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SEX-DEPENDENT DIFFERENCES ON THE IMPACT OF ANTI-INFLAMMATORY TREATMENT IN THE PROGRESSION OF CORONARY ARTERY DISEASE IN A MURINE MODEL OF LETHAL ISCHEMIC HEART DISEASE INDUCED BY DIET

Por

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Sex-dependent differences on the impact of anti-inflammatory treatment in the progression of coronary artery disease in a murine model of lethal ischemic heart disease induced by diet.

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Abstract

Background — Cardiovascular risk differs significantly between adult men and women. Therefore, it is expected that different treatments may affect both groups differently. We aim to compare sex-dependent differences on survival and systemic inflammation in response to anti-inflammatory treatment using a diet-induced myocardial infarction mouse model.

Methods— Male and female SR-B1$^{-/-}$ApoER61$^{h/h}$ mice, aged 2-3 months, were randomly assigned into two groups: Control (HFD-Control) and minocycline (HFD-MIN). Atherosclerosis was induced by feeding an atherogenic diet (15% fat, 1.25% cholesterol, 0.5% cholate). Minocycline was administered in the drinking water at a dose of 0.05 mg/mL. A survival test was performed to evaluate the impact of the anti-inflammatory intervention. Serum biomarkers of inflammation and monocyte sub-populations were studied at day 14 after the diet and treatment intervention.

Results— Female mice had a slightly better survival than male mice when fed an HFD (26.5 vs 22.5 days, p=0.12). Minocycline improved survival in male by 35% (30.4 vs 22.5 days, p=0.006) and by 33% in female (35.3 vs 26.5 days, p=0.01), without affecting total cholesterol levels. Male mice fed with HFD tended to have higher IL-6 levels than female mice (398.4 vs 291.9 pg/mL, respectively, p=0.08). Minocycline significantly reduced IL-6 levels (259.7 vs 398.4 pg/mL, p=0.04) and Ly6Chigh (35.9 vs 57%, p=0.006) and increased the Ly6Clow subset (64.1 vs 43 %, p=0.006). Male and female fed with HFD clustered in different groups by analyzing inflammatory parameters by PCA; however, after minocycline intervention, were indistinguishable.

Conclusion— High fat diet decreased survival and caused early death in this animal model, however, females had slightly better survival than male mice. Minocycline treatment improved survival in both groups although it did not affect their cholesterol levels. Males showed higher inflammatory serum biomarkers than females, and minocycline treatment showed a higher impact on systemic anti-inflammation in male mice than female, by reducing plasma IL-6 levels and shifting toward a more "reparative" phenotype on circulating monocyte subsets.

Highlights

- HFD caused early death in this model, however female mice had a slightly better survival than male mice, which tended to have a greater systemic inflammation associated with higher IL-6 plasma levels.
- Minocycline increased survival in male by 35% and by 33% in female mice, without affecting total cholesterol levels.
- Minocycline had an important anti-inflammatory effect in male but not in female mice by reducing plasma IL-6 levels and shifting toward a more “reparative” phenotype on circulating monocyte subsets.

- Under HFD male and female mice clustered in different groups by analyzing inflammatory parameters, however after Minocycline treatment were indistinguishable.

Keywords: Atherosclerosis, Gender, Inflammation, Monocytes/macrophages.

Plain language summary

Modern data shows that younger women have lower cardiovascular risk compare to men of the same age, but it increases considerably after menopause, however, the associated mechanisms remain incompletely understood. Our study aimed to compare sex-dependent differences of short-term anti-inflammatory treatment on systemic inflammation and survival in a murine model of accelerated atherosclerosis. Female and male mice were fed a high-fat, high-cholesterol diet (HFD) to induce disease and treated or not with minocycline, a potent anti-inflammatory. A survival test, serum biomarkers of inflammation and monocyte sub-populations were studied. Our results showed that HFD decreased the survival and caused early death in this model; but female mice had a slightly better survival than male mice, that tended to have a greater systemic inflammation (higher IL-6 levels). As expected, Minocycline improved survival in both: male by 35% and by 33% in female, without affecting total cholesterol levels, and additionally an important anti-inflammatory effect in male but not in female mice by reducing plasma IL-6 levels and shifting toward a more “reparative” phenotype on circulating monocyte subsets. These results contribute to guide drug therapy in patients where may not be necessary to observe high inflammatory parameters in women before initiation of anti-inflammatory treatment to improve survival if other risk factors are present.

1. Background

Atherosclerosis is a chronic inflammatory vascular disease and the major contributor to the global epidemic of cardiovascular disease, becoming the leading cause of death and disability worldwide (1). This lipid-driven disease arises from the accumulation of low-density lipoprotein (LDL) in the innermost layer of arteries with concomitant endothelial dysfunction, which head to atherosclerotic plaque or atheroma formation (2). Some of these plaques could be unstable and disrupt, thus contributing to of acute complications related to ischemic cardiovascular disease (CVD) (3): myocardial infarction (MI) and stroke.

There is evidence of sexual dimorphism in the incidence and complications of atherosclerosis (4). Modern epidemiological data shows that younger women have lower cardiovascular risk relative to men at the same age, but it increases considerably after menopause, even surpassing that of men suggesting an interaction between sex and age (5). This sex-dependent differences are probably associated with a lower prevalence of traditional risk factors in younger women (6) and also with a protective and pleiotropic effects of estrogens acting as an anti-inflammatory agent and enhancing relaxation in the vasculature through various mechanisms (7).

Development of atherosclerotic lesions is linked to the presence of several risk factors. In fact, a few decades ago, atherosclerosis was mostly considered a cholesterol storage disease (8). Currently, the causal relationship between LDL plasma levels and CVD is well established (9), so reducing LDL cholesterol with statins as a lipid-lowering drug class of choice (8), is currently the main goal in primary and secondary prevention (10). However, recurrent cardiovascular events still occur in more than half of these patients. Increasing evidence also points to a role of the immune system and

inflammation which link traditional and emerging risk factors promoting the progression and outcome of the disease (11,12) which could explain some of this residual risk (13).

Experimental evidence has described that the leukocyte recruitment into the intima and circulating proinflammatory cytokines are pivotally inflammatory processes that lead to the development and progression of the disease (11). Monocytes/macrophages are key effector cells in atherosclerosis-associated inflammation, leading to both plaque progression and instability (12). They accumulate quickly in response to injury or infection. Monocytes are a heterogeneous population and have been classified in two functional subsets. Ly6C^{high} cells are actively recruited into inflamed tissues (14) and exhibit phagocytic, proteolytic, and inflammatory functions (15), and Ly6C^{low} cells patrol vascular integrity (16), promote healing, and may be important in the resolution of inflammation (17). Additionally, the modulation of inflammation in clinical studies by direct anti-inflammatory therapies such as canakinumab, a therapeutic monoclonal antibody targeting interleukin-1β (18) and colchicine (19,20) have demonstrated unequivocally that can prevent the clinical complications related to major adverse cardiac events, therefore targeting inflammatory pathways appears to be a promising avenue for the management and treatment of atherosclerosis.

As well as the previous, Minocycline, a second-generation, semi-synthetic tetracycline with antibiotic properties against both Gram-positive and Gram-negative bacteria (21) has additional non-antibiotic properties, including anti-inflammatory activity with beneficial effects in experimental models of various inflammatory diseases such as atherosclerosis (22) where it has shown to reduce neointima formation following an acute vascular injury of the rat carotid artery (23), and plaque size and stenosis in ApoE$^{-/-}$ high fat-fed mice (24). In addition, it substantially reduced plaque metalloproteinase (MMP) activity, promoting plaque stabilization (25).

Despite these observations, fewer than half of the studies that include both sexes directly compare males versus females to consider this factor as an independent variable or the interaction of sex with a treatment (4). Thus, the underlying mechanisms associated with this biological variation remain incompletely understood. In fact, current therapies are validated primarily in males in preclinical animal models and are extrapolated to the female sex. Therefore, understanding the mechanisms of sex-associated differences in animal models of atherosclerosis is translationally relevant to mitigate CVD.

In this work, we used SR-B1$^{-/-}$ApoER61$^{h/h}$ mice, a mouse model that is susceptible to diet-induced occlusive coronary atherosclerosis, myocardial infarction (MI), and ultimately early death (26,27). The aim of this study was to compare sex-dependent differences of short-term minocycline administration on systemic inflammation, monocyte populations and survival in atherogenic diet-fed SR-B1$^{-/-}$ApoER61$^{h/h}$ male versus female mice.

2. Materials and Methods

2.1. Animals

SR-B1$^{-/-}$ApoER61$^{h/h}$ mice were produced by mating SR-B1$^{+/-}$ApoER61$^{h/h}$ females and SR-B1$^{-/-}$ApoER61$^{h/h}$ males from the founding colony originally obtained from Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA, USA). Mice were maintained in the animal facility at Pontificia Universidad Católica de Chile under light-, temperature-, and humidity-controlled conditions with free access to water and control chow diet (5% fat, 22% protein, 0.022% cholesterol,

4.0% fiber; Prolab RMH 3000; PMI Feeds Inc., Brentwood, CA, USA). Animals were supervised daily following institutional guidelines; and food intake and body weight were controlled and recorded throughout the study. All procedures were performed following recommendations from the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Ethics and Animal Welfare Committee from Pontificia Universidad Católica de Chile.

2.2. Intervention

For the survival study, female and male SR-B1$^{-/-}$ApoER61$^{h/h}$ mice, aged 2-3 months, were randomly assigned to 2 groups: Control (HFD-Control) and minocycline (HFD-MIN). Then, the animals were fed a high-fat and cholesterol diet (HFD) (57BB mouse diet: 15% fat, 1.25% cholesterol, 0.5% cholate, Test Diets, St. Louis, MO, USA) diluted with chow diet at 80% concentration with chow diet for 3-5 weeks to induce atherosclerosis. Minocycline was administered in the drinking water at a dose of 0.05 mg/mL concurrently with the HFD (28,29).

To evaluate systemic inflammation and monocyte population, another group of SR-B1$^{-/-}$ApoER61$^{h/h}$ mice -with the same characteristics as above- were randomly assigned to experimental or control groups and fed with HFD for 21 days, corresponding to the time point when MI mortality begun to be observed. Mice were euthanized with a mixture of ketamine:xylazine (150:10 mg/kg intraperitoneally) to obtain blood, plasma, and tissue samples. After blood collection, organs were perfused with saline solution by cardiac puncture to remove excess blood. Then, aorta and heart were removed, frozen in liquid nitrogen and stored to −80◦C. Liver, spleen and kidneys were also extracted and weighed in all animals.

2.3. Survival study

For the survival study, SR-B1$^{-/-}$ApoER61$^{h/h}$ mice were randomly assigned into 2 groups: female: HFD-Control (n= 8), HFD-MIN (n= 9) and male: HFD-Control (n= 9), HFD-MIN (n= 8). Animals were supervised daily in order to detect signs of life endpoint (e.g., hunched back, puckered coat, labored breathing, unsteady gait) according to the guidelines of the Ethics and Animal Welfare Committee of the Pontificia Universidad Católica de Chile). Survival analysis was evaluated by tabulating spontaneous death or humanitarian euthanasia of mice as a function of time by the Kaplan-Meier method as previously described in this animal model (30,31).

2.4. Blood flow cytometry

To assess systemic inflammation, monocyte populations were analyzed. Blood samples were collected by terminal cardiac puncture using citrate-coated tubes. Immediately after collection, blood was mixed with antibodies (mAbs) and incubated with a red blood cell lysis buffer (Beckman Coulter) to remove red blood cells. After centrifugation, mAbs-coated cells were resuspended in phosphate buffered saline (PBS) and finally fixed in 1% paraformaldehyde (PFA) and stored at 4°C until flow cytometry. For visualization of monocytes, blood was incubated with a cocktail of the following mAbs: Per-CP anti-mouse lymphocyte B220, APC anti-mouse F4/80, A488 anti-mouse CD115, Ef480 anti-mouse CD11b, and PE anti-mouse Ly-6C (eBiosciences, Frankfurt, Alemania; Invitrogen, Waltham, Massachusetts; and BD Pharmingen, San Diego, CA, USA). Monocytes were identified as a B220-, CD11b, and CD115-high population and reported as percentage of cells per sample. Within this population, Ly6C staining identified Ly6C^{high} and Ly6C^{low} subsets. The percentage

of each subset over total monocytes is reported. Fluorescence was detected by flow cytometry (FACS CANTO II, BD Biosciences, San Jose, CA,), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

2.5. Determination of circulating pro- and anti-inflammatory cytokines

To assess systemic inflammation, quantification of plasma levels of pro- and anti-inflammatory cytokines were determined by ELISA kits according to the manufacturer's instructions: IL-6 (Max™ Set Deluxe Kits, Biolegend, San Diego, CA, USA), IL-10, IL-1β, and TNF-α (R&D Systems, Minneapolis, MN). The data obtained for each sample were interpolated in a standard curve.

2.6. Determination of total plasma cholesterol

Total plasma cholesterol levels were measured in plasma samples through a colorimetric assay according to the manufacturer's instructions (Total Cholesterol Assay Kit, Cell Biolabs Inc, San Diego, CA, USA). The absorbance was measured at 540 nm in a plate reader and total cholesterol concentrations were determined using a standard curve.

2.7. Statistical analysis

For survival analysis, log-rank test was used to compare median survival among groups. For biomarker analysis, normality of data was determined with the Kolmogorov–Smirnov test and the difference between groups was analyzed using the Mann–Whitney U test for unpaired nonparametric data. Results are presented as mean ± standard error (SE). $P<0.05$ was used to define statistical significance. SPSS v20 software (IBM Corp, Armonk, NY, USA) and GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA, USA) were used for statistical data analysis. Principal component analysis (PCA) followed by a hierarchical cluster analysis upon data from plasma measurements of different mouse groups were created using R package version 4.0.2.

3. Results

3.1. Minocycline attenuated premature death in both female and male SR-B1$^{-/-}$ApoER61$^{h/h}$ mice

Feeding the high-fat and cholesterol atherogenic diet caused early death because of myocardial infarction (Supplementary Figure 1) in both female and male SR-B1$^{-/-}$ApoER61$^{h/h}$ mice. However, female mice had a slightly better survival than male mice when fed an atherogenic diet (26.5 vs 22.5 days, respectively, p=0.12, Figure 1A). Minocycline administration significantly improved survival in males by 35% (30.4 days vs 22.5 days, p=0.006, Figure 1B, Supplementary Figure 2) and female by 33% (35.3 days vs 26.5 days, p=0.01, Figure 1C, Supplementary Figure 2). In addition, we found a trend to longer survival in females versus males, but the difference did not reach statistical significance.

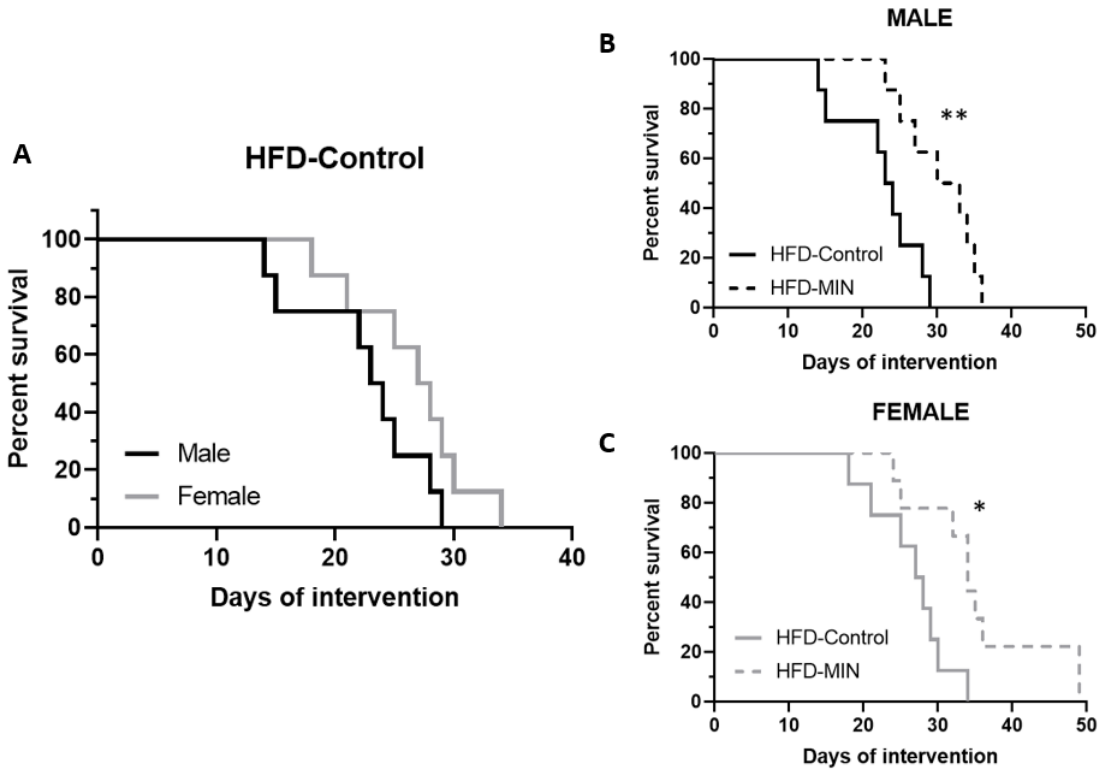

Figure 1. Effect of minocycline on survival of male and female SR-B1$^{-/-}$ApoER61$^{h/h}$ mice fed atherogenic diet. Kaplan–Meier survival curves of (A) male (n= 8) and female (n= 8) HFD-Control groups after fed with an atherogenic diet, (B) Male: HFD-Control (n= 8), HFD-MIN (n= 8) and (C) Female: HFD-Control (n= 8), HFD-MIN (n= 9). *p<0.05 and ** p <0.005

3.2. Minocycline reduced high circulating levels of IL-6 induced by HFD in male, but female, SR-B1$^{-/-}$ApoER61$^{h/h}$ mice

To assess the systemic inflammatory response, we evaluated plasma levels of biomarkers of inflammation: IL-6, IL-10, IL-1β and TNF-α. After feeding an atherogenic diet, male SR-B1$^{-/-}$ApoER61$^{h/h}$ mice tended to have higher IL-6 levels than female mice (398.4 vs 291.9 pg/mL, respectively, p=0.08, Figure 2A); but similar IL-10 (Figure 2B) and TNF-α levels (Figure 2C). Minocycline administration did not change plasma levels of IL-6 between male and female mice (Figure 2A), IL-10 (Figure 2B) and TNF-α (Figure 2C). Minocycline significantly reduced IL-6 levels in males (259.7 ± 42.8 pg/mL vs 398.4 ± 45.2 pg/mL, p=0.04, Figure 2A), but not in female mice (Figure 2A). No changes in IL-10 levels in both male and female mice (Figure 2B) neither in TNF-α levels in male and female mice (Figure 2C) were seen after minocycline administration. Plasma levels of inflammatory IL-1β were below detectability in this murine model.

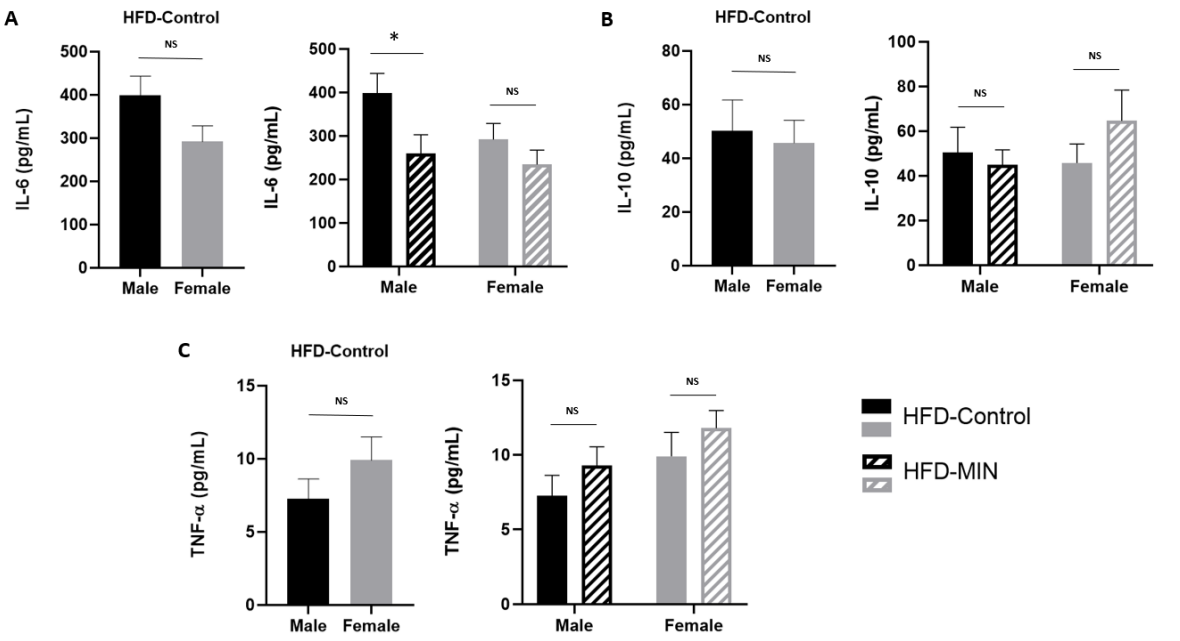

Figure 2. Effect of Minocycline on plasma levels of pro and anti-Inflammatory cytokines in Diet-fed female and male SR-B1$^{-/-}$ApoER61$^{h/h}$. (A) IL-6 and (B) IL-10 in Male: HFD-Control (n= 9), HFD-MIN (n= 9) and Female: HFD-Control (n= 10), HFD-MIN (n= 13) and (C) TNF-α levels in Male: HFD-Control (n= 10), HFD-MIN (n= 11) and Female: HFD-Control (n= 10), HFD-MIN (n= 13) after 21 days of feeding HFD and minocycline administration. * p <0.05. NS: "non significant".

3.3. Minocycline decreases Ly6C^{high} and increased Ly6C^{low} monocytes in male SR-B1$^{-/-}$ApoER61$^{h/h}$ mice

To assess the systemic monocyte response, we also evaluated blood monocyte sub-populations after minocycline treatment. Combined male and female mice had similar content of total circulating monocytes after feeding a HFD and after minocycline administration (Figure 3B). Separated by sex, treatment with minocycline also did not modify circulating monocytes in either male or female mice (Figure 3B).

On the other hand, HFD-fed male and female mice had similar percentages of monocyte subpopulations and also after minocycline treatment (Figure 3C-D). Interestingly, minocycline resulted in a significant reduction of Ly6C^{high} monocytes in male mice (35.9 ± 2.7% vs 57 ± 6.4%, p= 0.006, Figure 3C), along with an increase in Ly6C^{low} monocytes (64.1 ± 2.7% vs 43 ± 6.4%, p= 0.006, Figure 3D). However, there were no significant changes in monocyte subsets of female mice upon minocycline administration, for both Ly6C^{high} (Figure 3C) and Ly6C^{low} monocytes (Figure 3D).

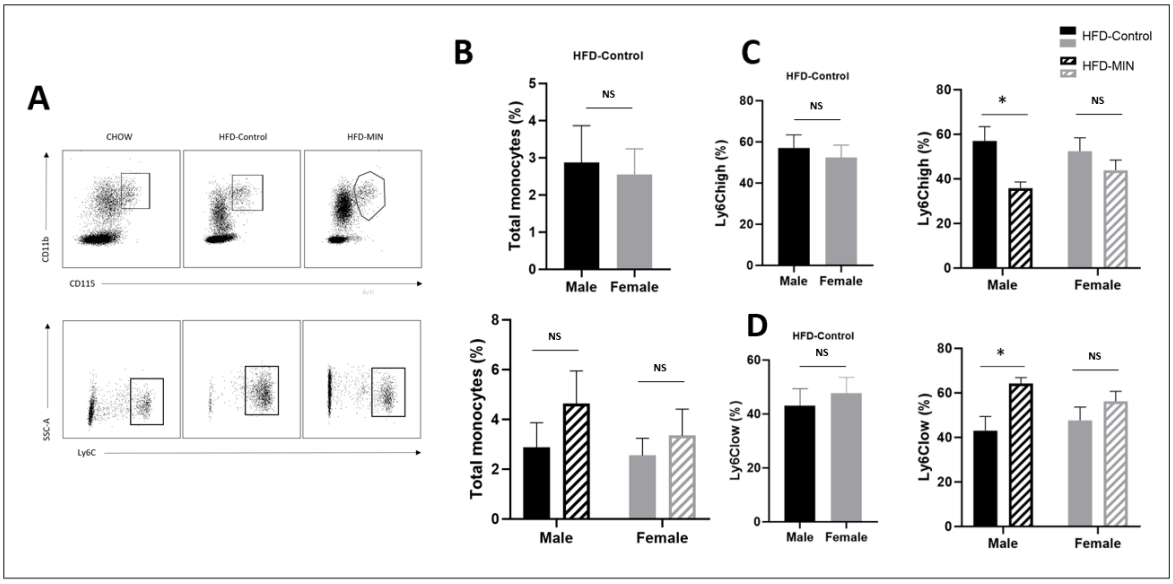

Figure 3. Effect of Minocycline on Blood Monocyte content and Subsets in male and female SR-B1$^{-/-}$ApoER61$^{h/h}$ mice fed atherogenic diet. (A) Representative flow cytometry images of the gating strategy employed to quantify monocytes and Ly6C^{high} and Ly6C^{low} monocyte subsets in CHOW, HFD-MIN and HFD-Control at 21 days. **(B)** Flow cytometric quantification of % monocytes in the sample **(C)** Flow cytometric quantification of Ly6C^{high} and **(D)** Ly6C^{low} monocyte subsets expressed as percentage of cells over total monocytes. CHOW: female SR-B1$^{-/-}$ApoER61$^{h/h}$ mice fed with regular chow. Male: HFD-Control (n= 9), HFD-MIN (n= 10) and Female: HFD-Control (n= 9), HFD-MIN (n= 10). Data were represented as mean ± SEM. *p<0.05. NS: "non significant".

3.4. Minocycline improved inflammatory conditions in males leading to an inflammatory pattern similar to female SR-B1$^{-/-}$ApoER61$^{h/h}$ mice.

In order to identify clusters of systemic inflammatory response to HFD and minocycline treatment, we integrated all the inflammatory biomarkers (Total monocytes, Ly6C^{high}, Ly6C^{low}, IL-1, and IL-10) using a reduction dimensionality analysis with PCA and hierarchical clustering (32). Male and female clustered in two different groups showing a different pattern of systemic inflammation. However, after treatment with minocycline, both groups were indistinguishable, showing that after treatment both join in a similar pattern of inflammatory markers (Figure 4).

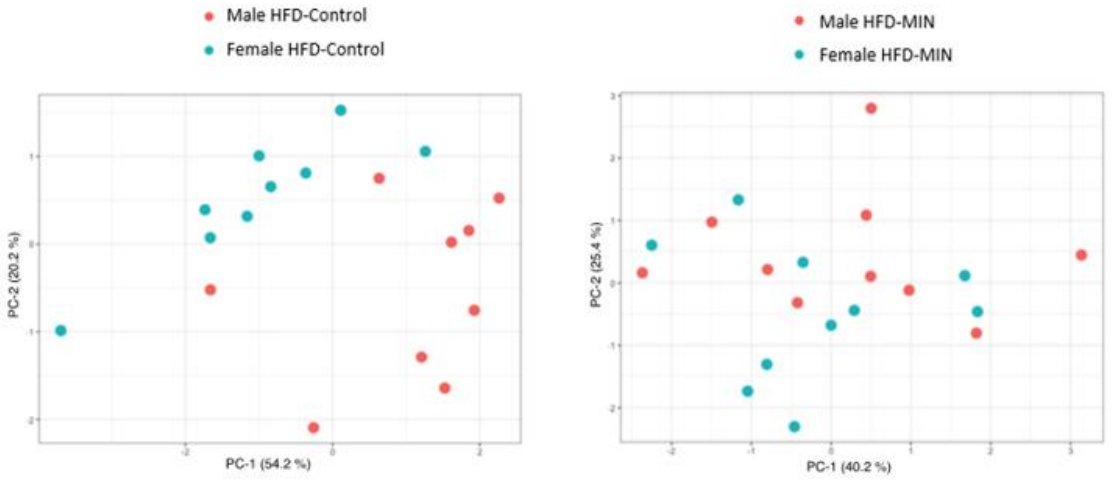

Figure 4. Simultaneous analysis of all inflammatory parameters by Principal Components Analysis. Males and females fed with HFD clustered in two different groups, however when treated with minocycline, both genders clustered similarly making both groups indistinguishable.

3.5. Minocycline did not affect total plasma cholesterol levels

To determine if the impact on survival was due to the anti-inflammatory effect of minocycline independent of changes blood total cholesterol levels, we evaluated plasma cholesterol after 21 days of feeding HFD and minocycline administration. At baseline, there were no differences between male and female mice after feeding HFD and after minocycline administration (Figure 5A). Minocycline did not affect total cholesterol levels in males neither in female mice (Figure 5A).

We also evaluated changes in body weight and observed that in the HFD group at day 21 there was a significant 8.9% reduction in body weight in male (24.7 vs 22.5 grs, p=0.03, Figure 5B) and of 8.4% in female mice (23.7 vs 21.7 grs, p=0.02, Figure 5C). On the other hand, body weight remained stable throughout minocycline treatment in both male (Figure 5B) and female mice (Figure 5C). At the end of the intervention, male mice fed with HFD had a significantly lower weight compared to those treated with minocycline (26.6 ± 2.5 grs vs 22.5 ± 2.9 grs, p<0.005, Figure 5B), not so in females (Figure 5C).

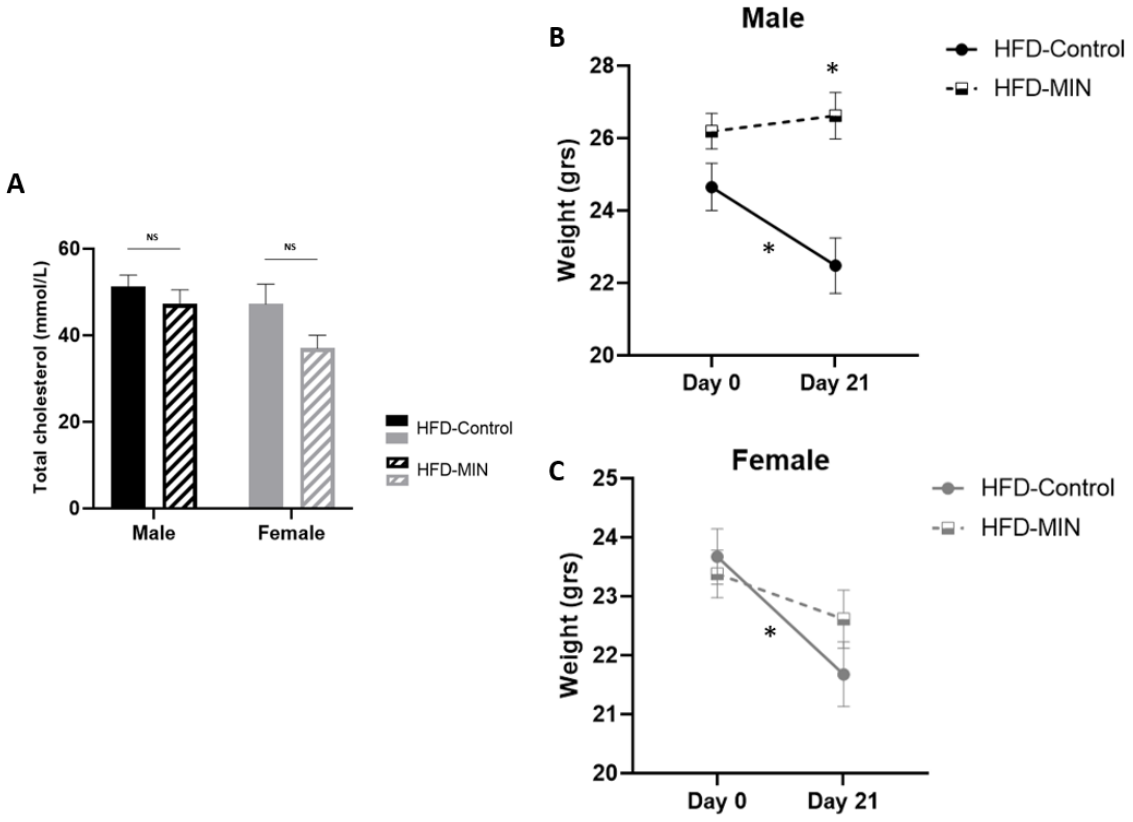

Figure 5. Effect of Minocycline on total plasma cholesterol levels and body weight in diet-fed female and male SR-B1$^{-/-}$ApoER61$^{h/h}$ mice. To total plasma cholesterol levels, Male: HFD-Control (n= 8), HFD-MIN (n= 8) and Female: HFD-Control (n= 12), HFD-MIN (n= 18), and Body weight, Male: HFD-Control (n= 15), HFD-MIN (n= 15) and Female: HFD-Control (n= 15), HFD-MIN (n= 15), after 21 days of feeding HFD and minocycline administration. *p<0.05. NS: "non significant".

4. Discussion

This study was the first to perform a survival study and compare sex-dependent differences on systemic inflammation and monocyte population and its response to anti-inflammatory minocycline treatment in male and female mice using a mouse model of lethal coronary heart disease that recapitulates the initiation and progression of atherosclerosis as well as an its fatal ischemic complications and death of the disease (31). Using the SR-B1$^{-/-}$ApoER61$^{h/h}$ strain, our results showed: 1) reduced survival of HFD-fed mice due to atherosclerotic cardiovascular diseases, with females having slightly better survival than males; 2) a trend to higher plasma IL-6 levels in male versus females at after feeding HFD, but no sex-dependent differences were found in plasma IL-10 levels, total blood monocytes, and monocyte sub-populations; 3) minocycline improved survival both in males and females, even when serum cholesterol levels were not affected by this treatment; 4) minocycline had a greater impact on reducing systemic inflammation in males by decreasing plasma IL-6 levels and shifting circulating monocyte subsets towards a more "reparative" phenotype, without affecting total blood monocytes; and 5) male and female mice fed a high-fat diet clustered in different groups based on integrative blood inflammatory biomarker analysis, but after minocycline intervention, they were indistinguishable, indicating that minocycline treatment attenuated the inflammatory status in males, mimicking that found in female mice.

We confirmed HFD-induced early death in male SR-B1$^{-/-}$ApoER61$^{h/h}$ mice, as previously reported (31). For the first time, we also observed a similar effect in female mice, but with slightly better survival rates, which aligns with modern epidemiological data showing that younger women have lower rates of cardiovascular disease and myocardial infarction compared to men (33). Notably, HFD-induced monocytosis has been found to correlate with acute MI in clinical studies (34). Furthermore, Ly6C^{high} monocyte subset is increased without changes in the Ly6C^{low} population in ApoE$^{-/-}$ mice (35). This suggests that circulating Ly6C^{high} monocytes play a critical role in chronic inflammation.

Although we found no sex-dependent differences in inflammatory biomarkers, male SR-B1$^{-/-}$ApoER61$^{h/h}$ mice tended to have higher plasma IL-6 levels after feeding HFD, which could exacerbate disease progression. IL-6 is produced in response to multiple stimuli, promoting inflammatory processes (36). Experimental studies have demonstrated the diverse roles of this central signaling cytokine in upregulating cell adhesion molecules (37), altering vascular permeability and endothelial barrier function (38), and promoting fatty lesion development in ApoE$^{-/-}$ mice (39). In humans, serum IL-6 levels have been strongly associated with atherothrombosis and future vascular events, independent of traditional risk factors (40).

Menopause is linked to an inflammatory state due to elevated levels of proinflammatory cytokines, including IL-6 (41-43). Female hormones, especially estrogens, seem to have cardioprotective effects, even though their impact on inflammation is still debated (44). Estrogen has been shown to have pleiotropic effects, acting as an anti-inflammatory agent and enhancing relaxation in the vasculature through various mechanisms (7). Therefore, we speculate that increased plasma IL-6 levels in male mice could contribute to increased systemic inflammation, which would favor the progression and complications of the disease, finally contributing to cardiac infarction and death. Also, estrogens may have a natural anti-inflammatory effect, inhibiting vascular inflammation and

partially explaining better survival in HFD-fed SR-B1$^{-/-}$ApoER61$^{h/h}$ females. This is in agreement with our PCA analysis, which suggests that both sexes have distinct inflammatory phenotypes under atherogenic conditions that may influence disease development.

Minocycline significantly improved survival in both male and female SR-B1$^{-/-}$ApoER61$^{h/h}$ mice without affecting total cholesterol levels. This is consistent with results seen in other animal models of atherosclerosis under anti-inflammatory treatments (45). Atherosclerosis is a chronic inflammatory disease, and inflammation plays a crucial role in its development through various pathophysiological mechanisms (46), involving the activation of endothelial cells (47), recruitment of monocytes and leukocytes to the atheroma (48), formation of foam cells (49), secretion of inflammatory cytokines and other mediators (48), degradation of extracellular matrix, and erosion/rupture of a weakened fibrous cap, ultimately leading to acute ischemic complications (50).

Although both sexes showed improved survival, minocycline had a greater anti-inflammatory effect in males, reducing plasma IL-6 levels and promoting a "more reparative" monocyte subset. These results are consistent with our prior studies demonstrating minocycline's systemic anti-inflammatory effects as well as within atheromatous plaques (29). In ApoE$^{-/-}$ mice with remote atheroma, minocycline decreased circulating and lesion monocyte content, shifted monocyte polarization, and reduced serum IL-6 levels (29). Our findings suggest that minocycline requires a higher threshold of systemic inflammation in order to generate an anti-inflammatory effect. In the case of HFD-fed SR-B1$^{-/-}$ApoER61$^{h/h}$ males, they responded to higher IL-6 levels compared to those detected in females. The observed reduction in IL-6 levels is consistent with previous studies showing that minocycline treatment suppresses IL-6 expression in ovarian cancer cells (51) and acts as a neuroprotective agent in the central nervous system (52). Additional studies are required to establish whether baseline and post-minocycline treatment led to changes in size and/or quality in atheromatous lesions.

We hypothesize that the mechanism underlying the improved survival in females following minocycline treatment differs from that of males, possibly due to the interaction between anti-inflammatory effect of minocycline and estrogens. Regardless of the mechanism, our PCA analysis indicated that minocycline modulated inflammatory conditions in HFD-fed SR-B1$^{-/-}$ApoER61$^{h/h}$ males resembling and female status make them indistinguishable groups. Future studies are needed to elucidate the molecular and cellular pathways by which minocycline decreases IL-6 expression and modulates monocyte polarization and their differential performance in both male and female mice.

5. Perspectives and signifcance

In summary, we found that HDF-fed young male and fertile female mice with SR-B1$^{-/-}$ApoER61$^{h/h}$ have distinct basal inflammatory characteristics, influencing atherosclerotic progression, development, and outcomes. Furthermore, treatment with minocycline produced a differential anti-inflammatory effect, primarily in males, by reducing IL-6 levels and modulating monocyte polarization. This led to an attenuated systemic inflammatory state resembling that of females, resulting in improved survival. The mechanism underlying improved survival in females may be linked to estrogen differing from that observed in males. Further studies are needed to elucidate the mechanisms through which minocycline acts on female mice and its possible interaction with

estrogens. These results contribute to the understanding of the mechanisms driving sex as a biological variable in atherosclerotic disease and may shed light to future medical strategies and how to guide drug therapy in patients. For instance, it may not be necessary to observe high inflammatory parameters in women before initiation of anti-inflammatory treatment to improve survival if other risk factors are present. Further studies are needed to elucidate the mechanisms through which minocycline acts on female mice and its possible interaction with estrogen, determine whether baseline and post anti-inflammatory treatment differences are observed at the atherosclerotic plaque level and also address mechanisms underlying age as a biological variable in atherosclerosis in an "old" male and female animal model.

Declarations

Ethics approval and consent to participate

All procedures were performed following recommendations from the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Ethics and Animal Welfare Committee from Pontificia Universidad Católica de Chile.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

All authors declare no competing interests.

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Authors' contributions

LP, LG and MA contributed to the conception and study design. LP and LG were responsible for the management, supervision, experimental procedures and sampling of the animals. LP and MA performed the acquisition, analysis and interpretation of data. LP drafted the manuscript. LP, LG, MA, AR and SU critically revised the manuscript. All authors read and approved the final manuscript.

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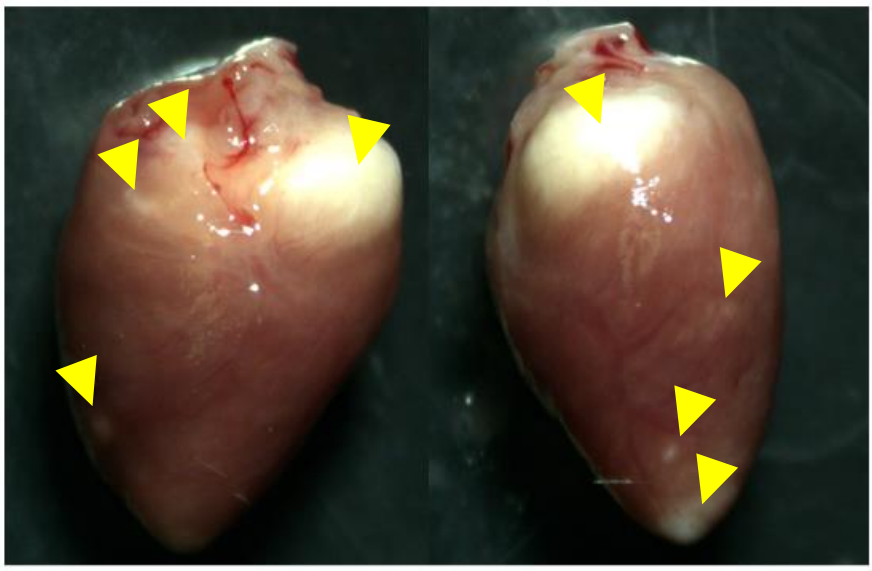
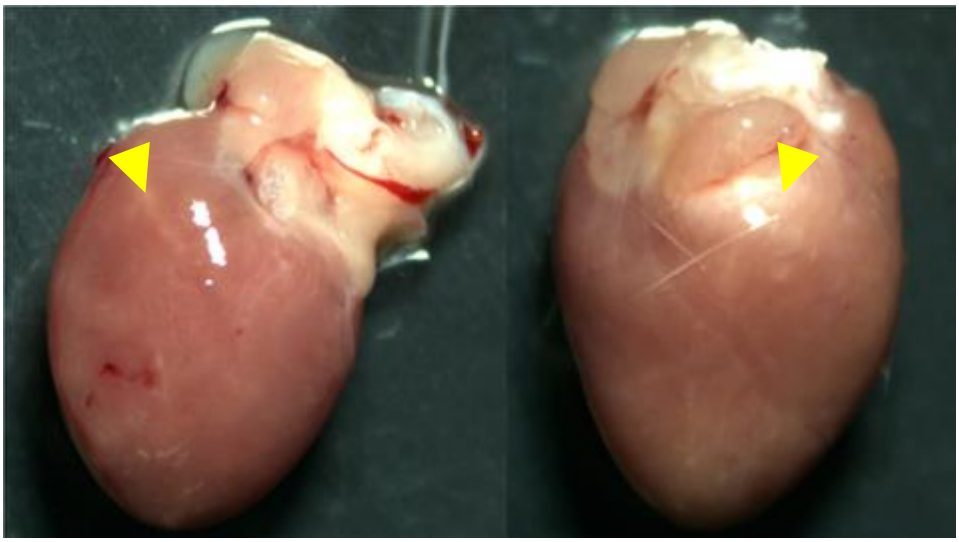
Supplementary Material



Supplementary figure 1. Representative image of infarcted hearts of SR-B1$^{-/-}$ApoER61$^{h/h}$ HFD-fed mice. Yellow arrows indicate infarcted areas.



Supplementary figure 2. Representative image of infarcted hearts of SR-B1$^{-/-}$ApoER61$^{h/h}$ HFD-fed mice treated with minocycline. Yellow arrows indicate infarcted areas.