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REVIEW ARTICLE



# Cobalt and its compounds: update on genotoxic and carcinogenic activities

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**ABSTRACT**

This article summarizes recent experimental and epidemiological data on the genotoxic and carcinogenic activities of cobalt compounds. Emphasis is on the respiratory system, but endogenous exposure from Co-containing alloys used in endoprostheses, and limited data on nanomaterials and oral exposures are also considered. Two groups of cobalt compounds are differentiated on the basis of their mechanisms of toxicity: (1) those essentially involving the solubilization of Co(II) ions, and (2) metallic materials for which both surface corrosion and release of Co(II) ions act in concert. For both groups, identified genotoxic and carcinogenic mechanisms are non-stochastic and thus expected to exhibit a threshold. Cobalt compounds should, therefore, be considered as genotoxic carcinogens with a practical threshold. Accumulating evidence indicates that chronic inhalation of cobalt compounds can induce respiratory tumors locally. No evidence of systemic carcinogenicity upon inhalation, oral or endogenous exposure is available. The scarce data available for Co-based nanosized materials does not allow deriving a specific mode of action or assessment for these species.

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## Table of contents



## 1. Introduction

Cobalt is an important industrial chemical, technologically and economically. Workers from industries producing or using cobalt compounds can develop respiratory toxicity if they inhale excessive dust concentrations (Lison 2015). In 2001, we reviewed the genotoxic and carcinogenic activities of cobalt compounds, focusing on occupational exposures and the respiratory system. Different modes of action were highlighted among the cobalt compounds (Lison et al. 2001).

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Here, we review new data published since this evaluation. Peer-reviewed papers appeared in the open literature after 2001, providing information on doses and speciation of cobalt compounds, are included. Cobalt compounds are grouped in two classes: (1) those for which the genotoxic activity is essentially mediated by the solubilization of Co(II) ions; and (2) metallic materials for which both surface corrosion and release of Co(II) ions act in concert to exert a genotoxic activity (Figure 1). As bioavailability is an important determinant of the genotoxic and carcinogenic activities of metals (Beyersmann and Hartwig 2008), the capacity of the different compounds to release Co(II) ions is first analyzed. *Bioavailability:* A useful estimate of bioavailability can be provided by the *in vitro* measurement in artificial or natural biological fluids of the fraction of metal available (soluble ions), namely, metal bioaccessibility. These measurements can be conducted with a range of surrogate human tissue fluids mimicking oral (saliva, synthetic gastric or intestinal fluids), inhalation (alveolar, interstitial, lysosomal fluids), dermal exposure (dermal sweat), or internal implantation (lysosomal, cytosolic) (Brock and Stopford 2003; Hillwalker and Anderson 2014). Next, new data on the genotoxicity and other mechanisms relevant for carcinogenicity are summarized. New information on the carcinogenic activity of cobalt compounds includes experimental inhalation data published by the National Toxicology Program (NTP) for cobalt metal (NTP 2014); and additional epidemiological data collected in cobalt and hard metal workers. Cobalt-based nanomaterials, oral exposures to cobalt compounds, as well as endogenous exposure to alloys used in prostheses are also considered.

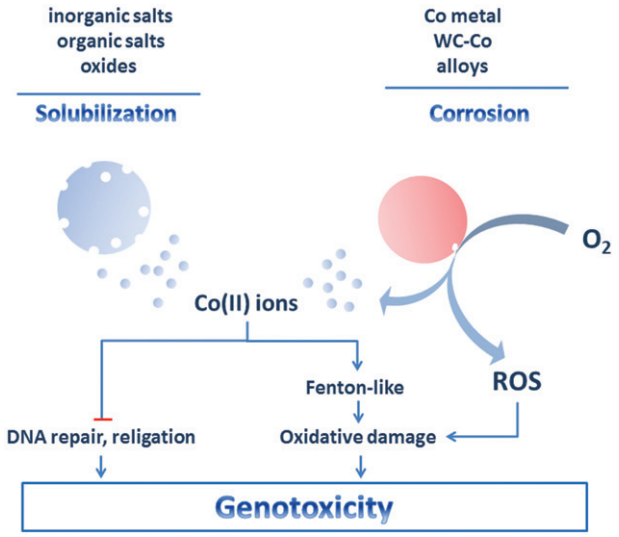


**Figure 1.** Cobalt compounds can be grouped according to the mechanisms of their genotoxic activity. Extracellular and/or intracellular solubilization of salts and oxides releases Co(II) ions that can inhibit enzymes and proteins involved in the maintenance and repair of DNA and produce reactive oxygen species (ROS) through a Fenton-like reaction in the presence of hydrogen peroxide. For metallic materials, surface corrosion is the source of extracellular Co(II) ions, coupled with the reduction of oxygen to produce ROS which contribute to genotoxicity, independently of a Fenton-like reaction.

## 2. Inorganic and organic salts and oxides

Human exposure to cobalt salts and oxides can occur in industrial settings where these materials are produced or used in diverse applications. Occupational exposure is essentially by inhalation of dusts. Oral exposure of the general population is possible through food contamination (Lison 2015).

### 2.1. Bioaccessibility of Co(II) ions

Bioaccessibility data are available for a range of soluble and less soluble cobalt compounds (Table 1). Inorganic cobalt salts, such as Co(II) sulfate or chloride, are highly bioaccessible. All cobalt carboxylates and salts tested are highly/completely soluble in gastric fluid and in intracellular lysosomal equivalents. The solubility of cobalt octoate is quite similar to that of cobalt sulfate in the interstitial and alveolar equivalent fluids as well as in serum, but bioaccessibility is lower for neodecanoate and naphthenate under the same conditions. Cobalt carbonate is more stable than cobalt chloride or cobalt sulfate in the neutral pH surrogate fluids, but is almost completely solubilized in intracellular lysosomal equivalent fluid. Cobalt spinels show minimal dissolution. Co(II) oxide powder is highly soluble at the low pH of the gastric fluid. Co(II) oxide is also almost completely solubilized in

**Table 1.** Bioaccessibility (% soluble cobalt extracted) of cobalt salts and oxides (see references for compound characteristics and testing conditions).

| | Extraction fluids | | | | | | |
|---|---|---|---|---|---|---|---|
| | Ingestion | | Inhalation | | | | |
| | Gastric (pH 1.5) | Intestinal (pH 7.4) | Alveolar (pH 7.4) | Interstitial (pH 7.4) | Serum | Lysosomal (pH 4.5) | |
| Sulfate ($CoSO_4 \cdot 7H_2O$) | 100 | >83.3 | >51.4 | 82.8 | >81.7 | >83.3 | [1] |
| Chloride ($CoCl_2$) | >91.6 | >79.4 | >68 | 78.4 | >85 | >89.6 | [1] |
| Naphthenate ($Co(C_{11}H_7O_2)_2$) | >85.7 | 45.4 | 35.4 | 40 | 42.9 | >79.1 | [1] |
| Octoate ($C_{16}H_{30}CoO_4$) | 100 | 50.8 | >59.6 | >80.4 | >66.9 | 100 | [1] |
| Neodecanoate ($Co(C_{10}H_{19}O_2)$) | 100 | 30.8 | 26.1 | 43.1 | 46.6 | >78.1 | [1] |
| Carbonate ($CoCO_3$) | >92 | 4.1 | 2.9 | 2.2 | 10.1 | >96 | [1] |
| Oxide(II) (CoO) | >91.8 | 2.1 | 2.4 | 9.9 | 19.9 | 92.4 | [1] |
| | | | | | | 92.65(np) | [2] |
| Mixed oxide (II,III) ($Co_3O_4$) | <1 | | | <1 | | 1.6-2 | [3] |
| | | | | | | 11.46(np) | [2] |
| Aluminum spinel ($CoAl_2O_4$) | 0.089 | 0.011 | 0.015 | 0.006 | 0.095 | 0.089 | [1] |

[1]: Stopford et al. (2003).
[2]: Jeong et al. (2015).
[3]: Collier et al. (1992); Henderson et al. (2014); Ortega et al. (2014).
np: nanoparticles.

intracellular lysosomal equivalent fluids. Mixed Co(II,III) oxide and cobalt spinel are sparsely soluble in all fluid equivalents and in serum.

Overall, bioaccessibility measurements allow identifying three distinct groups of cobalt compounds: (1) high bioaccessibility compounds (most inorganic and organic salts), (2) compounds bioaccessible at acidic pH only (gastric or lysosomal context), and (3) low bioaccessibility compounds (mixed oxide(II, III) and spinels). This information helps characterizing how these compounds are absorbed and interact with cells and whether Co(II) ions can mediate a genotoxic and/or carcinogenic activity.

Additional data are available for cobalt oxides. Ortega et al. (2014) showed that the *in vitro* cytotoxic activity of low-solubility cobalt (II,III) oxide particles (aggregates 100–400 nm in size by TEM) is mediated by a Trojan horse mechanism in BEAS-2B epithelial cells, leading to the intracellular release (under acidic conditions in phagolysosomes) of a very small fraction of Co(II) ions (0.07% of the total extracellular dose). Using ICP-MS and micro-PIXE techniques, they found that the intracellular solubilized Co(II) ions content was similar in BEAS-2B cells exposed to equi-cytotoxic doses (LC25 after 72 h) of low-solubility Co(II,III) oxide (6.5 fg/cell for an extracellular dose of 50 μgCo/ml) or completely soluble cobalt chloride (5.4 fg/cell for an extracellular dose of 2.9 μg Co/ml). These results suggest that, although the cytotoxic activity of those compounds was markedly different based on extracellular dose, cytotoxicity might be similarly mediated by intracellular Co(II) ions.

Other uptake mechanisms for Co(II) ions, involving pathways shared with calcium, have recently been described in human red blood cells (Simonsen et al. 2011). The relevance of this uptake pathway for other cell types and its importance for the toxic activity of cobalt compounds have, however, not been documented (Figure 2).

Bioaccessibility does, however, only partly reflect the biologically effective dose of metals. For cobalt in particular, solubilized Co(II) ions precipitate with phosphates or carbonates, complex with different biological ligands, or bind to proteins such as albumin. As a consequence, only a small fraction (about 10%) of solubilized Co(II) ions is estimated to be biologically effective (Bresson et al. 2013; Paustenbach et al. 2013). Whether Co precipitates are taken up by the cells via endocytosis pathways has not been explored. As these processes are complex and difficult to analyze experimentally, an integrative approach to evaluate biologically effective doses of Co(II) ions might, thus, be to measure a specific cellular response to intracellular Co(II) ions. Hypoxia-induced factor alpha (HIF-1α) might represent a specific cellular sensor for this aim. HIF is a heterodimeric transcription factor consisting of two subunits: a ubiquitously expressed α-subunit (oxygen regulated) and a constitutively expressed β-subunit. The HIF-α subunit has three isoforms among which HIF-1α is the best studied. Under normoxic conditions, HIF-1α is rapidly degraded in the cell by the ubiquitin–proteasome pathway; its degradation being mostly controlled through the hydroxylation of two prolyl residues by prolyl hydroxylases. Under hypoxic conditions, HIF-1α is stabilized, dimerizes with HIF-β, and binds to hypoxia-responsive elements of target genes to activate their transcription. Those genes code for proteins and enzymes involved in erythropoiesis, glycolysis, angiogenesis, cell proliferation, autophagy, and apoptosis. Interestingly, HIF-1α is upregulated in many human cancers, including pheochromocytomas (Jochmanova et al. 2013). Co(II) ions are known to block the iron-binding site of dioxygenases, including proline hydroxylases. This activity mimics hypoxia by blocking the iron-binding site of proline hydroxylases, thus stabilizing HIF-1α (Epstein et al. 2001). There is also evidence that Co(II) ions directly bind to HIF-1α and prevent its degradation (Yuan et al. 2003). As a consequence, Co(II) ions induce a coordinated upregulation of a wide series

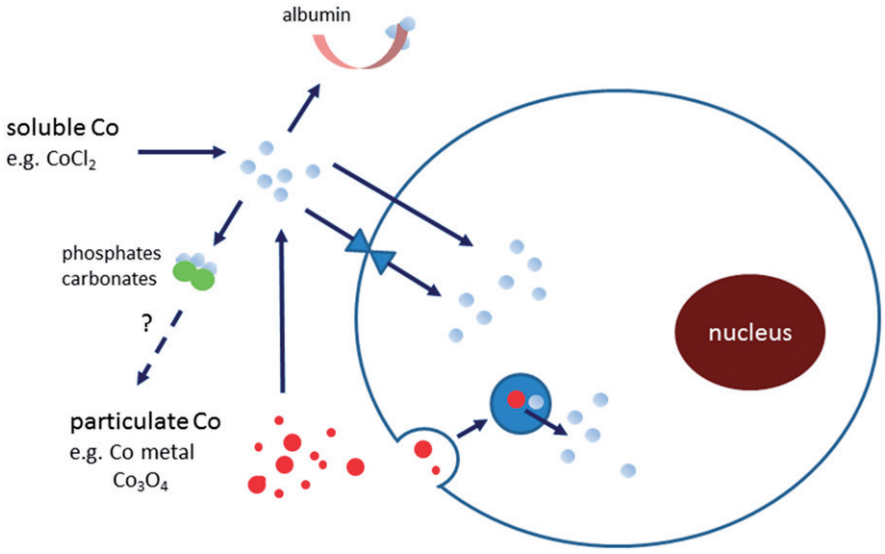


**Figure 2.** The cellular uptake of cobalt compounds. Co(II) ions released extracellularly can precipitate with phosphates/carbonates or bind to the N-terminus of albumin. Free Co(II) ions are available for cellular uptake either via filtration or transporter-mediated uptake. Particulate compounds are taken up by endocytosis, and are dissolved in phagolysosomes and release of Co(II) ions. The respective contribution of each pathway varies with the specie considered (adapted from Beyersmann and Hartwig (2008)).

of adaptive cellular responses to hypoxia, including vascular, metabolic shift, and inflammatory effects relevant for a carcinogenic potential (reviewed in Simonsen et al. (2012)). Saini et al. (2010) showed, in a lung-specific, inducible HIF-1α-deficient mouse model, that after 10 daily administrations of cobalt chloride (30 or 60 μg/mouse by oropharyngeal aspiration), HIF-1α deficiency was associated with increased overall cellularity. A significant alteration of the inflammatory profile shifted from neutrophilic to eosinophilic (biased toward a Th2-mediated reaction) was also recorded, suggesting that HIF-1α has an important role in regulating the lung epithelium response to cobalt-induced inflammation. As HIF-2α appears to be the dominant hypoxia-induced isoform in the human lung, both in epithelial cells and widely throughout the parenchyma, it was hypothesized that loss of HIF-2α in lung epithelium was also involved in cobalt-induced lung inflammation. Proper et al. (2014) showed, with a similar protocol as Saini et al. (2015), that selective HIF-2α deficiency in club and alveolar type II epithelial cells also leads to enhanced eosinophilic (Th2 biased) inflammation and increased goblet cell metaplasia after cobalt chloride treatment. While control mice showed less severe lesions after 10 than after 5 daily doses, this apparent recovery was not observed in HIF-2α-deficient mice, suggesting a role for epithelial HIF-2α in repair mechanisms. These results highlight the important role played by epithelial HIF-1α and HIF-2α signaling to modulate inflammatory and repair responses in the lung. HIF-1α and downstream genes and pathways can, therefore, not only serve as biomarkers of cellular response to Co(II) ions, but their biological functions are also relevant for evaluating the carcinogenic activity of Co(II) ions (Figure 3).

## 2.2. Genotoxicity and other mechanisms

### 2.2.1. In vitro genotoxicity

The genotoxic activity of Co(II) ions is generally associated with their capacity to generate reactive oxygen species (ROS) via a Fenton-like reaction (Lison et al. 2001). The toxicological relevance of this mechanism is, however, unclear because it requires high concentrations of Co(II) ions (millimolar range), in the presence of chelators (anserine, GSH) and hydrogen peroxide (Paustenbach et al. 2013).

A second mechanism accounting for the genotoxic activity of Co(II) ions is their capacity to interact with cellular proteins or enzymes, notably zinc finger motifs that mediate the interactions of many proteins with nucleic acids and/or other proteins, including those involved in DNA repair. Zn(II) ions, bound to cysteine thiol and/or histidine imidazole groups, are crucial for the function of these proteins. Zinc fingers in DNA repair proteins can be altered by a substitution of Zn(II) with another metallic cation, including Co(II). This molecular mechanism accounts for the inhibition of the nucleotide excision repair (NER) pathway in mammalian cells (Lison et al. 2001). A new mechanism, based on altered activity of human topoisomerase II, has been reported as contributing to the genotoxic activity of Co(II) ions. Topoisomerases are responsible for the maintenance of the topological structure of the DNA, namely by correcting overwinding or underwinding of the double helix. This function is, in particular, important during DNA replication and transcription as well as for chromosome structure and segregation. Topoisomerases bind to double-stranded DNA, cut either one or both DNA strands, allowing the DNA to be untangled or unwound. Finally, the DNA helix is resealed through the ligation function of these enzymes. The activity of topoisomerases is dependent on

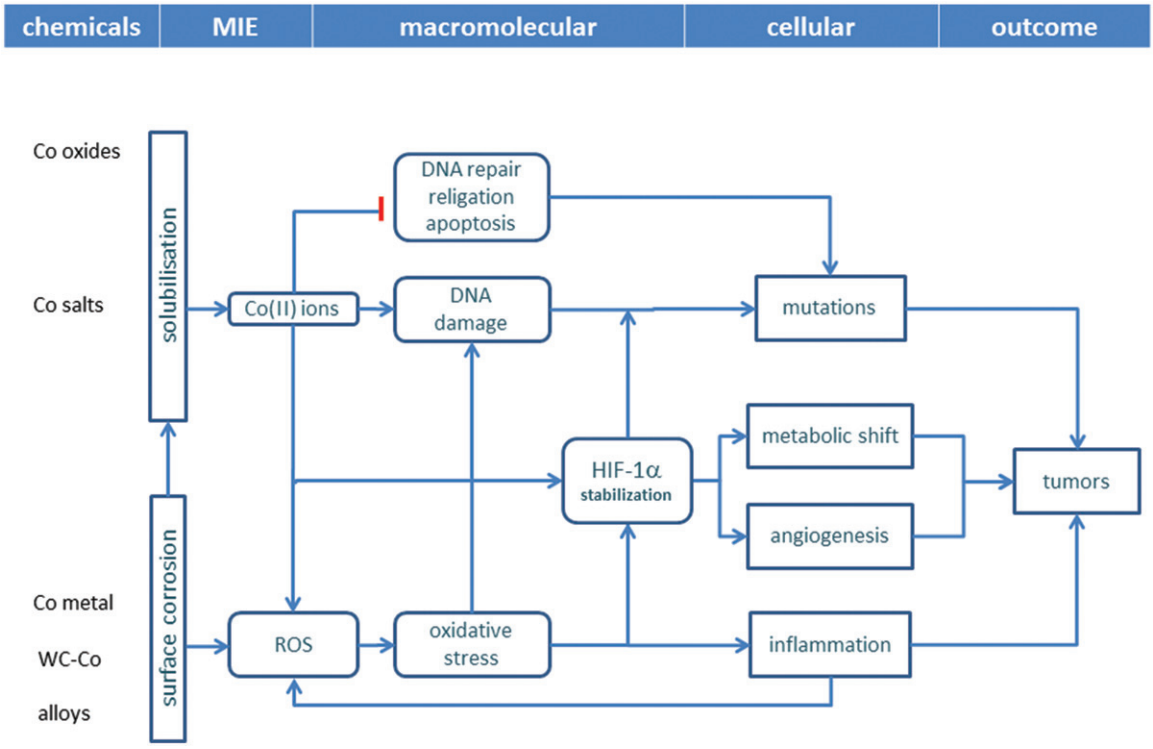


**Figure 3.** Tentative adverse outcome network for the carcinogenic activity of cobalt compounds. MIE: molecular initiating event.

Mg(II) ions intimately coordinated in their structure. Topoisomerase II covalently binds to DNA and forms cleavage complexes which, in low amount, are well tolerated by eukaryotic cells. In humans, topoisomerase IIα is the primary type II isoform. Similar to anti-cancer drugs such as etoposide, mitoxantrone, or doxorubicin, and Co(II) ions (1–10 mM) increase the level of DNA cleavage mediated by purified human topoisomerase IIα in an acellular system. This effect is attributed to a slower religation rate of topoisomerase IIα in the presence of Co(II) ions. A similar effect was reproduced in human breast adenocarcinoma (MCF-7) cells treated with Co(II) ions (200 μM for 24 h) (Baldwin et al. 2004). This new mechanism further contributes to explain the clastogenic activity of Co(II) ions.

Since our initial review, several studies have confirmed the genotoxic activity of Co(II) ions, often associated with moderate or low levels of cytotoxicity. Cobalt chloride induced aneugenic effects (chromosomes gain or loss) in human fibroblasts *in vitro* at doses between 0.005 and 0.100 μM (Figgitt et al. 2010). A series of OECD-compliant studies has been performed with different cobalt compounds in several laboratories (Kirkland et al. 2015). No biologically significant response was recorded in gene mutation tests in bacterial or mammalian cells *in vitro* with soluble compounds. Soluble compounds (cobalt chloride, cobalt sulfate heptahydrate) were able to induce DNA strand breakage *in vitro*, probably through the production of ROS. Tests for oxidative damage showed evidence of dose-dependent increases in DCF fluorescence in A549 cells treated with cobalt sulfate heptahydrate (and cobalt octoate). In addition, DNA strand breaks were significantly aggravated in the comet assay when treated with hOGG1. The main conclusions of this work are consistent with a genotoxic activity of cobalt compounds with a high bioaccessibility of Co(II) ions. The investigators concluded that soluble cobalt compounds can induce the formation of ROS, inhibit DNA repair, and enhance DNA cleavage by topoisomerase (Kirkland et al. 2015).

As the lung is the main target for cobalt toxicity, several authors have specifically studied the genotoxic activity of bioaccessible and less bioaccessible cobalt compounds in human lung cells *in vitro*. Patel et al. (2012) investigated the effect of cobalt chloride (alone and in association with nickel chloride) on cell survival, apoptotic mechanisms, and the generation of ROS in H460 human lung epithelial cells. The clonogenic survival assay (0, 50, 100, 200, and 300 μM cobalt chloride; 24h) resulted in a dose-dependent reduction of survival, and the calcein assay resulted in time-dependent decrease in viability from 200 μM. Signs of apoptosis (poly(ADP-ribose) polymerase (PARP) cleavage) were not observed until 300 μM. Exposure to cobalt chloride led to activation of executioner caspases 3 and 7 at 200 μM. Genotoxic effects were also reported after cobalt chloride exposure: an increased production of ROS (2.5-fold over control) was observed, as well as double-strand breaks with a dose-dependent increase in γ-H2AX expression (pronounced phosphorylation observed at 300 μM). Double-strand breaks were significantly reduced following pretreatment with N-acetylcysteine (NAC), suggesting that ROS are involved in the formation of this genotoxic lesion. The authors concluded that induction of apoptosis by cobalt chloride is due to the production of ROS, which leads to the formation of DNA double-strand breaks. Co-exposure to nickel chloride was significantly more toxic than single exposure (Patel et al. 2012).

Xie et al. (2016) used primary human bronchial epithelial cells (NHBE; isolated from normal donor airways) exposed to cobalt chloride hexahydrate (100, 175, and 250 μM; 24h) and Co(II) oxide (unspecified particle size, 0.1, 0.5, 1, and 5 μg/cm$^2$; 24h) selected as soluble and insoluble (particulate) species, respectively. Exposure to both compounds resulted in a concentration-dependent increase in cytotoxicity and genotoxicity (mainly chromatid lesions). Based on intracellular cobalt concentrations, both compounds induced similar cytotoxicity, whereas cobalt chloride was slightly more genotoxic than Co(II) oxide. With a very similar experimental design, based on intracellular cobalt concentrations, higher cytotoxicity for cobalt chloride hexahydrate but similar genotoxic activity (chromosome damage) was recorded for both compounds in WTHBF-6 lung fibroblasts and hTU1-38 urothelial cells (Smith et al. 2014; Speer et al. 2017). These results are consistent with those of Patel et al. (2012), collectively supporting the hypothesis that cytotoxicity and genotoxicity of cobalt salts and Co(II) oxides are mainly mediated by Co(II) ions through similar mechanisms. Qualitative differences among compounds, however, exist: for soluble compounds, Co(II) ions enter the cells by filtration or using cellular transporters (such as calcium channels or a divalent metal ion transporter), whereas less soluble Co(II) oxides require particle–cell contact and uptake through endocytosis where they release Co(II) ions under acidic conditions (Smith et al. 2014).

The picture is less clear for mixed Co(II,III) oxide particles which might exert a specific genotoxic activity independently of Co(II) ions. Uboldi et al. (2015) compared the genotoxic activity of mixed Co(II,III) oxide particles and cobalt chloride in BEAS-2B cells. Test compounds were exactly the same as in the study by Ortega et al. (2014) discussed above and the higher cytotoxic activity of cobalt chloride compared to Co(II,III) oxide was confirmed. At non-cytotoxic Co-equivalent extracellular doses (1.25–10 μg/ml), both compounds surprisingly exerted almost similar genotoxic activity: 10 and 6% tail DNA after 2h; 3 and 2 γ-HAX foci per cell after 24-h exposure; and 38 versus 47 micronucleated per 1000 binucleated cells after 24-h exposure to 10 μg Co/ml of Co(II,III) oxide and cobalt chloride, respectively. Some evidence was provided that DNA damage and double-strand breaks induced by both compounds were mediated by ROS (Fpg and hOGG1 treatment and protection by NAC, respectively). Since, in BEAS-2B cells, the intracellular Co(II) ion content was at least 10 times lower upon exposure to Co(II,III) oxide than after cobalt chloride (Ortega et al. 2014), these investigators deduced that Co(II,III) oxide induced genotoxic damage by a mechanism independent of Co(II) ions. This interpretation is, however, not completely supported by the data as intracellular levels of Co(II) ions were not determined after 2- or 24-h exposure at extracellular doses used in this study.

DNA damage (comet assay) was also observed in HepG2 cells exposed during 24 or 48 h to Co(II,III) oxide nanoparticles (28 nm in size, 3.5–10.5 μg Co/ml), which could not be reproduced by soluble Co(II) ions at doses equivalent to the

fraction released from Co(II,III) oxide nanoparticles after incubation in the culture medium (Alarifi et al. 2013). This study did, however, not assess whether Co(II,III) oxide particles were taken up by HepG2 cells and released Co(II) ions intracellularly. Co(II,III) oxide nanoparticles (about 22 nm in size) induced direct and oxidative DNA damage (comet assay w/o and with Fpg) in A549 and BEAS-2B cells after 2- and 24-h incubation with doses ranging from 1 to 40 μg/ml (0.7 to 28 μg Co/ml) (Cavallo et al. 2015). The bioaccessibility of these nanoparticles was not documented and a comparison with a soluble cobalt compound was not available. The apparent difference in genotoxic activity of Co(II) and Co(II,III) oxides, which both release Co(II) ions intracellularly via a Trojan horse mechanism, deserves further studies. Several possibilities exist to explain a specific genotoxicity of Co(II,III) oxide particles *in vitro*, including mechanisms such as oxidative stress involving other mediators than solubilized Co(II) ions, or a specific cellular distribution or availability of Co(II) ions after exposure to the mixed(II,III) oxide.

Co(II) ions can also affect epigenetic homeostasis. In A459 and BEAS-2B cells, Co(II) ions (12 μg Co/ml as cobalt chloride, 24 h) increased H3K4me3, H3K9me2, H3K9me3, H3K27me3, H3K36me3, uH2A, and uH2B, but decreased acetylation at histone H4 (AcH4). Different mechanisms were apparently involved, including inhibition of JMJD2A demethylase (H3K9me3, H3K36me3), probably through a competition with iron for binding to the enzyme active site, and inhibition of deubiquitinating enzyme activity (uH2A, uH2B) (Li et al. 2009). Co(II) ions might also affect the activity of several dioxygenases involved in histone methylation processes (e.g. demethylases, TET protein) but this has not been studied yet. How these alterations may lead to altered programs of gene expression and carcinogenesis remains to be elucidated.

Other non-genotoxic mechanisms might also contribute to the carcinogenic activity of Co(II) ions. The human *p53* tumor suppressor gene encodes a multifunctional transcription factor that mediates cellular responses to diverse stimuli, including DNA damage and hypoxia. Hypoxia-mimicking concentrations of cobalt chloride (1–100 μM, 16 h) or HIF-1α alone have been shown to repress the transcription of human p53. The site responsible for repression is the E-box element in the promoter of p53 (Lee et al. 2001). In view of the crucial role of p53 in carcinogenesis, this effect might contribute to the carcinogenic effect of Co(II) ions.

Co(II) ions can also affect other important carcinogenic pathways, including apoptosis. Cobalt chloride (150 μM) reduced the apoptotic death of HepG2 cells induced by t-BHP and serum deprivation, as measured by DNA fragmentation. The hypoxia-mimetic activity of Co(II) ions mediated this effect (Piret et al. 2002). In H460 human lung epithelial cells, normal human lung fibroblasts, and primary human bronchial epithelial cells, Co(II) ions (100–500 μM) accumulated much more than Ni(II) ions, but activated p53 more slowly and more weakly as measured by its accumulation, Ser15 phosphorylation, and target gene expression. As a result, caspase activation and cell death were delayed in cells treated with Co(II) ions compared to Ni(II) ions. Co(II) ions were ineffective in downregulating the p53 inhibitor MDM4 (HDMX) (Green et al. 2013). This low apoptotic response to Co(II) ions might lead to a high survival of cobalt-loaded cells, permitting accumulation of genetic and epigenetic abnormalities contributing to carcinogenesis.

In mouse embryonic fibroblasts exposed to mixed cobalt metal (85–90%): cobalt (II,III) oxide (10–15%) nanoparticles (average size 20 nm; 0.625–10 μg/ml), the growth arrest and DNA damage-inducible 45α protein (Gadd45α), a key player in the cellular responses to a variety of DNA damaging agents, was upregulated under the control of HIF-1α (Feng et al. 2015).

### 2.2.2. In vivo *genotoxicity*

*Local genotoxicity:* No publication exploring the local genotoxic activity of Co(II) ions *in vivo*, for example in the respiratory tract upon inhalation exposure, was located.

*Systemic genotoxicity:* Limited and early experimental studies in hamsters, rats, or mice have shown that acute exposure to near lethal doses of Co(II) ions (cobalt chloride) administered orally or intraperitoneally can induce chromosomal aberrations in bone marrow and/or germ cells (Farah 1983; Palit et al. 1991), micronucleated polychromic erythrocytes in bone marrow (Suzuki et al. 1993), and oxidative DNA damage in renal, hepatic, and pulmonary tissue (Kasprzak et al. 1994). These studies were already critically reviewed in Lison et al. (2001) and more recently in Paustenbach et al. (2013).

In more recent *in vivo* studies applying OECD-compliant protocols with various cobalt compounds, no significant genotoxic response was recorded (Kirkland et al. 2015). No significant increase in bone marrow micronuclei was observed after oral administration of two organic salts, Co(II) acetylacetonate (125, 250, and 500 mg/kg/d orally twice; 23% Co) and Co(II) resinate (187.5, 375, 750, and 1500 mg/kg/d orally twice; 9% Co), in mice. No increase in bone marrow chromosomal aberrations (CA) was observed after oral administration for 5 consecutive days of cobalt sulfate (100, 300, and 1000 mg/kg/d; 21% Co), Co(II) oxide (200, 600, and 2000 mg/kg/d; 78.2% Co), or mixed Co(II,III) oxide (200, 600, and 2000 mg/kg/d; 73.3% Co) in rats. No significant increase in CA or polyploid cells was recorded in spermatogonia of rats treated with cobalt chloride hexahydrate orally by daily gavage for 28 days (3, 10, and 30 mg/kg/d; 24.74% Co). In these assays, the compounds were administered up to the maximum tolerated dose and evidence was available that they reached the target tissue, excluding any possibility of false-negative finding. These investigators concluded that, while the threshold for the genotoxic activity of Co(II) ions can be exceeded at high concentrations *in vitro*, it may not be reached even at high doses *in vivo* (Kirkland et al. 2015).

Chronic exposure of adult male zebrafishes (13 days to a sub-lethal concentration of 25 mg Co/L added as cobalt chloride or sulfate) resulted in DNA strand breaks in sperm cells as well as induction of DNA repair genes (rad51, xrcc5, and xrcc6) in testes. DNA damage was reversible after 6 days in clean water (Reinardy et al. 2013).

### 2.3. Carcinogenic activity

#### 2.3.1. Local carcinogenicity

Previous studies had demonstrated the capacity of Co(II) ions to induce tumors, mainly in tissues locally exposed to high concentrations, but not at distance of the portal of entry (Lison et al. 2001). In the NTP inhalation study conducted with cobalt sulfate heptahydrate, tumors of the respiratory tract were observed in rats and mice (NOEL lung tumors 0.06 and 0.200 mg Co/$m^3$, respectively). No other study exploring the local carcinogenic activity of Co(II) ions was located.

#### 2.3.2. Systemic carcinogenicity

The increased incidence of pheochromocytomas recorded during the NTP study in rats is clearly a secondary phenomenon associated with respiratory toxicity and/or the hypoxia-like effect of Co(II) ions (Ozaki et al. 2002; Greim et al. 2009). Hemangiosarcomas recorded in male mice were linked to *H. pylori* infection in these animals (Bucher et al. 1999). Chronic neoangiogenesis resulting from HIF-1 stabilization by Co(II) ions also appears as a plausible mechanism to explain the excess of hemangiosarcomas. Early carcinogenicity studies with Co(II) ions using dietary exposures or intraperitoneal injections were recently reviewed (Christian et al. 2014). The authors concluded that none of these studies reported a statistically significant increased incidence of primary systemic tumors at any of the tested doses, with one exception reporting systemic fibrosarcomas in Wistar rats (9.6 mg Co/kg/d administered as 10 injections of cobalt chloride over 19 days) but without reporting tumor rates in the control group.

#### 2.3.3. Epidemiology

An incidence study conducted among workers from the Finnish cobalt industry, exposed to cobalt metal, sulfate, carbonate, oxide, and/or hydroxide, in association with nickel and sulfur dioxide, did not record an increased risk of cancer in general or of any specific cancer, including lung cancer ($n = 995$, 6 cases of lung cancer; SIR 0.50; 95%CI 0.18–1.08) (Sauni et al. 2017).

### 2.4. Summary: inorganic and organic cobalt salts and oxides

Recent evidence further supports the paradigm that Co(II) ions released by dissolution either extracellularly or intracellularly drive the genotoxic activity for cobalt salts and oxides. Information on the bioaccessibility of Co(II) ions is, therefore, useful for interpreting or predicting the genotoxic activity of cobalt compounds. A notable exception appears to be mixed Co(II,III) oxide particles that might exhibit a genotoxic activity independently of Co(II) ions (Uboldi et al. 2015). The limited data available for some nanosized materials (Alarifi et al. 2013; Cavallo et al. 2015; Feng et al. 2015) does not allow deriving a specific genotoxic activity or mode of action for these species. The mechanisms mediating the genotoxic and carcinogenic activity of Co(II) ions, including indirect genotoxicity (ROS production, inhibition of DNA repair, and maintenance), altered epigenetic homeostasis, p53 dysregulation, reduced apoptosis, HIF-1α stabilization, and inflammation (Figure 3), are all non-stochastic in nature and thus expected to exhibit a threshold. Inorganic and organic cobalt and Co(II) oxide should thus be considered as genotoxic carcinogens with a practical threshold (Bolt and Huici-Montagud 2008). Consistent with this concept, while the genotoxic activity of Co(II) ions can be detected *in vitro*, no systemic genotoxic response was recorded *in vivo* after oral or parenteral administration. Local *in vivo* genotoxic responses, for example in the respiratory epithelium upon inhalation exposure, remain to be explored. The carcinogenic activity of Co(II) ions appears limited to local tumors (mainly in the respiratory tract upon inhalation exposure), and there is no evidence supporting a systemic carcinogenic activity, notably after oral exposure to Co(II) ions. There is no epidemiological evidence of an increased risk for cancer in workers exposed to inorganic or organic cobalt salts or oxides.

## 3. Metallic cobalt

### 3.1. Cobalt metal particles

Human exposure to cobalt metal particles can occur in industrial settings where these materials are produced or used, mainly for the manufacture of alloys or hard metals. Exposure essentially occurs by inhalation (Lison 2015).

#### 3.1.1. Bioaccessibility of cobalt ions

Metallic Co powder is soluble at the low pH of intracellular lysosomal equivalents (Stopford et al. 2003; Hillwalker and Anderson 2014) (Table 2).

#### 3.1.2. Genotoxicity and other mechanisms

Previous experimental studies showed that cobalt metal particles induced DNA breaks (1.5–15 μg/ml) and micronuclei (0.6–6 μg/ml) in human lymphocytes *in vitro*. The mechanisms involved in this genotoxic activity include ROS generated by surface corrosion and possibly through a Fenton-like reaction, and inhibition of DNA repair (Lison et al. 2001). New studies have been published in recent years.

**3.1.2.1. In vitro *genotoxicity*.** Colognato et al. (2008) compared the genotoxic response of human peripheral leukocytes to cobalt (0.6–6 μg Co/ml) in two forms, metal particles

Table 2. Bioaccessibility (% available cobalt extracted) of cobalt metal particles (see references for test material characteristics and testing conditions).

| | Extraction fluids | | | | |
|---|---|---|---|---|---|
| | Inhalation | | | | |
| | Alveolar (pH 7.4) | Interstitial (pH 7.4) | Serum | Intracellular (pH 4.5) | |
| Extra fine HDFP | 4.8 | 4 | 11.3 | >91.1 | [1] |
| Co metal powder | | | | 30 | [2] |
| Co metal powder | – | 1.2 | – | 64.5 | [3] |

[1]: Stopford et al. (2003).
[2]: Hillwalker and Anderson (2014).
[3]: Henderson et al. (2014).

(median size, 246 nm) and Co(II) ions (cobalt chloride). A significant cellular uptake was observed with cobalt metal particles (about 500 fg/cell after 24-h exposure to 1.2 μg Co/ml), not with Co(II) ions (<10 fg/cell). Significantly higher DNA damage (comet assay) was recorded in cells exposed to cobalt metal particles compared to Co(II) ions. In contrast, similar dose-dependent increases in micronuclei were observed in cells exposed to cobalt metal or Co(II) ions. These findings were reproduced with an apparently different preparation of cobalt metal particles (20–500 nm in size). Cobalt cellular uptake was significantly greater after cobalt metal particles than after Co(II) ions, and cobalt metal particles were more rapidly cytotoxic to BALB/3T3 cells than Co(II) ions (0.006–0.6 μg Co/ml, 2 and 24 h); no difference in cytotoxicity was seen after 72-h incubation. Cobalt metal particles were more genotoxic than Co(II) ions, as assessed by the micronucleus test, but not with the comet assay. No clear dose–response relationship was observed for these effects. A clear interpretation of the latter results is not immediately evident; the difference between mutational events (micronuclei) and primary DNA damage (comet assays) remains difficult to explain. In both studies, the uptake and toxicity of cobalt metal particles were more rapid and/or more intense than with Co(II) ions, consistent with a Trojan horse mechanism for the cellular uptake of cobalt metal (nano)particles in non-phagocytic cells.

In the frame of the inhalation toxicity and carcinogenicity study with cobalt metal, NTP performed a genetic toxicology study in bacteria. Cobalt metal was reported to weakly increase the number of revertants in *S. typhimurium* TA98 in the absence of metabolic activation (S9), although with little or weak dose–response relationship. No mutagenic activity was recorded in the presence of S9. No mutagenic activity of cobalt metal was observed in *S. typhimurium* TA100 or in *E. coli* WP2 uvrA/pkM1010. These results are not consistent with the earlier NTP study conducted with cobalt sulfate heptahydrate in which increased mutation rates were recorded in *S. typhimurium* TA100 (with and without S9) but not in TA98 or TA1535 strains (NTP 1998). A mutagenic activity of cobalt metal in the TA98 strain could not be reproduced in other studies in three laboratories (Kirkland et al. 2015). In mammalian cells, cobalt metal powder did not induce biologically relevant HPRT mutations in mouse lymphoma L5178Y cells when tested up to cytotoxic concentrations in the absence of S9 (50 μg/ml), but did induce weak, reproducible mutagenic effects in the presence of S9 (>20 μg/ml). The authors concluded that there is no convincing or consistent evidence of induction by cobalt metal of gene (point) mutations in either bacteria or mammalian cells *in vitro* (Kirkland et al. 2015).

In addition to a clastogenic activity reported in parallel, Co metal particles (2–6 μg/ml) induced a rapid (15 min) apoptotic response in human lymphocytes *in vitro*, as measured by the Annexin-V and DNA fragmentation assays (Lombaert et al. 2004).

The genotoxic and transforming capacities of cobalt metal nanoparticles (mean diameter, 28 nm) were investigated in mouse embryonic fibroblasts (MEF) after acute and chronic exposure. Short-term exposure experiments (24 h) confirmed that Co nanoparticles produced ROS, and Ogg1-deficient cells were more sensitive to acute cytotoxicity and oxidative DNA damage. Chronic exposure (12 weeks to sub-cytotoxic doses of 0.05 or 0.1 μg Co/ml) induced cell transformation, as assessed morphologically and by increased secretion of metalloproteinases and anchorage-independent cell growth. Ogg1-deficient cells were also more sensitive to cobalt metal nanoparticles in the transformation assay (Annangi et al. 2015).

***3.1.2.2. In vivo genotoxicity.*** *Local genotoxicity:* Mutation of Kras, Egfr, and p53, three genes relevant for human lung cancers, was analyzed in lung tumors in rats and mice exposed to cobalt metal in the NTP study (Hong et al. 2015). The main finding was frequent G→T transversions in codon 12 of exon 1 in Kras (31 and 67% of cobalt metal induced lung tumors in rats and mice, respectively). No Kras mutation was found in spontaneous tumors in control rats (historical not concurrent controls). G→T transversions correlated with 8-hydroxyguanosine adducts, possibly representing a signature of inflammation and oxidative stress associated with cobalt metal exposure. In view of the weak, if any, activity of cobalt metal in gene mutation assays *in vitro*, it appears likely that these mutations reflect secondary genotoxic phenomena associated with inflammatory and oxidative stress induced in the lung upon cobalt metal particles exposure. No increase in peripheral erythrocyte micronuclei was noted in mice from the 3-month dose ranging NTP study (0.625–10 mg/m$^3$).

*Systemic genotoxicity:* No publication exploring the systemic genotoxicity of cobalt metal particles was located.

### 3.1.3. Carcinogenicity

***3.1.3.1. Experimental carcinogenicity.*** *Local carcinogenicity:* In the NTP study, F344/NTac rats and B6C3F1/N mice were exposed by inhalation to 0, 1.25, 2.5, or 5 mg cobalt metal/m$^3$ for 6 h per day, 5 days a week for up to 105 weeks (whole body exposure; mass median aerodynamic diameter, 1–3 μm; >98% pure, cubic, and hexagonal phases) (NTP 2014). In rats, chronic active and suppurative inflammation, respiratory metaplasia, epithelial atrophy, hyperplasia, basal cell hyperplasia, necrosis of the olfactive epithelium, and atrophy of the turbinates were observed already at the lowest dose. Non-neoplastic lung toxicity, including alveolar/bronchiolar epithelial hyperplasia, alveolar proteinosis, and chronic active inflammation, was also recorded from the lowest dose. Cobalt metal dose dependently induced alveolar and bronchiolar adenomas and carcinomas. No NOEL for carcinogenic effects was recorded. In mice, similar non-neoplastic lesions of the respiratory tract, including the larynx, were induced by cobalt metal already in the lowest dose group (0.125 mg/m$^3$). Alveolar/bronchiolar adenoma and carcinoma were induced by cobalt metal at 2.5 mg/m$^3$ and above (Figure 4).

In the earlier NTP study conducted with cobalt sulfate heptahydrate (NTP 1998), exposure was actually to cobalt sulfate hexahydrate (23% Co), and the highest dose tested of 3 mg/m$^3$ corresponds to 0.69 mg Co equivalent/m$^3$, which is less than the lowest dose tested in the cobalt metal study (1.25 mg/m$^3$). The respective carcinogenic potency of both compounds can, therefore, not be formally compared,

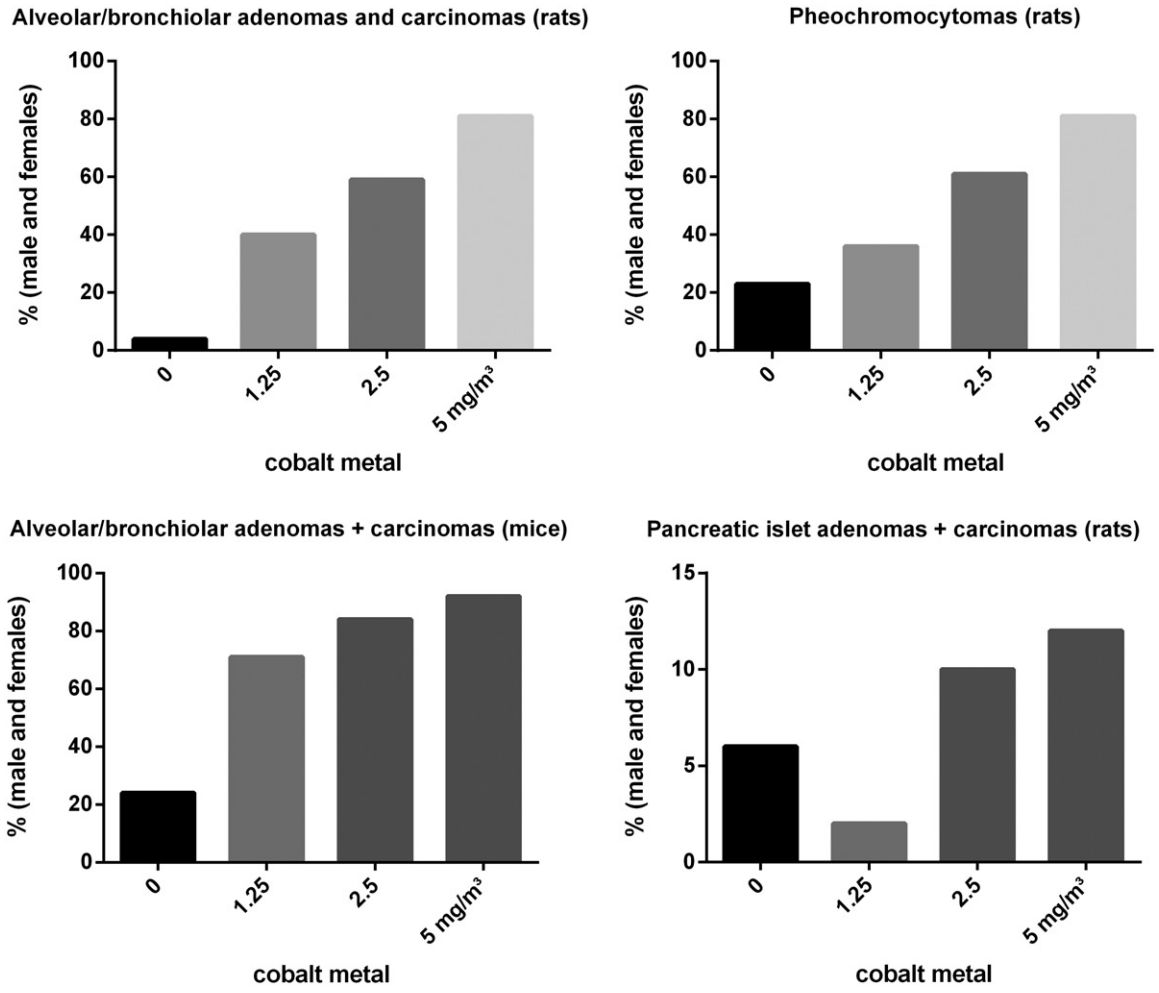


**Figure 4.** Tumor incidences in the 2-year NTP study with cobalt metal (males and females combined).

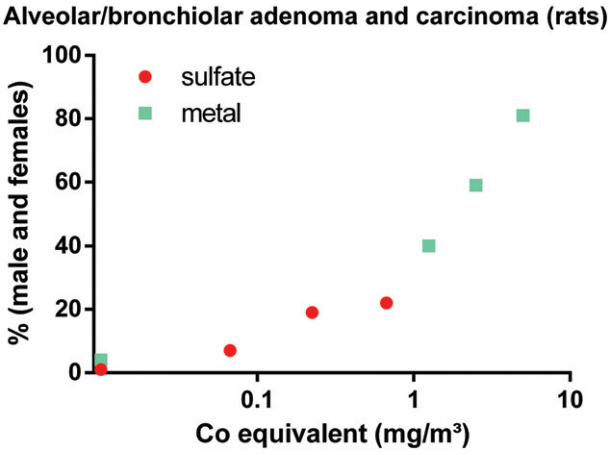


**Figure 5.** Incidences of alveolar/bronchiolar adenoma and carcinoma in NTP inhalation studies (105 weeks) with cobalt sulfate heptahydrate and cobalt metal in rats.

although major differences are not immediately evident (Figure 5). However, the alveolar/bronchiolar neoplastic response to cobalt metal mainly consisted of carcinomas (carcinoma/adenoma ratio, 25/17 and 63/20 in rats and mice, respectively), whereas adenomas were more frequent than carcinoma in the cobalt sulfate study (carcinoma/adenoma ratio 7/15 and 20/28, respectively). This might suggest that cobalt metal is more carcinogenic than cobalt sulfate. A striking difference between both studies is, however, the inflammatory respiratory status of control rats, as reflected by the much higher incidence of lung and nose inflammation (Table 3), which might have contributed to aggravate the carcinogenic response in rats exposed to cobalt metal particles. In a similar comparison analysis, the upper dose in the cobalt sulfate study was considered closely equivalent (1.14 mg/m$^3$) to the lowest dose used in the cobalt metal study (1.25 mg/m$^3$) (Behl et al. 2015), but the hydration of cobalt sulfate was apparently not integrated in calculations.

**Table 3.** Incidence % (severity score) of respiratory inflammation in control rats in the 2-year NTP carcinogenicity studies conducted with cobalt sulfate heptahydrate (NTP 1998) and cobalt metal particles (NTP 2014).

| | Cobalt sulfate heptahydrate | | Cobalt metal | |
|---|---|---|---|---|
| | Males | Females | Males | Females |
| Lung inflammation | 4 (1) | 18 (1) | 44 (1.1) | 40 (1) |
| Nose chronic inflammation | 0 (–) | 0 (–) | 56 (1.2) | 44 (1.3) |
| Nose suppurative inflammation | 0 (–) | 0 (–) | 18 (1) | 12 (1.2) |

Rats implanted intramuscularly with cobalt metal nanoparticles (60–100 mg; average size, 120 nm) developed malignant

mesenchymal tumors (sarcomas) at the site of implantation after 8 months (Hansen et al. 2006).

*Systemic carcinogenicity:* An increased incidence of pancreatic islets tumors was recorded in (mainly male) rats in the NTP inhalation study with cobalt metal (Figure 4). These tumors were considered as some or equivocal evidence of a systemic carcinogenic activity of cobalt metal particles in males or females, respectively. An increased incidence of pheochromocytomas was also recorded in rats after cobalt metal (also after cobalt sulfate) exposure. The occurrence of pheochromocytomas in rats is not uncommon in NTP studies conducted with particulates (Ozaki et al. 2002) and is considered to reflect a chronic catecholamine response of the adrenal medulla to hypoxia induced by lung pathology, but here possibly also by the hypoxia-like effect of Co(II) ions (Greim et al. 2009). These tumors should, therefore, be considered as secondary to the respiratory toxicity of cobalt metal particles. Mononuclear cell leukemias were also recorded in female rats but these were considered as unlikely related to exposure as it is a common spontaneous neoplasm in F344 rats (NTP 2014).

***3.1.3.2. Epidemiology.*** A case–control study nested in a cohort of Norwegian refinery workers has been conducted to verify whether the lung cancer risk among nickel refinery workers could be explained by occupational exposures of other chemicals than nickel, including arsenic, asbestos, sulfuric acid mists, and cobalt (Grimsrud et al. 2005). The multivariate regression analyses did not report dose-related increase in risk. All individuals exposed to nickel were also exposed to cobalt. The cancer incidence study in Finnish workers form the cobalt industry (see Section 2.3.3 under Organic and Inorganic Salts, Oxides) did not report an increase in cancer risk (Sauni et al. 2017).

### 3.1.4. Summary: cobalt metal particles

Cobalt metal particles are more rapidly and/or more intensely taken up by non-phagocytic cells than Co(II) ions, suggesting a Trojan horse mechanism for their cellular toxicity. The capacity of cobalt metal particles to damage DNA through the production of ROS was confirmed for particles in the nanosize range, but no data exist for comparison with micrometric particles. Cobalt metal particles do not appear to induce gene mutations in prokaryotic or eukaryotic cells *in vitro*. Gene mutations recorded in lung tumors from animals exposed to cobalt metal particles by inhalation are attributed to a secondary genotoxic mechanism driven by inflammatory cells. The genotoxic activity of cobalt metal particles derives from their capacity to generate ROS through a surface corrosion process. This mechanism is non-stochastic in nature and thus expected to exhibit a threshold. Cobalt metal particles should thus be considered as genotoxic carcinogens with a practical threshold (Bolt and Huici-Montagud 2008). Chronic inhalation of cobalt metal particles induced an increased incidence of local tumors (alveolar/bronchiolar adenoma and carcinoma) in rats and mice, with a NOAEL for carcinogenicity $<1.25$ mg Co/m$^3$. Evidence of systemic carcinogenicity was not strong in these studies. There is no epidemiological evidence of an increased risk for cancer in workers exposed to cobalt metal.

## 3.2. Hard Metals (Cemented Carbides, WC-Co)

Human exposure to WC-Co particles can occur in industrial settings where these materials are produced or used for their extreme hardness properties. Exposure essentially occurs by inhalation of dusts (Lison 2015).

### 3.2.1. Bioaccessibility

Hard metal particles are soluble in surrogate intracellular lysosomal fluids. Bioaccessibility is higher than cobalt metal particles, but lower than Co sulfate, in the neutral pH fluids (Table 4).

### 3.2.2. Genotoxicity and other mechanisms

***3.2.2.1. In vitro genotoxicity.*** Several new studies have confirmed the capacity of WC-Co (nano)particles to generate ROS and genotoxicity, and clarified their capacity to induce oxidative stress and subsequent activation of cell proliferation and inflammatory signaling pathways in diverse cellular models. The elective cytotoxicity of WC-Co particles was confirmed with a preparation in the nanosize range (mean particle size of 145 nm, 50–300 nm) on a broad range of cell types exposed for 3 days to 8.25–33 μg WC-Co/ml. Astrocytes and colon epithelial cells (Caco-2) appeared the most sensitive, although the relevance of these cell types is not immediately evident. The cytotoxic activity of these particles could not be reproduced by an equivalent dose of Co(II) ions (cobalt chloride), confirming that surface corrosion drives the cytotoxic potential of WC-Co particles (Bastian et al. 2009). The ability of nanosized (average 80 nm in size; specific surface area (SSA) 2.73 m$^2$/g; 15% Co w/w) and fine-sized (average 4 μm in size; SSA 0.16 m$^2$/g, 6% Co w/w) WC-Co particles

**Table 4.** Bioaccessibility (% available cobalt extracted) of cemented carbide particles (see reference for test material characteristics and testing conditions).

| | Extraction fluids | | | | |
|---|---|---|---|---|---|
| | Inhalation | | | | |
| | Alveolar (pH 7.4) | Interstitial (pH 7.4) | Serum | Intracellular (pH 4.5) | |
| Tungsten carbide pre-sintered | 2.6 | 8.3 | 21.7 | 25.9 | [1] |
| Tungsten carbide post-sintered | 3.6 | 12.4 | 14.7 | 26.7 | [1] |

[1]: Stopford et al. (2003).

to form ROS and their propensity to activate the transcription factors AP-1 and NF-kappa B, along with stimulation of mitogen-activated protein kinase (MAPK) signaling pathways, was compared in a mouse epidermal cell line (JB6 P(+)). WC-Co nanoparticles generated more hydroxyl radicals (about twofold), induced greater oxidative stress, as evidenced by a decrease in GSH levels (<2-fold), and caused faster JB6 P(+) cell growth/proliferation (about twofold) than observed after exposure to fine WC-Co (25–50 μg/cm$^2$). In addition, nano-WC-Co (25–150 μg/cm$^2$ for 24 h) activated AP-1 and NF-kappa B more efficiently in JB6(+/+) cells as compared to fine WC-Co (up to about fourfold and 28-fold, respectively). Experiments using AP-1-luciferase reporter transgenic mice confirmed the activation of AP-1 after topical application of nano-WC-Co particles on the skin (1 mg/mouse suspended in 0.3 ml acetone). Nano- and fine-sized WC-Co particles also stimulated MAPKs, including ERKs, p38, and JNKs with significantly higher potency of nano-WC-Co. Finally, involvement of oxidative stress induced by WC-Co particles was suggested by a decreased AP-1 activation and phosphorylation of ERKs, p38 kinase, and JNKs in the presence of N-acetyl-cysteine. This study confirmed that WC-Co particles induce ROS generation, cell proliferation, and activation of specific cell signaling pathways in mammalian cells (Ding et al. 2009). The greater biological activity of nanosized particles is likely related to their higher Co content and/or SSA. The relevance of investigating these mechanisms in an epidermal cell line is unclear. It was also reported by the same group that nanosized WC-Co particles can activate Nrf2 and downstream genes, including glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1), in JB6 cells. These responses appeared mediated by the generation of ROS in the presence of WC-Co nanoparticles (Zhang et al. 2010). An additional study in the same cell line indicated that WC-Co nanoparticles can induce miR-21 generation, but inhibit the production of its downstream target, the tumor suppressor gene programmed cell death 4 PDCD4. This effect also appeared mediated by ROS and ERK pathways (Hou et al. 2013).

The genotoxic capacity of WC-Co particles has been extended to the nanosize range. WC-Co nanoparticles (8% Co in weight; 20 to 160 nm by TEM) induced dose-dependent DNA damage and mutations in human lymphocytes as measured by the induction of micronuclei after 24 h (20–80 μg WC-Co/ml) and comet assay after 4 h (40–120 μg WC-Co/ml). The mechanisms of micronucleus induction by WC-Co nanoparticles involved both aneugenic and clastogenic events (Moche et al. 2015). In parallel experiments conducted with L5178Y cells, no DNA damage could be observed, and a weak micronucleus response and no mutagenic activity in the mouse lymphoma assay (MLA-TK) were recorded. The authors provided several possible explanations for the difference of sensitivity between both cell types, including differences in antioxidant capacity, p53 proficiency (L5178Y cells are p53-deficient), and origin (human versus murine) (Moche et al. 2014). Cell-specific sensitivity to the same WC-Co nanoparticles (1–150 mg WC-Co/ml) was further examined in a subsequent publication. WC-Co nanoparticles were readily taken up in human renal (Caki-1) and liver (Hep3B) cells but poorly in A549 alveolar epithelial cells. ROS production, DNA double-strand breaks, and cell cycle arrest were recorded in renal and liver cells, but not in A549 cells. The varying sensitivity to WC-Co nanoparticles according to cell type appeared mainly related to immediate (15 min exposure) WC-Co NP uptake and ROS induction (Paget et al. 2015).

In the study by Lombaert et al. (2004) (see cobalt metal), WC-Co particles (2–6 μg Co-equivalent/ml) induced a late and higher apoptogenic response than Co metal particles alone, which is consistent with a higher biological activity of these particles in terms of ROS production (Lison et al. 1995). A subsequent study confirmed the apoptotic response, involving both extrinsic and intrinsic pathways, induced by WC-Co particles (both nano- and microsized) in JB6 cells and rat lung macrophages. Activation of pro-apoptotic factors including Fas, Fas-associated protein with death domain (FADD), caspases 3, 8, and 9, BID, and BAX was recorded 24 h after WC-Co (3.75–15 and 1.5–6 μg Co/ml, for nano- and microsized particles, respectively). Cytochrome c and apoptosis-inducing factor (AIF) were also upregulated and released from mitochondria to the cytoplasm. On a mass basis, WC-Co nanoparticles exhibited higher apoptotic induction than fine particles (Zhao et al. 2013).

In a first study aiming at identifying pathogenic pathways that might contribute to explain the stronger genotoxic and apoptotic responses induced by WC-Co compared to cobalt metal particles in human lymphocytes, Lombaert et al. recorded an upregulation of apoptosis and stress/defense responses, as well as a downregulation of genes involved in the immune response, after 24-h incubation with WC-Co (33.3–100.0 μg WC-Co/ml, equivalent to 2–6 μg Co/ml). No apparent difference between WC-Co and cobalt metal particles was, however, observed (Lombaert N et al. 2004). Next, they focused on a much earlier time point (15 min) and recorded in the same cell type that 6 μg Co equivalent/ml of WC–Co, as well as metallic Co particles although with slower kinetics, induced a temporally ordered cascade of events implying p38/MAP kinase activation, HIF-1α stabilization, HMOX1 transcriptional activation, and ATM-independent p53 stabilization. These events, and in particular HIF-1α stabilization (Lombaert et al. 2008), could contribute to explain the carcinogenic activity of cobalt metal and WC–Co dusts. Interestingly, in this study, the responses to WC-Co or Co were assessed against an equivalent dose of Co(II) ions as control (not vehicle). This cascade of events reflects, therefore, the specific cellular response to the early (15 min) oxidative burst resulting from surface corrosion of cobalt metal (Figure 1) enhanced by the presence of WC particles, not the effect of Co(II) ions released. It further illustrates the higher impact of ROS produced by surface corrosion than compared to those arising from a potential Fenton-like reaction catalyzed by Co(II) ions. If the latter phenomenon exists, it occurs at much higher Co equivalent doses.

WC-Co nanoparticles can also induce angiogenic responses, critical for the carcinogenic process, by producing ROS and activating AKT and ERK1/2 signaling pathways. In BEAS-2B human bronchial epithelial cells, WC-Co nanoparticles (5 μg/cm$^2$) induced ROS production and activated AKT and ERK1/2 signaling pathways after 24 h. WC-Co treatment

also increased transcriptional activation of AP-1, NF-κB, and VEGF as assessed in cell reporter assays. The capacity of WC-Co nanoparticles to induce angiogenesis was confirmed in the chicken chorioallantoic membrane (CAM) assay performed with BEAS-2B and A459 cells pretreated with WC-Co nanoparticles (5 μg/cm$^2$ for 24 h) (Liu et al. 2015). The characteristics and composition of the WC-Co particles used in this study were not reported.

***3.2.2.2. In vivo genotoxicity.*** *Local genotoxicity:* To evaluate the *in vivo* genotoxic activity of micrometric WC-Co dust in the lung, DNA breaks/alkali-labile sites (alkaline comet assay) and chromosome/genome mutations (*ex vivo* micronucleus) were assessed in type II pneumocytes after a single intra-tracheal instillation of 16.6 mg WC-Co/kg body weight in rats (equivalent to 1 mg Co metal/kg b.w.) (De Boeck et al. 2003). This dose produced mild pulmonary inflammation. WC-Co induced a statistically significant increase in tail DNA (12 h) and in micronuclei (72 h). No systemic genotoxicity was recorded as assessed by DNA damage or micronuclei in circulating blood cells. The authors interpreted the local genotoxic response recorded in lung cells as the result of the primary genotoxic activity of the WC-Co dust, but possibly also as secondary genotoxicity produced by inflammatory cells recruited in the lung (De Boeck et al. 2003).

*Systemic genotoxicity:* No other publication exploring the systemic genotoxicity of WC-Co particles was located.

### 3.2.3. Carcinogenicity

***3.2.3.1. Experimental carcinogenicity.*** No publication exploring the carcinogenicity of WC-Co particles in experimental system was located.

***3.2.3.2. Epidemiology.*** Epidemiological studies from cohorts of hard metal workers in Sweden and France were already available in 2001 (Hogstedt and Alexandersson 1990; Lasfargues et al. 1994; Moulin et al. 1998; Wild et al. 2000). In France, increased mortality by lung cancer was recorded in the cohort of workers employed for at least 6 months in the hard metal industry ($n = 7459$; 63 lung cancers; SMR 1.30; 95%CI 1.00–1.66). All hard metal plants in the country were included. A case–control study nested in this cohort showed a significant association with cumulative exposure to hard metal dust. Simultaneous exposure to cobalt and tungsten carbide (WC-Co) was associated with a lung cancer risk of about 2 (Wild et al. 2009).

More recently, a large international study sponsored by the International Tungsten Industry Association (ITIA) analyzed the causes of mortality in hard metal workers, with a specific focus on lung cancer. Data from 17 manufacturing sites in 5 countries (USA, Germany, UK, Austria, and Sweden) were pooled to assemble a cohort of 32,354 workers employed for at least 1 day in this industry (Marsh et al. 2017). Increased mortality by lung cancer was recorded (459 lung cancers; SMR 1.20; 95%CI 1.09–1.31), but this increased risk was essentially limited to short-term workers (<1 year employment) and no clear dose–response relationship with cumulative or mean exposure to cobalt or tungsten (assessed separately) could be evidenced in long-term workers. In this pooled analysis, a "healthy plant" effect cannot be excluded because only a selection of plants was investigated, exposure to cobalt associated to tungsten carbide (WC-Co) could not be assessed, Co exposure levels were generally lower than in the French industry especially in the last years, and personal protective equipment worn by the most heavily exposed workers was not taken into account. Collectively, these elements may have contributed to obscure a carcinogenic activity of WC-Co exposure in this study.

### 3.2.4. Summary: hard metal particles

The capacity of WC-Co particles to generate ROS and genotoxicity was confirmed and extended to WC-Co particles in the nanosize range. WC-Co nanoparticles appear more active than their micrometer equivalents. Activation of cell proliferation and inflammatory signaling pathways induced by WC-Co (nano)particles, and relevant for their carcinogenic properties, was clarified in diverse cellular models *in vitro*. The local genotoxic activity of WC-Co particles recorded experimentally in the rat lung can result from both primary and secondary mechanisms. The genotoxic activity of WC-Co particles is driven by their elective capacity to generate ROS through a surface corrosion process. This mechanism is non-stochastic in nature and thus expected to exhibit a threshold. WC-Co particles should thus be considered as genotoxic carcinogens with a practical threshold (Bolt and Huici-Montagud 2008). Consistent with this notion, a genotoxic activity of WC-Co was detected *in vitro* and locally in the respiratory epithelium upon inhalation administration, but no systemic genotoxic response was recorded. The carcinogenic activity of WC-Co appears limited to local tumors of the respiratory tract upon inhalation exposure.

## 3.3. Cobalt-containing endoprostheses

Joint replacement (hip, knee, shoulder) is a common surgical procedure using a large variety of implants. In modern hip prostheses, the pairing of cobalt–chromium (CoCr) head and cup is used in total hip arthroplasty (THA) and hip resurfacing arthroplasties. While cobalt (>34%) and chromium (>19%) are the major constituents of alloys used in the manufacture of these devices (e.g. stellite, cast ASTM F75, forged F799m or wrought F1537), other metals (nickel, molybdenum, tungsten …) are often included to improve mechanical properties. Stellite alloys are also used for the manufacture of dental prostheses and in industrial applications. Thus, Co and Cr exposure from surgical devices occurs as a result of particles and ions released by the wear and/or corrosion of the implant locally, and metals released from the prostheses may gain access to different body compartments (Keegan et al. 2008). Systemic endogenous exposure to cobalt and chromium has been documented by measuring the elements in blood and/or urine in patients with CoCr metal-on-metal (MoM) implants (Kuzyk et al. 2011; Jantzen et al. 2013; Pahuta et al. 2016). Very high serum cobalt concentrations (>50 μg/L) were measured (Hartmann et al. 2013). Local and systemic adverse manifestations have been associated with

Table 5. Bioaccessibility (% available cobalt extracted) of cobalt alloys (see references for material characteristics and testing conditions).

| | Extraction fluid | |
|---|---|---|
| | Synovial (pH 7.4) | |
| Stellite 21 powder | 0.0018 | [1] |
| Stellite rods | 0.0013 | [1] |

[1]: Stopford et al. (2003).

the release of cobalt ions and nanoparticles, including inflammatory and other immune reactions in the directly exposed tissues as well as changes in the peripheral blood (evidence of oxidative stress) and alteration of circulating immune cells (reviewed in Czarnek et al. (2015); Cheung et al. (2016)). The physicochemical properties of CoCr particles determine local toxicity; CoCr micron-sized particles are more biopersistent *in vivo*, resulting in local inflammatory responses that are not seen with similar mass concentrations of nanoparticles (Madl et al. 2015a; Madl et al. 2015b).

### 3.3.1. Bioaccessibility

Co alloys used in medical implants show minimal dissolution in bioaccessibility tests. Two physical forms of stellite were tested in synovial fluid equivalent and were found to be almost completely stable over the entire extraction period (72 h) (Table 5). In the presence of proteins (e.g. albumin) and/or tribological stress (tribocorrosion), the alloys release significantly higher amounts of cobalt ions and metal particles (Gil and Munoz 2011; Mathew et al. 2012). The local release of wear debris particles further contributes to degrading the prosthesis surface(micro-abrasion) (Sadiq et al. 2015).

### 3.3.2. Genotoxicity and other mechanisms

**3.3.2.1. In vitro *genotoxicity*.** DNA damage, assessed by the comet assay, was recorded in primary human fibroblasts exposed during up to 5 days to CoCr alloy particles (2.9 μm in size; $5.1 \times 10^{-8}$ to 1.02 mg/cm$^2$). A dose-dependent increased tail moment was recorded after 24 h, with significantly increased damage starting at 1 μg/cm$^2$; the dose–response relationship was less clear after 5 days of exposure. This effect was probably not mediated by released ions (Co(II) and/or Cr(III)) because it could not be reproduced by exposing the cells to a leachate obtained by incubating the culture medium with the particles during 24 h. A leachate obtained after 1 month of incubation with the particles induced DNA damage, and this was reduced by NAC, suggesting the participation of ROS at high doses of ions. NAC did not protect against the clastogenic effect of particles after 24 h. Chronic exposure (15 days) to the CoCr particles (0.5–1 μg/cm$^2$) caused a high level of both structural and numerical chromosome aberrations in proliferating cultures (Papageorgiou et al. 2007b). A subsequent study from the same group showed that 24-h exposure to CoCr nanoparticles (about 30 nm) induced more severe DNA damage and aneuploidy (micronucleus assay) than volume-equivalent doses (μm$^3$/cell) of micrometric alloy particles (about 3 μm in size). The authors related the difference in genotoxic activity between both particles to a different cell uptake and intracellular localization. The nanoparticle aggregates rapidly dissolved or corroded within vacuoles in the cell cytoplasm. In contrast, the micron-sized particles dissolved less rapidly and persisted for at least 5 days after exposure. Moreover, microparticles were preferentially clustered around the nucleus. This pattern suggests that, after uptake, the nanoparticles might possibly deliver a high concentration of metal spread more evenly around the cell cytoplasm within the first 24 h after exposure. In contrast, the microparticles may deliver a slower release of metal particularly around the nucleus of the cell. Nanometric particles also released more ROS in an acellular EPR assay than micrometric particles (Papageorgiou et al. 2007a). In another study designed to compare the genotoxic activity of CoCr and alumina ceramic ($Al_2O_3$) particles, CoCr particles (0.1–10 mg/T-75 flask for 5 days) induced double-strand breaks (γ-H2AX foci), micronuclei, and mainly numerical chromosome aberrations in primary human fibroblasts. CoCr particles were significantly more genotoxic than ceramic particles (Tsaousi et al. 2010). A series of *in vitro* experiments to explore mechanisms of genotoxicity was performed with CoCr nanoparticles produced from a tribometer (30 nm) or thermal plasma technology (20, 35, and 80 nm) in primary human fibroblasts. The results indicated a role of mitochondrial ROS-dependent and independent responses, particle size, amount of ion release, and phagocytosis (Raghunathan et al. 2013). Interestingly, CoCr nanoparticles (36 or 360 μg/cm$^2$ of 30 nm or 3 μm in size) were able to induce DNA damage (alkaline comet assay and γ-H2AX foci) in primary human fibroblasts across an intact cellular barrier of human trophoblast choriocarcinoma-derived cells. DNA damage appeared mediated by the transmission of purine nucleotides (such as ATP) and intercellular signaling within the barrier through connexin gap junctions or hemichannels and pannexin channels (Bhabra et al. 2009).

Overall, the results of these studies are difficult to interpret because, in the end, it is not possible to formally discriminate the role of solid particulates from that of solubilized ions, and the respective role of Co(II) and Cr(III) ions cannot be differentiated. *In vitro* studies conducted with ionic solutions showed, however, that Cr(III) ions were consistently less genotoxic than Co(II) ions, suggesting a higher contribution of the latter in mediating the genotoxic activity of CoCr particles (Christian et al. 2014).

**3.3.2.2. In vivo *genotoxicity*.** *Local genotoxicity*: No animal study evaluated Co or Cr particulate individually. Chromosomal aberrations and DNA damage were observed in femur bone marrow of mice dosed four times intra-articularly (knee joint) with 0.05 mg/kg/day over 18 weeks with nano- or micron-sized CoCr particles (reviewed in Christian et al. (2014)). Patients with worn CoCr hip replacement implants have increased chromosome aberrations in the bone marrow adjacent to the implant and increased chromosome translocations and aneuploidy in peripheral blood mononucleated cells (Keegan et al. 2008).

*Systemic genotoxicity:* Increased chromosome translocations and aneuploidy were recorded in peripheral blood lymphocytes from patients at 6, 12, and 24 months after

receiving MoM hip arthroplasty. These changes were accompanied by a progressive increment in blood Co and Cr content (about 1 μg/L for both elements after 12 or 24 months) (Ladon et al. 2004). Whether these chromosome aberrations were induced by soluble or particulate metal or to some other aspects related to joint arthroplasty surgery cannot be answered by this study. A 2.5-fold increase in aneuploidy and a 3.5-fold increase in chromosomal translocations were found in circulating lymphocytes of patients with CoCr prostheses undergoing revision arthroplasty (predominantly MoM total hip replacements) compared with those at primary arthroplasty after adjustment for smoking, gender, age, and diagnostic radiographs (Doherty et al. 2001). Oxidative DNA damage, using 8-hydroxydeoxyguanosine (8-OHdG) as a marker, was measured in the urine of patients with MoM total hip replacement. 8-OHdG was higher in patients with implants 3–4 years old as compared to patients with implants 1–2 years old. No correlation with Co (or Cr) levels in whole blood (0.77–37.80 μg Co/L) and urine (2.59–166.94 μg Co/24 h) was, however, evident (Pilger et al. 2002). Christian et al. (2014) noted that the Co/Cr particle concentrations ($10^4$–$10^9$ μg/g) as well as the concentrations of ionic solutions required to induce genotoxic effects in human cells were several orders of magnitude higher (at least 1000-fold) than the particle concentration or blood Co and Cr levels typically present in patients with stable CoCr hip implants. Chronic inflammation is an important determinant of the genotoxic process (Colotta et al. 2009), especially inflammatory cells when particulate materials are involved (Schins and Knaapen 2007). The relationship between chronic inflammatory responses at the implant site with Co(II) ion release or to Co-Cr (nano)particles remains, however, unclear because these reactions are seen in the presence or in the absence of excessive metal-wear debris and none of the available studies has measured Co(II) ion concentrations (only total Co in tissue and body fluids) (Paustenbach et al. 2013).

#### 3.3.3. Carcinogenicity

***3.3.3.1. Experimental carcinogenicity.*** Several animal bioassays involving particulate Co, Cr, and CoCr alloy exposures have been published (reviewed by Christian et al. (2014)). Exposure pathways included intraperitoneal, intra-articular, intra-thoracic, intra-femoral, intramuscular, intra-renal, and subcutaneous injections of particles (micron-sized; diameters: 0.5–250 μm) in rats, mice, rabbits, and/or guinea pigs. None of these studies reported a statistically significant increase in primary systemic tumors; some studies noted a significant increase in local tumors at the site of injection or, in few cases, systemic tumors as a result of metastasis from the site of injection. Six animal studies evaluated the carcinogenic effects of implanted CoCr alloy. Four of them reported tumors at the implant site and none reported a statistically significant increased incidence of primary systemic tumors (Christian et al. 2014).

***3.3.3.2. Epidemiology.*** Cases with systemic toxicity attributed to metal release from hip prostheses have been reviewed by Devlin et al. (2013) and more recently by Bradberry et al. (2014). Only few individual cases reporting systemic toxicity manifestations were retrieved ($n = 18$). Systemic features were first reported months and often several years after placement of metal-containing implant, consisting of neuro-ocular toxicity (peripheral neuropathy, sensorineural hearing loss, cognitive decline, visual impairment), cardiotoxicity, and/or thyroid toxicity. Removal of the prosthesis (undertaken in all but two patients) was usually associated with a drop in circulating Co concentration and improvement in some or all adverse manifestations.

Overall, epidemiologic research conducted among hip prosthesis patients over various geographic, temporal, and demographic settings failed to demonstrate an increased risk of cancer (reviewed in Paustenbach et al. (2013)). Risk of cancer in patients with MoM total hip replacement or after MoM (first or second generation) total hip arthroplasty was assessed by several authors who did not report increased incidence of systemic tumors (Makela et al. 2012; Onega et al. 2006; Visuri et al. 2010; Smith et al. 2012). The latency periods in these studies may, however, not have been sufficient, highlighting the need for continued follow-up of patients with implants (Onega et al. 2006; Visuri et al. 2010; Lalmohamed et al. 2014; Pijls et al. 2016).

#### 3.3.4. Summary

In agreement with Christian et al. (2014), it can be concluded that *in vitro* and *in vivo* studies, as well as human data, suggest that materials released from CoCr hip implants can cause genotoxic effects (DNA damage/mutations) locally and possibly systemically (circulating lymphocytes in patients). Epidemiological studies did not record an increased incidence of local or systemic cancers in CoCr hip implant patients but the latency period might not be sufficient to formally exclude an increased risk. The exact mechanism of the genotoxic/carcinogenic activity of CoCr is not clearly defined and might involve CoCr particulate materials, Co(II) and/or Cr(III) ions.

## 4. Conclusions

Interactions of solubilized cations with proteins, for example with zinc fingers, are generally more relevant for the carcinogenic activity of metals than interactions with DNA. Thus, the genotoxic activity of these metals is driven by indirect mechanisms including disrupted cellular redox homeostasis (oxidative stress), causing oxidative DNA damage, activation of intracellular signaling cascades and stimulation of cell growth; inhibition of DNA repair mechanisms, resulting in genomic instability and accumulation of mutations relevant for carcinogenicity; and deregulation of cell proliferation and/or apoptosis by triggering signaling pathways or inactivating growth controls (Beyersmann and Hartwig 2008). *In vivo*, especially for inhaled particles, inflammatory processes induced by metallic elements can further elicit secondary genotoxicity and contribute to carcinogenesis (Schins and Knaapen 2007). For cobalt compounds, the present review confirmed the role of Co(II) ions in mediating indirect genotoxicity through interactions with proteins involved in DNA

repair and maintenance systems. The novel mechanism based on alteration of topoisomerase II by Co(II) ions further contributes to clarify their clastogenic activity. The relative importance of the Fenton-like activity of Co(II) ions for their genotoxic activity appears limited, certainly *in vivo*. Several studies demonstrated the capacity of Co(II) ions to trigger intracellular signaling pathways relevant for carcinogenesis, including apoptotic responses. All these mechanisms are non-stochastic and expected to follow thresholded dose–response relationships. All cobalt compounds release, to some extent, Co(II) ions extracellularly and/or intracellularly. Assessing the capacity of cobalt compounds to release these ions and make them available for cells is, therefore, an important dimension for interpreting and predicting their genotoxic and carcinogenic activities. Bioaccessibility data are available in various simulated biological fluids to provide this information. The biologically effective dose of Co(II) ions can be sensed by monitoring HIF-1$\alpha$ expression in cellular systems.

Surface corrosion in biological fluids is an additional mode of action for metal-based compounds (cobalt metal, WC-Co, and Co-based alloys) which, along with solubilization of Co(II) ions, drives their genotoxic and carcinogenic activities. This phenomenon leads to a burst of ROS production and oxidative stress resulting in indirect DNA damage which contribute to genotoxicity and inflammatory reactions. Again, these mechanisms are expected to follow thresholded dose–response relationships.

The genotoxic and/or carcinogenic activities of cobalt compounds (ionic and metallic) are essentially limited to the respiratory system upon inhalation and locally at the site of implantation. This conclusion is consistent with a characterization of cobalt and its compounds as genotoxic carcinogens with a practical threshold (Bolt and Huici-Montagud 2008).

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