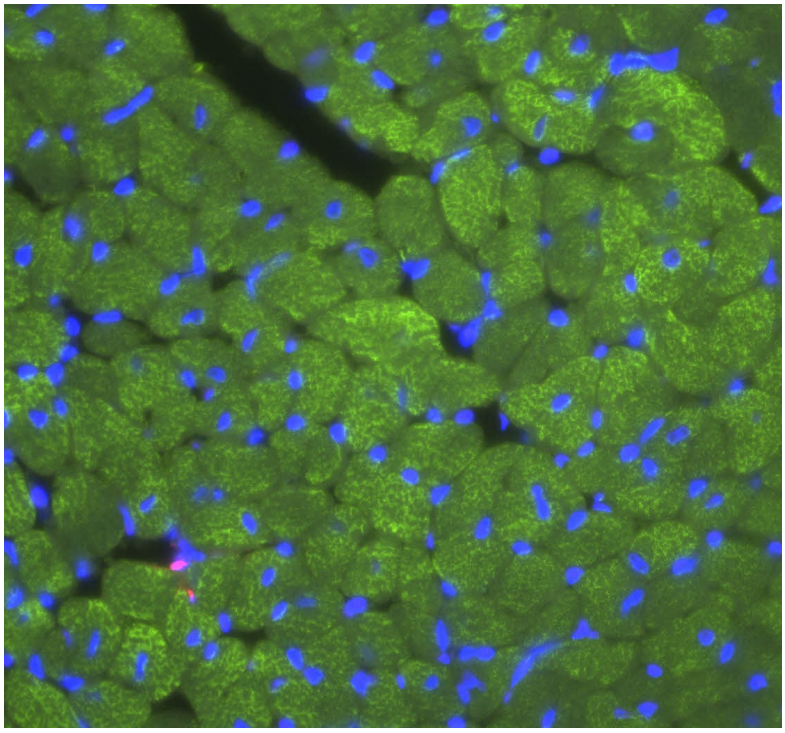
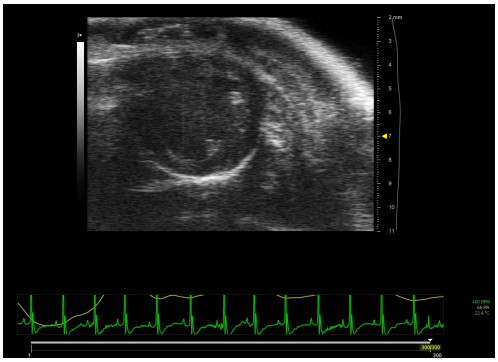
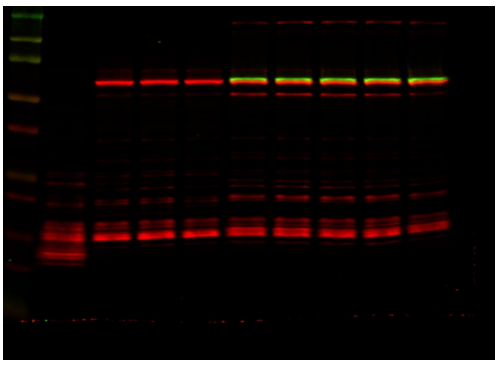
International PhD program in Cardiovascular Pathophysiology and Therapeutics

CardioPaTh
Cardiovascular
Pathophysiology
& Therapeutics







Novel Roles of GRK2 and GRK5 in Cardiovascular Diseases

PhD thesis

Federica Marzano

Università degli Studi di Napoli
Federico II

Temple
University

Novel Roles of GRK2 and GRK5 in Cardiovascular Diseases

PhD thesis

Federica Marzano

16-03-1992 Naples (Italy)

Promotor: Prof. Antonio Rapacciuolo

Federico II University of Naples, Naples, Italy

Co-promotor: Prof. Walter J. Koch

Temple University, Philadelphia, USA

Naples, 10/01/2022

University Federico II of Naples, Faculty of Medicine,

Via Pansini n. 5, 80131 Naples, Italy

“Above all, don't fear difficult moments. The best comes from them.”

Rita Levi-Montalcini

Table of contents

CHAPTER 1

General introduction and outline of the thesis

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide, with a heavy burden on global healthcare systems. Commonly, CVDs are a group of disorders that involves the heart or blood vessels, including, stroke, heart failure (HF), coronary heart disease, peripheral arterial disease, and other cardiac and vascular conditions (Mensah, et al., 2019; Amini, et al., 2021). Common risk factors for CVDs include core health behaviors (smoking, physical inactivity, unhealthy diet, overweight and obesity) and other factors (cholesterol, blood pressure, and glucose control) that are related to cardiovascular health (Virani, et al., 2021).

Among CVDs, HF constitutes a significant public health concern, with a prevalence estimated at 1% to 2% of the general adult population in developed countries (Groenewegen, et al., 2020). HF is a heterogeneous clinical syndrome, and it is characterized by reduced myocardial pump function and increased pressure load. The dysregulation of neurohormonal systems, including hyperactivity of the sympathetic nervous systems (SNS) and of the renin–angiotensin–aldosterone system (RAAS), exerts a pivotal role in HF pathogenesis (Bencivenga, et al., 2021).

Hyperactivity of the SNS, that provides increasing levels of circulating catecholamines (CAs), causes defects in β-adrenergic receptors (β-ARs) regulation in the heart, due to the upregulation of G protein-coupled receptor (GPCR) kinases (GRKs) (Sato, et al., 2015*a*; Mangmool, et al., 2018). Among GRKs, GRK2 and GRK5, are the major isoforms expressed in the heart, and the levels of these kinases are elevated in human failing myocardium (Dzimiri, et al., 2004).

Significant progress has been made in understanding the pathological role of these kinases in the heart, both as GPCR kinases and as molecules that can act independently of GPCRs, in a “non-canonical” manner. GRKs are capable to regulate several cellular processes, often through translocation to distinct intracellular compartments, such as the mitochondria for GRK2 or the nucleus for GRK5 (Schumacher and Koch, 2017).

Therefore, elucidating the role of GRKs in the modulation of these cellular processes may provide novel insight into molecular mechanisms involved in cardiac physiology and pathophysiology.

During my Ph.D. fellowship, I was involved in several research studies in these fields, aiming at uncovering novel molecular mechanisms underlying cardiac dysfunction, thus identifying new molecular targets and improve HF therapy.

Outline of the thesis

The thesis is divided into four parts:

Part I: Introduction to Cardiac Pathophysiology

In the first part of the thesis, we provide a general introduction to cardiac physiology and pathophysiology, focusing on GPCR signaling and GRKs.

Part II: New Roles of G Protein-Coupled Receptor Kinase 2 (GRK2) In the Heart

In this section, we explore the effects of non-canonical activities of GRK2 in the heart. In particular, we tested the impact of high aldosterone levels, and the role of GRK2, on both insulin and β-ARs signaling in in the heart.

Part III: G Protein-Coupled Receptor Kinase 5 (GRK5) in Chronic Degenerative Diseases

This section of the thesis is a collection of our research studies aimed at investigating the role of GRK5 in chronic degenerative diseases, including cancer, neurological and cardiovascular disease. Finally, we explore the effects of inactivating the catalytic site of GRK5, with a particular focus on its role in governing basal cardiac function and response to stress.

Part IV: Discussion and conclusions

The last section of the thesis is a broad discussion of the addressed topics with the conclusions.

Part I:

Introduction to Cardiac Pathophysiology

CHAPTER 2

Pathophysiology of Heart Failure

Heart failure (HF) is a global pandemic affecting about 1% to 2% of the population worldwide, with a further increase of prevalence expected over the next decade.

Despite the significant advances in therapies and prevention, it is still a leading cause of mortality and morbidity, with a dramatic clinical, societal, and economic burden (Savarese and Lund, 2017).

The pathogenesis of HF is complex, with several possible etiologies and multiple risk factors, including age, genetics, lifestyle habits and underlying health conditions, such as obesity, hypertension, or diabetes mellitus. HF constitutes the end-stage condition of several structural and functional CVDs, including ischemic heart disease, coronary artery disease, cardiomyopathies, valvular diseases, congenital malformations, and others.

Physiologically, HF is characterized by reduced ability of the heart to provide sufficient perfusion to meet metabolic requirements and accommodate systemic venous return.

Current guidelines have classified HF into three subtypes according to left ventricular ejection fraction (LVEF): HF with preserved LVEF (typically considered as ≥50%; HFpEF), HF with reduced LVEF (typically considered as <40%; HFrEF) and mid-range HF (typically in the range of 40 – 49%; HFmrEF) (Ponikowski, et al., 2016).

As the heart fails, patients develop symptoms which include dyspnea, persistent coughing or wheezing, and buildup of excess fluid in body tissues, due to altered venous return. Nausea, lack of appetite, tiredness, and fatigue are also common constitutional symptoms.

The failing heart initially undergoes adaptive and compensatory changes to maintain adequate function. These include increased cardiac output, progressive myocardial remodeling, and activation of neurohormonal systems. Although initially beneficial, the long-term effects of these compensatory mechanisms turn to be deleterious, thus further worsening cardiac function and HF progression (Kemp and Conte, 2012).

A principal hallmark of the progression to HF is a maladaptive structural remodeling of the heart, which consists of cellular and molecular changes that directly influence left ventricular (LV) mass, volume, and shape. These changes include progressive loss of myocytes and reorganization of the extracellular matrix, with deposition of interstitial collagen that leads to electrical disruption and decreased cardiac function (Hartupee and Mann, 2017). Another important biological change by which the heart attempts to normalize decreased systemic perfusion include hypertrophic growth by enlarging individual myocytes. The result of both fibrosis and hypertrophy is a change in the structure of the heart, with dilation of the LV chamber, and thickening or thinning of the walls of the heart (van Berlo, et al., 2013).

The most important neurohormonal adaptations in HF are activation of the SNS, that provides increasing levels of circulating CAs, and the RAAS, which maintain cardiac output through increased sodium retention, peripheral arterial vasoconstriction, and contractility.

In normal conditions, the SNS controls cardiac physiology, through the modulation of atrioventricular conduction, heart rate, cardiac contractility and relaxation. Conversely, in pathological conditions, such as HF, the increased levels of norepinephrine (NE) and

epinephrine (EPI) determine a persistent activation of β-ARs, thus leading to dysfunction in the signaling pathway and to depressed cardiac function (Bencivenga, et al., 2021).

On the other hand, the pathophysiologic effects of the RAAS are primarily driven by angiotensin II and aldosterone. Indeed, chronic exposure to excessive levels of these hormones has been linked to adverse effects, including ventricular hypertrophy, vasoconstriction, and sodium retention (Adams, 2004).

Such a dysregulation of these neurohormonal systems has been shown to have a central role in HF progression, through the promotion of myocardial remodeling, cardiac hypertrophy, and fibrosis. Treatment strategies have been developed based upon the understanding of these compensatory mechanisms.

Common medical therapies for the management of afflicted patients include β-ARs antagonists (β-blockers), that counteract the excessive sympathetic nervous system activity; angiotensin-converting enzyme (ACE) inhibitors, Angiotensin II receptor blockers (ARB), and mineralocorticoid receptor (MR) antagonists, that counteract the effects of RAAS hyperactivation.

Although considerable improvements have been made in the treatment of HF, some people with a severe and progressive pathology can't be helped by medications; in such cases, a heart transplantation may be the only effective option. However, there are also some surgical procedures for the management of HF, including cardiac resynchronization therapy (CRT), implantation of ventricular assist devices (VAD) or implantable cardioverter defibrillator (ICD), valve surgeries and others (Nicolini and Gherli, 2009).

Despite significant understanding of the pathophysiology of HF, the mortality is still elevated, and further research is needed to implement therapeutic approaches and improve survival among patients with HF.

CHAPTER 3

G Protein-Coupled Receptor Signaling in the heart

G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors in mammalian genomes, and they regulate multiple intracellular signaling cascades in response to proteins, hormones, neurotransmitters, ions, and other stimuli.

Such a variety of extracellular ligands is reflected in the structural diversity of more than 800 GPCRs, that have been classified into five main subfamilies, based on their sequence homology and functional characteristics (Fredriksson, et al., 2003; Katritch, et al., 2013). GPCRs are expressed in virtually all tissues, and they play a pivotal role in both physiological and pathological processes. Therefore, they represent attractive targets for pharmacological drugs (Hilger, et al., 2018).

The hallmark of GPCRs structure is the seven-transmembrane (7TM) domain, that spans the cell membrane with a ligand-binding domain at its extracellular side, and a carboxy-terminal intracellular domain (Lefkowitz, 2007; Zhou, et al., 2017).

In its resting state, the GPCR intracellular domain is bound to inactive heterotrimeric G proteins, comprising a GDP-bound Gα subunit, as well as Gβ and Gγ subunits. Upon binding their ligands, GPCRs undergo conformational changes in the 7TM, which triggers the replacement of GDP with GTP within the Gα subunit and the dissociation of the heterotrimer from the activated receptor. These events are followed by detachment of Gα subunit from the Gβγ subunits, thus allowing both subunits to modulate the activity of different downstream effector proteins. (Oldham and Hamm, 2008; Pfleger, et al., 2019). The heterotrimeric G proteins are generally divided into four main classes, which differentially control the signal transduction cascade: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$.

$G\alpha_s$ activates the enzyme adenylyl cyclase (AC), which in turn stimulates the production the second messenger cyclic adenosine monophosphate (cAMP). The latter binds to protein kinase A (PKA), thus triggering the phosphorylation of downstream target proteins. In contrast, $G\alpha_i$ has an inhibitory effect on AC activity. The $G\alpha_q$ subunit activates phospholipase C (PLC), which generates inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), thus activating protein kinase C (PKC) and increasing calcium efflux from the endoplasmic reticulum. Finally, the $G\alpha_{12/13}$ subunit binds to Rho guanine nucleotide exchange factors (RhoGEF) (Pfleger, et al., 2019) **(Figure 1)**.

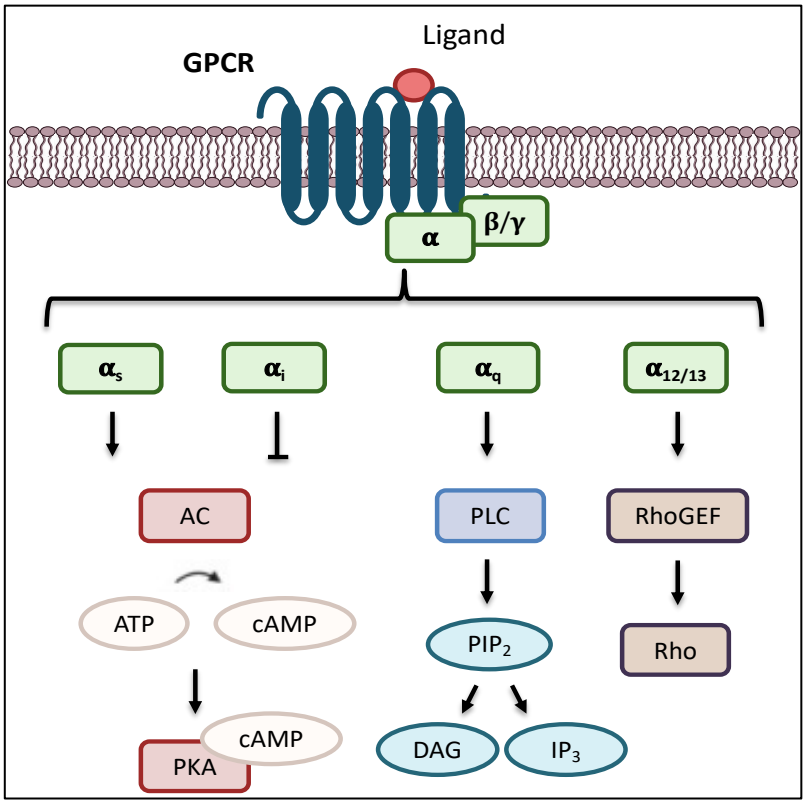


Figure 1. G protein-coupled receptors (GPCRs) signaling pathway. Upon ligand binding to G protein-coupled receptors (GPCRs), inactive G protein heterotrimers dissociate into separate, active Gα and Gβγ subunits. Depending on the subclasses of Gα, various downstream signaling pathways are activated. Gαs activates adenylyl cyclase (AC), whereas Gαi inhibits its activity. Gαq activates phospholipase C (PLC), which produces inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2). Finally, Gα12/13 activates Rho guanine nucleotide exchange factor (RhoGEF).

Following receptor activation and signal transduction, a complex regulatory mechanism is initiated to attenuate the cellular responses mediated by GPCRs, a process termed desensitization. Activated GPCRs are rapidly desensitized through phosphorylation of intracellular serine and threonine residues by specific kinases (GRKs).

Phosphorylation of agonist occupied GPCRs by GRKs facilitates the recruitment of cytosolic proteins called β-arrestins, resulting in the uncoupling of GPCR from G proteins, thus preventing further agonist responsiveness of the receptor (Penela, et al., 2003). In addition, β-arrestins have been shown to directly participate in the clathrin-mediated endocytic pathway, that results in either receptor degradation in lysosomes or recycling to the plasma membrane (Claing, et al., 2002; Pierce, et al., 2002). β-arrestins may also regulate GPCRs internalization and degradation by regulating their ubiquitination and they are involved in the modulation of mitogen-activated protein kinase (MAPK) cascades (Shenoy and Lefkowitz, 2011).

In the cardiovascular system, GPCRs are expressed on various cell types, including cardiomyocytes, fibroblasts, endothelial cells (ECs), and vascular smooth muscle cells (VSMCs), where they regulate cardiac physiology by modulating critical processes. Under pathological conditions, these receptors undergo aberrant changes in expression levels and localization, thus affecting downstream signaling pathways. Among the GPCRs, β-ARs are the predominant subtypes expressed in the heart and they play an important role in the pathophysiology of human heart diseases. As such, they are common targets for therapeutic drugs.

Three different isoforms of β-ARs have been identified in the human heart, β1-AR, β2-AR, and β3-AR, which are differentially expressed. β1-ARs are the predominant subtypes expressed in cardiomyocytes, whereas β2-ARs and β3-ARs are only present in around 5% of them (Myagmar, et al., 2017). β2-ARs are most abundant in cardiac fibroblasts, ECs and VSMCs, where they are involved in fibrosis and vasodilation (Wang, et al., 2018). β1-ARs are traditionally activated by both epinephrine and norepinephrine and their activation increases cardiac contractility and cellular growth.

Both β1-ARs and β2-ARs are coupled to the downstream $G\alpha_s$ protein, which promotes the activation of signaling effector AC and the generation cAMP, leading to increased cardiac contractility, through PKA-mediated phosphorylation of different target substrates (including voltage-gated L-type Ca2+ channel, cardiac ryanodine receptor, phospholamban, and troponin I).

Besides $G\alpha_s$, β2-ARs are also coupled to $G\alpha_i$ protein, allowing for inhibitory effects on cardiac contractile function (Salazar, et al., 2007; Wang, et al., 2018). This different signaling cascade might explain why β2-AR signaling has antiapoptotic and cardioprotective effects, whereas β1-AR activation enhances cardiomyocytes apoptosis, exerting detrimental effects on the heart (Communal, et al., 1999). Although β3-ARs are the least abundant subtype in cardiomyocytes, they have been shown to have a role in regulating cardiac function (Dessy and Balligand, 2010).

Modulation of the adrenergic pathway is crucial for the proper functioning of the cardiovascular system. It is widely recognized that chronic stimulation of the β-ARs exerts toxic effects on the heart and plays a pivotal role in HF progression.

Following cardiac injury several neurohormonal systems are hyperactive, such as the SNS, which increases circulating levels of CAs, thus inducing selective downregulation of β1-ARs, as opposed to β2-ARs. Nevertheless, both β1-ARs and β2-ARs are functionally uncoupled from G proteins and desensitized in HF (Bristow, et al., 1986; Lymperopoulos, et al., 2013). In conclusion, over- stimulation of β-ARs results in their dysregulation and, therefore, induces detrimental dysfunction in downstream signaling pathways.

CHAPTER 4

G Protein-Coupled Receptor Kinases

The desensitization and downregulation of agonist-activated GPCRs are primarily mediated by a family of serine/threonine protein kinases, called GRKs. There are seven known members of the GRK family that have been divided into three different subfamilies, depending on their sequence homology and structural similarities: the rhodopsin kinases subfamily (GRK1 and GRK7), the β-adrenergic receptor kinase (βARK) subfamily (GRK2 and GRK3), and the GRK4-like subfamily (GRK4, GRK5, and GRK6) (Marzano, et al., 2021).

Essentially, GRKs are expressed ubiquitously with varying degrees of expression depending on the type of tissue or cell. For instance, GRK1 and GRK7 are restricted to the retina, where they primarily act as rhodopsin and cone opsin kinases (Weiss, et al., 2001). GRK4 is mostly expressed in the testes, although it is also present in brain, kidneys, and in the uterine myometrium (Virlon, et al., 1998; Brenninkmeijer et al., 1999; Felder et al., 2002; Villar et al., 2009; Sallese et al., 2000). GRK2, GRK3 and GRK5 are predominant in the heart (Belmonte and Blaxall, 2011), with GRK5 also appreciably expressed the brain (Kunapuli and Benovic, 1993).

GRKs possess several protein domains, that affect their subcellular localization. For this reason, GRKs have been considered “multifunctional” proteins, that interact with both GPCRs and non-GPCR-related factors, depending on their location and specific circumstances. When present at the plasma membrane, GRKs exerts “canonical” effects, regulating the function of several GPCRs.

Accordingly, almost all the seven GRK isoforms are localized in specific subcellular compartments that afford them proximity and access to GPCRs. However, extensive work has revealed the existence of “non-canonical” and GPCR-independent roles for GRKs. For GRK2, these activities involve localization within mitochondria, while GRK5 has non-canonical activity within the cytosol or nucleus of the cells.

All GRKs share a common and very conserved structure consisting of three main modular domains: a short N-terminus (NT) region, that is implicated in GPCR binding and it also contains a regulator of G protein signaling (RGS) homology (RH) domain (Ribas et al., 2007); a centrally located catalytic domain, which is a Serine/Threonine kinase domain (KD) that belongs to the AGC family of kinases; and a C-terminus (CT) region, that is variable in length among the different GRKs and is responsible for membrane localization (Inglese et al., 1993; Sato et al., 2015*a*).

GRK1 and 7 have short CT prenylation sequences (Inglese, et al., 1992), GRK2 and GRK3 contain pleckstrin homology (PH) domains that bind anionic phospholipids and βγ subunits of G proteins (Pitcher, et al., 1992; Koch, et al., 1993; DebBurman, et al., 1996), while GRK4 and GRK6 carry CT palmitoylation sites and lipid-binding elements (Stoffel et al., 1994; Jiang et al., 2007). Although GRK5 shares a common structure with the other GRKs, it possesses distinct structural features.

It has been suggested that GRK5 contains a group of hydrophobic amino acids within its CT, that forms an amphipathic helix and, along with PIP2 binding, mediates its localization to plasma membrane (Thiyagarajan et al., 2004). Furthermore, GRK5 localization to the membrane is regulated by the calcium sensor protein calmodulin (CaM). GRK5 possesses CaM binding sites at each terminal domain, within residues 20–

39 of its NT and residues 540-578 of its CT (Pronin et al., 1997). GRK5 also contains a functional nuclear localization signal (NLS, amino acids 388–395), that enhances its ability to translocate into the nucleus and to bind DNA (Johnson et al., 2004). The presence of a functional NLS is a common feature in the GRK4 subfamily; however, the NLS sequence of GRK5 and GRK6 differ in terms of regulation and DNA-binding ability from the one of GRK4. Moreover, a functional nuclear export sequence (NES, amino acids 259–265), has been identified within GRK5 catalytic domain (Johnson et al., 2013). Finally, GRK5 contains specific caveolin binding motifs, and this characteristic is also shared by GRK2 and GRK3, that through their PH and NT domains, interact with caveolins (Carman, et al., 1999) **(Figure 2)**.

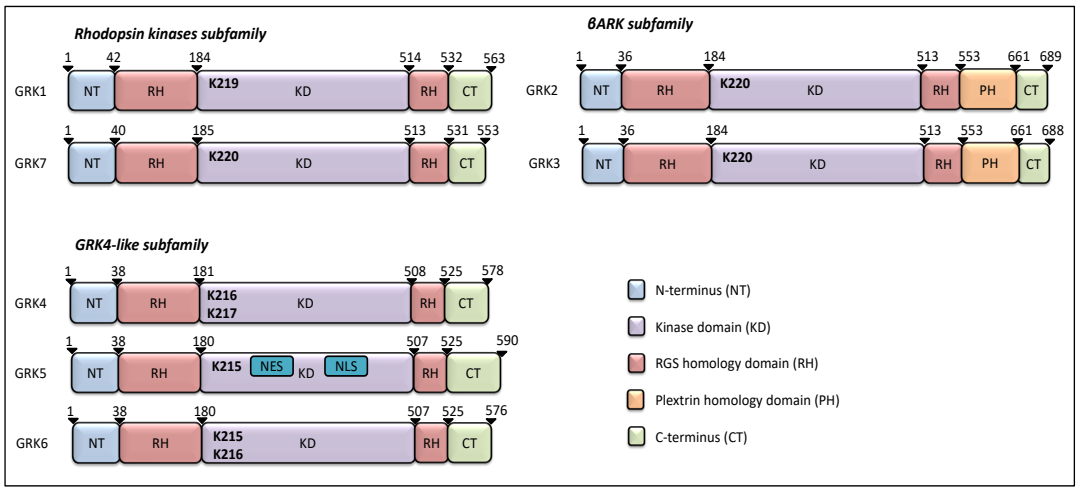


Figure 2. Schematic representation of GRKs structure. All GRKs share a common structure, comprising a NT region, followed by an RH domain, which includes the kinase domain (KD). Catalytic activity is dependent on lysine (K) residues, that are specific for each isoform. The CT region is variable among the different subfamilies.

CHAPTER 5

Canonical and Non-Canonical Activities of GRK2 and GRK5 in the heart

As mentioned above, GPCRs play a pivotal role in the regulation of cardiovascular function. Upon agonist stimulation, GPCRs become activated, leading to downstream signaling pathway via heterotrimeric G proteins. Cellular responses mediated by GPCRs are rapidly attenuated, through a complex regulatory mechanism, termed desensitization. This process is mediated by GRKs, that phosphorylate activated GPCRs, resulting in β-arrestin binding and uncoupling from G proteins. In physiological conditions, GRK2 is primarily located into the cytosol. Upon β-ARs activation, GRK2 translocates to the plasma membrane and binds to Gβγ subunits through its CT domain, thus initiating receptor desensitization. Unlike GRK2, GRK5 is constitutively located at the plasma membrane, and it does not need to translocate before phosphorylating the receptor (Huang, et al., 2014).

In the heart, β-ARs are typical GPCRs that, following CAs binding, activate intracellular signaling that regulates cardiac rate and myocyte contractile force. Of note, GRK2 and GRK5 are highly expressed in the heart, and they have been shown to be both upregulated in HF (Dzimiri, et al., 2004).

Following cardiac stress/injury, elevated SNS activity and increased circulating levels of CAs provide chronic hyperstimulation of β-ARs and GRK2 upregulation. Initially, this increase in GRK2 activity and expression helps to prevent β-ARs over-stimulation, but, in the long term, results in massive β-AR desensitization, thus contributing to the progression of HF (Woodall, et al., 2014).

Studies in transgenic mice showed that in vivo cardiac-specific GRK2 overexpression leads to impaired cardiac response due to an excessive β-ARs dysfunction. Interestingly, inhibiting the Gβγ-mediated translocation of GRK2 has been demonstrated to be an effective strategy to prevent receptors desensitization both in vitro and in vivo. The βARKct, a polypeptide comprising the last 194 amino acids of GRK2, is able compete with endogenous GRK2 for Gβγ binding and membrane translocation, thus blocking β-ARs desensitization (Koch, et al., 1995; Rockman, et al, 1998). In addition, studies in humans have shown that GRK2 levels in failing myocardium are mirrored by levels in peripheral lymphocytes and may reflect the degree of cardiac dysfunction and the severity of the syndrome. Therefore, peripheral measuring of levels of this kinase may represent a novel and early biomarker for HF diagnosis and prognosis (Iaccarino, et al., 2005; Rengo, et al., 2016).

GRK2 activity is not only restricted to β-ARs, but it is also capable to regulate other GPCRs, including sphingosine 1-phosphate (S1P) receptor 1 (S1PR1). In particular, following MI, GRK2 upregulation induced reciprocal downregulation of both β1AR and S1PR1, thus contributing to adverse remodeling and progression toward HF (Cannavo, et al., 2013).

Growing evidence has demonstrated that GRK2 can act independently of GPCRs, in a "non-canonical" manner. In this context, GRK2 can phosphorylate at the serine 307, the insulin receptor (IR) substrate-1 (IRS1), thus modulating glucose uptake and contributing to insulin resistance in the failing heart (Ciccarelli, et al., 2011).

GRK2 is also a regulator of fatty acid metabolism. In particular, GRK2 overexpression in cardiomyocytes impairs β-oxidation rates, leading to increased superoxide levels and reduced ATP production (Sato, et al., 2015*b*).

Interestingly, GRK2 can bind the chaperone protein heat shock protein 90 (HSP90), that facilitates its mitochondrial translocation and negatively affects cardiomyocyte survival (Chen, et al., 2013; Cannavo, et al., 2018a).

Among these non-canonical activities, GRK2 can act downstream aldosterone and MR. In response to aldosterone stimulation, GRK2 translocates to mitochondria, leading to augmented reactive oxygen species (ROS) generation and activation of pro-death signaling pathways in cardiomyocytes (Cannavo, et al., 2016).

Similarly to GRK2, GRK5 expression is elevated in patients with various cardiovascular diseases, including HF (Iaccarino, et al., 2005; Agüero, et al., 2012), dilated cardiomyopathy (Dzimiri, et al., 2004) and volume overload disease (Dzimiri, et al., 2002). As mentioned before, GRK5 is anchored at plasma membrane, exerting its "canonical" activities through phosphorylation and desensitization of activated GPCRs, including β-ARs. In pre-clinical models of HF, overexpression of GRK5 in mice leads to enhanced cardiac hypertrophy and early HF, whereas cardiac deletion of GRK5 is associated to reduced hypertrophy and remodeling, along with preserved cardiac function, in response to pressure overload, via transverse aortic constriction (TAC), or chronic administration of phenylephrine (PE) (Martini, et al., 2008; Gold, et al., 2012).

In addition to these deleterious effects, GRK5-dependent cardioprotective effects have been also reported. GRK5 has been shown to play a role in the transactivation of β1-AR and epidermal growth factor receptor (EGFR). Indeed, following β1AR stimulation,

GRK5 is able to transactivate the EGFR, which in turn is responsible for the activation of mitogenic signaling pathways, enhancing cell survival against chronic isoproterenol-induced cardiac damage (Noma, et al., 2007).

The presence of a NLS within its catalytic domain allows GRK5 to interact with cytosolic or nuclear non-GPCRs related molecules (Gurevich, et al., 2012; Hullmann, et al., 2016). Importantly, GRK5 activity into the nucleus of cardiomyocytes can promote maladaptive cardiac hypertrophy and HF. In response to pro-hypertrophic stimuli, GRK5 translocates into the nucleus, where it acts as a class II histone deacetylase (HDAC) kinase, phosphorylating HDAC5 and leading to de-repression of myocyte enhancer factor 2 (MEF2) mediated hypertrophic gene transcription (Martini et al., 2008). Moreover, GRK5 within the nucleus can act as a co-facilitator of the pathological transcription factor Nuclear factor of activated T-cells (NFAT). Importantly, this facilitation of NFAT activity was dependent on GRK5 DNA-binding ability, suggesting kinase-independent nuclear functions of GRK5 (Hullmann et al., 2014; Traynham et al., 2015). In addition to the above non-canonical activities of GRK5, this kinase can also act downstream of aldosterone and MR, promoting maladaptative pro-hypertrophic signaling (Cannavo et al., 2016).

Despite the plethora of effects attributed to GRK2 and GRK5, the role played by these kinases in governing physiological and pathological processes is still under intensive investigation. Hence, in this thesis, we report several studies which contributed to increase the knowledge in these fields.

Part II:

New Roles of G Protein-Coupled Receptor Kinase 2 (GRK2) In the Heart

CHAPTER 6

Aldosterone Jeopardizes Myocardial Insulin and β-Adrenergic Receptor Signaling via G Protein-Coupled Receptor Kinase 2

(Published in Front Pharmacol. 2019;10:888.)

As previously mentioned, dysregulation RAAS has a central role in HF progression. Aldosterone is a corticosteroid hormone secreted by the adrenal cortex, in response to renin-angiotensin system activation or high potassium levels. High aldosterone levels and the subsequent mineralocorticoid receptor (MR) hyperactivation have been associated to HF development and progression and to the onset of insulin resistance, which is a well-recognized risk factor of CVDs. Therefore, targeting aldosterone/MR system represents an effective therapeutic strategy (Hitomi, et al., 2007; Wada, et al., 2009; Cannavo, et al., 2018b).

Hyperaldosteronism impairs cardiac function, leading to adverse LV remodeling either via increased fibrosis deposition or mitochondrial dysfunction.

In this context, we have recently reported novel mechanisms whereby aldosterone may affect cardiac function. More in details, some of the effects of aldosterone in the heart can be attributed to a linkage with GPCR signaling. Accordingly, we have found that hyperaldosteronism induces cardiac activation of GRK2, which is followed by increased cell death and mitochondrial dysfunction (Cannavo et al., 2016).

In addition, this kinase is known to dysregulates both β-ARs and insulin signaling (Cannavo, et al., 2018a). Yet, whether aldosterone modulates cardiac insulin sensitivity and β-ARs function remains untested. Nor is it clear if this eventual modulation requires GRK2. Hence, in this study, we tested the impact of aldosterone stimulation, and the

role of GRK2, on both insulin and β-ARs signaling, in vitro, in 3T3 fibroblasts, and in vivo, in two murine models of hyperaldosteronism.

We demonstrated that, after chronic treatment of 3T3 cells with aldosterone, a marked inhibition of insulin signaling occurs, as documented by the increased negative phosphorylation of IRS1 at the serine in position 307 and by the subsequent reduction of Akt activity. Similarly, aldosterone impairs β-AR function, by reducing the activation of extracellular signal-regulated kinase (ERK) and the production of cAMP in response to isoproterenol. Of note, all these effects were markedly blunted in the presence of the GRK2 inhibitor, CMPD101, thus preventing both insulin and β-AR signaling dysfunction **(Figure 3A-D)**.

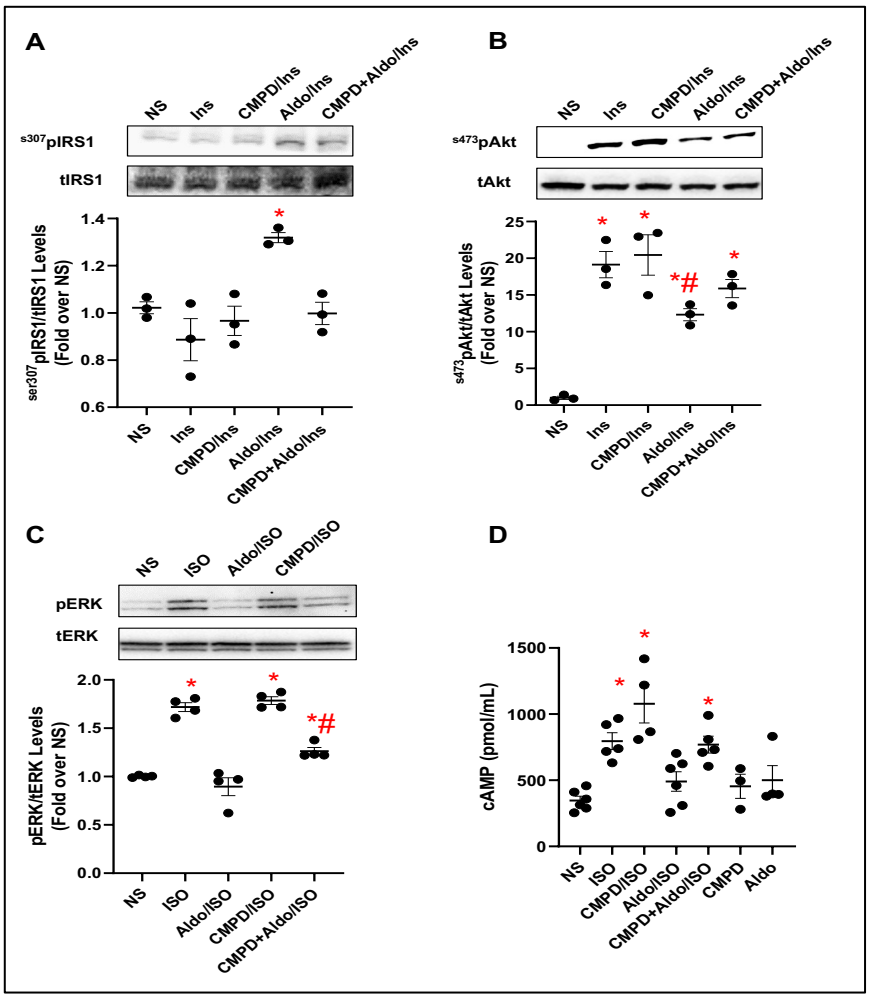


Figure 3. Aldosterone impairs insulin and β-ARs signaling *in vitro* in fibroblasts. A-B) Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple independent experiments to evaluate **(A)** IRS1 phosphorylation levels (s307pIRS1), as a ratio of inactivated IRS1 to total IRS1 (tIRS1), and **(B)** Akt phosphorylation levels (s473pAkt) as a ratio of activated Akt to total Akt (tAkt). The cells were either NS or stimulated with Aldo (1 μM) and/or CMPD101 (3 μM) for 12 h. After Aldo and/or CMPD101 treatment, cells were stimulated with insulin (Ins, 100 nM) for 15 min. Tukey's post hoc test. *p < 0.05 vs NS. #p < 0.05 vs Ins. **C)** Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple independent experiments to evaluate extracellular signal-regulated kinase (ERK) 1/2 phosphorylation (pERK) as a ratio of activated ERK to total ERK (tERK). The cells were either NS or stimulated with aldosterone (Aldo, 1 μM) and/or CMPD101 (3 μM) for 12 h. After Aldo and/or CMPD101 treatment, cells were stimulated with isoproterenol (ISO, 10 μM) for 15 min. Tukey's post hoc test. *p < 0.05 vs NS; #p < 0.05 vs ISO. **D)** Dot plots showing levels of cyclic adenosine 3',5'-monophosphate (cAMP, pmol/ml) in 3T3 fibroblasts NS or stimulated with ISO (10 μM) for 15 min. Prior ISO stimulation some groups of cells were pretreated with Aldo (1 μM) and/or CMPD101 (3 μM) for 12 h. Tukey's post hoc test. *p < 0.05 vs NS.

Next, to confirm our in vitro findings in an in vivo model of hyperaldosteronism, we used wild-type (WT) mice undergoing chronic infusion of aldosterone (2 μg/mouse/day for 4 weeks). In these mice, we observed a marked GRK2 upregulation that was paralleled by a massive β1-AR downregulation and augmented levels of phosphorylated IRS1.

Interestingly, these negative effects of aldosterone were wholly abolished in cardiac-specific GRK2-knockout mice (cGRK2KO) **(Figure 4A-C)**.

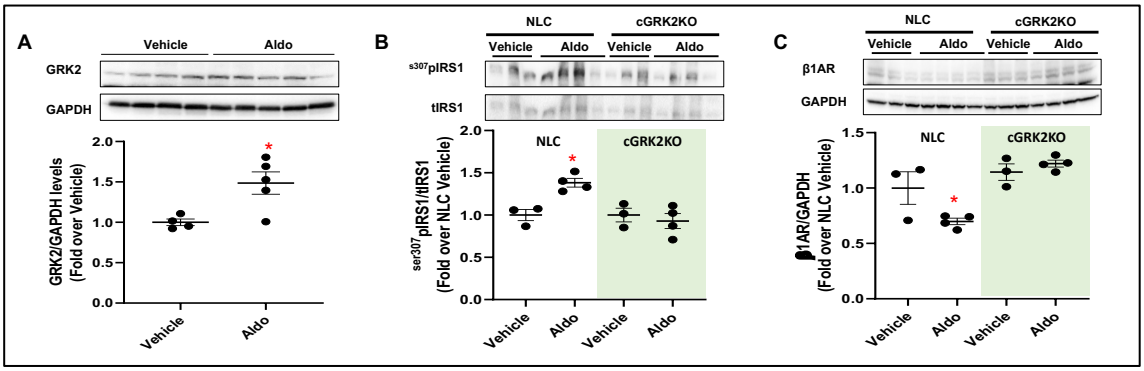


Figure 4. In vivo effects of chronic aldosterone infusion on murine myocardium. A) Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) showing levels of GRK2 in total cardiac lysates from mice treated with Aldosterone (Aldo) or Vehicle for 4 weeks. GAPDH levels were used as loading control; Mann–Whitney test. *p < 0.05 vs Vehicle. **B-C)** Representative immunoblots (upper panels) and quantitative data showing levels of **(B)** phosphorylated IRS1, at serine 307 (s307pIRS1) and **(C)** β1-adrenergic receptor (β1AR) in total cardiac lysates from NLC and cardiac GRK2KO mice either treated with Aldo or Vehicle for 4 weeks. GAPDH levels were used as loading control; Dunnet's post hoc test. *p < 0.05 vs NLC Vehicle.

Finally, we used WT mice subjected to surgical-induced myocardial infarction (MI), another model of hyperaldosteronism. After 4-week MI, echocardiographic analysis showed a substantial deterioration of cardiac function, with increased LV dilation and fibrosis deposition. At a molecular level, GRK2 levels were significantly increased, while insulin and β1-ARs signaling pathway were impaired. Treating MI mice with the MR antagonist, spironolactone (20 mg/kg/day), prevented aldosterone noxious effects, thus reducing IRS1 negative phosphorylation, β1-ARs downregulation, and GRK2 upregulation **(Figure 5A-C)**. In addition, MR antagonism by spironolactone ameliorated post-MI LV dysfunction and reduced cardiac fibrosis and apoptosis.

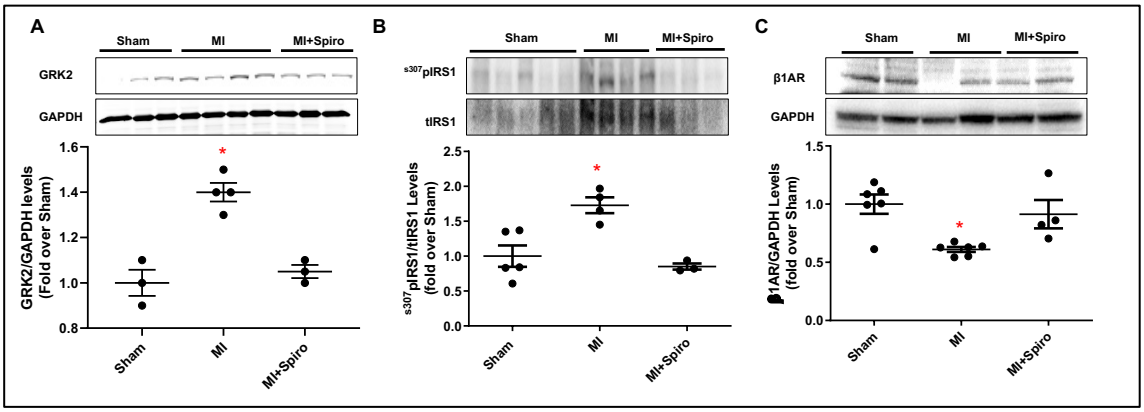


Figure 5. Aldosterone/mineralocorticoid receptor (MR) blockade prevents GRK2 upregulation in postischemic failing hearts and abolishes Insulin and βAR signaling downregulation. A–C) Representative immunoblots (upper panels) and quantitative data (lower panels) showing levels of **(A)** GRK2, **(B)** ser307pIRS1 and **(C)** β1AR in total cardiac lysates of Sham, MI, and MI + Spiro mice (n = 3–6 mice per group). tIRS1 and GAPDH levels were used as loading control; Tukey's post hoc test. *$p < 0.05$ versus Sham.

Our study reveals that GRK2 activity is a critical mediator of the noxious effects downstream of the aldosterone signaling pathway. Therefore, combining the benefits of a direct MR antagonism to those elicited by the pharmacological inhibition of GRK2, is an attractive strategy to prevent cardiac dysfunction that is associated to hyperaldosteronism **(Figure 6)**.

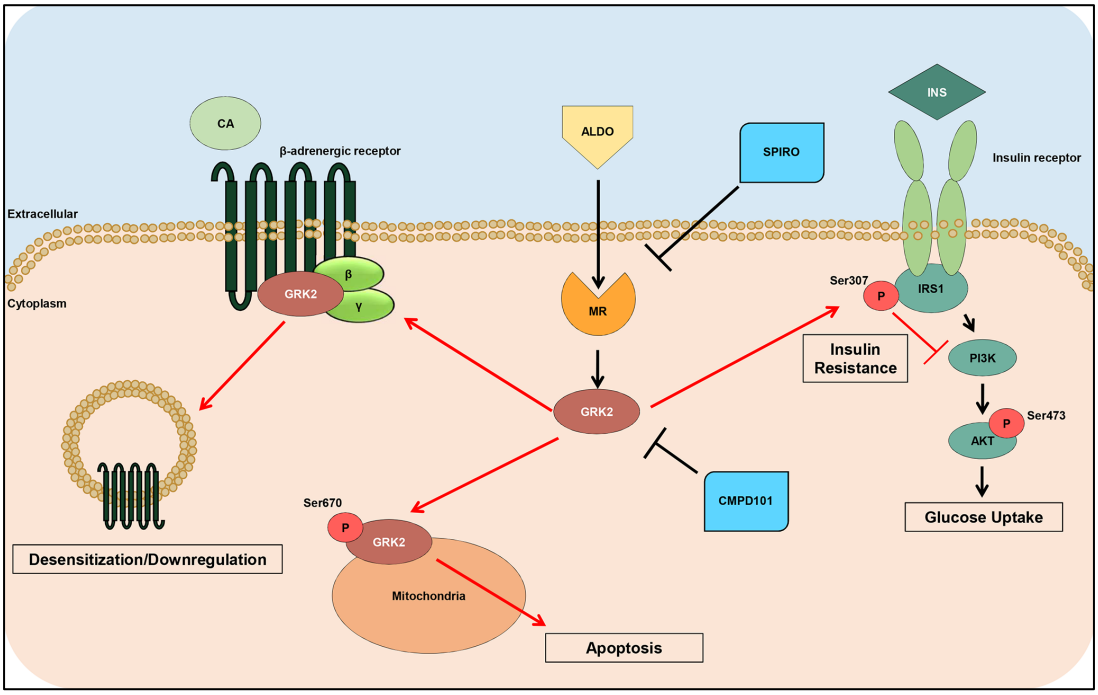


Figure 6. Schematic representation of GRK2 noxious effects downstream Aldosterone. High levels of Aldosterone induce the hyperactivation of MR leading to the upregulation of GRK2, which in turn, induces the negative phosphorylation of IRS1 (ser307pIRS1), with a consequent impaired response to insulin (INS) and reduced Akt activation, and downregulates βARs that do not response to catecholamine (CA) stimulation. Further, this kinase, when phosphorylated at ser670, is able to translocate to mitochondria, where it increases myocyte apoptosis. Of note, either spironolactone or CMPD101 (GRK2-inhibitor) can block the expression and noxious effects of GRK2 downstream aldosterone.

Part III:

G Protein-Coupled Receptor Kinase 5 (GRK5) in Chronic Degenerative Diseases

CHAPTER 7

Targeting GRK5 for Treating Chronic Degenerative Diseases

(*Published in Int J Mol Sci. 2021;22(4):1920.*)

As discussed above, GRK5 is a multifunctional protein, expressed in different cell types and subcellular compartments. For instance, when anchored to the plasma membrane, GRK5 exerts its canonical function, regulating several GPCRs. However, under certain conditions, it can translocate to the nucleus of cells, where it interacts with non-GPCR-related proteins as well as DNA, thus promoting “non-canonical” signaling, including gene transcription.

Interestingly, several studies have demonstrated that GRK5 has a pivotal role in the pathogenesis of chronic-degenerative disorders, such as cancer, cardiovascular, and neurological diseases. For this reason, targeting GRK5 could be an attractive therapeutic strategy to prevent the development or progression of these diseases.

In the last decades, GRK5 activities have been extensively studied in CVDs; however, since its expression is also prominent in the brain, in the limbic system, in this review article, we have also detailed the impact of GRK5 on neuronal pathophysiology, with a particular focus on its role in the pathogenesis of Alzheimer’s disease (AD) and Parkinson's disease (PD).

Importantly, given the ability of GRK5 to phosphorylate GPCRs, it is not surprising that this kinase interacts with the muscarinic cholinergic receptor, that is involved in the modulation of normal cognitive function (Kunapuli, et al., 1994; Bubser, et al., 2012). Indeed, GRK5 deficiency resulted in impaired cholinergic activity (Gainetdinov, et al., 1999), along with cognitive impairment and very early accumulation of soluble β-

amyloid (Aβ) (Suo, et al., 2004; Suo, et al., 2007). In addition, GRK5 activity in the brain is mainly dependent on its subcellular compartmentalization. Indeed, GRK5 translocation from the membrane to the cytosol facilitated the phosphorylation of the AD-associated protein tau, which is a molecular hallmark of AD (Zhang, et al., 2014). Further, GRK5 deficiency has also been shown to promote tau hyperphosphorylation through the activation of GSK3β, thus contributing to the pathogenesis of AD (Zhao, et al., 2019).

The importance of GRK5 in the pathogenesis of AD has also been suggested by genetic sequencing, that have identified two functional SNPs in the *grk5* gene, linked to AD risk (Zhang, et al., 2017). Although, these data suggest that GRK5 depletion contributes to neurodegeneration, high levels of GRK5 have been associated to the pathogenesis of PD. Indeed, GRK5 can phosphorylate α-synuclein at Serine in position 129, thus promoting its aggregation and the formation of soluble oligomers (Arawaka, et al., 2006). In addition, α-synuclein enhanced GRK5 nuclear accumulation, thus inhibiting BCL-2 transcription and increasing the apoptosis of neuronal cells (Liu, et al., 2010).

Emerging evidence demonstrated that GRK5 has a pivotal role in cancer development and progression, but also in its inhibition, depending on the cellular localization. As previously mentioned, GRKs are not only involved in modulating GPCR activities, but they can also interact with many non-GPCR proteins, including cell cycle regulators. In this context, GRK5 can phosphorylates p53 at a threonine residue at position 55, acting as a direct inhibitor of this tumor suppressor (Chen, et al., 2010).

In line with these data, several studies reported that GRK5 overexpression is linked to tumor cell growth, aggressiveness, drug resistance and worse survival rate in patients

with different types of cancer (Kim, et al., 2012; Kaur, et al. 2013; Lagman, et al., 2019). However, the role of GRK5 in tumor suppression appears controversial, since many studies have demonstrated that GRK5 can negatively affect cancer progression, through the desensitization of GPCRs and non-GPCR- receptors (Gambardella, et al., 2016). Therefore, GRK5 exerts opposite functions depending on subcellular localization. Indeed, when at the plasma membrane, GRK5 negatively affects tumor progression, whereas, when in the nuclei and the cytosol, it enhances cancer progression **(Figure 7)**.

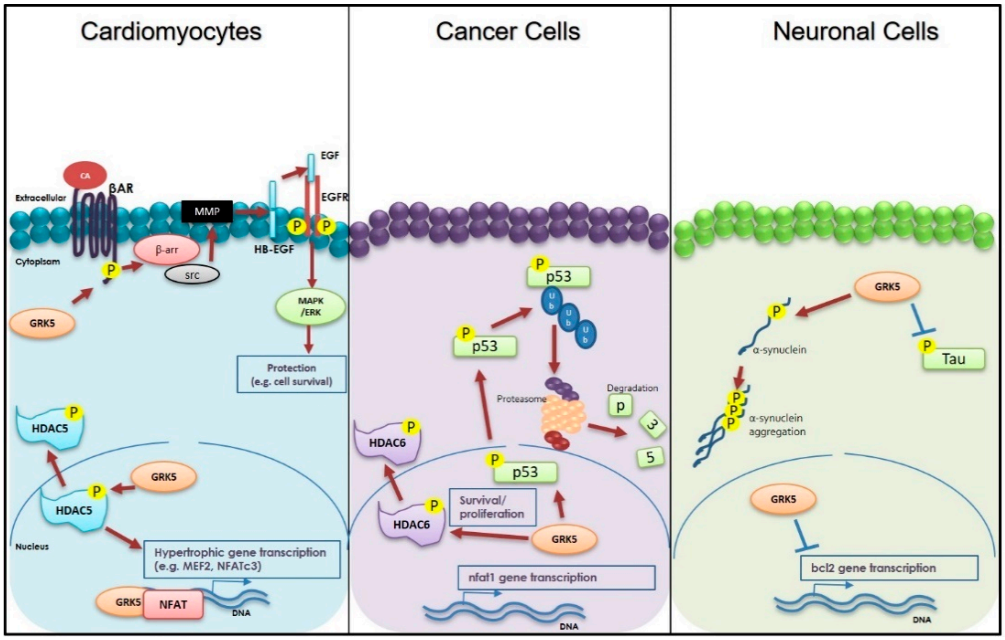


Figure 7. Schematic representation of GRK5 activities within cardiomyocytes, cancer and neuronal cells. In cardiomyocytes: GRK5 is involved in phosphorylation of GPCRs, including the β-adrenergic receptor (βAR). Importantly, following catecholamine (CA) stimulation of βAR GRK5 is also able to induce the transactivation of the Epidermal Growth Factor (EGF) receptor (EGFR) via β-arrestin (β-arr)/src/MMP pathway activation. EGFR in turn activates a MAPK/ERK protective pathway. In the nuclei, GRK5 can either bind to and phosphorylate, HDAC5 or bind the DNA and NFAT thus enhancing hypertrophic gene transcription. In cancer cells: GRK5 regulates p53 via direct phosphorylation and degradation. Moreover, into the nuclei, GRK5 can phosphorylate HDAC6 or can increase NFAT1 expression. In neuronal cells: GRK5 inhibits Tau phosphorylation. Conversely, this kinase can increase α-synuclein phosphorylation promoting its aggregation. Finally, in the nucleus GRK5 inhibits bcl2 gene transcription increasing the apoptosis.

Given the involvement of GRK5 in these chronic degenerative diseases, targeting this kinase could be an attractive therapeutic strategy in the treatment of cardiac and neuronal disease, as well as of cancer.

CHAPTER 8

Genetic catalytic inactivation of GRK5 impairs cardiac function in mice via dysregulated p53 levels

(Submitted)

We have previously mentioned that GRK5 is a multifunctional protein that can elicit either protective or deleterious effects, depending on its localization within the cell. For instance, when anchored to the plasma membrane, this kinase can regulate specific GPCRs via canonical phosphorylation, thus providing cardioprotection. Typically, following β1AR-phosphorylation, GRK5 recruits β-arrestin and Src to the plasma membrane, leading to metalloproteinase (MMP) activation and release of the heparin-bound epidermal growth factor (HB-EGF), which in turn transactivate EGFR. Activation of this signaling pathway protects the myocardium against chronic isoproterenol-induced cardiac damage (Noma, et al., 2007).

However, due to the presence of a NLS within its catalytic domain (Johnson, et al., 2013), GRK5 can localize to the nucleus, where it can drive pathological hypertrophic gene transcription. In this context, nuclear GRK5 can act as a HDAC kinase, specifically phosphorylating HDAC5 and leading to MEF2 de-repression and activation of its hypertrophic gene transcriptional activity (Martini, et al., 2008). Further, nuclear GRK5 has been shown to be a facilitator of NFAT-mediated pathological gene transcription. Interestingly, the latter effect may occur in a catalytic-independent manner (Hullmann, et al., 2014). GRK5 also has non-canonical apoptotic activity involving nuclear and cytosolic targets. Interestingly, in cancer cells GRK5 can directly bind to and

phosphorylate p53 at threonine in position 55, thus promoting its degradation and the inhibition of p53-mediated apoptotic response (Chen, et al., 2010).

Since some of the above evidence appears to involve interactions of GRK5 independent of its catalytic activity, it is of interest to specifically determine whether the catalytic activity of GRK5 is liable for these undesired pro-hypertrophic effects, or rather if it is involved in the protective ones, such as countering apoptosis.

The role played by the catalytic activity of GRK5 in the heart has not been fully elucidated and, for that reason, in this study we sought to assess the in vivo consequences of inactivating the catalytic site of GRK5 with an initial focus at examining the basal cardiac phenotype and response to stress.

To determine the overall significance of the catalytic activity of GRK5, we used CRISPR/Cas9 technology to generate a novel knock-in mouse model, with the ATP binding lysine (K) 215 in GRK5 catalytic site replaced by an arginine (R). This mutation (GRK5-K215R) causes the complete loss of kinase activity of GRK5.

We characterized the cardiac phenotype of these mice basally and after ventricular pressure overload and HF development.

Compared to age-matched WT littermates, GRK5-K215R mice were found to have significant deterioration of cardiac function, with high levels of apoptosis and fibrosis basally **(Figure 8A-E)**. Certainly, the loss of cardiac cells with replacement fibrosis could explain the loss of contractile force and dilatation observed via echocardiography.

Surprisingly, GRK5-K215R knock-in mice did not show significant cardiac hypertrophy compared to WT controls **(Figure 8F)**.

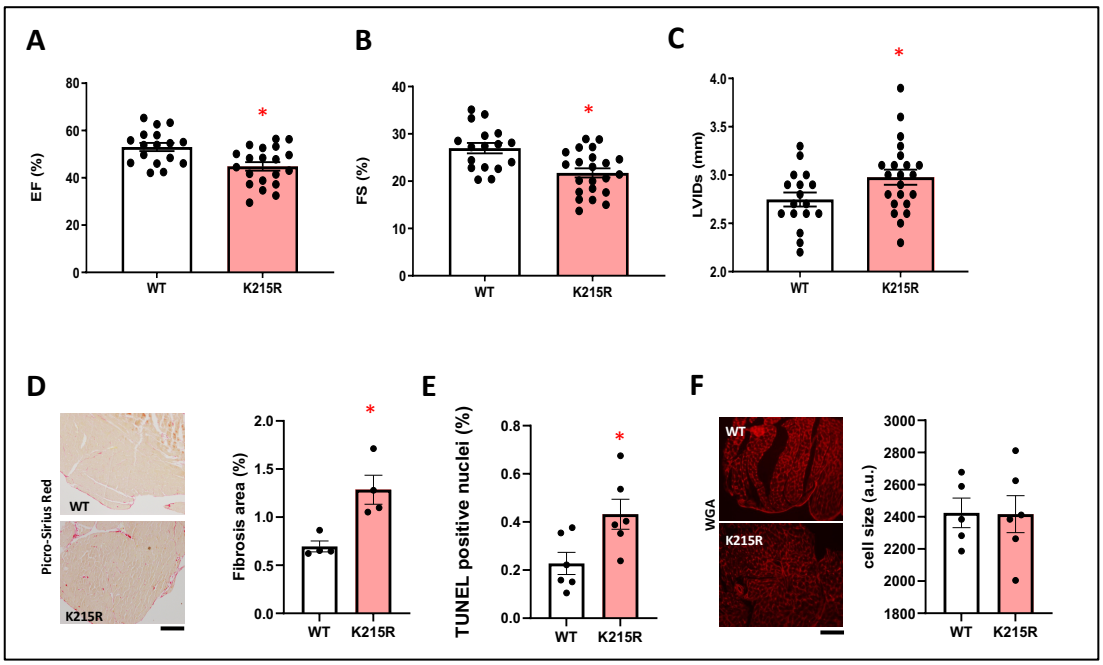


Figure 8. GRK5-K215R mice display basal cardiac dysfunction with increased interstitial fibrosis and apoptosis A-C) Scatter plot with bar graph showing echocardiographic analysis of **(A)** LV Ejection Fraction (EF, %), **(B)** Fractional Shortening (FS, %) and **(C)** LV internal diameter in systole (LVIDs) of individual mice from the WT and GRK5 K215R groups. **(D)** Representative images (left) and quantitative data (right) showing percentage of cardiac fibrosis (Picro-Sirius red staining, scale bar 100 µm) in WT and GRK5-K215R mice. **E)** Scatter plot with bar graph showing percentage of cell death (TUNEL staining) in WT and GRK5-K215R mice. **F)** Representative panels (left) and quantitative analysis (right) showing myocyte cell size (WGA staining, scal bar 100 µm) in cardiac sections from WT and GRK5-K215R mice.

Molecularly, mutant knock-in mice displayed basal increased p53 gene expression (both at mRNA and protein levels), thus suggesting that catalytic activity is not needed for GRK5 to upregulate p53 expression **(Figure 9A-B)**.

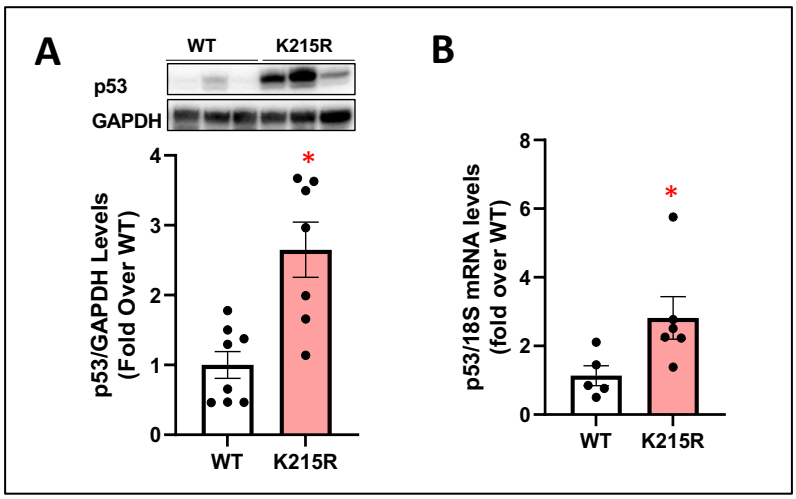


Figure 9. GRK5-K215R mice present increased p53 expression A) Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple independent experiments to evaluate p53 levels in total LV lysates from WT and GRK5-K215R group of mice. GAPDH was used as loading control. **B)** Scatter plot with bar graph showing mRNA levels of p53 in in total LV lysates from WT and GRK5-K215R group of mice. two-tailed unpaired Student's t-test. *p<0.05 vs WT.

In addition, all these effects translated into increased susceptibility to stress such as LV pressure overload. Indeed, GRK5-K215R mice had a heightened pathological response to TAC with even more dysfunction that included fibrosis **(Figure 10A-E)**.

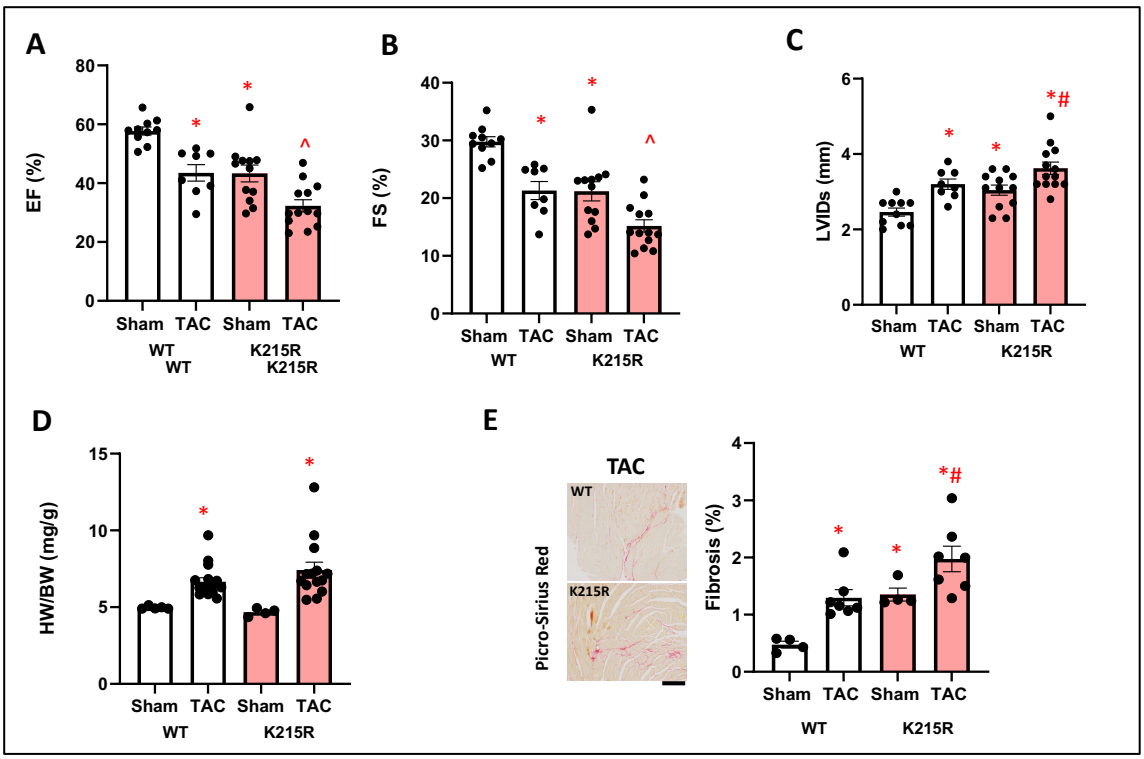


Figure 10. GRK5-K215R mice display worsened cardiac function in response to stress. A-B-C) Scatter plot with bar graph showing echocardiographic analysis of **(A)** LV Ejection Fraction (EF, %), **(B)** Fractional Shortening (FS, %) and **(C)** LV internal diameter in systole (LVIDs) of individual mice from the WT and GRK5 K215R groups (Sham and TAC). **D)** Scatter plot with bar graph showing heart weight (HW) and body weight (BW) ratio in WT and K215R groups of mice. **E)** Representative images (left) and quantitative data (right) showing percentage of cardiac fibrosis (Picro-Sirius red staining, scale bar 100 µm) in WT and GRK5 K215R mice (Sham and TAC). ANOVA with Tukey's multiple comparisons post hoc test: *p<0.05 vs WT Sham; ^p<0.05 vs all; #p<0.05 vs TAC.

To better understand whether and how the nuclear activity of GRK5 in cardiomyocytes is related to p53 regulation, we next explored the effects of GRK5 nuclear localization on p53 in H9c2 cells, a surrogate model of cardiomyocytes. Notably, we found that overexpression of GRK5-ΔNES, a mutant confined in the nucleus, heightened apoptosis along with p53 expression. In contrast, in the presence of the GRK5 mutant lacking the NLS (GRK5-ΔNLS), both mRNA and protein levels of p53 were significantly reduced compared to those observed in cells expressing the WT isoform **(Figure 11A-E)**.

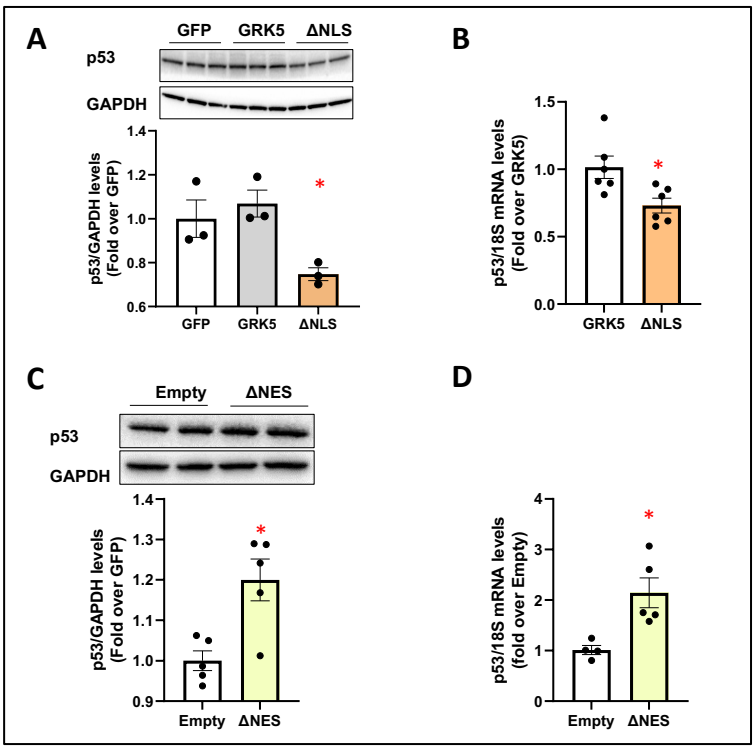


Figure 11. Nuclear GRK5 Serves as a Critical p53 Regulator *in vitro* A) Representative immunoblots (upper panel) and densitometric quantitative analysis (lower panels) of multiple independent experiments to evaluate p53 levels in H9c2 cells transduced with Adenovirus (Ad) encoding for GFP, GRK5 and the mutant form of GRK5 lacking the NLS (GRK5-ΔNLS). ANOVA with Tukey's

multiple comparisons post hoc test: *p<0.05 vs GFP. **B)** Scatter plot with bar graph showing mRNA levels of p53 in H9c2 cells expressing GRK5 and GRK5-ΔNLS. *p<0.05 vs GRK5. **C)** Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple independent experiments to evaluate p53 levels in H9c2 cells expressing an empty vector or the mutant form of GRK5 lacking the nuclear exportation signal (GRK5-ΔNES). GAPDH was used as loading control. *p<0.05 vs Empty. **D)** Scatter plot with bar graph showing mRNA levels of p53 in H9c2 cells expressing GRK5 and GRK5-ΔNES. *p<0.05 vs GRK5.

Overall, these findings add significantly to the understanding of GRK5 in gene transcription. This transcriptional regulation, influenced by nuclear GRK5, may occur via either kinase-dependent effects or kinase-independent actions (via DNA binding). Therefore, we analyzed the ability of the K215R mutant to bind the DNA, in vitro in 3T3 cells. Intriguingly, this mutation did not influence the subcellular localization of GRK5, but positively impacted its DNA-binding ability and increased p53 gene transcription and protein expression with an augmented apoptotic response **(Figure 12A-E)**.

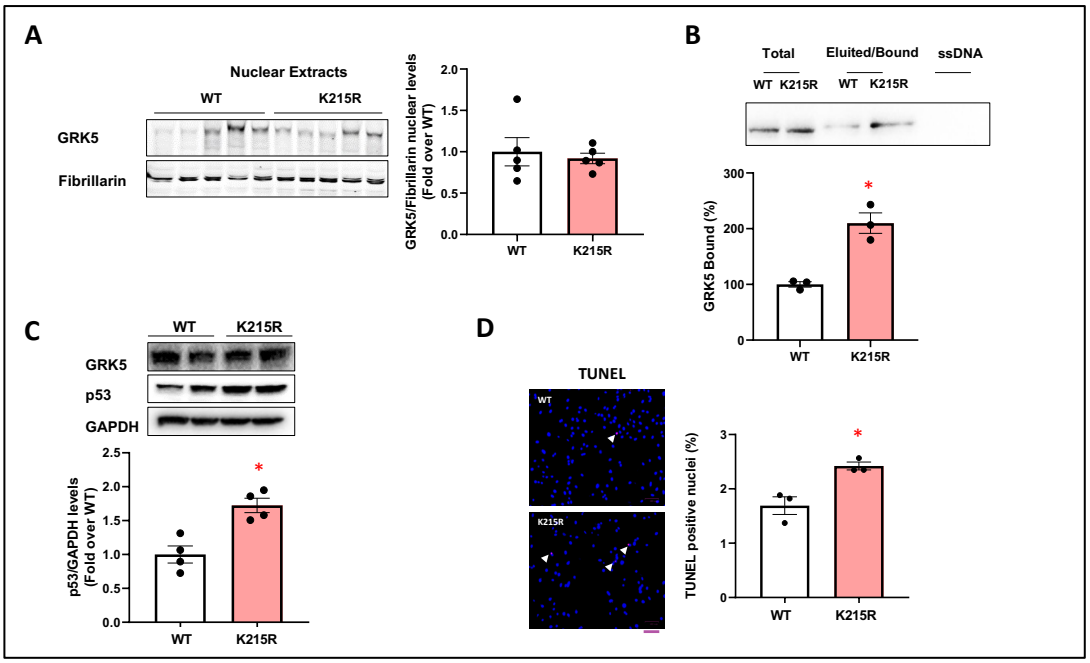


Figure 12. K215R mutation influences DNA binding and p53 expression *in vitro* A) Representative immunoblots (left) and densitometric quantitative analysis (right) of multiple independent experiments to evaluate GRK5 levels in nuclear extracts purified from both 3T3 cells WT or stable expressing GRK5-K215R mutant. Fibrillarin was used as loading control. **B)** Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple independent experiments to evaluate the percentage of GRK5 bound to DNA in 3T3 cells (WT-GRK5 vs stable expressing GRK5-K215R mutant). **C)** Representative immunoblots (upper panels) and

densitometric quantitative analysis (lower panel) of multiple independent experiments to evaluate p53 and GRK5 levels in 3T3 cells, either WT or expressing GRK5-K215R mutation. GAPDH was used as the loading control. *p<0.05 vs WT. **D)** Representative images (left) and quantitative analysis (right) showing the percentage of cell death (TUNEL/DAPI staining, scale bar: 50 µM) in 3T3 cells WT or GRK5-K215R. 2-tailed unpaired Student's t-test: *p<0.05 vs WT.

Our results reveal clear connections between the catalytic activity of GRK5 in the nucleus and p53-mediated apoptosis that profoundly influence cardiac function and the heart's response to stress. More in detail, basal GRK5 catalytic activity is essential to preserve myocardial function since its inactivation impairs p53 regulation, resulting in higher rates of apoptosis in the heart **(Figure 13)**.

The present study provides novel insight into the actions of GRK5 in the heart, widening the spectrum of biological actions attributed to this kinase.

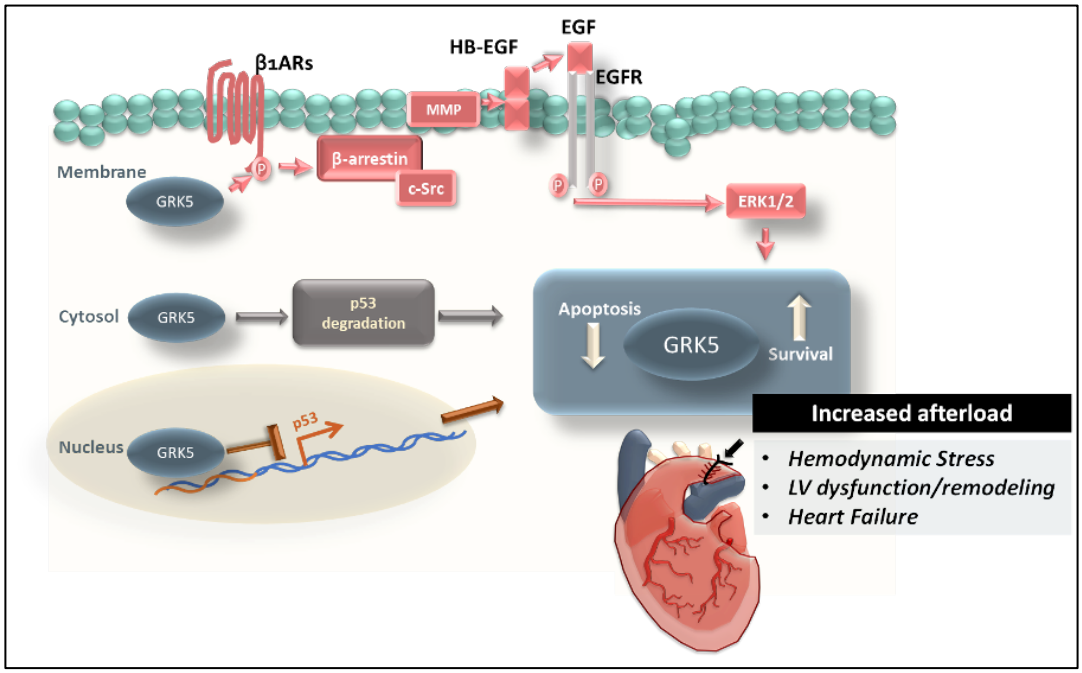


Figure 13. Schematic representation of GRK5 activities in cardiomyocytes. When at the plasma membrane, GRK5 regulates the function of several GPCRs, including beta-adrenergic receptors (βARs). Following β1-ARs phosphorylation, GRK5 recruits β-arrestin and Src to the plasma membrane, leading to metalloproteinase (MMP) activation and release of the heparin-bound epidermal growth factor (HB-EGF), which transactivate EGF receptor (EGFR) and promote pro-survival signaling. When in the cytosol and nuclei, GRK5 directly controls p53 expression/activity by controlling its degradation. Inhibition of GRK5 catalytic activity increases p53 expression and cardiomyocyte cell death.

Part V:

Discussion and conclusions

General Discussion

Despite significant progresses that have been made in understanding the pathophysiology of CVDs, the latter are still the leading cause of global mortality and a significant burden on global healthcare systems. Among CVDs, HF has dramatically increased in incidence and represents the heart disease with the highest degree of morbidity and mortality.

The current therapeutic armamentarium against HF (e.g., β-blockers, ACE inhibitors, angiotensin receptor blockers, etc.) has the aim to improve the clinical outcome, thus reducing hospitalization and mortality rates. However, these treatments do not cure the disease, but rather slow its progression, improving the quality of life of HF patients.

Therefore, a matter of great interest is further understanding the molecular mechanisms involved in HF progression, to identify new potential therapeutic targets.

GPCRs play a pivotal role in regulation of heart function, by modulating a range of physiological responses in various cardiovascular cell types. GPCRs are finely regulated by GRKs, that phosphorylate activated receptors, leading to their desensitization and downregulation. Among GRKs, GRK2 and GRK5 are highly expressed in the heart, and these kinases are known to be upregulated in HF. While the canonical role of GRKs is to phosphorylate agonist bound GPCRs located within the plasma membrane, these kinases can act independently of GPCRs, in a “non-canonical” manner. Indeed, GRKs are capable to regulate several cellular processes, often through localization into different intracellular compartments, such as the mitochondria for GRK2 or the nucleus for GRK5. Over the last 20 years, the role of these kinases not only in the heart, but also in other chronic-degenerative diseases, has been widely studied.

Given that increased expression and activity of GRK2 and GRK5 contribute to β-ARs dysregulation in HF patients, inhibition of overactive GRKs has been proposed as a novel therapeutic approach to restore cardiac function in the failing heart.

Several approaches have been used to modulate GRK2 activity in a wide variety of animal models and cell culture systems. GRK2 inhibition is commonly achieved through genetic expression of the peptide βARKct, which competes with endogenous GRK2 for Gβγ binding and membrane translocation, thus reducing β-ARs desensitization (Koch, et al., 1995).

Transgenic overexpression of βARKct has shown efficacy in preventing myocardial dysfunction and adverse remodeling and in increasing survival in experimental models of HF. Similarly, viral-mediated βARKct delivery, using adenovirus or adeno-associated virus vectors, was able to improve cardiac function by reversing β-AR desensitization in HF. Even though viral-mediated gene delivery of βARKct is a promising therapeutic strategy to modulate GRK2 activity in the treatment of HF, there are still major obstacles before clinical development. Alternatively, there are several ongoing efforts to develop more advantageous small-molecule inhibitors of GRK2 activity (such as M119, gallein, etc.), which would presumably avoid all the issues related to clinical gene therapy application. Likewise, Paroxetine, that is a selective serotonin uptake inhibitor antidepressant, has been identified as a GRK2 binding partner, capable of inhibiting agonist-induced β-ARs internalization (Pfleger, et al., 2019).

Although there are several inhibitors for GRK2, far fewer selective compounds for GRK5 have been identified. An important compound recently proposed as a GRK5-selective inhibitor is an anti-inflammatory and anti-allergic immunomodulator, called amlexanox.

This compound has been reported as a low micromolar inhibitor of this kinase, that can inhibit GRK5-mediated activation of MEF2 transcriptional activity (Homan, et al., 2014). Since the ability of GRK5 to promote maladaptive cardiac hypertrophy and HF, several therapeutic strategies have been proposed to inhibit these harmful effects. In this regard, intracardiac delivery of an adenovirus encoding for the NT of GRK5 (GRK5-NT), which is implicated in GPCR binding and transcription regulation in the nucleus, has been recently proposed as a novel therapeutic approach to inhibit and reduce left ventricular maladaptive hypertrophy (Sorriento, et al., 2010; Sorriento, et al., 2018). In addition, transgenic expression of GRK5-NT resulted in substantial reduction of maladaptive cardiac hypertrophy and HF in a mouse model of pressure overload (Coleman, et al., 2021).

Of course, further investigations are urgently needed in this field, to identify new therapies and interventions that could potentially improve cardiac function and outcome in HF patients.

In this context, as the result of the investigations at the basis of the present thesis, we elucidated novel roles of GRK2 and GRK5 in the modulation of cellular processes involved in cardiac dysfunction. Further, we also proposed new potential therapeutic strategies that might be useful in counteracting the deleterious effects of GRKs in HF.

The hyperactivation of neurohormonal systems, including SNS and RAAS, is a primary pathogenic driver of HF. Hyperaldosteronism is associated to cardiac dysfunction and to adverse cardiac remodeling via increased fibrosis and mitochondrial dysfunction. These harmful effects are mediated, at least in part, by the activation of GRK2.

Previous evidence has attested the existence of an association between aldosterone and insulin signaling dysfunction. However, no studies have assessed the relevance of this cross talk on heart function. Nor is it clear whether GRK2 has a role in the modulation cardiac insulin sensitivity and β-AR function, downstream of aldosterone. We have demonstrated, for the first time, that aldosterone impairs insulin and β-AR signaling, in a GRK2 dependent manner, confirming the role of GRK2 downstream mineralocorticoid receptor (MR) hyperactivation and its inhibition as a potential therapeutic strategy to prevent cardiac dysfunction associated to hyperaldosteronism.

Our study suggests the unique opportunity of combining the benefits of a direct MR antagonism to those elicited by the pharmacological inhibition of GRK2, a novel, highly translational perspective that warrants further, in-depth investigation.

Subsequently, we sought to assess the role played by GRK5 catalytic activity in the heart. GRK5 is a multifunctional protein, that can have either protective or deleterious effects, depending on its cellular localization. Previous evidence has indicated that, besides its canonical activities at plasma membrane level, this kinase can accumulate into the nucleus, where it can drive pathological hypertrophic gene transcription. Interestingly, the latter effects may not be kinase dependent.

Overall, our data shows that inactivation of GRK5 catalytic activity impairs its nuclear regulation of p53. This results in higher expression levels of p53 and higher rates of apoptosis in the heart, leading to significant basal cardiac dysfunction and an intolerance to stress. Further, our study provides crucial new comprehensions on how nuclear GRK5 activity influences myocardial signaling and function, widening the spectrum of the biological actions attributed to this kinase. Moreover, since this study suggests a

potential beneficial role of GRK5 on cardiomyocyte cell survival, perhaps targeting specific functional domains of GRK5, to limit its nuclear translocation, would be the best way to offer therapeutic avenues for heart disease.

These intriguing discoveries warrant future in-depth investigation.

Conclusions

In this long-lasting research journey, we investigated the therapeutic potential of interfering with canonical and non-canonical activities of GRK2 and GRK5.

GRK2 inhibition appears to be a novel therapeutic strategy to attenuate cardiac dysfunction. New data are emerging, that limiting GRK2 non-canonical activities could have beneficial effects in the heart. Here, we showed that combining the direct MR antagonism to the pharmacological inhibition of GRK2, could be a highly translational perspective in the treatment of cardiac structural dysfunction that accompany any clinical condition associated to hyperaldosteronism.

Similarly, GRK5 is also an emerging novel target due to its non-canonical role in promoting pathological hypertrophic response. In addition, given the ability of this kinase to modulate many physiological and pathological responses in different cell types, targeting GRK5 could be an attractive therapeutic strategy in the treatment of neurodegenerative, and neoplastic disorders.

Our study demonstrated that inactivation of GRK5 kinase activity in mice was associated with cardiac dysfunction, either in basal conditions or in response to stress. Moreover, GRK5 catalytic activity has been shown to be essential to preserve cardiomyocyte survival, by preventing p53-induced apoptosis. Therefore, selective targeting of the deleterious effects of this kinase would be the best way to offer therapeutic avenues for heart disease.

Overall, our data significantly contributed to the identification of novel molecular mechanisms underlying cardiac dysfunction and provided interesting new molecules that could be potentially targeted in the treatment of HF and other CVDs.

List of abbreviations

AC: adenylyl cyclase

ACE: angiotensin-converting enzyme

AD: Alzheimer's disease

ARB: Angiotensin II receptor blockers

Aβ: β-amyloid

β-ARs: β-adrenergic receptors

βARK: β-adrenergic receptor kinase

CaM: calmodulin

cAMP: cyclic adenosine monophosphate

CAs: catecholamines

cGRK2KO: cardiac-specific GRK2-knockout mice

CRT: cardiac resynchronization therapy

CT: C-terminus

CVDs: Cardiovascular diseases

DAG: diacylglycerol

ECs: endothelial cells

EGFR: epidermal growth factor receptor

EPI: epinephrine

ERK: extracellular signal-regulated kinase

GPCR: G protein-coupled receptor

GRKs: G protein-coupled receptor kinases

HB-EGF: heparin-bound epidermal growth factor

HDAC: histone deacetylase

HF: heart failure.

HFmrEF: mid-range heart failure

HFpEF: heart failure with preserved ejection fraction

HFrEF: heart failure with reduced ejection fraction

HSP90: heat shock protein 90

ICD: implantable cardioverter defibrillator

IP_3: inositol-1,4,5-trisphosphate

IR: insulin receptor

IRS1: IR substrate 1

KD: kinase domain

LV: left ventricular

LVEF: left ventricular ejection fraction

MAPK: mitogen-activated protein kinase

MEF2: myocyte enhancer factor 2

MI: myocardial infarction

MMP: metalloproteinase

MR: mineralocorticoid receptor

NE: norepinephrine

NES: nuclear export sequence

NFAT: Nuclear factor of activated T-cells

NLS: nuclear localization signal

NT: N-terminus

PD: Parkinson's disease

PE: phenylephrine

PH: pleckstrin homology

PIP_2: phosphatidylinositol 4,5-bisphosphate

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

RAAS: renin–angiotensin–aldosterone system.

RGS: regulator of G protein signaling

RH: RGS homology

RhoGEF: Rho guanine nucleotide exchange factors

ROS: reactive oxygen species

SNS: sympathetic nervous system

S1P: sphingosine 1-phosphate

S1PR1: S1P receptor 1

TAC: transverse aortic constriction

VAD: ventricular assist devices

VSMCs: vascular smooth muscle cells

WT: wild type

7TM: seven-transmembrane

Bibliography

1. Adams KFJ. Pathophysiologic role of the renin-angiotensin-aldosterone and sympathetic nervous systems in heart failure. *Am J Health Syst Pharm*. 2004;61 Suppl 2:S4-13. doi:10.1093/ajhp/61.suppl_2.S4

2. Agüero J, Almenar L, Montó F, et al. Myocardial G protein receptor-coupled kinase expression correlates with functional parameters and clinical severity in advanced heart failure. *J Card Fail*. 2012;18(1):53-61. doi:10.1016/j.cardfail.2011.10.008

3. Amini M, Zayeri F, Salehi M. Trend analysis of cardiovascular disease mortality, incidence, and mortality-to-incidence ratio: results from global burden of disease study 2017. *BMC Public Health*. 2021;21(1):401. doi:10.1186/s12889-021-10429-0

4. Arawaka S, Wada M, Goto S, et al. The role of G-protein-coupled receptor kinase 5 in pathogenesis of sporadic Parkinson's disease. *J Neurosci*. 2006;26(36):9227-9238. doi:10.1523/JNEUROSCI.0341-06.2006

5. Belmonte SL, Blaxall BC. G protein coupled receptor kinases as therapeutic targets in cardiovascular disease. *Circ Res*. 2011;109(3):309-319. doi:10.1161/CIRCRESAHA.110.231233

6. Bencivenga L, Palaia ME, Sepe I, et al. Why Do We Not Assess Sympathetic Nervous System Activity in Heart Failure Management: Might GRK2 Serve as a New Biomarker? *Cells*. 2021;10(2). doi:10.3390/cells10020457

7. Brenninkmeijer CB, Price SA, López Bernal A, Phaneuf S. Expression of G-protein-coupled receptor kinases in pregnant term and non-pregnant human myometrium. *J Endocrinol*. 1999;162(3):401-408. doi:10.1677/joe.0.1620401

8. Bristow MR, Ginsburg R, Umans V, et al. Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective beta 1-receptor down-regulation in heart failure. *Circ Res*. 1986;59(3):297-309. doi:10.1161/01.res.59.3.297

9. Bubser M, Byun N, Wood MR, Jones CK. Muscarinic receptor pharmacology and circuitry for the modulation of cognition. *Handb Exp Pharmacol*. 2012;(208):121-166. doi:10.1007/978-3-642-23274-9_7

10. Cannavo A, Bencivenga L, Liccardo D, et al. Aldosterone and Mineralocorticoid Receptor System in Cardiovascular Physiology and Pathophysiology. Penna C, ed. *Oxidative Medicine and Cellular Longevity*. 2018;2018:1204598. doi:10.1155/2018/1204598

11. Cannavo A, Komici K, Bencivenga L, et al. GRK2 as a therapeutic target for heart failure. *Expert Opin Ther Targets*. 2018;22(1):75-83. doi:10.1080/14728222.2018.1406925

12. Cannavo A, Liccardo D, Eguchi A, et al. Myocardial pathology induced by aldosterone is dependent on non-canonical activities of G protein-coupled

receptor kinases. *Nature Communications*. 2016;7(1):10877. doi:10.1038/ncomms10877

13. Cannavo A, Rengo G, Liccardo D, et al. β1-adrenergic receptor and sphingosine-1-phosphate receptor 1 (S1PR1) reciprocal downregulation influences cardiac hypertrophic response and progression to heart failure: protective role of S1PR1 cardiac gene therapy. *Circulation*. 2013;128(15):1612-1622. doi:10.1161/CIRCULATIONAHA.113.002659

14. Carman CV, Lisanti MP, Benovic JL. Regulation of G protein-coupled receptor kinases by caveolin. *J Biol Chem*. 1999;274(13):8858-8864. doi:10.1074/jbc.274.13.8858

15. Chen M, Sato PY, Chuprun JK, et al. Prodeath signaling of G protein-coupled receptor kinase 2 in cardiac myocytes after ischemic stress occurs via extracellular signal-regulated kinase-dependent heat shock protein 90-mediated mitochondrial targeting. *Circ Res*. 2013;112(8):1121-1134. doi:10.1161/CIRCRESAHA.112.300754

16. Chen X, Zhu H, Yuan M, Fu J, Zhou Y, Ma L. G-protein-coupled receptor kinase 5 phosphorylates p53 and inhibits DNA damage-induced apoptosis. *J Biol Chem*. 2010;285(17):12823-12830. doi:10.1074/jbc.M109.094243

17. Ciccarelli M, Chuprun JK, Rengo G, et al. G protein-coupled receptor kinase 2 activity impairs cardiac glucose uptake and promotes insulin resistance after myocardial ischemia. *Circulation*. 2011;123(18):1953-1962. doi:10.1161/CIRCULATIONAHA.110.988642

18. Claing A, Laporte SA, Caron MG, Lefkowitz RJ. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog Neurobiol*. 2002;66(2):61-79. doi:10.1016/s0301-0082(01)00023-5

19. Coleman RC, Eguchi A, Lieu M, et al. A peptide of the N terminus of GRK5 attenuates pressure-overload hypertrophy and heart failure. *Sci Signal*. 2021;14(676). doi:10.1126/scisignal.abb5968

20. Communal C, Singh K, Sawyer DB, Colucci WS. Opposing effects of beta(1)- and beta(2)-adrenergic receptors on cardiac myocyte apoptosis : role of a pertussis toxin-sensitive G protein. *Circulation*. 1999;100(22):2210-2212. doi:10.1161/01.cir.100.22.2210

21. DebBurman SK, Ptasienski J, Benovic JL, Hosey MM. G protein-coupled receptor kinase GRK2 is a phospholipid-dependent enzyme that can be conditionally activated by G protein betagamma subunits. *J Biol Chem*. 1996;271(37):22552-22562. doi:10.1074/jbc.271.37.22552

22. Dessy C, Balligand J-L. Beta3-adrenergic receptors in cardiac and vascular tissues emerging concepts and therapeutic perspectives. *Adv Pharmacol*. 2010;59:135-163. doi:10.1016/S1054-3589(10)59005-7

23. Dzimiri N, Basco C, Moorji A, Afrane B, Al-Halees Z. Characterization of lymphocyte beta 2-adrenoceptor signalling in patients with left ventricular volume overload disease. *Clin Exp Pharmacol Physiol*. 2002;29(3):181-188. doi:10.1046/j.1440-1681.2002.03625.x

24. Dzimiri N, Muiya P, Andres E, Al-Halees Z. Differential functional expression of human myocardial G protein receptor kinases in left ventricular cardiac diseases. *Eur J Pharmacol*. 2004;489(3):167-177. doi:10.1016/j.ejphar.2004.03.015

25. Felder RA, Sanada H, Xu J, et al. G protein-coupled receptor kinase 4 gene variants in human essential hypertension. *Proc Natl Acad Sci U S A*. 2002;99(6):3872-3877. doi:10.1073/pnas.062694599

26. Fredriksson R, Lagerström MC, Lundin L-G, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol*. 2003;63(6):1256-1272. doi:10.1124/mol.63.6.1256

27. Gainetdinov RR, Bohn LM, Walker JK, et al. Muscarinic supersensitivity and impaired receptor desensitization in G protein-coupled receptor kinase 5-deficient mice. *Neuron*. 1999;24(4):1029-1036. doi:10.1016/s0896-6273(00)81048-x

28. Gambardella J, Franco A, Giudice CD, et al. Dual role of GRK5 in cancer development and progression. *Transl Med UniSa*. 2016;14:28-37.

29. Gold JI, Gao E, Shang X, Premont RT, Koch WJ. Determining the absolute requirement of G protein-coupled receptor kinase 5 for pathological cardiac hypertrophy: short communication. *Circ Res*. 2012;111(8):1048-1053. doi:10.1161/CIRCRESAHA.112.273367

30. Groenewegen A, Rutten FH, Mosterd A, Hoes AW. Epidemiology of heart failure. *Eur J Heart Fail*. 2020;22(8):1342-1356. doi:10.1002/ejhf.1858

31. Gurevich EV, Tesmer JJG, Mushegian A, Gurevich VV. G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. *Pharmacol Ther*. 2012;133(1):40-69. doi:10.1016/j.pharmthera.2011.08.001

32. Hartupee J, Mann DL. Neurohormonal activation in heart failure with reduced ejection fraction. *Nat Rev Cardiol*. 2017;14(1):30-38. doi:10.1038/nrcardio.2016.163

33. Hilger D, Masureel M, Kobilka BK. Structure and dynamics of GPCR signaling complexes. *Nat Struct Mol Biol*. 2018;25(1):4-12. doi:10.1038/s41594-017-0011-7

34. Hitomi H, Kiyomoto H, Nishiyama A, et al. Aldosterone suppresses insulin signaling via the downregulation of insulin receptor substrate-1 in vascular smooth muscle cells. *Hypertension*. 2007;50(4):750-755. doi:10.1161/HYPERTENSIONAHA.107.093955

35. Homan KT, Wu E, Cannavo A, Koch WJ, Tesmer JJG. Identification and characterization of amlexanox as a G protein-coupled receptor kinase 5 inhibitor. *Molecules*. 2014;19(10):16937-16949. doi:10.3390/molecules191016937

36. Huang ZM, Gao E, Chuprun JK, Koch WJ. GRK2 in the heart: a GPCR kinase and beyond. *Antioxid Redox Signal*. 2014;21(14):2032-2043. doi:10.1089/ars.2014.5876

37. Hullmann JE, Grisanti LA, Makarewich CA, et al. GRK5-mediated exacerbation of pathological cardiac hypertrophy involves facilitation of nuclear NFAT activity. *Circ Res*. 2014;115(12):976-985. doi:10.1161/CIRCRESAHA.116.304475

38. Hullmann J, Traynham CJ, Coleman RC, Koch WJ. The expanding GRK interactome: Implications in cardiovascular disease and potential for therapeutic development. *Pharmacol Res*. 2016;110:52-64. doi:10.1016/j.phrs.2016.05.008

39. Iaccarino G, Barbato E, Cipolletta E, et al. Elevated myocardial and lymphocyte GRK2 expression and activity in human heart failure. *Eur Heart J*. 2005;26(17):1752-1758. doi:10.1093/eurheartj/ehi429

40. Inglese J, Freedman NJ, Koch WJ, Lefkowitz RJ. Structure and mechanism of the G protein-coupled receptor kinases. *J Biol Chem*. 1993;268(32):23735-23738.

41. Inglese J, Koch WJ, Caron MG, Lefkowitz RJ. Isoprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. *Nature*. 1992;359(6391):147-150. doi:10.1038/359147a0

42. Jiang X, Benovic JL, Wedegaertner PB. Plasma membrane and nuclear localization of G protein coupled receptor kinase 6A. *Mol Biol Cell*. 2007;18(8):2960-2969. doi:10.1091/mbc.e07-01-0013

43. Johnson LR, Scott MGH, Pitcher JA. G protein-coupled receptor kinase 5 contains a DNA-binding nuclear localization sequence. *Mol Cell Biol*. 2004;24(23):10169-10179. doi:10.1128/MCB.24.23.10169-10179.2004

44. Katritch V, Cherezov V, Stevens RC. Structure-function of the G protein-coupled receptor superfamily. *Annu Rev Pharmacol Toxicol*. 2013;53:531-556. doi:10.1146/annurev-pharmtox-032112-135923

45. Kaur G, Kim J, Kaur R, et al. G-protein coupled receptor kinase (GRK)-5 regulates proliferation of glioblastoma-derived stem cells. *J Clin Neurosci*. 2013;20(7):1014-1018. doi:10.1016/j.jocn.2012.10.008

46. Kemp CD, Conte JV. The pathophysiology of heart failure. *Cardiovasc Pathol*. 2012;21(5):365-371. doi:10.1016/j.carpath.2011.11.007

47. Kim JI, Chakraborty P, Wang Z, Daaka Y. G-protein coupled receptor kinase 5 regulates prostate tumor growth. *J Urol*. 2012;187(1):322-329. doi:10.1016/j.juro.2011.09.049

48. Koch WJ, Inglese J, Stone WC, Lefkowitz RJ. The binding site for the beta gamma subunits of heterotrimeric G proteins on the beta-adrenergic receptor kinase. *J Biol Chem*. 1993;268(11):8256-8260.

49. Koch WJ, Rockman HA, Samama P, et al. Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a beta ARK inhibitor. *Science*. 1995;268(5215):1350-1353. doi:10.1126/science.7761854

50. Kunapuli P, Benovic JL. Cloning and expression of GRK5: a member of the G protein-coupled receptor kinase family. *Proc Natl Acad Sci USA*. 1993;90(12):5588. doi:10.1073/pnas.90.12.5588

51. Kunapuli P, Onorato JJ, Hosey MM, Benovic JL. Expression, purification, and characterization of the G protein-coupled receptor kinase GRK5. *J Biol Chem*. 1994;269(2):1099-1105.

52. Lagman J, Sayegh P, Lee CS, et al. G protein-coupled receptor kinase 5 modifies cancer cell resistance to paclitaxel. *Mol Cell Biochem*. 2019;461(1-2):103-118. doi:10.1007/s11010-019-03594-9

53. Lefkowitz RJ. Seven transmembrane receptors: something old, something new. *Acta Physiol (Oxf)*. 2007;190(1):9-19. doi:10.1111/j.1365-201X.2007.01693.x

54. Liu P, Wang X, Gao N, et al. G protein-coupled receptor kinase 5, overexpressed in the alpha-synuclein up-regulation model of Parkinson's disease, regulates bcl-2 expression. *Brain Res*. 2010;1307:134-141. doi:10.1016/j.brainres.2009.10.036

55. Lymperopoulos A, Rengo G, Koch WJ. Adrenergic nervous system in heart failure: pathophysiology and therapy. *Circ Res*. 2013;113(6):739-753. doi:10.1161/CIRCRESAHA.113.300308

56. Mangmool S, Parichatikanond W, Kurose H. Therapeutic Targets for Treatment of Heart Failure: Focus on GRKs and β-Arrestins Affecting βAR Signaling. *Front Pharmacol*. 2018;9:1336. doi:10.3389/fphar.2018.01336

57. Martini JS, Raake P, Vinge LE, et al. Uncovering G protein-coupled receptor kinase-5 as a histone deacetylase kinase in the nucleus of cardiomyocytes. *Proc Natl Acad Sci U S A*. 2008;105(34):12457-12462. doi:10.1073/pnas.0803153105

58. Mensah GA, Roth GA, Fuster V. The Global Burden of Cardiovascular Diseases and Risk Factors: 2020 and Beyond. *J Am Coll Cardiol*. 2019;74(20):2529-2532. doi:10.1016/j.jacc.2019.10.009

59. Myagmar B-E, Flynn JM, Cowley PM, et al. Adrenergic Receptors in Individual Ventricular Myocytes: The Beta-1 and Alpha-1B Are in All Cells, the Alpha-1A Is in a Subpopulation, and the Beta-2 and Beta-3 Are Mostly Absent. *Circ Res*. 2017;120(7):1103-1115. doi:10.1161/CIRCRESAHA.117.310520

60. Nicolini F, Gherli T. Alternatives to transplantation in the surgical therapy for heart failure. *European Journal of Cardio-Thoracic Surgery*. 2009;35(2):214-228. doi:10.1016/j.ejcts.2008.11.003

61. Noma T, Lemaire A, Naga Prasad SV, et al. Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *J Clin Invest*. 2007;117(9):2445-2458. doi:10.1172/JCI31901

62. Oldham WM, Hamm HE. Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol*. 2008;9(1):60-71. doi:10.1038/nrm2299

63. Penela P, Ribas C, Mayor FJ. Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cell Signal*. 2003;15(11):973-981. doi:10.1016/s0898-6568(03)00099-8

64. Pfleger J, Gresham K, Koch WJ. G protein-coupled receptor kinases as therapeutic targets in the heart. *Nat Rev Cardiol*. 2019;16(10):612-622. doi:10.1038/s41569-019-0220-3

65. Pierce KL, Premont RT, Lefkowitz RJ. Seven-transmembrane receptors. *Nature Reviews Molecular Cell Biology*. 2002;3(9):639-650. doi:10.1038/nrm908

66. Pitcher JA, Inglese J, Higgins JB, et al. Role of beta gamma subunits of G proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors. *Science*. 1992;257(5074):1264-1267. doi:10.1126/science.1325672

67. Ponikowski P, Voors AA, Anker SD, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC)Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur Heart J*. 2016;37(27):2129-2200. doi:10.1093/eurheartj/ehw128

68. Pronin AN, Satpaev DK, Slepak VZ, Benovic JL. Regulation of G protein-coupled receptor kinases by calmodulin and localization of the calmodulin binding domain. *J Biol Chem*. 1997;272(29):18273-18280. doi:10.1074/jbc.272.29.18273

69. Rengo G, Pagano G, Filardi PP, et al. Prognostic Value of Lymphocyte G Protein-Coupled Receptor Kinase-2 Protein Levels in Patients With Heart Failure. *Circ Res*. 2016;118(7):1116-1124. doi:10.1161/CIRCRESAHA.115.308207

70. Ribas C, Penela P, Murga C, et al. The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling. *Biochim Biophys Acta*. 2007;1768(4):913-922. doi:10.1016/j.bbamem.2006.09.019

71. Rockman HA, Chien KR, Choi DJ, et al. Expression of a beta-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice. *Proc Natl Acad Sci U S A*. 1998;95(12):7000-7005. doi:10.1073/pnas.95.12.7000

72. Salazar NC, Chen J, Rockman HA. Cardiac GPCRs: GPCR signaling in healthy and failing hearts. *Biochim Biophys Acta*. 2007;1768(4):1006-1018. doi:10.1016/j.bbamem.2007.02.010

73. Sallese M, Salvatore L, D'Urbano E, et al. The G-protein-coupled receptor kinase GRK4 mediates homologous desensitization of metabotropic glutamate receptor 1. *FASEB J*. 2000;14(15):2569-2580. doi:10.1096/fj.00-0072com

74. Sato PY, Chuprun JK, Ibetti J, et al. GRK2 compromises cardiomyocyte mitochondrial function by diminishing fatty acid-mediated oxygen consumption and increasing superoxide levels. *J Mol Cell Cardiol*. 2015;89(Pt B):360-364. doi:10.1016/j.yjmcc.2015.10.002

75. Sato PY, Chuprun JK, Schwartz M, Koch WJ. The evolving impact of g protein-coupled receptor kinases in cardiac health and disease. *Physiol Rev*. 2015;95(2):377-404. doi:10.1152/physrev.00015.2014

76. Savarese G, Lund LH. Global Public Health Burden of Heart Failure. *Card Fail Rev*. 2017;3(1):7-11. doi:10.15420/cfr.2016:25:2

77. Schumacher SM, Koch WJ. Noncanonical Roles of G Protein-coupled Receptor Kinases in Cardiovascular Signaling. *J Cardiovasc Pharmacol*. 2017;70(3):129-141. doi:10.1097/FJC.0000000000000483

78. Shenoy SK, Lefkowitz RJ. β-arrestin-mediated receptor trafficking and signal transduction. *Trends in Pharmacological Sciences*. 2011;32(9):521-533. doi:10.1016/j.tips.2011.05.002

79. Sorriento D, Santulli G, Ciccarelli M, et al. The Amino-Terminal Domain of GRK5 Inhibits Cardiac Hypertrophy through the Regulation of Calcium-Calmodulin Dependent Transcription Factors. *Int J Mol Sci*. 2018;19(3). doi:10.3390/ijms19030861

80. Sorriento D, Santulli G, Fusco A, Anastasio A, Trimarco B, Iaccarino G. Intracardiac injection of AdGRK5-NT reduces left ventricular hypertrophy by inhibiting NF-kappaB-dependent hypertrophic gene expression. *Hypertension*. 2010;56(4):696-704. doi:10.1161/HYPERTENSIONAHA.110.155960

81. Stoffel RH, Randall RR, Premont RT, Lefkowitz RJ, Inglese J. Palmitoylation of G protein-coupled receptor kinase, GRK6. Lipid modification diversity in the GRK family. *J Biol Chem*. 1994;269(45):27791-27794.

82. Suo Z, Cox AA, Bartelli N, et al. GRK5 deficiency leads to early Alzheimer-like pathology and working memory impairment. *Neurobiol Aging*. 2007;28(12):1873-1888. doi:10.1016/j.neurobiolaging.2006.08.013

83. Suo Z, Wu M, Citron BA, Wong GT, Festoff BW. Abnormality of G-protein-coupled receptor kinases at prodromal and early stages of Alzheimer's disease: an association with early beta-amyloid accumulation. *J Neurosci*. 2004;24(13):3444-3452. doi:10.1523/JNEUROSCI.4856-03.2004

84. Thiyagarajan MM, Stracquatanio RP, Pronin AN, Evanko DS, Benovic JL, Wedegaertner PB. A predicted amphipathic helix mediates plasma membrane localization of GRK5. *J Biol Chem*. 2004;279(17):17989-17995. doi:10.1074/jbc.M310738200

85. Traynham CJ, Cannavo A, Zhou Y, et al. Differential Role of G Protein-Coupled Receptor Kinase 5 in Physiological Versus Pathological Cardiac Hypertrophy. *Circ Res*. 2015;117(12):1001-1012. doi:10.1161/CIRCRESAHA.115.306961

86. Traynham CJ, Hullmann J, Koch WJ. "Canonical and non-canonical actions of GRK5 in the heart". *J Mol Cell Cardiol*. 2016;92:196-202. doi:10.1016/j.yjmcc.2016.01.027

87. van Berlo JH, Maillet M, Molkentin JD. Signaling effectors underlying pathologic growth and remodeling of the heart. *J Clin Invest*. 2013;123(1):37-45. doi:10.1172/JCI62839

88. Villar VAM, Jones JE, Armando I, et al. G protein-coupled receptor kinase 4 (GRK4) regulates the phosphorylation and function of the dopamine D3 receptor. *J Biol Chem*. 2009;284(32):21425-21434. doi:10.1074/jbc.M109.003665

89. Virani SS, Alonso A, Aparicio HJ, et al. Heart Disease and Stroke Statistics-2021 Update: A Report From the American Heart Association. *Circulation*. 2021;143(8):e254-e743. doi:10.1161/CIR.0000000000000950

90. Virlon B, Firsov D, Cheval L, et al. Rat G Protein-Coupled Receptor Kinase GRK4: Identification, Functional Expression, and Differential Tissue Distribution of Two Splice Variants*. *Endocrinology*. 1998;139(6):2784-2795. doi:10.1210/endo.139.6.6078

91. Wada T, Ohshima S, Fujisawa E, Koya D, Tsuneki H, Sasaoka T. Aldosterone inhibits insulin-induced glucose uptake by degradation of insulin receptor substrate (IRS) 1 and IRS2 via a reactive oxygen species-mediated pathway in 3T3-L1 adipocytes. *Endocrinology*. 2009;150(4):1662-1669. doi:10.1210/en.2008-1018

92. Wang J, Gareri C, Rockman HA. G-Protein-Coupled Receptors in Heart Disease. *Circ Res*. 2018;123(6):716-735. doi:10.1161/CIRCRESAHA.118.311403

93. Weiss ER, Ducceschi MH, Horner TJ, Li A, Craft CM, Osawa S. Species-specific differences in expression of G-protein-coupled receptor kinase (GRK) 7 and GRK1 in mammalian cone photoreceptor cells: implications for cone cell phototransduction. *J Neurosci*. 2001;21(23):9175-9184. doi:10.1523/JNEUROSCI.21-23-09175.2001

94. Woodall MC, Ciccarelli M, Woodall BP, Koch WJ. G protein-coupled receptor kinase 2: a link between myocardial contractile function and cardiac metabolism. *Circ Res*. 2014;114(10):1661-1670. doi:10.1161/CIRCRESAHA.114.300513

95. Zhang Y, Zhao J, Yin M, et al. The influence of two functional genetic variants of GRK5 on tau phosphorylation and their association with Alzheimer's disease risk. *Oncotarget*. 2017;8(42):72714-72726. doi:10.18632/oncotarget.20283

96. Zhang Y, Chen L, Shen G, Zhao Q, Shangguan L, He M. GRK5 dysfunction accelerates tau hyperphosphorylation in APP (swe) mice through impaired cholinergic activity. *Neuroreport*. 2014;25(7):542-547. doi:10.1097/WNR.0000000000000142

97. Zhao J, Li X, Chen X, et al. GRK5 influences the phosphorylation of tau via GSK3β and contributes to Alzheimer's disease. *J Cell Physiol*. 2019;234(7):10411-10420. doi:10.1002/jcp.27709

98. Zhou XE, Melcher K, Xu HE. Understanding the GPCR biased signaling through G protein and arrestin complex structures. *Curr Opin Struct Biol*. 2017;45:150-159. doi:10.1016/j.sbi.2017.05.004

Curriculum Vitae

Federica Marzano

Date of birth: 16/03/1992 | **Nationality:** Italian | **Gender** Female | (+39) 3319926248 | federica.marzano@outlook.com | Viale Augusto, 62, 80125, Napoli, Italy

EDUCATION AND TRAINING

01/11/2018 – CURRENT
PHD STUDENT IN CARDIOVASCULAR PATHOPHYSIOLOGY AND THERAPEUTICS (CARDIOPATH) – University of Naples "Federico II", Italy; Temple University, Philadelphia, USA

03/2018 – Benevento, Italy
QUALIFICATION AS A PROFESSIONAL BIOLOGIST – University of Sannio

01/04/2015 – 23/06/2017 – Napoli, Italy
MASTER DEGREE IN MEDICAL BIOTECHNOLOGIES – University of Naples "Federico II"

110/110 cum laude | Circulating Sphingosine 1-Phosphate levels in patients with systolic heart failure

29/09/2011 – 16/12/2014 – Napoli, Italy
BACHELOR DEGREE IN BIOTECHNOLOGY FOR HEALTH – University of Naples "Federico II"

WORK EXPERIENCE

10/2020 – CURRENT – Philadelphia, United States
VISITING PHD STUDENT – TEMPLE UNIVERSITY

09/2017 – 10/2018
POST-GRADUATE TRAINING

Research activity in cardiovascular field at at University of Naples "Federico II", Department of Translational Medical Sciences.

LANGUAGE SKILLS

Mother tongue(s): ITALIAN

Other language(s):

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken production	Spoken interaction	
ENGLISH	C1	C1	C1	C1	C1

Levels: A1 and A2: Basic user; B1 and B2: Independent user; C1 and C2: Proficient user

HONOURS AND AWARDS

Honours and awards

- PROGRAMMA STAR PLUS LINEA D'INTERVENTO 2 - MOBILITÀ GIOVANI RICERCATORI - Annualità 2020 - compagnia San Paolo - University of Naples "Federico II", Italy
- Travel Award - ASPET Annual Meeting at Experimental Biology (EB) 2021
- Travel Grant - XXII Meeting of the Italian Society of Cardiovascular Research (SIRC), 2019

NETWORKS AND MEMBERSHIPS

Memberships

- 2021 - American Heart Association (AHA)
- 2021 - Heart Failure Association of the ESC (HFA)
- 2021 - American Society for Pharmacology and Experimental Therapeutics (ASPET)
- 2019 - WG on Myocardial Function (ESC)
- 2018 - Italian Society of Geriatrics and Gerontology (SIGG)
- 2018 - Italian Society of Cardiovascular Research (SIRC)

ATTENDANCE AT NATIONAL/INTERNATIONAL MEETINGS

Attendance at National/International Meetings

- **F. Marzano**, A. Rapacciuolo, W. J. Koch, A. Cannavo - Inactivating GRK5 Impairs Basal Cardiac Function and Survival via p53 Modulation - Basic Cardiovascular Sciences (BCVS) Scientific Sessions, 2021
- **F. Marzano**, A. Rapacciuolo, W. J. Koch, A. Cannavo - GRK5 controls basal cardiac function and survival via direct p53 modulation - Heart Failure (HFA), 2021
- **F. Marzano**, A. Rapacciuolo, W. J. Koch, A. Cannavo - GRK5-Dependent p53 Activity Controls Basal Cardiac Function and Survival - ASPET Annual Meeting at Experimental Biology (EB), 2021
- **F. Marzano**, G. Rengo, A. Rapacciuolo, W. J. Koch, A. Cannavo - GRK5 via direct modulation of p53 levels controls basal cardiac function and remodeling - Heart Failure Association Winter meeting, 2020 - **Young Investigator Award Finalist**
- **F. Marzano**, D. Liccardo, N. Ferrara, A. Cannavo, G. Rengo - Aldosterone impairs cardiac insulin and beta-adrenergic receptor signaling in a GRK2 dependent manner - 64° National Meeting of the Italian Society of Geriatrics and Gerontology (SIGG), 2019
- **F. Marzano**, D. Liccardo, W. J. Koch, G. Rengo, A. Cannavo - GRK2 downstream Aldosterone and Mineralocorticoid receptor system induces cardiac insulin-resistance and β-adrenergic dysfunction - XXII Meeting of the Italian Society of Cardiovascular Research (SIRC), 2019
- **F. Marzano**, G. Rengo, A. Rapacciuolo, A. Elia ,W. J. Koch, A. Cannavo GRK5 catalytic activity controls basal cardiac function and remodeling via p53 and EGFR signaling modulation - ESC Working Groups on Myocardial Function & Cellular Biology of the Heart, 2019 - **AWARD Session: Best abstracts in translational science.**
- **F. Marzano**, A. Rapacciuolo, G. Rengo, A. Cannavo - Aldosterone impairs insulin and β-adrenergic receptor signaling in a GRK2 dependent manner - ESC Working Groups on Myocardial Function & Cellular Biology of the Heart 2019 - **Best Poster Selection.**
- **F. Marzano**, A. Cannavo, N. Ferrara, G. Rengo - "Aldosterone induces insulin-resistance and beta-adrenergic dysfunction via GRK2 activation, contributing to HF development", 63° National Meeting of the Italian Society of Geriatrics and Gerontology (SIGG), 2018
- **F. Marzano,** A. Cannavo, D. Liccardo, N. Ferrara, Walter J. Koch, Giuseppe Rengo - High aldosterone levels induce cardiac insulin-resistance and β-adrenergic dysfunction contributing to post-ischemic heart failure in mice – 4th SIRC FORUM "New Roads in cardiovascular research", 2018

List of all pubblication

- Del Giudice, C.; Vaia, E.; Liccardo, D.; **Marzano, F.**; Valletta, A.; Spagnuolo, G.; Ferrara, N.; Rengo, C.; Cannavo, A.; Rengo, G. Infective Endocarditis: A Focus on Oral Microbiota. *Microorganisms* **2021**, *9* (6). https://doi.org/10.3390/microorganisms9061218.

- **Marzano, F.**; Rapacciuolo, A.; Ferrara, N.; Rengo, G.; Koch, W. J.; Cannavo, A. Targeting GRK5 for Treating Chronic Degenerative Diseases. *Int J Mol Sci* **2021**, *22* (4). https://doi.org/10.3390/ijms22041920.

- Liccardo, D.; **Marzano, F.**; Carraturo, F.; Guida, M.; Femminella, G. D.; Bencivenga, L.; Agrimi, J.; Addonizio, A.; Melino, I.; Valletta, A.; Rengo, C.; Ferrara, N.; Rengo, G.; Cannavo, A. Potential Bidirectional Relationship Between Periodontitis and Alzheimer's Disease. *Front Physiol* **2020**, *11*, 683. https://doi.org/10.3389/fphys.2020.00683.

- Cannavo, A.; **Marzano, F. (co-first)**; Elia, A.; Liccardo, D.; Bencivenga, L.; Gambino, G.; Perna, C.; Rapacciuolo, A.; Cittadini, A.; Ferrara, N.; Paolocci, N.; Koch, W. J.; Rengo, G. Aldosterone Jeopardizes Myocardial Insulin and β-Adrenergic Receptor Signaling via G Protein-Coupled Receptor Kinase 2. *Front Pharmacol* **2019**, *10*, 888. https://doi.org/10.3389/fphar.2019.00888.

- Cannavo, A.; Bencivenga, L.; Liccardo, D.; Elia, A.; **Marzano, F.**; Gambino, G.; D'Amico, M. L.; Perna, C.; Ferrara, N.; Rengo, G.; Paolocci, N. Aldosterone and Mineralocorticoid Receptor System in Cardiovascular Physiology and Pathophysiology. *Oxid Med Cell Longev* **2018**, *2018*, 1204598. https://doi.org/10.1155/2018/1204598.

Acknowledgments

Foremost, I would like to thank Prof. Emanuele Barbato and Prof. Raffaele Izzo, for guiding us during the course of this PhD program, for carefully organizing all the related activities and for always encouraging us to improve our scientific achievements.

I wish to deeply and sincerely thank Prof. Antonio Rapacciuolo, who has been my advisor since my master's degree. I am extremely grateful for his invaluable advice, continuous support, and patience during my PhD study.

I would like to thank Prof. Alessandro Cannavo, who has been my mentor in science and professional life. I will always be grateful to him for allowing me to participate in several scientific projects and for helping me grow as an independent researcher. His energy and his immense knowledge have been for me a source of inspiration and great admiration.

Also, I wish to thank Prof. Giuseppe Rengo for giving me the chance to work in his research laboratory for all these years.

I would like to extend my sincere thanks to all the researchers and collaborators I had the pleasure to work with in Naples, because all of them gave me something from a personal and professional point of view.

Coming to my experience in the US, I would like to express my deep and sincere gratitude to Prof. Walter J. Koch for welcoming me at the Center for Translational Medicine of Temple University. I am proud and honored for having had the privilege to join his laboratory and work under his direct guidance. From the beginning, he has always been encouraging and supportive, and to me, he will always be a role model.

Furthermore, I would like to thank all the research fellows and collaborators that I met during this experience abroad. They have always been truly friendly and collaborative and all of them contributed to my personal and professional growth. I will always look back on this period with great memories.

Last but not the least, I would like to thank my loving family, for always supporting and encouraging me, beyond distances and difficulties. I am grateful to have you in my life.

A special thanks to my fiancé Riccardo, my greatest supporter in life. Thank you for always stand by my side and continuously believing in me, even when I don't. You are my real strength in this life.