g., through oxidative stress that overwhelms natural antioxidant defence mechanisms).

Therefore, *scientifically*, the available data support the notion of a thresholded mechanism since the key events and mechanisms for the carcinogenic effects are suggested as being thresholded.

However, at present, data do not allow for identification of a threshold as genotoxic and carcinogenic responses have occurred *in vivo* in an inhalation study with cobalt(II) sulphate heptahydrate down to the lowest exposure level tested. Also, a full documentation of the suggested modes of action supported by data on cobalt(II) is still lacking.

Thus, in the context of a risk management decision under REACH, the scientific weight of evidence has to be weighted against the remaining uncertainties. The REACH Guidance R.8[3] emphasises that "*the decision on a threshold and a non-threshold mode of action may not always be easy to make, especially when, although a biological threshold may be postulated, the data do not allow identification of it. If not clear, the assumption of a non-threshold mode of action would be the prudent choice*". Thus, lack of sufficient documentation and existence of remaining uncertainties would lead to the use of the most cautious approach for assessing genotoxic carcinogens, i.e. the non-threshold approach.

Overall, it can be concluded:

- carcinogenicity data are only available for local tumours in the respiratory tract in relation to inhalation exposure, thus dose response estimations can only be made for inhalation exposure.
- the current scientific findings and mode of action considerations support the notion that water soluble cobalt substances may be threshold carcinogens although there are some uncertainties related to initiation by catalytic ROS generation and direct oxidative DNA damage. In addition, the genotoxicity data may indicate a non-threshold mechanism.
- thresholds have, however, not been identified for the cobalt salts in relation to the carcinogenicity and genotoxicity in the respiratory tract.

Therefore at present, due to lack of identified thresholds and due to remaining uncertainties regarding the mechanisms involved, the water soluble cobalt salts are considered as genotoxic carcinogens and are to be assessed using a non-threshold approach.

[2] Guidance on information requirements and chemical safety assessment, Chapter R.7a: Endpoint specific guidance (version 3.0)

[3] Guidance on information requirements and chemical safety assessment, Chapter R.8: Characterisation of dose [concentration]-response for human health (version 2.1)

5 DOSE-RESPONSE ANALYSIS AND QUANTITATIVE CANCER RISK ASSESSMENTS

It is concluded that cancer risk estimates can only be made in relation to the inhalational exposure route, as carcinogenicity data only pertain to inhalation exposure and local tumours of the respiratory tract. Also, it is concluded that the cobalt salts may be considered genotoxic carcinogens using a non-threshold approach for risk assessment.

The point of departure (POD) for the dose response assessment is based on the findings from the NTP (1998) inhalation studies in which mice and rats were exposed to cobalt sulphate heptahydrate by inhalation. From these data, OECD (2014a) calculated benchmark doses (BMD) using the US EPA BMD software (Version 2.0) with the Gamma Model (Version 2.13). The numbers of alveolar/ bronchiolar adenoma or carcinoma in the lungs of rats and mice were selected as benchmark response. The 95 % lower confidence limit of the BMD for a treatment-related increase in response of 10 % was calculated (BMDL10). The lowest BMDL10 value of 0.414 mg/m³ was found for female rat tumours.

When converting this dose level to cobalt(II)-levels, it further has to be taken into account that chemical analysis showed that exposure in fact was to cobalt sulphate *hexahydrate* and not the heptahydrate (NTP 1998). Thus, using the molecular weights of cobalt sulphate hexahydrate (263.10 g/mol) and cobalt (58.83 g/mol) a BMDL10 of 0.093 mg Co/m³ was derived by OECD (2014a).

As the animals in the NTP (1998) were exposed to cobalt sulphate particle with a MMAD (Mass Median Aerodynamic Diameter) in the range of 1 µm – 3 µm, and as the lung tumours from which the BMDL10 level were derived were located in the deeper part of the lung, the dose-response relationships below are related to the respirable fraction of the particles.

Inhalable particles would - for the particle fraction above the size of the respirable range – to a great extent be deposited in the upper part of the respiratory tract. Data from the NTP (1998) indicate that both rats and mice develop hyperplasia, metaplasia and atrophy in epithelial cells of the nose, and metaplasia of the squamous epithelium of the larynx. Although inhalable particles should also be considered as carcinogenic the dose-response related to this metric is far more uncertain as this will very much depend of the content of respirable particles. Thus, the most valid dose-response relationship for carcinogenicity is to be based on an exposure metric for respirable particles.

Dose response relationships were derived by linear extrapolation, which is to be considered as a very conservative approach, especially at very low exposure levels. It is acknowledged therefore that excess risks in the lower exposure range might be overestimated following this approach.

5.1 Inhalation exposure

5.1.1 Worker exposure, conversion of dose metric

The BMDL10 value of 0.093 mg Co/m³ was calculated in association to lifetime exposure of female rats (6h/d, 5d/week, for 105 weeks).

For conversion of the daily exposure concentration, the converted BMDL10 value can be calculated according to REACH Guidance R.8 by use of the following factor:

BMDL10 conv (daily exposure) = BMDL10 (conc.) x (6h/d / 8h/d) x (6.7 m³* /10m³ **)

*average inhalation volume of humans during 8h (comparable to situation of the experimental animals)

**inhalation volume of worker during 8h light activity

BMDL10 conv (daily exposure) = 0.093 mg Co/m^3 x (6h/d / 8h/d) x (6.7 m^3/10m^3)

BMDL10 conv (daily exposure) = 0.047 mg Co/m^3

5.1.2 General population exposure, conversion of dose metric

The BMDL10 value of 0.093 mg Co/m^3 was calculated in association to exposure of female rats 6h/d, 5d/week, for 105 weeks (lifetime).

Thus, this dose metric has to be converted to daily lifetime exposure for the general population, i.e. the conversion shall consider population exposure 24h/d, 7d/week during lifetime.

For conversion of the daily exposure concentration, the converted BMDL10 value can be calculated according to REACH guidance R8 by use of the following factors:

BMDL10 conv (daily exposure) = BMDL10 (conc.) x (6h / 24h) x (5d / 7d)

BMDL10 conv (daily exposure) = 0.093 mg Co/m^3 x (6h / 24h) x (5d / 7d) = 0.017mg Co/m^3

5.2 Non-threshold approach, dose-response

5.2.1 Non-threshold approach, Dose response, Workers

The linearized approach described by the REACH Guidance R.8 will be used for the non-threshold approach. When making risk calculations for occupational exposure levels, a correction has to be done to account for the fact that workers are only exposed during a fraction of their life (48 weeks per year during 40 years of work life) compared to the experimental animals that were exposed throughout their lifetime).

BMDL10 conv (occup exp) = BMDL10 (daily exp) x (52w / 48w) x (75y / 40y)

BMDL10 conv (occup exp) = 0.047 mg Co/m^3 x (52w / 48w) x (75y / 40y) = 0.095 mg Co/m^3

This BMDL10 conv (occup exp) should not be subject to the use of further assessment factors before scaling down to low level exposure, as an allometric assessment factor is only used for dose metrics expressed in mg/kg/d and not inhalational dose metrics expressed in mg/m^3.

Thus, from a risk level of 0.1 at a dose of 0.095 mg Co/m^3, a linear extrapolation for the dose response relationship for excess cancer risk can be made down to zero risk and zero exposure.

The risk can be calculated by the slope of the curve = 0.1 / 0.095 mg Co/m^3 = 1.05 (mg Co/m^3)$^{-1}$, thus:

Excess risk = dose level x 1.05 (mg Co/m^3)$^{-1}$

Using this relationship, the following levels of excess risk can be calculated in relation to 8h average worker exposure:

8-h TWA cobalt concentration (mg Co/m^3) as respirable particles	**Excess lung tumour risk in workers (x10^{-4})**
0.1	1 050
0.095	1 000
0.01	105
0.005	53
0.001	10.5
0.0001	1.1

5.2.2 Non-threshold approach, Dose response, General population

The linearized approach described by the REACH Guidance R.8 will be used for the non-threshold approach. According to this method the BMDL10 (daily exposure) value calculated above should not be subjected to the use of further assessment factors before scaling down to low level exposure, as an allometric assessment factor is only used for dose metrics expressed in mg/kg/d and not inhalational dose metrics expressed in mg/m^3.

Thus, from a risk level of 0.1 at a dose of 0.017 mg Co/m^3, a linear extrapolation for the dose response relationship for excess cancer risk can be made down to zero risk and zero exposure.

The risk can be calculated by the slope of the curve = 0.1 / 0.017 mg Co/m^3 = 5.88 (mg Co/m^3)$^{-1}$, thus:

Excess risk = dose level x 5.88 (mg Co/m^3)$^{-1}$

Using this relationship, the following levels of excess risk can be calculated in relation to 24h average population exposure:

24-h TWA cobalt concentration (mg Co/m^3) as respirable particles	**Excess lung tumour risk in the general population (x10^{-4})**
0.02	1 176
0.017	1 000
0.01	588
0.001	59
0.0001	5.9
0.00001	0.6
0.000001	0.06

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Appendix A

Mutagenicity data

Mutagenicity data, *in vitro*

IARC (2006) tabulated a large amount of mutagenicity data (both *in vitro* and *in vivo*) on cobalt an cobalt substances in table 15 of the monograph. Below the data on the soluble cobalt substances are presented from this table.

Table 15. Genetic and related effects of cobalt

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cobalt compounds				
Co(II) salts				
Cobalt(II) acetate				
Inhibition of repair of UV-induced pyrimidine dimers, nucleoid sedimentation, human HeLa S-3 cells, *in vitro*	+		100 µM	Snyder *et al.* (1989)
Enhancement of cell transformation by simian adenovirus SA7, Syrian hamster embryo cells, *in vitro*	+		0.2 mM	Casto *et al.* (1979)
DNA base damage (products of hydroxyl radical attack), female and male Fischer 344/NCr rats, *in vivo*	+ (kidney > liver > lung)		ip, single, 50 µM/kg	Kasprzak *et al.* (1994)
Cobalt(II) chloride				
Reduction of fidelity of DNA replication by substitution of Mg^{2+} *Escherichia coli* DNA polymerase, sea-urchin nuclear DNA polymerase, avian myeloblastosis virus DNA polymerase	+		1 mM [130 µg/mL]	Sirover & Loeb (1976)
Prophage induction, *Escherichia coli*	–		~ 320 µM[f] [415 µg/mL]	Rossman *et al.* (1984)
Escherichia coli $WP2_s$ inhibition of protein synthesis	+		6.25 µg/mL	Leitão *et al.* (1993)
Escherichia coli AB1886, inhibition of protein synthesis	+		6.25 µg/mL	Leitão *et al.* (1993)
Salmonella typhimurium TA100, reverse mutation	–		NG	Ogawa *et al.* (1986)
Salmonella typhimurium TA102, reverse mutation	–	–	40 ppm [40 µg/mL]	Wong (1988)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA1535, reverse mutation	–		NG	Arlauskas *et al.* (1985)
Salmonella typhimurium TA1535, reverse mutation	–	–	40 ppm [40 µg/mL]	Wong (1988)
Salmonella typhimurium TA1537, reverse mutation	–		NG	Arlauskas *et al.* (1985)
Salmonella typhimurium TA1537, reverse mutation	–		1000 µmol/plate [130 000 µg/plate]	Ogawa *et al.* (1986)
Salmonella typhimurium TA1537, reverse mutation	+	–	40 ppm [40 µg/mL]	Wong (1988)
Salmonella typhimurium TA1538, reverse mutation	–		NG	Arlauskas *et al.* (1985)
Salmonella typhimurium TA98, reverse mutation	–		NG	Arlauskas *et al.* (1985)
Salmonella typhimurium TA98, reverse mutation	–		NG	Ogawa *et al.* (1986)
Salmonella typhimurium TA98, reverse mutation	+	–	40 ppm [40 µg/mL]	Wong (1988)
Salmonella typhimurium TA2637, reverse mutation	–		1000 µmol/plate [130 000 µg/plate]	Ogawa *et al.* (1986)
Salmonella typhymurium, TA97 preincubation assay	+		100 µM [13 µg/mL]	Pagano & Zeiger (1992)
Salmonella typhimurium, TA97 preincubation assay	r[d]		100 µM [13 µg/mL] + DEDTC 420 µM	Pagano & Zeiger (1992)
Escherichia coli SY1032/pKY241 transfected with pUB_3, *supF* tRNA locus, mutation	+		20 µM [2.6 µg/mL]	Ogawa *et al.* (1999)
Bacillus subtilis rec strains H17/M45, growth inhibition	–		[325 µg/plate]	Nishioka (1975)
Bacillus subtilis rec strain H17, growth inhibition	+		[325 µg/plate]	Kanematsu *et al.* (1980)
Saccharomyces cerevisiae SBTD-2B, 'petite' mutation, respiratory deficiency	+		2 mM [260 µg/mL]	Prazmo *et al.* (1975)
Saccharomyces cerevisiae, strain 197/2d, 'petite' mutation	+		4 mM [520 µg/mL]	Putrament *et al.* (1977)
Saccharomyces cerevisiae, strain 197/2d, erythromycin-resistant mutation	–		4 mM [520 µg/mL]	Putrament *et al.* (1977)
Saccharomyces cerevisiae, 'petite' mutation, respiratory deficiency	(+)		640 µg/mL	Egilsson *et al.* (1979)
Saccharomyces cerevisiae D7, *ilv* gene mutation	–		10 mM [1300 µg/mL]	Fukunaga *et al.* (1982)
Saccharomyces cerevisiae D7, trp gene conversion	+		10 mM [1300 µg/mL]	Fukunaga *et al.* (1982)
Saccharomyces cerevisiae D7, *ilv* gene mutation	–		100 mM [13 000 µg/mL]	Singh (1983)
Saccharomyces cerevisiae D7, trp gene conversion	(+)		100 mM [13 000 µg/mL]	Singh (1983)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Saccharomyces cerevisiae D7, trp gene conversion	(+)		1500 µg/mL [11.5 mM]	Kharab & Singh (1985)
Saccharomyces cerevisiae D7, *ilv* gene mutation	(+)		3000 µg/mL [23 mM]	Kharab & Singh (1985)
Saccharomyces cerevisiae D7, 'petite' mutation, respiratory deficiency	+		750 µg/mL [5.76 mM]	Kharab & Singh (1987)
Drosophila melanogaster, gene mutation or mitotic recombination, wing spot test mwh/flr	+		2 mM [260 µg/mL]	Ogawa *et al.* (1994)
Drosophila melanogaster, gene mutation or reduced mitotic recombination, wing spot test mwh/TM3	–		8 mM [1040 µg/mL]	Ogawa *et al.* (1994)
DNA strand breaks, alkaline sucrose gradient, Chinese hamster ovary cells, *in vitro*	+		2 mM [260 µg/mL]	Hamilton-Koch *et al.* (1986)
DNA strand breaks, nucleoid sedimentation assay, Chinese hamster ovary cells, *in vitro*	–		10 mM [1300 µg/mL]	Hamilton-Koch *et al.* (1986)
DNA strand breaks, nucleoid sedimentation, human HeLa cells, *in vitro*	+		50 µM [65 µg/mL]	Hartwig *et al.* (1990)
DNA-protein cross links, rat Novikoff ascites hepatoma cells, *in vitro*	+		1 mM [130 µg/mL]	Wedrychowski *et al.* (1986)
Gene mutation, Chinese hamster V79 cells, *Hprt* locus, *in vitro*	(+)		0.2 mM [26 µg/mL]	Miyaki *et al.* (1979)
Gene mutation, Chinese hamster V79 cells, *Hprt* locus, *in vitro*	+		100 µM [13 µg/mL]	Hartwig *et al.* (1990)
Gene mutation, Chinese hamster V79 cell line, *Gpt* locus, *in vitro*	–		100 µM [13 µg/mL]	Kitahara *et al.* (1996)
Gene mutation, Chinese hamster transgenic cell line G12, *Gpt* locus, *in vitro*	+		50 µM [6.5 µg/mL]	Kitahara *et al.* (1996)
Sister chromatid exchanges, mouse macrophage-like cells P388D$_1$, *in vitro*	+		100 µM [13 µg/mL]	Andersen (1983)
Cell transformation, C3H10T1/2 mouse fibroblast cells, *in vitro*	+[c]		38 µM [5 µg/mL]	Doran *et al.* (1998)
Reduction in colony forming, V79 Chinese hamster cells, *in vitro*	+ (42%)		180 µM [24 µg/mL]	Kasten *et al.* (1992)
Reduction of cloning efficiency, Chinese hamster ovary cells, *in vitro*	+ (50%)		4 mM [520 µg/mL]	Hamilton-Koch *et al.* (1986)
Displacement of acridine orange from DNA, calf thymus DNA and *Micrococcus luteus* DNA	+		0.33 mM [43 µg/mL]	Richardson *et al.* (1981)
Formation of metal–DNA complex, calf thymus B-DNA	+		NG	Aich *et al.* (1999)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Induction of reporter gene expression under the control of the promoter region of the metallothionein gene, chick embryo liver cells transfected with luciferase or chloramphenicol acetyl transferase, *in vitro*	+		112 µM [15 µg/mL]	Lu *et al.* (1996)
Production of reactive oxygen species (fluorescence from oxidation of DCFH-DA), A549 cells, human lung cells, *in vitro*	+		200 µM [26 µg/ml]	Salnikow *et al.* (2000)
Production of reactive oxygen species (fluorescence from oxidation of DCFH-DA), A549 cells, human lung cells, *in vitro*	r[d]		300 µM [39 µg/mL] + 2-mercapto-ethanol	Salnikow *et al.* (2000)
Production of reactive oxygen species (fluorescence from oxidation of DCFH-DA), A549 cells, human lung cells, *in vitro*	r[d]		300 µM [39 µg/mL] + vitamin E	Salnikow *et al.* (2000)
DNA strand breaks, fluorescence analysis of DNA unwinding, human white blood cells, *in vitro*	+		50 µM [6.5 µg/mL]	McLean *et al.* (1982)
DNA strand breaks, alkaline sucrose gradient, human diploid fibroblasts, *in vitro*	+		5 mM	Hamilton-Koch *et al.* (1986)
DNA strand breaks, nick translation, human diploid fibroblasts, *in vitro*	+		10 mM [1300 µg/mL]	Hamilton-Koch *et al.* (1986)
DNA strand breaks, nucleoid sedimentation, human diploid fibroblasts, *in vitro*	–		10 mM [1300 µg/mL]	Hamilton-Koch *et al.* (1986)
DNA strand breaks and alkali-labile sites, alkaline Comet assay, human mononuclear leukocytes, *in vitro*	+[c]		0.3 µg/mL	De Boeck *et al.* (1998)
Induction of gene expression (*Cap43*), A549 cells, human lung cells, *in vitro*	+		100 µM [13 µg/mL]	Salnikow *et al.* (2000)
Induction of gene expression (*Cap43*), A549 cells, human lung cells, *in vitro*	s[d]		100 µM [13 µg/mL] + 2-mercapto-ethanol	Salnikow *et al.* (2000)
Induction of gene expression (*Cap43*), A549 cells, human lung cells, *in vitro*	s[d]		100 µM [13 µg/mL] + H_2O_2	Salnikow *et al.* (2000)
Sister chromatid exchange, human lymphocytes, *in vitro*	+		10 µM [1.3 µg/mL]	Andersen (1983)
Aneuploidy, human lymphocytes, *in vitro*	+		3.7 µg/mL	Resende de Souza-Nazareth (1976)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Aneuploidy, pseudodiploidy and hyperploidy, bone marrow of male hamsters, *in vivo*	+		400 mg/kg bw ip[g]	Farah (1983)
Aneuploidy, pseudodiploidy and hyperploidy, testes of hamsters, meiosis 1, *in vivo*	+		400 mg/kg bw ip[g]	Farah (1983)
Inhibition of binding of p53 protein to p53 consensus sequence on linear DNA fragment	+ (full)		> 100 µM (300 µM)	Palecek *et al.* (1999)
Inhibition of binding of p53 protein to supercoiled DNA	+		600 µM	Palecek *et al.* (1999)
Affinity of reconstituted apopolypeptide (Zn finger protein) with estrogen response element consensus oligonucleotide	r		NG (K_D 0.720 µM)[h]	Sarkar (1995)
Inactivation of bacterial Fpg protein (with Zn finger domain), conversion of supercoiled bacteriophage PM2 DNA into open circular form, electrophoresis	–		1000 µM	Asmuss *et al.* (2000)
Inhibition of XPA (with Zn finger domain) binding to UV-irradiated oligonucleotide, gel mobility shift analysis	+		50 µM [6.5 µg/mL]	Asmuss *et al.* (2000)
Cobalt(II) chloride hexahydrate				
Lysogenic induction, *Escherichia coli* $WP2_s$ (λ)	r[i]		(10 µg /mL)[f] + UV	Leitão *et al.* (1993)
Lysogenic induction, *Escherichia coli* K12 ABI886 (λ)	r[j]		(10 µg/mL)[k] + UV	Leitão *et al.* (1993)
Lysogenic induction, *Escherichia coli* ABI157 (λ)	+		100 µg/mL – Mg	Leitão *et al.* (1993)
Phage reactivation, *Escherichia coli* ABI157 (λ)	–		250 µg/mL – UV	Leitão *et al.* (1993)
Phage reactivation, *Escherichia coli* ABI157 (λ)	e[i]		62.5 µg/mL + UV	Leitão *et al.* (1993)
Escherichia coli WP2, reverse mutation	–[11]		20 µg/mL [84 µM]	Kada & Kanematsu (1978)
Escherichia coli WP2 *uvrA*, reverse mutation	–		NG	Arlauskas *et al.* (1985)
Escherichia coli $WP2_s$ gene mutation	–[11]		50 µg/mL [210 µM]	Leitão *et al.* (1993)
Escherichia coli $WP2_s$ gene muation	r[i]		50 µg/mL + UV	Leitão *et al.* (1993)
Saccharomyces cerevisiae D7, 'petite' mutation, respiratory deficiency	+		[130 µg/mL]	Lindegren *et al.* (1958)
Salmonella typhimurium TA100, reverse mutation	–		100 mM [23 800 µg/mL]	Tso & Fung (1981)
Salmonella typhimurium TA100, reverse mutation	–		NG	Arlauskas *et al.* (1985)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA1538, reverse mutation	–[12]		20 µg/mL [84 µM]	Mochizuki & Kada (1982)
Salmonella typhimurium TA98, reverse mutation	–[12]		20 µg/mL [84 µM]	Mochizuki & Kada (1982)
Bacillus subtilis strain NIG 1125, reverse mutation	–[13]		30 µg/mL [126 µM]	Inoue *et al.* (1981)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus, *in vitro*	–		57.11 µg/mL	Amacher & Paillet (1980)
Gene mutation, Chinese hamster V79 cells, *8AG* locus, *in vitro*	–		9 µM [2 µg/mL]	Yokoiyama *et al.* (1990)
Gene mutation, Chinese hamster V79 cells, *8AG* locus, *in vitro*	r[i]		3 µM [0.7 µg/mL] + γ rays	Yokoiyama *et al.* (1990)
Micronucleus formation, BALB/c mouse bone marrow, *in vitro*	–	–	50 µg/mL [385 µM]	Suzuki *et al.* (1993)
DNA strand breaks, alkaline elution, human lymphocytes, *in vitro*	–		102 µM [25 µg/mL]	Anard *et al.* (1997)
Inhibition of nucleotide excision repair (incision and polymerization steps) of UV-induced DNA damage, alkaline unwinding, VH16 human fibroblasts	+		50 µM [12 µg/mL]	Kasten *et al.* (1997)
Inhibition of nucleotide excision repair (ligation step) of UV-induced DNA damage, alkaline unwinding, VH16 human fibroblasts	–		200 µM [48 µg/mL]	Kasten *et al.* (1997)
Inhibition of UV-induced cyclobutane pyrimidine dimers (incision step), alkaline unwinding + T4 endonuclease V, VH16 human fibroblasts	+		150 µM [86 µg/mL]	Kasten *et al.* (1997)
Micronucleus formation, male BALB/c AnNCrj mouse bone marrow, *in vivo*	+		50 mg/kg bw	Suzuki *et al.* (1993)
Micronucleus formation, male BALB/c AnNCrj mouse bone marrow, *in vivo*	e[i]		50 mg/kg + DMH 20 mg/kg	Suzuki *et al.* (1993)
Micronucleus formation, male BALB/c AnNCrj mouse bone marrow, *in vivo*	e[i]		50 mg/kg + benzo(a)pyrene 50 mg/kg	Suzuki *et al.* (1993)
Micronucleus formation, male BALB/c AnNCrj mouse bone marrow, *in vivo*	e[i]		50 mg/kg + 2-naphthylamine 200 mg/kg	Suzuki *et al.* (1993)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cobalt(II) molybdenum(VI) oxide				
Enhancement of cell transformation by simian adenovirus SA7, Syrian hamster embryo cells, *in vitro*	+		250 µM [55 µg/mL]	Casto *et al.* (1979)
Cobalt(II) nitrate				
Chromosome aberrations (numerical), human diploid fibroblasts WI38 and MRC_5, *in vitro*	–		0.08 µM[g] [0.015 µg/mL]	Paton & Allison (1972)
Chromosome aberrations (numerical), human mononuclear leucocytes, *in vitro*	–		0.8 µM[g] [0.15 µg/mL]	Paton & Allison (1972)
Cobalt(II) nitrate hexahydrate				
Drosophila melanogaster (*flr³/In(3LR)TM3, $ri^1 p^P Sep\ bx^{34e} e^5 Ser$*) × (*mwh*).*mwh* and *flr³*, gene mutations, chromosomal deletion, non disjunction or mitotic recombination (small single spots and large single spots), SMART test	+		1 mM [291 µg/mL]	Ye°ilada (2001)
Drosophila melanogaster (*flr³/In(ELR)TM3, $ri^1 p^P Sep\ bx^{34e} e^5 Ser$*) × *(mwh).mwh and flr³*, mitotic recombination (twin spots), SMART test	+		10 mM [2910 µg/mL]	Ye°ilada (2001)
Cobalt(II) sulfate				
Allium cepa, chromosomal aberrations	+		20 µM [3 µg/mL]	Gori & Zucconi (1957)
Allium cepa, aneuploidy	+		100 µM [15 µg/mL] for 5 days + H_2O for 3 days	Gori & Zucconi (1957)
Production of reactive oxygen species (degradation of 2-deoxyribose), malondialdehyde assay	+		1 µM [0.155 µg/mL]	Ball *et al.* (2000)
Production of reactive oxygen species (degradation of 2-deoxyribose), malondialdehyde assay	r[d]		50 µM [7.8 µg/mL] + desferrioxamine 1 mM	Ball *et al.* (2000)
Chemical changes in DNA bases GC/MS-SIM, calf thymus DNA	–		25 µM [4 µg/mL]	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, calf thymus DNA	+[d]		25 µM [4 µg/mL] + H_2O_2 208 mM	Nackerdien *et al.* (1991)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Bacillus subtilis rec strain H17, growth inhibition	(+)		388 µg/plate	Kanematsu *et al.* (1980)
Cytoskeletal perturbation of microtubules and microfilaments, mouse cells SWISS 3T3, *in vitro*	+		100 µM [15.5 µg/mL]	Chou (1989)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	–		25 µM [4 µg/mL]	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	+[d]		25 µM + H_2O_2 208 mM	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	r[m]		25 µM [4 µg/mL] + H_2O_2 208 mM + EDTA 120 µM	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	r[m]		25 µM + H_2O_2 208 mM + mannitol 50 mM	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	r[m]		25 µM + H_2O_2 208 mM + DMSO 50 mM	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	r[m], e[m]		25 µM + H_2O_2 208 mM + glutathione 1 mM	Nackerdien *et al.* (1991)
Chemical changes in DNA bases GC/MS-SIM, isolated human chromatin, K562 cells	e[m]		25 µM + H_2O_2 208 mM + SOD 200 units/mL	Nackerdien *et al.* (1991)
Induction of human metal-inducible genes (*MT-IIA*, *hsp70*, *c-fos*), HeLa human cervical carcinoma cells, *in vitro*	+[n]		500 µM	Murata *et al.* (1999)
Metal responsive element (MRE)-DNA binding activity, HeLa human cervical carcinoma cells, *in vitro*	–		500 µM	Murata *et al.* (1999)
Heat shock element (HSE)-DNA binding activity, HeLa human cervical carcinoma cells, *in vitro*	?		500 µM	Murata *et al.* (1999)
Cobalt(II) sulfate monohydrate				
Cell transformation, Syrian hamster embryo cells, *in vitro*	+		0.125 µg/mL [0.75 µM]	Kerckaert *et al.* (1996)

Table 15 (contd)

Test system	Result[a]		Dose[b] (LED/HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cobalt(II) sulfate heptahydrate				
Salmonella typhimurium TA100, reverse mutation	+	–[c]	100 µg/plate	Zeiger *et al.* (1992)
Salmonella typhimurium TA98, TA1535, reverse mutation	–	–	10 000 µg/plate	Zeiger *et al.* (1992)
Induction of p53, ELISA assay, NCTC929 mouse fibroblasts, *in vitro*	+		50 µg/mL [178 µM]	Dujerksen-Hughes *et al.* (1999)
CO(II)acetate tetrahydrate				
Chromosomal aberrations, human lymphocytes, *in vitro*	–		0.6 µg/mL [2.4 µM]	Voroshilin *et al.* (1978)
Co(III)hexaamine ions and Co(III) amine complexes				
Conformational changes of DNA oligonucleotides, circular dichroism and NMR spectroscopy	+		µM range (< 24 µM)	Bauer & Wang (1997)

IARC (2006) summary regarding *in vitro* data:

> *"Cobalt (II) chloride was found to be inactive in the λ prophage induction assay, and gave conflicting results in the Bacillus subtilis rec+/– growth inhibition assay; when a cold preincubation procedure was used, positive results were observed (Kanematsu et al., 1980). Lysogenic induction and phage reactivation was found in Escherichia coli in the absence of magnesium. Also in E. coli, reduction of fidelity of DNA replication by substitution of magnesium and inhibition of protein synthesis were observed. Cobalt(II) chloride was inactive in all but two bacterial mutagenicity tests. One study gave positive results in the absence, but not in the presence, of an exogenous metabolic system, and in the second study, a preincubation procedure was used.*
>
> *In bacteria, cobalt(II) chloride has been reported to reduce the incidence of spontaneous mutations and to inhibit mutations induced by N-methyl-N'-nitrosoguanidine and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole. It was found to be comutagenic with several heteroaromatic compounds such as benzo(a)pyrene and naphthylamine.*
>
> *In Saccharomyces cerevisiae, cobalt(II) chloride induced gene conversion and petite ρ- mutation in mitochondrial DNA but no other types of mutation.*
>
> *In mammalian cells cultured in vitro, positive results were obtained for induction of DNA–protein cross-linkage, DNA strand breakage and sister chromatid exchange in most studies. Cobalt (II) chloride induced mutations at the Hprt locus in Chinese hamster V79 cells, but not at the 8AG and the Gpt loci. At the same Gpt locus in a transgenic Chinese hamster V79 G12 cell line, lower concentrations of cobalt (II) chloride did induce gene mutations. In a single study, at the Tk locus in mouse lymphoma L5178Y cells, the results were negative.*
>
> *In most studies, in cultured human cells in vitro, positive results were obtained for inhibition of protein-DNA binding activities, inhibition of p53 binding to DNA and for induction of gene expression* (in *Cap43* in human lung cells), *induction of DNA strand breakage and sister chromatid exchange. Chromosomal aberrations were not observed in cultured human cells (IARC, 1991). [The Working Group noted the low concentrations employed.] Cobalt(II) chloride induced aneuploidy in cultured human lymphocytes."*

For cobalt (II) sulphate IARC (2006) summarised:

> *"Cobalt sulphate has been shown to induce chromosomal aberrations and aneuploidy in plant cells, chemical changes in bases in purified calf thymus DNA and in isolated human chromatin in the presence of hydrogen peroxide, and cytoskeletal perturbation of micro-tubules and microfilaments and p53 protein in mouse fibroblasts treated in vitro. Cell*

transformation of Syrian hamster embryo cells has been induced by cobalt sulphate in vitro.

A number of mammalian genes (metallothionein MT-IIA, heat-shock proteins hsp70, c-fos) are transcriptionally regulated by a cis-acting DNA element located in their upstream regions. This DNA element responds to various heavy metals, including cobalt, to stimulate the expression of these genes (Murata et al., 1999). MT-IIA and hps70 but not c-fos RNA transcripts were increased in HeLa S3 cells exposed to high concentrations of cobalt sulphate (> 10 µM). Metal response element (MRE)-DNA binding activity was not inhibited by cobalt sulphate in Hela cells in vitro while the results for heat shock element (HSE)-DNA binding activity were inconclusive. It is unknown whether MT-IIA and hps70 induction plays a role in the pathophysiological processes involved in cobalt carcinogenesis."

For cobalt (II) acetate IARC (2006) summarised:

"*it was found that this substance induced cell transformation in vitro and enhanced viral transformation in Syrian hamster embryo cells*"

ECHC (2011) made a more condensed overview of the *in-vitro* data:

"In vitro mutagenicity assays in bacteria with soluble cobalt (II) salts were primarily negative both with and without activation. Mixed results were obtained in bacterial indicator assays for DNA damage (Rec assay in B. subtilis). In yeast, mainly positive results were obtained in mutagenicity and gene conversion assays in S. cerevisiae strains without activation. In mammalian cells in vitro, mutagenicity and cell transformation assays gave mixed results. However, cobalt chloride induced DNA damage (strand breaks and DNA-protein cross links), chromosome damage (micronuclei and sister chromatid exchanges) and aneuploidy in rodent and human cells in culture. Negative results were obtained for cobalt acetate and cobalt nitrate in chromosomal aberration assays in human cells in culture. Elemental cobalt particles induced DNA damage (strand breaks) and chromosome damage (micronuclei) in vitro."

Appendix B

Mode of action and threshold/ non-threshold conclusions made by the various national and international expert groups

ATSDR (2004)

This report on cobalt is in the series of "Toxicological profile for…" elaborated and published by U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry.

Mode of action

Very little is said on this issue but some possible mechanisms for effects in the respiratory tract are mentioned in the summary section of human health effects:

"A number of these effects are believed to be the result of the generation of oxidants and free radicals by the cobalt ion. In vitro exposure to soluble cobalt increases indices of oxidative stress, including diminished levels of reduced glutathione, increased levels of oxidized glutathione, activation of the hexose monophosphate shunt, and free-radical-induced DNA damage."

Threshold/ non-threshold

Although the carcinogenic effects seen in the NTP (1998) study were acknowledged, no further discussions regarding threshold/ non-threshold and description of carcinogenic dose response consideration were given.

ATSDR (2004) considered reduced lung function as the most critical effect in relation to inhalation exposure to cobalt and cobalt substances. Based on a NOAEL of 0.0053 mg Co/m^3 in workers (Nemery et al. 1992), a minimal risk level for chronic exposure of 0.001 mg Co/m^3 was derived for the general population using uncertainty factors and a threshold approach for this effect.

Swedish Work and Health, SWH (2005a+b)

The Criteria Document for Swedish Occupational Standards on Cobalt and Cobalt Compounds (SWH 2005a) was elaborated as a scientific background document for further discussion and evaluation by the Criteria Group of the Swedish National Institute for Working Life. This group makes overall conclusions on the dose-response effect relationship and the critical effect in relation to occupational exposure to specific chemical substances (SWH 2005b).

Mode of action

SWH (2005a+b) simply concluded that:

"Co is genotoxic presumably via an indirect mechanism involving reactive oxygen species. Genotoxic potential in vitro has been shown for Co ions and Co metal particles."

"Production of activated oxygen species is a common mechanism of genotoxicity for cobalt(II) ions, cobalt metal and hard metal (Co plus tungsten carbide), and cobalt and cobalt compounds are believed to act as indirect genotoxicants due to the formation of activated oxygen species. In addition, Co(II) ions are known to inhibit DNA repair. Co(II) ions are formed during the production of activated oxygen species from cobalt and hard metal (68). In a population (n= 78) of workers selected for Cd exposure, but also exposed to Co (mean air level: 2.0 µg Co/m3, state of Co not defined) and Pb, levels of DNA-SSB (single strand breaks) in mononuclear blood cells correlated strongly to Co levels in air (personal sampler) and in blood. Increased levels of SSB were recorded at Co levels of between 4–10 µg/m^3. An inhibition of repair activity of DNA adducts (8-oxoguanine) in blood from these workers was also reported, but referred to as unpublished data. The authors conclude that Co was the strongest determinant, but that interactions with Cd and/or Pb seem likely (34)."

Threshold/ non-threshold

Although the carcinogenic effects seen in the NTP (1998) study were acknowledged, no further discussions regarding threshold/ non-threshold and description of carcinogenic dose response consideration were given.

In the conclusion by SWH (2005a+b), the critical effect of occupational exposure to Co and Co compounds was identified as irritation of eyes, nose and throat. This was found at a mean Co exposure of 3 µg Co/m^3. Other effects on the respiratory system were said to appear at slightly higher levels.

IARC (2006)

The purpose of this evaluation was to update the previous IARC (1991) evaluation in which both metallic cobalt and cobalt substances were concluded as possibly carcinogenic to humans (Group 2B). IARC in 1991 found inadequate evidence for the carcinogenicity of metallic cobalt and cobalt compounds in humans and limited evidence for cobalt (II) chloride (the only water-soluble cobalt substance concluded upon) for the carcinogenicity in experimental animals.

Mode of action

IARC (2006) made several considerations as regards the genotoxic mechanisms of metallic cobalt and the cobalt(II)-cation:

"It had been assumed that, as for other metals, the biological activity of cobalt-metal particles, including their genotoxic effects, were mediated by the ionic form of cobalt and could be revealed by testing soluble compounds. However, Lison et al. (1995) demonstrated in vitro that cobalt metal, and not its ionic (II) species, was thermodynamically able to reduce oxygen in ROS independently of the Fenton reaction. During this process, soluble cobalt ions are produced which have several major cellular targets for induction of genotoxic effects and may, in turn, take part in a Fenton reaction in the presence of hydrogen peroxide. Moreover, since metallic cobalt forms particles which can be inhaled, assessment of genetic effects should also take into consideration: (i) that the primary production of ROS is related to the specific surface properties of the particles or the presence of transition metals, together with other parameters such as particle size, shape and uptake; and (ii) that excessive and persistent formation of ROS by inflammatory cells can lead to secondary toxicity. Since the mechanisms leading to the genotoxic effects of metallic cobalt are complex, assessment of its mutagenic effects should not be restricted to the genetic effects of metallic cobalt alone but should be complemented by those of cobalt in association with carbides, and of cobalt salts.

The results of genotoxicity assays with cobalt salts demonstrate clearly their mutagenic potential. Recent experimental studies have contributed to better delineate the molecular mechanisms involved in the genotoxic (and carcinogenic potential) of cobalt ions. These mechanisms may conceivably apply to soluble cobalt compounds — for example, cobalt chloride or sulphate — and also to cobalt-metal or hard-metal particles, which are readily solubilized in biological media. In vivo, however, the bioavailability of cobalt(II) is relatively limited because these cations precipitate in the presence of physiological concentrations of phosphates ($Co_3(PO_4)_2$); Ks: 2.5 × 10^{-35} at 25 °C) and bind to proteins such as albumin.

In vitro in mammalian cells, two mechanisms seem to apply:

(1) a direct effect of cobalt(II) ions causing damage to DNA through a Fenton-like mechanism;

(2) an indirect effect of cobalt(II) ions through inhibition of repair of DNA damage caused by endogenous events or induced by other agents.

In vitro, cobalt(II) has been shown to inhibit the excision of UV-induced pyrimidine dimers from DNA in a dose-dependent fashion. Inhibition of repair by cobalt(II) resulted in the accumulation of long-lived DNA strand breaks suggesting a block in the gap-filling stage (DNA polymerization) of repair. Ability to inhibit repair was not correlated with cytotoxicity. It has been shown that repair of X-ray-induced DNA damage is not sensitive to cobalt. All inhibitory metals inhibited closure of single-strand DNA breaks (Snyder et al., 1989).

In vitro, ionic cobalt(II) was shown to inhibit nucleotide excision repair processes after ultraviolet (UV) irradiation as measured by the alkaline unwinding method. A concentration as low as 50 µM cobalt chloride inhibited the incision as well as the polymerization step of the DNA repair process in human fibroblasts treated with UV light. As the repair of DNA damage is an essential homeostatic mechanism, its inhibition may account for a mutagenic or carcinogenic effect of cobalt(II) ions. Concentrations less than 1 mM cobalt chloride did not affect the activity of bacterial fpg but significantly reduced the DNA binding activity of the mammalian damage recognition protein XPA. Competition with essential magnesium ions and binding to zinc finger domains in repair proteins have been identified as potential modes of indirect genotoxic activity of cobalt(II) ions. It has also been reported that the DNA binding activity of the p53 protein, which is a zinc dependent mechanism, can be modulated by cobalt(II) ions (Kasten et al. 1997; Palecek et al. 1999; Asmuss et al. 2000).
This indirect mutagenic effect of cobalt on repair enzymes is not restricted to cobalt salts but has been shown to apply also to in-vitro exposure to metallic cobalt.

…..

Since the previous IARC evaluation of cobalt in 1991, additional information has been obtained on the genotoxicity of the various cobalt species. Cobalt(II) ions have been shown to substitute for zinc in the zinc-finger domain of some important proteins, such as those controlling cell cycling and/or DNA repair processes in animal and human cells."

Threshold/ non-threshold

The evaluation by IARC (2006) does not discuss whether the carcinogenicity of cobalt metal and cobalt compounds should be considered a threshold or non-threshold phenomenon, as this is not a part of the task of IARC.

WHO/CICAD (2006)

This evaluation is elaborated in the series of "Concise International Chemical Assessment Documents" under WHO.

Mode of action

The following MoA considerations were made in connection with the cobalt involving ROS generation oxidative stress, induction of hypoxia and inhibition of with DNA repair:

"Several studies have demonstrated that a hard metal alloy of tungsten carbide and cobalt matrix is more toxic than either tungsten carbide or cobalt alone. In a proposed mechanism, tungsten carbide facilitates the oxidation of cobalt metal to ionic cobalt (Co^{2+}) by transferring electrons from the cobalt atom to molecular oxygen (Lison et al., 1995, 1996). This causes an increase in the solubility of cobalt, relative to cobalt metal, and the generation of reactive oxygen species. The ionic cobalt may be transported by blood throughout the body, causing adverse effects by the generation of reactive oxygen species.

Cobalt toxicity may also be caused through oxidant-based and free radical-based processes. Exposure to soluble cobalt leads to increased indices of oxidative stress, diminished levels of reduced glutathione, increased levels of oxidized glutathione, activation of the hexose monophosphate shunt, and free radical-induced DNA damage (Lewis et al., 1991; Kasprzak et al., 1994; Zhang et al., 1998; Hoet et al., 2002). In the presence of hydrogen peroxide, cobalt(II) stimulates in vitro formation of 8-hydroxy-2'-deoxyguanosine (Ivancsits et al., 2002). A Fenton-type mechanism causes cobalt to generate oxygen radicals, such as superoxide, in both in vitro and in vivo studies (Moorhouse et al., 1985; Kadiiska et al., 1989; Kawanishi et al., 1994; Lloyd et al., 1997). Exposure of rats and guinea pigs to cobalt results in liver lipid peroxidation and reduced levels of glutathione, superoxide dismutase, catalase, haem oxygenase, and glutathione peroxidase (Sunderman & Zaharia, 1988; Christova et al., 2001, 2002). Cobalt accumulation in cardiac tissues is believed to stimulate carotid body chemoreceptors, which mimics the action of hypoxia (Di Giulio et al., 1990, 1991; Hatori et al., 1993; Morelli et al., 1994). Cobalt exposure also affects genes that are sensitive to oxidant status, such as hypoxia-inducible factor 1, erythropoietin, vascular endothelial growth factor, catalase, and monooxygenase enzymes (Yasukochi et al., 1974; Dalvi & Robbins, 1978; Legrum et al., 1979; Goldberg et al., 1988; Di Giulio et al., 1991; Goldberg & Schneider, 1994; Ladoux & Frelin, 1994; Semenza et al., 1994; Ho & Bunn, 1996; Bunn et al., 1998; Daghman et al., 1999; Hoet et al., 2002). These effects may also lead to the induction of apoptosis, through either these genes or other pathways (Zou et al., 2001).

….

Cobalt ions, in the presence of oxidants such as UV radiation or hydrogen peroxide, can cause increased levels of DNA damage in vitro (Hartwig et al., 1991; Nackerdien et al., 1991; De Boeck et al., 1998). Cobalt is hypothesized to inhibit DNA repair, particularly the steps of incision and polymerization, by interacting with zinc finger DNA repair proteins (Sarkar, 1995; Kasten et al., 1997; Asmuß et al., 2000)".

Threshold/ non-threshold

With respect to dose-response relationship in the NTP (1998) carcinogenicity study, WHO/CICAD (2006) used the US EPA 2003 benchmark dose software to calculate a lower limit for the 10% benchmark dose concentration level (BMCL10) of 0.358 mg/m^3 from the data from male mice.

When setting a tolerable exposure level, WHO /CICAD (2006) used a threshold approach in relation to reduced lung function that was considered the most critical effect. From a NOAEC of 0.0053 mg Co/m^3 in workers occupational exposed to cobalt dust (Nemery et al. 1992), a tolerable continuous exposure level for the general public of 0.0001 mg/m^3 was estimated.

This level of 0.0001 mg/m^3 was then compared to the BMDL10 value for cancer and was based on linear scaling estimated to represent a lifetime cancer risk level of 3 x 10^{-5}.

However, it was not clearly stated or discussed by WHO/CICAD (2006) whether the carcinogenic effect should be considered a threshold/non-threshold effect.

MAK (2007) and MAK (2009)

Expert groups under the German MAK-Commission have generated the following documentation as regards occupational limit values/ classification of cobalt and cobalt compounds (MAK 2007) and dermal absorption (MAK 2009)

Mode of action

MAK (2007) discussed possible modes of action in relation to oxidative stress and DNA-repair inhibition and co-carcinogenic effects:

"Cobalt and cobalt compounds were genotoxic in vitro and in the bone marrow of mice and hamsters. The genotoxic effect of cobalt ions probably takes place via the production of radical oxygen species, as 5 of 9 of the mutations found in tumour tissue in the carcinogenicity study of the NTP with cobalt sulfate in mice were G-T transversions in codon 12 of the K-ras oncogene. The authors interpret this transversion as supportive evidence that cobalt sulfate heptahydrate may indirectly damage DNA by oxidative stress (NTP 1998). This hypothesis is supported by direct proof that radical oxygen species are produced by the interaction between cobalt, tungsten carbide and oxygen in hard metal dusts (Lison et al. 1995). In human lymphocytes, the clastogenic effect of cobalt dust was weaker than that of a combination of cobalt and tungsten carbide (Anard et al. 1997, de Boeck et al. 1998, van Goethem et al. 1997).

The genotoxicity of other mutagenic agents was increased by cobalt ions (Beyersmann and Hartwig 1992) and cobalt dusts (de Boeck et al. 1998). Attention was drawn to this relationship by the inhibitory effect of cobalt ions on the repair of DNA damage (Kasten et al. 1997). In particular, the function of the XPA protein from mammalian cells participating in the nucleotide excision repair is inhibited by cobalt(II) ions (Asmuß et al. 2000). Evidence of a co-carcinogenic effect in animal studies, in which cobalt(II) oxide increased the carcinogenic effect of benzo(a)pyrene, corresponds to the comutagenic properties found (Steinhoff and Mohr 1991). Empirical evidence in humans also provides evidence for disturbed DNA repair due to cobalt. Both an increased number of DNA single strand breaks and reduced repair capacity for oxidative DNA damage in lymphocytes were found in a subgroup of 11 subjects from a group of 78 metal workers, who were exposed to > 4 g/m3 cobalt at the work site (Oesch et al. 1999)."

MAK (2009) further addressed the skin absorption rates of cobalt and cobalt compounds and in that context concluded that as genotoxic carcinogens dermal exposure to cobalt substances may lead to an increased cancer risk. MAK (2009) referred to an *in vitro* study by Sartorelli et al. (2004) that found a penetration of cobalt chloride dissolved in artificial sweat of 3.3 % into the receptor liquid after 24 hours of exposure.

Threshold/ non-threshold

MAK (2007) concluded that a threshold level that protects against genotoxic and carcinogenic effects could not be established.

EFSA (2009) and EFSA (2012)

The EFSA (2012) opinion on the safety of cobalt compounds as feed additives refers to the more detailed health assessment made by EFSA (2009) in its opinion on the use of cobalt compounds as additives in animal nutrition.

Mode of action

EFSA (2009) especially discussed a ROS and a DNA-repair mode of action for cobalt:

"Concern has been raised by the potential genotoxic and carcinogenic activity of Co(II). The results of in vitro-assays performed with soluble Co(II) salts including acetate, chloride, and sulfate (IARC 2006) clearly demonstrate the full mutagenic potential of the several salts in mammalian cells, whereas most of the tests performed in bacteria were negative. Two major mechanisms seem to be involved, namely (i) the generation of reactive oxygen species through a Fenton-like mechanism and (ii) the inhibition of DNA repair mechanisms (Beyersmann and Hartwig, 2008). Several published in vivo studies indicate that Co salts (chloride or acetate) are capable of inducing a variety of genotoxic

alterations (DNA damage, gene mutations, micronuclei formation, chromosomal aberration) in laboratory species exposed by oral or parenteral routes. Although experimental data indicate some evidence of a genotoxic potential for Co in human lymphocytes in vitro (De Boeck et al., 1998; Lison et al., 2001), inconclusive evidence of Co-mediated genotoxicity in humans has been provided by studies conducted in workers exposed to Co dusts (De Boeck et al., 2000) or in individuals bearing orthopaedic joint replacements made of Co-containing alloys (Keegan et al., 2008).

Several experiments performed in laboratory animals support the in vivo carcinogenicity of Co salts when administered by different routes, and namely local tumours (sarcomas) at injection sites and lung tumours after intratracheal instillation (Bucher et al., 1999). No published studies were found concerning oral route.

Lison et al. (2001) concluded that the genotoxic potential of Co(II) cations is demonstrated in vitro and there is substantial evidence that Co(II) cations exert genotoxic as well as carcinogenic effects in animals; moreover, it seems reasonable to consider that all soluble Co(II) salts (chloride, sulfate, acetate) share this carcinogenic potential."

Threshold/ non-threshold

EFSA (2009) concluded on a tolerable daily oral intake of 600 µg cobalt to protect from the known threshold-related adverse effects from oral exposure. This value was derived from a LOAEL of 1 mg/kg in humans in relation to polycythaemia.

However, EFSA (2009) did not further discuss whether carcinogenicity following inhalational exposure should be considered a threshold or non-threshold effect.

Environment Canada, Health Canada 2011 (ECHC 2011)

This screening assessment on elemental cobalt, cobalt chloride and cobalt sulphate was conducted by the Canadian authorities (Environment Canada and Health Canada).

Mode of action

For the genotoxic mode of action, a ROS mediated pathway as well as a pathway by inhibition of DNA repair were highlighted:

"It is considered likely that cobalt induces DNA damage through the generation of reactive oxygen species (ROS) and increased cellular oxidative stress. Some of the supporting evidence is described below. Both elemental cobalt particles and Co2+ ions have been shown to generate ROS under biologically relevant conditions. An aqueous suspension of elemental cobalt particles (0.1 to 1.5 µm) was found to react with dissolved oxygen, forming a strong oxidant, likely Co-O-O•, and in the presence of either superoxide dismutase or Fe2+ ions the oxidant was found to release hydroxyl radicals (Leonard et al 2006). In pH 7.4 phosphate buffer, free Co2+ ions promoted the conversion of hydrogen peroxide to the superoxide anion; however in the presence of chelating peptides such as glutathione, conversion of hydrogen peroxide to hydroxyl radicals was observed (Hanna et al. 1992; Shi et al 1993). This Fenton-type mechanism generated ROS in both in vitro and in vivo studies (Moorhouse et al., 1985; Kadiiska et al.,1989; Kawanishi et al., 1994; Lloyd et al., 1997 – all cited in IPCS 2006).

In vitro and in vivo, exposure to soluble cobalt leads to increased indices of oxidative stress (Lewis et al., 1991 cited in IPCS 2006; Hoet et al., 2002 cited in IPCS 2006). In the presence of hydrogen peroxide, cobalt(II) stimulates in vitro formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) (Ivancsits et al. 2002), and cobalt sulfate induces DNA cross-links (Lloyd et al 1997). In vivo, cobalt acetate induced oxidative DNA damage in the liver, kidney, and lungs of rats given a single intraperitoneal injection (Kasprzak et al 1994).

Additional suggestive evidence of an oxidative stress mechanism of DNA damage in tumour induction also comes from the examination of tumours from cobalt sulfate-exposed mice, in which the frequency of base pair transversion (guanine to thymine) in codon 12 of the K-ras oncogene was 55 % compared with none in the lung tumours of the control mice (NTP 1998).

A second potential mechanism contributing to the indirect genotoxicity of cobalt is the inhibition of DNA repair processes, possibly through competition with other essential ions and binding to zinc finger domains in DNA repair proteins. In vitro, cobalt (II) inhibits the mammalian repair protein Xeroderma pigmentosum group A (XPA), which contains zinc finger domains (Asmuss et al 2000; Kopera et al 2004). Cobalt chloride and cobalt acetate inhibited DNA repair following UV-induced DNA damage in human cells in culture, by inhibiting the incision and polymerization steps, but not the ligation step (Snyder et al 1989; Kasten et al 1997). In a small epidemiological study in which workers were exposed to cobalt dust, individuals with variations in several DNA repair genes had higher incidences of genotoxicity markers in the lymphocytes (Mateuca et al 2005). (reviewed in IARC 2006, IPCS 2006, Beyersmann and Hartwig 2008).

…….

In vitro and in vivo genotoxicity data on elemental cobalt and soluble cobalt (II) salts indicate that these substances can cause DNA and chromosome damage. However, these effects are likely mediated by indirect mechanisms including the generation of reactive oxygen species, increased oxidative stress, and inhibition of DNA repair enzymes. As the tumours observed in experimental animals are unlikely to have resulted from direct interaction with genetic material, a margin of exposure approach is used to assess risk to human health".

Threshold/ non-threshold

As indicated in the above section, the cobalt(II)-ion is not considered to have a direct interaction with genetic material, and thus an indirect and a threshold-mediated mechanism for the genotoxicity of cobalt(II) is concluded.

It was noted that in mice and rats, respiratory tract lesions were observed following 2-years of inhalation exposure to cobalt sulphate at all tested concentrations (0.11 to 1.14 mg Co/m^3), and further a concentration-dependent increase in lung tumours was observed (significant at 1.14 mg Co/m^3 in male mice and rats and at 0.38 and 1.14 mg Co/m^3 in female mice and rats). The lowest dose at which significantly increased tumours were observed in rodents was 0.38 mg Co/m^3.

Further, ECHC (2011) concluded on a human NOAEC for repeated inhalational exposure of 0.0053 mg Co/m^3 in relation to eye, nose and throat irritation and cough, and reduced lung function from the Nemery et al. (1992) study.

Danish EPA (2013)

This report on "Cobalt(II), inorganic and soluble salts Evaluation of health hazards and proposal of a health based quality criterion for drinking water" was elaborated for the Danish EPA in order to establish a health based limit value for drinking water.

Mode of action

Danish EPA (2013) referred to proposed MoA such as oxidative stress and inhibition of DNA-repair:

"Exposure to soluble cobalt increases indices of oxidative stress, including decreased levels of reduced glutathione, increased levels of oxidized glutathione, activation of the

hexose monophosphate shunt and free-radical-induced DNA damage (Hoet et al. 2002, Kasprzak et al. 1994, Lewis et al. 1991, Zhang et al. 1998a – quoted from ATSDR 2004).

…

The results of genotoxicity assays have indicated a genotoxic potential of cobalt(II) compounds. In mammalian cells in vitro, two mechanisms seem to operate: 1) a direct effect of cobalt(II) ions causing DNA damage through a Fenton-like mechanism, and 2) an indirect effect of cobalt(II) ions through inhibition of repair of DNA damage caused by endogenous events or induced by other agents. As the repair of DNA damage is an essential homeostatic mechanism, this inhibition may account for a mutagenic or carcinogenic effect of cobalt(II) ions. Competition with essential magnesium ions and binding to zinc finger domains in repair proteins have been identified as potential modes of the indirect genotoxic activity (IARC 2006)."

Further, it was concluded that:

"It should be noted that the local lung tumours observed following inhalation exposure are of no relevance for an evaluation of systemic carcinogenicity following oral exposure to cobalt compounds.

No conclusion can be drawn regarding a carcinogenic potential following oral exposure to cobalt compounds based on the available data; however, a carcinogenic potential cannot be excluded as the available genotoxicity data indicate a genotoxic potential of cobalt(II) compounds."

Threshold/ non-threshold

No discussion was included in the report with respect to a threshold/ non-threshold level for the carcinogenic effects of inhalation.

A tolerable daily intake (TDI) value for oral exposure was established using a threshold approach based on a LOAEL of 1 mg Co/kg bw/day for polycythaemia from an oral human voluntary study with cobalt chloride (Davis and Fields 1958).

NTP (2013)

This NTP (2013) report is a peer-reviewed *draft-report* on a 2-year inhalation carcinogenicity study on metallic cobalt, but also considers background information from other cobalt compounds.

Mode of action

The findings on mutations on the K-*ras* gene from inhalational exposure to metallic cobalt in mice were compared with the findings from inhalational exposure to cobalt sulphate:

Mutations within codon 12 of K-ras were observed in both spontaneous alveolar/bronchiolar carcinomas [27% (34/124), (Hong et al., 2008)] as well as alveolar/bronchiolar carcinomas from cobalt metal-exposed mice [67% (46/69)]. However, alveolar/bronchiolar carcinomas from cobalt metal-exposed mice had predominantly G→T transversions [77% (23/30)], whereas the spontaneous carcinomas had G→A transitions [70% (14/20)] in codon 12. The G→T transversions were also the most predominant mutations in alveolar/bronchiolar carcinomas from mice chronically exposed to cobalt sulfate heptahydrate aerosols (NTP, 1998), as well as other chemicals such as ozone, ethylene oxide, and cumene. This suggests that these chemicals target guanine or cytosine bases suggesting that these chemicals induce mutations at multiple sites and tissues by a common mechanism. Interestingly, G→T transversions are one of the more common K-ras mutations in human lung cancer (Rodenhuis et al., 1987). G→T K-ras

mutations were reported to correlate with 8-hydroxydeoxyguanine adducts that result from oxidative stress. In the current study, these transversion mutations were seen almost exclusively in murine alveolar/bronchiolar carcinomas from cobalt exposure but not in spontaneous alveolar/bronchiolar carcinomas.

Threshold/ non-threshold

No further discussion was provided as to whether the carcinogenic response should be considered a threshold or non-threshold phenomenon.

NTP (2014)

Carcinogenicity

The NTP Report on Carcinogens (2014) concluded that "cobalt sulfate is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in experimental animals."

Mode of action

"The mechanism by which cobalt ions cause cancer has not been determined. It has been suggested that cobalt may replace other essential divalent metal ions (e.g., magnesium, calcium, iron, copper, or zinc), thus altering important cellular functions. Other potential mechanisms include inhibition of DNA repair and interaction with hydrogen peroxide to form reactive oxygen species that can damage DNA (Beyersmann and Hartwig 1992, Lison et al. 2001)."

Threshold/ non-threshold

No further discussion on this issue.

OECD (2014 (a+b))

The OECD SIDS initial assessment report (OECD 2014a) and the condensed OECD SIDS initial assessment profile (OECD 2014b) are recent assessments on water soluble cobalt salts (cobalt carbonate was not included) elaborated under the OECD Chemical Assessment programme. The documents have been agreed upon by the competent authorities in the OECD member states.

Mode of action

OECD (2014b): "*In summary, soluble cobalt salts do not elicit any mutagenic activity either in bacterial or mammalian test systems. However they induce some genotoxic effects in vitro, mainly manifest as DNA strand or chromosome breaks, which are consistent with a reactive oxygen mechanism, as has been proposed by various authors. A weight-of-evidence approach was applied, considering positive as well as negative in vivo clastogenicity studies and the absence of such chromosome damage in humans that are occupationally exposed to inorganic cobalt substances. It was concluded that effective protective processes exist in vivo to prevent genetic toxicity with relevance for humans from the soluble cobalt salts category."*

Threshold/ non-threshold

As OECD (2014b) concluded on the absence of *in vivo* genotoxicity by relevant human exposure routes, the carcinogenic effects could be considered as thresholded. A benchmark approach was suggested for the indication of a carcinogenic POD:

"*Following chronic inhalation exposure of cobalt sulfate in rats and mice at concentrations of 0, 0.3, 1 and 3 mg/m³. Respiratory tract tumours developed in rats and mice of both sexes at concentrations ≥ 0.3 mg/m³ cobalt sulfate heptahydrate (equivalent to ≥ 0.067 mg Co/m³), thus this concentration represents a LOAEC for inhalation carcinogenicity.*

Taking into account the lack of a NOAEC in the concentration-response assessment of cobalt sulphate a benchmark dose (BMD) was calculated using the US EPA BMD software (Version 2.0) with the Gamma Model (Version 2.13). The numbers of alveolar/bronchiolar adenoma or carcinoma in the lung of rats and mice were selected as benchmark response. The 95% lower confidence limit of the BMD for a treatment-related increase in response of 10% was calculated (BMDL10). The lowest BMDL10 value was that for female rat tumours with 0.414 mg/m³ cobalt sulphate heptahydrate."

ANSES (2014)

Mode of action:

ROS generated DNA repair inhibition was briefly mentioned as genotoxic mode of action of the cobalt(II)-ion. However, no further discussion on MoA in relation to the carcinogenic effects is included in the report

Threshold/ non-threshold:

ANSES (2014) concluded that the carcinogenic effects observed in experimental animals after inhalation should be considered a stochastic effect, i.e. a non-threshold effect where a linear dose-response relationship should be applied.

However, ANSES (2014) concluded that the data and dose-responses on carcinogenic effects were too limited to establish an occupational limit value for cobalt and cobalt substances and instead concluded on a pragmatic occupational limit value by using a BMDL10 value and assessment factors based on the inflammatory responses in rats from the NTP (1998) study.

Thus, a BMDL10 value of 0.07 mg Co/m^3 was estimated and an 8-h occupational limit value of 2.5 µg/m^3 was established by using an interspecies factor of 10 and an intraspecies factor of 3.

It was mentioned that at levels protecting against non-carcinogenic effects, the risk of cancer could not be ruled out.

REACH registrations (2014)

The lead REACH registrations on the five cobalt salts have by ECHA been sent to the contractor (submission dates from 17/03/2014 – 24/11/2014). The substances were evaluated as a group and therefore the background data, the interpretation and the conclusions are identical in CSR reports for all five substances.

Mode of action

With respect to the clastogenic effects as identified in *in vitro* assays, the MoA in relation to *in vivo* exposure was further discussed:

"Clastogenicity (chromosome breakage) can often be caused by oxidative damage, or by indirect mechanisms such as excessive cytotoxicity, disruption of non-DNA targets etc. Such mechanisms would be expected to have a threshold. The clastogenic potential of cobalt salts in vitro, as seen in chromosomal aberration, micronucleus and tk mutation (small colony mutants) assays, has been satisfactorily addressed by negative in vivo

bone marrow micronucleus and chromosomal aberration results with cobalt chloride, cobalt 2-ethyl hexanoate, cobalt acetyl acetonate and cobalt resinate. Further, a survey in workers occupationally exposed to cobalt, inorganic cobalt substances did not detect significant increases of genotoxic effects (micronuclei and DNA damage in peripheral blood) in workers exposed to cobalt-containing dust at a mean level of 20 µg Co/m³".

Appendix C

Extracts of data submitted by CDI/CoRC (2015)

CDI/CoRC (2015) submitted detailed information on discussions regarding mode of action and threshold/ non-threshold approach for the carcinogenicity of the cobalt salts.

It is noted that this type of data and discussion have not been included in the OECD (2014) assessment nor in the REACH-registrations of the substances.

Mode of action:

CDI/CoRC information describes the mechanisms of ROS generation, oxidative stress and cytotoxicity towards lung tissue as the most relevant mode of actions for the carcinogenic response of cobalt salts.

"The generation of reactive oxygen species (ROS) as an effect of exposure to cobalt is well described in the literature. Several in vitro as well as in vivo experiments report ROS-related effects upon cobalt exposure in biological and chemical targets (see, e.g. Moorhouse et al., 1985, and Christova et al. 2002). Kadiiska et al. (1989) demonstrated ROS production by cobalt di-nitrate using a spin trapping agent for hydroxyl radicals, in an in vitro cell free system. ROS formation was inhibited by SOD, catalase, by chelating agents such as EDTA, as well as by hydroxyl radical scavengers.

A combined in vivo and in vitro study was conducted by Lewis et al. (1991), where lung tissue was exposed to cobalt dichloride by intratracheal installation and by incubation of lung tissue slides, respectively. Changes were observed which are indicative of generation of ROS, mainly oxidation of thiol residues in proteins and lipids.

In an in vitro study by Ivancsits et al. (2002) calf thymus DNA and human diploid fibroblast DNA were exposed to cobalt-dichloride. A subsequent induction of 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is a marker of ROS-related DNA damage, was observed. This effect was only seen in the presence of hydrogen peroxide, not by cobalt alone. Lloyd et al (1997) made similar observations of signs of ROS-related damage ("bulky DNA lesions") after salmon sperm DNA had been incubated with cobalt-sulphate and hydrogen peroxide.

In both NTP inhalation studies (with Co metal and Co sulphate), a larger than usual number of G to T transversions was observed at the second base of codon 12 of those mouse lung neoplasms carrying a mutated K-ras gene. This finding is consistent with oxidative injury. The authors of both NTP cobalt inhalation studies came to the conclusion that cobalt probably induces tumours by increasing oxidative stress (Bucher 1998; Behl and Hooth 2013)."

…..

"ROS play a twofold job as both beneficial and toxic compounds to the living system. At moderate or low levels, ROS have beneficial effects and involve in various physiological functions such as in immune function (i.e. defense against pathogenic microorganisms), in a number of cellular signaling pathways, in mitogenic response and in redox regulation. But at higher concentration, ROS generate oxidative stress, causing potential damage to the biomolecules. Oxidative stress is developed when there is an excess production of ROS on one side and a deficiency of enzymatic and non enzymatic antioxidants on the other side. Most importantly, excess ROS can damage the integrity of various biomolecules… (Phaniendra et al., 2015)."

……

"Endogenous agents are responsible for several hundred DNA damages per cell per day. The majority of these damages are altered DNA bases (e.g. 8-oxoguanine and thymine glycol) and AP sites. The cellular processes that lead to DNA damage are oxygen consumption that results in the formation of reactive oxygen species (e.g. superoxide

.O2, hydroxyl free radicals .OH and hydrogen peroxide) and deamination of cytosines and 5-methylcytosines leading to uracils and thymines, respectively. The process of DNA replication itself is somewhat error-prone and an incorrect base can be added by replication polymerases. The frequencies of these endogenously produced DNA damages can be increased by exogenous (genotoxic) agents (Casarett & Doull's, 2008)."

Based on this, it is concluded that the ROS generation and the DNA damage associated to this is an initiating event for the development of cancer.

For the progression of the carcinogenic processes the cytotoxicity of the cobalt(II)-ion is considered crucial:

"Chronic inhalation exposure of rats and mice cobalt sulfate resulted in inflammation, hyperplasia, and formation of tumors in the lung. The available toxicological data include long-term, sub-chronic and sub-acute inhalation studies in animals, data on generation of reactive oxygen species (ROS) by cobalt, with corresponding DNA oxidative damage, as well as inhibition of DNA repair enzymes at high doses of cobalt. At exposures and doses achieved in vivo in animals, inhibition of DNA repair is unlikely to be a predominant factor in the development of cancer. The in vivo data give support to the hypothesis that the cancer MoA for cobalt-induced lung tumors involves cytotoxicity, inflammation, alveolar proteinosis, hyperplasia of the alveolar and bronchiolar epithelia, alveolar/bronchiolar adenoma, and alveolar/bronchiolar carcinoma.

The postulated MoA is mainly based on observations of consistent concentration-response relationships for the key events inflammation, hyperplasia, and formation of carcinoma."

…..

"Considering that benign and malignant tumors finally occurred in the lung, alveolar proteinosis, chronic inflammation, hyperplasia of the alveolar epithelium and hyperplasia of the bronchiolar epithelium could be interpreted to represent site-specific, early steps in the cascade of tumorigenic events induced by cobalt. This sequential occurrence of key events is in line with the assumed MoA in which cobalt induced alveolar/bronchiolar adenoma and carcinoma through generation of ROS, which in turn causes DNA damage (Behl and Hooth 2013)"

…..

"Thus, the finding of occurrence of bronchiolar epithelial hyperplasia in 2-week and 3-month studies before appearance of tumors (adenomas and carcinomas) after two-year exposure can be regarded as strong support of the postulated MoA".

It is though acknowledged that:

"Uncertainties remain as to the exact mechanisms of the alterations in the alveolar and bronchiolar epithelia and the disturbances of the control of regenerating cell proliferation leading to carcinogenesis. A high level of reparative cellular proliferation could amplify the background mutation rate and thereby may ultimately lead to tumor formation".

Threshold/non-threshold:

"The apparent balance between background ROS levels and antioxidant defense mechanisms has driven Zastrow et al to their search for a 'free radical threshold value'.

Three evolutionary sources create 'primary' reactive oxygen species (ROS) and 'secondary' lipid oxygen species (LOS), forming the human body's 'free radical ground state'. We present evidence for the existence of a universal free radical threshold value (FRTV), defining the borderline between advantageous and adverse effects of free

radicals observed above the free radical ground state. […] we investigated whether this threshold is also existent in internal organs by extending our experiment to fresh porcine liver. Based on the determination of ROS/LOS below and above the FRTV, ROS > LOS was characterized as beneficial and LOS > ROS as deleterious to the organism, respectively. Results of the experiments using porcine liver confirmed the appearance of the FRTV at radical generation ∼3.5 × 10 12 rad/mg. The relation ROS/LOS before and after the FRTV was consistent with the results determined for the skin. We conclude that the FRTV, theoretically calculated and experimentally confirmed, should be considered as a new 'universal body constant' (Zastrow et al., 2015). Although the physiological relevance still needs to be verified, this 2015 paper by Zastrow et al. is the first attempt to quantify a threshold for making a distinction between the physiological (beneficial) effects of ROS and the deleterious effects that may induce and stimulate toxicity. The authors made use of the correlation between radicals and ratio between reactive oxygen species and lipid oxidation."

….

"Also from an EU regulatory perspective it has been accepted that 'Genotoxic mechanisms based on reactive oxygen' have at least a practical threshold.

It is difficult to state at the present time the precise role of ROS-induced DNA damage in carcinogenesis and how genetic and epigenetic events induced by ROS interact with cell transformation and malignant progression. Many aspects have been elucidated so far indicating that at low levels of ROS adaptive responses, on the side of repair and antioxidative defense, strengthen non-linear dose–response relationships between low and high levels of ROS.

In general, the idea is receiving more and more support from the scientific community that ROS-mediated processes of carcinogenesis have at least practical thresholds (Bolt et al., 2004.)"

"In the case of cobalt, its effect on the (binding-) activity of DNA repair enzymes (proteins) is reported in the literature for several examples of such enzymes/proteins. Several aspects have been demonstrated in the literature:

- Cobalt inhibits some DNA repair enzymes, generally to a lesser extent than other metals tested in the same study.
- The inhibition by cobalt appears to be following a sigmoidal shape, meaning that there is a threshold for the response.
- Each report in the public domain on DNA repair enzyme inhibition demonstrates a threshold for the cobalt effect at or above the doses tested.
- There is no report of a non-thresholded effect by cobalt on DNA repair enzymes.

The following DNA repair enzymes have been tested in combination with cobalt:

***Ape1** (apurinic/apyrimidinic endonuclease 1) – repair of apurinic/apyrimidinic sites No inhibition by cobalt (up to 100 µM), inhibition by other metals with threshold or as dose-response (McNeill, 2004)*

***MPG** (N-methylpurine-DNA glycosylase) - removal of alkylated bases. No inhibition by cobalt up to 1000 µM, other metals displaying dose-response from lowest dose tested (50 µM) (Wang, 2006; CoSO4)*

***MTH1** ("human nucleotide pool sanitization enzyme") – 8-oxo-dGTPase, elimination/ removal of 8-hydroxyguanine (8-HG, a result of oxidative stress, and resulting in G □ T transversions) IC50 for cobalt 376 µM (Porter, 1997; CoCl2)*

p53, p63 and p73 *("tumour suppressor protein family") – conservation of DNA stability, preventing of genome mutations. Inhibition of DNA binding by the different proteins was evaluated: inhibition by cobalt was observed as follows: of p53 at ≥ 300 µM (test range 10 – 600 µM) (Palecek, 1999; CoCl2), inhibition of p63 at ≥ 600 µM and of p73 at ≥ 300 µM (test range 50 – 2000 µM) (Adamik, 2015; CoCl2)*

PNK *(polynucleotide kinase) - base excision repair (BER) and nonhomologous end-joining (NHEJ) DNA repair. No inhibition by cobalt at 200 µM (Whiteside, 2010)*

XPA *(Xeroderma pigmentosum group A) - damage recognition factor in exision repair. Cobalt concentration range tested 1 – 1000 µM; lowest inhibitory concentration 100 µM (= threshold for inhibitory effect was 100 µM) (Asmuß, 2000b, Tox Lett); Inhibition of XPA enzyme activity at 200 µM (tested were 50, 100, 200, 500 µM) (Asmuß, 2000a Carcinogenesis; CoCl2)"*

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