### Title

Metal reactivity is present in dogs with tibial plateau leveling osteotomy and total hip replacement implants.




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# Metal reactivity is present in dogs with tibial plateau leveling osteotomy and total hip replacement implants

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**OBJECTIVE**
Determine whether dogs with well-functioning orthopedic metal implants can develop metal reactivity.

**SAMPLE**
Client-owned dogs that had tibial plateau leveling osteotomy (TPLO) or total hip replacement (THR) implants for 12 months or more and control dogs with no implants.

**PROCEDURES**
Lymphocyte transformation testing was performed by exposing peripheral blood lymphocytes to nickel (Ni), chromium (Cr), cobalt (Co), or a combination of these metals. Lymphocyte proliferation was assessed with flow cytometry. Lymphocyte stimulation indexes (SIs) were calculated. A SI > 2 was considered reactive. Median SIs of dogs in response to metal exposure were compared statistically.

**RESULTS**
Samples from 10 dogs with TPLO, 12 dogs with THR, and 7 control dogs were analyzed. Six dogs out of 22 with metal implants had a reactive SI to 1 or more metals, while 2 of 7 control dogs had a SI > 2 when exposed to nickel only. When all metals were considered, no differences in metal reactivity were found between TPLO, THR, and control groups.

**CLINICAL RELEVANCE**
Metal reactivity is present in dogs and can be identified using lymphocyte transformation testing. Reactivity to Ni is present in dogs with and without metal implants. Reactivity to Co and Cr occurs in some dogs with metal implants.

Hypersensitivity reactions, including dermal metal hypersensitivity, are a common form of delayed type-IV hypersensitivity (DTH) in humans.[1–6] The prevalence of dermal metal hypersensitivity ranges from 10 to 17%.[2–7] Nickel (Ni), cobalt (Co), and chromium (Cr) are common metal sensitizers.[2–4,6–8] All metals implanted in the human body during orthopedic surgery can release particles that may cause a DTH reaction.[9–11] Metal implant DTH is postulated to result from the release of metal particles into tissues due to corrosion, wear, and dissolution.[12–15] Soluble metal ions and, to a lesser degree, particulate metals have been shown to induce a macrophage response, triggering an adaptive cell-mediated immune response by causing metal-specific lymphocyte activation.[15,16] Hypersensitivity can be diagnosed using patch testing, intradermal testing, or a lymphocyte transformation test (LTT). While patch and intradermal testing are routinely used to diagnose contact dermatitis, it is unclear whether these tests can accurately diagnose hypersensitivity to metal implants.[15,17] A LTT, however, provides a more complete picture of the potential immune response to metal implants than skin testing and allows for the identification of involved cellular subpopulations and secretory cytokine profiles.[18–21]

Hypersensitivity reactions, including food hypersensitivity and atopic dermatitis, are well documented in dogs, but it is unclear whether metal hypersensitivity occurs. The literature includes limited evidence suggesting the presence of metal hypersensitivity. In 1 report[22] describing 6 hairless

dogs housed in stainless steel cages, chromium-induced contact hypersensitivity was suspected based on the reaction to potassium dichromate during patch testing and on histopathological findings closely resembling those of chromium-induced contact dermatitis in humans. Dogs with metal implants can also exhibit clinical signs that could result from metal hypersensitivity, such as licking the skin covering metal implants, pain on palpation at the site of the metal implant, and lameness following surgery. The resolution of these signs after implant removal has been considered suggestive of metal hypersensitivity in cases where other causes, such as implant motion and infection, were not present.[23] To our knowledge, no studies have investigated lymphocyte reactivity in dogs with metal implants.

The aim of this study was to use LTTs to determine whether dogs with well-functioning orthopedic metal implants develop metal reactivity. We hypothesized that metal reactivity occurs in dogs with well-functioning metal implants. We further hypothesized that dogs with metal implants would be more reactive to chromium, cobalt, and nickel alone or in combination compared to control dogs without implants.

# Materials and Methods

## Pilot study

The project received institutional animal care and use committee approval (No. 21185). Owners signed informed consent. Three staff-owned dogs without surgical implants and no known allergic disease were enrolled in a pilot study to develop the LTT protocol.

## Lymphocyte isolation and freezing

Twenty to 25 milliliters of peripheral blood was collected from each dog via peripheral venipuncture into heparinized tubes. Peripheral blood mononuclear cell (PBMC) isolation was carried out using a lymphocyte isolation medium (Histopaque 1119 [Sigma-Aldrich] and Ficoll-Paque [GE Healthcare]) diluted with tissue culture water for a final specific gravity of 1.066, based on a previously optimized protocol.[24] Freezing media (90% FBS and 10% DMSO) was added to the lymphocyte pellet and distributed evenly to freeze the cells at 5E6 cells/aliquot. Cryovials were gradually frozen to −80 °C over 24 hours using a freezing system (Cryo-Safe −1 °C freezing container; ULAB Scientific) and then transferred to liquid nitrogen.

## Lymphocyte proliferation

The contents of each PBMC cryovial were thawed, added to 9 mL of Dulbecco phosphate-buffered saline, and centrifuged at 400 X *g* for 10 minutes with slow acceleration and deceleration. The PBMCs were resuspended in lymphocyte culture medium (RPMI 1640, 10% FBS, 1% GlutaMax, 1 mmol sodium pyruvate, 2 mmol HEPES, 1 µl/mL MEM NAA, and 55 µmol b-mercaptoethanol) and quantified using an Act Diff Coulter Counter (Beckman Coulter Inc). Based on cell count, PBMCs were plated at a density of 1,333 cells/µL using 24-, 48-, or 96-well plates. Each test was conducted in duplicate, triplicate, or quadruplicate, depending on the recovered lymphocyte count. For the metal challenges, the media were supplemented with 0.1 mMol of $CrCl_3$ (Cr), $NiCl_2$ (Ni), $CoCl_2$ (Co), the combination of $CoCl_2$ and $CrCl_3$ (Co + Cr), or the combination of all three metals (Co + Cr + Ni) in separate wells for a total of 5 days.[10] For negative controls, lymphocytes were incubated in unsupplemented media. For proliferation-positive controls, the media were supplemented with 5 mg/mL concanavalin A (ConA; Sigma-Aldrich) at a final concentration of 50 µg/mL. After 84 hours of culture with ConA or metal antigen present, lymphocyte proliferation was assessed via 5-ethynyl-2′-deoxyuridine (EdU) incorporation per manufacturer's instructions (Click-iT Plus EdU Alexa Fluor488 flow cytometry assay kit; Thermo Fisher Scientific). Twelve hours later, the cells were fixed, permeabilized, and labeled with anti-EdU conjugated with a fluorescent dye. The cells were analyzed using a flow cytometer (FACSCalibur; BD Biosciences), and data were analyzed with commercial software (FlowJo; BD Biosciences).

The lymphocyte proliferation rate was calculated by dividing the fluorescing cell count by the total cell count. Mean proliferation rates of repeated assays were calculated. The stimulation index (SI) was calculated by dividing the mean proliferation rate of metal-treated cells or ConA-stimulated cells (positive control) by the mean proliferation rate of unstimulated cells (negative control). Assays for individual dogs were considered valid if the SI was > 10 when challenged with the ConA-positive control.[10] Assays with an SI < 10 when exposed to ConA were excluded from analyses because the expected lymphocyte stimulation did not occur. For metal challenges, individual dog assays with SIs > 2 were considered reactive to metal.[10,15]

## Study animals

Canine patients treated by the orthopedic surgery service at the University of California-Davis Veterinary Medical Teaching Hospital were eligible for inclusion if they weighed > 20 kg, underwent unilateral or bilateral tibial plateau leveling osteotomy (TPLO) or total hip replacement (THR) at least 12 months before enrollment, and had no postoperative complications. All TPLO plates and screws were purchased from a single manufacturer (DePuy Synthes). Plates and screws were made of stainless steel (ASTM F138). All THR implants were cementless components from two manufacturers (BioMedtrix, n = 10; and Kyon AG, 2). The BioMedtrix acetabular shells, femoral stems, and lateral bolts were made of titanium alloy (ASTM F136), and the heads were made of Co-Cr alloy (ASTM F799). The Kyon AG femoral stems, screws, and head and neck components were made of titanium alloy (ASTM F136), and the acetabular shells were made of commercially pure titanium (ASTM F67). Metal composition is listed **(Supplementary Table S1)**. Dogs with confirmed or suspected orthopedic implant infection and dogs receiving medication to treat allergic

disease, including corticosteroids and immunomodulatory medications, were excluded from enrollment. Age, breed, date, and type of surgery were recorded. Eight dogs owned by staff or students without a history of either allergic disease or surgery involving implant placement were enrolled as control dogs. These dogs were not used in the pilot study. Control dogs' breed, sex, and age were selected to match those of dogs with metal implants. Lymphocyte isolation, proliferation, and SI calculation were performed as previously described.

## Statistical analysis

Descriptive data collected (age of the dogs and time of exposure to implant in dogs with and without reactivity) were expressed as median (minimum, maximum) and analyzed using Mann-Whitney *U* test (https://www.socscistatistics.com/tests/mannwhitney/). The remaining analyses were performed using commercial software (SAS version 9.4; SAS Institute). The sample size was determined using a priori power analysis ($\beta = 0.80$; $\alpha = 0.05$) based on a previous report.[10] Dogs with implants were compared to dogs with no implants (control group), and the effect of group (controls or with implants) and metal type on the log-transformed SI data was analyzed with a mixed-model ANOVA with post hoc pairwise comparisons using Tukey-Kramer test. Statistical significance was set at $P < .05$.

# Results

Thirty-six dogs were enrolled: 14 that had undergone TPLO surgery, 14 that had undergone THR surgery, and 8 control dogs. Four samples from TPLO dogs and 1 from a THR dog could not be assayed due to a lack of cellular proliferation. Two dogs (1 THR and 1 control) had an SI < 10 when exposed to ConA and were excluded from the analysis. Characteristics of the 10 dogs with TPLO, 12 dogs with THR, and 7 control dogs that were analyzed are described **(Table 1)**. There was no difference in age between the control dogs and dogs with implants ($P = .337$).

When considering all metal types combined, there was no difference between control dogs and implanted dogs nor between types of implants **(Table 2)**. In the control group, the SI from Ni was larger than any of the other metals. In the TPLO group, there were no differences among metals. In the THR group, the SI from Co, Co + Cr, or Co + Cr + Ni was less than Cr, and the SI from Ni was greater than Co + Cr and Co + Cr + Ni. The statistical comparisons were reanalyzed without the 2 THR dogs with titanium-only implants. There was no change in the significant differences with and without the 2 dogs in the THR group.

Two control dogs and 1 THR dog were metal reactive (SI > 2) to nickel only. Three dogs with TPLO

**Table 1**—Signalment of 29 dogs used to evaluate reactivity to metals.

| Subject group | Number of subjects | MC | FS | F | Median (range) age (y) | Median (range) implant time in situ (mo) |
|---|---|---|---|---|---|---|
| Control dogs | 7 | 3 | 3 | 1 | 6.5 (2.0–7.0) | N/A |
| G Retriever (1)<br>L Retriever (1)<br>Pit Bull Terrier (1)<br>Border Collie (1)<br>Boxer (1)<br>GSD mix (1)<br>BMD (1) | | | | | | |
| TPLO* dogs | 10 | 5 | 5 | 0 | 8.5 (2.0–14.0) | 32 (14–62) |
| L Retriever (2)<br>Mastiff/Boxer mix (1)<br>1 GSD (1)<br>GSD mix (1)<br>G Retriever mix (1)<br>Border Collie (1)<br>Pit Bull Terrier (1)<br>Pit Bull Terrier mix (1) | | | | | | |
| THR† dogs | 12 | 6 | 5 | 1 | 6.5 (3.0–11.0) | 18 (12–31) |
| G Retriever (2)<br>L Retriever mix (2)<br>GSD (2)<br>GSD mix (1)<br>G Retriever mix (1)<br>Border Collie (1)<br>Border Collie mix (1)<br>A Shepherd (1)<br>Akita (1) | | | | | | |

A Shepherd = Australian Shepherd dog. BMD = Bernese Mountain dog. F = Female. FS = Female spayed. G Retriever = Golden Retriever. GSD = German Shepherd dog. L Retriever = Labrador Retriever. MC = Male castrated. N/A = Not applicable. THR = Total hip replacement. TPLO = Tibial plateau leveling osteotomy

*Bone plates and screws were manufactured by DePuy Synthes. †Total hip replacement stems, head, and cups were manufactured by BioMedtrix (BFX cups and stems, 10 dogs) and Kyon AG (Zurich Cementless implants, 2 dogs).

**Table 2**—Median (minimum, maximum) stimulation indexes of dogs in response to metal exposure.

| Metal | Control | TPLO | THR |
|---|---|---|---|
| All | 0.95 (0.21, 6.83)[a] | 0.84 (0.15, 6.40)[a] | 0.61 (0.02, 11.04)[a] |
| Cr | 1.16 (0.31, 1.30)[a,1] | 0.80 (0.31, 2.87)[a,1] | 0.90 (0.40, 4.91)[a,1] |
| Co + Cr | 0.64 (0.22, 1.45)[a,1] | 0.75 (0.23, 2.82)[a,1] | 0.44 (0.08, 4.15)[a,2,3] |
| Co + Cr + Ni | 0.86 (0.23, 1.83)[a,b,1] | 0.72 (0.15, 4.06)[a,1] | 0.43 (0.04, 5.90)[b,2,5] |
| Co | 0.98 (0.21, 1.56)[a,1] | 0.94 (0.25, 2.16)[a,1] | 0.73 (0.09, 2.31)[a,2,6] |
| Ni | 1.76 (0.49, 6.83)[a,2] | 1.06 (0.51, 6.40)[a,1] | 0.84 (0.02, 11.04)[a,1,2,4,6] |

Co = Cobalt. Cr = Chromium. Ni = Nickel. *See* Table 1 for remainder of key.

[a,b]Within a row, median values with different superscript letters differ significantly ($P < .05$).

[1–6]Within a column, median SI values after exposure to various metals with different superscript numbers differ significantly ($P < .05$).

**Table 3**—Number of dogs reactive (stimulation index > 2) to metal exposure.

| Group (n) | Ni | Co | Cr | Co + Cr | Co + Cr + Ni | Total number of reactive dogs |
|---|---|---|---|---|---|---|
| Control dogs (7) | 2 | 0 | 0 | 0 | 0 | 2 |
| TPLO dogs* (10) | 2 | 1 | 1 | 1 | 2 | 3 |
| THR dogs† (12) | 3 | 1 | 1 | 1 | 2 | 3 |

*See* Table 2 for key.

*Two dogs in the TPLO group and 2 dogs in the THR group were metal reactive to 2 or more metals, while a third dog in each group was only metal reactive to 1 type of metal. †All THR dogs with reactivity had BioMedrix implants.

plates and 3 with THR implants had an SI > 2 to 1 or more metals **(Table 3)**. All dogs that reacted in the THR group had BioMedtrix implants. Duration of exposure to the implant did not differ between reactive dogs and nonreactive dogs ($P = .582$).

## Discussion

In the current study, 27% of dogs with long-term metal implants had evidence of metal reactivity identified using LTT. We accepted the hypothesis that metal reactivity can be identified in dogs with metal implants. Metal reactivity has previously not been reported in dogs with orthopedic implants. The metal reactivity rate in dogs in the current study matched the 25% rate of lymphocyte reactivity reported in humans with well-functioning THR implants.[10] In human patients, with no evidence of metal allergy before total hip replacement, 18% developed metal sensitivity within 36 months of surgery, and the probability of metal hypersensitivity was higher in cases with implant loosening and implant failure.[20,25] Duration of exposure is needed to develop reactivity. Therefore, dogs with implants in place > 1 year were enrolled and there was no difference in the duration of exposure between reactive dogs compared to nonreactive dogs. As the dogs with metal implants in the current study were not tested before surgery; it is unknown if any dog had a preexisting metal allergy.

We rejected the hypothesis that dogs with metal implants were more reactive to Cr, Co, and Ni alone or in combination than control dogs without implants. When all metal combinations were considered, no difference in the SI was detected between the different groups. The lack of difference may have been the result of a relatively low reactivity rate in dogs with implants (27%) and the presence of reactivity in some control dogs. The combined SI for all metal challenges and all groups was less than the negative control. This could indicate a general suppression of lymphocytes when exposed to metals and could be due to metal toxicity. Toxicity and subsequent suppression have been demonstrated in humans when exposing lymphocytes to various concentrations of metals.[10]

Importantly, reactivity to Co and Cr was only detected in dogs with metal implants. These results match findings in humans with metal implants, where Co and Cr are commonly implicated as a source of metal reactivity or hypersensitivity after orthopedic surgery.[26–29] Seven of the 8 dogs with metal reactivity were also reactive to Ni. In the control group, the 2 dogs with metal reactivity were reactive to Ni only, while 1 dog in the THR group was reactive to Ni only. In humans, Ni exposure and reactivity are the most common causes of dermal metal hypersensitivity and are linked to nickel-containing jewelry, including earrings.[6,30] Dogs may be exposed to Ni from bowls and collars made of stainless steel, but stainless steel also contains Co and Cr. It is possible that Co and Cr are more immunoreactive when present internally compared to externally, but this would have to be further investigated. In this study, there was a limited number of PBMC, and each assay was performed with a minimum of duplicates, including positive, negative, and metal challenge assays. Therefore, not all metal combinations were tested. Our results show that metal reactivity is present in dogs with metal implants and that Cr and Co alone or in combination, along with Ni, are likely sensitizers.

The clinical impact of metal reactivity in dogs with implants is unknown and was beyond the objective of the current study. In human medicine, it is unclear whether metal reactivity and metal hypersensitivity contribute to implant failure or if abnormal implant wear and/or implant failure leads to hypersensitivity.[2,26] Human metal implant hypersensitivity is

diagnosed by exclusion. Infection, implant mechanical failure, and loosening are excluded first as they cause symptoms similar to metal hypersensitivity. Radiographic changes associated with metal hypersensitivity are nonspecific and consist of osteolysis without bone atrophy.[26] Patients with no osteolysis or mild osteolysis following THR were more reactive to Cr than the control group with no implants, while patients with moderate or severe osteolysis were more reactive to Co than the control group.[26] People with hypersensitivity to metal implants exhibit signs of pain at the surgical site, persistent drainage from the surgical wound, dermatitis, implant loosening, and implant failure.[31–33] These clinical signs have been sporadically reported in veterinary patients with complications after orthopedic surgery. It is therefore plausible that dogs develop these types of clinical signs due to metal hypersensitivity in response to implants. Investigating metal hypersensitivity in dogs with implant loosening or implant failure is warranted: it will improve our understanding of the immune response to permanent metal implants in dogs and may inform the development of implants used in the future.

Hypersensitivity is diagnosed using different testing strategies. Patch testing is readily available and considered the gold standard for diagnosing Ni allergy in humans, while LTT has equal or higher sensitivity than dermal tests to diagnose metal hypersensitivity in people with metal implants.[18,21,34] Using in vitro testing like the LTT may be clinically more applicable to evaluate the peri-implant environment and immune response to the metal compared to a dermal patch test, as primary antigen-presenting cells will differ.[26,34] In addition, the dermal tests have marked reproducibility variation.[26,34] The LTT was first described in veterinary medicine in 1982 and has been used in veterinary medicine for diagnosis and monitoring of treatment of food hypersensitivity.[35–37] Our laboratory successfully developed a LTT protocol for dogs using frozen PBMC, which allows for more flexibility during the testing process as the blood sampling did not dictate the time of assay completion. Despite the loss of five samples due to lack of proliferation, most frozen PBMCs were successfully tested, indicating that the freezing, thawing, and proliferation processes developed were acceptable. Thus, a LTT using frozen cells can be used to diagnose metal reactivity in dogs with orthopedic metal implants and should be further investigated as a diagnostic tool in dogs with clinical signs consistent with metal hypersensitivity. While the apparent utility of the LTT supports its use for the diagnosis of metal reactivity, disadvantages such as cost, equipment, supplies, and time make LTT less convenient than dermal tests.[34] The SI threshold of 2 was selected in the current study based on several human studies.[10,21,38] Increasing the SI threshold to 3 would have decreased the number of positive cases to 2 TPLO dogs, 2 THR dogs, and 1 control dog. Conversely, lowering the SI threshold to 1.5 would have increased the number of dogs considered to be reactive by 3, 1 in each group. Currently, no consensus on a SI threshold in human lymphocyte testing exists.[26] Further research is needed to determine the optimal discriminating SI value, for example, by basing SI thresholds on metal hypersensitivity rates.

In conclusion, we demonstrated the presence of metal reactivity in dogs and that this can be identified using a LTT. Cobalt and Cr reactivity was detected in a subset of dogs with metal implants, and Ni reactivity was identified in subsets of dogs with and without metal implants.

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There are no conflicts of interest to declare.

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## Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org