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Targeting the CD39/CD73 pathway: New insights into cardiac fibrosis and inflammation in female cardiac surgery patients

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ABSTRACT

Women undergoing cardiac surgery suffer from worse outcomes than their male counterparts. The reasons for this disparity are multifactorial, but the loss of the protective effects of estrogen likely plays a role. Estrogen acts on the CD39/CD73 purine pathway, and loss of estrogen effects may contribute to the increased inflammation seen in post-menopausal women. We aimed to compare CD39/CD73 expression and downstream fibrosis, and inflammation in men and women undergoing cardiac surgery and then used an ovariectomy/high fat diet mouse model to approximate women who present for cardiac surgery to test therapeutics. We found decreased CD39 and CD73 in women compared to men, which was associated with increased fibrosis. Apyrase supplementation (a CD39 mimetic) improved ejection fraction and decreased E/e'. Increased CD73 function (via dipyridamole) decreased fibrosis. This study demonstrates the importance of purinergic dysfunction in cardiovascular disease in women and presents two potential therapeutics to improve cardiac health via manipulation of purine pathways.

1. Introduction

Cardiovascular disease is the leading cause of mortality in women both in the United States and globally [1]. Despite the burden of disease, women suffer from higher mortality, morbidity, and prolonged recovery following cardiac surgery [2–4]. The American Heart Association highlighted the need for strategies to address these disparities in outcomes in 2011 [5], but little progress has been made [6]. Societal biases, including under-recognition of disease because of "atypical" symptoms, less aggressive treatment [7], and lower rates of referral for surgery [1] certainly play a role in these outcomes. However, estrogen loss during the menopause transition also plays a pivotal role in the development of cardiovascular disease [8]. Postmenopausal women suffer from accelerated chronic low-grade inflammation [9,10], which is accompanied with an increased incidence of metabolic sydnrome [11] and impaired mitochondrial function [12] – this constellation of findings likely contributes to an impaired stress response that contributes to the disparities observed for women undergoing cardiac surgery.

Chronic inflammation drives the development of microvascular disease, fibrosis, and the resulting increase in diastolic dysfunction that contributes to poor outcomes for women [13]. The purinergic pathway is critical for inflammation modulation and is modulated by estrogen under hypoxic conditions [14], making it a promising subject for investigation to explain the differences in inflammation seen in women compared to men during cardiac surgery. CD39 is the enzyme responsible for conversion of ATP to adenosine monophosphate (AMP) and CD73 is, in turn responsible for the conversion of AMP to adenosine. Adenosine is then cleared via ENT1 [15]. This CD39-CD73-ENT1 pathway is responsible for the clearance of extracellular ATP (eATP) and is an essential component of balancing pro- and anti-inflammatory responses in humans and are influential in cardiac protection and estrogen signaling [16,17]. Estrogen signaling boosts expression of

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adenosine receptors (ADORA) and enhances the expression of CD39 [18]. The interaction between adenosine and its receptors initiates intracellular signaling necessary for protective microvascular responses, including barrier integrity and inflammatory adaptation in tissues such as the kidney and skin [19–21]. Adenosine is generally considered protective to the myocardium [22]. Conversely, lower levels of CD39 are associated with heart failure [23]. The specific role of purinergic signaling and cardiac disease in older females has not been described.

Estrogen plays a positive role in dynamically regulating purinergic pathways, which could potentially explain the increased risk of developing cardiac dysfunction in postmenopausal women. In this study, we aim to define the role of purinergic signaling in women compared to men presenting for cardiac surgery and explore treatment options aimed at these pathways using a mouse model. We conducted a prospective analysis assessing fibrosis and purinergic expression of patients undergoing cardiac surgery at a single academic center. We hypothesize that in cardiac surgery, CD39 and CD73 levels are decreased in response to stress, with a disproportionate decrease in the expression of CD39 in females leading to worse outcomes. To model women presenting for cardiac surgery, we used an ovariectomy and high-fat diet female mouse model. We used this model to test therapeutics to restore purine homeostasis and cardiac function with apyrase, a CD39 agonist, and dipyridamole, an ENT inhibitor, in mice.

2. Methods

This study was approved by the Institutional Review board of Beth Israel Deaconess Medical Center. All methods were carried out in accordance with relevant guidelines and regulations. Data involving human research participants was de-identified and performed in accordance with the Declaration of Helsinki. Patients undergoing elective coronary artery bypass graft or aortic valve replacement that required use of CPB were prospectively recruited in this study over a span of three years from August 2018 to August 2021. Patients under the age of 18 or patients undergoing emergency cardiac surgeries were excluded. All patients provided written informed consent prior to enrollment in the study. Females on hormone replacement therapy were excluded. For the purposes of the study, sex was defined as self-reported sex at birth. All patients reported sex and gender at the time of surgery that was congruent with their self-reported sex at birth. Myocardial blush grade from preoperative cardiac catheterization was assessed by an experienced interventional cardiologist (MFP). All patients underwent a pre-defined echocardiography protocol based on the 2016 American Society of Echocardiography Guidelines for the assessment of diastolic dysfunction. Diastolic dysfunction was defined as two or more abnormal left ventricular filling indices based on e', E, their ratio (E/e'), LA volume index (LAVI), and peak tricuspid regurgitation jet velocity.

2.1. Sample collection and processing

Ten mL of blood was collected through an arterial catheter after induction of general anesthesia and prior to incision. Pre-cardiopulmonary bypass right atrial tissue was harvested during cannulation (before administration of cardioplegia). Post-cardiopulmonary bypass right atrial tissue sample was harvested from between the purse strings during removal of the venous cannula following separation from cardiopulmonary bypass. Samples were snap frozen in liquid nitrogen and subsequently stored in a secure -80 °C freezer. Tissue lysates were prepared through homogenization with commercially prepared RIPA buffer. For electrophoresis, 30 μg of protein was loaded and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking, membranes were incubated with CD39 (Invitrogen, PA5–80587), CD73 (cell signaling, 13160S), adenosine receptor 2 A (ADORA2A) (abcam, ab3461), adenosine receptor 2B (ADORA2B) (abcam, ab40002). HRP-linked secondary antibody and chemiluminescent substrates were used to visualize signal strength of membranes using Chemi Doc. Image J (National Institutes of Health, Bethesda, MD) was used to perform densitometry analysis for quantification of signal intensity. Western blot images were quantified and normalized using densitometry of housekeeping proteins glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and/or beta-actin. Immunohistochemical staining was performed on formalin fixed, paraffin embedded human atrial tissue sections. Slides were stained for fibrosis with Direct Picrosirius stain after slide section (Sigma, Cat # 365548). Slides were separated into four distinct regions and analyzed at 10× magnification using ImageJ (National Institutes of Health, Bethesda, MD) for quantification of areas of fibrosis. P2X7 distribution in atrial tissue was also performed on sectioned tissues (Invitrogen, Cat # PA5–28020) Serum adenosine deaminase activity was measured using Sigma Aldrich ADA colorimetric assay (EPI023).

2.2. Cell culture and extracellular ATP assessment

Primary Human Umbilical Vein Endothelial Cells (HUVEC) isolated from human umbilical vein were purchased from Gibco™ in cryopreserved vials and grown in the endo cell growth medium (Fisher Scientific). Cultured HUVEC were first seeded at a density of 10,000 cells per well in a 96-well opaque-walled culture plates and were exposed to the same volume of saline (control), estrogen (1 μM) or tamoxifen (10 μM) solution for 30 min (Group: Control, Estrogen, Tamoxifen, in each group, $n = 16$). Then, cobalt chloride ($CoCl_2$) was added to half of the wells of each group to create the hypoxia environment (Subgroups: Control Hypoxia, Estrogen Hypoxia, and Tamoxifen Hypoxia, in each group, $n = 8$). RealTime-Glo™ Extracellular ATP Assay (Promega) was used to measure eATP level in all subgroups. Briefly, it uses a bioluminescence detection chemistry to measure ATP that has been released into the cultured cell environment as a result of cell death, stress or activation. Luminescence measurements were taken every 2 min until the extracellular ATP levels normalized using a multimodal plate reader.

2.3. Mouse study design

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at BIDMC and adhered to the National Institutes of Health guidelines for the care and use of laboratory animals under the Animal Welfare Act and followed ARRIVE guidelines.

Forty female C57BL/6 mice were obtained from Charles River Labs (Wilmington, MA). Six-week-old mice were housed in box cages, maintained on a 12:12-h light-dark cycle, and fed for 24 weeks either a normal diet (LFD: D09100304; 10 % of total calories from fat, which consists of 5.5 % of total calories from soybean oil and 4.5 % kcal from lard) or High-Fat Diet (60 % of total calories from fat, which consists of 5.5 % from soybean oil and 54.5 % from lard) purchased from Research Diets (D09100310i, New Brunswick, NJ). At 12 weeks, twenty mice underwent ovariectomy (OVX). Ten of these mice were continually fed a high-fat diet (HFD). There were four groups in total, each consisting of 10 mice: a control (wild type), HFD, OVX and OVX + HFD group.

At week 24, animals were anesthetized with isoflurane at 2.5 %. Fat mass, lean mass, and total body weight were measured with EchoMRI 3-in-1 (EchoMRI; Echo Medical Systems, Houston, TX). Echocardiography was performed to assess cardiac function using a 40-MHz MXX550D linear array transducer (FUJIFILM Visual-Sonics) by trained echocardiographers (YB, RM). The mice were then euthanized, and plasma was collected via whole blood terminal cardiac puncture. Cardiac tissue was snap frozen in liquid nitrogen and stored in a -80 °C freezer. Mouse tissue lysates were prepared through homogenization of whole cardiac tissue in RIPA buffer. Human tissue lysates were prepared through homogenization of atrial tissue with commercially prepared RIPA buffer. 15 μg of protein was loaded for electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking, membranes were incubated with CD73 (cell signaling 13,160), and CD 39 (cell signaling 14,481). ENT1 (Invitrogen, PA5–116468), ADA (Abcam, ab217846).

HRP-linked secondary antibody and chemiluminescent substrates were used to visualize signal strength of membranes using Chemi Doc. Image J 3.0 (National Institutes of Health, Bethesda, MD) was used to perform densitometry analysis for quantification of signal intensity. Western blot images were quantified and normalized using densitometry of housekeeping proteins glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.4. Rescue experiments

2.4.1. Dietary intervention and ovariectomy

39 C57BL/J6 mice were split into 2 groups and fed a high fat diet ($n = 29$) and control diets ($n = 10$) starting at 8-weeks of age. At 12 weeks 2 groups were ovariectomized and continued the designated high fat or control diet until cardiac puncture.

2.4.2. Purinergic pathway treatments

At week 29–32, the high-fat diet and ovariectomized group were separated into two treatment groups and experimental control (saline). These groups were exposed to 7 days of IP injections once a day of saline control ($n = 6$), apyrase 200 U/Kg ($n = 9$) or dipyridamole 20 mg/kg (n = 9). Apyrase (#A6410, Sigma Aldrich Inc. St. Louis, MO. USA) increases CD39, a transmembrane protein breaking down eATP to ADP and AMP. Dipyridamole (#D9766, Sigma Aldrich Inc) inhibits the bidirectional adenosine transmembrane protein ENT, and prevents uptake of adenosine.

2.4.3. Data acquisition

EchoMRI and echocardiography data were gathered as described above. Cardiac puncture and tissue harvesting were also performed as previously described. Immunohistochemical staining was performed on formalin fixed mice cardiac tissue sections. Slides were stained for fibrosis with Direct Picosirius stain after slide section (Sigma, Cat # 365548) and were separated into four distinct regions and analyzed at 10× magnification using ImageJ for quantification of areas of fibrosis. Extracellular adenosine levels were determined enzymatically with the help of a fluorometric adenosine assay (abcam, Cat# ab211094). (Figs. 5 and 6).

2.5. Statistical analysis

Data analysis and visualization was performed using SPSS (Version 27, IBM Corporation) and/or Prism GraphPad 9.0 (GraphPad Software, Inc., La Jolla, CA, USA). A descriptive analysis of all demographic variables including features of hospital stay was conducted. Shapiro-Wilk test for normality ($p > 0.05$) was utilized to assess all continuous demographic and echocardiographic variables to satisfy the assumption of normality between genders. For normally distributed data, mean ± standard deviation was used to describe central tendencies while median (interquartile range) was used to describe central tendencies for skewed data. Independent sample *t*-test was used to compare means between male and female patients, while dependent *t*-tests were used to compare samples pre- and post-bypass. To assess differences in dichotomous variables, Pearson's chi-squared test was utilized. A *p*-value <0.05 was considered statistically significant.

For mice and cytokine data, independent sample t-test for two independent groups was used for data expressed using means. Mann Whitney *U* tests were used for medians and IQR if the data was not normally expressed. Results of western blotting, immunohistochemistry and quantitative assays were compared between pre- and post-CPB patients using an independent sample *t*-test unless stated otherwise. Scatter plots were created to represent individual data points.

3. Results

Forty female and 40 male patients presenting for elective cardiac surgery were enrolled in the study. The male and female groups were of similar age, body mass index, and underwent similar types of surgery. The groups had similar comorbidities, cardiopulmonary bypass and aortic cross clamp times. Women had a significantly longer length (5.45 ± 1.92 days 7.98 ± 6.31 days, $p = 0.021$) of stay and were significantly more likely to be diagnosed with diastolic dysfunction (25 % vs 55 %, $p = 0.034$) (Table 1).

Women also had significantly more fibrosis and a corresponding lower myocardial blush grade, which implies worse microvascular tissue perfusion (Fig. 1A, B). There was a significant decrease in CD39 expression post-bypass in both men and women, but women ended with significantly lower CD39 expression. Both men and women had a decrease in CD73 expression post bypass, but women had a lower expression than men both before and after bypass. Men had significantly higher ADA expression (Fig. 1C). While not significant, women had a trend towards increased P2X7 while men had a trend towards decreased P2X7 following bypass (Fig. 1D). IL-22, a wound healing a tissue regeneration cytokine [24], was higher in men ($p < 0.01$) (Fig. 1E). Pro-inflammatory markers MDC, leptin, and ENA-78 were higher in women (Fig. 1E, F).

We were unable to measure extracellular ATP in vivo due to its short half-life. In lieu of in vivo data, we performed a cell culture experiment to assess the effect of estrogen on eATP production in the setting of hypoxia. Hypoxia in the setting of estrogen blockade via tamoxifen increases eATP in HUVEC cells (Fig. 2).

To approximate women presenting for cardiac surgery, we treated female C57/BL6 mice with ovariectomy (estrogen loss) and high-fat diet. High fat diet increased body weight, decreased systolic function, and increased LV mass. High fat diet plus ovariectomy increased fibrosis, and leptin was increased in both high-fat diet and ovariectomy (Fig. 3).

After validating the mouse model, we tested two possible therapeutic options. Apyrase, a CD39 agonist, and dipyridamole, an ENT-1 inhibitor. Both apyrase and dipyridamole were associated with lower weight, lower fat mass, and higher lean mass (Fig. 4A). Apyrase was additionally associated with decreased ratio of heart to body weight, better systolic

Table 1
Patient demographic and clinical characteristics.

	Male ($n = 40$)	Female ($n = 40$)	p-value
General characteristics			
Age, yr	66.43 ± 8.27	63.70 ± 9.71	0.18
Body mass index, kg/m2	30.24 ± 5.99	30.39 ± 6.50	0.92
Type of surgery			
CABG	39 (97.5)	33 (82.5)	0.24
AVR	1 (2.5)	5 (12.5)	
CABG + AVR	2 (5.0)	2 (5.0)	
Comorbidities			
Hypertension	36 (90.0)	35 (87.5)	0.72
Diabetes mellitus	14 (35.0)	21 (52.5)	0.12
Heart failure	4 (10.0)	7 (17.5)	0.33
Coronary artery disease	35 (87.5)	27 (67.5)	0.032
Transient ischemic attack	6 (15.0)	5 (15.0)	1.00
Renal failure	5 (12.5)	7 (17.5)	0.53
Hypothyroidism	4 (10.0)	9 (22.5)	0.13
CPB			
CPB time, min	96.06 ± 27.46	92.83 ± 27.65	0.60
Clamp time, min	79.05 ± 24.54	73.91 ± 22.83	0.34
Postoperative outcomes			
Atrial fibrillation	17 (42.5)	15 (37.5)	0.65
Cardiac arrest	0 (0.0)	2 (5.0)	0.15
Reintubation	1 (2.5)	1 (2.5)	0.99
Renal failure[a]	0 (0.0)	3 (7.5)	0.077
Length of stay, day	5.45 ± 1.92	7.90 ± 6.31	0.021
Diastolic dysfunction	10 (25.0)	22 (55.0)	0.034

Data presented as mean ± standard deviation or n (%). AVR = aortic valve replacement; CABG = coronary artery bypass graft; CPB = cardiopulmonary bypass.

[a] Criteria for renal failure = fold change in serum creatinine >2.5 from baseline or the need for temporary/permanent dialysis.

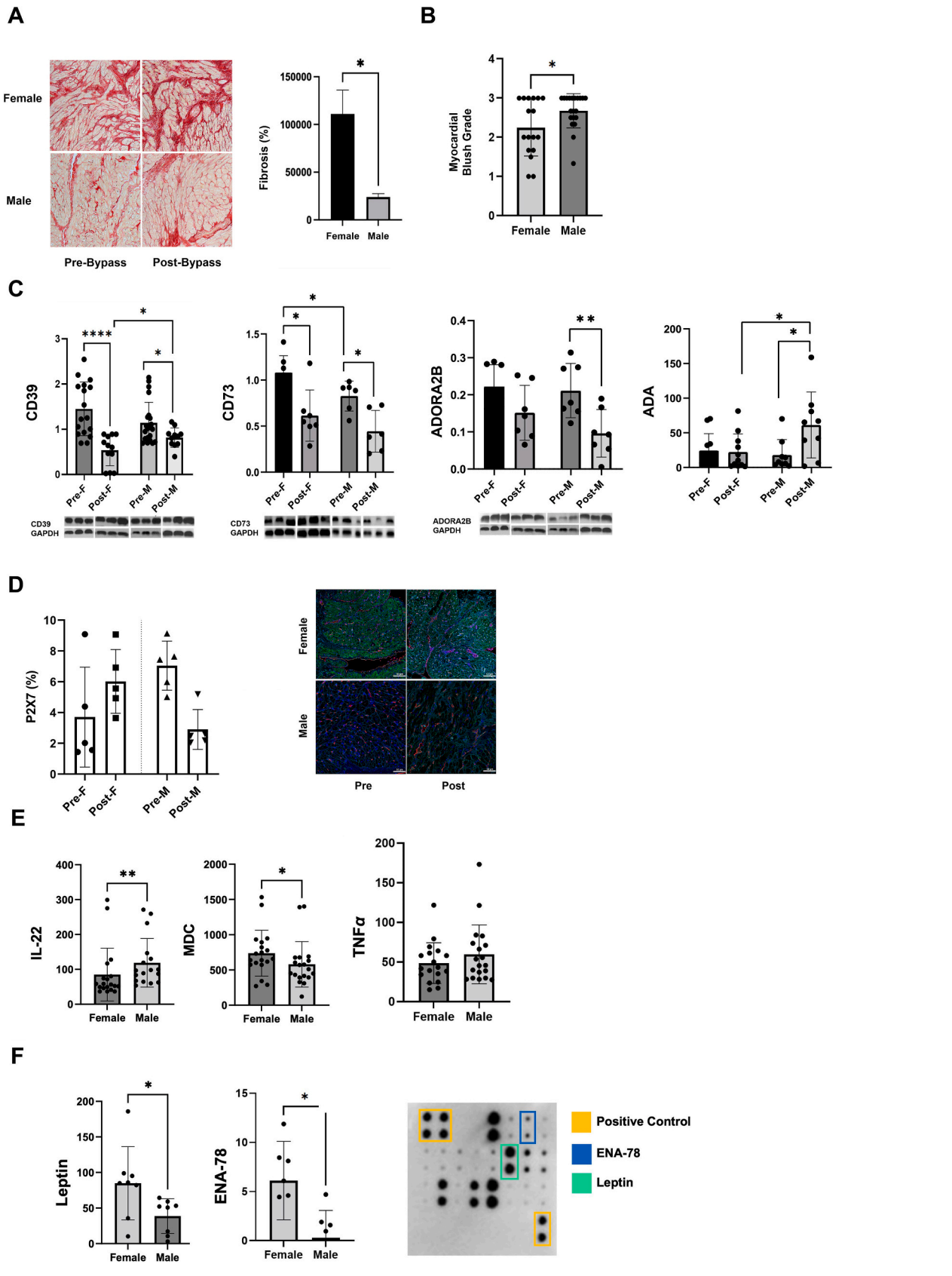

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Fig. 1. Gender differences in atrial fibrosis and myocardial Blush Grades among patients undergoing cardiac surgery correspond to the altered purinergic pathway and inflammation profile in females.
A, PicoSIRIUS fibrosis staining on atrial tissue. Images taken at 20× on atrial tissue before CBP. B, myocardial Blush Grade assessed preoperatively. C, quantification of purinergic signaling markers pre- and post-CPB from human atrial tissue. (Western blotting for CD39, CD73, and ADORA2B; ADA assay for serum ADA levels). D, Immunofluorescence staining of P2X7 on atrial tissue. Scale bar = 50 μm. E and F, quantification of inflammatory markers on human samples. Data represented as median ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. CBP = cardiopulmonary bypass; CD39 = cluster of differentiation 39 (ecto-nucleoside triphosphate diphosphohydrolase 1); CD73 = cluster of differentiation 73 (Ecto 5'nucleotidase); ADORA2B = adenosine receptor 2B; ADA = adenosine deaminase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IL-22 = interleukin 22; MDC = macrophage-derived chemokine; TNFα = tumor necrotizing factor α; ENA-78 = epithelial neutrophil-activating protein 78. In A, $n = 40$ for both female and male participants. In B, $n = 17$ for both female and male participants. In C n = 17 for each group in CD39, $n = 7$ for each group in CD73 and ADORA2B, $n = 9$ for each group in ADA. In D, $n = 5$ for Pre—F, Post-F, Pre-M, and Post-M in P2X7. In E, $n = 19$ for each group in IL-22, MDC, and TNFα. In F, $n = 8$ for each group in Leptin, $n = 6$ for each group in ENA-78. In all cases, n corresponds to one participant.

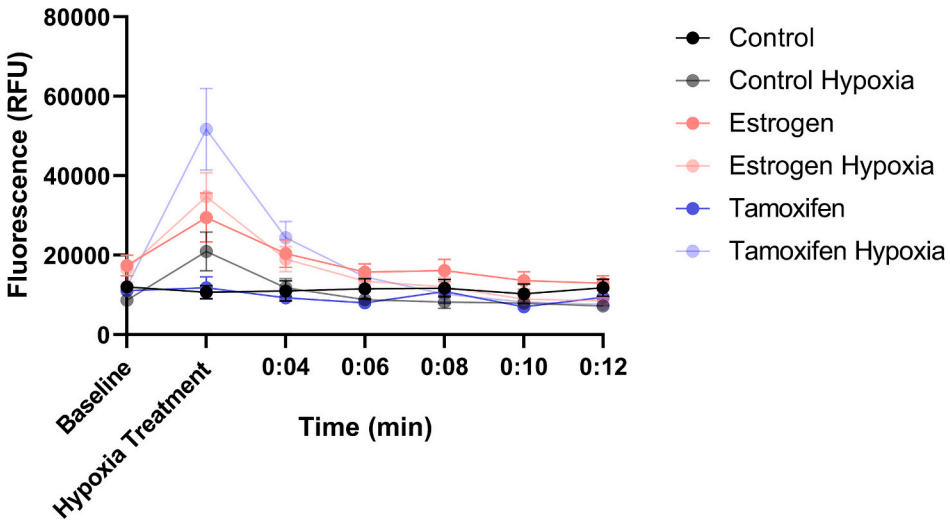

Fig. 2. Under hypoxia, the inhibition of estrogen, rather than estrogen treatment or saline control, demonstrated the highest extracellular ATP level.
Human umbilical vein endothelial cells (HUVEC) were cultured with saline (control), estrogen, or tamoxifen for 30 min first (Baseline, each subgroup $n = 8$). Then, cobalt chloride was added to the Hypoxia subgroups to create hypoxia environment (Control Hypoxia, Estrogen Hypoxia, and Tamoxifen Hypoxia). Measurements were taken every 2 min until the extracellular ATP levels normalized. Data are represented as mean ± SD. In each group, $n = 8$. In all cases, n corresponds to one well of a 96-well cell culture plate.

function, and better diastolic function (Fig. 4A and B). Dipyridamole was associated with lower fibrosis (Fig. 4C).

4. Discussion

Lower CD39 expression is associated with more fibrosis in women undergoing cardiac surgery:

We observed that women present for cardiac surgery with more advanced cardiac fibrosis than men and describe CD39 loss and eATP as key factor driving the adverse remodeling seen in women. Metabolic disturbances (obesity and diabetes) and hemodynamic load (hypertension and aortic stenosis) are known to contribute to the development of fibrosis via microvascular injury and resulting inflammation [25]; however, we present the CD39/CD73 eATP-adenosine pathway as another possible contributing factor to the development of cardiac fibrosis. This is pathway gives new insight to the well described inflammation – microvascular disease – fibrosis axis [25], and acts in a sex dependent manner.

4.1. Estrogen exerts protective effects in premenopausal women

Estrogen protects pre-menopausal women from developing microvascular disease via its positive effects on the endothelium [26], but estrogen loss during menopause likely contributes to an over-representation of women in the patient cohort of heart failure with preserved ejection fraction [27]. We have previously shown that estradiol exposure significantly increases CD39 expression and exerts a positive angiogenesis effect in hypoxic endothelial cells, and that estrogen loss has a negative angiogenic effect [14]. This is mediated through the effects of ERα, which activates transcription of CD39. Impaired angiogenesis has been linked to production of transforming growth factor-beta (TGF-β) and increased fibrosis in the liver [28]. In this study, we confirm the importance of CD39 and correlate lower expression with increased fibrosis in female cardiac tissue. The importance of these biological and hormonal differences are becoming better appreciated, but are not routinely implemented in the treatment of patients. Hormone replacement therapy (HRT) with estrogen supplementation, which would be a good first-line treatment, has long been known to reduce in the incidence of coronary artery disease and mortality from cardiovascular disease in women. [29] As a result, HRT was widely used in the 1990s, but its use dramatically decreased following the publication of the results from the Women's Health Initiative, which concluded "… (HRT) should not be initiated or continued for primary prevention of coronary heart disease" due to the increased risk of breast cancer, thrombosis, and stroke [30].. This report, which has been criticized since its publication, led to a significant decrease in prescription of HRT [31]. With this study, we aimed both identify the importance of the ERα dependent enzyme CD39 in cardiovascular health and propose it as a potential specific therapeutic target to improve cardiovascular outcomes.

4.2. Dysregulated CD39 and CD73 may drive fibrosis and inflammation via eATP

The role of purinergic signaling and purine receptors, particularly their implications in cardiac disease, is a growing field of study [28].

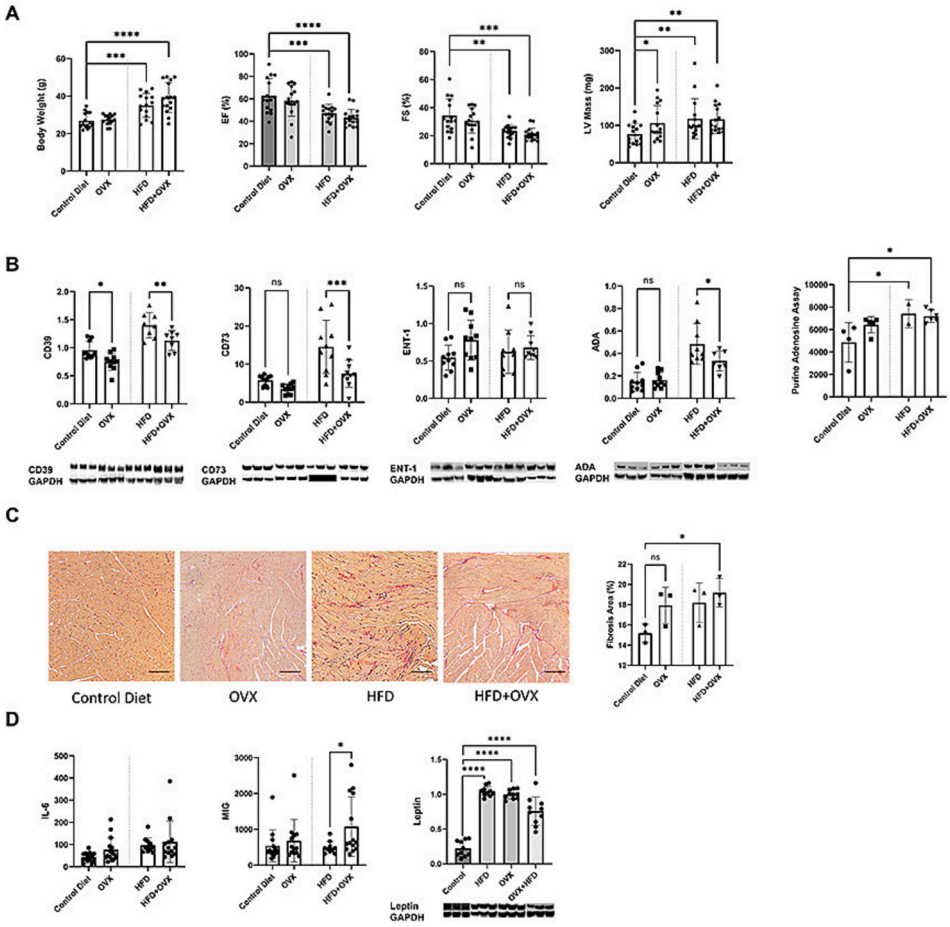

Fig. 3. The mouse ovariectomy model demonstrated the same purinergic pathway changing pattern compared with human females both on normal or high-fat diets, which contributed to the elevating extracellular ATP levels, leading to higher inflammation markers, myocardial fibrosis, and ultimately systolic dysfunction.
A, Body weight (g), EF (%), FS (%), and calculated LV mass (mg) among four groups of mice: female mice fed with control diet (Control Diet); female ovariectomized mice, control diet (OVX); female mice with high-fat diet (HFD); female ovariectomized mice with high-fat diet (HFD + OVX). B, Quantification of purinergic pathway markers and extracellular adenosine level among four groups. C, PicoSIRIUS fibrosis staining on mice myocardial tissue. Scale bar = 100 μm. D, Quantification of inflammatory markers. Data are represented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. EF = ejection fraction; FS = fractional shortening; LV = left ventricle; CD39 = cluster of differentiation 39 (ecto-nucleoside triphosphate diphosphohydrolase 1); CD73 = cluster of differentiation 73 (Ecto 5'nucleotidase); ENT-1 = equilibrative nucleoside transporter 1; ADA = adenosine deaminase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IL-6 = interleukin 6; MIG = Monokine induced by gamma- interferon. In A, $n = 10$ for each group in Body Weight, EF, FS, and LV Mass. In B, n = 10 for each group in CD39, CD73, ENT-1, and ADA, n = 4 for Purine Adenosine Assay. In C, $n = 3$ for Control Diet, OVX, HFD, and HFD + OVX. In D, n = 10 for each group in IL-6, MIG, and Leptin. In A, B, and D, n corresponds to one mouse. In C, n corresponds to slices from different mouse in the same group.

Under physiological conditions, extracellular ATP exists in micromolar concentrations, but the concentration increases during cellular stress such as with CPB associated myocardial ischemia [29–31]. Extracellular ATP is catalyzed by CD39 to adenosine monophosphate and then by CD73 to generate adenosine, which in turn regulates metabolic activity through AMP-activated protein kinase (AMPK) related pathways and decreases inflammation. Dysregulation at any step in the catabolism of ATP can shift the balance between ATP and adenosine, resulting in altered ADORA receptor expression and cause fibrosis and increased inflammatory response [32,33]. Adenosine Deaminase (ADA) is responsible for the breakdown of adenosine into its inosine, and ADA bound to A2A receptor and DDPIV transports adenosine into the cell [34]. In our cohort, women and men present with similar levels of CD39 prior to cardiopulmonary bypass but have a larger decrease and significantly lower CD39 expression following cardiopulmonary bypass, suggesting that women have an impaired ability to metabolize eATP. Men had significantly higher ADA levels post bypass suggesting that they were better able to clear and metabolize adenosine, which may be due to improved eATP metabolism leading to higher adenosine concentration. Older, post-menopausal women may generate more eATP and have an impaired ability to metabolize it and its metabolites.

Extracellular ATP is released in times of stress and binds to purine receptor P2X7 to induce a pro-inflammatory cascade [32]. Females show higher levels of, MDC, and GM-CSF compared to males, indicating a more pronounced inflammatory response. Similarly, ENA-78, a chemokine that activates neutrophils, is also increased in women, reinforcing their heightened inflammatory response. Leptin, a hormone associated with abnormal fat metabolism and inflammation, is elevated in females compared to males. Estrogen exerts a key role in immune cell regulation and protects the heart from mitochondrial dysfunction. The age associated loss of estrogen regulation in metabolism, immune control and purine control explains the observed cellular data and our clinical observations.

4.3. High fat diet and estrogen loss drive inflammatory disturbances via purinergic dysregulation in a mouse model

We utilized both ovariectomized and high fat diet mice to independently and jointly assess the effects of estrogen loss and metabolic dysregulation on purinergic signaling and cardiac function. This model

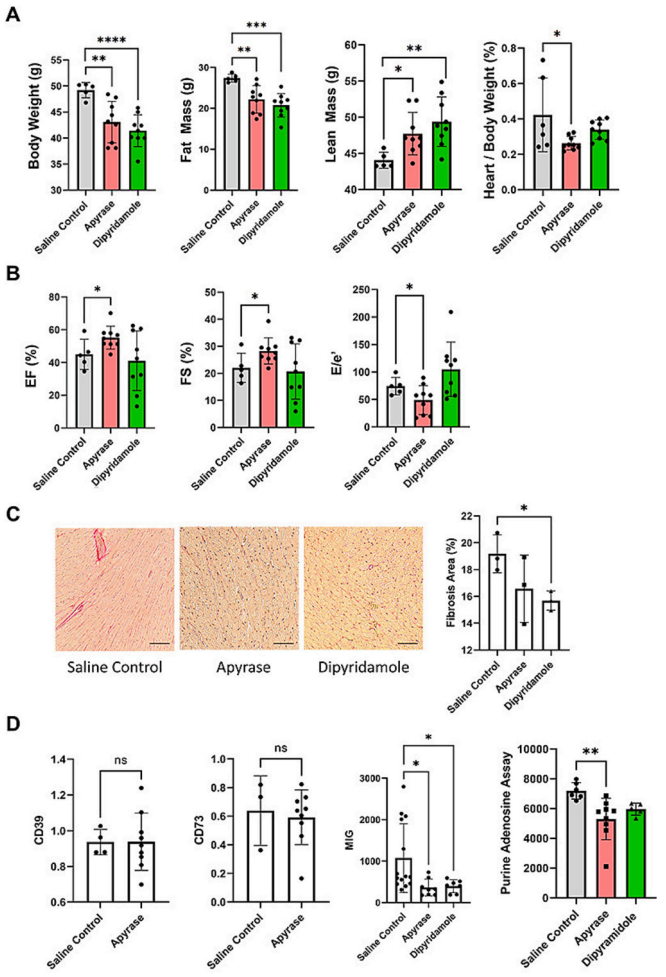

Fig. 4. Targeting the purinergic pathway alleviated cardiac dysfunction by reducing inflammation and extracellular ATP levels.
A, Body weight (g), fat mass (g), lean mass (g), heart mass/body weight ratio (%) among three groups of female ovariectomized mice fed with high-fat diet: peritoneal injection with saline (Saline Control), apyrase or dipyridamole. B, Transthoracic echocardiography data compared between the three groups. C, PicoSIRIUS fibrosis staining on mice myocardial tissue. Scale bar = 100 μm. D, Quantification of purinergic pathway markers and extracellular adenosine level among three groups. Data are represented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. EF = ejection fraction; FS = fractional shortening; E/e' = ratio between early mitral inflow velocity and mitral lateral annular early diastolic velocity; CD39 = cluster of differentiation 39 (ecto-nucleoside triphosphate diphosphohydrolase 1); CD73 = cluster of differentiation 73 (Ecto 5'nucleotidase); GAPDH = glyceraldehyde 3-phosphate dehydrogenase; MIG = Monokine induced by gamma- interferon. In A and B, $n = 5$ for Saline Control, $n = 9$ for Apyrase and Dipyridamole in Body Weight, Fat Mass, Lean Mass, Heart/Body Weight, EF, FS, and E/e'. In C, n = 3 for Saline Control, Apyrase and Dipyridamole. In D, n = 4 for Saline Control, n = 9 for Apyrase in CD39 and CD73; $n = 14$ for Saline Control, n = 8 for Apyrase, $n = 7$ for Dipyridamole in MIG; $n = 6$ for Saline Control, n = 9 for Apyrase, n = 5 for Dipyridamole in Purine Adenosine Assay. In A, B, and D, n corresponds to one mouse. In C, n corresponds to slices from different mouse in the same group.

represents an attempt to approximate the condition of women who present for cardiac surgery and does not approximate any surgical stress or the effects of cardiopulmonary bypass. We believe that this model is an acceptable approximation based on the observed decrease in CD39, CD73, and ADA, worse cardiac function and higher fibrosis burden seen in the ovariectomy plus high fat diet mice compared to our controls. This phenotype was similar to that seen in women presenting for surgery. The combination of a high-fat diet and ovariectomy results in a more pronounced adverse effect on weight, fractional shortening (FS), and ejection fraction (EF%) compared to ovariectomy alone. Taken together, these results highlight that the loss of CD39 and CD73 activity are associated with the metabolic and inflammatory disturbances observed in the combined high-fat diet and estrogen loss model.

4.4. Apyrase improves cardiac function and Dipyridamole Improves fibrosis

We evaluated the effects of these two therapeutics on our previously described ovariectomy plus high fat diet mouse model. Apyrase was selected because it approximates CD39 activity by catalyzing the breakdown of eATP to AMP and appears to be safe based on phase 1 human clinical trials [33,34]. Dipyridamole is an FDA approved medication that is indicated as an adjunct antiplatelet agent in patients with mechanical valve replacement. It increases extracellular adenosine concentration by inhibiting its uptake [35]. Both apyrase and dipyridamole improved body weight and heart mass. We did not observe changes to CD39 or CD73 in our treated mice, which was expected since these molecules catalyze the same reactions but do not serve as translational activators. Apyrase improved cardiac function, and dipyridamole improved fibrosis.

To date, no non-hormonal therapeutic target to selectively reverse the adverse cardiac remodeling associated with estrogen loss has been described. We demonstrate that administration of apyrase improves both ejection fraction and fractional shortening in the ovariectomized and high-fat diet groups. Apyrase also improves several diastolic parameters: it reduces the myocardial performance index, shortens the isovolumetric relaxation time, enhances the ratio of early mitral inflow velocity to early diastolic mitral annular velocity, and improves the ratio of early to late ventricular filling velocities, all of which reflect improved diastolic function and ventricular filling dynamics. Additionally, apyrase and dipyridamole administration improved body composition by reducing fat mass and increasing lean mass, suggesting both cardiovascular and metabolic benefits. Dipyridamole may regulate extracellular adenosine levels by inhibiting ENT-1 channels resulting in decreased inflammation and significantly decreased fibrosis.

4.5. Limitations

There are several limitations that must be mentioned. Our mouse model does not account for the ischemic disease present in patients presenting for coronary artery bypass surgery, however, we see the expected expression of CD39 and CD73 and therefore believe that it is an acceptable approximation of women who present for surgery. Additionally, the model is unable to approximate the stress of surgery, so it only applies to the preoperative state. Extracellular ATP and adenosine are notoriously difficult to measure due to instability [36], and we were unable to measure their concentrations in vivo, which would have strengthened our findings. We were able to demonstrate increased eATP production in HUVEC cells exposed to hypoxia and estrogen blockade. In the future we plan to replicate these findings in cardiomyocytes to strengthen the findings. We present an association between CD39/CD73 expression and fibrosis in female human tissue and demonstrate the potential benefit of the CD39 and CD73 analogues apyrase and dipyridamole and posit that this is related to excess eATP and P2X7 activity, but these data do not prove an underlying mechanism. Finally, while apyrase and dipyridamole are promising medications with an acceptable safety profile, they both exhibit anti-thrombotic properties, which may limit their use in clinical settings. Additionally, inherent sex differences in immune and metabolic responses likely play a role in modulating CD39/CD73 expression. Future studies comparing premenopausal and postmenopausal women will be critical in further elucidating the direct impact of estrogen on purinergic signaling and cardiac inflammation.

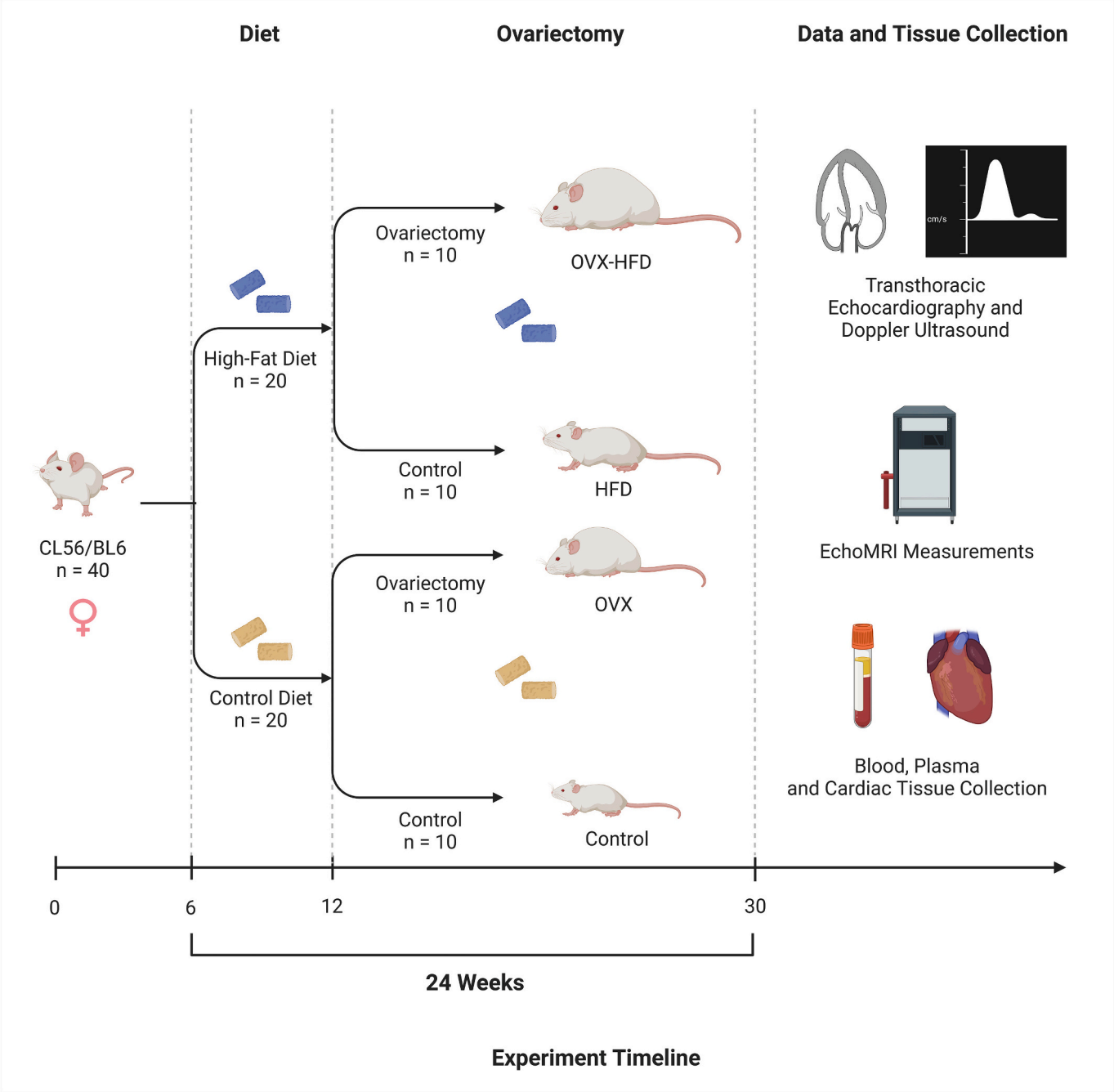

Fig. 5. Experimental Timeline for the Ovariectomy and High-Fat Diet Mouse Model.
Female C57BL/6 mice (n = 40) were divided into two dietary groups at 6 weeks of age: high-fat diet (HFD, $n = 20$) and control diet (n = 20). At 12 weeks, half of each group underwent ovariectomy (OVX, n = 10 per diet), while the remaining mice underwent a sham procedure (control, n = 10 per diet). This created four groups: OVX + HFD, HFD, OVX, and Control. Mice were maintained on their respective diets for a total of 24 weeks, after which cardiac function and body composition were assessed using transthoracic echocardiography, Doppler ultrasound, and EchoMRI measurements. Blood, plasma, and cardiac tissue samples were collected for biochemical and histological analyses. This model was used to approximate postmenopausal women with metabolic dysfunction and assess the effects of estrogen loss and purinergic signaling dysregulation on cardiac function and fibrosis.

5. Conclusions

Women present for cardiac surgery with more fibrosis and worse microvascular perfusion than men. This may be a contributing factor to the worse outcomes that have been consistently observed in female patients. We present decreased CD39/CD73/ADA expression leading to increased eATP as a possible mechanism and provide pre-clinical evidence for both apyrase and dipyridamole as potential therapeutics to target this pathway and improve outcomes for women undergoing cardiac surgery. In conclusion, our experimental observations strongly suggest a connection between estrogen deficiency and purinergic dysfunction, and we demonstrate the ability to reverse the consequences of this dysfunction with apyrase or dipyridamole.

Author disclosure

None.

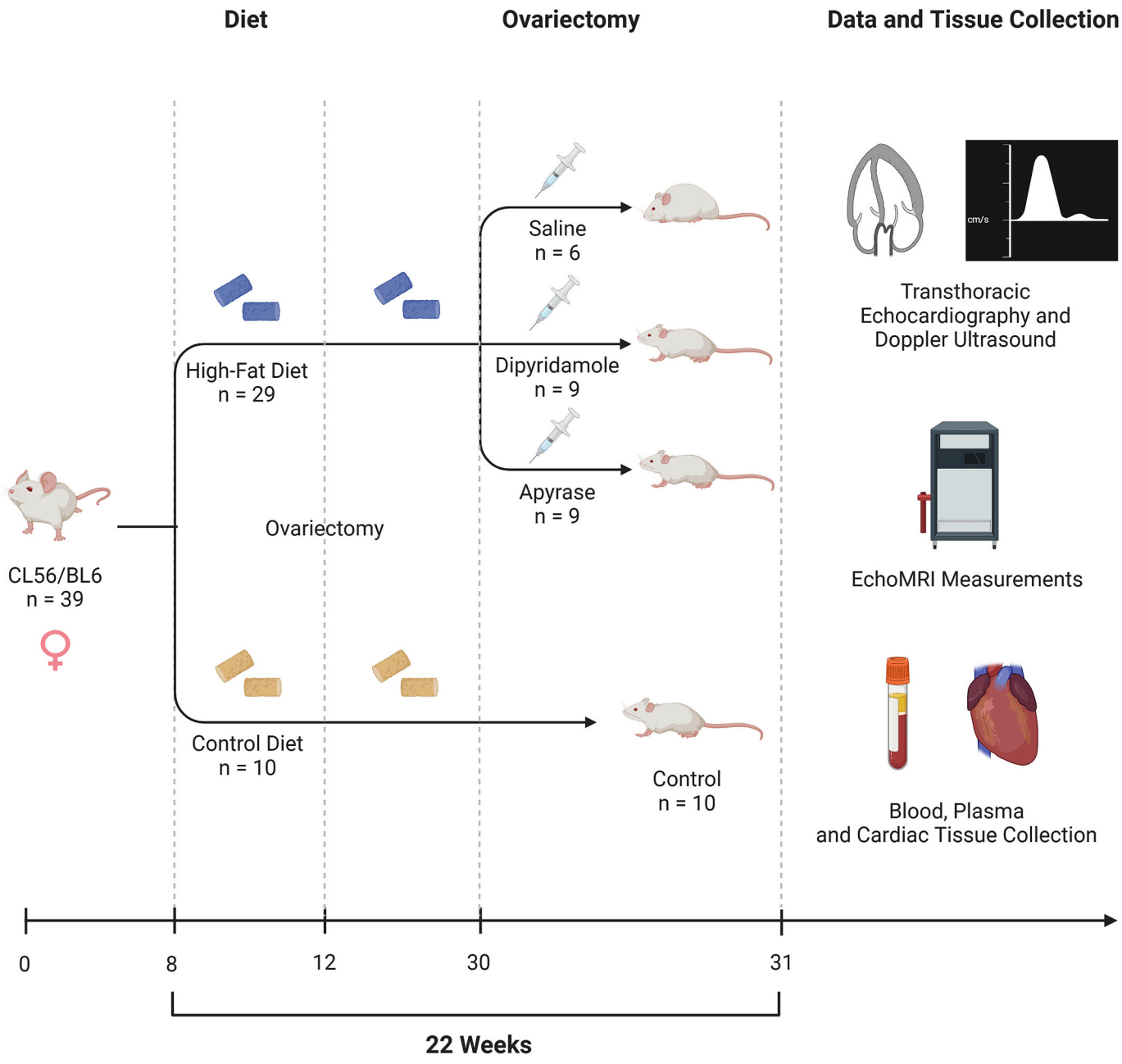

Fig. 6. Experimental Timeline for the Ovariectomy and High-Fat Diet Mouse Model with Purinergic Pathway Interventions.
Female C57BL/6 mice (n = 39) were divided into high-fat diet (HFD, n = 29) and control diet (n = 10) groups at 8 weeks of age. At 12 weeks, all HFD-fed mice underwent ovariectomy (OVX), while control diet-fed mice remained intact. At 30 weeks, OVX-HFD mice were further divided into three treatment groups: Saline (n = 6), Dipyridamole (CD73 enhancer, n = 9), and Apyrase (CD39 mimetic, n = 9). Control diet-fed mice (n = 10) served as a baseline comparison. Cardiac function was assessed using transthoracic echocardiography and Doppler ultrasound, and body composition was measured via EchoMRI. Following the experimental period, blood, plasma, and cardiac tissues were collected for further analysis. This model was designed to evaluate the impact of CD39/CD73 modulation on cardiac fibrosis and dysfunction in an estrogen-deficient, high-fat diet environment.

AI disclosure

The authors did not use generative AI or AI-assisted technologies in the development of this manuscript.

CRediT authorship contribution statement

Eitezaz Mahmood: Validation, Methodology, Funding acquisition, Conceptualization. **Mark Robitaille:** Methodology, Investigation, Formal analysis, Conceptualization, Writing – original draft. **Yifan Bu:** Visualization, Methodology, Formal analysis, Data curation. **Adnan Khan:** Methodology, Investigation, Formal analysis. **Marie-France**

Poulin: Formal analysis. **Feroze Mahmood:** Funding acquisition, Conceptualization, Writing – review & editing. **Ruma Bose:** Methodology, Funding acquisition, Conceptualization. **Kamal R. Khabbaz:** Investigation, Data curation, Conceptualization. **Simon C. Robson:** Supervision, Project administration, Conceptualization. **Robina Matyal:** Methodology, Investigation, Formal analysis, Conceptualization, Writing – original draft.

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Declaration of competing interest

Robina Matyal reports financial support was provided by National Institutes of Health. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmccpl.2025.100294.

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