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BRIEF REPORT

Testosterone Is Protective in the Sexually Dimorphic Development of Arthritis and Lung Disease in SKG Mice

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Objective. **Rheumatoid arthritis (RA) is a sexually dimorphic inflammatory autoimmune disease with both articular and extraarticular disease manifestations, including RA-associated interstitial lung disease. Low levels of testosterone have been linked to disease severity in men with RA, and supplemental testosterone has been shown to improve RA symptoms in both postmenopausal women and men with low levels of testosterone. The mechanisms by which sex and sex steroids affect the immune system and autoimmunity are poorly understood. The purpose of this study was to examine the protective effects of testicular-derived sex hormones on the development of joint and lung disease in an autoimmune mouse model.**

Methods. **Arthritis prevalence and severity were assessed in orchiectomized, sham-orchiectomized, and intact male SKG mice as well as in female SKG mice over a 12-week period after intraperitoneal injection of zymosan. Lung tissues were evaluated by quantifying cellular accumulation in bronchoalveolar lavage fluid, collagen levels, and histologic changes. An antigen microarray was used to evaluate autoantibody generation under each experimental condition.**

Results. **Female SKG mice developed arthritis and lung disease at increased prevalence and severity as compared to intact male mice. The absence of testosterone after orchiectomy led to increased arthritis, lung disease, and autoantibody generation in orchiectomized male mice as compared to intact male mice.**

Conclusion. **SKG mice represent an authentic sexually dimorphic mouse model of both the joint and lung disease seen in humans with RA. Testosterone protects against the development of joint and lung disease in male SKG mice.**

Rheumatoid arthritis (RA) is a systemic disease with both articular and extraarticular manifestations that preferentially affects women (1). Because of the predominance of autoimmunity in women, the role of estrogen in immunity and autoimmunity has been studied much more extensively than that of testosterone. Estrogen has been shown to influence T cell and B cell maturation and to promote a Th2 CD4+ T cell phenotype, which can lead to increased antibody production by plasma cells (for review, see ref. 2). Male sex hormones have been shown to play an important role in immune regulation as well, and may contribute to the sex differences seen in RA. For example, testosterone inhibits the secretion of inflammatory cytokines such as tumor necrosis factor α and interferon-γ from stimulated human peripheral blood leukocytes (3). Macrophages from orchiectomized mice have higher cell surface expression of Toll-like receptor 4 (TLR-4), rendering the mice more susceptible to endotoxic shock as compared to their intact counterparts (4).

Cross-sectional analysis in humans indicates that men with RA have a lower mean serum testosterone level as compared to that in healthy men (5). Such cross-sectional studies are limited, in that they cannot determine if low testosterone levels reflect a primary

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risk factor for disease development, an effect of inflammation, or the disease process itself. Taken together, these and other studies suggest that testosterone may play an important immunoregulatory role in autoimmune diseases such as RA.

Extraarticular disease manifestations, including pulmonary disease, are an important source of morbidity and mortality in patients with RA. Progressive RA-associated interstitial lung disease occurs in nearly 10% of RA patients and is associated with significantly reduced rates of survival (6). Little is known about the mechanisms by which lung disease develops in the context of RA, and even less is known about the role of sex hormones in disease development. To address this issue, we studied the role of testicular-derived sex hormones on the development of arthritis, interstitial pneumonia, and anti–citrullinated protein antibodies (ACPAs) in SKG mice. We found that female SKG mice developed arthritis and interstitial pneumonia with more rapid onset and with increased prevalence and severity as compared to male SKG mice. We used a surgical orchiectomy approach to prospectively investigate the effects of testosterone on the development of arthritis, interstitial lung disease, and autoantibody formation. Our findings are presented herein.

MATERIALS AND METHODS

Animals. SKG mice were maintained in a specific pathogen–free environment in the animal colony at National Jewish Health. All experiments were approved by the Institutional Animal Care and Use Committee of the National Jewish Health system.

Surgical orchiectomy and measurement of testosterone levels. Anesthetized and surgically prepared male mice (4–6 weeks) were placed in dorsal recumbency. Ventral midline incisions measuring 1–2 cm were made at the scrotum, and the skin was retracted to expose the tunica. The tunica was pierced, and the testes were pushed out one at a time. The testes were raised to expose the underlying blood vessels and tubules. Forceps were used to pull off each testicle. Any minor bleeding was controlled by direct pressure using forceps. All deferential vessels and ducts were placed back into the tunica. Skin incisions were closed with stainless steel wound clips (removed after 7–10 days) or with absorbable suture material. Sham surgical orchiectomy, with and without testicular manipulation, was performed and resulted in universally reduced testosterone levels. Additional sham surgery at a remote site located on the dorsum of the mice also resulted in reduced testosterone levels. Therefore, sham orchiectomy was determined to be an inappropriate control for surgical intervention alone.

Serum testosterone levels were measured prior to surgery and at 12 weeks following zymosan or saline injection. A commercially available testosterone enzyme-linked immunosorbent assay (ELISA) kit was used according to the manufacturer's instructions (Cayman Chemical).

Induction of arthritis and pulmonary disease. All mice (8–12 weeks old) were given a single intraperitoneal injection of 5 mg of zymosan (Sigma-Aldrich) to induce arthritis and lung disease, as previously described (7). Control mice were injected with saline. Arthritis scores were determined weekly as previously described (7).

Determination of lung disease. Cells obtained by bronchoalveolar lavage (BAL) were assessed as previously described (7). The left lung was inflated with 10% neutral buffered formalin at a pressure of 20 cm of H_2O, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin. For stereologic examination, lung tissue was cut into 2-mm sections and randomly arranged into paraffin blocks. Sections were cut at 5-μm intervals. Three slides per mouse were examined, with a total of 15 images captured at 10× magnification in a blinded manner using an Olympus BX51 microscope. Quantitative analysis was performed using stereologic grid-counting techniques to assess areas of disease, as previously described (7). All coefficients of error were calculated at less than $P < 0.05$ (7). Representative images were obtained of mice with the median stereology score in each of the zymosan-injected groups. To determine lung collagen content, the right upper lobe was homogenized in phosphate buffered saline and incubated overnight with an equal volume of 12*N* HCl at 100°C. Lung collagen levels were evaluated by quantifying hydroxyproline content as previously described (7).

Antigen array analysis. Bead-based antigen arrays were used to characterize a panel of serum autoantibodies, as previously described (8). Briefly, 43 putative RA-associated autoantigens were conjugated to spectrally distinct beads (Bio-Rad) and mixed with diluted mouse serum. After washing, beads were incubated with phycoerythrin-conjugated goat anti-mouse antibody, and after another series of washes, the bead mixture was passed through a laser detector (Luminex 200) that identifies beads based on dye fluorescence. The amount of antibody bound to each bead was determined by fluorescence intensity.

Statistical analysis. Data are presented as the mean ± SEM. Differences between data points were examined using an unpaired *t*-test with Welch's correction or using one-way analysis of variance with Newman-Keuls multiple comparison test. Antigen array data were analyzed using Significance Analysis of Microarrays (SAM) software (version 3.08) to identify autoantibody reactivities that exhibited significant differences between intact males, orchiectomized males, and female zymosan-treated SKG mice. The antigen array reactivities for each mouse were arranged using hierarchical cluster analysis (Cluster 3.0 software) and displayed as a heatmap (Java TreeView software version 1.1.3).

RESULTS

Arthritis in SKG mice is sexually dimorphic, and the absence of testosterone increases the prevalence and severity of arthritis. To investigate how testosterone affects the development of arthritis, male SKG mice were either surgically orchiectomized, left intact, or

underwent a sham surgical procedure. After full recovery from surgery, the sham-operated and the orchiectomized mice as well as the intact male mice and the female mice were injected intraperitoneally with 5 mg of zymosan or saline (control). Female SKG mice developed arthritis that was of increased severity and at increased prevalence as compared to their intact male counterparts (Figures 1A and B). Surgical orchiectomy of male mice, as confirmed by low serum testosterone levels (Figure 1C), resulted in increased arthritis severity and prevalence as compared to intact male mice (Figures 1A and B). There was no significant difference between the severity of arthritis in the female mice and the orchiectomized male mice ($P > 0.17$). Interestingly, a sham surgical procedure at the testes, both with and without testicular manipulation, as well as a surgical procedure at a remote site on the dorsum of the mice, resulted in low testosterone levels that were not statistically different from those in female mice or in castrated male mice (Figure 1C). Mice that received a sham surgical procedure developed arthritis that was not statistically different from that in either the castrated or the intact male mice (Figure 1A). However, they developed significantly less arthritis than the female mice at later time points (Figure 1A).

Testosterone protects against the development of interstitial lung disease. We next investigated how testosterone affects the development of lung disease in female, intact male, and orchiectomized male mice. Given the lack of significant difference in arthritis prevalence and severity or testosterone between sham surgical and orchiectomized mice, we did not include sham surgical mice in further analyses. At 12 weeks, the lung disease in the zymosan-injected female and orchiectomized male SKG mice was characterized by patchy subpleural and peribronchovascular mixed inflammation as compared to lung tissues from normal mice and from saline-injected controls (Figure 2A). At higher magnification, severe infiltration of macrophages, neutrophils, and lymphocytes within alveolar septae and airspaces was observed (Figure 2A). Very few areas of disease were identified in the lung samples from the zymosan-injected intact male mice.

Using quantitative stereologic techniques, we evaluated the penetrance and extent of lung involvement in female, orchiectomized male, and intact male mice. There was a statistically significant increase in the degree of lung disease in the zymosan-injected female and zymosan-injected orchiectomized male mice as compared to the saline controls ($P < 0.05$) (Figure 2B). No increase in the amount of lung disease was seen in

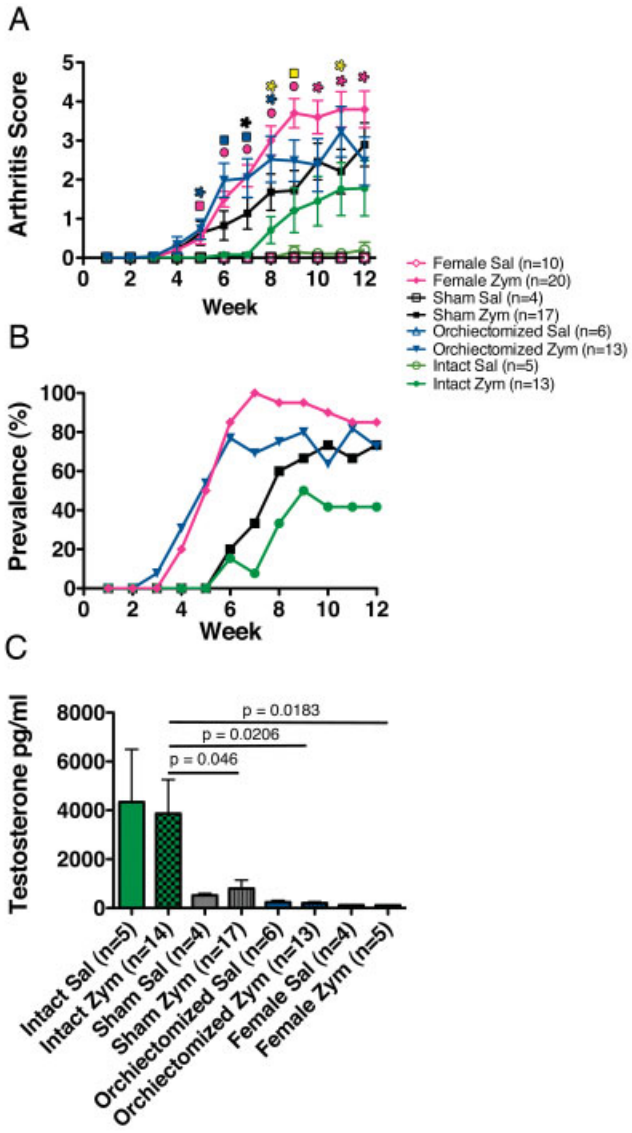

Figure 1. Development of arthritis in SKG mice is sexually dimorphic and is decreased in the presence of testicular-derived sex hormones. **A,** Arthritis scores were assessed in orchiectomized, sham-orchiectomized, and intact male SKG mice as well as in female SKG mice that were subsequently injected with zymosan (zym) or saline (sal; control). There was increased arthritis severity in female and orchiectomized mice as compared to intact male control mice (* = $P < 0.5$; ■ = $P < 0.01$; ● = $P < 0.001$; blue = orchiectomized mice versus intact male mice; pink = female mice versus intact male mice; black = sham-operated male mice versus intact male mice; yellow = sham-operated male mice versus female mice). There was no difference in arthritis severity between female mice and orchiectomized mice at any time point. **B,** Female and orchiectomized male mice developed arthritis at an increased prevalence as compared to intact male control mice. **C,** Testosterone levels were significantly increased in intact male control mice as compared to orchiectomized and sham-operated male mice as well as female mice. Values in **A** and **C** are the mean ± SEM.

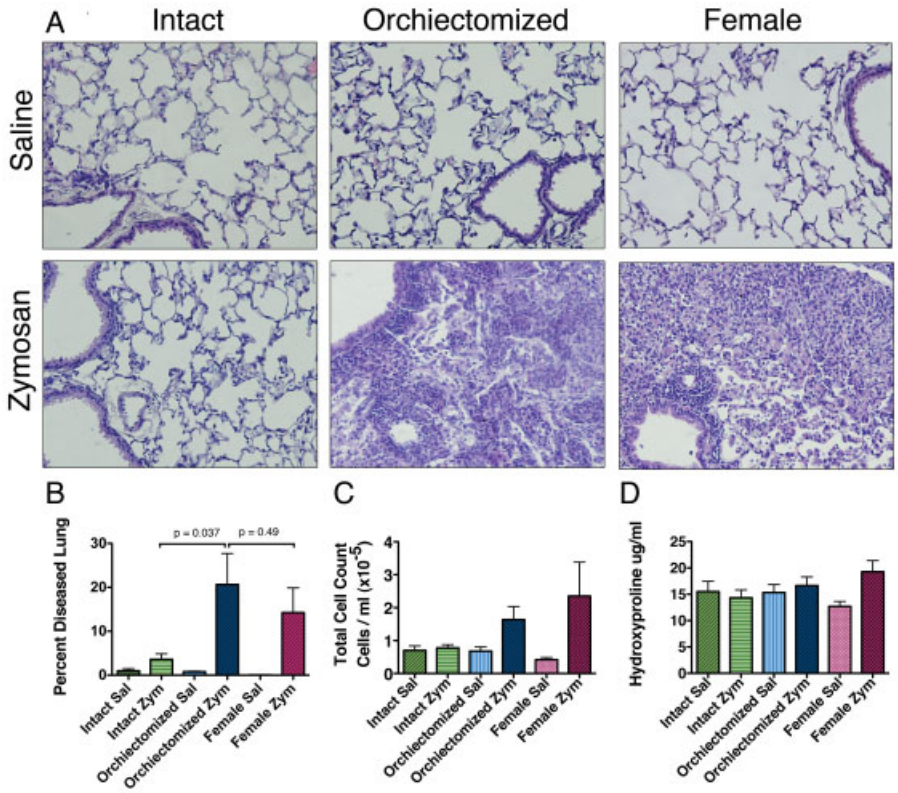

Figure 2. Development of lung disease in SKG mice is sexually dimorphic and is decreased in the presence of testicular-derived sex hormones. **A,** Lung architecture is normal in tissue samples from saline (sal)–injected mice and from the zymosan (zym)–injected intact male mouse. Lung samples from the zymosan-injected orchiectomized male mouse and from the female mouse demonstrate mixed peribronchoalveolar inflammation rich in macrophages, neutrophils, and lymphocytes. Hematoxylin and eosin stained; original magnification × 20. **B,** An increased percentage of zymosan-injected orchiectomized male mice had diseased lungs as compared to the percentage of zymosan-injected intact male mice. There was no significant difference between the zymosan-injected orchiectomized male mice and the zymosan-injected female mice. **C,** Bronchoalveolar lavage performed 12 weeks after zymosan injection showed a nonsignificant trend toward increased airspace cells in orchiectomized male mice and in female mice as compared to intact male mice. **D,** There was no significant increase in the collagen content of the lung, as determined by hydroxyproline levels. Values in **B–D** are the mean ± SEM of 4 saline-treated intact male mice, 7 zymosan-treated intact male mice, 4 saline-treated orchiectomized mice, 12 zymosan-treated orchiectomized mice, 10 saline-treated female mice, and 20 zymosan-treated female mice.

the intact male mice as compared to the saline controls ($P = 0.15$) (Figure 2B). However, there was a significant increase in the amount of lung disease in the zymosan-injected orchiectomized male mice as compared to the zymosan-injected intact male mice ($P < 0.05$) (Figure 2B). There was no significant difference between the amount of lung disease in zymosan-injected orchiectomized male mice and the zymosan-injected female mice. Bronchoalveolar lavage 12 weeks after zymosan injection showed a nonsignificant trend toward increased numbers of airspace inflammatory cells in orchiectomized male and female mice as compared to intact zymosan-injected male mice ($P = 0.49$) (Figure 2C). There was no difference in the numbers of macrophages, lymphocytes, or neutrophils between the groups of zymosan-injected mice. There was no evidence of significant collagen deposition, as measured by the hydroxyproline content (Figure 2D).

The absence of testosterone results in increased production of antibodies to citrullinated peptides. Finally, we investigated how testosterone affects the development of ACPAs in zymosan-treated female, intact male, and orchiectomized male mice. At 12 weeks, there was an increase in the level of 24 autoantibodies (of 43 evaluated) in the female and orchiectomized male mice ($q < 5.2\%$) (Figure 3). Multiclass SAM analysis revealed highly different ACPA levels across all 3 groups, with the highest levels in female mice, followed by the orchiectomized male mice, and the lowest levels in the intact male mice. Increased reactivity to a broad range of citrulli-

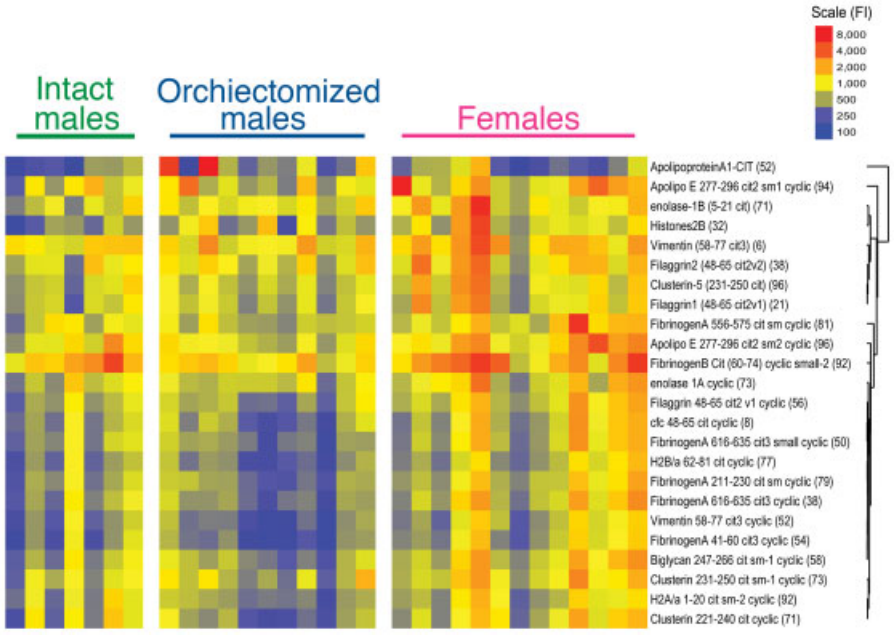

Figure 3. Development of disease in SKG mice is associated with the production of antibodies to citrullinated proteins. Multiclass Significance Analysis of Microarrays analysis revealed an increase in the level of 24 autoantibodies (43 evaluated) in zymosan-injected female mice and zymosan-injected orchiectomized male mice as compared to zymosan-injected intact male control mice ($q < 5.2\%$).

nated antigens was noted, including those derived from fibrinogen, vimentin, enolase, histone H2B, clusterin, biglycan, apolipoproteins A and E, as well as filaggrin. No increased reactivity was noted among noncitrullinated control antigens, including native fibrinogen and vimentin. Included among the highly targeted antigens are several proteins that are themselves TLR agonists, including fibrinogen, biglycan, and histone H2B (9,10), suggesting that orchiectomy may enhance inflammation through both innate and adaptive immune mechanisms.

DISCUSSION

Rheumatoid arthritis is a progressive systemic autoimmune disease that affects ~1% of the population, with a sex bias of at least 3:1 favoring females to males. Herein, we have shown that SKG mice represent an authentic, sexually dimorphic mouse model of human RA. Similar to RA in humans, the arthritis is more prevalent and more severe in female SKG mice as compared to male SKG mice and can be associated with progressive interstitial lung disease. Furthermore, we have shown that testosterone protects against the development of joint and lung disease in male SKG mice and is associated with alterations in the production of antibodies to citrullinated protein antigens.

One of the primary goals of this study was to investigate the role of testosterone in an autoimmune mouse model of RA. Low levels of testosterone have been linked to disease severity in men with RA (5), and supplemental testosterone has been shown to reduce disease severity in both postmenopausal women and men with low testosterone levels (11,12). It remains unclear whether low testosterone levels reflect a primary risk factor for the development of RA or whether it represents an effect of chronic inflammation. This was demonstrated by the universally low testosterone levels seen in the mice that received surgical sham procedures. It is important not to underestimate the impact of stress-induced changes in the hypothalamic–pituitary–adrenal axis on hormone production, since it represents a confounder in any surgical procedure designed to induce low testosterone levels. Interestingly, clinical studies in humans have also shown decreased testosterone levels related to general anesthesia, surgical stress, and other forms of inflammation, including sepsis and end-stage renal disease (13–15).

Sex steroids likely play an important role in the sex differences seen in sexually dimorphic diseases such as RA. However, the mechanisms by which sex and sex steroids affect the immune system and autoimmunity

remain poorly understood. Estrogen receptors are expressed in many cells of the immune system, including lymphocytes, neutrophils, macrophages, natural killer cells, and dendritic cells (2). Estrogen has been shown to have multiple effects on the immune system, including promotion of Th2 T cell skewing, increasing numbers of regulatory CD4+ T cells and B cells, and increased immunoglobulin production (2). Androgen receptors are also found in lymphocytes, and testosterone itself has been associated with Th1 T cell skewing and B cell tolerance (2). Additionally, androgen receptor–knockout mice have increased numbers of B cells and are more susceptible to collagen-induced arthritis (16). Taken together, these findings suggest that sex hormones play a significant role in fine-tuning the immune system and affect the development of autoimmunity in part by altering B cell biology, T cell skewing, and immunoglobulin production.

Accumulation of citrullinated proteins occurs in the context of many inflammatory conditions. However, it is the presence of a specific antibody response to citrullinated proteins that is characteristic of RA (17). Proteins become citrullinated when there is a posttranslational modification of the positively charged amino acid arginine to the neutral amino acid citrulline. This can lead to changes in protein structure and the creation of neoepitopes presenting altered self antigens, which can lead to breaches in immune tolerance. Citrullinated fibrinogen/fibrin, vimentin, type II collagen, filaggrin, and α-enolase have been identified as specific autoantigens in human RA (17,18). In mice with low-grade collagen-induced arthritis, infusion of antibodies to citrullinated fibrinogen has been shown to increase arthritis severity, which suggests a direct pathogenic role of these antibodies (18). In the present study, we demonstrated that zymosan-injected arthritic SKG mice produce clinically relevant antibodies to citrullinated proteins, including fibrinogen, vimentin, filaggrin, and enolase. In addition, we showed that the presence or absence of these hormones is a modifying factor in the production of ACPAs in SKG mice.

In summary, testosterone protects against the development of autoimmune arthritis and lung disease in male SKG mice. The removal of testosterone increases both lung and joint disease in male mice, creating a phenotype that is intermediate between that of intact males and that of females. We speculate that this is due in part to the ability of testosterone to modulate the development of antibodies against citrullinated proteins. Thus, we conclude that SKG mice represent a useful model that will allow future investigations of sex differences that contribute to the development of both inflammatory arthritis and associated interstitial pneumonia. This model may additionally provide a unique opportunity to investigate the role of ACPAs in the preclinical phase of disease as well as in the progression of joint and lung disease.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Riches had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Keith, Edelman, Redente, Sakaguchi, Riches.

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Analysis and interpretation of data. Keith, Sokolove, Edelman, Lahey, Redente, Holers, Robinson, Riches.

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