Prevention and reversal of hypertension-induced coronary microvascular dysfunction by a plant-based diet

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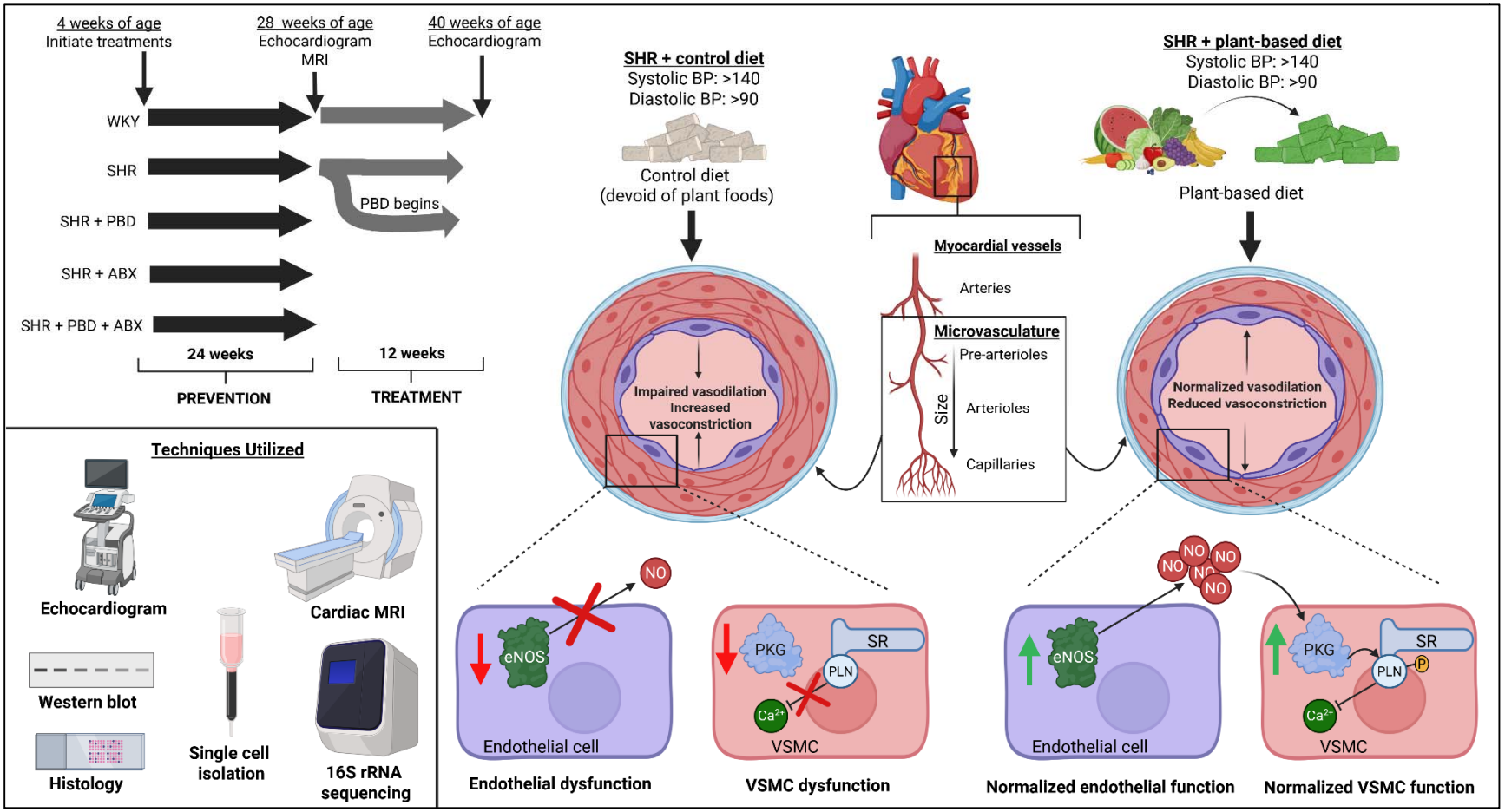
Abstract

Background and aims: Coronary microvascular dysfunction (CMD) is associated with adverse cardiovascular outcomes. CMD is driven by endothelial and vascular smooth muscle cell (VSMC) dysfunction. We aimed to test whether CMD could be mitigated by a plant-based diet (PBD) in an animal model of hypertension.

Methods: We compared 28- and 40-week-old female normotensive Wistar-Kyoto and spontaneously hypertensive (SHR) rats, maintained, from age 4 weeks, on a control refined diet or a PBD, comprised of 28% fruits, vegetables, nuts and legumes. A subset of control SHRs were switched to the PBD at 28 weeks. CMD was assessed by coronary flow reserve via echocardiogram. Cardiac microvascular endothelial function was assessed via cMRI. Endothelial and VSMC function were assessed in the left ventricle (LV) or in isolated VSMCs. The role of gut microbiota was probed via 16S sequencing and antibiotics. Cardiac inflammation, oxidative stress, and fibrosis were also explored.

Results: SHRs had endothelial and VSMC dysfunction. PBD did not ameliorate their hypertension but, nonetheless, prevented and reversed CMD. PBD's mitigation of CMD was associated with improved endothelial nitric oxide synthase function and NO-mediated VSMC signaling, as well as reductions in LV oxidative stress, inflammatory signaling, and fibrosis. PBD altered the gut microbiota, although antibiotic studies failed to establish its importance in ameliorating CMD.

Conclusions: A PBD prevented CMD development and reversed established CMD in SHRs. Such benefits of PBD, which occurred without alleviating hypertension, were likely due to improved endothelial and VSMC function. These results support clinical trials to test PBDs in human CMD.



Graphical Abstract. A plant-based diet prevented and reversed CMD without attenuating hypertension. Such amelioration of CMD was not negated by antibiotics and correlated with improved endothelial and VSMC function. Legend: ABX, antibiotics; BP, blood pressure; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PBD, plant-based diet; PKG, protein kinase G; PLN, phospholamban; SHR, spontaneously hypertensive rat; SR, sarcoplasmic reticulum; VSMC, vascular smooth muscle cell.

1. Introduction

Chest pain (angina pectoris) affects ~112 million people globally and is classically associated with ischemic heart disease due to obstructive atherosclerosis[1]. Interestingly, up to two-thirds of women and one-third of men with angina who undergo coronary angiography are found to lack evidence of a blockage[2]. This condition is termed ischemia with non-obstructive coronary artery disease (INOCA)[3], and is linked to increased risk of major adverse cardiovascular events, including myocardial infarction and heart failure[3]. Women are also more likely to have angina[4], and are four times more likely than men to be re-hospitalized within six months following discharge[3]. An underlying pathological driver of INOCA is coronary microvascular dysfunction (CMD), an impairment of the cardiac microvessels, which are responsible for 80% of total vascular resistance[5]. CMD is diagnosed by a reduction in coronary flow reserve (CFR), the ratio of coronary blood flow at rest versus maximal blood flow. A major risk factor for CMD is hypertension, which is associated with reduced CFR[6]. CMD is associated with dysfunction of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs)[7]. EC dysfunction largely results from reduced nitric oxide (NO) bioavailability due to downregulated expression of endothelial nitric oxide synthase (eNOS), which produces NO, and/or increased production of superoxide ($O_2^{\cdot-}$), which reduces NO bioavailability via generation of peroxynitrite ($ONOO^-$). VSMC dysfunction includes impaired vasodilation due to improper calcium handling via sarcoplasmic reticulum (SR) stress, and reactive oxygen species (ROS)-mediated inhibition of guanylyl cyclase, limiting protein kinase G activation (PKG)[8].

CMD is treated by controlling cardiac risk factors, in combination with the use of anti-atherosclerotic, anti-anginal medications such as statins, angiotensin converting enzyme inhibiters, beta-blockers, calcium channel antagonists, and ranolazine for symptom management. However, CMD therapeutic strategies are not clearly defined, with patients having persistent symptoms and poor response to usual therapies in a subset[9 5]. Furthermore, this multi-pharmacological approach is burdensome to patients and associated with a variety of adverse effects/intolerances and compliance issues. Thus, new approaches are urgently needed.

Consumption of diets rich in fruits and vegetables has long been known to be health-promoting, especially with respect to CVD prevention. Indeed, plant-based diets (PBDs) are associated with a reduced risk of ischemic heart disease[10,11]. This association appears unique to “healthy” PBDs[11,12], characterized by increased intake of unprocessed plant-foods, such as whole fruits, vegetables, nuts, seeds, legumes, and whole grains, compared with “unhealthy” PBDs comprising processed foods (e.g., refined grains and added sugars)[12]. Some of the benefits PBDs stem from components not found on standard nutritional labels, including phytochemicals such as polyphenols, secondary metabolites of plants, which have beneficial molecular effects in attenuating CVD[13]. Once consumed, these phytochemicals undergo gut microbiota metabolism[14], and these metabolites can exert their effects on target tissue. Indeed, in interventions

that maximize the intake of unrefined plant-foods, reversal of atherosclerosis can be observed as well as improved myocardial perfusion in patients with coronary artery disease (CAD)[15-17]. Thus, both the American Heart Association (AHA) and Academy of Nutrition and Dietetics (AND) recommend the consumption of a mostly PBD to reduce the risk of cardiovascular events[18,19].

The extent to which PBD might mitigate CMD is largely unknown. Indeed, only a single published study to date used a dietary approach to attenuate CMD, namely calorie restriction, with poor efficacy[20]. Thus, we sought to examine the potential of a plant-based diet (PBD) in CMD. We developed approaches to measure CMD, which was indeed present in SHRs, and found a PBD prevented and reversed this disorder.

Methods

2.1 Study Design

All animal use and procedures were approved by Georgia State University's Institutional Animal Care and Use Committee (protocol #: A23025). Female animals were used to align with the fact that CMD afflicts women more severely than men[21-23]. Three-week-old, female, normotensive Wistar-Kyoto (WKY) rats and SHRs were purchased from Inotiv (West Lafayette, IN, USA) within the same respective in-bred colonies. Upon arrival, rats were doubly housed in an environmentally controlled animal care facility and maintained on 12-h light/dark cycles. Rats had free access to water and were maintained on a compositionally-defined open-source diet (Table S1 and Table S2; D23061303, Research Diets). After one week, SHRs either 1) continued the diet, 2) were placed on antibiotic (ABX) treatment to deplete the gut microbiota, 3) began the consumption of a PBD (Table S1 and Table S2; D23061304, Research Diets), or 4) consumed a PBD along with ABX treatment. WKY continued the control diet and did not consume ABX or PBD. To assess the potential of a PBD to treat CMD, a subset of SHRs on the control diet were switched to a PBD at 24 weeks of age and monitored for 12 additional weeks. A subset of WKY and SHR also continued the control diet for 12 weeks. Figure 1 outlines the animal study design.

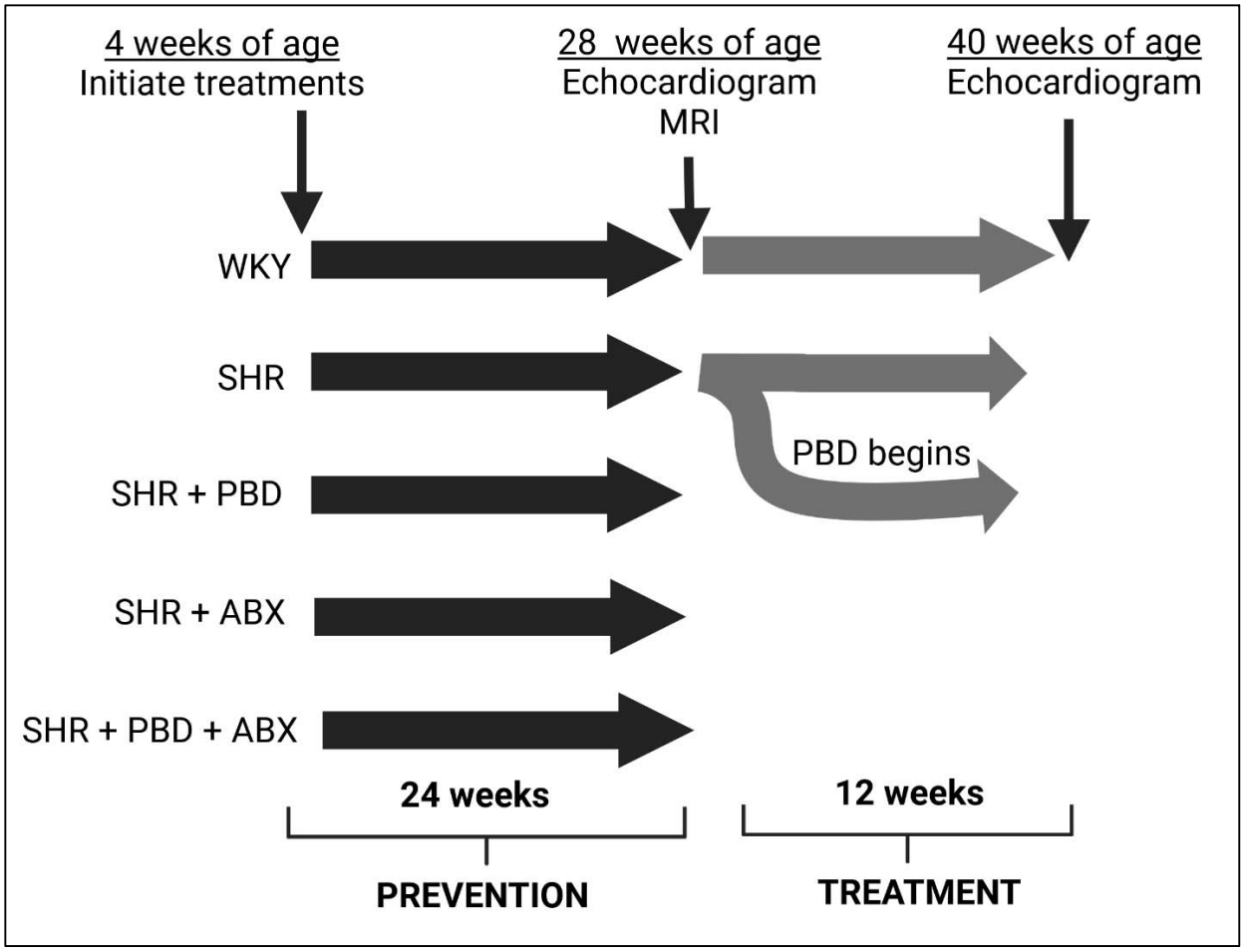


Figure 1. Animal study design.

The PBD comprised 28% (w/w) seven different plant foods: walnuts, black beans, red bell pepper, sweet potato, blueberries, Brussels sprouts, and lemon (4% each). These foods were selected for their high polyphenol content within their respective food categories[24] (citrus, cruciferous vegetables, legumes, etc.) and their common consumption in the United States. Soy protein was used in place of casein in the PBD. The control diet was matched to the PBD in both macronutrient and micronutrient composition, including in both soluble (inulin) and insoluble (cellulose) fiber (Table S1 and Table S2). Thus, the main known difference between the PBD and control diet was phytochemical content. The control diet did not contain any plant foods.

Regarding ABX treatments, the first 3 days of antibiotic treatment comprised of 1 g/L of ampicillin, neomycin sulphate, metronidazole, and 0.5 g/L of vancomycin in drinking water followed by a ⅓ concentration maintenance dose for the remainder of the study[25]. Sucrose was added (0.5%) to increase palatability. This ABX water was changed weekly. Preliminary testing revealed that after 4 weeks of the maintenance ABX dose, 10^{-5} fewer bacteria were in feces (~4,602,842 bacteria/mg vs ~57 bacteria/mg); thus, the ABX successfully reduced gut microbes.

The remaining methods for all procedures can be found in the Supplementary Materials. Sample sizes for measures are indicated in figure legends.

Results

PBD did not ameliorate hypertension or impact cardiac function in SHRs

Tracking of body weight indicated that, in accord with other studies[26], SHRs exhibited moderately reduced weight gain relative to age-matched WKYs (Figure 2A & B). This difference was accompanied by SHRs exhibiting a slight reduction in cumulative food intake during this period (Figure 1C & D). PBD and/or ABX did not significantly impact weight at 28 weeks, when heart function was to be assessed.

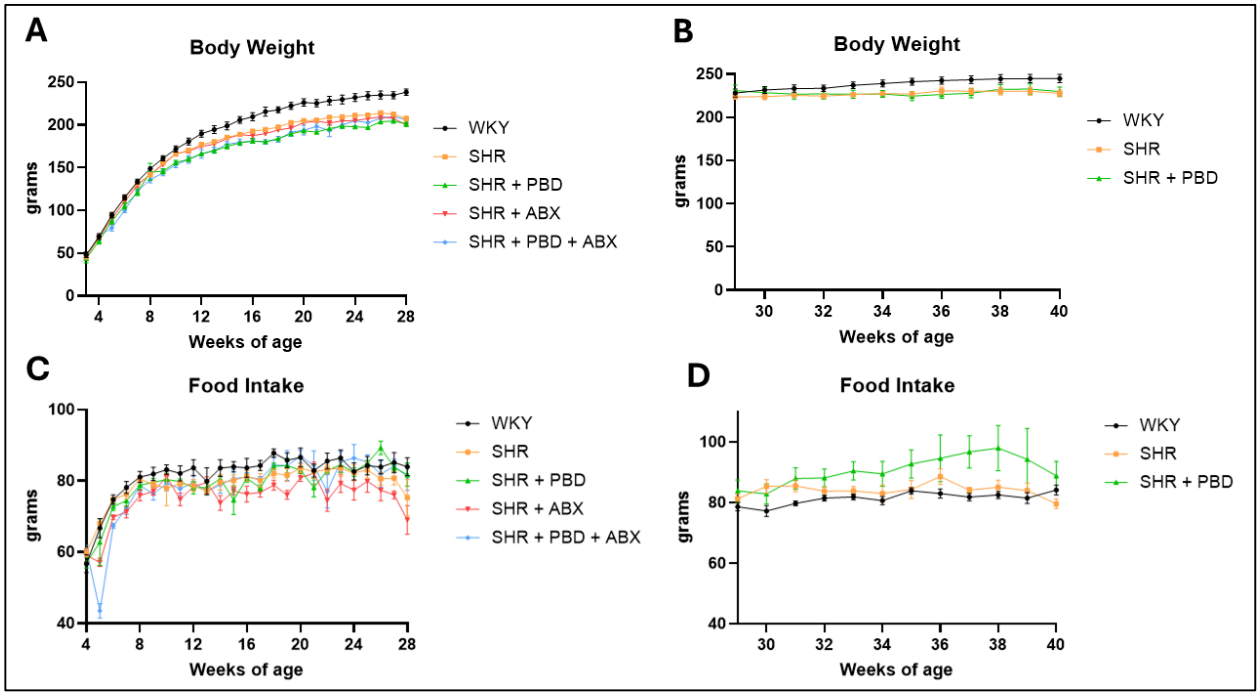


Figure 2. Body weight and food intake. Wistar-Kyoto (WKY) rats or spontaneously hypertensive rats (SHR) consumed a purified diet or 28% (w/w) plant-based diet (PBD) for 24 weeks with or without antibiotics (ABX). After 24 weeks, a subset of SHRs were switched from the control diet to PBD, while a subset of SHRs and WKY continued the control diet for 12 additional weeks. (A, B) Body weight and (C, D) food intake were measured weekly, and the average of both animals per cage was used (n = 8–14/group). Data are expressed as mean ± SD.

The assessment of microbiota composition via 16S rRNA sequencing indicated that, as expected, microbiomes of SHRs randomized to receive the control or PBD were similar at baseline (Figure 3A). However, microbiomes of rats who were fed the control and PBD clearly differed from each other at all timepoints assessed following diet initiation (Figure 3A & B; Figure S1). Despite this, these changes did not significantly impact alpha diversity between groups (Figure S2). A similar pattern of PBD-induced changes

in the microbiome was observed for animals that began consuming PBD at 24 weeks of age (Figure 3B). PBD-induced changes in the microbiome included changes in several genera whose abundance was significantly (adjusted p-value<0.01) enriched (blue) or depleted (orange) following PBD invitation at weeks 24 or 36 (Figure 3C & D).

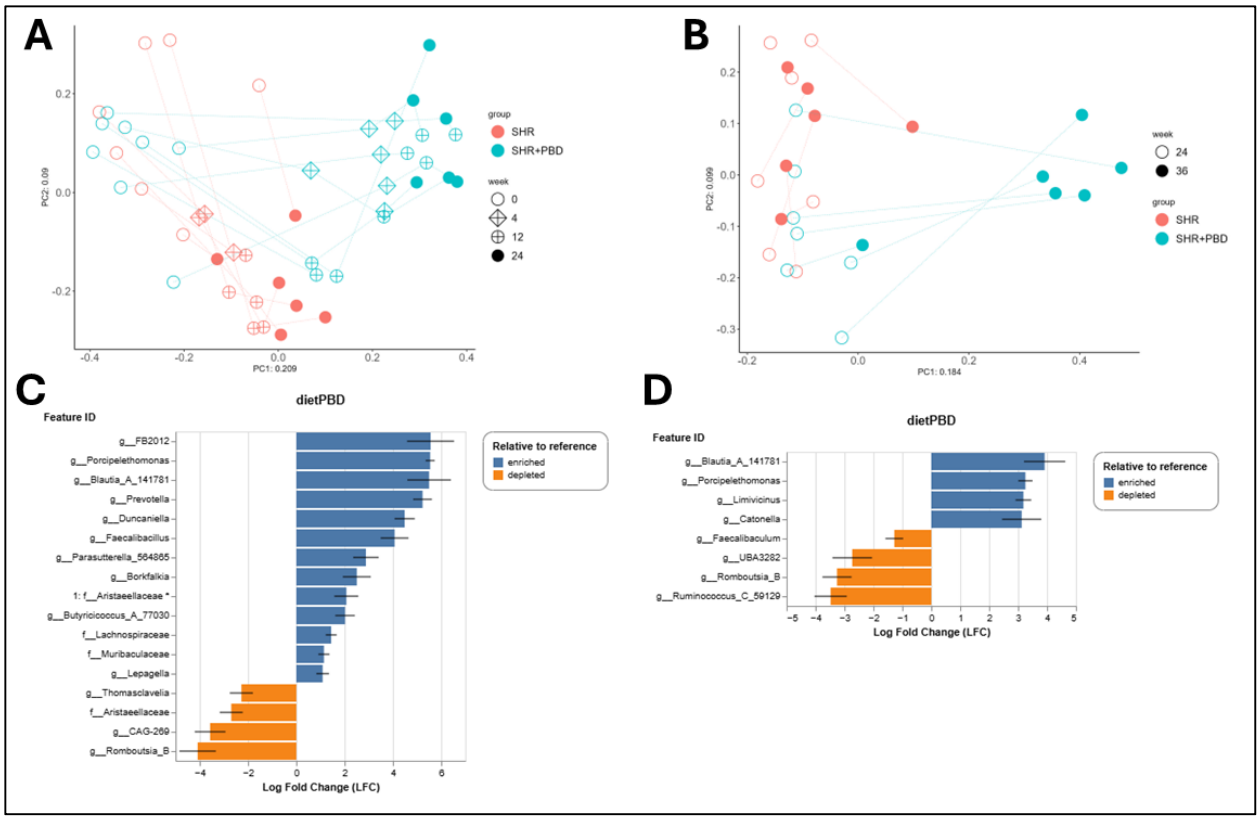


Figure 3. Gut microbiota beta diversity and bacterial taxa of differential abundance in SHRs consuming a control diet or PBD. Spontaneously hypertensive rats (SHR) consumed a purified diet or 28% (w/w) plant-based diet (PBD). After 24 weeks, a subset of SHRs were switched from the control diet to PBD, while a subset of SHRs continued the control diet for 12 additional weeks. Feces were collected at weeks 0, 4, 12, 24, and 36 for 16S rRNA sequencing. (A, B) PCoA plot of Bray-Curtis distance and (C, D) enrichment or depletion of bacterial genus after PBD diet is illustrated. Panels A and C reflect weeks 0-24 during the prevention phase, while panels B and D reflect weeks 24-36 during the treatment phase.

Blood pressure measurements revealed that, as expected, WKY exhibited normal blood pressure while SHRs had significantly increased systolic and diastolic blood pressure. Or: Neither PBD nor antibiotics ameliorated hypertension (Figure 4).

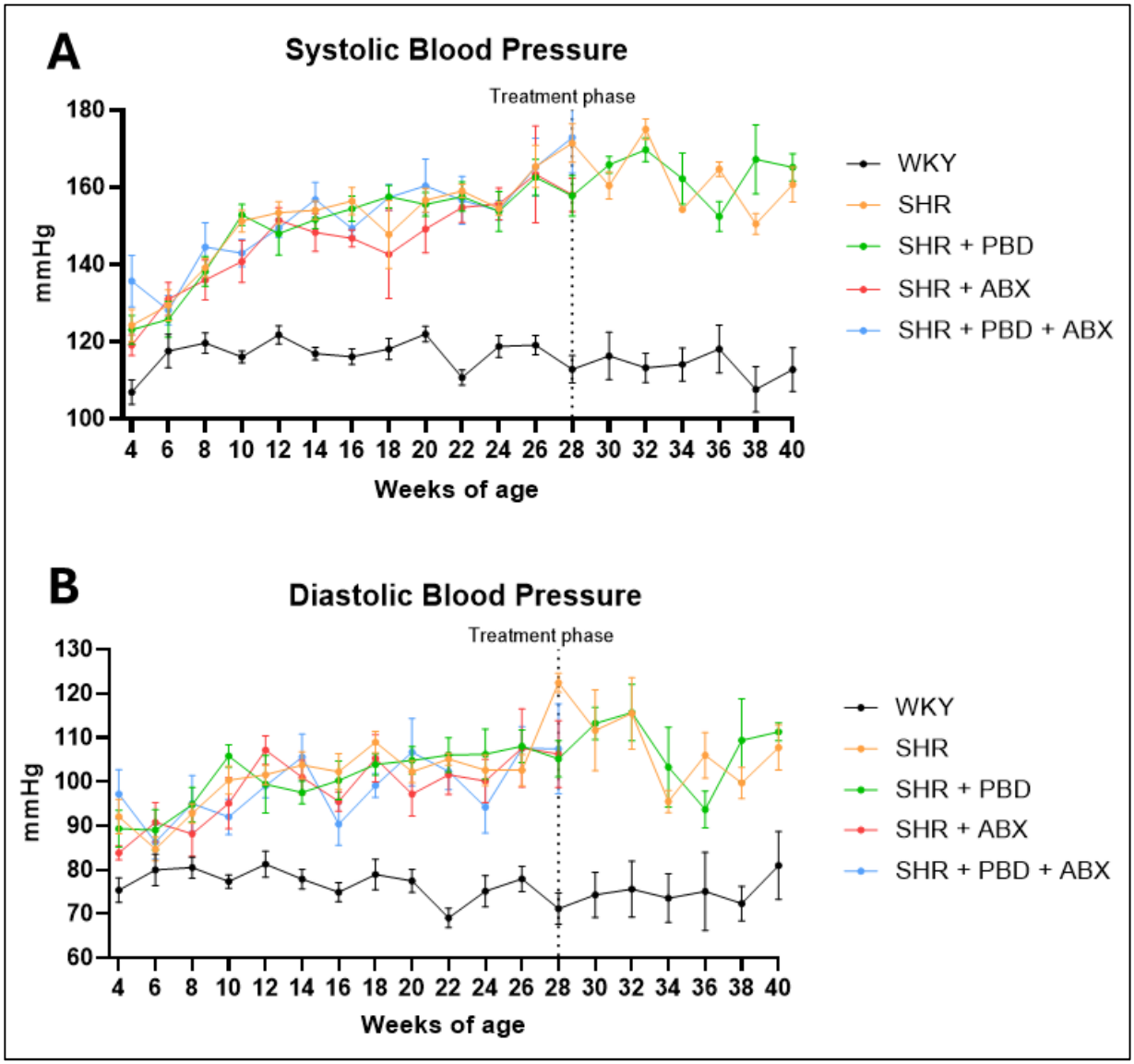


Figure 4. Blood pressure over time. Wistar-Kyoto (WKY) rats or spontaneously hypertensive rats (SHR) consumed a purified diet or 28% (w/w) plant-based diet (PBD) for 24 weeks with or without antibiotics (ABX). After 24 weeks, a subset of SHRs were switched from the control diet to PBD, while a subset of SHRs and WKY continued the control diet for 12 additional weeks. (A) Systolic blood pressure and (B) diastolic blood pressure was measured every two weeks with tail plethysmography (*n* = 5–14/group. Data are expressed as mean ± SEM.

A plant-based diet prevented CMD development and reversed established CMD

Cardiac function of our cohort of 28-week-old female SHRs was assessed by measuring ejection fraction (EF) via echocardiography (Figure 5A, B). While EF values of female SHRs did not fall within the dysfunctional range, a result expected for their age[27], they were nevertheless significantly reduced compared to age-matched WKYs. Consistent

with the absence of marked cardiac dysfunction, there was also no significant difference in LV mass normalized to body weight among WKYs and SHRs (Figure 5A, C). Nonetheless, at week 24, both LV internal diameter during systole (LVIDs) and during diastole (LVIDd) was significantly increased in SHRs compared to WKY (Figure 5A, D, E), indicating LV dilation. None of these measures in SHRs were impacted by PBD, ABX, or the combination thereof, nor did PBD impact these parameters during the treatment phase of the study (Figure 6).

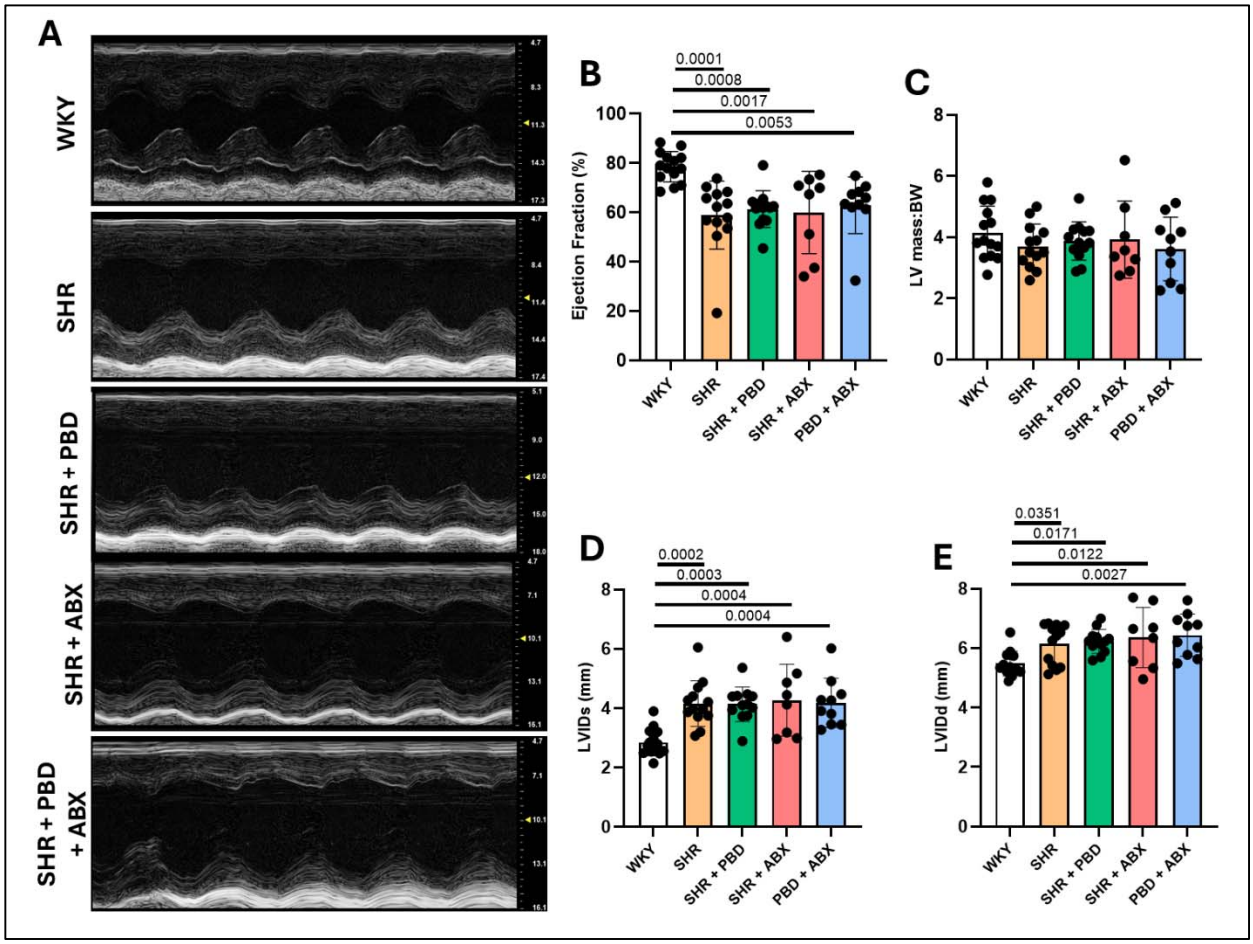


Figure 5. Cardiac function and morphological characteristics during the prevention phase (week 24). Wistar-Kyoto (WKY) rats or spontaneously hypertensive rats (SHR) consumed a purified diet or 28% (w/w) plant-based diet (PBD) for 24 weeks with or without antibiotics (ABX). (A) Cardiac function was assessed by (B) ejection fraction (EF) with echocardiogram. (C) Left ventricle (LV) morphology was assessed with LV mass normalized to body weight (BW), as well as (D) LV internal diameter during systole (LVIDs) and (E) diastole (LVIDd). Data were statistically assessed with one-way ANOVA and Dunnett's post-hoc test (n = 8–14/group). Significance ($P \leq 0.05$) is denoted in the graphs with exact *P*-value. Data are expressed as mean ± SD.

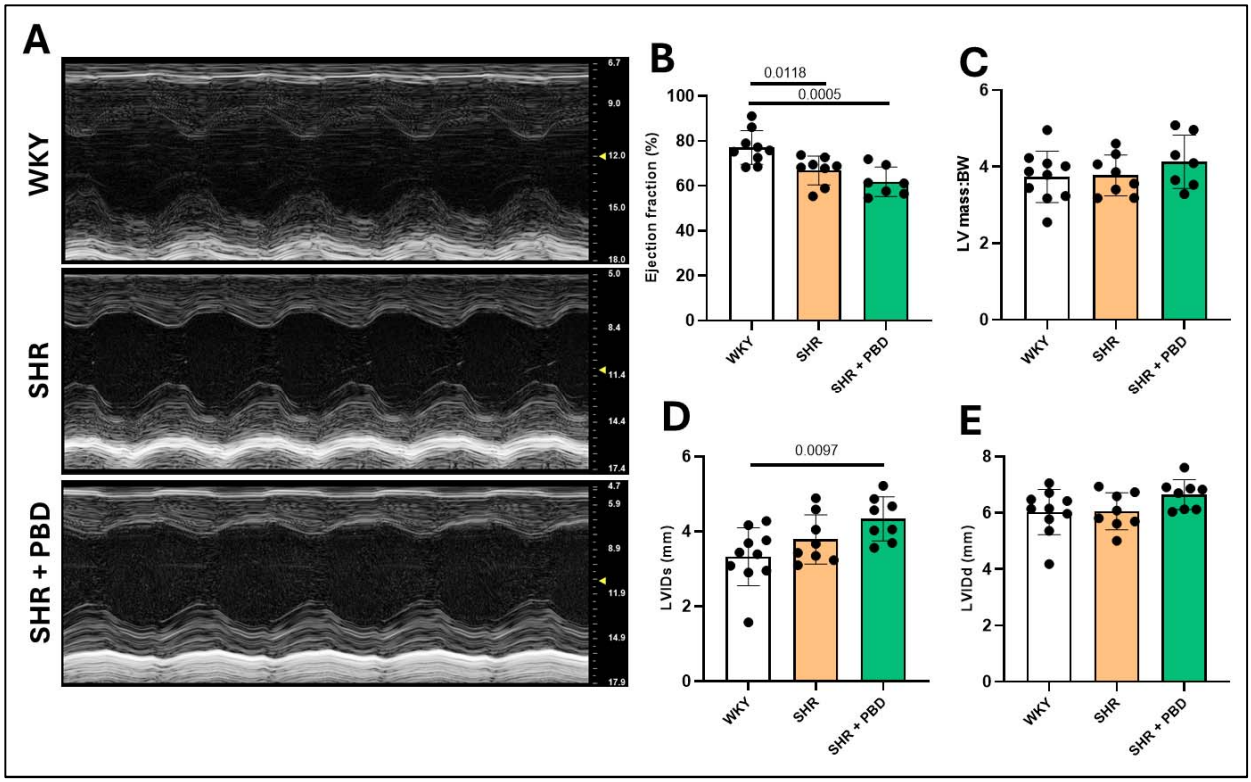


Figure 6. Cardiac function and morphological characteristics during the treatment phase (week 36). Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) consumed a purified diet for 24 weeks. After 24 weeks, a subset of SHRs were switched from the control diet to PBD, while a subset of SHRs and WKY continued the control diet for 12 additional weeks. (A) Cardiac function was assessed by (B) ejection fraction (EF) with echocardiogram. (C) Left ventricle (LV) morphology was assessed with LV mass normalized to body weight (BW), as well as (D) LV internal diameter during systole (LVIDs) and (E) diastole (LVIDd). Data were statistically assessed with one-way ANOVA and Dunnett's post-hoc test (n = 7–9/group). Significance ($P \leq 0.05$) is denoted in the graphs with exact *P*-value. Data are expressed as mean ± SD.

The hallmark of CMD is a reduction in CFR, which we measured by echocardiography. While a normal range of CFR in rats has not been well established, CFR levels were indeed lower in SHRs than WKYs (CFR of WKYs and SHRs was 2.25 ± 0.38 vs. 1.70 ± 0.39, respectively p-0.01) supporting our presumption that SHRs indeed develop CMD (Figure 7A, B). Most importantly, PBD significantly increased CFR, restoring it to the levels of WKYs (CFR of PBD-fed SHRs was 2.32 ± 0.54, p=0.01 for SHRs fed control vs PBD diet) supporting our hypothesis that PBD could prevent CMD. The extent to which gut microbiota contributed to the benefits of PBD was less clear. Specifically, CFR of control SHRs with ABX did not differ significantly from WKY or control SHRs without ABX. Thus, determining the role of the gut microbiota in this model will require future studies.

Echocardiograms performed on the second cohort of SHRs in the treatment study showed low and similar levels of CFR, at 24 weeks, in groups of animals that were to remain on the control diet or be switched to PBD. In accordance with the notion that hypertension-induced CMD continues to worsen with time, mean CFR levels declined between 24 and 36 weeks in SHRs fed the control diet in 5 of 8 SHRs assessed (Figure 8A). In contrast, CFR levels increased over this period in PBD-fed SHRs resulting in significantly higher CFR level in PBD-fed SHRs compared to SHRs fed the control diet (Figure 8B, C). The increase in CFR following PBD consumption resulted in CFR levels that were similar to WKYs, indicating that PBD had reversed CMD (Figure 8D, E).

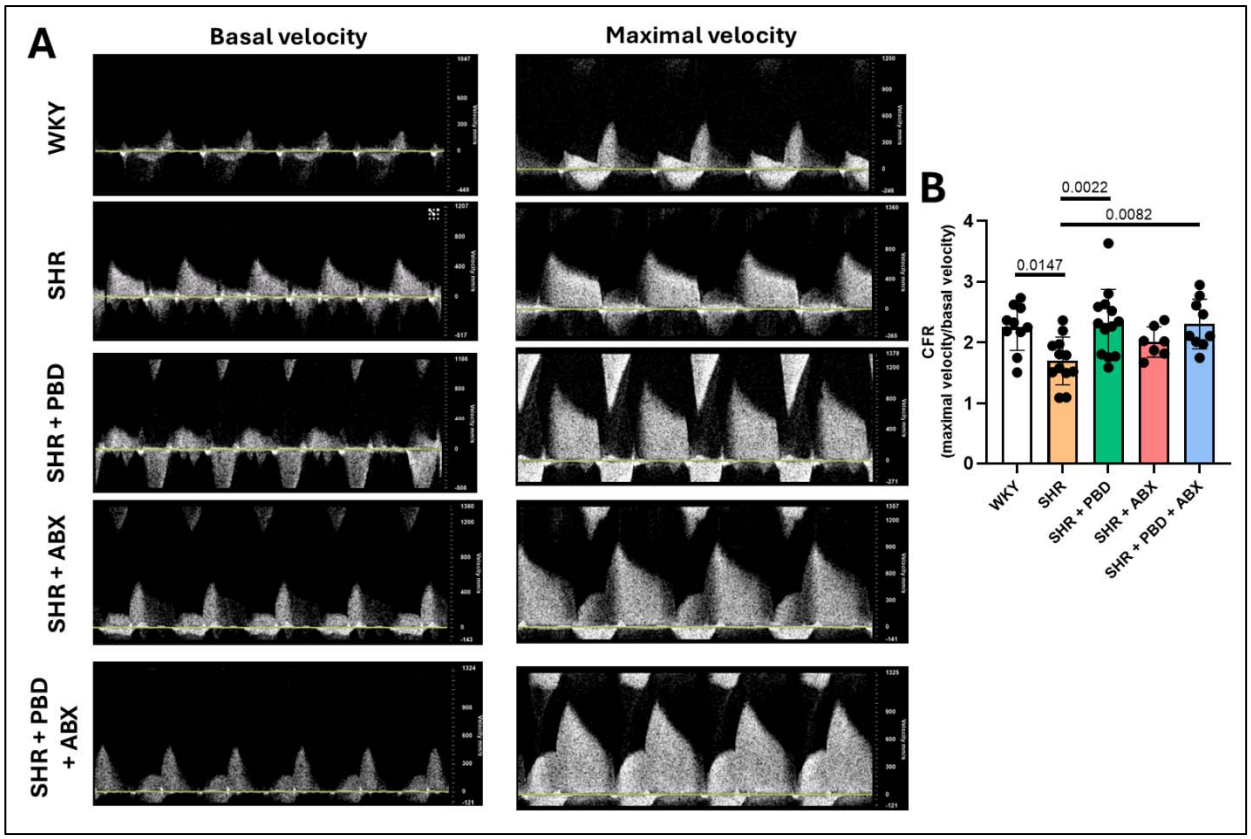


Figure 7. Coronary microvascular function during the prevention phase (week 24). Wistar-Kyoto (WKY) rats or spontaneously hypertensive rats (SHR) consumed a purified diet or 28% (w/w) plant-based diet (PBD) for 24 weeks with or without antibiotics (ABX). Coronary flow reserve (CFR) was calculated as maximal velocity prior to adenosine infusion ÷ basal velocity during adenosine infusion (0.25 mg/kg/min). Data were analyzed with one-way ANOVA and Dunnett's post-hoc test (n = 7–13/group). Significance ($P \leq 0.05$) is denoted in the graphs with exact *P*-value. Data are expressed as mean ± SD.

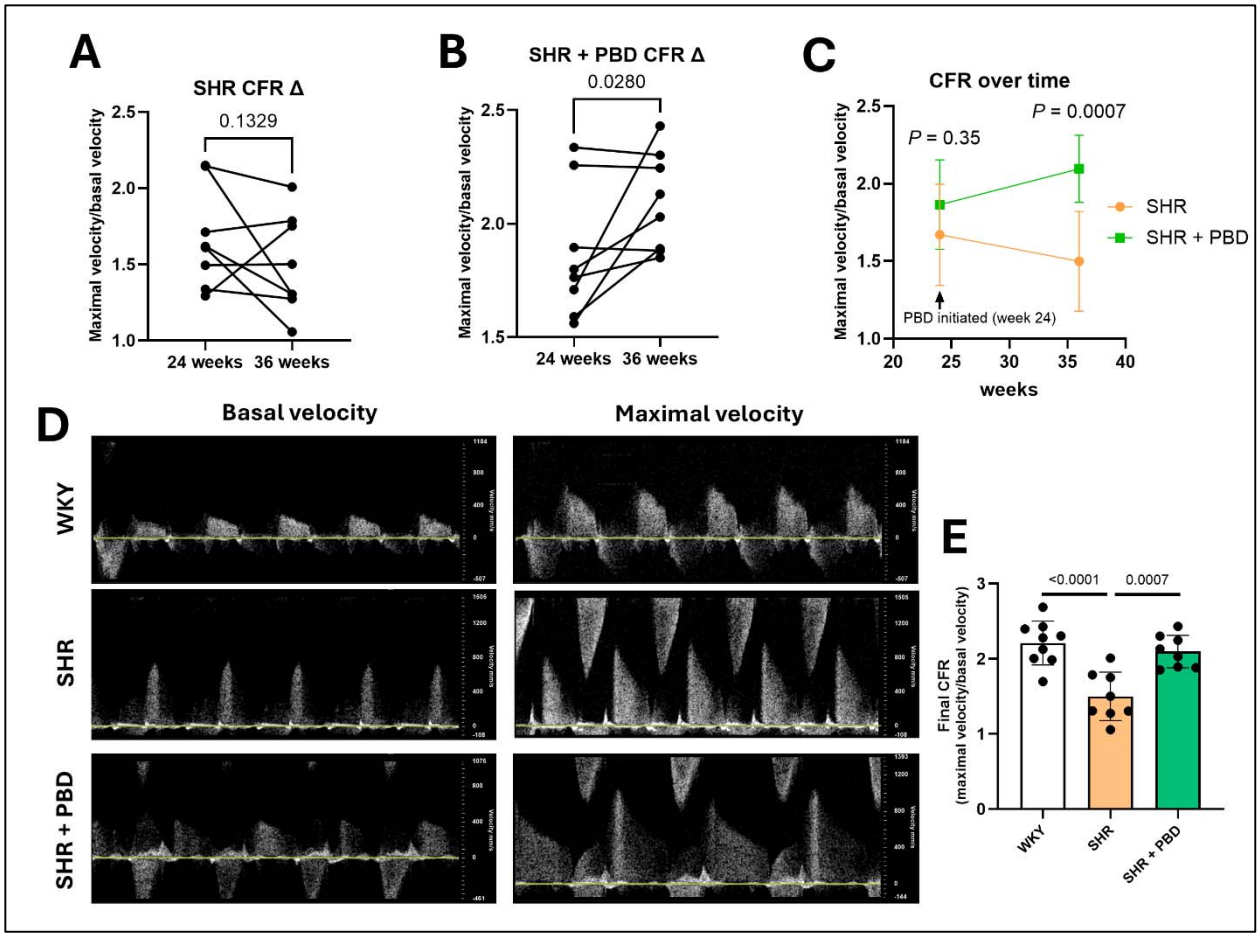


Figure 8. Coronary microvascular function during the treatment phase (week 36). Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) consumed a purified diet for 24 weeks. After 24 weeks, a subset of SHRs were switched from the control diet to PBD, while a subset of SHRs and WKY continued the control diet for 12 additional weeks. Coronary flow reserve (CFR) was calculated as maximal velocity prior to adenosine infusion ÷ basal velocity during adenosine infusion (0.25 mg/kg/min). Data was statistically assessed with (A, B) Student's paired t-test, (C) two-way ANOVA with Šídák's multiple comparisons test, or (E), one-way ANOVA with Dunnett's post-hoc test (n = 8–9/group). Significance ($P \leq 0.05$) is denoted in the graphs with exact *P*-value. Data are expressed as mean ± SD.

A plant-based diet treats CMD in an endothelial-dependent and -independent manner.

To investigate the cellular mechanisms by which PBD consumption prevented CMD, cardiac magnetic resonance imaging (cMRI) was employed (Figure 9). We evaluated myocardial blood flow (MBF) before and after the intravenous injection of 75 mg/kg BW L-NG-Nitroarginine methyl ester (L-NAME), an eNOS inhibitor. The rationale for this approach was that if eNOS was functioning normally, then L-NAME would dramatically reduce MBF. If eNOS was impaired, the provision of L-NAME would have little impact in

reducing MBF[28]. This was assessed by observing intensity changes of the myocardium (Figure 9A). Indeed, L-NAME injection resulted in a visually appreciable reduction in myocardial blood flow by 8 min in WKYs but not SHRs (Figure 9B). Image analysis-based quantitation confirmed this, as WKYs exhibited a stark drop in MBF in response to L-NAME injection while injection of PBS did not impact this parameter, indicating the experimental approach was working as expected (Figure 9C, D). In contrast, SHRs consuming the control diet exhibited only a modest reduction in MBF in response to L-NAME, indicating endothelial dysfunction. PBD-fed SHRS exhibited L-NAME-induced reductions in MBF that were at least as robust as that of WKYs indicating that PBD had fully prevented hypertension-induced cardiac microvascular endothelial dysfunction. ABX did not alter L-NAME induced changes in MBF in control- or PBD-fed SHRs, arguing that this aspect of PBD's benefit was not microbiota-mediated. The preservation of eNOS activity in SHRs consuming PBD was associated with increased levels of NO metabolites, irrespective of ABX (Figure S3).

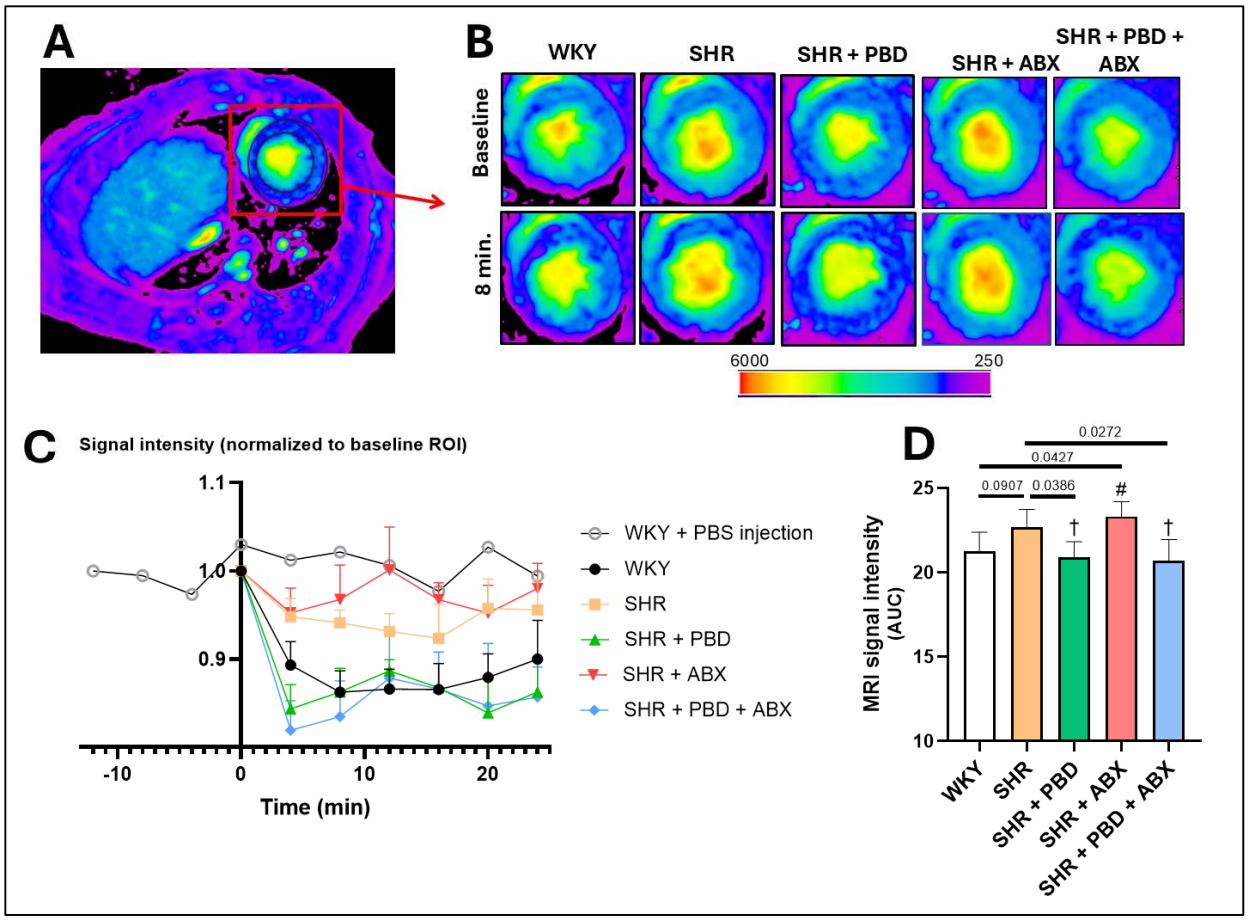


Figure 9. Coronary microvascular endothelial function via cMRI. Wistar-Kyoto (WKY) rats or spontaneously hypertensive rats (SHR) consumed a purified diet or 28% (w/w) plant-based diet (PBD) for 24 weeks with or without antibiotics (ABX). (A) The region of interest (ROI) of the myocardium used for analysis is outlined in black. A baseline MRI scan was acquired, followed by L-NAME injection, and subsequent scans

every 4 min for a total of 24 min. In addition, one WKY was scanned every 4 min for 12 min prior to PBS injection, and every 4 min for 24 min after injection. (B) Representative cardiac MRI images are provided for baseline and 8 min. (C) Signal intensity over time, (D) which was statistically quantified with AUC analysis followed by one-way ANOVA and Dunnett's post-hoc analysis (n = 4–6/group). Significance ($P \leq 0.05$) is denoted in the graphs with exact *P*-value. Data are expressed as (C) mean ± SEM or (D) mean ± SD.

eNOS is a critical mediator of endothelial function and thus might have played a role in mediating the beneficial impacts of PBD. To probe this notion, we assessed LV eNOS and phospho-eNOS (Ser^{1177}) levels via western blot in animals from the treatment study. We found that, PBD-fed SHRs exhibited elevated eNOS and phospho-eNOS relative to SHRs that consumed the control diet (Figure 10A–C). We envisaged that PBD's potentiation of eNOS might impact VSMCs. Hence, we measured ventricular VSMC expression of phospho-vasodilator-stimulated phosphoprotein (VASP), which is downstream of PKG, activated by NO signaling, in cells treated or not, with sodium nitroprusside (SNP), a compound that releases NO. Phospho-VASP expression was markedly increased following SNP treatment compared to untreated cells (Figure S4), supporting the role of SNP in NO-induced signaling. Relative to WKYs, SHRs exhibited a marked decrease in NO-induced phospho-VASP levels, while PBD treatment fully restored phospho-VASP (Figure 10D, E). Analysis of the cell prep on which this assay was performed revealed both VSMCs (54.3%) and fibroblasts (45%) (Figure S5). Thus, to validate NO signaling in VSMCs, phosphorylation of the VSMC-specific sarcoplasmic reticulum marker, phospholamban (PLN), a downstream target of PKG resulting from NO activity, was assessed (absent in fibroblasts)[29]. Phosphorylation of PLN results in the sequestration of Ca^{2+}, facilitating VSMC relaxation[30]. Mirroring phospho-VASP, phospho-PLN in VSMC was significantly increased in SHRs consuming PBD compared to SHRs consuming the control diet (Figure 10D, F).

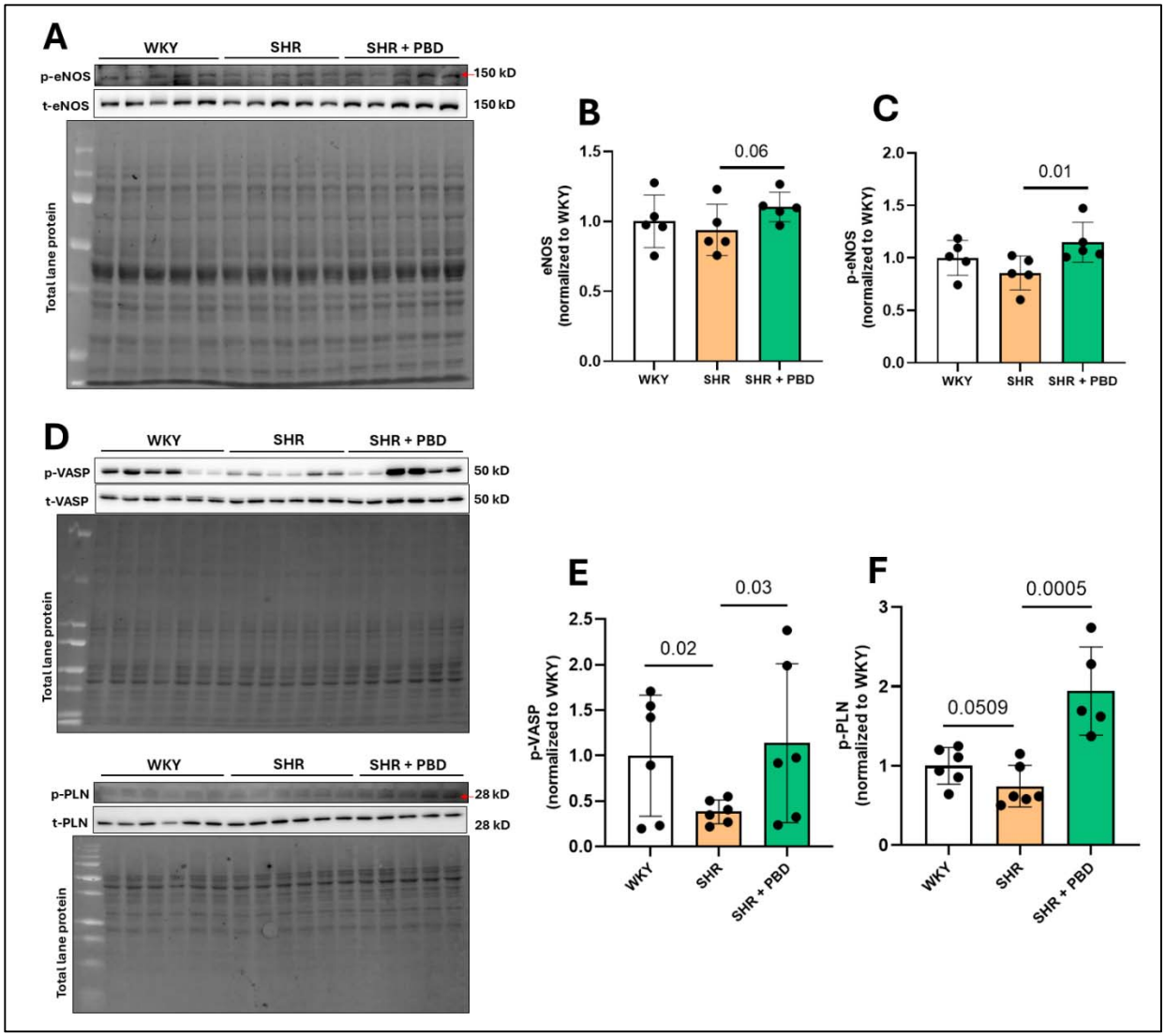


Figure 10. Cardiac endothelial- and VSMC-dependent cellular mechanisms. Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) consumed a purified diet for 24 weeks. After 24 weeks, a subset of SHRs were switched from the control diet to PBD, while a subset of SHRs and WKY continued the control diet for 12 additional weeks. (A) Left ventricular protein was isolated and (B) endothelial nitric oxide synthase (eNOS), as well as phospho-eNOS (C) was probed via western blot. Vascular smooth muscle cell (VSMC)-comprised cells were isolated from the heart. Following culture and confluence, cells were placed in starvation medium (10% of the growth supplement; 0.5% FBS) for 24 h. Cells were then lysed at passage 0 in supplemented RIPA after treatment with freshly prepared sodium nitroprusside (100 nM) for 5 min to induce nitric oxide (NO)-mediated signaling. (D) Protein from VSMC was probed for (E) vasodilator-stimulated phosphoprotein (VASP) and (F) phospholamban (PLN) phosphorylation. Statistical comparisons were made with the Student's t-test between WKY and SHR or SHR and SHR + PBD (n = 6/group). Data are expressed as mean ± SD.

A plant-based diet differentially impacted redox status and inflammation

Oxidative stress is a key factor in driving EC and VSMC dysfunction[31,32]. This led us to explore whether proteins involved in the redox pathway were impacted. Superoxide-neutralizing proteins, superoxide dismutase (SOD)1 and SOD2, were significantly increased in LV tissue in SHRs consuming PBD compared to SHRs consuming the control diet (Figure 11A–C). Similarly, catalase (CAT), a hydrogen peroxide-neutralizing protein, was also significantly increased in SHRs consuming PBD, but glutathione peroxidase (GPx1) was not (Figure 11A, D, E). Nuclear factor erythroid 2-related factor (NRF)2, a master regulator of endogenous antioxidant defense, was significantly reduced in nuclear LV extracts in SHRs consuming the control diet compared to WKY, and SHRs consuming PBD (Figure 12A, B). Nuclear extraction purity was validated (Figure S6) with the expression of nuclear marker, specific protein (SP)1, in nuclear and cytosolic extracts. Glutathione is known to be regulated by NRF2, and reduced glutathione was also significantly increased in LV tissue of SHRs consuming PBD compared to SHRs consuming the control diet (Figure 11C). In isolated cells (54.3% VSMC, 45% fibroblast), the pro-oxidant-associated NADPH-oxidase subunit, p22phox, was significantly reduced in SHRs consuming PBD versus SHRs consuming the control diet, as was the lipid peroxidation marker, 4-hydroxy-2-nonenal (4-HNE) (Figure S7). These results suggest that PBD improved cardiac oxidative stress management.

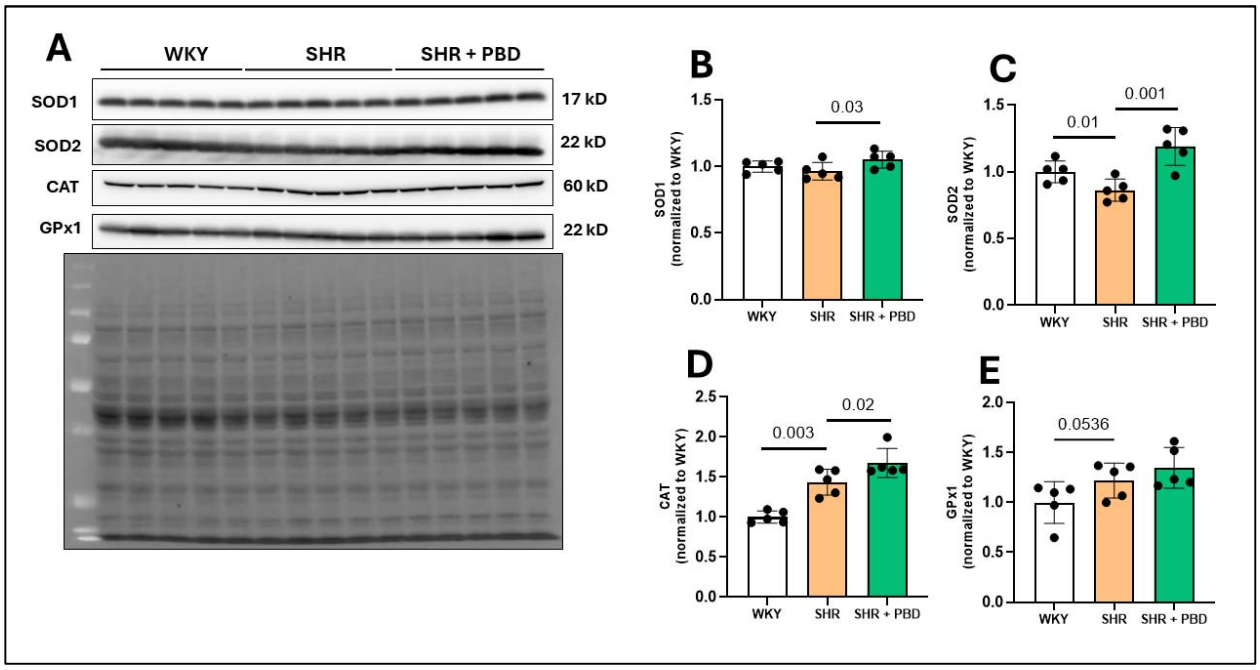


Figure 11. Cardiac endogenous antioxidant defense. Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) consumed a purified diet for 24 weeks. After 24 weeks, a subset of SHRs were switched from the control diet to PBD, while a subset of SHRs and WKY continued the control diet for 12 additional weeks. (A) Left ventricular protein was isolated and (B) superoxide dismutase (SOD)1, (C) SOD2, (D) catalase (CAT), and (E) glutathione peroxidase (GPx)1 was probed via western blot. Statistical

comparisons were made with Student's t-test between WKY and SHR or SHR and SHR + PBD (n = 5/group). Data are expressed as mean ± SD.

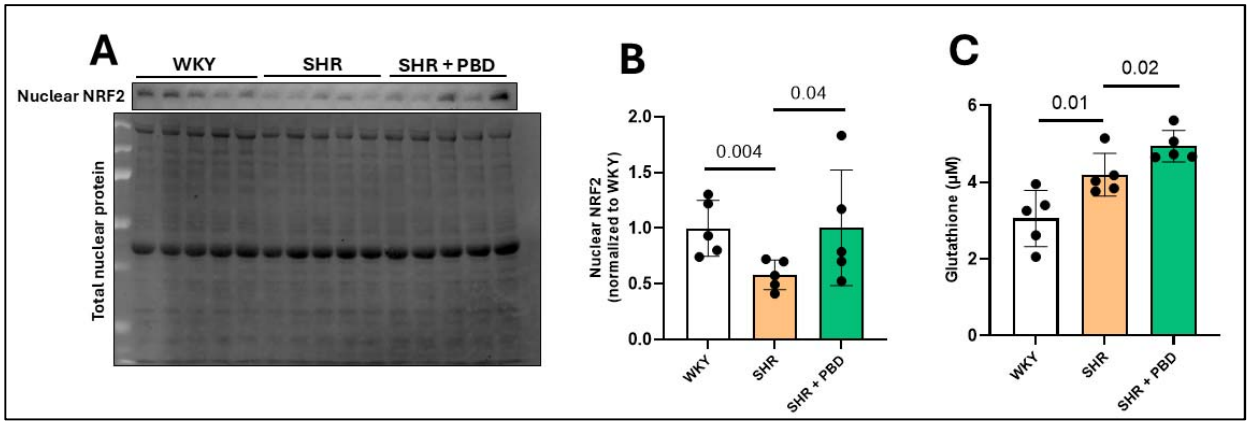


Figure 12. Cardiac antioxidant regulation. Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) consumed a purified diet for 24 weeks. After 24 weeks, a subset of SHRs were switched from the control diet to PBD, while a subset of SHRs and WKY continued the control diet for 12 additional weeks. (A) Left ventricular nuclear protein was isolated and (B) nuclear factor erythroid 2-related factor 2 (NRF2) was probed via western blot. (C) Glutathione concentrations in the LV were determined with a commercially available kit. Statistical comparisons were made with Student's t-test between WKY and SHR or SHR and SHR + PBD (n = 5/group). Data are expressed as mean ± SD.

Next, we examined the impact of PBD on cardiac inflammatory signaling, anticipating that PBD might reverse detrimental changes and/or enhance beneficial counter-regulatory changes that had ensued in SHRs. Expression of phospho-p65, which is known to reflect activation of canonical NF-κB signaling, followed the latter pattern in that it was lower in SHRs, relative to WKYs, and further reduced in PBD-fed SHRs (Figure 13A, B). In contrast, levels of phospho-p38 were lower in SHRs compared to WKYs but were restored by PBD (Figure 13A, C) and SHRs consuming PBD. Analogous to results for phospho-p65, expression of phospho-SAPK/JNK and c-Jun were lower in SHRs and even lower in PBD-fed SHRs although only the differences between SHRs fed the control and PBD reached statistical significance (Figure 13A, C, D). While p38 and SAPK/JNK both can regulate c-Jun[33,34], SAPK/JNK appeared to be a more specific regulator in this context. PBD did not attenuate pro-oxidative and inflammatory processes in isolated cardiac white blood cells (Figure S8), nor did we observe significant effects of PBD in the hypothalamus, a region of the brain involved in mediating sympathetic output in hypertension[35] (Figure S9). These results are in accordance with reduced cardiac inflammatory signaling playing a role in mediating benefits of a PBD.

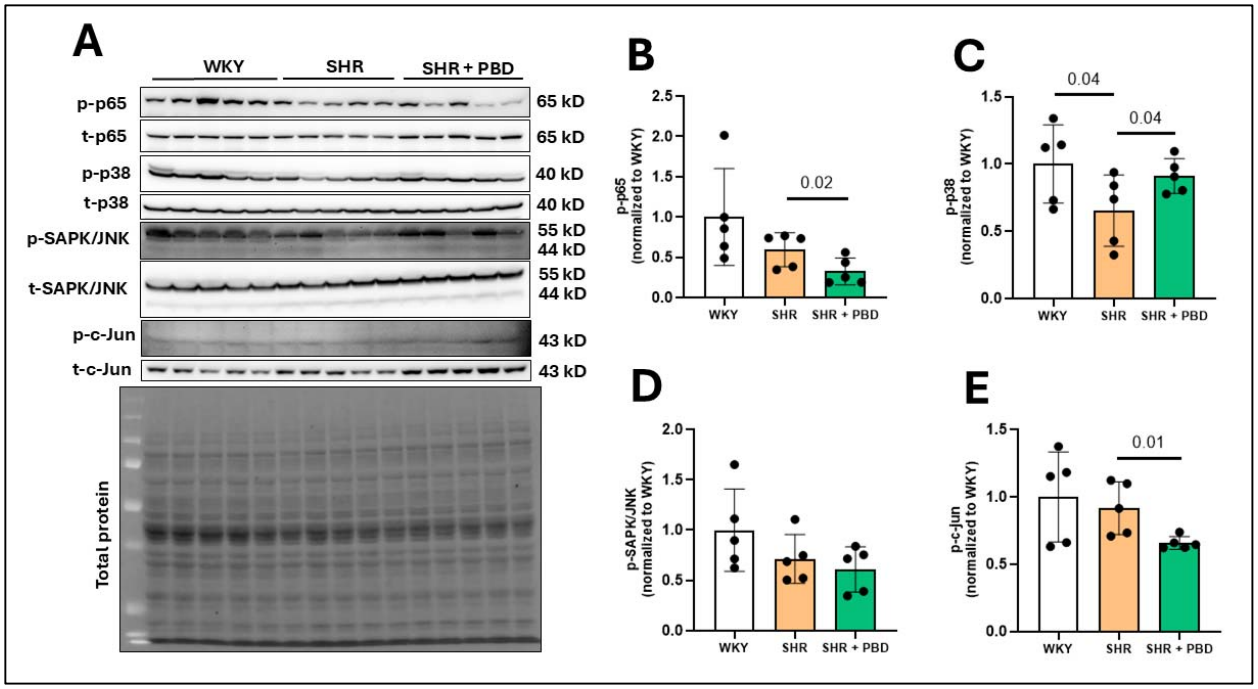


Figure 13. Cardiac inflammatory signaling. Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) consumed a purified diet for 24 weeks. After 24 weeks, a subset of SHRs were switched from the control diet to PBD, while a subset of SHRs and WKY continued the control diet for 12 additional weeks. (A) Left ventricular protein was isolated and probed for (B) p38 mitogen-activated protein kinase (MAPK), (C) stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK), (D) c-Jun, and (E) p65, a nuclear factor-kappa B (NF-κB) subunit phosphorylation. Statistical comparisons were made with Student's t-test between WKY and SHR or SHR and SHR + PBD (n = 5/group). Data are expressed as mean ± SD.

A plant-based diet treated LV fibrosis in CMD

Fibrosis is a cause, consequence, and amplifier of hypertension-induced cardiac dysfunction. While analysis of cardiac tissue via hematoxylin and eosin staining did not reveal abnormalities in SHRs (Figure S10), this did not rule out the possibility of subtle, early-stage fibrosis. Accordingly, we investigated this notion by visualizing collagen via Sirius red staining of coronal sections of the LV. Despite not having reached definitive levels of heart failure, collagen-dense regions were apparent in SHRs but not in WKYs at 24 weeks of age. Accordingly, quantitation of collagen staining indicated over a 100% increase in Sirius red staining in SHRs relative to WKYs. Meanwhile, PBD prevented fibrosis, nearly completely, by these measures. Furthermore, a very similar pattern of results was observed at 40 weeks even though the PBD was only initiated at 24-weeks of age, indicating that PBD not only prevented, but also reversed early-stage fibrosis in SHRs.

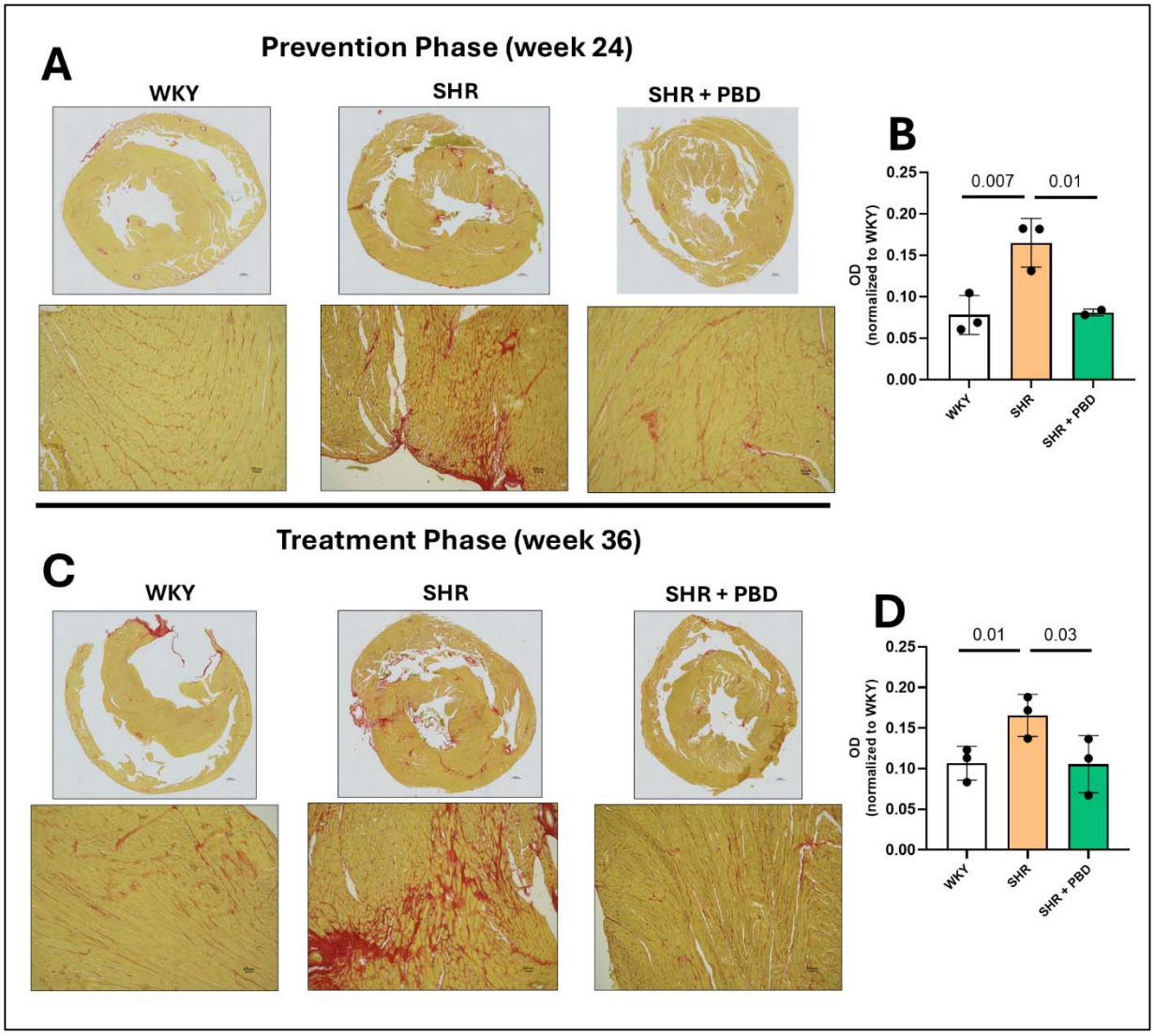


Figure 13. Left ventricular fibrosis. Wistar-Kyoto (WKY) rats or spontaneously hypertensive rats (SHR) consumed a purified diet or 28% plant-based diet (PBD) for 24 weeks with or without antibiotics (ABX). After 24 weeks, a subset of SHRs were switched from the control diet to PBD, while a subset of SHRs and WKY continued the control diet for 12 additional weeks. Hearts were processed for histological analysis and stained for collagen with Sirius red dye. Areas of peak fibrosis (10x magnification) were used for quantification. Blood vessels and collagen attachment sites were avoided for the analysis. Data were analyzed with ImageJ and FIJI plugin. Statistical comparisons were made with Student's t-test between WKY and SHR or SHR and SHR + PBD (n = 2-3/group). Data are expressed as mean ± SD.

4. Discussion

Hypertension is a predominant risk factor and an important contributor to CMD. Accordingly, we hypothesized that SHRs might develop CMD and thus serve as a clinically relevant model of this disorder. We report confirmation of this hypothesis.

Furthermore, our use of this tractable model to investigate the impacts of diet on CMD revealed that a PBD both prevented and reversed CMD. Such benefits of a PBD occurred without alleviation of hypertension, a finding we did not anticipate, but which may have reflected that the congenital hypertension of SHRs is difficult to alleviate. In any case, it highlights the potential of PBDs to mitigate CMD irrespective of impacts on blood pressure. The beneficial effects of the PBD correlated with, and were likely mediated by, both endothelial- and VSMC-dependent mechanisms (Figure 14).

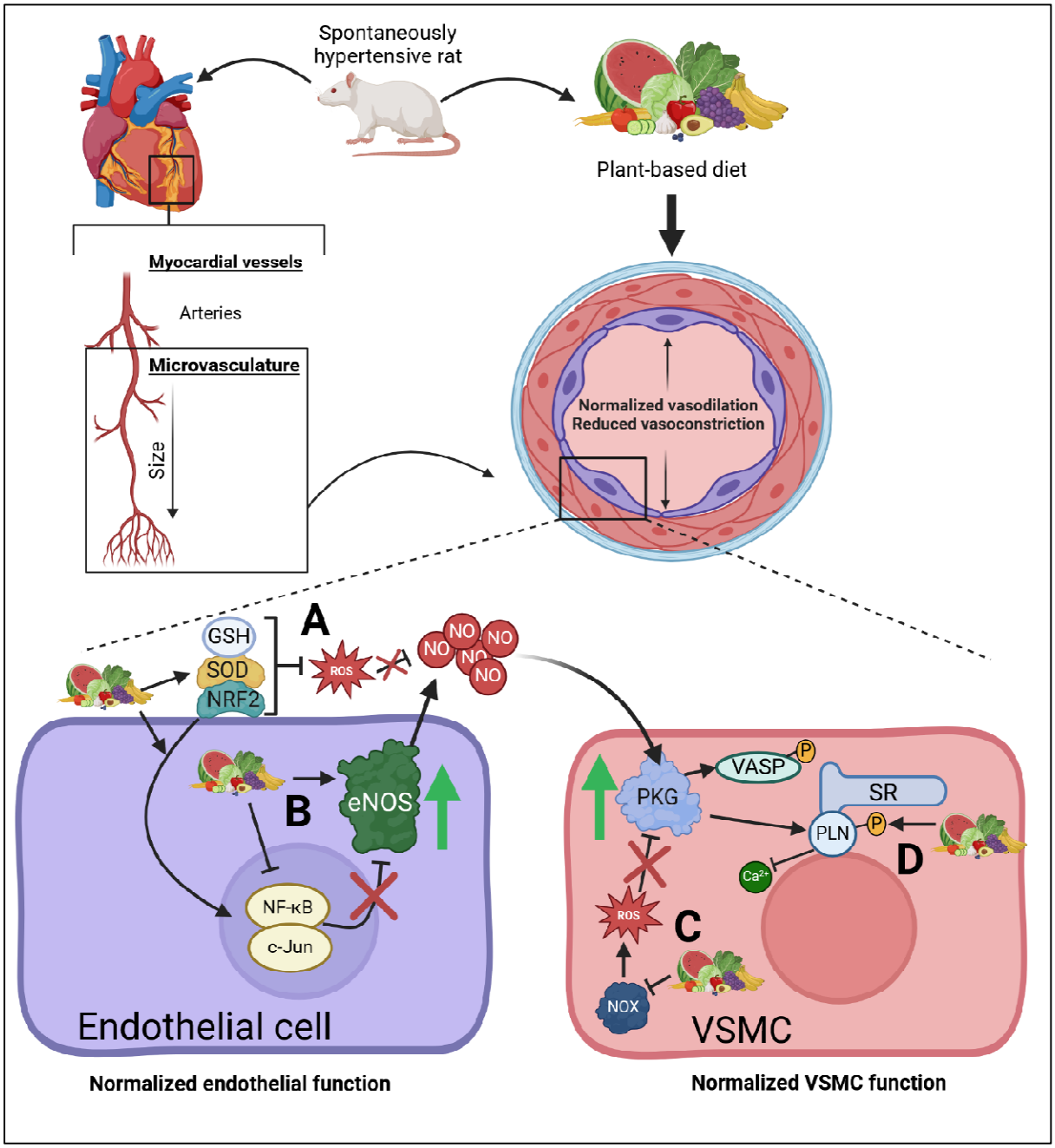


Figure 14. Model by which a PBD targets coronary microvascular dysfunction (CMD) in hypertension. Notable cellular differences within the microvasculature of the

cardiac ventricles of spontaneously hypertensive rats (SHR)s fed a plant-based diet (PBD) compared to a control diet are illustrated. (A) PBD enhanced endogenous antioxidant defense by increasing superoxide dismutase (SOD), glutathione (GSH), and increasing nuclear translocation of nuclear factor erythroid 2-related factor (NRF)2. The upregulation of these proteins leads to the direct and indirect neutralization of reactive oxygen species (ROS), preserving nitric oxide (NO) bioavailability. (B) a PBD also enhanced and preserved endothelial nitric oxide synthase (eNOS) expression and function in the endothelium, a protein which synthesizes NO. A PBD also reduced the expression of pro-inflammatory transcription factors, nuclear factor kappa B (NF-κB) and c-Jun, which can downstream reduce eNOS expression and activity. (C) protein kinase G (PKG) activity was notably increased in VSMCs due to increased vasodilator-stimulated phosphoprotein (VASP) phosphorylation. Activation of PKG can be impeded by increased ROS, and a PBD reduced the expression of p22phox, a subunit of NADPH-oxidase (NOX) enzymes which produce ROS, potentially (D) as a consequence of improved PKG activity, a PBD was able to increase phospholamban (PLN) phosphorylation of the sarcoplasmic reticulum (SR), a protein involved in sequestration of calcium ions, resulting in vascular smooth muscle cell (VSMC) relaxation.

VSMC relaxation is driven by NO, which is produced by eNOS. Thus, increased VSMC relaxation in PBD-fed animals may have reflected increased NO and/or increased responsiveness of VSMC to NO. A PBD resulted in increased eNOS phosphorylation (Figure 10A, C), indicating increased eNOS activity. Indeed, the L-NAME-induced reduction in myocardial blood flow, which reflects loss of eNOS activity, was reduced in SHRs, compared to WKYs, and restored by PBD-feeding, suggesting increased eNOS activity in PBD-fed SHRs (Figure 9). Additionally, a PBD resulted in increased levels of serum NO metabolites (Figure S3), likely reflecting that this diet is rich in nitrates, which may have contributed to increased NO generation[36]. Collectively, these data indicate impaired endothelial function which was rescued by PBD consumption. Consistent with this mechanism, reversal of CMD by PBD via 12-week course of PBD feeding restored NO-mediated signaling in VSMCs, which concomitantly led to increased PLN phosphorylation (Figure 10D, F). Thus, both the endothelium and VSMCs were favorably impacted by the PBD. These findings may have high translatability to clinical settings wherein CMD needs to be treated rather than merely be prevented.

Preservation of endothelial and VSMC function may have been a consequence of an improved redox state, as endogenous antioxidant defense was upregulated by PBD (Figure 11-12; Figure S7). Increased oxidative stress and inflammation can facilitate eNOS uncoupling, reducing NO availability, resulting in endothelial dysfunction[37]. Similarly, PKG signaling as a result of NO can be impaired in the presence of oxidative stress and inflammation, resulting in increased VSMC contraction[8,38]. Oxidative stress and inflammation can also drive pro-fibrotic processes[39,40], which was attenuated and reversed in the LV by the consumption of a PBD in the present study (Figure 13). While fibrosis is not classically thought of as reversible, early stage fibrosis may be[41], as was the case in the present study. Indeed, extensive LV fibrosis would be detrimental to

cardiac function and lead to heart failure. Heart failure does not typically manifest in female SHRs until 24 months of age[27], thus, an extension of this study may have resulted in more pronounced fibrosis.

The persistence of hypertension in PBD-fed SHRs was likely due to the multifaceted mechanisms within the SHR that drive hypertension. These include structural abnormalities of the vasculature, VSMC hyperreactivity, overactive sympathetic output, as well as renin-angiotensin system upregulation [42-46]. These factors may make dietary changes insufficient in overcoming this pronounced hypertensive phenotype. In contrast in humans, we have shown that a PBD could treat hypertension in a mostly female cohort of patients and significantly reduce the need for blood pressure-managing medications[47]. Thus, it is noteworthy that in the present model, CMD was attenuated and reversed despite hypertension.

Considering the essential role of the gut microbiota in metabolizing phytochemicals, such as polyphenols, it was expected that any beneficial effects of the PBD would be negated by ABX. In contrast, a PBD improved CMD and endothelial function even with ABX (Figures 7 & 9). Parent phytochemical compounds are in much lower concentrations in serum following the consumption of phytochemical-rich foods compared to their metabolites. These compounds undergo various hydrolysis, deglycosylation, dehydroxylation, and demethylation reactions facilitated by gut microbiota to increase absorption[14]. Indeed, unique polyphenol profiles in serum can be observed 2 h following the consumption of red raspberry compared with 24 h which closely tied with improved macrovascular function at both time-points[48]. The consumption of a single 500 mg dose of cyanidin-3-glucoside, a common anthocyanin found in blueberries and blackberries, resulted in the presence of 33 different metabolites, each with distinct times of appearance in serum at 1, 6, and 24 h post-consumption[49]. These postprandial profiles independently were effective at physiological concentrations in attenuating endothelial cell inflammation[50]. Further, gut microbe populations have differing metabolic capabilities, with *Bacteroides* conferring negative health effects compared with *Prevotella* which are beneficial to health[51]. Indeed, this favorable shift occurs rapidly within 24 h of consuming a PBD in humans, and reverses once the PBD is ceased[52,53]. Thus, polyphenols are known for modulating microbial populations[14]. Microbial analysis in the present study revealed increased *Prevotella* in SHRs consuming a PBD at week 24 (Figure 2C), a microbe associated with PBDs[54]. Nonetheless, we cannot draw firm conclusions regarding the role the microbes in mediating benefits of PBD in that ABX treatment did not lead to clear differences in CMD.

Several limitations in the present investigation exist. While we aimed for this research to be as clinically relevant as possible, it is unclear whether the unique genetic phenotype of the SHR is translatable to humans. For example, angiotensin II infusion and Dahl salt sensitive rats may result in different outcomes. Furthermore, this animal model of CMD is newly established[55], thus, more established models of CMD utilizing metabolic

syndrome characteristics[56] require investigation. Another limitation is the lack of purity in the VSMC culture. Initially, one aim of this study was to isolate ECs. However, these cells, despite being $CD31^+$ and $CD90^-$, would not successfully culture. It was identified that VSMCs could be isolated with these methods, however. Initial labelling with MYH11 antibody (50-6400-80, ThermoFisher) a VSMC-specific marker, with flow cytometry revealed ~99% purity. However, it was later discovered that this antibody was nonspecific and of poor quality. Additional testing with α-SMA and CD90 antibodies revealed that the VSMC culture was contaminated with fibroblasts (Figure S5). Since this VSMC data had already been collected, it was not possible to go back and remove fibroblasts with CD90 magnetic beads. However, since PLN is VSMC-specific, we are confident that we have captured the relevant data illustrating preserved VSMC function with SNP. As emphasized throughout, women are more likely to be impacted by CMD, particularly those that are post-menopausal. However, since we used cycling female rats, this does not fully reflect the human phenotype. Further, since we only used female animals in this study, sex-differences could not be investigated. Future studies should evaluate the effects of PBD in both sexes, especially those in older animals.

In conclusion, a PBD was able to both prevent CMD development and reverse CMD once established in an animal model of hypertension. These effects were likely due to improved endothelial and VSMC function. This occurred independent of hypertension. Pilot clinical studies evaluating the effects of a PBD in CMD are warranted.

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Disclosure of interest

None to declare

Data availability statement

Raw western blot images are provided on Figshare, an online repository with the following DOI: 10.6084/m9.figshare.28790810. Microbiota 16s sequencing data can be found at the following DOI on Figshare: 10.6084/m9.figshare.28781054. Other data can be provided by the corresponding author upon reasonable request.

Ethical Approval

This study was approved by the Institutional Animal Care & Use Committee (IACUC) at Georgia State University. The approval number for this study is A23025.

CRediT authorship contribution statement

Rami S. Najjar: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization, Funding acquisition, Project administration. **Khan Hekmatyar**: Writing – review & editing, Methodology, Investigation. **Yanling Wang**: Writing – review & editing, Methodology, Formal analysis. **Vu Ngo**: Writing – review & editing, Methodology, Formal analysis. **Hannah L. Lail**: Writing – review & editing, Methodology. **Juan P. Tejado**: Writing – review & editing, Methodology, Formal analysis. **Jessica P. Danh**: Writing – review & editing, Investigation. **Desiree Wanders**: Writing – review & editing, Resources. **Rafaela G. Feresin**: Writing – review & editing, Resources. **Puja K. Mehta**: Writing – review & editing, Methodology. Conceptualization. **Andrew T. Gewirtz**: Writing – review & editing, Funding acquisition, Methodology, Supervision, Resources.

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