

In vivo response of heme-oxygenase-1 to metal ions released from metal-on-metal hip prostheses

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Abstract. Metal ion release and accumulation is considered to be a factor responsible for the high failure rates of metal-on-metal (MoM) hip implants. Numerous studies have associated the presence of these ions, besides other factors, including a hypoxia-like response and changes in pH due to metal corrosion leading to the induction of the oxidative stress response. The aim of the present study was to verify whether, in patients with a MoM hip prosthesis, mRNA and protein expression of HMOX-1 was modulated by the presence of metal ions and whether patients without prostheses exhibit a different expression pattern of this enzyme. The study was conducted on 22 matched pairs of patients with and without prostheses, for a total of 44 samples. Ion dosage was determined using inductively coupled plasma mass spectrometry equipped with dynamic cell reaction. *HMOX-1* gene expression was quantified by reverse transcription-quantitative polymerase chain reaction and HMOX-1 protein expression was analyzed using an enzyme-linked immunosorbent assay. The results demonstrated that although there were significant differences in the metallic ion concentrations amongst the two groups of patients, there was no correlation between circulating levels of cobalt (Co) and chromium (Cr), and HMOX-1 gene and protein expression. Additionally, there was no significant difference in the protein expression levels of HMOX-1 between the two

groups. In conclusion, it was demonstrated that circulating Co and Cr ions released by articular prosthetics do not induce an increase in HMOX-1 mRNA and protein expression at least 3.5 years after the implant insertion. The present study suggests that involvement of HMOX-1 may be excluded from future studies and suggests that other antioxidant enzymes, including superoxide dismutase, glutathione peroxidase and reductase should be investigated.

Introduction

Since the 2010 voluntary withdrawal of DePuy ASR Hip Resurfacing System and ASR XL Acetabular System prompted by several studies showing high failure rates of these hip implants (1-3), careful attention has been given to metal-on-metal (MoM) hip prostheses. The European community (4), in line with the international scientific community (5) and the Consensus Statement (6), has decided to stop the use of MoM big head stemmed implants (diameter ≥ 36 mm).

The high failure rate of these devices is well asserted by all national registers (7-10). One of the factors considered to be responsible for this, was the release and the systemic accumulation of surface released microparticles, nanoparticles and ions (articular and trunnion) (11). These prostheses were also associated with local aseptic lymphocytic vasculitis, pseudo-tumours and necrosis of surrounding tissues with consequent prosthetic failure (12-14).

The MoM alloys are usually composed of chromium (Cr, 26-30%), molybdenum (Mo, 5-7%) and cobalt (Co) (for balancing ISO 5832-12:2007 High-Carbon-Alloy).

The accumulation of Co leads to a pathological condition, defined as cobaltism, predominantly affecting the nervous, cardiac and thyroid systems (15). The biological activity of Co is dictated by the concentration of unbound ionic Co (II) (16-18). Amongst the categories at risk of cobaltism are patients with big head MoM prostheses, in addition to reported

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cases of occupational or iatrogenic exposure investigated by toxicology experts (19).

While risk levels have already been established for cases of occupational exposure (20), those for patients with prosthetics have only been suggested by The Medicine and Healthcare products Regulatory Agency (MHRA) (21) and by the Consensus Statement (6). They have been suggested to be 7 µg/l for Cr and Co circulating ions, although certain authors have proposed 4 µg/l as a precaution (22). In addition, the risk levels for urinary ions have not been established yet.

Numerous studies have correlated the presence of metal ions with the formation of reactive oxygen species (ROS) (23), whose systemic and local effects are well known in different tested models (24). The metal ions Cr (III) and Co (II) catalyze the conversion of hydrogen peroxide into reactive hydroxyl radicals by the Fenton reaction (25). In response to oxidative stress, the organism protects itself by upregulating several enzymes, including heme-oxygenase-1 (HMOX-1) (26).

HMOX-1 is a member of the oxidoreductase family and catalyses the degradation of heme in carbon monoxide, divalent iron and biliverdin. It is then converted in bilirubin, the most abundant endogenous antioxidant in mammalian tissues, responsible for a number of antioxidant activities (26).

HMOX-1 represents the inducible isoform of the antioxidant system of heme-oxygenase and its induction is due to the action of multiple oxidation factors, including certain heavy metals (27), such as Co and Cr.

As it is known that Co (II) can induce the expression of HMOX-1 to counteract oxidative stress, the aim of the present study was to verify whether mRNA and protein expression of HMOX-1 was modulated by the presence of metal ions in patients with a MoM prosthesis and whether patients without a prosthesis exhibited a different expression pattern.

Materials and methods

Patient enrolment. This study was approved by the Institutional Review Board of the Rizzoli Orthopaedic Institute (Bologna, Italy). All investigations were conducted in conformity with ethical principles of research, and informed consent for participation in the study was obtained from all enrolled patients. This parallel cohort study was designed in order to evaluate *HMOX-1* expression in patients with/without MoM prosthetics, in correlation with Co and Cr levels in the blood and urine. It has been registered at clinicaltrials.gov with the identification number: NCT02427984.

Patients with primary coxarthrosis, on a waiting list for primary hip prosthesis intervention, were enrolled in the study as a control group (non-prosthetic group; n=22). These 22 patients were coupled with patients with aseptic loosening MoM hip prostheses (prosthetic group; n=22), matched for gender, age and smoking habits. The recruitment period was from March 2014 to October 2014. The exclusion criteria were the presence of other articular prostheses, sepsis or suspected sepsis, hematologic pathologies and rheumatoid arthritis. Each group (prosthetic and non-prosthetic) contained 17 women and 5 men, of which 4 were smokers and 18 were non-smokers or ex-smokers (who had not smoked for >10 years). The mean age \pm standard error of the mean of the patients in the

prosthetic group was 64.9 ± 1.9 years and of the patients in the non-prosthetic group was 64.2 ± 2.1 (Table I).

Sample collection. Peripheral blood samples (total, 18 ml) were obtained using a disposable intravenous cannula, the first 3 ml were discarded to eliminate possible contamination by metals caused by the sampling system, then 10 ml of blood were withdrawn and transferred into two separate trace element vacutainer tubes (5 ml/tube) containing ethylenediaminetetraacetic acid (BD Biosciences, Franklin Lakes, NJ, USA) for whole blood. An additional 5 ml of blood aliquot was transferred into a trace element serum vacutainer tube and centrifuged at 800 x g for 7 min at 4°C to obtain blood serum. Next, 1 ml samples of whole blood and serum were immediately frozen and stored at -80°C for the ion analysis. The remaining 4 ml aliquot of blood was collected to isolate white cells using a density gradient separation medium Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's protocol. The blood sample was diluted 1:1 in PBS and was layered on 4 ml of the Histopaque-1077 medium and centrifuged at 400 x g for 30 min at room temperature. The ring of white cells was collected and washed with 10 ml of PBS centrifuging at 250 x g for 10 min at room temperature. The cell pellet was resuspended in 1 ml of TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to preserve the white cell lysates, which were stored at -80°C until RNA extraction.

Clean-catch urine samples (10 ml) were collected in universal sample pots. These samples were frozen and stored at -20°C until the analysis was conducted.

Determination of ionic circulating and urinary levels of Co and Cr. Inductively coupled plasma mass spectrometry (ICP-MS; Perkin Elmer Inc., Waltham, MA, USA) equipped with dynamic cell reaction (ELAN DRC II, Perkin Elmer Inc.) was used for the measurements. A reaction system with ammonia gas was used for the elimination of spectral interferences.

Blood samples were diluted (1:20) with 0.05% Triton X-100 while urine samples were diluted with bi-distilled water, for inorganic trace analysis (Merck KGaA, Darmstadt, Germany).

The calibration curve and the sample solutions were pumped in the spray chamber using a peristaltic pump. Blank samples were used to correct for any contamination in each batch. The concentration of metal ions was expressed as µg/l. The calibration curve was prepared by dilution of a standard solution ranging from 0.5 to 1,000 mg/l (cobalt in HNO₃ 2% mono elemental standard solution, Carlo Erba Reagenti, Milano, Italy; chromium in HCl atomic absorption standard solution, Sigma-Aldrich). The procedure followed was previously described (28,29).

The accuracy of the method was verified by comparison with certified reference materials for blood obtained from the German External Quality Assessment Scheme (Institute for Occupational, Social and Environmental Medicine, Erlangen, Germany). The coefficients of variation ranged from 4 to 8% and the limit of detection, calculated as three standard deviations of the background signal obtained on 10 blinded samples, was 0.05 µg/l in all matrices (whole blood and urine).

Table I. Patients demographic characteristics and metal ions distribution.

Characteristic	Non-prosthetic group	Prosthetic group	P-value
Age, years (mean±SEM)	64.2±2.1	64.9±1.9	-
Gender			
Male	5	5	-
Female	17	17	-
Smoking habit			
Non-smokers (n)	18	18	-
Smokers (n)	4	4	-
Time from implant (years) range	3.5-15	-	-
Co-blood (µg/l) range	0.09-0.65	0.40-35.70	0.0001
Cr-blood (µg/l) range	0.03-2.03	0.05-12.50	0.0001
Co-urine (µg/l) range	0.20-1.50	2.00-867.00	0.0001
Cr-urine (µg/l) range	0.08-0.90	1.00-138.20	0.0001

SEM, standard error of the mean; Co, cobalt; Cr, chromium.

The exclusion criteria of the American Conference of Governmental Industrial Hygienists recommendation for very diluted (creatinine concentrations less than 0.3 g/l) or very concentrated (creatinine concentration greater than 3.0 g/l) urine samples were adopted (30). Urinary creatinine was determined by a modified Jaffè reaction (ILab 350 Clinical Chemistry System, Instrumentation Laboratories SpA, Bedford, MA, USA).

RNA extraction and reverse transcription. From the white cell lysates, the aqueous phase containing RNA was isolated using TRIzol and total RNA was purified following the clean-up protocol of the RNeasy Mini kit (Qiagen, Valencia, CA, USA). RNA quantity and quality was analysed using a spectrophotometer (Nanodrop ND 1000; Thermo Fisher Scientific, Inc.) and genomic DNA contamination was excluded by RNA gel electrophoresis in 1% agarose gel in 1X TAE (Merck & Co., Whitehouse Station, NJ, USA) stained with 0.5 µg/ml ethidium bromide (Sigma-Aldrich) and visualized with UV-light.

RNA was subjected to reverse transcription using the following: 1 µg total RNA, 200 units Moloney murine leukaemia virus reverse-transcriptase (Promega Corporation, Madison, WI, USA; used with companion buffer), 2.5 µM oligo dT-15 (Sigma-Aldrich), 2 µM random hexamers (Sigma-Aldrich) and 500 µM dNTPs (Takara Biotechnology Co., Ltd., Shiga, Japan). RT reaction was performed in a final volume of 25 µl for 60 min at 37°C. In order to verify that the RT reaction was successful, amplification of the human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was performed, using specific primers (*GAPDH* forward: 5'-GAA ATCCCATCACCATCTTCCAG-3' and reverse: 5'-AGGAGA CCACCTGGTGCTCAGTGTAGC-3'). *GAPDH* amplification was performed in a final volume of 25 µl, containing 1 µl cDNA, 0.2 µM each primer, 12.5 µl BioMix Red (Bioline, Taunton, MA, USA) under the following conditions: Initial denaturation for 2 min at 94°C; 25 cycles of 30 sec at 94°C, 30 sec at 61°C (annealing temperature of *GAPDH* primers), 30 sec at 72°C followed by a final extension for 7 min at 72°C.

Amplicon detection was performed by gel electrophoresis in 1.5% agarose gel as aforementioned.

Quantitative -polymerase chain reaction (qPCR). qPCR was performed using the CFX-96 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Amplification of 5 µl diluted cDNA (i.e. 25 ng) were amplified in 20-µl reactions using Sso Advanced SYBR Green Supermix (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. Following an initial denaturation step at 95°C for 2 min, temperature cycling was initiated. Each cycle consisted of 95°C for 5 sec, and 60°C for 30 sec repeated 40 times with the fluorescence being read at the end of this step. The primers were obtained from the PrimePCR SYBR Green Assay (Bio-Rad Laboratories, Inc.) and were specific for human *HMOX-1*, *GAPDH*, hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and TATA-box binding protein (*TBP*). Every sample was amplified as a technical duplicate and its specificity was evaluated with the melting curves, performed from 65 to 95°C for 2 sec every 0.5°C.

The quality of technical duplicates was established setting a Cq value of 0.3 as the limit for the standard deviation. The quality of the reference genes was evaluated based on their M value (<0.5), calculated by the CFX Manager software (version 3.1, Bio-Rad Laboratories, Inc.).

HMOX-1 relative expression was determined using the $2^{-\Delta\Delta Cq}$ method (31) with *GAPDH*, *HPRT1* and *TBP* as reference genes.

Analysis of *HMOX-1* protein expression. The concentration of *HMOX-1* in the serum was measured using an anti-human *HMOX-1* enzyme-linked immunosorbent assay kit (Enzo Life Sciences, Inc. Farmingdale, NY, USA), whose detection range for *HMOX-1* concentration was 0.78-25 ng/ml, according the manufacturer's instructions for undiluted samples. This analysis was conducted on 39 out of 44 total samples due to of lack of samples or reagents.

Statistical analysis. In order to evaluate the differences between the prosthetic and non-prosthetic groups in circulating

and urinary Co and Cr values, the Mann-Whitney test was used. The same test was used to analyze the difference in serum protein levels of HMOX-1 between patients with circulating values $>7 \mu\text{g/l}$ (high) and $<7 \mu\text{g/l}$ (low), this threshold was selected in agreement with previous studies (6,21). The same test was used to analyze difference of expression levels of HMOX-1, between prosthetic and non-prosthetic patients, or between those with high and low ion levels. For the correlation between Co and Cr levels in the blood and urine and the gene and protein levels of HMOX-1 the Pearson's correlation test was used. $P < 0.05$ was considered to indicate a statistically significant difference.

Statistical analysis and graphs were conducted using SPSS software (version 14.0; SPSS Inc., Chicago, IL, USA).

Results

Difference in circulating and urinary Co and Cr levels in the prosthetic and non-prosthetic groups. Circulating blood Co levels ranged between 0.09 and 0.65 $\mu\text{g/l}$ and urine levels ranged between 0.2 and 1.5 $\mu\text{g/l}$ in controls, while in patients from the prosthetic group these values ranged between 0.4 and 35.7 $\mu\text{g/l}$ in blood and between 2 and 867.1 $\mu\text{g/l}$ in urine, in this group 15 out of 22 patients had Co $<7 \mu\text{g/l}$; the difference between controls and prosthetic patients was significant ($P < 0.0001$) as determined using the Mann-Whitney test. Circulating blood Cr levels ranged between 0.03 and 2.03 $\mu\text{g/l}$ in controls, while in the prosthetic group these values ranged between 0.05 and 12.50 $\mu\text{g/l}$. In urine samples the Cr values ranged between 0.08 and 0.90 $\mu\text{g/l}$ in controls and between 1.00 and 138.20 $\mu\text{g/l}$ in the prosthetic group; in this group 17 out of 22 patients had Cr $<7 \mu\text{g/l}$. The difference between controls and patients in the prosthetic group was significant ($P < 0.0001$) using the Mann-Whitney test. These results are summarized in Table I.

Difference in gene expression of HMOX-1 between the prosthetic and non-prosthetic groups. Gene expression of HMOX-1 in patients in the prosthetic group compared with controls, regardless of Co and Cr levels, did not differ significantly using the Mann-Whitney test ($P = 0.581$). Even when samples were stratified by Co levels, no statistically significant differences were observed ($P = 0.837$) using the Mann-Whitney test. In subjects with high levels of Co, HMOX-1 expression was 1.05 ± 0.15 folds the paired controls value, while in subjects with low levels of Co HMOX-1 expression was 1.02 ± 0.13 folds the paired controls value (Fig. 1).

The same analysis was conducted based on circulating Cr values. HMOX-1 expression in prosthetic patients with high levels of Cr compared to those with low levels of Cr was not identified to be statistically different ($P = 0.802$) using the Mann-Whitney test. The relative mRNA levels in patients with low levels of Cr was 1.00 ± 0.04 fold compared with controls, and 1.10 ± 0.20 fold compared with controls in patients with high levels of Cr (Fig. 2). In summary, for high Cr and Co groups and for low Cr and Co groups, the HMOX1 gene expression was increased, compared with the respective coupled control groups.

In addition, HMOX-1 expression was also evaluated in the samples stratified by gender ($P = 0.901$), age ($P = 0.413$) and

smoking habits ($P = 0.598$), but no significant differences were observed.

Difference in protein expression of HMOX-1 between the prosthetic and non-prosthetic groups. Protein expression of HMOX-1 in serum ranged from 1.8 to 7.7 ng/ml in patients in the prosthetic group, while it ranged from 2.4 to 9.2 ng/ml in controls with median values of 5.5 and 4.7 ng/ml, respectively (Fig. 3). Protein expression of HMOX-1 was not statistically different among prosthetic patients and controls ($P = 0.143$), as well as among patients with high circulating metal ions and low circulating metal ions ($P = 0.494$) using the Mann-Whitney test.

Correlation between Co and Cr levels in the blood and urine, and the gene and protein levels of HMOX-1. Finally, the Pearson test did not identify any correlation between gene and protein expression of HMOX-1 ($r = -0.06$; $P = 0.74$), nor between gene and protein HMOX-1 expression and Co blood ($r = 0.11$; $P = 0.48$ and $r = 0.01$; $P = 0.93$) and urinary ($r = -0.1$; $P = 0.52$ and $r = -0.06$; $P = 0.74$) levels in the studied sample.

There was no significant correlation between gene and protein expression of HMOX-1 and the Cr blood ($r = 0.22$; $P = 0.16$ and $r = 0.09$; $P = 0.59$) and urine ($r = 0.02$; $P = 0.92$ and $r = 0.02$; $P = 0.90$) values.

Discussion

The accumulation of metal ions is considered, together with other factors, responsible for the high failure rates of MoM big head hip devices. In a number of studies, the presence of these ions was associated with the induction of oxidative stress (32-40).

Since HMOX-1 is one of the most important antioxidant enzymes to be induced by the presence of metal ions, the aim of the present study was to verify whether, in patients with MoM hip prosthesis, mRNA and protein expression of HMOX-1 was correlated with the level of released metal ions. This was investigated by comparing with patients without prostheses and intentionally not considering implant manufacturers, diameters and performances of the devices, but only the level of metal released.

mRNA and protein expression of HMOX-1 was not identified to be statistically different between patients in the prosthetic and non-prosthetic groups, as well as between patients with high and low ion levels. Moreover, no correlation was identified between the expression of the HMOX-1 gene and its relative protein. This may be due to the use of white blood cells to determine gene expression and the use of the serum alone for the protein assays. Despite the significant differences identified in the ion values between patients in the prosthetic and non-prosthetic groups, there was no correlation between Co and Cr levels and HMOX-1 gene expression.

HMOX-1 production (the predicted physiological response) is induced by the increase of metallic ions; however, it is limited in the high ions group. This production is often not enough to avoid circulating ions contributing to the formation of ROS, which may lead to cellular damage and later, the symptoms reported by patients with prosthetic hips.

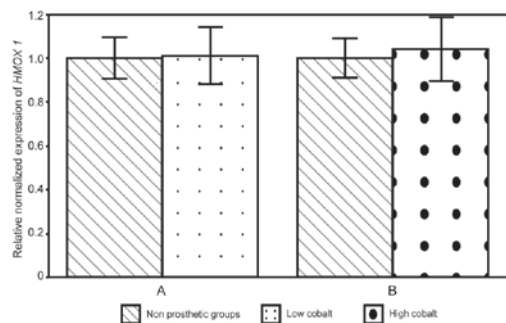


Figure 1. *HMOX-1* gene expression differences between patients with varying concentration of Co in the blood. *HMOX-1* gene expression in the patients from the prosthetic group with (A) low levels of cobalt ($<7 \mu\text{g/l}$) and (B) high levels of cobalt ($>7 \mu\text{g/l}$) compared with their non-prosthetic controls. y-axis, fold change (\pm standard error of the mean).

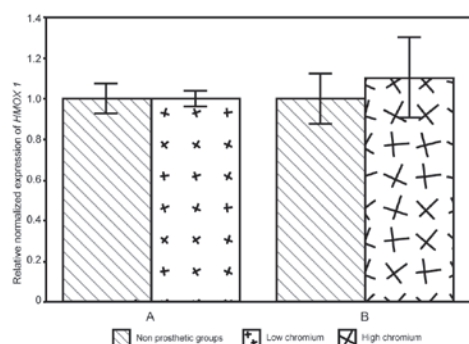


Figure 2. *HMOX-1* gene expression differences between patients with varying concentration of Cr in the blood. *HMOX-1* gene expression in patients from the prosthetic group with (A) low levels of chromium ($<7 \mu\text{g/l}$) and (B) high levels of chromium ($>7 \mu\text{g/l}$) compared with their non-prosthetic controls. y-axis, fold change (\pm standard error of the mean).

The levels of HMOX-1 identified in the present study were lower than expected in high Co patients, this may be due to the fact that in the current study, the exposure to Co was from an internal source, whereas in other studies where HMOX-1 was overexpressed, the source of Co was external (36,38,41). In the present study conditions, the stimulus that should induce oxidative stress, is the internal continuous chronic release of ions as the patients have had the prosthesis for at least 3.5 years. However, in a previous study subjects ingested a bolus or have received injection/drugs with high concentrations of Co (42).

HMOX-1 was selected as an enzyme involved in oxidative stress response, as there are numerous studies in the literature that support the correlation between HMOX-1 and metal ion concentration. *In vitro* studies demonstrated that Co (II) dose- and time-dependently induces HMOX-1 expression in different cell lines (33,40). In addition, *in vivo* studies that demonstrated HMOX-1 induction by Co, were conducted predominantly in the seventies and eighties (36-38), while the most recent studies were conducted in animal models (32,34,35,39). In these studies Cr appears to exhibit a different role on HMOX-1, depending on whether it is in the Cr (III) or Cr (VI) form. Indeed, it has been demonstrated that Cr (III) can be reduced to Cr (II) by biological reductants (i.e. l-cysteine and NADPH), which in turn react with hydrogen peroxide via the Fenton reaction to produce hydroxyl radicals.

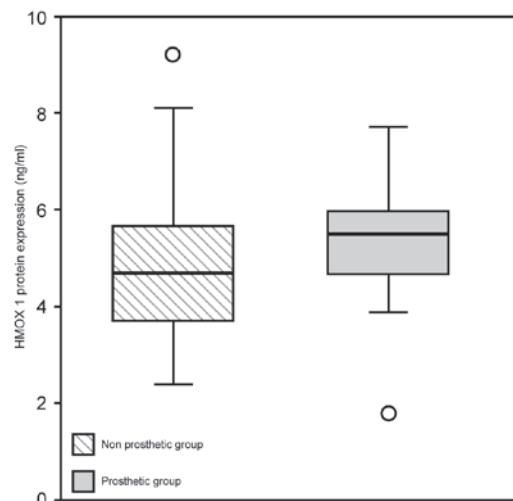


Figure 3. Box plot for protein expression of HMOX-1. Boxes are limited by values of the 25th and 75th percentile. The horizontal line crossing the box represents the median value. The vertical lines are extended from min to max value. Outliers are indicated by \circ , depending on their distance from 75th percentile (greater than 3 interquartile differences or greater than 1.5).

However, Cr (VI)-induced cytotoxicity and overexpression of *HMOX-1* were shown to be dependent on the glutathione level (43).

Therefore, it cannot be excluded that the molecular mechanisms involved in the present study could be different or differently regulated from those observed in other studies. For that reason it would be noteworthy in future studies to measure HMOX-1 levels present in the synovial fluid, where a regulation of the expression similar to that found in this study cannot be ruled out. The discrepancy between the results in the present study and previous literature is possibly due to the small sample size, which had a few uncommon cases, that may have influenced the results.

In the current study, the expression level of HMOX-1 was not affected by the presence of Co, this may be due to the species of Co that was investigated here, the majority of the evidence of interactions between HMOX-1 and Co is in relation to the Co (II) species; however, it is possible that in the present study the Co metallic form (Co^0) may also be involved. Occupational exposure to hard metal dust (WC-Co) induced effects similar to those of exposure to Co (II) via a different molecular mechanism which does not involve HMOX-1 (44,45). Metallic Co is able to produce ROS; however, the kinetics of this process is slower due to the reduced capacity of oxygen to bind to the surface of the metallic particles (46). In addition, Co^0 does not react with H_2O_2 via the Fenton reaction (43) and for this reason, if Co^0 was the predominant species circulating, this could explain the results of the present study.

Conversely, as far as the lack of effect of circulating Cr on HMOX-1 induction is concerned, this is probably due to the fact that only Cr (III) was circulating and does not appear to exert any direct effect on HMOX-1 (43). Previous studies (47,48), have demonstrated that the Cr released by MoM prostheses and present in circulation is in the Cr (III) form. This was confirmed by preliminary evaluations of a small group of samples, in which the chemical speciation was determined by hyphenated techniques (HPLC-ICP-MS), investigating the concentration

of Cr (III) and Cr (VI) in the synovial fluid of patients with prostheses, confirming that the only species present is Cr (III) (unpublished data from Laboratory of Toxicology and Industrial Hygiene, University of Brescia, Italy). Therefore, the results of this study confirm the requirement for greater comprehension of the following for Co and Cr: Ion transport within the organism once released by MoM prosthesis, the identity of the species involved, movement of the ions and the mechanisms of elimination. This has also been suggested by Paustenbach *et al* (49) who hypothesized the existence of a subjective susceptibility to Co (possibly correlated with low albumin levels), which may explain its varied response and transport within the organism. In this case, the identification of individual susceptibility markers, detectable in the peripheral blood, would be an innovative element for investigation of the mechanism by which a patient with a Co-Cr prosthesis may react to Co ions.

Despite the limitations highlighted, the methodology in the present study was robust and accurate. The preliminary results obtained here may be extrapolated to a wider context and suggest that Co and Cr ions, released by articular prostheses, do not induce an increase in HMOX-1 gene and protein expression at least 3.5 years following the insertion of the implant. However, the involvement of other metal-induced oxidative stress enzymes cannot be excluded and will be the subject of future studies.

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