

ORIGINAL ARTICLE

Protective effect of hydroxychloroquine on rheumatoid arthritis-associated atherosclerosis

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Abstract

Background: Patients with rheumatoid arthritis (RA) have an increased risk for cardiovascular disease. We examined the effect of gut microbiota in a mouse model of RA that develops atherosclerosis.

Methods: We created three groups of K/BxN female mice that were positive for the anti-glucose-6-phosphate isomerase (GPI) antibody: control diet (CD), high fat diet (HFD), and HFD with hydroxychloroquine (HFD + HCQ). Serological tests were used to detect the serum levels of total cholesterol (TCHO), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), anti-GPI antibody titers, and serum cytokines. Atherosclerotic plaque was determined by histological analysis, and gut microbiota were determined by 16sV4 sequencing.

Results: Relative to mice given the CD, those receiving the HFD had increased serum levels of LDL-C, TCHO, and TG, decreased serum levels of HDL-C, increased atherosclerotic lesions in the aortic root, and altered gut microbiota. Addition of HCQ to HFD decreased the serum levels of LDL-C, TCHO, and TG, increased serum levels of HDL-C, and decreased the atherosclerotic lesions in the aortic root. Mice receiving HFD + HCQ also had the greatest bacterial diversity among the three experimental groups. Moreover, HCQ treatment significantly increased the abundance of *Akkermansia* and *Parabacteroides*, and decreased the abundance of *Clostridium sensu stricto* cluster 1, and therefore may be responsible for the reduced RA-associated atherosclerosis and dyslipidemia.

Conclusion: Our mouse model of RA indicated that HFD increased ankle width and aggravated atherosclerosis and dyslipidemia, and that HCQ alleviated the dyslipidemia and atherosclerosis, but had no effect on ankle width.

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KEYWORDS

atherosclerosis, autoimmunity, hydroxychloroquine, intestinal microbiota, rheumatoid arthritis

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by chronic and erosive synovitis, especially of the peripheral joints.¹ Patients with RA have increased rates of cardiovascular (CV) morbidity and mortality due to accelerated formation of atherosclerotic lesions that are more prone to rupture.²⁻⁵ This elevated CV risk is associated with traditional cardiometabolic risk factors and with the presence of systemic inflammation, which may promote atherosclerosis.^{4,6} Hydroxychloroquine (HCQ), a weak basic 4-aminoquinoline compound, can improve the survival rates of patients with some inflammatory diseases, including RA.⁷⁻⁹ Additional data suggest that HCQ could protect against CV disease (CVD) because of its effects on lysosomes, which appear have reduced insulin degradation¹⁰ and cholesterol synthesis¹¹ following HCQ treatment.

RA is also associated with chronic inflammation and significant alterations in the composition of intestinal microbiota. The abnormal changes of gut microbiota may correlate with chronic inflammation, and together increase the risk of CVD.¹² One study indicated that gut microbiota can metabolize dietary L-carnitine into trimethylamine N-oxide (TMAO), and this can accelerate atherosclerosis.¹³ Treatment of RA patients with probiotics may help to restore intestinal health, reduce inflammation, and decrease the incidence of CVD.¹² An appropriate animal model could help scientists to better understand the relationships of inflammatory arthritis and atherosclerosis in RA.

Mice expressing the KRN T cell receptor transgene and the MHC class II molecule Ag7 (K/BxN mice) develop severe inflammatory arthritis that mimics RA.¹⁴ Injection of serum from these mice causes similar signs and symptoms in a wide range of other mouse strains, because of the presence of pathogenic autoantibodies against glucose-6-phosphate isomerase (GPI).¹⁵ Other research indicated that the K/BxN mouse is predisposed towards atherosclerosis.¹⁶ Thus, this model appears to be a useful tool for investigation of the development of RA combined with atherosclerosis.

We investigated the effect of a high fat diet (HFD) on the development of atherosclerosis in K/BxN mice, and the effect of HCQ on amelioration of the symptoms of RA and atherosclerosis, and alterations in gut microbiota.

2 | MATERIALS AND METHODS

2.1 | Animal model

KRN mice were a gift from the colony of Dr Diane Mathis and Christoph Benoist at Harvard University (USA), NOD/LtJ mice were purchased from HuaFuKang Bioscience Co., Ltd (Beijing), and

K/BxN mice were obtained by crossing KRN mice with NOD/LtJ mice that were homozygous for the A^{g7} allele. The first cross generated K/BxN mice expressed the T cell receptor (TCR) transgene KRN and the MHC class II molecule A^{g7}, and develop severe inflammatory arthritis. Injection of serum from these mice into a variety of other mice strains can cause symptoms that mimic RA because the serum contains autoantibodies against glucose-6-phosphate isomerase (GPI).

All mice were housed and maintained in individual ventilated cages (IVCs) in specific pathogen-free (SPF) conditions in the Institute of Laboratory Animal Sciences (ILAS) at the Chinese Academy of Medical Sciences & Peking Union Medical College. They were given control (standard) food (CD), a high fat diet (HFD), or a high fat diet with hydroxychloroquine (HFD + HCQ) beginning 8 weeks after birth. Experiments were performed on mice aged 8-24 weeks (weight 23.1-25.3 g; Research License No. SYXK (Beijing) 2015-0035). All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committees of the ILAS (ILAS-PG-2015-002).

2.2 | Reagents

The HFD (Botaihongda, Beijing) consisted of 15.8% fat, 1.25% cholesterol, and 0.5% cholate, and the control diet (HuaFuKang, Beijing) consisted of 0% fat, 0% cholesterol, and 0% cholate. All antibodies (APC-CY7-conjugated anti-CD45R, FITC-conjugated anti-AA4.1, PE-CY7-conjugated anti-CD19, PE-conjugated anti-CD23, PE-conjugated anti-CD95, Alexa Flour 647-conjugated anti-CD38, percp-CY5.5-conjugated anti-B220, APC-conjugated anti-CD11c, FITC-conjugated anti-CD11b, PE-conjugated anti-CD5, APC-CY7-conjugated anti-CD3, APC-CY7-conjugated anti-CD45R, APC-conjugated anti-CD138, PE-conjugated anti-CD3, and FITC-conjugated anti-CD4) were from BD Bioscience.

2.3 | Animal groups and methods

The K/BxN female mice, all of which were anti-GPI antibody positive, were given one of three treatments: control diet (CD), high fat diet (HFD), or HFD with HCQ (10 mg/kg/d in drinking water). The dose of HCQ was selected based on the ranges used in previous research (2.5-10 mg/kg/d).¹⁷⁻¹⁹ Swelling of hind limbs was determined weekly using a Vernier caliper.

2.4 | Blood and aortic root collection

At 10 weeks after initiation of the different treatments, blood was collected from the cheeks of the mice and centrifuged at 2504 g at 4°C for 10 minutes. After 16 weeks of treatment, mice were

anesthetized and euthanized, the aortic roots were embedded with tissue freezing medium by freezing to -80°C and slicing into sections.

2.5 | Measurement of cytokines, plasma lipoproteins, and anti-GPI antibody

Serum levels of TNF- α , IL-6, IL-23P19, IL-17A, IL-10, and IFN- γ were determined using a Luminex 200 Mouse Magnetic 6 plex Assay (LXSAMSM-06) according to the manufacturer's instructions. Serum levels of LDL-cholesterol (LDL-C), total cholesterol (TCHO), triglyceride (TG), and HDL-cholesterol (HDL-C) were determined using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing) in accordance with the manufacturer's instructions. Serum levels of anti-GPI antibodies were determined using an enzymatic assay in which phosphoglucose isomerase (Sigma, USA) was used to coat the wells of 96-well plates, blocking was performed using bovine serum albumin (Sigma), and detection was performed using goat anti-mouse IgG (Southern Biotech, USA), with measurement of absorbance at 405 nm.

2.6 | Oil Red O staining of aortic roots

The aortic roots were cut into 7- μm sections using a freezing microtome (CM 3050S) from Leica (Germany), starting from the three valve cusps of the aortic sinus, at 35- μm intervals. The sections were stained with Oil Red O for measurement of plaque using Image-Pro Plus 6.0 software. Results are expressed as the percentage of stained area: (oil red O positive/aorta cross section) \times 100%.

2.7 | Flow cytometry

Red cells were removed from splenocytes prior to flow cytometry. To assess the distribution of T1 and T2 B cells, the APC-CY7-conjugated anti-CD45R, FITC-conjugated anti-AA4.1, PE-CY7-conjugated anti-CD19, and PE-conjugated anti-CD23 were used for staining. A

flow cytometer (Aria I) from Becton Dixon (USA) was used for detection and the data were analyzed using Flow Jo version 10 (USA).

2.8 | 16S rDNA sequencing

Mice feces were collected after 10 weeks of treatment, total DNA was isolated using the QIAamp DNA Stool Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions, and the 16S V4 region was amplified using PCR. Then, a library was built using TruSeq[®] DNA PCR-Free Sample Preparation Kit, and quantification was performed using Qubit and Q-PCR, with sequencing by an Illumina HiSeq2500 PE250. Operational taxonomic units (OTUs) were clustered (97% identity effective tags) using the UPARSE pipeline, and representative sequences were assigned to a taxonomic identity using the RDP classifier, with a default confidence threshold of 0.8 to 1.0. Mothur and SILVA SSU database 119 were used to annotate and analyze the OTUs. This analysis determined the number of observed-species. Community alpha diversity was measured by Simpson's index.

2.9 | Statistical analysis

All values are presented as means \pm standard deviations (SD). Statistical computations were performed using GraphPad Prism 6.0 (San Diego, USA). Student's *t* test was used for two groups comparison, and 95% confidence intervals (CIs) were calculated. Results were considered statistically significant if the *P* value was below 0.05.

3 | RESULTS

3.1 | HFD triggers joint swelling and alters splenic lymphocyte populations in K/BxN mice, while HCQ has no affect on these responses

The joints of K/BxN mice undergo swelling that mimics RA, and this is related to the presence of serum autoantibodies against GPI.

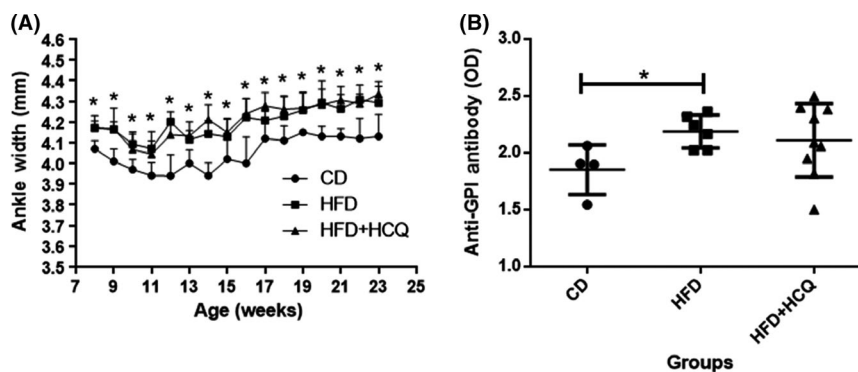


FIGURE 1 Ankle width and anti-GPI antibody titers of K/BxN mice after treatment. Ankle widths of hind limbs (A) and anti-GPI antibody titers (B) of K/BxN mice in the different treatment groups. Ankle widths were measured weekly from 8 to 24 wk, and serum anti-GPI antibody titers were determined by ELISA after 10 wk of treatment. Here and below, each group had 4-9 mice

*Statistically significant difference between the HFD and CD groups ($P < 0.05$)

Thus, we performed weekly measurements of hind limb swelling and determined serum anti-GPI antibody titers of the different groups. The results show that the HFD group had significantly greater ankle width and anti-GPI antibody titers than the CD group, and that HCQ had no observable effect on either parameter (Figure 1). Measurement of serum cytokines (TNF- α , IL-6, IL-23P19, IL-17A, IL-10, IFN- γ) indicated no significant differences among the three groups (data not shown). These data indicate that HFD exacerbates joint inflammation in this mouse model of RA, but HCQ had no effect on this response.

We next examined why HFD increased anti-GPI antibody titers by use of flow cytometry. Transitional B cells can be divided into T1 and T2 subsets. Thus, we used specific markers (reactivity to the AA4.1 and surface expression of CD23) to identify T1 B cells (B220⁺AA4.1⁺CD23⁻) and T2 B cells (B220⁺AA4.1⁺CD23⁺). Our results indicate that mice in the HFD group had higher percentages of splenic T1 B cells and T2 B cells than the CD group (Figure 2).

HFD had no effect on lymphocyte distribution in the aorta, whole blood, or bone marrow (data not shown). HCQ had no impact on the percentages of splenic T1 B cells or T2 B cells. Thus, although HCQ is used clinically to treat RA, it did not down-regulate distribution of B cells and did not reduce paw swelling in RA mice associated with atherosclerosis.

3.2 | HFD increases serum levels of TCHO, LDL-C, and TG in K/BxN mice, decreases serum levels of HDL-C, while HCQ partially reverses these effects

We also examined the effect of the HFD on the symptoms of atherosclerosis in K/BxN mice. The results showed that HFD significantly increased the serum levels of LDL-C, TCHO, and TG, decreased the serum levels of HDL-C, and that HCQ partially reversed these effects (Figure 3A-D). Our results also found that plaque at the root of aortic

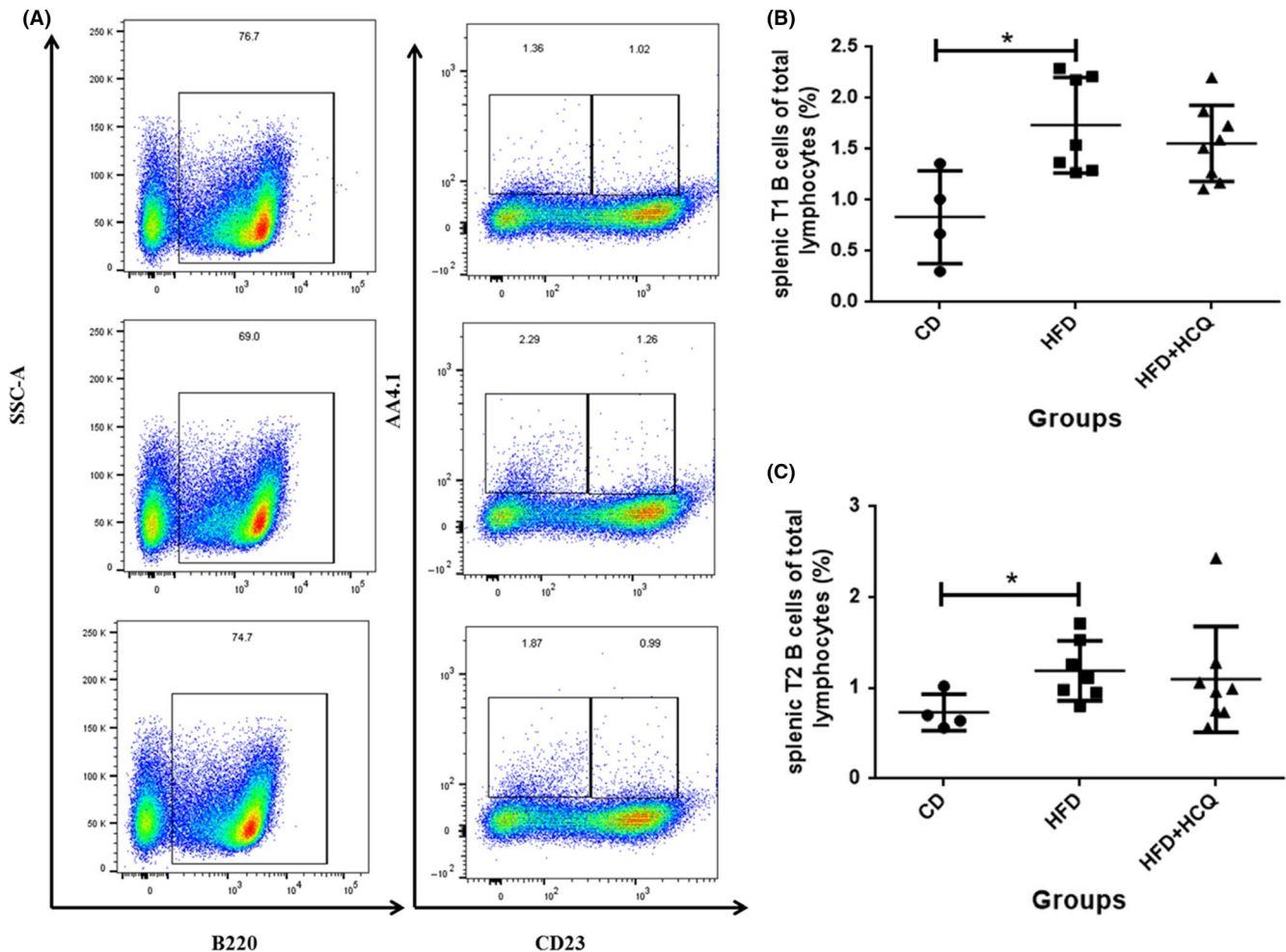


FIGURE 2 Splenic transitional T1 B and T2 B cells of total lymphocytes (%). Flow cytometry plots of splenic T1 B cells and T2 B cells of K/BxN mice in the different treatment groups after 16 wk (A) and quantification of these results (B, C). Cells were gated, and plotted onto forward scatter FCS-A and SSC-A. B cells were plotted onto B220 and side scatter (SSC), splenic transitional T1 and T2 B cells were plotted with B220, AA4.1, and CD23. B220⁺AA4.1⁺CD23⁻ T1 B cells and B220⁺AA4.1⁺CD23⁺ T2 B cells were then identified

*Statistically significant difference between the HFD and CD groups ($P < 0.05$)

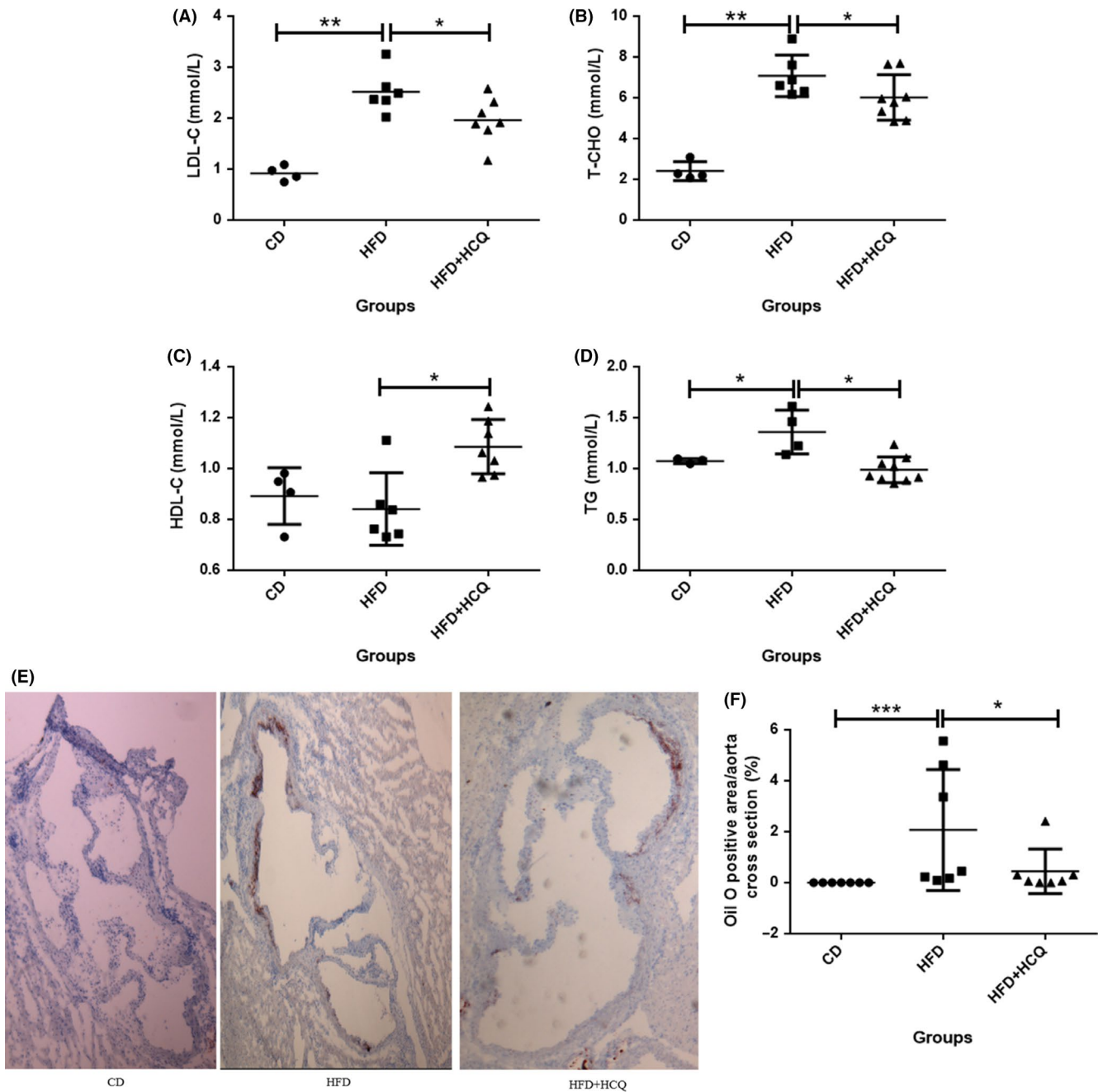


FIGURE 3 Detection of atherosclerosis index. Dyslipidemia (A, B, C, D) and atherosclerosis in the aortic root (E, F) of K/BxN mice in the different treatment groups after 10 wk of treatment. Magnification: 40×

*Statistically significant difference between the HFD and CD groups ($P < 0.05$), and between the HFD and HFD + HCQ groups ($P < 0.05$)

**Statistically significant difference between the HFD and CD groups ($P < 0.01$)

***Statistically significant difference between the HFD and CD groups ($P < 0.001$)

valve was greater in mice in the HFD group than the CD group, and that HCQ partially reversed this effect (Figure 3E-F).

3.3 | HFD leads to abnormal intestinal flora and decreased diversity of intestinal microbiota in K/BxN mice, while HCQ alters this effect

There is some evidence that use of probiotics may reduce the incidence of CVD. Thus, we next examined the effect of different

treatments on the gut microbiota of K/BxN mice using 16S rDNA amplicon Illumina sequencing. The results indicate the three groups had significant differences in overall OTUs. More specifically, all three groups had 430 identical OTUs, the HFD group had the greatest variability in the number of OTUs and slightly more OTUs than the CD group, and the HFD + HCQ group had the greatest mean number of OTUs (Figure 4A). Evaluation of community alpha diversity in the three groups indicated greater diversity in the HFD + HCQ treatment group, and that the HFD and CD groups had similar diversity (Figure 4B).

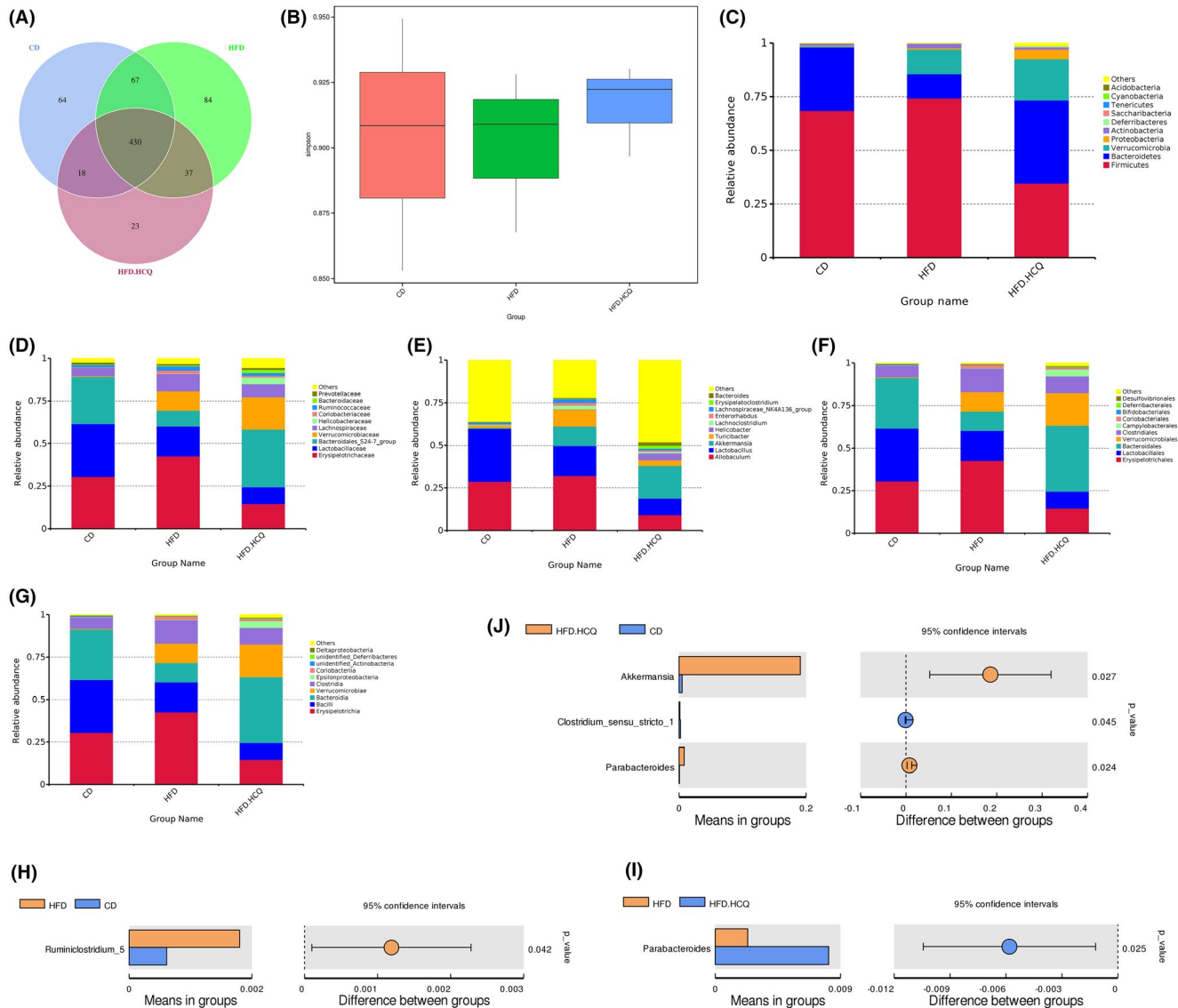


FIGURE 4 Intestinal microbiota of K/BxN mice in the different treatment groups after 10 wk of treatment. 16S V4 region rDNA gene sequences were analyzed for determination of shared and independent bacterial OTUs (Venn diagram, A), Simpson's index of community diversity (B), and abundance of dominant bacteria at the level of the phylum (C), family (D), genus (E), order (F), and class (G). Student's *t* test was used to compare the abundance of *A. muciniphila* ($P < 0.05$), *Clostridium sensu stricto-1* ($P < 0.05$), *Ruminiclostridium* ($P < 0.05$) and *Parabacteroides* ($P < 0.05$) in the CD, HFD and HFD + HCQ groups (H-J)

Further analysis of the intestinal flora indicated that differences among the three groups occurred at the levels of the phylum, order, class, family, and genus (Figure 4C-G). At the phylum level, the five major intestinal microbiomes were *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Proteobacteria* and *Actinobacteria*. The intestinal microbiota composition of all three groups was also altered. For example, at the genus level, relative to mice in the CD group, those in the HFD group had more *Ruminiclostridium_5* (Figure 4H). Relative to mice in the HFD group, those in the HFD + HCQ group had more *Parabacteroides* (Figure 4I). Relative to mice in the CD group, those in the HFD + HCQ group had more *Akkermansia muciniphila* and *Parabacteroides*, and fewer *Clostridium sensu stricto-1* (Figure 4J). Thus, HCQ treatment of this mouse model of RA alleviated the

HFD-induced dyslipidemia and atherosclerosis, and altered the composition of intestinal flora, suggesting a possible relationship of these responses.

4 | DISCUSSION

Our study of a mouse model of RA indicated that relative to mice in the CD group, mice in the HFD group had aggravation of dyslipidemia and atherosclerosis, increased joint swelling, and altered gut microbiota. Mice in the HFD + HCQ group had a normalized lipid profile, alleviated symptoms of atherosclerosis, and altered gut microbiota (possibly explaining its impact on atherosclerosis). However, HCQ

had no impact on joint swelling or the percentages of splenic T1 B cells or T2 B cells.

HCQ can normalize the lipid profiles of patients with RA and systemic lupus erythematosus (SLE),^{7,20-22} and of mice in our model of RA. This is due to the hydrolysis of internalized cholesterol esters mediated by the lysosomotropic action of HCQ, which stimulates LDL-receptors and the expression of related genes. HCQ can also directly affect insulin metabolism.⁹ Our results indicate that HCQ alleviates the symptoms of atherosclerosis, possibly because it suppresses platelet aggregation, releases arachidonic acid from activated platelets, and reduces antiphospholipid antibody production.⁹ However, we found that HCQ had no impact on paw swelling in K/BxN mice, presumably because it had no impact on the level of anti-GPI antibodies. GPI plays an important role in the pathophysiology of RA, in that it stimulates cell proliferation, inhibits apoptosis, and increases the secretion of pro-inflammatory cytokines by fibroblast-like synoviocytes.²³ The articular manifestations in K/BxN mice are the result of high titers of anti-GPI antibodies.²⁴ Mice in the HFD group had alterations in the percentages of splenic T1 B cells and T2 B cells, but HCQ had no impact on this response, which is consistent with its lack of an effect on anti-GPI antibody titer and paw swelling.

Gut microbiota play an important role in the etiology of RA²⁵ and atherosclerosis.²⁶ Relative to individuals with fibromyalgia, RA patients have decreased levels of *Bifidobacteria*, the *Bacteroides- Porphyromonas-Prevotella* group, and the *Bacteroides fragilis* subgroup.²⁷ An increased level of *Prevotella histicola* bacteria can increase the LPS concentration, which activates toll-like receptor-4 (TLR4). TLR4 activation is associated with the secretion of interleukin-4 (IL-4) and the failure of T cells to properly differentiate, both of which contribute to the chronic inflammatory state that is characteristic of patients with CVD and RA.¹² Many disease-modifying antirheumatic drugs can reverse the dysbiosis associated with RA.²⁵

Our research is consistent with these clinical results, in that mice in the HFD + HCQ group had the greatest intestinal bacterial diversity. Thus, the beneficial effects of HCQ on the colon may be explained by its ability to adjust the disruptions of intestinal flora caused by or associated with certain diseases.²⁸ Our results also indicate that HCQ treatment significantly increased the numbers of *Akkermansia muciniphila* in the gut. This species is in the *Verrucomicrobia* phylum, and is a human intestinal mucin-degrading bacterium and an important component of the gut microbiota.²⁹ Some studies showed that *A muciniphila* has an anti-atherosclerotic effect, due to its ability to control the gut barrier and other processes important for maintaining homeostasis, and due to its anti-inflammatory activity.^{30,31} Moreover, *A muciniphila* can prevent obesity and related complications in mice and humans.^{30,34} These effects are due to its ability to adhere to enterocytes that line the intestinal tract, which increases the integrity of the epithelial layer, prevents intestinal leakage, and activates intestinal immunity. *A muciniphila* also releases enzymes into the intestinal tract that modulate the function of mucin, the main surface component of gastrointestinal mucosa.³⁵ The mucus layer has a critical role in preventing the adhesion of

harmful bacteria.³⁶ A recent study isolated a specific protein from the outer membrane of *A muciniphila* that interacts with TLR2 and improves the intestinal barrier.³⁴ *A muciniphila* also preserves the gut barrier by increasing the expression of two tight junction proteins that protect against atherosclerosis in ApoE^{-/-} mice.³²

Our results indicated that HCQ treatment significantly increased the level of intestinal *Parabacteroides*, decreased the serum levels of LDL-C and TCHO, and alleviated atherosclerosis. Previous researchers found that feeding lingonberries to ApoE^{-/-} mice decreased triglyceridemia, reduced atherosclerosis, and increased cecal abundance of *Parabacteroides*.³⁷

Our results also show that HCQ treatment significantly decreased the abundance of *Clostridium sensu stricto-1*. Previous researchers reported that expression of IL-1 β and TNF- α mRNAs were positively associated with the abundance of *Clostridium sensu stricto-1* in the colon of sheep, thus suggesting that enrichment of these bacteria in the intestinal mucosa of sheep fed a high glucose diet may be responsible for the epithelial inflammation.³⁸ Another study reported that *Clostridium spp* should be considered a "conditioned pathogen" that functions as a pathogen in goat intestinal enteric diseases.³⁹ This may be partly explained by the adverse effect of *Clostridium* on the gut barrier.²⁸ Thus, an enrichment of intestinal *Clostridium sensu stricto-1* may be responsible for the aggravation of symptoms in our mouse model of RA, and an HCQ-mediated decrease in the abundance of these bacteria may explain the beneficial effects of HCQ on atherosclerosis and dyslipidemia.

4.1 | Conclusions

This study of a mouse model of RA indicates that HFD altered the intestinal microbiota, increased joint swelling, and aggravated atherosclerosis and dyslipidemia. HCQ treatment increased the abundance of intestinal *A muciniphila* and *Parabacteroides*, and decreased the abundance of *Clostridium sensu stricto-1*, an effect that may explain its amelioration of dyslipidemia and atherosclerosis.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

NS performed the experiments, analyzed the data, and prepared the manuscript. GS provided suggestions for manuscript revision. SZ

performed some animal experiments. ML assisted with design of the study. JC assisted with data interpretation and preparation of the manuscript. HN was responsible for the overall design of the study, analysis and interpretation of the data, and manuscript preparation. All authors read and approved the final manuscript.

DATA AVAILABILITY

The data sets generated and analyzed for the present study are available from the corresponding author on reasonable request.

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