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**INTERLEUKIN-18 AS A THERAPEUTIC TARGET IN WESTERN-DIET INDUCED CARDIOMYOPATHY**

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# INTERLEUKIN-18 AS A THERAPEUTIC TARGET IN WESTERN-DIET INDUCED CARDIOMYOPATHY

A thesis submitted in partial fulfillment of the requirements for the degree of the Master of Science in Physiology and Biophysics at Virginia Commonwealth University School of Medicine.

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# DEDICATION

I dedicate this thesis to my mother for her extraordinary support of my pursuits. I cannot put into words how grateful I am to have your support, without which I would not have come this far. You have always believed in me and taught me about commitment, hard work and to never surrender in adverse circumstances. Thank you for always being there for me.

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## ABSTRACT

INTERLEUKIN-18 AS A THERAPEUTIC TARGET IN WESTERN-DIET INDUCED CARDIOMYOPATHY

By Pratyush Narayan, B.S.

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Virginia Commonwealth University School of Medicine, 2017.

Director: Stefano Toldo, PhD. Assistant Professor of Medicine, Division of Cardiology.

*Background:* Heart Failure (HF) is characterized by impaired cardiac function with symptoms of dyspnea, fatigue and exercise intolerance. Western diet (WD), a diet high in saturated fats and sugars, causes systemic increase in IL-18, a cytokine elevated in HF, which induces abnormal myocardial structure and diastolic dysfunction. IL-18 binding protein(IL-18BP) binds IL-18, preventing the binding to the IL-18-receptor. We investigated a therapeutic intervention using recombinant-murine IL-18BP to improve the WD-induced cardiomyopathy in C57BL/6J mice.

*Methods:* Echocardiography with pulse-wave Doppler and tissue imaging was performed to assess cardiac function. Oral glucose tolerance test was performed to assess metabolic parameters and IL-18 plasma levels were quantified with ELISA.

*Results:* WD-fed mice had worsened isovolumetric relaxation time (IVRT) and myocardial performance index (MPI), indicating cardiac dysfunction. Daily intraperitoneal injections of 0.5mg/kg IL-18BP for two weeks, significantly improved both IVRT and MPI.

*Conclusions:* IL-18BP treatment improves cardiac function in a model of WD-induced cardiomyopathy.

# INTRODUCTION

Heart Failure (HF) is clinically characterized by impaired cardiac function with symptoms of shortness of breath, dyspnea, fatigue and poor exercise tolerance[1,2]. HF occurs because of an impairment of the heart in filling and/or ejecting blood because of structural and/or functional impairments[4]. The poor left ventricle (LV) function results in insufficient oxygen supply to the rest of the body. HF patients accounted for approximately one million hospital discharges in 2010[1]. In recent years, the patients with HF have been divided in two groups, based on their ejection fraction (EF): HF with preserved ejection fraction (HFpEF) and HF with reduced ejection fraction (HFrEF). HFpEF is used to designate HF with EF greater than 50% and HFrEF is used to designate HF with an EF less than 50%[7,8]. These designations are dependent on the systolic function and were made because the patients of these two populations respond differently to medical therapies. In fact, current therapies, decrease mortality and improve the survival of patients with lower systolic function, but have little effect on patients with normal systolic function[7–10].

Virtually all HF is associated with diastolic dysfunction regardless of the quality of the systolic function[11]. Normally, diastole is characterized by a rapid myocardial relaxation that allows adequate LV filling at a relatively low pressure. Diastolic dysfunction is the result of impaired filling which is assessed by an increase in the time of relaxation [the isovolumetric relaxation time (IVRT)], and/or by an increase in filling pressures. An increase in the time required to relax elevates filling pressures and leads to diastolic dysfunction[11,12]. In HFpEF, the diastolic dysfunction is the leading source of symptoms.

Worldwide, 38 million people are affected by HF with 5.7 million people in the United States suffering from the disease[2,4]. In the U.S. there are 915,000 new cases of HF every year and approximately half of the patients who develop HF, die within 5 years of their diagnosis[3,4]. For these reasons, it is appropriate that we continue to explore the pathophysiological mechanisms that contribute to HF with the goal of preventing it as well as improving disease outcomes.

HF is associated with low-grade but, chronic systemic inflammation. We and others believe that the inflammation contributes to the cardiac function impairment in HF[4]. It is well known that inflammation is a central component of healing in the body, however, prolonged inflammation appears to contribute to heart disease because proinflammatory cytokines promote LV dysfunction[1]. One such cytokine is interleukin-18 (IL-18). IL-18 is part of the IL-1 family of cytokines and it is constitutively expressed in virtually every cell in its inactive form, pro-IL-18. During a pathological condition, such as heart disease, IL-18 expression is significantly increased systemically and locally in the myocardial tissue[1,4,13]. High levels of IL-18 correlate with increased risk of cardiovascular disease (CVD) and worse prognosis in the patient population[1].

IL-18 levels are increased in patients with HF, and IL-18 has been associated with both structural and functional diastolic impairments[4]. IL-18 drives chronic profibrotic and pro-hypertrophic effects in cardiomyocytes[1]. Increased fibrosis and hypertrophy of cardiac tissue reduce the ventricular compliance and impair LV filling[1]. In HF patients, serum levels of IL-18 were associated with increased mortality[1].

**The IL-18 Pathway**

IL-18 was first identified as an inducer of interferon gamma (IFN-γ) and found to act synergistically with IL-12 since IL-12 induces the expression of the IL-18 receptor (IL-18R) on T-cells[1,14]. Similar to IL-1β, another cardiodepressive pro-inflamamtory cytokine, IL-18 has a secondary structure largely consisting of β-sheets, making it part of the IL-1 family of cytokines[1].

Similar to IL-1β, IL-18 is produced as an inactive zymogen (pro-IL-18)[1]. It is cleaved and activated by caspase-1[1]. Caspase-1 itself is produced as an inactive protein, pro-caspase-1, and it is activated by autocatalytic cleavage within the inflammasome[1]. The inflammasome is a multimolecular structure which consists of NOD-like receptor protein 3 (NLRP3), apoptosis speck-like protein with a caspase recruiting domain (ASC), and pro-caspase-1[1]. The oligomerization of these proteins into the inflammasome leads to the formation of a high output platform for cytokine production[4].

IL-18 binds to its receptor, the IL-18R. IL-18R is a dimer composed of IL-18Rα and IL-18Rβ[1]. Although, IL-18Rα has a low affinity for IL-18, it is the first to bind the cytokine. The IL-18Rβ follows by binding to the IL-18/IL-18Rα complex[1]. This triggers the activation of the intracellular signaling, which involves the intracellular MyD88, IRAK1, TRAF6 pathway[13]. MyD88 is an adaptor molecule which is involved in signaling of various receptors such as toll-like receptors (TLRs), IL-1 receptor (IL-1R), and notably IL-18R. The MyD88 modulation of IL-18R signaling is STAT3 independent[13]. Mice lacking IRAK or MyD88 show impaired response to IL-18[1].

## Cardiac Effects of IL-18

Chronically, IL-18 induces abnormal myocardial structure and diastolic dysfunction[5,6]. In obese patients, IL-18 is elevated[4]. IL-18 exerts profibrotic effects through increase in osteopontin (OPN) and fibronectin expression in cardiac tissue as well as through the increase in interstitial collagen content in cardiac tissue[1]. OPN, an extracellular matrix protein, regulates collagen levels in the heart and can be induced both in vivo and in vitro by IL-18 in murine cardiac fibroblasts[1]. Increased collagen is a marker for increased fibrosis which is followed by increased wall stiffness[1].

In experimental models, recombinant IL-18 (rIL-18) causes systolic and diastolic dysfunction. IL-18 causes contractile dysfunction through the activation of the p38 MAPK or IL-1β[1]. IL-18 appears to have a cell-specific role in apoptosis[1]. While it does not cause neutrophil apoptosis, IL-18 does increase apoptosis of human cardiac microvascular endothelial cells[1]. In vitro, calcium transients are increased in the presence of IL-18, which alters the contraction and relaxation of cardiomyocytes[6]. When the protein phospholamban (PLN) is dephosphorylated, it suppresses sarcoendoplasmic reuptake calcium ATPase (SERCA) pump's ability to reuptake of calcium from the cytoplasm into the sarcoplasmic reticulum. PLN phosphorylation releases its suppression of SERCA allowing for the reuptake of calcium, limiting duration of contraction. When PLN is dephosphorylated, it prolongs the time of calcium in the sarcoplasm and therefore potentiates the muscle contraction and impairs the relaxation. The IL-18 dependent dephosphorylation of PLN is dependent upon phosphatase2A (PP2A) activity[15].

## Role of IL-18 in metabolism

Since HF is a clinically heterogenous syndrome, it is complicated by a variety of risk factors such as obesity and diabetes. Diastolic dysfunction is associated with comorbidities such as obesity and diabetes[11,12,16,17]. IL-18 levels are elevated in patients suffering from obesity and diabetes, both conditions which are associated with HFpEF[2,6,7,18]. This increase can either be an attempt of the body to counteract weight gain or similar to hyperlipidemia in obesity, the high levels of IL-18 may suggest resistance or defect in IL-18 signaling[4].

Interestingly, the lack of IL-18 causes hyperphagia and increased weight gain[4]. IL-18-/- mice increase their food intake results in these mice having a more robust increase in weight than in control mice[13]. IL-18-/- mice become obese due to excess accumulation of body fat and increased percentage of adipose tissue due to lipid abnormalities[13]. IL-18-/- mice also become diabetic and have impaired glucose tolerance[13]. The effects of IL-18 absence on glucose metabolism is a result of increased food intake and weight gain[13].

It has been hypothesized that IL-18 targets the hypothalamus in the CNS[13]. When IL-18-/- mice are injected with rIL-18, their insulin sensitivity increases as seen by improvement in glucose tolerance[13]. Reduction in food intake has been reported in both IL-18-/- and IL-18+/+ mice following intracerebral administration of rIL-18 but not intravenous administration[13]. This suggests that IL-18 plays a role in the satiety signaling process centrally and that the influence of rIL-18 on food intake may occur in the CNS[4,19]. IL-18-/-, IL-18R-/- and overexpression of IL-18BP, in a global transgenic mouse model, have been shown to cause obesity, hyperphagia and

hyperglycemia in mice as well[13]. Thus, the absence of IL-18 signaling negatively impacts metabolism.

It has been reported that exogenous IL-18 influences glucose metabolism by activating the STAT3 phosphorylation, correlating with reports associating STAT3 deficiency with insulin resistance and obesity[13]. IL-18R dependent intracellular signaling involves the phosphorylation of STAT3[13]. Insulin, insulin-like growth factor, and adiponectin are endogenous hormones known to exhibit hypoglycemic effects[13]. From the data present, it is possible that IL-18 also has hypoglycemic potential[13].

High concentration of IL-18 in patients with type 2 diabetes mellitus has been reported[13]. Similar to the hyperleptinemic state and leptin resistance in obese individuals, increased concentrations of IL-18 may indicate that diabetic patients have reduced sensitivity to IL-18 and IL-18 is increased in an attempt to reduce hyperglycemic states[13,20]. While the absence of IL-18 contributes to impaired glucose clearance and insulin resistance, it does not induce leptin resistance[13].

**Western Diet**

The Western Diet is a high saturated fat, high sugar diet resembling the average American diet (Table 1). Metabolic diseases are risk factors for HF and WD is a risk factor for developing metabolic diseases[4]. Diets high in fat and sugars have been associated with increased risk of obesity and heart disease[2]. The sugars and saturated fats in the WD are known to induce systemic

inflammation[2]. Free fatty acids (FFAs) act as chronic stressors leading to low and chronic inflammation[21]. FFAs and simple carbohydrates (sugars), abundant elements of the WD, increase systemic levels of IL-18[22–24].

The link between WD and IL-18 is through the Toll Like Receptors (TLRs) which are involved in the innate immune response. TLR2 and TLR4 are activated by the FFAs, subsequently contributing to local and systemic inflammation[15,22,25–28]. The activation of TLR2 and TLR4 leads to increased expression of pro-IL-18. The WD increases the mRNA levels of IL-18 in the heart and the protein in plasma[4].

**Effect of WD on diastolic function**

Previously our lab has shown that WD-fed mice develop progressive diastolic dysfunction over a period of 8 weeks[2]. In the same study, there was a mild but significant decrease in the left ventricular ejection fraction (LVEF), indicative of systolic dysfunction[2]. The study did not find any sex-related differences.

We have established that IL-18 mediates cardiac dysfunction in WD-fed mice through a IL-18 knock out (IL-18KO) mouse model[4]. WD-fed IL-18KO mice do not develop cardiac dysfunction despite having higher food intake and higher increase in body weight than WT mice on WD[4]. The hyperphagia and higher increase in body weight in the absence of IL-18 is consistent with past studies[13].

By using the IL-18KO model, our lab has shown that IL-18 is sufficient to impair cardiac function and established causality. In WD-fed mice, there is less myocardial fibrosis in IL-18KO compared to WT mice[4]. Moreover, there was preservation of both the diastolic and systolic function on IL-18KO mice. Since we have found that IL-18KO mice are cardio-protected when fed with WD, it suggests a causative role of IL-18 in WD induced cardiomyopathy.

**IL-18 blocking strategies**

Active IL-18 levels are strictly controlled by IL-18 binding protein (IL-18BP), which is naturally produced in humans and mice[13]. There are six naturally occurring isoforms of IL-18BP[1]. Of these six, in humans, IL-18BPa has the highest affinity for IL-18[1]. In the circulation, IL-18 can exit as both "free" and bound to IL-18BP[1]. The "free" IL-18 levels are less than the total IL-18 in circulation and it is the "free" IL-18 which correlates with disease severity[1]. In response to rising IL-18 levels, IL-18BP levels in the circulation also rise[1]. This data and since the rise in IL-18BP mRNA is delayed compared to the rise of pro-IL-18 and IL-18 mRNA, suggests that there may be a feedback system between IL-18BP levels and the production of IL-18.

Recombinant IL-18BP (rIL-18BP) and IL-18 blocking antibody have been developed with rIL-18BP being tested in clinical trials for psoriasis and rheumatoid arthritis while IL-18 antibody is being investigated in type 2 diabetes[1]. IL-18BP has been studied in animals and in humans[1]. In models of ischemia/reperfusion (IR), using human atrial tissue, adding IL-18BPα in the perfusate to compete for IL-18 binding, improved contractile function[1]. In acute myocardial infarction (AMI) blocking IL-18 activity has been shown to improve contractile function and decrease infarct

size[1]. Blockade of IL-18 with an antibody prevented LPS-induced myocardial dysfunction[1]. When IL-18BP was used to block endogenous IL-18, it prevented the occurrence of diastolic dysfunction[29].

Blockade of IL-18 in the context of HF is interesting to study since increase in IL-18 has been correlated with HF and because IL-18 mediates contractile dysfunction, hypertrophy and fibrosis of cardiac tissue[1].

**Hypothesis**

Based on our knowledge of the role of WD and IL-18 in HF there is strong evidence to support IL-18, as a link between systemic inflammation and HF. I hypothesize that WD causes chronic systemic inflammation through the upregulation of IL-18 which subsequently leads to HF. Therefore, the pharmacological IL-18 blockade using murine recombinant IL-18BP will reverse established diastolic dysfunction.

## METHODS

The experiments were conducted under the guidelines of laboratory animals for biomedical research published by National Institutes of Health (No. 85-23, revised 2011). The study protocol was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

### Study Design

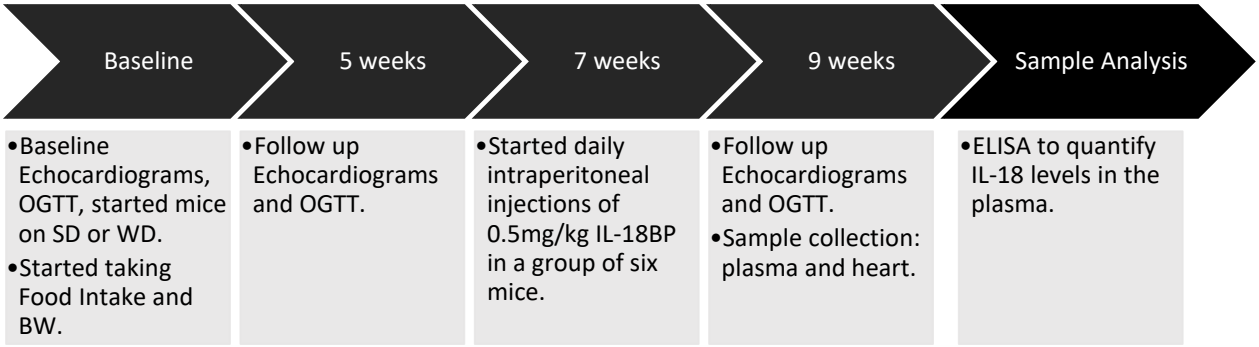


**Figure 1: A visual timeline of our study design.**

We divided 29 WT C57BL/J6 mice (Jackson Laboratories), 10 weeks old, into three groups: Standard Diet (SD group), Western Diet (WD group), WD with delayed IL-18BP administration as a rescue approach (treatment group). There were 15 mice in the SD group, 8 in the WD group, 6 in the treatment group. The SD group were fed SD (Teklad LM-485; Harlan) and the WD groups were fed WD (TD.88137; Harlan) for the entirety of the study. A comparison of the composition of the Standard Diet is and the Western Diet, fed to our mice, are presented in Table 1. The treatment group of mice (in whom WD-induced diastolic dysfunction was established

at 5 weeks) received daily intraperitoneally (IP) injections of 0.5mg/kg of IL-18BP starting at the 7-week time point until the end of the study at 9 weeks. The lyophilized recombinant mouse IL-18BP (IL-18BP), His-tagged (IL-18BP-767M; Creative Biomart) was diluted in saline prior to injections. Insulin syringes (0.5cc; Becton Dickinson) were used for the injections because of their minimal residual volume.

| Components in %kcal | Standard Diet | "Western Diet" | American Diet |
|---|---|---|---|
| Total fat | 17 | 42 | 32.8 |
| Saturated fat | 0.8 | 12.8 | 10.6 (among adults >20 years) |
| Monounsaturated fat (MUFA) | 1.3 | 5.6 | - |
| Polyunsaturated fat (PUFA) | 2.9 | 1 | - |
| Proteins | 25 | 15.2 | 15.4 |
| Total carbohydrates | 58 | 42.7 | 51.8 |
| Sucrose | 0 | 34 | 8 |
| Refined Sugars | - | - | 18.6 |
| Cholesterol | 0 | 0.2 | - |
| Sodium | 0.3 | 0.1 | 9.6 |
| Energy density (kcal/g) | 3.1 | 4.5 | 2.34 per eating occasion |

**Table 1: Diet composition.** Comparison of the composition of the Standard diet, Western diet, and American diet[30–32].

**Echocardiography**

All mice underwent transthoracic echocardiography (TTE) with pulse wave Doppler (PWD), and tissue Doppler imaging (TDI) using the Vevo770 imaging system (VisualSonic Inc, Toronto, Ontario and 30-MHz probe), at baseline (0 weeks), 5 weeks and 9 weeks[33,34]. The echocardiography was done under anesthesia with pentobarbital at a dose of 50-70 mg/kg. The mice were kept on a warming pad after anesthesia was administered and until they woke up. Hair removal lotion (Nair) was used to remove fur from the axilla to the abdomen exposing the skin of the chest of the anesthetized mouse. The mouse was placed in a supine position onto the echocardiographic heated platform which maintains the body temperature of the animal during the procedure[33]. The limbs of the mice were taped down and a toe or tail pinch was used to check for proper sedation. Small increments of additional anesthetic were given if the mouse was not well sedated. Preheated ultrasound gel was applied to the left anterior side of the chest overlying the heart. The transducer was placed in this same position.

The heart was visualized in B-mode from parasternal short-axis view to obtain a 2-dimensional (2D) mid-ventricular position. A mid-ventricular image was obtained at the level of the papillary muscles below the tip of the mitral valve[34]. Once this image was successfully obtained, we entered M-mode and placed the cursor 90 degrees to the anterior and posterior walls of the LV. In M-mode we measured the systolic parameters in 1-dimension (1D). We measured the LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), LV anterior wall thickness during systole and diastole (LVAWST and LVAWDT), LV posterior wall thickness during systole and diastole (LVPWST and LVPWDT) and Heart Rate (HR). The Vevo770 imaging

system calculated the fractional shortening (FS), ejection fraction (EF), LV mass, LV end diastolic volume (LVEDV) and LV end systolic volume (LVESV). The FS and EF can also be manually calculated as follows: FS = [(LVEDD-LVESD)/(LVEDD)]*100; the EF is calculated with the Teichholz equation[34]. Stroke volume (SV) and cardiac output (CO) were calculated separately with the following equations: SV = LVEDV-LVESV and CO = HR X SV.

Next, PWD was used to measure flow of blood from the left atria (LA) into the LV. With the PWD, we measured the velocity of blood flow across the mitral valve by placing the cursor on the mitral valve. The first peak labelled E corresponds to the velocity of blood flow from the LA to the LV during the early phase of filling. This is after the isovolumetric relaxation and the pressure in the atria is high enough to cause the opening of the mitral valve. The slope of the E wave is known as the deacceleration time (DT) which corresponds to the velocity at which flow returns to baseline at the end of passive filling of the LV. Atrial systole, which contributes to the final phase of the LV filling, is indicated by the A wave which is the velocity of blood flow during the active emptying of blood from the LA to the LV. After the atrial systole, ventricular filling is complete and the mitral valve closes when the LV pressure starts rising during the systole. As the LV starts ventricular systole to build up the pressure needed to open the aortic valve, there is zero blood flow in or out of the LV. This phase is called the isovolumetric contraction. When the intraventricular pressure equals the pressure in the aorta, it causes the aortic valve to open and the blood is ejected. This results in a negative flow velocity, due to the blood leaving the LV. The time between the end of the A wave and the start of ejection is the isovolumetric contraction time (IVCT). The duration of the flow measured during the ejection corresponds to the ejection time (ET). The ET flow is retrograde because blood is leaving the ventricle, therefore an outflow is

being measured whereas, E and A measured an inflow of blood into the ventricle. After the ET, the LV pressure drops and when it becomes lower than the aortic pressure, the aortic valve closes. This is a period of ventricular relaxation at which time the ventricle relaxes prior to the opening of the mitral valve and the beginning of the passive ventricular filling (E wave). It is therefore, called the isovolumetric relaxation phase. The duration of time between the end of ET and start of E is the isovolumetric relaxation time (IVRT). Therefore, the PWD images acquired from the echo probe show a repeat cycle of waves in the following order: E, DT, A, IVCT, ET, IVRT. The sum of IVCT + ET + IVRT = AE.

Following the PWD measurements, we used TDI to determine the velocity of the mitral valve. The cursor was placed on the annulus of the mitral valve. In the image received from the probe, E’ is the early ventricular diastolic velocity of the mitral annulus and A’ is the late ventricular diastolic velocity of the mitral annulus[33,34]. Both E’ and A’ appear retrograde because the annulus of the mitral valve moves towards the RA as the ventricle is being filled. The anterograde velocity wave is S' which is the systolic velocity of the annulus when the atria contracts causing the annulus of the mitral valve to move anterograde with respect to the LV.

In the same TDI mode, we moved the cursor to the tricuspid valve to measure the systolic velocity of the tricuspid valve denoted as S’RV. Similar to S’ the S’RV is an anterograde measurement. By moving our cursor to the annulus of the tricuspid valve, in M mode we measured the Tricuspid annular plane systolic excursion (TAPSE). The TAPSE is a measure of right ventricle (RV) function (apex to base shortening) and it closely correlates with RVEF[35]. TAPSE is determined by measuring the distance of movement of the tricuspid annulus.

Echocardiography with TTE, PWD, and TDI was used to measure both the systolic and diastolic cardiac function of the mice. The above measurements were taken in all the mice and the data was inserted into an excel database. The FS and EF determined the systolic function and the IVRT determined the diastolic function. Myocardial Performance Index (MPI), also known as the Tei index is calculated for the LV by using either (IVCT+IVRT)/ET or (AE –ET)/ET. The MPI takes into account both systolic and diastolic parameters of the LV, resulting in an index of myocardial performance.

**Oral glucose tolerance test**

We waited two to three days for the mice to recover from anesthesia after each echocardiography. Then we performed an oral glucose tolerance test (OGTT) to obtain the 12-hours fasting glucose as well as perform glucose tolerance in the mice at the baseline, 5-week and 9-week time points in the study. The OGTT assay was performed to determine whether the mice showed signs of diabetes and metabolic dysfunction due to the consumption of WD. The OGTT was performed using (Clarity Diagnostics). The mice were fasted in clean cages for 12 hours, while having access to water. The cages were changed prior to fasting to ensure the removal of food particle and excrement from the bedding of the cages. Prior to the measurement of fasting glucose, a glucose solution was prepared at 20% volume per weight in $H_2O$ for the OGTT.

Fasting glucose was measured by obtaining a small drop of blood from the tail vein of the mice. Following fasting glucose measurements, the mice were fed 2g/kg of the 20% glucose

solution with a 1cc syringe (Becton Dickinson) that had an oral gavage needle. Glucose levels were measured at 15 minutes, 30 minutes, 60 minutes and 120 minutes following the oral gavage of glucose. The glucose measurements were collected into an excel database. At the end of the 120 minutes, the OGTT was complete and food was placed on the cage tops.

Oral gavage simulates the manner in which the mouse and human would normally obtain glucose therefore controlling for any variabilities in glucose digestion and absorption that may occur during the digestive processes.

**Food intake and body weight**

Food Intake (FI) was taken daily for the first two weeks of the study and weekly for the rest of the study while body weight (BW) was monitored weekly. By collecting FI, we were able to estimate the weekly energy/caloric intake of mice. Collecting the BW allowed us to monitor any change in weight due to the WD and make comparisons to the mice fed with SD.

**Sample collection**

All mice were euthanized under deep anesthesia (sodium pentobarbital 70-100 mg/kg) through exsanguination after we performed echocardiography and OGTT at the 9-week time point. We collected blood from the inferior vena cava (IVC) using 1cc tuberculin syringe and a 25 gauge 5/8-inch needle (Becton Dickinson). The blood was collected in Vacutainer® PST Gel Lithium Heparin tubes (light-green top tubes; Becton Dickinson) and put on ice. The light-green top tubes

were centrifuged at 2000 revolutions per minute (rpm) for 10 minutes at 4˚C to separate plasma from the rest of the components of blood. The plasma was then collected into 1.7ml tubes, snap frozen in liquid nitrogen and stored at -80˚C.

At the time of sacrifice, the heart was collected in addition to the blood. It was cut into two parts, one part was snap frozen in liquid nitrogen then stored at -80˚C, the second part was fixed in 10% formalin.

**ELISA**

Systemic levels of IL-18 were measured in the in the plasma using an enzyme-linked immunosorbent assay (ELISA). The plasma IL-18 levels were measured with a commercially available murine IL-18 ELISA kit (MBL International) sandwich assay.

ELISA is a plate-based assay which designed for the detection and quantification of various substances including cytokines. In the IL-18 kit, 96 well plates come pre-coated with a monoclonal antibody against murine IL-18. The kit comes with a standardization reagent which allows us to calibrate our assay. We made our calibration solution (the standard) with known concentrations of IL-18 and added 100µL from each of our plasma samples to the pre-coated wells. After incubating the pre-coated wells with the standard and our plasma samples, we washed the wells. At this point only the murine IL-18 bound to the coated monoclonal antibody remained in the wells. A second anti-mouse IL-18 monoclonal antibody, conjugated to a Peroxidase enzyme is added to the wells and allowed to incubate with the IL-18 bound to the pre-coated antibody. The wells are washed

again, leaving behind only the peroxidase conjugated antibody that is bound to the IL-18. A peroxidase substrate mixed with a chromogen is added to the wells. The reaction between the peroxidase and its substrate results in the development of color. The more the IL-18 in the well, the more the peroxidase conjugated antibody and therefore the more color developed in the well. The enzyme reaction was stopped by the addition of an acid solution. The optical density (OD) of the wells was measured at 450 nanometer (nm) with a microplate reader. The results were entered into excel and by using the slope of the OD of the wells containing the known concentration of IL-18 standard, we quantified the amount of IL-18 in each of our samples.

**Statistical Analysis**

The statistical analysis was performed using SPSS 24.0 package for Windows. Differences within each group were analyzed using the Student's T test for paired data, whereas differences between groups were assessed using Student's T test for unpaired data. Unadjusted two-tailed P values $\leq 0.05$ were considered statistically significant.

## RESULTS

### Food and Energy Intake in the SD, WD and treatment groups.

The WD group (N=8) had a 12% (P≤0.01) lower food intake (g) than the SD group (N=11-15). The treatment group (N=6) had a 10% (P≤0.01) lower food intake than the SD group. However, both the WD and the treatment group had higher energy intake (kcal). The WD group had a 32% (P≤0.01) higher caloric intake and the treatment group had a 31% (P≤0.01) higher caloric intake than the SD group.

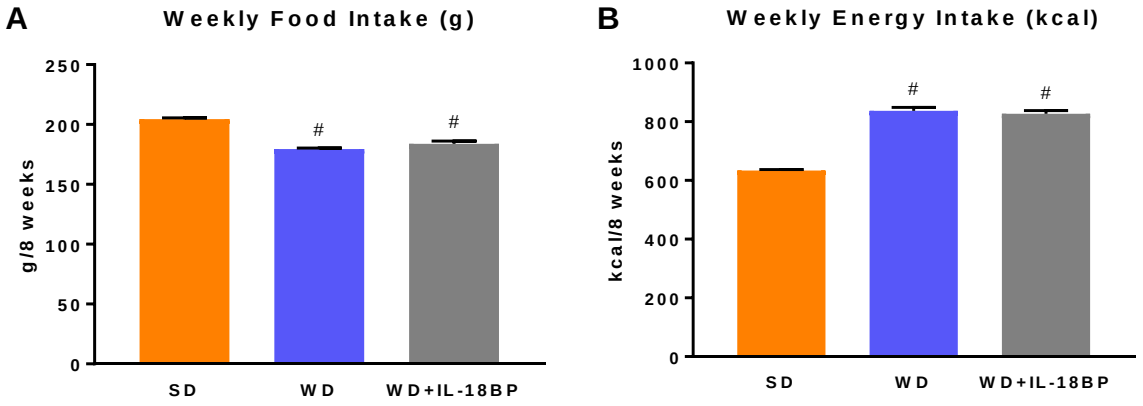


**Figure 2: Average weekly food intake (A) and caloric intake (B) per mouse.** #P≤0.01 vs SD.

### Weekly body weight measurements in the SD, WD and treatment groups.

The weight of the WD group (N=8) increased by 23% (P≤0.01) and the weight of the treatment group (N=6) increased by 22% (P≤0.01) whereas the weight of the SD group increase by 12% from baseline. At the end of 9 weeks, the SD group (N=15) gained 2.9g, the WD group gained 7.2g, and the treatment group 7.3g from baseline. Therefore, the WD group gained +153%

or 4.4g more weight than the SD group (P≤0.01) and the treatment group gained 156% or 7.3g more than the SD group (P≤0.01).

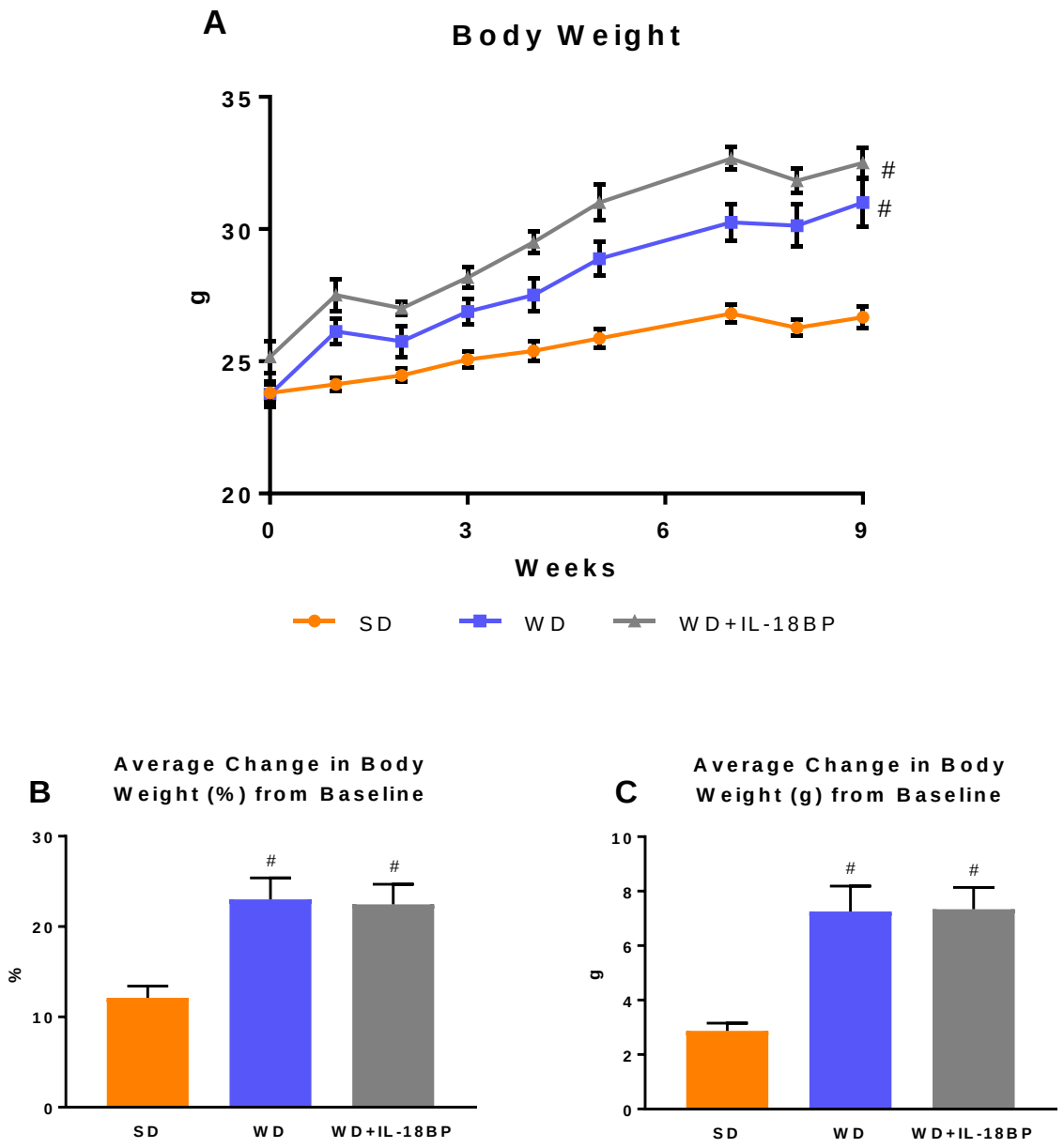


**Figure 3: Weekly body weight.** The body weight was maintained weekly (A), and the change in body weight from baseline was calculated for each group in percentage (B) and in grams (C). #P≤0.01 vs SD.

**Oral glucose tolerance test area under the curve in SD, WD and treatment groups.**

The AUC calculated during the OGTT gives an overall measure of the capability of the organism to handle a glucose load. Increased AUC indicates glucose intolerance. At baseline, the mice in the WD group had lower glucose values at all the time points. At 5 weeks and 9 weeks, a higher peak plasma glucose level was found in the WD groups, and the IL-18BP treatment did not alter the glucose levels at all time points. These were reflected in a significant lower AUC of the WD group when compared to the SD group at baseline ($P \leq 0.01$). At 5 weeks, however, we did not find any significant differences when comparing the WD groups to the SD group, and both the WD group and the WD+IL-18BP treatment group had higher AUCs than the SD group at 9 weeks (Figure 4).

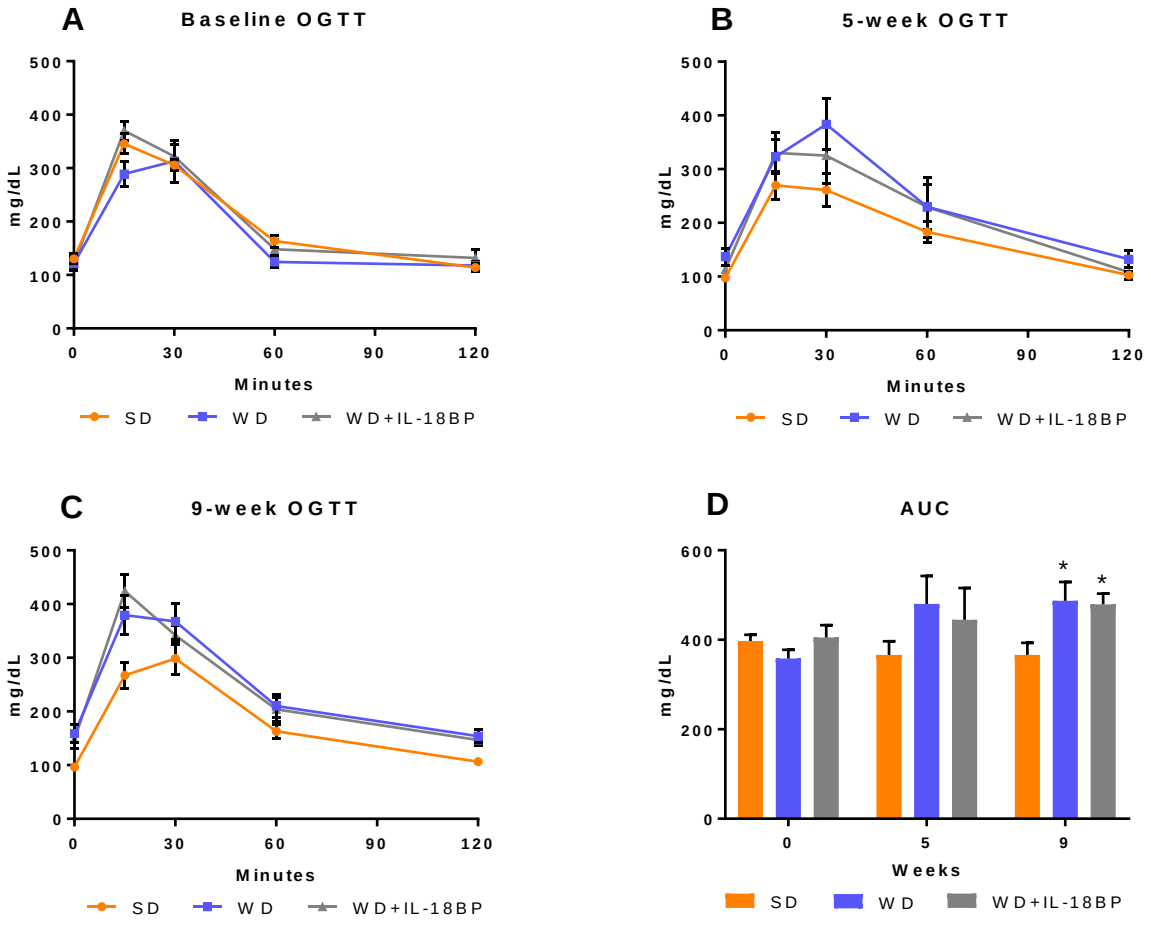


**Figure 4: OGTT and AUC.** The oral glucose tolerance tests were graphed at baseline (A), 5 weeks (B), 9 weeks (C) in addition to the area under the curve of each curve (D). *P≤0.05 vs SD; #P≤0.01 vs SD.

**Glycemia levels in SD, WD and treatment groups.**

The fasting glycemia and 2-hour glycemia recorded during the OGTT assay are presented in Figure 5. After 5 weeks of the study, there was a significant increase in the fasting glycemia of the WD group (N=8) when compared to the SD group (+40%, P≤0.01). Although, the fasting glycemia of the treatment group (N=4-6) was not significantly different than the SD group (N=13-

15), it was 15% higher at 5 weeks. The 2-hour glycemia of both the WD group (+28%) and the treatment group (+5%) were higher than the SD-fed mice at 5 weeks. After 9 weeks, the WD group had a 64% higher fasting glycemia than the SD group (P≤0.01). The fasting glycemia of the treatment group was 56% higher than the SD group (P≤0.01) at 9 weeks. At the same time point, the 2-hour glycemia of the WD group (+45%; P≤0.01) and treatment group (+38%; P≤0.01) were both significantly higher than the SD group. The fasting and 2-hour glycemia values of each group at baseline, 5 weeks and 9 weeks are shown in Table 2 and 3**.**

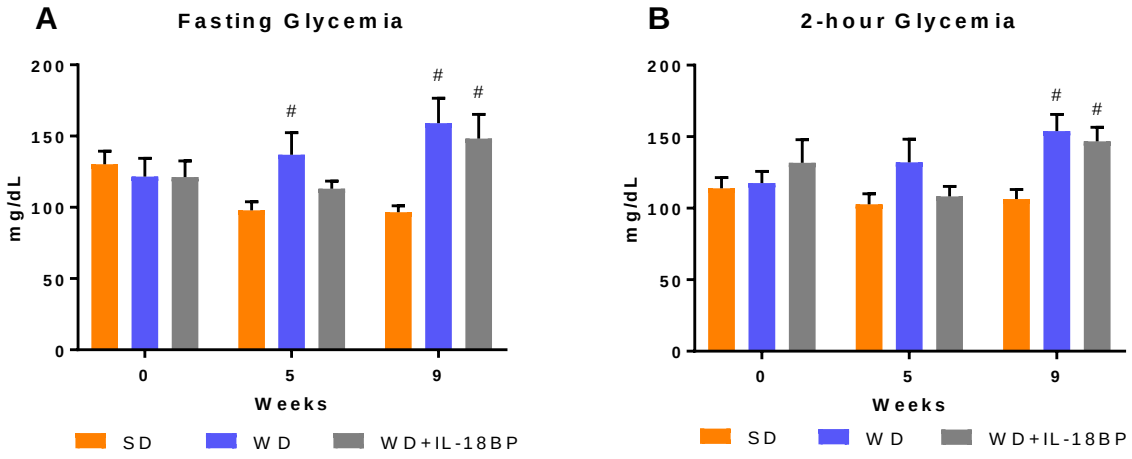


**Figure 5: Glycemia.** Effects of WD on the fasting (A) and the 2-hour glycemia (B), taken at baseline, 5 weeks and 9 weeks. Groups fed with WD had higher glycemia than the SD group. #P≤0.01 vs SD.

**Fasting Glycemia**

| Groups | Baseline | 5 weeks | 9 weeks |
|---|---|---|---|
| SD | 135±10 | 98±6 | 97±5 |
| WD | 122±13 | 137±16 # | 159±18 # |
| Treatment Group | 121±11 | 113±5 | 148±17 # |

**Table 2: Fasting Glycemia at baseline, 5 weeks and 9 weeks (mg/dL).** A glycemia under 100mg/dL is within the normal range. Glycemia of 100 to 125mg/dL is diagnostic of prediabetes/impaired fasting glucose and glycemia above 126mg/dL is diagnostic of diabetes[36–38]. #P≤0.01 vs SD.

**2-hour Glycemia**

| Groups | Baseline | 5 weeks | 9 weeks |
|---|---|---|---|
| SD | 114±8 | 102±7 | 106±7 |
| WD | 117.5±8 | 132±16 | 154±12 # |
| Treatment Group | 132±16 | 108±7 | 147±10 # |

**Table 3: 2-hour Glycemia values at baseline, 5 weeks and 9 weeks (mg/dL).** 2-hour glycemia under 140mg/dL is within the normal range. 2-hour glycemia of 140 to 199mg/dL is diagnostic of prediabetes/impaired glucose tolerance and above 200 mg/dL is diagnostic of diabetes[36–38]. #P≤0.01 vs SD.

**Measurement of plasma IL-18 in the SD group and WD group.**

We tested the ability of WD to induce IL-18 in the mouse plasma. This was a confirmation experiment that we performed in the SD group (N=8) and the WD group (N=8). We found that the WD had a 91% ($P \leq 0.05$) higher IL-18 plasma levels than the SD-fed mice.

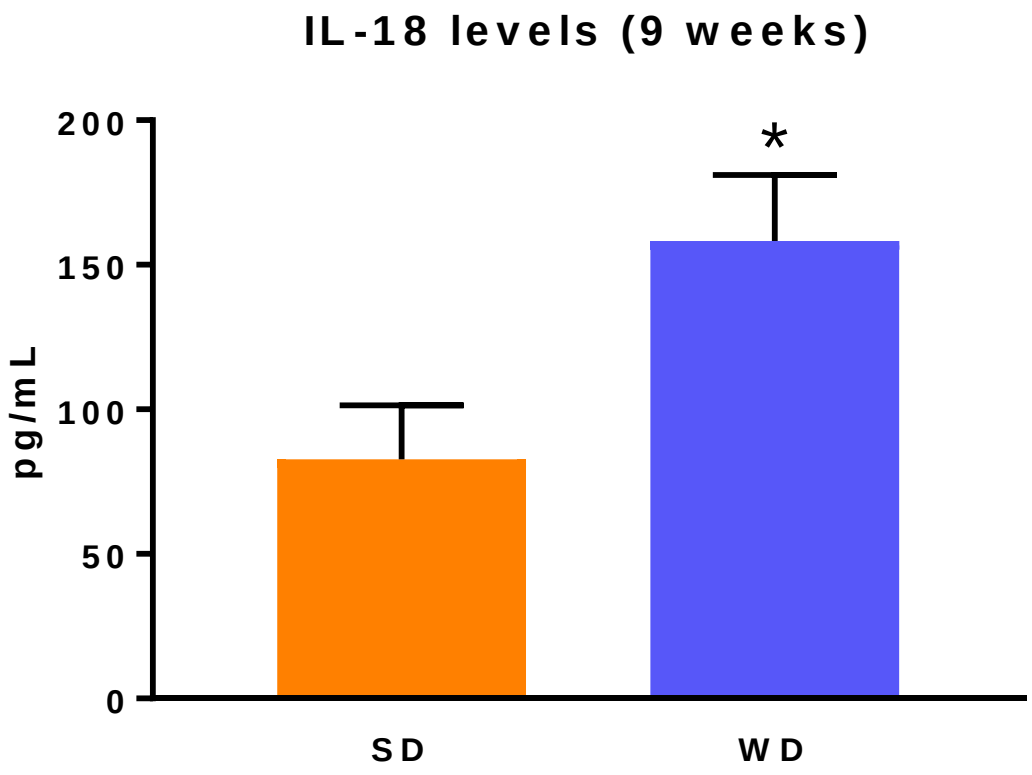


**Figure 6: Plasma IL-18.** Plasma IL-18 levels were measured using an ELISA for murine IL-18. *****$P \leq 0.05$ vs. SD.

**IL-18BP treatment improves the isovolumetric relaxation time.**

The IVRT is a measure of the diastolic function. The WD induced a significant increase in the IVRT, at 5 and 9 weeks, when compared to the baseline or to the SD group (Figure 7). The administration of the IL-18BP reverted this trend. In fact, in the group later treated with the IL-18BP from week 7 to 9, the IVRT was significantly decreased compared to the same group at 5 weeks (-41%, P≤0.01) and to the WD group at 9 weeks (Figure 7). Moreover, the IVRT was no longer significantly different from the SD group.

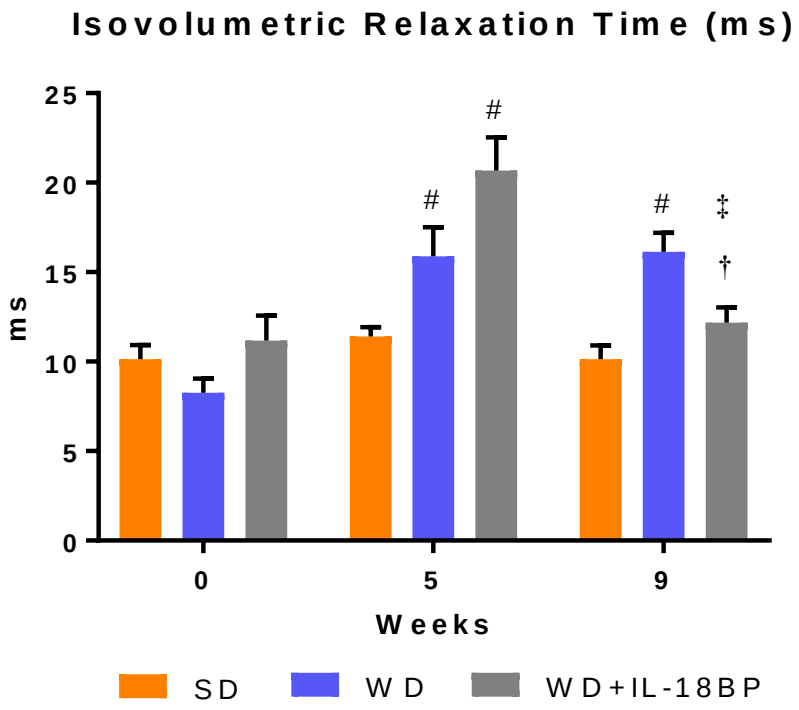


**Figure 7: Isovolumetric Relaxation Time.** Effects of Western Diet on the Isovolumetric Relaxation Time, an indicator of diastolic function. Nine weeks of WD produced an increase in IVRT. A two-week treatment with IL-18BP in the treatment group decreased the IVRT. #P≤0.01 vs SD; †P≤0.01 treatment group at 5 weeks vs treatment group at 9 weeks; ‡P≤0.01 vs WD.

**IL-18BP treatment improves the myocardial performance index.**

The MPI is a measure of both systolic and diastolic function. An increase of the MPI indicates a worsening of function. The WD group had a significant increase in MPI compared to the baseline value and to the SD group at 5 and 9 weeks (Figure 8). Similarly, in the WD+IL-18 BP treatment group, the MPI significantly increased at 5 weeks. Following treatment with IL-18BP from week 7 to 9, the MPI was significantly decreased compared to 5 weeks and to the WD group. These results reflected those observed in the IVRT.

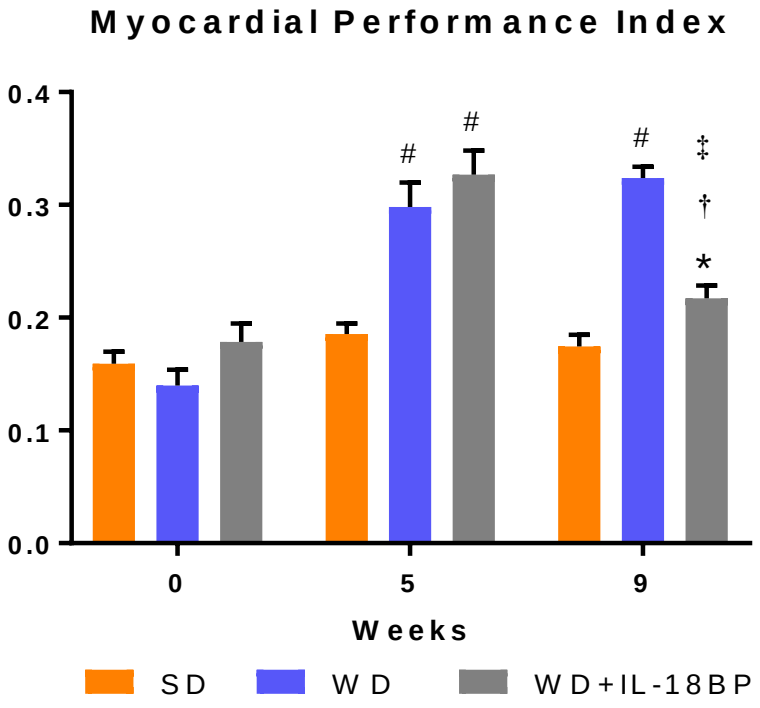


**Figure 8: Myocardial Performance Index.** Effects of Western Diet on the Myocardial Performance Index ((IVCT+IVRT)/ET), an indicator of both systolic and diastolic function. Nine

weeks of WD produced an increase in MPI. A two-week treatment with IL-18BP in the treatment group decreased the MPI. *P≤0.05 vs SD; #P≤0.01 vs SD; †P≤0.01 treatment group at 5 weeks vs treatment group at 9 weeks; ‡P≤0.01 vs WD.

**Measurement of left ventricular ejection fraction in WD-induced cardiomyopathy.**

The LVEF is a direct measure of the contractility of the LV and is a measure of systolic function. We did not detect any significant differences between the systolic function, measured by the LVEF through echocardiography, between the SD group, WD group and treatment group.

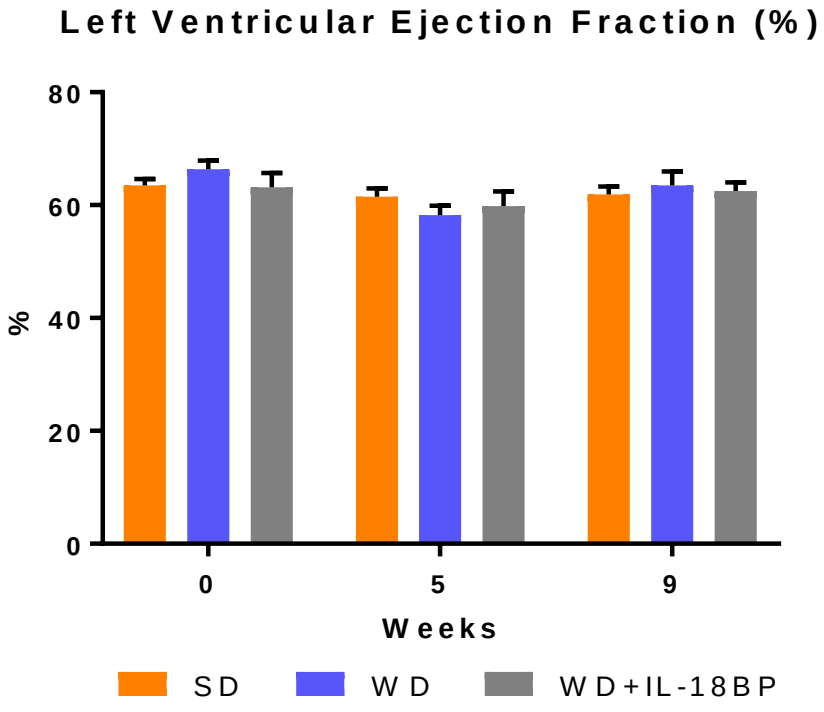

**Figure 9: Left Ventricular Ejection Fraction.** Effects of WD on the left ventricular ejection fraction (LVEF), an indicator of systolic function. Neither nine weeks of WD, nor a two-week treatment with IL-18BP in the treatment group significantly affect the LVEF.

**BW and IL-18 plasma levels have a positive correlation with the IVRT and MPI.**

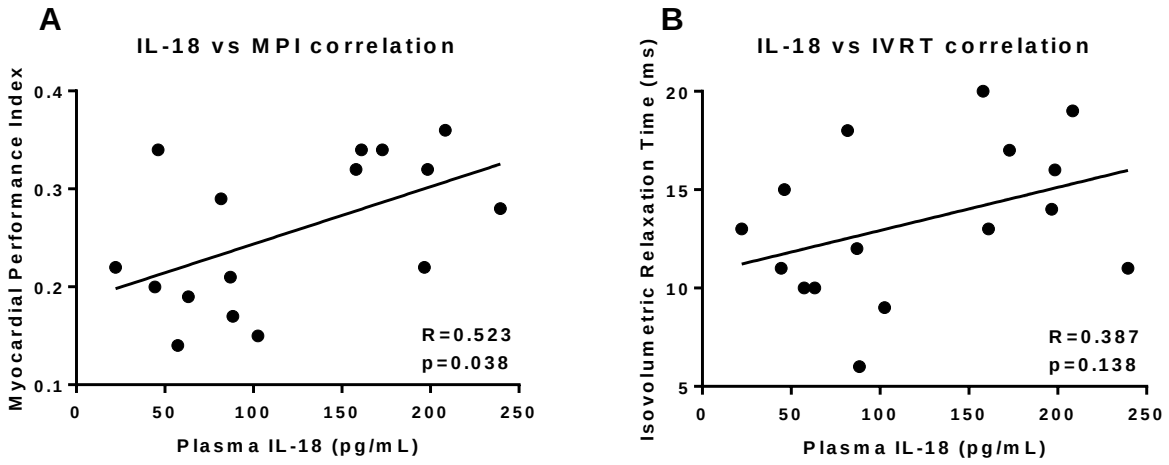


**Figure 10: IL-18 correlations with MPI (A) and IVRT (B).** A Pearson correlation was performed on the levels of IL-18 of the SD group (N=8) and WD group mice (N=8) with the MPI and IVRT. There was a significant positive correlation between the levels on IL-18 and the MPI. There was a non-significant positive trend between the IL-18 levels and IVRT.

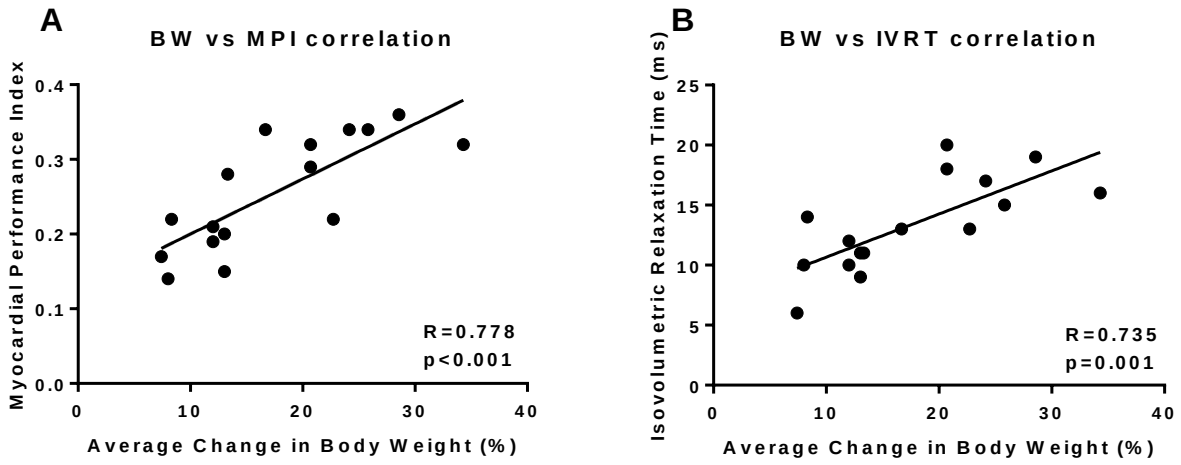


**Figure 11: BW correlations with MPI (A) and IVRT (B).** A Pearson correlation was performed on the BW of the SD group (N=8) and WD group mice (N=8) with the MPI and IVRT. There was a significant positive correlation between the BW and both the MPI and the IVRT.

## DISCUSSION

A chronic state of inflammation is a constant factor in the development of HF. The WD promotes systemic inflammation which contributes to HF[4]. There are many inflammatory markers which contribute to systemic inflammation. One of these is IL-18, as observed in animal studies using WD and in obese patients. Here we confirmed that a diet rich in saturated fat and sugar increases obesity, glucose intolerance and induces the pro-inflammatory cytokine IL-18.

In our lab, using a IL-18 knock out (KO) mouse model, we have established that the lack of IL-18 is sufficient to prevent WD-induced cardiac dysfunction, both diastolic and systolic. Although the KO models help us to investigate the mechanism, the clinical relevance of this type of approach is minimal.

Herein, we investigate a possible translational approach of treating HF with pharmacological IL-18 blockade using rIL-18BP. The use of IL-18BP in the context of WD-induced cardiac dysfunction has not been tested before. We modelled the average American diet and expected that the systemic inflammation driven by a WD would induce cardiac dysfunction (specifically diastolic dysfunction leading to HFpEF) driven by an increase in IL-18[30–32]. In fact, free fatty acids (FFAs) in the WD activate the IL-18 pathway. Previous studies have shown that IL-18 is a cardio-depressive proinflammatory cytokine which induces cardiac dysfunction, both diastolic and systolic and knocking it out reduces this dysfunction[2].

We decided to use a dose of 0.5mg/kg of IL-18BP in the treatment group (N=6). The treatment was started after 7 weeks and IL-18BP was administered daily through IP injections until week 9, allowing for two weeks of therapeutic intervention.

At the end of the two weeks of interventions, the IVRT of the treatment group was significantly lower than the WD group and no longer significantly different from the SD group. An increase in the time needed for the left ventricle to relax, the IVRT, is caused by impaired relaxation which is an indicator of diastolic dysfunction. The higher the IVRT, the worse the diastolic function. The IVRT of the WD+IL-18BP group at 9 weeks was significantly lower than its IVRT at 5 weeks, and was significantly improved versus the WD only. Moreover, there was no difference between the WD+IL-18BP and the SD group at 9 weeks.

Following in the same trend, we saw significant increase in the myocardial performance index (MPI) at 5 weeks and an even higher increase in MPI by 9 weeks in the WD group compared to the SD group. MPI is an index for both the systolic and diastolic function. The higher the MPI, the worse the cardiac function. At 9 weeks, the MPI of the treatment group was still significantly higher than the SD, however it was significantly lower than the WD group. The MPI at 9 weeks of the treatment group was also significantly lower than its MPI at 5 weeks. This may suggest the need for a longer time of intervention or a better titration of the IL-18BP dose.

The IL-18BP dose of 0.5 mg/kg was chosen based on a different basis. First, it was in the effective range of the doses (between 0.1 and 1 mg/kg) that have been used in our lab in a previous study using a model of diastolic dysfunction induced by angiotensin II, a vasoactive neurohormone

that is elevated during HF. Furthermore, it is in the effective dose range that has been shown to work in a mouse model of arthritis[14,39–41]. Interestingly, in this model, doses of 1 mg/kg and above loose effectiveness compared to 0.5 mg/kg. We confirmed this in a pilot study in which we started the treatment with 1 mg/kg of IL-18BP with the WD. We found a neutral effect of IL-18BP treatment when compared to WD only, which was in accordance with the arthritis study. Therefore, we opted for the 0.5 mg/kg dose as a rescue strategy to treat the WD-induced cardiomyopathy.

These results are consistent with other studies which have shown the manifestation of heart failure induced by WD[2,4,42]. However, unlike past studies, we did not see any significant differences in the left ventricular ejection fraction (LVEF) due to WD. In fact we have previously reported that the WD leads to a mild yet significant decrease in LVEF after 8 weeks of WD[2,4]. There are a few possibilities to explain this discrepancy. The strain of the mice used in one of these studies was CD-1 and in the other study we used C57BL/6J mice, as in the present protocol[2,4]. However, both strains of mice came from a different vendor and had different baseline characteristics (higher body weight and bigger heart diameters). In our present study, however, the smaller mice may have eaten a little less than in the prior study, reducing the effect of the WD on the systolic function. These variabilities may contribute to the differences in LVEF in our study and past studies[2,4]. Nevertheless, the WD was still able to induce diastolic dysfunction.

Treatment with 0.5mg/kg IL-18BP did not affect the metabolic parameters. At 9 weeks, the glycemia of the treatment group had significantly increased from week 5 and was significantly higher than the SD group. The AUC of the OGTT curve was significantly higher than week 5 and also significantly higher than the SD group at 9 weeks. The treatment group mice were

hyperglycemic and had impaired glucose tolerance. The group's metabolic parameters had worsened from week 5, consistent with previous evidence that the administration of IL-18BP into the systemic circulation does not affect metabolic homeostasis[19].

The food intake and energy intake of the treatment group were not affected by the treatment with IL-18BP, as they were not statistically different from the WD group. The food intake was significantly lower than the SD group while the energy intake was significantly higher as expected in WD-fed mice. The gain in body weight was very similar for the WD and treatment groups. Treatment with IL-18BP did not affect food intake, energy intake or body weight.

Fasting plasma glucose is used clinically as a tool to determine if a patient is non-diabetic, pre-diabetic or diabetic[36–38]. A fasting glycemia below 100mg/dL is within the normal range, between 100 and 125mg/dL is diagnostic of impaired fasting glycemia and fasting glycemia above 126mg/dL is diagnostic of diabetes[36–38]. The WD group had significant increase in glycemia at 5 weeks and 9 weeks compared to the SD group. The WD group had an increased glycemia at 5 weeks, and the glycemia level increased further at 9 weeks. Although not significantly higher, the treatment group which was also fed with WD, had a higher glycemia, which was in the impaired fasting glycemia range, compared to the SD group at 5 weeks.

Oral glucose tolerance test is an assay performed to access the glucose tolerance of mice. Impaired glucose tolerance signifies metabolic dysfunction. The AUC of the WD group was higher, although not significantly, than the SD group at 5 weeks. By 9 weeks, the AUC of the WD group was significantly higher than the SD group. The AUC of the treatment group at 5 weeks,

was also not significantly different but still higher than the SD group. The glycemia and OGTT data implicate WD as the driver in the development of diabetes and impaired glucose tolerance.

WD has been shown to cause obesity and higher weight in mice than the SD[2,4]. In our study, both the WD group and the treatment group had a significantly higher percentage weight gained from baseline than the SD group. Even though the WD and treatment groups had a significantly lower food intake (g) than the SD group, they had a significantly higher energy/caloric intake (kcal) as the WD has a much higher energy density than the SD. This data suggests that WD causes obesity.

The IVRT and MPI of the SD group and WD group together had a positive Pearson correlation with the levels of plasma IL-18, with the MPI correlation being significant. This is in accordance with our hypothesis that the levels of IL-18 drive the cardiac dysfunction. Additionally, the body weight of the SD group and WD group together had significant positive correlation with both IVRT and MPI. This direct correlation shows a cause and effect between the WD-induced obesity and the development of cardiac dysfunction. These correlations taken together confirm prior results and are in accordance with our data that increase in IL-18 and BW lead to cardiomyopathy.

**Limitations**

This study, as any preclinical study, has inherent limitations. Our results should be viewed as preliminary findings into the attenuation of HF using IL-18BP as pharmacological blocker of

IL-18 in an animal model. Our assessment of HF was limited to echocardiography parameters which are limited by observer variability. Moreover, we cannot access other symptoms of HF such as dyspnea, fatigue, exercise intolerance in rodents as we can in humans. However, in prior studies we have found that 8 weeks of WD induce an increase in LV end diastolic pressure, a sign of HF. Although heart function was improved in the treatment group, we cannot assess whether HF was completely rescued as we could not quantify the other symptoms which characterize HF. Future studies are needed to answer this question, looking at longer treatment with the WD and IL-18BP, with different doses of IL-18BP and by measuring the hemodynamic parameters with LV catheterization.

# CONCLUSIONS

In conclusion, the Western diet, rich in high saturated fats and sugars, induces cardiomyopathy in the mouse as a result of IL-18 driven systemic inflammation. As hypothesized, daily exogenous IL-18BP administration, at the appropriate dose, can improve WD-induced cardiomyopathy. From a translational point of view, since diastolic dysfunction exists in both HF with preserved or with reduced ejection fraction, reversing diastolic dysfunction by intervening on IL-18 signaling, which is cardio-depressive, may have important therapeutic implications in both types of HF. The IL-18BP dose needs to be further fine-tuned to determine which dose would be best for treating HF in WD-induced cardiomyopathy. Further studies are needed to explore the mechanisms underlying the local and systemic inflammatory response to the WD and the role of IL-18 in response to WD and its role in HF. It remains also to be determined whether pharmacological interventions to rising IL-18 levels in response to WD can be suppressed to improve outcomes in patients with HF.

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## VITA

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