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# Papel da dipeptidil peptidase IV na fisiopatologia da insuficiência cardíaca

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Orientador: Adriana Castello Costa Girardi

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# Original Papers

This thesis is based on the following papers:

- dos Santos L, Salles TA, Arruda-Junior DF, Campos LC, Pereira AC, Barreto AL, Antonio EL, Mansur AJ, Tucci PJ, Krieger JE, and Girardi AC. Circulating dipeptidyl peptidase IV activity correlates with cardiac dysfunction in human and experimental heart failure. *Circ Heart Fail* 6: 1029-1038, 2013.

- Salles TA, dos Santos L, Barauna VG, and Girardi AC. Potential role of dipeptidyl peptidase IV in the pathophysiology of heart failure. *Int J Mol Sci* 16: 4226-4249, 2015.

- Salles TA, Zogbi C, Lima TM, Carneiro CG, Faria DP, Barbeiro HV, Antonio EL, Pereira AC, Tucci PJ, Soriano FG, Girardi AC. Interplay between Dipeptidyl Peptidase IV (DPPIV) and inflammation in heart failure. *Manuscript*

# Abstract

Salles TA. *Role of dipeptidyl peptidase IV in the pathophysiology of heart failure [thesis].* São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2015.

**Aim:** The present study aimed to test the hypothesis that the activity and/or expression of dipeptidyl peptidase IV (DPPIV), an enzyme that inactivates cardiorenal protective peptides including glucagon-like peptide-1 (GLP-1) and brain natriuretic peptide (BNP), would be associated with poorer outcomes in heart failure (HF). **Methods:** Experimental HF was induced in male Wistar rats (200–250 g) by left ventricular (LV) myocardial injury after radiofrequency catheter ablation. Rats were divided in three groups: Sham, HF and HF+DPPIV inhibitor (sitagliptin 200mg/kg/b.i.d). Six weeks after surgery, animals were individually housed in metabolic cages during 3 days for assessment of renal function. Plasma and heart DPPIV activity/expression were measured spectrophotometrically and by immunoblotting respectively. For evaluation of cardiac function a pressure-volume catheter was positioned into the LV cavity. Histological analysis was performed for morphometric parameters. Plasma DPPIV activity was also measured in patients (n = 190) with heart failure. **Results:** Plasma DPPIV activity and abundance were increased in animals with HF compared to Sham. Additionally, plasma DPPIV activity positively correlated with ventricular end diastolic volume ($R^2$ =0.517; $p<0.001$) and lung/body weight ($R^2$ =0.492; $p<0.01$). A negative correlation between plasma DPPIV activity and ejection fraction was also observed ($R^2$ =0.602; $p<0.001$). Interestingly, HF animals also exhibited an increase of expression and activity of DPPIV in heart tissue, especially in endothelial cells. Six-week treatment with the DPPIV inhibitor sitagliptin attenuated cardiac dysfunction, mitigated cardiac hypertrophy, interstitial fibrosis, lung congestion and macrophage infiltration. Sitagliptin also raised the plasma levels of active GLP-1, increased activation of cardioprotective signaling pathways including PKA, and Akt; and reduced the levels of apoptosis and pro-inflammatory biomarkers compared to non-treated HF rats. Despite the higher circulating total BNP, renal PKG activity was lower

in HF rats compared with sham and sitagliptin-treated rats, suggesting a decrease in active/total BNP ratio. Renal function did not differ between groups, but glomerular filtration rate was modestly, but significantly increased by Sitagliptin compared to HF. Plasma DPPIV activity in patients was also increased compared to healthy subjects and correlations was found with ejection fraction ($R^2$ =-0.20; p=0.009) and the chemokine Ccl2 ($R^2$ =0.30; p<0.01). **Conclusions:** Taken together, our results demonstrate that circulating DPPIV activity correlates with poorer cardiovascular outcomes in *human and experimental HF and might play an important role in the pathophysiology of HF.*

***Keywords:*** *Heart failure; Dipeptidyl peptidase 4; Glucagon-like peptide 1; Inflammation; Natriuresis; Rats, Wistar*

# Resumo

Salles TA. *Papel da dipeptidil peptidase IV na fisiopatologia da insuficiência cardíaca [tese].* São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2015.

**Introdução**/**Objetivo:** Este estudo teve como objetivo testar a hipótese de que a atividade e/ou expressão da dipeptidil peptidase IV (DPPIV), uma enzima que inativa peptídeos com ações cardioreno protetoras, como o peptídeo-1 semelhante ao glucagon (GLP-1) e o peptídeo natriurético cerebral (BNP), estaria associada a um pior prognóstico na insuficiência cardíaca (HF). **Métodos:** Injúria do miocárdio foi realizada através da ablação do ventrículo esquerdo (VE) por radiofrequência em ratos Wistar machos (200-250 g). Os ratos foram divididos em três grupos: Sham, HF e HF + inibidor de DPPIV (sitagliptina 200mg/kg/b.i.d). Seis semanas após a cirurgia, os animais foram alojados individualmente em gaiolas metabólicas durante 3 dias para avaliação da função renal. Atividade e expressão da DPPIV no plasma e coração foram medidas por espectrofotometria e por immunoblotting, respectivamente. Para a avaliação da função cardíaca um cateter de pressão-volume foi posicionado dentro da cavidade do VE. A análise histológica foi realizada para os parâmetros morfométricos. A atividade da DPPIV no plasma também foi medida em pacientes com HF (n = 190). **Resultados:** A atividade DPPIV e sua abundância estavam aumentadas em animais com HF em comparação com Sham. Além disso, a atividade de DPPIV no plasma se correlacionou positivamente com o volume diastólico final (R = 0,517; p <0,001) e o peso do pulmão/peso corporal (R = 0,492; p <0,01). Uma correlação negativa entre a atividade DPPIV plasmática e a fração de ejeção também foi observada (R = 0,602; p <0,001). Curiosamente, os animais HF também exibiram um aumento da expressão/atividade de DPPIV no tecido cardíaco, especialmente em células endoteliais. Seis semanas de tratamento com o inibidor de DPPIV sitagliptina atenuou a disfunção cardíaca, fibrose intersticial, congestão pulmonar e infiltração de macrófagos. O tratamento com sitagliptina também

elevou os níveis plasmáticos de GLP-1 ativo, e aumentou a ativação de vias de sinalização cardioprotetoras como PKA e Akt; e reduziu os níveis de apoptose e marcadores pró-inflamatórios em comparação com ratos não tratados. Ratos com HF apresentaram maiores níveis circulantes de BNP, contudo a atividade da PKG renal foi mais baixa nesses animais em comparação com o grupo tratado com sitagliptina, sugerindo uma diminuição da razão BNP ativo/total. A função renal não diferiu entre os grupos, mas o ritmo de filtração glomerular estava ligeiramente aumentado no grupo tratado em comparação com os animais HF. Pacientes com HF apresentaram uma maior atividade plasmática da DPPIV e correlações foram encontradas com a com a fração de ejeção ($R = -0,20$; $p = 0,009$) e a quimiocina Ccl2 ($R^2 = 0,30$; $p<0.01$). **Conclusões:** Em conjunto, nossos resultados demonstram que a atividade plasmática da DPPIV se correlaciona com um pior prognóstico em pacientes e animais com HF e que a DPPIV possui um papel importante na fisiopatologia desta doença.

**Descritores:** Insuficiência Cardíaca; Dipeptidil peptidase 4; Peptídeo 1 Semelhante ao Glucagon; Inflamação; Natriurese; Ratos Wistar

# Abbreviations

| | |
|---|---|
| ANP | Atrial Natriuretic Peptide |
| BAX | Bcl-1–associated X protein |
| BCl-2 | B-cell CLL/lymphoma 2 |
| BNP | Brain Natriuretic Peptide |
| cAMP | Cyclic Adenosine Monophosphate |
| cGMP | Cyclic Guanosine Monophosphate |
| CO | Cardiac Output |
| DPPIV | Dipeptidyl Peptidase IV |
| eGFR | estimated Glomerular Filtration Rate |
| EX-4 | Exendin-4 |
| EXAMINE | Examination of Cardiovascular Outcomes with Alogliptin vs. Standard of Care |
| GFR | Glomerular Filtration Rate |
| GLP-1 | Glucagon Like Peptide-1 |
| GLP-1R | GLP-1 Receptor |
| GLUT-4 | Glucose Transporter-4 |
| HF | Heart Failure |
| HR | Heart Rate |
| hsCRP | high-sensitivity C-reactive protein |
| IL10 | Interleukin 10 |
| IL1β | interleukin 1 beta |
| IL6 | interleukin 6 |
| IRF5 | Interferon regulatory factor 5 |
| LPS | Lipopolysaccharides |
| LV | Left ventricule |
| LVEF | Left Ventricular Ejection Fraction |
| LVSP | Left ventricule systolic pressure |
| MAP | Mean arterial pressure |
| MAPK | Mitogen-Activated Protein Kinase |

| | |
|---|---|
| MI | Myocardial Infarction |
| NEP | Neutral Endopeptidase |
| NHE3 | Na+/H+ exchanger isoform 3 |
| NPR-A | Natriuretic peptide-A receptor |
| NPR-C | Natriuretic peptide-C receptor |
| NYHA | New York Heart Association |
| PBMNC | Peripheral blood mononuclear cells |
| PKA | Protein Kinase A |
| PKG | Protein Kinase G |
| PVDF | Polyvinylidene fluoride |
| SAVOR-TIMI 53 | Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus—Thrombolysis in Myocardial Infarction 53 |
| SD | Standard Deviation |
| SDF-1α | Stromal Cell-Derived Factor- 1α |
| SEM | Standard Error of Mean |
| SPECT | Single-Photon Emission Computed Tomography |
| SW | Stroke Work |
| TECOS | Trial Evaluating Cardiovascular Outcomes with Sitagliptin |
| TNFα | Tumor necrosis factor-α |
| TPR | Total peripheral resistance |
| TUNEL | Terminal transferase-mediated dUTP nick end labeling |
| USP | University of Sao Paulo |
| VEGF | Vascular Endothelial Growth Factor |
| VIVIDD | Vildagliptin in Ventricular Dysfunction Diabetes |

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# Summary

# Introduction

Heart failure (HF) is a complex syndrome characterized by the inability of the heart to pump sufficient amounts of blood to the circulation, or it can only do so by elevating ventricular filling pressures. The current pathophysiological concept of this syndrome is complex and involves a progressive disorder consisting of ventricular remodeling and inflammatory and neurohormonal responses, resulting from single or multiple causal events, which culminate in fatigue, dyspnea, exercise intolerance and fluid retention (1-3). Although the etiologic keystones of HF can be diverse, diseases such as hypertension, myocardial infarction (MI) and diabetes are important risk factors. Taking into account that cardiac diseases are the leading cause of mortality in the modern world and that the prevalence of HF increases considerably with age, it is expected that HF will continue to be an important health and economic burden (4). Such aspects justify the effort to obtain a better understanding of the HF syndrome, particularly with regard to enabling the development of novel therapeutic and preventive approaches.

Dipeptidyl peptidase IV (DPPIV), also known as CD26, is a widely expressed serine peptidase that exists on the surface of various cell types; however, its expression level differs greatly among cells. High levels of DPPIV-mRNA and abundant protein levels are found in the kidneys, small intestine and lung; moderate levels exist in the pancreas, liver and spleen; low levels are found in the stomach and heart, and no detectable expression exists in the brain and skeletal muscles (5). The kidney is the main source of DPPIV, where it is one of the major brush border membrane proteins (6). Within the kidneys, DPPIV is also present in the glomerular podocytes and capillaries (7). In the systemic vasculature, DPPIV is expressed in the endothelial cells of venules and particularly in the capillaries. In fact, in different organs and tissues such as the lung, muscle and heart, almost all tissue DPPIV activity is due to its presence in the microvasculature (7, 8). DPPIV is also found in cells of the hematopoietic system, especially those involved in the immune response such as T, B and NK cells (7). In the immune system, DPPIV is associated with T cell

signal transduction as a co-stimulatory molecule (9, 10). Notably, a soluble form of DPPIV can be found in plasma and other body fluids (7, 11). There are very few studies available in the literature concerning the origin of soluble DPPIV. Some studies support the notion that soluble DPPIV is generated from cleavage of the DPPIV expressed at the membrane of peripheral lymphocytes, especially T lymphocytes, through the catalytic action of a yet unidentified "sheddase" (*i.e.*, an enzyme that cleaves the extracellular portion of transmembrane proteins, releasing them into the extracellular medium) (7, 12).

Transmembrane and soluble forms of DPPIV preferentially cleave dipeptides from the amino terminus of polypeptides with a proline or alanine at the second position. DPPIV catalyzes the release of dipeptides from numerous substrates with known biological effects, including hormones, chemokines, neuropeptides and growth factors (7). The most widely studied DPPIV substrate is incretin hormone glucagon like peptide-1 (GLP-1), which plays a pivotal role in the maintenance of systemic glucose homeostasis. In 2000, a seminal study by Marguet and colleagues (13) showed that the circulating intact insulinotropic form of GLP-1 (14) is preserved in DPPIV knockout mice and that specific genetic deletion or pharmacological inhibition of DPPIV improves insulin secretion and glucose tolerance. Not long after that, the first DPPIV inhibitor, sitagliptin, was approved by the FDA for managing glucose homeostasis in type II diabetic patients. Currently, seven DPPIV inhibitors, known as gliptins, have been approved for use as anti-diabetic drugs worldwide.

Interestingly, recent studies suggest that GLP-1 and other DPPIV substrates possess cardiorenal protective actions that go beyond glycemic control and might be useful for treating cardiac dysfunction. Since DPPIV has a wide variety of substrates, below we list some that in our belief possess promising effects in treating cardiovascular dysfunction.

## DPPIV Substrates with Cardiorenal Protective Effects:

### *Glucagon like peptide-1 (GLP-1):*

GLP-1 is an incretin hormone secreted from intestinal L-cells in response to nutrient ingestion that potentiates glucose-dependent insulin secretion, suppresses glucagon levels and improves β-cell function (15). Because native

GLP-1 is rapidly degraded by DPPIV, its therapeutic use is limited. Thus, DPPIV inhibitors and GLP-1 receptor (GLP-1R) agonists that are resistant to DPPIV degradation have been developed and are currently in use as anti-diabetic agents (16, 17).

In addition to its effect on glucose homeostasis, several independent lines of evidence have demonstrated that GLP-1 exerts beneficial renal and cardiovascular actions independent of its glucose-lowering actions (18-22). Acute diuretic and natriuretic actions of GLP-1 have been consistently demonstrated by a variety of studies in rodents (23-26) and humans (27-30). The mechanisms underlying the natriuretic effects of GLP-1 involve the inhibition of Na+/H+ exchanger isoform 3 protein (NHE3)-mediated renal proximal tubule sodium reabsorption (24-26). In fact, stationary *in situ* microperfusion experiments have demonstrated that GLP-1 is capable of directly inhibiting NHE3 via the cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA) signaling pathway (24). GLP-1 may also be involved in increasing urinary sodium excretion through indirect mechanisms because the GLP-1R agonist liraglutide has been shown to induce atrial natriuretic peptide (ANP) secretion in mice (31). Interestingly, in a double-blind, single-day study, GLP-1 infusion induced diuresis and natriuresis in healthy subjects; however, these renal effects were not accompanied by significant changes in plasma proANP concentrations (27). The effects of GLP-1 on sodium and water homeostasis may also involve hemodynamic mechanisms because GLP-1 infusion is known to increase the glomerular filtration rate and renal plasma flow. DPPIV inhibitors also induce diuresis and natriuresis in rodents; however, the effects of DPPIV inhibition on renal sodium and water handling may occur through both GLP-1 dependent and independent mechanisms, given that infusion of a gliptin was capable of inducing natriuresis in GLP-1R knockout mice (25). Notably, GLP-1 as well as GLP-1R agonists also confer renoprotection by reducing albuminuria and ameliorating renal damage in numerous experimental models of cardiovascular and renal diseases (32-36).

The cardioprotective actions of GLP-1 independent of glucose control have also been reported in both preclinical and clinical studies (21, 37-42). *In vitro*, GLP-1R agonists activate cytoprotective pathways and reduce

cardiomyocyte apoptosis in response to diverse stimuli such as ceramide, palmitate, staurosporine and tumor necrosis factor-α (TNFα) (21, 43). Additionally, native GLP-1 attenuates infarct size after ischemia/reperfusion in *in vivo* and isolated perfused hearts (37), and liraglutide improves MI outcomes in both diabetic and non-diabetic mice (39). Furthermore, these preclinical results are supported by clinical data because GLP-1 and exenatide treatment significantly improves cardiac function and the myocardial salvage index in patients with acute MI and left ventricular dysfunction independently of the history of diabetes (38, 41).

Interestingly, similar to DPPIV, GLP-1R is abundantly expressed in the vasculature, and GLP-1 has vasoactive properties. Vasodilatory actions of GLP-1 have been reported in several vessels as GLP-1 induces vasorelaxation in the aorta and the femoral, renal and pulmonary arteries (44, 45). More detailed information about the vascular properties of GLP-1 can be found in recent review articles (42, 46).

*Brain Natriuretic Peptide (BNP):*

BNP is produced in myocardial cells and secreted in response to distention of the cardiac chambers. Originally synthesized in the heart as the 108 amino acid precursor $(\text{pro-BNP})_{1-108}$, pro-BNP undergoes posterior processing, which culminates in the release of the biologically active form $\text{BNP}_{1-32}$ and the *N*-terminal $\text{proBNP}_{1-76}$ (47). Active $\text{BNP}_{1-32}$ binds to the natriuretic peptide-A receptor (NPR-A), which, via cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG), mediates its vasodilatory and natriuretic effects. Importantly, BNP is either cleared by the natriuretic peptide-C receptor (NPR-C) or degraded by neutral endopeptidase (NEP) or DPPIV (48).

BNP plays an important role in regulating extracellular fluid homeostasis and blood pressure by counteracting the actions of the sympathetic nervous system and the renin-angiotensin aldosterone system (49-51). BNP exerts its natriuretic effects by both renal hemodynamic and tubular effects. In the glomerulus, BNP causes afferent arteriolar dilation together with efferent arteriolar vasoconstriction, thereby increasing the glomerular filtration rate

(GFR). In the inner medullary collecting ducts, it decreases sodium chloride reabsorption, thereby increasing natriuresis (50). Moreover, BNP also decreases aldosterone and renin release (49).

Plasma levels of BNP are increased in patients with HF and positively correlate with the degree of left ventricular dysfunction (52-54). Indeed, BNP has been widely used as a reliable prognostic indicator for HF patients in all stages of the disease (55, 56). However, despite exceedingly high circulating levels of BNP measured by commercially available immunoassays, HF patients continue to experience fluid retention, increased peripheral vascular resistance and edema (57, 58). Several mechanisms have been proposed to explain the hyporesponsiveness to BNP in HF (58, 59), including an increase in the proximal tubule sodium reabsorption with a resultant decrease of sodium delivery to the distal nephron where the BNP receptor is located, increased activity of peptidases that degrade and inactivate these peptides and/or decreased activity of peptidases that activate the peptides. Indeed, a report by Inoue *et al. (60)* demonstrated that NHE3 transport activity is significantly higher in the renal proximal tubules of an experimental model of post-myocardial injury-induced HF than in sham-operated animals. In addition, the endocrine BNP paradox has also been attributed to a deficiency of the active form of BNP in HF patients (61, 62). In fact, quantitative mass spectrometric analysis has demonstrated that the intact form of BNP is absent in the plasma of patients with severe chronic HF [New York Heart Association (NYHA) class IV] (61). Interestingly, des-serine-proline $BNP_{3–32}$, the cleaved form of BNP yielded by *N*-terminal dipeptide removal by DPPIV (63), displays remarkably reduced natriuretic actions and a lack of vasodilatory activity compared to $BNP_{1–32}$ (64).

*Stromal Cell-Derived Factor-1α (SDF-1α) or Cxcl12:*

SDF-1α, also known as chemokine Cxcl12, is a potent chemoattractant protein that plays a fundamental role in leukocyte recruitment to inflammatory sites. Cxcl12 effects are thought to be mediated mainly by binding to the G protein-coupled receptor CXCR4, although binding to CXCR7 has also been described (65). Due to the prominent effects of this chemokine in leukocyte and stem cell recruitment to injury sites, several groups have studied its role after

cardiac injury. It has been well described that after a cardiac injury, similar to MI, Cxcl12 expression rapidly increases, and due to the higher gradient, several types of cells migrate to the injured heart tissue with the aim of improving cardiac repair and remodeling (65-67). Among the cells that migrate to the injured heart tissue, bone marrow and circulating $CXCR4^+$ progenitor cells are crucial for increasing cardiac angiogenesis and reducing cardiac remodeling (68). Accordingly, several studies have shown that Cxcl12 is a potent angiogenic factor *in vitro (68)*. Therapeutic use of Cxcl12, in a similar manner to that of native GLP-1, is also challenged by its rapid degradation by DPPIV and matrix metalloproteinase-2. Indeed, a protease-resistant variant of Cxcl12 significantly improves blood flow in a model of peripheral artery disease and exhibits greater potency for cardioprotection than wild-type Cxcl12 after MI (67, 69, 70). Moreover, synergism between granulocyte-colony stimulating factor and DPPIV inhibition significantly improves stem cell mobilization, angiogenesis, cardiac function and survival after MI in rodents (71). Notably, co-treatment with the CXCR4 antagonist AMD3100 reverses the recruitment of $CD34^+/CXCR4^+$ cells into the heart and mitigates the improvement in cardiac function (72).

Elevated levels of total Cxcl12 and low migratory activity of circulating progenitor cells were both independent predictors of death or repeat acute MI and new-onset HF in patients with acute MI (73, 74). Interestingly, four-week treatment with sitagliptin significantly increased the levels of circulating endothelial progenitor cells in type 2 diabetic patients (75). Moreover, after adjusting for traditional cardiovascular risk factors, Cxcl12 was associated with HF and all-cause mortality risk in Framingham Heart Study participants (74).

**DPPIV and Inflammation:**

As mentioned earlier, DPPIV is widely expressed in the hematopoetic system. In fact, before the discovery of the incretin hormones and their respective modulation by DPPIV, most studies have focused on the role of DPPIV on T cells and inflammation. Interestingly, some studies have demonstrated that adding the DPPIV inhibitor, sitagliptin, to the usual metformin care treatment in diabetic patients significantly reduced the levels of

inflammatory biomarkers such as TNFα, interleukin 6 (IL6) and high-sensitivity C-reactive protein (hsCRP) (76-79). Because DPPIV inhibition is an effective therapy for obtaining adequate glycemic control, it is reasonable to assume that the reduced levels of inflammatory biomarkers were secondary to the improvement in blood glucose. Yet the effects of DPPIV inhibition on these biomarkers do not seem to be so simple. In an experimental model of sepsis, linagliptin displayed huge antioxidant, and anti-inflammatory effects independent of its glucose-lowering properties (80). Moreover, DPPIV inhibition significantly attenuated monocyte migration *in vitro* and reduced inflammation in an experimental model of atherosclerosis (81). Thus, DPPIV might be a viable target for diseases that have an inflammatory component.

**Is there a role for DPPIV in the pathophysiology of HF?**

As mentioned above, several studies demonstrated that peptides like GLP-1, BNP and Cxcl12 have cardiorenal protective properties that can be exploited for the treatment of HF. In addition to possess beneficial actions one thing that these peptides have in common is the fact that they are all truncated by DPPIV. Moreover, DPPIV inhibition also appears to have anti-inflammatory effects. Since HF is a complex heterogeneous syndrome characterized by activation of different neurohumoral, metabolic and also immune mechanisms, in this work we evaluated if DPPIV plays a role in the pathophysiology of HF and if DPPIV inhibition attenuates cardiac dysfunction in an experimental model of HF.

# Objectives

The main goal of this work was to evaluate if DPPIV plays a role in the pathophysiology of HF.

**Specific Objectives:**

- Test the hypothesis that DPPIV activity is increased in the plasma of HF patients and HF animals.

- Evaluate whether chronic DPPIV inhibition increases the half-life of cardio- and renoprotective peptides and mitigates cardiac dysfunction and development of HF in an experimental model of cardiac injury.

- Evaluate whether chronic DPPIV inhibition attenuates cardiac inflammation in an experimental model of cardiac injury.

# Material and Methods

*Selection of patients with HF:*

One hundred ninety HF patients from an ongoing inception cohort from the General Outpatient Clinic of the Heart Institute of University of Sao Paulo (USP) were included in this study (Table 1). The ascertainment period was from 2005 to 2010. After enrollment, the serum samples were frozen at -80°C until analysis. The HF diagnosis was made according to previously published criteria (82) and the classification of HF etiology followed previous recommendations (83). Patients with symptomatic HF of varying etiology and a Left Ventricular Ejection Fraction (LVEF) < 45% on two-dimensional transthoracic Doppler echocardiography were eligible for enrollment into the cohort. We excluded patients with cardiomyopathy due to valvular heart disease who would be candidates for conventional surgical treatment, such as valve repair or replacement, including patients with hypertrophic cardiomyopathy, chronic obstructive pulmonary disease, recent myocardial infarction and/or unstable angina. Patients with severe renal or hepatic dysfunction, severe peripheral artery disease, cerebrovascular disease, active infection, coexisting neoplasm or active peptic ulcer disease were excluded. In addition, 42 healthy subjects with no prior history of HF or cardiovascular disease were selected as controls.

**Table 1: Clinical characteristics of the studied HF population**

| | | |
|---|---|---|
| **Number** | | 190 |
| **Mean Age (SD)** | | 56(13) |
| **Gender** | *Male (%)* | *70.5* |
| | *Female (%)* | *29.5* |
| **Ethnicity (%)** | *White* | *60.4* |
| | *Mulatto* | *12.7* |
| | *Black* | *12.7* |
| **Body mass index (SD)** | | 25.6 (5.4) |
| **Left ventricular diameter (SD)** | *Systolic* | 54(14) |
| | *Diastolic* | 65(11) |
| **Interventricular septum thickness (SD)** | | 10(2) |
| **Ejection Fraction (SD)** | | 37(15) |
| **Etiology (%)** | *Valvular* | 14 |
| | *Hypertensive* | 25 |
| | *Ischemic* | 29 |
| | *Idiopathic* | 10 |
| | *Chagas* | 12 |
| | *Other* | 10 |
| **Serum Sodium** | | 137(4) |
| **Hemoglobin** | | 13.4(2.2) |

*Animals protocols and surgical procedures:*

All procedures and animal care were conduct in accordance with the guidelines established by the Brazilian College for Animal Experimentation and approved by the institutional animal care and use committee (CEUA #211/13). Male Wistar rats (2-3months old; 200-250g) were submitted to myocardial injury by ablation of the left ventricle (LV) by radiofrequency catheter as described previously (84). Briefly, rats were anesthetized with halothane and the heart was exposed after a left thoracotomy at the fourth intercostal space. A catheter was placed on the LV anterolateral wall and lesions were created by high-frequency currents (1,000 KHz, 12 watts, during 10 s) generated by a conventional radiofrequency generator (model TEB RF10; Tecnologia Eletrônica Brasileira, São Paulo, Brazil). Sham-operated animals underwent the same procedure but were mock-ablated. Sitagliptin was administered by oral gavage (200mg/kg/b.i.d) for six weeks after cardiac injury surgery.

*Sitagliptin Extraction:*

Januvia tablets (Merck & Company, Inc.) containing 100 mg of sitagliptin monophosphate were purchased from a local pharmacy. Briefly, four 100 mg Januvia tablets were added to 16 ml of water and incubated in the refrigerator for 1 hour to dissolve the tablets. The suspension was vortexed and centrifuged at 2.000 *g* for 10 minutes to remove the majority of the excipients. The supernatant was collected and used for the treatment of animals (0.8mL/100g).

*DPPIV Measurement:*

Plasma and heart DPPIV activity were measured spectrophotometrically by measuring the release of p-nitroaniline resulting from the hydrolysis of glycylproline p-nitroanilide tosylate, as previously described (Pacheco, 2011, Dipeptidyl peptidase IV inhibition attenuates blood pressure rising in young spontaneously hypertensive rats). Briefly, 15µL of plasma or diluted heart protein sample was added to 185µL of Tris-HCl (10mM; pH=7.6) buffer containing 4mM of glycylproline p-nitroanilide tosylate for one hour at 37ºC. After that, 500µL of acetate buffer (0.1mM) was added to terminate the reaction. Samples were measured colorimetrically in the spectrophotometer at 405nm.

Heart DPPIV activity was normalized by total protein assessed by the Lowry method. DPPIV levels were evaluated in the culture medium by ELISA (Cloud-Clone Corp., Houston, TX).

*Evaluation of cardiac function:*

Surgical procedures for hemodynamic assessment were performed as previously described (60). In brief, anesthetized rats (ketamine 50 mg/kg and xylazine 10 mg/kg, i.p.) were placed on a heated rodent operating table (37°C). Thereafter, a microtip Pressure-Volume catheter (Mikro-Tip® 1.4 F SPR 839, Millar Instruments Inc., Houston, TX) was positioned into the LV cavity by means of right carotid artery catheterization. After 10-15 minutes of measurement under steady-state conditions, LV performance was evaluated by determination of P-V relationships during gradual changes in preload obtained by gently compressing the inferior cava vein with a swab. At the end of each experiment, 50 µl of hypertonic saline was injected intravenously, and from the shift of P-V signals, parallel conductance volume (VP) was calculated and used for correction of cardiac mass volume. Data were acquired for computer analysis (PVAN Software, Millar Instruments Inc., Houston, TX) using the LabChart 7 Software System (PowerLab, ADInstruments, Bella Vista, NSW, Australia). At the end of the hemodynamic measurements, rats were killed by decapitation, and their hearts and lungs were immediately removed.

*Radiosynthesis and assessment of cardiac perfusion by Single-photon emission computed tomography (SPECT) imaging:*

Sodium [$^{99m}$Tc]pertechnetate ($Na^{99m}TcO_4$) was eluted from a $^{99}Mo/^{99m}Tc$ generator (IPEN-TEC) and used for labeling the molecule MIBI (methoxyisobutylisonitrile) (Cardiolite®, DuPont-Merck Pharmaceutical Co., Inc.). $Na^{99m}TcO_4$ (1.11-1.85 GBq in 2 mL) was added to the lyophilized kit and heated in boiling water for 10 min, and then allowed to reach room temperature. The quality control was performed as recommended in the insert package of the product and injected only when the radiochemical purity was higher than 95%. After synthesis of the radiosynthesis, animals were anesthetized with isoflurane 2-3% in oxygen and injected intravenously (penile vein) with 20-35 MBq of

$^{99m}$Tc-MIBI. After radiopharmaceutical injection, the animals were allowed to wake up for a better radiopharmaceutical distribution. Animals were anesthetized again (30 min after $^{99m}$Tc-MIBI injection) and positioned (based on CT image) with the heart in the center of the field of view (FOV) of the SPECT for small animal imaging equipment (Triumph$^{TM}$ Trimodality Gama Medica Ideas) using a 5 pinhole collimator. Images were acquired in 64 projections of 30 seconds each and reconstructed using OSEM algorithm. Image analysis was performed by PMOD$^{TM}$ software and the results of heart perfusion expressed as relative percentage of maximum uptake.

*Morphometric analysis:*

Histological analyses were performed to evaluate tissue remodeling. The observer was blinded to the experimental group. Five-micrometer sections of paraffin-embedded tissue were mounted onto slides and stained with hematoxylin and eosin for myocyte analysis. Picrosirius red was used to evaluate fibrosis and quantify the injury scar. A computerized image acquisition system (Leica Imaging Systems, Bannockburn, IL) was used for the analyses. As an estimate of myocyte hypertrophy, the average nuclear volume was determined in 50−70 cardiomyocytes cut longitudinally (acquired in 5 randomized 400× magnification fields per animal) and calculated according to the following equation: nuclear volume = π × D 6 × d2 / 6 (d = shorter nuclear diameter; D = longer diameter), as previously described (Gerdes, 1994, Changes in nuclear size of cardiac myocytes during the development and progression of hypertrophy in rats)(Serra, 2008, Exercise training prevents beta-adrenergic hyperactivity-induced myocardial hypertrophy and lesions). Interstitial fibrosis in the remodeled LV was evaluated as the area occupied by collagen fibers, excluding stained ablation scar and perivascular fibers. After digitalization, the red-stained areas were quantified as the average percentage of the total area from each of 5 randomized 200× magnification fields per animal. Myocardial lesion was quantified as the percentage of the LV perimeter containing scar tissue. In addition, the thickness of the scarred myocardial wall was determined in the midportion of the injury in 4 transverse LV histological sections.

*Preparation of heart homogenates:*

Hearts from rats were minced with razor blades and homogenized in a Polytron PT 2100 homogenizer (Kinematica, AG, Switzerland) in an ice-cold buffer pH 7.4 containing 150mM NaCl; 7.2mM $Na_2HPO_4$; 2.8mM $NaH_2PO_4$; 15mM NaF; 50mM $Na_4O_7P_2 \cdot 10H_2O$; and Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL). The homogenate was centrifuged at 2000g for 10 min at 4ºC and supernatant was aliquoted and stored at -80ºC. The protein concentration was determined by the Lowry method.

*SDS-PAGE and immunoblotting:*

Protein samples were solubilized in Laemmli sample buffer, separated by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA). For immunoblotting, membranes were incubated with 5% non-fat dry milk or 5% bovine serum albumin and 0.1% Tween 20 in PBS, pH 7.4 solution for 1 hour to block nonspecific antibody binding followed by overnight incubation in primary antibodies. The monoclonal antibody against DPPIV, clone 5E8, and the polyclonal antibodies against GLP-1 receptor, BCl2, and Bax were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The monoclonal antibody against actin JLA20 was from Calbiochem (San Diego, CA). Antibodies against total and phospho-Akt (Ser473) were from Cell Signaling Technology (Beverly, MA). Antibodies against total and phospho-p38 (1:1000) purchased from Cell Signaling. The membranes were then washed five times with the blocking solution and incubated for 1 h with horseradish peroxidase-conjugated immunoglobulin secondary antibody (1:2000). Subsequently, membranes were washed again and then rinsed in PBS. An enhanced chemiluminescence system (GE Healthcare) was used for visualization of the bands. The visualized bands were scanned using the ImageScanner (GE HealthCare) and quantified using the Image J Software.

*RNA isolation and real time RT-PCR reaction:*

The gene expression was performed by quantitative RT-PCR. Total RNA isolated with Trizol Reagent (Invitrogen) according to the manufacturer's

instructions. First-strand cDNA synthesis was performed with Super-Script III Reverse Transcriptase following the manufacturer's guidelines. The oligonucleotides were designed into different exons with a large intron between them to avoid that genomic DNA was amplified by our protocol. The quantitative RT-PCR reactions were performed using SYBR Green PCR Master Mix-PE (Applied Biosystems) in an ABI QuantStudio 12K flex (Applied Biosystems). All samples were assayed in duplicate. The control gene Gapdh was used to normalize the results. Data were analysed by the $2^{-\Delta\Delta}$CT method. Table 2 shows the oligonucleotide primers used in Real Time RT-PCR.

**Table 2: List of Primers**

| Gene | Foward | Reverse |
|---|---|---|
| **Arg1** | TCCAAGCCAAAGCCCATAGA | AGCTTTCCTTAATGCTGCGG |
| **Ccl2** | TGCCCACTCACCTGCTGCT | TGGGGTCAGCACAGATCTCTCTCT |
| **Cxc12** | CATCAGTGACGGTAAGCCAG | AGCCTCTTGTTTAAGGCTTTGT |
| **Gapdh** | ATGGTGAAGGTCGGTGTG | GAACTTGCCGTGGGTAGAG |
| **Il10** | TGGGAGAGAAGCTGAAGACC | AGATGCCGGGTGGTTCAAT |
| **Il1b** | TGAAGCAGCTATGGCAACTG | ATCTTTTGGGGTCTGTCAGC |
| **Il6** | CTGGTCTTCTGGAGTTCCGT | GCCACTCCTTCTGTGACTCT |
| **Irf5** | ACCAATACCCCACCACCTT | TTGAGATCCGGGTTTGAGA |
| **Nos2** | CCTGTGTTCCACCAGGAGAT | CACCAAGACTGTGAACCGGA |
| **Tnfa** | GCGTGTTCATCCGTTCTCTA | GAGCCACAATTCCCTTTCTAA |

*Immunohistochemistry and immunofluorescence:*

Hearts were fixed with formaldehyde, paraffin-embedded and sectioned. Hearts were fixed with formaldehyde, paraffin-embedded and sectioned. An antigen retrieval step was used in all experiments, by heating samples in a citrate buffer (Spring-Bioscience) to 95°C for 30 min. After pre-incubation of heart sections with PBS solution-2% casein, sections were incubated for an overnight period with anti-CD68 antibody (Abcam, Cambridge, UK), then washed and incubated with secondary antibody. Immunostaining was visualized under a light microscope. Infiltration of $CD68^+$ cells were estimated by analyzing six-200x magnification fields per animal near to the lesion area. Positive

staining was quantified and normalized by field area with Image J. For dual-fluorescence primary anti-CD68, anti-GLP1R (Santa Cruz Biotechnology, Inc.,Dallas, TX) and anti-DPPIV antibody and anti-von Willebrand factor (Abcam) antibody antibodies were used. Alexa Fluor 555 and Alexa Fluor 488 (LifeTechnologies Corporation; Carlsbad, CA) were used as secondary antibodies. Immunofluorescence staining was detected using a Carl Zeiss 510 LMS confocal system connected to an Axiovert microscope.

*Terminal transferase-mediated dUTP nick end labeling (TUNEL) assay:*

DNA fragmentation was detected using an in situ cell death detection kit, AP (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions. Briefly, tissue sections were deparaffinized in citrisolv (Fisher Scientific Company, Pittsburgh, PA), rehydrated in serial alcohol dilutions and permeabilized with 0.5% triton X-100 in 0.1% sodium citrate. The reaction with terminal deoxynucleotidyl transferase and alkaline phosphatase conversion was performed and the cross-sections were examined by light microscopy. The percentage of TUNEL-positive nuclei was quantified by an observer blinded to the three conditions using Leica Qwin 2.2 Q500IW software.

*Determination of the Plasma Concentrations of Active GLP-1 and Total BNP:*

The plasma levels of intact GLP-1 (7–36 amide) and total BNP were measured using ELISAs from Linco Research (St. Charles, MO) and Bachem (Torrance, CA), respectively, in accordance with the manufacturer's instructions.

*Renal function:*

Rats were anesthetized with a mixture of ketamine, xylazine, and acepromazine (64.9, 3.20, and 0.78 mg/kg subcutaneously, respectively) and placed on a heated surgical table to maintain body temperature. After a tracheostomy, polyethylene catheters were inserted into the jugular vein and the urinary bladder for inulin infusion and urine collection, respectively. To control mean arterial pressure and allow blood sampling, a PE-60 catheter was inserted into the right carotid artery. Glomerular filtration rate was determined by

measuring the clearance of inulin as follows. First, a loading dose of inulin (100 mg/kg in 0.9% saline) was administered. Subsequently, continuous infusions of inulin (10 mg/kg in 0.9% saline) were given at 0.04 ml/min. Three consecutive 30-min periods of urine collection were performed. Blood samples were obtained at the beginning and the end of the experiment. Plasma and urine sodium concentrations were measured by flame photometry (Micronal B262, São Paulo, SP, Brazil), and inulin was determined using the anthrone method (85).

*Primary macrophage isolation and cell culture:*

Male Wistar rats (200-250g) were injected i.p with 3mL of 4% Thioglycolate solution. After 96hrs, rats were anesthetized and peritoneal cavity was washed twice with 20mL of RPMI culture medium (Cultilab, Campinas, Brazil). After a gentle massage of the abdominal wall, the peritoneal fluid was collected and centrifuged at 300g for 5 min at 4ºC. A cell pellet was formed and suspended in 1 mL of lysis solution pH 7.4 containing 150mM $NH_4Cl$; 10mM $NaHCO_3$; 0.1mM EDTA for 10 min at 4ºC. Thereafter, cells were centrifuged at 300g for 30 seconds at 4ºC and suspended in 1 mL of RPMI medium twice for posterior use. $1x10^6$ cells/mL were plated in P24 wells and after 1hr adherent cells were identified as macrophages. Macrophages were stimulated with 1µg/mL of LPS (Escherichia coli 055:B5 Sigma) for 3 hours after 30min pre-incubation with 30nM of Exendin-4 (Abcam). Levels of TNF-α, IL-1β and IL-6 were evaluated by ELISA (R&D Systems; Minneapolis, MN) in the culture medium according to the manufacturer's instructions.

*Splenocytes Isolation:*

Ten weeks after LV radiofrequency surgery, rats were anesthetized and the spleen was removed. Spleen was gently dissociated with autoclaved slides in a phosphate buffer solution. After complete dissociation the homogenate was centrifuged at 300g for 10min at 4ºC. The supernatant was discarded and the cell pellet was suspended in a lysis solution for 10 min on ice for erythrocytes removal. Cells were centrifuged at 300g for 10 min at 4ºC and the cell pellet was suspended in PBS. Thereafter, cells were centrifuged again at 300g for 5

min at 4ºC and resuspended maintained in RPMI-1640 medium in addition to 10% FBS.

*Peripheral Blood Mononuclear Cells Isolation:*

Blood was diluted (1:1) in phosphate-buffered saline (PBS), pH=7.4, and this suspension was layered into Histopaque-1077 and centrifuged for 30 min, at 800g and 4ºC. Peripheral blood mononuclear cells (PBMNC) were collected from the interphase, suspended in lysis solution for erythrocytes removal and washed twice with PBS. The PBMNC were maintained in RPMI-1640 medium in addition to 10% of fetal bovine serum (FBS) (Life Technologies, CA).

*Assessment of pro-inflammatory markers in HF patients:*

The concentration of TNFα and Ccl2 was evaluated in the serum of HF patients by Milliplex® MAP kit technology (Millipore Corporation, MA).

*Statistical Analysis:*

All data are expressed as the mean±SEM. All data was tested to evaluate if values come from a gaussian distribution. For comparisons between two groups an unpaired *t*-test was used. If more than two groups were compared, results were analyzed by 1-way ANOVA followed by Bonferroni's post-hoc test. Correlation between DPPIV activity and the parameters of HF were assessed by Pearson Correlation test. $P<0.05$ was considered statistically significant.

# Working hypothesis and Results

**Working hypothesis #1:**

HF is characterized by cardiac dysfunction, increased vascular resistance and water and sodium retention. Since DPPIV inactivates several peptides with cardiorenal protective actions we hypothesize that DPPIV activity is increased in the plasma of HF patients.

*Circulating DPPIV Activity in Patients with HF:*

The general demographic characteristics of the studied population are shown in Table 1 . In the patients with HF, the measured DPPIV activity followed a normal distribution (Figure 1A). The mean (±SD) serum DPPIV activity in the 190 selected patients with HF was significantly higher than that of the normal subjects (n=42; P<0.001; Figure 1B)

A significant negative correlation was found between serum DPPIV activity and LV ejection fraction in patients with HF (r=−0.20; P=0.009). Interestingly, we also observed statistically significant correlations between serum DPPIV activity and age (r=−0.19; P=0.02), serum sodium (r=0.22; P=0.004) and hemoglobin (r=0.20; P=0.01) (Figure 2). The serum DPPIV activity in patients with HF did not significantly correlate with body weight index, heart rate, systolic or diastolic blood pressure, serum potassium, total cholesterol, serum creatinine, or serum glucose (data not shown).

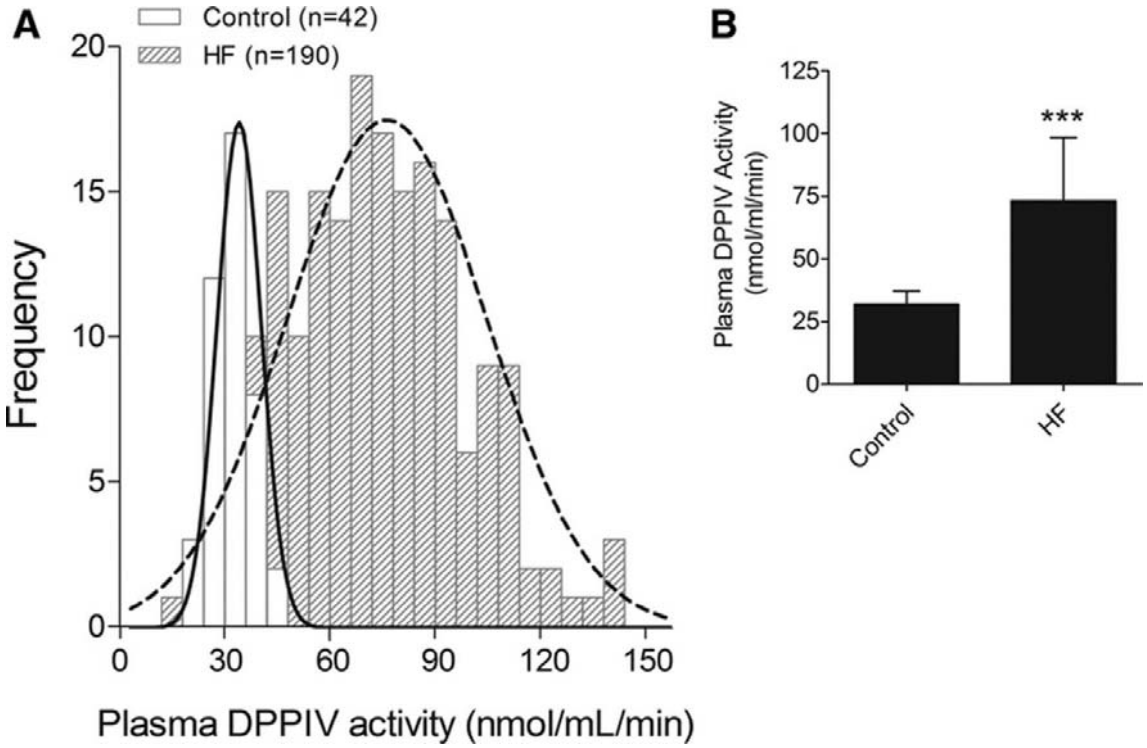


**Figure 1: Circulating dipeptidyl peptidase IV (DPPIV) activity in patients with heart failure (HF) and normal subjects.**

**(A)** Frequency distribution of serum DPPIV activity from 190 patients with HF and 42 control subjects without cardiovascular disease. In both groups, serum DPPIV activity exhibited a Gaussian distribution. **(B)** Average serum DPPIV activity in patients with HF and control subjects. The values are the means±SD. ***P<0.001 vs. control.

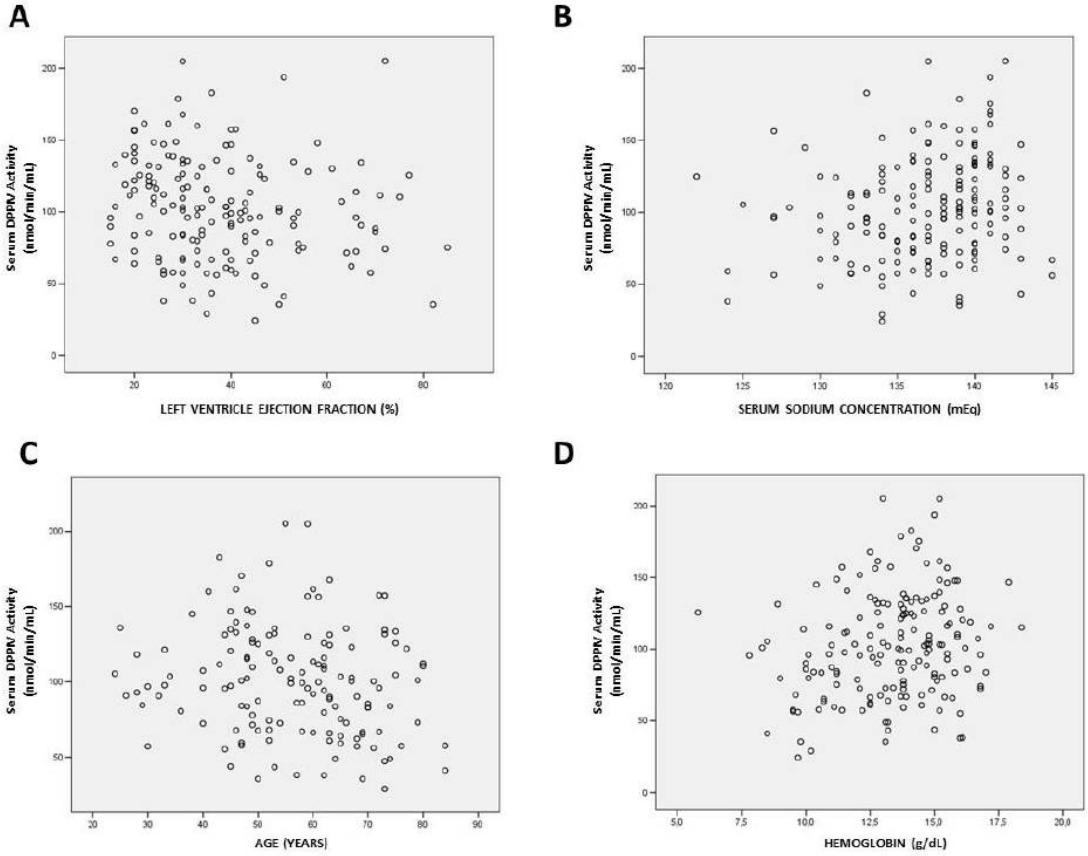


**Figure 2: Serum DPPIV activity in 190 HF patients plotted against different parameters from the respective patient.**

DPPIV activity exhibited significant correlations with **(A)** LVEF (r=−0.20; P=0.009) **(B)** age (r=−0.19; P=0.02), **(C)** plasma Na+ concentration (r=0.22; P=0.004) and **(D)** hemoglobin (r=0.20; P=0.01).

**Working hypothesis #2:**

Consistent with our working hypothesis #1, we have found that HF patients exhibit an increase in DPPIV plasma activity compared to healthy subjects. Thus, our next approach was to evaluate if DPPIV is increased in HF animals and if chronic treatment with the DPPIV inhibitor sitagliptin was able to improve cardiac function in an experimental model of HF.

**Experimental Design:**

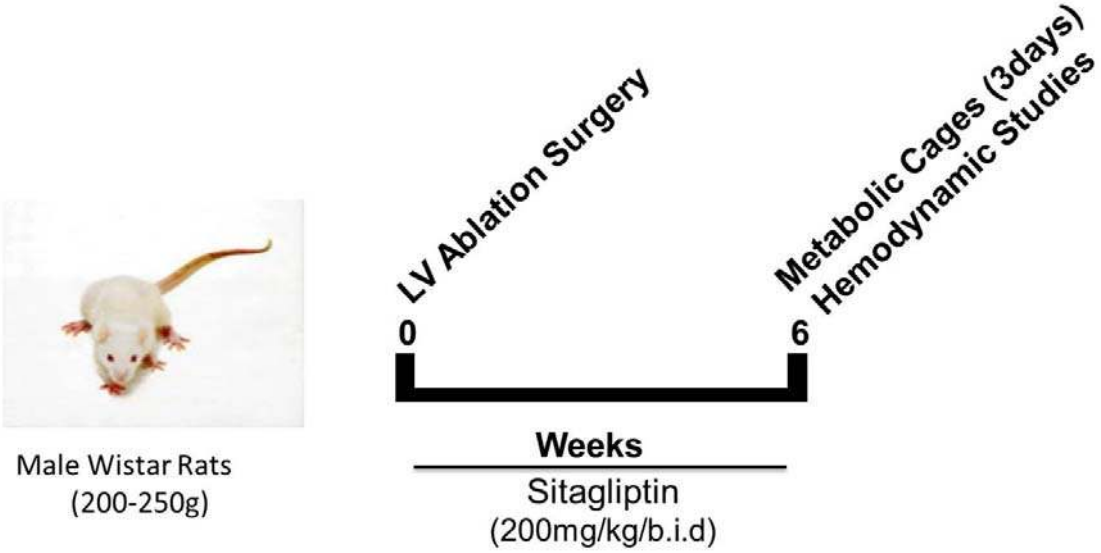


**Figure 3: Schematic illustration of the protocol used to assess the effects of DPPIV inhibition treatment after myocardial injury.**

*Determination of DPPIV Activity and Abundance in Sham-Operated and Experimental HF Rats Treated or Not With the DPPIV Inhibitor Sitagliptin*

Figure 4 demonstrates that rats with HF displayed higher DPPIV activity in the plasma (Figure 4A) and heart (Figure 4B) compared with sham animals. The average plasma DPPIV activity in radiofrequency LV-ablated rats treated with sitagliptin for 6 weeks was 6.77±1.05 nmol/mL per minute, which corresponded to ≈90% inhibition compared with HF rats and 85% inhibition compared with sham rats (Figure 4A). Moreover, treatment with sitagliptin significantly inhibited DPPIV heart activity in HF rats (Figure 4B). In agreement with the pattern of enzymatic activity, the abundance of DPPIV, as assessed by immunoblotting, increased both in the plasma (Figure 4C) and in the heart (Figure 4D) from the HF rats compared with the sham rats. As depicted in Figure 4C and 4D, sitagliptin not only inhibited DPPIV catalytic activity but also decreased the abundance of the enzyme both in plasma and heart. To determine in which cardiac cell type the upregulation of DPPIV expression occurred, heart sections were analyzed by immunofluorescence using specific cell markers. As demonstrated in Figure 4E, costaining for von Willebrand factor with DPPIV indicated that upregulated DPPIV expression was confined to the surface of heart endothelial cells.

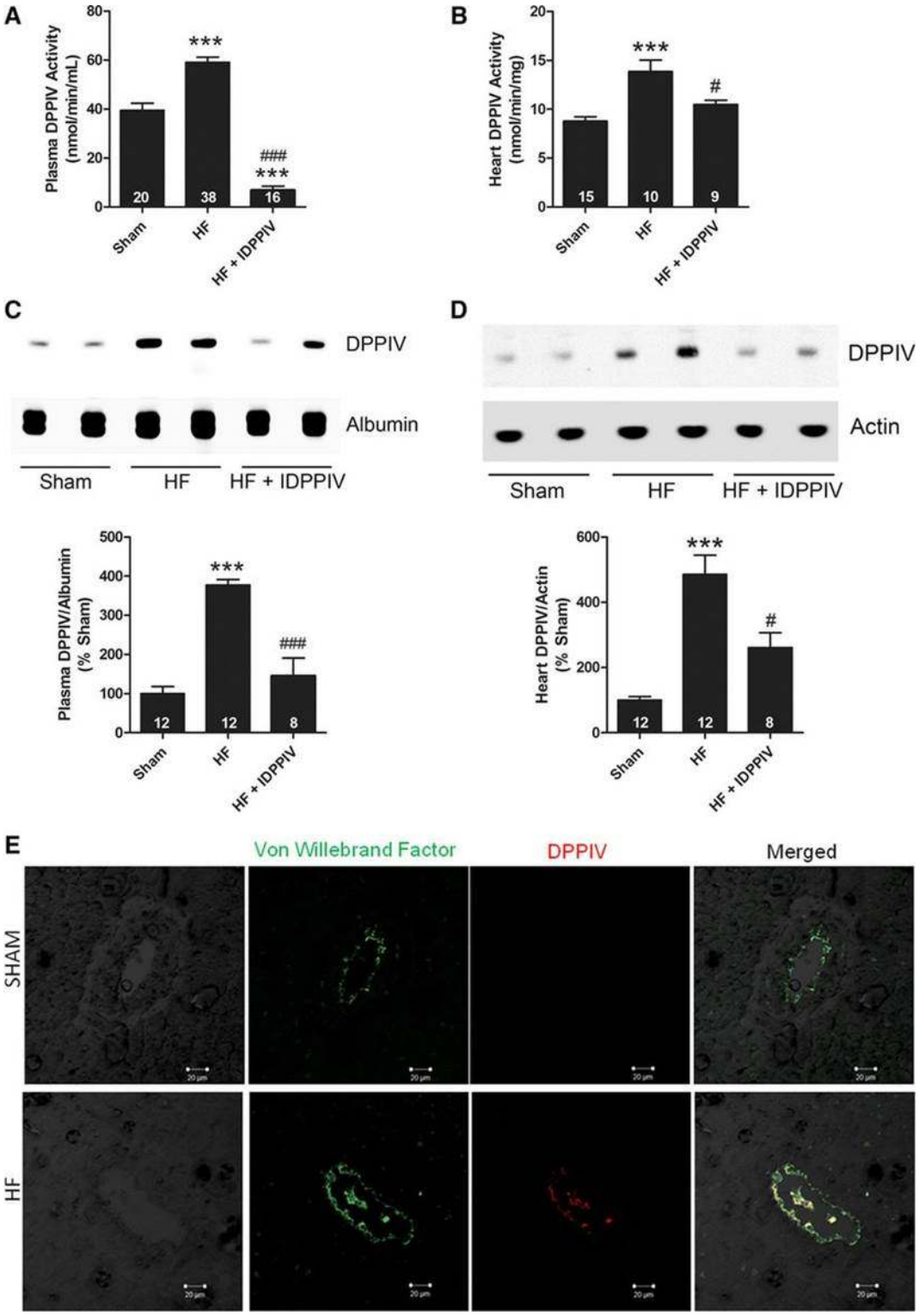


**Figure 4: Dipeptidyl peptidase IV (DPPIV) activity and expression in the plasma and heart of a rat model of chronic heart failure (HF).**

DPPIV activity in **(A)** plasma and **(B)** heart from radiofrequency left ventricular (LV) ablation-induced HF rats treated with the DPPIV inhibitor sitagliptin

(HF+IDPPIV) or not (HF) and sham rats. **(C)** An equal volume of plasma (0.5 µL) from each animal was subjected to SDS-PAGE, transferred to polyvinylidene fluoride membranes and incubated with an antibody against DPPIV. The membranes were stained with Ponceau S before antibody incubation, and albumin was used as an internal control. **(D)** Equal amounts of protein (50 µg for DPPIV and 2.5 µg for actin) from the heart membrane fractions isolated from sham or HF rats were subjected to immunoblotting for DPPIV and actin; the latter was used as an internal control. The values are the means±SEM. The number of animals analyzed in each group is indicated within the bar. $^{**}P<0.01$ and $^{***}P<0.001$ vs sham; $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ vs HF. **(E)** Representative images of the heart sections from radiofrequency LV ablation-induced HF rats (HF) and sham rats costained with antibodies against DPPIV (red) and the endothelial cell marker von Willebrand factor (green). The scale bar is 20 µm.

*Correlation of plasma DPPIV activity with cardiac dysfunction and congestion in an experimental model of HF:*

Similar to what was observed in patients with HF, there were significant correlations between plasma DPPIV activity and different parameters of cardiac dysfunction and congestion in the rat model of HF (Figure 5). Plasma DPPIV activity correlated negatively with LV ejection fraction (Pearson r=−0.70; P<0.01; Figure 5A) and positively with LV end-diastolic pressure (Pearson r=0.72; P<0.001; Figure 5B) and with the lung/ body weight index (Pearson r=0.78; P<0.001; Figure 5C).

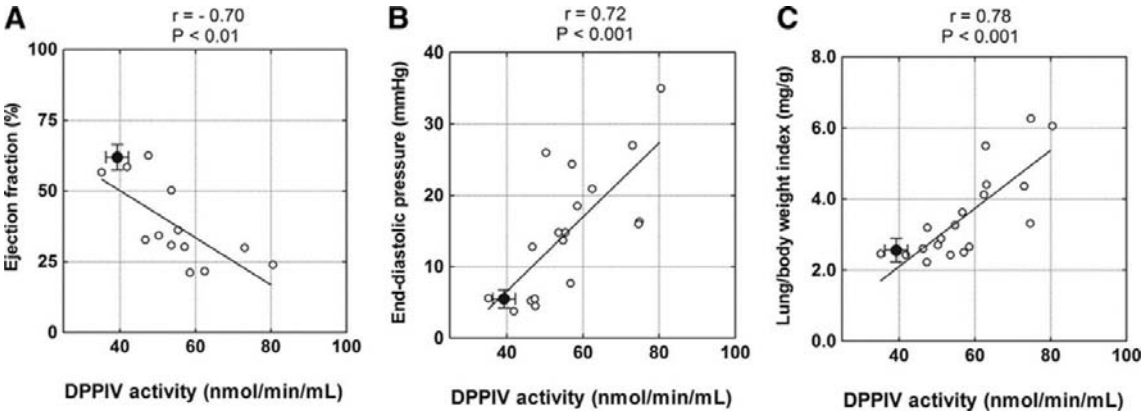


**Figure 5: Plasma dipeptidyl peptidase IV (DPPIV) activity correlates with the severity of cardiac dysfunction and congestion in the rat model of heart failure.**

Correlations between plasma DPPIV activity and **(A)** ejection fraction, **(B)** end-diastolic pressure, and **(C)** lung weight indexed by body weight in radiofrequency left ventricular ablation-induced HF rats. The filled symbols represent the mean±SEM of the sham group. The correlation coefficients and P values were obtained using Pearson correlation test, and the lines represent linear regression plotting.

*Treatment with sitagliptin attenuated cardiac dysfunction:*

Consistent with previous studies (60, 84) six weeks after myocardial injury rats that were subject to LV radiofrequency ablation surgery presented several markers of HF such as cardiac hypertrophy, pulmonary congestion, decreased ejection fraction and increased levels of LV end-diastolic pressure (Figure 6A-D and Table 3). Noteworthy, treatment with the DPPIV inhibitor, sitagliptin, showed a cardioprotective profile since it was able to mitigate cardiac hypertrophy, pulmonary congestion and cardiac dysfunction.

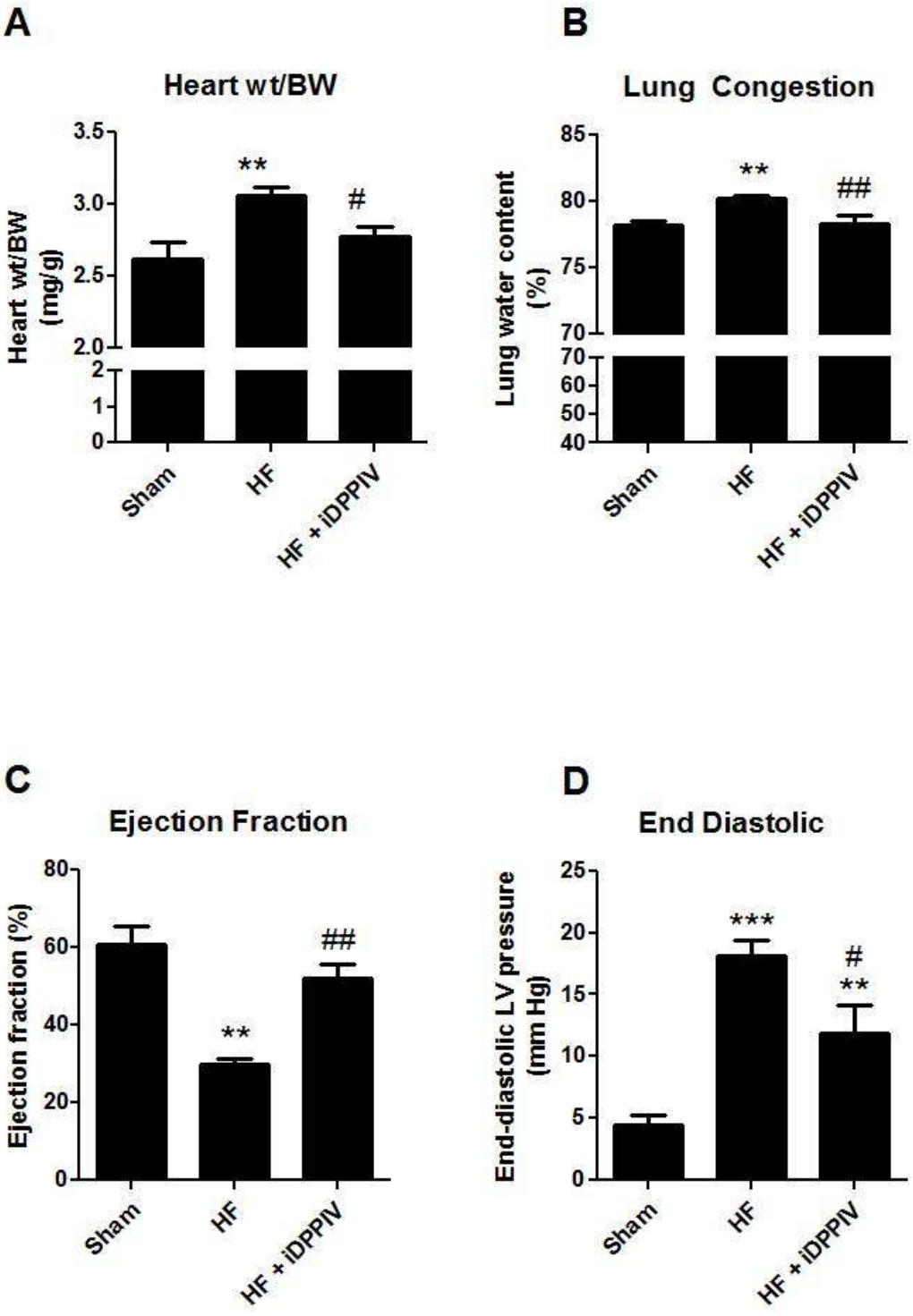


**Figure 6: Evaluation of cardiac function in HF animals.**

Treatment with sitagliptin mitigates cardiac hypertrophy **(A)**, pulmonary congestion **(B)**, ejection fraction **(C)** and end-diastolic LV pressure **(D)**. **P<0.01 and ***P<0.001 vs Sham; #P<0.05, ##P<0.01 and ###P<0.001 vs HF

**Table 3: Biometric and Hemodynamic Parameters of Sham and Experimental Heart Failure Rats Treated With the DPPIV Inhibitor Sitagliptin (HF+IDPPIV) or Untreated (HF)**

| | Sham (n=9-12) | HF (n=12-16) | HF+IDPPIV (n=11-16) |
|---|---|---|---|
| **Biometry** | | | |
| Body weight, g | 400±9 | 406±12 | 391±16 |
| Lung/BW, mg/g | 2.69±0.11 | 3.84±0.29** | 3.03±0.13## |
| **Hemodynamics** | | | |
| HR, beats/min | 260±10 | 254±11 | 248±5 |
| MAP, mmHg | 99±4 | 101±2 | 100±4 |
| LVSP, mmHg | 121±3 | 112±3 | 110±3 |
| CO, mL/min | 37±2 | 25±1* | 31±2# |
| SW, mmHg/mL | 12.4±1.1 | 7.3±0.5** | 9.9±0.4**## |
| $+dP/dt_{max}$, mmHg/s | 9058±211 | 7072±264* | 8097±217*# |
| $-dP/dt_{max}$, mmHg/s | -7885±429 | -5347±297* | -5891±308* |
| τ, ms | 10.4±0.4 | 16.8±0.7* | 13.3±0.3*# |
| TPR, mmHg/mL per minute | 3.17±0.13 | 4.89±0.27** | 4.04±.0.39 |
| SV, µL | 126±4 | 90±4* | 109±7# |
| EDV, µL | 191±23 | 335±16 | 261±14*# |

Values are means±SEM. +dP/dt max and –dP/dt max indicate maximal rate of LV pressure increment and decrement, respectively; BW, body weight; CO, cardiac output; DPPIV, dipeptidyl peptidase IV; EDV, end-diastolic volume; HF, heart failure; HR, heart rate; LVSP, left ventricular (LV) systolic pressure; MAP, mean arterial pressure; SV, stroke volume; SW, stroke work; TPR, total peripheral resistance; and τ, time constant of LV pressure decay. *P<0.05 and **P<0.01 vs Sham; #P<0.05 and ##P<0.01 vs HF.

*Effects of DPPIV Inhibition in cardiomyocytes hypertrophy, fibrosis and apoptosis:*

Histological analysis of the remodeled myocardium far from the scar demonstrated a significant increase in the average cardiomyocyte nuclear volume in the HF rats compared with the sham rats, which was significantly reduced by DPPIV inhibition (Figure 7A). In addition, the increased interstitial collagen in reminiscent tissues evidenced in the HF group was significantly attenuated by sitagliptin treatment (Figure 7B) compared with samples from similar regions.

As depicted in Figure 6C, the apoptosis rate was higher in HF rats compared with sham rats. The extent of apoptosis was attenuated, but not normalized, by sitagliptin.

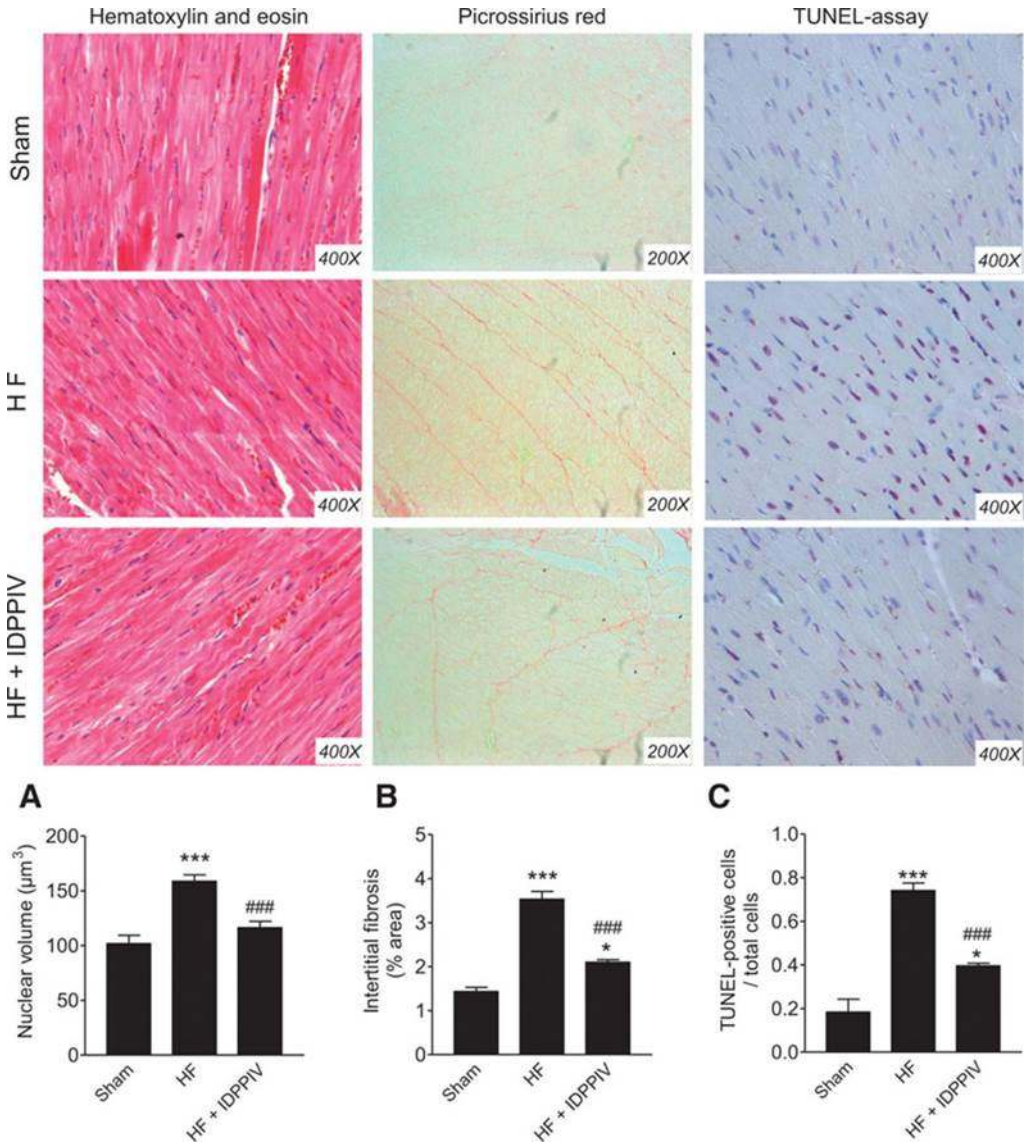


**Figure 7: Effect of dipeptidyl peptidase IV (DPPIV) inhibition on cardiac remodelling and apoptotic rates in the rat model of heart failure (HF).**

**(A)** Myocardial hypertrophy was assessed by measuring the cardiomyocyte nuclear volumes in hematoxylin-eosin–stained heart samples. **(B)**, Interstitial collagen was evaluated in equivalent regions of the remote myocardium from each group. **(C)**, The number of apoptotic nuclei was evaluated at ×40 magnification in 6 random fields per section and expressed as TUNEL (terminal deoxy(d)-UTP nick end labeling)-positive nuclei per total nuclei. *P<0.05, **P<0.01, and ***P<0.001 vs sham; ##P<0.01 and ###P<0.001 vs HF.

*Effect of DPPIV Inhibition on GLP-1 Circulating Level, on Heart GLP-1 Receptor Expression, and on the Stimulation of Cardioprotective Signaling Pathways:*

The plasma level of active GLP-1 was 3.2 times greater in the sitagliptin-treated radiofrequency LV-ablated rats than in the HF animals and 2.4 times greater than in the sham rats (Figure 8A). Additionally, there was a significant decrease in plasma GLP-1 in HF rats (≈25%) compared with the sham rats (Figure 8A). The level of the GLP-1 receptor in the heart was examined using immunoblotting and normalized to actin. As depicted in Figure 8B, the GLP-1 receptor was significantly more abundant in the HF rats than in the sham rats. Sitagliptin remarkably increased cardiac GLP-1 receptor expression compared with the sham rats. The signaling pathways transduced downstream of the cardiac GLP-1 receptor were examined using ELISA and immunoblotting. Sitagliptin treatment increased cardiac protein kinase A activity compared with HF and sham rats, suggesting the activation of the cAMP-protein kinase A pathway (Figure 8C).

Similarly, the ratio of phosphorylated to total Akt increased in the hearts of the sitagliptin-treated radiofrequency LV-ablated rats compared with the HF rats and compared with the sham rats (Figure 8D). The expression of B-cell CLL/lymphoma 2 (Bcl-2) and Bax (Bcl-1–associated X protein), apoptosis-related proteins downstream of Akt, were also examined (Figure 8E). Cardiac Bcl-2 expression was decreased in HF rats relative to sham and to sitagliptin-treated radiofrequency LV-ablated rats. Conversely, Bax expression was increased in the heart of HF rats relative to sham and to sitagliptin-treated radiofrequency LV-ablated rats. Consistent with the data shown in Figure 6C, the Bcl-2 to Bax ratio was decreased in HF rats compared with sham, and this reduction was significantly mitigated by treatment with sitagliptin.

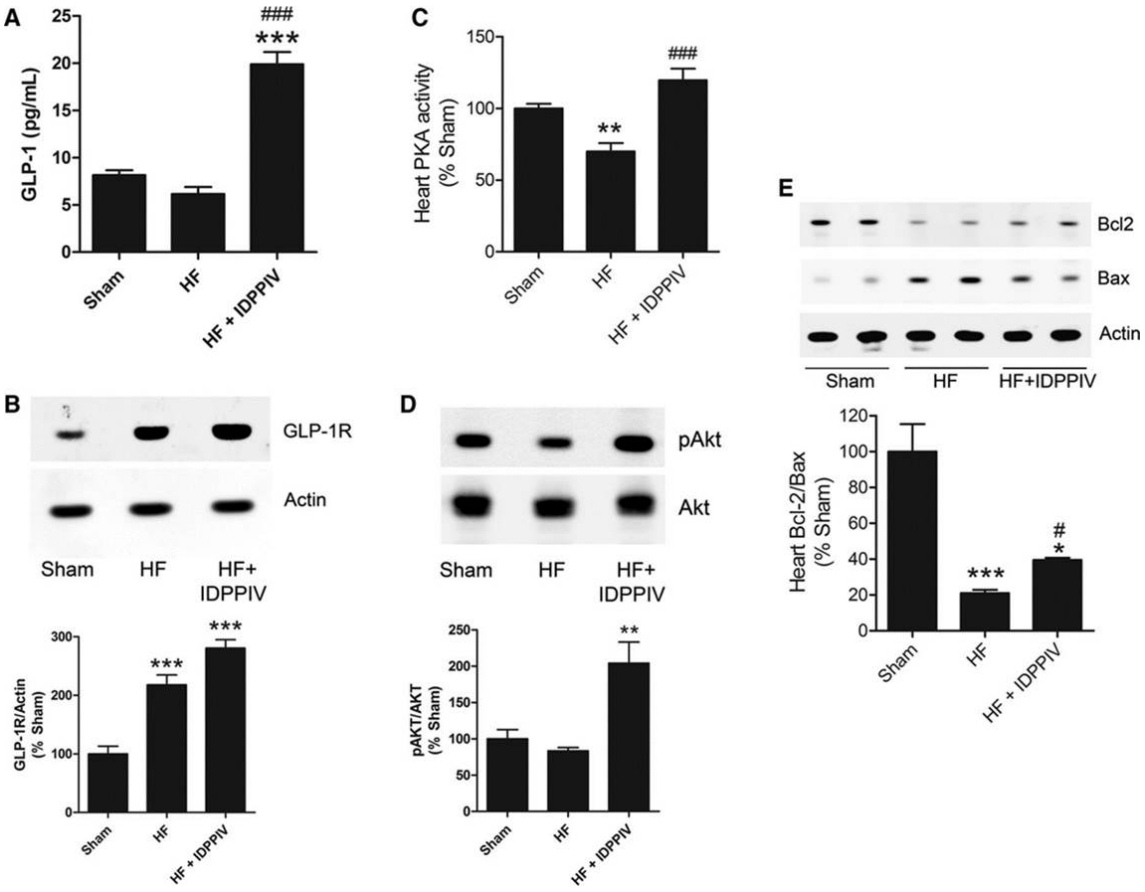


**Figure 8: Effects of dipeptidyl peptidase IV (DPPIV) inhibition on glucagon-like peptide-1 (GLP-1) circulating level, GLP-1 receptor expression in the heart and the activation of cardioprotective signaling pathways.**

**(A)** Circulating active GLP-1 (7–36) was measured using ELISA in sham, radiofrequency LV ablation-induced heart failure rats (HF), and LV-ablated rats treated with sitagliptin for 6 weeks (HF+IDPPIV). **(B)** Representative immunoblots and graphical representation of heart proteins isolated from sham rats, HF rats, or HF+IDPPIV rats probed with an antibody against the GLP-1 receptor. **(C)** protein kinase A (PKA) activity was measured by ELISA. **(D)** and **(E)**, Representative immunoblots and graphical representation of heart proteins from the 3 groups of rats probed with **(D)** antibodies against phosphorylated Akt (pAkt) and total Akt and **(E)** antibodies against Bcl-2 and Bax. Antiactin was used as an internal control. The values are the means±SEM. n=6 rats/group. *P<0.05, **P<0.01, and ***P<0.001 vs sham. #P<0.05 and ###P<0.001 vs HF.

*Effect of DPPIV Inhibition on Total BNP Circulating Level, on Kidney Function, and on the Stimulation of Renoprotective Signaling Pathway:*

Plasma total BNP was greater in the HF rats than in the sham rats and the radiofrequency LV-ablated rats treated with sitagliptin (Figure 9A). Despite the higher circulating total BNP, renal protein kinase G activity (pathway activated by BNP) was lower in HF rats compared with sham and with sitagliptin-treated radiofrequency LV-ablated rats (Figure 9B). As previously shown (60), urinary output (Figure 9C), urinary sodium (Figure 9D), and GFR (Figure 9E) were not significantly different between HF and sham rats. Treatment with sitagliptin did not alter urinary flow or fractional sodium excretion compared with sham and HF rats. However, as shown in Figure 9E, GFR was modestly but significantly increased by sitagliptin compared with HF rats.

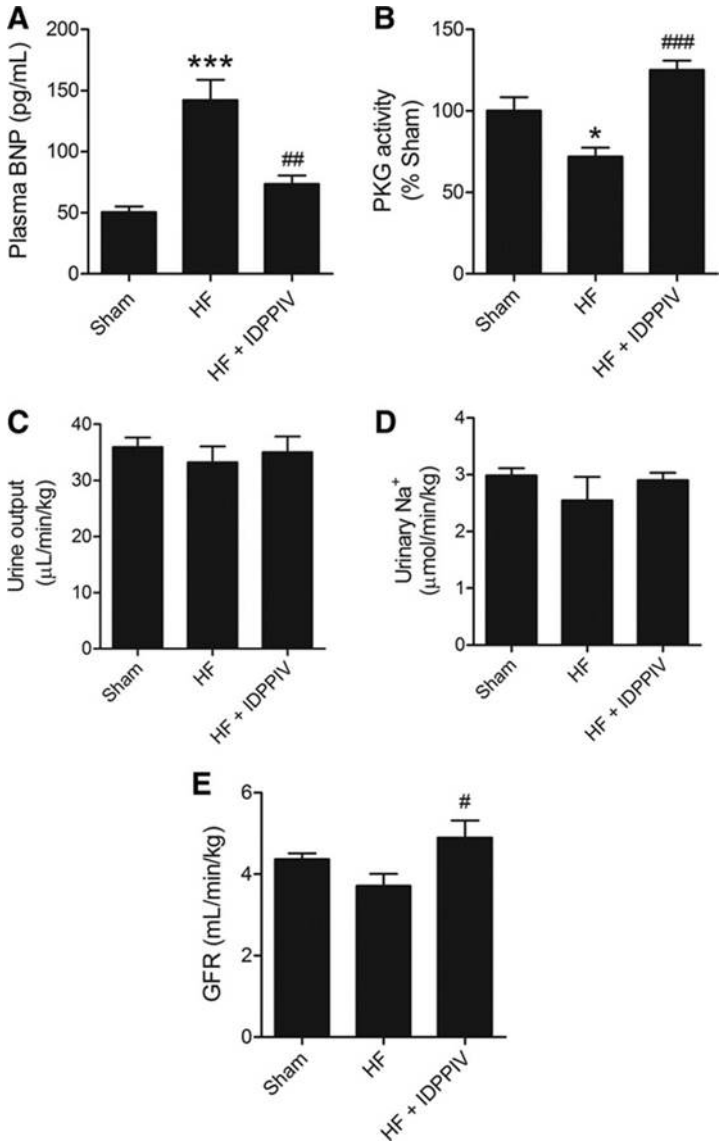


**Figure 9: Effect of dipeptidyl peptidase IV (DPPIV) inhibition on total brain natriuretic peptide (BNP) circulating level, PKG activity, and renal function.**

**(A)** Circulating total BNP. **(B)** Activity of PKG in renal cortex of sham, radiofrequency LV ablation-induced heart failure rats (HF), and LV-ablated rats treated with sitagliptin for 6 weeks (HF+IDPPIV) was measured by ELISA. **(C)** Urine output was measured gravimetrically. **(D)** Urinary sodium. **(E)** The inulin clearance was used to measure glomerular filtration rate (GFR). The values are the means±SEM. The number of animals analyzed in each group is indicated within the bar. **P<0.01 and ***P<0.001 vs sham. #P<0.05, ##P<0.01, and ###P<0.001 vs HF.

**Working hypothesis #3:**

The results demonstrated above show that increased DPPIV activity in plasma significantly correlates with poorer prognostic in human and experimental HF. Interestingly, in a similar fashion, inflammatory markers such as tumor necrosis factor-α (TNFα), interleukin 6 (IL6) and Ccl2 are also associated with poorer outcomes in HF patients (86, 87). Since DPPIV is expressed in cells of the hematopoietic system and it was originally known as a T cell differentiation antigen, we hypothesized that the cardioprotective effects of DPPIV inhibition after myocardial injury in rats were associated with reduced cardiac inflammation.

**Experimental Design:**

We used the same experimental design as described in Figure 3

*Chronic DPPIV inhibition attenuated cardiac inflammation:*

Six weeks after cardiac injury, HF rats displayed increased phosphorylation levels of p38 mitogen-activated protein kinase (MAPK) (Figure 10A) which was attenuated by Sitagliptin treatment. Since p38 is usually related to stress and inflammation we evaluated the numbers of macrophages in those hearts. Interestingly, macrophages levels were significantly increased in HF rats compared to sham animals and treatment with the DPPIV inhibitor reduced those levels (Figure 10B). Corroborating these data, levels of pro-inflammatory markers such as tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL1β), interleukin 6 (IL6) and the chemokine Ccl2 were all increased in the hearts of HF animals; and with the exception of IL6, DPPIV inhibition significantly attenuated all this inflammatory markers (Figure 11A-D). The levels of the anti-inflammatory cytokine interleukin 10 did not differ between the groups (Figure 11E).

The phenotypes M1 and M2 describe the two major and opposing activities of macrophages. In order to get a better understanding of the processes taking place in the hearts of those animals, we evaluated the levels of M1 and M2 markers (Figure 12A-B). According to these results, although the levels of the M2 marker Arginase-1 did not differ between the groups, HF animals exhibited increased levels of the M1 marker iNOS, and a trend to a lower M2/M1 ratio compared to healthy animals (Figure 12C). Moreover, the expression of interferon regulatory factor 5 (Irf5), which is usually associated with M1 polarization (88), was also increased in HF and treatment with sitagliptin significantly improved this pro-inflammatory profile (Figure 12D).

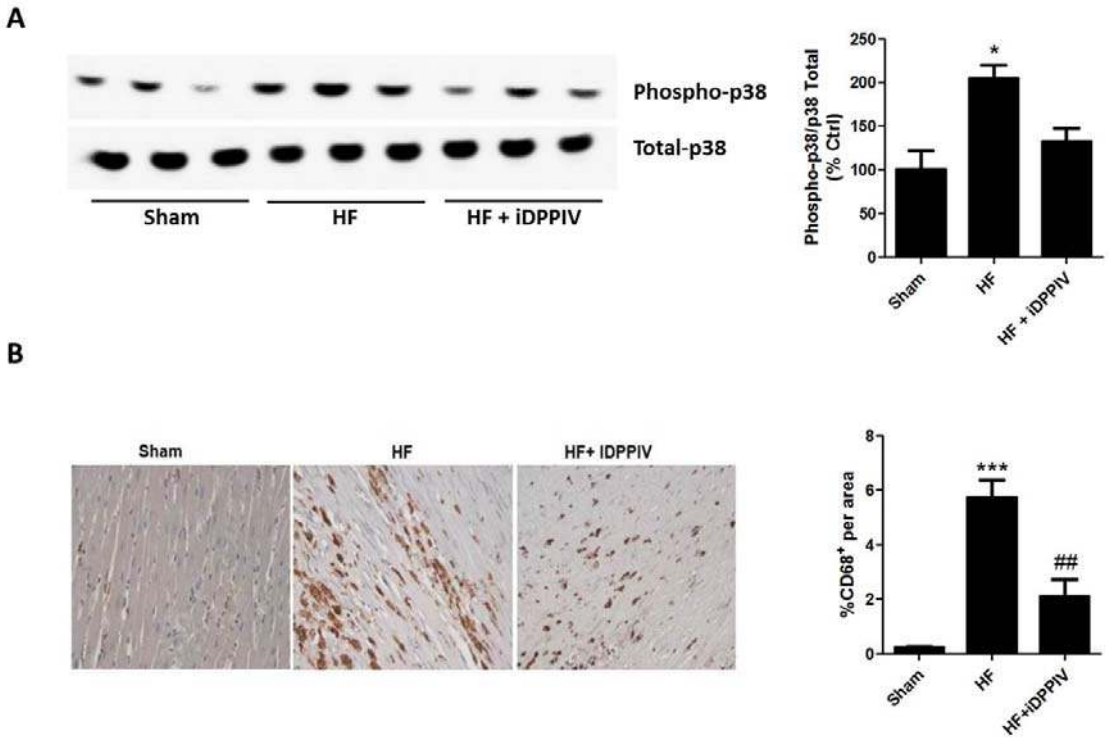


**Figure 10: Evaluation of p38 phosphorylation and macrophage infiltration in the heart of HF and treated rats.**

Representative Immunoblotting showing that chronic DPPIV inhibition attenuated cardiac phosphorylation of p38 **(A)**. Heart sections were stained for $CD68^+$ to evaluate the macrophage infiltrate. HF animals exhibited an increase in $CD68^+$ compared to sham and sitagliptin treated rats **(B)**. *P<0.05 and ***P<0.001 vs Sham; ##P<0.01 vs HF.

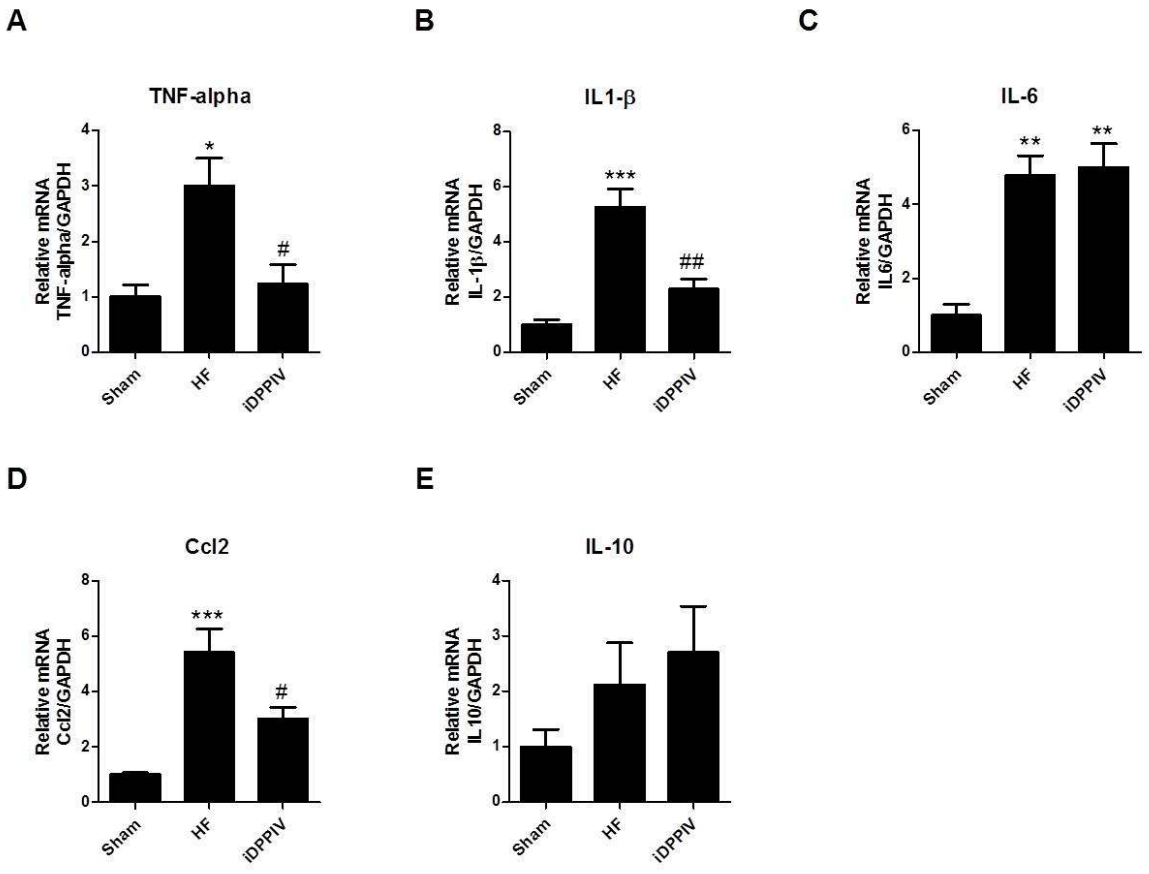


**Figure 11: Cardiac expression of inflammatory markers six weeks after cardiac injury.**

HF rats exhibited higher levels of TNFα **(A)**, IL1β **(B)**, Ccl2 **(C)** and IL6 **(D)** compared to sham animals. With the exception of IL6 treatment with sitagliptin attenuated all this markers. No difference between the three groups was found in IL-10 expression **(E)**. *P<0.05, **P<0.01 and ***P<0.001 vs Sham; #P<0.05 and ##P<0.01 vs HF.

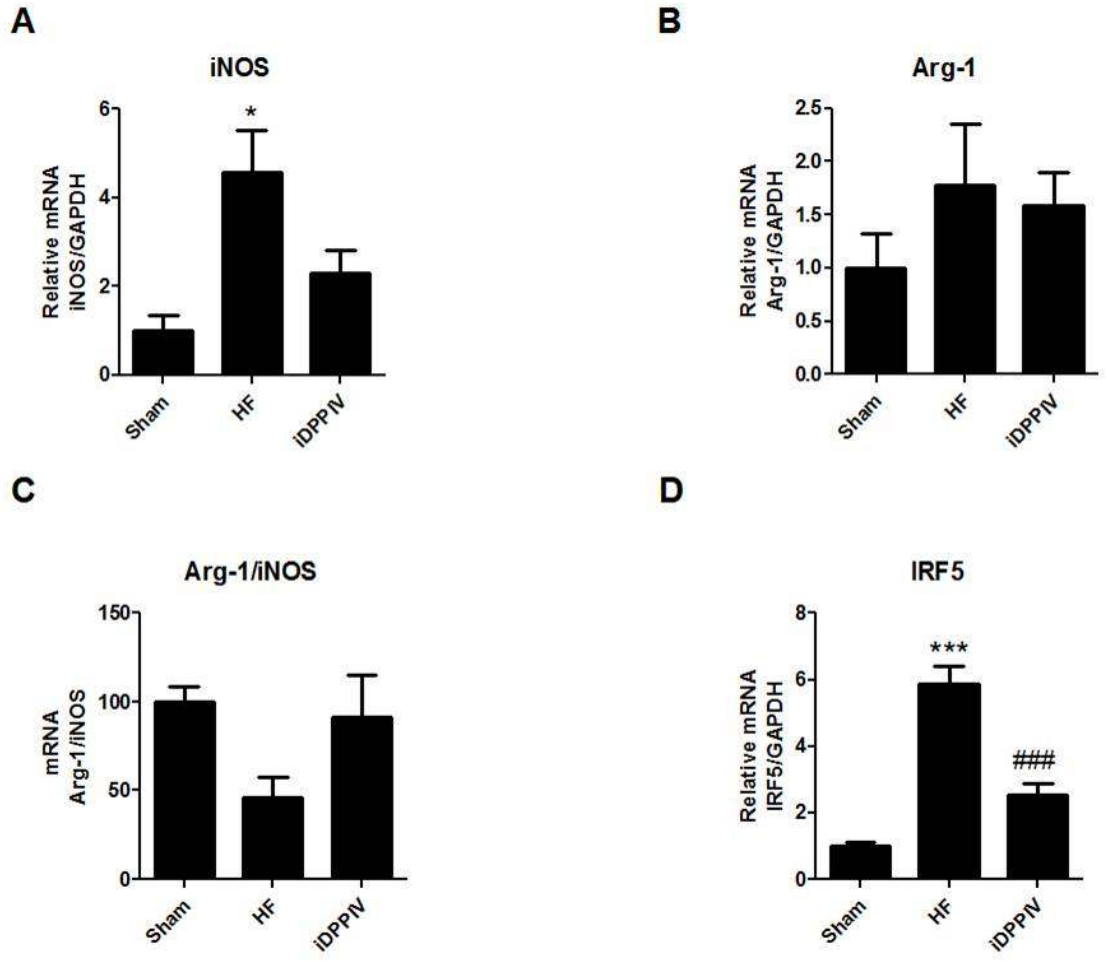


**Figure 12: Cardiac expression of M1 and M2 markers.**

Cardiac expression of the macrophage markers iNOS **(A)**, Arg-1 **(B)**, Arg-1/iNOS ratio **(C)** and IRF5 **(D)**. *P<0.05 and ***P<0.001 vs Sham; ###P<0.001 vs HF.

*Cardiac perfusion:*

Since M2 macrophages play a role in tissue repair, combined with the fact that DPPIV could inactivate Cxcl12, an important chemokine associated with angiogenesis (66, 71), we also evaluated if chronic treatment with sitagliptin might improve cardiac perfusion and angiogenesis. Firstly we assessed the cardiac perfusion by micro-SPECT. As shown in Figure 13A-B, animals subjected to myocardial injury displayed a significant decrease in cardiac perfusion compared to sham animals. However, animals treated with sitagliptin exhibited a significantly increase in cardiac perfusion compared to HF animals. Interestingly, no difference was found in the levels of Vascular Endothelial Growth Factor (VEGF) and Cxcl12, analyzed ELISA and by real time RT-PCR respectively (Figure 13C-D).

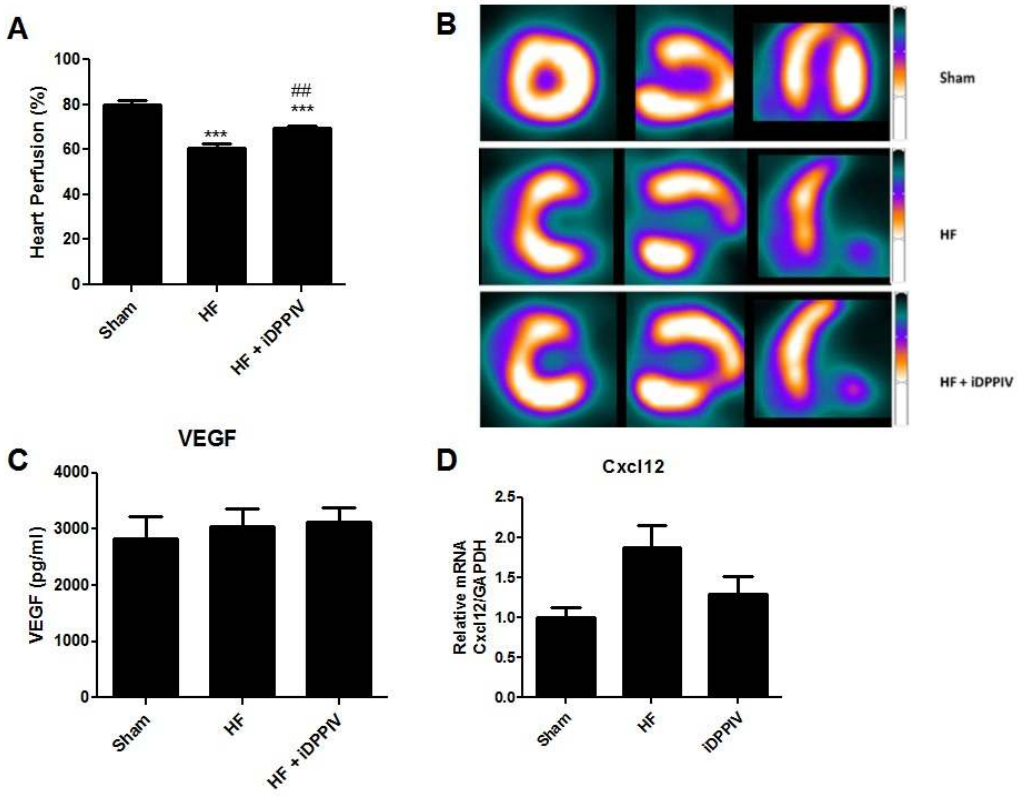


**Figure 13: Assessment of heart perfusion and biomarkers involved in angiogenesis.**

Six weeks after LV ablation surgery HF rats exhibited a significantly decrease in total heart perfusion compared to sham and treated rats **(A-B)**. Despite the difference in perfusion observed between the groups, no difference was found in the Vascular Endothelial Growth Factor (VEGF) **(B)** or Cxcl12 **(C)**. ***P<0.001 vs Sham; #P<0.05 vs HF.

*Macrophage expression of GLP-1 receptor (GLP-1R) and anti-inflammatory effects of GLP-1R agonist:*

As previously mentioned, DPPIV may inactivate several peptides. In order to get a better understanding of the possible mechanisms involved in the anti-inflammatory effects of sitagliptin we assessed if GLP-1, the main substrate of DPPIV *in vivo*, plays a role in this context. As described earlier in Figure 8A, rats treated with sitagliptin exhibited higher levels of active GLP-1 in plasma compared to non-treated rats. Costaining of GLP-1R with CD68 indicated that cardiac macrophages express GLP-1R in their surface (Figure 14A). Interestingly, although we found the GLP-1R in cardiac macrophages, we did not find co-localization of CD68 and DPPIV (data not shown). Taken together, these results suggested that GLP-1 could act directly in those cells. Thus, we evaluated the effects of the GLP-1R agonist, Exendin-4 (Ex-4), in primary macrophages *in vitro*. In line with our hypothesis, we have found that treatment with Ex-4 significantly attenuated LPS-mediated TNFα, IL1β and IL6 secretions in those cells (Figure 14B-D).

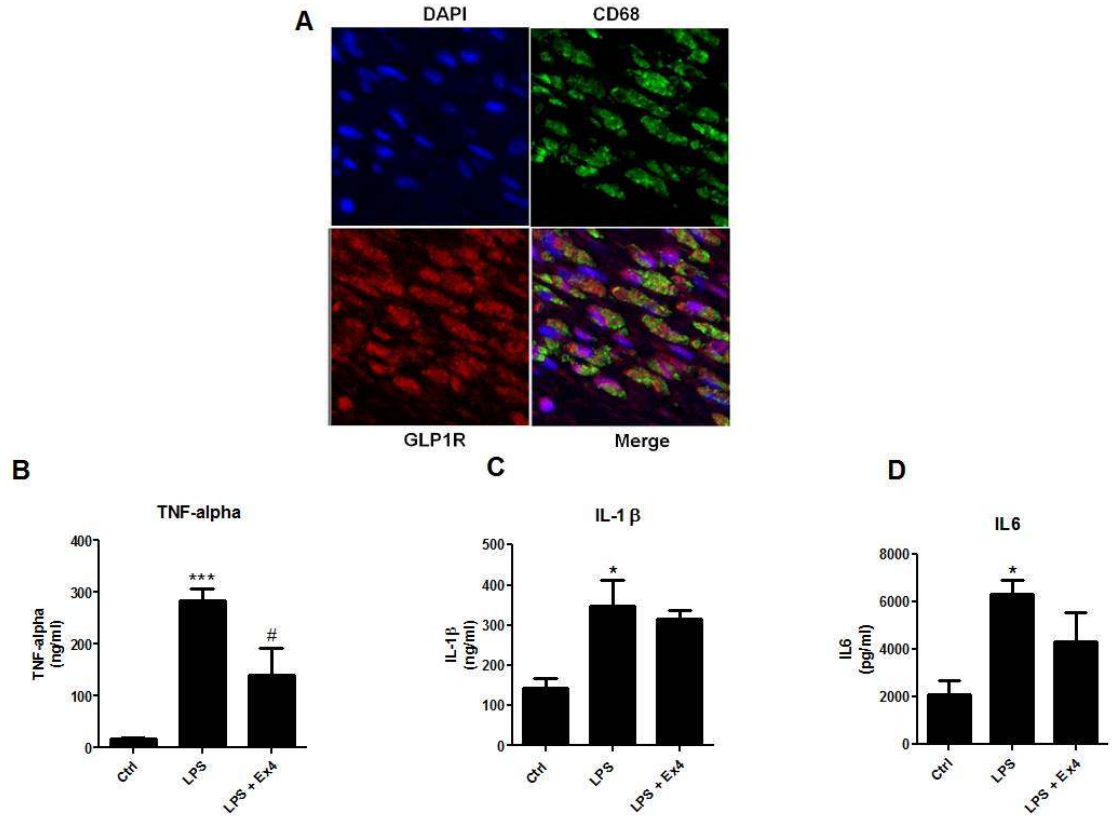


**Figure 14: Evaluation of GLP-1R expression on cardiac macrophages and effects of GLP-1R agonist (Ex-4) in primary macrophages *in vitro*.**

Immunofluorescence in a heart section slide demonstrating a co-staining of the macrophage marker CD68 and GLP-1R **(A)**. Treatment with Ex-4 attenuated the LPS-mediated TNFα **(B)**, IL1β **(C)** and IL6 **(D)** secretion in vitro. *P<0.05 and ***P<0.001 vs Ctrl; #P<0.05 vs LPS.

**Working hypothesis #4:**

In line with our hypothesis, treatment with the DPPIV inhibitor, sitagliptin, significantly improves cardiac inflammation. Since inflammation is usually associated with worse prognosis and we showed that DPPIV activity correlates with poorer outcomes in HF, we hypothesize that DPPIV activity correlates with inflammatory markers in HF patients.

***Experimental Design:***

To evaluate if the levels of the inflammatory markers in HF patients correlates with DPPIV activity we selected 76 patients of the 190 HF patients that were used to test the working hypothesis #1. To ensure homogeneous distribution patients were distributed in deciles groups and we selected 7-9 patients per decile.

*DPPIV correlates with Ccl2 in HF patients:*

Serum DPPIV activity and the plasmatic level of TNFα and Ccl2 were assessed in 76 patients with HF. As shown in Figure 15, DPPIV activity did not correlate with TNFα (r=-0.14; P=0.19), however we found a significantly correlation with the levels of Ccl2 (r=0.30; P<0.01).

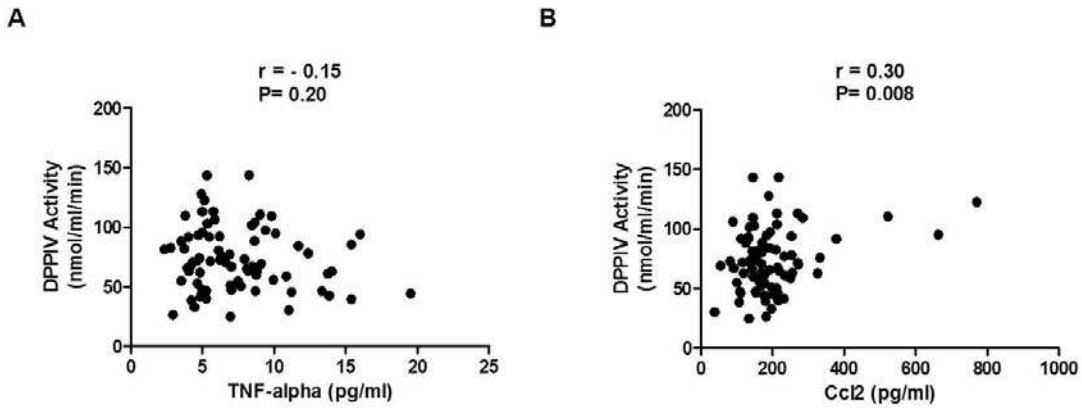


**Figure 15: Correlation between DPPIV activity and in inflammatory markers in HF patients.**

Correlations between plasma DPPIV activity and serum TNFα **(A)** and Ccl2 **(B)** in HF patients. (n = 76) Pearson Correlation test was used.

**Working hypothesis #5:**

Over the past years, the importance of the spleen as an extramedullary hematopoiesis site and source of inflammatory cells that migrate to the injury heart has been revealed (89-92). Since Ccl2 plays a fundamental role in leukocyte recruitment, combined with the facts that DPPIV activity is increased in HF patients and positive correlates with this chemokine, we hypothesized that circulating mononuclear cells and/or the spleen might play a role in the increased levels of DPPIV in HF.

*DPPIV release is increased by HF splenocytes:*

Peripheral blood mononuclear cells (PBMNCs) and splenocytes from HF and sham animals were isolated. After 5 days in cell culture we evaluated the levels of DPPIV in the culture medium. As shown in Figure 16A, DPPIV release did not differ between PBMNCs derived from HF animals or sham; however, HF splenocytes released almost 70% more DPPIV than splenocytes derived from healthy animals (Figure 16B). Additionally, to ratify the link between inflammation and DPPIV we assessed if HF splenocytes also release more pro-inflammatory cytokines than the ones derived from sham animals. As showed in Figure 16C, in addition to release more DPPIV, HF splenocytes also release more IL1β. Unexpectedly, no difference was found in TNFα secretion compared to sham animals (Figure 16D).

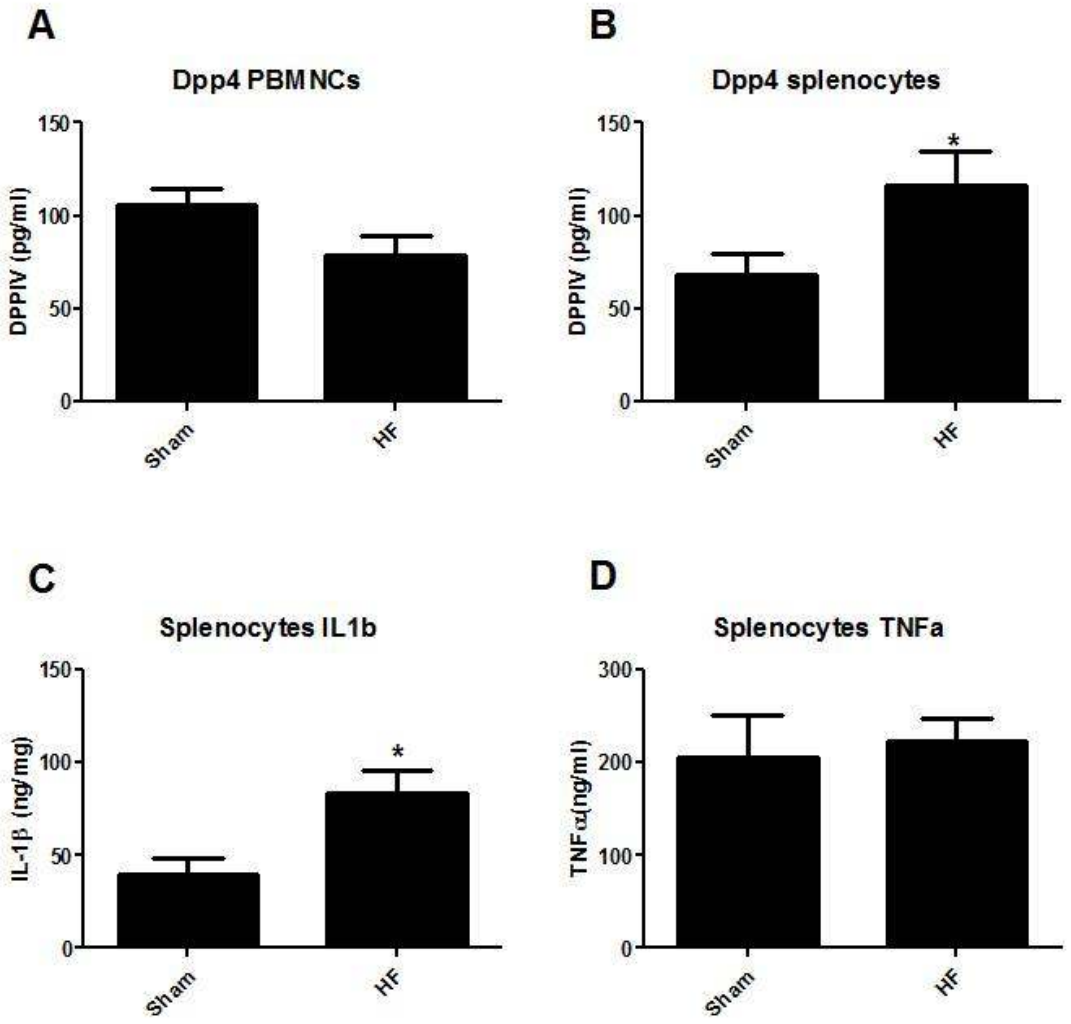


**Figure 16: Assessment of DPPIV release by PBMNCs and splenocytes derived from HF and healthy rats.**

No difference was found in the release of DPPIV by HF and sham PBMNCs **(A)**; however HF splenocytes release more DPPIV than the one derived from sham animals **(B)**. Additionally, HF splenocytes also release more IL1β **(C)** than splenocytes derived from sham rats. No difference was found in the release of TNFα **(D)** [(n = 8-11) per group].

# Discussion

The present work demonstrates that circulating DPPIV activity correlates with poorer cardiovascular outcomes in human and experimental HF. Moreover, the upregulation of DPPIV activity and expression in serum and cardiac endothelial cells in HF rats suggests that this peptidase may be directly involved in cardiac dysfunction. Furthermore, we determine that treating radiofrequency LV-ablated rats with the DPPIV inhibitor sitagliptin significantly attenuates HF-related cardiac remodeling and dysfunction.

The higher DPPIV activity observed in HF suggests that this condition may involve greater degradation of a wide range of DPPIV substrates that possess cardioactive, vasoactive, and renal effects. The reduced bioavailability of these molecules after myocardial injury may lead to HF aggravation and decompensation. Indeed, DPPIV inhibition was able to mitigate cardiac dysfunction, and despite the fact that DPPIV inactivates several peptides, in this work we were able to elucidate some of the mechanisms responsible for improving cardiac function.

GLP-1 is the most studied DPPIV substrate mainly because it pivotal role in the maintenance of glucose homeostasis; however, GLP-1 also seems to play a role in the cardioprotective effects observed in our model of cardiac injury. Long-term treatment of radiofrequency LV-ablated rats with sitagliptin increased circulating active GLP-1 by ≈3-fold. Moreover, the hearts of radiofrequency LV-ablated rats treated with sitagliptin expressed significantly higher levels of the GLP-1R compared with the sham and HF rats. These findings suggest that enhanced coupling of GLP-1 to its cardiac receptor may occur, and this enhanced coupling may represent one possible mechanism underlying the observed sitagliptin-induced cardioprotection. Accordingly, we observed that the cardioprotective signaling pathways transduced downstream of the GLP-1R (93), including PKA, Akt, and the antiapoptotic protein Bcl-2, were activated by sitagliptin treatment. Interestingly, in addition to the effects described above, GLP-1 also seems to exert anti-inflammatory effects since we found a co-staining of the macrophage marker CD68 and GLP-1R and

treatment with the GLP-1R agonist, exendin-4, significantly attenuated the macrophage LPS-mediated secretion of TNFα, IL1β and IL6 *in vitro*. Shiraishi *et al* suggested that GLP-1 induces M2 polarization of human macrophages via STAT3 activation (94); however, although we did not measure the levels of STAT3 phosphorylation we do found a decrease in M1 markers such as iNOS and Irf5 in the hearts of treated animals compared to HF animals. Thus, it is possible that the GLP-1R pathway might interfere in more than one way in cardiac protection; both as acting directly in the cardiomyocytes and vessels and through modulation of inflammation.

Our results also suggest that BNP is involved in the protective effects of the sitagliptin treatment. From a renal point of view, given that $BNP_{3-32}$, the yielded N-terminally cleaved form of $BNP_{1-32}$ by DPPIV, displays reduced natriuretic actions and a lack of vasodilatory activity compared to $BNP_{1–32}$ (64), it would be expected that treatment with the DPPIV inhibitor increase the levels of $BNP_{1–32}$. Unfortunately, we weren't able to distinguish among the different forms of BNP in plasma. However, our results do suggest that treatment with sitagliptin improves the ratio of active/ total BNP. At first, we demonstrated that sitagliptin-treated rats display a remarkable reduction in congestive HF parameters compared with LV-ablated rats not given sitagliptin; (2) the sitagliptin treated rats exhibit lower circulating total BNP but higher renal cortical PKG activity than in the HF rats; and (3) treated rats exhibited higher GFR and cardiac perfusion compared to HF animals. In this regard, Gomez *et al* (95) demonstrated that acute intravenous administration of $BNP_{1-32}$ to sitagliptin-treated HF pigs improved cardiac performance and contractility, whereas no beneficial effect was observed when $BNP_{1-32}$ was administered to HF pigs treated with placebo. Moreover, they found that sitagliptin-treated pigs display higher GFR than those treated with placebo. Of note, BNP might also play a role in cardiac inflammation. In fact, BNP is upregulated at the transcriptional and translational levels by pro-inflammatory cytokines such as TNFα and IL1β in a p38 dependent manner in ventricular cardiomyocytes (96). Curiously, unlike TNFα and IL1β, no effect was found in BNP regulation/secretion after IL6 stimulation (96). These data are in accordance with our results, since with the exception of IL6, DPPIV inhibition was able to reduce these inflammatory

markers and also reduce the levels of total plasma BNP in HF animals. Moreover, BNP inhibits Ccl2-induced monocyte migration *in vitro* (97) and treatment with sitagliptin significantly reduced the levels of macrophages in the failing heart.

Another DPPIV substrate that might also be involved in the cardioprotective effects of DPPIV inhibition is the chemokine Cxcl12. Cxcl12 is a potent chemoattractant protein that plays a fundamental role in leukocyte recruitment to inflammatory sites and angiogenic processes. Interestingly, although some studies having been shown that DPPIV inhibition significantly increase the levels of Cxcl12 and improves vascular density after MI (71, 72), we found no difference in the levels of this chemokine or the levels of VEGF six weeks after chronic DPPIV inhibition. Of note, Cxcl12 usually increases after an acute ischemic event such as MI and then return the basal levels after a few days (71), thus it is possible that six weeks after cardiac injury Cxcl12 levels were already normalized. Interestingly, despite the fact that we did not evaluate the number of vessels per se, heart perfusion was significantly increased in treated rats compared to HF animals. Thus, it is possible that at least part of the observed increase in cardiac perfusion might be due to this peptide and increased vascular density.

Besides describing the beneficial effects of DPPIV inhibition after cardiac injury, in this work we also demonstrate that DPPIV activity is in increased in HF animals and patients. Moreover, DPPIV activity also correlates with poor outcomes such as pulmonary congestion, ejection fraction and the inflammatory chemokine Ccl2. Ccl2 play a crucial role in leukocyte recruitment to injured and inflamed tissues as the heart in HF. High levels of Ccl2 might increase the number of inflammatory cells and combined with pro-inflammatory mediators might promote cardiomyocyte cell death, cardiac remodelling and modulate fibroblast phenotype deteriorating the cardiac function (98-100).

The source of the inflammatory cells that migrate to heart might be diverse. Although the classic view suggests that leukocyte derives mainly from the bone marrow, extramedullary source such as the spleen has drawn attention. Interestingly, besides the role of the spleen in acute inflammation such as MI (89, 90, 92), recently it was suggest that the spleen might also play

a role in chronic inflammation and HF progression (91). In fact, in the present work, we found that besides the increase release of IL1β, splenocytes derived from HF animals release 70% more DPPIV than those derived from healthy animals. Interestingly, the same pattern was not observed in PBMNCs, suggesting that DPPIV release occurs before spleen emigration or through a distinct population that is present in the spleen and not from circulating mononuclear cells. The exact source of serum DPPIV is still under debate and might be diverse. However, since the spleen is basically comprised of myeloid cells and lymphocytes; combined with the fact that DPPIV expression in dendritic cells is usually restricted to a subpopulation present in draining lymph nodes of the intestine and skin (7), and the absence of DPPIV expression in $CD68^+$ cells. We might speculate that spleen lymphocytes may contribute to the increase in plasmatic DPPIV in HF. Thus, the spleen has an important role in the progression of HF, since it may aggravate cardiac function by increasing the output of inflammatory cells to the heart and by increasing the levels of soluble DPPIV, which in turn inactivates several cardiorenal protective peptides such as GLP-1, BNP and Cxcl12. Of note, plasma DPPIV is also increased in other diseases such as obesity and hypertension (101, 102), and recent studies have shown that the spleen has an important role in the pathophysiology of these disorders (103-105). Since the immune system plays a role in these diseases such as in HF, it is plausible to hypothesize that the immune system and possibly the spleen might also be responsible for the increase levels of soluble DPPIV in those illnesses. In agreement, studies have shown that not only plasma DPPIV is increased but also that DPPIV inhibition was able to reduce inflammation and end organ damage in obesity and hypertension (76, 101, 106-108).

## DPPIV inhibitors and Cardiovascular Outcomes: Clinical Studies and Perspectives:

Despite our promising data, documenting that DPPIV inhibition is beneficial for treating cardiac dysfunction; conflicting results have been found when translating these promising findings from preclinical animal models to clinical therapy.

In accordance with the pre-clinical studies, small pilot studies have reported positive effects of DPPIV inhibitors in patients with cardiac disease. In a small study, fourteen patients with coronary artery disease and preserved left ventricular function awaiting revascularization received an oral load of 75 g of glucose after a single dose of 100 mg of sitagliptin or placebo. Dobutamine stress echocardiography was conducted with tissue Doppler imaging at rest, during peak stress, and after 30 min of recovery. Interestingly, patients treated with the DPPIV inhibitor exhibited an improvement in global left ventricular function at peak stress, and after a 30 min recovery. Moreover, sitagliptin mitigated post-ischemic stunning dramatically compared to the placebo (109). Because an oral load of glucose was administered to patients in this study, one can infer that GLP-1 may be the major DPPIV substrate responsible for the observed cardioprotective effects.

The failing heart undergoes an intense metabolic remodelling, switching its primary energy substrate to glucose. In this regard, DPPIV inhibition seems to exert a positive effect on myocardial energy metabolism because four-week treatment with sitagliptin significantly increased myocardial glucose uptake in a cohort of nondiabetic patients with nonischemic dilated cardiomyopathy (110). These findings may be attributed, at least in part, to the fact that sitagliptin is capable of increasing the protein and mRNA expression of glucose transporter-4 (GLUT-4) in the heart (111), at least in part, due to a GLP-1-dependent mechanism because this incretin directly enhances GLUT4 expression in isolated cardiomyocytes *in vitro* (111).

Since 2008, regulatory agencies have demanded that all new anti-diabetic drugs undergo cardiovascular safety assessments. Until now, three major clinical trials assessing the benefits and risks of DPPIV inhibitors in high-cardiovascular risk patients with diabetes had their results published. The Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus—Thrombolysis in Myocardial Infarction 53 study (SAVOR-TIMI 53) (112), the Examination of Cardiovascular Outcomes with Alogliptin *vs.* Standard of Care (EXAMINE) (113) and the Trial Evaluating Cardiovascular Outcomes with Sitagliptin (TECOS) Study Group (114).

The SAVOR-TIMI 53 study was a multicenter, randomized, double-blind, placebo-controlled, phase 4 trial. A total of 16,492 patients with a history of documented type 2 diabetes mellitus, a glycated hemoglobin level of 6.5% to 12.0%, and either a history of established cardiovascular disease or multiple risk factors for vascular disease were randomly assigned to receive the DPPIV inhibitor saxagliptin at a dose of 5 mg daily (or 2.5 mg daily in patients with an estimated GFR of ≤ 50 mL/min) or a placebo. The primary endpoints consisted of cardiovascular death, nonfatal MI or nonfatal ischemic stroke. The secondary endpoints included hospitalization for HF, coronary revascularization, or unstable angina. The median follow-up period was 2.1 years. As expected, patients treated with saxagliptin exhibited lower levels of fasting plasma glucose and glycated hemoglobin. Notably, the saxagliptin group presented a better albumin-to-creatinine ratio than the placebo group, suggesting a positive effect on renal function. Unexpectedly, this trial showed a 27% increased relative risk of hospitalization for HF in patients assigned to the saxagliptin group (3.5% *vs.* 2.8% in placebo; $p = 0.007$) (112). Further analysis showed that patients with a high overall risk of HF (*i.e.*, a history of HF, impaired renal function, or elevated baseline levels of *N*-Terminal proBNP) were more susceptible to the detrimental effects of the DPPIV inhibitor (115).

The EXAMINE trial was a multicenter, randomized, double-blind trial (113). Unlike the SAVOR-TIMI 53 study, patients were eligible for enrollment if they had type 2 diabetes mellitus, a glycated hemoglobin level of 6.5% to 11.0%, and had an acute coronary syndrome within 15 to 90 days before randomization. Acute coronary syndromes included acute MI and unstable angina requiring hospitalization. The patients were assigned to receive alogliptin or a placebo. Because alogliptin is cleared by the kidneys, dose adjustment in patients with diabetes and chronic kidney disease was required. Patients with normal renal function or mild renal insufficiency, *i.e.*, levels of estimated GFR (eGFR) > 60 mL/min received 25 mg, patients with an eGFR of 30 to less than 60 mL/min received 12.5 mg and patients with an eGFR < 30 mL/min received 6.25 mg. The mean follow-up was 18 months, and the primary outcomes were cardiovascular death, nonfatal MI and nonfatal stroke. A total of 5380 patients were evaluated, and similar to the SAVOR-TIMI 53 study, no significant

differences in primary cardiovascular outcomes between the placebo and alogliptin groups were observed (113). Further analyses regarding HF and the EXAMINE trial were published, and despite the similar history of HF in both groups, alogliptin neither induced new onset of HF nor worsened the outcomes in patients with prior HF (116).

At last, in 2015 the results of TECOS were published (114). The TECOS study was also a multicenter, randomized, double blind trial. To be eligible for enrollment patients should had type 2 diabetes with a glycated hemoglobin level of 6.5 to 8.0%, established cardiovascular disease and were at least 50 years of age. Established cardiovascular disease was defined as a history of major coronary artery disease, ischemic cerebrovascular disease, or atherosclerotic peripheral arterial disease. Patients were excluded if they had taken a DPPIV inhibitor, GLP-1R agonist, or thiazolidinedione (other than pioglitazone) during the preceding 3 months; if they had a history of two or more episodes of severe hypoglycemia (defined as requiring third-party assistance) during the preceding 12 months; or if the eGFR was less than 30 ml per minute per 1.73 $m^2$ of body-surface area at baseline. A total of 14.671 patients assigned to add either sitagliptin or placebo to their existing therapy. During a median follow up of 3 years adding sitagliptin to usual care treatment did not increase the risk of hospitalization for heart failure or major adverse cardiovascular events such as MI or stroke.

A smaller but ongoing study evaluating DPPIV inhibitor and cardiac outcomes is the Vildagliptin in Ventricular Dysfunction Diabetes (VIVIDD) trial. In the VIVIDD trial, 254 patients with type 2 diabetes mellitus, with glycated hemoglobin of 6.5% to 10%, and chronic HF (NYHA class I to III) were randomized to receive vildagliptin (50 mg b.i.d) or a placebo (117). The ejection fraction (primary endpoint) was measured at baseline and after 52 weeks of follow-up. No significant difference in the ejection fraction was found between the groups; however, patients taking vildagliptin exhibited a significant increase in left ventricular end-diastolic volume, end systolic volume and stroke volume. Interestingly, despite the increased volume, after 52 weeks, BNP levels decreased by 14% relative to baseline in the placebo group *vs.* 28% in the vildagliptin group. These data suggest a decrease in cardiac stress. According

to new findings reported at the American Diabetes Association 2014 Scientific Sessions, patients treated with the DPPIV inhibitor vildagliptin exhibit no significant difference in the incidence of hospitalization for HF compared to the placebo group (118).

Taken together these data suggest that adding DPPIV inhibitors in the therapeutic armamentarium is viable choice for reducing the levels of glycated hemoglobin in diabetic patients, however unlike the pre-clinical studies no clear benefits beyond glycemic control were observed. Of note, the medium follow up of these studies was relative small to evaluated huge differences in major cardiovascular events. In this regard, The Cardiovascular Outcome Study of Linagliptin *vs.* Glimepiride in Patients with Type 2 Diabetes (CAROLINA) which is a multicenter, randomized, double blind, head-to-head trial that has been ongoing since November 2010, and with primary completion estimation date to September 2018 (119), will help to clarify whether DPPIV inhibitors play a role in terms of cardiovascular outcomes in a clinical perspective.

# Conclusions

Collectively our data suggest that DPPIV may play a role in the progression and the pathophysiology of HF (Figure 17) by potentiating the degradation of cardiorenal peptides such as GLP-1 and BNP. Moreover, we conclude that the spleen may be one of the sources of increased plasma DPPIV in HF and as such might play a role in the inactivation of these peptides.

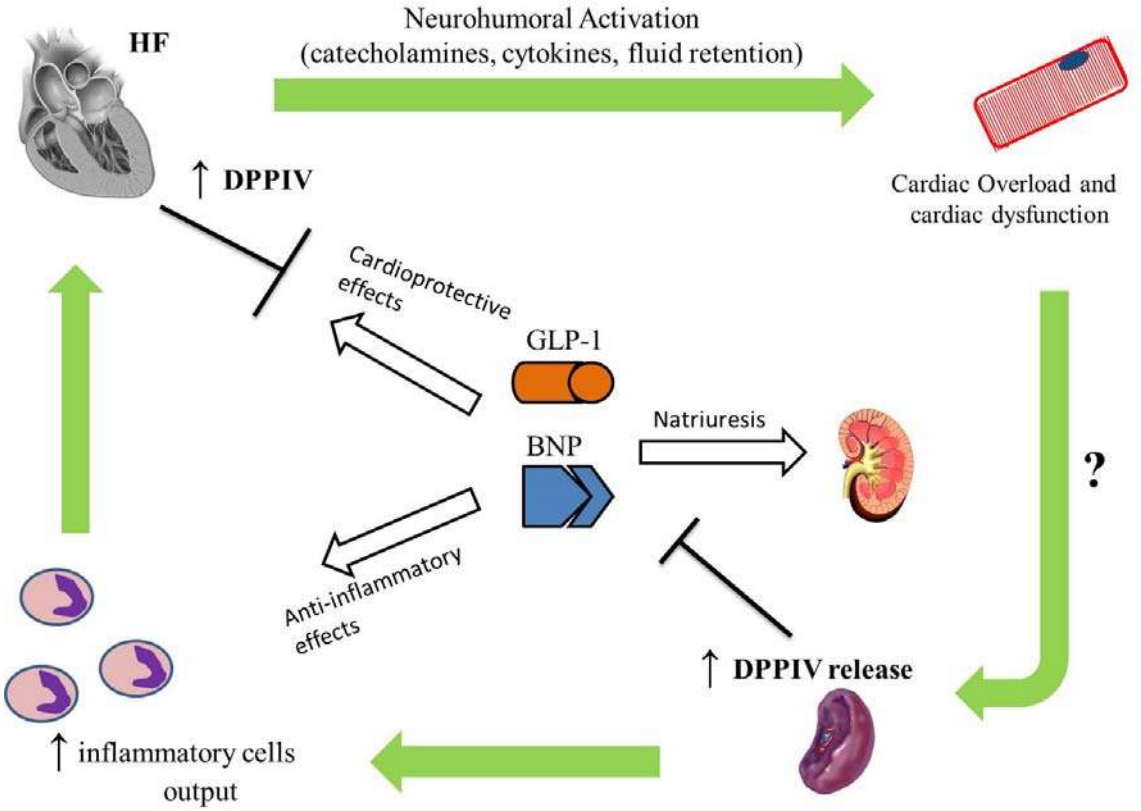


**Figure 17: Schematic representation of the role of DPPIV in the pathophysiology of heart failure (HF).**

Cardiac dysfunction leads to increased neurohumoral activation and HF. Through an unknown mechanism HF splenocytes contribute to the increase in the levels of soluble DPPIV which in turns leads to increased degradation of peptides with cardioprotective and natriuretic actions such as GLP-1 and BNP. Low levels of active GLP-1 and BNP lead to increased fluid retention, cell death and inflammation aggravating cardiac dysfunction and HF.

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**COMITÊ DE ÉTICA EM PESQUISA**

**A CEUA** do Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de São Paulo, em sessão de **31/07/2013, APROVOU** o Protocolo de Pesquisa nº **211/13** intitulado**: "IMPACTO DA INIBIÇÃO DA ENZIMA DIPEPTIDIL PEPTIDASE IV SOBRE AS ALTERAÇÕES CARDÍACAS E RENAIS DE RATOS SUBMETIDOS À INJÚRIA DO MIOCÁRDIO: AVALIAÇÃO DOS EFEITOS PREVENTIVOS E TERAPÊUTICOS"** que utilizará **50** animais da espécie **Ratos Wistar Adultos de 250-300g,** apresentado pela **COMISSÃO CIENTÍFICA DO INCOR.**

Cabe ao pesquisador elaborar e apresentar ao CEP-FMUSP, o relatório final sobre a pesquisa, (Lei Procedimentos para o Uso Científico de Animais - Lei Nº 11.794 -8 de outubro de 2008).

**Pesquisador (a) Responsável: Adriana Castello Costa Girardi**
**Pesquisador (a) Executante: Thiago de Almeida Salles**

**CEP-FMUSP, 01 de Agosto de 2013.**

**Dr. Eduardo Pompeu**
**Coordenador**
**Comissão de Ética no Uso de Animais**

**Prof. Dr. Roger Chammas**
**Coordenador**
**Comitê de Ética em Pesquisa**