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Heart failure with preserved ejection fraction and its comorbidities: role of adenosine pathway

Tutor

Prof. Armando Ialenti

PhD student:

Rossella Bilancia

Coordinator:

Prof. Maria Valeria D'Auria

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Abstract

Heart failure (HF) is a systemic, multifactorial disease that affects most of the world's population. It causes a large increase in hospitalizations so affecting the health economics. About 50% of patients with HF symptoms have a normal left ventricular ejection fraction (HFpEF) and its prevalence continues to increase compared to heart failure with a reduced ejection fraction (HFrEF).

Current research suggests that HFpEF is characterized by cardiac fibrosis and remodeling, and occurs when chronic medical conditions (e.g. obesity, hypertension, diabetes mellitus, coronary artery disease, chronic kidney disease, chronic obstructive pulmonary disease, thrombosis) damage the heart and other organ systems. These diseases are thought to gradually change the structure and function of the heart over time.

There is evidence that an imbalance of extracellular purine levels may be associated with increased cardiovascular risk. Adenosine is an endogenous nucleoside with autocrine/paracrine functions, acting as signal molecule to preserve host defense and tissue integrity during inflammation and trauma. Adenosine is produced primarily from sequential dephosphorylation of extracellular adenosine triphosphate (ATP) to AMP by activity of CD39 (ectonucleoside triphosphate diphosphohydrolase), followed by ecto-5'-nucleotidase CD73. There is much evidence for a role of adenosine in cardiac fibrosis associated with the progression of heart failure. Endogenous adenosine appears to play a significant role in reshaping the microenvironment during inflammatory processes through the interaction with four subtypes of cell surface G-protein-coupled adenosine receptors. These receptors are widely expressed on cardiac cells including fibroblast, endothelial cells, smooth muscle cells and leukocytes, all with a cardioprotective role. Scientific evidence has shown that the expression of CD39 and CD73 are involved in the extracellular adenosine accumulation, were upregulated in human circulating leukocytes of heart failure patients, suggesting that HF could benefit from adenosine-based drug therapy.

It is well recognized that patients with HF have an increased risk of venous thromboembolism, stroke, and sudden death. The increased cardiovascular risk may be associated with an imbalance of extracellular purine levels.

Platelets are an important source of purine nucleotides and nucleosides. In case of stress, adenosine is released in large quantities by the CD39 enzyme. It is expressed on the endothelium, circulating blood cells, and smooth muscle cells. Changes in CD39 expression and activity affect the thrombogenic potential of a tissue. Gender difference in the cardiovascular risk has been extensively observed; however, while the age-dependent difference in the prevalence of cardiovascular events between men and women has been attributed to the loss of the protective effect of estrogens in the postmenopausal period, the physiological mechanism behind gender disparity is still unclear. In the light of these considerations, the aims of this study were:

1) to investigate comparatively platelet functionality in male and female rats and the possible link to CD39 enzyme

2) to evaluate the changes in the adenosine pathway in Dahl salt-sensitive hypertensive rat, a model of heart failure with preserved ejection fraction.

We found a reduced *in vitro* response to ADP of female compared to male platelets, associated to increased platelet CD39 expression and activity. Platelet response to ADP was strongly increased by incubation (10 min) with the CD39 inhibitor, ARL67156, while male platelet response was unaffected. Rat treatment with clopidogrel (30 mg/kg, per os) inhibited ex vivo platelet aggregation. Bleeding time was prolonged in female compared to male.

Dahl rats, feeding with a 8% NaCl diet, progressively developed hypertension, compared to LS animals that remained normotensive. While systolic parameters were not altered, diastolic parameters were changed in high salt animals. Indeed, hemodynamic analysis showed a decreased dP/dt min, increased end-diastolic pressure, longer time constant Tau and steeper slope of the end-diastolic pressure-volume relationship. In addition, our data showed an increase of LV systolic and diastolic posterior wall thickness in the HS group of animals at both 5 and 13 weeks, respectively. In our animal model of HFpEF we observed, in the myocardium of 13 HS animals, an increase in perivascular fibrosis due to collagen accumulation and an increase in reactive oxygen species and nitrogen. Western blot analysis showed a reduced production of adenosine in rats with HFpEF, an increased production of inosine, which has pro-inflammatory action, and a greater expression of the A_{2B} receptors which, with their pro-fibrotic, cause cardiac rigidity in the hearts of Dahl rats at 13 weeks and thus worsening the inflammatory state in the latter group.

Our results suggest that the sex variability in platelet ATPase and ADPase activity should be taken into account for understanding more in depth the molecular mechanisms behind gender difference in cardiovascular risk.

These preliminary results suggest, also, that adenosine pathway may represent an attractive target to test potential therapeutic strategies to prevent and/or delay the progression of HFpEF syndrome.

1. INTRODUCTION

Inflammation, or phlogosis, is a non-specific defense mechanism that the body puts in place to neutralize a phenomenon deemed harmful such as, for example, the invasion of pathogens or cell and tissue damage caused in turn by trauma or by stimulation by an antigen (Nathan, 2002). The first to delineate the typical signs of inflammation was Celsus in the first century AD. He described that the inflammation caused *rubor* (redness, due to increased blood flow), *calor* (heat, due to an increase in the temperature of the inflamed area due to the increased blood flow), *dolor* (pain, due to biochemical alterations of the tissues), *tumor* (swelling, caused by exudate that occupies the intercellular space). Later in the 1850s, Rudolf Virchow added the fifth sign of inflammation *functio lesa* (functional impairment, due to edema and pain). These events are triggered by mediators and cells that characterize inflammation. Inflammation is defined by an interplay between soluble, cellular, and vascular factors endothelium. In particular, there is a delicate balance between pro-and anti-inflammatory mediators, which are produced during acute inflammation, that regulates the duration of the inflammatory response and the timing of tissue resolution (Fredman G L. Y., 2021), (Chiang N F. G., 2012). Short-term acute inflammation is the first defense mechanism acting against harmful agents. Initially, acute inflammation is characterized by the extravascular accumulation of neutrophils (PMN) and edema formation. Subsequently, mononuclear cells and macrophages head, at the site of inflammation, to help restore homeostatic balance (Serhan, 2007). It is important to highlight that in this phase the main mediators are the pro-inflammatory ones that increase the acute response. Among the pro inflammatory mediators, we remember eicosanoids. They originate from arachidonic acid (acid 5,8,11,14-eicosatetraenoico; AA) through a series of metabolic processes (Samuelsson B., 1979-1980). Cytosolic A_2 phospholipase ($cPLA_2$) is the major enzyme responsible for the release of AA from membrane phospholipids. When the AA is hydrolysed by membrane phospholipids from $cPLA_2$ it can be substrate of three different enzymes: a) the lipoxygenases (LOXs) for the production of leukotrienes (LTs) (5-LO), lipoxins (LXs) and related hydroxylated metabolites of AA (12- and 15-LO, respectively); b) cyclooxygenases

(COXs), enzymes that lead to the formation of prostaglandins (PGs) and thromboxane (TX) and c) CYP450 for the formation of epoxy-diic-metetraenic acids (SET) and HETEs (Smith, 1989) (Fig.2). LTs are involved in several immune and inflammatory diseases such as cardiovascular disease, asthma, atherosclerosis, allergic rhinitis, cancer, and autoimmune diseases (JZ., 2018), (Duquette P, 1992).

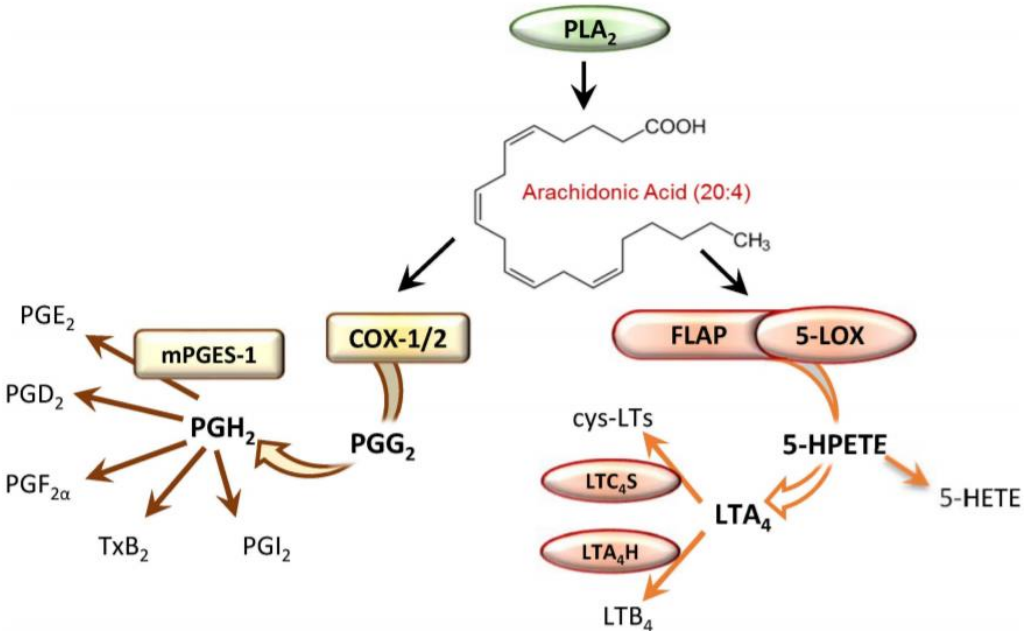


Figure 2. Biosynthesis of lipid mediators. From Pace S, Werz O. Impact of Androgens on Inflammation-Related Lipid Mediator Biosynthesis in Innate Immune Cells. Front Immunol. 2020.

Pro-inflammatory mediators are counterbalanced by endogenous anti-inflammatory signals such as corticosterone, which serve to limit the duration of the early onset phase. As the inflammation progresses, there are some "stop signs" to prevent further trafficking of leukocytes into the tissues. Among the stop signs, we can mention the anti-inflammatory mediators which we will discuss in the paragraph *1.1*. However, when the acute inflammatory response does not resolve and persists, chronic inflammation sets in. The immune cells involved in this phase are macrophage- and lymphocyte-accumulated leukocytes (Chen M, 2015) and various other cellular components. Furthermore, since inflammation is an evolutionary process of conservation, the problem is not how often this happens but how many times it does not resolve. Indeed, non-resolving inflammation is the cause of the

onset of chronic diseases such as cardiovascular disease, atherosclerosis, obesity, cancer, chronic obstructive pulmonary disease, asthma, inflammatory bowel disease, neurodegenerative disease (Karp CL, 2004), (Levy BD & Severe Asthma Research Program, 2005), (Fredman G O. S., 2011).

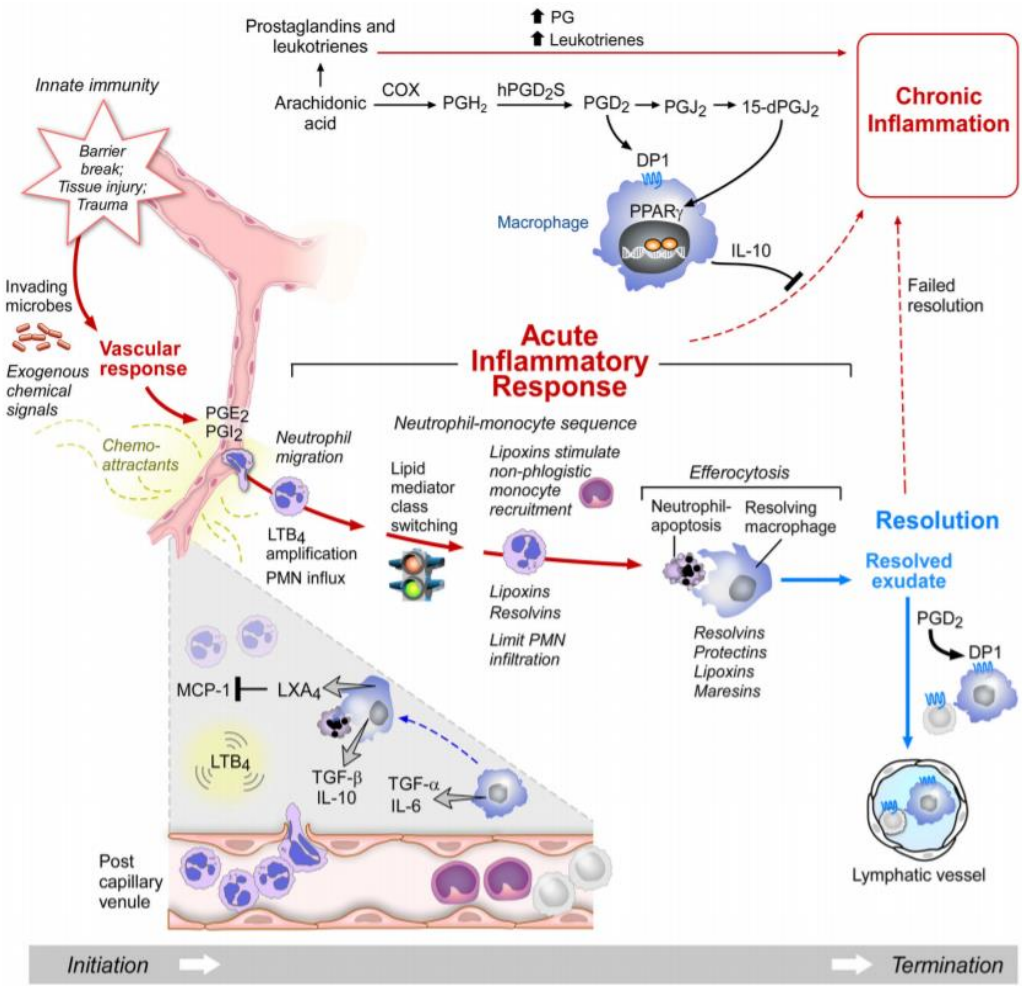


Figure 1. Time-course illustration of the key roles of lipid mediators in the initiation and resolution of acute inflammation. From Serhan CN. Treating inflammation and infection in the 21st century: new hints from decoding resolution mediators and mechanisms. FASEB J. 2017

1.1 Anti-inflammatory mediators

The resolution of inflammation is an active process in which new mediators, cells, and endogenous control mechanisms are involved (Serhan, 2007). There are several classes, and they are briefly reported.

Among the cytokines that resolve inflammation, we can mention Interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β) (Mosser DM, 2008). IL-10 and

TGF-β are confluence points for other interacting cytokines that play important roles in the phase of the resolution of the inflammatory. For example, the resolution of TNBS-induced colitis in mice required IL-13, which served to augment the production of IL-10 and TGF-β (Fichtner-Feigl S, 2008).

As gaseous signals, small molecules as reactive oxygen intermediates (ROI) and nitrogen intermediates (RNI) are important anti-inflammatory factors although they have pro-inflammatory effects like lethal pneumonia in mice infected with the flu virus (Karupiah G, 1998). RNI is produced from nitrogen monoxide (NO), the main product of iNOs (NOS2), NOS1, and NOS3. NO is capable of antagonizing cell adhesion to the endothelium, inhibit caspases, and thus the generation of Interleukin-1β (IL-1β) and Interleukin-18 (IL-18) and suppress the clonal expansion of T lymphocytes (Bogdan, 2001).

IL-10 and the stress-induced heme oxygenase-1 enzyme produce bilirubin and carbon monoxide (CO). CO can mediate some anti-inflammatory effects of IL-10 (Lee TS, 2002), improve the expression of IL-10, and suppresses LPS-induced production of TNF-α (Otterbein LE, 2000).

Another class of anti-inflammatory mediators is nitrated fatty acids. They can inhibit NF-kB, neutrophil activation and they can induce expression of iNOS and VCAM-1; to activate the peroxisome proliferator-activated receptor-γ (PPARγ); and to induce heme oxygenase-1 (Freeman BA, 2008).

Many studies have discovered a new kind of mediator called specialized pro-resolving mediators (SPMs). SPMs are derived from essential fatty acids, including AA (C20: 4n-6), eicosapentaenoic acid (EPA; C20: 5n-3), and docosahexaenoic acid (DHA; C22: 6n-3) through LOX enzymes. During the phase of acute inflammation, through a series of signals, the metabolism of AA switches from the production of prostaglandins and leukotrienes to those of lipoxins (LXs), the lead

family of pro-resolving mediators (Levy BD C. C., 2001). LXs are formed via leukocyte-derived 5-LOX, platelet-derived 12 LOX in the vasculature (Serhan CN S. K., 1990), eosinophil, or monocyte-derived 15-LOX by epithelial cell (Levy BD R. M., 1993) (Serhan CN H. M., 1984). Another way through which lipoxins can be formed is that of aspirin-mediated acetylation of cyclooxygenase 2 (COX 2). This leads to the switch of AA to 15(R)-hydroxyeicosatetraenoic acid (15(R)-HETE), a substrate for 5-LOX-mediated conversion to 15-epi-lipoxins (Clària J S. C., 1995). It is important to note that even in the absence of aspirin the 15(R)-HETE is produced by the cytochrome P450 enzymes (Clària J L. M., 1996), (Chiang N., 1998). In addition to LXs, other molecules include resolvins (Rvs), protectins (PDs) and maresins (MaRs) (Serhan CN., 2002). The resolvins (E-series and D-series) are produced from EPA and DHA, respectively. Biosynthesis of E-series Rvs starts with the addition of an oxygen atom at the carbon-18 position of EPA, which is converted into RvE1 and E2 by LOX or into RvE3 by 15 LOX. Conversely, biosynthesis of D-series Rvs begins from DHA is converted to 17S-HpDHA and subsequently in Rv-epoxide intermediates by 15 and 5-LOX, respectively (Oh SF, 2012). The intermediates are transformed to RvD1–D6. 17-HpDHA is also the precursor to the 16,17-epoxide-PD intermediate, which is converted to NPD1/PD1 and related PDs (Mukherjee PK, 2004). MaRs are produced by macrophages 12 LOX from DHA. SPM act via specific receptors that are expressed on different cell types, giving rise to tissue selectivity and evoke powerful anti-inflammatory mechanisms (Arita M, 2005), (Chiang N D. J., 2015). They can counter the production of PGs, LTs, PAF formation; they can regulate COX-2 expression; counter the pro-inflammatory cytokines; increase IL-10 (Serhan, 2007). They can regulate NF-kB gene products and ultimately lead to the regulation of edema (Arita M, 2005).

Another powerful anti-inflammatory pathway is the one represented by the vagus nerve. Vagus nerve terminals release acetylcholine, which acts on α7 nicotinic cholinergic receptors to suppress the release of IL-8, IL-1β, TNF-α, IL-6, and HMGB1 by macrophages in the liver and spleen (Tracey, 2007).

An example of a protease inhibitor with anti-inflammatory effects secretory leukocyte protease inhibitor (SLPI). SLPI is secreted by macrophages in response to LPS (Jin FY, 1997). SLPI blocks the activation of neutrophils by suppressing TNF-α and it helps in wound healing (Zhu J, 2002).

Another anti-inflammatory mediator is represented by adenosine. Adenosine is an endogenous nucleoside that has been recognized to be a molecule with autocrine/paracrine functions. It is defined as "a guardian angel" for its properties as signal molecule to preserve host defense and tissue integrity during inflammation and trauma (Caiazzo E, 2016), (Haskó G. C. B., 2004), (Newby, 1985) (Fig. 3).

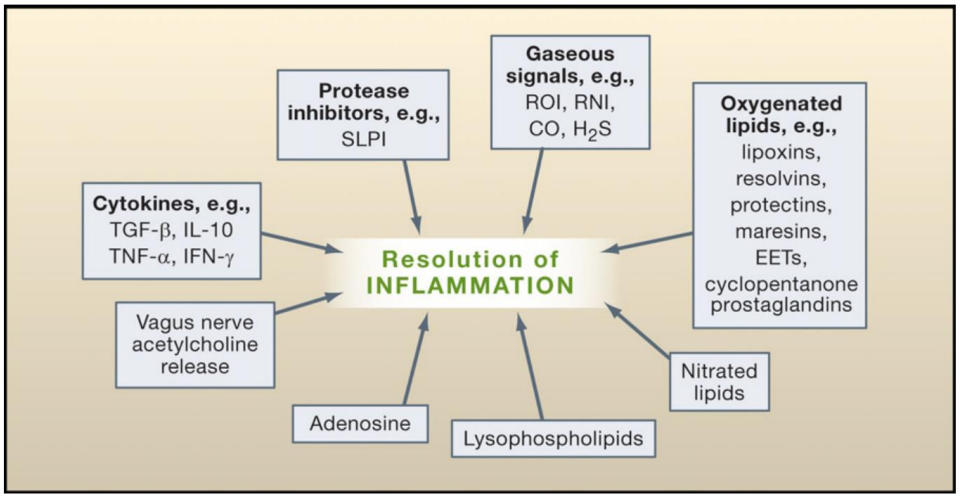


Figure 3. Main soluble factors that are involved in the resolution of inflammation. From Nathan C. and Ding A. "Nonresolving Inflammation". Cell 140, 2010, 871–882.

1.1.1 Adenosine pathway

Adenosine comes from the dephosphorylation of adenine nucleotides and is constitutively present at low concentrations both intracellularly and extracellularly. In homeostatic conditions the levels of extracellular adenosine are between 30 and 200 nM (Ballarín M, 1991) and the maintenance of these concentrations depends on the balance between the removal of the nucleoside by the membrane enzymes/transporters and its formation, starting from adenosine triphosphate (ATP) through the action of ectonucleotidases. In the extracellular space, adenosine is produced by the hydrolysis of extracellular ATP through a two-step enzymatic process by a series of cellular ectoenzymes. There are four enzymes of the ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) family that are located on the cell surface with an outward-facing catalytic site (NTPDase 1, 2, 3, 8). The four

enzymes can be differentiated according to the preference of the substrate, the use of divalent cations, and the formation of the product. All surface localized NTPDases require Ca^{2+} or Mg^{2+} ions in millimolar concentration, for maximum activity, and are inactive in their absence (Zimmermann H., 2001). All enzymes hydrolyze ATP. While NTPDase, also known as CD39, hydrolyzes ATP and adenosine diphosphate (ADP) in the same way, NTPDase3 and NTPDase8 reveal a preference for ATP. NTPDase2 stands out for its high preference for nucleoside triphosphates and therefore was previously also classified as ecto-ATPase (Kukulski F, 2005). Another family involved in the metabolization of ATP is that of the ecto-nucleotides pyrophosphatase-phosphodiesterase (E-NPP). It is composed of seven structurally related molecules that hydrolyze the pyrophosphate and phosphodiesterase bonds in various substrates (Schetinger MR, 2007) (Stefan C, 2005) (Yegutkin G. , 2008). Among the seven members of the family, E-NPP1, E-NPP2, and E-NPP3 are the main ones that hydrolyze triphosphate nucleotides (Bollen M, 2000). Mainly, E-NTPD 1 hydrolyzes ATP to ADP and adenosine 5'-monophosphate (AMP), while ecto-5'-nucleotidase (e-5'NT, also known as CD73) hydrolyzes AMP to adenosine (Zimmermann H., 2001). A decrease in the production of extracellular adenosine occurs due to the alkaline phosphatase and enzymes of the pyrophosphatase and phosphodiesterase family, which hydrolyze the pyrophosphate and phosphodiester bonds of the nucleotides (Yegutkin G. , 2014). The intracellular production of adenosine occurs with a mechanism similar to the extracellular one in which the AMP is transformed into adenosine by a cytoplasmic 5'-nucleotidase or by the hydrolysis of S-adenosyl-L-homocysteine (SAH) by the action of adenosyl- L-homocysteine hydrolase (SAHH) (Broch OJ, 1980). Following damage, stress, increased metabolic demand or as in the case of ischemia and inflammation, the extracellular levels of adenosine can increase respectively up to 1200 nM and up to 500-600 nM; this occurs mainly when ATP is released, either through a lytic or non-lytic mechanism, to be transformed into adenosine (Fredholm B.B., 2007). Under physiological conditions, the average life of adenosine in human plasma is very short, ranging from 0.6 to 1.5 seconds (Möser GH, 1989); this is due to the uptake of adenosine by specific transporters. Indeed, adenosine, being hydrophilic and non-permeable, does not passively cross the cell membrane and is transported using bidirectional transporters for facilitated diffusion, thus balancing its intra- and extracellular levels; these sodium-

independent transport proteins are called "Equilibrative Nucleoside Transporter" (ENT1 e ENT2). The average life of adenosine is also influenced by its degradation, which takes place by adenosine deaminase (ADA), which has dipeptidyl peptidase IV (DPP4) as a cofactor, which metabolizes it to inosine (Eltzschig HK, 2006), or by its internalization and subsequent phosphorylation to AMP, by adenosine kinase (AK). In pathological conditions, an ATP-dependent induction of the signaling activity of adenosine and its receptors was found and at the same time, a suppression of the enzymes involved in its transformation, such as adenosine kinase (Chen JF, 2013).

1.1.2 Adenosine receptors

Adenosine signaling is evoked through interaction with its four distinct and widely diffused membrane receptors: A_1, A_{2A}, A_{2B}, A_3. These receptors, also known as P1 purinoreceptors, are G protein-coupled receptors (GPCRs), which transmit signals by activating heterotrimeric G proteins. This family of receptors have a central domain formed by seven transmembrane α-helices, composed of 20-27 amino acids, with the extracellular amino-terminal portion and the intracellular carboxy-terminal portion. The expression levels of all adenosine receptors are transcriptionally regulated and can change rapidly with various stimuli. A_1 and A_{2A} receptors have a high affinity for adenosine (K_m <30 nM), while A_{2B} and A_3 are low affinity receptors (K_m 1-20 µM) and are activated only in conditions of high metabolism and cellular stress (Fredholm BB., 2001). The adenosine A_1 receptor (A1AR) is distributed and expressed in the central nervous system (neocortex, cerebellum, hippocampus), adipose, skeletal and muscle tissue, liver, heart, kidney, salivary glands, lungs and inflammatory cells such as neutrophils (Polosa, 2002). A1AR is coupled to G proteins belonging to the G_i/G_q family, and its activation inhibits adenylate cyclase (AC) and reduces the activity of protein kinase A (PKA) (van Calker D, 1978). At the cardiac and neuronal level, the receptor can activate pertussis-sensitive potassium channels, as well as ATP-dependent ones, and inhibit calcium channels of type N, P and Q; the activation of A1AR can also lead to an increase in the intracellular levels of Ca^{2+} through the stimulation of phospholipase C (PLC), which splits the phosphatidylinositol 4,5-bisphosphate into diacylglycerol

and inositol 1,4,5-triphosphate, with consequent activation of enzymes such as protein kinase C, phospholipase D and phospholipase A_2. The A_2 adenosine receptor is divided into A_{2A} and A_{2B}, respectively with high and low affinity for the substrate. The A_{2A} adenosine receptor was found in large quantities in the striatum, immune cells of the spleen, thymus, heart, leukocytes and platelets; it is coupled to a G_s protein in peripheral tissues or to a G_{olf} protein in the brain. High levels of cAMP stimulate PKA, which in turn activates calcium and potassium channels, CREB proteins, PLC and MAP kinase; the latter is responsible for an increase in collagen production. It has also been shown that A2AAR can exist both as a homodimeric and heterodimeric receptor, coupled to other receptors such as the dopamine D_2 receptor (Fredholm B.B., 2007). The A_{2B} adenosine receptor (A2BAR) is widely expressed in the brain, gastrointestinal tract, heart, bladder and endothelial and muscle cells, mast cells and fibroblasts (Polosa, 2002). The activation of this receptor stimulates the AC and the PLC through the activation, respectively, of G_s and G_q proteins (Feoktistov I, 1995). The adenosine A_3 receptor (A3AR) has been identified in many types of tissues, including kidneys, testes, lungs, heart and cerebral cortex and in various types of cells, such as mast cells, eosinophils and neutrophils. At the heart level, this receptor mediates cardioprotective effects through the activation of ATP-dependent potassium channels, protecting cardiomyocytes from events such as ischemia. Activation of A3AR involves inhibition of AC, stimulation of PLC and PLD and mobilization of calcium (Zhou QY, 1992), (Abbracchio MP, 1995); like the other adenosine receptors, the A3ARs are also coupled to the MAP kinase and mediate the stimulation of ERK1/2 (Fig.4).

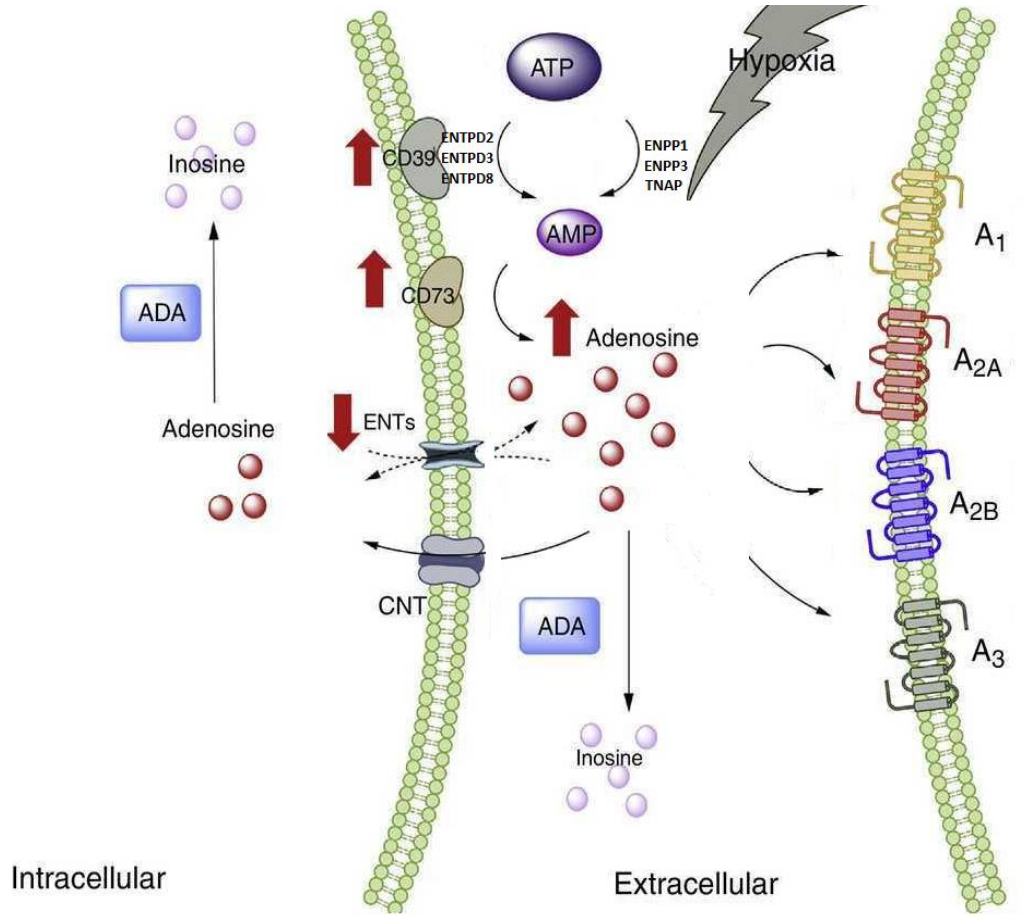


Figure 4. Adenosine pathway. Adapted from Meriño M, et al. "Role of adenosine receptors in the adipocyte–macrophage interaction during obesity". Endocrinol Diabetes Nutr. 2017.

1.1.3 Pathophysiological role of adenosine

The wide distribution of adenosine receptors in most of the body's cells makes them an important target for the development of drugs for many diseases. In case of metabolic stress or cellular damage, adenosine accumulates in the extracellular space and its rapid release has a dual role in maintaining homeostasis: firstly, extracellular adenosine represents one of the main alarm molecules that signal any tissue damage, by means of an autocrine and paracrine mechanism, to the surrounding tissues; moreover, extracellular adenosine generates a tissue response

that can be considered organ-protective, so as to maintain homeostatic balance (Caiazzo E, 2016), (Haskó G. C. B., 2004). Adenosine receptors are expressed in the cells of the immune system, which suggests a role of adenosine in immune-inflammatory states (Cekic C, 2016); in particular, adenosine has been found to exert effects on neutrophils, lymphocytes, macrophages and dendritic cells (Mills JH, 2012). At the level of macrophages, the A_{2A} receptor is the main adenosine receptor involved in inhibiting the release of tumor necrosis factor (TNF-α) and increasing the production of IL-10, while the A_{2B} receptor has a lower impact in the production of TNFα and IL-10, but induces the release of interleukin 6 (IL-6) by macrophages (Haskó G. K. D., 2000), (Kreckler LM, 2006), (Ryzhov S, 2008). Adenosine, at the level of mature dendritic cells, activates the A_{2A} receptors, modifying the production of cytokines; indeed, the activation of this receptor decreases the production of pro-inflammatory cytokines, such as interleukin 12 (IL-12), IL-6 and interferon α (INF-α) and increases the production of anti-inflammatory cytokines, such as IL-10 (Panther E, 2001). On the other hand, adenosine, acting on the A_1 and A_3 receptors, promotes the recruitment of immature dendritic cells to the inflammation site (Schnurr M, 2004). At the level of neutrophils, adenosine regulates the production of ROS and phagocytic activity (Cronstein BN. K. S., 1983) (Cronstein BN. R. E., 1985). On the A_{2A} receptors, adenosine inhibits the adhesion of neutrophils to the endothelium, while its action on the A_1 receptors promotes it, through various adhesive molecules present on the endothelium and other surfaces. On the surface of mast cells, the A_{2A}, A_{2B} and A_3 receptors are present; recently, adenosine has been observed to stimulate the release of interleukin 13 (IL-13), the main cytokine involved in asthma, by mast cells of wild-type mice of the A_{2B} receptor, but not by mast cells of mice knockout (Ryzhov S, 2008). Adenosine also has an action at the level of lymphocytes, in fact, some studies conducted, using animal models of A_{2A}-knockout, have shown that the activation of these receptors inhibits the secretion of IL-12 by $CD4^+$ T lymphocytes (Naganuma M, 2006), so as to reduce their proliferation following stimulation of the T cell receptor (TCR) (Sevigny CP, 2007).

Regarding the harmful role of adenosine in chronic inflammatory conditions, the activity of the A_{2B} receptor is important, as it is activated by μM concentrations of adenosine (Fredholm B.B., 2007), (Bartels K, 2013); in particular, the expression of A_{2B} increases in hypoxia in response to inducible factors of hypoxia (HIF) (Eckle

T, 2014). Furthermore, the A_{2B} and A_3 receptors in chronic inflammatory processes are more involved in the modulatory effects of adenosine in processes such as angiogenesis and fibrosis, increasing the production of the vascular endothelial growth factor (VEGF), of IL-6 and IL-8. A_{2B} receptors also play a role in asthma and chronic obstructive pulmonary diseases (Haskó G. L. J., 2008), (Kolachala V, 2005). Activation of this receptor on human lung fibroblasts promotes their differentiation into myofibroblasts, suggesting that adenosine may participate in lung fibrosis and remodelling during asthma and chronic obstructive pulmonary disease (Reutershan J, 2007). Similarly, the A_3 receptors expressed in human lungs, on mast cells and on lipid-rich macrophages, the so-called foam cells, play a role in the regulation of inflammation typical of lung and atherosclerotic diseases (Hua X, 2008), (Borea PA, 2015). In 1929, Drury and Szent-Györgyu were the first to recognize the role of adenosine in reducing heart rate, blood pressure and inducing coronary vasodilation (Drury AN, 1929); for this reason, since the 1980s, adenosine was used to reduce heart rate in patients with supraventricular tachycardia (diMarco JP, 1985). At the cardiovascular level, adenosine has a greater role in controlling hypertension, rather than in vascular remodelling; however, adenosine receptors are important in changes in vascular tissues associated with hypertension, as demonstrated by vascular remodeling following inhibition of endogenous adenosine (Matias A, 1991). Some studies have revealed coronary vascular protection mediated by adenosine receptors, which enhances the role of this molecule in cardiovascular diseases; exogenous adenosine receptor agonists can limit coronary dysfunction and damage resulting from an ischemic insult (Maczewski M, 1998), (Zatta AJ, 2006). Adenosine has both anti- and pro-arrhythmic action inducing, for example, in animal and human models, atrial flutter or fibrillation; these models also support the involvement, in particular, of the A_1 receptor in atrial fibrillation during a heart attack (Bertolet BD, 1997), (Yavuz T, 2004). An etiological agent of many cardiovascular diseases is atherosclerosis and various studies have shown that inhibition of the A_{2B} and A_3 receptors can be useful in preventing the development of atherosclerotic plaque (Gessi S, 2010). Recently, it has been shown that blocking the A_{2B} receptor during hypoxia significantly reduces proliferation and renal fibroblastic activation with a reduction in the release of profibrotic cytokines (Tang J, 2015); furthermore, the inhibition of the A_3 receptor, in mouse models of renal and cardiovascular diseases, prevents the

development of hypertension and attenuates cardiac hypertrophy and fibrosis, together with the reduction of kidney damage and proteinuria (Yang T, 2016). From this, it can be assumed that the A_{2B} and A_3 receptor antagonists may represent an interesting new therapeutic strategy for the development of new drugs that prevent tissue remodelling after ischemia and can be used in the treatment of fibrotic diseases such as heart failure.

1.2 Heart failure with preserved ejection fraction and its comorbidities

Heart failure (HF) is a systemic, multifactorial disease that affects most of the world's population. It causes a large increase in hospitalizations so affecting the health economics (Lindenfeld J, 2010). In 1933 Lewis defined HF as "a condition in which the heart fails to discharge its contents adequately" (Lewis, 1933), while in the modern definition of HF it means "an inability of the heart to pump blood to the body at a rate commensurate with its needs or to do so only at the cost of high filling pressures" (Braunwald, 1992).

HF is a common pathology in the adult population (1–3%) and its prevalence increases exponentially after 70 years of age (Dunlay, 2017). HF patients present dysfunction of cardiac contractility (systolic dysfunction) and/or cardiac relaxation (diastolic dysfunction). Depending on the trend, HF can have an acute or chronic course. The most common classification of heart failure currently in use stratifies patients into:

- HF with reduced ejection fraction (**HFrEF**), also known as heart failure with left ventricular ejection fraction ≤ 40% (systolic HF)
- HF with preserved ejection fraction (**HFpEF**), also known as heart failure with left ventricular ejection fraction ≥ 50% (diastolic HF) (Taylor R, 2014).

Recently the European Society of Cardiology (ESC) described a third type: HF with medium-range ejection fraction (**HFmrEF**) (Ponikowski P, 2016).

Population-based epidemiological studies reported that 50% of patients with HF symptoms have a normal left ventricular ejection fraction (HFpEF) and its

prevalence continues to increase compared to HFrEF at a rate of 1% per year (Borlaug, 2015). Signs and symptoms are the same in both types of disease but there are differences in pathophysiology and treatment. Patients, with HFrEF, present with left ventricular contraction problems, exercise intolerance, and fatigue. Their reduced contractility involves the accumulation of blood in the left ventricle causing an increase in end-diastolic pressure. This increased pressure is transferred to the pulmonary and peripheral circulation causing pulmonary, peripheral edema (Van Aelst, 2018).

HFpEF is a complex, heterogeneous, multi-organ systemic syndrome, characterized by numerous etiologies and pathophysiological alterations. ESC guidelines base the diagnosis of HFpEF on typical HF symptoms and signs (impaired left ventricular filling, diastolic dysfunction, and elevated brain natriuretic peptides) (**Tab.1**). Patients with HFpEF show cardiac alterations such as impaired atrial function (Melenovsky V, 2007), diastolic dysfunction, atrial fibrillation, altered chronotropic effect, structural cardiomyopathy, and an increase in B-type natriuretic peptide (BNP) (Paulus WJ, 2007) (Vasan RS, 2000) (McMurray JJ, 2012).

	European Study Group on Diastolic Heart Failure	**Framingham**	**ESC Guidelines**
	Lung crepitations, pulmonary oedema, ankle swelling, hepatomegaly, dyspnoea on exertion, and fatigue. Distinguish between effort related or nocturnal dyspnoea. Objective evidence of reduced exercise performance includes reduced peak oxygen consumption (<25 mL/kg/min) or 6-minute walking test distance<300 m	Clinical signs and symptoms, supportive laboratory test (e.g.chest X-ray), and a typical clinical response to treatment with diuretics, with or without documentation of elevated LV filling pressure (at rest, on exercise, or in response to a volume load) or a low cardiac index	Symptoms (e.g. breathlessness, ankle swelling, and fatigue) and signs (e.g. elevated jugular venous pressure, pulmonary crackles, and displaced apex beat) typical of HF
LV systolic function		LVEF ≥50% within 72h of HF	Normal or only mildly reduced LVEF and LV not dilated
Diastolic dysfunction	Symptoms or signs	Assessment of diastolic function is not needed for probable diagnosis	Relevant structural heart disease (LV hypertrophy/ LA enlargement) and/or diastolic dysfunction

Table 1. Diagnostic criteria for HFpEF. From Senni M, Paulus WJ, Gavazzi A, Fraser AG, Díez J, Solomon SD, Smiseth OA, Guazzi M, Lam CS, Maggioni AP, Tschöpe C, Metra M, Hummel SL, Edelmann F, Ambrosio G, Stewart Coats AJ, Filippatos GS, Gheorghiade M, Anker SD, Levy D, Pfeffer MA, Stough WG, Pieske BM. New strategies for heart failure with preserved ejection fraction: the importance of targeted therapies for heart failure phenotypes. Eur Heart J. 2014; 2797-815.

On the other hand, extracardiac anomalies and comorbidities such as atrial fibrillation, chronic kidney disease, chronic obstructive pulmonary disease, anemia, type 2 diabetes mellitus (20-45%) (Dhingra A, 2014), obesity (84%) (Haass M, 2011), hypertension (60-80%) (Dhingra A, 2014) and thrombosis (Serebruany V, 2002) can be associated/contribute to the onset of the disease (Reddy YN, 2016).

These diseases are thought to gradually change the structure and function of the heart over time (myocardial hypertrophy and cardiomyocyte rigidity) (van Heerebeek L, 2014). Studies conducted on endomyocardial biopsies have revealed that diastolic dysfunction in HFpEF is caused by myocardial interstitial fibrosis, hypertrophy, and stiffness of cardiomyocytes (Mohammed SF, 2015), (Heinzel FR, 2015), (Zile MR, 2015). In 1988 Brutsaert and his collaborators discovered that cardiomyocytes were affected by the NO-sGC-cGMP pathway (Brutsaert DL, 1988). In basal conditions nitric oxide (NO) and natriuretic peptides activate a particular guanylate cyclase which produces cGMP which in turn activates protein kinase G (PKG) (Takimoto, 2012), (Kovács Á, 2016). PKG regulates Ca^{2+} homeostasis, influencing the contractility of cardiomyocytes, and acts as an inhibitor of hypertrophy and promotes left ventricular relaxation. Titin is a sarcomeric protein and exists in two isoforms the larger one (N2BA ventricular compliance and relaxation by phosphorylating troponin I and titin (Takimoto, 2012)) and the smaller and more rigid N2B (LeWinter MM, 2016). PKG is one of the factors that reduces titin stiffness via phosphorylation (LeWinter MM, 2016). Therefore, the rigidity of cardiomyocytes depends on the degree of phosphorylation of titin and on the expression of its isoforms (Krüger M, 2009), (LeWinter MM, 2016). In the heart failure with preserved ejection fraction disease, there is less phosphorylation than in HFrEF which results in greater rigidity of the cardiomyocytes (Hamdani N, 2013). Hypophosphorylation is caused by a downregulation of cGMP and PKG due to an increase in microvascular inflammation (van Heerebeek L., 2012). Indeed, animal HFpEF models and cardiac biopsies of HFpEF patients exhibit an increased cardiomyocyte stiffness which is reversed by PKG administration (van Heerebeek L., 2012) and related to microvascular endothelial inflammation (Sorop O, 2018). It was found that patients with HFpEF and concurrently presenting other comorbidities had elevated levels of inflammatory biomarkers such as IL-1, IL-6, TNFα, C reactive protein (CRP), growth differentiation factor 15 (GDF15) a plasma level (Paulus WJ., 2013). Circulation of inflammatory cytokines increases oxidative stress with increased ROS production, at the endothelium, and reducing the bioavailability of NO and cGMP causing stiffness of cardiomyocytes (van Heerebeek L., 2012). Furthermore, myocardial remodeling is caused by an increase in the expression of adhesion molecules (VCAM) and E selectin at the endothelial level. These molecules attract

leukocytes which infiltrate and secrete transforming growth factor β (TGF-β), which converts fibroblasts into myofibroblasts with increased interstitial deposition of collagen (Paulus WJ., 2013) (Fig.5).

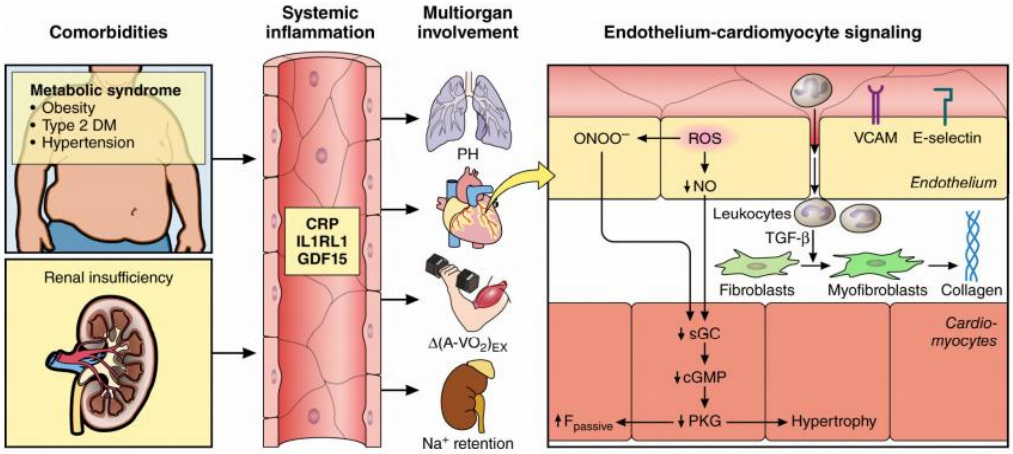


Figure 5. Role of inflammation in cardiac remodeling. From Shah SJ, Kitzman DW, Borlaug BA, van Heerebeek L, Zile MR, Kass DA, Paulus WJ. Phenotype-Specific Treatment of Heart Failure with Preserved Ejection Fraction: A Multiorgan Roadmap. Circulation. 2016.

Regarding therapeutic approaches, there are American, Australian and European clinical guidelines on HFrEF. In contrast, there are no specific guidelines on medical therapies for HFpEF. The therapeutic deficit constitutes a huge unmet public health need. The results of clinical trials have demonstrated that while neurohumoral antagonists such as beta-blockers (Packer M., 1996) , angiotensin-converting enzyme (ACE) inhibitors, and angiotensin receptor blockers (ARBs), MRAs (mineralocorticoid receptor antagonists) as well as cardiac resynchronization are effective in HFrEF, these therapies do not decrease morbidity and mortality in HFpEF (Schwartzenberg S, 2012).

Recent studies have shown that patients affected by this pathology are mainly women (50-84 %) (O'Meara E, 2019). This sex difference can be attributed to the age distribution of the population a risk of HFpEF, as women have a higher life expectancy (Dunlay SM, 2017). The higher incidence of this disease in the female population can be explained by anatomical differences, risk factors and demographic differences. The molecular mechanisms underlying these differences

are not entirely clear. Studies point out that endothelial dysfunction and arterial stiffness is less evident in women (Beale AL, 2018), but on the other hand women are more prone to cardiac hypertrophy and fibrosis (Regitz-Zagrosek V, 2010).

It is well recognized that patients with HF have an increased risk of venous thromboembolism, stroke, and sudden death. Platelets play a pivotal role in the control of vascular homeostasis and thrombosis and they are an important source of purine nucleotides and nucleosides. Scientific evidence has shown that the increased cardiovascular risk is due to an imbalance of extracellular purine levels (Ajjan R, 2006). In damaged tissue, extracellular ADP released by activated platelets stimulates other platelets via the P2Y1 and P2Y12 receptors (Burnstock G., 2017). As previously explained (see paragraph *1.1.1*) the production of ATP and ADP is regulated by CD39, while CD73 hydrolyzes adenosine monophosphate (AMP) into adenosine (Antonioli L, 2013). CD39 is expressed on the endothelium, circulating blood cells and smooth muscle cells and can almost directly hydrolyze ATP into AMP (Behdad A, 2009). Changes in CD39 expression and activity affect the thrombogenic potential of a tissue (Covarrubias R, 2016), (Anyanwu, 2019). For example, a reduction in CD39 expression and activity has been shown to be associated with thrombotic disorders (Deaglio S, 2011), (Samudra AN, 2018). Gender is emerging as an important variable in various pathophysiological conditions (Segnaposto1), (Rossi A. R. F., 2019); sex differences in thrombosis and bleeding risks have been described (Breet NJ, 2011). There is evidence of a higher prevalence of cardiovascular diseases (CVD) in younger men than in women as well as of dramatically increase in cardiovascular risk in postmenopausal women (Regitz-Zagrosek V., 2006), (Kim E.S., 2009) age-dependent difference in the prevalence of cardiovascular events between males and females has been attributed to the loss of the protective effect of estrogens in the postmenopausal period, the physiological mechanism behind gender disparity is still unclear (Iorga A, 2017). Moreover, the gender difference in the effect of cardiovascular drugs has also been reported (Stolarz AJ, 2015). Antiplatelet therapy represents the cornerstone in the prevention of cardiovascular risk (Patrono C, 2017). Change in platelet reactivity plays a preeminent role in thrombus formation and may affect the response to therapy.

2. AIM OF THE STUDY

Current research suggests that HFpEF is characterized by cardiac fibrosis and remodeling, and occurs when chronic medical conditions (e.g. obesity, hypertension, diabetes mellitus, coronary artery disease, chronic kidney disease, chronic obstructive pulmonary disease, thrombosis) damage the heart and other organ systems (Reddy YN., 2016). These diseases are thought to gradually change the structure and function of the heart over time.

Unfortunately, the results of clinical trials have demonstrated that while neurohumoral antagonists such as beta-blockers, angiotensin-converting enzyme (ACE) inhibitors, and angiotensin receptor blockers (ARBs) as well as cardiac resynchronization are effective in HFrEF, these therapies do not decrease morbidity and mortality in HFpEF.

There is much evidence for a role of adenosine in cardiac fibrosis associated with the progression of heart failure. Endogenous adenosine appears to play a significant role in reshaping the microenvironment during inflammatory processes through the interaction with four subtypes of cell surface G-protein-coupled adenosine receptors. These receptors are widely expressed on cardiac cells including fibroblast, endothelial cells, smooth muscle cells and leukocytes, all with a cardioprotective role. Scientific evidence has shown that the expression of CD39 and CD73 are involved in the extracellular adenosine accumulation, were upregulated in human circulating leukocytes of heart failure patients, suggesting that HF could benefit from adenosine-based drug therapy.

It is well recognized that patients with HF have an increased risk of venous thromboembolism, stroke, and sudden death. The increased cardiovascular risk may be associated with an imbalance of extracellular purine levels.

Platelets are an important source of purine nucleotides and nucleosides. In case of stress, adenosine is released in large quantities by the CD39 enzyme. It is expressed on the endothelium, circulating blood cells, and smooth muscle cells. Changes in CD39 expression and activity affect the thrombogenic potential of a tissue. Gender difference in the cardiovascular risk has been extensively observed; however, while

the age-dependent difference in the prevalence of cardiovascular events between men and women has been attributed to the loss of the protective effect of estrogens in the postmenopausal period, the physiological mechanism behind gender disparity is still unclear. In the light of these considerations, the aims of this study were:

1) to investigate comparatively platelet functionality in male and female rats and the possible link to CD39 enzyme

2) to evaluate the changes in the adenosine pathway in Dahl salt-sensitive hypertensive rat, a model of heart failure with preserved ejection fraction.

3. Materials and methods

3.1.1 Animals

All experiments were performed on male and female Wistar rats (8 weeks of age, Charles River, Calco, Italy). The animals were maintained at a room temperature of 22 ± 2° on a 12-h/12 h light/dark cycle and were housed in a specific pathogen-free environment and fed standard rodent chow and water ad libitum. All procedures were carried out following the principles of the Basel Declaration and recommendation according to the European (n.63/2010/UE) and to Italian (DL26/ 2014) regulations on the protection of animals used for experimental and other scientific purposes. The protocol was approved by the Italian Ministry of Health (according to DL 26/ 2014; protocol project no. 459/2019-PR).

3.1.2 Preparation of platelet-rich plasma (PRP) and platelet aggregation

Blood was withdrawn by cardiac puncture from rats slightly anaesthetized with enflurane, and anticoagulated with 3.8 % (w/v) trisodium citrate (1:9 v/v). Platelet-rich plasma (PRP) was obtained by centrifugation at 200 x g for 15 min at 25° C. Platelet-poor plasma (PPP) was prepared from the remaining blood by centrifugation at 600 x g for 15 min at 25 °C. Platelet count in PRP was performed by a cell counter (Beckman Coulter s.r.l., Milano, Italy) and adjusted to 3 x 10^5 platelets/µl with autologous PPP. Platelet aggregation was monitored by a lumiaggregometer (Chrono-Log, Coorporation, Mod.490, USA) according to Born (1963) by measuring changes in turbidity of 0.25 ml of re-calcified ($CaCl_2$ 1mM) PRP warmed at 37°C and under continuous stirring. A single concentration response curve (1-30 µM) to ADP was evaluated. The maximum platelet aggregation rate was recorded within 16 min with continuous stirring at 37 °C. In

some experiments, platelets were also pre-incubated (10 minutes) with CD39 inhibitor, ARL67156 trisodium salt (100 μM, Tocris Bioscience, Bristol, U.K.). Platelet aggregation was determined by software AGGRO/LINK (Chrono-Log, Havertown, PA, USA) and evaluated in terms of amplitude (maximum % of aggregation).

3.1.3 Rat treatment

Different groups of animals were treated with (+)-Clopidogrel hydrogen sulfate (30 mg/kg; Tocris Bioscience, Bristol, UK) or vehicle (0.5% sodium carboxymethyl cellulose) per *os* (by gastric gavage), 2 h before blood sampling and tail transaction. Dosage of clopidogrel was estabilished based on literature (Ogawa T, 2009), Xu et al., 2018).

3.1.4 Bleeding time

The bleeding time was determined using a cutting-tail rat model. Rats were anesthetized by intraperitoneal injection of a mixture of zoletil® (30 mg/kg) and xilazine (10 mg/kg). The rat tail was transectioned with a scalpel at a point 3 mm from the tip and immersed in 37 °C saline. The tail was pre-warmed by immersion in saline for 3 min before the cut. Blood flowing from the incision was carefully monitored and bleeding time was recorded as the time between the blood overflowed and the very first moment when the bleeding stopped.

3.1.5 Western Blot analysis

Platelet suspension was washed with PBS and centrifuged at 600 x g for 15 min at 25 °C. The platelet pellet was then lysed in the radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor (Sigma-Aldrich, Italy). Total protein concentration was determined by Bradford assay, using BSA (bovine serum albumin) as standard. Protein samples (50 μg) were separated by 8% SDS-PAGE and then transferred onto a nitrocellulose membrane using standard procedure. The membranes were saturated by incubation with 5 % non-fat dry milk in PBS supplemented with 0.1 % Tween-20 (PBS-T) for 1 h at room temperature and primary antibody was incubated overnight at 4° C. Antibodies used on different membranes were: anti-CD39 goat (1:200; Santa Cruz Biotechnology, Italy), anti-cyclooxygenase 1 rabbit (COX-1, 1:1000; Cell Signaling Technology Inc., USA), anti-CD73 goat (1:200; Santa Cruz Biotechnology, Italy), anti- $P2Y_1$ mouse (1:500; Invitrogen Carlsbad, CA); anti-$P2Y_{12}$ rabbit (1:2000; Abcam, Cambridge, UK). After washing, membranes were incubated with the appropriated secondary antibody. Protein bands were normalized over the intensity of the housekeeping β-actin. Immunoreactive bands were detected using the enhanced chemiluminescence (ECL) detection kit and Chemidoc Imaging System (Bio-Rad Laboratories Inc.). Densitometry was performed with ImageLab software (Bio-Rad Laboratories Inc.).

3.1.6 Measurement of specific ATP - and ADPase activity

Platelet ATP - and ADP - ase activity was determined by measuring the concentrations of inorganic phosphate (Pi) with the Malachite Green assay kit (CliniSciences, Italy). Briefly, platelet lysates (50 μg) were pre incubated in 200 μl of reaction buffer containing NaCl (10 mM), KCl (5 mM), glucose (60 mM), $CaCl_2$ (5 mM) and Tris-HCl (50 mM), pH 7.5, at 37°C for 10 minutes. The enzyme reaction was started by the addition of ATP or ADP to a final concentration of 1

mM; after 40 minutes at 37°C, the reaction was stopped by the addition of 200 µl of trichloroacetic acid (TCA). The concentration of inorganic phosphate (P*i*) released during the hydrolysis of ATP and ADP was measured using the Malachite Green assay according to the manufacturer's instructions. To determine specificity, experiments were also performed in the presence of the CD39 inhibitor, ARL67156 trisodium salt (100 µM, Tocris Bioscience, Bristol, U.K.). For these experiments, samples were incubated with ARL67156 in assay medium for 30 min at 37°C before adding ATP or ADP. To have the net value of P*i* produced following enzymatic reaction, non-specific P*i* released in the presence of ARL67156 in each sample was subtracted from the value obtained following incubation with the substrate. Results were expressed as P*i* released pmol/min/µg protein.

3.2.1 Animal model of heart failure with preserved ejection fraction (HFpEF).

Seven-week-old Dahl rats (salt-sensitive rats) (Charles River Laboratories, Wilmington, MA, USA) were subjected to a 12-hour light and 12-hour dark cycle in a controlled temperature and humidity room. The animals were fed food containing 8% NaCl (High-Salt Diet, HS) to induce hypertension. The animals were subjected to this hypersodic diet for a total of 13 weeks to develop heart failure with preserved ejection fraction due to hypertension. In the control group, however, the rats were fed a low-salt diet (Low-Salt diet, LS), containing 0.3% NaCl. The animals were sacrificed at five and thirteen weeks and their hearts were removed. Both the housing and the treatment to which the animals were subjected complied with the Italian rules on the protection of animals used for experiments or other scientific purposes (DM 26/2014) and with EEC regulations (OJ of EC L 358/1 18/12 / 1986) (Fig.6).

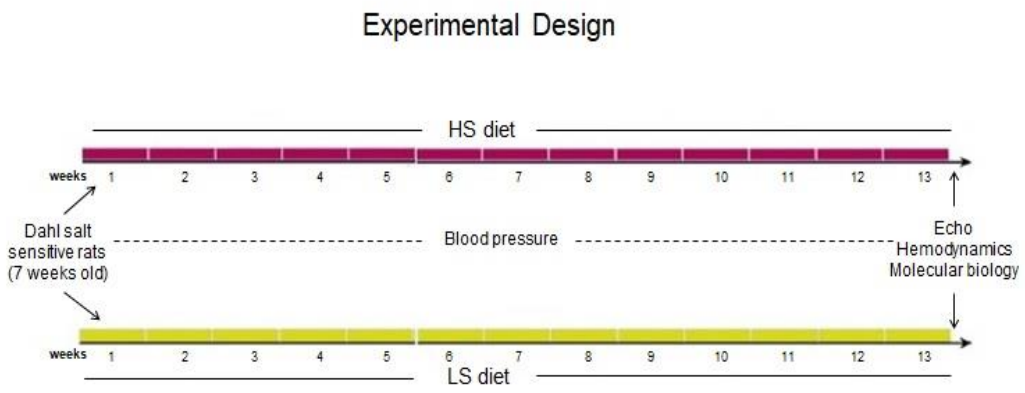


Figure 6. The time course of HFpEF experiments.

3.2.2 Blood pressure and heart function

Mean blood pressure was measured weekly in conscious animals using the tail-cuff method. Echocardiography was performed with a high-resolution Micro-Ultrasound System equipped with a 25-MHz linear transducer (Vevo 770, VisualSonics Inc., Ontario, Canada). Rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg b.w., i.p.) and medetomidine (0.25 mg/kg) and body temperature was maintained at ~37 °C with a heating pad. Serial M-mode images were recorded along the minor axis at the level of the papillary muscles to measure diastolic left ventricle (LV) diameter and wall thickness and to calculate ejection fraction (EF) and fractional shortening (FS). To avoid inter-operator variability, a single investigator, blinded to the animal groups, performed all image acquisitions and offline measurements. Prior to sacrifice, hemodynamic parameters were collected. In anesthetized animals the right carotid artery was cannulated with a microtip pressure transducer (SPR-612, Millar Instruments, Houston, TX, USA) connected to an A/D converter (iWorx 214) and a computer system. The catheter was advanced into the LV cavity for the evaluation of LV pressures and + and − dP/dt in the closed-chest preparation.

3.2.3 Tissue harvesting

After completion of the functional measurements, the heart was arrested in diastole by injection of 100 mM $CdCl_2$. After perfusion with 10% phosphate-buffered formalin, the heart was dissected and weighed. Finally, tissue specimens were embedded in paraffin, and 5 µm thick histological sections were cut. Alternatively, the heart was fixed in 4% paraformaldehyde for 1 h, immersed in a 30% sucrose solution overnight at 4°C and then embedded in tissue freezing medium (OCT). Tissue sections of 10 µm in thickness were cut (Di Meglio *et al.*, 2012; De Angelis *et al.*, 2015).

3.2.4 Oxidative stress

ROS generation was first measured by DHE staining with flow cytometry analysis. ROS production was detected by DHE staining. DHE is freely permeable to cells. In the presence of ROS such as superoxide anion, DHE is oxidized to ethidium bromide (EtBr) with red fluorescence, and it is trapped by intercalating with the DNA. DHE (5 μM) was applied to heart cross-sections (10 μm) and incubated in a light-protected and humidified chamber at 37°C for 30 min. In situ fluorescence was assessed using fluorescence microscopy.

Peroxynitrite formation was assessed by using an anti-3-nitrotyrosine antibody. FITC conjugated was used as secondary antibody. Samples were analysed with a Leica DM5000B (Leica Microsystems) microscope and a Zeiss LSM700 confocal microscope (Zeiss, Oberkochen, Germany).

3.2.5 Molecular biology analysis

The protein analysis of the adenosine pathway was performed on the hearts of Dahl rats using the Western Blot method. The samples were homogenized in a buffer containing 20 mM HEPES pH 7.6, 1.5 mM $MgCl_2$, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 15 μg / ml trypsin inhibitor, 3 μg / ml pepstatin, 2 μg / ml leupeptin, 40 μM benzamidine, 1% Nonidet P-40, 20% glycerol and 50 mM NaF. The lysate thus obtained was centrifuged at 10000 × g for 15 minutes at 4 ° C and the supernatant on which the protein determination was carried out using the Bio-Rad protein assay (Bio-Rad, Richmond CA) was taken using bovine serum albumin as standard (BSA). Protein samples (50 μg) were mixed in a 1: 1 ratio with a "loading buffer" (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol and 2 mg / mL bromophenol), boiled to 3 minutes, centrifuged at 10,000 × g for 10 minutes. Subsequently, they were separated by 8% SDS-polyacrylamide gel electrophoresis; the proteins were then transferred to nitrocellulose membranes (Hybon ECL

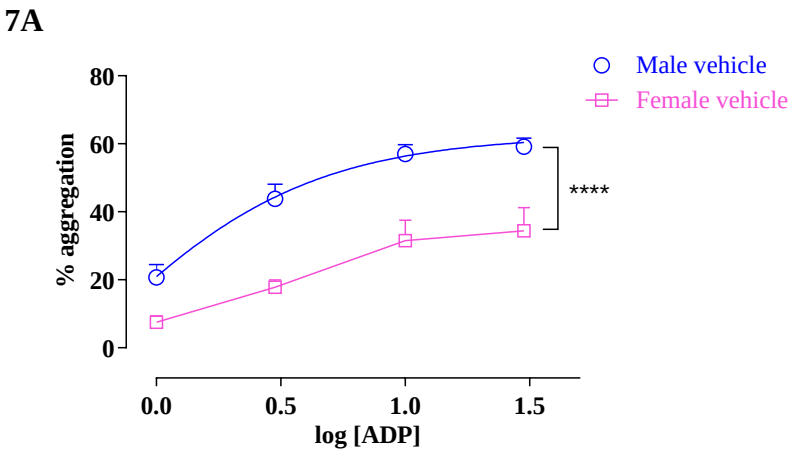
Nitrocellulose, Amersham). The membranes were subjected to blocking of the non-specific sites by washing with 0.1% PBS-Tween containing 5% w / v nonfat dry milk for one hour at room temperature; after blocking, the membranes were incubated with the relevant primary antibody overnight at 4 ° C. The antibodies used were anti-CD39 (diluted 1: 200), anti-ENTPD2 (diluted 1: 1000), anti-ENTPD3 (diluted 1: 500), anti-ENPP1 (diluted 1: 1000), anti-ENPP3 (diluted 1: 1000), anti-CD73 (diluted 1: 200) and anti-A_{2B} (diluted 1: 2000), ADA (diluted 1: 500), DPP4 (diluted 1: 10000), GAPDH (diluted 1: 500). After incubation, the membranes were washed three times with 0.1% PBS-Tween and incubated for an hour and a half with the secondary anti-rabbit antibody (diluted 1: 10000 in 5% milk and PBST) and anti-mouse. (diluted 1: 10000 in 5% milk and PBST), conjugated with horseradish peroxidase (Dako Cytomation). The protein bands were normalized on the intensity of GAPDH, while the immunoreactive bands were identified using the enhanced chemiluminescence detection kit (ECL) and the Chemidoc imaging system (Bio-Rad Laboratories Inc.). Densitometry was performed with ImageLab software.

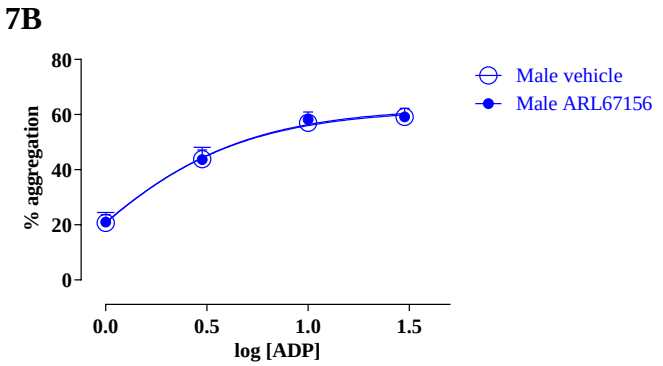
4. Results

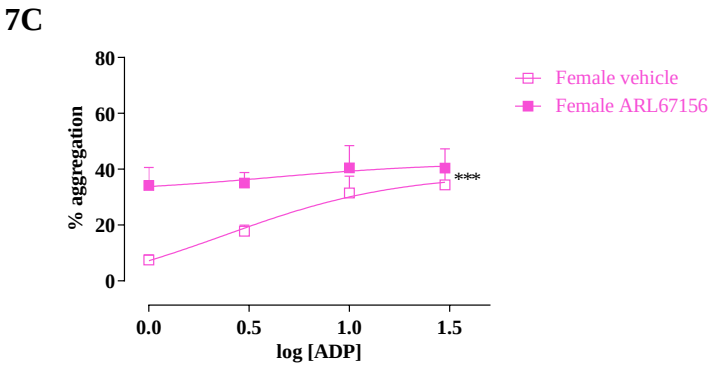
4.1.1 CD39 Inhibition Increases Female Platelet Aggregation

(Data of this thesis have been published: "Ectonucleoside Triphosphate Diphosphohydrolase-1/CD39 Affects the Response to ADP of Female Rat Platelets". Caiazzo E, Bilancia R, Rossi A, Ialenti A and Cicala C. Front Pharmacol. 2020)

Platelets isolated from male and female rats were stimulated at different concentrations of ADP (1 - 30 μM). Our analysis of concentration-response curves showed that platelets from female rats aggregated much less than platelets from male rats at each ADP concentration (Emax was 61.88 ± 3.55 % for male and 38.40 ± 6.58 % for female rats) (Fig.7A). In addition, even when platelets of both sexes were pre-incubated with a CD39 inhibitor, ARL67156 (100 μM, 10 min), it caused a decrease in ADP response in platelets isolated from female rats while it had no effect on platelets from male rats as shown by our data (Fig. 7B-7E).







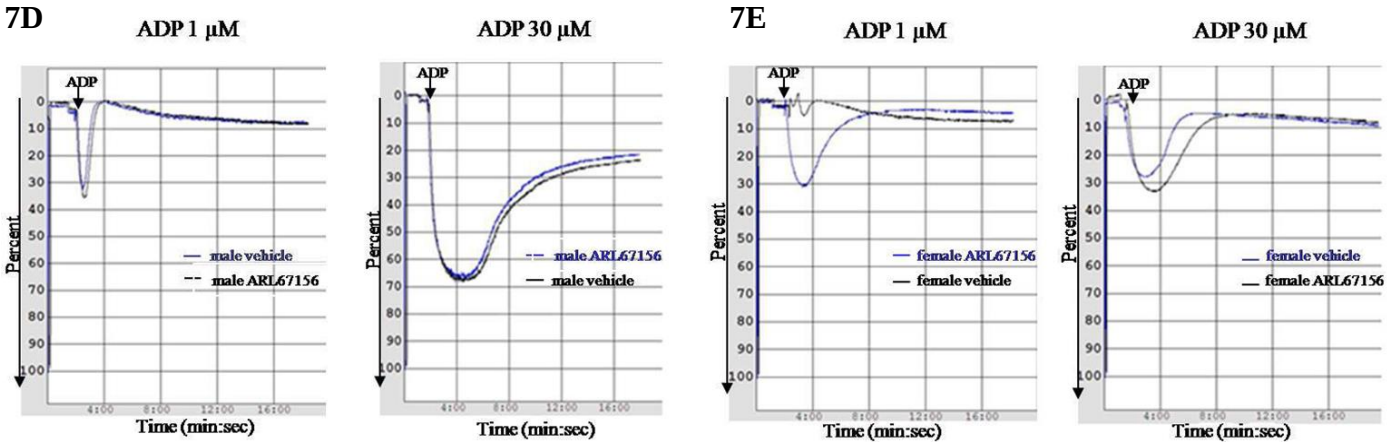
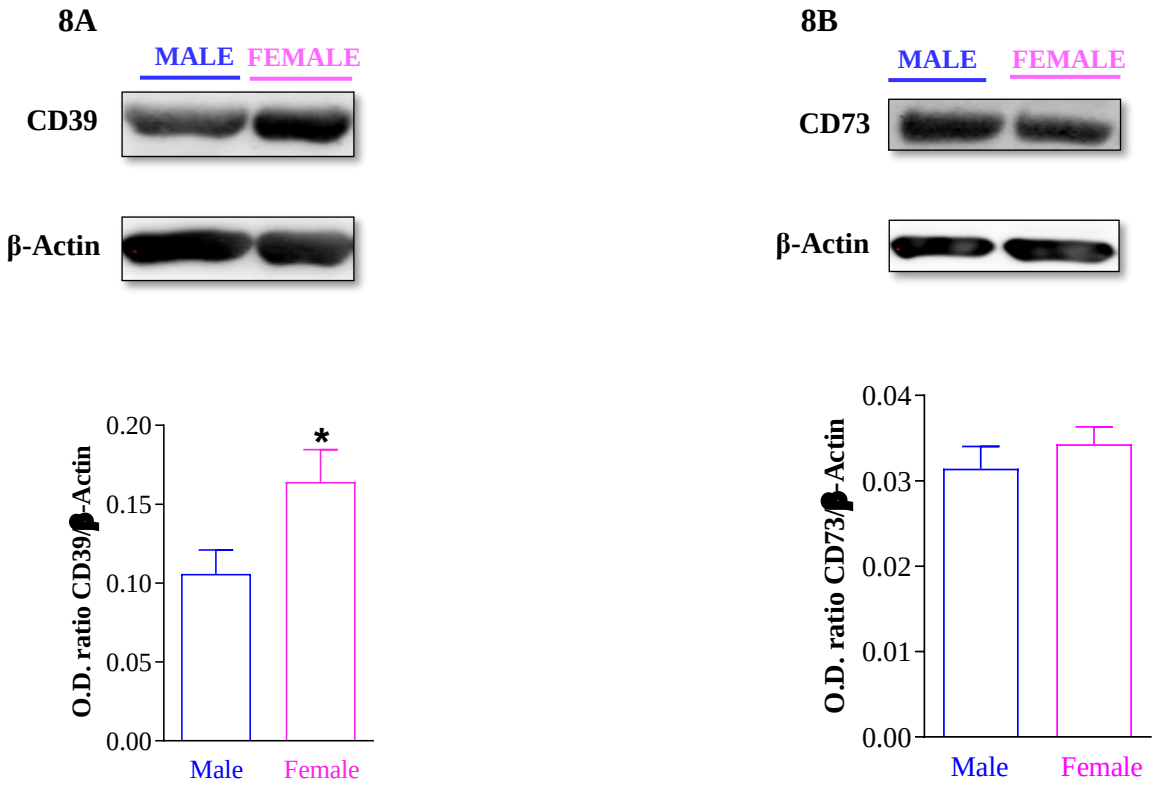


Fig. 7. Sex difference in platelet aggregation in response to ADP (1–30 µM) (A) and the *in vitro* effect of CD39 inhibitor (ARL67156) was evaluated (7B, 7C). Platelet-rich plasma (PRP) from male (7B) and from female (7C) rats was incubated with ARL67156 (100 µM, 10 min) or with the vehicle (distilled water) before a single ADP concentration. Aggregation was monitored over a 16 min period, and then quantified and expressed as a percentage of maximum amplitude. Curves were analyzed by nonlinear

regression. ****p < 0.001, two-way ANOVA, N = 6.* Typical records of platelet aggregation showing the effect of ARL67156 are also reported (7D, 7E).

4.1.2 Female platelets show increased CD39 expression.

A portion of the isolated non-stimulated platelets was used for the analysis of the basal expression of CD39, CD73, COX-1, $P2Y_1$, $P2Y_{12}$. Platelets from female rats showed significant expression of CD39 (8A). On the contrary, no sex difference was found in the expression of CD73 (8B). In addition, our data showed a greater, albeit insignificant, expression of the $P2Y_1$ receptor on female platelets but not of $P2Y_{12}$ (8C, 8D). To demonstrate that the sex difference in aggregation was due to the involvement of the adenosine pathway, we also evaluated the basal expression of a key COX-1 aggregation enzyme. Our data showed that, even in this case, there was no gender difference in the expression of this enzyme (8E).


8A
MALE
FEMALE
CD39
β-Actin
O.D. ratio CD39/β-Actin
0.20
0.15
0.10
0.05
0.00
*
Male
Female
8B
MALE
FEMALE
CD73
β-Actin
O.D. ratio CD73/β-Actin
0.04
0.03
0.02
0.01
0.00
Male
Female
8D
MALE
FEMALE
P2Y1
β-Actin
O.D. ratio P2Y1/β-Actin
2.0
1.5
1.0
0.5
0.0
Male
Female
8E
MALE
FEMALE
P2Y12
β-Actin
O.D. ratio P2Y12/β-Actin
1.5
1.0
0.5
0.0
Male
Female

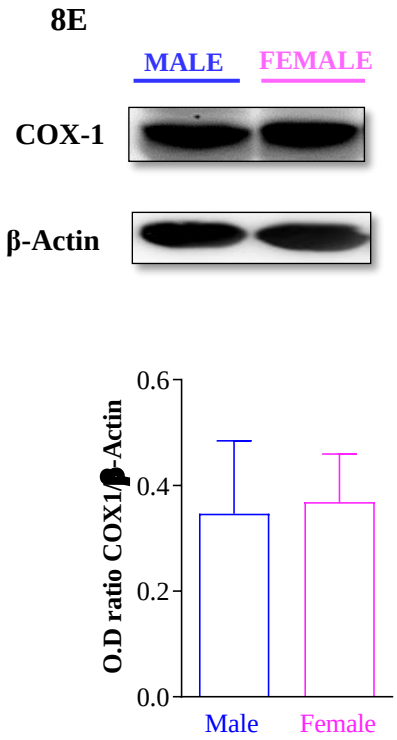


Figure 8. Basal expression of CD39, CD73, cyclooxygenase-1, $P2Y_1$, $P2Y_{12}$ (A-B-C-D-E) was evaluated by Western blot analysis in platelet lysates from male and female rats. Densitometry of specific bands has been normalized to β-actin expression. * *p < 0.05 versus male, Two-tailed t test, N=6.*

4.1.3 Female platelets show enhanced ATP-ase and ADP-ase activity

In agreement with the previously shown data of a higher baseline expression of CD 39, we also evaluated its activity. As shown in Figure 9A and 9B, increased ATP-ase and ADPase activity was evident in platelets isolated from female.

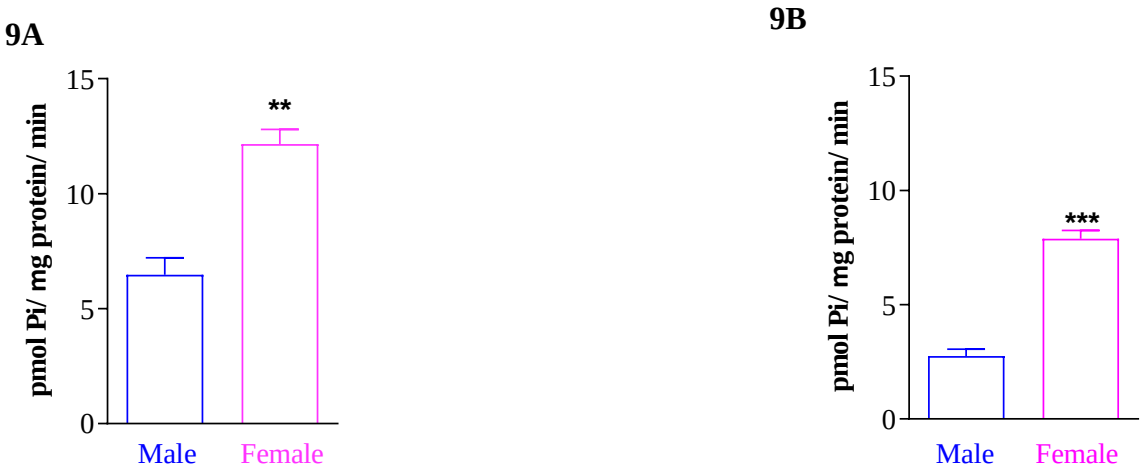


Figure 9. Sex difference in ATP – (9A) and ADP (9B) – hydrolysis mediated by CD39. ATPase and ADPase activity was evaluated on unstimulated platelet lysate from male and female rats by measuring P_i released following incubation with ATP or ADP, respectively. ** *p<0.01 and* *** *p<0.001 versus male, Two- tailed t test, N=6.*

4.1.4 Clopidogrel inhibits ex vivo platelet response to ADP but does not affect CD39 activity

After 2 hours that the animals were treated with clopidogrel (30 mg/kg, p.o.) or with the vehicle (0.5% sodium carboxymethyl cellulose, p.o.), we evaluated platelet aggregation in response to ADP (1-30 μM). Our data showed reduced platelet reactivity following treatment with clopidogrel in both platelets isolated from male rats (Emax, 73.27 ± 6.01 to 57.37 ± 4.73; n = 5-6) and those isolated from female rats (Emax, from 49.72 ± 5.01 % to 27.73 ± 5.3 %, n = 5-6) (Fig. 10A e 10B). Rat treatment with clopidogrel did not affect CD39 activity (Fig.10E, 10F).

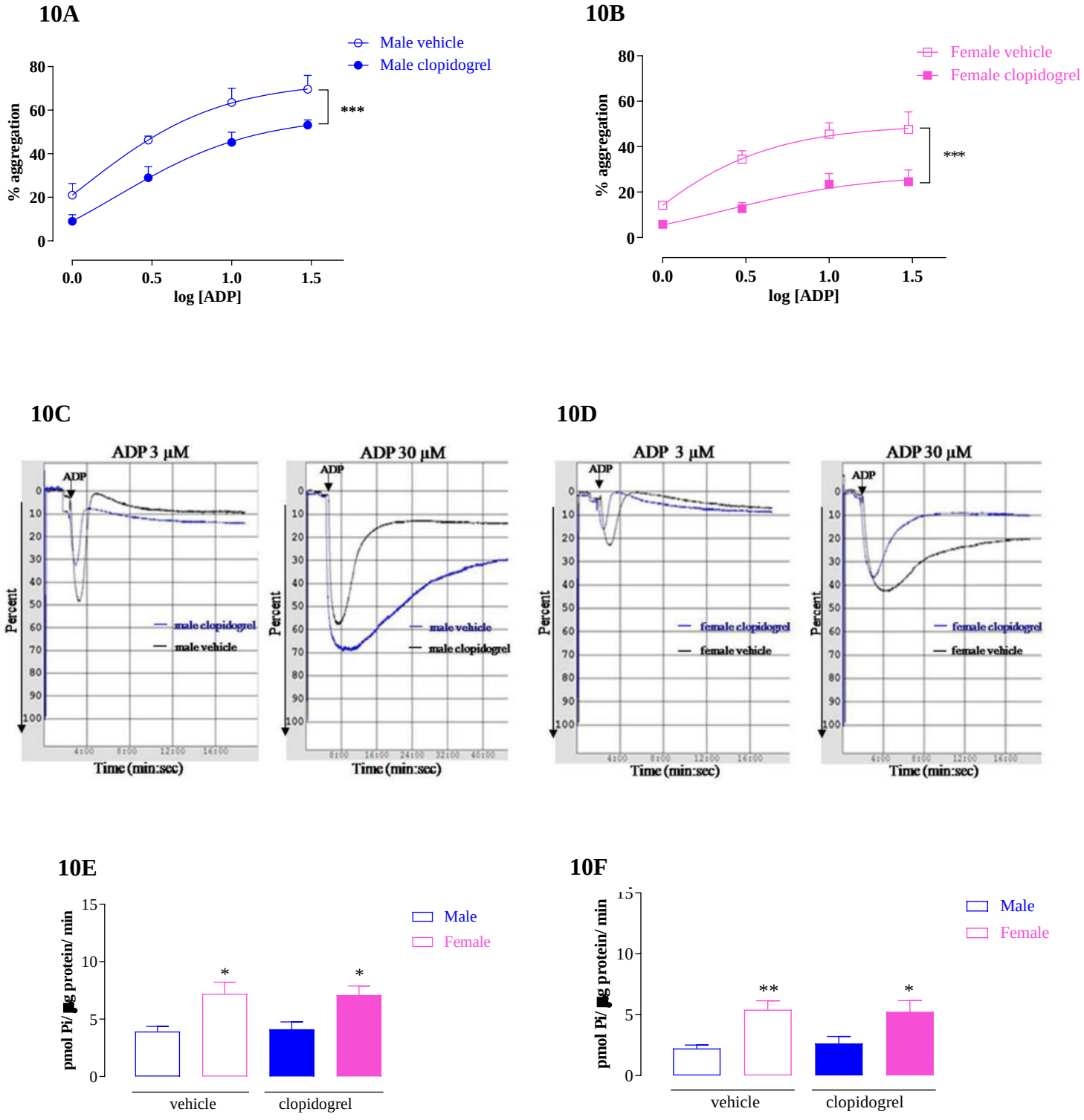


Figure 4. Ex vivo effect of treatment with clopidogrel on ADP-induced platelet aggregation, in male and female rats. Aggregation was quantified and expressed as a percentage of maximum amplitude (A, B) *** *p <0.001, Two Ways ANOVA, N=6.* Typical records of platelet aggregation showing the ex vivo effect of clopidogrel are also reported (C, D). ATPase (E) and ADPase (F) activity evaluated on platelet lysate from male and female rats administered with clopidogrel or with the vehicle by measuring P_i released following incubation with ATP or ADP, respectively. **p<0.05 and **p<0.01 versus male, Two-tailed t test, N=5*

4.1.5 Female rats have prolonged bleeding time

In another set of experiments two hours after the oral administration of the vehicle or clopidogrel, the bleeding time was evaluated. Blood loss was increased in female compared to male rats (female, 326 ± 21.39 sec vs. male, 129 ± 32.52 sec); this sex difference was observed also in clopidogrel – treated groups (female, 491.8 ± 33 sec vs. male, 245 ± 38.14 sec).

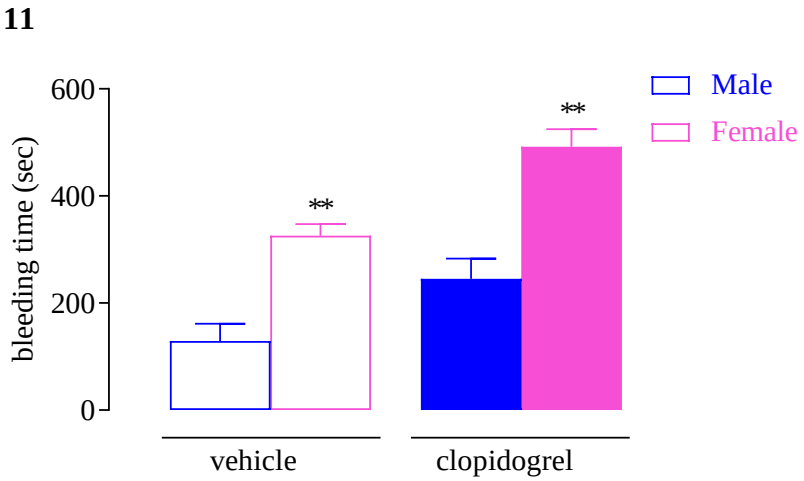


Figure 11. Sex difference in bleeding time (sec) evaluated 2 hours after vehicle (0.5% sodium carboxymethyl cellulose, p.o) or clopidogrel (30 mg/kg, p.o.) administration. ***p<0.01 versus male, Two-tailed t test, N=5.*

4.2 Heart Failure with Preserved Ejection Fraction

4.2.1 Blood pressure, survival and body weight

The unpublished data shown was performed in collaboration with the research team of Prof. Berrino of the Luigi Vanvitelli University of Naples.

Dahl rats, fed the HS diet, developed significant hypertension starting from the fifth week of treatment, compared to LS rats who instead maintained normal blood pressure for all 13 weeks (Fig. 13A). The development of this hypertension occurred together with a worsening of the general condition, such as to cause a decrease in weight (Fig. 13B) and an increase in mortality in HS rats (Fig. 13C).

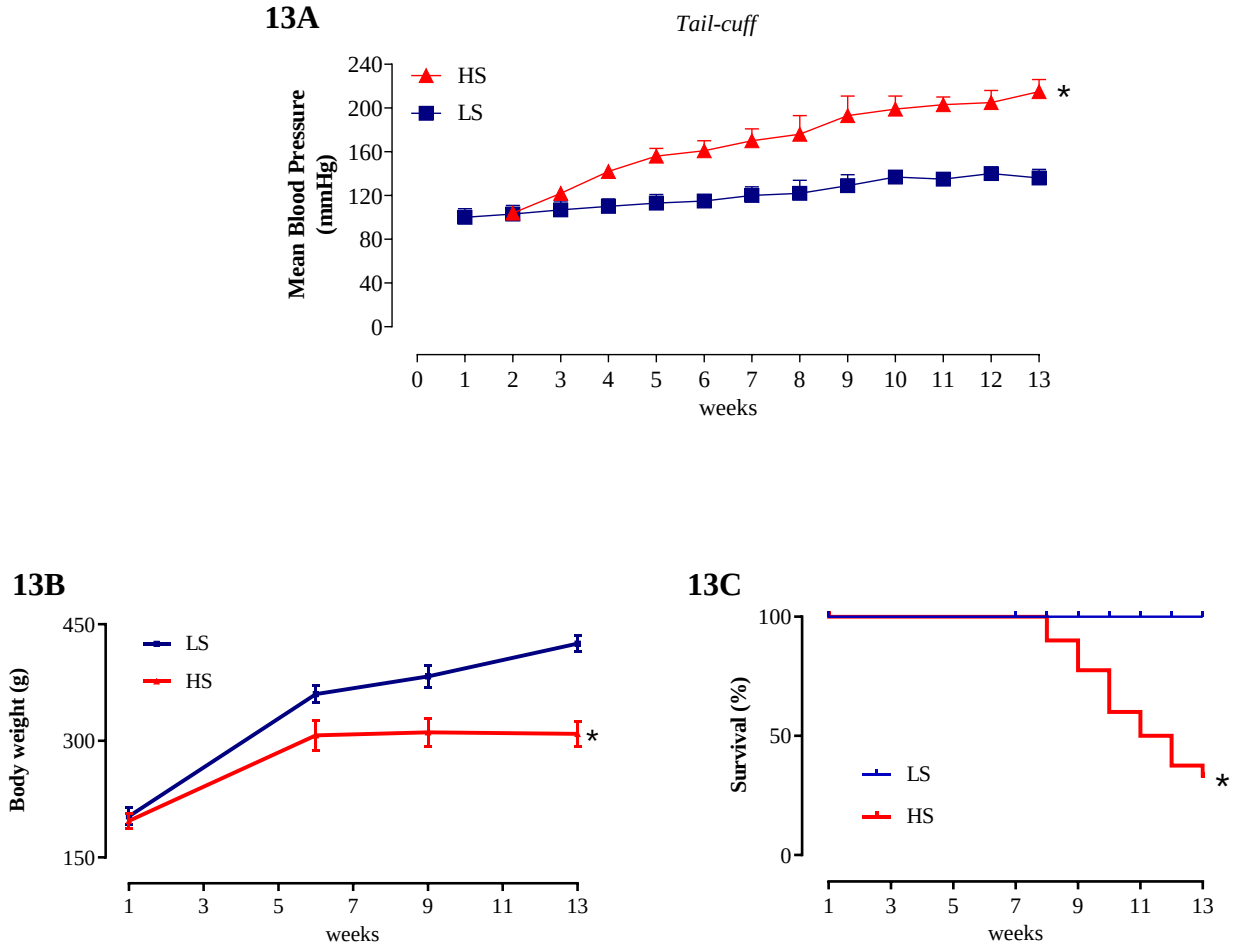


Figure 13. Sex difference in bleeding time (sec) evaluated 2 hours after vehicle (0.5% sodium carboxymethyl cellulose, p.o) or clopidogrel (30 mg/kg, p.o.) administration. ***p<0.01 versus male, Two- tailed t test, N=5.*

4.2.2 Diastolic function

Confirming the heart failure model with preserved ejection fraction, diastolic parameters were evaluated before sacrifice at the fifth and thirteenth weeks, which were significantly different between the two groups. The data indicated an increase in final diastolic pressure (Fig. 14A), a decrease in the dP / dt min ratio (Fig. 14B), an increase in the time constant Tau (Fig. 14C) and in the final diastolic pressure-volume ratio (Fig. 14D).

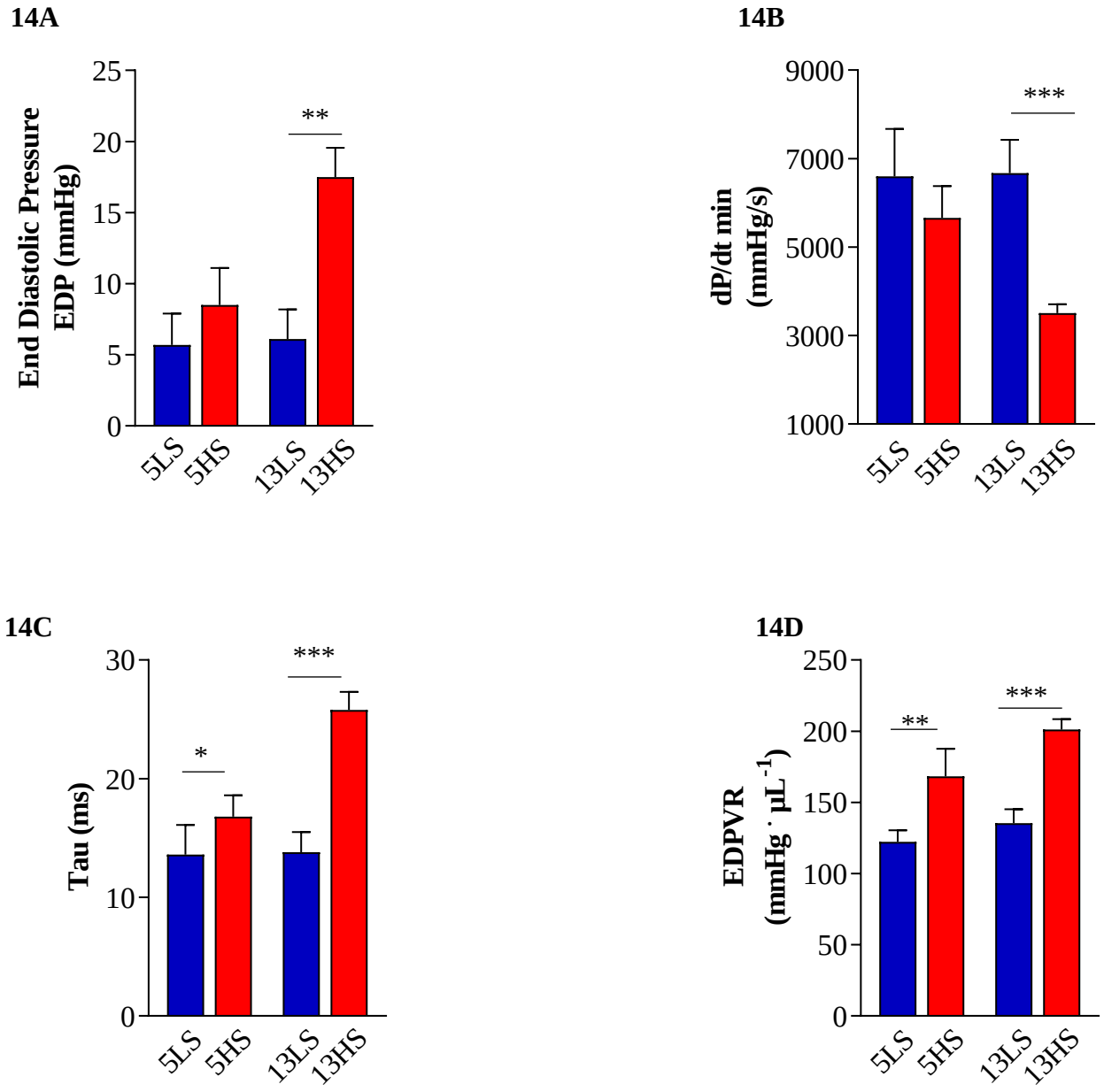


Figure 14. Diastolic function: EDP (A), dP / dt min (B), constant Tau (C) and EDPVR (D). Results are expressed as mean ± S.D. **p<0.05, **p<0.01 *** p <0.001* (n = 6 in each group).

4.2.3 Systolic function

As previously explained, HFpEF is a pathology characterized by diastolic dysfunction while systolic function is preserved. Our data show that in LS and HS rats, at both 5 and 13 weeks, systolic function did not show differences; in fact, the ejection fraction (FE) (Fig. 15A) and the shortening fraction (FS) (Fig. 15B) are the same in both groups.

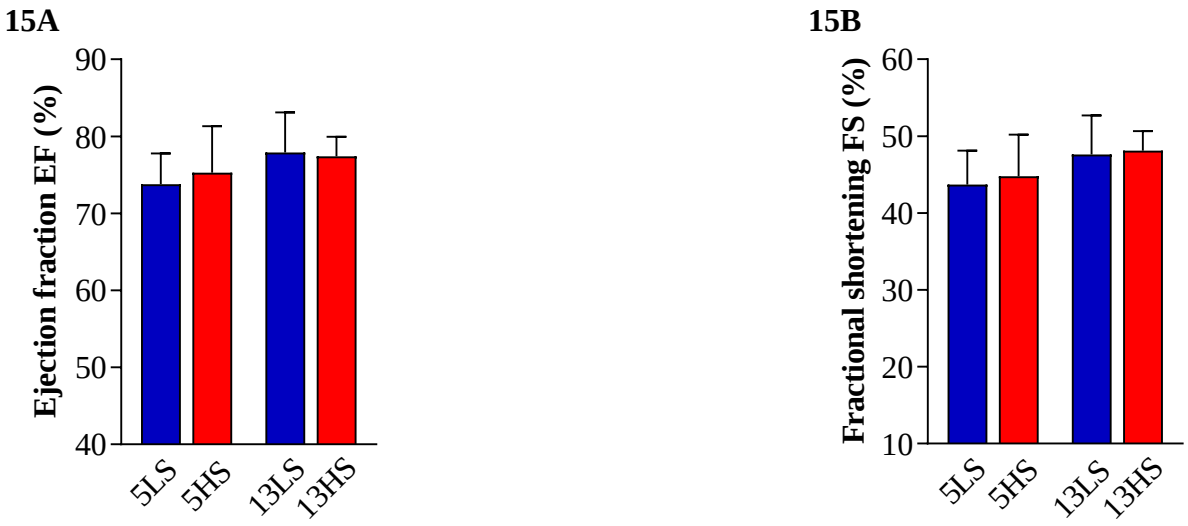


Figure 15. Ejection fraction (EF) and shortening fraction (FS) (A and B). Results are expressed as mean ± S.D. (n = 6 in each group).

4.2.4 Echocardiographic parameters

From echocardiographic analyzes our data showed a significantly increase of LV systolic and diastolic posterior wall thickness in the HS group of animals at both 5 and 13 weeks respectively (Fig.16A and 16B).

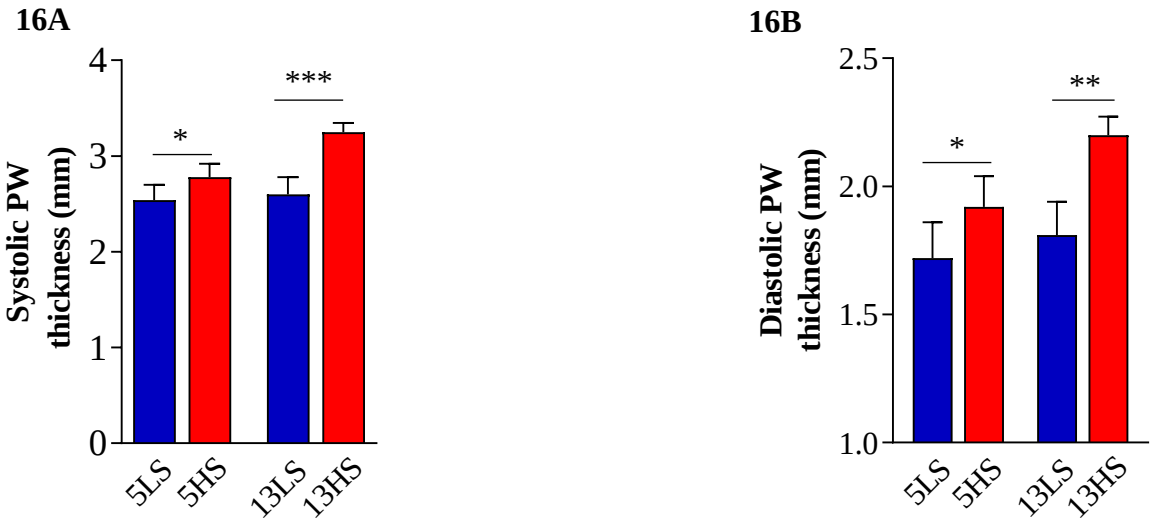
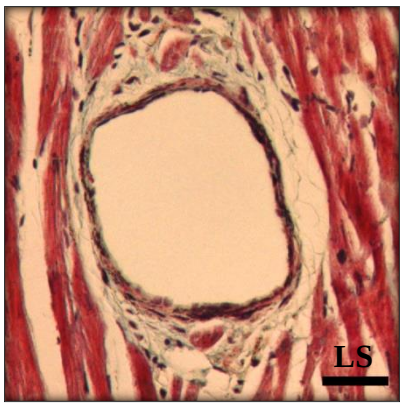


Figure 16. **Echocardiographic parameters.** Systolic Posterior Wall thickness (A) and diastolic Posterior Wall thickness (B). Results are expressed as mean ± S.D. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, significantly different from LS (n = 6 in each group).

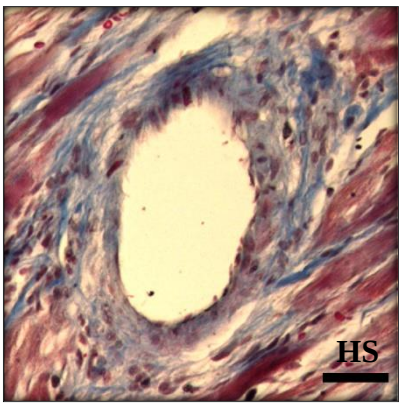
4.2.5 Myocardial remodelling

As already described in section 1.2, oxidative stress and inflammatory state contribute to the onset of fibrosis. In our animal model of HFpEF we observed, in the myocardium of 13 HS animals, an increase in perivascular fibrosis due to collagen accumulation, shown by Masson's trichrome staining (17A-17C).

17A



17B



17C

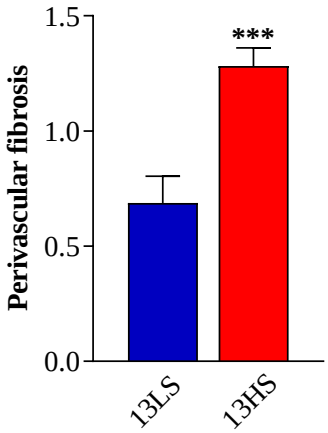
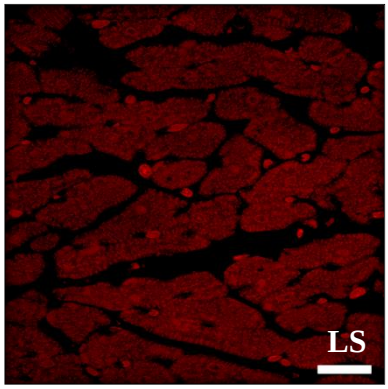


Figure 17. Myocardial fibrosis. Masson's trichrome staining showing collagen deposition (blue) in the myocardium of LS (left panel), HS (right panel) animals (A-B). Perivascular collagen fraction (D). Results are expressed as mean ± S.D. ****p < 0.001*, significantly different from LS (n = 6 in each group).

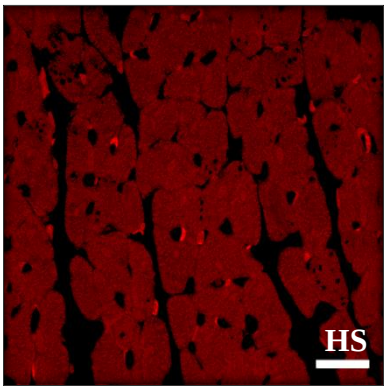
4.2.6 Oxidative/Nitrative stress

In our model of HFpEF, the involvement of myocardial oxidative stress was also addressed. To ascertain whether ROS levels are increased in the hearts of the Dahl rats, we measured the levels of DHE fluorescence in LS and HS heart sections. When compared with LS hearts, HS hearts displayed an increase in EtBr fluorescence, reflecting an increase in superoxide levels (Fig.19A and 19B). Regarding nitrative stress, also in this case our data showed greater formation of reactive nitrogen species (RNS), as documented by the 3-nitrotyrosine staining in the HS group at 13 weeks, compared to the levels in the LS rats (Fig.19C and 19D).

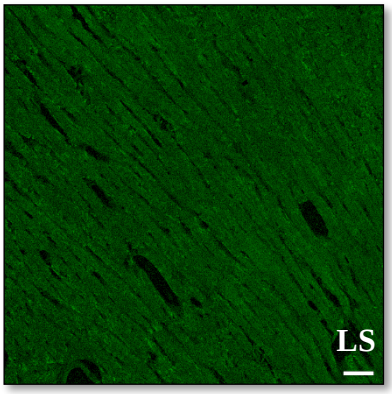
19A



19B



19C



19D

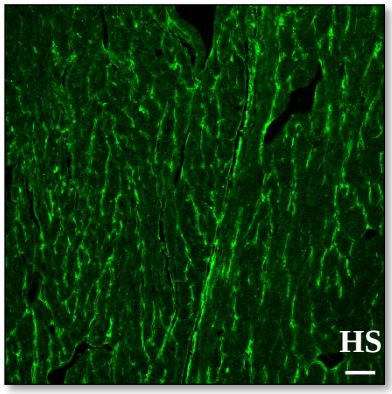


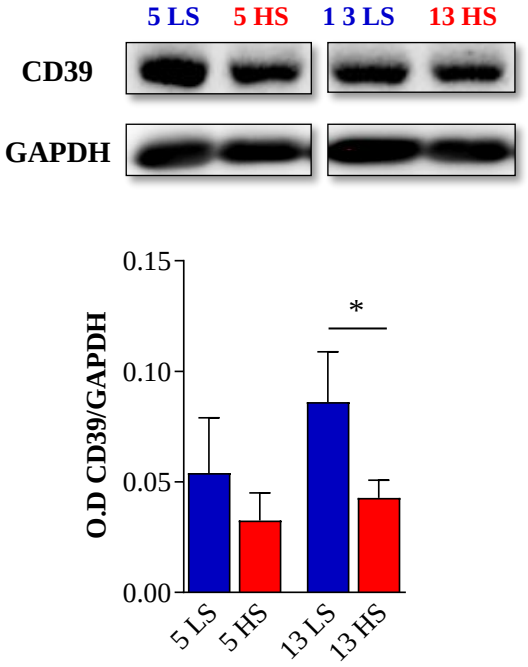
Figure 19. Oxidative/nitrative stress. Levels of ROS production as revealed by DHE (red) fluorescence (A and B) in LS (left panel) and HS (right panel) myocardium. 3-nitrotyrosine (green) in the endothelium in hearts from LS (left panel) and HS (right panel) rats (C-D). Data represent the mean ± SD (n = 6 in each group).

4.2.7 Western blot analysis of the adenosine pathway

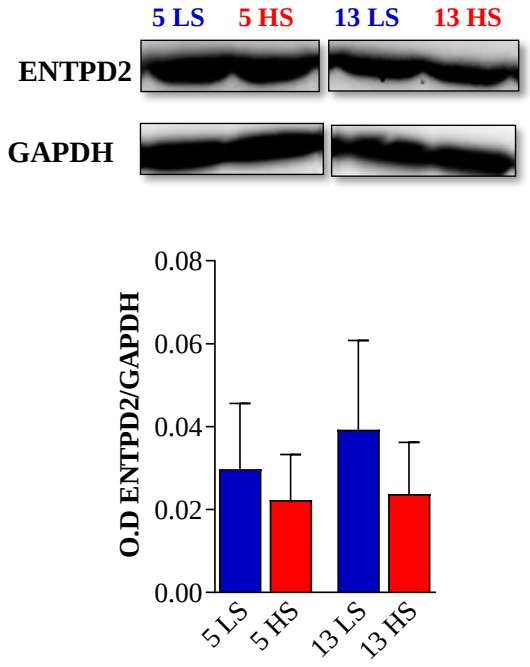
The enzymatic components of this pathway were evaluated by Western Blot method on the hearts of Dahl rats, analyzing the changes at the two time points: at five weeks, when hypertension begins to appear, and at thirteen weeks of treatment, when established heart failure.

A first evaluation was performed on the enzymes involved in the metabolization of ATP to AMP; these are part of the ENTPDase family and the expression of the enzymes ENTPD1, also known as CD39, (Fig. 20A) ENTPD2 (Fig. 20B) and ENTPD3 (Fig. 20C) was evaluated. These analyzes highlighted a lower expression of these enzymes in HS rats at thirteen weeks compared to the control group, suggesting a lower production of adenosine in conditions of heart failure with preserved ejection fraction.

20A



20B



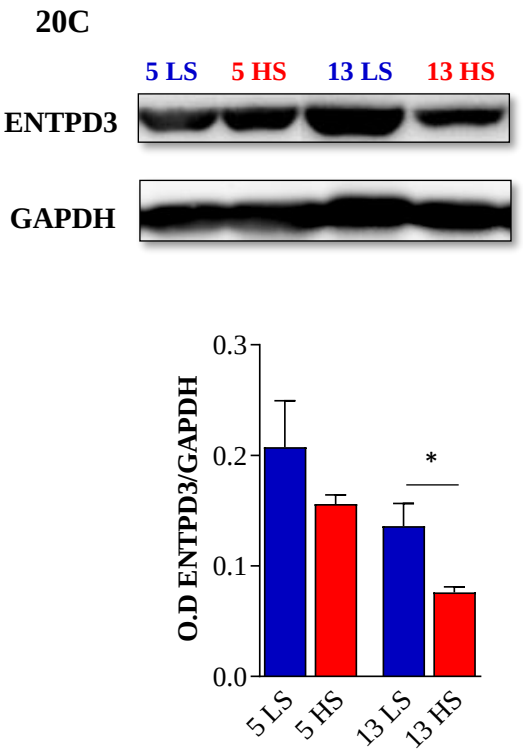


Figure 20. Expression of CD39 (A), ENTPD2 (B) and ENTPD (C) by Western blot analysis. Results are express as mean ± S.D. * $p < 0.05$ (n = 5 in the LS group and n = 5 in the HS group).

Subsequently, the expression, in the two groups of Dahl rats of the enzymes belonging to the family of ENPPs, of ENPP1 (Fig. 21A) and ENPP3 (Fig. 21B), also involved in the metabolization of the AMP.

Also in this case, an alteration of the expression of enzymes in HS rats was seen; in particular, a significant increase in ENPP3 can be observed in HS rats at thirteen weeks, compared to the LS group, thus suggesting a possible compensatory action of these enzymes in the production of AMP, following the decrease in the expression of ENTPDases.

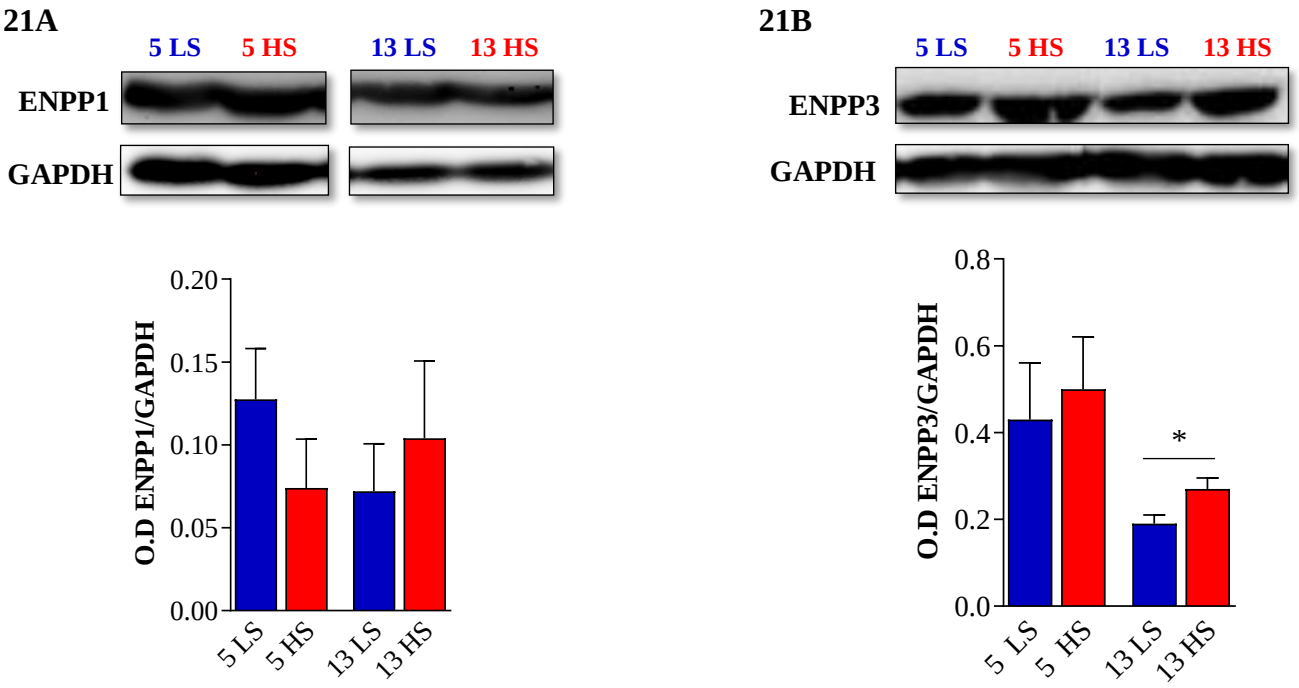


Figure 21. Expression of ENPP1 (A) and ENPP2 (B) by Western blot analysis. Results are expressed as mean ± S.D. * $p < 0.05$ (n = 5 in the LS group and n = 5 in the HS group).

The adenosine formed is metabolized by the ADA enzyme and its DPP4 cofactor to inosine, which has a pro-inflammatory action. For this reason, the expression levels of these two enzymes were also evaluated. As can be seen from Figures 22A and 22B, the expression of these two enzymes increases in HS rats as the disease progresses, supporting the hypothesis of an inflammatory process underlying heart failure with preserved ejection fraction.

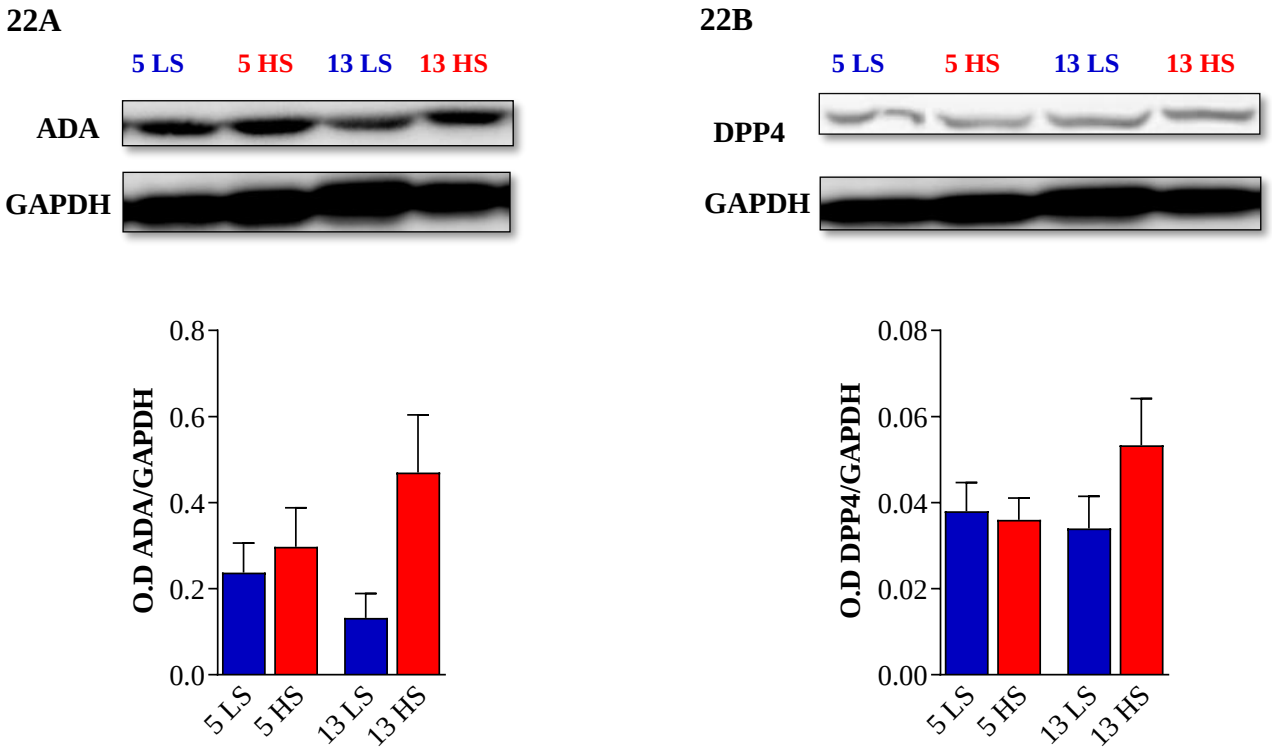


Figure 22. Expression of ADA (A) and DPP4 (B) by Western blot analysis. Results are expressed as mean ± S.D. (n = 5 in the LS group and n = 5 in the HS group).

The last enzyme involved in the cascade of adenosine formation starting from ATP is ecto-5-nucleotidase, also known as CD73, which metabolizes AMP to adenosine (Fig. 23). Western Blot analyzes showed a decrease in the expression of this enzyme in HS rats compared to the LS group, suggesting also in this case, a decrease in the production of adenosine in this pathology.

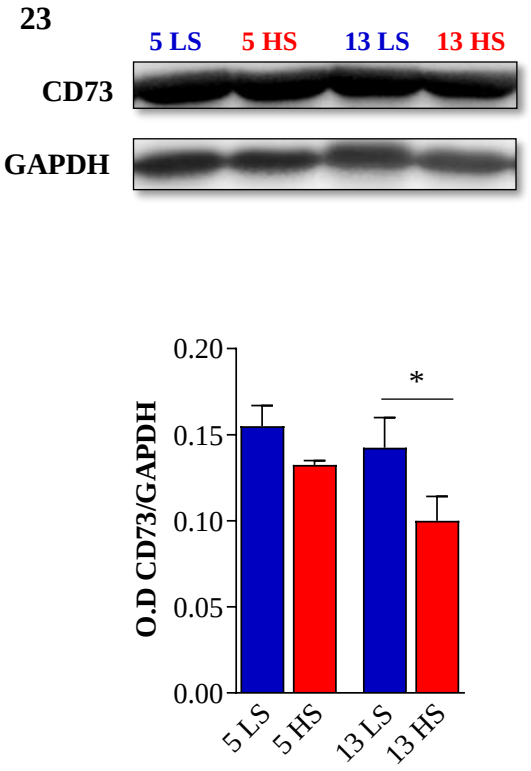


Figure 23. Expression of CD73 by Western blot analysis. Results are expressed as mean ± S.D. * $p < 0.05$ (n = 5 in the LS group and n = 5 in the HS group).

Finally, the expression of the A_{2B} receptor was evaluated, which is the main adenosine receptor involved in cardiac remodeling. This receptor, in fact, is involved in the processes that lead to cardiomyocyte rigidity and cardiac fibrosis.

The analyzes showed a significant increase in the expression of this adenosine receptor in HS rats at thirteen weeks of treatment (Fig.24), thus confirming the harmful role of A_{2B} activation in the fibrotic process during heart failure with preserved ejection fraction.

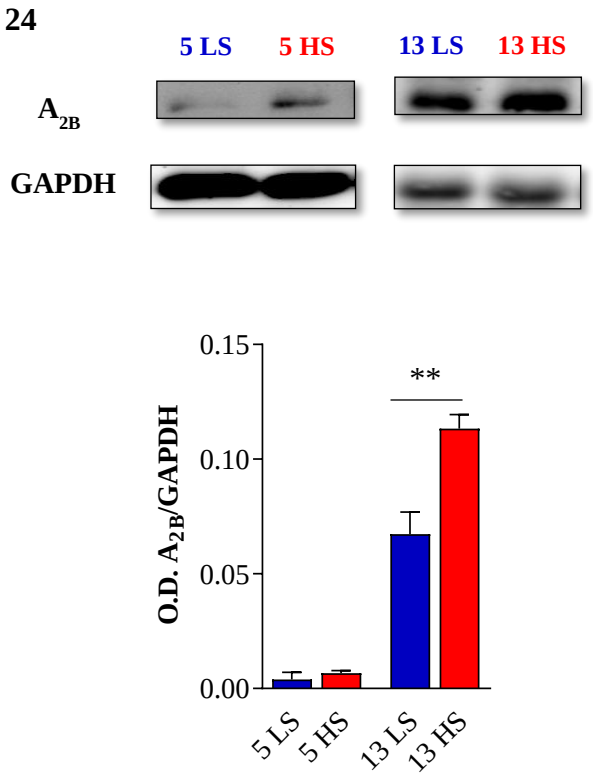


Figure 24. Expression of A_{2B} by Western blot analysis. Results are expressed as mean ± S.D. ** *p <0.01* (n = 5 in the LS group and n = 5 in the HS group).

5. Discussion

5.1 Ectonucleoside triphosphate diphosphohydrolase-1/CD39 affects the response to ADP of female rat platelets

Heart failure (HF), which affects over 23 million people worldwide, is defined as a "complex clinical syndrome" characterized by comorbidities such as hypertension, chronic kidney disease, obesity, diabetes and lung disease. Recent studies have shown that inflammation plays an important role in the emergence and progression of heart failure and a correlation has been shown between high circulating levels of pro-inflammatory mediators and adverse clinical outcomes. Heart failure patients are known to have an increased risk of venous thromboembolism and alterations in platelet function, in patients with HF, are reported in the literature.

Scientific evidence has shown that the increased cardiovascular risk is due to an imbalance of extracellular purine levels. Platelets are the main source of nucleotides and nucleosides and they play a pivotal role in the control of vascular homeostasis and thrombosis. The hydrolysis of nucleotides and nucleosides is regulated by two main enzymes, NTPDase-1/CD39 and Ecto-5'-NT/CD73. CD39 is expressed in several cells, including platelets and endothelial cells. It hydrolyzes pro-inflammatory and pro-thrombotic molecules such as ATP and ADP in AMP. CD39 is essential in maintaining antithrombotic balance. CD73 controls extracellular adenosine levels which, in turn, through its receptors, exercises antithrombotic, anti-inflammatory, and cardioprotective effects.

Today, sex is emerging more and more as a relevant variable in epidemiology, physiology, pathology, clinical manifestations, disease progression, and response to treatment, calling for more consideration of sex differences in biomedical research (Biomedical research. Of mice and women: the bias in animal models, 2012) (Pollitzer, 2013). Numerous studies have shown that there are significant sex differences in cardiovascular diseases with a higher incidence in men, and in particular the leading cause of death is represented by coronary events. However, such the difference also depends on age, since in older women, cardiovascular events become more frequent and have a worse prognosis than men.

Here, we reveal that platelets from female rats had reduced, *in vitro*, reactivity in response to ADP, compared with platelets from male rats. The % of maximum amplitude of platelet aggregation was reduced in female rats compared with the effect observed in platelets in male rats. There are also sex differences in platelet aggregation. It is interesting to underline that in the literature there are discordant data on the effect of sex on platelet aggregation and varies between species (Haque SF, 2001), (Patti G, 2014). Numerous *ex vivo* functional tests have shown, for example, that women have greater platelet reactivity than men, in terms of platelet aggregation, adhesion to fibrinogen, and interaction with leukocytes (Caulin-Glaser T, 1997). Other studies, however, show that testosterone increases the density of the thromboxane A2 receptor and therefore suggests greater platelet aggregation in males (Ajayi AA, 2005). It is therefore clear that the molecular and cellular mechanisms underlying this difference are not yet fully known. Platelets from ovariectomized rats have been shown to show decreased ATP, ADP and AMP hydrolysis suggesting that sex hormones may influence the hydrolysis of nucleotides (Pochmann D, 2005). Here, we demonstrated increased basal expression of the CD39 enzyme and an increase in its ATPase and ADPase activity in female platelets, concerning the expression and activity evaluated on male platelets. Many studies are showing the antiplatelet effect CD39 might have. In a murine model over-expressing CD39, reduced ex vivo platelet aggregation and resistance of thrombus formation have been demonstrated (Huttinger ZM, 2012). For this reason, it could be hypothesized that greater expression and activity of CD39 contribute to the reduced response of female platelets to ADP. This hypothesis is supported by the fact that when platelets from female rats were pre-incubated with the CD39 inhibitor, ARL67156, ADP-induced aggregation was strongly increased, while ARL67156 did not affect male platelets. At the platelet level, ADP can bind to two G protein coupling receptors. P2Y1 and P2Y12 are metabotropic receptors to which a Gq and Gi protein is underlying, respectively. The P2Y1 receptor, once activated, leads to the change of shape and reversible platelet aggregation, while P2Y12 activation leads to irreversible platelet aggregation. Both receptors are needed for a physiological response to ADP (Hechler B, 2011). Clopidogrel is a widely used antiplatelet drug (Lischke S, 2011). Here, we highlighted how platelet reactivity to ADP following treatment with clopidogrel in both male and female rats.

We found that clopidogrel treatment inhibited *ex vivo* platelet aggregation in response to ADP in rats of both sexes. The effect was evident with a high ADP concentration, consistent with inhibition of the P2Y12 receptor; however, in vivo treatment with clopidogrel did not abolish the difference in platelet reactivity in response to ADP between male and female rats, suggesting that clopidogrel treatment inhibits ex vivo platelet aggregation regardless of basal platelet reactivity.

Bleeding time is a test to evaluate platelet function; It is affected by changes in platelet reactivity and / or changes in the molecules involved in the recruitment of platelets to the vessels. We found that the bleeding time in female rats is longer than in males. Also, this result would be congruent with a greater expression and activity of CD39 and activity in platelets of female rats. Indeed, there is a study showing that increased CD39 activity also affects bleeding time (Degen H, 2017). We also found that female platelets showed a slight increase in P2Y1 receptor expression compared to the expression found on male platelets. We do not know if such a difference may explain the different functionality we observed between male and female platelets; however, it has been described that the P2Y1 and P2Y12 receptors cross-communicate and, on one hand, P2Y12 receptor contributes to the P2Y1-mediated calcium response through phosphoinositolo-3 kinase activation but, on the other hand, P2Y1 negatively regulates P2Y12 activity. Thus, it is possible that in platelets from female rats the balance between CD39 and P2Y receptor expression, which dictates ADP availability and signaling, respectively, leads platelets to a reduced response to ADP.

5.2 Heart failure with preserved ejection fraction and adenosine pathway

The prevalence of heart failure with preserved ejection fraction continues to increase in the developed world. The management of HFpEF differs from the management of HFrEF given differences in the evidence base for therapy. The results of clinical trials have demonstrated that while neurohumoral antagonists such as beta-blockers, angiotensin-converting enzyme (ACE) inhibitors, and angiotensin receptor blockers (ARBs) as well as cardiac resynchronization are effective in HFrEF, these therapies do not decrease morbidity and mortality in HFpEF. These data suggest that there is a difference in the pathophysiology underlying HFrEF versus HFpEF (Schwartzenberg S, 2012).

There are many comorbidities of this disease, including hypertension, diabetes, some chronic kidney diseases, and obesity, although it is not yet clear whether these diseases are comorbidities or may also be the etiological causes of heart failure with preserved ejection fraction. The fact that hypertension is the most common comorbidity in heart failure with preserved ejection fraction validates the model selected for our experiment, as shown by the data obtained (Teo LY, 2016). This type of heart failure is also referred to as diastolic heart failure, as it is characterized more by a dysfunction of the diastole; the left ventricular ejection fraction, on the other hand, remains preserved with a value greater than or equal to 50%. In fact, our data show a worsening of diastolic properties such as an increase in final diastolic pressure, a decrease in the dP / dt min ratio, an increase in the time constant Tau and in the final diastolic pressure-volume ratio. The overall picture emerging from our results is in line with a new paradigm of HFpEF as a systemic inflammatory condition partly mediated by co-morbidities. These diseases are thought to gradually change the structure and function of the heart over time (Reddy YN, 2016). Of note, our hypertensive animals with diastolic dysfunction had a significantly increase of LV systolic and diastolic posterior wall thickness in the HS group of animals at both 5 and 13 weeks, respectively. In our animal model of HFpEF we observed, in the myocardium of 13 HS animals, an increase in perivascular fibrosis due to collagen accumulation. In addition, our data show an increase in reactive oxygen species and nitrogen in hearts of rats fed

the hypersodic diet at 13 weeks. For this reason, our study focused on the role of the adenosine pathway in this pathology. Adenosine is defined as “a guardian angel” for its properties as signal molecule to preserve host defense and tissue integrity during inflammation and trauma (Caiazzo E, 2016). Endogenous adenosine appears to play a significant role in reshaping the microenvironment during inflammatory processes through the interaction with four subtypes of cell surface G-protein-coupled adenosine receptors (Ars) named A_1, A_{2A}, A_{2B}, and A_3. These receptors are widely expressed on cardiac cells including fibroblast, endothelial cells, smooth muscle cells and leukocytes, all with a cardioprotective role (Fredholm B.B., 2007). Interestingly, it has also been demonstrated that mRNA expression of ARs as well as of both CD39 and CD73 that are involved in the extracellular adenosine accumulation, were upregulated in human circulating leukocytes of heart failure patients, suggesting that HF could benefit from adenosine-based drug therapy.

Furthermore, it is known that hypertension is the greatest comorbidity in HFpEF, adenosine can regulate coronary blood flow and exerts potent vasodilatory effects (BERNE R.M., 1963). Our preliminary data suggest a reduced production of adenosine in rats with heart failure with preserved ejection fraction, an increased production of inosine, which has pro-inflammatory action, and a greater expression of the A_{2B} receptors which, with their pro-fibrotic, cause cardiac rigidity in the hearts of Dahl rats at 13 weeks and thus worsening the inflammatory state in the latter group.

6. Conclusions

In conclusion, the data shown in this thesis highlight the role of adenosine as an anti-inflammatory mediator at the cardiovascular level. At the platelet level, the importance of CD39 as a potential anti-aggregating factor. Although additional studies are required to predict gender difference in platelet reactivity, we suggest that the sex variability in platelet ATPase and ADPase activity should be considered for understanding more in depth the molecular mechanisms behind gender difference in cardiovascular risk. In our HFpEF model, although further studies are needed to confirm our data, the adenosine pathway could represent a therapeutic target for slowing/preventing the progression of heart failure with preserved ejection fraction.

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