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Modulation of Macrophage Activity During Fracture Repair has Differential Effects in Young Adult and Elderly Mice

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Abstract

Objectives—Advanced age is a factor associated with altered fracture healing. Delays in healing may increase the incidence of complications in the elderly, who are less able to tolerate long periods of immobilization and activity restrictions. The following study sought to determine if fracture repair could be enhanced in elderly animals by inhibiting macrophage activation, blocking the M-CSF receptor c-fms, and inhibiting monocyte trafficking using CC chemokine receptor-2 (CCR2) knockout mice.

Methods—Closed, unstable tibial shaft fractures were produced in mice aged four, 12 and 78 weeks. Mice were then fed a diet containing PLX3397 or a control diet from days 1–10 post-injury. Fractures were similarly made in CCR2−/− mice aged 78 weeks. The fracture callus was collected during fracture healing and was assessed for its size and the presence of macrophages, both of which were evaluated using the Mann-Whitney U test.

Results—PLX3397 treatment resulted in a decrease in the number of macrophages in the fracture callus at Day 5. Calluses in juvenile mice trended towards being smaller compared to elderly mice (p=0.08). There was also a trend toward larger callus size and increased bone formation in PLX3397-treated elderly animals compared to those of the control animals (p=0.12). Similar increases in bone formation (p=0.013) and decreases in cartilage within the callus (p=0.03) were seen at Day 10 in $CCR2^{-/-}$ mice.

Conclusions—The inhibition of macrophages in elderly mice may lead to an acceleration of fracture healing. Altering macrophage activation after fracture may represent a therapeutic strategy for preventing delayed healing and nonunion in the elderly.

Keywords

Aging; Fracture Healing; Inflammation; Macrophage

Introduction

The global incidence of fractures is growing due to increased rates of age-related bone fragility. Approximately 10% of all fractures exhibit impaired healing or nonunion.[1]Several populations, including the elderly, are at risk for delayed bone healing after fracture.[2–4] Nonunions in elderly patients present specific treatment challenges to orthopaedic surgeons,

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Conflicts of Interest
All other authors have no conflicts.

given the patients' diminished bone mineral density, increased risk of complications from treatment, and failure to adhere to activity restrictions. Therefore, developing strategies to prevent or treat altered bone healing in this population is imperative.

Fracture healing progresses through a series of overlapping anabolic and catabolic phases resulting in bone union.[5] During the earliest stage of fracture repair, a hematoma forms at the fracture site providing molecular signals that recruit inflammatory cells necessary for the initiation of healing. Normal fracture healing includes inflammation, which is in part modulated by macrophages at the site of injury. Macrophages in the surrounding tissues and from the bloodstream are key regulators of osteoprogenitor cells in the endosteum and periosteum, as evidenced by their production of trophic factors such as BMP-2.[6] A previous study has demonstrated that altering the number of macrophages at the fracture site affects the normal healing response.[7; 8]

Dysregulation of the inflammatory phase of bone healing can result in impaired fracture healing, as is seen in diabetics and smoker.[9; 10] Aging is also characterized by an increase in systemic inflammation.[11] A previous study demonstrated that engraftment of juvenile bone marrow into elderly animals, which produced a shorter duration of inflammation in the recipient animals, resulted in accelerated fracture healing compared to controls.[12]

A novel drug, PLX3397, [13] blocks the kinase domain of the colony-stimulating factor 1 receptor (CSF-1R), inactivating signaling by colony-stimulating factor 1 (csf-1), a factor necessary for the activation of macrophages. The goal of this study was to assess the effects of PLX3397 on recruitment of macrophages and subsequent fracture healing in an elderly murine tibia fracture model. In a preliminary study, we also compared the healing responses that occurred after treating aged animals with PLX3397 to those of aged animals lacking the chemokine receptor-2 (CCR2) gene, as these mice have impaired recruitment of macrophages to sites of injury.

Materials and Methods

Aged Animals

Male wild-type C57BL/6L mice were bred at our Institution and CCR2−/− mice were purchased (Jackson Laboratories, Bar Harbor, ME) for use in this study. The CCR2−/− strain has previously been shown to have an 80% decrease in macrophage density at the fracture site.[7] All animals were maintained in a climate-controlled facility with a 12-hour light/dark cycle and were supplied food and water *ad libitum*. Juvenile male mice were aged for four weeks prior to treatment. The mice in the adult group were aged for 12 weeks and mice in the elderly group were aged for 18 months prior to treatment. All procedures were approved by the UCSF Institutional Animal Care and Use Committee.

PLX3397 Treatment

After reaching the proper age, wild type mice in each age group were divided into two groups based on exposure to the compound PLX3397. The control group was fed chow (Group 1), and the treatment group was fed the same chow supplemented with PLX3397 from day 1–10 post-fracture (Group 2).

Fracture Creation and Tissue Harvest

After induction of anaesthesia with a mixture of ketamine and metedomidine, unstable, closed diaphyseal tibial fractures were created in the right tibia by three-point bending.[14] Animals were recovered on a heating pad and supplied with Buprenorphine as needed for analgesia. Animals were allowed to bear weight as tolerated. At scheduled timepoints, mice

were terminally anaesthetized using Avertin and sacrificed by cervical dislocation and the calluses were collected. Callus tissue was fixed overnight at 4C in 4% formaldehyde and decalcified in 19% EDTA for 14 days. After being dehydrated and embedded in paraffin, the samples were sectioned. Serial sections 10μm thick were mounted for histologic evaluation.

Histomorphologic Evaluation

Every thirtieth section was stained with Milligan's Trichrome following an established protocol. Milligan's trichrome allows the visualization of bone and cartilage during fracture healing. Bone volume and cartilage volume were then estimated using an Olympus CAST system (Olympus, Center Valley, Pa) and Visiopharm software (Visiopharm, Horsholm, Denmark).

Macrophage Quantitation

Sections (every 30^{th}) spanning the callus were used for macrophage quantitation. Sections were incubated with a macrophage-specific antibody, F4/80. A secondary antibody conjugated with horseradish peroxidase was employed to visualize macrophages in the callus. The number and density of macrophages in the callus were estimated using the Olympus CAST system and Visiopharm software.

Data Analysis

Data were entered directly into Microsoft Excel (Redmond, WA). The Student's t-statistic was used to compare mean macrophage numbers and callus component volumes. A p-value less than 5% was deemed significant. All statistical analysis was performed in Stata I/C version 12 (College Station, TX).

RESULTS

PLX3397 Effect on Macrophage Population at the Fracture Site

Employing immunohistochemical techniques, we were able to quantify macrophages at the fracture site in PLX3397-treated and control elderly mice five days after fracture. The mean number of macrophages in the fracture calluses of treated animals (1008 cells; 95% C.I. 598 – 1418) was lower than that of the control animals (7231 cells; 95% C.I. 1747 – 12714). The mean macrophage density in the fracture calluses of treated animals (53 cells/mm^2; 95% C.I. 47 – 60) was also lower than that of control animals (296 cells/mm^2; 95% C.I. 193 – 399; Fig. 1).

Age-dependent Differences Exist in Bone Formation after Tibia Fracture in Juvenile and Elderly Mice

Calluses trended toward smaller volumes in juvenile mice compared to elderly mice (mean difference −11.20mm^3; p=0.083) at Day 10. The total volume of cartilage (mean difference 2.99mm^3; p=0.27), as well as the proportion of the callus that was comprised of cartilage (mean difference 12.74%; p=0.19) were similar in the two groups. Total bone volume (mean difference 7.06mm^3; p=0.027) and the proportion of bone in the callus (mean difference 17.78%; p=0.0056) were increased in juvenile control animals compared to elderly control animals.

Age-dependent Inhibition of Bone Formation after treatment with PLX 3397

Treatment of elderly animals with PLX3397 for ten days starting at the time of fracture resulted in an average callus volume of 85.44mm^3 compared with a callus volume of 53.82mm^3 in control animals (p=0.12, n=2 per group; Fig. 2). This trend for increased callus size was not observed in juvenile animals. PLX3397-treated mice had a mean callus volume

of 41.94mm^3 relative to control animals that had a mean callus volume of 42.63mm^3 (p=0.83, n=3 per group; Fig. 2). The composition of the callus in elderly animals receiving PLX-3397 trended toward increased cartilage (mean volume 26.05 vs. 11.16mm^3; p=0.12; Fig. 2) and bone volume (mean volume 5.64 vs. 3.07mm^3; p=0.12; Fig. 2) when compared to elderly control animals. In juvenile animals, these trends were not observed. Mean cartilage volume (16.93 vs. 14.15mm^3; p=0.40; Fig. 2) and bone volume (10.12 vs. 8.53mm^3; p=0.36; Fig. 2) were similar in treated and control animals.

Effects of CCR2 KO on Fracture Healing in Elderly Animals

The genetic inhibition of macrophage recruitment by the deletion of the CCR2 gene did not affect the size of the fracture callus at days 10 (p=0.81) or 14 (p=0.51) in elderly animals (Fig. 3). The composition of the callus in CCR2$^{-/-}$ mice did show increased maturity at day 10 with decreased cartilage (mean volume 5.32 vs. 15.69mm^3; p=0.03; Fig. 3) and increased bone (mean volume 4.83 vs. 1.70mm^3; p=0.013; Fig. 3) compared to wild type mice. A trend toward decreased cartilage content in the callus of CCR2$^{-/-}$ animals was noted at day 14 (mean volume 12.36 vs. 22.28mm^3; p=0.12; Fig. 3), although bone formation in CCR2$^{-/-}$ and wild-type animals was nearly equivalent (mean volume 7.96 vs. 6.46mm^3; p=0.49; Fig. 3).

DISCUSSION

Bone possesses a remarkable capacity to regenerate throughout life. While the processes of embryonic bone development, including cell proliferation and differentiation, osteogenesis, chondrogenesis, and angiogenesis, are similar to those of adult repair, healing differs in that adult repair relies on an inflammatory process while embryonic development does not.[15; 16] In some cases the dysregulation of the immune response, as observed in diabetes, polytrauma, smoking, and increased age, may be responsible for delayed healing or nonunion.[17]

While the precise mechanisms underlying a sustained inflammatory state are largely unknown, the relationship between age-related immune dysregulation and the production of a disease state is well-documented.[18] We have previously demonstrated that aging reduces the rate of fracture healing,[4] and that a non-resolving inflammatory state is associated with age-related delays in healing.[12]

The inflammatory phase of fracture healing is characterized by communication between the innate immune system and local cells involved directly in the formation of bone. Macrophages play an important part in the innate immune response at the fracture site.[8; 19] In addition, there is a complex communication between macrophages and bone-forming cells, suggesting that macrophages may play a major role in providing trophic signals to chondroprogenitor and osteoprogenitor cells.[20]. In vitro isolation of osteoblasts from tissue-resident macrophages and in vivo depletion of tissue-resident macrophages resulted in inhibition of bone formation.[20] These results provide compelling evidence that the interactions between the innate immune system and musculoskeletal tissues are essential to normal fracture healing, and may be a therapeutic target to accelerate fracture healing in at-risk populations including the elderly.

Alterations in the activity of macrophages, which are a key component of the inflammatory response following a skeletal fracture, have been implicated in age-related changes in the response to injury. This study investigated the effect of inhibited macrophage activation on fracture healing through pharmacologic and genetic means. Our preliminary data suggest that decreased macrophage activity in elderly animals may result in accelerated callus

maturation, even though previous research in young animals has shown that macrophages stimulate fracture healing.[7; 8]

Although an inflammatory response has been shown to be crucial for normal healing, persistent or non-resolving inflammation can inhibit healing.[21; 22] Normally, inflammatory cells are mobilized to an injured area to eliminate bacteria and debris and to regulate tissue repair. However, in conditions where inflammation persists beyond the initial response to fracture, there may be destruction of viable tissue and sustained production of pro-inflammatory cytokines that can impair healing. A characteristic common to many conditions associated with impaired bone healing, including smoking, obesity, diabetes, polytrauma, and aging [17; 23; 24] is an impaired ability to resolve the inflammatory cascade and achieve homeostasis. Previous work by Wallace, et al. [25] suggests that removal of interleukin-6 (IL-6) results in osteoclast-driven impairments in early fracture healing. In agreement with these results, mice lacking Toll-like receptor-4 (TLR-4), one of the primary receptors initiating the innate immune response, showed accelerated healing of calvarial defects.[26] In these mice, the levels of mRNA expression of inflammatory cytokines IL-1β, IL-6, Tumor Necrosis Factor –α (TNF-α), and Receptor Activator of NFκB Ligand (RANKL) were increased, demonstrating the importance of cytokines produced by the innate immune response in fracture healing. Further work is required to determine if alterations in the magnitude and timing of inflammation could be targeted to improve fracture healing in at-risk patients.

There are several limitations to our current study. These results should be considered preliminary given that the sample sizes were too small to draw definitive conclusions regarding the effects of PLX3397 and the loss of CCR2 gene activity on fracture healing in elderly mice. Additionally, only histomorphometric data were employed in the determination of callus maturity, and other analyses, such as immunohistochemistry, are needed to confirm the progression of the fracture healing observed in this study. Future work will address these issues. Overall, these data suggest the possibility that the modulation of macrophage activity may provide a mechanism to enhance fracture healing in the elderly. Blocking macrophage activation pharmacologically resulted in trends towards increased callus volume and bone formation, results similar to those noted in elderly $CCR2^{-/-}$ mice that genetically lack the ability to mobilize macrophages. Further study is necessary to confirm these preliminary results and to translate them into clinical practice.

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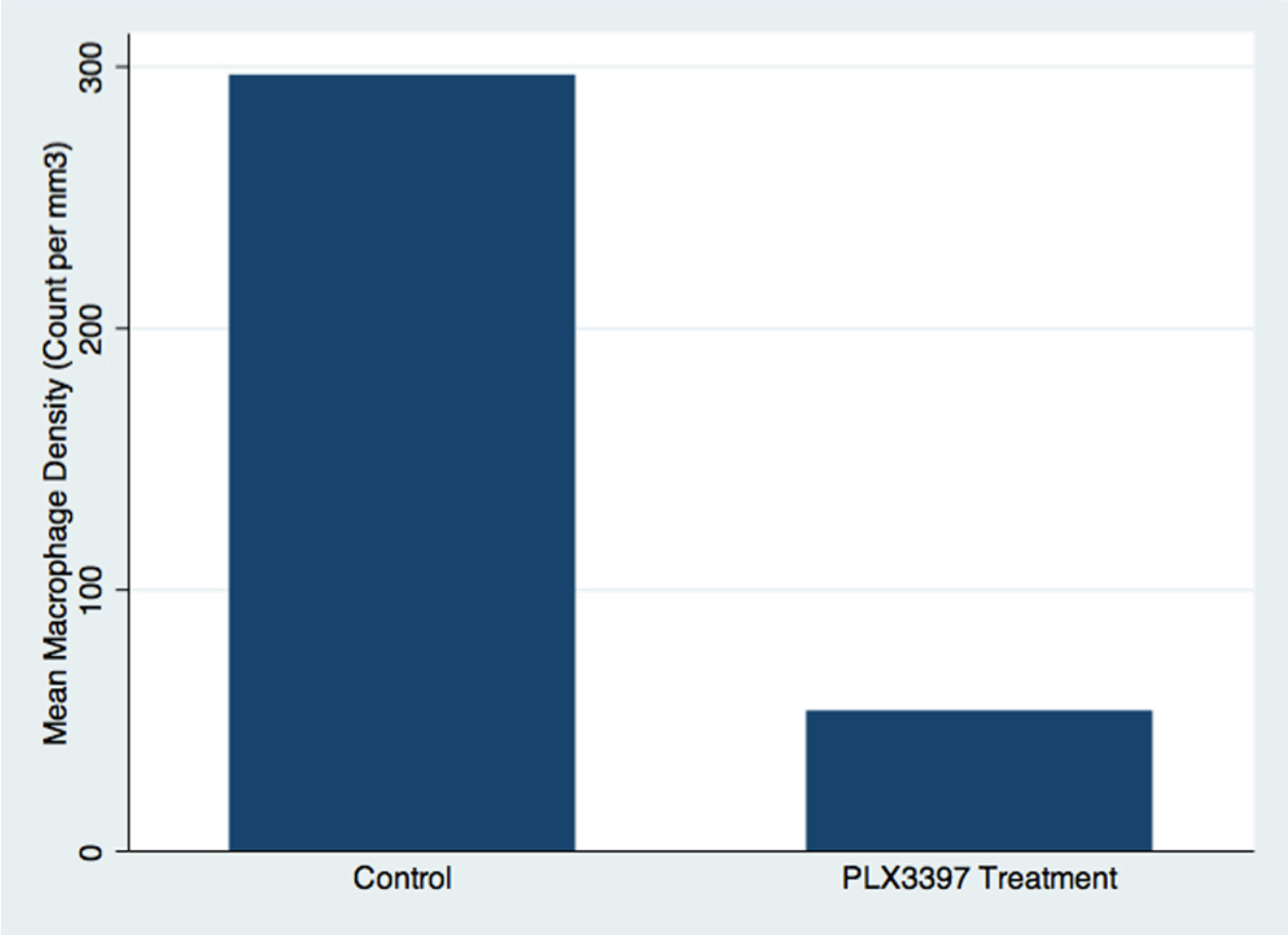

Figure 1.
The effect of PLX3397 on the population of macrophages at the fracture site five days after fracture. The macrophage density per callus volume is depicted.

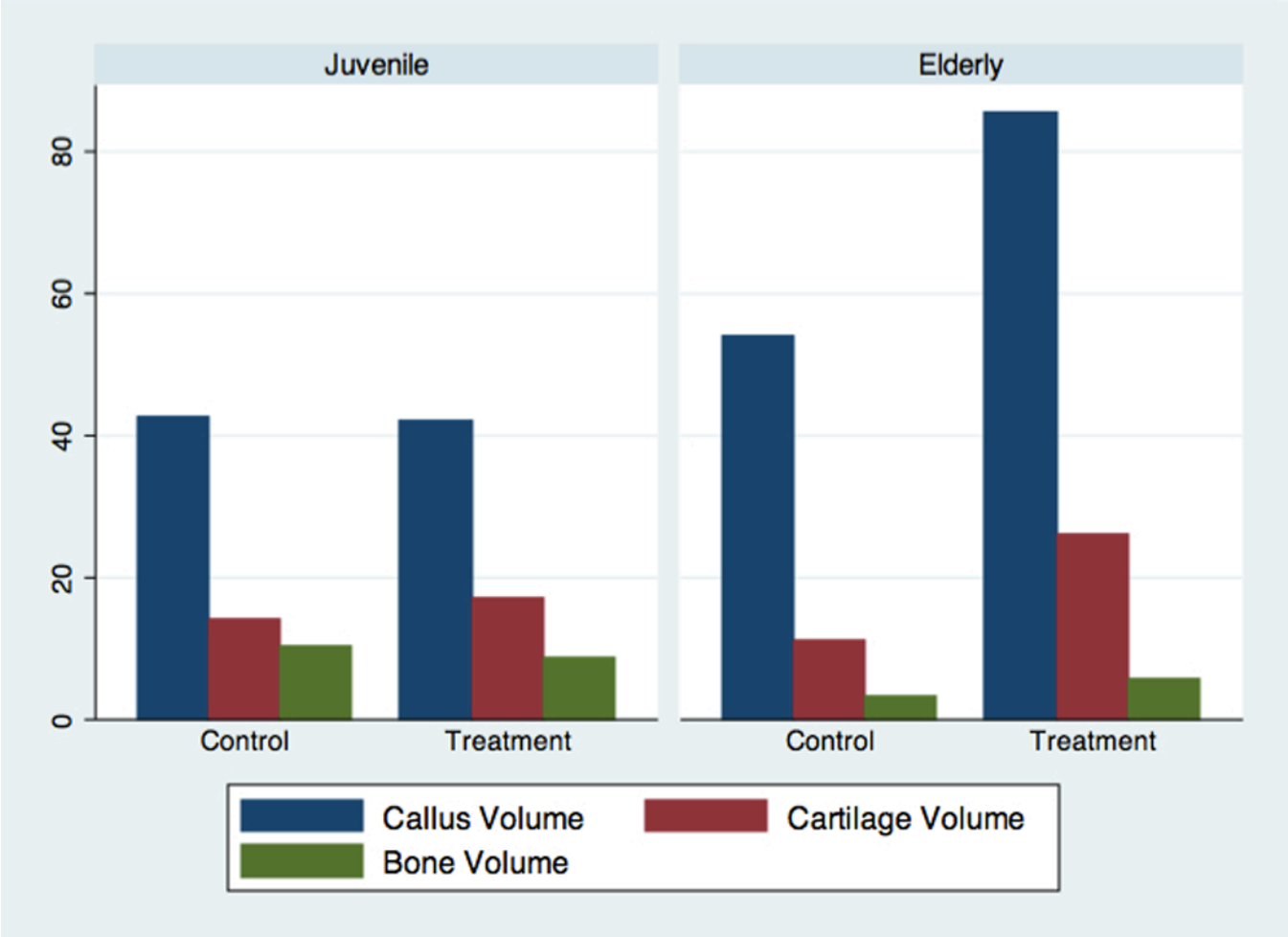

Figure 2.
Callus parameters in young and elderly animals treated with PLX3397 immediately following fracture.

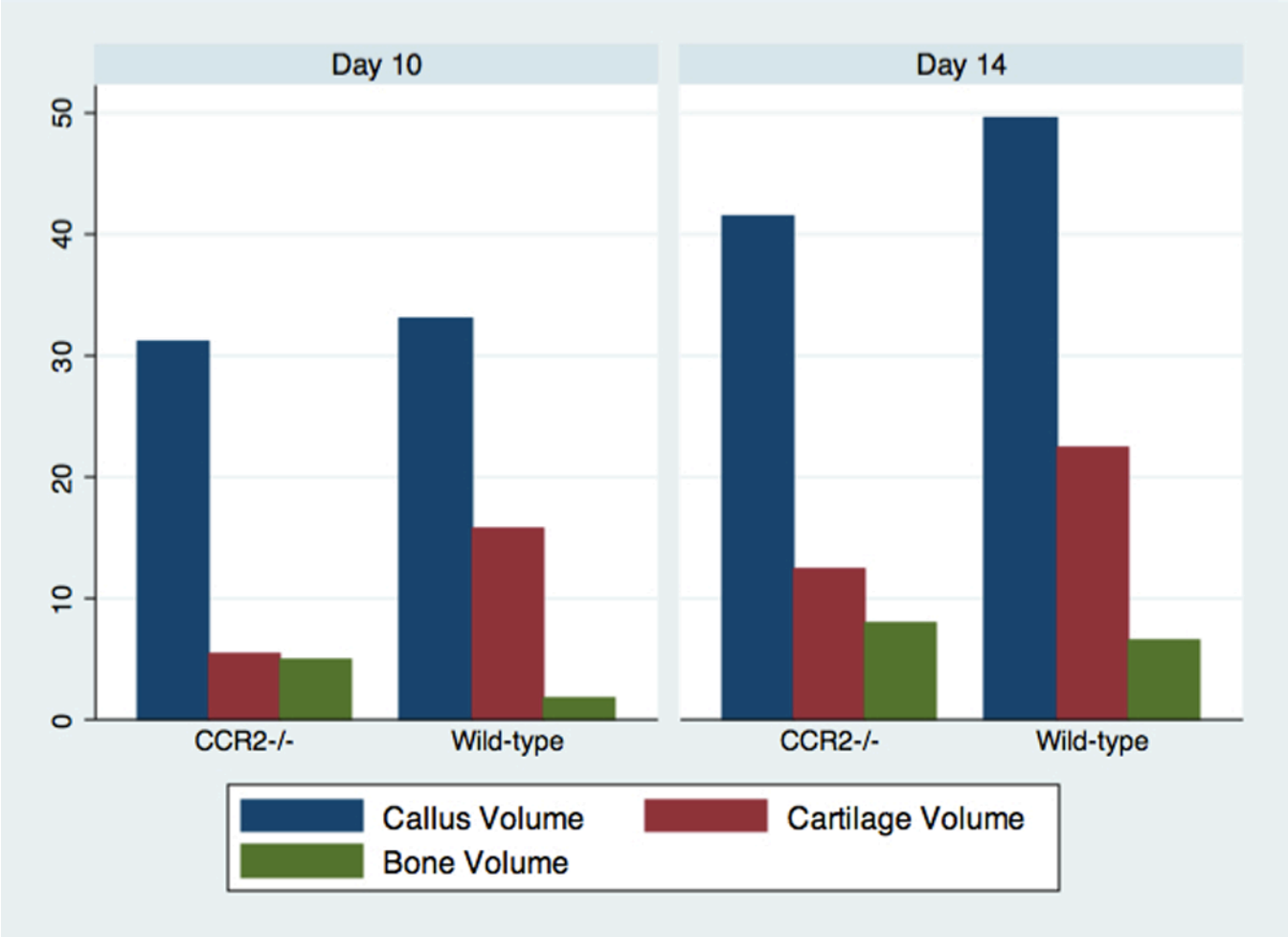

Figure 3.
Callus parameters in aged Ccr2−/−mice compared to wild type mice.