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Original Research

Local and Systemic Changes Associated with Long-term, Percutaneous, Static Implantation of Titanium Alloys in Rhesus Macaques (*Macaca mulatta*)

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Metal alloys are frequently used as implant materials in veterinary medicine. Recent studies suggest that many alloys induce both local and systemic inflammatory responses. In this study, 37 rhesus macaques with long-term skull-anchored percutaneous titanium alloy implants (duration, 0 to 14 y) were evaluated for changes in their hematology, coagulation, and serum chemistry profiles. Negative controls (*n* = 28) did not have implants. Macaques with implants had higher plasma D-dimer and lower antithrombin III concentrations than nonimplanted animals. In addition, animals with implants had higher globulin and lower albumin and calcium concentrations compared with nonimplanted macaques. Many of these changes were positively correlated with duration of implantation and the number of implants. Chronic bacterial infection of the skin was present around many of the implant sites and within deeper tissues. Representative histopathology around the implant site of 2 macaques revealed chronic suppurative to pyogranulomatous inflammation extending from the skin to the dura mater. X-ray fluorescence microscopy of tissue biopsies from the implant site of the same 2 animals revealed significantly higher levels of free metal ions in the tissue, including titanium and iron. The higher levels of free metal ions persisted in the tissues for as long as 6 mo after explantation. These results suggest that long-term skull-anchored percutaneous titanium alloy implants can be associated with localized inflammation, chronic infection, and leaching of metal ions into local tissues.

Abbreviation: ATIII, antithrombin III

Cephalic implants comprising titanium alloys are commonly used in many animal models for neurologic research. Although these implants are essential to researcher, the chronic presence of a large metallic foreign body presents its own obstacles, including leaching of alloys, promotion of chronic inflammation, and a nidus for infection and biofilm formation. Percutaneous implants of various materials are prone to infection in many human specialties, including oral, orthopedic, and neurosurgery,[10,17] even under optimal conditions.

The most common metal in cephalic implants in research settings is titanium. This transition metal is noted for its corrosion resistance and biocompatibility within the human body.[11,35] When titanium is used as a prosthetic material, such as in periodontal implants, prosthetic joints, or fracture repair, its mechanical and chemical properties and its interaction with the host are of prime importance for long-term clinical success of the implant. Titanium alloys are more commonly used than pure titanium, to improve the tensile strength and yield strength of the implant.[19] Previous generations of titanium alloys were composed of vanadium and aluminum (for example, Ti–6Al–4V), although research has shown that these implants may have toxic effects resulting from the leaching of these components.[20,31,46,51,61] Newer titanium alloy compositions show improved biocompatibility and stability and include niobium, zirconium, and molybdenum (for example, Ti–13Nb–13Zr and Ti–12Mo–6Zr),[20,55,70,73] and new ways of synthesizing high-strength titanium are being developed. Modifications to the surface coating of the titanium and related alloys are also being developed, to both improve biocompatibility and decrease biofilm formation.[18,26,42,58,65] These improved titanium-based alloys have dramatically improved the stability and longevity of implants and reduced the amount of associated local tissue inflammation[16,37]

Recent literature has revealed the metal–tissue interaction of titanium within the host and suggests that galvanism, the corrosion of the alloy, may induce and sustain a local host immune response, jeopardizing the stability of the surgical implant as well as the health of the local tissue.[7] In addition, serum titanium levels

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often are significantly elevated in patients with implants and are correlated with the type of the implant and the duration of implantation.[14,36,40] Neurobiology research often uses percutaneous skull-anchored implants of various conformations, frequently with rhesus macaques (*Macaca mulatta*) as the experimental subjects.[1,2] To date, no studies have evaluated the systemic effects of these long-term implants in rhesus macaques.

In this study, we evaluated the effects of long-term skull-anchored percutaneous titanium alloy implants in rhesus macaques. Histologic evaluation revealed chronic pyogranulomatous inflammation, and X-ray fluorescence microscopy demonstrated metal ions that were distributed within the granulation tissue around the implant site and that persisted for as long as 6 mo after explantation. In addition, chronic bacterial infection frequently was present at the site of implantation. Furthermore, basic blood analysis and assessment of coagulation parameters revealed a trend toward a prothrombotic and proinflammatory state.

Materials and Methods

Animals. Chinese-origin rhesus macaques (*Macaca mulatta*; n = 56; 37 with implants, 28 without implants; 45 male, 20 female; age, 6 to 19 y) were evaluated for this study. Animals had one or more skull-anchored percutaneous titanium alloy implants types as a consequence of their use in cognitive neuroscience research. The composition of the titanium alloys varied depending on the laboratory and implant distributor; other metals in the alloy included, but were not limited to, iron, aluminum, niobium, molybdenum, and vanadium. Macaques were divided into groups according to implant status: cephalic restraint pedestal only, n = 18 (16 male, 2 female); cephalic restraint pedestal and a cephalic recording chamber, n = 19 (16 male, 3 female); and no implant, n = 28 (13 male, 15 female). Briefly, the cephalic restraint pedestals are percutaneous titanium alloy posts that are anchored to the skull with titanium alloy screws; the cephalic recording chambers are percutaneous recording chambers that lead to the brain and are usually made of acrylic or CILUX plastic and anchored to skull by either ceramic or titanium alloy cortical screws.[1,2] The duration of implantation ranged from 0 to 14 y (0 y signifies more than 6 mo but less than 1 y of implantation). Chronic bacterial infections were frequently present around the base of the cephalic restraint pedestals or within the cephalic recording chambers. The implants were cleaned as frequently as necessary with sterile saline, 0.05% chlorhexidine solution, or 1% to 2% povidone–iodine solution, according to the recommendation of the Division of Comparative Medicine veterinary staff, to reduce the animals' infectious and inflammatory burden and to decrease stress. More specifically, all cephalic implants were inspected at least once weekly for any signs of infection or contamination. If mild but chronic signs were evident, a minimum of twice weekly (3 to 4 d apart) inspection and cleaning was required. For infected implant margins or for margins with obvious signs of inflammation, dehiscence, or necrosis, the veterinary staff was consulted, given that these cases needed to be assessed regarding the need for daily care to treat the infection and prevent tissue devitalization. Bacterial cultures and antibiotic susceptibility testing were performed, and macaques received oral or injectable antibiotics as needed.

All study animals were indoor-housed (either singly or paired) in an AAALAC-accredited facility. Commercial primate chow (Lab Diet 5038, PMI Nutrition International, St Louis, MO) was fed twice daily. Environmental enrichment was provided daily in the form of toys, videos, and seasonal fruits, vegetables, and other treats. While quarantined, macaques were screened for endoparasites, *Salmonella* spp., *Shigella* spp., other enteric pathogens, tuberculosis, and a battery of viral agents including simian retrovirus, *Macacine herpesvirus* type 1 (B virus), simian T-lymphotrophic virus 1, measles virus, and SIV. With the exception of scattered positive measles antibody titers, all of the animals were free of these agents. Macaques were tested and confirmed negative for enteric pathogens annually and for tuberculosis, fecal endoparasites, and B virus semiannually. All of the macaques were on IACUC-approved experimental protocols and were not implanted solely for the purposes of the current study.

Blood collection, processing, and testing. All macaques were sedated with ketamine (10 mg/kg IM; Zoetis, Florham Park, NJ) or tiletamine–zolazepam (5 mg/kg IM; Zoetis). Blood collected from the femoral vein was dispensed into 2 (2.9-mL) sodium citrate (3.2%) tubes, a 1.2-mL EDTA tube, and a serum-separator vacuum phlebotomy tube (Sarstedt, Nümbrecht, Germany). The first sodium citrate tube was discarded to minimize the chance that endothelial and platelet activation from the initial venipuncture would result in spurious biomarker readings (that is, '2 tube technique').[41] Platelet-poor plasma was prepared within 4 h of phlebotomy. Platelet-poor plasma was collected from the citrate tubes by 2 sequential centrifugations at 1900 × g for 10 min, separated into 200-µL aliquots, and stored at –80 °C until analyzed. CBC analysis was performed inhouse (Hemavet 950 FS, Drew Scientific, Oxford, CT), and a serum biochemistry profile was performed by a commercial laboratory (IDEXX Laboratories, Westbrook, ME). Assessment of coagulation biomarkers, including protein C, antithrombin III (ATIII), D-dimer, and soluble P-selectin (that is, sCD62P), were performed as previously described.[23] Briefly, bead-based fluorescence technology was used in previously validated sandwich ELISA to obtain quantitative outputs for coagulation biomarkers.

Histopathology. Postmortem tissue samples were collected from the implant area; the pathologist was not blinded to sample collection or evaluation. At the time of this study, representative samples were collected from only 3 macaques, because the number of animals available at the end of the study limited collection. Biopsies were not collected from additional animals, because this study was designed to be as minimally invasive as possible, with no testing or sample collection performed outside of health-screening events. Rhesus 1 had no implant during specimen collection but 6 mo prior had a grade 2 titanium cephalic restraint pedestal (President Titanium, Hanson, MA) with Ti cortex self-tapping screws (diameter, 2.0 to 2.7 mm) made from both commercially pure grade 4 titanium and Ti–6Al–7Nb alloys (Synthes, West Chester, PA). Rhesus 2 had 2 implants for a total duration of 6 y—a grade 2 titanium cephalic restraint pedestal (President Titanium, Hanson, MA) with 7-mm cortical screws made from Ti–6Al–4V alloy (Veterinary Orthopedics Implants, St. Augustine, FL) and a CILUX plastic recording chamber, which contained no metal (Crist Instruments, Hagerstown, MD). Rhesus 3 had no implant and served as the negative control. The samples were fixed in 10% formalin, embedded in paraffin, and processed routinely. All tissue blocks were sectioned at 4 µm and stained with hematoxylin and eosin; sections were evaluated by a board-certified veterinary pathologist. Selected slides were also stained with special stains, including Brown and Brenn, Fontana–Masson, Perls Prussian blue, and rubeanic acid.[5]

X-ray fluorescence microscopy. X-ray fluorescence microscopy was performed as previously described on the same tissue

specimens that were evaluated for histopathology.[38] Briefly, paraffin-embedded tissue samples were cut into sections (thickness, 9 µm), placed onto polyethylene nahphalate slides, and deparaffinized. Laser-capture microscopy was performed (Arcturus Veritas platform, ThermoFisher Scientific, Waltham, MA) to excise areas of tissue with black pigment, which subsequently were mounted on Si_3N_4 membrane grids (2.0 × 2.0 mm). The samples were excited with incident synchrotron X-rays of 10 keV for elemental Kα characteristic emission lines. Elemental profiles were obtained by using synchrotron scanning X-ray fluorescence microscopy at the Advanced Photon Source of the Argonne National Laboratory (Lemont, IL).

Statistical analysis. Because of the large numbers of nonimplanted and implanted animals and the difficulty in obtaining baseline data for each animal, statistical analysis was performed to compare nonimplanted with implanted macaques in terms of serum biochemistry, CBC, and coagulation biomarker quantitative data. The data were analyzed (GraphPad Software, La Jolla, CA) by using 2-tailed Student *t* tests assuming either equal or unequal variance, depending on the result of an F test, with a *P* value of less than 0.05 on the F test considered to signify unequal variance between the 2 groups. A *P* value of less than 0.05 was considered significant in the Student *t* test. Prior evaluation of the coagulation biomarkers panel confirmed minimal to no effect of age or sex on the plasma values of protein C, ATIII, D-dimer, and sCD62P.[23] Pearson correlation analysis verified the lack of correlation between animal age and number of implants and between animal age and duration of implantation, signifying that all statistically significant differences in hematologic parameters reported here are most likely related to implant status.

Results

Local tissue effects of implantation. Histopathology was performed on tissue surrounding the implant site in rhesus macaques to examine the cellular changes associated with chronic titanium implantation. At time of tissue collection, chronic purulent exudate was on the skin around the implant site of rhesus 2 and was occasionally found within deeper tissues. In rhesus 1, the dura mater was disrupted by black granular material surrounded by woven bone fragments and few multinucleated giant cells (foreign body type; Figure 1 A and B). The dura mater of rhesus 1 had black accellular material surrounded by degenerate neutrophils and proteinaceous material (Figure 1 D). In addition, gram-positive bacteria surrounded by inflammatory cells were present in the dura mater (Figure 1 E). In rhesus 2, the epidermis was expanded by acanthosis and orthokeratosis. The epidermis was covered by fibrin, hemorrhage, neutrophils, and macrophages. The superficial dermis was infiltrated by macrophages and lymphocytes (Figure 1 C and D). The samples from these 2 macaques were both compared with cranial tissue biopsies from the normal control (macaque 3), which had no abnormal changes or inflammatory cell infiltrates. Histopathology around the implant site of both implanted animals revealed chronic pyogranulomatous inflammation extending from the skin to the dura mater.

Fontana–Masson staining of biopsy sections confirmed that the black pigment was foreign material and not melanin. Perls Prussian blue and von Kossa stains were performed to confirm the presence of iron and mineral, respectively, in the biopsy sections. These results indicate the presence of a black foreign material in the tissue surrounding the implant site and an acute and chronic pyogranulomatous inflammation associated with this foreign material. These 2 implanted animals also had both intra- and extracellular bacteria in deeper tissues and superficial purulent material. In fact, many of the animals had chronic bacterial infections at the site of implantation, which were confirmed with routine culture of either the skin surrounding the cephalic restraint pedestal or within the cephalic recording chamber.[6,69]

X-ray fluorescence microscopy of tissues surrounding the implant site. X-ray fluorescence microscopy was used to examine whether the black foreign material in the tissue around the implant site was of metal origin, and if so, to determine the metal composition (Figure 2, Table 1) Both rhesus 1 and 2 had a large amount of titanium and iron deposition in the tissue samples. Although the increase in iron of both rhesus could be due to the composition of the titanium alloy, the large amount of iron in rhesus 2 also correlated with the presence of RBC. In the negative control, no titanium was identified in the tissue samples, and only traces of iron were present. The presence of titanium and a small amount of iron was confirmed in the areas of the black material (as seen in the brightfield image) of the 2 macaques with titanium implants that were evaluated. Imaging confirmed the leakage of Ti ions from the implants into local tissues; the results also indicated that other metals, such as Cu, also leaked into surrounding tissues.

Association of implantation with a proinflammatory state. Macaques with implants had lower albumin and calcium concentrations than those without implants (3.7 ± 0.4 g/dL and 9.1 ± 0.4 mg/dL compared with 4.2 ± 0.3 g/dL and 9.5 ± 0.6 mg/dL, respectively; $P < 0.01$); these differences were also significantly negatively correlated with the number of implants involved and the duration of implantation (Figure 3 A through C; Tables 2 and 3). In addition, globulin levels were significantly elevated in implanted compared with control animals (3.3 ± 0.6 g/dL compared with 2.7 ± 0.3 g/dL; $P < 0.01$); these differences were also positively correlated with the number of implants and duration of implantation (Figure 3 D through F; Tables 2 and 3).

Other clinical chemistry alterations observed in the implanted compared with control macaques included significantly higher creatinine concentrations (1.1 ± 0.3 mg/dL compared with 0.9 ± 0.3 mg/dL; $P < 0.01$); these differences were positively correlated with the number of implants but not the duration of implantation (Figure 4 A through C; Tables 2 and 3). In addition, cholesterol was lower in animals with implants (122 ± 26 mg/dL compared with 159 ± 33 mg/dL; $P < 0.01$); these differences were negatively correlated with the number of implants but not the duration of implantation (Figure 4 D through F; Tables 2 and 3). Urinalyses were not performed on these animals; therefore renal function was not thoroughly evaluated in this study.

Pearson correlation analysis of these clinical chemistry parameters showed no significant correlation of age and biomarker concentration (Table 4). There was slight positive correlation between macaque age and the number of years with an implant but no significant correlation between albumin or globulin level and age (Table 4).

Association between implantation and a procoagulant state. Analysis revealed that macaques with implants had higher plasma D-dimer and lower ATIII concentrations (360.6 ± 312.5 ng/mL and 117.4 ± 12.9 µg/mL, respectively) than did nonimplanted animals (178.1 ± 137.5 ng/mL and 128.4 ± 12.4 µg/mL, respectively). In addition, the number of implants, but not the dura-

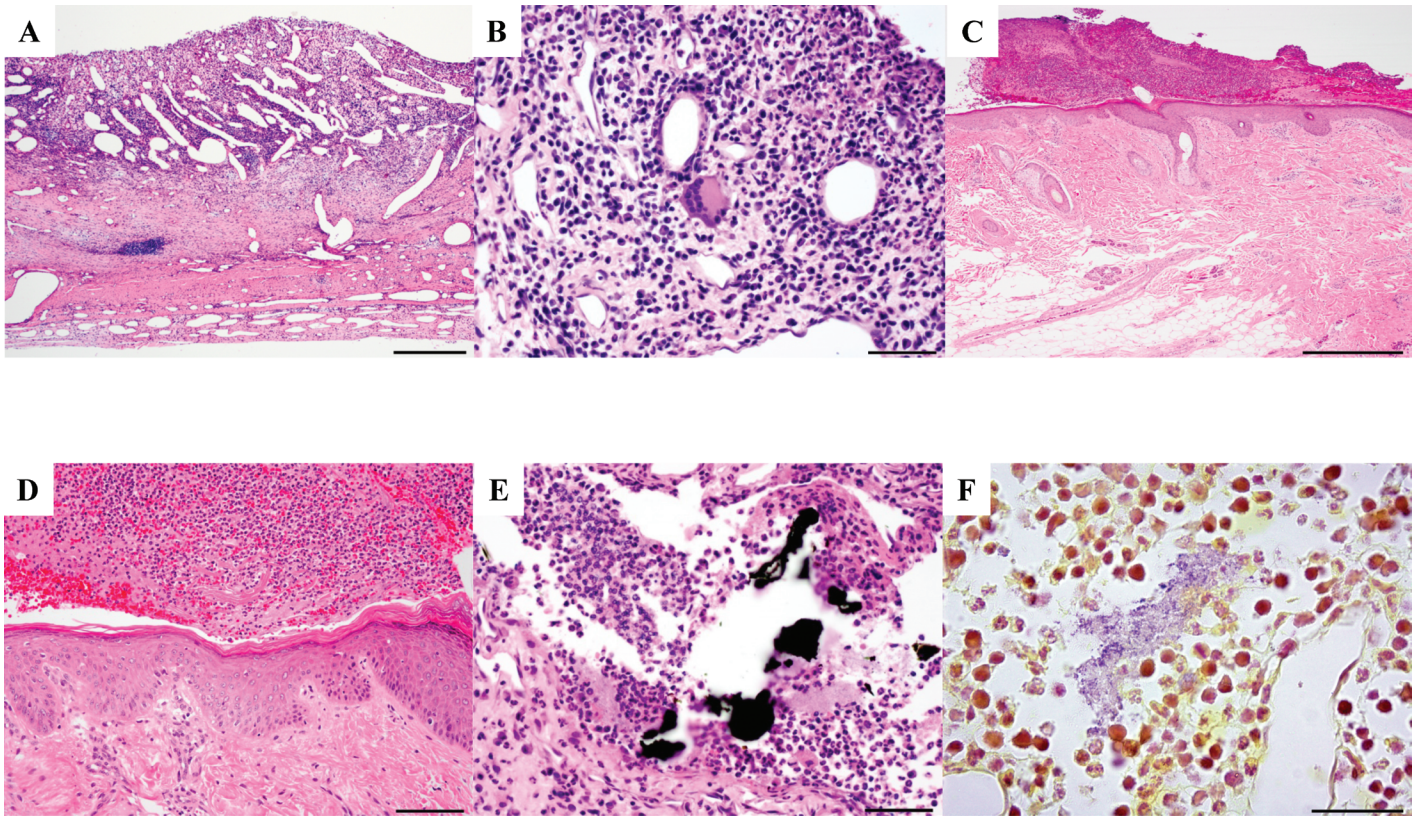

Figure 1. Histopathology of tissue surrounding the implant site. (A) The epidermis was covered by serocellular exudate (crust). Magnification, 4× (scale, 500 µm). (B) The epidermis was expanded by acanthosis and orthokeratosis and covered by serocellular exudate composed of fibrin, hemorrhage, and inflammatory cells. Magnification, 40× (scale, 50 µm). (C) The dura mater was expanded by diffuse chronic–active pyogranulomatous inflammation and ectatic blood vessels. Magnification, 10× (scale, 200 µm). (D) A higher-magnification image from panel C shows inflammation composed of a mixture of granulocytes, mononuclear cells, and few multinucleated giant cells. Magnification, 40× (scale, 50 µm). (E) The dura mater is disrupted by multiple, variably shaped patches of black acellular material surrounded by degenerate neutrophils and proteinaceous material. Magnification, 40× (scale, 50 µm). Hematoxylin and eosin stain (panels A through E). (F) Aggregates of gram-positive bacteria are surrounded by inflammatory cells within the dura mater. Modified Brown and Brenn stain; magnification, 40× (scale, 50 µm).

tion of implantation, had a significant effect on levels of D-dimer and ATIII. (Figure 5, Tables 2 and 3). Concentrations of protein C and sCD62P did not differ between controls and animals with implants. Pearson correlation analysis of these markers showed no significant effect of age on biomarker concentration (Table 4). The increased D-dimers (a by-product of fibrin degradation) and decreased ATIII level (a major endogenous anticoagulant) suggest a hypercoagulable state associated with chronic titanium implantation and infection.

Discussion

In this study, we have shown that percutaneous, static, chronic titanium alloy implants are associated with localized bacterial infection and inflammation, leaching of metal ions into the surrounding tissues, and various associated systemic hematologic changes. We have previously documented isolation of mixed bacterial infections from cephalic implants in this population.[6,69] In addition, recent case reports have documented life-threatening infections associated with cephalic implants in rhesus macaques from other colonies.[34]

The development of physiologically inert synthetic materials for orthopedic implantation is of major importance. A variety of metals are used in surgical implantation, and over time, appear to be correlated with adverse local and systemic effects. Of note, cobalt–chromium alloys, previously used in total hip arthroplasties, can experience long-term corrosion and wear of the implant, resulting in local tissue necrosis, osteolysis, and implant failure.[13,47,64] In addition, these alloys have the capability to release metal ions both in the local tissue environment as well as into the systemic circulation, resulting in metallosis.[32,33,36] New metal alloys and synthetic polymers have been developed to prevent these adverse effects.[66]

Titanium alloys were previously perceived to be relatively inert within the host, until recent biomaterials research elucidated the potential for titanium ion leakage and its associated adverse effects. Like cobalt–chromium alloys, titanium can result in elevated serum metal ion levels after implantation.[36,39,45,57] A growing body of literature in oral surgery research shows that release of titanium ions from implant surfaces results in increases in inflammation and alveolar bone resorption.[63] In addition, titanium ions have been associated with carcinogenic and mutagenic activity within the oral cavity.[4,12,54,59,62,68] One recent case report demonstrated the development of a pseudotumor caused by titanium particles from a total hip prosthesis, suggesting that titanium, like cobalt–chromium, may result in metallosis and its associated

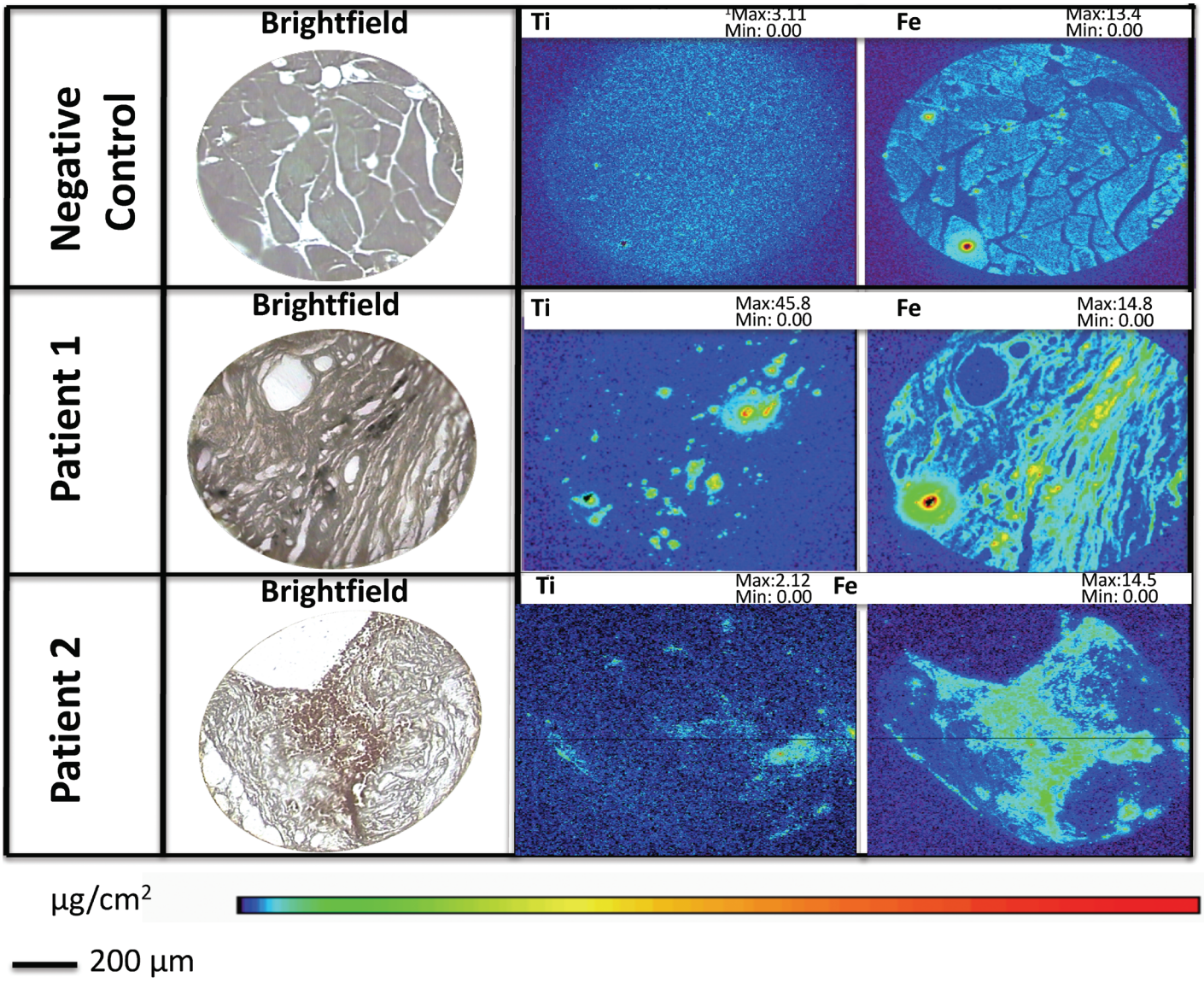

Figure 2. X-ray fluorescence microscopy of tissue surrounding the implant site. The negative control (rhesus 3, top) show no Ti metal deposition. Rhesus macaques 1 (middle) and 2 (bottom) show a moderate amount of Ti deposition around the tissues as well as small to moderate amounts of Fe deposition.

Table 1. Total metal content (fg) according to X-ray fluorescent microscopy

	Ti	Fe
Rhesus 1	15×10^4	17×10^4
Rhesus 2	3.7×10^4	21×10^4
Rhesus 3	2.4×10^4	3.4×10^4

Rhesus macaque 3 was the negative control.

adverse systemic effects.[56] Although most studies attribute ion-leakage to implant wear, a recent study on cochlear implants used X-ray fluorescence microscopy to demonstrate 'passive' surface deterioration of medical titanium in the absence of wear.[3]

Numerous mechanisms could mediate degradation of metal implants within the body, including wear and corrosion. The macaques we studied had static cranial implants and persistent localized bacterial infection; therefore, corrosion is the most likely cause of metal-ion leaching. Local inflammation, pH, and bacterial load all play important roles in the corrosion of titanium implants.[7,9] Histopathology was performed in only 3 animals in this study; however, those evaluations of tissue from around the implants showed that the areas of metal deposition appear to be a focus of acute and chronic inflammation (Figure 1 A and B). Free metal ions have been shown to cause local pain and swelling, with or without infection, in the region of implant insertion.[68]

The implanted macaques in the current study displayed chronic low-grade bacterial infection and inflammation surrounding the implant sites. Studies have shown that titanium can serve as a nidus for bacterial biofilm formation.[15,22,30,52] A physiologic pH of 4 to 7 in the local environment has an additive effect with LPS, promoting the corrosion of titanium and its alloys, suggesting that the presence of bacteria around the implant site could pro-

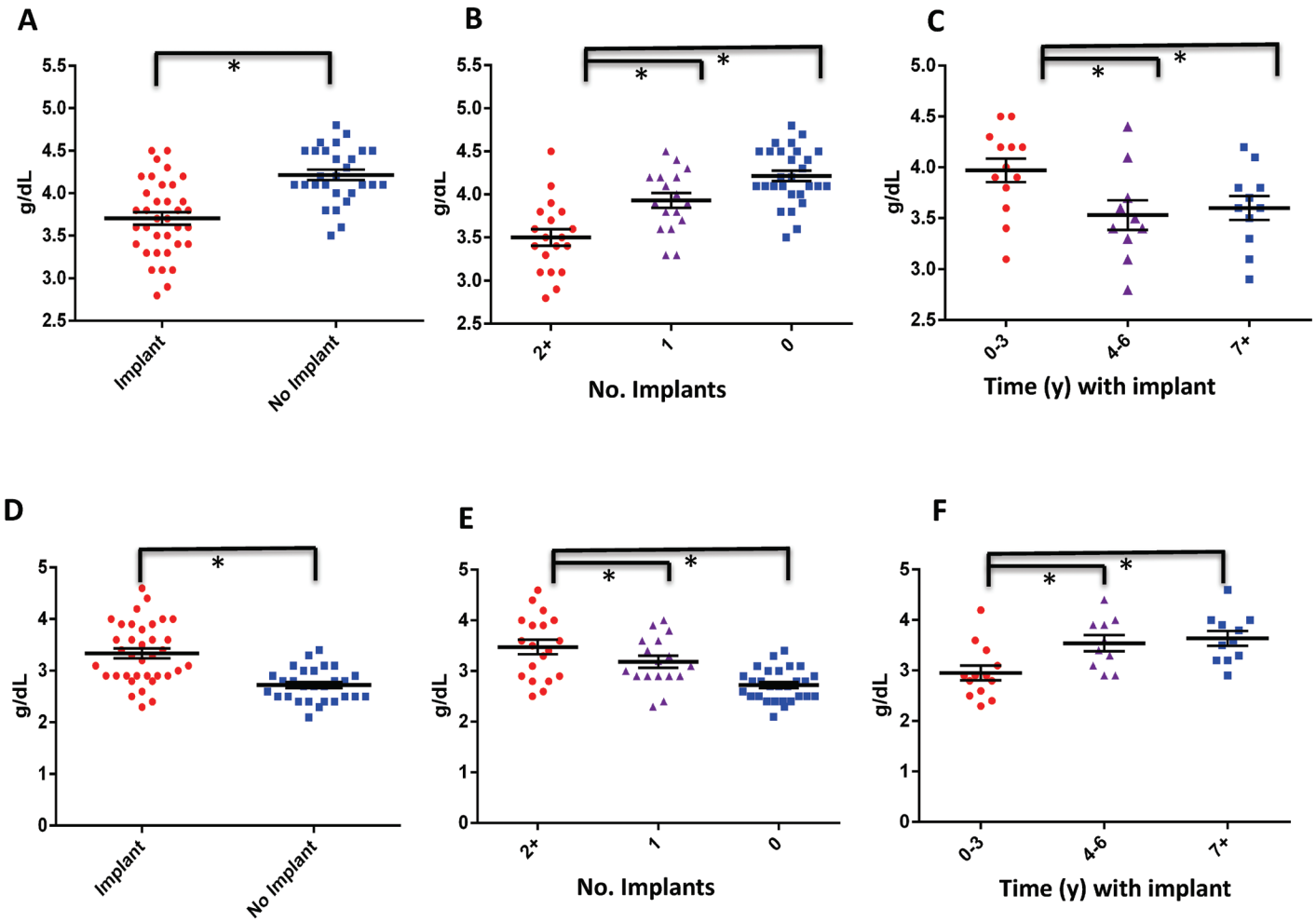

Figure 3. Titanium implantation is likely associated with a proinflammatory state. (A through C) Albumin and (D through F) globulin concentrations (mean ± SE) were compared (A and D) between implanted and nonimplanted macaques, (B and E) between animals that had 2 or more implants, 1 implant, or no implant, and (C and F) between animals that were implanted for 0 to 3 y, 4 to 6 y, or 7 y or more. *, Statistically significant relationship ($P < 0.05$).

duce an environment conducive to implant surface corrosion.[71] Implant corrosion and subsequent metal ion leakage activate host complement, and phagocytized metal particles induce the release of inflammatory cytokines from macrophages, which potentially resulted in sustained inflammation and loosening of implants in our animals.[7,59] In this cohort of macaques with cephalic implants, we are unable to determine whether the leaching of the titanium ions incited inflammation and predisposed the animals to chronic infection, or whether the presence of chronic infection caused a local environmental change that promoted ion leakage. Based on the current evidence, we cannot conclude that the implant material served as a chronic source of infection for the implanted animals, but it likely plays an important role.[21,24,25,50]

Although the local effects of titanium alloy implantation with concurrent chronic bacterial infection have been studied in both humans and animals, the systemic effects in general are not well studied. In the current study, macaques with titanium alloy implants had biochemical alterations that can be consistent with chronic inflammation and that were not seen in nonimplanted animals; these alterations included increased levels of globulin, which can be a positive acute-phase protein, and decreased concentrations of albumin, which is a negative acute-phase protein. Globulins represent a wide spectrum of proteins, and increases in several types of globulins are consistent with inflammation. Determining the specific types of globulins present was beyond the scope of this study. Because albumin is the main carrier protein for calcium, calcium levels are often decreased when albumin levels are low, as in the current case.[15] We think the decreased albumin concentrations in these macaques were due to inflammation because other common causes of decreased albumin, such as endoparasites and hepatic or renal pathology, were unlikely given the routine screening for endoparasites and lack of strong convincing biochemical evidence indicative of hepatic or renal pathology. In addition, note that creatinine was elevated, but BUN was not,[16] and neither liver function nor urine parameters were assessed; therefore other causes of decreased albumin cannot be entirely ruled out. Given that the 2 implanted macaques that underwent histopathologic evaluation had inflammatory changes and that many of the animals with implants had superficial bacterial infections, the combination of low albumin and high globulin levels in implanted macaques is likely due to local inflammation. Furthermore, systemic amyloi-

Table 2. Summary of biomarker statistics

	No implant	Implant	P^a	1 implant	P^b	2 or more implants	P^c
D-dimer (ng/mL)	178.1 ± 137.5	360.6 ± 312.5	0.01	317.0 ± 287.6	0.04	406.9 ± 340.1	0.00
Antithrombin III (μg/mL)	128.4 ± 12.4	117.4 ± 12.9	0.00	117.1 ± 15.8	0.01	117.7 ± 9.4	0.01
Protein C (μg/mL)	3.4 ± 0.7	3.6 ± 1.0	0.38	3.4 ± 1.0	0.85	3.8 ± 1.0	0.15
sCD62P (ng/platelet × 10^6)	0.14 ± 0.0	0.15 ± 0.1	0.52	0.14 ± 0.0	0.42	0.1 ± 0.1	0.73
WBC (×10^3/μL)	7.09 ± 3.40	7.0 ± 2.3	0.92	6.4 ± 2.6	0.49	7.6 ± 2.0	0.60
ALP (IU/L)	205.7 ± 154.3	153.8 ± 92.9	0.10	171.7 ± 111.0	0.43	143.2 ± 72.9	0.11
ALT (IU/L)	31.9 ± 20.9	25.5 ± 19.0	0.20	26.1 ± 18.7	0.35	24.5 ± 20.1	0.23
AST (IU/L)	30.4 ± 7.8	29.2 ± 7.2	0.52	29.4 ± 6.6	0.67	29.1 ± 8.0	0.57
Albumin (g/dL)	4.2 ± 0.3	3.7 ± 0.4	0.00	3.9 ± 0.4	0.01	3.5 ± 0.4	0.00
Globulin (g/dL)	2.7 ± 0.3	3.3 ± 0.6	0.00	3.2 ± 0.5	0.00	3.5 ± 0.4	0.00
Total protein (g/dL)	6.9 ± 0.4	6.9 ± 1.2	0.74	7.1 ± 0.5	0.20	7.0 ± 0.4	0.74
BUN (mg/dL)	14.6 ± 5.3	15.2 ± 4.4	0.62	15.5 ± 4.2	0.55	14.3 ± 4.0	0.86
Creatinine (mg/dL)	0.9 ± 0.3	1.1 ± 0.3	0.00	1.1 ± 0.2	0.01	1.1 ± 0.3	0.01
Glucose (mg/dL)	64.6 ± 12.9	63.2 ± 8.6	0.60	63.5 ± 5.5	0.73	63.1 ± 11.0	0.67
Calcium (mg/dL)	9.5 ± 0.6	9.2 ± 0.4	0.00	9.2 ± 0.4	0.07	9.1 ± 0.3	0.00
Phosphorus (mg/dL)	3.5 ± 1.2	3.4 ± 0.9	0.82	3.4 ± 1.1	0.76	3.4 ± 0.8	0.95
Platelets (×10^3/μL)	382.5 ± 86.7	394.1 ± 97.9	0.62	379.3 ± 86.5	0.91	409.0 ± 109.7	0.36
Hct (%)	38.5 ± 4.3	39.2 ± 5.1	0.58	40.7 ± 4.3	0.10	37.7 ± 5.6	0.59
MPV (fL)	13.3 ± 2.4	13.1 ± 2.5	0.74	13.2 ± 2.5	0.89	13.0 ± 2.5	0.69
MCV (fL)	71.6 ± 4.4	69.3 ± 7.7	0.17	71.44 ± 5.6	0.94	67.3 ± 8.9	0.04
MCHC (g/dL)	31.7 ± 0.8	31.3 ± 1.6	0.20	31.0 ± 1.7	0.05	31.6 ± 1.5	0.71
Cholesterol (mg/dL)	158.6 ± 33.0	122.1 ± 25.7	0.00	120.8 ± 25.3	0.00	123.3 ± 26.8	0.00
Total bilirubin (mg/dL)	0.2 ± 0.1	0.14 ± 0.1	0.03	0.16 ± 0.1	0.25	0.13 ± 0.1	0.01
Lipase (U/L)	34.5 ± 30.1	33.7 ± 29.3	0.91	28.2 ± 20.6	0.44	38.8 ± 35.5	0.65
Creatine kinase (U/L)	541.2 ± 346.8	513.6 ± 306.7	0.74	494.9 ± 377.9	0.68	523.5 ± 244.4	0.85
Amylase (U/L)	275.7 ± 101.6	273.8 ± 78.6	0.93	272.3 ± 91.8	0.91	273.2 ± 68.2	0.99
γ-Glutamyl transferase (IU/L)	58.4 ± 24.1	51.8 ± 16.8	0.20	54.3 ± 17.1	0.54	49.4 ± 16.6	0.16

[a]Unpaired Student *t* test between no implant and implant (regardless of number)
[b]Unpaired Student *t* test between no implant and 1 implant
[c]Unpaired Student *t* test between no implant and 2 or more implants

Table 3. Summary of statistics regarding duration of implantation

	0 to 3 y	P^a	4 to 6 y	7 y or more	P^b	P^c
D-dimer (ng/mL)	255.73 ± 171.48	0.19	435.38 ± 399.02	462.31 ± 358.73	0.11	0.88
Antithrombin III (μg/mL)	115.98 ± 12.84	0.77	117.54 ± 9.57	118.85 ± 13.23	0.63	0.82
Albumin (g/dL)	3.97 ± 0.42	0.03	3.53 ± 0.46	3.60 ± 0.39	0.04	0.71
Globulin (g/dL)	2.95 ± 0.53	0.01	3.54 ± 0.49	3.64 ± 0.48	0.00	0.66
Creatinine (mg/dL)	1.12 ± 0.31	0.54	1.19 ± 0.23	1.13 ± 0.24	0.92	0.57
Calcium (mg/dL)	9.29 ± 0.32	0.05	9.01 ± 0.31	9.19 ± 0.41	0.50	0.27
MCV (fL)	70.04 ± 6.05	0.94	69.86 ± 5.56	67.26 ± 11.43	0.46	0.52
MCHC (g/dL)	30.73 ± 2.41	0.22	31.71 ± 0.48	31.80 ± 0.96	0.18	0.79
Cholesterol (mg/dL)	127.46 ± 29.68	0.43	118.20 ± 23.90	119.00 ± 26.91	0.48	0.94
Total bilirubin (mg/dL)	0.15 ± 0.07	0.88	0.15 ± 0.05	0.13 ± 0.05	0.28	0.31

[a]Unpaired Student *t* test between 0 to 3 y and 4 to 6 y
[b]Unpaired Student *t* test between 0 to 3 y and 7 y or more
[c]Unpaired Student *t* test between 4 to 6 y and 7 y or more

dosis, an indicator of chronic systemic inflammation, is common in captive macaques and has been frequently noted in our animal population, with 2 cases reported in animals with cephalic implants.[53] Taken together, these changes suggest that static, cephalic, titanium-alloy implants are likely associated with systemic inflammation.

Although many publications in both humans and animals show that acute implantation with metal alloys is associated with changes in systemic coagulation, most of those studies

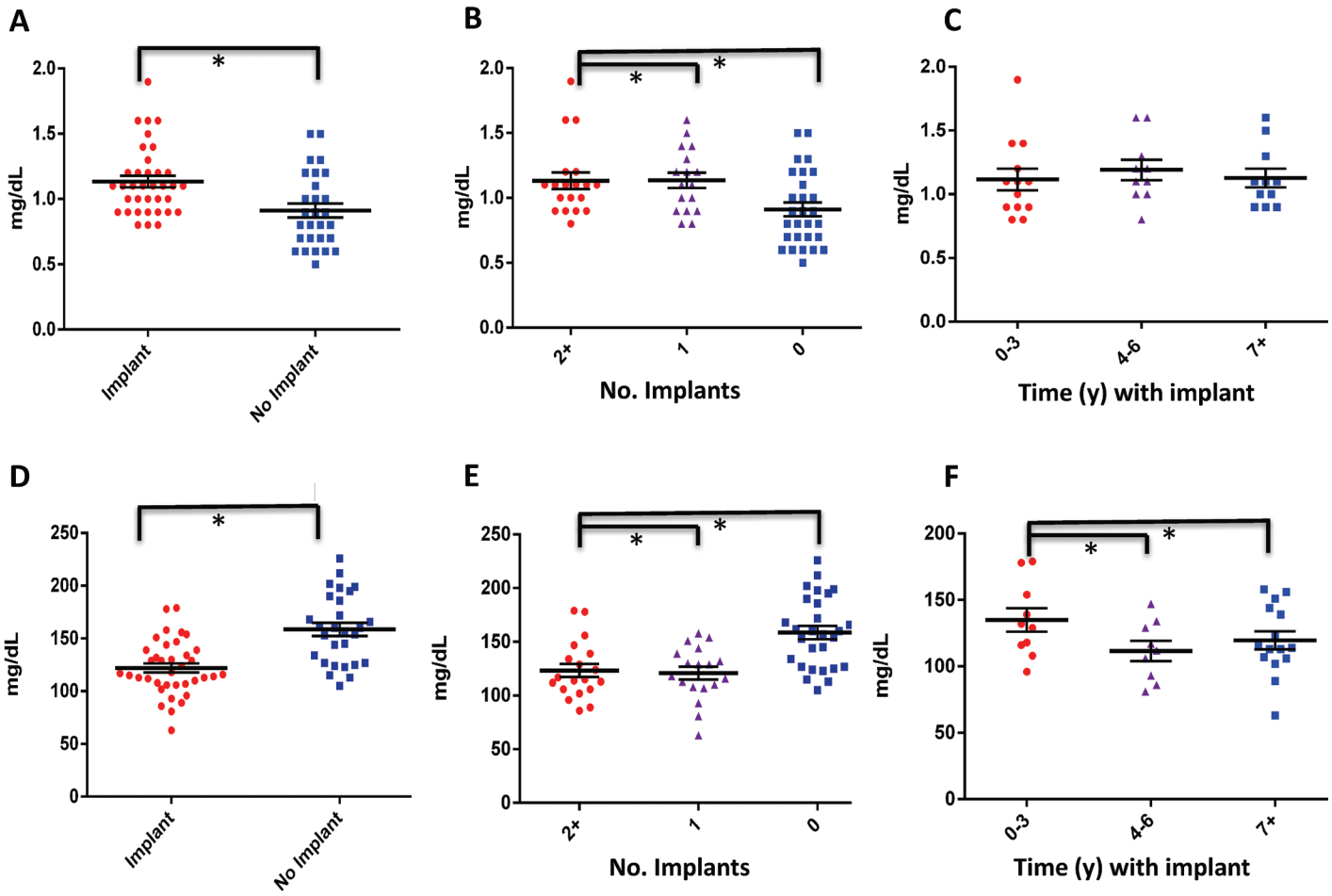

Figure 4. Titanium implantation is likely associated with a proinflammatory state. (A through C) Creatinine and (D through F) cholesterol were compared (A and D) between implanted and nonimplanted macaques, (B and E) between animals that had 2 or more implants, 1 implant, or no implant, and (C and F) between animals that were implanted for 0 to 3 y, 4 to 6 y, or 7 y or more. *, Statistically significant relationship ($P < 0.05$).

were performed in peracute and acute settings, with follow-up being limited to a few weeks or months after surgery.[27,60,70] The etiology of coagulation status changes in those cases might reflect tissue trauma during surgery, anesthesia, metal implantation, or a combination thereof. The current study evaluated coagulation biomarkers at 1 to 14 y after implantation, and, to our knowledge, is the first to measure long-term changes in coagulation parameters after metal implantation. The implanted animals in this study displayed both a significant elevation in D-dimer and a significant decrease in ATIII levels. D-dimer is a fibrin degradation product that forms when crosslinked fibrin is cleaved by plasmin and is therefore an indicator of fibrinolytic activity; elevations in D-dimer are suggestive of ongoing clot lysis or a hyperfibrinolytic state.[67] ATIII, an anticoagulant that is synthesized in the liver, binds and inhibits several coagulation factors, including factors II and X.[49] These changes in D-dimer and ATIII levels suggest that chronic titanium alloy implant-induced bacterial infection may be associated with a systemic procoagulant state. Although D-dimer values were significantly increased in our macaques (Figure 2), the data range widely, including a few outliers with very elevated D-dimer levels, which might influence the overall analysis. ATIII is considered a negative acute-phase protein; therefore the chronic–active inflamma-

Table 4. Pearson correlation between animal age and biomarker

	Pearson correlation coefficient	Strength of association
Antithrombin III	0.04	Negligible
D-dimer	–0.30	Weak negative
Albumin	–0.24	Negligible
Globulin	0.61	Weak positive
Creatinine	–0.16	Negligible
Calcium	–0.43	Weak negative
MCV	–0.29	Negligible
MCHC	0.27	Negligible
Total bilirubin	0.12	Negligible
No. of implants	0.12	Negligible
Duration (y) of implantation	0.57	Weak positive

Strength of association: Pearson R of –0.7 to –0.3, weak negative association; –0.3 to +0.3, negligible or no association; +0.3 to +0.7, weak positive association

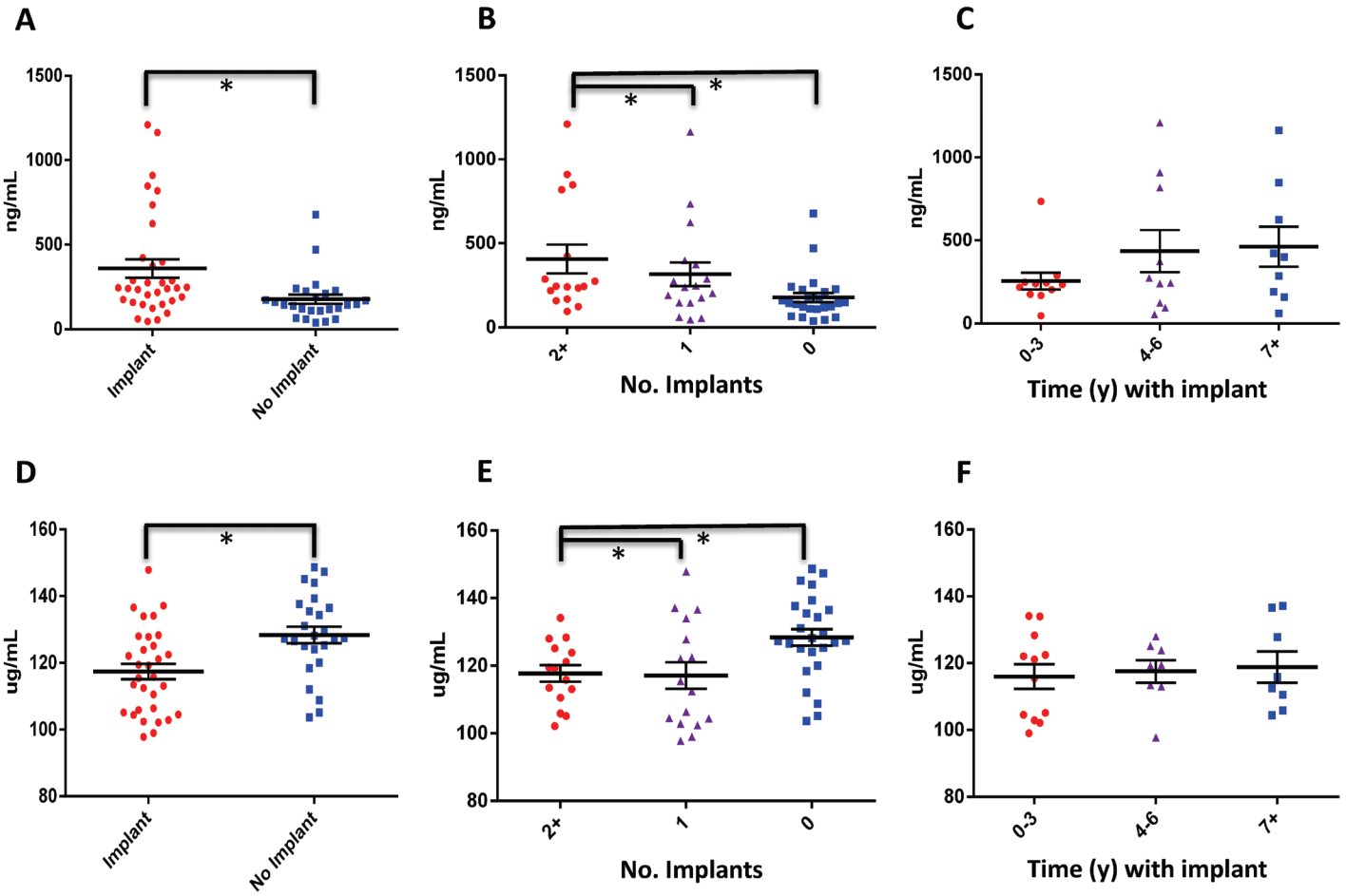

Figure 5. Titanium implantation is associated with a likely procoagulant state. (A through C) D-dimer and (D through F) antithrombin III concentrations were compared (A and D) between implanted and nonimplanted macaques, (B and E) between animals that had 2 or more implants, 1 implant, or no implant, and (C and F) between animals that were implanted for 0 to 3 y, 4 to 6 y, or 7 y or more. *, Statistically significant relationship ($P < 0.05$).

tion and infection may be the reason for the lower ATIII levels. The loss of ATIII through the kidneys or decreased production of ATIII by the liver cannot entirely be ruled out because, as previously stated, urinalyses and liver function tests were not done.[44] In addition, when interpreting these findings, one must take into account that presence of chronic bacterial colonization and inflammation associated with the implants in this colony. These findings may have a major effect on the monitoring and follow-up care of animals with chronic metal implants, and future studies evaluating coagulation markers in these subjects are warranted to evaluate whether they have an increased risk for thrombus formation.

The implants we evaluated are similar to other titanium alloy implants, which are exposed to the external environment, such as external fixators and cochlear implants. Although many of our animals had implants with concurrent bacterial infections, it was not possible to discern whether the systemic changes in inflammation and coagulation were due solely to implant status, the presence of bacterial infection, or another unrelated etiology. Bacterial contamination and biofilm formation is common in implants that are exposed to the external environment.[6,28,29,43,48] Implants for deep brain stimulation—which are made from a variety of metal alloys, protected from the outside environment, and placed in the same anatomic location as the implants our animals—are similarly subject to biofilm formation.[8] No comprehensive studies to date evaluate either the long-term effects of implantation on coagulation status or the effects of biofilm presence on implants upon the coagulation system. Research in this area may improve our understanding of the pathophysiology of metal ion leakage, local tissue inflammation or infection, and system changes in inflammation and coagulation status, and could thereby eventually provide clinical utility.

The current study determined the systemic effects of chronic, percutaneous, static cranial implantation of macaques with titanium alloys. The findings indicate that chronic bacterial infection and likely a proinflammatory and procoagulant state are common in rhesus macaques with cephalic implants. Biopsy and histopathologic evaluation of tissue surrounding the implant site showed evidence of simultaneous active, chronic inflammation and bacterial infection. X-ray fluorescence microscopy confirmed the leaching of titanium ions into the local tissue, which appeared to persist for as long as 6 mo after removal of the implant. Some limitations in this study include the lack of sham surgery controls and the inability to measure serum titanium levels in these animals, because serum for metal analysis was not collected at the time of this study. Local bacterial infection in the area surround-

ing the implant site was also common in the macaques and might be a confounding factor in this study.

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