Comments on the „Peer-Review Draft: Report on Carcinogens (RoC) Monograph on Cobalt“ (published June 5, 2015),

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Introduction

The Cobalt Development Institute (CDI) is a non-profit trade association composed of producers, users, recyclers, and traders of cobalt. We promote the sustainable and responsible production and use of cobalt in all its forms.

The CDI acts as a knowledge center for governments, agencies, industry, the media and the public on all matters concerning cobalt and cobalt containing substances. It represents the voice of the cobalt industry on cobalt related health, safety, and environmental issues. We also promote cooperation between members, especially on issues of the environment and human health, and provide a mechanism for the development of independent information concerning the resources, production and safe use of cobalt.

The research that the CDI has conducted on cobalt and its compounds over the years supports a number of points made by the extensive NTP draft Report on Carcinogens (RoC) monograph on cobalt and cobalt substances. The CDI research programme mainly supports the requirements of EU REACH, but more recently also those of the OECD CoCAM review and other reviews in various jurisdictions, including Canada and Australia.

Some of the CDI data do not, however, lead to the same conclusions and we appreciate the opportunity to comment on this draft RoC monograph.

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Comments on tonnages, uses and human exposure:

Page 5, last paragraph states that "Cobalt and several cobalt compounds are high-production volume chemical based on their production or importation into the United States in quantities of 1 million pounds or more per year." This equates to only around 450mt of cobalt (assuming the initial number refers to contained cobalt) which is a very low figure by comparison to other metals such as copper and aluminium. To put this into perspective global refined cobalt production is around 92,000mt (source CDI Refined Cobalt Statistics 2014 – Cobalt News April 2015) whereas refined copper is ~25million mta and aluminium is ~40million mta. Cobalt is essentially a by-product of cobalt and nickel mining and rarely found as a primary metal. Total apparent demand for cobalt in the USA is ~7,600mta (source World Bureau of Metal Statistics 2013).

We would like to ask for the addition of the following uses in the following paragraphs:

- Page 7, section 2.2.3 - Chemical Uses
  'Cobalt compounds are also used in biotechnology as trace mineral additives for fermentation processes for the production of bio-molecular precursors for pharmaceuticals and *in vitro* diagnostics.'
- Page 8, section 2.2.4. – Electronics and 'green' energy.
  The following sentence could be added at the end of the paragraph:
  'It is also used in fermentation processes to produce biogas (energy) from waste.'

Page 9, section 2.3.1 – Evidence of Exposure (and again on p 17 - Summary and synthesis):
Whilst the source of (lower) exposure levels in both the urine and the hair of general public is described as 'unknown', it is stated in the Summary and Synthesis (page 18) that: '[… for the majority of the general public the primary source of cobalt exposure is food;…]'.
This should be reflected in the body of the text.

Page 18 - Summary (last paragraph):
We are aware that the RoC relates to Co compounds, not including cobalamin (vitamin B12). However, the intake of Co substances in scope of the RoC is essential for ruminant animals and bacteria. Both generate vitamin B12, which is in turn essential to humans. This results in an essential intake of Co (as part of cobalamin) of around 0.1 µg Co/day, and it should be clarified that the essentiality of Co results in "non-zero" levels of Co in food and in all tissues of humans.

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General comments on hazard conclusion:

We agree with the RoC conclusion that there is evidence for local carcinogenic activity in rodents by inhalation of Co sulfate heptahydrate (and by extension Co dichloride, Co dinitrate and Co acetate), and Co metal powder. This hazard is reflected by a harmonized classification of 5 soluble Co salts in the EU as CLP[1] carcinogens category Carc. 1B "Presumed to have carcinogenic potential for humans", limited to the inhalation route by the hazard statement H350i (may cause cancer by inhalation). Within 3 months of the release of the Co metal NTP (National Toxicology Program) Study (draft TR 581, released in September 2013), the CDI and its members self-classified Co metal with the same UN GHS category and hazard statement as the soluble salts (Cat 1B carcinogen; H350 i). This new self-classification was widely communicated with down-stream users and customers.

Further, we agree that the evidence for carcinogenic activity in humans is not conclusive, and that hazard conclusions need to be based purely on animal evidence. We are aware of two current epidemiological studies, one updating the "Kokkola cohort" (see previous work by Sauni and Roto; this cohort is currently being updated by the Finnish Institute of Occupational Health). Further, several companies of the hard-metal sector are currently conducting the largest-to-date epidemiological study with over 35,000 subjects (1950 – 2008), including 12 US sites and 9 EU sites. Due to the large study population size, it is expected that there will be opportunities for contrasting cohort attributes, mainly processes and exposures where workers were exposed to Co in the absence of the hard-metal components W and C, in processes upstream of the formation of the Co-WC metal matrix composite. Both studies will be published, and are expected to be available by mid-2016.

These studies are anticipated to increase the statistical power of the database on cancer cases in the Co industry, and are expected to increase the precision of the risk estimates, such as derived no-effect levels (DNELs) and occupational exposure levels (OELs) for cancer in humans.

Several conclusions in the RoC and the related publication (Behl, 2015) have raised significant concerns, however. It is stated in section 6.2.1 of the RoC monograph that "Genotoxicity assays with Co salts and Co metal demonstrate a mutagenic potential…", and the abstract of Behl (2015) concludes that "Taken together, these findings suggest that both forms of Co, soluble and insoluble, appear to be multi-site rodent carcinogens following inhalation exposure."

In the following detailed comments, we highlight relevant new information in the areas of genotoxicity and bioelution testing. We discuss our concerns related to the topics

1. **Genotoxicity and cancer mode of action;**
2. **Grouping of Co substances (by solubility and other *in vitro* markers);**
3. **Interpretation of non-portal of entry neoplasms after inhalation exposure to Co.**

[1] CLP = Regulation (EC) N° 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures.

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Detailed comments

1 - GENOTOXICITY and CANCER MODE ACTION

Genotoxicity of Co substances

The CDI has been investigating possible modes of action of Co related carcinogenicity. Starting in 2010, the database on Co and Co compounds and genotoxicity (public domain as well as industry studies) was reviewed by an external expert (Prof. D. Kirkland). This review highlighted several datagaps and areas for improvement, for example cases where an endpoint was covered only by unreliable information. Based on the first review, the role of genotoxicity in the development of Co-related lung cancer could not be concluded.

A research program was initiated to repeat unreliable studies, or to conduct new studies as needed. All tests were conducted on well-characterized substances, with guideline compliant protocols carried out under Good Laboratory Practice (GLP). These new data were summarized and submitted for publication[2], where they are reviewed in the context of the existing studies.

The abstract of the manuscript is as follows:

> "The genotoxicity of cobalt metal and cobalt compounds has been widely studied. Several publications show induction of chromosomal aberrations, micronuclei or DNA damage in mammalian cells *in vitro* in the absence of S9. Mixed results were seen in gene mutation studies in bacteria and mammalian cells *in vitro*, and in chromosomal aberration or micronucleus assays *in vivo*. To resolve these inconsistencies, new studies were performed with soluble and poorly soluble cobalt compounds according to OECD-recommended protocols. Induction of chromosomal damage was confirmed *in vitro*, but data suggest this may be due to oxidative stress. No biologically significant mutagenic responses were obtained in bacteria, Tk+/- or Hprt mutation tests. Negative results were also obtained for chromosomal aberrations (in bone marrow and spermatogonia) and micronuclei at maximum tolerated doses *in vivo*. Poorly soluble cobalt compounds do not appear to be genotoxic. Soluble compounds do induce some DNA and chromosomal damage *in vitro*, probably due to reactive oxygen. The absence of chromosome damage in robust GLP studies *in vivo* suggests that effective protective processes are sufficient to prevent oxidative DNA damage in whole mammals. Overall, there is no evidence of genetic toxicity with relevance for humans of cobalt substances and cobalt metal."

[2] As of July 8, 2015, the status of the manuscript is *"accepted for publication in "Regulatory Toxicology and Pharmacology" with minor revisions"*

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The same dataset was submitted to the OECD (Organisation for Economic Cooperation and Development) for peer-review under CoCAM (Cooperative Chemicals Assessments Meeting) in October 2014, concluding the following:

> In summary, soluble cobalt salts do not elicit any mutagenic activity either in bacterial or mammalian test systems. However they induce some genotoxic effects *in vitro*, mainly manifest as DNA strand or chromosome breaks, which are consistent with a reactive oxygen mechanism, as has been proposed by various authors. A weight-of-evidence approach was applied, considering positive as well as negative *in vivo* clastogenicity studies and the absence of such chromosome damage in humans that are occupationally exposed to inorganic cobalt substances. It was concluded that effective protective processes exist *in vivo* to prevent genetic toxicity with relevance for humans from the soluble cobalt salts category.
>
> (http://webnet.oecd.org/hpv/ui/handler.axd?id=e5e60085-1f3f-4df5-92f6-8f32c26c3082).

The thorough investigation of the genotoxic properties of a variety of Co substances has allowed the CDI to conclude that (i) Co compounds are not directly mutagenic, and (ii) genotoxicity is not the predominant mode of action (MoA) of cancer. Based on the genotoxicity database update, as well as on all other available data, a MoA for cancer was postulated following the International Programme on Chemical Safety (ICPS) framework for the assessment of carcinogens (Sonich-Mullin, Fielder et al. 2001, Boobis, Cohen et al. 2006, Meek 2008). Below is an extract of the MoA determination, focusing on *key events with associated critical parameters*, *concordance of dose-response relationships*, *temporal association and strength, consistency, and specificity of association of key events and tumor response.*

The Postulated Mode of Action (MoA)

Chronic inhalation exposure of rats and mice to Co metal and -sulfate resulted in inflammation, hyperplasia, and formation of tumors in the lung. The available toxicological data – long-term inhalation studies in animals, generation of reactive oxygen species (ROS) and subsequent DNA damage – give support to the hypothesis that the cancer MoA for Co metal-induced lung tumors involves inflammation, alveolar proteinosis, hyperplasia of the alveolar and bronchiolar epithelia, alveolar/bronchiolar adenoma, and alveolar/bronchiolar carcinoma. It has been hypothesized that Co metal induces alveolar/bronchiolar adenoma and carcinoma through Co-mediated generation of ROS, which subsequently might cause oxidative damage to DNA.

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

Generally, the key events and parameters were the same in both NTP studies (Bucher 1998, Behl and Hooth 2013). Due to the higher exposure regimen, the effects were more pronounced in the Co metal study, which is referred to below.

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Key events, and associated critical parameters

The inflammation and non-neoplastic alterations in the lower regions of the lung observed in the toxicity and carcinogenicity studies after repeated/prolonged inhalation exposure give an indication that Co metal-induced cytotoxicity in target cells contributes to the development of lung tumors in rats and mice (cf. section 2.1; (Behl and Hooth 2013)). Non-neoplastic lesions (alveolar proteinosis, chronic active inflammation) and pre-neoplastic lesions (hyperplasia in the alveolar and bronchiolar epithelia) were found in the lungs of Co-exposed male and female rats. The spectrum of these non- and pre-neoplastic lesions invariably occurred together and presented as a complex mixture of changes, with the difficulty to separate the individual components at times. Subsequently, the most decisive alterations in lung tissue of rats regarded as key events will be detailed based on results of histopathological examinations.

Alveolar epithelial hyperplasia is a multifocal and sometimes focally extensive, discrete, randomly distributed but frequently sub-pleural lesion characterized by proliferation of flat to cuboidal to low columnar epithelial cells lining the alveolar septa; however, the underlying alveolar architecture was generally maintained in the Co studies. Alveolar proteinosis included accumulations of brightly eosinophilic, homogeneous, proteinaceous material, frequently containing acicular cholesterol crystals or cleft-like spaces. These lesions were invariably accompanied by chronic active inflammation consisting predominantly of macrophages and lymphocytes mixed with a lesser number of neutrophils within the alveolar spaces and septa. Bronchiolar epithelial hyperplasia was characterized by proliferation and disorganized crowding of ciliated, cuboidal columnar to pleomorphic epithelia cells lining terminal bronchioles with an extension into adjacent alveolar septa.

Alveolar/bronchiolar adenoma were discrete, expansive, densely cellular masses composed of relatively well-differentiated, uniform, cuboidal to columnar cells supported by a fine fibrovascular stroma and arranged in solid nests or papillary fronds that projected into alveolar space. Alveolar/bronchiolar carcinoma were larger, irregular, poorly circumscribed, un-encapsulated, expansive, locally invasive masses that effaced the lung parenchyma. They were composed of poorly differentiated, moderately to markedly pleomorphic cuboidal, columnar, or polygonal cells with pleomorphic nuclei. The cells were arranged in single to multiple layers, forming irregular papillary or acinar structures and/or solid sheets supported by fibrovascular stroma. Large numbers of inflammatory cell infiltrates, mostly macrophages, accumulated frequently around the alveolar/bronchiolar neoplasms.

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

A number of *in vivo* and *in vitro* studies (Lewis, Demedts et al. 1991, Bucher 1998, Ivancsits, Diem et al. 2002, Behl and Hooth 2013) have shown that Co catalyzes the generation of different reactive oxygen species. Out of them, hydroxyl radicals and singlet oxygen cause mainly oxidative DNA damage. Hydroxyl radicals were able to generate DNA single-strand breaks, whereas DNA single-base damage was caused by the specific reaction of singlet oxygen with guanine producing 8-hydroxy-7,8-dihydroguanine (8-oxo-guanine) thus leading to G→T transversion mutations (Klaunig, Kamendulis et al. 2010).

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

Concordance of dose-response relationships

Considerably increased incidences of alveolar/bronchiolar carcinoma were already noted in male and female rats at the lowest exposure concentrations of 1.25 mg/m^3 in the NTP study on Co metal (38.2% and 21.3%, respectively) (Behl and Hooth 2013). A NOAEC for pulmonary tumor formation was not established in the 2-year bioassays in rats and mice. Incidences for alveolar/bronchiolar carcinomas were 76.8% and 80.6% for male rats and 42.0% and 69.2% for female rats at higher exposures of 2.5 mg/m^3 and 5 mg/m^3, respectively.

With respect to the dose-response of hyperplastic effects assumed to be precursors of tumor development, no NOAEC was noted. In lieu thereof, nearly maximal incidences in the range between 88% and 100% for bronchiolar epithelial hyperplasia and alveolar epithelial hyperplasia were found in rats of each sex at the lowest exposure concentrations of 1.25 mg/m^3. At both higher exposure levels, the nearly maximal incidences remained elevated for both types of hyperplasia.

With respect to inflammation it is to be noted that hyperplastic lesions were invariably accompanied by chronic inflammation of moderate severity in all Co-exposed rats of each sex, while its incidence in controls amounted to about 40%.

In summary, the neoplastic changes co-occurred locally with the non-neoplastic lesions. A plateau-like concentration-response relationship for hyperplastic changes considered as precursor lesion and a dose-response relationship for the development of alveolar/bronchiolar carcinomas were identified. A maximum incidence of hyperplastic lesions of nearly 100% became evident at the LOAEC for tumor formation of 1.25 mg/m^3 with an incidence of 38.2% and 21.3% in male and female rats, respectively.

Thus, the hypothesis that inflammation and hyperplasia of the alveolar and bronchiolar epithelia are key events in the induction of alveolar/bronchiolar carcinomas is supported by the observation that the incidence of the preceding changes is greater than for the latter (carcinomas) at a similar dose. The dose dependency of the increase in the incidence of hyperplasia in the alveolar and bronchiolar epithelia is in concordance with the ultimate incidence of alveolar/bronchiolar carcinomas.

Temporal association

Temporal concordance (reworded according to (Meek 2008)) refers to the observation of key events in sequential order as described in the hypothesized MoA.

A temporal relationship of cytotoxicity-related tumor growth can be assumed for the tumors of the lung (alveolar/bronchiolar carcinomas), because early non-neoplastic lesions (alveolar proteinosis), chronic active inflammation, and hyperplastic findings were seen in rat studies with 16-day and 90-day duration at the same concentrations of Co metal (NTP 2013). In the 16-day exposure study, chronic active inflammation (lung) and bronchiolar hypertrophy (lung) were observed at 2.5 and 5 mg Co metal. Minimal to mild bronchiolar epithelial hyperplasia occurred in all male and female rats

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exposed to 1.25 mg/m^3 Co metal or greater. The severity of the effects generally increased with increasing exposure concentrations.

Thus, the finding of occurrence of bronchiolar epithelial hyperplasia in 2-week and 3-month studies before appearance of tumors (adenomas and carcinomas) after two-year exposure can be regarded as strong support of the postulated MoA. Two-year carcinogenicity assays with satellite groups for interim evaluation and recovery studies are however not available.

Strength, consistency, and specificity of association of key events and tumor response

There is a good correlation between key events and regional tumor incidences and tumor sites. Chronic inflammation, alveolar proteinosis, and hyperplasia of the alveolar and bronchiolar epithelia were seen in that region of the lower respiratory tract where adenoma and carcinoma have been observed.

Consistency was redefined to reflect support of the pattern of effects across species, strains, organs, and test systems for the hypothesized MoA (Meek 2008).

The observation of statistically significant increases in alveolar/bronchiolar carcinomas in both sexes of rats and mice in two-year carcinogenicity studies on analogous Co substances (Co metal and Co(II) sulfate (NTP, 1998), cf. 3.6) and in different strains of rats (Co metal: F344/NTac rats; Co sulfate: F344/N rats) is consistent with the hypothesized MoA.

In order to reflect the described proposed MoA of cancer, further CDI investigations have focused on non-genotoxic modes of action of cancer (generation of ROS, inflammatory response). These studies are ongoing.

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Proposed amendment in RoC monograph on cobalt:

We propose inclusion of a statement clarifying that Co compounds, independent of their solubility in water or artificial biological fluids, have been shown not to induce gene mutations in bacteria or mammalian cell systems, or clastogenic and aneugenic events *in vivo*. Consequently there is no evidence of genetic toxicity with relevance for humans of Co substances and Co metal.

We propose inclusion of a clarifying statement that the MoA of cancer is not genotoxic, but is predominantly driven by local chronic inflammation, alveolar proteinosis, and hyperplasia of the alveolar and bronchiolar epithelia.

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2 - GROUPING of Cobalt compounds

"Soluble" and "insoluble" Co compounds

There is a general concern about the definition and specificity of the terms "soluble" and "insoluble". With regard to the Co compounds, the definition of these terms should include a specification of the relevant fluid. While Co metal powder is poorly soluble in water at 20℃, it is moderately to highly soluble in all physiologically relevant fluids ("moderately to highly bioaccessible"), and known to be highly bioavailable *in vivo*. The following terms are proposed as a more precise way to communicate the behavior of Co compounds in various media:

"Solubility" = solubility in water at 20℃

"Bioaccessibility", expressed in appropriate units* = *in vitro* solubility in artificial biological fluids at 37℃, which is a conservative predictor of bioavailability

"Bioavailability" = Dose to target organ *in vivo*

"Bioelution" = *in vitro* test to determine bioaccessibility of metal compounds

"Poorly soluble particles" = in the context of lung toxicity, particles which are poorly soluble in artificial alveolar or interstitial fluid

*Bioaccessibility can be expressed in different units, common units being "percentage release" ("% release"), "release concentration" or "release rate". The following examples are based on an experimental set-up where 100 mg substance is incubated in 50 ml receptor fluid. The substances have a range of Co contents, from 100% (Co metal powder) to 21% in the case of Co sulfate heptahydrate or even less in the case of the Co carboxylates (often less than 10% Co content).

"Percentage release" is the percentage of the available Co in the compound being tested that is dissolved into the receptor fluid after the standardized incubation time. For example, 79% of a Co powder may be bioaccessible in gastric fluid, whereas Co sulfate heptahydrate (with contains 21% Co) is 100% bioaccessible. Although the % release of Co sulfate heptahydrate is higher than that of Co metal powder, its absolute Co release is smaller. Percentage release is useful to compare a substance's behavior across different fluids, or to apply bioelution information to values such as Reference Doses (RfDs), or other risk assessment determinants.

"Release concentration" is the absolute value of the Co concentration observed in the receptor fluid after the standardized incubation time. In the above example, the release concentration for Co metal powder is around 1.6 mg Co/ml, and for Co sulfate heptahydrate it is 0.43 mg Co/ml. This value often corresponds better to *in vivo* observations, where the same mass-based doses of substances are tested (e.g. to determine acute LD50s). The release concentration better reflects what a tissue may be exposed to assuming equal mass-based dosing of different Co compounds. This unit works best, if one compares "like with like", that is, if powders of similar particle size distribution are compared. In the case of the Co compounds, 28 very different substances are being compared, due to the large variety of substances on the market. In order to adjust for the differences in individual particle size, sometimes, the surface area of the substances is introduced in the unit, by expressing the "release rate".

The release rate is the release concentration divided by the surface area and exposure time. This unit is useful when comparing diverse substances with diverse physical properties, and also may be a better reflection of shorter or limited exposure times, such as a 2 hour "incubation" in the stomach. The examples Co metal powder and Co sulfate heptahydrate above have release rates of 22.3 µg Co/cm^2/h and 3.4 µg Co/cm^2/h, respectively.

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Water solubility is often chosen as an initial screening parameter for bioavailability. However, it is considered to be a poor surrogate for the solubility of metals under physiological conditions, since this is strongly influenced by pH, redox conditions and the presence of various anionic species. For this reason, a more refined approach for the assessment of bioavailability of metals was applied by investigating the bioaccessibility of cobalt substances in various artificial biological fluids *in vitro* (e.g., gastric, or lung fluids). Both Co sulfate and Co metal powder are readily bioaccessible in artificial lung fluids, and therefore both substances represent the same, highly bioavailable, group. Extensive CDI bioelution testing has shown that Co metal powder is in fact amongst the most bioaccessible Co compounds across all biological media, and that Co metal powder is not representative for an "insoluble, poorly bioavailable substance".

The bioelution data underline the necessity to distinguish between highly bioavailable (such as cobalt dichloride and also cobalt metal powder) and poorly bioavailable (such as tricobalt tetraoxide) cobalt substances. Such grouping is corroborated by several repeated dose toxicity (RDT) oral tests, conducted with substances of high and low bioavailability. In a recent guideline compliant GLP 90-day repeated dose toxicity study with Co_3O_4 in rats, the sole observed adverse effect was an increased hematocrit level as a consequence of induced erythropoiesis at the limit dose of 1000 mg Co_3O_4/kg bw/day. No adverse effects were observed in any organ, sex hormone levels or neurological/behavioral parameters.

A significant amount of work has been done and is currently ongoing at the CDI, aimed at determining for all toxicological endpoints which substances belong to the highly bioaccessible or "reactive" group, and which ones belong to the poorly bioaccessible or "inert" group.

The first step of these investigations was the complete assessment of the bioaccessibility of 28 Co compounds, in 6 different artificial physiological fluids (gastric-, intestinal-, interstitial-, alveolar-, lysosomal- fluid and artificial sweat). The data for water, gastric as well as for all lung-relevant fluids is shown in figure 2.1 through figure 2.8. Importantly, the data on gastric fluid has been correlated with effect levels of RDT oral studies, and the study NOAELs are plotted with the bioelution data in figure 2.2. The *in vivo* NOAELs confirm the expected low toxicity of the less bioaccessible substances Co_3O_4 and CoS, and the high expected toxicity of $CoCl_2$ and Co metal powder. The group of the Co carboxylates is not included in the grouping paradigm for oral endpoints, as they cause gastro-intestinal irritation at low doses, irrespective of their bioaccessibility of Co.

The carboxylate NOAEL is based on an apparently Co-independent local gastrointestinal effect. The NOAELs for the inorganic compounds are based on a Co-related systemic effect, an increase in hematocrit and erythrocytes. This effect is observed at the limit dose only for the representative of the poorly bioavailable group (Co_3O_4), resulting in a high NOAEL for the group of the poorly bioaccessible (gastric) inorganic Co compounds. The same hematological effect is observed for $CoCl_2$ (highly bioavailable) at a dose three orders of magnitude lower than that of Co_3O_4, resulting in a low NOAEL for the group of the highly bioaccessible (gastric) inorganic Co compounds.

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In the draft listing of the RoC (chapter 7, preliminary listing recommendation) it is stated that the listing applies to "those cobalt compounds – including soluble and poorly water-soluble cobalt compounds and particles – that can release cobalt ions *in vivo*..." Relating the bioelution data to the RoC draft listing, there is evidence for a group of poorly soluble Co substances that do not release appreciable amounts of Co ions *in vitro*, and that may not release sufficient amounts of Co ions *in vivo* to generate the observed adverse effect. For example, it was demonstrated that Co_3O_4 is poorly soluble/insoluble in lysosomal fluid and that therefore it is not likely that Co_3O_4 will reach the intracellular toxicity threshold.

In order to address toxicological endpoints which may result from Co-ion related as well as local effects, such as inhalation, a second set of *in vitro* experiments is currently ongoing. Here, the "reactivity" of Co compounds with alveolar cells is investigated in terms of their ability to generate ROS, or induce HIF1α, cytokines or markers of cytotoxicity. These studies are currently ongoing.

Further, an *in vivo* inhalation RDT testing of Co_3O_4 is currently ongoing, with a range finder (2-weeks) and a planned 28-day study. Based on the outcome of the *in vitro* work and the 28-day study, the need for a longer-term testing program will be determined.

Until these data are available, the overall toxicological profile of Co_3O_4 can be considered as a demonstration of the fundamental differences between Co substances. Table 1 summarizes the differences in classifications and other properties between Co SO_4, Co metal, and Co_3O_4. The aim of this table is to demonstrate the disparity between a "reactive" group of Co substances (including of $CoSO_4$, Co metal) and an "inert" group of Co substances (represented by Co_3O_4), as well as to demonstrate that Co metal is not a representative of the "insoluble", or "inert" Co substances. Based on all available data, it appears inappropriate to group all Co substances into one group for any toxicological endpoint.

Lysosomal dissolution and lung clearance

There are more inhalation data available for the "reactive" group of Co substances, as opposed to the "inert" Co substances. Potential pathways of lung toxicity for Co_3O_4 were investigated in an *in vitro* study by Ortega et al (2014). This paper reports a mechanism of "trojan horse toxicity" with Co_3O_4 particles in the 100 – 400 nm size range. Based on this study, Co_3O_4 particles enter epithelial cells *in vitro* by clathrin-mediated pathways. Only the clathrin-mediated pathway was shown to be involved in cellular uptake (vesicle size approx. 100 nm), although larger particles were observed intracellularly after exposure.

From the Ortega study, it cannot be concluded whether epithelial cells would be able to ingest poorly soluble particles above the sub-micron size, e.g. by activating phagocytosis. The phenomenon of epithelial uptake of micron-sized particles has been observed *in vitro* and *in vivo*. It is unclear, however, to which extent it plays a quantitative role *in vivo*, especially in situations where a strong inflammatory response is observed, as with exposure to Co compounds. *In vivo*, the presence of poorly soluble particles will immediately recruit immune cells (neutrophils, macrophages), also referred to as "professional phagocytes", to the lung. It is generally assumed that the majority of the particles are taken up by the "professional

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phagocytes", and that these cells return the ingested material to the liver for detoxification. This is in line with the observation that Co levels rise significantly in the liver after 2-week inhalation of Co metal powder. Co metal is moderately soluble in alveolar and interstitial fluid, and causes a strong inflammatory response, thereby triggering the recruitment of immune cells to the lung.

Co metal powder is highly bioaccessible in lysosomal fluid, indicating that it will become bioavailable in the lysosome of a macrophage as well as of an epithelial cell. In both cell types, cell death, membrane degradation and release of the Co ions to neighboring cells may occur. In the case of Co_3O_4, however, Co release in lysosomal fluid is minimal, and would not be expected to lead to cell death of the phagocyte. Instead, removal from the lung and distribution to the liver or lymph nodes for detoxification is expected.

The Ortega paper shows a high overall observed Co content (by ICP-mass spectrometry) in the epithelial cells after *in vitro* exposure to Co_3O_4. However, only a very small portion of the high overall Co content in the cells becomes solubilized in the lysosome. This is in agreement with our bioelution findings (see figure 2.7 and figure 2.8). It is further a confirmation that exposure to very high levels of Co_3O_4 generates very small "dose-to-target" amounts of cobalt ion in the cells (only a fraction of the contained Co is bioavailable). Ortega et al conclude that "cobalt oxide particles would produce significant toxic effects only at very high concentrations, which are not relevant to environmental or even accidental occupational exposures."

Figure 2.9. represents an amended proposed MoA, including micron-sized poorly soluble particles, and the alternative pathway of phagocytosis, which is predominantly relevant for lesser-soluble (in alveolar and interstitial fluid) compounds. All particles can be ingested by either phagocytes or epithelial cells, whereby phagocytosis is thought to be the predominant pathway. Phagocytosis may lead to removal from the lung and detoxification, or to cell death of the phagocyte, and neighboring epithelium may be exposed to Co ions. Solubility of a Co compound in lysosomal fluid may predict which pathway is entered.

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Proposed amendment in RoC monograph on cobalt:

We propose an amendment of the grouping of the Co substances, stating that Co sulfate heptahydrate and Co metal powder both belong to the same, highly bioaccessible, group.

We propose wording to highlight the presence of a separate group, the poorly bioaccessible compounds, represented by Co_3O_4. Members of this group are poorly bioaccessible in all physiological fluids, including lysosomal fluid. Support for the existence of this group with a fundamentally different toxicological profile comes from *in vivo* oral RDT toxicity tests, *in vivo* acute oral and inhalation data, and from *in vitro* testing (bioelution data).

We propose wording stating that there is insufficient information to classify for inhalation carcinogenicity the poorly bioavailable, inert Co substances, represented by Co_3O_4.

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3 - DISTAL SITE NEOPLASMS

In the two NTP studies, rats developed non-portal of entry cancers upon inhalation of Co sulfate heptahydrate and Co metal powder; in some cases the cancers were sex-specific. Mice did not develop any distal-site cancers after inhalation exposure to either form of Co. The tissues of concern were adrenal gland in male (M) and female (F) rats (Co metal powder and Co sulfate heptahydrate), and for Co metal powder only bone marrow/blood (mononuclear cell leukemia; MNCL) in F rats, and pancreas in M rats. The findings of neoplasms in the pancreas for F rats and in the kidney for M rats are reported as being equivocal (Co metal powder study).

Pheochromocytoma

In the publication by Bucher et al. 1999, it is highlighted that historical control rates of pheochromocytoma do not appreciably differ between inhalation and feed studies, but a positive response is more likely to occur in inhalation studies than in studies using other routes of exposure. This finding is corroborated by the complete absence of adverse effects towards the adrenal medulla (benign and malignant pheochromocytoma, hyperplasia) observed in a 90-day repeated dose toxicity via the oral route in rats with cobalt dichloride (Hansen 2015).

In a statistical re-evaluation for chronic active inflammation, interstitial fibrosis, alveolar epithelial hyperplasia, squamous metaplasia, proteinosis, and histiocytosis and the association of pheochromocytoma was investigated over a range of nine recent 2-year NTP particulate inhalation studies. It was concluded that there is an overall association between chronic pulmonary fibrosis and inflammation and the elevated incidence of adrenal pheochromocytoma in the male F344 rat in the NTP inhalation studies (Ozaki, Haseman et al. 2002, Greim, Hartwig et al. 2009).

Taken together, the exposure concentration-dependent increases in the incidences of benign and malignant pheochromocytoma (combined) in all Co metal-exposed male and female rats appear to be related to the inhalation exposure of Co. In addition, exposure to Co sulfate heptahydrate at the highest concentration of 1.14 mg Co/m^3 resulted in an increased incidence of benign and malignant pheochromocytoma in male F344/N rats and to a lesser extent in female F344/N rats. However, a dose dependency could not be demonstrated for the male animals. The mechanistic data suggest that there is strong evidence for a secondary mechanism on the formation of pheochromocytoma, following chronic pulmonary fibrosis and inflammation. This is corroborated by an absence of such findings in sub-chronic studies with oral exposure. Consequently, the elevated incidences of pheochromocytoma in rats after inhalation exposure towards Co sulfate and Co metal are not considered as substance related but rather exposure related. An absence of such findings in mice is indicative for a rat-specific response.

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Occurrence of tumors in the pancreatic islets

In general, it has to be noted that historical control data are limited, as they are based on a dataset of only 100 F344/NTac rats from two carcinogenicity studies. Thus, a sound interpretation of background incidences of spontaneous tumors is not possible.

For comparison, a gender dependent spontaneous incidence of pancreatic islet tumors in F344 rats became obvious from an analysis of two-year inhalation carcinogenicity studies carried out by the NTP (Haseman, Hailey et al. 1998). Accordingly, the spontaneous incidences of adenoma and carcinoma in pancreas islets of male F344 rats were given to be 6.2% (range 2-16%) and 2.7% (range 0-8%), respectively. In female F344 rats, the respective incidences were calculated to be 2.4% (range 0-11%) and 0.7% (range 0-4%). More recently, (Kuroiwa, Ando et al. 2013) reported on an increase of the incidence of pancreatic islet cell adenoma in male F344 rats over the years based on the analysis of control groups of carcinogenicity studies conducted in the same Japanese test facility. The incidence of that pancreatic adenoma in males tended to increase from around 2000 and remained high in recent years (10.5%, 17.1% and 20.5% in 1990-1999, 2000-2004 and 2005-2009, respectively).

These tumors are rare and they were seen for the first time in NTP studies. They were also not observed in other studies with Co substances. In this context, it needs to be considered that the historical control data for this strain and exposure route in this institution are weak. The exact mechanism for induction of these tumors is not well understood and they may result from chronic inflammation of the endocrine hormone producing tissue of the pancreas. However, no evidence of pancreatic inflammation was seen in the other studies with Co. Therefore, these findings should be interpreted with caution, and it does not appear plausible that a causal relationship between Co inhalation exposure and formation of pancreatic islets tumors can be established.

Occurrence of kidney tumors

The neoplasms in the kidney were slightly above the historical control data, but not statistically significant and no overall positive trend was established. They were seen only in one sex (males) and not in females, nor in mice. Also in this context the weakness of the historical control data need to be taken in consideration. Based on the above information, it does not appear plausible that a causal relationship between Co inhalation exposure and formation of kidney tumors can be established.

Mononuclear cell leukemia

This single species, single sex result is of questionable relevance for an assessment of human cancer risk because it was obtained in a rat strain highly predisposed for developing MNCL. The finding of a statistically significant increase of MNCL in female rats is unlikely to be related to Co exposure.

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Distal-site cancer in the context of the proposed MoA of cancer

We would further like to review the non-portal of entry cancer findings in the context of the postulated MoA of cancer. According to the proposed cancer MoA, an increase of cellular Co levels in the target cell is a requirement for the development of cancer. If the distal-site cancers were directly Co-related, there should be evidence for a temporal, local and dose-response association between Co levels and neoplasms.

Tissue Co levels were measured in a 2-week range finding study by the NTP (Co metal study), and neoplasms were observed after a 2-year exposure. According to the proposed MoA (by NTP as well as by CDI), an increase in intracellular Co levels is the first event in the pathway to cancer. In terms of a temporal concordance, Co levels are therefore expected to rise very early in the pathway to cancer. In terms of a local relationship, Co levels are expected to increase in the cell-type and tissue giving rise to neoplasms further down the pathway. Finally, to draw conclusions on a causal relationship between Co and the origin of the neoplasm, it would be expected to observe a dose-response relationship between Co levels and the magnitude of the effect (cancer).

The criterion of temporal concordance is fulfilled for all cases of observed cancers, local and distal-site, as the Co tissue level data stem from 2 week exposures, and the neoplasms were observed after 2 years. This means that in all cases the change in Co tissue levels occurred "upstream" of the occurrence of cancer. The exposure levels were higher in the 2-week experiment than in the 2-year study, to account for differences in dose-to-target, which is dependent on exposure level as well as exposure duration. The exposure levels can therefore not be compared "numerically", but are plotted as low-, mid- and high-exposure group. In figures 3.1 through 3.4, the local- and dose-response relationships are examined by overlaying the incidences of cancer with the Co levels in the respective tissues. On the y1 axis, the Co tissue levels are plotted (blue), on the y2 axis the % incidence of cancer is plotted (red).

It can be seen in figure 3.1, that in the case of the lung carcinoma, the local- and dose-response relationship are evident, giving support to the proposed MoA for lung cells. Further, the proposed MoA is corroborated by observations of inflammatory response, hyperplasia as well as other pre-neoplastic lesions as key events.

In figure 3.2. to figure 3.4., there is no observed local concordance or dose-response relationship. The rise of Co levels in kidney, femur and blood is not associated with the incidence of cancer, and there is insufficient evidence to conclude that these cancers are directly related to Co. Since the key required criterion of an increase in intracellular Co levels in the target cell is not met, there is insufficient evidence to conclude on a Co-related MoA of cancer. Co levels in the pancreas and adrenal medulla were not examined, and it is therefore impossible to conclude whether or not these cancers were directly Co-related or secondary effects.

Finally, the highest increase in Co tissue levels was observed in the liver, in both M and F rats and mice. While it is acknowledged that liver tissue may be less sensitive to ROS-related cancer, due to its high antioxidative capacity, it is interesting to note the complete absence of

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liver neoplasms. The absence of distal-site cancer in the tissue with the highest Co levels, together with the lack of concordance between Co levels and cancer incidence in the tissue with distal-site cancers, indicates that there is little evidence for a direct carcinogenic effect of Co beyond the portal of entry.

****

Proposed amendment in RoC monograph on cobalt:

We propose the inclusion of a statement clarifying that evidence for the proposed MoA of cancer, with its sequential key events (rise in Co tissue levels, inflammation, hyperplasia) has only been observed in the lung.

We propose inclusion of an acknowledgement that the distal-site cancers (kidney and MCL) do not fulfil the key criteria indicating a Co-related MoA, based on the lack of an increase in intracellular Co levels in the target tissues/cells.

We propose inclusion of an acknowledgement that there is insufficient evidence to conclude a direct causal relationship between the distal-site cancers (pancreas and adrenal medulla) and exposure to Co.

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Figures

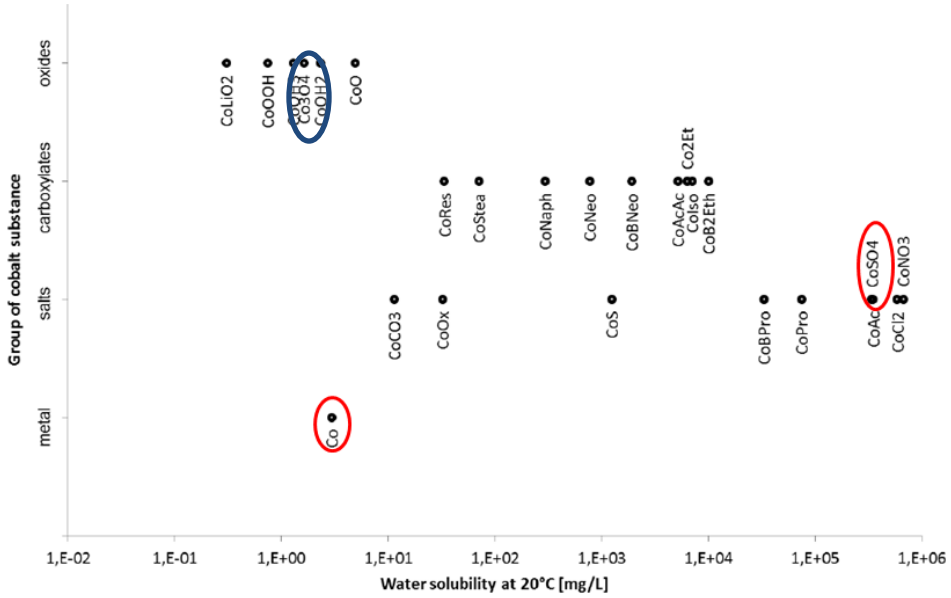

Figure 2.1. Water solubility (according to OECD 105 at 20℃) of Co substances (abbreviated substance names given in Table 2)

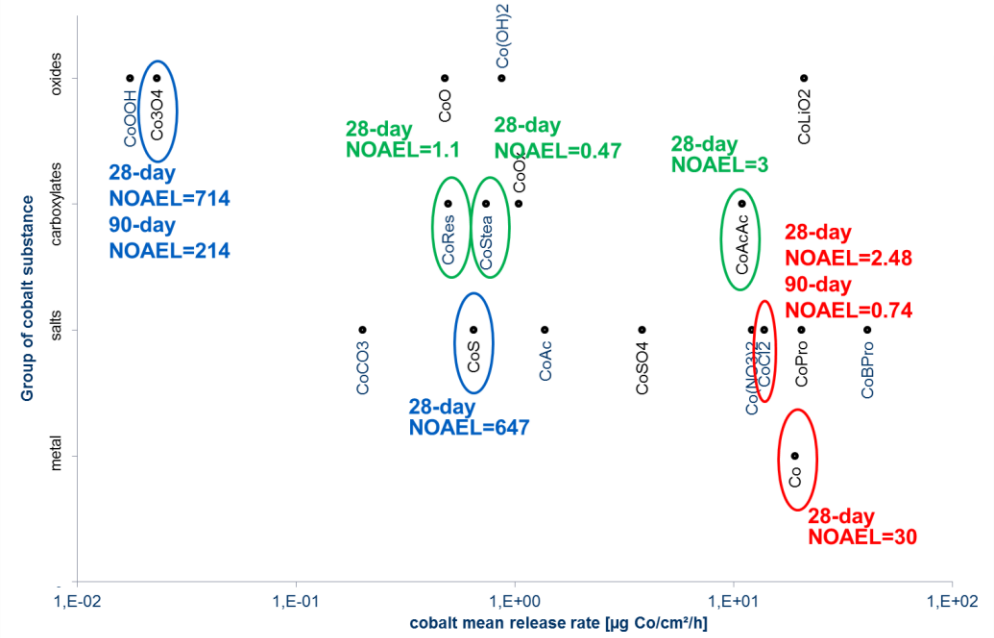

Figure 2.2 Co release rate of Co substances in gastric fluid (2h or 5h exposure, expressed as mean release rate normalized to time and surface area (BET measurement) in µg cobalt per cm² and hour) with *in vivo* oral RDT NOAELs

NOAELs are given in mg/kg bw/day as equivalent Co. Inorganic Co substances: Highly bioaccessible, toxic substances are circled red. Poorly bioaccessible, inert substances are circled blue. The group of Co salts of organic acids (Co carboxylates) displays low NOAELs based on gastrointestinal effects; the local low NOAEL is independent of Co bioaccessibility (abbreviated substance names given in Table 2).

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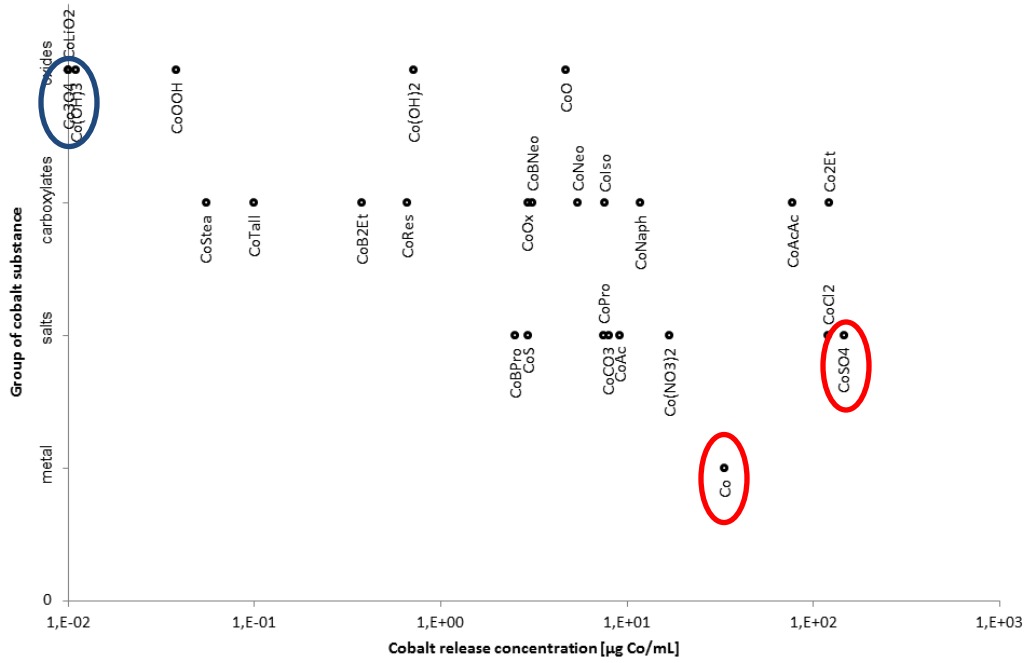

Figure 2.3 Co release concentration in interstitial fluid (2h or 5h exposure)
Co metal powder and $CoSO_4$ are circled red, Co_3O_4 is circled blue (abbreviated substance names given in Table 2).

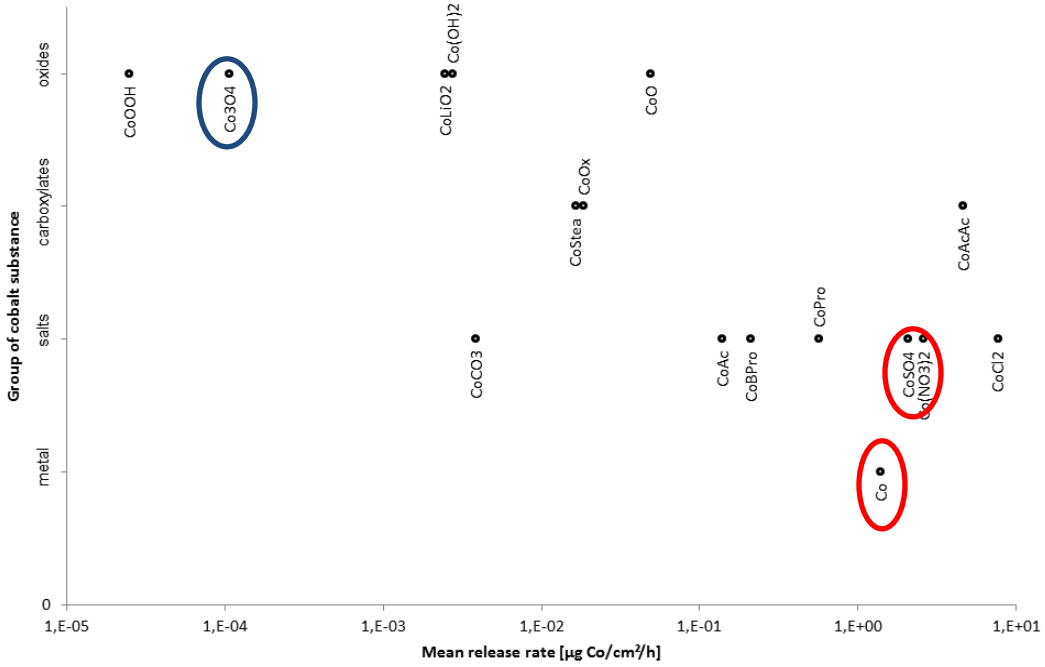

Figure 2.4 Co release rate in interstitial fluid (2h or 5h exposure)
Co metal powder and $CoSO_4$ are circled red, Co_3O_4 is circled blue (abbreviated substance names given in Table 2).

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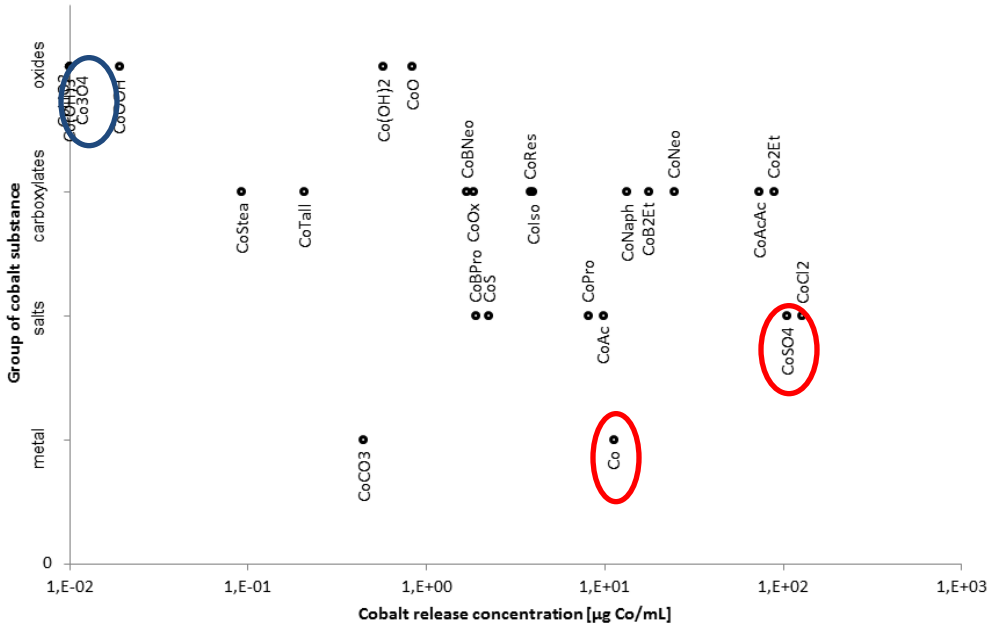

Figure 2.5 Co release concentration in alveolar fluid (2h or 5h exposure)
Co metal powder and $CoSO_4$ are circled red, Co_3O_4 is circled blue (abbreviated substance names given in Table 2).

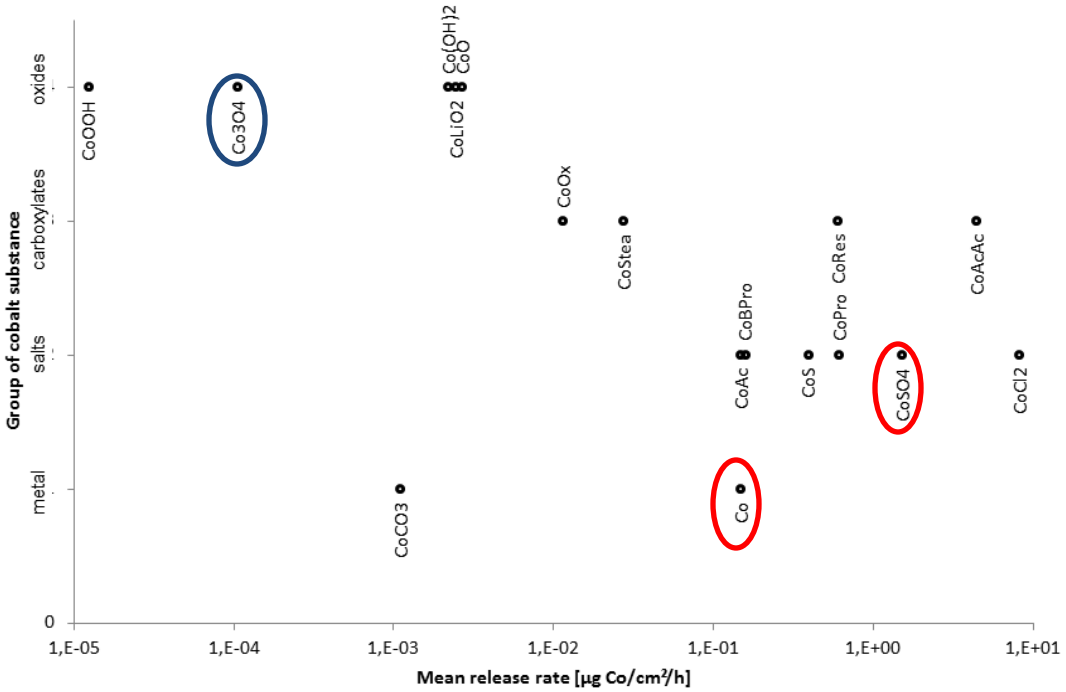

Figure 2.6 Co release rate in alveolar fluid (2h or 5h exposure)
Co metal powder and $CoSO_4$ are circled red, Co_3O_4 is circled blue (abbreviated substance names given in Table 2).

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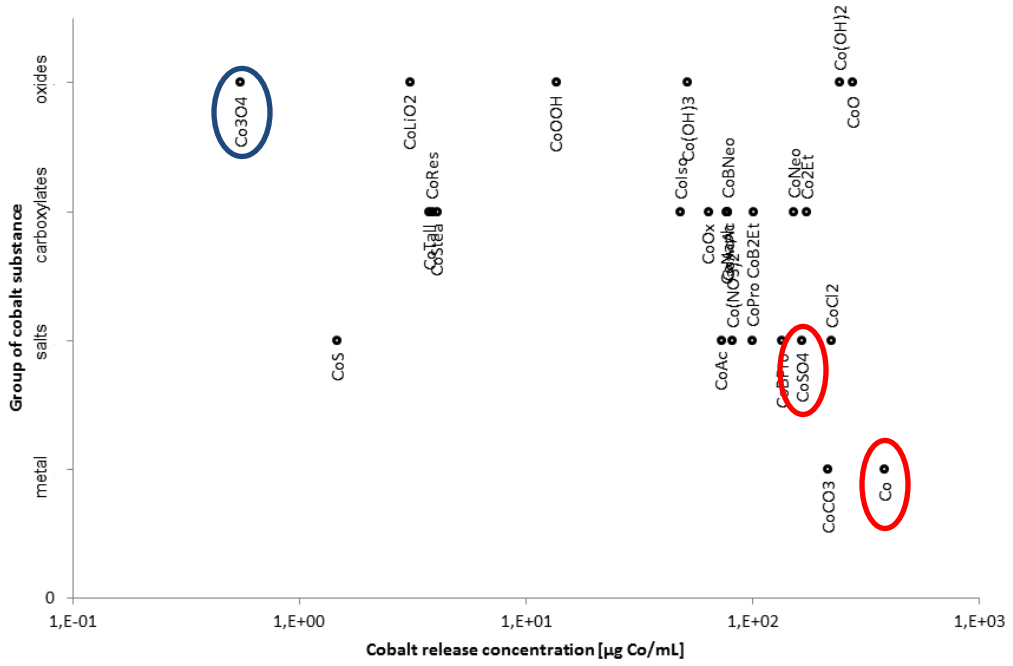

Figure 2.7 Co release concentration in lysosomal fluid (2h or 5h exposure)

Co metal powder and $CoSO_4$ are circled red, Co_3O_4 is circled blue (abbreviated substance names given in Table 2).

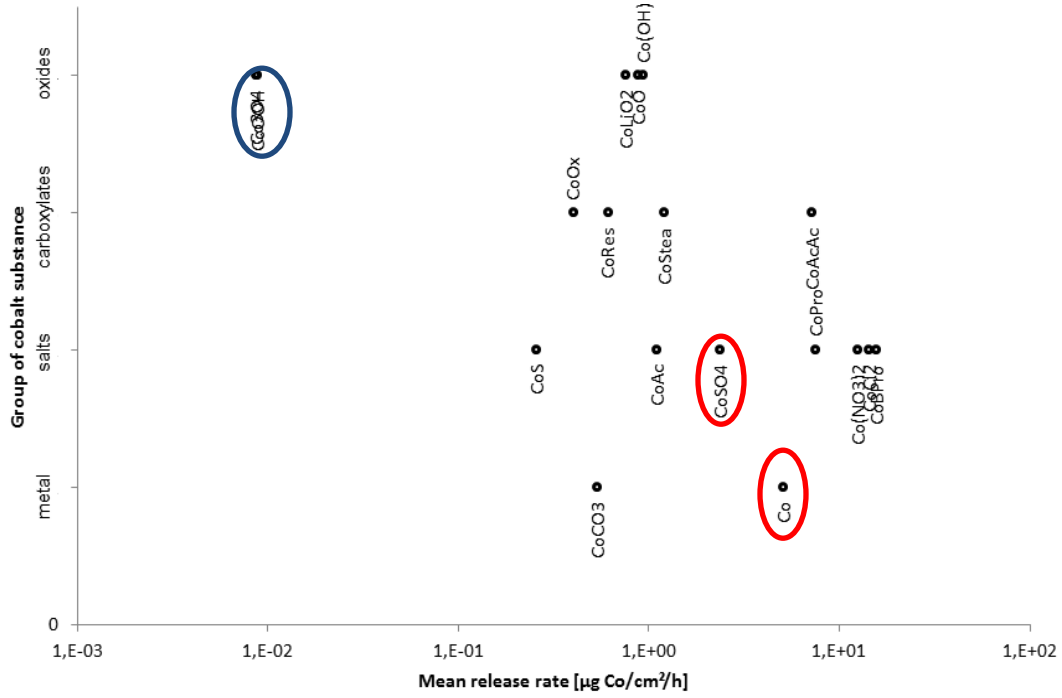

Figure 2.8 Co release rate in lysosomal fluid (2h or 5h exposure)

Co metal powder and $CoSO_4$ are circled red, Co_3O_4 is circled blue (abbreviated substance names given in Table 2).

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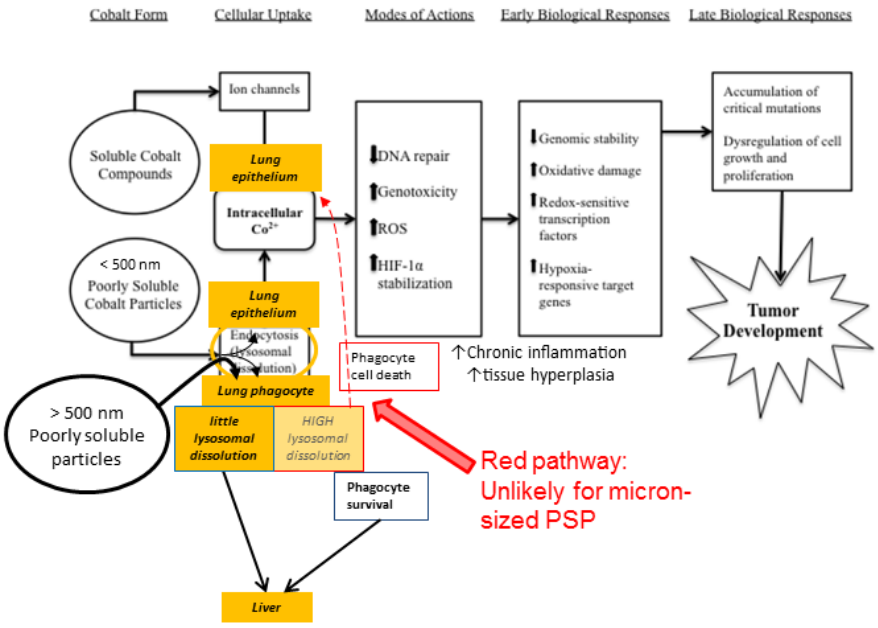

Figure 2.9 Proposed Mode Of Action for Co compounds, including micron-sized poorly soluble particles and the alternative pathway of phagocytosis and lung clearance.

Note: High lysosomal dissolution and phagocyte cell death induced by poorly soluble particles (red boxes) is highly unlikely.

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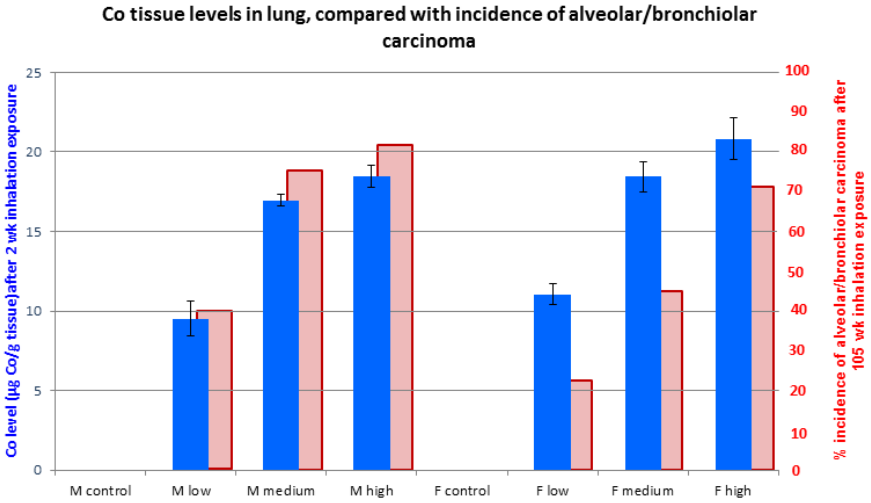

Figure 3.1 Co tissue levels in lung, compared with incidence of alveolar/bronchiolar carcinoma
Data from NTP TR 581, inhalation exposure of rats to Co metal powder. Co lung tissue levels after a 2-week inhalation exposure are plotted on the y1 axis, incidence of lung carcinoma after 2-year exposure is plotted on the y2 axis. Exposure levels (x-axis) were low, medium and high corresponding to 0, 2.5, 5 and 10 mg Co/m3 and 0, 1.25, 2.5 and 5 mg Co/m3 for the 2-week and 2-year exposures, respectively, in males (M) and females (F).

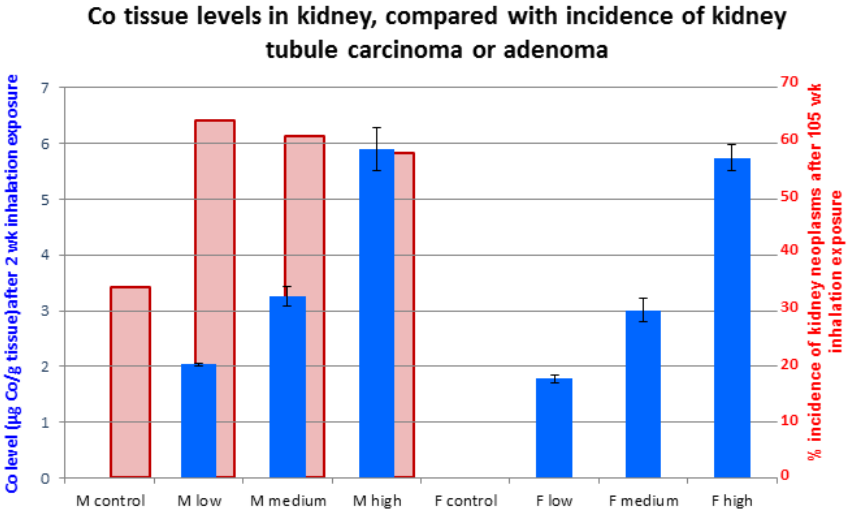

Figure 3.2 Co tissue levels in kidney, compared with incidence of kidney tubule carcinoma or adenoma
Data from NTP TR 581, inhalation exposure of rats to Co metal powder. Co kidney tissue levels after a 2-week inhalation exposure are plotted on the y1 axis, incidence of lung carcinoma after 2-year exposure is plotted on the y2 axis. Exposure levels (x-axis) were low, medium and high corresponding to 0, 2.5, 5 and 10 mg Co/m3 and 0, 1.25, 2.5 and 5 mg Co/m3 for the 2-week and 2-year exposures, respectively, in males (M) and females (F).

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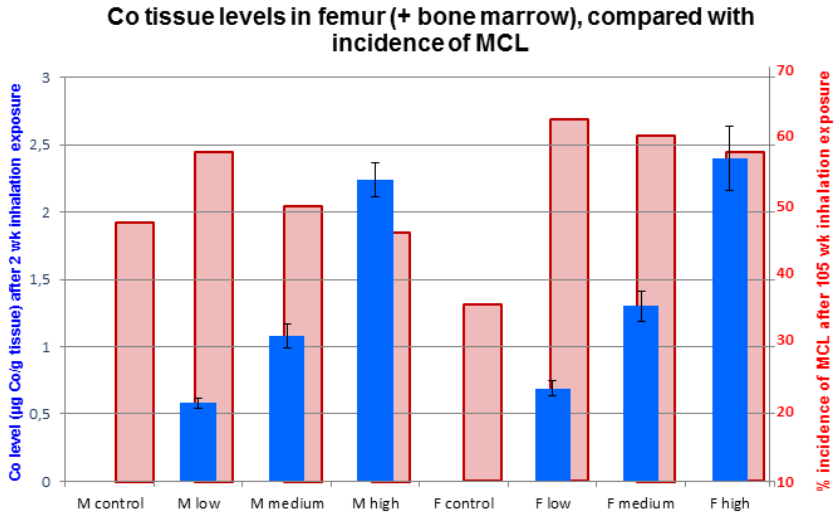

Figure 3.3 Co tissue levels in femur (bone+marrow), compared with incidence of mononuclear cell leukemia (MCL)

Data from NTP TR 581, inhalation exposure of rats to Co metal powder. Co femur tissue levels after a 2-week inhalation exposure are plotted on the y1 axis, incidence of lung carcinoma after 2-year exposure is plotted on the y2 axis. . Exposure levels (x-axis) were low, medium and high corresponding to 0, 2.5, 5 and 10 mg Co/m3 and 0, 1.25, 2.5 and 5 mg Co/m3 for the 2-week and 2-year exposures, respectively, in males (M) and females (F).

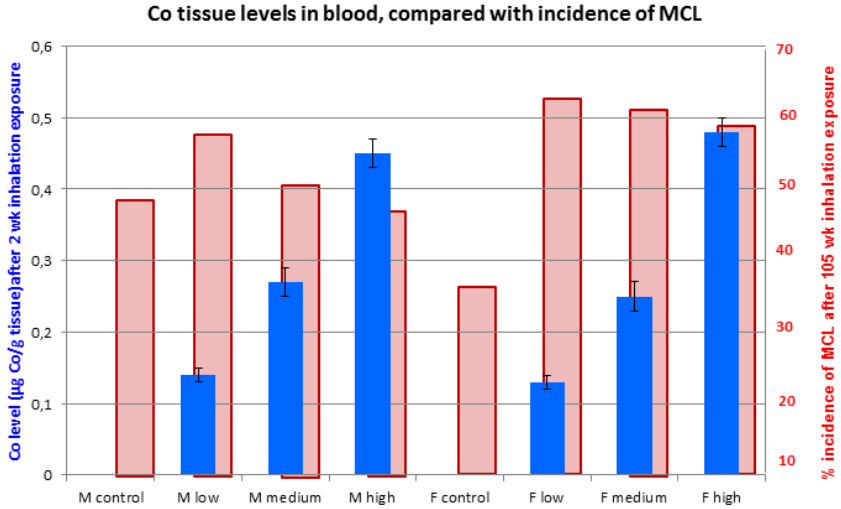

Figure 3.4 Co blood levels, compared with incidence of mononuclear cell leukemia (MCL)

Data from NTP TR 581, inhalation exposure of rats to Co metal powder. Co blood levels after a 2-week inhalation exposure are plotted on the y1 axis, incidence of lung carcinoma after 2-year exposure is plotted on the y2 axis. Exposure levels (x-axis) were low, medium and high corresponding to 0, 2.5, 5 and 10 mg Co/m3 and 0, 1.25, 2.5 and 5 mg Co/m3 for the 2-week and 2-year exposures, respectively, in males (M) and females (F).

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Tables

Table 1: Overview of classifications and other properties of Co SO_4, Co metal, and Co_3O_4

Endpoint	$CoSO_4$ classification or toxicological behavior	Co metal classification or toxicological behavior	Co_3O_4 classification or toxicological behavior	Data / testing method
Serious eye damage / eye irritation	UN GHS: Eye Irrit. 2; H319	UN GHS: Eye Irrit. 2; H319	Conclusive but not sufficient for classification[#]	OECD 437 and OECD 405; guideline compliant GLP testing.
Skin sensitisation	**UN GHS: Skin Sens. 1A; H317**	**UN GHS: Skin Sens. 1; H317**	Conclusive but not sufficient for classification[#]	OECD 429 (Local Lymph Node Assay); guideline compliant GLP testing.
Acute toxicity - oral	**UN GHS: Acute Tox. 4; H302**	Conclusive but not sufficient for classification[#]	Conclusive but not sufficient for classification[#]	OECD 425; guideline compliant GLP testing.
Acute toxicity - inhalation	Waived (stable inhalation test atmosphere cannot be generated)	industry self-classification acute tox Cat 1 for "respirable powders" (> 0.01% respirable fraction)	Conclusive but not sufficient for classification[#]	OECD 436; guideline compliant GLP testing.
Potential to produce ROS (reactive oxygen species) *in vitro*	No ROS generation. (As a control, high ROS generation was observed by Co metal powder)	High ROS generation	No ROS generation. (As a control, high ROS generation was observed by Co metal powder)	Compounds were incubated with human lung cells (A549), in the presence of a radical scavenger (TEMPO), and subsequent ROS measurement by ESR.§
Reproductive toxicity – fertility	**UN GHS: Repr. 1B; H360F**	UN GHS: Repr. 2; H361F, based on read-across from the harmonised classification; not test data on Co metal.	Conclusive but not sufficient for classification[#]	OECD 408 with reproductive toxicity screening; guideline compliant GLP testing: no reproductive effects observed up to limit dose (1000 mg Co_3O_4/kg bw/day), Co metal: industry self-classification (no data); Co sulfate: CLP harmonised classification (literature data)
Reproductive toxicity – developmental	Testing of $CoCl_2$ as representative for highly bioavailable group; no developmental effects in presence of maternal toxicity	Testing of $CoCl_2$ as representative for highly bioavailable group; no developmental effects in presence of maternal toxicity	Testing of Co_3O_4; no developmental effects and complete absence of maternal toxicity at limit dose (1000 mg Co_3O_4/kg bw/day)	OECD 414; guideline compliant GLP testing;
Potential to become bioavailable (in various artificial biological fluids), expressed as % of Co contained in substance	CoSO4 (% release) Gastric 100.0 Intestinal 69.0 Alveolar 50.0 Lysosomal 79.0 Interstitial 72.0 Sweat 97.0	Co metal (% release) Gastric 79.0 Intestinal 10.0 Alveolar 3.0 Lysosomal 93.0 Interstitial 4.0 Sweat 3.0	Co3O4 (% release) Gastric 0.00 Intestinal 0.00 Alveolar 0.00 Lysosomal* 2.00 Interstitial 0.00 Sweat 1.00	Tests carried out at Kirby Memorial Health Center; Wilkes-Barre, PA, USA according to published protocols (Brock and Stopford 2003)

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Endpoint	$CoSO_4$ classification or toxicological behavior	Co metal classification or toxicological behavior	Co_3O_4 classification or toxicological behavior	Data / testing method
Potential to become bioavailable (in different artificial biological fluids), as release concentration (µg Co/ml in receptor fluid) after incubation of equal amounts of substance	CoSO4 (release concentration in µg Co/ml receptor fluid) Gastric 429 Intestinal 258 Alveolar 214 Lysosomal* 331 Interstitial 301 Sweat 408	Co metal (release concentration in µg Co/ml receptor fluid) Gastric 1600 Intestinal 7.6 Alveolar 52.7 Lysosomal* 1907 Interstitial 73 Sweat 58.1	Co3O4 (release concentration in ug Co/ml receptor fluid) Gastric 2.90 Intestinal 0.80 Alveolar 0.14 Lysosomal* 28.00 Interstitial 0.07 Sweat 10.10	Tests carried out at Kirby Memorial Health Center; Wilkes-Barre, PA, USA according to published protocols (Brock and Stopford 2003)

Classification decision based on test data; any classification endpoints without test data are not reported
§ CDI testing on *in vitro* markers (ROS, HIF1α, several cytokines and markers of cytotoxicity); currently ongoing.

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Table 2: Substance name abbreviations

Substance name	Substance abbreviation
Cobalt	Co
Cobalt carbonate	CoCO3
Cobalt di(acetate)	CoAc
Cobalt dichloride	CoCl2
Cobalt dinitrate	CoNO3
Cobalt oxalate	CoOx
Cobalt sulfate	CoSO4
Cobalt sulfide	CoS
Cobalt(2+)propionate	CoPro
Cobalt, borate propionate complexes	CoBPro
Cobalt bis(2-ethylhexanoate)	Co2Et
Cobalt(2+)isononanoate	CoIso
Cobalt(II) 4-oxopent-2-en-2-olate	CoAcAc
Cobalt, borate 2-ethylhexanoate complexes	CoB2Eth
Cobalt, borate neodecanoate complexes	CoBNeo
Fatty acids, tall-oil, cobalt salts	CoTall
Naphthenic acids, cobalt salts	CoNaph
Neodecanoic acid, cobalt salt	CoNeo
Oleic acid, cobalt salt	CoOlea
Resin acids and Rosin acids, cobalt salts	CoRes
Stearic acid, cobalt salt	CoStea
Cobalt dihydroxide	CoOH2
Cobalt hydroxide oxide	CoOOH
Cobalt lithium dioxide	CoLiO2
Cobalt oxide	CoO
Cobalt trihydroxide	CoOH3
Dicobalt trioxide	Co2O3
Tricobalt tetraoxide	Co3O4

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References

Behl, M. and M. J. Hooth (2013). Toxicology studies of cobalt metal (CAS NO. 7440-48-4) in F344/N rats and B6C3F1/N mice and toxicology and carcinogenesis studies of cobalt metal in F344/NTac rats and B6C3F1/N mice (inhalation studies), National Toxicology Program. NTP TR 581

Boobis, A. R., S. M. Cohen, V. Dellarco, D. McGregor, M. E. Meek, C. Vickers, D. Willcocks and W. Farland (2006). "IPCS framework for analyzing the relevance of a cancer mode of action for humans." Crit Rev Toxicol 36(10): 781-792.

Brock, T. and W. Stopford (2003). "Bioaccessibility of metals in human health risk assessment: evaluating risk from exposure to cobalt compounds." J Environ Monit 5(4): 71N-76N.

Bucher, J. R. (1998). Toxicology and carcinogenesis studies of cobalt sulfate heptahydrate (CAS NO. 10026-24-1) in F344/N rats and B6C3F1 mice (inhalation studies) National Toxicology Program. NTP TR 471.

Greim, H., A. Hartwig, U. Reuter, H. B. Richter-Reichhelm and H. W. Thielmann (2009). "Chemically induced pheochromocytomas in rats: mechanisms and relevance for human risk assessment." Crit Rev Toxicol 39(8): 695-718.

Hansen, B. (2015). "Repeated dose 90-day oral toxicity Study of cobalt dichloride hexahydrate in rats; According to OECD guideline 408, EC B.26. -." GLP Study for REACH compliance Final draft report.

Haseman, J. K., J. R. Hailey and R. W. Morris (1998). "Spontaneous neoplasm incidences in Fischer 344 rats and B6C3F1 mice in two-year carcinogenicity studies: a National Toxicology Program update." Toxicol Pathol 26(3): 428-441.

Ivancsits, S., E. Diem, A. Pilger and H. W. Rudiger (2002). "Induction of 8-hydroxy-2'-deoxyguanosine by cobalt(II) and hydrogen peroxide in vitro." J Toxicol Environ Health A 65(9): 665-676.

Klaunig, J. E., L. M. Kamendulis and B. A. Hocevar (2010). "Oxidative stress and oxidative damage in carcinogenesis." Toxicol Pathol 38(1): 96-109.

Kuroiwa, Y., R. Ando, K. Kasahara, M. Nagatani, S. Yamakawa and S. Okazaki (2013). "Transition of historical control data for high incidence tumors in f344 rats." J Toxicol Pathol 26(2): 227-230.

Lewis, C. P., M. Demedts and B. Nemery (1991). "Indices of oxidative stress in hamster lung following exposure to cobalt(II) ions: in vivo and in vitro studies." Am J Respir Cell Mol Biol 5(2): 163-169.

Meek, M. E. (2008). "Recent developments in frameworks to consider human relevance of hypothesized modes of action for tumours in animals." Environ Mol Mutagen 49(2): 110-116.

Ortega, R., C. Bresson, C. Darolles, C. Gautier, S. Roudeau, L. Perrin, M. Janin, M. Floriani, V. Aloin, A. Carmona and V. Malard (2014). "Low-solubility particles and a Trojan-horse type mechanism of toxicity: the case of cobalt oxide on human lung cells." Part Fibre Toxicol 11: 14.

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Ozaki, K., J. K. Haseman, J. R. Hailey, R. R. Maronpot and A. Nyska (2002). "Association of adrenal pheochromocytoma and lung pathology in inhalation studies with particulate compounds in the male F344 rat--the National Toxicology Program experience." Toxicol Pathol 30(2): 263-270.

Sonich-Mullin, C., R. Fielder, J. Wiltse, K. Baetcke, J. Dempsey, P. Fenner-Crisp, D. Grant, M. Hartley, A. Knaap, D. Kroese, I. Mangelsdorf, E. Meek, J. M. Rice, M. Younes and S. International Programme on Chemical (2001). "IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis." Regul Toxicol Pharmacol 34(2): 146-152.

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