Introduction

Western-type diets, high in fat and sugars, lead to obesity[1]. Obesity in turn is associated with chronic inflammation, and thought to be a risk factor for the onset and increased rate of progression of metabolic osteoarthritis (OA) in joints[2]. Emerging evidence suggests that intrinsic inflammatory mediators secreted by body fat, or adipose tissue, including cytokines, adipokines, and advanced glycation end products, may be sufficient to lead to onset and progression of OA[3,4]. It appears that these obesity-associated, intrinsic inflammatory factors define a metabolic subtype of osteoarthritis[5–7]. Characterizing the factors that comprise this unhealthy metabolic phenotype is critical to understanding the influence of obesity on OA. Furthermore, establishing the "indirect" role of the microbiota and the gut is required to fully understand the initiators and drivers of metabolic OA.

Experimental high fat diets (45-60% of energy derived from lard-based fat rich in saturated fatty acids) have been associated with an increase in OA independent of gains in body weight[8–11]. However, these diets do not accurately represent the typical human Western-type diet, as the high percentage of fat (>50% kcal from fat) would be considered extreme. Rather, the obesity epidemic in North America appears driven by processed foods high in fat and simple carbohydrates, which is better modeled by a high fat/high sucrose (HFS) diet[12].

When fed a HFS diet, Sprague-Dawley rats, as well as Wistar rats and C57BL/6C mice exhibit obesity prone (top ~50% of weight gainers) and obesity resistant (bottom ~50% of weight gainers) phenotypes, where obesity resistant animals grow normally and the prone become obese[13]. This feature allows for experimental evaluation wherein animals are all exposed to the

same obesogenic diet but develop disparate body weight[13]. However, the effect of HFS diet-induced obesity on OA in prone and resistant animals has yet to be experimentally evaluated.

Although the extent to which inflammatory mediators contribute to metabolic OA remains unknown, several cytokines and adipokines detected in serum and synovial fluid of obese animals have been implicated [8,10,11,14–17]. In particular, leptin has been reported to be elevated in serum and synovial fluid of obese individuals and animals with OA[18]. However, the detailed molecular origins of such low-grade systemic inflammation remain unknown[19]. Systemically, visceral adipocytes, macrophages, and adipose tissue mast cells have been suggested to contribute to this chronic inflammatory state[20]. In knee joints, the infrapatellar fat pad and synovial fluid are thought to contribute to the local inflammatory environment[21–23].

Recently, a link between low-grade inflammation and changes in the composition and activity of the microbes that reside in the gastrointestinal tract, collectively termed the gut microbiota, has been established. Notably, high fat diets enhance translocation of the bacterial membrane component lipopolysaccharides (LPS) into the bloodstream, initiating obesity and insulin resistance[24]. New evidence suggests that the gut microbiota, through activating innate immune responses that lead to systemic inflammation, represent a possible mechanistic link to metabolically induced OA[25]. As metabolic OA may be one manifestation of a systemic metabolic disorder, our objective is to evaluate gut microbiota changes induced by a HFS diet in the context of OA. Specifically, we want to identify potential associations between gut microbial profiles, serum and synovial fluid cytokine and adipokine profiles, and knee joint damage.

We **hypothesize** that HFS diet-induced obesity, regardless of weight gain, will result in increased Modified Mankin Scores when compared with chow-fed control animals. Furthermore,

we explore the associations between gut microbiota, systemic LPS levels, serum and local inflammatory profiles, and joint damage in HFS animals in order to better understand the development of Metabolic OA.

Method

Animals. Thirty-two male, 8-12-week old Sprague Dawley rats, housed individually on a 12 hour dark/light cycle, were purchased from a specific pathogen free facility (Charles River Laboratories) and maintained at the University of Calgary with standard monitoring thereafter. Animals were allocated to the HFS diet-induced obesity group (DIO, 40% of total energy from fat, 45% of total energy from sucrose, n=21, Diet #102412, Dyets, Inc), or the standard control chow diet group (12% fat, 3.7% sucrose n=11, Lab Diet 5001) for a 28-week *ad libitum* feeding intervention[26]. The HFS diet consisted of (g/100g): casein (20.0), sucrose (49.9), soybean oil (10.0), lard (10.0), Alphacel (5.0), AIN-93M mineral mix (3.5), AIN- 93 vitamin mix (1.0), DL-methionine (0.3), and choline bitartrate (0.25). The energy densities of the HFS and chow diets were 4.60 kcal/g and 3.34 kcal/g respectively. All experiments were approved by the University of Calgary Life and Environmental Sciences Animal Care Committee. After a 12-week obesity induction period, DIO animals were stratified into tertiles according to changes in body mass, resulting in an Obesity Prone group (DIO-P, top 33% of animals by change in body mass, n=7), an Obesity Resistant group (DIO-R, bottom 33% of animals by change in body mass, n=7), and a middle tertile group, which is not further considered here.

Body Composition. Animals were followed for 28 weeks (36-40 weeks old), at which point they were euthanized by barbiturate overdose (Euthanyl®, MTC Animal Health Inc., Cambridge, Ontario, Canada). Immediately after sacrifice, body composition was measured

using Dual Energy X-ray Absorptiometry with software for small animal analysis (Hologic QDR 4500; Hologic, Bedford, MA).

Preparation of Knee Joints. Joints were harvested by cutting the femur and tibia/fibula 2cm above and below the joint line. Decalcification and processing of the joints was conducted according to previously described methods[7]. Serial, sagittal plane sections of 8μm thickness were obtained using a Leica RM 2165 rotary microtome. Sections were mounted on Super Frost plus slides (Fisher Scientific) and allowed to dry at 40˚C for 4 days. Sampling was done approximately every 80μm, thus alternate slides were stained sequentially with haematoxylin, fast green and safranin-O stains (Fisher Scientific) using an auto stainer (Leica ST 5010). Sections were then dehydrated in a graded series of alcohols, cleared in xylene, and mounted with cytoseal 60 mounting media (Richard Allan) using an auto cover slipper (Leica CV 5030). Slides were dried at room temperature for several days before being evaluated using a light microscope (Zeiss Axiostar plus, Carl Zeiss Inc., Toronto, Ontario, Canada). Images were digitized using a Zeiss Axiocam® Icc 5 camera and analyzed using the Zen 2011 Zeiss imaging system. Sections were examined under 10x and 25x objectives and scored for OA degeneration using a Modified Mankin scoring system[27].

Osteoarthritis Scoring. A Modified Mankin Score was used to describe the volumetric damage in each joint. Five areas were evaluated: the medial and lateral tibial plateau, the medial and lateral femoral condyle, and the patella. These five sites were assigned a score based on the standard 14-point Mankin scale[27]. Subchondral bone and synovium were then assessed using a 5 and 4 point criteria, respectively, that was adapted from the rat-specific OARSI metric[28]. Meniscal damage was scored on a scale of 0-5. The final Modified Mankin score was obtained by adding the five site-specific Mankin scores, the two corresponding OARSI scores, and the

meniscal damage score[27–29]. The inter-rater reliability between two independent assessors was r>0.95.

Cytokine, Growth Factor, Adipokine and LPS Measurements. Animals were sacrificed following a 12 hour fast, and blood was collected immediately via cardiac puncture. Serum was stored at -80°C until analysis. Synovial fluid was collected shortly after sacrifice using the Whatman chromatography paper method[30]. Samples were weighed, diluted 1:30, centrifuged at 13,500 rpm, and stored at -20°C overnight. Samples were aliquoted 24 hours later and stored at -80°C until analysis.

Twenty-seven serum and synovial fluid cytokines and adipokines were quantified using a Rat 27 Multiplex Discovery Assay with Luminex®xMAP technology (Eotaxin, EGF, Fractalkine, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IP-10/CXCL10, GRO/KC, IFN-γ, TNF-α, G-CSF, GM-CSF, MCP-1, leptin, LIX, MIP-1α, MIP-2, RANTES, VEGF; Eve Technologies, Calgary, AB). Synovial fluid from the left and right limbs of each animal was pooled for quantification. Urea was evaluated in duplicate in serum and synovial fluid using ELISA (Sigma Aldrich Urea Kit, Eve Technologies, Calgary, AB) while accounting for volumetric differences in synovial fluid concentration[31]. Systemic LPS was evaluated using EndoZyme Recombinant Factor C Assay (Hyglos GmbH, Germany).

Gut Microbiota profiling using qPCR. Microbial profiling of 12 microbial groups (total bacteria, *Bacteroides/Prevotella* species (spp.), *Clostridium coccoides* (cluster XIV), *Clostridium leptum* (cluster IV), *Clostridium* clusters XI and I, *Roseburia* spp., *Lactobacillus* spp., *Bifidobacterium* spp., *Methanobrevibacter* spp., Enterobacteriaceae, *Akkermansia muciniphila*) was performed according to our previous work and microbial abundance was measured as the

number of 16S rRNA gene copies per 20 ng total genomic DNA (primers provided in Supplementary Table 1)[32,33]. Briefly, total microbial DNA was extracted from fecal samples using the FastDNA Spin Kit for Feces (MP Biomedicals, Lachine, QC, Canada) and quantified using PicoGreen DNA quantification kit (Invitrogen, Carlsbad, CA, USA). Samples were then diluted to 4 ng/µl and stored at −20°C until analysis. Amplification and detection were conducted in 96-well plates with SYBR Green 2 × qPCR Master Mix (BioRad). Samples were assessed in duplicate with a final volume of 25 µl containing 0.3 µM primer and 20 ng template genomic DNA. The specificity of the primers and the limit of detection were determined according to previously reported methods[34]. Purified template DNA from reference strains was used to generate standard curves for each primer set using serial dilutions of DNA. Standard curves were normalized to copy number of 16S rRNA genes (ng genomic DNA converted into genome copies according to calculations outlined in the following webpage http://cels.uri.edu/gsc/cndna.html and values for 16S rRNA gene copies/genome obtained from the following database http://rrndb.mmg.msu.edu).

Statistical Analysis. Right knee joints from all 32 animals were scored and analyzed. Blood, synovial fluid, and feces were collected from all 14 DIO animals and 6 chow animals. Levene's test for equality of variance was conducted on all outcomes. If significant ($p<0.05$), Kruskal-Wallis non-parametric tests were used to evaluate between phenotype/diet groups (DIO-P, DIO-R, Chow), and between strictly dietary groups (all DIO, Chow). Differences in Modified Mankin scores, synovial fluid analytes, sera analytes, fecal microbes, and LPS were evaluated. If equal variances were found, ANOVAs were performed to assess differences between groups. Bonferonni corrections were utilized to control for multiple testing error. Multiple linear regression equations were used to evaluate each analyte with body fat to predict Modified

Mankin Scores. Pearson correlations and multiple linear regression equations were used to associate Modified Mankin scores with gut microbes, synovial fluid inflammatory markers, and serum inflammatory markers by diet using IBM SPSS Statistics 20 (α=0.05).

Results

DIO-P rats were heavier than both DIO-R and chow-fed controls by week nine of the obesity induction period and remained heavier through the remainder of the study ($p=0.03$, Fig. 1a). At sacrifice, DIO-R and chow-fed controls had similar mass ($p=0.25$, Fig. 1b), but DIO-P and DIO-R animals had increased body fat compared to control group animals ($p<0.001$, Fig. 1c).

Joint scores

DIO group animals had greater Modified Mankin scores than chow-fed control animals ($p=0.002$, Fig. 1d). There was a positive relationship ($r=0.60$, $p=0.001$) between body fat percentage and Modified Mankin scores across all animals, but this relationship was not significant when each dietary group was evaluated independently. No statistically significant relationship was found between body mass and Modified Mankin scores. Despite having more mass, DIO-P animals had similar Modified Mankin Scores to the DIO-R group ($p=0.81$, Figure 2). Of the categories summed to create the total Modified Mankin Score, medial tibial plateau and groove scores were significantly increased in DIO-P and DIO-R compared to chow ($p=0.013$ and $p=0.002$, respectively, Table 1).

Inflammatory Markers

Nineteen synovial fluid analytes were increased in DIO animals compared with chow-fed control animals (Table 2). There were no detectable differences in synovial fluid analytes between DIO-P and DIO-R animals. Five serum analytes (leptin, IP-10, GRO-KC, MIP-2 and MIP-1α) were increased in DIO animals compared to chow-fed control animals. These serum analytes were also significantly associated with increased synovial fluid analytes in DIO animals compared to chow-fed control animals (leptin: associated with 11/18 increased synovial fluid analytes; IP-10: 15/18; GRO-KC: 3/18; MIP-1α: 6/18). Finally, synovial fluid IL-1α was positively associated with Modified Mankin Scores across all animals (r=0.48, p=0.029). An association between Modified Mankin Score and synovial fluid IL-1β approached significance (r=0.40, p=0.067).

Gut Microbiota

The overall composition of gut microbes differed between DIO and chow-fed control animals (Fig. 3). DIO animals demonstrated an increased ratio of Firmicutes (sum total of *C. coccoides*, *C. leptum*, *Clostridium* clusters XI and I, *Roseburia* spp., *Lactobacillus* spp.) to Bacteroidetes (*Bacteroides/Prevotella* spp.) due to a lower abundance of *Bacteroides/Prevotella* spp. (p=0.016), as the total abundance of Firmicutes was similar between groups (all group data shown in Supplementary Table 2). However, within the Firmicutes phylum, the relative composition of microbiota differed between DIO animals and chow-fed control animals, mainly within the lactobacilli and clostridial bacterial groups (Fig. 3). Overall, the profile of Firmicutes in DIO-P and DIO-R animals was similar.

Serum LPS levels were higher in DIO animals (n=14) compared to chow-fed control animals (n=6, DIO 2.18 (2.13-2.23) log EU/mL, chow 2.06 (1.91-2.20) log EU/mL, p=0.031).

Furthermore, LPS levels were increased in DIO-P animals compared with DIO-R (P: 2.24 (2.17-2.30) log EU/mL, R: 2.13 (2.07-2.18) EU/mL, p=0.008).

Relationships between Modified Mankin Scores, Inflammation, and Microbiota

LPS concentration was associated with body fat (r=0.62, p=0.003) and *Lactobacillus* spp. abundance (r=-0.60, p=0.009). Additionally, a significant negative relationship between *Lactobacillus* spp. abundance and Modified Mankin Score was found **(**-0.45, p=0.04, Supplementary Table 2**).** Despite no difference in *Methanobrevibacter* spp. abundance between dietary groups, there was a strong positive relationship between *Methanobrevibacter* spp. abundance and Modified Mankin Score (r=0.51, p=-0.02, Supplementary Table 2). Together, using linear regression, *Methanobrevibacter* spp. and *Lactobacillus* spp. abundance had a strong predictive relationship with Modified Mankin Scores (r^2=0.50, p<0.001, Figure 4, Table 3).

Both microbial groups demonstrated significant relationships with synovial fluid and serum inflammatory mediators. *Methanobrevibacter* spp. abundance had a significant positive relationship with synovial fluid [IL-1β (r=0.47, p=0.03), LIX (r=0.54, p=0.01), MIP-2 (r=0.47, p=0.03) and RANTES (r=0.44, p=0.04)], but was not associated with any of the serum inflammatory mediators. *Lactobacillus* spp. abundance was negatively associated with synovial fluid leptin (r=-0.50, p=0.01), IL-12 (r= -0.50, p=0.02), IL-13 (r= -0.48, p=0.029), IFN-γ (r= -0.57, p =0.008), MCP-1 (r=-0.47, p=0.30), LIX (r= -0.50, p=0.02), TNF-α (r = -0.50, p=0.04), serum leptin (r= -0.46, p=0.04), and positively associated with IL-4 (r=0.50, p=0.02), EGF (r=0.57, p=0.009), and IL-17 (r=0.44, p=0.05).

Discussion

The aim of this study was to identify potential cross-sectional associations between inflammation, gut microbiota and knee joint damage in the context of HFS diet-induced obesity. Our primary finding was that DIO animals, independent of body weight, had similar Mankin scores that were greater than those found in chow-fed control animals. Since body fat was higher in DIO-P and DIO-R animals compared to control group animals, while body mass was the same for DIO-R and control group animals, it appears that body fat, rather than body mass, is a risk factor for joint damage. Another key finding was the detection of a distinct inflammatory signature in synovial fluid and serum of DIO group animals which was more pronounced in synovial fluid. The abundance of the gut microbes *Lactobacillus* spp. was negatively and *Methanobrevibacter* spp. positively associated with Mankin Scores and pro-inflammatory mediators in serum and synovial fluid. Finally, increased levels of serum LPS may provide a link between the associations measured between gut microbes, chronic inflammation and increased joint damage.

Mankin Scores were not significantly related to total body mass; a finding that has been reported previously[9–11,14,35,36]. Typically, obesity-induced OA has been associated with increased joint loading. However, we found that DIO-R animals had significantly higher Mankin Scores than chow-fed control animals despite similar body weight. Moreover, DIO-P and DIO-R animals, which both consumed the HFS diet, had similar Mankin scores, despite the significantly higher body weight in the DIO-P than DIO-R group animals. Furthermore, we found a positive relationship between body fat percentage and Mankin scores, supporting the idea that OA onset and progression may be affected by intrinsic factors related to metabolic disturbance rather than

joint loading, although more detailed studies of how differences in body composition affect joint loading are warranted[10,37].

In order to understand the profile of a diet-induced systemic inflammatory environment, blood serum was assessed for 27 cytokines, growth factors, and hormones. Five serum analytes associated with adipose inflammation were increased in all DIO animals compared to chow-fed control group animals[38]. Two of these, GRO-KC and MIP-1α, have been linked to insulin resistance and are regulated via autocrine signalling by adiponectin, an adipocytokine that is decreased in obesity[39]. Although not directly measured, adiponectin may play a protective anti-inflammatory role in OA[40]. The increase in serum GRO-KC and MIP-1α suggests that adiponectin may be decreased in the serum of all DIO animals, but not necessarily in the synovial fluid. Our data suggest that factors downstream of adiponectin, like GRO-KC and MIP-1α, may be potential treatment targets in metabolic OA.

Leptin, a satiety hormone and key inflammatory mediator, has been widely explored in humans and animals to explain the effect of obesity on onset and progression of osteoarthritis[14,18]. In contrast to others, we did not find a relationship between OA severity and serum levels of leptin; however, leptin and a mediator selectively activated by leptin, IP-10, were increased in the serum of DIO animals[18]. Given that visceral adipose tissue, which secretes inflammatory mediators more actively than subcutaneous adipose tissue, is associated with hand OA in men, there is the possibility that adipose-derived factors influence the joint environment through the synovial fluid or local blood supply in the infrapatellar fat pad and bone[41].

The synovial fluid cytokine, growth factor, and hormone profiles in DIO animals were distinct from chow-fed control animals. Despite a higher body fat percentage in DIO-P compared

with DIO-R group animals, there were no significant differences in the 27 synovial analytes measured here, indicating similar inflammatory environments in the knee. This result implies that a HFS diet is sufficient to modify the knee inflammatory environment, a finding that may be explained partly by an altered gut microbial profile. Of the nineteen inflammatory markers increased in DIO synovial fluid, IL-1α was the only analyte that had a significant positive relationship with the Mankin scores. IL-1α and IL-1β are considered gatekeepers of inflammation, and have been implicated in early OA changes[42]. Leptin, in conjunction with IL-1α and TNF- α, has been shown to directly induce cartilage damage[23]. Furthermore, it has been demonstrated that the infrapatellar fat pad can be a significant contributor of leptin levels in knees, and that leptin may be critical to disturbing homeostasis in joints when present in sufficiently high concentrations[15,23]. Serum and synovial fluid leptin levels were increased in DIO animals, in agreement with reported findings[15]. Despite similar synovial fluid levels of IL-1β in DIO and chow animals, the positive association between IL-1β and OA was approaching significance in this dataset. Time-course data may clarify the respective roles of IL-1α and IL-1β in this model.

While the effect of obesity on peripheral circulation is impacted by many tissues and pathways, obesity's impact in the local environment may differ across tissues and joints. Concordant with previous reports, increases in the magnitude of local inflammatory markers in synovial fluid compared to serum were observed[15]. Moreover, all of the increased serum inflammatory markers in our DIO animals were positively associated with at least three synovial fluid inflammatory markers suggesting an effect of systemic inflammation on the joint.

In order to further understand factors affecting the peripheral inflammatory environment, gut microbiota composition was assessed. Gut microbiota composition and activity, especially in

individuals consuming a Western-type diet, has been linked with the rising incidence of metabolic disease, including obesity and type 2 diabetes[1]. Consistent with evidence that obesity in animal models is generally associated with an increase in Firmicutes with a concomitant decrease in Bacteroidetes, we found that DIO animals exhibited an increased Firmicutes:Bacteroidetes ratio[43].

Furthermore, *Lactobacillus* spp. and *Methanobrevibacter* spp. abundance may directly or indirectly explain a moderate amount of the Mankin Score. Although a strong predictive association between these microbes and Mankin Score was demonstrated, this relationship warrants further investigation to elucidate linkages with local and systemic inflammatory marker concentrations. To provide context of this relationship, the predictive strength of leptin levels in the serum and synovial fluid were calculated, as leptin has been identified as a marker strongly associated with OA[18]. Although the confidence intervals for predictive values were similar from both models, the standard error of estimate achieved from the model using *Methanobrevibacter* spp. and *Lactobacillus* spp. was lower. This data indicates that these microbial groups could be examined further, as they are similar in predictive power to leptin in both fluids.

Though not consistent across all strains, certain lactobacilli have been associated with health-promoting properties, including attenuated weight gain, reduced recruitment of inflammatory macrophages in adipose tissue, and improved gut barrier function[44,45]. Furthermore, reduced lactobacilli abundance has been reported in animals consuming diets high in saturated fat[46]. Here, the higher levels of plasma LPS in DIO group animals suggest that HFS-fed animals have increased gut permeability, which may be due in part to observed decreases in lactobacilli abundance. Importantly, oral administration of *Lactobacillus casei* in an experimental rat model of OA led to reduced inflammation and cartilage degradation and has

been suggested as a possible treatment for Metabolic OA[47]. Animal studies have also linked *Methanobrevibacter* spp. abundance with increased weight gain and adipose tissue growth, Surprisingly, despite the positive relationship between *Methanobrevibacter* spp. abundance and Mankin Scores, *Methanobrevibacter* spp. abundance was not different between dietary groups[48]. However, the overall pattern of gut microbial composition can impact host-microbial interactions[49]. Hence, the combination of decreased lactobacilli and the potential obesity-associated effects of *Methanobrevibacter* spp. may partly explain the association of *Methanobrevibacter* spp. with Mankin Scores despite the lack of statistical difference in abundance.

There are several limitations to this work. As designed our study is not able to establish causality. However, with this dataset, mechanistic considerations between the contributions of the gut microbiota or changes in diet in OA development can be approached with more understanding. Factors such as mobility and joint loading have not been examined here, as kinetic data are difficult to collect and interpret given the marked increase in abdominal adiposity in our DIO animals. Lastly, the middle tertile of DIO animals were not considered here. Future studies should carefully address the interaction of obesity and joint loading in determining the contributions of metabolic and mechanical factors to knee osteoarthritis. Moreover, future considerations of the spectrum of DIO animals could be used to assess the potential threshold and/or the linear relationship between body fat and Metabolic OA.

The goal of this work was to evaluate systemic influences of diet-induced obesity on the knee joint inflammatory environment. We show that body fat, not body mass, is associated with OA damage in the knee, and that a distinct inflammatory serum and synovial fluid signature differentiates between obese and chow-fed control animals. The source of this inflammation

remains to be clarified. We propose that a potential link between body fat inflammation, microbiota, and OA damage may be increased translocation of bacterial LPS into the bloodstream (Fig. 5). LPS-induced inflammation in adipose tissue could precipitate systemic changes in cytokines, adipokines, and growth factors, including leptin, IP-10 and IL-1α, that ultimately contribute to OA development by affecting the local inflammatory environment inside the knee[50]. Whether circulating LPS directly impacts the secretion of inflammatory cytokines from cells of the infrapatellar fat pad remains to be examined. Future work evaluating inflammatory markers in serum and synovial fluid over time, in conjunction with measuring inflammatory marker gene expression in the infrapatellar fat pad, synovium and fat depots from various locations, will help determine the potential sources of the inflammation measured here. The present series of experiments suggest that systemic factors beyond what has currently been shown or proposed, including the gut microbiota, may play a role in metabolic OA and warrant further investigation.

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Contributions

KHC was responsible for design of the study, execution of the study, data collection, data analysis, interpretation of data, drafting the manuscript, revising the manuscript and approving the final submitted version.

HAP was responsible for data analysis, interpretation of data, drafting the manuscript, revising the manuscript, and approving the final submission.

RAR contributed to design of the study, interpretation of data, revising the manuscript and approving the final submitted version.

DAH contributed to the design of molecular aspects of the studies, interpretation of the results, and revising the manuscript.

RAS contributed to data collection, analysis, and revising the manuscript, and approving the final submitted version.

WH contributed to study design, interpretation of the data, writing the manuscript, revising the manuscript, and approving the final submission.

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