OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT

Air Toxics Hot Spots Program

Cobalt and Cobalt Compounds

Cancer Inhalation Unit Risk Factors

Technical Support Document for Cancer Potency Factors
Appendix B

Scientific Review Panel Draft

September 2019

Air, Community, and Environmental Research Branch

Office of Environmental Health Hazard Assessment

California Environmental Protection Agency

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Introduction

This document summarizes the carcinogenicity and derivation of cancer inhalation unit risk factors (IURs) for cobalt and cobalt compounds. Cancer unit risk factors are used to estimate lifetime cancer risks associated with inhalation exposure to a carcinogen.

The Office of Environmental Health Hazard Assessment (OEHHA) is required to develop guidelines for conducting health risk assessments under the Air Toxics Hot Spots Program (Health and Safety Code Section 44360 (b) (2)). In implementing this requirement, OEHHA develops cancer inhalation unit risk factors for carcinogenic air pollutants listed under the Air Toxics Hot Spots program. The cobalt and cobalt compounds IURs were developed using the most recent "Air Toxics Hot Spots Program Technical Support Document for Cancer Potency Factors", finalized by OEHHA in 2009. Literature summarized and referenced in this document covers the relevant published literature for cobalt and cobalt compounds through the spring of 2019.

Several government agencies or programs currently list cobalt metal and cobalt compounds as carcinogens. Under the California Proposition 65 program, cobalt metal powder, cobalt sulfate, cobalt sulfate heptahydrate, and cobalt(II) oxide are listed as chemicals known to the state to cause cancer (OEHHA, 2018a). Cobalt metal and soluble cobalt(II) salts are listed separately by the International Agency for Research on Cancer (IARC) as Group 2B carcinogens, *i.e.*, possibly carcinogenic to humans (IARC, 2006). The National Toxicology Program (NTP) listed cobalt and cobalt compounds that release cobalt ions *in vivo* in the 14th Report on Carcinogens, which identifies substances that either are known to be human carcinogens or are reasonably anticipated to be human carcinogens, and to which a significant number of persons residing in the United States are exposed (NTP, 2016).

NTP conducted inhalation carcinogenicity bioassays with cobalt sulfate heptahydrate, a soluble cobalt compound, in rats and mice of both sexes in 1998 (NTP, 1998). NTP subsequently conducted inhalation carcinogenicity bioassays with cobalt metal in rats and mice of both sexes in 2014 (NTP, 2014). These studies provided evidence of carcinogenicity for cobalt sulfate heptahydrate and for cobalt metal in rats and mice of both sexes. Due to chemical, physical, and toxicological differences between cobalt metal and various cobalt compounds, separate IURs were derived for water soluble cobalt compounds (based on studies with cobalt sulfate heptahydrate) and cobalt metal and insoluble cobalt compounds (based on studies with cobalt metal).

Most cobalt is used industrially in the form of cobalt metal powder as an alloying component and in the preparation of cobalt salts (NTP, 2016; HSDB, 2019). Cobalt salts and oxides are used as pigments in the glass and ceramics industries, as catalysts in the oil and chemical industries, as paint and printing ink driers, and as trace metal additives

in agriculture and medicine. Other significant cobalt uses are as a catalyst or component in green energy technologies (*e.g.*, solar panels), and as a primary component in lithium- and nickel-based rechargeable batteries. The presence of cobalt in some electric and electronic devices may also result in exposure to cobalt in the E-waste recycling industry (Leyssens *et al.*, 2017).

Cobalt occurs naturally in the Earth's crust but is usually in the form of arsenides and sulfides (Baralkiewicz and Siepak, 1999). Natural levels of cobalt in air generally range from 0.0005 to 0.005 nanograms per cubic meters (ng/m^3). In major industrial cities, levels of cobalt may reach as high as 6 ng/m^3. The California Air Resources Board collects air monitoring data for numerous pollutants found in urban areas, including cobalt and other metals (CARB, 2018). In southern California, mean cobalt concentrations at air monitoring sites in 2017 ranged from 1.3 to 1.97 ng/m^3, with maximum levels between 2.9 and 5.6 ng/m^3. However, cobalt concentrations were often below the limit of detection (1.3 ng/m^3).

Emissions estimates of cobalt in California are collected and presented in the California Toxics Inventory, or CTI (CARB, 2013). Potential sources include stationary (point and aggregated point), area-wide, on-road mobile (gasoline and diesel), off-road mobile (gasoline, diesel, and other), and natural sources. The primary emission source for cobalt in 2010 was area-wide sources, at 55.2 tons per year. Stationary point sources released 2.2 tons of cobalt per year while the remaining sources were small or negligible. Area-wide sources are source categories associated with human activity, and emissions take place over a wide geographic area. Such sources include consumer products, fireplaces, farming operations and unpaved roads. Stationary sources include point sources provided by facility operators and/or districts pursuant to the Air Toxics "Hot Spots" Program (AB 2588).

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List of Acronyms

8-OHdG	8-hydroxydeoxyguanosine	IARC	International Agency for Research on Cancer
AIC	Akaike Information Criterion	IUR	Inhalation unit risk
$BMDL_{05}$	The 95% lower confidence bound at the 5% response rate	IR	Inhalation rate
		LDH	Lactate dehydrogenase
BMD	Benchmark dose	MMAD	Mass median aerodynamic diameter
BMD_{05}	BMD 5% response rate	µg/L	Micrograms per liter
BMDS	Benchmark dose modelling software	µg/ml	Micrograms per milliliter
		µm	Micrometer
BMR	Benchmark response	µM	Micromole per liter
BNMN	Binucleated micronucleated	mg/m^3	Milligrams per cubic meter
BR	Breathing rate	mg/kg-BW	Milligrams per kilogram of bodyweight
BW	Body weight		
CEBS	Chemical effects in biological systems	mM	Millimole per liter
		NCE	Normochromatic erythrocytes
CF	Conversion factor	NP	Nanoparticle
CKE	Cystic keratinizing epithelioma	NTP	National Toxicology Program
		O_2^-	Superoxide radical
Co	Cobalt	OECD	Organisation for Economic Co-operation and Development
$CoSO_4 \cdot 7H_2O$	Cobalt sulfate heptahydrate		
CPF	Cancer potency factor	OEHHA	Office of Environmental Health Hazard Assessment
CSF	Cancer slope factor		
CTI	California Toxics Inventory	PCE	Polychromatic erythrocytes
DMSO	Dimethyl sulfoxide	ROS	Reactive oxygen species
DNA	Deoxyribonucleic acid	SHE	Syrian hamster embryo
Fpg	Formamido-pyrimidine glycosylate	SIR	Standardized incidence rate
		SMR	Standardized mortality ratio
GSD	Geometric standard deviation	SPF	Specific pathogen free
H_2O_2	Hydrogen peroxide	TWA	Time-weighted average
HL	Human lymphocyte	UV	Ultraviolet
hOOG1	Human 8-hydroxyguanine DNA-glycosylate 1	US EPA	United States Environmental Protection Agency

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COBALT AND COBALT COMPOUNDS

I. PHYSICAL AND CHEMICAL PROPERTIES

(Kyono *et al.*, 1992; Hillwalker and Anderson, 2014; NTP, 2016)

Molecular formula	Co (elemental form)
Molecular weight	58.93
Description	Gray, hard, magnetic, ductile, somewhat malleable metal
Density	8.92 g/cm^3
Boiling point	2927ºC
Melting point	1495ºC
Vapor pressure	Not applicable
Odor	Cobalt metal powder or fumes are odorless
Solubility	Metallic cobalt particles in the micrometer size range or larger are considered poorly water soluble. Soluble in dilute acids.
Conversion factor	Not applicable

II. HEALTH ASSESSMENT VALUES

Cobalt metal and water-insoluble cobalt compounds

Unit Risk Factor	8.0×10^{-3} $(\mu g/m^3)^{-1}$
Inhalation Slope Factor	28 $(mg/kg\text{-}day)^{-1}$

Water-soluble cobalt compounds (normalized to cobalt content)

Unit Risk Factor	8.6×10^{-4} $(\mu g/m^3)^{-1}$
Inhalation Slope Factor	3.0 $(mg/kg\text{-}day)^{-1}$

Insolubility of a cobalt compound in water is defined in this document as having a water solubility of ≤100 mg/L at 20˚C (MAK, 2007; USP, 2015). Cobalt compounds that have a water solubility of >100 mg/L at 20˚C are considered water-soluble. The cancer potency factors (unit risk and inhalation slope factors) for cobalt metal applies to insoluble cobalt compounds and the cancer potency factors for cobalt sulfate heptahydrate applies to soluble cobalt compounds. This definition of solubility is only applicable to this document for regulatory purposes, and does not apply to other OEHHA documents and programs.

III. CARCINOGENICITY

Bioaccessibility of the cobalt ion following inhalation is considered to be the primary factor for cancer risk (NTP, 2016). Thus, any inhaled cobalt compound that releases cobalt ion in pulmonary fluids presents an inhalation cancer risk. Water-soluble cobalt compounds reaching the alveoli following inhalation will dissolve in the alveolar lining

fluid and release the cobalt ion (Kreyling *et al.*, 1986; Stopford *et al.*, 2003). Water-insoluble cobalt compounds (*e.g.*, cobalt oxides) and cobalt metal reaching distal airways and alveoli are taken up by macrophages and other epithelial cells by endocytosis and dissolve intracellularly in the acidic environment (pH 4.5 to 5) of lysosomes (Kreyling *et al.*, 1990; Ortega *et al.*, 2014).

Differences in cellular uptake between soluble and insoluble forms of cobalt have been proposed as a reason for differences in cancer potency (Smith et al. 2014). *In vitro* studies observed that insoluble cobalt nanoparticles interacted with proteins on the surface of cells and were readily taken up, resulting in a considerably greater intracellular concentration of cobalt ion (following release in lyosomal fluid) when compared to uptake of extracellular ions from soluble cobalt compounds (Ponti et al., 2009; Colognato et al., 2008).

The IUR values derived by OEHHA apply to metallic cobalt, water-soluble cobalt compounds, and water-insoluble cobalt compounds that have some solubility in lysosomal fluid. The IURs and cancer slope factors are intended for use in the evaluation of cancer risk due to the inhalation of cobalt and cobalt compounds. They are not intended to be used for the evaluation of cancer risk due to cobalt and cobalt compound exposure by the oral route. There is currently inadequate evidence for carcinogenicity of cobalt and cobalt compounds by the oral route of exposure. Commercially significant cobalt compounds include, but are not limited to, the oxide, hydroxide, chloride, sulfate, nitrate, carbonate, acetate, and oxalate forms (Table 1). The cobalt IURs do not apply to cobalt alloy particles (*e.g.*, cobalt-tungsten hard metal and cobalt in stainless steel and super alloys), cobalt aluminum spinel, or the cobalt-containing essential nutrient vitamin B12.

Table 1. Water solubility of some commercially important cobalt compounds (IARC, 1991; Stopford *et al.*, 2003; Hillwalker and Anderson, 2014; NTP, 2016; Lison *et al.*, 2018; HSDB, 2019)

Molecular Formula	Molecular Weight	Form of Cobalt (Metal or Cobalt Compound)	CAS #	Water Solubility
Co	58.9	Cobalt metal particles/dust	7440-48-4	2.9 mg/L
$CoSO_4$	281.1	Sulfate (heptahydrate)	10026-24-1	604,000 mg/L
Co_3O_4	240.8	Oxide(II,III) [a]	1308-06-1	1.6 mg/L
$Co(OH)_2$	93.0	Hydroxide [a]	21041-93-0	3.2 mg/L
CoS	91.0	Sulfide [a]	1317-42-6	3.8 mg/L
CoO	74.9	Oxide(II) [a]	1307-96-6	4.9 mg/L
$CoCO_3$	118.9	Carbonate [a]	513-79-1	11.4 mg/L
CoC_2O_4	147.0	Oxalate [a]	814-89-1	32.2 mg/L
$C_8H_{16}O_2$:1/2Co	344.9	Octoate [b]	136-52-7	40,300 mg/L
$Co(C_2H_2O_2)_2$	249.1	Acetate (tetrahydrate) [b]	71-48-7	348,000 mg/L
$CoCl_2$	129.9	Chloride (hexahydrate) [b]	7646-79-9	450,000 mg/L
CoN_2O_6	182.9	Nitrate (hexahydrate) [b]	10141-05-6	670,000 mg/L

[a] The IUR value for cobalt metal applies to this cobalt compound (insoluble in water (≤100 mg/L at 20˚C))

[b] The IUR value for cobalt sulfate heptahydrate (normalized to cobalt content) applies to this cobalt compound (soluble in water (≥100 mg/L at 20˚C)).

The mechanism of action for cobalt genotoxicity and carcinogenicity probably involves release of cobalt ions leading to cobalt-mediated generation of free radicals and cellular oxidative stress (Hanna *et al.*, 1992; Lison, 1996; Valko *et al.*, 2005). Cobalt-generated reactive oxygen species (ROS) result in oxidative damage to deoxyribonucleic acid (DNA) and inhibition of DNA repair. Cobalt and several other transition metals, such as nickel, copper, vanadium, and chromium, likely participate in ROS generation (*e.g.*, hydroxyl radical formation) through a Fenton-type reaction (Valko *et al.*, 2005). Work by Green *et al*. (2013) found that lung cells have a high tolerance (i.e., delayed apoptosis and cell death) for cobalt loading (as cobalt chloride), when compared to nickel (Ni^{2+}). High cobalt loading of the cells led to accumulation of genetic and epigenetic abnormalities. Exposure of lung cells to Ni^{2+} led to comparatively greater overall cell death and apoptosis and less genotoxicity. These investigators proposed that lung

carcinogenicity may result from tolerance to cobalt cell loading, which allows cell replication and survival despite the presence of cobalt-mediated accumulation of genetic damage.

NTP Carcinogenicity Bioassays

Cobalt Metal

NTP conducted lifetime rodent inhalation carcinogenicity studies for cobalt metal (NTP, 2014a). The mass median aerodynamic diameter (MMAD) ± geometric standard deviation (GSD) of the inhaled particles, recorded monthly, was in the range of 1.4-2.0 micrometers (μm) ± 1.6-1.9. This particle size was noted by NTP to be within the respirable range of the rodents. Groups of F-344/NTac rats and $B6C3F_1$/N mice (50/group/sex/species) were exposed to the cobalt metal aerosol via whole-body inhalation at concentrations of 0, 1.25, 2.5 or 5 milligrams per cubic meter (mg/m^3), for 6.2 hrs/day, 5 days/week for up to 105 weeks. These nominal concentrations were within 1% of the analytical concentrations. The daily exposures include the 6 hr exposure time at a uniform aerosol concentration plus the ramp-up time of 12 min (0.2 hrs/day) to achieve 90% of the target concentration after the beginning of aerosol generation. The decay time to 10% of the target concentration at the end of the exposures was about 9.4 min.

In rats, body weights of males and females in the 2.5 and 5 mg/m^3 groups were reduced (≥10%) compared to controls. In the 5 mg/m^3 groups, body weights were reduced starting after weeks 12 and 21 for males and females, respectively. In the 2.5 mg/m^3 groups, body weights were reduced after weeks 99 and 57 in males and females, respectively. Survival was significantly reduced in the mid-dose 2.5 mg/m^3 female rats compared to controls (p=0.038, life table pairwise comparison) (NTP, 2014a). However, significant differences in survival between the 2.5 mg/m^3 group and controls were not apparent until after week 85 of the study. Most of the female rats in the 2.5 mg/m^3 group had died with treatment-related tumors (42 of 50 (84%)), many of which were considered the primary cause of death (13 of 50 [26%]).

The statistically significant and/or biologically noteworthy tumor incidences in male and female rats are shown in Table 2. The incidences of pulmonary alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma, and alveolar/bronchiolar adenoma or carcinoma (combined) were statistically significantly increased in nearly all cobalt-exposed groups. Positive trends for these tumors, both individually and combined, were observed in both males and females.

The rats also exhibited a generally increasing trend of multiple alveolar/bronchiolar adenoma and carcinoma with increasing exposure concentration. Squamous cell neoplasms of the lung, which were predominantly cystic keratinizing epitheliomas (CKE),

were observed in several cobalt-exposed females and in two cobalt-exposed males, but did not reach statistical significance in either sex. CKE is a rare chemically-induced pulmonary tumor that has been observed in rats exposed to certain particulate compounds (Behl *et al.*, 2015). CKE originates from a different lung cell type from that of alveolar/bronchiolar adenoma and carcinoma, and is considered separately for tumor dose-response analysis (McConnell *et al.*, 1986; Brix *et al.*, 2010). One female rat in the high exposure group had a squamous cell carcinoma, which is believed to be part of the continuum of lesions progressing from CKE. NTP considered the increase in squamous cell neoplasms of the lung to be a treatment-related effect in female rats due to its rarity and exceedance in incidence when compared to the historical control range for all routes of administration. The incidence of lung squamous cell neoplasms in male rats was lower, resulting in an equivocal finding of carcinogenicity by NTP (2014a).

Increased incidences of benign and malignant pheochromocytoma, and benign or malignant pheochromocytoma (combined) of the adrenal medulla were observed in male and female rats. The incidences of these adrenal medulla neoplasms, both individually and combined, were statistically significantly increased at 2.5 and 5 mg/m^3 in male rats. The same was true for female rats, with the exception of a lack of increased incidence in malignant pheochromocytoma at 2.5 mg/m^3. NTP (2014a) also noted a trend-related increased incidence of bilateral pheochromocytoma, both benign and malignant, in male and female rats.

In male rats, a positive trend for pancreatic islet cell carcinoma, and pancreatic islet cell adenoma or carcinoma (combined), was observed following cobalt metal exposure. A borderline positive trend (p=0.0501) for pancreatic islet cell adenoma was noted. At 2.5 mg/m^3, the incidence of adenoma was significantly increased compared to controls. A significantly greater incidence of adenoma or carcinoma (combined) was observed at both 2.5 and 5 mg/m^3. In female rats the incidence of islet cell neoplasms was slightly increased at 5 mg/m^3 (two rats with a carcinoma, and one with an adenoma and a carcinoma), but was not statistically significant. However, islet cell tumor incidence in high exposure females did exceed the historical control incidences for all routes of administration. NTP concluded there was equivocal evidence of pancreatic islet cell carcinoma in female rats due to the absence of statistically significant trends or pairwise comparisons. NTP stated this was the first time that the pancreas was a target organ of carcinogenicity in NTP inhalation studies.

Standard kidney evaluation, in which only one section of each kidney is microscopically examined, revealed a slightly increased incidence of renal tubule adenoma or carcinoma (combined) in 5 mg/m^3 male rats. Although not statistically significant, this finding suggested a treatment-related effect due to exceedance of historical control ranges for all routes of administration. An extended evaluation of the kidneys with step-sectioning at 1 mm intervals subsequently revealed more tumors in the 5 mg/m^3 rats but also more

in the control group. Thus, pairwise test comparison was still not significant. In addition, no supporting nonneoplastic lesions were found in the kidneys. Nevertheless, NTP concluded that due to the relative rarity of these tumors, there is equivocal evidence that these tumors are related to cobalt exposure.

Lastly, female rats had an increased incidence of mononuclear cell leukemia in all exposure groups. NTP considered the increased incidence of this leukemia to be related to cobalt exposure.

Table 2. Tumor incidences[a] in male and female rats in the two-year NTP (2014a) inhalation studies of cobalt metal

Tumor	Cobalt Concentration (mg/m^3)			
	0	1.25	2.5	5.0
Male Rats				
Lung				
Alveolar/bronchiolar adenoma	2/50†	10/50*	10/50*	14/50**
Alveolar/bronchiolar carcinoma	0/50‡	16/50**	34/50**	36/50**
Alveolar/bronchiolar adenoma or carcinoma	2/50‡	25/50**	39/50**	44/50**
Cystic keratinizing epithelioma	*0/50*	*1/50*	*0/50*	*1/50*
Adrenal medulla				
Benign pheochromocytoma	15/50‡	23/50	37/50**	34/50**
Malignant pheochromocytoma	2/50‡	2/50	9/50*	16/50**
Benign or malignant pheochromocytoma	17/50‡	23/50	38/50**	41/50**
Pancreatic Islets				
Adenoma	0/50	1/50	6/48*	3/49
Carcinoma	2/50†	1/50	5/48	6/49
Adenoma or carcinoma	2/50‡	2/50	10/48*	9/49*
Kidney				
Adenoma or carcinoma				
standard evaluation	*0/50*	*1/50*	*0/50*	*4/50*
standard + extended evaluation	*3/50*†	*1/50*	*1/50*	*7/50*
Female Rats				
Lung				
Alveolar/bronchiolar adenoma	2/50‡	7/50	9/50*	13/50**
Alveolar/bronchiolar carcinoma	0/50‡	9/50**	17/50**	30/50**
Alveolar/bronchiolar adenoma or carcinoma	2/50‡	15/50**	20/50**	38/50**
Squamous cell tumors (predominantly cystic keratinizing epithelioma)[b]	0/50	4/50	1/50	3/50
Adrenal medulla				
Benign pheochromocytoma	6/50‡	12/50	22/50**	36/50**
Malignant pheochromocytoma	0/50‡	2/50	3/50	11/50**
Benign or malignant pheochromocytoma	6/50‡	13/50	23/50**	40/50**
Pancreatic Islets				
Adenoma or carcinoma	*1/50*	*0/50*	*0/50*	*3/50*
Immunologic System				
Mononuclear cell leukemia	16/50	29/50**	28/50*	27/50*

Tumor type and incidence data in italics: equivocal finding of carcinogenicity by NTP (2014a)

* $p<0.05$, ** $p<0.01$ for statistical difference from control, poly-3 test

† $p<0.05$, ‡ $p<0.01$ for positive trend for tumor type, poly-3 test conducted by NTP

[a] Denominator represents number of animals examined

[b] Includes one squamous cell carcinoma in the 5 mg/m^3 group

Nonneoplastic findings in the rats included various pulmonary lesions (alveolar epithelium hyperplasia, alveolar proteinosis, chronic active inflammation and bronchiole epithelium hyperplasia), which were observed in the animals at all exposure levels (data not shown). A spectrum of nonneoplastic nasal lesions was also observed in all exposed groups.

In mice exposed to cobalt metal for two years, body weights of males and females at the highest exposure were reduced ≥10% compared to controls. The body weights in these groups were reduced starting after weeks 85 and 21 for males and females, respectively. Survival of male mice was significantly reduced in the 2.5 and 5 mg/m^3 males compared to controls. However, most of the male mice in the two groups died late in the study resulting in mortality rates that were not significantly different than controls until after week 85. Most of the male mice in these two exposed groups died with treatment-related lung tumors (43/50 (86%) and 47/50 (94%) in the 2.5 and 5 mg/m^3 groups, respectively). For the males that died prior to terminal sacrifice, the primary cause of death were lung tumors in most cases (13 of 21 (62%) at 2.5 mg/m^3 and 25 of 28 (89%) at 5 mg/m^3).

The tumor incidences resulting from two-year exposure to cobalt metal in mice are presented in Table 3. Treatment-related tumors in mice were confined to the lungs. The incidences of pulmonary alveolar/bronchiolar carcinoma and alveolar/bronchiolar adenoma or carcinoma (combined) were statistically significantly increased in both males and females in all cobalt-exposed groups, and showed positive trends with exposure in both sexes (Table 3). Statistically significantly increased alveolar/bronchiolar adenomas were observed in male mice in the 2.5 mg/m^3 group, and in female mice in the 5 mg/m^3 group. The incidences of multiple alveolar/bronchiolar carcinomas were statistically significantly increased in both males and females in all cobalt-exposed groups.

Table 3. Tumor incidences[a] in male and female mice in the two-year NTP (2014a) inhalation studies of cobalt metal

Tumor	Cobalt Concentration (mg/m^3)			
	0	1.25	2.5	5.0
Male Mice				
Lung				
Alveolar/bronchiolar adenoma	7/50	11/49	15/50*	3/50
Alveolar/bronchiolar carcinoma	11/50‡	38/49**	42/50**	46/50**
Alveolar/bronchiolar adenoma or carcinoma	16/50‡	41/49**	43/50**	47/50**
Female Mice				
Lung				
Alveolar/bronchiolar adenoma	3/49†	9/50	8/50	10/50*
Alveolar/bronchiolar carcinoma	5/49‡	25/50**	38/50**	43/50**
Alveolar/bronchiolar adenoma or carcinoma	8/49‡	30/50**	41/50**	45/50**

* $p<0.05$, ** $p<0.01$ for statistical difference from control, poly-3 test
† $p<0.05$, ‡ $p<0.01$ for positive trend for tumor type, poly-3 test conducted by NTP
[a] Denominator represents number of animals examined

Nonneoplastic findings in the mice were mainly confined to the lungs, including alveolar/bronchiolar epithelium hyperplasia and cytoplasmic vacuolization, alveolar epithelium hyperplasia, proteinosis, and infiltration of cellular histiocytes within alveolar spaces, which were observed at all exposure levels (data not shown). The incidences of bronchiole epithelium hyperplasia, bronchiole epithelium erosion, and suppurative inflammation occurred at mid- and/or high-exposure levels in one or both sexes. Additionally, nonneoplastic lesions in the nose, larynx and trachea were observed in males and females in all exposed groups.

Overall, NTP (2014a) concluded there was clear evidence of carcinogenic activity of cobalt metal in male and female rats and mice. The lung was the primary site for carcinogenicity in rats and mice exposed to cobalt metal, with concentration-related increases in alveolar/bronchiolar adenoma and carcinoma, including multiple adenomas and carcinomas, observed in males and females of both species.

Cobalt Sulfate Heptahydrate

Groups of F-344/N rats and B6C3F$_1$ mice (50 group/sex/species) were exposed to 0, 0.3, 1.0 or 3.0 mg/m^3 cobalt sulfate heptahydrate aerosol via whole-body inhalation for 6.2 hrs/day, 5 days/week, for 105 weeks (NTP, 1998a; Bucher *et al.*, 1999). The MMAD, recorded monthly, was within the range of 1 to 3 µm. Generation of the aerosol particles to which the rodents were exposed resulted in formation of primarily cobalt sulfate hexahydrate, although it is expected that environmental exposures to hydrated cobalt sulfate would be the heptahydrate form. The heptahydrate reportedly does not

dehydrate to the hexahydrate until a temperature of 41.5º C is reached. The daily exposures included the 6 hr exposure time at a uniform aerosol concentration plus the ramp-up time of 12 min (0.2 hr/day) to achieve 90% of the target concentration after the beginning of aerosol generation. The decay time to 10% of the target concentration at the end of the exposures was in the range of 11-13 min.

In rats, survival and body weights of cobalt sulfate heptahydrate-exposed animals remained similar to that of controls throughout the studies. The statistically significant and/or biologically noteworthy tumor incidences in male and female rats are shown in Table 4. The tumor incidence of alveolar/bronchiolar adenoma or carcinoma (combined) was statistically significantly increased in male rats exposed to 3.0 mg/m^3, and showed a positive trend with exposure. In addition, the incidence of alveolar/bronchiolar adenoma at 3.0 mg/m^3, and alveolar/bronchiolar carcinoma at 1.0 mg/m^3 exceeded historical control ranges in the males. Female rats at the two highest exposures showed statistically significantly increased incidences of alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma, and alveolar/bronchiolar adenoma or carcinoma (combined). A positive trend for these lung tumors was also present in the female rats.

One female rat in each of the 1.0 and 3.0 mg/m^3 exposure groups had a squamous cell carcinoma in the lungs at terminal necropsy. These tumors were included with the alveolar/bronchiolar adenoma or carcinoma (combined) for determination of the effective tumor incidence. Squamous cell carcinoma generally arises from a lung tissue different from that of alveolar/bronchiolar adenoma and carcinoma. However, NTP (1998a) noted that squamous lesion differentiation was a variable component of other alveolar/bronchiolar proliferative lesions, including the fibroproliferative lesions (some of which were diagnosed as alveolar/bronchiolar carcinomas) observed in this study. Therefore, NTP combined the two squamous cell carcinomas identified in cobalt-exposed female rats with the observed alveolar/bronchiolar adenomas and carcinomas in assessing treatment-related lung tumors.

A significant increase (p = 0.045) in the incidence in the adrenal medulla of benign, complex or malignant pheochromocytoma (combined), was observed in 1.0 mg/m^3 male rats. There was also some evidence for an increased incidence of bilateral pheochromocytoma in the cobalt sulfate heptahydrate-exposed male rats. However, lack of increased severity of hyperplasia and lack of increased neoplasms in the 3.0 mg/m^3 group led to an equivocal finding of carcinogenicity in male rats by NTP. In female rats, statistically significantly increased incidences of benign pheochromocytoma, and benign, complex or malignant pheochromocytoma (combined) were observed in the 3.0 mg/m^3 exposure group. Positive trends were observed for both benign pheochromocytoma and for the combined adrenal medulla neoplasms.

Table 4. Tumor incidences[a] in male and female rats in the two-year NTP (1998) inhalation studies of cobalt sulfate heptahydrate

Tumor Type	$CoSO_4 \cdot 7H_2O$ Concentration (mg/m^3)			
	0	**0.3**	**1.0**	**3.0**
Male Rats				
Lung				
Alveolar/bronchiolar adenoma	1/50	4/50	1/48	6/50
Alveolar/bronchiolar carcinoma	0/50	0/50	3/48	1/50
Alveolar/bronchiolar adenoma or carcinoma	1/50†	4/50	4/48	7/50*
Adrenal medulla				
Benign pheochromocytoma[b]	*14/50*	*19/50*	*23/49*	*20/50*
Benign, complex or malignant pheochromocytoma[b]	*15/50*	*19/50*	*25/49**	*20/50*
Benign bilateral pheochromocytoma	*1/50*	*4/50*	*6/49*	*5/50*
Female Rats				
Lung				
Alveolar/bronchiolar adenoma	0/50‡	1/49	10/50**	9/50**
Alveolar/bronchiolar carcinoma	0/50†	2/49	6/50*	6/50*
Alveolar/bronchiolar adenoma, carcinoma, or squamous cell carcinoma	0/50‡	3/49	16/50**	16/50**
Adrenal medulla				
Benign pheochromocytoma	2/48‡	1/49	3/50	8/48*
Benign, complex or malignant pheochromocytoma	2/48‡	1/49	4/50	10/48*

Tumor type and incidence data in italics: equivocal finding of carcinogenicity by NTP (1998)
* p<0.05, ** p<0.01 for statistical difference from control
† p<0.05, ‡ p<0.01 for positive trend for tumor type, logistic regression test conducted by NTP
[a] Denominator represents number of animals examined
[b] Includes benign bilateral pheochromocytoma

Nonneoplastic pulmonary lesions (alveolar epithelium metaplasia, proteinosis, granulomatous inflammation, and interstitial fibrosis) were observed in nearly all cobalt sulfate heptahydrate-exposed rats of both sexes, and the severity generally increased with dose (data not shown). Squamous metaplasia of the larynx and a spectrum of nonneoplastic lesions in the nose were also observed in all cobalt-exposed groups.

In mice, two-year exposure to cobalt sulfate heptahydrate aerosol did not affect the survival rate. Body weights of 3.0 mg/m^3 males were slightly reduced compared to controls starting at week 96. Body weights of cobalt sulfate heptahydrate-exposed female mice were similar to, or slightly greater, than body weights of controls.

Neoplastic findings in mice included statistically significantly increased incidences of alveolar/bronchiolar adenoma and alveolar/bronchiolar carcinoma in both 3.0 mg/m^3 males and females (Table 5). The incidences of alveolar/bronchiolar adenoma or carcinoma (combined) were statistically significantly increased in both 3.0 mg/m^3 males

and females, and also in 1.0 mg/m^3 females. Positive trends were observed for these pulmonary neoplasms, both individually and combined.

The incidence of hemangiosarcoma was increased above the historical control range in all cobalt sulfate heptahydrate-exposed male mice, and was significantly increased (p = 0.050) above control mice in the 1.0 mg/m^3 group. However, the presence of *Helicobacter hepaticus* infection in the males, and in some females, compromised the liver tumor findings in these studies, leading to equivocal findings of carcinogenicity by NTP.

Table 5. Tumor incidences[a] in male and female mice in the two-year NTP (1998) inhalation studies of cobalt sulfate heptahydrate

Tumor	$CoSO_4 \cdot 7H_2O$ Concentration (mg/m^3)			
	0	0.3	1.0	3.0
Male Mice				
Lung				
Alveolar/bronchiolar adenoma	9/50†	12/50	13/50	18/50*
Alveolar/bronchiolar carcinoma	4/50‡	5/50	7/50	11/50*
Alveolar/bronchiolar adenoma or carcinoma	11/50‡	14/50	19/50	28/50**
Liver				
Hemangiosarcoma	*2/50*	*4/50*	*8/50**	*7/50*
Female Mice				
Lung				
Alveolar/bronchiolar adenoma	3/50†	6/50	9/50	10/50*
Alveolar/bronchiolar carcinoma	1/50‡	1/50	4/50	9/50**
Alveolar/bronchiolar adenoma or carcinoma	4/50‡	7/50	13/50*	18/50**
Liver				
Hemangiosarcoma	*1/50*	*0/50*	*3/50*	*0/50*

Tumor type and incidence data in italics: equivocal finding of carcinogenicity by NTP (1998)
* p≤0.05, ** p≤0.01 for statistical difference from control
† p≤0.05, ‡ p≤0.01 for positive trend for tumor type, logistic regression test conducted by NTP
[a] Denominator represents number of animals examined

Non-neoplastic lesions of the bronchi, nasal tissue and larynx were observed either in the two highest exposure groups or in all exposed groups in both studies (data not shown). Similar to rats, squamous metaplasia of the larynx was observed in mice, and was considered one of the most sensitive tissue responses to cobalt sulfate heptahydrate exposure.

Overall, NTP (1998a) concluded that there is “clear evidence” for a treatment-related increase in carcinogenic activity in female rats exposed to cobalt sulfate heptahydrate due to the increased lung and adrenal tumors. The weaker tumor response in cobalt

sulfate heptahydrate-exposed male rats resulted in a lower finding of "some evidence" for carcinogenic activity in male rats. In mice, NTP concluded there was "clear evidence" for treatment-related lung tumors in both males and females.

Other Supporting Cancer Bioassays

Inhalation

In an early chronic inhalation study, male Syrian golden hamsters (51/group) were exposed whole-body to 0 or 10.1 mg/m^3 aerosolized cobalt(II) oxide 7 hr/day, 5 days/week for their life span (Wehner *et al.*, 1979). The particle size was 0.45 µm ± 1.9 (MMAD ± GSD). Exposures began at 2 months of age. No difference in survival was observed between the two groups throughout the study. However, approximately 50% of the animals in both groups had died by 15-16 months of age, and the maximum survival was about 22 months. The normal average life span of Syrian golden hamsters is 2 to 2.5 years. Noncancer effects due to cobalt(II) oxide exposure included interstitial pneumonitis, diffuse granulomatous pneumonia, and emphysema. No differences were observed in the total incidence of neoplasms between cobalt(II) oxide-exposed animals (3/51) and control animals (3/51), which IARC (1991) suggested may be partly related to the overall poor survival rate. Only one of these tumors was specifically identified as a lung tumor (adenoma in the control group) by the authors. Compared to rats, Syrian golden hamsters appear to be more resistant to respiratory tract tumors following exposure to carcinogenic metals (*e.g.*, nickel) (Wehner *et al.*, 1979; NTP, 1996; 2014a).

Intratracheal instillation

Two additional sets of chronic exposure studies exposed the respiratory tract of animals via intratracheal instillation. Groups of male and female hamsters (25/sex/group) received weekly doses of 0 or 4 mg cobalt(II, III) oxide powder suspended in gelatin/saline vehicle via intratracheal administration for 30 weeks (Farrell and Davis, 1974). The animals were then observed for another 68 weeks. The size range of the particles were described as 0.5 to 1.0 µm. Two of 50 hamsters receiving cobalt oxide developed pulmonary alveolar tumors, and one of 50 hamsters receiving gelatin-saline control developed a tracheal tumor.

Steinhoff and Mohr (1991) administered cobalt(II) oxide to specific pathogen free (SPF)-bred male and female Sprague Dawley rats by intratracheal instillation every 2-4 weeks over a period of two years. Exposure groups in these studies consisted of 50 rats/sex/dose given either nothing (untreated control), saline (vehicle control), 2 mg/kg-body weight (BW) cobalt(II) oxide (total dose 78 mg/kg), or 10 mg/kg-BW cobalt(II) oxide (total dose 390 mg/kg). Approximately 80% of the cobalt particles instilled were said to be in the range of 5-40 µm. In males, no pulmonary tumors were found in the untreated controls or the saline controls, one benign squamous epithelial lung tumor was found in

the low dose group, and 2 bronchioalveolar adenomas, 1 bronchioalveolar adenocarcinoma, and 2 adenocarcinomas (cell type not specified) were observed in the high dose group. The increase in combined pulmonary tumors in the high dose group was statistically significant ($p = 0.02$) by pairwise comparison with controls. The authors concluded that under the conditions of this study, cobalt(II) oxide is weakly carcinogenic by the intratracheal instillation route. In females, no pulmonary tumors were found in the untreated controls or the saline controls, one bronchoalveolar adenoma was found in the low dose group and one bronchoalveolar carcinoma was found in the high dose group.

Subcutaneous, intraperitoneal and intramuscular administration

Subcutaneous and intraperitoneal injections of rats with cobalt(II) oxide resulted in local tumors (Steinhoff and Mohr, 1991). In SPF male Sprague Dawley rats (10/group), subcutaneous injection of saline (control), 2 milligrams per kilogram of bodyweight (mg/kg-BW) cobalt(II) oxide five times per week, or 10 mg/kg-BW cobalt(II) oxide once per week over a two-year period resulted in no tumors in controls and 9/20 malignant tumors in treated rats ($p<0.001$). In the intraperitoneal injection study, male and female SPF rats (10/sex/dose) were injected with saline (control) or 200 mg cobalt(II) oxide 3 times at intervals of 2 months. Tumors were reported for males and females combined at the end of two years: 1/20 control rats developed malignant tumors (1 malignant histiocytoma) compared to 14/20 cobalt-treated rats (10 histiocytomas, 3 sarcomas, 1 mesothelioma) ($p<0.001$).

Using a rodent implantation model, ten male Sprague-Dawley rats were implanted bilaterally with cobalt nanoparticles (NPs) (surface area to volume ratio: 5×10^4 mm^{-1}) intramuscularly, and bulk cobalt particles (surface area to volume ratio: 4.73 mm^{-1}) subcutaneously on the contralateral side (Hansen *et al.*, 2006). The specific cobalt compound was not identified by the authors, but was likely cobalt metal. On the cobalt NP side, malignant mesenchymal tumors were found in one of four rats sacrificed after six months of exposure, and in five out of six of the remaining rats sacrificed after eight months of exposure. On the cobalt bulk material side, inflammation was observed after six months, and one preneoplastic lesion out of six rats after eight months. The authors concluded that the physical properties of cobalt (NP vs. bulk form) could have a significant influence on the acceleration of the neoplastic process.

Earlier non-inhalation studies summarized by IARC (1991) also suggest that cobalt metal and cobalt compounds are carcinogenic by the subcutaneous and intramuscular routes of administration, mainly producing local sarcomas.

Toxicokinetics

Human Toxicokinetics and Comparison to other Mammalian Species

For cobalt metal and salts, good correlations were found between the cobalt concentration in the breathing zone of workers and the concentration of cobalt in post-shift urine and blood (Swennen *et al.*, 1993; Lison *et al.*, 1994; Hutter *et al.*, 2016). For every 1 mg/m^3 cobalt in air, there was an excretion of approximately 200 micrograms per liter (µg/L) in urine of cobalt workers (Hutter *et al.*, 2016). In cobalt oxide workers, concentrations of cobalt in blood and urine were higher than in non-exposed subjects, but no correlation with air concentration was found with post-shift urine and blood concentrations (Lison *et al.*, 1994) The authors suggested the lack of correlation was due to lower pulmonary absorption of cobalt oxides compared to more soluble cobalt compounds.

Inhalation studies in workers and volunteers exposed to cobalt metal or cobalt oxides have shown that cobalt elimination from the lungs is multiphasic with reported half-lives of 2 to 44 hrs, 10 to 78 days, and a long-term phase lasting many months to years (Newton and Rundo, 1971; Foster *et al.*, 1989; Apostoli *et al.*, 1994; Beleznay and Osvay, 1994). These elimination phases likely involve an initial rapid elimination from the tracheobronchial region via mucociliary clearance, an intermediate phase of macrophage-mediated clearance, and long-term retention and clearance probably due to cobalt bound to cellular components in the lung. Approximately 1 to 10% of the inhaled cobalt deposited in lung is subject to long-term retention and is predominantly cleared by translocation to blood (Bailey *et al.*, 1989). The pattern of elimination appears to be independent of the level of exposure (Apostoli *et al.*, 1994).

In a study of human volunteers (n=4) inhaling cobalt(II,III) oxide (as $^{57}Co_3O_4$), about 20% of the initial lung burden was eliminated after 10 days (Foster *et al.*, 1989). However, about 40% of the initial lung burden was still retained in the body 100 days following exposure. The clearance half-time of the slow phase, a result of lung to blood translocation of cobalt, was in the range of 150-250 days. Two volunteers each had inhaled cobalt particles with different mass median aerodynamic diameters (MMAD) of 0.8 and 1.7 µm. Fractional deposition averaged 52% for the 0.8 µm particles and 78% for the 1.7 µm particles. However, differences in the elimination rates and retention rates could not be detected.

Oral intake of soluble cobalt, as cobalt chloride, by human volunteers resulted in intermediate half-times (32 days) and long-term half-times (80-720 days) that were consistent with intermediate and long half-times resulting from inhalation exposure to cobalt metal or cobalt oxides (Holstein *et al.*, 2015). An initial rapid half-time phase

(mean = 0.71 days) following oral intake reflected loss through fecal excretion during the first week after ingestion.

Translocation rates of inhaled radiolabeled cobalt(II,III) oxide ($^{57}Co_3O_4$) from lung to blood show considerable interspecies variation (Bailey *et al.*, 1989). Excluding the initial rapid phase of mucociliary clearance from the tracheobronchial tree, rats, mice, dogs, hamsters, and guinea pigs exhibited 90% or greater lung clearance of cobalt six months after exposure. However, humans and baboons showed much slower lung clearance with only 50% and 70% cleared by six months, respectively. The translocation rate of dissociated ^{57}Co from the lung to the blood in humans and baboons was 0.2 to 0.6% day^{-1}. In other mammalian species, this translocation rate was greater (up to 2.4% day^{-1}) but varied considerably in some species over time. The maximum difference in the translocation rate was up to seven-fold between rats and humans for 0.8 µm particles (Bailey *et al.*, 1989; Kreyling *et al.*, 1991b). Kreyling *et al*. (1991b) considered that for cobalt oxide particles retained in the lung, the rate-determining process for translocation to blood is the intracellular particle dissolution in the macrophage since transfer of the dissociated material to blood is fast and almost quantitative. However, interspecies phago-lysosomal pH differences in alveolar macrophages were not found and do not appear to be the cause of translocation rate differences among mammalian species (Kreyling *et al.*, 1991a).

Lung retention is generally greater for larger cobalt particles (as Co_3O_4) than smaller particles (Kreyling *et al.*, 1986; Bailey *et al.*, 1989; Leggett, 2008). However, cobalt metal and radiolabeled cobalt oxide (^{57}Co) had whole body clearance times very similar to that of clearance times from the lung indicating cobalt does not translocate or accumulate appreciably in other tissues (Rhoads and Sanders, 1985; Bailey *et al.*, 1989; Patrick *et al.*, 1994; NTP, 2014a).

For soluble cobalt compounds (as $CoCl_2$ or $Co(NO_3)_2$), Patrick *et al*. (1994) found that the fraction of instilled or inhaled cobalt remaining in the lung of mammalian species (rat, dog, baboon, guinea pig, hamster) averaged 0.13-0.58% 100 days after exposure. This finding suggests lung clearance of cobalt may be faster with soluble compounds compared to poorly soluble or insoluble compounds such as Co_3O_4.

NTP Tissue Burden Studies of Cobalt Metal in Rats and Mice

Tissue burden and concentration were assessed by NTP (2014a) in rats and mice exposed by inhalation to cobalt metal (1.25, 2.5 or 5 mg/m^3) for up to two years. Tissue burden (µg Co/tissue), rather than tissue concentration (µg Co/g tissue), was generally preferred to express levels of cobalt in the organs due to significant changes in organ weights caused by cobalt exposure. In the 2-year exposure studies, lung cobalt concentrations and burdens in rats and mice increased with increasing cobalt

concentrations, but appeared to reach steady state by day 184. Little change in lung burden was observed through day 548, and then the burden steadily decreased following cessation of cobalt exposure. The modeled pulmonary clearance of cobalt showed a two-phase elimination. The half-life of the rapid phase ranged from 1.53 to 2.94 days in rats and 1.1 to 5.2 days in mice. The slow clearance phase in rats produced a half-life estimate ranging from 83 to 167 to days. In mice, the slow clearance half-life was 409, 172, and 118 days with increasing exposure concentration. The majority of the deposited lung cobalt was cleared during the fast elimination phase (>95% in rats and >82% in mice).

Cobalt concentrations and burdens in exposed rats and mice increased in all other tissues examined by NTP (2014a) indicating absorption and systemic distribution occurs by the inhalation route. In 13-week studies, blood cobalt levels increased proportionally to exposure concentration in rats and mice. Blood cobalt in exposed groups of animals reached steady state at the earliest time point (day 5) measured in rats, and at about day 12 in mice. In both rats and mice, blood cobalt then rapidly decreased below the level of detection following cessation of cobalt exposure. Cobalt burdens and concentrations in liver also increased with increasing cobalt concentration up to day 26 in both rodent species. However, cobalt burdens by day 40 were generally lower than at day 26. Liver cobalt burdens approached, or even exceeded, the lung cobalt burdens at days 26 and 40.

Overall, normalized lung tissue burdens (measured as µg Co/total lung per mg Co/m^3) did not increase with increasing exposure even though cobalt lung concentrations increased with increasing exposure (NTP, 2014a). Cobalt concentrations (µg Co/g tissue) in rats showed the following order: lung > liver >kidney > femur > heart > serum > blood. Tissue cobalt burdens (µg Co/tissue) showed similar order with the exception that liver accumulated more cobalt than lung, and the heart accumulated more cobalt than the femur. With minor exceptions, the order for tissue concentration and burden were similar in mice.

Cellular Toxicokinetics of Cobalt Nanoparticles

Cobalt oxide NPs are finding increasing use in commercial and industrial applications, leading to interest in conducting genotoxicity studies to examine their effects in various human and animal cells (Alarifi *et al.*, 2013). NPs have diameters of 0.1 µm or less. Compared to fine-scale particles, or micrometer-sized particles, NPs have a larger specific surface area, higher physical and chemical activity, and thus higher biological activity (Horie *et al.*, 2012). For poorly soluble cobalt oxide NPs, *in vitro* studies in human keratinocyte HaCaT cells suggested that the most important cytotoxic factor of these particles is cobalt ion (Co^{2+}) release. Horev-Azaria *et al*. (2011) and Cappellini et al. (2018) came to a similar conclusion following *in vitro* studies with cobalt metal NPs.

Cappellini et al. (2018) found the high genotoxic activity of cobalt metal NPs was related to intracellular corrosion (i.e., oxidation) generating both Co^{2+} ions and ROS. However, when accounting for differences in surface area, the toxicity of Co (average primary size 25 nm) and CoO (primary size 43 nm) NPs was similar based on surface area dose rather than of mass dose.

The cellular uptake of radiolabeled cobalt(II) oxide NPs (^{60}Co) and cobalt chloride ($^{57}Co^{2+}$) were investigated *in vitro* in Balb/3T3 mouse fibroblasts (Ponti *et al.*, 2009) and human peripheral blood leukocytes (Colognato *et al.*, 2008). In both studies, cobalt NPs showed a 50- to 140-fold greater uptake compared to $^{57}Co^{2+}$. The authors postulated a "Trojan horse"-type mechanism was involved, in which cobalt NPs interacted with proteins on the surface of the cells and were more readily taken up (Ponti *et al.*, 2009). This led to the observed increase in cytotoxicity and genotoxicity. Further research suggests internalized cobalt metal nano- and micro-particles diffuse to subcellular organelles and release cobalt ion in millimolar concentrations in nuclei and mitochondria (Sabbioni *et al.*, 2014a; Sabbioni *et al.*, 2014b).

Epidemiological Studies

Limited information is available to assess the carcinogenic risk to workers exposed to cobalt and cobalt compounds.

In studies of workers primarily exposed to cobalt compounds, Mur *et al*. (1987) performed a retrospective mortality investigation of 1,143 workers at a French electrochemical plant producing cobalt and sodium. Of these, 110 workers had at least one year of service between 1950 and 1980 in the facility producing cobalt metal and some cobalt oxides and salts. Using male mortality in France as a reference, a Standardized Mortality Ratio (SMR) of 1.29 was found for the cobalt worker cohort. The relative high death rate was attributed, in part, to higher lung cancer cases (SMR=4.66, $p<0.05$, 4 cases). However, this study had several limitations or confounders, including too small a number of lung cancer cases to reliably establish a link to occupational risk, no smoking assessment, possible (but not quantified) co-exposure to the carcinogenic metals arsenic and nickel, and no findings of nonneoplastic pulmonary diseases usually associated with cobalt exposure.

Follow-up by Moulin *et al*. (1993) at the French electrochemical plant extended mortality surveillance from 1981-1988. The study did not find excess mortality due to lung cancer in the cobalt worker cohort (SMR= 0.85 including all cobalt workers; SMR=1.16 including only French-born workers). This disparate finding was due to the lack of additional lung cancer deaths during the 1981-1988 follow-up and improved collection of causes of death by examining death certificates. Use of death certificates rather than medical records lowered the proportion of unknown causes from 20% observed by Mur *et al*.

(1987) to 11% in the later study, though the number of lung cancer cases did not increase. Neither Mur *et al*. (1987) nor Moulin *et al*. (1993) provided an estimate of the airborne cobalt concentrations the workers were exposed to.

The incidence of lung cancer among Danish women plate painters was investigated in a retrospective study at two porcelain factories in which workers sprayed cobalt blue dye onto plates (Tuchsen *et al.*, 1996). Only trace exposure to other carcinogenic metals was said to have occurred. Participation for the study entailed employment between 1943 and 1987 at Factory 1 (n=382), and 1962 and 1987 at Factory 2 (n=492). The last year of follow-up occurred for both factories in 1992. A referent group consisted of 520 women working in another part of Factory 1 without exposure to cobalt. Cancer incidence rates for all Danish women were used to calculate the expected number of cancer cases.

Exposure at the porcelain factories was to insoluble cobalt-aluminate spinel with a cobalt content of 25%. The factories switched over to soluble cobalt silicate dye in 1972 (Factory 1) and 1989 (Factory 2). The authors reported the latency period was too short and number exposed too low to assess cancer risk for the soluble dye exclusively (Tuchsen *et al.*, 1996). Christensen and Poulsen (1994) observed that exposure of porcelain plate painters to the insoluble dye resulted in lower levels and slower excretion of cobalt in urine compared to painters exposed to the soluble dye. Limited personal sampling in 1982, before improvements in industrial hygiene, showed a mean airborne cobalt concentration of 1,356 nanomoles per cubic meter (0.08 mg/m^3) for painters exposed to the soluble silicate dye. Since 1982, personal exposures were 372-593 $nmol/m^3$ (0.03-0.04 mg/m^3).

Tuchsen *et al*. (1996) found a statistically significant increase in lung cancer incidence for the exposed group (8 observed, 3.41 expected, standardized incidence rate (SIR = 2.35, 95% confidence interval (95% CI) = 1.01 - 4.61) compared to all Danish women. Lung cancer in the reference group was also elevated, although not significantly (7 observed, 3.51 expected, SIR = 1.99, 95% CI 0.8 - 4.1), compared to all Danish women. Comparison of the exposed group with the reference group resulted in a relative risk of 1.2. No association was found between length of employment and lung cancer incidence. The authors noted that smoking information was incomplete, but suggested the increased risk was likely not due to differences in smoking. However, the women plate sprayers consisted of unskilled manufacturing workers who are known to have a higher lung cancer incidence rate compared to the general Danish population. The authors concluded follow-up is needed to determine if there is a true effect of lung cancer in the cobalt-exposed painters.

Stopford *et al*. (2003) found that *in vitro* bioaccessibility of cobalt aluminate spinel was very low in all physiological fluids tested, including artificial interstitial, alveolar and

lysosomal fluids. Thus, the equivocal increased cancer risk noted by Tuchsen *et al*. may be related to the lack of significant *in vivo* release of cobalt ion from cobalt aluminate spinel. Presently, no cancer assessment for exposure exclusively to the soluble cobalt silicate dye has been performed.

In a recent retrospective study by Sauni *et al*. (2017), 995 male workers at a Finnish cobalt plant were assessed for cancer incidence during the period of 1968 to 2004. Workers were employed at the plant at least one year and the mean follow-up was 26.2 years. An average duration of exposure was not provided. Cancer incidence was determined as SIRs that compared the observed worker cancer incidence to the expected incidence of the population in the same region using the Finnish Cancer Registry, a population-based nationwide database. The cohort was also subdivided into low, moderate, high and variable exposure groups based on exposure by department. Respirators were available for use, but not mandatory, during the study period. Airborne levels of cobalt and other compounds were consistently measured several times per year over the study period (Linna *et al.*, 2003; Sauni *et al.*, 2017).

Highest cobalt exposures were in the reduction and powder production departments and sulfating-roasting department where mean cobalt levels during 1968-2003 were between 0.06 and 0.10 mg/m^3 (Sauni *et al.*, 2017). In the roasting department, dust in the ambient air contained 15-20% iron, 1% zinc, 0.4% cobalt and 0.2% nickel, with cobalt and nickel in the form of water-soluble sulfates. The concentration of nickel was usually ≤0.04 mg/m^3. In the reduction and powder production facility, cobalt was mainly in the form of cobalt powder and fine powder. Moderate exposures to cobalt (0.02-0.03 mg/m^3) as sulfates, carbonates, oxides and hydroxides occurred in the chemical department, whereas low exposure (≤0.02 mg/m^3) to cobalt sulfides and sulfates occurred in the leaching and solution purification building. Nickel compounds (as sulfates, carbonates, oxides and hydroxides) were also present in the chemical department, but at lower levels compared to the cobalt compounds (Linna *et al.*, 2003).

Neither total cancer risk incidence (SIR 1.00; 95% CI 0.81-1.22) nor lung cancer incidence (SIR 0.50; CI 0.18-1.08) were increased in this cohort of Finnish cobalt workers (Sauni *et al.*, 2017). For workers with over five years of exposure, the total cancer risk (SIR 1.08; 95% CI 0.85-1.34) and lung cancer incidence (SIR 0.52; 95% CI 0.17-1.22) were likewise not significantly elevated. In addition, none of the exposure subgroups with over one year of employment had lung cancer SIRs significantly different from 1.0. Three cases of tongue cancer were observed in the cobalt worker group, which was significantly greater than expected (SIR 7.39; 95% CI 1.52-21.6). However, all were smokers. The authors suggested a synergistic action of cobalt exposure with smoking, although the excess may have occurred through chance alone. Bladder cancer among the workers was nearly twice the expected number (SIR 1.88; 95% CI 0.86-3.56), but not statistically significant. Six out of the nine total cases were in the low

exposure group, only one of which was a non-smoker. The authors noted that the SIR of 0.5 for lung cancer was likely not a result of lower smoking prevalence because the cobalt worker smoking prevalence (31.8%) was greater than the control population prevalence (18 to 25%, depending on educational class). The authors concluded that at the cobalt levels measured, lung cancer risk and overall cancer risk is not increased in the cobalt workers.

Occupational exposure to combined cobalt and tungsten carbide powders in the hard metal refinery industry has resulted in excess lung cancer cases, and is also known to cause a severe noncarcinogenic lung disease known as hard metal disease (Hogstedt and Alexandersson, 1987; Lasfargues *et al.*, 1994; Lison, 1996; Moulin *et al.*, 1998; Wild *et al.*, 2000). Mixed tungsten carbide-cobalt hard metal powders are categorized by IARC (2006) in Group 2A (probably carcinogenic to humans). The cobalt metal powder content used in the presintering process usually ranges from 5-15% while tungsten carbide usually exceeds 80% (Keane *et al.*, 2002). Co-exposure to other pulmonary system carcinogens (nickel, hexavalent chromium, asbestos) in the hard metal industry has been reported. Nickel is sometimes added as a binding agent for the sintering of hard metal, but is normally found in only trace amounts in tungsten (Yamada *et al.*, 1987; Scansetti *et al.*, 1998).

Studies suggest an interaction between cobalt and tungsten carbide that produces activated oxygen species that is markedly greater than that produced by cobalt metal alone. Tungsten carbide alone appears to have no carcinogenic action or ability to generate ROS (Lison, 1996). The genotoxicity of tungsten carbide-cobalt powder is also considerably greater than cobalt metal alone (Anard *et al.*, 1997; Lloyd *et al.*, 1997; Van Goethem *et al.*, 1997; De Boeck *et al.*, 2003). Zanetti and Fubini (1997) suggest that the two metals together act like a new compound with different physico-chemical properties from those of cobalt and tungsten carbide alone. Clinical and epidemiological evidence support this interaction of cobalt and tungsten leading to pulmonary injury, while cobalt metal on its own is not as potent (Lison, 1996). Consequently, OEHHA recommends that a cancer potency factor for cobalt and cobalt compounds not be applied in estimating risks from cobalt-tungsten carbide exposure related to the hard metal refinery industry, as it may underestimate the cancer risk resulting from this metal-on-metal interaction.

Genotoxicity

Soluble and Insoluble Cobalt Compounds, Not Including Cobalt Metal

Early studies examined the genotoxicity of soluble cobalt(II) compounds, since it was thought that bioavailable cobalt ions were a leading cause of genetic damage (IARC, 1991; Lison, 1996). More recent studies compared the genotoxicity of soluble and

insoluble cobalt compounds (particularly NP cobalt). Thus, soluble and insoluble cobalt studies are presented together in this section. In *in vitro* mammalian cell systems, soluble and insoluble cobalt compounds were found to produce altered DNA bases, DNA strand breaks, DNA crosslinks, micronuclei, chromosomal aberrations, aneuploidy, gene mutations, and inhibition of DNA repair. However, *in vivo* studies show mixed results for induction of chromosomal aberrations in bone marrow cells.

DNA strand-break and cross-linking tests

The comet assay is a commonly used method to identify DNA lesions (*e.g.*, breaks or alkali-labile sites) following exposure of an isolated cell culture with a genotoxin. When DNA lesions are present, this electrophoretic technique at high pH results in streaming of cellular DNA towards the anode giving the appearance of a comet. The comet effect is only seen when DNA contains breaks, or when DNA lesions are converted to breaks under alkaline conditions. This assay measures premutagenic lesions, which, in intact cells, can be removed by DNA repair processes if the repair occurs prior to DNA replication. Thus, positive assay data for a given compound do not necessarily indicate that the compound will induce mutations.

De Boeck *et al*. (1998) showed that cobalt chloride (0.3 to 6.0 micrograms per milliliter [µg/ml] Co-equivalents) induced DNA damage in isolated human lymphocytes (HLs) from three donors by the alkaline comet assay. DNA damage occurred in both a dose- and time-dependent manner.

Cobalt chloride induced DNA double strand breaks in a cancer-derived H460 human lung epithelial cell line (Patel *et al.*, 2012). Increased double strand break formation was determined by examining histone H2AX phosphorylation in Western blot analysis. The production of double strand breaks correlated with the intracellular generation of ROS; a 2.5-fold induction of ROS at 300 µM cobalt chloride resulted in a measurable increase in double strand break formation. Pretreatment of the cells with N-acetyl cysteine to inhibit ROS generation reduced the production of double strand breaks.

Cobalt sulfate produced DNA double strand breaks in *E. coli* as measured by the pulse field gel electrophoresis method (Kumar *et al.*, 2017). However, generation of ROS could not be detected using two different ROS-sensing dyes (2',7'-dichlorodihydrofluorescein diacetate and dihydroethidium) in *E. coli* cultured with cobalt sulfate, suggesting to the authors that oxidative stress did not cause the DNA damage.

Cobalt chloride was found to inhibit the removal of pyrimidine dimers in HeLa cells exposed to UV light, even though cobalt chloride by itself does not induce these DNA lesions (Hartwig *et al.*, 1991). This suggested to the authors that cobalt interferes with DNA repair processes. At a cobalt chloride concentration that did not cause strand

breaks (100 µM), nucleoid sedimentation showed a greater accumulation of strand breaks when UV irradiation was combined with cobalt chloride treatment. Chromatin structures are repaired 3-5 hrs after UV alone, but the process was delayed with combined cobalt chloride-UV treatment indicating an interference with the completion of repair events.

Non-cytotoxic doses of cobalt chloride (50 to 200 µM as Co(II), or 3.0 to 12 µg Co/ml) were used to investigate DNA repair of lesions induced by low UVC rays (200 to 280 nm in wavelength) in cultured human fibroblasts (Kasten *et al.*, 1997). Employing the alkaline unwinding technique, cobalt was observed to inhibit both the incision and polymerization step of nucleotide excision repair, but did not interfere with the ligation step.

The comet assay was also used to examine the genotoxicity of cobalt(II, III) oxide NPs (5 to 15 µg/ml) in human hepatocarcinoma (HepG2) cells (Alarifi *et al.*, 2013). A dose- and time-related increase in DNA damage, measured as increased percentage of tail DNA and increased olive tail moment, was observed in the HepG2 cells. The authors confirmed that a small percentage of Co^{2+} ions were released from cobalt(II, III) oxide in the suspensions, which is considered to be the factor responsible for genotoxicity. Similar levels of soluble cobalt chloride (10 and 15 µg/ml as Co^{2+}) in cell suspension also produced a statistically significant increase in DNA damage, although the genotoxic response was less than that of cobalt(II, III) oxide. The authors also observed that cobalt(II, III) oxide NPs caused a reduction of glutathione in HepG2 cells with a concomitant increase in lipid hydroperoxides, ROS generation, and increased superoxide dismutase and catalase activity.

In a similar *in vitro* study using HLs, cobalt(II, III) oxide NPs caused a significant increase in percentage tail DNA damage in the comet assay (Rajiv *et al.*, 2016). The level of exposure used (100 µg/ml for 24 hrs) also led to a significant reduction in cell viability (<30% viability), and increases in cellular LDH leakage and ROS levels.

Cobalt NPs (likely as cobalt(II) oxide) and cobalt chloride were compared in their ability to cause DNA damage in human peripheral blood leukocytes by means of the comet assay (Colognato *et al.*, 2008). Incubation time was 2 hrs and subtoxic concentrations used were 10, 50 and 100 µM (0.6, 3 and 6 µg/ml as Co^{2+}). A dose-dependent increase in percent tail DNA was observed for cobalt NPs, which was significantly greater ($p <$ 0.05) than controls at the two highest doses. Cobalt chloride did not induce significant changes over control levels, which the authors thought could be a result of the short incubation time used and the longer uptake time needed for cobalt ions.

Ponti *et al*. (2009) compared cobalt(II) oxide NPs and cobalt chloride for induction of DNA damage in Balb/3T3 mouse fibroblast cells by the comet assay at doses of 1, 5,

and 10 µM 0.075, 0.37 and 0.75 µg/ml for cobalt(II) oxide, and 0.13, 0.65 and 1.3 µg/ml for cobalt chloride. Incubation time was 2 hrs. A comparable genotoxic response was observed for the two cobalt forms, including formation of single- and double-strand breaks. However, a dose-dependent increase in DNA damage was only seen for cobalt chloride, probably a result of increased cytotoxicity at the higher doses of cobalt NPs, which masked the genotoxic potential. Differences in results compared to work by Colognato *et al*. (2008) were suggested by the authors to be related to the sparse data on NP cobalt and different *in vitro* models used.

The genotoxicity of cobalt(II, III) oxide NPs was investigated by use of the comet assay in four different human cell lines: A549 lung carcinoma cells, HepG2 hepatocarcinoma cells, Caco-2 colorectal adenocarcinoma cells, and SH-SY5Y neuroblastoma cells (Abudayyak *et al.*, 2017). DNA damage was induced only in the A549 lung cell line, and was induced in a concentration-dependent manner over a range of 0.1 to 100 µg/ml. Additionally, cell viability was tested in all four cell types and only A549 cell viability was decreased by cobalt(II, III) oxide NPs (IC_{50} = 409.2 µg/ml). Oxidative damage was also demonstrated in A549, HepG2, and SH-SY5Y cell lines (but not in Caco-2 cells) resulting in increased malondialdehyde and 8-hydroxydeoxyguanosine levels and decreased GSH levels. The authors concluded that A549 lung carcinoma cells were the most sensitive cell line to DNA damage from cobalt(II, III) oxide NPs.

In isolated salmon sperm DNA exposed to a Fenton-type oxygen radical-generating system, including cobalt sulfate (25 micromoles per liter (µM) to 1 millimole per liter (mM), or 1.5 to 59 µg Co/ml) with hydrogen peroxide, bulky DNA lesions were produced suggestive of free radical-mediated intrastrand cross-linking (Lloyd *et al.*, 1997). However, unlike other transition metals tested by the authors, cobalt sulfate did not cause DNA strand breaks up to 1 mM.

<u>Oxidative DNA damage tests</u>

Neither superoxide radical (O_2^-) nor hydrogen peroxide (H_2O_2) reacts chemically with DNA. However, a number of transition metal ions catalyze hydroxyl radical (•OH) formation in the presence of O_2^- and H_2O_2, which can modify purine and pyrimidine bases and cause strand breaks. In the presence of H_2O_2, cobalt sulfate (25 µM) was observed to cause DNA damage to isolated chromatin from human K562 cells (Nackerdien *et al.*, 1991). The altered base products (*e.g.*, cytosine glycol, formamidopyrimidines, 8-hydroxypurines) were typical of hydroxyl radical attack suggesting that the hydroxyl radical was generated in a Fenton-type reaction with cobalt and H_2O_2. Addition of scavengers of hydroxyl radical, mannitol and dimethyl sulfoxide (DMSO), led to a partial inhibition of DNA damage.

Human A549 alveolar adenocarcinoma cells and bronchial BEAS-2B normal cells were exposed to concentrations of 1 to 40 µg/ml cobalt(II, III) oxide NPs *in vitro* to investigate differences in cyto-genotoxic effects (Cavallo *et al.*, 2015). No cytotoxicity was found in A549 cells, whereas BEAS-2B cells showed reduced viability at 40 µg/ml. In A549 cells, direct and oxidative DNA damage occurred at 20 µg/ml and greater as measured by the Formamido-pyrimidine glycosylate (Fpg)-modified comet assay. The Fpg enzyme recognizes and cuts the oxidized DNA bases for evaluation of oxidative DNA damage. BEAS-2B cells showed peak oxidative DNA damage at a lower concentration of 5 µg/ml, but direct DNA damage only at 40 µg/ml. The authors suggested that the transformed A549 cells are more resistant to cytotoxicity, but have a lower capacity for DNA repair compared to BEAS-2B cells.

Induction of DNA strand breaks and oxidative DNA lesions by cobalt octoate (50, 200 and 800 µg/ml of original substance mass) and cobalt sulfate heptahydrate (800 µg/ml of original substance mass) were determined in A549 cells using the human 8-hydroxyguanine DNA-Glycosylate 1 (hOGG1) modified comet assay (Kirkland *et al.*, 2015). Oxidative DNA-base modifications can only be detected in the comet assay if lesion-specific repair enzymes (*e.g.*, hOGG1) are incorporated. For the assay, the cobalt compounds were suspended in artificial alveolar fluid to simulate dissolution in physiological environments. Cobalt octoate showed high solubility in this fluid. Both cobalt compounds induced a significant increase in mean tail intensity in the absence of hOGG1. In the presence of hOGG1, mean tail intensity was further enhanced indicating induction of oxidative DNA-base lesions. However, DNA strand breaks and oxidative DNA-base lesions seemed to coincide with cytotoxic activity (*i.e.*, reduced cell number). The results suggested to the authors that cobalt solubility and the cobalt cations are important determinants of the observed DNA damaging effect.

Soluble cobalt acetate administered intraperitoneally in rats at a dose of 50 or 100 µmol/kg produced oxidative DNA damage in renal, hepatic and pulmonary chromatin (Kasprzak *et al.*, 1994). The altered DNA bases (*e.g.*, 5-hydroxycytosine) were the same as those found due to hydroxyl radical attack on DNA, supporting ROS generation by cobalt *in vivo*. Some of the altered bases, including 5-(hydroxymethyl)uracil and 7,8-dihydro-8-oxoguanine, have been shown to be promutagenic.

<u>Tests for reduction in DNA replication and repair</u>

Kumar et al. (2017) observed that cobalt sulfate produced double strand breaks in *E. coli*, but could not demonstrate the generation of ROS and subsequent oxidative stress by the methods used. Rather, cobalt sulfate was found to reduce the rate of DNA replication in *E. coli* and inhibited the SOS repair pathway, which is the bacteria's response to DNA damage. The authors proposed that direct binding of cobalt to the

DNA caused conformational changes in the DNA, as measured in a circular dichroism experiment, and led to replication fork stalling and the DNA damage observed.

Bacterial and mammalian cell gene mutation tests

A number of early prokaryotic assays were performed with soluble cobalt(II) salts and are reported in IARC (1991). Most of these studies were negative for mutagenicity, which may be related to poor bioavailability of cobalt(II) in these *in vitro* systems (Beyersmann and Hartwig, 1992; Kirkland *et al.*, 2015).

NTP (1998a) evaluated the genotoxicity of cobalt sulfate heptahydrate in bacteria, using *Salmonella typhimurium* strains (TA98, TA100, TA1535) either in buffer or S9 mix obtained from Arochlor 1254-induced liver of Sprague-Dawley rats or Syrian hamsters. Cobalt sulfate heptahydrate (3 to 10,000 µg/mL) was mutagenic in *S. typhimurium* TA100 with and without S9, but was not mutagenic in TA98 or TA1535 strains with or without S9.

To resolve inconsistencies in previous bacterial mutation assays, cobalt chloride was tested in strain TA97a and cobalt sulfate was tested in strain TA100 independently in three different laboratories using Organisation for Economic Co-operation and Development (OECD)-recommended guidelines (Kirkland *et al.*, 2015). Neither soluble cobalt compound produced a mutagenic response up to 5000 µg per plate, with and without S9, using either plate incorporation or pre-incubation methodology. Negative assay results in five strains of *S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537, with and without S9) were also found with two poorly soluble cobalt salts/compounds, cobalt acetyl acetonate and cobalt resinate.

Cobalt hydroxide and cobalt oxalate were tested for induction of *Hprt* mutations in mouse lymphoma L5178Y cells (Kirkland *et al.*, 2015). The cobalt compounds were incubated for 3 hr either in the absence or presence of S9. An extended 24 hr treatment in the absence of S9 was also performed with cobalt sulfate, cobalt sulfide and cobalt(II) oxide in order to detect any mutagenic effects that might only manifest after being in contact with the cells for a full cell cycle. *Hprt* enzyme activity is important for DNA synthesis. Incubation of the cells with a chemical that leads to mutations, which destroy the functionality of the *Hprt* gene and/or protein are detected by positive selection using a toxic analogue. *Hprt*-negative mutants are seen as viable colonies. Although some equivocal results were obtained, overall it was concluded by the authors that the cobalt compounds did not induce *Hprt* mutations when tested in the absence or presence of S9.

Chromosomal damage

Micronucleus tests

The frequency of micronucleated cells was examined following exposure of human blood

leukocytes *in vitro* to 0.01 to 0.5 µg/ml (0.0006 to 0.03 µM as Co) cobalt chloride (Capomazza and Botta, 1991). In general, the micronucleus test can detect DNA lesions that have survived at least one mitotic cycle. Micronuclei rates increased with cobalt chloride concentration up to a maximal level of 77 micronuclei per 1000 binucleated cells, corresponding to a dose of 0.1 µg/ml. This dose was considered subtoxic due to a marginal 10-20% decrease of the mitotic index. In the *in vitro* micronucleus test in Syrian hamster embryo (SHE) cells, cobalt sulfate heptahydrate also tested positive (Gibson *et al.*, 1997). A dose-dependent, significant increase in the percentage of binucleated micronucleated (BNMN) cells occurred at multiple concentrations (1.0 to 4.0 µg/ml) of cobalt sulfate heptahydrate.

Chromosomal aberrations

Human primary fibroblasts were examined for translocations and aneuploidy following 24-hour *in vitro* exposure to cobalt chloride at concentrations of 1.3, 25 and 50 ppb (0.005, 0.105 and 0.210 µM) (Figgitt *et al.*, 2010). Aneuploidy occurs during cell division when the chromosomes do not separate properly between the two cells. The lowest concentration tested was considered a physiological concentration, as it resulted in a cobalt ion concentration equivalent to that found in patients with well-functioning metal-on-metal cobalt chrome alloy hip implants. Cobalt chloride caused a dose-dependent increased incidence of total chromosomal aberrations that was statistically significant ($p<0.05$) at the lowest dose compared to controls. The types of chromosomal aberrations present were predominantly numerical (aneuploidy). Structural aberrations (translocations) were not observed.

Figgitt *et al.* (2010) also investigated the delayed effects of cobalt chloride up to 30 days post-exposure in order to monitor the repair of any lesions induced in human primary fibroblasts. Simple aneuploidy (cells displaying gains or losses involving only 3 chromosomes) was increased ($p<0.001$) one-day post-exposure in the 25 and 50 ppb groups, but had resolved by Day 10 post-exposure. Complex aneuploidy (numerical aberrations in excess of 49 chromosomes) was observed only at the highest dose one-day post-exposure, and none of the cobalt treatments led to chromosome fragments.

In human lung fibroblast cells, the genotoxic and cytotoxic potency of soluble cobalt chloride and insoluble cobalt(II) oxide (average size of 1 µm) was compared *in vitro* (Smith *et al.*, 2014). Genotoxicity was determined by treating cell cultures with varying concentrations of the soluble cobalt (50 to 500 µM, or 3 to 30 µg Co/ml) or insoluble cobalt (0.1 to 5 µg/cm^2), and then harvesting for metaphases to look for chromosome aberrations. Using intracellular cobalt ion levels for comparison, both soluble and insoluble cobalt induced similar levels of aberrations per 100 metaphases that were dose-dependent. The most common aberrations for both cobalt forms were simple chromatid lesions, with no complex lesions such as dicentrics and chromatid exchanges.

However, soluble cobalt induced cell cycle arrest, indicated by a lack of metaphases, at much lower intracellular concentrations compared to insoluble cobalt. The authors concluded that both cobalt forms have similar levels of genotoxicity, but that soluble cobalt induces more cytotoxicity than insoluble cobalt.

Further *in vitro* research by Smith *et al*. (2014) observed that uptake of cobalt(II) oxide particulate by human lung fibroblast cells requires particle-cell contact, indicating that the primary mechanism for cobalt ion release is from the internal dissolution of phagocytized particles rather than uptake of extracellular ions. The researchers concluded that solubility appears to play a role in cobalt-induced lung cell genotoxicity and suggests soluble and insoluble forms of cobalt may have different carcinogenicity potentials.

The genotoxicity and cytotoxicity of cobalt(II) oxide (0.1 to 5 µg/cm^2) and cobalt chloride (100 to 250 µM) were investigated in normal primary human bronchial epithelial cells (Xie *et al.*, 2016). Both cobalt compounds induced a concentration-dependent increase in cytotoxicity and chromosomal aberrations, most commonly seen as chromatid lesions. However, based on intracellular cobalt concentrations, cobalt chloride induced more chromosome damage than cobalt(II) oxide in the cells. In terms of cytotoxicity, intracellular levels of cobalt indicated no significant difference between the two cobalt compounds. The difference in genotoxicity between the two cobalt compounds was suggested by the authors to be a result of insoluble cobalt taking a longer time to reach genotoxic intracellular concentrations compared to soluble cobalt. In comparing similar work with human lung fibroblast cells (Smith *et al.*, 2014), the primary human bronchial epithelial cells were less efficient in taking up cobalt ions than fibroblasts. However, chromosome damage was similar after soluble cobalt treatment despite lower intracellular cobalt levels in the epithelial cells.

The chromosomal aberration test was conducted *in vitro* with cobalt acetyl acetonate, cobalt resinate, and cobalt oxyhydroxide suspended separately in cultures of HLs (Kirkland *et al.*, 2015). Cobalt acetyl acetonate induced a clastogenic response in the cells in both the absence (34 to 150 µg/ml) and presence (17 to 100 µg/ml) of S9. Cobalt resinate induced chromosomal aberrations with S9 (75 to 300 µg/ml), although cobalt precipitation may have been a confounding factor. The biological relevance of the results for cobalt oxyhydroxide was unclear due to the presence of a persistent cobalt compound precipitate on the cell layer resulting in cytotoxic and genotoxic effects.

Due to urinary elimination being the main route of excretion for absorbed cobalt, a human urothelial cell line (hTUI-38) was used by Speer *et al.* (2017) to investigate the genotoxicity and cytotoxicity of cobalt(II) oxide and cobalt chloride. Based on intracellular cobalt ion levels, both compounds induced similar levels of chromosomal aberrations primarily in the form of chromatid breaks and chromatid gaps. However, cobalt chloride was more cytotoxic at similar intracellular levels and induced cell cycle

arrest that was not observed after treatment with cobalt(II) oxide. The authors concluded that both cobalt compounds were cytotoxic and genotoxic to human urothelial cells and solubility may play a role in cobalt-induced toxicity.

Cobalt chloride (10 to 100 µM, or 0.6 to 6 µg Co/ml) enhanced the number of UV-induced sister chromatid exchanges in V79 Chinese hamster cells (Hartwig *et al.*, 1991). The increase was significantly higher than the expected values from individual treatments of UV irradiation or cobalt alone.

Nanoparticle chromosomal damage tests

The frequency of BNMN cells was examined following exposure of human peripheral blood leukocytes to cobalt NPs (likely as cobalt(II) oxide) and cobalt chloride by means of the cytokinesis-block micronucleus assay (Colognato *et al.*, 2008). Both cobalt NPs and cobalt chloride increased the frequency of BNMN with increasing dose, which was statistically significant at 400 µM (24 µg Co/ml). Ponti *et al*. (2009) compared cobalt(II) oxide NPs and cobalt chloride *in vitro* with Balb/3T3 mouse fibroblast cells using the micronucleus test at doses of 1, 5, and 10 µM (0.06, 0.3 and 0.6 µg Co/ml), corresponding to 50% plating efficiency (a measure of cytotoxicity). Cobalt NPs caused chromosomal aberrations at all concentrations, although not dose-dependently, while cobalt chloride was not genotoxic under the conditions used.

Exposure of HLs to cobalt(II, III) oxide NPs (100 µg/ml) *in vitro* has also resulted in increased chromosomal aberrations in the form of greater numbers of chromosome breaks and deletions compared to controls (Rajiv *et al.*, 2016). Oxidative stress was observed in the cells, measured as increased ROS and lipid peroxidation, depletion of catalase, and reduced glutathione and superoxide dismutase. The authors concluded oxidative stress resulting from cobalt(II, III) oxide NP exposure led to DNA damage and chromosomal aberrations in the HLs.

In vivo chromosomal damage tests

Chromosomal aberrations in bone marrow cells of mice have been induced by cobalt chloride *in vivo* (Palit *et al.*, 1991). Mice orally administered cobalt chloride at high doses of 20, 40 and 80 (1/10 LD_{50}) mg/kg body weight resulted in a dose-related increase in aberrations, including chromosomes with and without gaps and breaks per cell.

Farah (1983) administered daily injections of cobalt chloride to male Syrian hamsters intraperitoneally over nine days (total dose: 0.04 g/100 g body weight). Bone marrow cells showed a significant increase ($p<0.001$) in pseudodiploidy and hyperdiploidy. An increased frequency ($p<0.01$) of meiotic cells with abnormal chromosome numbers during metaphase 1 was found in testicular preparations of the male hamsters.

Cobalt resinate and cobalt acetyl acetonate were tested for induction of micronuclei *in vivo* in mouse bone marrow cells (Kirkland *et al.*, 2015). Mice were administered the cobalt compounds up to the maximally tolerated dose by oral gavage (1500 and 500 mg/kg-d for cobalt resinate and cobalt acetyl acetonate, respectively) on two occasions 24 hr apart. Polychromatic erythrocytes (PCE) from bone marrow were counted to determine the micronucleus frequency, and total erythrocyte count was used to determine the ratio of PCE to normochromatic erythrocytes (NCE). Neither cobalt compound produced significant increases in micronucleus frequency up to the maximally tolerated dose, although cobalt resinate caused bone marrow toxicity with a significant decrease in PCE:NCE ratio.

Kirkland *et al*. (2015) also conducted an *in vivo* bone marrow chromosomal aberration study in rats with single-dose and multi-dose oral administration (3 dose levels each per sex) of cobalt sulfate, cobalt(II) oxide, and tricobalt tetroxide. In the single dose study, no increase in chromosomal aberrations was seen in the bone marrow with any cobalt compound up to the maximally tolerated dose (1000 or 2000 mg/kg-d). In the multi-dose phase of the study, rats were orally administered the same cobalt compounds daily for up to 5 days. The authors found no biologically significant induction of chromosome aberrations in bone marrow with any cobalt compound at or above the maximally tolerated dose. Kirkland *et al*. (2015) also orally administered daily doses of cobalt chloride (3, 10 and 30 mg/kg/day) to male rats for 28 days up to the maximally tolerated dose to look for chromosomal aberrations in spermatogonia. No reduction in the mitotic index was found and there was no increase in the frequency of chromosomal aberrations.

1031 **Table 6. Genotoxicity testing summary for soluble and insoluble cobalt**
1032 **compounds, not including cobalt metal**

Cell type or species/strain	Cobalt compound	Metabolic Activation		Reference
		without	with	
DNA strand-break tests (comet assay, or other DNA damage assay)				
Human lymphocytes	Cobalt chloride	+	NA	De Boeck *et al.* (1998)
H460 human lung epithelial cells	Cobalt chloride	+	NA	Patel *et al*. (2012)
Human HepG2 cells	Cobalt chloride	+	NA	Alarifi *et al*., 2013
	Cobalt(II, III) oxide NP	+	NA	
Human lymphocytes	Cobalt(II, III) oxide NP	+	NA	Rajiv *et al*. (2016)
Human peripheral blood leukocytes	Cobalt(II) oxide NP	+	NA	Colognato *et al*. (2008)
	Cobalt chloride	-	NA	
Balb/3T3 mouse fibroblast cells	Cobalt(II) oxide NP	+	NA	Ponti *et al*. (2009)
	Cobalt chloride	+	NA	
Human cell lines:	Cobalt(II, III) oxide NP			Abudayyak *et al*. (2017)
A549 lung carcinoma		+	NA	
HepG2 hepatocarcinoma		-	NA	
Caco-2 colorectal		-	NA	
SH-SY5Y neuroblastoma		-	NA	
HeLa cells; enhanced UV-caused strand breaks	Cobalt chloride	+	NA	Hartwig *et al*. (1991)
Human fibroblasts Inhibit DNA repair by UVC	Cobalt chloride	+	NA	Kasten *et al*. (1997)
Salmon sperm DNA	Cobalt sulfate	+	NA	Lloyd *et al*. (1997)
Human K562 cells	Cobalt sulfate	+	NA	Nackerdien *et al*. (1991).
E.coli bacteria	Cobalt sulfate	+	NA	Kumar et al., 2017
Oxidative DNA Damage Tests				
Human A549 alveolar adenocarcinoma and bronchial BEAS-2B cells	Cobalt(II, III) oxide NP	+	NA	Cavallo *et al*. (2015)
Human A549 cells	Cobalt octoate	+	NA	Kirkland *et al.* (2015)
	Cobalt sulfate heptahydrate	+	NA	
Rat *in vivo* IP - renal, hepatic and pulmonary chromatin	Cobalt acetate	+	NA	Kasprzak *et al*. (1994)
Test for Reduction in DNA Replication and Repair				
E.coli bacteria	Cobalt sulfate	+	NA	Kumar *et al*., 2017

1033 +/-: equivocal NA: not applicable NP: nanoparticles
1034

Table 6. Genotoxicity testing summary for soluble and insoluble cobalt compounds, not including cobalt metal (continued)

Cell type or species/strain	Cobalt compound	Metabolic Activation		Reference
		without	with	
Bacterial and mammalian cell gene mutation tests (continued)				
S. typhimurium TA100	Cobalt sulfate $7H_2O$	+	+	NTP (1998)
S. typhimurium TA98, and TA1535		-	-	
S. typhimurium TA97a	Cobalt chloride	-	-	Kirkland *et al*. (2015)
S. typhimurium TA100	Cobalt sulfate	-	-	
S. typhimurium TA98, TA100, TA 102, TA1535, TA1537	Cobalt acetyl acetonate and cobalt resinate	-	-	
L5178Y mouse lymphoma cells	Cobalt hydroxide	-		Kirkland *et al.* (2015)
	Cobalt oxalate	-	-	
	Cobalt sulfate	-	-	
	Cobalt sulfide	-	-	
	Cobalt(II) oxide	-	-	
Chromosomal Damage – Micronucleus test				
Human blood leukocytes	Cobalt chloride	+	NA	Capomazza and Botta (1991)
SHE cells	Cobalt sulfate heptahydrate	+	NA	Gibson *et al.* (1997)
Chromosomal Damage – Chromosomal Aberrations				
Human peripheral blood leukocytes	Cobalt(II) oxide NP	+	NA	Colognato *et al.* (2008)
	Cobalt chloride	+	NA	
Balb/3T3 mouse fibroblast cells	Cobalt(II) oxide NP	+	NA	Ponti *et al.* (2009)
	Cobalt chloride	-	NA	
Mouse bone marrow cells (*in vivo*)	Cobalt resinate	-	NA	Kirkland *et al.* (2015)
	Cobalt acetyl acetonate	-	NA	
Human primary fibroblasts cells	Cobalt chloride	+	NA	Figgitt *et al.* (2010)
Human lung fibroblast cells	Cobalt chloride	+	NA	Smith *et al.* (2014)
	Cobalt(II) oxide	+	NA	
Human lung bronchial epithelial cells	Cobalt chloride	+	NA	Xie *et al*. (2016)
	Cobalt(II) oxide	+	NA	
Human lymphocytes	Cobalt acetyl acetonate	+	+	Kirkland *et al.* (2015)
	Cobalt resinate	-	+	
	Cobalt oxyhydroxide	+/-	+/-	
Human urothelial cells	Cobalt chloride	+	NA	Speer *et al*. (2017)
	Cobalt(II) oxide	+	NA	
Human lymphocytes	Cobalt(II, III) oxide NP	+	NA	Rajiv *et al.* (2016)

+/-: equivocal NA: not applicable NP: nanoparticles

Table 6. Genotoxicity testing summary for soluble and insoluble cobalt compounds, not including cobalt metal (continued)

Cell type or species/strain	Cobalt compound	Metabolic Activation		Reference
		without	with	
Chromosomal Damage – Chromosomal Aberrations (continued)				
Mouse bone marrow cells (*in vivo*)	Cobalt chloride	+	NA	Palit *et al.* (1991)
Syrian hamster bone marrow cells (*in vivo*)	Cobalt chloride	+	NA	Farah (1983)
Rat bone marrow cells (*in vivo*)	Cobalt sulfate	-	NA	Kirkland *et al.* (2015)
	Cobalt(II) oxide	-	NA	
	Tricobalt tetroxide	-	NA	
Male rat spermatogonia (*in vivo*)	Cobalt chloride	-	NA	Kirkland *et al.* (2015)
Chinese hamster V79 cells Enhanced UV-induced sister chromatid exchanges	Cobalt chloride	+	NA	Hartwig *et al*. (1991)

+/-: equivocal NA: not applicable NP: nanoparticles

Cobalt Metal, Including Comparisons with Soluble and Insoluble Cobalt Compounds

Investigation of the metal form of cobalt for genotoxicity has been explored more recently. Some of these studies compared the genotoxicity of cobalt metal with a soluble cobalt compound. Cobalt metal produced mixed results for mutations in bacterial tests. In *in vitro* mammalian cell systems, cobalt metal caused DNA strand breaks, chromosomal aberrations, gene mutations, and inhibition of DNA repair. Cobalt metal did not cause chromosomal aberrations *in vivo* in HLs or murine bone marrow.

DNA strand break tests

Employing the alkaline comet assay, cobalt metal (median particle size 4 µm) induced a dose-dependent increase in tail lengths and moments (0.6 to 6.0 µg/ml) in isolated HLs that were statistically significant at 4.5 µg/ml (Anard *et al.*, 1997). These changes occurred without a significant effect on cell viability. A modified alkaline elution assay also showed a dose-dependent increase (1.5 to 15 µg/ml) in production of DNA breaks in isolated lymphocytes exposed to cobalt metal. The increase in number of breaks was statistically significant starting at 3 µg/ml. The alkaline elution assay measures the rate of DNA elution through a filter membrane and the amount of DNA single strand breaks or lesions converted to breaks under alkaline conditions. DNA breaks are estimated by the increase in DNA elution rate. When cobalt chloride was substituted for cobalt metal in the alkaline elution assay, production of DNA breaks was not different from controls.

Similar strand break results as that obtained with HLs were observed when mouse 3T3 fibroblast cells were exposed to cobalt metal in the alkaline elution assay.

In a similar study, De Brock *et al*. (1998) examined the ability of cobalt metal (median particle size: 4 µm) and cobalt chloride to induce DNA damage in isolated HLs from three donors using the alkaline comet assay. In this case, however, both cobalt compounds over a range of 0 to 6.0 µg Co-equivalent/ml showed comparable responses in inducing DNA damage in a dose-dependent and time-dependent manner. Relatively large inter-experimental and inter-donor variability in the response was observed. This finding plus differences in comet assay methodologies may explain the different results obtained by Anard *et al*. (1997).

Cobalt metal may also have an indirect genotoxic action through inhibition of DNA repair. Isolated HLs were exposed to methyl methanesulphonate (MMS) followed by a 2-hour post-incubation recovery period, or MMS followed by a 2-hour treatment with a non-genotoxic dose of 1.2 µg/ml cobalt metal particles (De Boeck *et al.*, 1998). Cobalt metal was observed to inhibit the repair of MMS-induced DNA damage.

The genotoxicity of cobalt metal, cobalt(II) oxide, and cobalt(II, III) oxide NPs were compared *in vitro* using human lung cells in culture (Cappellini *et al.*, 2018). All three cobalt-containing NPs showed efficient uptake in A549 type II epithelial cells, although only cobalt metal NPs were cytotoxic after 24 hrs. Cobalt metal NPs significantly increased DNA damage in A549 (at 40 µg/ml) and HBEC (human bronchial epithelial cells) (20 and 40 µg/ml) cell types by the alkaline comet assay. Cobalt(II) oxide NPs also produced significant DNA damage in these cell types at the higher dose of 60 µg/ml. Cobalt(II, III) oxide NPs caused no DNA damage at any concentration. The Fpg assay used in A549 cells showed that DNA damage caused by cobalt metal and cobalt(II) oxide NPs was related to oxidative stress. The Fpg assay was negative for cobalt(II, III) oxide NPs.

Cappellini et al. (2018) also tested the three cobalt-containing NPs in the ToxTracker reporter assay to investigate the mechanisms of genotoxicity. The ToxTracker assay is a mouse embryonic stem cell-based genotoxicity assay employing six green fluorescent protein reporters specific for DNA damage, oxidative stress, protein damage, and cellular stress response. Cobalt metal NPs, and to a lesser extent cobalt(II) oxide NPs, caused an induction of the Srxn1-GFP reporter related to generation of ROS that can lead to DNA single strand breaks during the repair of oxidative DNA lesions. Cobalt metal and cobalt(II) oxide NPs also activated the Rtkn-GFP genotoxicity reporter that is associated with induction of DNA strand breaks. Cobalt(II, III) oxide NPs were inactive. Overall, the authors concluded that the primary mechanism of genotoxicity by cobalt metal and cobalt(II) oxide NPs, but not cobalt(II,III) oxide, was induction of oxidative stress that can lead to DNA strand breaks.

Wan et al. (2017) intratracheally instilled 50 µg cobalt NPs (85-90% metal cobalt and 10-15% cobalt(II, III) oxide) per mouse to determine if these NPs result in DNA damage and DNA mutation. Results on DNA mutation are discussed in "Gene Mutation Analysis". DNA damage was measured four months after treatment by immunohistochemical staining for γ-H2AX, which is involved in DNA repair activities for specific types of DNA damage such as double-strand breaks, and as levels of 8-hydroxydeoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage caused by ROS. At day 7 following exposure, during the acute inflammatory phase of pulmonary injury, treated mice showed a significant increase in γ-H2AX-positive nuclei as compared to saline-instilled controls. An increase in γ-H2AX-positive nuclei was still apparent at 4 months following exposure, during which the pulmonary injury in the treated mice progressed to pulmonary interstitial fibrosis and continued infiltration of inflammatory cells. The level of 8-OHdG was also significantly higher in genomic DNA of lung tissues of treated mice compared to saline controls. 8-OHdG formation was suggested by the authors to result in the G:C to T:A transversion, which was observed at higher frequencies in cobalt-treated tissues (also discussed in "Gene Mutation Analysis").

Bacterial and mammalian cell gene mutation tests

NTP (2014a) evaluated the genotoxicity of cobalt metal particulate (1.7-1.8 µm) in bacteria, using *Salmonella typhimurium* strains (TA98, TA100) and *Escherichia coli* (WP2 uvrA/pKM101) either in buffer or S9 mix obtained from Arochlor 1254-induced liver of rats. Five doses of cobalt metal (100 to 5000 µg/plate) were examined, with the highest concentration resulting in toxicity. Without S9, cobalt produced an equivocal response with *S. typhimurium* TA100, but was weakly mutagenic with the TA98 strain. With S9, no mutagenic activity was observed in either *S. typhimurium* strain. No mutagenic activity was observed, with or without S9, in *E. coli*. Hong *et al*. (2015) suggested the lack of mutagenicity in *S. typhimurium* with S9 could be related to radical scavenging enzymes (*e.g.*, glutathione peroxidase) contained within the S9 mix and/or binding of cobalt to S9 proteins.

Kirkland *et al*. (2015) conducted the Ames test with cobalt metal following OECD-recommended guidelines. Cobalt metal powder (median diameter: 2.9 µm) was suspended in DMSO and tested in strain TA98 independently in three different laboratories up to maximum test concentrations of 1000 to 5000 µg/plate. A mutagenic response was not produced, with and without S9, using either plate incorporation or pre-incubation methodology.

Cobalt metal powder (median diameter: 3.4 µm) was tested for induction of *Hprt* mutations in mouse lymphoma L5178Y cells (Kirkland *et al.*, 2015). The metal was incubated for 3 hr both in the absence and presence of S9 at treatment concentrations ranging from 0 to 250 µg/ml. An extended 24 hr treatment in the absence of S9 was also

performed with cobalt metal powder extract in order to detect any mutagenic effects that might only manifest after being in contact with the cells for a full cell cycle. Although some equivocal results were obtained, overall it was concluded by the authors that cobalt metal and the metal extract did not induce *Hprt* mutations when tested in the absence or presence of S9. Undissolved cobalt metal was present in the culture medium of the cobalt metal experiment, but it was unclear how this influenced the toxic and mutagenic responses.

Chromosomal damage

The ability of cobalt to induce micronuclei *in vivo* in normochromatic erythrocytes (NCEs) of male and female $B6C3F_1$/N mice was determined by NTP (2014a) following inhalation exposure to cobalt metal particulate (MMAD 1.7-1.8 µm) for 14 weeks. The percentage of circulating polychromatic erythrocytes (reticulocytes) was also scored as a measure of bone marrow toxicity. Peripheral blood samples were collected from mice (5 animals/sex/group) exposed to cobalt by inhalation at concentrations of 0, 0.625, 1.25, 2.5, 5, and 10 mg/m^3 (6 hrs/day, 5 days/week). No increases in the frequencies of NCEs, or significant alterations in the percentages of reticulocytes, were observed. Under the conditions examined, NTP concluded cobalt metal did not cause bone marrow toxicity.

The chromosomal damaging capacity of cobalt metal powder (median particle size: 4 µm) was assessed by the cytokinesis-blocked micronucleus test *in vitro* on isolated human leukocytes (Van Goethem *et al.*, 1997). The cytokinesis-block micronucleus assay is a sensitive and simple indicator of chromosome damage, both chromosome loss and chromosome breakage, and provides information on cell cycle progression and cytotoxicity. Cobalt metal induced a dose-dependent and statistically significant increase (p<0.05) in micronucleated cytokinesis-blocked cells at all concentrations tested (0.6 to 6.0 µg/ml). Cell cycle delay and/or cytotoxicity were also observed at all doses tested. A concurrently run alkaline Comet assay on the same cell type by the authors showed a dose-dependent increase of DNA breaks characterized by increased tail lengths and/or moments that was statistically significant from control at all doses (0.3 to 12.0 µg/ml). Combined, these two genotoxicity tests (i.e.,Comet assay and cytokinesis-block micronucleus assay) show that a significant amount of DNA breakage translated into chromosome damage and/or gene mutations.

Gene Mutation Analysis

A mutation analysis of the lung neoplasms observed in rats and mice in the 2-year cobalt metal NTP studies was performed to look for the most commonly altered genes, the *Kras*, *Egfr* and *Tp53* genes, which are found in human lung cancer (NTP, 2014a; Hong *et al.*, 2015). The most frequent mutation found in the mouse alveolar/bronchiolar carcinomas was in the *Kras* gene (67%, 46/69). None of these mutations (*Kras*, *Egfr* or *Tp53*) were present in alveolar/bronchiolar tumors examined in the control mice, although *Kras* gene mutations are observed in historical controls (27%, 34/124). The majority of the *Kras* mutations were within codon 12 of lung carcinomas in metal cobalt-exposed mice, where mutations are also frequently localized in spontaneous alveolar/bronchiolar carcinomas of mice. The difference is that G→T transversions were primarily found in cobalt metal-exposed mice (80%, 24/30), while G→A transversions are most common in historical spontaneous carcinomas examined (70%, 14/20). The G→T transversion is seen in chemically-induced lung tumors, including cobalt sulfate heptahydrate (NTP, 1998a) and is thought to be related to ROS generation.

In rats, the most frequent mutation found in alveolar/bronchiolar carcinomas from cobalt metal treated animals was also in the *Kras* gene (31%, 15/48) (Hong *et al.*, 2015). *Kras* mutations were not found in spontaneous lung tumors of controls (0/10). Similar to mice, the majority of *Kras* mutations in cobalt-exposed rats were within codon 12, with the most common being G→T transversions.

Cobalt metal NPs were instilled intratracheally (50 µg) in guanine phosphoribosyl-transferase (*gpt*) delta transgenic mice to determine if exposure results in DNA damage and DNA mutation in the lung (Wan et al., 2017). The transgenic mice carry about 80 copies of the transgene, lambda EG10 DNA, on each chromosome 17. Four months after exposure, mutation frequencies of *gpt* genes in the lungs were significantly greater compared with saline instilled controls. The most common mutation was G:C to T:A transversion, which can be increased due to oxidative stress.

Genotoxicity tests in workers exposed to cobalt metal

Genotoxic endpoints were evaluated in workers (n = 35) exposed exclusively to cobalt metal dust in hard metal refineries (De Boeck *et al.*, 2000). Matched control workers (n = 35) were recruited from the same plants. A third group consisted of workers (n = 29) exposed to both cobalt and tungsten carbide dust. Exposure to cobalt was characterized as moderate, with a mean cobalt urine concentration of 21.5 µg/g creatinine on Friday at the end of the work week. Based on previous epidemiological studies by the authors, a urine concentration at this level equates to a time-weighted average (TWA) exposure of 20 µg/m^3 of cobalt. Lymphocytes from the blood of the workers were examined for DNA damage by the comet assay and for chromosomal aberrations by the micronucleus test.

In addition, the urine of the workers was examined for altered DNA (i.e., 8-OHdG). No significant increase in genotoxic effects could be detected in workers exposed to cobalt dust alone or combined cobalt-hard metal dusts. The authors suggested that the cobalt exposure may have been too low to detect genotoxic changes, and that analysis of respiratory cells that are in direct contact with inhaled particles may be more appropriate to examine for genotoxic effects.

In another study, sister chromatid exchange in blood lymphocytes was evaluated in 24 workers in a metal powder producing factory that were matched against 23 control workers by age and smoking status (Gennart *et al.*, 1993). Urinary cobalt levels in exposed workers were 23.6 µg/g creatinine, which was similar to that observed in hard metal refinery workers by De Boeck *et al*. (2000). However, it was not specified when the samples were collected during the week. The workers were also exposed to chromium and nickel powders and showed significantly higher levels of these metals in urine compared to controls. The mean sister-chromatid exchange score in lymphocytes was significantly greater ($p < 0.05$) in the exposed workers. Considering the weak carcinogenic action of cobalt, the researchers believed that the small amounts of chromium and nickel that were solubilized and absorbed caused the increased score. The levels of serum tumor markers (carcinoembryonic antigen and polypeptide antigen) were also increased in exposed workers, but did not reach statistical significance.

Exfoliated cells were collected from the buccal and nasal mucosa of electroplate workers that were exposed to cobalt (specific cobalt compounds not discussed) and hexavalent chromium to look for genotoxic effects (Wultsch *et al.*, 2017). The workers (n = 42) were matched with a control group (n = 43) with regard to gender, age, body mass index, alcohol consumption and smoking. No induction of micronuclei was detected in the collected cells that would suggest chromosomal aberrations. However, the electroplaters wore masks while working and blood levels of cobalt (0.85 µg/L plasma) were not statistically significantly elevated from the controls (0.80 g/L plasma). Air levels of cobalt were below the detectable limit (0.12 ng/m^3) while the mean level of hexavalent chromium was 0.2 µg/m^3.

Table 7. Genotoxicity testing summary for cobalt metal, including comparisons with soluble and insoluble cobalt compounds

Cell type or species/strain	Cobalt metal/compound	Metabolic Activation		Reference
		without	with	
DNA strand-break tests (comet assay or other DNA damage assay)				
Human lymphocytes (HL)	Cobalt chloride	-	NA	Anard *et al.* (1997)
	Cobalt metal	+	NA	
and mouse 3T3 fibroblast cells (3T3)	Cobalt metal	+	NA	
Human lymphocytes	Cobalt metal	+	NA	De Broeck *et al.* (1998)
	Cobalt chloride	+	NA	
Human leukocytes	Cobalt metal	+	NA	Van Goethem *et al*. (1997)
Blood lymphocytes of exposed workers (*in vivo*)	Cobalt metal	-	NA	De Boeck *et al.* (2000)
Lung tissue of *gpt* delta transgenic mice (*in vivo*)	Cobalt metal NPs	+	NA	Wan et al. (2017)
Human A549 and HBEC cells	Cobalt metal NPs	+	NA	Cappellini et al. (2018)
	Cobalt(II) oxide NPs	+		
	Cobalt(II, III) oxide NPs	-		
Oxidative DNA damage test (8-hydroxy-deoxyguanosine)				
Lung tissue of *gpt* delta transgenic mice (*in vivo*)	Cobalt metal NPs	+	NA	Wan et al. (2017)
Blood lymphocytes of exposed workers (*in vivo*)	Cobalt metal	-	NA	De Boeck *et al.* (2000)
Bacterial and mammalian cell gene mutation tests				
S. *typhimurium* TA98,	Cobalt metal	+	-	NTP (2014a)
S. *typhimurium* TA100,		+/-	-	
and *E. coli* (WP2 uvrA/pKM101)		-	-	
S. *typhimurium* TA98	Cobalt metal	-	-	Kirkland *et al.* (2015)
L5178Y mouse lymphoma cells	Cobalt metal	-	-	Kirkland *et al.* (2015)
	Cobalt metal extract	-	-	
Chromosomal damage				
Human leukocytes	Cobalt metal	+	NA	Van Goethem *et al.* (1997)
B6C3F1/N mouse reticulocytes (*in vivo*)	Cobalt metal	-	NA	NTP (2014a)
Blood lymphocytes of exposed workers (*in vivo*)	Cobalt metal	-	NA	De Boeck *et al.* (2000)
Buccal and nasal mucosa cells of exposed workers (*in vivo*)	Cobalt metal (likely)	-	NA	Wultsch *et al*. (2017)

+/-: equivocal NA: not applicable NP: Nanoparticle

Table 7. Genotoxicity testing summary for cobalt metal, including comparisons with soluble and insoluble cobalt compounds (continued)

Cell type or species/strain	Cobalt metal/compound	Metabolic Activation		Reference
		without	with	
Gene mutation analysis				
Kras gene mutations (*in vivo*):	Cobalt metal			NTP (2014a); Hong *et al*. (2015)
B6C3F$_1$/N mice		+	NA	
F-344/NTac rats		+	NA	
gpt gene mutation Lung tissue of *gpt* delta transgenic mice (*in vivo*)	Cobalt metal NPs	+	NA	Wan et al. (2017)

+/-: equivocal NA: not applicable NP: Nanoparticle

Morphological Cell Transformation and Tumor Suppressor Protein Induction

Positive cell transformation assays are suggestive of carcinogenic potential (Creton *et al.*, 2012). Cobalt sulfate was positive in the SHE cell transformation assay (Kerckaert *et al.*, 1996). SHE cells have been used to evaluate the potential carcinogenicity of a wide variety of chemical and physical agents. SHE cells display a multistage pattern of progression to cancer following acute (24 hr exposure in this study) carcinogen exposure that is similar to the multistage progression of *in vivo* carcinogenesis. At all concentrations tested (0.125 to 1 µg/ml), cobalt sulfate statistically significantly (p<0.05) increased the number of transformed colonies per total colonies, although a dose-response trend was not observed.

Crystalline cobalt sulfide (CoS_2) and amorphous cobalt sulfide (CoS) particles (1.25 to 2 µm) were observed to increase the incidence of morphological transformation in SHE cells (1 to 20 µg/ml), with the crystalline form showing a greater potency for cell-transforming activity (Costa *et al.*, 1982). Compared to findings of crystalline and amorphous nickel sulfides, the authors postulated that the crystalline cobalt sulfide form is more actively phagocytized by cells resulting in greater intracellular dissolution and ROS formation, and subsequently leading to greater cell transformation.

Balb/3T3 mouse fibroblast cells were used to evaluate the morphological transforming ability of cobalt(II) oxide NPs (1 to 30 µM) and cobalt chloride (1 to 70 µM) (Ponti *et al.*, 2009). Cobalt NPs were cytotoxic, but also increased morphological transformation at nearly all concentrations tested. Cobalt chloride was also cytotoxic but did not lead to morphological transformation at any concentration tested.

Wild-type mouse embryonic fibroblast cells (MEF *Ogg1*$^{+/+}$) and its isogenic *Ogg1 knockout* partner (MEF *Ogg1*$^{-/-}$) were exposed *in vitro* to low, subtoxic doses (0.05 and

0.1 µg/ml) of cobalt NPs for 12 weeks (Annangi *et al.*, 2015). MEF *Ogg1*$^{-/-}$ cells are unable to maintain genomic integrity by effectively repairing oxidative DNA damage lesions, such as 8-OH-dG lesions on DNA. At five weeks of exposure, there was an increased number of colonies formed by MEF *Ogg1*$^{-/-}$ cells. After 10 weeks of exposure, significantly increased colony formation was observed for both cell types. Additionally, cancer-like phenotypic hallmarks were also observed in the exposed cells, including morphological cell changes, significant increases in the secretion of metalloproteinases, and anchorage-independent cell growth ability, with MEF *Ogg1*$^{-/-}$ cells showing greater sensitivity to these changes. The cobalt NP compound used was not specified, but was likely a cobalt oxide.

Balb/3T3 mouse fibroblast cells were used to evaluate the *in vitro* morphological transforming ability of cobalt metal NPs and microparticles and cobalt chloride (Sabbioni *et al.*, 2014a). Both cobalt NPs and microparticles were cytotoxic and significantly positive (p<0.05) at most concentrations tested (1 to 10 µM) for morphological transformation, measured as the increase of type III foci. Cobalt chloride added to the culture medium displayed lower cytotoxicity and did not cause an increase in morphological transformation in the cells. Cobalt microparticles were more efficient than NPs in inducing both morphological transformation and oxidative stress, which conforms with the finding of greater cellular uptake of cobalt microparticles by the cells. The authors concluded that a high degree of internalization of cobalt particles and/or dissolution within cells could play an important role in inducing morphological transformation. On the other hand, cobalt ions released from soluble cobalt chloride do not become bioavailable to cells until after saturation of binding with culture medium components (>40 µM).

The NCTC 929 cell line derived from mouse fibroblast cells was treated *in vitro* with cobalt sulfate (1 to 100 µg/ml) to determine if there is a resulting induction of p53 protein (Duerksen-Hughes *et al.*, 1999). The p53 protein is a tumor-suppressor protein that increases following DNA damage. The protein prevents replication of damaged DNA, either by causing the cell to undergo a reversible growth arrest or by initiating a cell's apoptotic pathway. Cobalt sulfate strongly induced p53 at 6 hrs (50 and 100 µg/ml) and 17 hrs (20 and 50 µg/ml) with subtoxic doses. Cytotoxicity was evident in cells exposed to 100 µg/ml cobalt sulfate for 17 hrs.

Toxicogenomics

Mateuca *et al*. (2005) evaluated polymorphisms responsible for reduced DNA repair capacity among cobalt-only exposed workers and hard metal workers to look for associations with genotoxic endpoints resulting from cobalt-generated ROS. The gene variations examined were involved in base-excision (*hOGGI*, *XRCC1*) and double strand break (*XRCC3*) DNA repair. Lymphocytes were collected for genotyping from 21 cobalt-

exposed, 26 hard metal-exposed and 26 matched control male workers. The alkaline comet assay was used to look for DNA single strand breaks and the cytokinesis-block micronucleus test was used to look for chromosomal rearrangements. The presence of 8-OHdG in urine, suggestive of oxidative DNA damage, was also investigated. The only significant genotoxic endpoint found was a higher frequency of micronucleated mononucleates (p=0.01) in hard metal-exposed workers with the variant *hOGG1*^{326} genotype, which leads to a reduced ability to excise 8-OHdG and has been associated with increased risk of esophageal, lung and prostate cancers.

Multivariate analysis was also performed with a number of independent variables on cobalt and hard metal workers combined (Mateuca *et al.*, 2005). The presence of the variant *XRCC1*^{280} genotype was associated with higher Comet assay DNA breakage (p=0.053), and having both *XRCC3*^{241} and *hOGG1*^{326} variant genotypes was associated with greater micronucleated mononucleate frequency (p=0.020). Smoking status and type of plant (cobalt or hard metal) was also shown to have a significant impact on genotoxicity endpoints. The authors noted that the small number of subjects was a weakness of this study.

IV. CANCER HAZARD EVALUATION

The carcinogenicity of cobalt sulfate heptahydrate and cobalt metal were assessed by NTP in separate chronic inhalation rodent studies (NTP, 1998a; 2014a). *In vitro* studies suggest that different pathways of cellular uptake for soluble and insoluble forms of cobalt compounds are associated with differences in the intracellular concentration and distribution, which in turn may be reflected in distinct genotoxic and carcinogenic potencies (Colognato *et al.*, 2008; Ponti *et al.*, 2009; Smith *et al.*, 2014).

Based on the results of these NTP studies, cobalt exhibits carcinogenicity in multiple species, which corresponds with the greatest potential to induce tumors in other species including humans (Tennant and Spalding, 1996; NTP, 2014a; Behl *et al.*, 2015). Cobalt induced tumors at one or more sites in both rats and mice, and induced tumors at the same site (*i.e.*, lung) that are of the same histogenic type in both species. Similar toxicity results for cobalt metal and cobalt sulfate heptahydrate in the NTP studies point to a common mechanism of action.

Release of the cobalt ion in physiological fluids following inhalation is considered the primary factor for cancer risk. To compare cancer potencies of cobalt metal and cobalt sulfate heptahydrate, the exposure levels in the studies were calculated based on cobalt content alone. Thus, chamber concentrations of cobalt sulfate heptahydrate were normalized to the cobalt content. Since the rodents in the NTP study were actually exposed to the hexahydrate, the hydrated cobalt sulfate chamber concentrations of 0, 0.3, 1.0 and 3.0 mg/m^3 $CoSO_4 \cdot 6H_2O$ were normalized to 0, 0.067, 0.22 and 0.67 mg/m^3

Co, respectively. Thus, it might be expected that the lowest concentration of cobalt metal (1.25 mg/m^3 Co) would produce a greater incidence of tumors than the highest concentration of hydrated cobalt sulfate (0.67 mg/m^3 Co).

Comparing the two sets of NTP studies in this way, cobalt metal exposure at the lowest concentration (1.25 mg/m^3 Co) produced a greater incidence of pulmonary tumors in the mice and male rats, and proportionally more pulmonary carcinomas than adenomas, compared to the highest concentration of hydrated cobalt sulfate (0.67 mg/m^3 Co). In female rats, exposure to cobalt metal at the lowest concentration produced a similar incidence of pulmonary tumors compared to the highest concentration of cobalt sulfate hexahydrate.

Also in the lung, the rare chemically-induced squamous cell neoplasms (predominantly CKE neoplasms) were found only in rats exposed to cobalt metal. Pancreatic islet tumors in male rats were observed only with exposure to cobalt metal, although at comparatively higher Co concentrations (2.5 and 5 mg/m^3) than those used in the cobalt sulfate heptahydrate studies. In addition, an increased incidence of mononuclear cell leukemia in female rats was observed only with exposure to cobalt metal. On the other hand, cobalt sulfate in rats at the highest exposure (0.67 mg/m^3 Co) produced approximately the same number of benign, malignant and benign/complex/malignant pheochromocytomas (combined) as that produced by cobalt metal at the lowest exposure concentration (1.25 mg/m^3 Co).

Regarding the finding of pheochromocytomas in both studies, NTP has noted an association with the generation of these tumors in other inhalation studies that also produced extensive chronic non-neoplastic lung lesions (Ozaki *et al.*, 2002; NTP, 2014a; Behl *et al.*, 2015). However, it is unclear if pheochromocytoma is a secondary response to hypoxia, or a directly acting chemical response to cobalt exposure. It is hypothesized that large space-occupying tumors and nonneoplastic lesions, including fibrosis and chronic inflammation, may lead to systemic hypoxemia. This in turn chronically stimulates catecholamine secretion from the adrenal medulla causing endocrine hyperactivity. The result may be hyperplasia and neoplasia of adrenal gland tissue.

No conclusive inhalation carcinogenicity studies have been performed for water-insoluble cobalt particulate compounds (*e.g.*, cobalt oxides), although exposure to these compounds is prevalent in occupational settings. A cobalt(II) oxide carcinogenicity inhalation study in hamsters has been performed (Wehner *et al.*, 1979), but drawbacks with the experimental animal choice (*i.e.*, resistant to lung tumor development, unusually short life-span) prevent any conclusions regarding the carcinogenic potential for cobalt oxide. However, intratracheal instillation, subcutaneous injection and intraperitoneal injection studies with cobalt(II) oxide in animals suggest that this cobalt form is carcinogenic (IARC, 1991; Steinhoff and Mohr, 1991). In addition, cobalt oxide

compounds have been shown to release cobalt ions in pulmonary fluids, which then reach the bloodstream (Bailey *et al.*, 1989; Foster *et al.*, 1989; Kreyling *et al.*, 1991b; Lison *et al.*, 1994). Therefore, water-insoluble cobalt compounds that release cobalt ion in pulmonary fluids are considered to be an inhalation cancer risk by OEHHA.

Several epidemiology studies have been conducted, but were too limited or inadequate to assess the carcinogenic risk of cobalt in humans. A recent retrospective study by Sauni *et al*. (2017) did not find an increased total cancer risk or lung cancer incidence among 995 workers exposed to cobalt metal powder and cobalt compounds. However, the exposures for many of the workers appear to have been short (as low as one year), and respiratory protection was available, although the level of use was not specified. Additionally, in a direct comparison (i.e., without adjustment parameters such as inhalation rate and body weight), the highest cobalt levels the workers were exposed to (0.06 to 0.10 mg/m^3) were below the lowest cobalt sulfate heptahydrate concentration (0.3 mg/m^3) used in the NTP rodent studies. This was a concentration that did not result in an increased tumor incidence in the rodents.

Overall, cobalt in its various forms has been found to be genotoxic, particularly by *in vitro* DNA-breaking tests and chromosomal aberration tests. In particular, *in vitro* studies have shown cobalt oxide compounds to be genotoxic. Both cobalt(II, III) oxide and cobalt(II) oxide particles have been shown to cause DNA damage and chromosomal aberrations in human lung or lymphocyte cells (Alarifi *et al.*, 2013; Smith *et al.*, 2014; Rajiv *et al.*, 2016; Xie *et al.*, 2016; Abudayyak *et al.*, 2017; Cappellini *et al.*, 2018). Additionally, cobalt(II) oxide and cobalt sulfide particles have resulted in morphological cell transformation in mammalian cells (SHE cells and Balb/3T3 mouse fibroblast cells) *in vitro* (Costa *et al.*, 1982; Ponti *et al.*, 2009).

Several studies have pointed to ROS generation being involved in these types of genotoxicity studies. Positive morphological cell transformation findings in mammalian cells indicate a mutagenic action for cobalt metal and cobalt compounds. Recent rigorous *in vivo* studies (oral gavage and inhalation exposure) in cobalt-exposed rodents by Kirkland *et al*. (2015) and NTP (2014a) did not find evidence of chromosomal damage in bone marrow or erythrocytes, although *in vivo* chromosomal damage assays are regarded to be less sensitive than *in vitro* assays. The few genotoxicity tests conducted on blood lymphocytes of workers exposed to cobalt have been negative. Kirkland *et al*. (2015) suggest that protective processes that exist in whole animals compared to single cells are sufficient to prevent DNA damage resulting from ROS. Thus, other processes may be involved (*e.g.*, inhibition of DNA repair) in the genotoxicity of cobalt. However, cells exposed to cobalt at the point of contact (*i.e.*, pulmonary cells with inhalation exposure), as suggested by De Boeck *et al*. (2000), may be a better approach to investigate genotoxic damage caused *in vivo*. Cobalt metal NPs intratracheally instilled into lungs of mice have resulted in evidence of DNA damage in the lung cells (Wan *et al.*,

2017). In addition, the *in vivo* NTP (NTP, 1998a; 2014a) cobalt inhalation studies performed a mutation analysis of the lung neoplasms in the exposed rodents and observed a greater proportion of G→T transversions, which are thought to be chemically-induced and related to ROS generation.

In vitro and *in vivo* studies with cobalt NPs indicate that they are also genotoxic and possibly carcinogenic. However, the level of exposure to cobalt NPs in the general population is unclear since it appears to be largely limited to occupational exposure. In comparison studies with soluble cobalt compounds, cobalt NPs induced more cytotoxicity than cobalt ions while cobalt ions induced more micronuclei but fewer strand breaks than cobalt NPs (Colognato *et al.*, 2008; Ponti *et al.*, 2009). A separate *in vitro* study observed that soluble cobalt compounds induced more cytotoxicity than microparticles of water-insoluble cobalt compounds but with similar levels of genotoxicity (Smith *et al.*, 2014). Finally, an *in vitro* comparison of cobalt metal NPs and microparticles found that the cobalt metal microparticles are more efficient than NPs in inducing both morphological cell transformation and oxidative stress, which supported the finding of greater cellular uptake of cobalt metal microparticles compared to cobalt metal NPs (Sabbioni *et al.*, 2014a; Sabbioni *et al.*, 2014b). However, soluble cobalt compounds showed considerably lower cellular uptake than either cobalt metal NPs or microparticles, and induced no oxidative stress or morphological cell transformation.

The available carcinogenicity and genotoxicity data indicate that separate cancer slope factors (CSFs) and IURs should be used for water-soluble cobalt compounds and cobalt metal. Toxicity data are limited for poorly water-soluble cobalt compounds, but due to a similar particle uptake mechanism and intracellular distribution of cobalt ions released from these water-insoluble cobalt compounds, a CSF based on cobalt metal can also represent water-insoluble cobalt compounds. Similarities in how cells treat cobalt nano- and micro-particles indicate that a cobalt metal CSF based on microparticle exposure will also be relevant for exposure to cobalt metal NPs.

V. QUANTITATIVE CANCER RISK ASSESSMENT

Cobalt Metal

Effective Tumor Incidences

The effective tumor incidences in rats (Table 8) and mice (Table 9) were used to calculate the cancer potency factor (CPF) for cobalt metal. The effective tumor incidence is the number of tumor-bearing animals (numerator) over the number of animals alive at the time of first occurrence of the tumor (denominator). This method of tallying tumor incidence removes animals from the assessment that died before they are considered at risk for tumor development. For example, effective tumor incidences of tumor types that were only observed near the end of the rodents' lifespan will generally

have smaller denominators as a result of early deaths occurring before first appearance of the tumor. The NTP individual animal pathology data from the cobalt inhalation studies were obtained from the Chemical Effects in Biological Systems (CEBS) database (NTP, 2014b).

No treatment-related effects of cobalt metal on survival were observed in the male rat study. In the female rat study, a reduction in survival was observed in the 2.5 mg/m^3 exposure group compared to controls. However, significant survival differences in this group were not apparent until late in the study (after week 85). Thus, use of effective tumor incidences for cancer dose-response modeling were judged appropriate for both the male and female rat studies.

Table 8. Effective tumor incidences (number of animals alive at day of first tumor) of treatment-related lesions in rats in the two-year inhalation studies of cobalt metal (NTP, 2014a)

Tumor	Cobalt Concentration (mg/m^3)			
	0	1.25	2.5	5.0
Male Rats				
Lung				
Alveolar/bronchiolar adenoma	2/47‡	10/48*	10/50*	14/49**
Alveolar/bronchiolar carcinoma	0/47‡	16/48**	34/50**	36/49**
Alveolar/bronchiolar adenoma or carcinoma	2/47‡	25/48**	39/50**	44/49**
Adrenal medulla				
Benign pheochromocytoma	15/46‡	23/48	37/49**	34/46**
Malignant pheochromocytoma	2/37‡	2/37	9/39*	16/38**
Benign or malignant pheochromocytoma	17/46‡	23/48	38/49**	41/46**
Pancreatic Islets				
Adenoma	0/38	1/39	6/40*	3/39
Carcinoma	2/38†	1/39	5/40	6/39
Adenoma or carcinoma	2/38‡	2/39	10/40*	9/39*
Female Rats				
Lung				
Alveolar/bronchiolar adenoma	2/45‡	7/47	9/46*	13/44**
Alveolar/bronchiolar carcinoma	0/48‡	9/49**	17/48**	30/50**
Alveolar/bronchiolar adenoma or carcinoma	2/48‡	15/49**	20/48**	38/50**
Cystic keratinizing epithelioma[a]	0/45	4/43	1/40	3/40
Adrenal medulla				
Benign pheochromocytoma	6/45‡	12/47	22/46**	36/44**
Malignant pheochromocytoma	0/36‡	2/27	3/25	11/30**
Benign or malignant pheochromocytoma	6/45‡	13/47	23/46**	40/44**
Immunologic System				
Mononuclear cell leukemia	16/50†	29/50**	28/50*	

* $p<0.05$, ** $p<0.01$ for difference from control by Fisher's exact test (calculated by OEHHA)
† $p<0.05$, ‡ $p<0.01$ positive trend for tumor type by the Cochran-Armitage trend test (calculated by OEHHA
[a] Includes one squamous cell carcinoma in the 5 mg/m^3 group

No treatment-related effects of cobalt metal on survival were observed in the female mouse study. In the male mouse study significant reductions in survival were observed in the 2.5 and 5 mg/m^3 exposure groups, but the animal deaths occurred late in the study (after week 85). Thus, the use of effective tumor incidences for cancer dose-response modeling were appropriate for both the male and female mouse studies.

Table 9. Effective tumor incidences (number of animals alive at day of first tumor) of treatment-related lesions in mice in the two-year inhalation studies of cobalt metal (NTP, 2014a)

Tumor	Cobalt Concentration (mg/m^3)			
	0	1.25	2.5	5.0
Male Mice				
Lung				
Alveolar/bronchiolar adenoma	7/49	11/48	15/43*	3/44
Alveolar/bronchiolar carcinoma	11/50†	38/49**	42/49**	46/49**
Alveolar/bronchiolar adenoma or carcinoma	16/50†	41/49**	43/49**	47/49**
Female Mice				
Lung				
Alveolar/bronchiolar adenoma	3/46	9/49	8/49	10/48*
Alveolar/bronchiolar carcinoma	5/47†	25/49**	38/50**	43/49**
Alveolar/bronchiolar adenoma or carcinoma	8/47†	30/49**	41/50**	45/49**

* $p<0.05$, ** $p<0.01$ for statistical difference from control by Fisher's exact test (calculated by OEHHA)
† Positive trend ($p<0.01$) for tumor type by the Cochran-Armitage trend test (calculated by OEHHA)

Calculation of Single- and Multi-Site Tumor CSFs

For the derivation of the CSF, cobalt metal chamber concentrations of 0, 1.25, 2.5 and 5.0 mg/m^3 were time-adjusted (6.2 hrs/24 hrs × 5 days/7 days) to extrapolate from the intermittent chamber exposure conditions to a continuous exposure over the life span of the animals (*i.e.*, to simulate an annualized average air concentration). The time-adjusted concentrations were 0, 0.2307, 0.4613, and 0.9226 mg/m^3.

The average daily dose, in mg/kg BW-day, is used for calculating the cancer potencies. To calculate the daily dose, the average body weight of the rats and mice over the duration of the study is used to determine the inhalation rate (IR). The weighted average lifetime body weights for control animals in each study were calculated from data of

group mean body weights reported every 1 to 4 weeks during the 2-year exposure period. The average body weights were 453.8, 276.0, 48.5, and 52.7 g for the control male rats, female rats, male mice and female mice, respectively.

A comprehensive analysis of rat minute volume data was undertaken by OEHHA (2018b) to update the IR equation by Anderson (1983) and is shown below (Eq. 6-1a). The analysis incorporates studies since 1988 that more accurately reflect true resting IRs of rats. For mice, the IRs were determined using the equation (Eq. 6-1b) by Anderson (1983). These formulas reflect proportional differences of body weight ($BW^{2/3}$) on the respiratory rate within a species:

rats: IR (m^3/day) = 0.702 × $(BW)^{2/3}$ Eq. 6-1a

mice: IR (m^3/day) = 0.0345 m^3/day × (BW / 0.025 kg)$^{2/3}$ Eq. 6-1b

The calculated average daily IRs during the cobalt exposures are 0.4146, 0.2976, 0.05367, and 0.05672 m^3/day for male and female rats and male and female mice, respectively. The average daily doses (shown in Table 10) could then be calculated with the following equation:

Dose (mg/kg BW-day) = IR × C / BW Eq. 6-2

Where: C = time-adjusted cobalt metal concentration (mg/m^3)

Table 10. Calculated average daily exposed dose (mg/kg-day) of cobalt metal in the rats and mice during the two-year exposures (rounded to two significant figures in the final assessment).

Species **Sex**	**Cobalt Metal Chamber Concentration (mg/m^3)**			
	0	**1.25**	**2.5**	**5.0**
	Daily Exposed Dose (mg/kg-day)			
Rats				
Males	0	0.21	0.42	0.84
Females	0	0.25	0.50	1.00
Mice				
Males	0	0.26	0.51	1.02
Females	0	0.25	0.50	0.99

The US Environmental Protection Agency's (US EPA's) Benchmark dose (BMD) methodology (US EPA, 2017) and Benchmark Dose Modeling Software (BMDS) version 2.7 were used to perform dose-response extrapolation. The multistage-cancer model in BMDS was applied for analysis of single-site tumors for tumor types considered by OEHHA to be treatment-related.

Where tumors of the same histological cell type (*e.g.*, alveolar/bronchiolar adenomas and carcinomas) were observed at a single site and benign tumors were considered to have the potential to progress to malignant tumors, the combined incidence was used for dose-response assessment. These tumor types included alveolar/bronchiolar adenoma and carcinoma for rats and mice (both sexes), benign and malignant pheochromocytoma in male and female rats, pancreatic islets adenoma and carcinoma in male rats, and mononuclear cell leukemia in female rats.

In the cancer dose-response analysis of the female rat study, OEHHA did not include tumor findings judged by NTP to be equivocal (i.e., pancreatic islet adenoma or carcinoma), or the CKE tumors. CKE in female rats was considered a treatment-related tumor by NTP (2014a). However, increases in the incidence of CKE were relatively

small (0/45, 4/43, 1/40, 3/40 for control, low-, mid-, and high-dose groups, respectively) compared with increases in other treatment-related tumors, and were not statistically significant by trend test or pairwise comparison of cobalt-exposed group tumor incidence with controls.

The NTP (2014a) concluded that exposure to cobalt metal led to an increased incidence of mononuclear cell leukemia in female rats, although the trend test applied by the NTP (based on total number of animals examined) did not reach statistical significance (p = 0.118). Lack of a positive trend was likely a result of a plateau response for all non-control cobalt exposures. When converted to an effective tumor incidence by OEHHA, a significant positive trend (p = 0.0426) was observed with the Cochran-Armitage trend test supplied in the BMDS, version 2.7 (US EPA, 2017). Thus, BMD analysis was performed for the leukemia tumor data.

For large datasets such as those by NTP, OEHHA typically sets the benchmark response (BMR) equal to 5%, plus "extra risk" of a tumor response (OEHHA, 2008). The dose associated with this risk is defined as the BMD_{05} and the lower 95% confidence bound on that dose is defined as the $BMDL_{05}$. Instead of calculating an upper bound on β_1 directly, BMDS uses an approximation to calculate the upper bound on β_1 and reports this as the cancer slope factor: BMR/BMDL. The β_i are parameters of the model, which are taken to be constants and are estimated from the data (see Appendix A).

The multistage-cancer polynomial model was fit to the data, which fits most tumor data sets well. First- and second-degree polynomial multistage models were run for all tumor incidence data sets, and the most appropriate model was chosen based on BMD guidance (U.S. EPA, 2016). Briefly, a goodness-of-fit p-value > 0.05 indicates that the model fits the data well, and in cases where more than one model provides an adequate fit, the model with the lowest Akaike Information Criterion (AIC) value is often selected as the best fitting model. The BMD_{05} and $BMDL_{05}$ are shown in Table 11. The degree of polynomial chosen was 1 in all cases, except for adrenal medulla tumors in female rats where a 2nd degree polynomial provided the best fit to the data.

Male and female rats developed tumors in several organ systems following cobalt metal exposure. Basing cancer risk on only one tumor type may underestimate the carcinogenic potential of a chemical that induces tumors at multiple sites. Multisite tumor CSFs were calculated in both male and female rats using MS Combo Model (US EPA, 2017). The BMDS procedure for summing risks over several tumor sites uses the profile likelihood method. In this method, the maximum likelihood estimates (MLEs) for the multistage model parameters (q_i) for each tumor type are added together (i.e., Σq_0, Σq_1, Σq_2), and the resulting model is used to determine a combined BMD. A confidence interval for the combined BMD is then calculated by computing the desired percentile of

the chi-squared distribution associated with a likelihood ratio test having one degree of freedom.

For male rats, multisite tumor analysis was conducted for lung (alveolar/bronchiolar adenoma or carcinoma combined), adrenal medulla (benign or malignant pheochromocytoma combined), and pancreatic islet tumors (pancreatic islets adenoma and carcinoma). In female rats, multisite tumor analysis was conducted for lung (alveolar/bronchiolar adenoma or carcinoma combined), adrenal medulla (benign or malignant pheochromocytoma combined), and mononuclear cell leukemia. Some evidence suggests that pheochromocytoma of the adrenal medulla may be dependent on tumor formation in the lungs (see Cancer Hazard Evaluation section), although NTP (2014a) noted that the evidence is not clear. OEHHA therefore uses the health protective assumption that these two tumor types are independent and considered the lung and adrenal tumors as separate sites in the multi-site analysis. The treatment-related female rat CKE tumor data were not included in the dose-response analysis as they were judged not to contribute significantly to the CSF, based on the relatively small increased incidence (0/45, 4/43, 1/40, 3/40 for control, low-, mid-, and high-dose groups, respectively) compared with increases in other treatment-related tumors, and the absence of any apparent dose-related trend.

For male and female mice, single-site tumor analyses were conducted for lung (alveolar/bronchiolar adenoma or carcinoma combined) tumors.

At the effective dose producing a 5% tumor response, the CSF is calculated as $0.05/BMDL_{05}$ and is in units of $(mg/kg\text{-}day)^{-1}$ (Table 11). The rodent CSFs (CSF_a) were then converted to human equivalents (CSF_h) using body weight ($BW^{3/4}$) scaling:

$$CSF_h = CSF_a \times (BW_h / BW_a)^{1/4} \qquad \text{Eq. 6-3}$$

Using this interspecies scaling factor is preferred by OEHHA because it is assumed to account not only for pharmacokinetic differences (*e.g.*, breathing rate, metabolism), but also for pharmacodynamic considerations, *i.e.*, tissue responses to chemical exposure (U.S. EPA, 2005). Lifetime body weights for control rats and mice of both sexes were calculated from the NTP (2014a) study as described above. The default body weight for humans is 70 kg. The body weight scaling factor assumes that mg/surface area/day is an equivalent dose between species (OEHHA, 2009).

Comparison of the single-site and multisite CSFs in Table 11 shows that the lung tumor human CSF of $27\ (mg/kg\text{-}day)^{-1}$ based on male mice to be the most sensitive estimate of cancer risk (CSF rounded to two significant figures in the final assessment). Therefore, the cancer potency of cobalt metal will be based on this lung tumor response in male mice.

Table 11. BMD_{05}, $BMDL_{05}$, rodent CSFs, and human CSFs for single-site and multi-site tumors in rats and mice resulting from 2-year inhalation exposure to cobalt metal

Tumor type	AIC[a]	*p*-value	BMD_{05} (mg/kg-day)[a]	$BMDL_{05}$ (mg/kg-day)	CSFa - $(mg/kg\text{-}day)^{-1}$	CSFh - $(mg/kg\text{-}day)^{-1}$
Rats						
Alveolar/bronchiolar						
Males	173.14	0.54	0.01647	0.01364	3.66	12.91
Females	202.56	0.54	0.04162	0.03363	1.49	5.96
Adrenal medulla						
Males	217.47	0.27	0.02451	0.01853	2.70	9.51
Females	187.45	0.75	0.1287	0.04931	1.01	4.03
Pancreatic Islet cell						
Males	126.34	0.16	0.1636	0.1029	0.49	1.71
Mononuclear cell leukemia						
Females	277.81	0.065	0.1297	0.06698	0.74	2.95
Multisite: lung-adrenal-pancreatic tumors combined						
Males	NA[b]	NA	0.009291	0.007947	6.29	22.17
Multisite: lung-adrenal-leukemia combined						
Females	NA	NA	0.02828	0.01867	2.68	10.70
Mice						
Alveolar/bronchiolar						
Males	167.47	0.12	0.01446	0.01122	4.46	27.49
Females	188.20	0.57	0.01868	0.01506	3.32	20.04

[a] Akaike Information Criterion
[b] Not applicable

The Multistage model fit to the data and the resulting BMD and BMDL are shown in Figure 1 for alveolar/bronchiolar lung tumors in male mice.

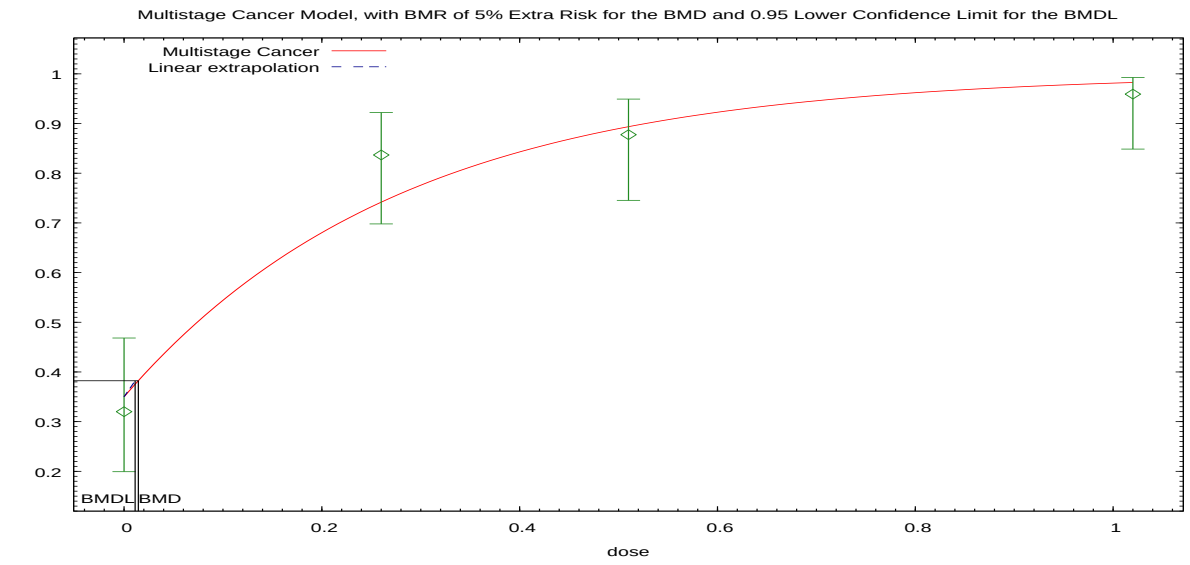

Figure 1. Multistage model fit to the male mouse lung tumor data for cobalt metal. (The benchmark used is the exposure concentration producing 5% tumor response (BMD) with the 95% lower confidence bound (BMDL) on the BMD.)

Figure 1 shows that the lowest non-zero dose is considerably greater than the BMD_{05}. A BMD_{05} well below the lowest administered cobalt metal dose may introduce model uncertainty and parameter uncertainty that increase with the distance between the data and the BMD_{05} (U.S. EPA, 2005). In such cases, using a BMR higher than 5% yields a BMD closer to the lowest non-zero dose. In these cases, OEHHA uses the following formula for the calculation of the cancer slope factor (upper bound on β_1): CSF = -ln(1-BMR)/BMDL. This conservative estimate is derived by solving for β_1 in the risk equation and inserting the result into the log-likelihood equation for β_1 to use it to profile the BMD and obtain the BMDL. The expression CSF = -ln(1-BMR)/BMDL is constant over different values of the BMR and this approach appropriately accounts for the increased curvature in the dose response relationship at higher doses and BMRs (see Appendix A for further discussion).

In deriving a measure of the cancer response to cobalt metal (per mg/kg-day) from the data on male mice, the BMD_{05} was over 10 times lower than the lowest non-zero dose used in the study. This is because a large fraction of the animals in each treatment group, including the lowest dose group, had lung tumors. Because of this, OEHHA calculated the "animal cancer slope factor (CSF_a)", or the "animal cancer potency", for male mice using the exact formula described above: -ln(1-BMR)/BMDL, at a higher BMR, in this case, 15%. As shown in Table 12 below, not only does setting the BMR to 15% result in a viable model from BMDS 3.1, but the choice of BMR has no effect on the value of the animal cancer slope factor when the exact formula is used to calculate the

CSF_a. Applying Eq. 6-3, the human cancer slope factor (CSF_h) is 28.17 $(mg/kg\text{-}day)^{-1}$ (rounded to 28 $(mg/kg\text{-}day)^{-1}$ in the final assessment."

Table 12. Results from BMDS 3.1 using the approximation (BMR/BMDL) and use of the exact formula

BMDS output using the approximation					Exact formula -ln(1-BMR)/BMDL
Model	**BMDL**	**CSF_a**	**BMDS "Recommen-dation"**	**BMDS "Recommendation notes"**	
BMR05 1st degree polynomial	0.01122	4.46	Questionable	BMD 3x lower than lowest non-zero dose BMDL 3x lower than lowest non-zero dose BMD 10x lower than lowest non-zero dose BMDL 10x lower than lowest non-zero dose	= -ln(1-0.05)/0.01122 = 4.57
BMR10 1st degree polynomial	0.02304	4.34	Questionable	BMD 3x lower than lowest non-zero dose BMDL 3x lower than lowest non-zero dose BMDL 10x lower than lowest non-zero dose	= -ln(1-0.10)/0.02304 = 4.57
BMR15 1st degree polynomial	0.03554	4.22	Viable - Recommended	BMD 3x lower than lowest non-zero dose BMDL 3x lower than lowest non-zero dose Lowest AIC	= -ln(1-0.15)/0.03554 = 4.57

Inhalation Unit Risk Factor

The Inhalation Unit Risk (IUR) describes the excess cancer risk associated with an inhalation exposure to a concentration of 1 $\mu g/m^3$ and is derived from the cobalt metal CSF. Using a human breathing rate (BR) of 20 m^3/day, an average human body weight (BW) of 70 kg, and a mg to µg conversion factor (CF) of 1,000, the IUR is calculated as:

$$IUR = (CSF \times BR) / (BW \times CF) \qquad \text{Eq. 6-4}$$

Use of the equation above with the cobalt metal CSF of 28 $(mg/kg\text{-}day)^{-1}$ results in a calculated IUR of 0.0080 $(\mu g/m^3)^{-1}$ or 8.0×10^{-3} $(\mu g/m^3)^{-1}$. Thus, the extra cancer risk associated with continuous lifetime exposure to 1 $\mu g/m^3$ cobalt metal is 8 in one thousand, or 8000 in a million.

Cobalt Sulfate Heptahydrate

Effective Tumor Incidences

The effective tumor incidences (number of tumor-bearing animals over the number of animals alive at the time of first occurrence of the tumor) for treatment-related tumors observed in the NTP studies conducted in rats and mice are shown in Tables 13 and 14, respectively. The NTP individual animal pathology data used to determine the tumor incidences for cobalt sulfate heptahydrate were obtained from the CEBS database (NTP, 1998b).

Table 13. Effective tumor incidences (number of animals alive at day of first tumor) of treatment-related lesions in rats in the two-year inhalation studies of cobalt sulfate hyptahydrate NTP (1998a)

Tumor Type	$CoSO_4 \cdot 7H_2O$ Concentration (mg/m^3)			
	0	0.3	1.0	3.0
Male Rats				
Lung				
Alveolar/bronchiolar adenoma	1/43†	4/44	1/43	6/40
Alveolar/bronchiolar carcinoma	0/24	0/28	3/34	1/25
Alveolar/bronchiolar adenoma or carcinoma	1/43†	4/44	4/43	7/40*
Female Rats				
Lung				
Alveolar/bronchiolar adenoma	0/39‡	1/33	10/37**	9/35**
Alveolar/bronchiolar carcinoma	0/44†	2/41	6/42*	6/46*
Alveolar/bronchiolar adenoma, carcinoma, or				
squamous cell carcinoma	0/44‡	3/41	16/42**	16/46**
Adrenal medulla				
Benign pheochromocytoma	2/39‡	1/37	3/38	8/38
Benign, complex or malignant pheochromocytoma	2/39‡	1/37	4/38	10/39*

* $p<0.05$, ** $p<0.01$ for statistical difference from control by Fisher's exact test (calculated by OEHHA)
† $p<0.05$, ‡ $p<0.01$ for positive trend for tumor type by the Cochran-Armitage trend test (calculated by OEHHA)
[a] Includes benign bilateral pheochromocytoma

Table 14. Effective tumor incidences (number of animals alive at day of first tumor) of treatment-related lesions in mice in the two-year inhalation studies of cobalt sulfate heptahydrate NTP (1998a)

Tumor	$CoSO_4 \cdot 7H_2O$ Concentration (mg/m^3)			
	0	0.3	1.0	3.0
Male Mice				
Lung				
Alveolar/bronchiolar adenoma	9/49†	12/50	13/49	18/48*
Alveolar/bronchiolar carcinoma	4/50†	5/50	7/49	11/48*
Alveolar/bronchiolar adenoma or carcinoma	11/50‡	14/50	19/49	28/48*
Female Mice				
Lung				
Alveolar/bronchiolar adenoma	30/40†	6/47	9/42	10/39*
Alveolar/bronchiolar carcinoma	1/49‡	1/49	4/49	9/45**
Alveolar/bronchiolar adenoma or carcinoma	4/49‡	7/49	13/49	18/45**

* $p<0.05$, ** $p<0.01$ for statistical difference from control by Fisher's exact test (calculated by OEHHA)

† $p<0.05$, ‡ $p<0.01$ for positive trend for tumor type by the Cochran-Armitage trend test (calculated by OEHHA)

Calculation of Single- and Multi-Site Tumor CSFs

For the derivation of the CSF, cobalt sulfate heptahydrate chamber concentrations of 0, 0.3, 1.0 and 3.0 mg/m^3, were time-adjusted (6.2 hrs/24 hrs x 5 days/7 days) to extrapolate from the intermittent lab exposure conditions to a continuous exposure over the life span of the animals (*i.e.*, to simulate an annualized average air concentration). The time-adjusted cobalt sulfate heptahydrate concentrations of 0, 0.055, 0.18, and 0.55 mg/m^3 were used to calculate the average daily dose in mg/kg BW-day.

To calculate the daily dose, the average body weight of the rats and mice over the duration of the study is used to determine the IR. The weighted average lifetime body weights for control animals in each study were calculated from the data o group mean body weights reported every 1 to 4 weeks during the 2-year exposure period. The average body weights were 435.8, 263.3, 41.7 and 40.2 g for the control male rats, female rats, male mice and female mice, respectively.

The IRs were estimated the same as that shown in Eq 6-1a and 6-1b above, where:

For rats, $IR\ (m^3/day) = 0.702\ (BW)^{2/3}$ (OEHHA, 2018b)

For mice, $IR\ (m^3/day) = 0.0345\ m^3/day\ (BW / 0.025\ kg)^{2/3}$ (Anderson, 1983)

The calculated average daily IRs during the cobalt exposures are 0.4035, 0.2884, 0.04852, and 0.04735 m^3/day for male and female rats and male and female mice,

respectively. The IR multiplied by the time-adjusted exposure concentration and divided into the animal body weight gives the dose (Eq. 6-2) in mg/kg BW-day (Table 15).

Table 15. Calculated average daily exposed dose (mg/kg-day) of cobalt sulfate heptahydrate in the rats and mice during the two-year exposures (rounded to two significant figures in the final assessment)

Species Sex	$CoSO_4 \cdot 7H_2O$ Chamber Concentration (mg/m^3)			
	0	0.3	1.0	3.0
	Daily Exposed Dose (mg/kg-day)			
Rats				
Males	0	0.051	0.17	0.51
Females	0	0.061	0.20	0.61
Mice				
Males	0	0.064	0.21	0.64
Females	0	0.065	0.22	0.65

US EPA (2017) BMD methodology (BMDS version 2.7) was applied for single-site tumors using the multistage-cancer model. US EPA BMD guidance (U.S. EPA, 2016) was used to choose the most appropriate model among multistage 1st, and 2nd degree polynomial models, similar as that described above for cobalt metal. Tumor incidences in the low dose groups of both rats and mice were very near or below a 5% tumor response. Combined with the large group sizes (n=48 to 50), a benchmark of 5% tumor response (BMD_{05}) is appropriate for determining the cancer potency (OEHHA, 2009).

The BMD_{05} and $BMDL_{05}$ were determined for treatment-related tumors in each of the studies. Specifically, these values were determined for lung alveolar/bronchiolar adenoma, carcinoma, or squamous cell carcinoma (combined) for rats of both sexes, for lung alveolar/bronchiolar adenoma or carcinoma (combined) for mice of both sexes, and for benign or malignant adrenal medulla tumors (combined) in female rats (Table 16). The incidence of these tumors showed a statistically significant increase above control values at one or more dose levels, and also exhibited a statistically significant positive trend across dose levels (See Tables 12 and 13).

Cobalt sulfate heptahydrate induced tumors at two sites in female rats (tumors in the lung and adrenal medulla). To avoid the potential underestimation of the true carcinogenic risk using a single tumor-site approach, a multi-site tumor risk analysis for female rats was performed, which included both alveolar/bronchiolar adenoma, carcinoma, or squamous cell carcinoma (combined) and benign, complex, or malignant pheochromocytoma (combined). The multi-site tumor CSFs were calculated using MS Combo Model (US EPA, 2017). A description of the MS Combo Model is provided in the cobalt metal CSF derivation above.

Some evidence suggests that pheochromocytoma of the adrenal medulla may be dependent on tumor formation in the lungs (see Cancer Hazard Evaluation section), although NTP (2014a) noted that the evidence is not clear. OEHHA therefore uses the health protective assumption that these two tumor types are independent and considered separate tumor sites for multi-site analysis.

Modeling the single-site tumor incidence data, in all cases the selected models were first degree polynomials either because the model defaulted to polynomial = 1 or because this degree of polynomial provided the best fit to the data (*i.e.*, lowest AIC value). The multistage polynomial model fit the tumor data well (goodness of fit *p*-value $p > 0.05$), except for the lung tumor incidence in female rats. The female rat lung tumor data exhibited a plateau response at the two highest dose groups, resulting in the lack of model fit ($p = 0.0065$). The high dose group was subsequently removed and the three remaining dose groups were rerun using the multistage model. An acceptable model fit to the data was achieved ($p = 0.57$) with these three dose groups (Figure 2). For comparison, a plot showing the multistage model fit to the male mice lung tumor data is given in Figure 3.

At the effective dose producing a 5% tumor response, the cancer slope factor (CSF) is calculated as $0.05/BMDL_{05}$ and is in units of $(mg/kg\text{-}day)^{-1}$ (Table 16). The animal (a) CSFs were then converted to human (h) equivalents using body weight $(BW)^{3/4}$ scaling as shown above in Eq. 6-3. Lifetime body weights for control rats and mice of both sexes were calculated from NTP (2015) as described above. The default body weight for humans is 70 kg.

Comparison of the single-site and multi-site human CSFs in Table 16 shows the human CSF of 13.41 $(mg/kg\text{-}day)^{-1}$ based on the female rat multi-site tumor data to be the most sensitive indicator of cancer risk for cobalt sulfate heptahydrate. Since the cobalt ion is considered to be the primary factor for cancer risk, the cobalt sulfate heptahydrate CSF is normalized to the content of cobalt. As discussed in Section III, generation of the aerosol particles to which the rodents were exposed resulted in formation of primarily cobalt sulfate hexahydrate, although it is expected that environmental exposures to hydrated cobalt sulfate would be to the heptahydrate form. Thus, the molecular weight of cobalt is divided into the molecular weight of cobalt sulfate hexahydrate (58.9 Co / 263.1 $CoSO_4 \cdot 7H_2O = 0.2239$) and multiplying by 13.41 $(mg/kg\text{-}day)^{-1}$ results in an adjusted CSF of 3.0 $(mg\ Co/kg\text{-}day)^{-1}$.

Table 16. BMD_{05}, $BMDL_{05}$, rodent CSFs, and human CSFs for single-site and multi-site tumors in rats and mice resulting from 2-year inhalation exposure to cobalt sulfate heptahydrate

Tumor type	AIC[a]	*p*-value	BMD_{05} (mg/kg-day)[a]	$BMDL_{05}$ (mg/kg-day)	CSF - Rodent $(mg/kg\text{-}day)^{-1}$	CSF - Human $(mg/kg\text{-}day)^{-1}$
Rats						
Alveolar/bronchiolar						
Males	105.27	0.53	0.1644	0.08383	0.60	2.14
Females[b]	80.53	0.57	0.02456	0.01717	2.91	11.75
Adrenal medulla						
Females	100.07	0.60	0.1295	0.07852	0.64	2.58
Multisite: lung/adrenal tumors combined						
Females	NA[c]	NA	0.02064	.01504	3.32	13.41
Mice						
Alveolar/bronchiolar						
Males	246.71	0.96	0.05161	0.03435	1.46	9.35
Females	189.87	0.70	0.07258	0.04819	1.04	6.72

[a] Akaike Information Criterion
[b] The high dose group was removed for benchmark dose modeling to achieve sufficient goodness of fit.
[c] Not applicable

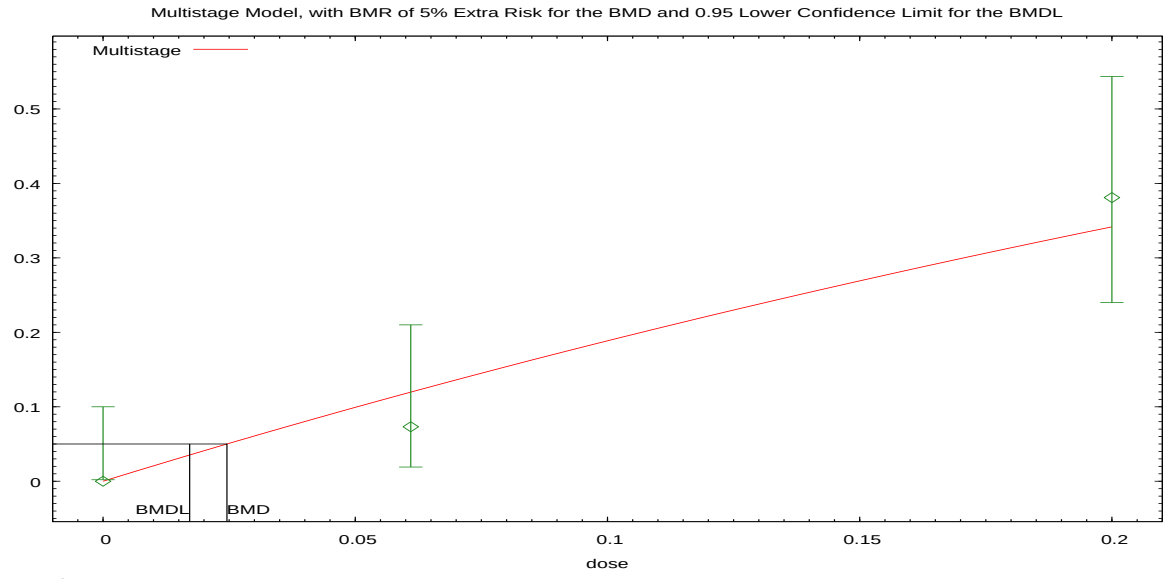

Figure 2. Multistage model fit to the female rat lung tumor data for cobalt sulfate heptahydrate (BMR = 0.05) (The benchmark used is the exposure concentration producing 5% tumor response (BMD) with the 95% lower confidence bound (BMDL) on the BMD)

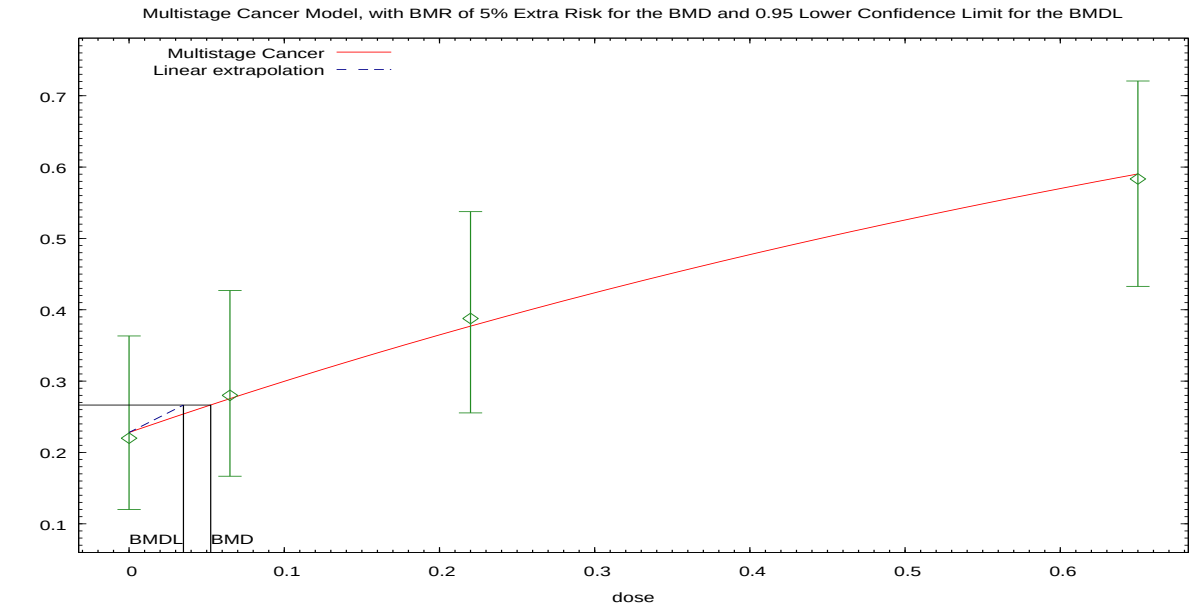

Figure 3. Multistage model fit to the male mice lung tumor data for cobalt sulfate heptahydrate (BMR = 0.05) (The benchmark used is the exposure concentration producing 5% tumor response (BMD) with the 95% lower confidence bound (BMDL) on the BMD.)

Inhalation Unit Risk Factor

The Inhalation Unit Risk (IUR) describes the excess cancer risk associated with an inhalation exposure to a concentration of 1 µg/m^3 and is derived from the cobalt sulfate heptahydrate CSF. Using a human breathing rate of 20 m^3/day, an average human BW of 70 kg, and a mg to µg conversion factor of 1,000, the IUR was calculated as shown in Eq. 6-4 (see above).

Using the cobalt normalized CSF of 3.0 (mg Co/kg-day)$^{-1}$ results in a calculated IUR of 0.00086 (µg Co/m^3)$^{-1}$ or 8.6 $\times$ 10^{-4} (µg Co/m^3)$^{-1}$. Thus, the extra cancer risk associated with continuous lifetime exposure to 1 µg/m^3 cobalt sulfate heptahydrate normalized to the cobalt content is 8.6 in ten thousand, or 860 in a million.

VI. CONCLUSIONS

Carcinogenicity studies conducted by NTP established clear evidence of carcinogenicity for cobalt metal and cobalt sulfate heptahydrate. Release of the cobalt ion in physiological fluids is considered the primary factor for cancer risk. The lungs were the primary site of tumor formation in both rats and mice, and both cobalt metal and cobalt sulfate heptahydrate induced tumors of the same histogenic type in lungs. Cobalt metal and cobalt sulfate heptahydrate exposure also induced tumors at multiple sites in rats.

Carcinogens that produce tumors in more than one species have the greatest potential to induce tumors in other species, including humans. For each cobalt compound, the CSF was based on the most sensitive species and sex. Derivation of an IUR for cobalt metal (8.0×10^{-3} $(\mu g/m^3)^{-1}$) is based on lung tumor formation in male mice. The IUR derivation for cobalt sulfate heptahydrate (8.6×10^{-4} $(\mu g/m^3)^{-1}$) is based on a multi-site analysis of lung and adrenal medulla tumors observed in female rats.

Additionally, *in vitro* studies suggest differences in how the cells internalize cobalt metal particles and water-insoluble cobalt compounds compared to cobalt ions (released by water-soluble cobalt compounds), which are then distributed within the cells. This may explain some of the different genotoxicity results observed for cobalt metal and insoluble cobalt compounds as compared to those observed for soluble cobalt compounds. The *in vitro* studies also suggest that insoluble cobalt compounds, such as cobalt oxides, are internalized and distributed in cells in a manner similar to that of cobalt metal particles.

With the available information, OEHHA recommends that the IUR derived from cobalt metal be used for cobalt metal exposure and for cobalt compounds, such as cobalt oxides, that are water insoluble (≤100 mg/L at 20˚C), but bioavailable in pulmonary fluids. The IUR derived for cobalt sulfate heptahydrate is recommended exclusively for water-soluble cobalt compounds (>100 mg/L at 20˚C), such as the chloride, acetate, and nitrate salts of cobalt.

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Appendix A - derivation of the equation CSF = -ln(1-BMR)/BMDL

Assume a 3rd degree polynomial multistage model is being fit to cancer dose-response data:

$p(d) = 1-\exp(-\beta_0-\beta_1 d- \beta_2 d^2- \beta_3 d^3)$

The cancer slope factor is estimated, using profile likelihood, as the 95% upper bound on β_1. There are different software programs available that can carry out these calculations and it is possible to fit the multistage model to data and to estimate the upper bound on β_1 without using BMDS. This means the estimate of the cancer slope factor should not depend on the choice of BMR. While other software calculates the cancer slope factor (upper bound on β_1) directly, BMDS estimates other values that can be used to calculate the cancer slope factor.

The BMR is defined as:

$$BMR = \text{Extra risk} = [p(d) - p(0)]/[1-p(0)] = 1-\exp(-\beta_1 d- \beta_2 d^2- \beta_3 d^3)$$

Solving for β_1 gives:

$$\beta_1 = -\ln(1-BMR)/d - \beta_2 d- \beta_3 d^2$$

This can be plugged into the log-likelihood equation for β_1 and used to profile the BMD and obtain the BMDL.

An expression for the upper bound on β_1 is:

$$UB\beta_1 = -\ln(1-BMR)/BMDL - \beta_2\, BMDL - \beta_3\, BMDL^2$$

where β_2 and β_3 are the estimates from the profile likelihood iteration used to get the BMDL. When the doses are normalized and the BMDL is small, the second two terms are very small relative to the first term in the expression. A conservative estimate of this upper bound on β_1 drops the two last terms

$$UB\beta_1 = -\ln(1-BMR)/BMDL$$

This expression for the upper bound on β_1 is constant over various choices of the BMR. This is demonstrated in Table 12 in Section V, which shows the results of using BMDS to fit the model to the data using different values for the BMR and using this expression to calculate the cancer slope factor.

Notice that the numerator in the above equation $-\ln(1-BMR) = BMR + BMR^2/2 + BMR^3/3 +\ldots \approx BMR$ when the BMR is small so that an alternate and simpler expression for the upper bound on β_1 is

$$BMR/BMDL$$

This approximation is what BMDS 3.1 uses to estimate the cancer slope factor.