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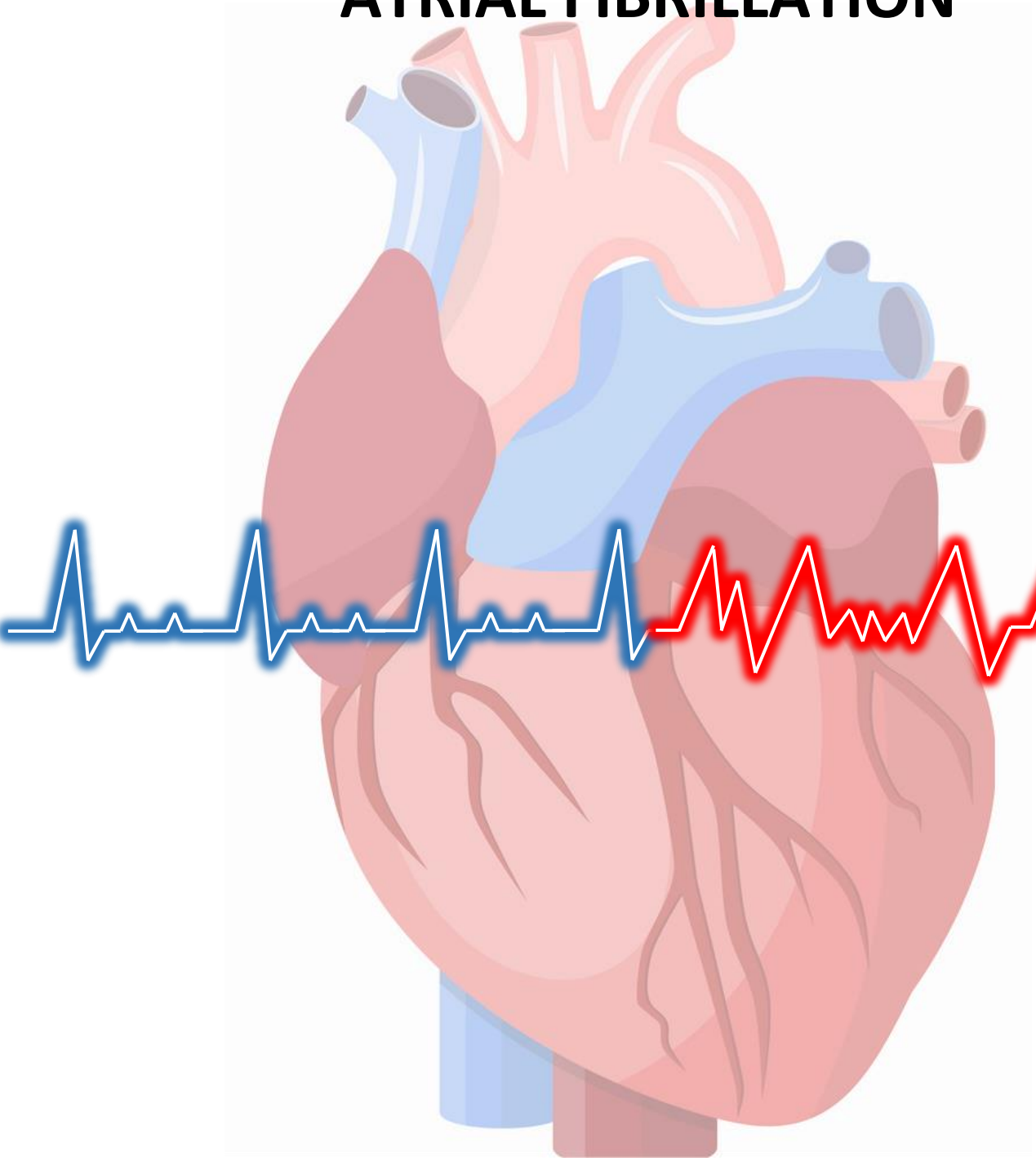
Adenosine receptors in Atrial Fibrillation

Héctor Godoy Marín

ADENOSINE RECEPTORS IN ATRIAL FIBRILLATION



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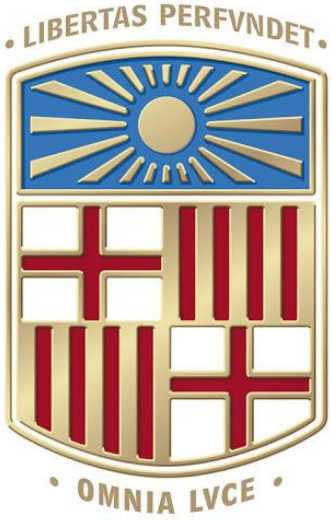

Universitat de Barcelona

This thesis has been submitted in the Doctoral program of Biotechnology.

Experimental work and manuscript have been performed by **Héctor Godoy Marín**, under the supervision of:

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Foundings

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List of publications

Part of the work presented in this thesis resulted in publications in international peer-reviewed journals:

1. **Godoy-Marín, H**., Duroux, R., Jacobson, K. A., Soler, C., Colino-Lage, H., Jiménez-Sábado, V., Montiel, J., Hove-Madsen, L., & Ciruela, F. (2021). Adenosine A_{2A} Receptors Are Upregulated in Peripheral Blood Mononuclear Cells from Atrial Fibrillation Patients. *International journal of molecular sciences*, *22*(7), 3467. https://doi.org/10.3390/ijms22073467

2. Herraiz-Martínez, A., Tarifa, C., Jiménez-Sábado, V., Llach, A**., Godoy-Marín, H**., Colino, H., Nolla-Colomer, C., Casabella, S., Izquierdo-Castro, P., Benítez, I., Benítez, R., Roselló-Díez, E., Rodríguez-Font, E., Viñolas, X., Ciruela, F., Cinca, J., & Hove-Madsen, L. (2021). Influence of sex on intracellular calcium homeostasis in patients with atrial fibrillation. *Cardiovascular research*, cvab127. Advance online publication. https://doi.org/10.1093/cvr/cvab127

During the course of my doctoral work, I actively participated in different projects that resulted in international peer-reviewed scientific journals:

3. Betari, N., Sahlholm, K., Morató, X., **Godoy-Marín, H**., Jáuregui, O., Teigen, K., Ciruela, F., & Haavik, J. (2020). Inhibition of Tryptophan Hydroxylases and Monoamine Oxidase-A by the Proton Pump Inhibitor, Omeprazole. *In Vitro* and *In Vivo* Investigations. *Frontiers in pharmacology*, *11*, 593416. https://doi.org/10.3389/fphar.2020.593416

Other academic publications are under preparation for submission:

4. Tarifa, C., Vallmitjana, A., Jiménez-Sábado, V., Marchena, M., Llach, A., Herraiz, A., **Godoy-Marín, H**., Nolla-Colomer, C., Ginel, A., Viñolas, C., Montiel, J., Echebarria, B., Ciruela, F., Benítez, R., Cinca, J., Hove-Madsen, L. Preferential distribution of calcium sparks at the sarcolemma potentiates afterdepolatizations in atrial fibrillation.

Other academic publications:

5. Martí-Solans J, **Godoy-Marín H**, Diaz-Gracia M, Onuma TA, Nishida H, Albalat R, Cañestro C. (2021). Massive Gene Loss and Function Shuffling in Appendicularians Stretch the Boundaries of Chordate Wnt Family Evolution. Front Cell Dev Biol. 2021 Jun 9;9:700827. doi: 10.3389/fcell.2021.700827. PMID: 34179025; PMCID: PMC8220140.

6. Martí-Solans, J., Ferrández-Roldán, A., **Godoy-Marín, H**., Badia-Ramentol, J., Torres-Aguila, N. P., Rodríguez-Marí, A., Bouquet, J. M., Chourrout, D., Thompson, E. M., Albalat, R., & Cañestro, C. (2015). Oikopleura dioica culturing made easy: a low-cost facility for an emerging animal model in EvoDevo. *Genesis (New York, N.Y. : 2000)*, *53*(1), 183–193. https://doi.org/10.1002/dvg.22800

I. Table of contents

List of abbreviations

ºC	Grades centigrade
µg	Micrograms
µL	Microliters
µM	Micromolar
µm	Micrometre
4-AA	4-aminoantipyrine
7AAD	7-actinomicine-D

A.

A_1R	Adenosine receptor type 1
$A_{2A}R$	Adenosine receptor type 2A
$A_{2B}R$	Adenosine receptor type 2B
A_3R	Adenosine receptor type 3
AAS	Acetylsalicylic acid
AC	Adenylate cyclase
ADA	Adenosine deaminase enzyme
ADORA	Adenosine receptors coding gene
AF	Atrial fibrillation
AK	Adenylate kinase
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Action potential

ARs	Adenosine receptors
ARA	Angiotensin receptor antagonist
AVN	Atrioventricular node

B.

BCA	Bicinchoninic acid
B-cell	Lymphocyte type B
BPM	Beats per minute
BRET	Bioluminescence resonance energy transfer

C.

Ca2+	Calcium ion
CaMKII	Calcium calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine
CCR	Chemokine receptor
CICR	Calcium induced calcium release
Cl^-	Chloride ion
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNT	Concentrative nucleoside transporters
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CRE	cAMP response element
CREB1	cAMP response element B
CSQ	Calsequestrin protein

C-T	Carboxi-terminal domain
CXCL	CXC chemokine
CXCR	CXC chemokine receptor

D.

D_2R	Dopamine receptor type 2
DAD	Delayed depolarization
DAG	Diacylglycerol
DCF	Diclorodihidrofluorescein
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dSR	Dilated sinus rhythm
DPCPX	Dipropylcyclopentylxanthine

E.

EAD	Early depolarization
ECG	Electrocardiogram
ECL	Extracellular loop
ECO	Echocardiogram
EDTA	Ethylenediaminetetraacetic acid
EHSPT	N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline
ENT	Equilibrate nucleoside transporter
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase
ERP	Refractory period

Eu-cAMP	Europium-attached cAMP

F.

FBSi	Inactivated fetal bovine serum
FRET	Förster resonance energy transfer

G.

GABA	γ-Aminobutyric acid
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate

H.

h	Hours
HBSS	Hank's balanced salt solution
HEK	Human embryonic kidney
HRP	Horseradish peroxidase

I.

ICL	Intracellular loop
i.e.	In example
IECA	Angiotensin-converting enzyme inhibitors
IKB	Nuclear factor of kappa light polypeptide
IL	Interleukin
IMP	Inosine monophosphate
InsP3R	Inositol triphosphate sensitive receptor
IP3	Inositol 1,4,5-trisphosphate

J.

JNK	C-jun N-terminal kinases

K.

K+	Potassium ion
KNCQ1	Potassium voltage-gated channel 1

L.

LAA	Lest atrial appendage
LTCaCs	L-type calcium currents
LTCC	L-type calcium channels
LV	Left ventricle

M.

MAPK	Mitogen activated protein kinases
MARKS	Mitogen activated protein kinases
mGlu	Metabotropic glutamate
min	Minutes
mL	Millilitre
mM	Millimolar
MMP-2	Metalloprotease type 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N.

n	Sample number
NA	Noradrenaline
NaCl	Sodium chloride
NanoBiT	NanoLuc binary technology

NCX	Sodium-Calcium exchanger
NDMA	*N*-methyl-D-aspartate
ndSR	Non-dilated sinus rhythm
NK	Natural killer lymphocyte
nm	Nanometer
nM	Nanomolar
NMR	Nuclear magnetic resonance
N-T	Amino-terminal domain

P.

P	P-value
PAM	Positive allosteric modulator
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDGF-A	Platelet derived growth factor
PEI	Polyethyleneimine
PI	Propidium iodide
PKA	Protein kinase A
PKC	Protein kinase C
PLB	Protein kinase B
PLC	Phospholipase C
PLD	Phospholipase D
PNP	Purine nucleoside phosphorylase
POD	Peroxidase
PPI	Protein:protein interactions

PRPPase	Ribose-phosphate diphosphokinase
PVDF	Polyvinylidene difluoride

Q.

qPCR	Quantitative PCR

R.

RA	Right atria
Raf	Rapid accelerated fibrosarcoma
RhoA	Ras homology family member
RhoGEFs	Rhodopsin guanine nucleotide exchange factors
RNA	Ribonucleic acid
ROS	Oxygen reactive species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RV	Right ventricle
RyR	Ryanodine receptors

S.

s	Seconds
SAH	S-Adenosyl-L-homocysteine
SAN	Sinoatrial node
SD	Standard deviation
SDS/PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERCA2	Sacro/endoplasmic reticulum calcium atpase
SR	Sarcoplasmic reticulum

T.

T-cell	Thymus-developed lymphocyte
TM	Transmembrane
TNF-α	Tumour necrosis factor

U.

U	Units

V.

Veh	Vehicle
v/v	Volume/volume

II. Introduction

1. G-protein-coupled receptors

1.1. General characteristics

G-protein-coupled-receptors (GPCRs) represent the largest family of signalling proteins. GPCRs mediate cellular responses to a huge number of molecules including hormones, metabolites, cytokines, and neurotransmitters. These receptors are encoded by more than 800 genes which constitute more than 1% of the human genome and represent more than 1000 proteins (Sriram et al., 2019). This family modulates process involved in behaviour (Van Den Burg & Neumann, 2011), blood pressure (Jara et al., 2019), cognition (Azam et al., 2020), immune response (D. Wang, 2018) and many more, becoming a rich source of drug targets in the last decades. All GPCR's share a common seven transmembrane (TM) architecture but they possess different extracellular N-terminal (N-T) domains and diverse ligand-binding pockets (Figure 1A). These receptors can adopt a series of different conformations influenced by association with ligands (Bissantz, 2003), post-transcriptional modifications such as glycosylation or phosphorylation (Hoffmann et al., 2008), other receptors (homo and heterodimerization) (Damian et al., 2006), regulatory proteins and environmental cues. This conformational flexibility allows that superfamily to recognize a huge variety of ligands for the same receptor (Jähnichen, 2006) (Figure 1B).

Much of the vertebrate physiology is based on GPCR signal transduction. When the ligand binds to GPCRs and activates the signalling pathway, the conformation is stabilized. At this moment, it can interact and modulate an heteromeric G-protein to promote the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) from the Gα subunit (Capper & Wacker, 2018). GTP-bound Gα dissociates from Gβγ, and Gα and Gβγ separately mediate downstream signalling activities (i.e. cyclic AMP (cAMP), inositol triphosphate (IP3), diacylglycerol (DAG), rhodopsin guanine nucleotide exchange factors (RhoGEFs) (Hur & Kim, 2002; Jong et al., 2018; Tuteja, 2009) that ends in the regulation of many cellular pathways. Regulation by GPCRs is necessary for a large variety of physiological roles such as inflammation, homeostasis or visual, gustatory and olfactory senses (Spehr & Munger, 2009).

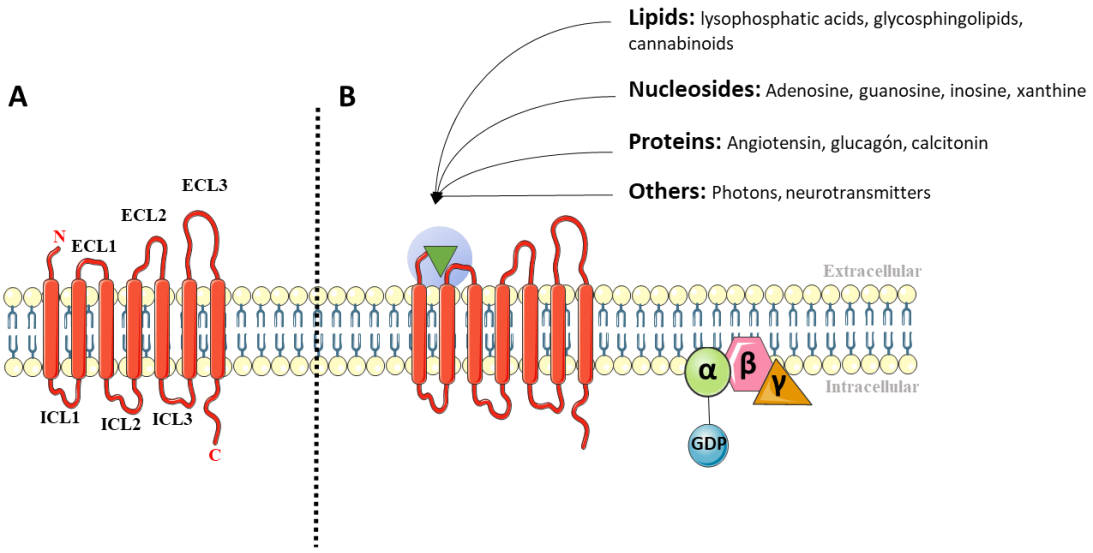


Figure 1. **General characteristics of GPCRs (A)** Main structural features of GPRCs: representation of the 7TM domains, three extracellular and three intracellular loops with the N-extracellular and the C-intracellular terminus. **(B)** Ligand diversity for GPCRs including lipids, nucleotides, proteins, and others that initiates the signalling cascade through G-coupled proteins stimulation.

1.2. Structural Features

As integral membrane proteins, GPCRs display seven hydrophobic transmembrane alpha-helix domains with three intracellular loops (ICL1, ICL2, ICL3), three extracellular loops (ECL1, ECL2 and ECL3), and C and N terminus (C-T and N-T) (Kobilka, 2007). Extracellular N-terminus is susceptible to modifications such as glycosylations or phosphorilations (Q. Chen et al., 2010). The extracellular loops contain two conserved cysteines to stabilize the structure by disulphide bonds. C-terminus can be also modified by phosphorylation or palmitoylation, used mainly for desensitization and internalization (Ng et al., 1994; X. Zhang et al., 2016). Even when the TM domains are relatively well conserved, the amino and the carboxy terminus present a high variability. The second extracellular loop and the third intracellular loop are also extremely flexible, which explain in part the higher functional differences between receptors (Kim et al., 2018). Unfortunately, GPCR crystallography represents a major challenge principally due to 2 inconvenient. First, the lower expression of GPCRs in native tissues, which is partially solved by recombinant protein expression. Second, the thermodynamic and proteolytic protein-stability problems, which are solved by mutating certain domains to increase stability or inserting fragments to improve the crystal forming contacts (Rosenbaum et al., 2009). Luckily, in the recent times and thanks to functional, structural, spectroscopic,

and computational studies, more and more information of GPCR is available. These advances allow the study of the conformational changes and the helical arrangements due to activation.

1.3. GPCRs classification

Currently, the classification system of GPCRs is based on the amino acid sequences and functional similarities (depending mostly on the 7TM domains). This classification includes GPCRs from vertebrates and invertebrates. Is divided in six classes from A to F based on sequence homology or five groups based on phylogenetic origin (Davies et al., 2007; Hu et al., 2017). The main groups are: class A (Rhodopsin-like) which is the largest one (about 80% of all GPCRs) with 7TM, eight helix and apalmitoylated cysteine in the C terminal end (Angel et al., 2009; Palczewski, 2006), class B (Secretin receptor family) with a long 120 residues N-terminus stabilized with disulphide bonds (Harmar, 2001; Miller et al., 2012), class C (Glutamate receptor family) with a very long N terminus end with 600 residues and a cysteine-rich loop in the TM (Wu et al., 2015), class D which includes fungal GPCRs (Eilers et al., 2005), class E where we find the cAMP receptors and class F, with the frizzled receptors (Huang & Klein, 2004). Within the classification based on phylogenetic origin (GRAFS classification) the five groups are Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin receptors (Schiöth & Fredriksson, 2005).

1.4. GPCRs oligomerization

G-protein-coupled receptors were, at the beginning, considered monomeric entities, but the evidence in the recent years demonstrated that can form homo and heteromeric structures (Bouvier, 2001; Park et al., 2004).

This does not mean that GPCRs have to be in homo or heterodimer form to be active. In example, the receptors of the class A can work in a monomeric entity but also as a dimer or even high-order oligomers (Szidonya et al., 2008). Nowadays, we can study the association between receptors due to new biophysical techniques such as resonance energy transfer (bioluminescence resonance energy transfer or BRET and fluorescence resonance energy transfer or FRET), fluorescence complementation and others (Kaczor & Selent, 2012). Oligomeric structures have been studied in more detail by nuclear

magnetic resonance (NMR) and X-ray crystallography, concluding that oligomerization is quite common in many different cell types (Park et al., 2004).

The association between receptors occur between two identical receptors (known as homodimer) or with two different ones (named heterodimer). In the same way, when are more than two receptors associated are named homo and heterooligomers (Figure 2).

Oligomerization is more than a way to modulate receptors in the membrane. This structures are involved in the trafficking from the endoplasmic reticulum (ER) to the membrane of many different molecules (Alguel et al., 2016; Torres et al., 2003). When proteins have been synthesised by ribosomes, proteins are integrated to the membrane of the ER. Then, proteins fold and are sent to the Trans-Golgi network where can mature and undergo post-transcriptional modifications. Finally, they can be destinated to the plasma membrane. In many cases, such as neurotransmitter transporters or γ-Aminobutyric acid (GABA) receptors, oligomerization is a crucial step to be released from ER (Balasubramanian et al., 2004). In this case, when neurotransmitter transporters suffer a mutation that disrupt the oligomer formation, they get stuck into ER. GABA receptors are synthesised but is when the different subunits are coupled forming the whole structure that they are released from the ER. The ER exerts an efficient quality control on misfolded proteins, and that control pass through oligomer formation.

GPCRs oligomerization has a remarkable effect on each individually GPCR function. The signalling derived from each receptor can be modulated in one way or another (Milligan et al., 2019). For example, adenosine 2A receptor ($A_{2A}R$) and dopamine D2 receptor (D_2R) heteromeric construct reduce the D_2R affinity for his ligands (Fastbom et al., 1998). In addition, oligomer formation has been shown to have a role in many diseases, such as Parkinson Disease, turning into a new target for therapeutic intervention (Fuxe et al., 2005; Jordan et al., 2003; Moreno et al., 2013).

Overall, the study and analysis of oligomers expression and localization is essential to understand the cell signalling and their function and impact on each specific tissue.

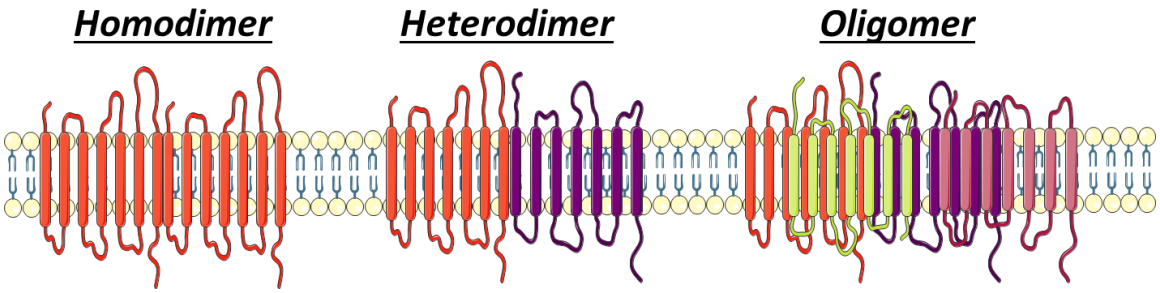


Figure 2. **Oligomerization of GPCRs**. GPRCs can couple to form homodimers binding two identical receptors, heterodimers if are two different receptors and higher order oligomers when the structure is formed by more than two identical or different receptors.

2. The adenosinergic system

2.1. Adenosine

The first evidence for the role of the adenosine dates from 1927, when adenosine was discovered to slow heart rate in extracts from cardiac tissue. Actually, it is known to be related due to his physiological role in many diseases like Parkinson (Nazario et al., 2017), Alzheimer (S. Y. Wang et al., 2020), autism (Masino et al., 2013), schizophrenia (Williams-Karnesky et al., 2012), and many heart diseases like atrial fibrillation (AF) and heart failure (Feldman et al., 2011). In the last years, knowledge of the role of adenosine in cellular physiology has improved. Actually, it is known to be involved in neuronal activity, vascular function, platelet aggregation and blood cell regulation among many others. In addition, as an ubiquitous molecule, can be found in nearly all tissues like heart, brain, kidney or spleen (Fredholm, 2011; Layland et al., 2014; H. Liu & Xia, 2015). Adenosine mediates its effect mainly through its interaction with four GPCRs named adenosine receptor type 1 (A_1R), adenosine receptor type 2A ($A_{2A}R$), adenosine receptor type 2B ($A_{2B}R$) and adenosine receptor type 3 (A_3R). Adenosine receptors (ARs) are also expressed in several cell and tissues through the body (Sheth et al., 2014).

2.2. Adenosine synthesis

Adenosine is a purine nucleoside composed by an adenine molecule and a ribose sugar moiety. If synthesized *de novo*, purines are synthetized in cells from the nucleotide inosine monophosphate (IMP) thanks to the ribose-phosphate diphosphokinase (PRPPase). Forming the IMP includes several steps were finally the purine is added to a

ribose-5-phosphate ring. IMP then is channelled into adenosine monophosphate (AMP) and guanosine monophosphate (GMP) by the inosine branch point. AMP is transformed then into adenosine diphosphate (ADP) and finally into adenosine triphosphate (ATP) (as well as GMP can be transformed into GDP and GTP) or can be turned into adenosine. IMP can be also synthetized from hypoxanthine, which is formed from inosine, the metabolite obtained from adenosine degradation. PRPPase also participates in the conversion to AMP and GMP from adenine and guanine (Hove-Jensen et al., 2017) (Figure 3A).

Adenosine can be produced both intracellularly and extracellularly through different sources when is not generated *de novo*. The origin from the intracellular way is mainly from the ATP breakdown and the conversion of the 5'-AMP by the 5'-nucleotidase into adenosine (K Yeung, 2014). Then adenosine can be turned into inosine and hypoxanthine or be transported to the extracellular space. Adenosine can be also turned back into 5'-AMP by adenosine kinases, turning in ADP and then ATP again. Adenosine can be produced alternatively by the transmethylation of the S-Adenosyl-L-homocysteine (SAH) by SAH hydrolase enzyme (Ernst et al., 2010). Extracellularly, is formed by the degradation of 5'AMP. Adenosine monophosphate comes from the cAMP released to the extracellular media via ectonucleoside triphosphate diphosphohydrolase and ecto-5′-nucleotidase (J. Zhang et al., 2018). Adenosine can be also pumped out or into the cell by equilibrate nucleoside transporters (ENTs) or by concentrative nucleoside transporters (CNTs), both with an ubiquitous tissue distribution (Pastor-Anglada & Pérez-Torras, 2018) (Figure 3B).

ATP metabolism is the base in the energy production by the cells as well as is required for most of the intracellular processes. In addition, is considered a signal of metabolic imbalance. The adenosine is released from metabolically compromised cells (physiological or pathological) to optimize the balance between energy waste and generation. However, ATP is constantly restored through ATP anabolism. When a lack of energy happens (often due to an excess of energy demand by the cell) it starts a reaction with the adenylate kinase which converts the ADP generated by the energy consumption into AMP and ATP (Bonora et al., 2012). That produces an excess of adenosine nucleoside which is transported to the extracellular media. This triggered amount of adenosine prompts the surrounding cells to decrease metabolism activity and control the energy waste (Cunha, 2001).

2.3. Adenosine degradation

Since adenosine signalling is involved in many physiological roles, excess of adenosine must be regulated by cells to guarantee a correct signalling. Intracellularly, adenosine can be turned back into AMP by the adenosine kinase (AK) in a reaction that expends ATP (Kroll et al., 1993) (Figure 3A-B). The other way to regulate adenosine amount is shared intracellularly and extracellularly and involves the Adenosine Deaminase (ADA) enzyme. ADA is expressed ubiquitously (at different levels depending on the tissue) and turns adenosine and 2'deoxyadenosine into inosine (Figure 3). The docking on the membrane of ADA is regulated by A_1 and A_{2B} receptors, and in certain cell types the anchoring to the membrane is also carried out by CD26, a glycoprotein associated to immune response and transduction signalling cascades (Whitmore & Gaspar, 2016). ADA plays a key role for the immune system regulation. Its deficiency initiates an accumulation of toxic purine degradation by-products affecting mostly lymphocytes which end in a severe immunodeficiency. Other effects on the lack of ADA are skeletal abnormalities, neurodevelopmental affects and pulmonary malfunctions associated with alveolar proteinosis (Flinn & Gennery, 2018). All this regulation provides the adenosine a physiological half-life below one second.

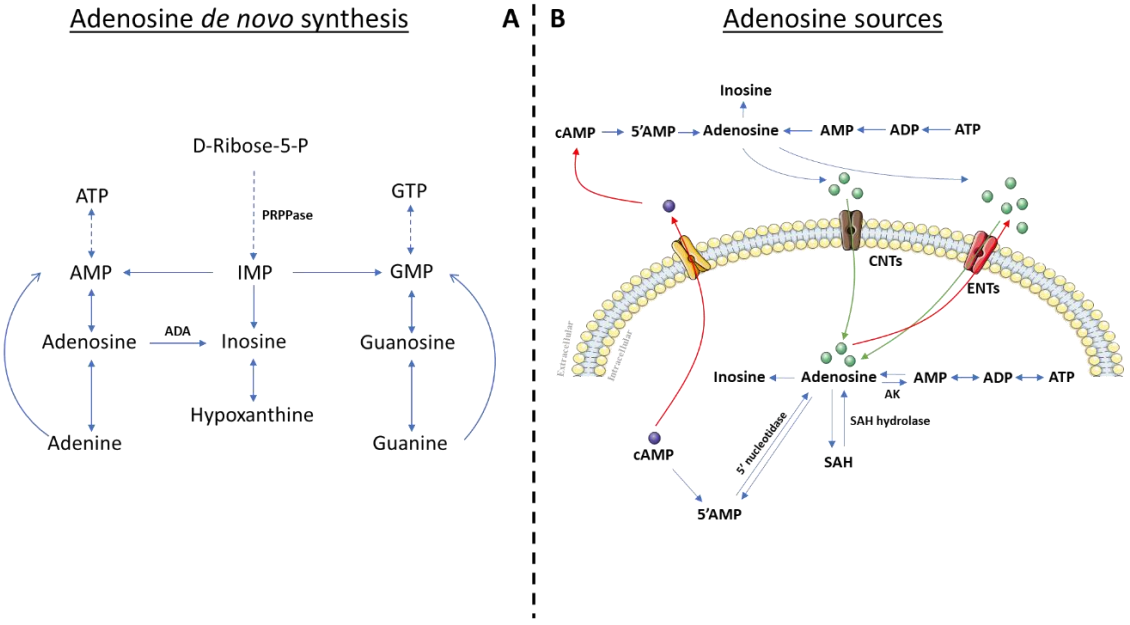


Figure 3. Adenosine synthesis, degradation, and intracellular and extracellular sources. (A) Adenosine is generated de novo from ribose molecule to IMP by PRPP synthase. IMP then is turned into AMP that can be transformed into Adenosine by 5' nucleotidases or into ATP by AK and AMPK kinases. Adenosine can be degraded into Inosine by ADA enzyme. (B) When is not generated the novo the adenosine sources can be adenosine introduced to the cell by CNTs and ENTs transporters, ATP consumption or from the molecules S-Adenosyl-L-Homocysteine and 5'AMP in a reaction carried by SAH hydrolase and 5'nucleotidases.

2.4. Adenosine in heart environment

Adenosine, through adenosine receptors expressed in heart tissue, is involved in many physiological roles such as modulation of vascular tone, heart rate, cardiac impulse generation and conduction (Hori & Kitakaze, 1991; Mubagwa et al., 1996). The adenosine is released from metabolically compromised cells and exerts a physiological control via adenosine receptors to the cellular energy state. In addition, adenosine input can modify the adenosine receptors expression and sensitization in membrane. In example, A_1R adenosine binding leads to a slow reduction of A_1R membrane density and increased intracellular A_1R localization. One way to mediate this can be through adenosine deaminase which can form complexes with A_1R. $A_{2A}R$ is another candidate to desensitize A_1R by reducing A_1R ligand affinity. It can also suffer a rapid desensitization by itself after adenosine input. A_{2B} receptors seems to desensitize and internalize similarly to the $A_{2A}R$, whereas A_3R seems to desensitize much faster than A_1R (Klaasse et al., 2008; Mundell & Kelly, 2011).

3. Adenosine receptors

Adenosine receptors belong to the class A (rhodopsin like family) of GPCRs. There are four types in humans named A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R. ARs are widely distributed along the human body participating in a large variety of physiological responses like pain, inflammation, or vasodilatation (Borea et al., 2018; To, 2001). Each one of these receptors have distinct localization, biochemical characteristics and signalling cascade. The similarity in the sequences is about 49% between the A_1 and the A_3, whereas the A_{2A} and A_{2B} share a 59% of sequence similarity (R. K. Y. Cheng et al., 2017). In addition, A_1, A_{2A} and A_{2B} receptors sequence homology among species is quite high, being the A_1R the most conserved one. The A_1R and the $A_{2A}R$ have higher affinity for the adenosine (nanomolar range) when compared to A_{2B} and the A_3 (micromolar range) (Sachdeva & Gupta, 2013). The structure among the adenosine receptors shows a remarkably similar pattern as all of them are GPCRs. All possess a core domain which cross the plasma membrane seven times (TM); each one is linked by to the next the three intracellular and extracellular loops. The third intracellular loop is the one able to couple with the G proteins (Piirainen et al., 2011). N-extracellular end have glycosylation sites which influences the trafficking to the membrane and the COOH intracellular terminus provides sites for palmitoylation and phosphorylation. A_1R and $A_{2A}R$ seem to have a key role in physiological conditions and the $A_{2B}R$ and the A_3R play a more supportive role. Activation of these two receptors occurs probably when adenosine reaches high concentrations or the main A_1 and A_{2A} receptors are not in an optimal state.

3.1. Adenosine 1 receptor

A_1R as a GPCR is coupled to a G protein by the third intracellular loop. In the case of the A_1R, the G protein is a Gi/o protein, which under activation of the A_1R induces the inhibition of the adenylate cyclase (AC) activity (Merighi et al., 2018). Thus far, at least nine different mammalian ACs have been recognized. All of them share a similar topology of a variable N-terminus end and two repeats of a membrane-spanning domain followed by a cytoplasmic domain. AC is an ATP-pyrophosphate lyase that challenge ATP to cAMP and pyrophosphate. cAMP is a common second messenger used for intracellular signal transduction. cAMP triggers the activation of the protein kinase A (PKA) by binding the specific locations on the regulatory units of the PKA (Das et al.,

2007). This protein is an inactive tetrameric holoenzyme consisting of two catalytic subunits bound to a regulatory subunit dimer, which are the target for cAMP binding, enhancing the PKA activity. PKA has many phosphorylation targets which have an Arginine-Arginine-X-Serine exposed. These targets can be proteins involved in many physiological events such as synaptic transmission, cell growth or contraction. Despite the classical cAMP/PKA pathway, other transduction systems have been described for the A_1R such as the phospholipase C (PLC) or the mitogen activated protein kinases (MAPK) signalling. In PLC pathway by expending GTP as energy source and coupling to a Gq protein PLC-βIII is activated. This generates two second messengers: inositol 1,4,5-trisphosphate (IP-3) and diacylglycerol (DAG), an activator of several PKC isoforms. IP-3 can diffuse through the cytoplasm to the endoplasmic/sarcoplasmic reticulum (ER or SR) where binds the inositol triphosphate sensitive receptor (InsP3R). InsP3R triggers the opening of calcium channels and the release of calcium into the cytoplasm. On the other side, DAG does not diffuse and remains in the plasmatic membrane because of its hydrophobic properties. In the membrane, DAG leads the activation of the protein kinase C which modifies the activity of a bunch of effector proteins. Some of them are MARKS/MAPK, rapidly accelerated fibrosarcoma (RAF) or C-jun N-terminal kinases (JNK), p38, extracellular-signal regulated kinase (ERK), nuclear factor of kappa light polypeptide (IkB), vitamin D3, receptors as calcitriol and many others (Goldsmith & Dhanasekaran, 2007). All these proteins are involved in many different roles depending on the tissue, like contraction, vascular tone regulation, ejaculation, or bronchoconstriction. The calcium released from the ER/SR can also activate through calmodulin activation the protein kinase-C (PKC) (Figure 4) (Merighi et al., 2018).

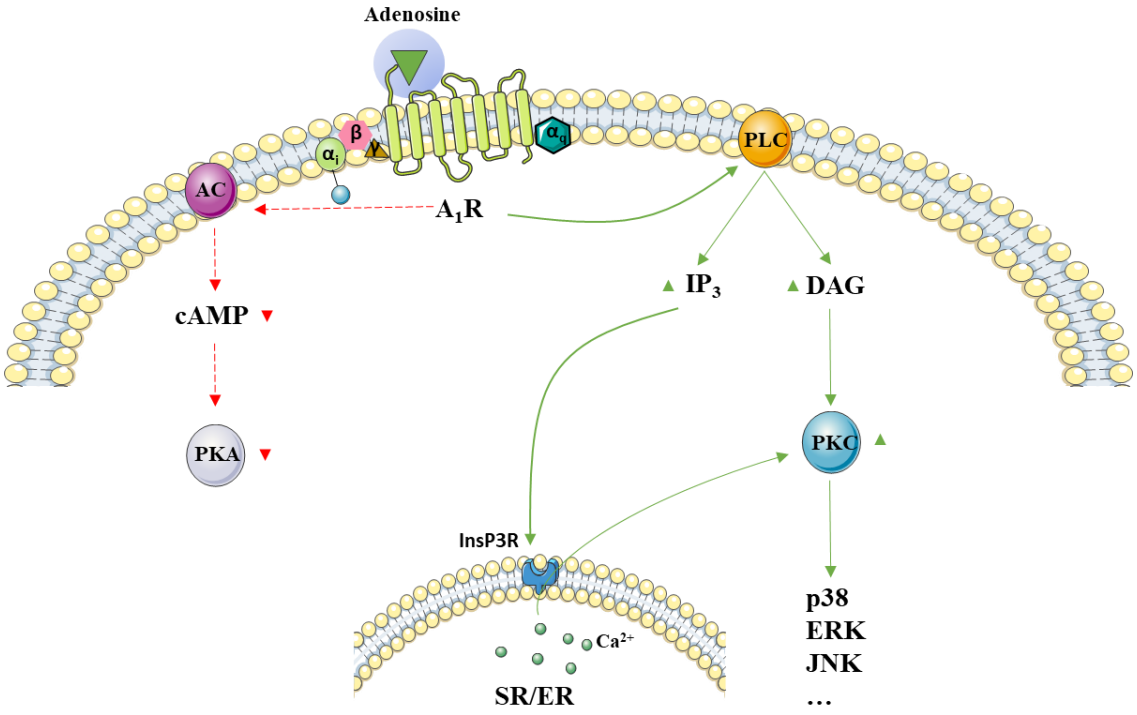


Figure 4. Schematic representation of A_1 adenosine receptor signalling pathways. A_1R activation induce a coupling to Gi proteins which inhibits AC activity and induce no cAMP accumulation and less PKA activation. In addition, it couples to Gq protein, leading the activation of phospholipase C which raises the concentrations of IP3 and DAG, which ends in a PKC increased activity and posterior phosphorylation and activation of many targets like JNK, ERK and p38.

Since A_1R is widespread through the body, the function of this receptor depends on the tissue that is located. In example, in brain regions A_1R activation induce via PLC the modification of ion channels and receptors including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors (Deng et al., 2011; Di Angelantonio et al., 2015). In the hippocampus, it leads the internalization of GluA1 and GluA2 subunits of AMPA receptors. In the reproductive system, the lack of A_1R decrease fertility since is necessary for sperm production (Minelli et al., 2004). In the digestive system, A_1R are involved in glycogenolysis, gluconeogenesis and gastric acid secretion (Christofi, 2008).

Focusing on heart tissue, A_1R displays key roles for the correct function of the heart. In the contraction rhythm, A_1R exerts a slowdown effect. A_1R acts in an indirect way by precluding the activation of PKA and modulating the Gs cycle from the β-adrenergic responses, avoiding the Ca^{2+} release from the sarcoplasmic reticulum. In addition, A_1R inhibits the release of noradrenaline (NA) from cardiac nerves in some situations such as ischemia. This effect will stop cardiac activation during high energy demand and high

adenosine levels episodes (Zhong et al., 2013). Cardiac impulse generation and conduction are also under control of A_1R. When A_1R is stimulated impulse conduction in the heart is slowed. The A_1R signalling inactivates the inward potassium (K^+) and calcium (Ca^{2+}) currents generating a deletion on the impulse generation and a delaying impulse conduction due to the altered ion concentrations. In cardiac pacemaker cells that ionic changes decrease the slope of phase four of the action potential reducing the spontaneous firing rate (negative chronotropy) and slowing the heart rate (Headrick et al., 2011). A_1R can also modify vascular tone and be involved in vasculogenesis, angiogenesis and vascular remodelling. This regulation is essential specially during oxygen supply: demand mismatch, inducing a contraction of the vessels through PLC pathway or by offsetting $A_{2A}R$ activity (Kunduri et al., 2013). A_1R also modulates myocardial substrate metabolism, being important in heart stress conditions such as hypoxia by modifying the glucose and fatty acids availability. The A_1R activation reduces the cAMP accumulation and lipolysis. This reduces the availability of fatty acids and helps to improve cardiac insulin resistance, glucose handling and dyslipemias (Koupenova & Ravid, 2013).

3.2. Adenosine 2_A receptor

Adenosine 2 receptor as well as A_1R is coupled to a G protein, in that case a G_s subunit that promotes the AC activity under $A_{2A}R$ activation by expending GTP. AC triggers the accumulation of cAMP which leads on the activation of PKA. PKA, as described previously, phosphorylates many different targets that are involved in many physiological roles. In example, glutamate and dopamine release in the brain, regulation of the blood flow in the heart or control the heart rate. There are not alternative direct pathways driven by $A_{2A}R$ activation, but the different downstream cAMP accumulation pathways are diverse. Apart from PKA activation, exist a complex signalling that ends on the activation of Erk kinases (MAPK1/3), cAMP response element B (CREB1) factor and the PI3 kinase which activate the protein kinase B (Akt) (Figure 5) (Merighi et al., 2018).

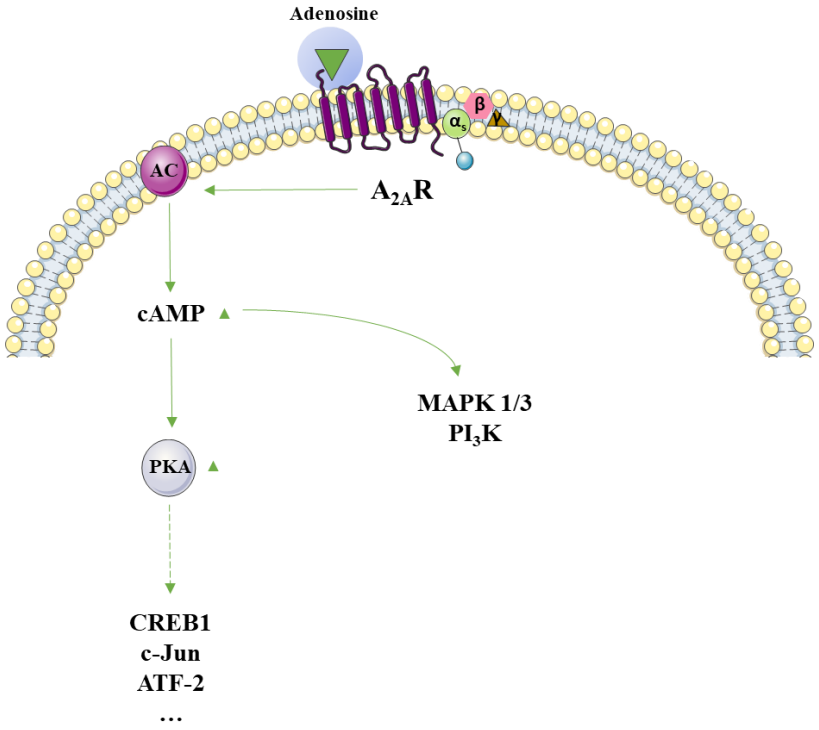


Figure 5. Schematic representation of A_{2A} adenosine receptor signalling pathways. $A_{2A}R$ activation induce a coupling to Gs proteins which stimulates AC activity and induce cAMP accumulation and PKA activation which phosphorylates many targets like CREB1, c-Jun and calcium handling proteins. cAMP accumulation itself induce through cAMP-GEF1 and PDZ-GEF1 the activation of many kinases like MAP1/3 kinases and Pi3 kinases.

The $A_{2A}R$ functions also depends on the tissue where $A_{2A}R$ is expressed. In example, in brain tissue exerts an effect on spontaneous locomotor activity (Collins et al., 2010). In the immune system, $A_{2A}R$ activation inhibits NK cell and efferent T-cells activity, whereas enhances the regulator T-cells activity (Linden, 2011). $A_{2A}R$ also participates in bone homeostasis, where its known to oppose the effect of A_1R by osteoblast activation (Ham & Evans, 2012). In wound healing, $A_{2A}R$ promote matrix production and neovascularization, key roles for healing and tissue repair (Johnston-Cox et al., 2012).

In the heart, most of the $A_{2A}R$ roles are opposite with the A_1R ones. Since one of them induce cAMP accumulation and the other inhibits it, its logic to think that they act in an opposite way. As well as A_1R, $A_{2A}R$ regulate contractility in cardiac tissue stimulating PKA activity and inducing phosphorylation on RyR channels, L-type calcium channels and other calcium regulatory proteins. This modulation triggers the release of Ca^{2+} to the sarcoplasm and leads to the contraction. In the same way and carried by calcium release, $A_{2A}R$ also participates and promotes in the generation and conduction of cardiac impulse (Headrick et al., 2011). In the vascular control, $A_{2A}R$ displays a more important role

compared with the A_1R. It seems to be highly expressed in smooth muscle and endothelium and directly promotes muscle relaxation (not just under O_2 hypoxia conditions). This effect helps on the regulation of coronary and peripheral vascular tone and has a great impact in the blood pressure (Johnston-Cox et al., 2012; C. Sun et al., 2019). Regarding the myocardial substrate metabolism, the hypothesis is that $A_{2A}R$ may increase the glucose and lactate levels in plasma, although more research about it is required (Z. Liu et al., 2017; Maeda & Koos, 2009). Finally, $A_{2A}R$ can also contribute to myocardial remodelling through control of cardiac fibroblast and endothelial and smooth muscle growth and death.

3.3. Adenosine 2_B receptor

In the case of the A_{2B} receptor, the signalling pathways and structure are similar to $A_{2A}R$. They are coupled to a G_s/G_q proteins and under activation of their ligands induce cAMP accumulation and activation of PKA. However, $A_{2B}R$ has a second signalling pathway that includes the coupling to G_q proteins and the PLC pathway. This double role of $A_{2B}R$ (PKA activation or PKC and calcium mobilization through PLC pathway) depends on the type of G protein coupling. The type of G protein is thought to be cell type dependent. This will explain that one and the other pathways seems to have opposite roles in many physiological processes. For example, Gs coupling leads an anti-inflammatory response, whereas G_q is pro-inflammatory (Figure 6) (Merighi et al., 2018).

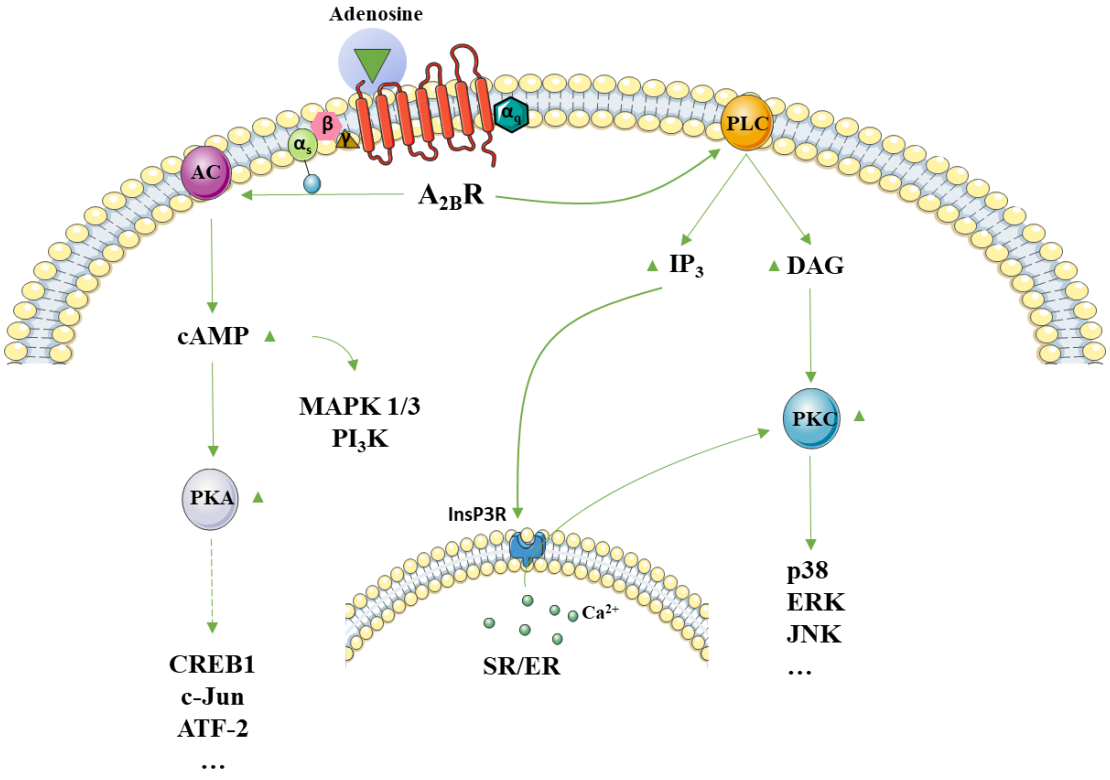


Figure 6. Schematic representation of A_{2B} adenosine receptor signalling pathways. $A_{2B}R$ activation induce a controversial response by a coupling to Gs proteins which stimulates AC activity and cAMP accumulation, PKA activation and phosphorylation of many targets, as well as MAPK and PI3K activation due to cAMP accumulation. In addition, it couples to Gq protein, leading the activation of phospholipase C which raises the concentrations of IP3 and DAG, which ends in a PKC increased activity and posterior phosphorylation and activation of many targets like JNK, ERK and p38, having a dual role similar to A_1 and A_{2A} receptors.

There are multiple roles where $A_{2B}R$ is known to be involved, but it mainly displays a supportive role for the $A_{2A}R$. Even when the major expression for the $A_{2B}R$ is in the vessels, it has been described that immune cells, brain tissue, pulmonary epithelia, intestine, heart, and many other tissues also express $A_{2B}R$. The $A_{2B}R$ expression is relatively low but can be highly influenced by environmental cues such as inflammation or hypoxia (Y. Sun & Huang, 2016). In immune cells, $A_{2B}R$ is related with migration, degranulation and cytokines and growth factors secretion (Haskó et al., 2009). In pulmonary system, is known to display a regulatory role during asthma and chronic obstructive pulmonary disease segregating pro-inflammatory cytokines (Wilson et al., 2009). In brain, is known to protect some brain areas during hypoxia and ischemia. In the intestines, can induce chloride secretion and contribute to colonic mobility, which helps during intestinal inflammation events, constipate or diarrhea (Rajagopal & Pao, 2010). They also play a protective role in kidneys, but that also depends on regulation the vascular tone.

In heart tissue, their expression is especially high in smooth muscle and endothelium, contributing to coronary dilatation (Berwick et al., 2010). $A_{2B}R$ is mainly involved in smooth muscle relaxation, as well as $A_{2A}R$. In addition, they contribute to mesenchymal stem-like cells scar formation in the recovery after surgery (Ryzhov et al., 2014). Finally, $A_{2B}R$ modulates the remodelling of myocardium by control of cardiac fibroblast, endothelial and smooth muscle growth and death (Vecchio et al., 2017). These are especially important in inflammatory processes where it is known to trigger IL-6 production with exerts pleiotropic effects on cardiac remodelling.

However, not many studies about signalling pathways or physiological roles have been done for the $A_{2B}R$. This is probably due to the low expression of the receptor and the lower affinity for adenosine (compared with A_1 or A_{2A} receptors). In consequence, we have a poor understanding (compared with the main adenosine receptors) of the complete functional scheme for that receptor.

3.4. Adenosine 3 receptor

A_3R is the other supporting character among adenosine receptors. It displays a similar signalling with the A_1R, being coupled to Gi/Gq proteins and having both downstream pathways: the PLC and the Gi/cAMP/PKA inhibition ones. In addition to this pathways, A_3R stimulation activates other ones like monomeric G protein RhoA and phospholipase D. Ras homolog family member A (RhoA) is a GTPase protein which activation is involved in actin organization, cell development, cell cycle and transcriptional control, among others. Also, is the responsible to activate phospholipase D, an enzyme that exchanges polar headgroups covalently attached to membrane-bound lipids. This pathway is related with alcohol metabolization, cytoskeletal organization and vesicular trafficking (Figure 7) (Borea et al., 2015).

This adenosine receptor is responsible of many physiological roles in the human body, usually supporting the A_1R activity. In the central nervous system (CNS), it can depress locomotor activity, promote hippocampal neuroprotection against excitotoxicity and have anti-inflammatory effects during high adenosine levels episodes (Stone et al., 2009). In the pulmonary system triggers a pro and anti-inflammatory responses depending on the cell type involved in (Polosa, 2010). Neutrophil immune cells are also strongly affected by A_3R activation through inhibition of the oxidative burst and chemotaxis. It induces anti-inflammatory activity and is related with NK cells activation, monocyte and

macrophage functions and many other events related with immune system (Terayama et al., 2018). Muscle system is also regulated by A_3R, leading a protective response reducing calcium overload in muscle cells. Remarkably, A_3R is also involved in pain where have been observed a nociceptive response and seems to reduce pain in certain animal models (Terayama et al., 2018).

In the cardiovascular system, we can also find A_3R activity. A_3R, as well as A_{2B} receptor, appears to have important and many times protecting functions in the heart. Despite the low expression of A_3R, one of its main effects is to generate a cardioprotective effect against heart ischemia and after that, during reperfusion (Rothermel & Hill, 2008). In addition, A_3R seems to play a role in the modulation of blood vessel tone, inducing contraction when activated (Ho et al., 2016). At the level of myocardial substrate, A_3R seems to partially mirror the A_1R activity by improving glucose uptake in myocardium (Guinzberg et al., 2006). However, further studies must prove it to confirm the evidence for preclinical models.

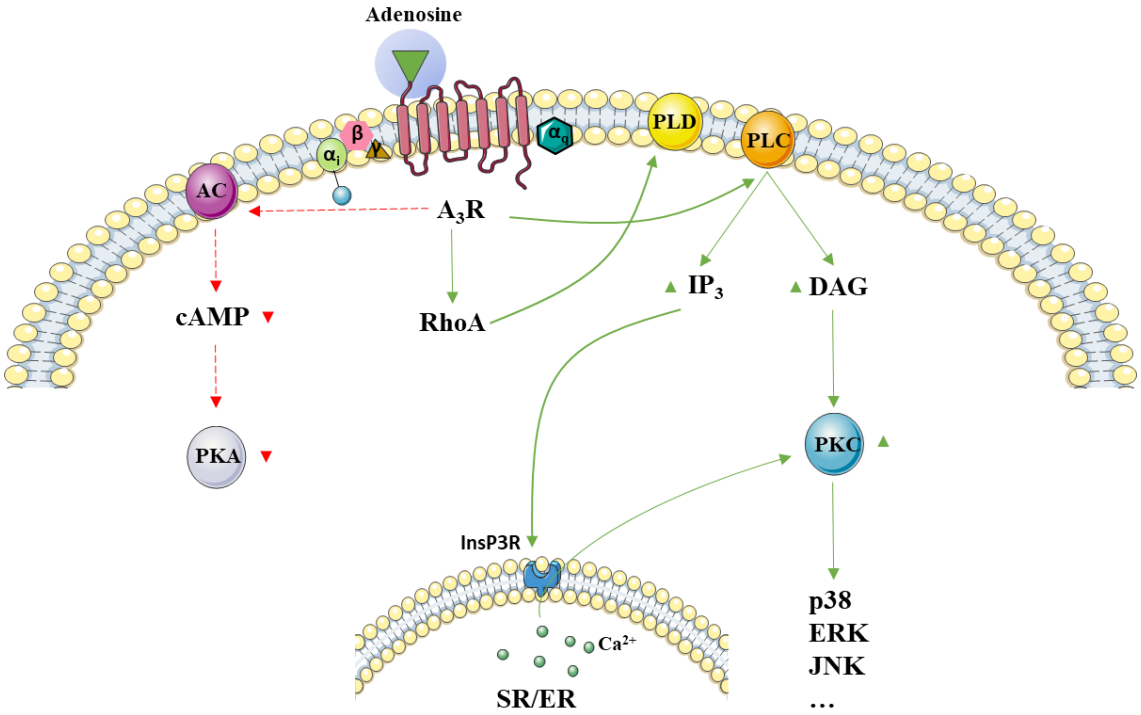


Figure 7. Schematic representation of A_3 adenosine receptor signalling pathways. A_3R activation induce a coupling to Gi proteins which inhibits AC activity and induce no cAMP accumulation and less PKA activation. In addition, it couples to Gq protein, leading the activation of phospholipase C which raises the concentrations of IP3 and DAG, which ends in a PKC increased activity and posterior phosphorylation and activation of many targets like JNK, ERK and p38. A_3R activation also induce G protein RhoA and PLD stimulation.

3.5. Adenosine receptors distribution

As described previously, adenosine receptors are widely distributed through the body, displaying a bunch of different roles. The expression levels and functions depend on the cell type and tissue where they are expressed (Sachdeva & Gupta, 2013). We can find them in central nervous, cardiac, circulatory, respiratory, gastrointestinal, and immune systems and in different organs or tissues including the kidney, bone, joints, eyes, and skin. The A_1R is strongly expressed in striatum, cortex, hippocampus, cerebellum, and thalamus in the brain (A. K. Dixon et al., 1996; Ribeiro et al., 2002). It is also greatly expressed in lung endothelial cells (Gazoni et al., 2010), in macrophages, neutrophils, and monocytes from the immune system as well as in pancreas and adipocytes (Haskó & Pacher, 2012; Hayashi, 2019). The expression levels of $A_{2A}R$ are high in brain (specially striatum and thalamus areas) (Ribeiro et al., 2002), and in peripheral immune cells like platelets, macrophages, neutrophils and lymphocytes (Mills et al., 2012). A_{2B} receptor has a lower expression compared with the other two and can be find mainly in some brain areas such as thalamus, hypothalamus or cortex, vessels and some immune cells like mast cells or lymphocytes (Y. Sun & Huang, 2016). Regarding A_3R, their main presence is also located in brain regions (mostly same as A_1R) (Brand et al., 2001), epithelial, immune, and inflammatory cells (Kumar & Sharma, 2009; Liang et al., 2001; Ren et al., 2014).

Regarding the heart tissue and the surrounding environment, we can find the four types of adenosine receptors. Nevertheless, the expression of each one depends on the area. The A_1R density varies, with a high density in left and right atria. The expression in ventricular myocardium differs but is lower when compared with the atria. A_1R has been also relative high expression levels in coronary arteries and most of the heart vessels as aorta or pulmonary veins. A_{2A} receptors are mainly expressed in atria respect ventricles. They are also especially present in coronary vessels , veins, and arteries, where adenosine exerts a relaxation effect, as well as A_1R is the main path to contract them. A_{2B} receptors as well the A_1R and the A_{2A} expression is widespread in the heart but in a lower level. The higher expression is found in arteries, veins and in the ventricles, where is reported to possibly modify the ventricular function. Finally, A_3R have the lowest levels of expression in heart among adenosine receptors but seems to be more expressed in coronary arteries and other vascular smooth muscle cells (Headrick et al., 2011; Mustafa et al., 2009).

Table 1. Adenosine receptors distribution in heart

Location	A_1R	$A_{2A}R$	$A_{2B}R$	A_3R
Right Atria	++++	++++	+	+
Left Atria	++++	++++	+	+
Right Ventricle	+++	+++	+	+
Left Ventricle	+++	+++	+	+
Aorta	+++	++++	++	++
Pulmonary Artery	+++	+++	++	++
Cava Vein	++	+++	++	+
Pulmonary Vein	+++	+++	++	+
Coronary Arteries	+++	++++	++	++
Coronary Sinus	+++	++++	++	+

3.6. Oligomerization of adenosine receptors

As previously described, GPCRs can form homo/heterodimeric and oligomeric structures between them and other receptors. Adenosine receptors are not different from the rest of GPCRs and can modulate the activity of other receptors by coupling them (Franco et al., 2000). Many examples of dimerization and oligomerization of adenosine receptors have been described in the literature. A_1R can form heteromers with D_2R, which are essential for receptor trafficking (Maggio et al., 2009) and can modulate the affinity for the dopamine of that receptor (Ginés et al., 2000). When coupled to $mGlu_{1\alpha}$, A_1R can prevent glutamate excitotoxicity (León-Navarro et al., 2018). $A_{2A}R$ and D_2R can regulate the function of GABAergic neurons and can form oligomeric structures interacting with cannabinoids receptors or metabotropic receptors (Ferre et al., 2008). Adenosine receptors themselves can form homo and heterodimers, having reports of A_1R, $A_{2A}R$ and A_3 (but not $A_{2B}R$) homodimers or even oligomers and A_1R-$A_{2A}R$ heteromers. They exert a fine-tuning modulation of many processes such as glutamatergic neurotransmission. This regulation depends on the affinity for adenosine and the activation of one or other receptor in physiological conditions (Ferré et al., 2007).

Although of considerable interest, the expression, and roles of endogenous adenosine receptor complexes in cardiovascular cells remain poorly defined. Some studies determined that heteromers between A_1R and δ and κ opioid receptors can be involved in

cardioprotection during ischemia events (Surendra et al., 2013). A_1R and adrenergic β1 and β2 receptors can modify the affinity for the ligands of the β units and ERK1/2 phosphorylation (Chandrasekera et al., 2013). It has also been reported that A_1R and A_3R as well as A_{2A} and A_{2B} receptors can form heteromers, but the function of this structures remains unclear. Also, as in many other tissues, there are no studies about adenosine receptors oligomerization in heart tissue.

4. Heart anatomy and contraction

4.1. Heart anatomy

The heart is the muscle of the body which pumps blood through blood vessels and distribute oxygen and nutrients as well as helps on removing metabolic wastes. Heart anatomy (Figure 8) consists of 4 main chambers, 2 upper ones and 2 lower ones called respectively Atria (left and right, LA and RA) and Ventricles (also left and right, LV and RV) constituted by muscle cells named cardiomyocytes. These cardiomyocytes are single cells that act as a syncytium and are the responsible to contract the heart. There are two circuits where blood circulates: pulmonary and systemic. In the pulmonary, deoxygenated blood travel to lungs via pulmonary artery from the right ventricle and return as oxygenated blood to the left atrium. In the systemic one, oxygenated blood leaves the heart from left ventricle to aorta and is distributed to tissues. Then deoxygenated blood returns to right atrium via cava veins and the cycle is repeated. The coronary arteries that wrap around the heart supply oxygenated blood to the heart (Feliciano & Henning, 1999).

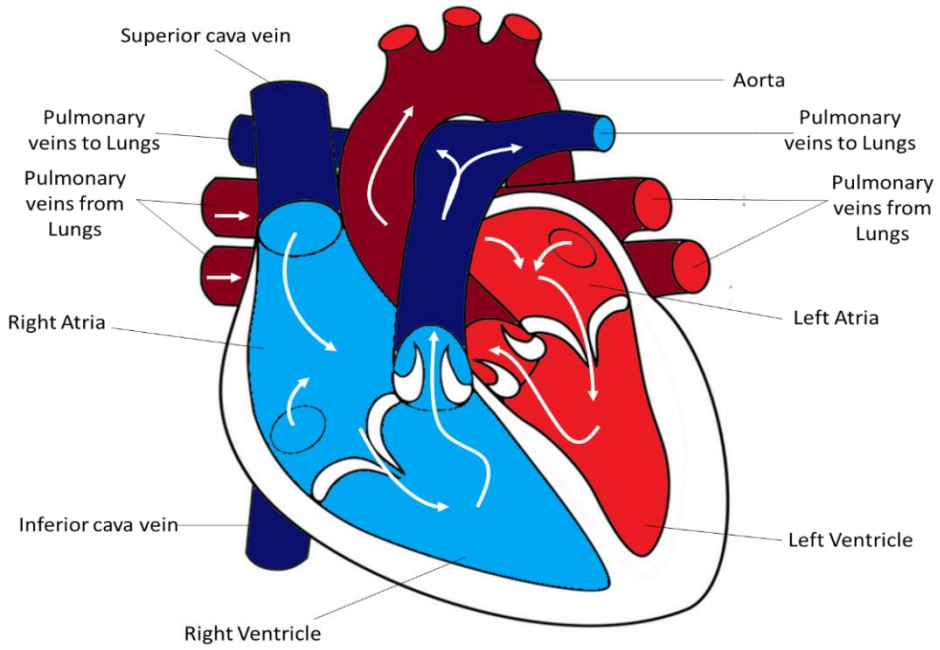


Figure 8. Schematic representation of heart anatomy. Anterior view of heart with atria located superiorly, with pulmonary veins and cava veins providing the oxygenated (red) and not oxygenated (blue) blood. Ventricles are located just down of the atria separated by tricuspid (right) and mitral (left) valves. From ventricles, oxygenated blood is distributed to the body through aorta, whereas non-oxygenated goes to lungs through pulmonary veins. Coronary arteries (not represented) wrap around the heart and provides oxygen to cardiac tissue.

4.2. Cardiac contraction

The cardiac contraction must be precisely regulated to ensure blood is pumped efficiently to the body. First, blood must flow to the atria, contract, and push the blood to the ventricles. Then the ventricles contract and send the blood to the whole body. The contraction starts with an electric signal produced in the sinoatrial node (SAN) which is located in the right atria (Noma, 1996). This electric signalling can be induced by the nervous system which promotes the contraction or can be spontaneously generated by peacemaker cardiomyocyte cells. This cells are mostly located in the sinoatrial node and can spontaneously depolarize the membrane and generate the action potential (AP) (Weisbrod et al., 2016). This action potential is propagated from these original cells to the rest of the heart leading the contraction. Through Bachmann's bundle is propagated to the left atria. Then, it propagates toward the atrioventricular node (AVN) to go through this bundle to the Purkinje fibres and then leads ventricle contraction (Weisbrod et al., 2016). This action potential is produced and conducted by ionic movements through ionic channels present in the membranes (Morad & Tung, 1982). The first phase (phase 0) is the depolarization, mainly characterized by the entry of sodium ions to the cells that fast depolarize the membrane. In peacemaker cells this depolarization is not mainly driven by sodium ions and is carried out by L-type calcium channels (that also contributes later). Then, the sodium channels rapidly inactivate while potassium channels start to open and lets the potassium leave the cell and partially repolarize the membrane potential (phase 1). After this phase the plateau phase starts, where L-type calcium channels contribute to the entry of calcium, that counters the exit of potassium, and the entry of chloride ions. These ionic movements and the activity of sodium/potassium exchanger as well as sodium/potassium pump keeps the membrane potential relatively constant (phase 2). In peacemaker cells this phase is not present and directly goes to the next one. In the last phase (phase 3), the complete repolarization occurs by the closure of the L-type calcium channels. In parallel, a constant flux of potassium leaves the cell. Altogether, these events it leads the cell again to the rest state (phase 4) (Amin et al., 2010; Gonotkov & Golovko, 2013; Mitra & Coller, 2016; Nerbonne & Kass, 2005). Finally, a refractory period starts, where the cell is not able to excite again due to changes in the state of sodium and potassium channels that preclude their activation. Finally, the cycle can be repeated again (Figure 9) (Ednie et al., 2013).

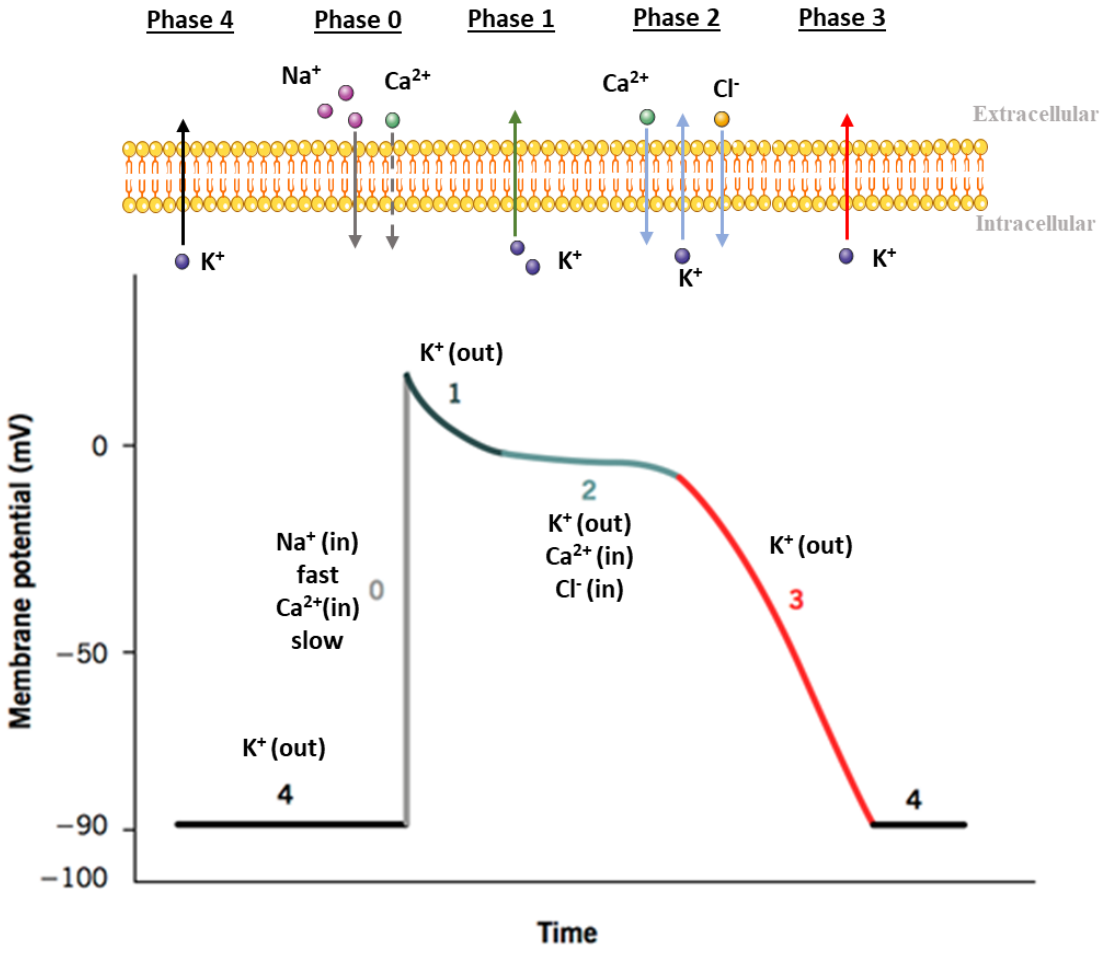


Figure 9. Cardiac action potential and ion movement. Cardiac action potential starts in phase 4 or rest state of the cell with outer K^+ potassium current. Next in phase 0 fast sodium inward current starts to depolarize the membrane with a small influx of calcium ions. At phase 1 opening of potassium channels and closing of sodium ones induce a partial repolarization where potassium is extruded to the extracellular medium . Membrane potential stabilizes at phase 2 by constant calcium and chloride ion inward flux by keeping the potassium ionic movement. Finally, in phase 3 ionic channels are close except the potassium ones which restores the resting membrane potential and left cell ready for a new action potential. Each phase and arrows indicating ionic movement are labelled with different colours: black (phase 4), grey (phase 0), green (phase 1), blue (phase 2) and red (phase 3).

Generation and conduction of action potentials makes the cells contract synchronically and allows to propagate it zone by zone in the correct order. At a physiological level, contraction is carried out by calcium ions. The ionic movements induced by action potential generates a calcium influx to the cell through L-type calcium channels (LTCCs) (Feng et al., 2018; Mangoni et al., 2003). These channels are located close to another protein named Ryanodine Receptor 2 (RyR2) due to T-tubules network invaginations. This receptor is the responsible for the opening and release of calcium from the sarcoplasmic reticulum (SR), the organelle where calcium is stored in the cardiomyocytes. This input of calcium towards L-type channels induce the calcium

induced calcium release (CICR) cycle. In CICR, calcium ions can directly open the RyR2 and lead the massive releasement of calcium from the SR and trigger the contraction (Endo, 2009; Y. S. Lee & Keener, 2008). The contraction initiates when calcium binds to cardiac troponin C, which moves the tropomyosin away from the actin binding site thus exposing it to the myosin heads. Myosin heads can then bind to actin and by expending one ATP molecule induce a power stroke that makes myosin pivots, pulling the actin and causing contraction. These can be repeated as long as the cell has enough ATP and calcium supply (Figure 10) (Lehman & Craig, 2008; Wakabayashi, 2015).

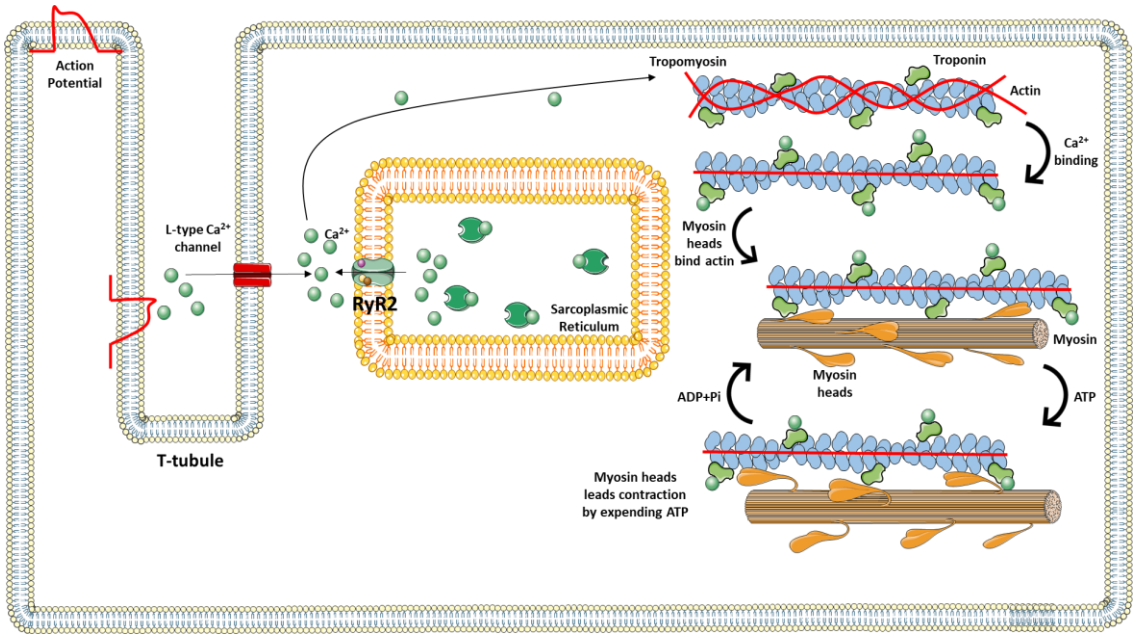


Figure 10. Schematic cardiac contraction cycle. Action potential (in red) induce the opening of LTCCs which triggers the entry of calcium that activate RyR2 receptors through CICR cycle and leads a huge calcium release from SR. Calcium then can bind to subunit C of tropomyosin, leading the conformational change that allows myosin heads to interact with actin. By expending ATP myosin can contract actin fibres. This cycle is repeated as much calcium and ATP is available. Calcium handling then restores the normal calcium levels, and the contraction stops. RyR2, ryanodine receptor 2, LTCCs, L-type calcium channels, SR, sarcoplasmic reticulum.

4.3. Calcium homeostasis

As mentioned before, the key for the contraction are ATP and calcium. ATP is restored constantly by the cell due to the role in almost all the energetic processes. Thus, calcium handling becomes a crucial step for the correct heart functioning (Fearnley et al., 2011; Griffiths, 2000). The levels of calcium inside of the cardiomyocyte are regulated mainly by two different ways: the NCX exchanger and the SERCA2/ATPase bomb. On the one

hand, during diastole calcium is returned to sarcoplasmic reticulum through SERCA2A/ATPase bomb (Kiess & Kockskämper, 2019), which expends 1 ATP to pump 2 Calcium molecules. On the other hand, calcium can be extruded to the extracellular medium by the sodium-calcium exchanger (NCX), which extrudes three sodium ion molecules for one of calcium (Sathish et al., 2011). Inside of the SR, the calsequestrin (CSQ) protein binds to the calcium until release. SERCA2A/ATPase activation is regulated by phospholamban (PLB). When phosphorylated by phosphokinase-A (PKA) or calmodulin (CaMKII) proteins, PLB can phosphorylate then SERCA2A and becomes active. PKA can also phosphorylate other proteins such as RyR2 or LTTCs, opening the channels for the calcium. PKA is directly under control of adenosine and adrenergic receptors, becoming one of the first steps in the calcium handling process (Figure 11) (Van Der Velden et al., 2000).

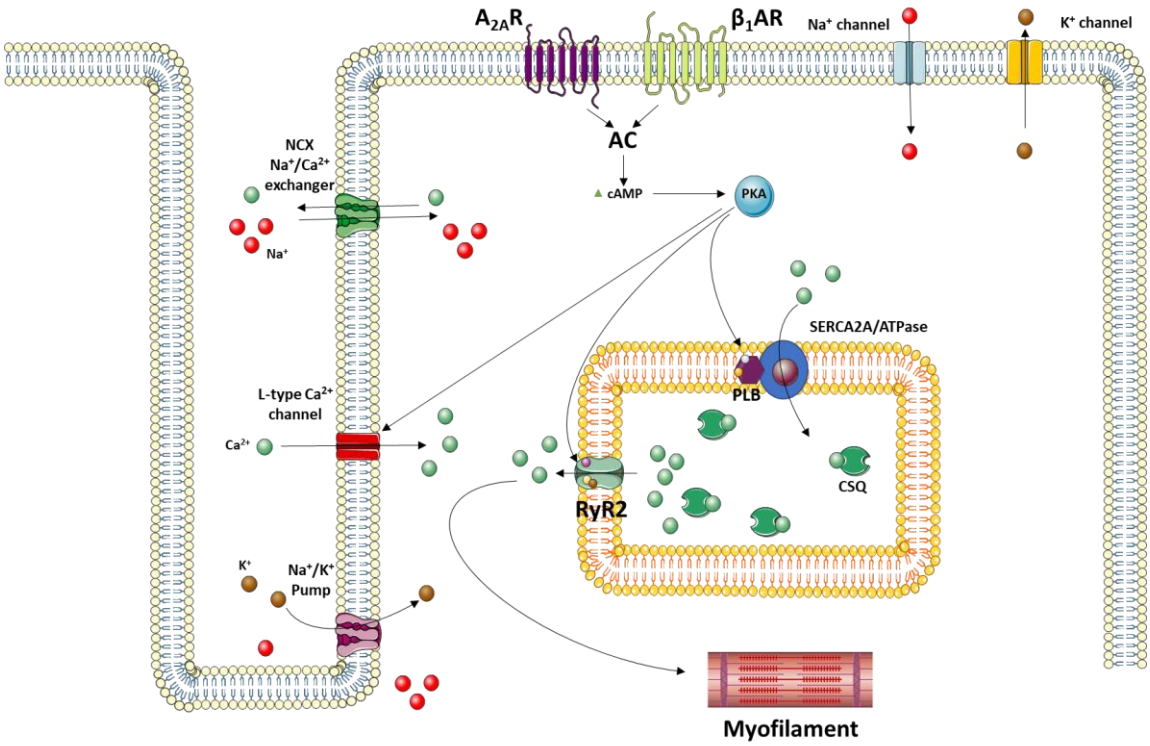


Figure 11. Calcium handling. Schematic representation of the network for calcium handling. Calcium is stored in SR by SRCA2A/ATPase bomb, which is under control of phospholamban (PLB) protein. Inside of the SR, calcium is attached to calsquestrin (CSQ) and remains there until released through RyR2 receptors. Calcium can also be regulated by NCX membrane exchanger which extrudes 3 sodium ions and introduce 1 calcium one. LTCCs, RyR2 and PLB are regulated by phosphokinase A which is activated by Gs coupled proteins like adenosine 2_A receptors.

4.4. Cardiac diseases

Heart diseases are diverse, they affect different parts of the organ and are originated by many different reasons. First, some are related with malformations present since birth, also called congenital heart diseases. This ones generate problems in the septal zone or can obstruct one or more chambers or vessels in the heart (R. R. Sun et al., 2015). Second, some tissues, like arteries, can be damaged or sick like arteries where cholesterol is deposited in plates and narrow them. Another possibility is the dilatation or thickening of the chambers, which makes difficult the pump of the blood through the body (Mathew et al., 2017). Heart can also directly stop working correctly or stop beating, like in heart failure or myocardial infarction (Inamdar & Inamdar, 2016; Saleh & Ambrose, 2018). Finally, heart cannot beat in the correct way, being altered the rhythm and developing an arrythmia. The origin of the arrythmias is usually the abnormal generation or conduction of action potentials (Austin et al., 2019). Arrythmias can be classified by rate in tachycardias (if the beating is faster than usual), bradycardias (if the beating is slower than usual) and fibrillations (if the beating is irregular). They can be also classified depending on the mechanism, duration, or site of origin. Regarding the last one, we have atrial, junctional, ventricular, and atrioventricular arrythmias. One of the most common arrythmias and heart diseases in the world is the Atrial Fibrillation (Benjamin et al., 1998).

5. Immune system

The immune system is a defense system within an organism that detects and neutralizes aberrant macromolecules. It also promotes their removal, being a powerful tool for evolutionary success and becoming a shield against other organism invasions (Nicholson, 2016). However, immune system is part of a complex network that regulates the correct functioning of the body. It is involved in other processes apart from protection against other organisms, and sometimes its harmful for the organism itself like autoimmune responses or immune reactions that provokes tissue damage (Godwin & Brockes, 2006; L. Wang et al., 2015). For that reason, understanding the complete role of the immune system is crucial for diagnose and treatment of many diseases. There are many roles described in different tissues in physiological and pathophysiological conditions. For example, in the brain it is known to remove neurons and synapses during development, being crucial for brain plasticity. It can also induce autoimmune attack, it is related with anxiety, offers neuroprotection in neurodegenerative diseases and many others (Mcgeer et al., 1989; Nautiyal et al., 2008; Tanabe & Yamashita, 2018; Tansey & Romero-Ramos, 2017). In the skin, it is related with the progression of skin cancer and psoriasis (Lowes et al., 2014). In the lungs, it can promote the progression of chronic obstructive pulmonary disease (COPD) and fibrosis (Bhat et al., 2015) through cytokines released from macrophages (L. Chen et al., 2018). This occurs when immune cells recognize a threat such as other organism or damaged tissue and release molecules to trigger a huge response from the immune system. This response is mainly based on microcirculatory events that include vascular permeability changes, leukocyte recruitment and releasement and accumulation of inflammatory mediators. This event is a common pathogenesis in many diseases such as cardiovascular diseases, diabetes, cancer, and arthritis (Schett & Neurath, 2018).

5.1. The Immune cells

The whole immune cells comes from a multipotential hematopoietic stem cell which can differentiate in a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP) (Donahue & Chen, 2003) (Figure 12).

The CMP can differentiate in four different cell types. The first one is the megakaryocyte, which will become thrombocytes or platelets, chiefly involved in inflammation, wound repair, and thrombosis, among others. Secondly, erythrocytes, which are the most common ones, and whose major function is to distribute oxygen to tissues. Thirdly, mast cells, that are related with dilatating blood vessels, inflammation, cell recruitment and wound healing. Finally, myeloblast which turns into basophils, neutrophils, eosinophils, and monocytes, that are involved in inflammation, cell recruitment, direct kill/inhibition of invasive organisms and allergic reactions. Monocytes can also differentiate again in two different cell types. The first type are the macrophages, which can phagocyte pathogens and cancer cells and stimulates the response of other immune cells. The second ones are dendritic cells, that presents antigens on its surface and triggers adaptative immunity (Donahue & Chen, 2003; Nahrendorf, 2018).

The CLP is the progenitor for all the lymphocytic cells which are the Natural Killer cells (NK), the T-lymphocytes and the B-lymphocytes. NK cells are mainly involved in cytotoxicity, by killing tumour and virus-infected cells. T and B cells participate in cell-mediated immunity. T-cells can subdivide in many groups being the most relevant the T-CD4 and the T-CD8 cells. This division depends on the expression of CD4 or CD8 on their surface. The function of $CD4^+$ cells are mainly to secrete cytokines that regulate and assist the immune response and the inflammatory process. They can also aid the differentiation and antibody production by B-cells and participate in defense against parasites and other pathogens. $CD8^+$ ones or cytotoxic T-cells directly supress virus-infected and tumoural cells. B lymphocytes have one major function and its antibodies production. B-cells can be activated by T cells mostly but can also activate independently by certain polysaccharides and DNAs. Once activated, B-cell can differentiate into a plasmacyte and produce antibodies against one specific antigen. B-cells mediate in other functions such as inflammation, tumour development and immunity, tissue rejection,

wound healing, tissue organization and neogenesis and autoimmunity (Donahue & Chen, 2003; Ingulli, 2010; Petersone et al., 2018; Yuen et al., 2017).

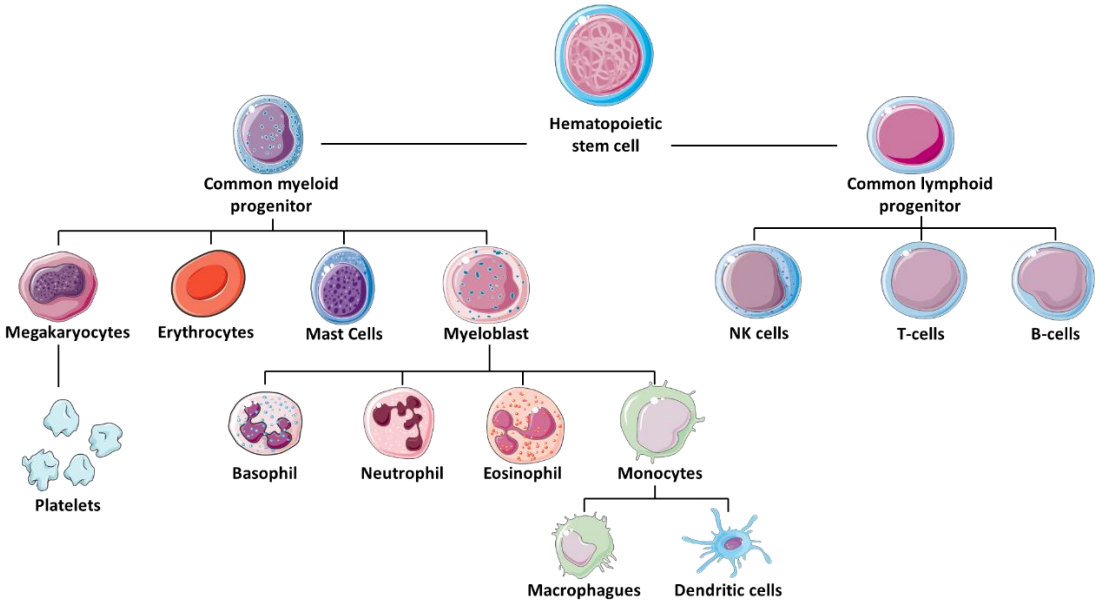


Figure 12. Main cells of immune system. Hematopoietic stem cells can differentiate in the myeloid and lymphocytic progenitors which will differentiate in the rest of the cell subtypes. Myeloid progenitor differentiates in megakaryocytes, erythrocytes, mast cells and myoblast, which will generate basophils, eosinophils, neutrophils, and monocytes which can turn into macrophages and dendritic cells. Lymphoid progenitor can divide in all the lymphocytic subtypes, mainly NK cells, T-cells, and B cells and all their subtypes.

5.2. Homing

Homing is the process by which lymphocytes leaves circulation and extravasate in a specific tissue (Ullah et al., 2019). This cell trafficking is crucial for many physiological roles such as inflammation or wound healing, where many types of immune cells are needed. The process is mediated first by selectins, which lead the tethering and rolling on the endothelium. This approach to the endothelium allows the cells to contact to chemokines displayed on the surface. This triggers integrin activation leading the arresting of cells in the endothelium. Then can transmigrate to the site of inflammation in response to chemotactic gradients (Figure 13). Each tissue releases different kind of chemokines for different type of cells, and many of them shares one or more of these chemokines. For example, lymphocytes are recruited to heart environment by expressing chemokine receptor (CCR) type 4 (CCR4) and CXC chemokine receptor (CXCR) 3 (CXCR3) receptors (Altara et al., 2016). These receptors can bind a huge bunch of

chemokines such as CCL2, CCL4, CCL5, CCL17, CCL22 and the CXCL4, CXCL9, CXCL10 and CXCL11. In addition, CXCR3 is an ubiquitous chemokine receptor for mostly of the inflammatory events in many tissues apart of heart, like brain or kidney (Panzer et al., 2007; Zhou et al., 2019). In skin, CCR4 and CCR10 are the most common ways to do the homing whereas intestine requires the CCR9 (Soler et al., 2003). CCR5 and CCR1 are used in the liver by binding the CCL3, CCL5, CCL7 and CCL23, one of them shared with CCR4 for skin and heart (Ullah et al., 2019).

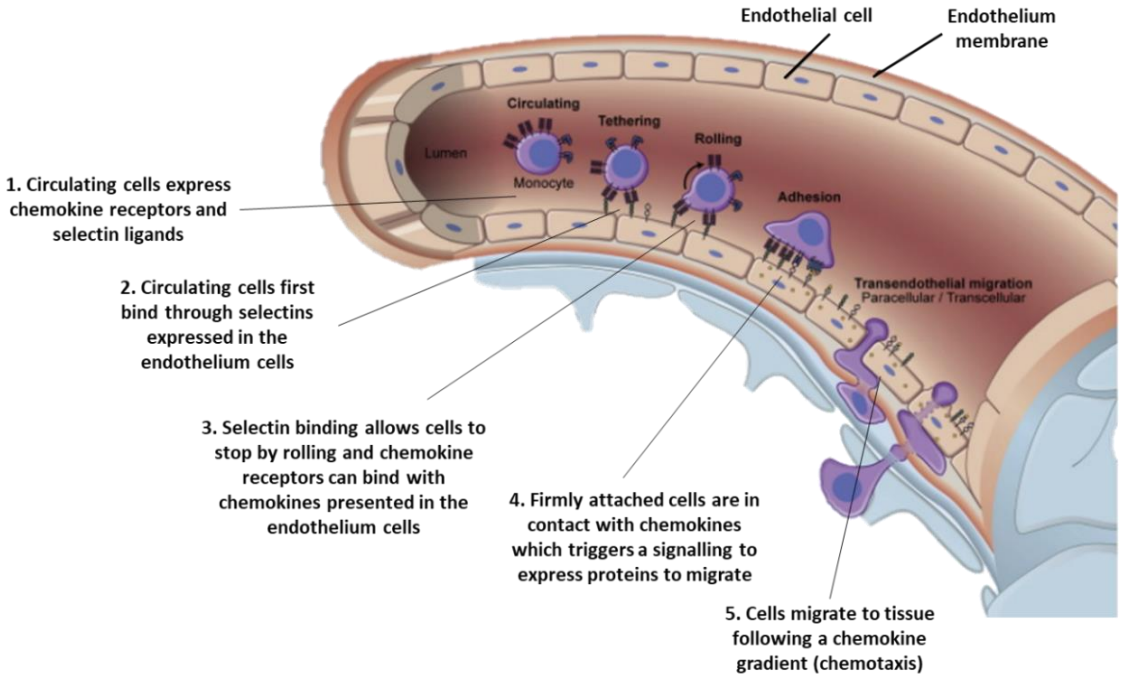


Figure 13. The homing processes. Extravasation to different damaged or inflamed tissues starts by an immune cell expressing chemokine and selectin receptors which will allow cells to partially bind and stop circulating in the blood vessel. Once firmly arrested on the endothelium surface, chemokines presented by endothelial cells will bind their receptors in the immune cell surface which will trigger the signalling to start the extravasation to the vessel. Once the interstitial migration starts, a chemokine gradient will guide the cell to the target tissue. Adapted from Lopes Pinheiro et al., 2016.

5.3. Immune system in heart

In the last years, researchers have identified complex interactions between immune system and heart tissue. It has been described that immune cell are resident in the heart, including lymphocytes T and B, neutrophils, and most of the rest of leukocyte cells. Among the non-leukocytary population, macrophages and a small presence of other dendritic-like cells have been found in heart environment (Bönner et al., 2012; Harari et al., 2017). These populations have a role not just during injury or damage state but also

during many other events. Some physiological roles that are involved in are cardiac homeostasis, development during embryogenesis, conduction and generation of cardiac action potential, changes produced by age or cytokine dysregulation. However, they also play a role during heart diseases, becoming a potential tool to work with in diagnosis and treatment of this disorders. In example, in Myocardial Infarction some immune cells are mobilized: mast cells release the granules and macrophages starts the cytokine production to induce inflammation. These events recruit monocytes and neutrophils to the heart tissue. B-cells are recruited to heart zone and induce the mobilization of proinflammatory monocytes, potentiating the inflammation effect. T-CD4 mimics the effect of B ones but not T-CD8, which drives anti-inflammatory responses and induce a cardioprotective effect. All these cells induce a huge inflammatory response, eliminates the damaged tissue, and remodels the extracellular matrix to heal the injury (Alwi, 2019; Gentek & Hoeffel, 2017; Latet et al., 2015; Saparov et al., 2017). In Myocarditis, its known that neutrophils and monocytes can infiltrate to heart. The concrete function is still unknown but presumably they induce inflammation and control viral infections via specific T and B cells. A similar process occurs in endocarditis, with the difference of the necessary macrophage intervention to recruit the leukocytary populations (Vdovenko & Eriksson, 2018). In Atrial Fibrillation, macrophages tends to accumulate in the atria leading the structural and electrical atrial remodelling (Z. Sun et al., 2016). Neutrophils increases the inflammatory response and induce atrial remodelling by releasing cytokines like interleukin 6 (IL-6), tumour necrosis factor alfa (TNF-α), metalloprotease type 2 (MMP-2), myeloperoxidases and reactive oxygen species (ROS) like hypochlorous acid (Friedrichs et al., 2014). Mast cells induce inflammation and atrial fibrosis by platelet-derived growth factor (PDGF-A) (Liao et al., 2010). T-CD4 lymphocytes are known to infiltrate in the left atrium of the heart and induce the expression of hypertrophic genes by activating calcineurin-nuclear factor. These genes enhance the efficacy of macrophages altering the conduction/generation of action potential and promotes the inflammatory process. B-cells can activate β-adrenergic receptors by producing antibodies against them. That induces a larger intra atrial activation times, shortly refractory periods and increasing arrhythmogenesis (Y. Liu et al., 2018).

6. Atrial Fibrillation

6.1. History

Atrial Fibrillation is one of the most common arrhythmias in the world, characterized by rapid and disorganized electrical activation of the atria. This leads to an uncoordinated contraction that affects 0.12%-0.14% of those younger than 49 years old, 3%-7% in people up to 65 years old and 8%-10% of octogenarians or older (Zoni-Berisso et al., 2014). In addition, it exists a gender-dependent effect, affecting more frequently in males than females with a 1.2:1 ratio. However, we have to consider that women represents the bulk of patients with AF due to their longer survival (Aggarwal et al., 2015; Michelena et al., 2010). In addition, AF has an estimated prevalence of 1.5-2% and is expected to double in the next decades, becoming a global medical challenge (Lippi et al., 2021).

The first documents associating an irregular pulse and mitral stenosis were first reported by Robert Adams in 1827. It was not until the 20th century when William Einthoven invented the electrocardiography (ECG) that allows to detect and record the Atrial Fibrillation events. Since that moment, the pathogenesis, and the impact in society of this disease gained appreciation until the 90s. In that moment, the epidemiological data on associated risk factors and clinical outcomes became critical. Nowadays, the investigation of AF prevention, diagnosis and treatment is increasing year by year.

6.2. Etiology of AF

The etiology of AF is still unclear. AF is characterised by an irregular and rapid heart rate due to the abnormal electrical signals in the upper chambers (atria) of the heart (X. Liu et al., 2019). That abnormal electrical signalling can be provoked by abnormalities or damage in the heart's structure. These can be generated by high blood pressure, heart attack, coronary artery disease, abnormal heart valves (congenital), overactivity in the thyroid gland, exposure to stimulants like caffeine in high doses, lung diseases, heart surgery, viral infections, sleep apneas and many others (Bosch et al., 2018; Brandes et al., 2018; Zimetbaum, 2017). However, some people spontaneously present AF without any damage or abnormalities in heart structure. There are also many risk factors that triggers AF events. Among them, alcohol, obesity, age, and family history are the most common ones (Brandes et al., 2018; Chang et al., 2017; Wasmer et al., 2017). Regarding the family

ascendants and the heritable condition of AF, some genetic factors must be considered. Potassium voltage-gated channel 1 (KCNQ1) gene was the first gene associated with familiar atrial fibrillation (Lundby et al., 2007). It affects the potassium ionic channels in the cardiomyocyte membrane and difficult the maintenance of the heart's normal rhythm. In general, genes involving potassium currents have been associated with AF, but also in sodium and calcium channel proteins. In addition, mutations concerning the inward sodium current, laminins, nuclear pore complex, connexins, or natriuretic peptide have been also linked to AF (Kalstø et al., 2019; Lubitz et al., 2010).

6.3. Pathophysiology of AF

The primary symptom in Atrial Fibrillation is an arrythmia produced principally due to focal ectopic firing and reentrant activity. When the contraction stimulus starts in an abnormal place is known as ectopic focal activity. That can be transient, ending in an isolated extrasystoles or self-limited tachycardias, but can contribute to the progression of AF. The reentry occurs when the waves promoted by focal or normal firing are sustained in time and induce constant and abnormal contractions in the heart (Czick et al., 2016; Wakili et al., 2011). AF is usually also classified in paroxysmal (when are self-terminating) or persistent (which ends with clinical intervention). When the duration increase, AF become long persistent and is usually permanent. The local firing usually promotes paroxysmal atrial fibrillation whereas reentry tends to produce more persistent forms.

6.3.1. AF initiation: Focal ectopic firing

Ectopic activity is understood as a disturbance of the cardiac rhythm frequently related to the electrical conduction system of the heart. Beats arise from fibres or group of fibres outside the region in the heart muscle ordinarily responsible for impulse formation: the sinoatrial node (SAN). Then, instead of contracting auricles and then ventricles, the contraction can start in another chamber (i.e., ventricles) or when the contraction cycle is not finished (Figure 14). The mechanism underlying the ectopic activity are not completely understood but all depends on the imbalance between inward and outward currents during phase 4 of action potential (Heijman et al., 2018). If calcium or sodium inward are increased, leads a spontaneous phase 4 depolarization that can reach the threshold and cause ectopic firing. Focal activity can also be produced by afterdepolarizations, subdivided in early (EADs, before phase 3) or delayed (DADs, after

full repolarization). The EADs are produced when the action potential is excessively prolonged. This is probably caused by L-type calcium currents (LTCaCs) . LTCaCs may have time enough to recover from the inactivation and carry an inward Calcium current to generate the EADs and promote the extrasystole. DADs are originated by calcium leak from the Sarcoplasmic Reticulum by the Ryanodine Receptors, leading the cardiomyocytes contraction. Then, SERCA2A/ATPase bomb reintroduce calcium in the Sarcoplasmic reticulum, inducing the relaxation of the cell. Also, the NCX exchanger removes the excess of cytosolic calcium, generating a depolarization and producing the DAD (Nattel et al., 2008; Nattel & Dobrev, 2012, 2017). RyR mutations, which typically cause catecholaminergic polymorphic ventricular tachycardias, also promote DAD-related AF (Nattel & Dobrev, 2016).

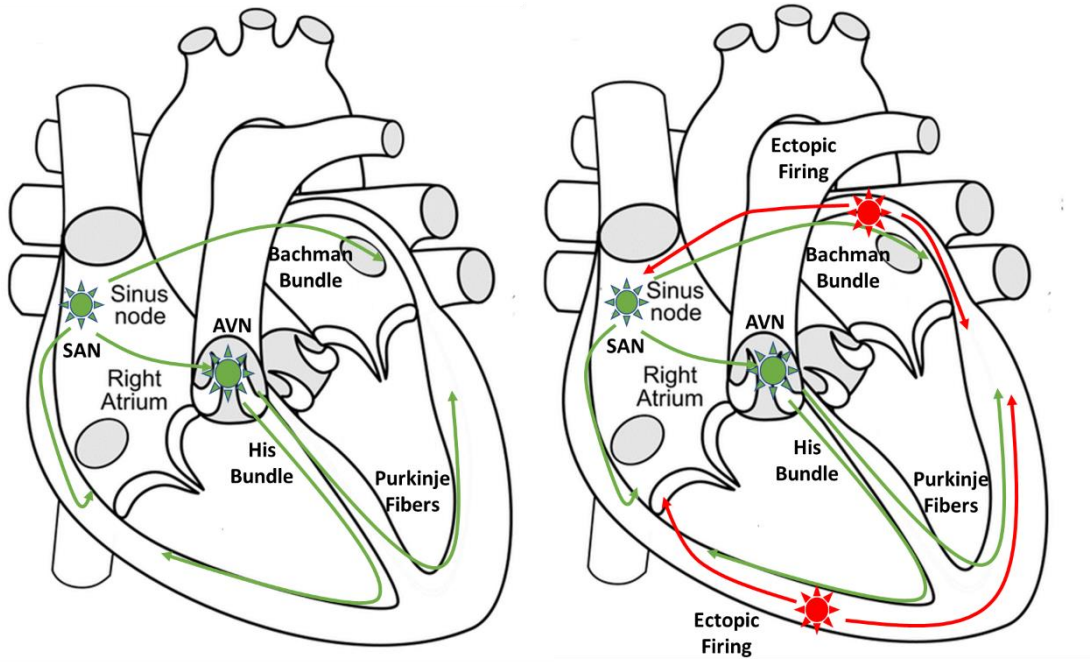


Figure 14. Schematic representation of ectopic firing. In green we have the generation and conduction of the normal electric signals, which starts in the sinoauricular node and propagates to contract first the atrias and then, going through the Bundle His and the Purkinje Fibbers, the ventricles. In red we can observe when the signal is not generated in the correct place and then propagates to the different chambers of the heart, generating an abnormal beating.

6.3.2. AF perpetuation: Reentry

The reentry can be explained by 2 different hypotheses. The first one is the leading-circle model, where the waves are reentrying around a central zone that is continuously activated by the activation wavefront (Waks & Josephson, 2014). The second model is the spiral-wave model, postulating that reentry is maintained by tissue excitability properties, which determines the period, excitability, and size of the wavefront. The electrical and structural remodelling characteristic of the AF contributes to the reentry generating longer conduction pathways for reentry. Tissue fibrosis slows conduction, makes conduction heterogenous, and create barriers that favours the reentry circuits that can sustain AF. For perpetuation of functional reentry, the propagating wavefront must complete one circus movement. In addition, this have to be done in a time period long enough for atrial tissue within that circuit to recover excitability (refractory period, ERP). Thus, slow conduction velocity and a short ERP promote reentry. Then, the shorter the wavelength is, larger is the number of simultaneous reentry circuits the atria can accommodate in. Increasing wavelengths reduces the number of possible circuits and the possibility of sustaining AF (Figure 15) (Cheniti et al., 2018; Iwasaki et al., 2011).

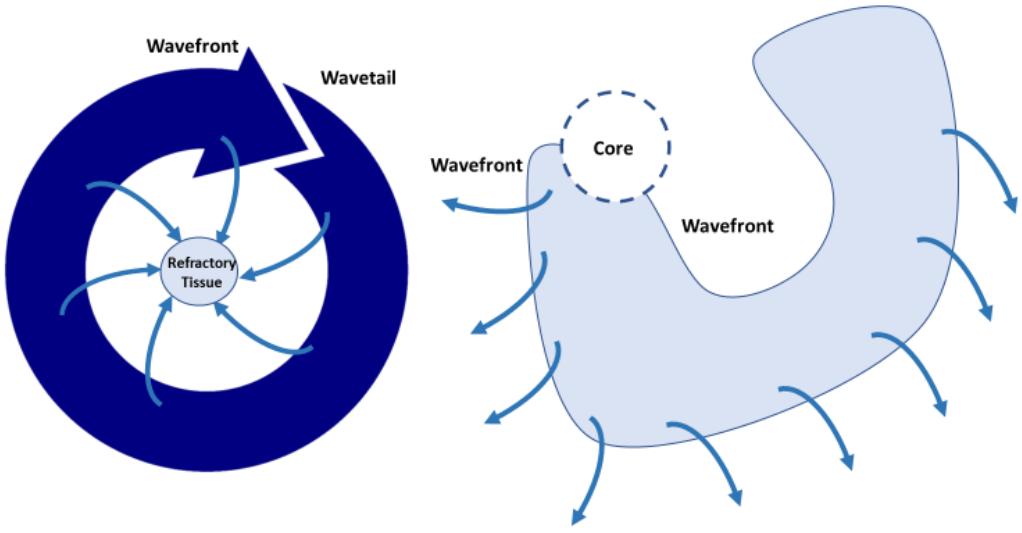


Figure 15. Models of AF perpetuation. (A) circle model of AF perpetuation that postulates that reentry is done around a central zone and is continuously activated by centripetal waves from the reentring wavefront (B) the spiral-wave model postulates that reentry is determined by excitability and wave curvature, determined by tissue excitability properties.

6.3.3. AF calcium handling

As described before, during the systole calcium enters in the cardiomyocytes through L-type voltage channels (LTCCs). That triggers the calcium induced calcium release (CICR) through the Ryanodine-2 receptors (RyR2) located in the Sarcoplasmic Reticulum (SR). During diastole, calcium is returned to Sarcoplasmic reticulum through SERCA2A/ATPase bomb, which expends 1 ATP to pump 2 calcium molecules. Calcium can be also extruded to the extracellular medium by the NCX exchanger, that exchanges 3 sodium ion molecules for 1 of calcium. Inside of the SR, calsequestrin (CSQ) protein binds to the calcium until release. SERCA2A/ATPase activation is regulated by phospholamban (PLB). When phosphorylated by phosphokinase-A (PKA) or calmodulin (CaMKII) proteins, SERCA2A becomes active. In addition, RYRs can be phosphorylated too to enhance their activity. Altogether means that AR and Adrenergic receptors play a key role in the calcium handling. When calcium homeostasis is lost, beating rhythm can be altered, prompting an arrythmia like AF (Ai, 2015; Denham et al., 2018; Dobrev & Nattel, 2008; Heijman et al., 2012; Yeh et al., 2008). In AF literature is described that adenosine receptor A_1 (Li et al., 2016) and A_{2A} (Llach et al., 2011) altered expression is linked to AF. This is probably due to the effect on AC activity and consequently the phosphorylation of PLB, RyR and LTCCs which will promote loss of calcium handling. Mutations or malfunctions in LTCCs, CSQ, SERCA and PLB have been also reported to induce AF or other cardiac diseases . The loss in the ability to control the restoring of calcium and the entry through membrane promotes the arrythmia. This similarly happens with NCX failure or overexpression, which is also reported to be linked with AF (Pott et al., 2011).

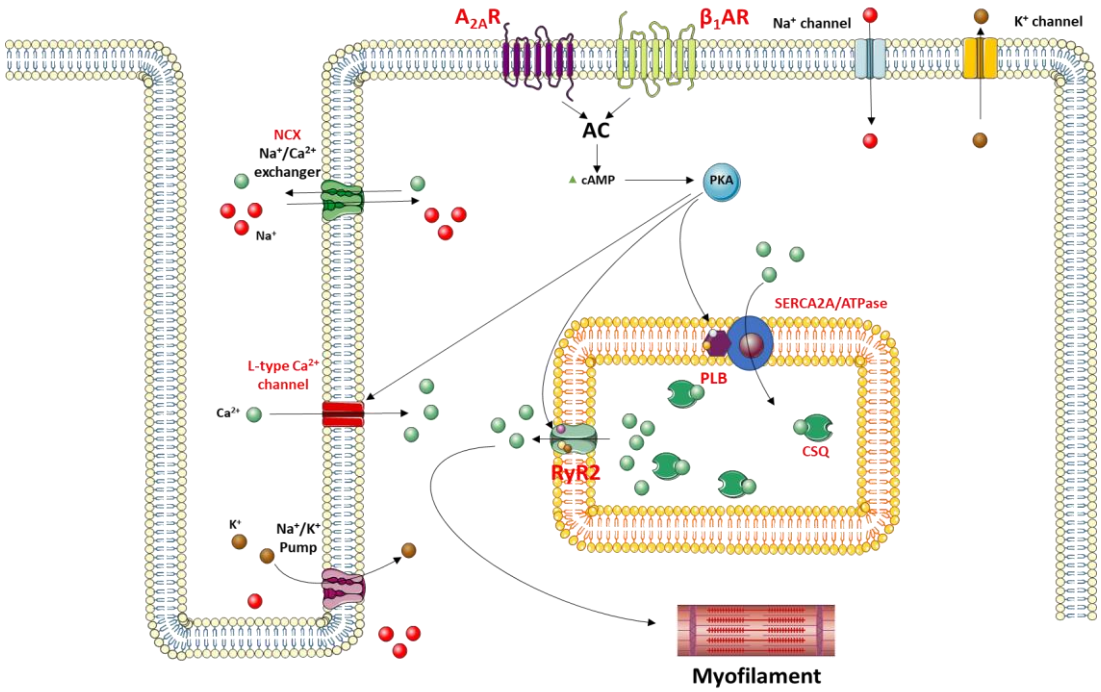


Figure 16. Loss of Calcium handling in AF. Calcium homeostasis is crucial for the correct heart beating. Loss of calcium handling can be one of the main problems during AF. Malfunctioning of the proteins labelled in red can induce the arrythmia: SERCA or PLB malfunctioning will not restore the calcium to the SR; CSQ related problems will not bind calcium in SR and this can be easily released to cytoplasm; RyR2, LTTCs and NCX dysregulations leads abnormal calcium amounts in the cytoplasm that will trigger contraction of cardiomyocytes. In addition, most of those proteins are under control of PKA which will be overactivated if adrenergic or adenosine 2_A receptors are overexpressed or overactivated; this will lead a higher level of phosphorylation in most of those proteins, generating a cascade that will finish in loss of calcium homeostasis and can generate AF.

6.4. Animal models in cardiac diseases

Animal models contributes to the study of most of the diseases in the world. For the study of the AF many animal models have been used: goat, dog, pig, guinea pig, rabbit, and mice (Nishida et al., 2010). If we aimed for an ideal animal for AF experimentation, we would look for concrete characteristics for that model. It should share a similar human heart anatomy and electrophysiology, allow easy housing and breeding at not awfully expensive cost. It also has to be possible to diagnose and operate similar to the humans and precise genetic modifications to generate customized animal models. None of the aforementioned models combine all the desired characteristics. However, pigs possess higher similarities to humans that makes them a good choice in the study of AF and most of heart diseases (Camacho et al., 2016a; J. A. Dixon & Spinale, 2009; Spannbauer et al., 2019). They possess a long lifespan (10-15 years), adult mass similar to humans, similar body temperature and heart:body mass ratio (0.4 vs 0.32). We have almost identical blood

pressure, heart rate, electrocardiograms, blood chemistry. When compared, we observe a similar cardiac anatomy (size, coronary anatomy, and circulation) and similar electrophysiology of the heart. Furthermore, all seasonally breeding with a large litter size and genetically modified animals are available and easy to handle. For that reasons, pig animal model is one of the most desirable model to study AF disease precisely (Clauss et al., 2019; A. M. Lee et al., 2016). Nevertheless, mice or rabbit can be a good choice for a first analysis or drug testing (Camacho et al., 2016b) (Figure 17).

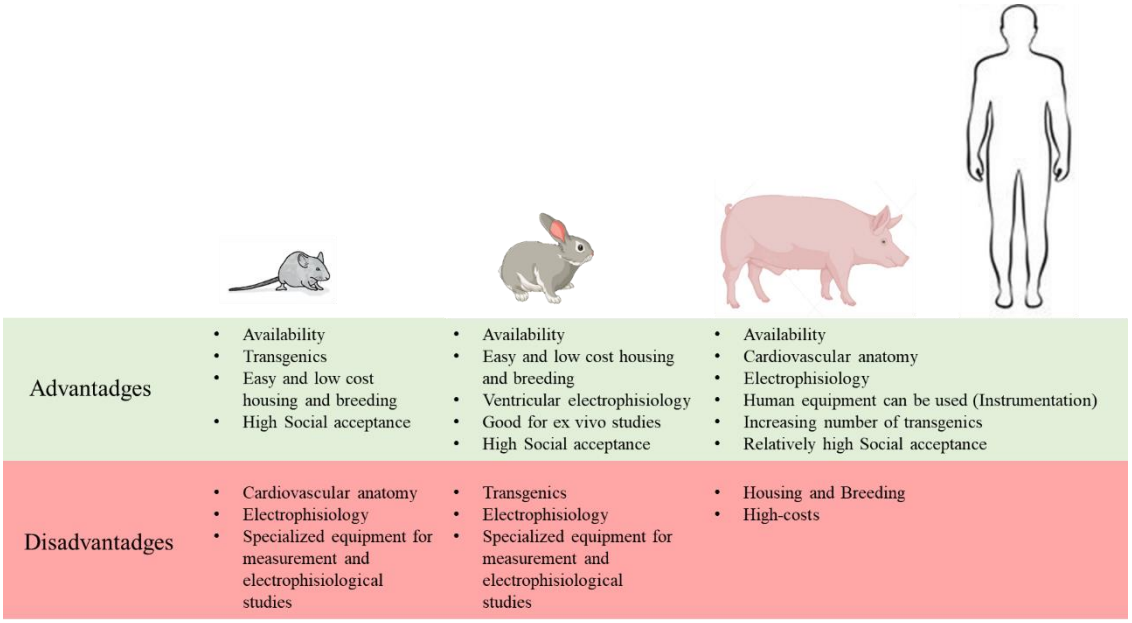


Figure 17. Animal models in Atrial Fibrillation. Overview of general properties, advantages, and disadvantages on using different species (mice, rabbit, and pig) for research in AF studies. Pigs are the most appropriate due to the similarity to humans but is expensive to work with so first studies can be done in mice and rabbits and then jump to pigs and finally translate it to humans. Adapted from Clauss et al., 2019.

6.5. Diagnosis

The diagnose of AF is mainly based on symptomatology, which is not always present in AF patients. The first criteria to infer on AF is the altered hearth rhythm, which should be confirmed by ECG that can reveal an arrythmia. The main alteration is the replacement of the normal consistent P waves (which represent synchronous atrial activation) with oscillatory or fibrillatory waves of different sizes, amplitudes, and timing. ECG is followed by a 24-hours Holter to measure cardiac rhythm and analyse the electrical heart signalling in prolonged time. Also, a blood test is desirable to rule out thyroid problems or substances that can lead to Atrial fibrillation (Van Marion et al., 2015). Another

possible symptomatology in AF are heart palpitations, lacking energy, shortness of breath, chest pain and dizziness (Dewar & Lip, 2007). If necessary, echocardiogram (ECO) or cardiac tomography can be done to determine if the anatomy of the heart and the muscular contraction and blood pumping are correct. ECO will be used to measure atrial diameter index, which will reveal muscular abnormalities as a consequence of AF. In addition, risk-factors must be considered: age, diabetes, hypertension, previous cardiopathy, heart inflammation, heart failure, sleep apnea, alcohol use, and lung or thyroid disease (Bosch et al., 2018; Brandes et al., 2018; Zimetbaum, 2017). Depending on the results, AF is classified in different types. First, paroxysmal AF, which occurs sometimes and then stops by itself. Recurrent AF, when are detected 2 or more episodes of AF. Persistent AF, with more than 7 days of AF events which requires medication or other treatments to return to normal rhythm. Finally, long standing persistent AF or permanent AF, that cannot be corrected (Ogawa et al., 2018). In addition, depending on the reason that causes the AF, can be classified also as lone AF and nonvalvular AF. Lone AF is when the patient is younger than 60 years old and have no clinical or echocardiographic causes are found. Nonvalvular AF is when is not caused by valvular disease.

6.6. Treatments

6.6.1. Antiarrhythmic drugs

Mostly of the patients do not need any aggressive treatment such as catheter ablation as in drug therapies can restore the normal heart rhythm (Dan & Dobrev, 2018). Calcium channel blockers, such as Verapamil or beta-blockers like Esmolol are the most effective drugs. They avoid nearly all of the side effects compared with other treatments like Digoxin and eliciting a quick response of sympathetic tone. After monitoring the patients for 48-72 hours, many of these patients (about 67%) spontaneously convert to sinus rhythm (Zimetbaum, 2012). This strategy should be applied depending on the background of each patient and other strategies should be considered.

Adenosine is also a classical drug to treat some cardiac diseases such as supraventricular tachycardia (PSTV) (Rankin et al., 1992). Its mechanism of action is to “temporary paralyze” the supraventricular tissue. Adenosine hyperpolarizes the cell by stimulating

inward potassium current and inhibiting calcium migration. However, this is extremely dangerous when unintentionally administered to AF patients. About 12-16% of the treated patients develop AF due to adenosine treatment (Li et al., 2016). In these cases, the heart rhythm quickly starts to degenerate and can end in the death of the patient. This is due to the blockade in the conduction through AVN . This makes the diagnose and the adenosine dosage critical points in the treatment of PSTV and other cardiac diseases.

6.6.2. Anticoagulant therapy

Anticoagulation significantly reduces the risk of embolic stroke but increases the risk of bleeding. Usually the benefits of the anticoagulation therapy exceed the risk of bleeding, but medical history of the patient should be taken in account (Katritsis et al., 2015). CHA_2DS_2-VASc score is the main guide to determine which type of anticoagulant should be used. If the score is 2 or greater, Warfarin is one of the most common anticoagulants even with his narrow therapeutic range. Oral anticoagulants can be used too like the Xa inhibitors such as Rivaroxaban, which presents less drug interactions compared with Warfarin. If the score is 1, a simple aspirin or even without antithrombotic therapy can be enough to revert AF (Dan & Dobrev, 2018).

6.6.3. Cardioversion

Cardioversion therapy is recommended when AF is unstable or not tolerated and is not responding to any drug therapy. This therapy is usually complemented with anticoagulant treatment (Klein & Trappe, 2015). There are 2 ways to attempt cardioversion: electrically or pharmacologically. The first one sends a direct-current shock to synchronize the QRS complex and restore the normal rhythm. Electric approach usually works in short term but not in the long term AF, where its desirable to use the second approach (Nusair et al., 2010). Pharmacological cardioversion uses drugs such as Amiodarone or Flecainide to restore normal rhythm and trigger the complete recuperation of the arrythmia. The main problem with this therapy is that long-term effectiveness is limited and includes some and dangerous side effects like thrombus or multifocal atrial tachycardia (Boriani et al., 2004).

6.6.4. Catheter ablation

This approach is used commonly when medical management is not enough or is not tolerated. Its specially recommended for persistent AF and systolic disfunction. As the name says, the ablation is done by a laser which partially destroy areas of the heart. That

isolates the areas where abnormal electrical signalling is originated (Mujović et al., 2017). In addition, a peacemaker should be implanted to maintain heart rate.

6.6.5. Surgery

Surgery is a high-risk approach and has to be considered just for patients which do not response to the rest of the treatments. Surgery involves maze procedure and left atrial appendage (LAA) obliteration (A. M. Lee et al., 2009). Maze procedure is based on use of incisions in the atrial wall to interrupt arrhythmogenic wavelet pathways and reentry circuits. LAA obliteration reduces stroke risk by percutaneous ligation or surgical removal of the LAA.

6.6.6. Hybrid ablation

New technologies are making possible to make surgeries with minimal invasion. This can be applied in a hybrid procedure known as hybrid ablation, where combines minimal invasive epicardial ablation and catheter based endocardial ablation (Vroomen & Pison, 2016). This approach can be done simultaneously or staged and provides access to areas of the atria not easily accessed like cavotricuspid isthmus or mitral isthmus. This last and most recent technique have demonstrated to be safe for lone AF with one-year results and antiarrhythmic drug-free success rate higher than isolated procedures (Driver & Mangrum, 2015).

III. Hypothesis & Objectives

Hypothesis

As Atrial Fibrillation is the most common arrythmia and is still having an insufficient treatment with high relapse after therapy, we postulate that an altered signalling from the membrane receptors and a defective calcium homeostasis are the origin of this disease. Therefore, the working hypothesis points that adenosine receptors can constitute a new source of therapeutic targets and tools in the Atrial Fibrillation research.

Objectives

Aim 1: To evaluate the impact of the disease in the adenosinergic system in AF subjects.

Aim 2: To ascertain the A_1R and $A_{2A}R$ expression and heteromer formation in cells, animal models and human atrial biopsies.

Aim 3: To assess the spatiotemporal A_1R and $A_{2A}R$ control using a photopharmacological approach.

IV. Materials & Methods

1. Cell culture

1.1. Hek-293T and HEK-293T-A_{2A}R-SNAP

Human embryonic kidney (HEK)-293T and (HEK)-293T stable cell line expressing A_{2A}R-SNAP (American Type Tissue Culture, Manassas, VA, USA) were manipulated in a biological safety cabinet class1. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U⁄mL streptomycin, 100 mg⁄mL penicillin and 5% (v⁄v) inactivated fetal bovine serum (FBSi) at 37ºC and 5% CO_2. Absence of mycoplasma was checked regularly to avoid mycoplasma contaminations.

1.2. HL-1

HL-1 Cardiac Muscle Cell Line is an immortalized mouse cardiomyocyte cell line able to continuously divide and spontaneously contract like an original cardiac muscle cell. HL-1 can be serially passaged without losing its differentiated cardiac myocyte phenotype, including morphological, biochemical, and electrophysiological properties (Chaudary et al., 2002). HL-1 cells were manipulated in a biological safety cabinet class1 and grown in Claycomb Medium (Sigma-Adrich) supplemented with FBSi 10% (v/v), 100 U/mL streptomycin, 100mg/mL penicillin, 0.1mM Norepinephrine and 2mM L-Glutamine at 37ºC and 5% CO_2. Absence of mycoplasma was checked regularly to avoid mycoplasma contaminations.

1.3. Cell membrane preparation

Cells were resuspended in ice-cold 10 mM Tris HCl, pH 7.4 buffer containing a protease inhibitor cocktail (Roche Molecular Systems, Belmont, CA, USA). Cells were homogenized using a Polytron (VDI 12, VWR, Barcelona, Spain) for three periods of 10 seconds each. The homogenate was centrifuged at 12,000 × g at 4ºC for 30 min. The membranes were dispersed in 50 mM Tris HCl (pH 7.4). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher

Scientific, Inc., Rockford, IL, USA) by doing duplicates of 10µL of BCA Standards from 0 to 2mg/ml of Albumin protein and the membrane samples in a 96-well plate. Then, 200uL BCA solution (1% (w/v) 4,4 dicarboxy-2,2 biquinoline disodium salt, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium potassium tartrate, 0.4% (w/v) sodium hydroxide, 0.95% (w/v) sodium bicarbonate pH 11.25, 0.08% (w/v) cooper sulphate) was added to each well. After 30 minutes at 37ºC in dark conditions, we read the absorbance at 495nm using a POLARStar plate reader. Membranes were stored at -20ºC for future AlphaLisa and Immunoblot assays.

2. Biological samples

2.1. Human samples

Human whole blood Samples (4 mL) from 28 non-dilated sinus rhythm (ndSR), 24 dilated sinusal rhythm (dSR) and 27 atrial fibrillation (AF) patients (Hospital Sant Pau Dos de Maig, Barcelona) were collected into lithium heparin tubes. In addition, atrial heart tissue from 11 ndSR and 12 AF were also collected. Heart atrium tissue were obtained from patients under cardiac surgery in the Unit of Cardiology and Cardiac surgery of the Hospital de la Santa Creu i Sant Pau of Barcelona. Specifically, atrial cardiac samples were obtained previously to the cannulation of the right atria in surgeries that requires extracorporeal circulation, where part of the tissue is usually discarded. To ensure the quality of the tissue and the confidence of the results, tissue was carried as fast as possible to the lab in a Tyrode solution without calcium, cold and oxygenated. This study is approved for the Etic Committee of Clinical Investigation from the Fundació de Gestió Sanitària de l'Hospital de la Santa Creu i Sant Pau of Barcelona. Relevant clinical and echocardiographic data as well as pharmacological treatments of these patients, included in the statistical analysis, are summarized in Table 1 (*see* Results chapter 1). Patients undergoing mitral valve replacement or repair were not included in this study to avoid potentially confounding effects of mitral valve disease. Each patient gave written consent to obtain a sample from the right atrial appendix that would otherwise have been discarded during the surgical intervention. The study was approved by the Ethics

Committee at Hospital de la Santa Creu i Sant Pau, Barcelona, Spain, and the investigation conforms to the principles outlined in the Declaration of Helsinki.

2.2. Pig samples

Pacemakers were implanted and turned on and the right atria (RA) was paced 420 beats per minute (bpm). The pigs were medicated with digoxin (250 µg/day) throughout the study starting 2-4 days before turning on the pacemaker in order to prevent heart failure symptoms following pacing. After 7 days of pacing, the ECG was monitored while the pacemaker was turned off. If a pig had AF lasting for at least 10 minutes, it was assigned to the study. If AF was converted at any point, the pacemaker was turned on (420 BPM) again. Thus, the pig was paced 420 bpm for 1-7 more days and the procedure was repeated. When AF could no longer be converted to sinus rhythm, the pacemaker was turned off under ECG monitoring. When AF lasting for at least 10 minutes had been confirmed, pigs were assigned again to the study. Then, animals were anesthetized and euthanized to collect cardiac tissue for Immunoblot and AlphaLisa assays.

All animal studies were performed under a license from the Danish Ministry of Environment and Food (license No. 2014-15-0201-00390) and in accordance with the Danish guidelines for animal experiments according to the European Commission Directive 86/609/EEC.

2.3. Human blood processing

Blood was collected into a tube containing ethylenediaminetetraacetic acid (EDTA). 1-unit volume of blood (4 ml) was diluted by the addition of 1-unit volume of 0.9% NaCl. Diluted blood was carefully layered over 1-unit volume of Lymphoprep TM (Stemcell technologies Vancouver, Canada) in a 15 ml centrifuge tube, avoiding its mixing of the diluted blood with the separation fluid. The tube was capped and centrifuged for 30 min at 750 x g at room temperature (20°C) in a centrifuge. The mononuclear cells forming a distinct band at the sample-medium interface were gently removed using a glass pipette without removing the upper layer. The upper phase corresponding to plasma was removed by pipetting and stored at -80ºC for future uses (Adenosine deaminase and Adenosine quantification). The obtained cells were diluted in 20mL of 1% inactivated bovine serum albumin (BSA, Sigma-Aldrich) in phosphate buffered saline (PBS; 8.07 mM Na2HPO4,

1.47 mM KH2PO4, 137 mM NaCl, 0.27 mM KCl, pH 7.2) in order to reduce the density of the solution. Cells were then centrifuged for 10 min at 250 x g at room temperature (20°C) (Figure 18). Cells were stored in Triazol for mRNA studies or 10% dimethyl sulfoxide (DMSO) in FBSi at -80ºC for future uses (RNA extraction and flow cytometry assays).

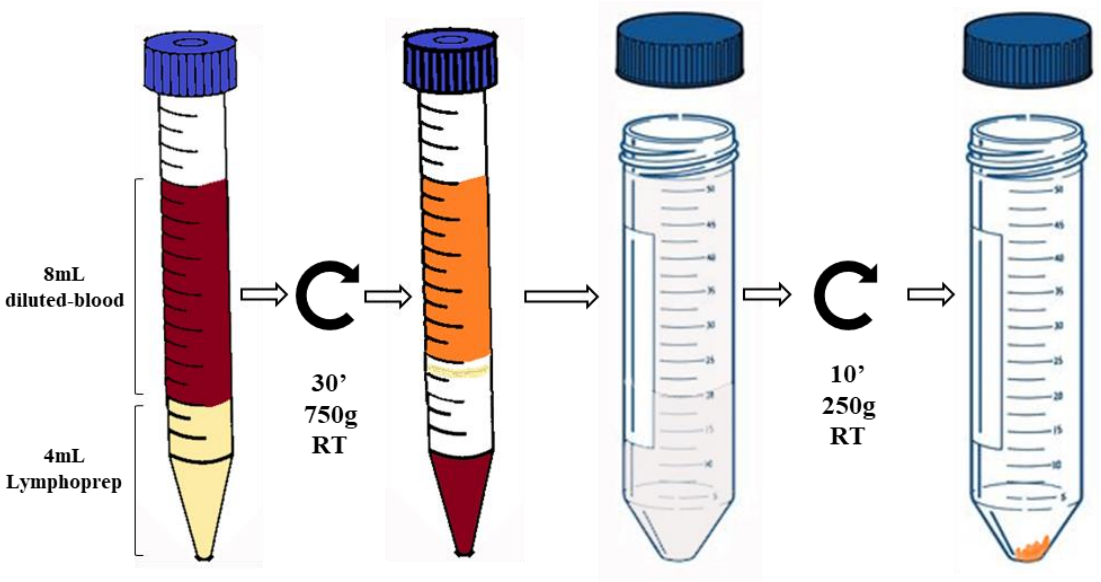


Figure 18. Steps showing the procedure for PBMCs and plasma isolation from whole blood.

2.4. Human and pig heart membrane preparation

Atrial samples from pig and humans were pulverized in liquid nitrogen and sonicated (Brandson Sonifier 250, ICN Hubber S.A.) in 500 µL of ice-cold 10 mM Tris HCl, pH 7.4 buffer containing a protease inhibitor cocktail (Roche Molecular Systems, Belmont, CA, USA). The sonicated tissue was further homogenized using a Polytron (VDI 12, VWR, Barcelona, Spain) for three periods of 10 seconds each. The homogenate was centrifuged at 12,000 × g at 4ºC for 30 min. The membranes were dispersed in 50 mM Tris HCl (pH 7.4). Total protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA) and stored at -20ºC until use for AlphaLisa and Immunoblot Assays.

3. Cellular assays

3.1. AlphaLISA

The AlphaLISA technology allows the detection of molecules of interest in serum, plasma, cell culture supernatants or cell membranes. An anti-analyte antibody which is biotinylated binds the streptavidin donor bead while another anti-analyte antibody is conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into proximity. The excitation of the donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in light emission (Figure 19).

Human, pig, and HL-1 cell membranes were obtained by the methods described before. Cells were also treated with ATX-II toxin to induce arrythmia and simulate AF events and with/without the different ARs antagonist. ATX-II is a toxin that delays Na^+ channels inactivation, disturbing the phase 1 of the cardiac action potential (Antzelevitch et al., 2014). The loss of sodium currents inactivation triggers spontaneous depolarizations and ends in an arrhythmic state (Lu et al., 2012). Also, incubation with different drugs was carried out in HL-1 cells to determine the contribution of each receptor to heterodimer formation. Drugs (Dipropylcyclopentylxanthine (DPCPX) and SCH442416) were incubated for 24 hours in a NE and adenosine free medium in presence of ADA (0.5 U/ml). Membrane pellets were resuspended in assay buffer (20 mM $MgCl_2$, 130 mM NaCl, 0.2 mM EDTA, 0.1 mg/mL saponin, and 0.5% immunoglobulin G (IgG)–free bovine serum albumin) to a final concentration of 1µg/µl. Subsequently, 10µL of each atrial or cell membrane in assay buffer was distributed in quadruplicate into a white 384-well plate. Then, the membranes were incubated by pipetting up and down with the specific $A_{2A}R$ and A_1R antibodies (1nM mouse anti-$A_{2A}R$, Santa Cruz Technologies and 1nM rabbit anti-A_1R, Santa Cruz Technologies) in assay buffer overnight at 4ºC. In the negative controls, only the anti-A_1R antibody was added, corresponding to the donor beads antibody. The volume corresponding to the $A_{2A}R$ antibody was compensated with assay buffer. Next day, plates were tempered at 22ºC and 5µl anti-Mouse acceptor beads (20 µg/µL) were added to each well for 1h at RT and mixed by pipetting up and down. Then, 5µL anti-Rabbit donor beads (20 µg/uL) were added, and the mix was again

homogenized by pipetting up and down carefully. Any prolonged light exposure was avoided. Finally, after 4h in dark incubation, the donor beads were excited (640-720nm) and acceptor beads emission (597-633) was measured using a CLARIOstar plate-reader (BGM Labtech, Durham, NC, USA). Data were fitted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

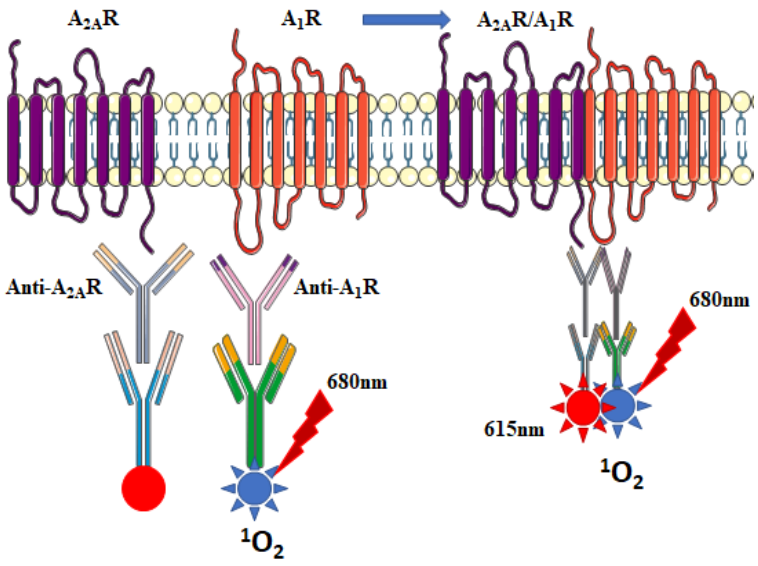


Figure 19. Schematic representation of AlphaLISA technique. Two receptors bind to two different specific antibodies attach to Donor and Acceptor beads, respectively. Then, if close enough, excitation of donor beads can produce a singlet oxygen that triggers a luminous and measurable signalling in the acceptor bead.

3.2. NanoBit

NanoLuc Binary Technology (NanoBiT) is a two-subunit system based on NanoLuc luciferase that can be used for intracellular detection of protein:protein interactions (PPI). LongBiT (LgBiT; 17.6kDa) and SmallBiT (SmBiT; 11 amino acids) subunits are fused to proteins of interest (A_1R, A_2AR and empty vector in this case) and when expressed, the PPI brings the subunits into close proximity to form a functional enzyme that generates a bright, luminescent signal. Empty vector fusion protein is generated to assess the unspecific light of the technique. These subunit pair weakly associates (Kd=190µM) yet retains the bright signal of NanoLuc luciferase. These Lg-SmBit interaction is reversible and can also detect dissociating proteins, which will be used to determine heterodimeric A_1-$A_{2A}R$ levels (Figure 20). Prior to the NanoBit, 1x10^6 HEK-293T cells were seeded in a 6 wells plate and stored overnight in the incubator with complete DMEM medium. Next, when the plates reach 90% of confluence, cells were transiently cotransfected using polyethyleneimine (PEI) (PEI condenses DNA into positively charged particles, which bind to anionic cell surface residues and are brought into cell via endocytosis) with the different DNA (encoding A_1R-LgBit and $A_{2A}R$-SmBit). For the

negative control we transfected an empty vector encoding the LongBit and a vector encoding the A_1R with the ShortBit subunit. For the positive control we mixed $A_{2A}R$ LongBit and $A_{2A}R$ ShortBit. For the heteromer quantification we used the A_1R-LongBit and the $A_{2A}R$-ShortBit. DNA/NaCL and PEI/NaCl mixtures were prepared in different 1.5mL eppendorfs tubes. Then, both solutions were mixed and incubated for 30min at room temperature and shacked each 5 minutes to generate the complexes. In one hand, we mixed 1.5µg of each ShortBit and LongBit genes (3µg of total DNA amount) with 47µl of NaCl. In the other hand, we prepared another Eppendorf with 14.5µL of PEI and 35.5µL of NaCl for each Small-LongBit. Then, we added 900µL of complete DMEM medium to each 100µL of DNA transfection mix and added drop by drop homogeneously to each well. After 4 hours at 37ºC, we replaced the mix for 2mL of complete DMEM medium for 48 hours in the incubator at 37ºC. If needed, drugs were added at 24 hours to determine the effect in the heterodimerization at long-term incubations. Then, we detached the cells with TryPle from the 6-well plate and centrifuged at 500g for 5 minutes. The pellet was resuspended in 1.5mL of HBSS and 1x10^5 cells were seeded in quintuplicate in a white 96-well plate. Immediately, we added 10 µL/well of 5uM coelenterazine and measured luminescence after 2 minutes. Drugs can be added in this point to measure the immediate effect on the heterodimerization (short-term incubation), as described previously, but directly into the 96-well plate. The excess of cells was stored to normalize the different wells by protein amount (BCA assay). NanoLuc intensity at 485nm in a CLARIOstar plate-reader (BGM Labtech, Durham, NC, USA). Data were fitted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

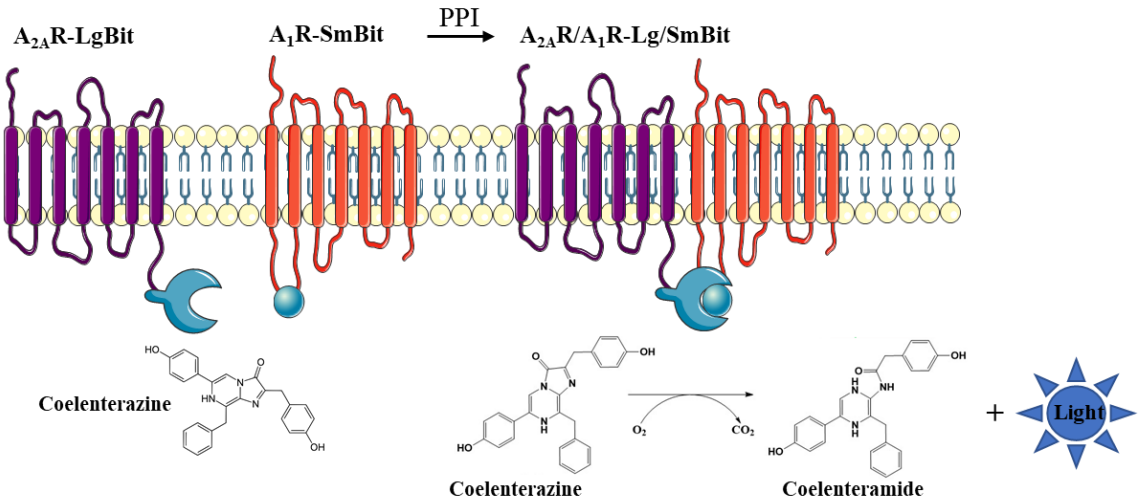


Figure 20. Schematic representation of NanoBit technique. Two quimeric receptors expressing the LongBit and SmallBit of the NanoLuc enzyme, when close enough, interact, and form the complete enzyme which can metabolize the substract coelenterazine and emit a light.

3.3. cAMP determination by CRE-luciferase system in HL-1 cells

This assay consists of use a luciferase reporter assay to indirectly detect variations of cAMP levels in transiently transfected HL-1 cells. This technique is based on the transfection of a vector carrying the luciferase gene under the control of the promoter CRE (cAMP response element), that responses to cAMP levels (Figure 21A). Accordingly, cells were seeded at a density of 4x10^5 cells/well in 6-well dishes. Then, we transfected with 2 µg of the plasmid encoding the cAMP response element−firefly luciferase fusion protein (pGL4-CRE- luc2p; Promega, Madison, WI, USA) with 5µL of Lipofectamine in a mix of 200µL of OptiMem Medium and 800µL of Claycomb transfection medium (Norepinephrine and Penicilin/Streptomicin free). Approximately 18 hours after transfection we added 1 mL of fresh Claycomb transfection medium and 6 hours later replaced everything with 2mL of fresh Claycomb transfection medium. Then, after 24 hours, cells were incubated with the different drugs and in dark and light conditions, if necessary, for 5 hours at 37ºC and 5%CO_2. In addition, to avoid endogenous adenosine and PDE interferences, cells were harvested with ADA (ADA, 0.5 U/ml; Roche Diagnostics, GmbH, Mannheim, Germany) and Zardaverine (100 µM; Calbiochem, San Diego, CA, USA). Cells were then washed twice with PBS, and the firefly luciferase luminescence was determined using the Bright-Glo luciferase assay system (Promega) following the manufacture's indications. Luminescence was measured

in a CLARIOstar Optima plate reader (BMG Labtech, Ortenberg, Germany) using a 30 nm bandwidth 535 nm filter setting. Data were fitted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

3.4. cAMP determination by TR-FRET in HL-1/HEK-293T cells

cAMP accumulation was measured using the LANCE Ultra cAMP kit (PerkinElmer, Waltham, MA, USA). In brief, HL-1 cells were detached with TryPle (Sigma-Aldrich) and incubated for 1h at 37°C in deprived ClayComb (Sigma-Aldrich, Norepinephrine free) supplemented with 0,1% BSA, adenosine deaminase (ADA, 0.5 U/ml; Roche Diagnostics, GmbH, Mannheim, Germany) and Zardaverine (100 µM; Calbiochem, San Diego, CA, USA). Cells (2x10^5 cells/2000µl). Subsequently, cells were incubated with the saline or the different drugs and then irradiated, if necessary, for 30 min at 37°C. Then, cells were harvested in a 384-wells plate in order to add the reagents of the kit (Eu-cAMP tracer and ULight™-anti-cAMP) which were prepared and added following manufacturer's indications. 384-wells plate was incubated 1 hour at room temperature in dark prior to be read on a CLARIOstar microplate reader (BMG Labtech). Measurements at 620 nm and 665 nm were used to detect the time resolved-förster/fluorescence resonance energy transfer (TR-FRET) signal and the concomitant cAMP levels were calculated following manufacturer's instructions (Figure 21B). Data were fitted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

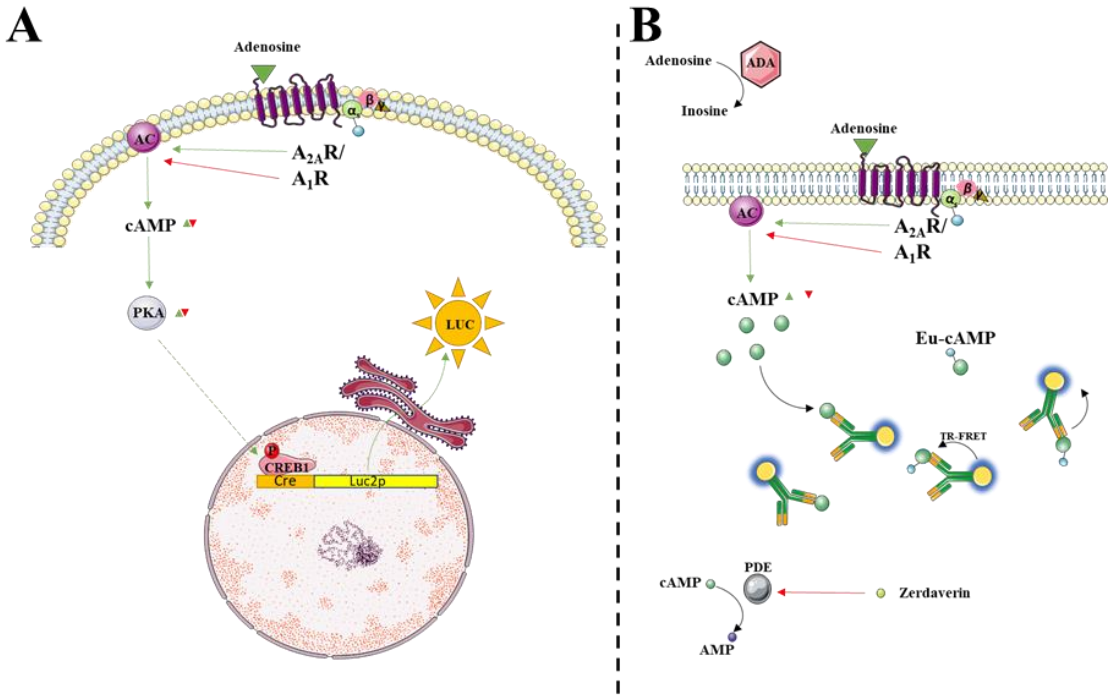


Figure 21. Schematic representation of cAMP determination by CRE and TR-FRET techniques. (A) cAMP determination by CRE luciferase system. Cells were transfected with Luc2P luciferase gene under control of the Cre promoter, which response to transcription factor CREB. CREB, once phosphorylated, induce the transcription of Luc2p and promotes the luminescence signal by luciferase (LUC) protein. Phosphorylation of CREB depends on PKA, a protein regulated by cAMP levels, which can rise or down depending on the adenosine receptor activated in each moment. (B) cAMP determination by TR-FRET. Once adenosine receptors are challenged with their agonist, can induce both accumulation (Gs receptors like $A_{2A}R$) or reduction (Gi receptors like A_1R) of cAMP. Then, cAMP competes with the Eu-cAMP probe for the U-light antibody, reducing the TR-FRET signalling and leading an inverse competitive reaction. Zardaverine inhibits the phosphodiesterase (PDE) and allows cAMP accumulation; adenosine deaminase (ADA) degrades adenosine to avoid noise from the adenosine remaining in the environment.

3.5. Cell confocal imaging

HEK-293T-$A_{2A}R$-SNAP cells and human PBMC's were incubated with 50nM MRS7396 in presence or absence of 100nM ZM241385 (Tocris) in Eppendorf tubes ($4x10^5$ cells) with 300µL of complete DMEM medium for HEK cells and Roswell Park Memorial Institute (RPMI) medium (Gibco) in rotation (25rpm WiseMix Rotator) during 16h at 4ºC. Then, cells were centrifuged at 500g 4ºC and rinsed in cold Hank's balanced salt solution (HBSS, Sigma) and seeded for 2 hours with DMEM or RPMI medium at RT both in an 8-well treated plate (Ibitreat 15µ-Slide 8 well Plate, Ibidi, Gräfelfing, Germany). 20 minutes before confocal imaging we stained the cells with of Hoechst 33342 (500nM, ThermoFisher Scientific) for in vivo nuclear staining. Confocal images were captured using ZEISS microscope (ZEISS LSM 880 Confocal Laser Scanning

Microscope, Zeiss, Oberkochen, Germany). Analysis was done with ZEN software (Zen 2 blue edition, Zeiss).

3.6. Cell viability assays

Three different metabolic assays were performed to test the effect of ATX-II in HL-1 cells: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (cell metabolic activity), Propidium Iodide (PI) assay, (membrane integrity) and diclorodihidrofluorescein (DCF) assay (production of ROS). HL-1 cells were cultured in 6-well plates and incubated in presence of 50nM of ATX-II for 24 hours. Next, cells were collected and incubated with MTT reagent from the MTT kit (MTT Assay for Cell Viability and Proliferation, Sigma) following the manufacturer's indications, propidium iodide (100 µg/mL) and DCF (1µM). Then, absorbance from MTT assay and luminescence from PI and DCF assays was read on a CLARIOstar microplate reader (BMG Labtech). Data were fitted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

3.7. Flow-Cytometry characterizations and competition binding assays

HEK-293T-A_2AR-SNAP cells and human PBMC's were incubated with 50nM MRS7396 in presence or absence of 100nM ZM241385 (Tocris) in Eppendorf tubes ($4x10^5$ cells/300µL of complete DMEM medium for HEK cells and RPMI medium for lymphocytes) in rotation (25rpm WiseMix Rotator) for 16h at 4ºC. Then, cells were centrifuged at 500g 4ºC and rinsed in cold Hank's balanced salt solution (HBSS, Sigma) to be used for flow cytometric measurements. Lymphocytes and monocytes were gated by size and shape, excluding the death cells, doublets or triplets and aberrant cells (Figure 5). In addition, anti-7-actinomicine-D-PE-Cyanine-5 (anti-7AAD) (Applied Biosystems) labelling was used to determine alive/dead cells (Figure 22). At least 10^4 cells (events) per tube were analysed using a BD FACSCanto II flow cytometer (Becton Dickinson, Heidelberg, Germany) with excitation at 488 nm and emission at 647 nm. Samples were maintained in the dark and at 4°C during the analysis to avoid photobleaching and ligand dissociation. The mean fluorescence intensity per event were obtained in the FL-1 channel in log mode; negative fluorescent events were obtained by fluorescence minus one method. Data were collected using FacsDiva Software v6 1.3 (BD, Franklin Lakes, NJ,

USA. Analysis was made using the FlowJo v10.1 software (Becton Dickinson, Ashland, Oregon). Data were fitted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

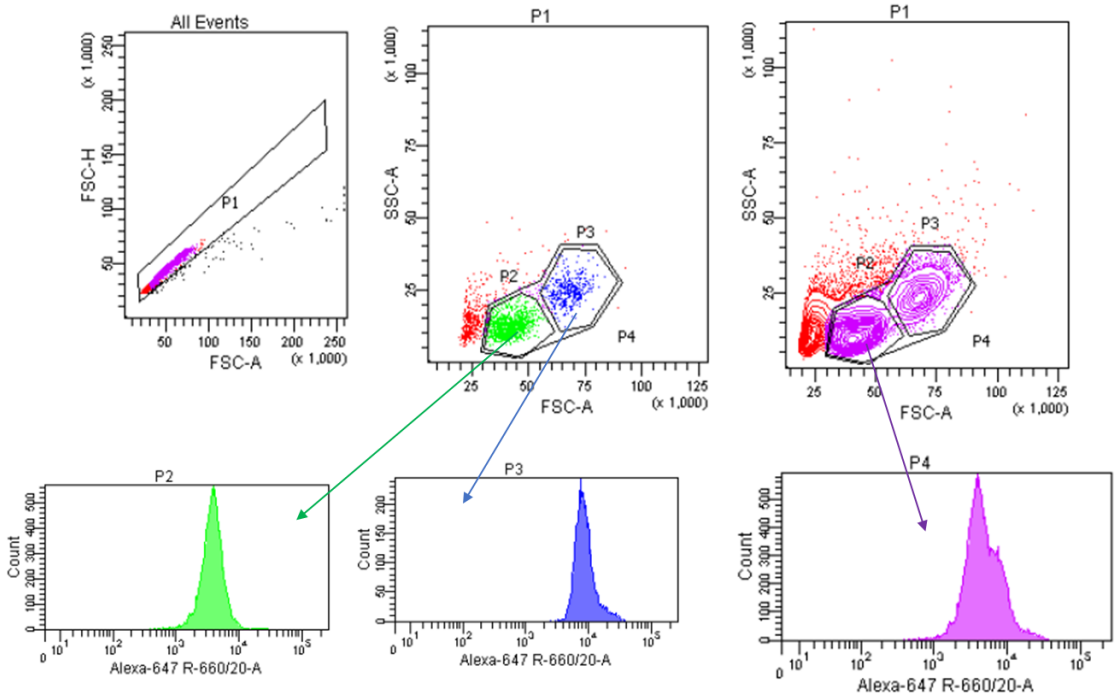


Figure 22. Schematic representation of Lymphocyte gating. Upper panels: cells are selected to discard doublets, triplets and debris present in the sample (Gate P1, All events). Then, in the gate P1 lymphocyte and monocyte populations are selected by size and shape (gate P2 and P3, respectively) discarding other residual blood populations (i.e., granulocytes). P4 represent the collective population of lymphocytes and monocytes. Dead cells from P2, P3 and P4 are separated by adding 7AAD specific marker to generate the specific signalling from the alive cells. Lower panels: immunofluorescence profiles from the MRS7396 obtained in the different populations: P2 lymphocytes, P3 monocytes and P4 both populations.

4. In vitro Assays

4.1. Gel electrophoresis and immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) was performed using 10% polyacrylamide gels. Proteins were transferred to Hybond®-LFP polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Chicago, IL, USA) using the Trans-Blot®TurboTM transfer system (Bio-Rad, CA, USA) at 200 mA/membrane for 30 min. PVDF membranes were blocked with 5% (wt/vol) dry non-fat milk in phosphate-buffered saline (PBS; 8.07 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 0.27 mM KCl, pH 7.2) containing 0.05% Tween-20 (PBS-T) during 1h at 20°C before being immunoblotted using, rabbit anti-A_1R (0.5 µg/ml, Santa Cruz Technologies), mouse anti-

$A_{2A}R$ (0.5 µg/ml, Santa Cruz Technologies), rabbit anti-α-actinin (0.5 µg/ml, Santa Cruz Technologies), anti-ATP2A2/SERCA2 (1:1000, D51B11, Cell Signaling Technlogy), anti-NCX1 (1:500, ab135735, Abcam), anti-Phospholamban (1:5000, A010-14, Badrilla), anti-Phospholamban pSer16 (1:5000, A010-12, Badrilla), anti-Phospholamban pTre11 (A010-10, Badrilla) and anti-Calsequestrin (1:1000, ab3516, Abcam) antibodies in blocking solution overnight at 4°C. PVDF membranes were washed with PBS-T three times (5 min each) before incubation with either a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (1/10,000) or HRP-conjugated goat anti-rabbit IgG (1/30,000) in blocking solution at 20°C during 2h. After washing the PVDF membranes with PBS-T three times (5 min each) the immunoreactive bands were developed using a chemiluminescent detection kit (Thermo Fisher Scientific, Waltham, MA, USA) and detected with an Amersham Imager 600 (GE Healthcare Europe GmbH, Barcelona, Spain). Data were fitted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

4.2. RT-qPCR assay

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by density centrifugation using Lymphoprep (Lymphoprep, Palex Medical, Madrid, Spain). PBMC's were stored at -80ºC in Triazol until use. Cell RNA was purified using RNeasy Plus Universal Mini Kit (Quiagen). Heart tissue was pulverized upon liquid nitrogen congelation and sonicated in 100 µL of Tris (pH 7.4) buffer and stored with 1ml of TriPure reagent (TriPure isolation Reagent, Roche) at -80ºC. Subsequently, the RNA was separated from DNA/proteins using TriPure (Sigma-Aldrich, St. Louis, MO, USA) and following manufacturer's instructions. RNA yield was determined using a NanoDrop® ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using 0.5-1µg of total RNA obtained from each sample using StaRT Reverse Transcription Kit (AnyGenes, Paris, France) according to the manufacturer's instructions. To quantify A_2AR and A_1R mRNA expression we used TaqMan single tube assay probe (ADORA2A Hs00169123_m1, ADORA1A Hs00181231_m1, Thermo Fisher Scientific). The TBP (TATA-box binding protein) probe (TBP Hs00427620, Thermo Fisher Scientific) was used to normalize mRNA expression from both lymphocytes and heart samples. Reaction conditions were done following manufacturer's instructions in a 384-well plate (MicroAmp Optical 384-well plate, Applied Biosystems).

The reaction was carried out in a total volume of 10µL per reaction. Reaction mix included 5µL TaqMAn Fast Advanced Master Mix, 0.5µL TaqMan Assay Probe, 3.5µL of Nuclease Free Water and 1µL of cDNA template (100 ng cDNA total amount) and were done in 7900HT Advanced Real-Time PCR instrument (Applied Biosystems). Amplification protocol started with 95ºC for 10 min, 42 cycles of 95ºC (10 sec) and 60ºC (30 sec), and a final cycle of 95ºC (10 sec) and finishing with a 60ºC (30 sec) step. The results are presented relative to those for the housekeeping gene TBP using the ΔCq method. Results were analysed with Expression Suit Software v1.2 (Thermo Fisher Scientifics). Data were fitted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

4.3. Adenosine Deaminase (ADA) determination

ADA determination was done using Diazyme's Adenosine Deaminase Assay. This technique is based on the enzymatic deamination of adenosine to inosine which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP). Hypoxanthine is then converted to uric acid and hydrogen peroxide (H_2O_2) by xanthine oxidase (XOD). H_2O_2 is further reacted with N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (EHSPT) and 4-aminoantipyrine (4-AA) in the presence of peroxidase (POD) to generate quinone dye which is monitored in a kinetic manner. From the whole blood obtained from the bunch of patients (ndSR, DSR and AF) plasma phase was isolated by density centrifugation using Lymphoprep (Lymphoprep, Palex) as described previously. The plasma was stored at -80ºC until use. ADA was determined by using the colorimetric ADA assay kit (Dyazime Laboratories Inc., Poway, CA, USA). In brief we have to mix 5µL of each plasma sample with 180µL reagent 1 and, after 3 minutes at 37ºC, add 90µL of Reagent 2. ADA activity in each plasma sample was determined in triplicate by measuring the absorbance at 550 nm using a POLARstar Omega plate reader after 10 minutes of incubation. Data were fitted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

4.4. Adenosine quantification MS-HPLC

Plasma samples from ndSR, DSR and AF patients were stored at -80ºC until used. Quantitative analysis of ribonucleosides (i.e., adenosine) was carried out by liquid chromatography technique coupled with tandem mass spectrometry (LC-MS/MS) using an Agilent 1290 Infinity UHPLC chromatograph (Santa Clara, CA, USA) coupled to a 6500 QTRAP mass spectrometer (ABSciex, Framingham, MA, USA) equipped with Ion Drive Turbo V ion source operating in positive ion mode. The column used was a Discovery HS F5-3 150 x 2.1 mm 3µm (Supelco, Bellefonte, CA, USA) at 40 ºC; autosampler temperature, 4 ºC; injection volume, 10µL; flow rate, 0.6 mL min^{-1}. Mobile phase was A) Ultrapure water with 0.1% HCOOH and B) Acetonitrile with 0.1 %HCOOH. The gradient program was as follows (t, %B): (0, 1), (0.2, 1), (3, 20), (3.5, 95), (6, 95), (6.5, 1), (10, 1). Mass spectrometry detection was performed by using the multiple reaction monitoring (MRM) mode using the following parameters: ion spray voltage, +5500 V; source temperature, 600 °C; curtain gas, 20 psi; ion source gas 1 and gas 2, 50 and 20 respectively; collision-activated dissociation gas, High; entrance potential, (+/-)10 V. The MRM transitions for adenosine were 268.3/136.1 (Declustering potential DP 65V and collision energy CE25V) for quantitative purposes and 268.3/119.1 (DP 60V, CE 25V) for confirmation purposes dA-d_3 was used as internal standard with a transition 230.9/115 (DP 20V, CE 15V). Calibration curve was constructed with adenosine standard solutions between 0.03-8 ng/ml diluted in water TCA30%. Linear regression was adjusted (1/x or $1/x^2$) to have accuracies between 80-120% for all the adenosine standards. Analyst 1.6.2 Software was used for data acquisition and MultiQuant 3.0.1 for data processing both from ABSciex (Framingham, MA, USA). Data were fitted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

5. Statistics

GraphPad Prism 7.00 (San Diego, CA, USA) software was used for statistical analysis. Statistical significance was accepted when p-value was <0.05. Data is represented as mean +- standard error of mean (SEM). The number of samples in each experiment is indicated in the corresponding figure legend. Data normality was guaranteed by Shapiro-Wilk normality test. Outliers were assessed by Grubb's tests. Comparisons among

experimental groups were performed by Student's T test. Otherwise, statistical analysis was performed by one- or two-way analysis of variance (ANOVA) followed by Bonferroni or Dunnett's multiple comparison post-hoc test.

V. Results

Chapter 1: **Assessing the status of the adenosinergic system in AF patients.**

AF is the most common cardiac arrhythmia in the world (Zoni-Berisso et al., 2014) and a relationship with the adenosinergic system has been established (Ishihara et al., 2020; Li et al., 2016). Accordingly, in this first chapter we aimed to evaluate the status of the adenosinergic system in AF by analysing the adenosine content, ADA activity and the expression of adenosine receptors in AF patients. Since $A_{2A}R$ has been postulated to the be related to AF pathophysiology (Leif Hove-Madsen et al., 2006; Llach et al., 2011) we assessed its expression both in right atrium biopsies and peripheral blood cells from the same subjects. In addition, the adenosine levels and ADA activity in plasma from these patients were evaluated.

1.1. $A_{2A}R$ expression in AF patients

Previous findings in the literature described that AF patients shows an increased expression of $A_{2A}R$ in right atrial tissue, thus suggesting a possible participation of $A_{2A}R$ in AF pathophysiology. Here, we aimed to explore a potential cardiac-peripheric connection of the adenosinergic system in AF. First, to determine $A_{2A}R$ density and mRNA expression levels in atrium we collected atrial tissue from non-atrial fibrillated patients (i.e., non-dilated sinus rhythm) and AF patients (see Table 1 for patient's clinical information). In brief, atrial diameter index and ECG values were used to classify the patients in the different groups (i.e., ndSR, dSR and AF). Other relevant information such as harmful habits or drug treatments have been considered too (Table 2).

Table 2. Patients' clinical information

	ndSR	dSR	AF
Number of patients	28	24	27
Weight (mean±SD) (Kg)	83.18±9.86	71.5±10.5	75.5±11.386
Height (mean±SD) (cm)	169.4±9.8	160.8±8.7	163.8±8.9
Age (mean±SD)	63.1±9.7	73.5±8.4	72.9±9.4
Sex (male/female)	23/5	17/7	14/13
Body Surface (mean±SD)(m^2)	1.93±0.14	1.75±0.15	1.81±0.15
LA diameter index (mean±SD)	1.99±0.17	2.6±0.2	2.65±0.55
LV diameter index (mean±SD)	2.7±0.5	2.83±0.54	2.82±0.42
Tabaquism (y/n/ex)	9/12/7	3/18/3	6/18/3
Enolism (y/n/ex)	0/28/0	1/23/0	1/26/0
Hipertension	25	22	20
Diabetes	11	12	9
Dislipemia	15	18	15
IECAs	16	12	12
Beta-bloquers	12	9	16
β1	3	2	8
β2	0	0	1
α	0	0	0
RyR-inhibitors	0	0	0
ARA-II	5	4	2
Ca^{2+} antagonist	6	3	4
Sintrom	3	1	16
AAS	17	15	6
Statins	17	18	15

LA, left atria. LV, left ventricle. IECAs, angiotensin-converting enzyme inhibitors. β1, β2 and α, anti-adrenergic drugs. ARA-II, angiotensin receptor-II antagonist. AAS, acetylsalicylic acid.

Accordingly, we confirmed a significant increase of $A_{2A}R$ density ($P < 0.0001$) in the right atrium from AF patients when compared to non-AF subjects (ndSR) (Figure 23A and B). Indeed, a concomitant significant ($P = 0.0207$) increase in $A_{2A}R$ mRNA expression in right atrial tissue from these patients was also found (Figure 23C), consistent with the immunoblot results (Figure 23A).

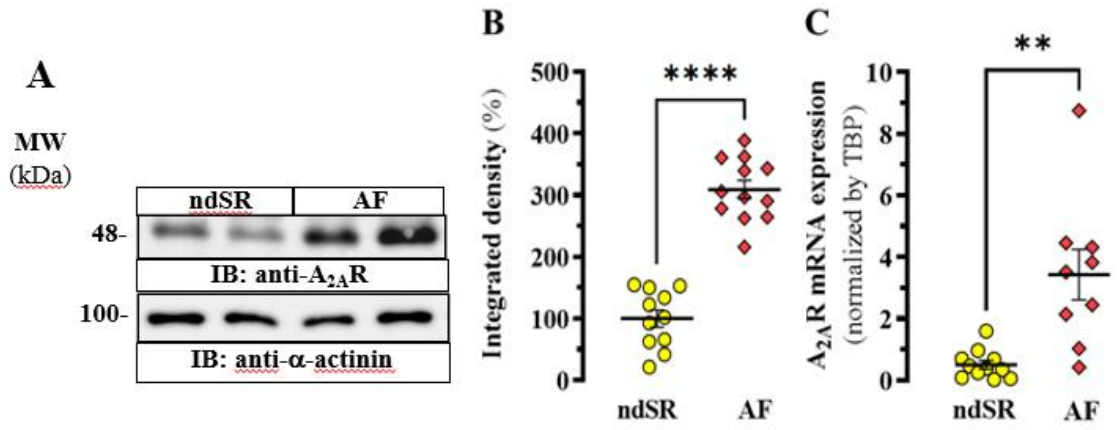


Figure 23. $A_{2A}R$ expression in human right atrium. (A) Representative immunoblot showing the expression of $A_{2A}R$ in right atrium from non-dilated sinus rhythm (ndSR) and atrial fibrillation (AF) patients; membranes from human atrium were analysed by SDS-PAGE (10 μg of protein/lane) and immunoblotted using goat anti-$A_{2A}R$ and rabbit anti-α-actinin antibodies (see Methods). (B) Relative quantification of $A_{2A}R$ density; the immunoblot protein bands corresponding to $A_{2A}R$ and α-actinin from ndSR (n = 11) and AF (n = 12) patients were quantified by densitometric scanning; values were normalized to the respective amount of α-actinin in each lane to correct for protein loading. (C) Relative expression of $A_{2A}R$ transcripts in human atrium. Mean ± SEM from ndSR (n=11) and AF (n=9) patients. ****$P < 0.0001$ and **$P < 0.01$,Student *t* test.****$P < 0.0001$ and **$P < 0.01$ Student *t* test.

1.2. Adenosine content and ADA activity in plasma from AF patients

Once we determined that $A_{2A}R$ expression in heart tissue was increased, we interrogated whether adenosine homeostasis was also altered in these patients. Adenosine deaminase regulates the excess of adenosine, which is critical in high energy demand episodes, like in AF events. Thus, we hypothesize that a decrease in the amount of adenosine deaminase in plasma, together with an increase of energy demand of the fibrillated heart, can lead to an increased adenosine plasma levels (APL). To this end, ADA activity and adenosine content in plasma from patients with and without AF were determined, including the ones analysed previously. In addition, we analysed a third group of subjects named dilated sinus rhythm patients (i.e., dSR, see Table 1). Dilatated sinus is one of the main conditions of the AF and points to a loss of the maintenance of the sinus rhythm and a possible future arrythmia (Seko et al., 2018). Importantly, while no significant alteration in ADA activity was found in dSR patients ($P = 0.1312$), a significant 38% reduction in ADA activity was found in plasma from AF patients when compared to ndSR subjects ($P = 0.0286$, $F_{(2, 66)} = 3.753$) (Figure 24A). Subsequently, when the APL was evaluated, the levels in patients with AF were ~2.5-fold higher when compared to ndSR and dSR subjects ($P < 0.0001$, $F_{(2, 40)} = 44.66$) (Figure 24B). Indeed, a negative correlation ($r = -0.4087$, $P = 0.0202$) between ADA activity and adenosine content within ndSR and AF patients was found

(Figure 24C). Collectively, these results suggested that concomitantly to an increased $A_{2A}R$ expression in right atrium, AF patients also show reduced ADA activity and increased APL, which is related with a worse prognosis in heart diseases.

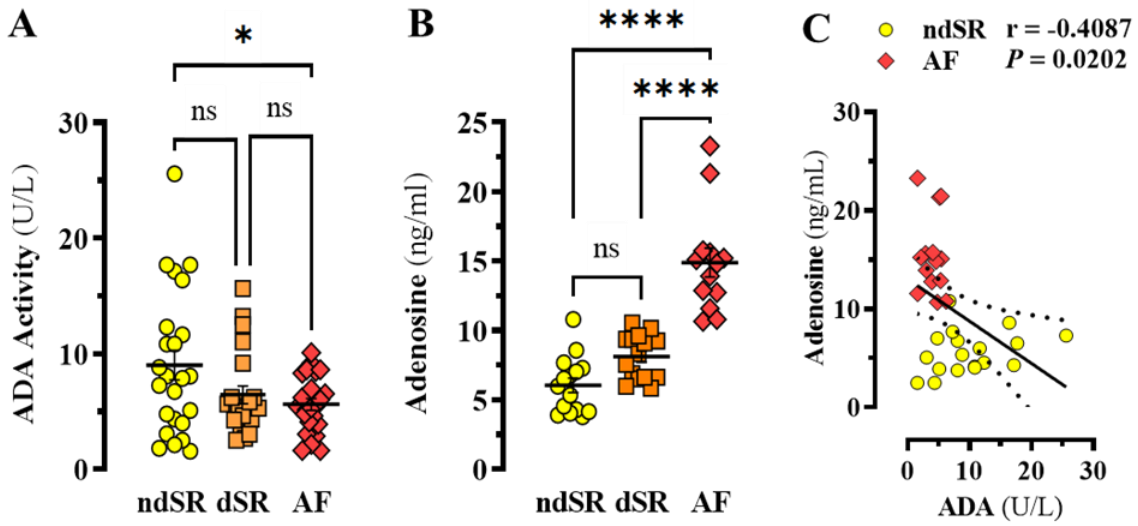


Figure 24. ADA activity and adenosine content in human plasma. (A) ADA activity in plasma from ndSR (n=24), dSR (n=22) and AF (n=23) patients was determined using the Dyazime kit. Two outliers were removed from the dSR group by the ROUT method (*see* Materials and Methods). (B) Adenosine plasma levels from ndSR (n=15), dSR (n=15) and AF (n=13) patients was determined by MS-HPLC. One outlier was removed from the AF group by the ROUT method (*see* Materials and Methods). (C) Correlation between ADA activity and adenosine content in ndSR (yellow, n=18) and AF (red, n=14) patients. The correlation coefficients (r) were calculated using the Pearson's two-tailed correlation test. Results are expressed as mean ± SEM. *$P < 0.05$ and ****$P < 0.0001$, one-way ANOVA with Tukey's *post-hoc* test.

1.3. Detection of $A_{2A}R$ in PBMCs

After establishing that $A_{2A}R$ expression, adenosine content and ADA activity are altered in AF patients, we next questioned whether this dysregulation may have any impact on $A_{2A}R$ density in PBMCs from these patients. To this end, we used a fluorescent (i.e., BODIPY630/650) $A_{2A}R$ antagonist (namely MRS7396) (Duroux et al., 2017), to monitor $A_{2A}R$ density in living PBMCs. Accordingly, we first validated the specificity of MRS7396 $A_{2A}R$ staining in HEK293-$A_{2A}R^{SNAP}$ cells by displacing its binding with ZM241385, a selective $A_{2A}R$ antagonist. Our results revealed that $A_{2A}R$ MRS7396 membrane staining disappear when cells were co-incubated with ZM24138 (Figure 25, upper panels). Next, we aimed to demonstrate the usefulness of using ZM24138 to detect $A_{2A}R$ in living cells (i.e., PNMC's) through flow cytometry. Thus, isolated PBMC's from a healthy subject (ndSR) were stained with MRS7396 in presence and absence of

ZM241328. Interestingly, we observed similar results compared with in HEK293-$A_{2A}R^{SNAP}$ cells (Figure 25, lower panels). The antagonist ZM241385 was able to displace the binding of the fluorescent $A_{2A}R$ ligand, thus validating the specificity of the detection. Overall, these results suggest that we can use this ligand to determine $A_{2A}R$ density by other assays such as flow cytometry.

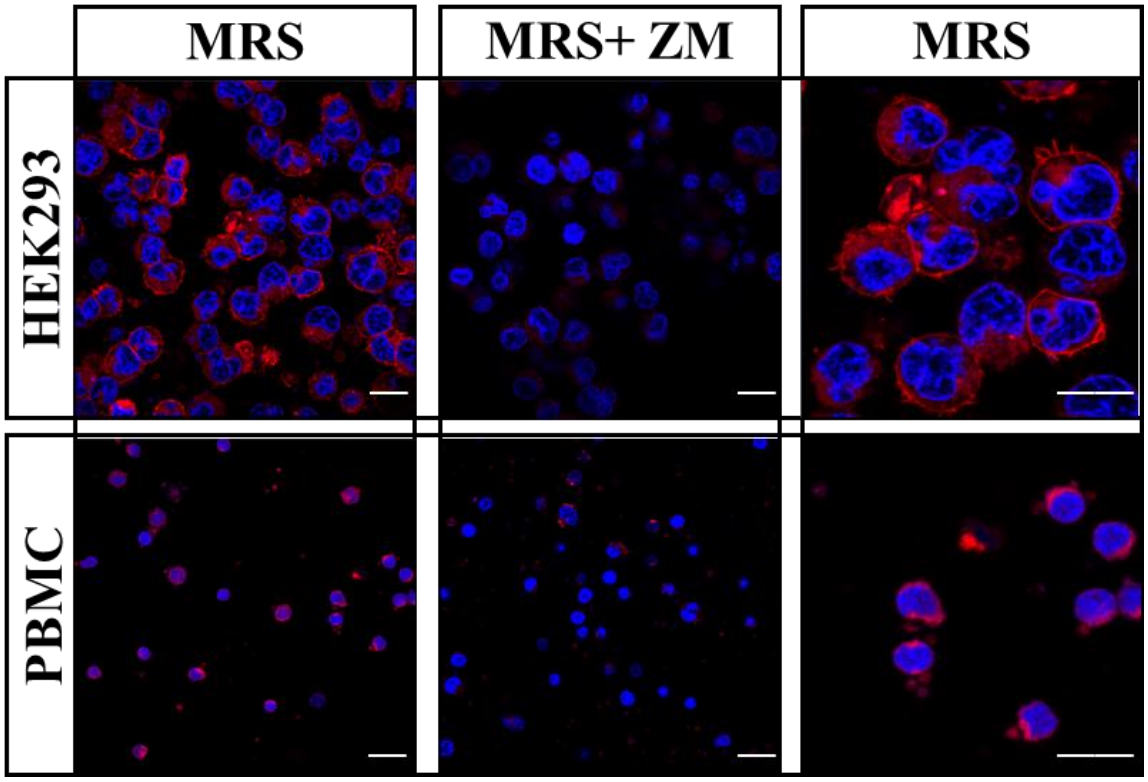


Figure 25. $A_{2A}R$ detection in living cells using a fluorescent ligand. HEK-293 cells permanently expressing $A_{2A}R$, and human peripheral blood mononuclear cells (PBMC) were incubated with 50 nM MRS7396 (MRS, red) in the absence or presence of 100 nM of ZM241385 (ZM). For live nuclear staining cells were incubated with 1µM Hoechst 33342 (blue). Scale bar: 20 µm.

1.4. Adenosine receptors expression in PBMCs

Once the specificity of MRS7396 staining was demonstrated, we aimed to assess $A_{2A}R$ density in PBMCs from ndSR, dSR and AF patients using flow cytometry. Accordingly, PBMCs from ndSR, dSR and AF patients (*see* 1.2) were obtained by Ficoll gradient and gated by flow cytometry. The gating was carried out based on morphology (size and shape) and discarding doublets, triplets, and dead cells. Selected populations mainly contain lymphocyte, monocyte or both type of cells. The PBMC's MRS7396 staining was determined in absence or presence of ZM241385 to define the $A_{2A}R$ specific binding. To

this end, PBMCs were incubated with 50 nM MRS7396 in the absence and presence of 100 nM of ZM241385 in constant rotation for 16h at 4°C before flow cytometry analysis. Interestingly, the $A_{2A}R$ density in lymphocytes from AF patients was significantly increased (~3.7-fold) when compared to both ndSR and dSR patients ($P < 0.0001$, $F_{(2, 39)} = 23.55$) (Figure 26A). In addition, $A_{2A}R$ density in monocytes from AF patients was also significantly increased (~2.5-fold) when compared to both ndSR and dSR patients ($P < 0.005$, $F_{(2, 35)} = 8.81$) (Figure 26B). Next, we assessed if the enhanced density of $A_{2A}R$ found in PBMCs from ndSR, dSR and AF patients correlated with an increased $A_{2A}R$ mRNA expression in these subjects. To this end, we carried out $A_{2A}R$ mRNA expression by RT-qPCR analysis. Importantly, a three-fold significant increase in $A_{2A}R$ mRNA expression in PBMCs from AF patients was found ($P < 0.0001$, $F_{(2, 24)} = 14.64$) (Figure 26C). These results point to an altered $A_{2A}R$ expression on the PBMC's in AF patients.

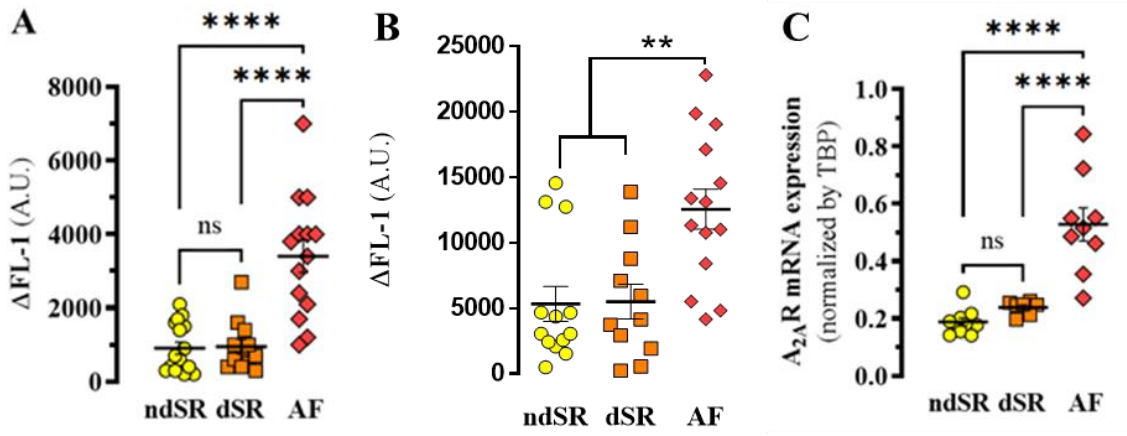


Figure 26. Detection of $A_{2A}R$ in human PBMCs. (A) The specific MRS7396 binding to lymphocytes from ndSR (yellow, n=16), dSR (orange, n=12) and AF (red, n=14)) patients was obtained by subtracting the nonspecific binding (i.e., 100 nM MRS7396 in the presence of 100 nM of ZM241385) and represented as ΔFL-1 (specific binding). (B) The specific MRS7396 binding to monocytes from ndSR (yellow, n=14), dSR (orange, n=10) and AF (red, n=14)) patients was obtained by subtracting the nonspecific binding (i.e. 100 nM MRS7396 in the presence of 100 nM of ZM241385) and represented as ΔFL-1 (specific binding). (C) Relative expression of $A_{2A}R$ mRNA in PMBCs. Mean ± SEM of ndSR (n=10), DSR (n=7) and AF (n=9) patients. ****$P < 0.0001$, **$P < 0.005$, one-way ANOVA with Tukey's *post-hoc* test.

1.5. $A_{2A}R$, ADA and adenosine relationships among atrial tissue, plasma and PBMCs from AF patients

Finally, we aimed to study if a correlation between the different players of the adenosinergic system could be established in AF. First, a positive correlation was found between $A_{2A}R$ density in PBMCs and right atrium tissue from the same patient (r = 0.7813, *P* = 0.001) (Figure 27A). This correlation could indicate that changes of peripheral $A_{2A}R$ expression in PBMCs mirror changes occurring in the fibrillated atrium. Indeed, these changes could be related to the adenosine and/or ADA alterations observed in these patients. Accordingly, we assessed the correlations between ADA and APLs with $A_{2A}R$ expression. Thus, a positive correlation between APLs and $A_{2A}R$ density in PBMCs (gated on lymphocytes) and in right atrium (r = 0.6851 , *P* < 0.0004 and r = 0.7679, *P* = 0.02, respectively) was found (Figure 27B and C). However, no correlation between plasma ADA activity and $A_{2A}R$ density in PBMCs and in right atrium was observed (Figure 27D and E). Besides, we repeated these analyses gating on monocyte populations. Results shown a positive correlation between $A_{2A}R$ expression in PBMC's gating lymphocytes and monocytes (r= 0.8257, *P* < 0.0001) (Figure 27F). Similar results were obtained for the rest of correlations when analysing the correlations with ADA and adenosine content (data not shown). Altogether, these results suggest a relationship between atrial and PBMCs $A_{2A}R$ expression, which might be related to adenosine content.

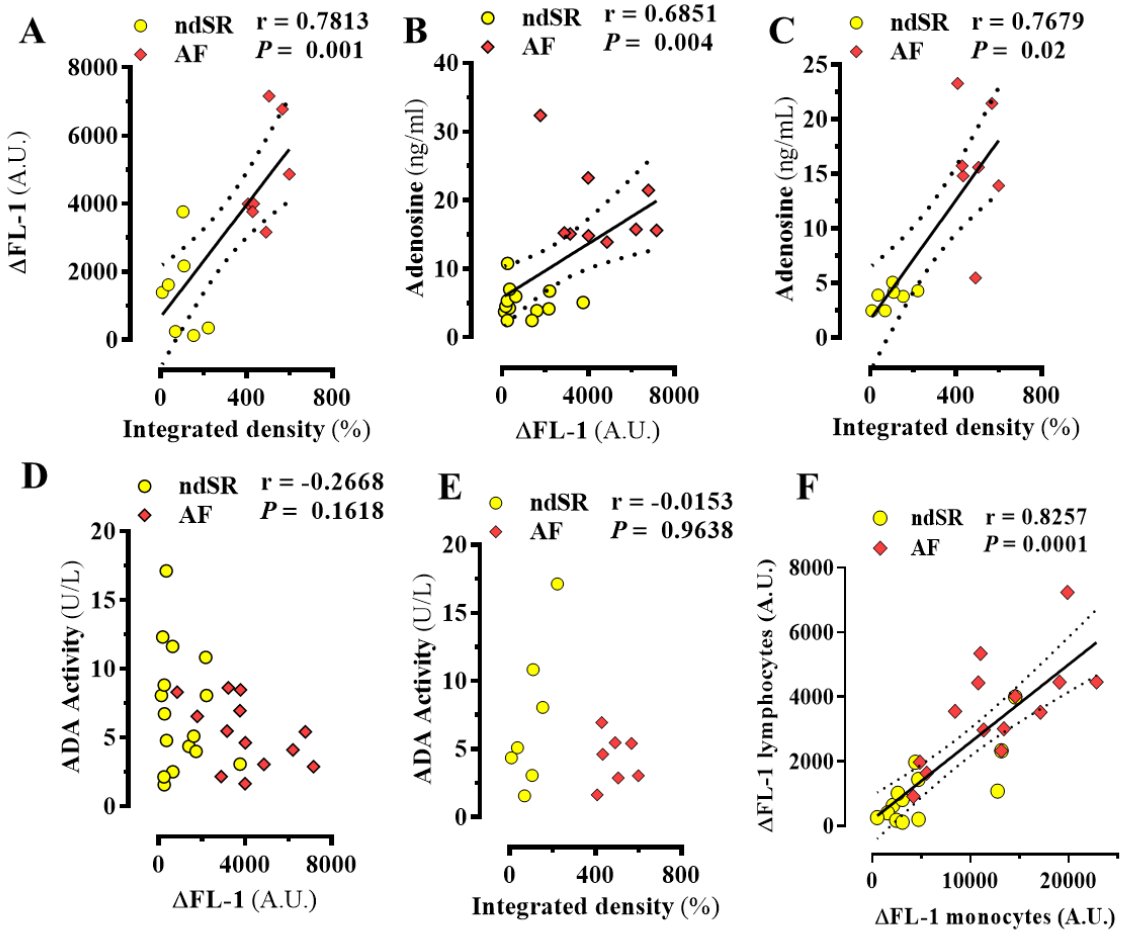


Figure 27. Correlation of atrial $A_{2A}R$ density and peripheric adenosinergic system. Correlation between the relative $A_{2A}R$ density in the right atrium determined by immunoblot (Integrated density, *see* Figure 1B) and the specific MRS7396 binding (ΔFL-1) to lymphocytes (A), adenosine content (C) and ADA activity (E) from ndSR (yellow) and AF (red) patients. Also, the correlation between the specific MRS7396 binding (ΔFL-1) to lymphocytes and the adenosine content (B) and ADA activity (D) from ndSR (yellow) and AF (red) patients was assessed. (F) Correlation between the specific MRS7396 binding to lymphocytes and monocytes. The correlation coefficient (r) was calculated using the Pearson (two-tailed correlation) test.

Chapter 2: **Adenosine receptors expression and heteromer formation in experimental models of AF**

In the first chapter, we demonstrated that ARs expression is altered in heart and PBMCs from AF patients. In addition, we also shown a disruption of adenosine homeostasis (i.e., ADA and adenosine content in plasma) in those patients. Interestingly, since ARs function may depend on its ability to heteromerize (Cristóvão-Ferreira et al., 2013) (Ciruela et al., 2006), in this second chapter we questioned whether $A_1R/A_{2A}R$ heteromer formation is altered in experimental models of AF. To this end, several AF models were used: **i)** a cardiomyocyte stable cell line (i.e., HL-1 cells); **ii)** a pig animal model of AF; and **iii)** human biopsies from AF patients. Furthermore, we aimed to determine whether calcium handling related proteins, namely SERCA2, NCX-1, CSQ-2, and PLB would be altered in these experimental models of AF.

2.1. A_1R and $A_{2A}R$ expression and heteromer formation in HL-1 cell line

HL-1 is an immortalized mouse cardiomyocyte cell line able to spontaneously contract while maintaining a differentiated cardiac phenotype. Expression of ARs have been characterized in these cells alongside many other cardiac features (Chaudary et al., 2002). Thus, we decided to assess the A_1R and $A_{2A}R$ expression and heteromer formation in HL-1 cells upon basal and *in vitro* induced arrythmia-like conditions. To induce an arrhythmic-like phenotype to HL-1 cells we challenged the cells with Anemonia toxin II (ATX-II), a cardiotoxic peptide toxin from the coelenterate *Anemonia viridis* that modulates voltage-gated Na^+ channel gating kinetics prolonging the action potential of excitable cells, thus triggering an arrhythmic-like status (Lu et al., 2012) (Antzelevitch et al., 2014). The extent of ATX-II-mediated HL-1 cells arrhythmia-like condition was carried out by assaying cell viability. Cell viability can be used as a method to determine toxin induced arrhythmogenic like state (F. Zhang et al., 2018). To this end, we implemented the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and diclorodihidrofluorescein (DCF) assays determining cell metabolic activity, membrane integrity and ROS formation, respectively, in HL-1 cells incubated in the absence and presence of ATX-II (30 nM) for 24h. Indeed, the viability of HL-1 cells treated with ATX-II was significantly reduced as determined by all three

cell viability assays (Figure 28), thus validating that ATX-II incubation compromises HL-1 cellular membrane potential and viability (i.e., pro-arrhythmic status).

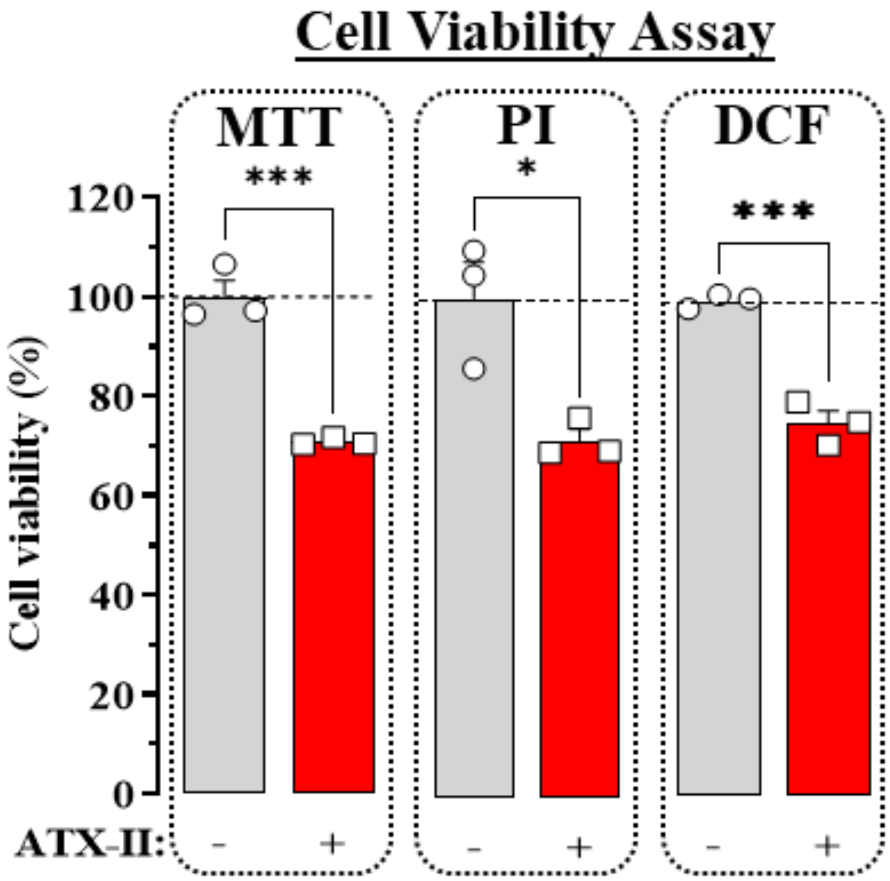


Figure 28. Cell viability assays in HL-1 cells. Effect of ATX-II on viability in HL-1 cells detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay (left column), Propidium iodide (PI) excitation assay (middle column) and dichlorodihydrofluorescin DiOxyQ or DCF assays (right column) (n=3 for each assay and condition). Results are expressed as percentage (mean ± SEM). **P* < 0.05, *** *P* < 0.001, Student's *t*-test.

After inducing the arrythmia-like status into HL-1 cells, we aimed to determine the impact of the ATX-II treatment in the endogenous A_1R and $A_{2A}R$ expression. To this end, we performed immunoblot experiments to assess A_1R and $A_{2A}R$ density in membrane extracts from HL-1 cells incubated in the absence and presence of ATX-II (30 nM) for 24h (Figure 29A). Interestingly, when HL-1 cells were treated with ATX-II a significant increment of ~ 2- and ~ 4-fold in the density of endogenous A_1R ($P < 0.05$) and $A_{2A}R$ ($P = 0.01$), respectively, was shown (Figure 29B). Thus, these results indicated that the ATX-II-induced arrhythmia-like condition in HL-1 cells boosted A_1R and $A_{2A}R$ expression.

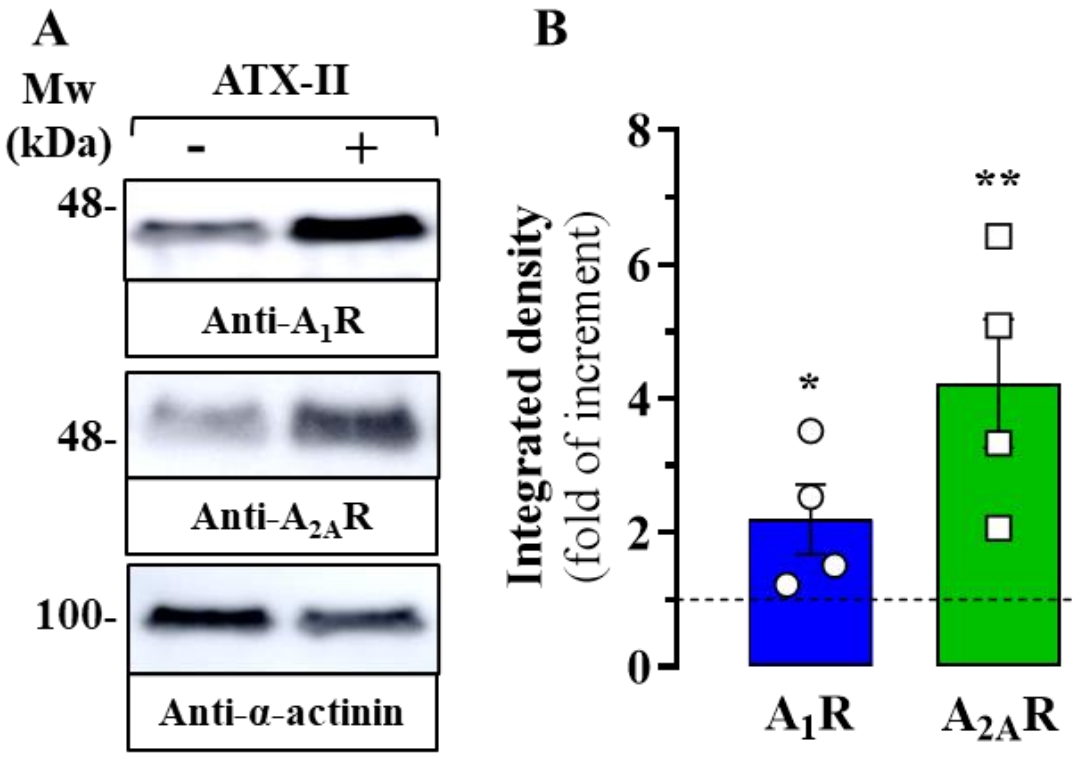


Figure 29. Endogenous A_1R and $A_{2A}R$ density in ATX-II treated HL-1 cells. (A) Representative immunoblot showing the expression of A_1R and $A_{2A}R$ in HL-1 cells incubated in absence or presence of ATX-II. Membranes extracts were analysed by SDS-PAGE (20 µg of protein/lane) and immunoblotted using mouse anti-$A_{2A}R$, mouse anti-A1R and rabbit anti-α-actinin antibodies. **(B)** Relative quantification of A_1R and $A_{2A}R$ density. The immunoblot protein bands corresponding to A_1R, $A_{2A}R$ and α-actinin from vehicle (n = 6) and ATX-II treated (n=4) cell cultures were quantified by densitometric scanning. Values were normalized to the respective amount of α-actinin in each lane to correct for protein loading. Results are expressed as fold change over the control (vehicle treated cells; dashed line) (mean ± SEM). **P* < 0.05, ***P* < 0.01, Student's *t*-test comparing with the vehicle treated cells (dashed line).

Subsequently, we aimed to determine the effect of ATX-II-induced HL-1 cells arrhythmia-like condition in endogenous $A_1R/A_{2A}R$ heteromer formation. To this end, we implemented the new AlphaLISA approach developed in our laboratory. (*see* Material and Methods) (Crans et al., 2020; Fernández-Dueñas et al., 2019; Valle-León et al., 2021) (Figure 30A). Therefore, HL-1 cells were incubated with ATX-II in the absence or presence of DPCPX (an A_1R antagonist), SCH442416 (a selective $A_{2A}R$ antagonist) or both simultaneously. Importantly, ATX-II-induced arrhythmia-like condition in HL-1 cells resulted in a significant 60% reduction in their $A_1R/A_{2A}R$ heteromer content ($P < 0.0001$) (Figure 30B). Interestingly, the ATX-II-induced $A_1R/A_{2A}R$ heteromer content reduction was partially but significantly precluded by either DPCPX ($P < 0.001$) or SCH442416 ($P < 0.0001$) treatment and abolished when HL-1 cells were treated with both antagonists simultaneously (Figure 3B).

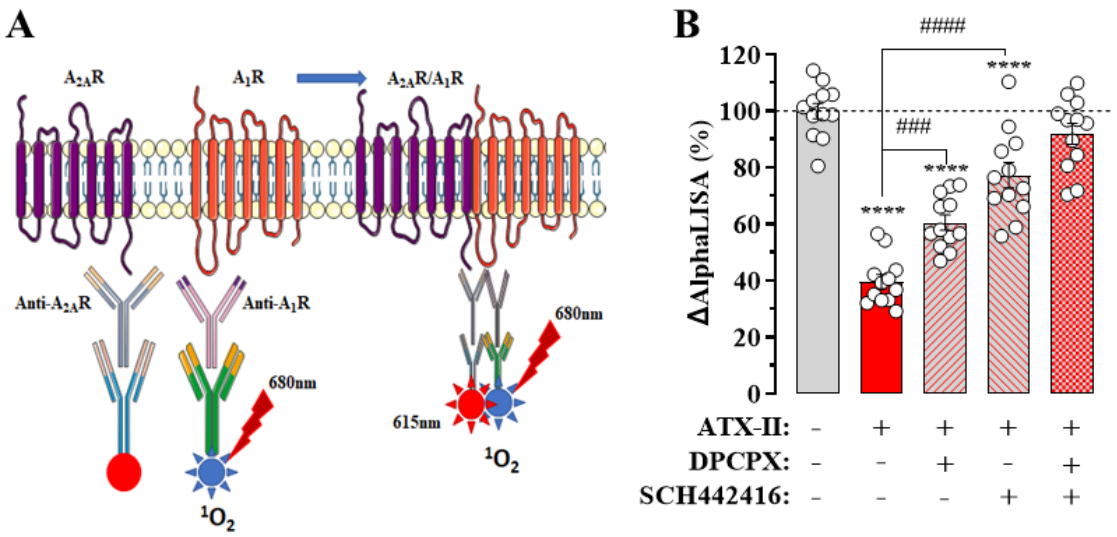


Figure 30. Effect of ATX-II on $A_1R/A_{2A}R$ heterodimer formation in HL-1 cells. (A) Schematic representation of AlphaLISA approach. **(B)** $A_1R/A_{2A}R$ heterodimer content in HL-1 cells. Cells were incubated in presence or absence of ATX-II (30 nM), DPCPX (100nM) and/or SCH442416 (100nM) (n=4 for each condition). The specific AlphaLISA signal (i.e., ΔAlphaLISA) was calculated as described in the 'Material and Methods' section and expressed as percentage (mean ± SEM) of either the control or treated cells in three independent experiments. ***$P < 0.001$, ****$P < 0.0001$, one-way ANOVA followed by Dunnet's post-hoc test when compared to vehicle treated cells (dashed line) and $^{###}P < 0.001$ and $^{####}P < 0.0001$ by Tukey's post-hoc test.

Overall, our results demonstrated that in the ATX-II-induced arrhythmia-like cell model the A_1R and $A_{2A}R$ expression is significantly increased whereas the $A_1R/A_{2A}R$ heteromer content is considerably reduced, thus suggesting a potential deregulation of adenosine signalling through a concomitant adenosine receptors unbalance. Interestingly, the ATX-II-mediated effect was precluded upon incubation with A_1R and/or $A_{2A}R$ antagonists, thus suggesting a major role for adenosine controlling $A_1R/A_{2A}R$ heteromer content in arrhythmia-like conditions.

2.2. A_1R and $A_{2A}R$ expression and heteromer formation in a pig model of AF

Once we characterized the status of A_1R and $A_{2A}R$ expression and heteromer formation in the arrhythmia-like cellular model we aimed to validate these results in an animal model of AF. Pigs have been used for a long time in cardiovascular research due to the advantages over other animal models like dog, sheep, mice, and rat (Tsang et al., 2016). Accordingly, we aimed to assess the status of A_1R and $A_{2A}R$ density and heteromerization in a pig model of AF. Thus, in collaboration with Leif-Hove Madsen (CSIC-Hospital Sant Pau, Barcelona, Spain), we get access to cardiac tissue from AF

tachypaced pigs (Diness et al., 2020). Hence, atrial membrane extracts from sham (not AF) and AF pigs were prepared and analyzed by immunoblot. First, to evaluate the extend of atrial remodeling of our pig model of AF we assessed the relative expression of atrial calcium handling proteins (i.e., SERCA, NCX-1, PLB and CSQ-2) as it has been described a potential alteration of these proteins in AF (Greiser et al., 2011). Interestingly, while immunoblot analysis (Figure 31A) of atrial membrane extracts from AF pigs revealed a significant reduction in the expression CSQ ($P = 0.0135$) and PLB ($P = 0.0138$; when compared to sham pigs, no significant differences were found for SERCA ($P = 0.4207$) and NCX ($P = 0.7599$) (Figure 4B). Thus, these results confirmed a subjacent atrial remodeling in our pig model of AF (Figure 31B).

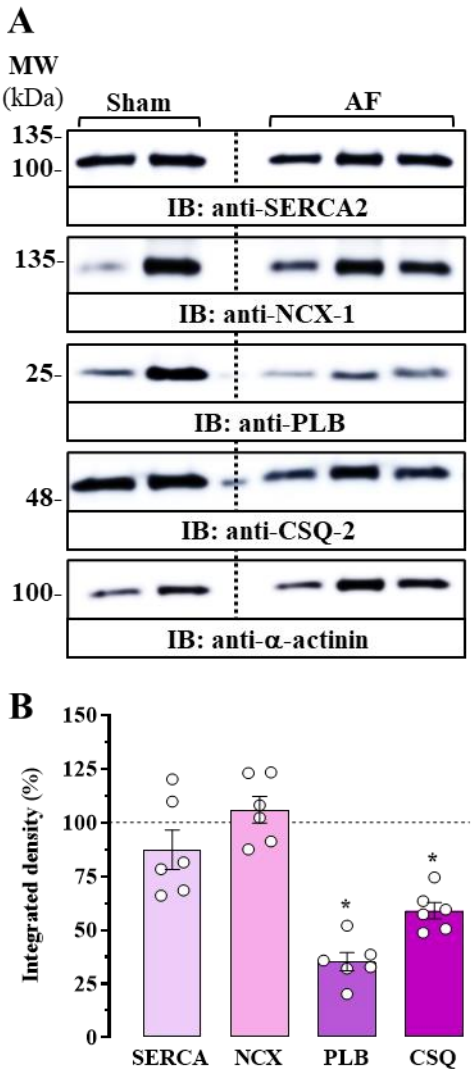


Figure 31. Ca^{2+}-regulatory proteins expression in the atrium of AF pig model. (A) Representative immunoblot showing the expression of calcium handling proteins. Atrial membranes extract from sham and fibrillated pigs were analysed by SDS-PAGE (10 µg of protein/lane) and immunoblotted using antibodies against SERCA2, Na^+-Ca^{2+} exchange protein (NCX-1), phospholamban (PLB) and calsequestrin-2 (CSQ-2) (*see* Material and Methods). **(B)** Relative quantification of SERCA2, NCX-1, PLB and CSQ-2 density. The immunoblot protein bands corresponding to SERCA2, NCX-1, PLB and CSQ-2 and α-actinin from sham (n = 4) and fibrillated pigs (n=6) were quantified by densitometric scanning. Values were normalized to the respective amount of α-actinin in each lane to correct for protein loading. Results are expressed as fold change over the control (vehicle treated cells; dashed line) (mean ± SEM). $*P < 0.05$, $**P < 0.01$, Student's *t*-test comparing with the vehicle treated cells (dashed line).

Subsequently, we aimed to determine A_1R and $A_{2A}R$ density and heteromer content through immunoblot experiments and AlphaLISA, respectively. Interestingly, our immunoblot results (Figure 32A) revealed that both A_1R and $A_{2A}R$ density was significatively increased in AF pigs when compared to sham animals (Figure 32B, $P = 0.0138$, $P = 0.0135$, respectively). Next, heteromer formation was determined by AlphaLISA technique by using the same pig atrial membranes. Importantly, the $A_1R/A_{2A}R$ heteromer formation was significantly reduced in AF pigs ($P = 0.0018$; Figure 32C). Overall, while AF pigs displayed enhanced ARs density in the

atrium, a reduced $A_1R/A_{2A}R$ heteromerization was showed, thus revealing an imbalance in the adenosinergic signalling. Alltogether, these results are in line to those obtained in HL-1 cultures, thus validating the usefulness of pigs as an animal model to study the adenosinergic system in AF.

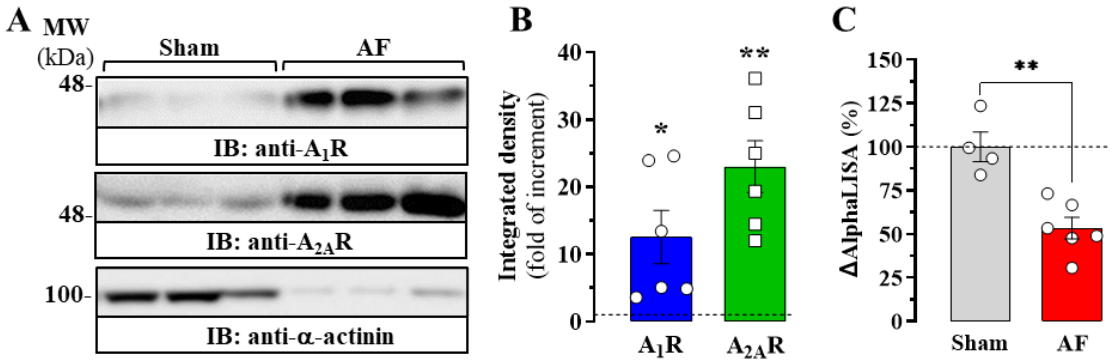


Figure 32. $A_1R/A_{2A}R$ density and heterodimer formation in pigs. (A) Representative immunoblot showing the expression of A_1R and $A_{2A}R$ in membranes of atrium pigs; membranes from pig atrium were analysed by SDS-PAGE (10 µg of protein/lane) and immunoblotted using mouse anti-A_1R, mouse anti-$A_{2A}R$ and rabbit anti-α-actinin antibodies. **(B)** Relative quantification of A_1R and $A_{2A}R$ density respectively; the immunoblot protein bands corresponding to A_1R, $A_{2A}R$ and α-actinin from Sham (n = 4) and AF (n = 6) pigs were quantified by densitometric scanning; values were normalized to the respective amount of α-actinin in each lane to correct for protein loading. Results are represented as fold of increment respect Sham pigs (mean ± SEM). **(C)** $A_1R/A_{2A}R$ heterodimer levels in pig atrium membranes of sham (n=4) and AF (n=6) pigs. The specific AlphaLISA signal (i.e., ΔAlphaLISA) was calculated as described in the 'Material and Methods' section and expressed as percentage (mean ± SEM) of either the control (Sham) or AF pigs in three independent experiments. **P*<0.05, ***P*<0.01, Student-T test.

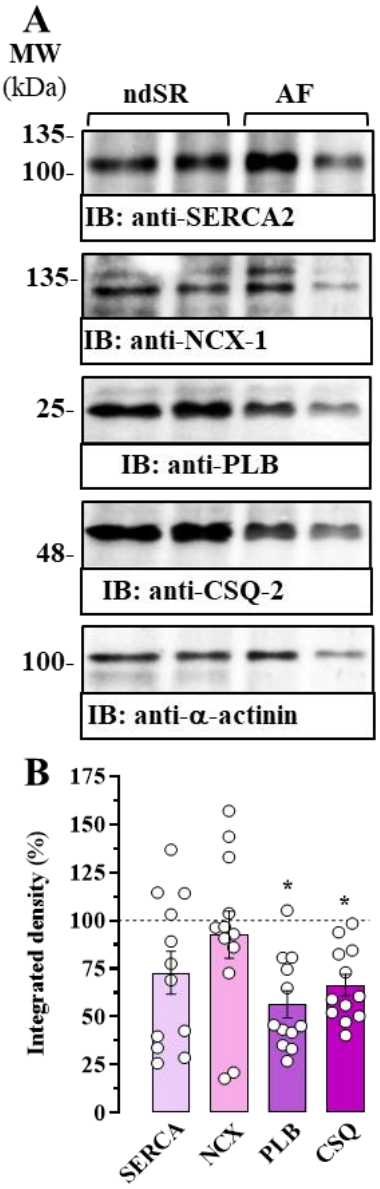
2.3. A_1R and $A_{2A}R$ expression and heteromer formation in AF patients

After we determined the A_1R and $A_{2A}R$ expression levels and heteromer formation in the arrhythmia-like cells and the pig animal model, we aimed to validate these results in AF patients. To this end, the status of A_1R and $A_{2A}R$ density and heteromerization in atrial membrane extracts from AF patients was assessed. In collaboration with Leif-Hove Madsen (CSIC-Hospital Sant Pau, Barcelona, Spain), we get access to cardiac tissue from AF patients (Table 3).

Table 3. Baseline demographic characteristics of AF patients

	ndSR	AF
Number of patients	11	9
Weight (mean±SD) (Kg)	79.75±8.62	72.01±12.01
Height (mean±SD) (cm)	168.8±8.7	162.92±8.06
Age (mean±SD)	64.7±9.9	70.9±10.5
Sex (male/female)	9/2	6/3
Body Surface (mean±SD)(m^2)	1.90±0.12	1.77±0.17
LA diameter (mean±SD) (mm)	41.1±5.9	47.2±7.21
LV diameter (mean±SD) (mm)	52.8±6.72	52.4±5.47

Abbreviations: LA, left atria. LV, left ventricle



Accordingly, atrial membrane extracts from ndSR (not AF) and AF patient's biopsies were prepared and analyzed by immunoblot. First, to confirm the AF status we assessed the relative expression of atrial calcium handling proteins (i.e., SERCA, NCX-1, PLB and CSQ-2, Figure 33A). Interestingly, immunoblot results showed that SERCA2 expression is slightly but not significantly reduced in AF patients compared with ndSR ones (Figure 33B). Also, NCX-1 expression is not significantly different among patients with and without AF (Figure 33B). However, PLB and CSQ-2 expression was significantly lower in AF patients than in those without AF ($P = 0.0261$, $P = 0.0016$, respectively) (Figure 33B).

Figure 33. Ca^{2+}-regulatory proteins expression in the atrium of AF patients. (**A**) Representative immunoblot showing the expression of calcium handling proteins. Atrial membranes extract from ndSR and AF patients were analysed by SDS-PAGE (10 μg of protein/lane) and immunoblotted using antibodies against SERCA2, Na^+-Ca^{2+} exchange protein (NCX-1), phospholamban (PLB) and calsequestrin-2 (CSQ-2) (*see* Material and Methods). **(B)** Relative quantification of SERCA2, NCX-1, PLB and CSQ-2 density. The immunoblot protein bands corresponding to SERCA2, NCX-1, PLB and CSQ-2 and α-actinin from ndSR (n = 11) and AF patients (n=p) were quantified by densitometric scanning. Values were normalized to the respective amount of α-actinin in each lane to correct for protein loading. Results are expressed as fold change over the control (dashed line) (mean ± SEM). *$P < 0.05$, **$P < 0.01$, Student's *t*-test comparing with the ndSR (dashed line).

Overall, these results demonstrate that AF has a strong impact on the expression of SR calcium buffering protein CSQ-2 and PLB total expression in the atrium of patients with AF. Also, it corroborates the AF phenotype in our subjects.

Subsequently, once confirmed the fibrillated status of our AF patients, we aimed to assess the density and heterodimer formation in these subjects. First, we performed immunoblot experiments (Figure 34A) to determine A_1R and $A_{2A}R$ density in atrial membranes from ndSR and AF patients. Again, we confirmed a significant increase of A_1R and $A_{2A}R$ density ($P = 0.0179$ and $P = 0.0001$, respectively) in AF patients when compared to ndSR (Figure 34B). In addition, $A_1R/A_{2A}R$ heterodimeric levels were determined again by AlphaLISA assay. Interestingly, the results in humans resemble the observed in cells and pigs, thus a significant ($P = 0.0002$) reduction in the heterodimer formation in AF patients was observed (Figure 34C). Overall, we confirmed that the A_1R and $A_{2A}R$ density and heteromer formation in three different AF models was altered, the confirming the potential usefulness of these models to study AF.

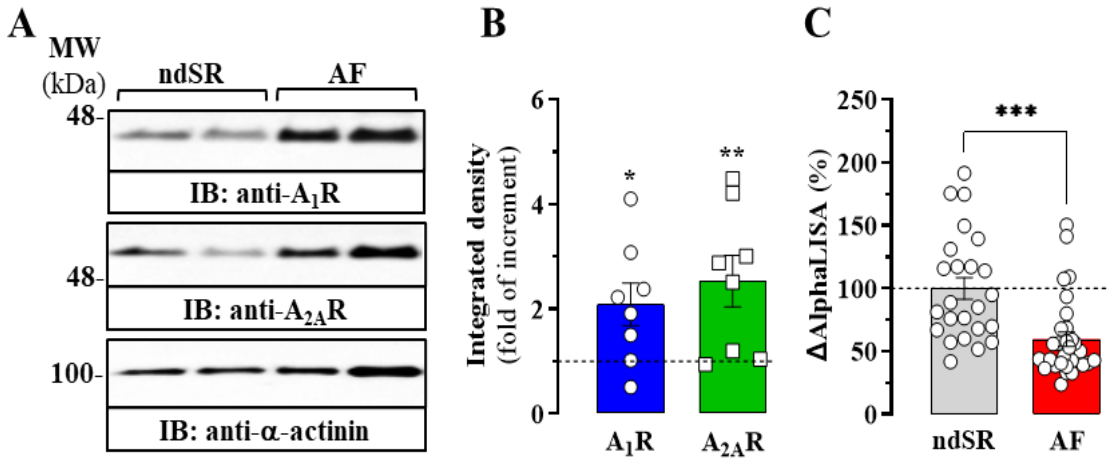


Figure 34. $A_1R/A_{2A}R$ expression and heterodimer formation in human heart atria. (A) Representative immunoblot showing the expression of A_1 and A_{2A} receptor in right atrium from non-dilated sinus rhythm (ndSR) and atrial fibrillation (AF) patients; membranes from human atrium were analysed by SDS-PAGE (10 µg of protein/lane) and immunoblotted using rabbit anti-A_1R, goat anti-$A_{2A}R$ and rabbit anti-α-actinin antibodies (see Material and Methods). **(B)** Relative quantification of A_1R and $A_{2A}R$ density respectively; the immunoblot protein bands corresponding to A_1R, $A_{2A}R$ and α-actinin from ndSR (n = 11) and AF (n = 9) pigs were quantified by densitometric scanning; values were normalized to the respective amount of α-actinin in each lane to correct for protein loading. Results are represented as fold of increment respect ndSR patients (mean ± SEM). **(C)** $A_1R/A_{2A}R$ heterodimer levels in human atrium membranes of ndSR and AF (n=10 each) patients. The specific AlphaLISA signal (i.e., ΔAlphaLISA) was calculated as described in the 'Material and Methods' section and expressed as percentage (mean ± SEM) of either the ndSR or AF subjects in three independent experiments. *$P < 0.05$, ***$P < 0.005$, ****$P < 0.0001$, Student-T test. *$A_{2A}R$ immunoblot results were discussed in chapter 1.

Chapter 3: **Modulating the adenosine receptors activity**

In the previous chapters, we have found alterations in the ARs expression and heterodimerization in arrhythmogenic cardiomyocytes, fibrillated pigs, and AF patients. In addition, these alterations are not restricted to heart atria, hitting also peripheral cells/tissues such as PBMCs. Both, A_1R and $A_{2A}R$, can regulate the calcium release and CICR cycle in the cardiomyocytes (Hleihel et al., 2006). Then, A_1R and $A_{2A}R$ play a key role in contraction, heart rhythm and many other cardiac events (Mustafa et al., 2009). For these reasons, A_1R and $A_{2A}R$ can constitute a promising therapeutic target in AF on two different approaches: reducing or blocking the $A_{2A}R$ activity or triggering the A_1R activity. Consequently, in the third chapter we intended to modulate the ARs activity and its heteromer formation. Accordingly, we will determine the impact on cAMP accumulation (receptors activity) and heterodimer formation of 4 different drugs on HL-1 and HEK-293T transfected cells. Selected drugs are an $A_{2A}R$ antagonist (SCH442416), an A_1R agonist (CPA), an A_1R positive allosteric modulator (T-62) and a non-selective AR agonist (chloro-adenosine). Also, we will try to replicate this modulation with the corresponding photodrugs.

3.1. $A_{2A}R$ antagonist: SCH442416

The first tested molecule was a selective $A_{2A}R$ full antagonist named SCH442416. To evaluate the effectiveness on blocking $A_{2A}R$ activity we first determined intracellular cAMP accumulation. cAMP is a second messenger that regulates the PKA activity and can be related directly with calcium releasement, heart rhythm and contraction. Thus, cAMP levels in HL-1 cells were evaluated by the Bright-Glo approach (*see* Material and Methods) (Z. Cheng et al., 2012).

Therefore, we induced cAMP accumulation by adding the selective $A_{2A}R$ full agonist CGS21680. Then, we aimed to tackle it with the SCH442416 antagonist. Interestingly, when HL-1 cells in presence of ADA were challenged with CGS21680 a significant ($P < 0.0001$) increase in cAMP accumulation was observed (Figure 35A), pointing that HL-1 cells effectively accumulate cAMP through $A_{2A}R$ stimulation. Indeed, this $A_{2A}R$-mediated cAMP accumulation was precluded by the simultaneous incubation with SCH442416 (Figure 35A), demonstrating that SCH442416 effectively block the $A_{2A}R$ activity.

Next, we evaluated the effect of SCH442416 in the heterodimer $A_1R/A_{2A}R$ formation by using the NanoBit technology in transfected HEK-293T cells (*see* Material and Methods) (Ohmuro-Matsuyama & Ueda, 2019). The sensitivity of that methodology allows us to determine the effect of our compounds at short-acute term (the first 50 minutes in presence of the drug) or at long-chronic term (after 24 hours of drug incubation).

Then, in long-term incubation non-treated cells and cells incubated with SCH442416 alone shows no reduction on the heterodimer formation (Figure 35B). In contrast, a significant reduction of the heterodimer was found in cells cultured with CGS. Finally, when challenged with both molecules we observe similar heterodimeric levels compared with the non-treated ones (Figure 35B). Similar results were observed in short-time incubations. CGS incubated cells reduce the heterodimeric $A_1R/A_{2A}R$ formation whereas SCH442416 alone does not affect heterodimeric levels. Nevertheless, when cells were co-incubated with CGS and SCH442416 heterodimeric levels remain stable (Figure 35C). Overall, these results point to a high effectiveness in the blocking of the $A_{2A}R$ activation, which could help in AF events. Also, heterodimeric structure remains stable when incubated with SCH442416 countering the effect of CGS. Future studies will determine the impact of this compound in in vivo models.

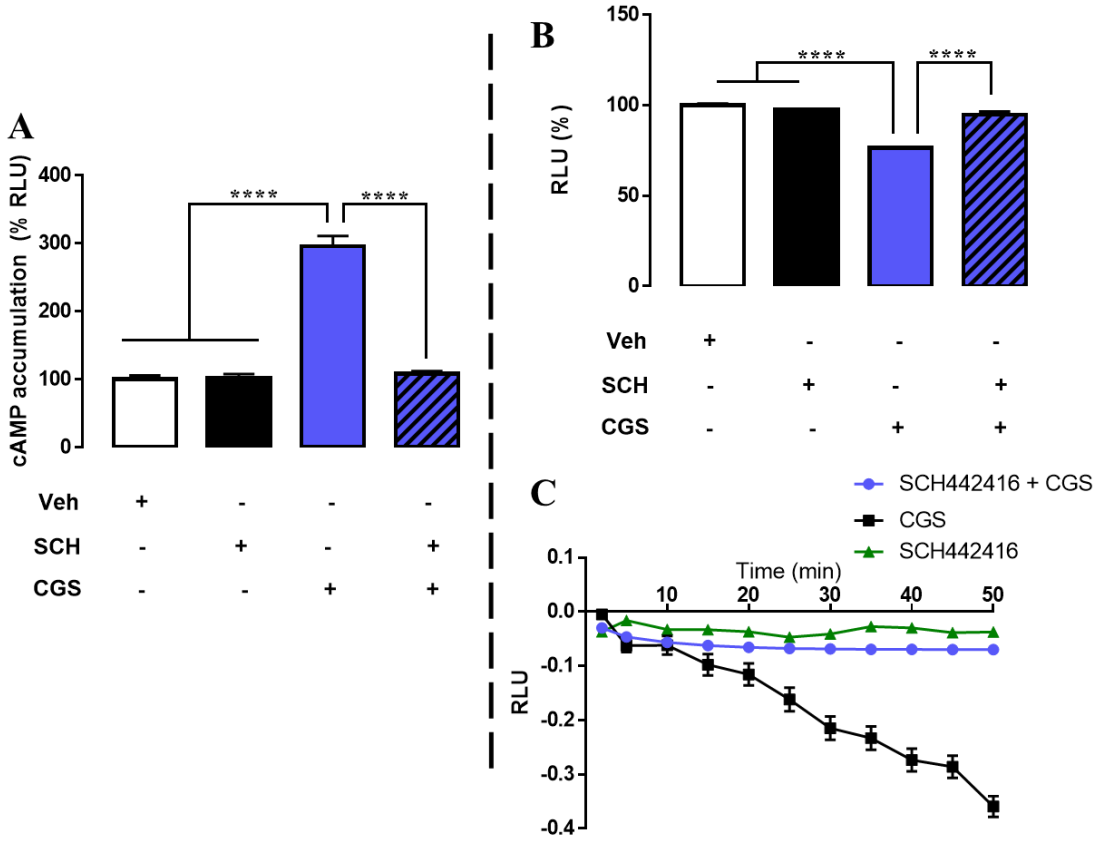


Figure 35. Modulation of A_{2A} receptor by SCH442416. (A) A_{2A}R-mediated cAMP accumulation in HL-1 cells. Cells were incubated with ADA, saline (veh), 400nM CGS21680 (CGS) and 400nM CGS21680 in the absence or presence of 1µM SCH442416 (SCH). (B) Long-term heterodimer formation in HEK-293T transiently transfected with the A_1RlgBit and A_{2A}RsmBit incubated for 24 hours with ADA and in presence or absence of CGS (400nM) and/or SCH442416 (1µM). Data is represented as a percentage of relative luminescence units (RLU) mean ± SEM (n= 3). (C) Short-term heterodimer formation in HEK-293T transiently transfected with the A_1RlgBit and A_{2A}RsmBit incubated for 24 hours with ADA. Heterodimer formation is measured at real-time in presence or absence of CGS (400nM) or SCH442416 (1µM) and CGS (400nM) coincubated with SCH442416 (1µM). The data was normalized for the basal luminescence, vehicle (Veh) and cell protein amount. Results are expressed as relative luminescence units (RLU) mean ± SEM (n= 3). The asterisks denote significant differences: ****P <0.0001, one-way ANOVA with Dunnett's multiple comparison test.

3.2. A_1R agonist: CPA

Once we have demonstrated that we can preclude the A_{2A}R activity, we focused on the A_1R, the physiological regulator of the A_{2A}R. In that case, as A_1R activity reduce the accumulation of cAMP and calcium release, we selected a selective A_1R full agonist named CPA. A_1R activation could equilibrate the adenosinergic dysregulation present in AF events and be useful in the treatment of the disease. To measure the cAMP levels, we used the Perkin Elmer cAMP kit (*see* Material and Methods).

Thus, ADA treated HL-1 cells were challenged with Forskolin to induce cAMP accumulation. Next, when challenged with CPA we can observe the effect of A_1R activation by precluding this accumulation. As expected, results shown that forskolin-CPA treated cells have a significative ($P < 0.0001$) reduction in cAMP accumulation when compared with forskolin-vehicle ones (control cells) (Figure 36A).

Next, we aimed to determine the effect of this drug on the $A_1R/A_{2A}R$ heterodimerization by using the NanoBit approach. As in the previous results, ADA treated cells were incubated for 1 hour or 24 hours in presence or absence of CPA. Results shows that at both long and short-term incubations we can observe a significative ($P < 0.0001$) reduction of the heterodimeric signal (Figure 36B-C respectively). Overall, these results reveals that we can reduce the effect on cAMP accumulation by using A_1R agonist. However, we may lose fine control due to the loss of heterodimer formation. Future studies will determine the effect on AF cells and the risk-benefit ratio of this compound in vivo.

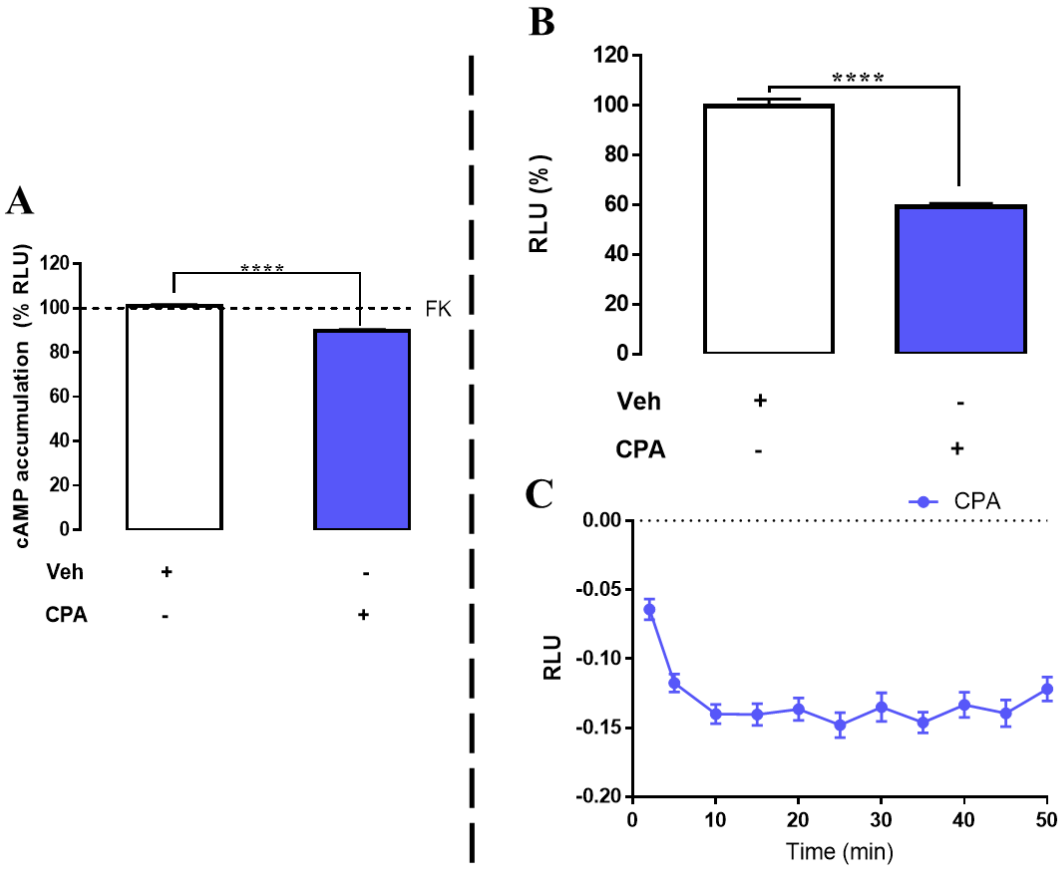


Figure 36. Modulation of A_1 receptor by CPA. (A) A_1R-mediated cAMP accumulation in HL-1 cells. Cells were incubated with ADA and Forskolin (500nM) in presence or absence (veh) of 3nM CPA. (B) HEK-293T transiently transfected with the A_1RlgBit and A_{2A}RsmBit incubated for 24 hours with ADA and in presence or absence of CPA (3nM). Data is represented as a percentage of relative luminescence units (RLU) mean ± SEM (n= 3). (C) HEK-293T transiently transfected with the A_1RlgBit and A_{2A}RsmBit incubated for 24 hours with ADA but incubated and measured at real-time the heterodimer in presence or absence of CPA (3nM). The data was normalized for the basal luminiscence, vehicle (Veh) and cell protein amount. Results are expressed as relative luminescence units (RLU) mean ± SEM (n= 3). The asterisks denote significant differences: ****P <0.0001, Student-t test.

3.3. A_1R Positive allosteric modulator: T-62

For the third drug, we pointed to modulate more accurately the A_1R activity by using a PAM (positive allosteric modulator) named T-62. Allosteric modulators can alter the response of its receptor for its agonist (Kew, 2004). In the case of the T-62, as a PAM, supports the A_1R agonist (i.e., CPA) to bind to the receptor. This triggers the A_1R activation without modifying the A_1R agonist input. Consequently, we have analysed the effect of T-62 in both cAMP accumulation and heterodimerization of the A_1R/ A_{2A}R.

Once again, cAMP accumulation was determined with the PerkinElmer cAMP kit. HL-1 cells incubated with ADA were challenged with Forskolin and CPA in presence or

absence of T-62. Importantly, we ended that while significant ($P < 0.005$), the PAM potency of T62 was moderate (with about a 30% induced increase of CPA activity) (Figure 37A), which represents a three times higher cAMP inhibition when compared with CPA alone.

Regarding the heteromer formation under T-62 activity, no effects were observed when challenged with ADA and T-62 alone for one or 24 hours, whereas CPA activity drastically reduce it. T-62 coincubated with CPA reduce heterodimer formation in a similar way than CPA alone at 24 hours (figure 37B) and is even higher the first 50 minutes (Figure 37C). Alltogether, these results points that using that PAM approach could be helpful in the treatment of AF, as well as can modullate A_1R activity without effects on heterodimer formation.

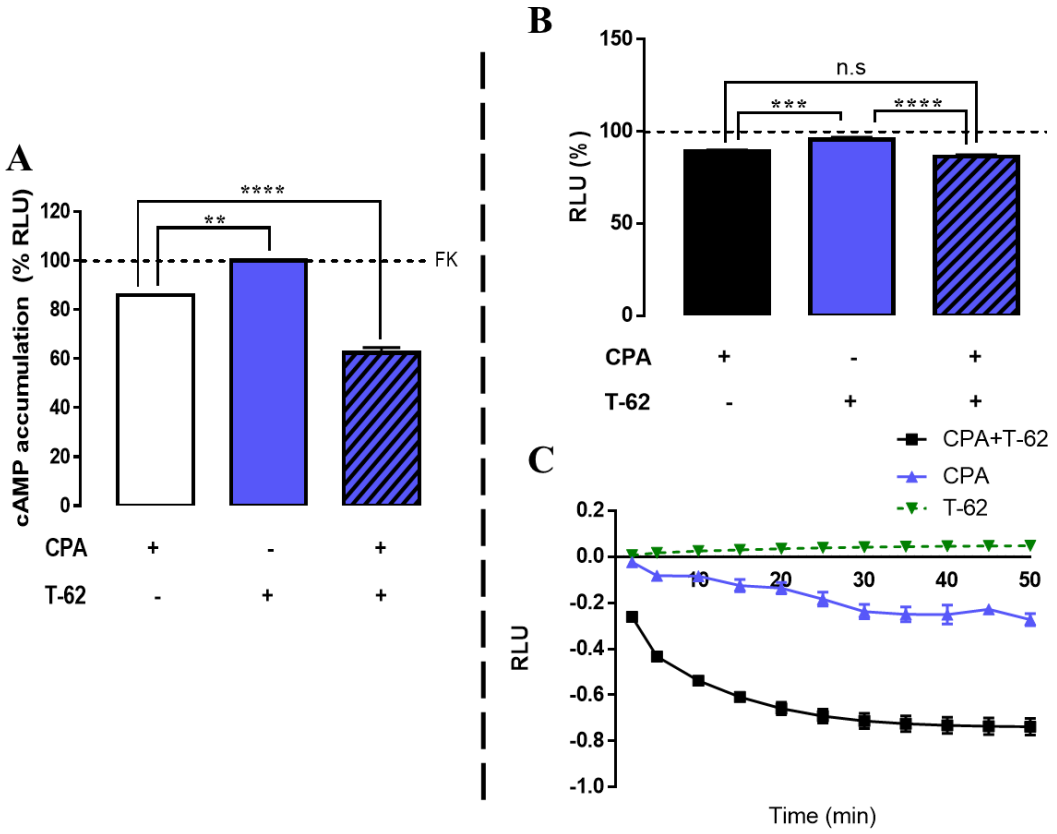


Figure 37. Modulation of A_1 receptor by T-62. (A) A_1R-mediated cAMP accumulation in HL-1 cells. Cells were incubated with ADA and Forskolin (500nM) and coincubated with saline (veh), 3nM CPA and CPA plus T-62 (100nM). (B) HEK-293T transiently transfected with the A_1RlgBit and A_{2A}RsmBit incubated for 24 hours with ADA and in presence or absence of CPA (3nM) and T-62 (100nM). Data is represented as a percentage of relative luminiscence units (RLU) mean ± SEM (n= 3). (C) HEK-293T transiently transfected with the A_1RlgBit and A_{2A}RsmBit incubated for 24 hours with ADA but incubated and measured at real-time the heterodimer in presence or absence of CPA (3nM) and T-62 (100nM). The data was normalized for the basal luminescence, vehicle (Veh) and cell protein amount. Results are expressed as relative luminescence units (RLU). The results are expressed as RLU mean ± SEM (n= 3). The asterisks denote significant differences: $^{**}P$ <0.01, $^{***}P$ <0.001 $^{****}P$ <0.0001, one-way ANOVA with Dunnett's multiple comparison test.

3.4. $A_1R/A_{2A}R$ Non-selective agonist: Adenosine

Adenosine is the endogenous agonist for the adenosine receptors and displays an important role in cardiac events and circulation (Mubagwa et al., 1996). Consequently, we aimed to determine the in vitro effect of adenosine on cAMP accumulation and heterodimer formation.

Thus, first we challenged HL-1 cells with ADA and chloro-adenosine (cADO, which is not affected by ADA activity) at different doses. Interestingly, chloro-adenosine tends to accumulate cAMP (when compared with saline and CGS incubated cells) at higher doses whereas have the opposite effect at lower ones (Figure 38A). This can be explained by the affinity of the adenosine receptors to adenosine, which is higher for the A_1R compared with the $A_{2A}R$.

Next, we determined the impact on the A_1R/ $A_{2A}R$ heteromer, using the same NanoBit approach described in the previous results. We used a fix dose of chloro-adenosine, in that case 30nM. cADO challenged cells (24 and one hours) coincubated with ADA reveals a significative reduction in the heterodimeric formation (Figure 38B-C). Overall, these results point to a loss of $A_{2A}R$ control by the heterodimeric structure when we have high doses of adenosine, like in an AF event.

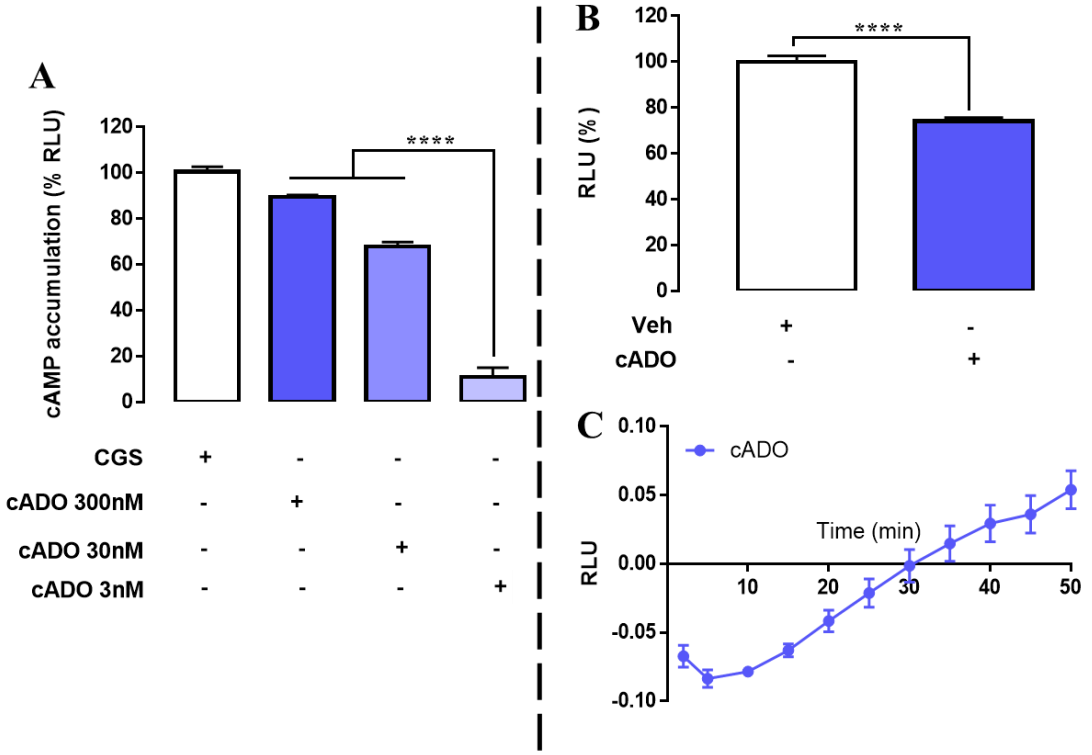


Figure 38. Modulation of A_1 and A_{2A} receptors by chloro-adenosine. (A) $A_1R/A_{2A}R$-mediated cAMP accumulation in HL-1 cells. Cells were incubated with ADA and 400nM CGS or chloro-adenosine (300nM, 30nM and 3nM). (B) HEK-293T transiently transfected with the A_1RlgBit and A_{2A}RsmBit incubated for 24 hours with ADA and in presence or absence of chloro-adenosine (30nM). Data is represented as a percentage of relative luminescence units (RLU) mean ± SEM (n= 3). (C) HEK-293T transiently transfected with the A_1RlgBit and A_{2A}RsmBit incubated for 24 hours with ADA but incubated and measured at real-time the heterodimer in presence or absence of Adenosine (30nM). The data was normalized for the basal luminescence, vehicle (Veh) and cell protein amount. Results are expressed as relative luminescence units (RLU) mean ± SEM (n= 3). The asterisks denote significant differences: ***P* <0.01, ****P* <0.001 *****P* <0.0001, one-way ANOVA with Dunnett's multiple comparison test and Student-t test.

3.5. The photodrugs

We designed four different drugs based on Photopharmacology approach (Figure 39) that are based on the same compounds tested before. These are the $A_{2A}R$ antagonist MRS7145 (SCH442416 attached to a coumarin), the A_1R agonist CPA-cage (CPA attached to a coumarin), the A_1R PAM T-62-cage (T-62 attached to a coumarin) and the switch compound AA3 (adenosine switch). Then, we aimed to replicate the results obtained in the previous experiments but with the cage and switch compounds.

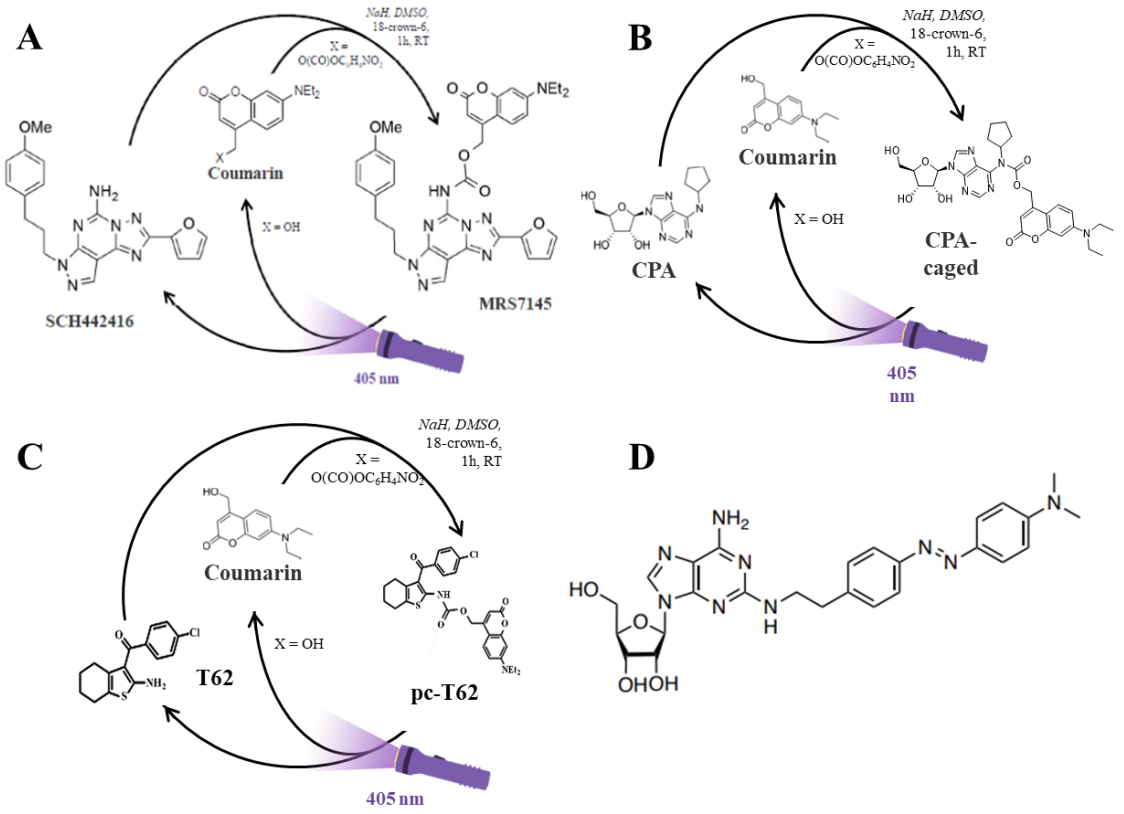


Figure 39. Schematic representation of the photodrugs and the photo-uncaging. (A) $A_{2A}R$ antagonist MRS7145 cage compound. (B) A_1R agonist CPA-cage compound. (C) A_1R PAM pc-T62 cage compound. (D) Non-selective adenosine receptors agonist AA3 switch compound.

First, we determined the cAMP accumulation in HL-1 cells with the different photodrugs. All results were compared with the non-light-sensitive molecules. Thus, we modulated the cAMP accumulation due to $A_{2A}R$ activity with the MRS7145 (Figure 40A), A_1R activity with the CPA-cage (Figure 40B) and the T-62-cage (Figure 40C), and by ARs activity with the AA3-switch (Figure 40D). Importantly, results shown that we have effectively done the uncaging/photoswitching for the three cage compounds (MRS7145, CPA-cage and T-62-cage) and for the photoswitch one (AA3) upon irradiation at 405nm. We obtained almost identical results in cAMP accumulation when compared with the non-light-sensitive drugs by blocking the accumulation in the case of the MRS7145 (Figure 40A), by reducing it with A_1R agonist CPA-cage and PAM T-62-cage (Figure 40B-C). Light-inactivated adenosine AA3 compound shows no cAMP accumulation (Figure 40D) as corresponds to the disabled form of the compound.

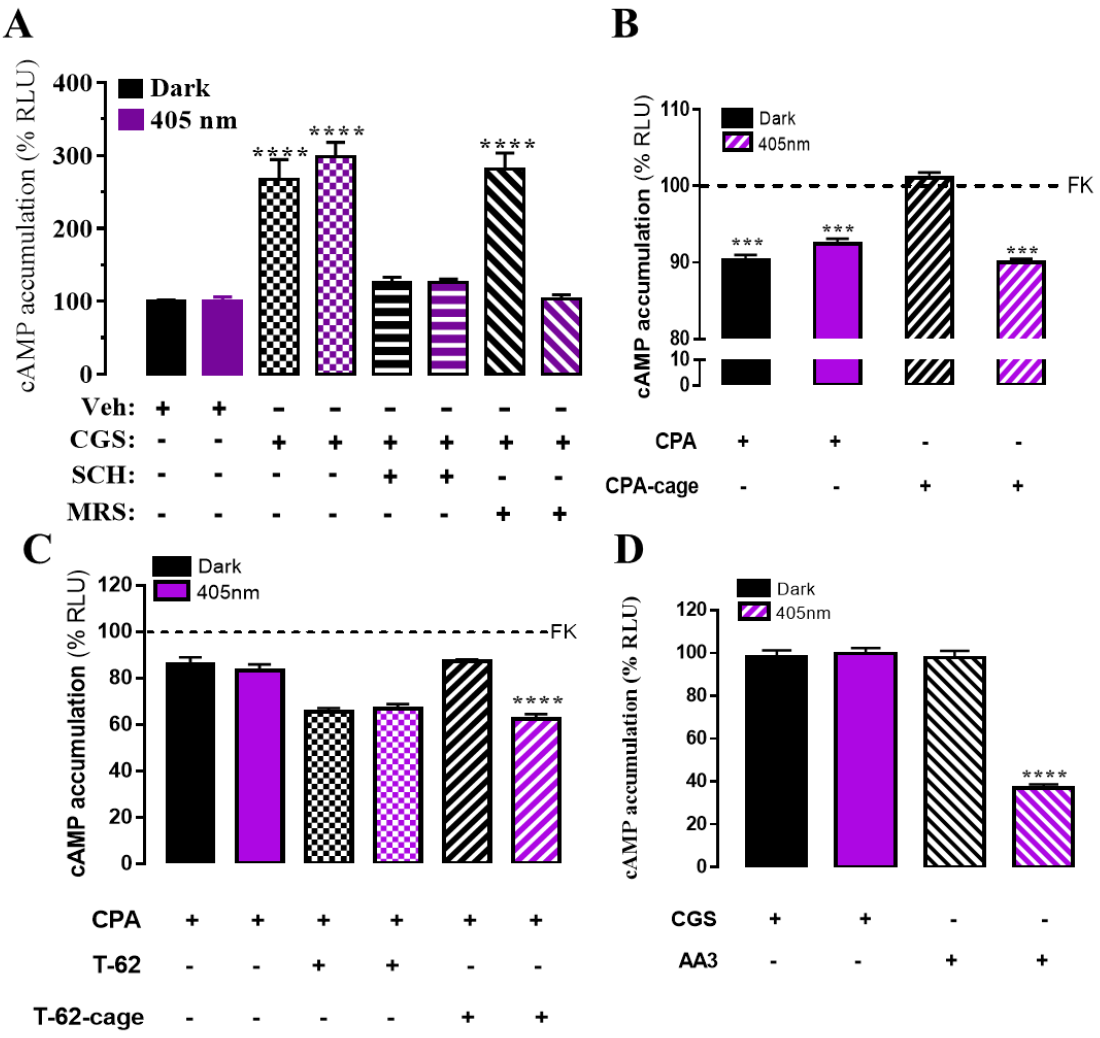
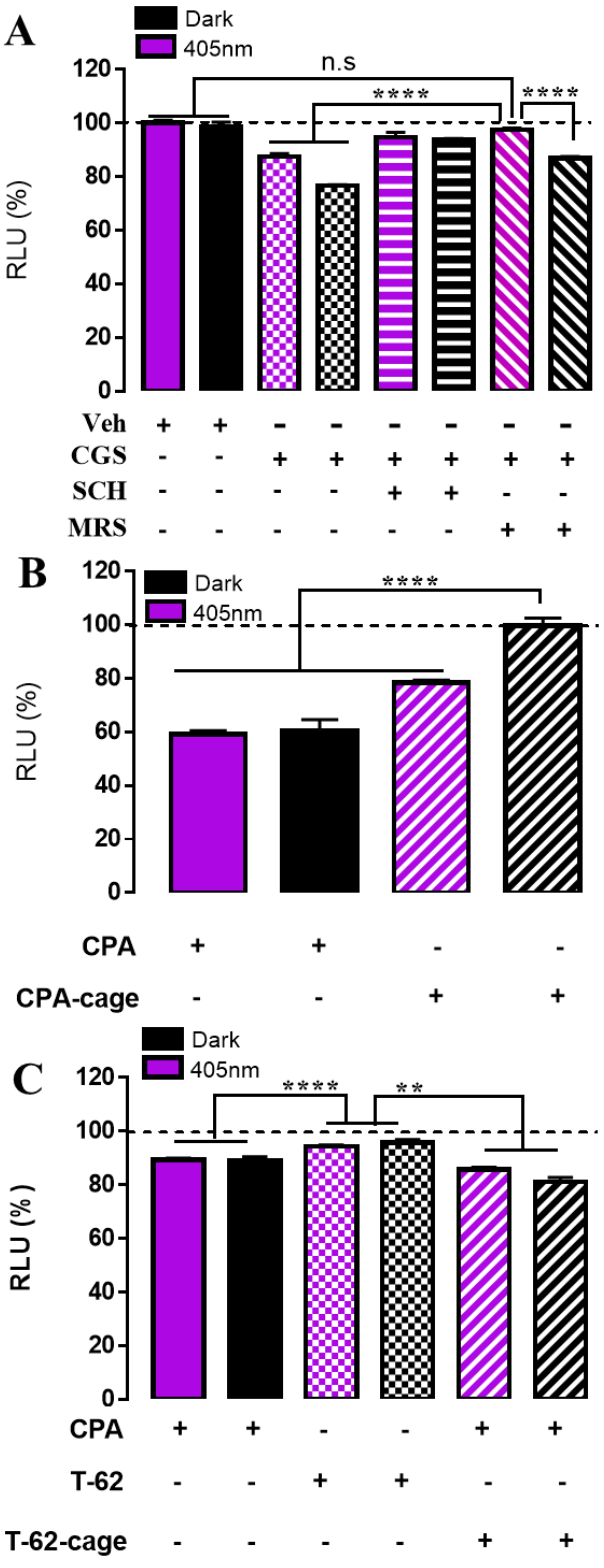


Figure 40. Photodrug modulation of adenosine receptors activity. (A) A_{2A}R-mediated cAMP accumulation in HL-1 cells. ADA treated cells were incubated with saline, 400nM CGS21680 (CGS) and 400nM CGS21680 in the absence or presence of either 1µM SCH442416 (SCH) or 1µM MRS7145 (MRS) in dark and upon 405 nm irradiation. The data was normalized for the vehicle (Veh) in dark condition and expressed as percentage of relative luminescence units (RLU). (B) A_1R-dependent inhibition of forskolin-mediated cAMP accumulation in HL-1 cells. ADA treated cells were incubated with 500nM of Forskolin and CPA (3nM) or CPA-Cage (300 nM) in dark and upon 405 nm irradiation. The data was normalized for cAMP accumulation by Forskolin (500nM) in dark condition and expressed as percentage of relative luminescence units (RLU). (C) A_1R-dependent inhibition of forskolin-mediated cAMP accumulation in HL-1 cells. Cells were incubated with 500 nM forskolin and CPA (3 nM) in the absence and presence of T62 (100 nM) or T-62-cage (100nM) in dark and upon 405 nm irradiation. The data was normalized for cAMP accumulation by Forskolin (500 nM) in dark condition and expressed as percentage of relative luminescence units (RLU). (D) ARs modulation determined by cAMP accumulation in ADA treated HL-1 cells. Cells were incubated with 400nM CGS or 30nM AA3 in dark and upon 405 nm irradiation. The data was normalized for cAMP accumulation by chloro-adenosine (30nM) in dark condition and expressed as percentage of relative luminescence units (RLU). The results are expressed as mean ± SEM (n= 3). The asterisks denote significant differences: ***P <0.001, ****P <0.0001, one-way ANOVA with Dunnett's multiple comparison test.



Next, measuring heterodimer formation after 24 hours of incubation, results revealed once again similar results when compared with the non-caged/switched compounds: MRS7145 does not affect the heteromer formation in both light and dark conditions. When uncaged by light, can bock the effect produced by CGS (Figure 41A). CPA-cage reduce heterodimeric structures in light conditions as well as do the non-caged CPA, whereas no effect was observed in dark conditions (Figure 41B). T-62-cage seems not to affect heterodimer formation in both dark and light conditions, revealing a similar pattern compared with the uncaged compound (Figure 41C). The switchable compound AA3 shows the same effect in light and dark conditions with 24 hours incubation (data not shown). This could be explained due to the natural conversion to the cis state after a short period of time next to light irradiation. Consequently, only heterodimer determination at short-time formation will be shown.

Figure 41. Photodrug heterodimer modulation of adenosine receptors in long-term incubations. (A) A_{2A}R-mediated heterodimer formation in ADA treated HL-1 cells. Cells were incubated with saline (veh), 400nM CGS21680 (CGS) and 400nM CGS21680 in the absence or presence of either 1µM SCH442416 (SCH) or 1µM MRS7145 (MRS) in dark and upon 405 nm irradiation. (B) A_1R-mediated heterodimer formation in ADA treated HL-1 cells. Cells were incubated with CPA (3nM) or CPA-Cage (300 nM) in dark and upon 405 nm irradiation. (C) A_1R-mediated heterodimer formation in ADA treated HL-1 cells. Cells were incubated with CPA (3nM), T-62 (100nM) and T-62-cage (100nM) in dark and upon 405 nm irradiation. The data was normalized for the vehicle (Veh) signal in dark conditions, the cell protein amount and expressed as a percentage of relative luminescence units (RLU). The results are expressed as mean ± SEM (n= 3). The asterisks denote significant differences: ***P* <0.01, *****P* <0.0001, one-way ANOVA with Dunnett's multiple comparison test.

Finally, we moved to determine heterodimer formation at short-term incubations. Then, HEK-293T transfected cells were challenged with the different photodrugs. As expected, MRS7145 shows a similar effect as SCH442416 when irradiated at 405nm. However, no effect was exerted by MRS7145 in dark conditions (Figure 42A). In the case of A_1R modulators (CPA and T62 cage), heterodimer determinations conclude that CPA-cage mimic the effect of CPA in light conditions but not in dark ones (Figure 42B). T-62-cage shown no effect on heterodimer formation in dark and light conditions as well as T-62 (Figure 42C). AA3, the adenosine switch compound, replicates the effect of adenosine in dark conditions (active state). When challenged by light, it changes in an inactive state, but as time progresses it converse to active state, starting to reduce heterodimer formation (as well as do adenosine) (Figure 42D). Overall, these data suggest that upon irradiation, cage and switch compounds imitate the effect of the classical drugs. This point to a potential use in future studies to treat AF episodes without all the side-off effects of these drugs.

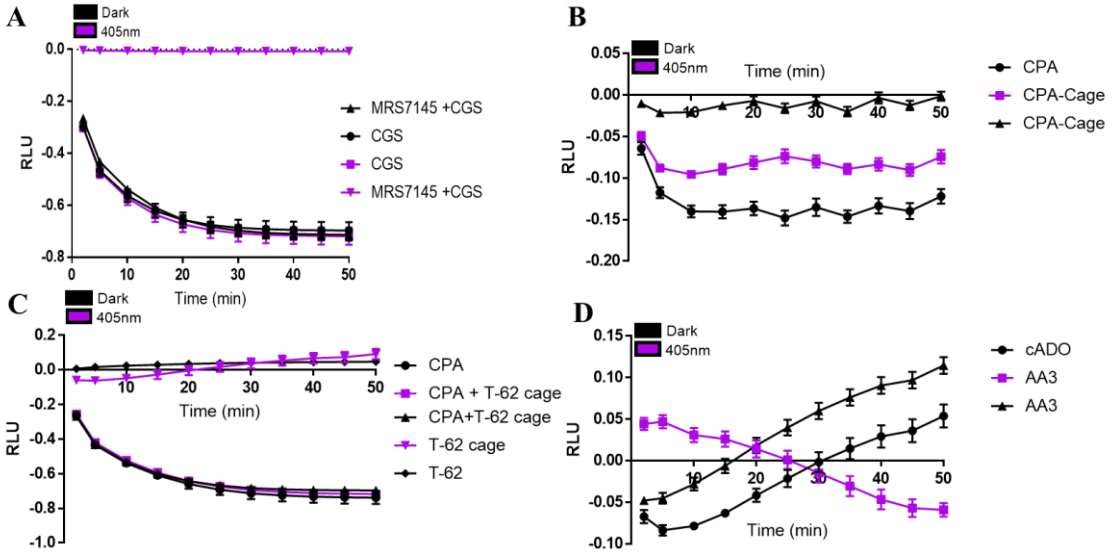


Figure 42. Photodrug heterodimer modulation of adenosine receptors in long-term incubations. (A) $A_{2A}R$-mediated heterodimer formation in ADA treated HL-1 cells. Cells were incubated with saline (veh) and 400nM CGS21680 in the absence or presence of either 1µM MRS7145 (MRS) in dark and upon 405 nm irradiation. (B) A_1R-mediated heterodimer formation in ADA treated HL-1 cells. Cells were incubated with CPA (3nM) or CPA-Cage (300 nM) in dark and upon 405 nm irradiation. (C) A_1R-mediated heterodimer formation in ADA treated HL-1 cells. Cells were incubated with CPA (3nM), T-62 (100nM) and/or T-62-cage (100nM) in dark and upon 405 nm irradiation. (D) ARs-mediated heterodimer formation in ADA treated HL-1 cells. Cells were incubated with chloro-adenosine (30nM) or AA3 (30nM) in dark and upon 405 nm irradiation. The data was normalized for the basal luminescence, vehicle (Veh) and cell protein amount. Results are expressed as relative luminescence units (RLU) mean ± SEM (n= 3).

VI. Discussion

Cardiovascular diseases (CVDs) constitute one of the leading causes of premature death and disability worldwide. Approximately 80% of population will be affected by CVDs at some point of their lives (Shi et al., 2016). One of the most frequent CVDs consist of arrythmias, described by an irregular heartbeat, circumscribed by impaired frequencies of beating (i.e. faster, slower or inconstant) when compared to healthy heartbeats. Among them, AF is the most prevalent diagnosed variety of arrhythmia globally, it is characterized by a rapid and irregular beating of atrial chambers of the heart (X. Liu et al., 2019). Actually, its harmful impact in the society makes AF research a public health challenge that will need to be assessed. Additionally, its prevalence is tend to double in the next decades and is expected to aggravate with time (Morillo et al., 2017). AF Pathophysiology is not completely understood, whereas it is described that its related primarily with the loss of calcium handling in atrial cardiomyocytes. Calcium handling proteins, as well as the occurrence and progression of AF, are tightly related with adenosinergic system (Li et al., 2016). Thus, we decided to analyse the role of adenosine receptors in subjects suffering of AF. However, since adenosine is a key molecule regarding all cells of the organism, the effect of AF could be affecting other than heart-related structures and milieu. This approach could be a critical point in understanding AF mechanisms, and could be useful to detect or prevent AF-related events. Therefore, we need more than human patients to carry out this research. For that reason, adequate and precise AF models must be developed. In the present work, we aimed for one *in vitro* model (i.e. cardiomyocyte cell line HL-1) and one animal model (i.e. AF pig). Moreover, the clinical management of this arrhythmia (i.e. anticoagulatory therapy, anti-arrhythmic drugs…) is frequently ineffective, thus relapse after some period of treatment is quite common. As ARs are related to calcium handling and AF, they constitute a promising source of targets related to AF treatment. In addition, the new field of photopharmacology could make us overcome the conventional treatments by its benefits on spatio-temporal control and its precluded side- effects.

1. Atrial fibrillation in human patients

The relationship of the adenosinergic system and adenosine receptors with AF has been well described in the scientific literature (Ishihara et al., 2020). Since arrythmia triggers a high energy demand state, it is reasonable to think that adenosine content is increased

in cells and heart environment, affecting heart cardiomyocytes but also surrounding cells and tissues. AR $A_{2A}R$ play a key role in many of the processes that involves the heart, by way of example coronary contraction. However, since ARs are related to calcium release from SR, these receptors can also influence the cardiac contraction and rhythm. Additionally, this type of receptor has been related with some cardiopathological conditions, such as the genesis of arrhythmias (Boknik et al., 2021; Llach et al., 2011). However, AR alterations could be not restricted to heart environment, due to its ubiquitously expression through the body. Furthermore, adenosine releasement from heart can have unintended consequences over ARs within other systems.

In the present work, we assessed the status of the peripheric adenosinergic system in AF patients. Firstly, we confirmed the AF phenotype of our subjects by immunoblotting and RT-qPCR, in concordance with the medical literature (L. Hove-Madsen et al., 2006). Then, we scrutinized for alterations in ARs within other cell types, such as PBMCs. PBMCs are involved in most of the physiological cardiac events and might be also related with AF progression and maintenance. As way of example, inflammation processes linked to AF recruit's different populations of PBMCs that may carry out atrial remodelling and fibrosis (Miyosawa et al., 2020). Interestingly, signalling through $A_{2A}R$ regulates PBMCs function (Gessi et al., 2000). Hence, we analysed the expression of $A_{2A}R$ in PMBCs from AF patients and we interestingly found that the mentioned receptor was increased when compared to ndSR and dSR subjects, thus mirroring what we observed in right atrium. Parallelly, increased levels of $A_{2A}R$ have been reported in patients with immunological diseases such as systemic lupus erythematosus (Bortoluzzi et al., 2016) and amyotrophic lateral sclerosis (Vincenzi et al., 2013), among many others. Furthermore, it is also described an increased $A_{2A}R$ expression in lymphocytes from patients diagnosed with coronary artery disease (Gariboldi et al., 2017) and chronic heart failure (CHF) (Varani et al., 2003). However, to date, no studies have been reported a relationship between $A_{2A}R$ expression in PBMCs and AF. Yet, the consequences of this increased AR density in PBMCs should be assessed in future studies.

Due to the increased energy spent in AF, we have also analysed possible changes in adenosine plasma content. Subsequently, we considered to analyse ADA levels in plasma, justified by its importance in adenosine catabolism. Then, we found increased adenosine levels in plasma from AF patients. This results also correlates with increased $A_{2A}R$ density in atrium and PBMCs surface, pointing to a potential relationship among

adenosine levels and cardiac function in a systemic level in both AF and physiological conditions.

Additionally, ADA levels are also altered in AF patients, increasing the understanding of the mechanism behind the origin, progression, and maintenance of that disease. Low levels of ADA have been previously reported in alternative heart diseases, such as in CHF (Khodadadi et al., 2014), unfortunately its alteration have never been related to AF. In the case of CHF, reduction of ADA and ADA2 activity could offer some cardioprotection against the disease. However, in the case of AF, seems to be potentially risky since the main pathway to regulate adenosine level is eradicated. Furthermore, it remains a controversy if ADA-altered levels are the main responsible of triggering adenosine content or if, alternatively, are just a consequence of AF. Similarly, it is doubted if ADA and adenosine alterations comprise the cause or the consequence of AF. Briefly, inflammation events and altered hearth rhythm can promote an increase in energy demand. That could be translated into a triggered adenosine content in plasma that in turn will alter peripheral tissues and cells. These abnormal levels can preclude the ADA catabolism and induce an altered adenosinergic signalling, which will fit with the results observed in PBMCs and right atria. Conversely, low levels of ADA could directly reduce the adenosine catabolism. Higher levels of adenosine could hit the cardiac environment and alter adenosinergic signalling, over activating adenosine receptors. This alteration could end in an arrhythmic phenotype that may develop on a future AF. However, not altered levels of ADA or adenosine have been found in dSR patients (despite the slightly but not significant reduced levels of ADA and raised levels of adenosine). Alternatively, another hypothesis could be that ADA and adenosine alterations are just another step into the development of AF. Coherently, ADA and adenosine content might be considered as another risk factor for the disease.

Interestingly, our data reveals a correlation between adenosine levels and $A_{2A}R$ expression rates. Higher adenosine levels would stimulate $A_{2A}R$ activity (indeed, its expression is also higher in AF). $A_{2A}R$ signalling cascade will activate PKA which is able to phosphorylate calcium handling related proteins (PLB and RyR2). As consequence of that, higher levels of calcium will be released from SR. This could contribute to a higher incidence of spontaneous calcium release-induced electrical activity and irregular beating reported in atrial myocytes from patients with AF (Llach et al., 2011; Molina et al., 2016). However, the mechanism that links $A_{2A}R$ density and adenosine remains unclear. A

reasonable explanation could be a positive regulatory loop between these two elements. Accordingly, the more adenosine is released, the more $A_{2A}R$ is synthetized to deal with the excess of adenosine content. In addition, more $A_{2A}R$ would be needed to maintain the fast beat of the arrythmia. Oppositely, another hypothesis could be that one of the first steps in AF progression is the enhanced $A_{2A}R$ expression. As a consequence, fast beating would be promoted due to the loss of calcium homeostasis. In consequence more energy would be needed, and more adenosine is produced due to ATP hydrolysis, which could explain the increased adenosine content in found in AF. Either way, further research is needed to reveal the mechanism underlying these results.

2. Atrial fibrillation models

In vitro assays and animal models comprise essential tools in biological and medical investigations repertoire. In the case of the *in vitro* models of AF, are mainly based on primary animal derived cardiomyocytes. However, due to its difficulties in production and maintenance of that cell lines, its use has been decreasing in the last years. One alternative able to overwhelm these difficulties could be an immortalized cardiomyocyte cell culture, such as HL-1 cells, which have been repeatedly proved to be a useful as an in vitro model for research in arrythmias (van Gorp et al., 2020).

To validate Hl-1 system in AF disease, we induced an arrhythmic state, by altering the sodium influx, in those cells. Thus, ARs expression and heterodimerization were measured. Interestingly, its effect on $A_{2A}R$ expression mirrors the results observed in humans, confirming the arrhythmic phenotype and the usefulness as a model to study AF alterations. Surprisingly, A_1R expression was increased too, but AlphaLISA measured heterodimer A_1R-$A_{2A}R$ level was reduced. This prompt HL-1 cells and AlphaLISA assays to be a desirable tools regarding the study of AF alterations on ARs. However, mechanism behind these alterations requires a deeper research.

In the case of the animal models, several options have been developed for cardiac research throughout the last century (Schüttler et al., 2020). Classically, the use of small rodents (i.e. mice and rats) are the most common. However, small rodents have a substantial difference in scale and cardiac electrophysiology when compared to humans. In general, the size of the cardiac chambers makes difficult to naturally maintain AF phenotype. Concerning large animal models, we found some common models like goats, dogs, and

sheep. Interestingly, the frequently used over the last years is the pig animal model. Pigs have similar heart size compared to humans, as well as electrophysiological conditions and coronary anatomy are equiparable and comparable.

Subsequently, we aimed to determine the viability of using pigs for AF research, focusing on the AF impact on ARs expression and heterodimerization. Additionally, we compared the impact of AF in calcium handling proteins. In the current doctoral thesis we have demonstrated that the typical alterations reported in calcium handling proteins are replicated in tachypaced pigs (Ai, 2015). Among them, alterations in CSQ and PLB proteins were significantly lower in AF, as well as in humans. In both cases, these alterations modulate RyR open probability and calcium buffering in the SR and could trigger calcium release in AF events. However, if its alteration is cause or consequence of AF remains unclear. This loss of calcium buffering in the SR can be persistent in time, becoming an AF. Finally, AF progression could affect, in some way, the expression of PLB and CSQ, becoming another step in the development of the disease. Further studies are needed to determine the mechanism behind this effect.

Regarding ARs, cardiac research mainly focused on $A_{2A}R$ alterations, especially in AF (L. Hove-Madsen et al., 2006). However, its impact on A_1R expression and heterodimer formation has been poorly studied previously in pigs or humans. Our results reveal that AF pigs and human manifest an increased A_1R and $A_{2A}R$ expression in membrane, as well as HL-1 arrhythmic cells. This increased expression in A_1R and $A_{2A}R$ can be an origin point or an aftereffect of AF disease. Probably, because of AF or elevated adenosine levels, $A_{2A}R$ expression is increased in AF patients. Accordingly, A_1R, act as a balancer of $A_{2A}R$ activity, and could have increased expression to regulate this $A_{2A}R$ overactivity and try to maintain the normal resting state. In addition, high adenosine contents will also affect A_1R expression and activity. As well as can be explained for $A_{2A}R$, the triggered adenosine content can exert the same positive turnover effect on A_1R.

Surprisingly, heterodimer formation is found to be reduced in all three conditions explained above. Remarkably, this is the first time that heterodimeric A_1R-$A_{2A}R$ formation have been detected by using a protein-protein interaction system in samples with endogenous expression of ARs. The reason could be the highly presence of adenosine receptors in the heart tissue, which will favour its detection, as well as new technologies developed on that field. Nevertheless, the comprehension of its role and the

impact of its reduction in AF it stays far of being clearly understood. Heterodimeric structures are reported to be a mechanism to finely-control the binding of the receptors for its ligand (Fuxe et al., 2007). In the case of adenosine and the reduction on the heterodimeric A_1R-$A_{2A}R$ levels, may have consequences in the progression or generation of AF by altering the Ca^{2+} release in atrial cardiomyocytes. Nonetheless, as the expression in membrane is increased, we expected the same for heterodimeric levels, but they are reworked in the opposite way. One possible explanation is that each receptor has more affinity for its self-type which will favour the formation of homodimeric clusters compared to heteromeric ones. Another possibility could be that as $A_{2A}R$ helps in the trafficking to the membrane, we have more presence of $A_{2A}R$ than A_1R. Deeper research is needed to understand the cause and consequences of that outcomes in AF models and subjects. Overall, these results contribute to a better understanding of AF and a valuable usage of pig and HL-1 models in the study of AF.

3. Atrial fibrillation treatment: Photopharmacology?

AF treatments involve many different approaches depending on the type, severity, and patient conditions. Among all the options, the most aggressive alternatives are the ones that requires medical surgery or invasive/harmful therapies (i.e., cardiac, or surgical ablation and electrical cardioversion) (J. Li et al., 2020). Regarding drug therapy, there are a lot of different drugs available to use depending on the method to deal with AF. Mainly, these drugs are related with blood coagulation, slow heart rate and regulate calcium channels. However, the current medications lack of long-term success due to two main problems. The former consists of inadequate consideration of atrial tissue and cellular-level abnormalities. The latter refers to coarse criteria for patient classification in clinical trials that does not adequately capture mechanistic heterogeneity. Furthermore, these treatments tend to have an early relapse usually followed by a progressive increase in AF recurrence (Ang et al., 2020). because of the exposed responses reason, new pharmacological approaches should be developed.

In this context, we demonstrated the influence and alterations of ARs in AF. In addition, no drug therapies for AF have been developed by targeting these receptors. Therefore, we aimed to demonstrate the efficacy of these drugs in *in vitro* models such as HEK-293T and HL-1 cells by precluding $A_{2A}R$ activation or potentiate A_1R activity. Thus, we have

effectively blocked $A_{2A}R$ functioning (determined by cAMP accumulation) by using a selective $A_{2A}R$ antagonist, named SCH442416. In addition, we also potentiated A_1R activity by using a selective A_1R agonist, named CPA, which induce the reduction on cAMP accumulation through A_1R signalling. Furthermore, PAM T-62 potentiate the effect of the A_1R signalling by triggering the binding to its ligands (i.e., CPA), reducing even more the cAMP accumulation. Finally, regarding the physiological ligand of ARs adenosine, low levels of adenosine can tackle cAMP accumulation, whereas high doses will promote it.

Also, we have to consider the impact on A_1R-$A_{2A}R$ heterodimer formation. Ideally, no effect on heterodimer formation or even an increased heterodimeric level would be desirable to keep the fine-tune regulation on adenosinergic signalling intact. In that case, SCH442416 and PAM T-62 would be the optimal propositions. No effect on heterodimeric levels were observed at short- or long-term treatments by NanoBit experiments. In addition, they can restore altered heterodimeric levels caused by A_1R and $A_{2A}R$ agonist (CGS and CPA). On the other side, A_1R agonist CPA and adenosine drastically reduce heterodimeric formation, which could have a negative impact on adenosinergic signalling and regulation.

Thus, SCH442416 and T62 reunites the characteristics to be considerate useful to treat AF. However, some points must be considered. As ARs expression can be found ubiquitously, drugs can hit other non-desired tissues. Consequently, its side-effects can be as harmful as the AF disease (J. F. Chen et al., 2013). In the case of $A_{2A}R$, blockade effects in other tissues like brain, can induce problems in neurotransmitter release and normal neuropsychological functioning. A_1R stimulation by CPA could have an impact on AMPA, NDMA and ATP-sensitive K^+ channels, becoming a potential problem in the adenosinergic signalling in brain and other tissues (Stockwell et al., 2017). Consequently, T-62, as a PAM, maybe could be the best option, due to it has no direct effect on A_1R signalling, and its effect depend on the concentration of the A_1R ligands (adenosine). Regarding chloro-adenosine, we can find both problems, as it can activate A_1R and $A_{2A}R$ depending on the dose. In addition, adenosine is also a dangerous approach since it has been demonstrated that can induce an arrythmia by himself.

Subsequently, to overcome these problematic outcomes, we studied the photopharmacology approach. In the last decade, the use of optical tools such as

optogenetics and photopharmacology have been increasing year by year, due to its inherent benefits (Chemi et al., 2019). In our case, it involves light-sensitive drugs that are active when and where are irradiated by light. They can act according two different ways: on the one hand i) the caged compounds share the structure of the classical drugs (as a moiety) but are chemically attached to another molecule by a light-sensitive bound. When irradiated, the link between the two molecules breaks and the parent compounds become active (Taura et al., 2018). In our case, all three cage compounds were attached to a type of violet-coumarin and can be released after light-irradiation at 405nm wavelength. On the other hand, ii) the swich compounds are synthetised in a specific way to change its chemical state from trans to cis or cis to trans when irradiated (Font et al., 2017). In our case, adenosine-switch (AA3) is in an active trans state and changes to cis (inactivated state) when irradiated at 405nm. This may be classified as a non-therapeutical approach but could be useful for future drug groups that will allow on/off behaviours. By using the light to determine where and when we want to activate our drugs, we will obtain an extremely precise tool to control the spatio-temporal release and activation of our drugs. This can be helpful to avoid off-targets and increase the efficiency of our treatments. In the four tested drugs, we have been able to obtain the same effect with light-sensitive compounds when irradiated compared with normal drugs. However, in the case of the CPA cage, the dose has to be greater compared with classical CPA. This is probably due to our uncaging system or the breaking of the coumarin bond, which is not completely effective and does not allow the full release of the drug. Moreover, impact on heterodimer formation were also replicated by the sensitive lights, both cage and switch. These results point toward a future use in animal models and, possibly, in human subjects. However, more research is needed to determine the risk-benefit of this approach in in vivo models. Also, new technologies have to be developed in order to be able to irradiate heart area in alive animals and humans, allowing the uncaging-switching but without damaging heart or other peripherical tissues.

4. Concluding Remarks

In conclusion, the present work has deepened into the understanding and treatment of AF. We have revealed new insights related to AF through ADA and Adenosine alterations found in plasma. In addition, we have revealed new impacts of AF in surrounding cells that offer a new course to study or prevent and detect AF. We have additionally provided

evidence for the use of relevant tools for research about AF in *in vitro* and in animal models using HL-1 cells and pigs. That will facilitate the future analysis of AF causes, effects, and treatment. Besides, novel findings of AF impacts have been found in the three conditions through the heterodimeric A_1-A_{2A}R reduced formation and the alterations in A_1R expression. Finally, we showed new potential approaches to pharmacologically tackle this disease, either by antagonizing A_{2A}R or potentiate A_1R activity. In addition, we have demonstrated that the emerging field of optopharmacology could give us the chance to overcome the limitations of the classical pharmacology and possibly be useful for the development of light-dependent treatments in the near future.

VII. Conclusions

Conclusions

1. Atrial fibrillation impact on adenosinergic system can be detected on heart atrial myocytes but also in ARs expression of PBMCs and ADA and adenosine content in plasma. Consequently, these alterations might be used in prevention, detection, and risk-stratification of atrial fibrillation.

2. A_1R and $A_{2A}R$ expression have been detected successfully in the three different samples (HL-1 cells, pigs, and humans) in both control and arrhythmic phenotypes. Also, endogenous expression of A_1R and $A_{2A}R$ heterodimer has been detected for the first time by using a direct protein interaction assay in cells, animals, and human samples.

3. Reported changes in A_1R and $A_{2A}R$ expression and heterodimeric levels are identical in arrhythmic HL-1 cells, AF pigs and AF human patients. Also, calcium handling proteins are altered in the same way in both pigs and humans. This highlights the usefulness of this models in the AF research.

4. We effectively controlled A_1R and $A_{2A}R$ activity using a pharmacological approach in HL-1 cells by using the compounds SCH442416, CPA, T-62, and chloro-adenosine. Also, we determined its impact on heterodimer formation in HEK-293T cells, concluding that SCH442416 and T-62 will have no negative impact on heterodimer formation in vitro.

5. Photocaged compounds have been designed correctly and they positively replicate the effect of the classical drugs upon 405 nm irradiation. This field open a new way to potentially treat AF in the near future with all the additional benefits that photopharmacology offers.

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IX. Acknowledgements

"Certeza de muerte… mínima esperanza de éxito… ¿a qué esperamos?" – Gimli, Hijo de Gloin. El señor de los anillos, la comunidad del anillo, 2001.

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Primero y más importante, los tres pilares externos a mi entorno laboral: el primero de todos, mi padre **Juan Godoy Herrero**. Veintiún años a tu lado no fueron suficientes y aun así te sigo teniendo presente cada día. Tú me enseñaste las virtudes del esfuerzo, la perseverancia y el no rendirme nunca, y quizás lo más importante de todo que es a reírme siempre y a ser feliz pase lo que pase. Hoy en día sigo intentándolo con todas mis fuerzas y si estoy donde estoy ahora escribiendo estas palabras es en gran parte gracias a ti. Quiero que sepas que estoy orgulloso de ser tu hijo, y que sé que estés donde estés, tú también estás orgulloso de mí por este momento de mi vida. Te quiero y siempre te llevaré en mi corazón.

Como no hay yin sin yang, creo que hay que hablar de la persona que quizás ha tenido un impacto más fuerte en los últimos 10 años, mi madre **Esther Marín Martínez**. Gracias por estar siempre ahí, a pesar de las muchas veces que no hemos estado de acuerdo, y por dejar que cometiera errores, aun cuando sabías que me iba a equivocar y que tú ibas a tener que recoger mis pedacitos después. Agradezco cada consejo y cada momento que pasamos juntos, y estoy muy, muy feliz de poderte llamar mi madre. Habéis sido unos padres 10 y tendré suerte si algún día me llego siquiera a parecer a vosotros. Lo único que puedo decir es gracias por la vida que me habéis dado y la que me das a día de hoy, no podría ser mejor.

Finalmente, la persona que navega por las tormentas de mi corazón: **Natalia Hughes Cueto**, alias "*El pichón*". Creo que has sido la persona que más ha sufrido todos los altibajos emocionales que he tenido durante este período, y has estado ahí para apoyarme

en los momentos malos y para disfrutar conmigo los momentos buenos. Cuantas tardes hablando de los experimentos que no salían, y que, aunque no acababas de entender, siempre me ayudabas a solucionar. Cuántas horas los fines de semana de "tengo que ir un rato corto, unas tres o cuatro horas, a revelar un western" o de "ay, no puedo, es que saldré a las nueve de la noche". Siempre recordaré las veces que me has dicho "tranquilo, merecerá la pena". Que no te quepa la menor duda, has sido el apoyo más importante en los últimos años y, aunque no te lo creas, esta tesis también es en gran parte tuya. Tu amor incondicional hacia mí, y hacia mi trabajo, me ha permitido superar los más duros momentos y las más grandes crisis que he sufrido, y has sido no solo el pilar que necesitaba, sino también la inspiración que me ha hecho falta durante los últimos 3 años. Me has aportado un millón de cosas que me han hecho crecer como persona, y para mí es un orgullo quererte y poderte llamar mi prometida (y pronto mi mujer); jamás podría elegir a nadie mejor que tú ni a nadie a quien yo admire tanto. Gracias por hacer que mi vida sea un cuento de hadas día a día y hacer que cuando hable con los demás, pase lo que pase, siempre pueda contestar a todo con una sonrisa en la cara y en el corazón. Te quiero, te querré siempre y quiero que sepas que eres y serás la mejor y la persona más importante en mi vida.

Segundo, y cubriendo quizás los aspectos más "científicos" de este apartado, tenemos a la gente del laboratorio, que se podría considerar casi mi segunda familia puesto que los he visto más a ellos que a prácticamente nadie de mi entorno. En primer lugar, querría dar las gracias tanto a Paco como a Víctor, las *almas mater* de este laboratorio. A **Paco** me gustaría agradecerle primero, de forma un poco más seria, la oportunidad de realizar la Tesis en su laboratorio, puesto que sinceramente, era el último intento que hacía de hacer la tesis. Si no salía aquí, ya nunca más lo habría intentado y hoy en día no estaría escribiendo estas palabras. También me gustaría agradecerle todos los momentos buenos que hemos pasado juntos y las dos ideas principales que me ha transmitido en este tiempo: la primera, cómo ser un buen investigador, me ha enseñado que no debo rendirme con los resultados, la frustración y que hay que saber pedir ayuda cuando algo no sale bien, en vez de seguir intentándolo hasta el agotamiento. La segunda, quizás no tan relacionada con la ciencia, su particular forma de ser y de plantear la vida, quedándome con una frase que dijo una vez de "*un día sin reír es un día desperdiciado, hay que reír siempre"*. A **Víctor** le quería agradecer su paciencia infinita cuando le llegaba con resultados que no sabía por dónde coger y que, aunque al final fueran un completo desastre y tuviera que

repetirlo todo, su forma de reírse ante la adversidad te hacía tomarte las cosas con más filosofía y buen humor del que yo solo era capaz de tener. Gracias a ambos por hacerme crecer como científico y como persona.

Por seguir con los elementos "senior" del laboratorio, agradecer también todo lo que me han aportado Ester, Alex, Laura y Salut. **Ester**, gràcies per tot el que has fet per mi, des de la part més científica en seminaris, consells als experiments i demés, fins a d'altres més personals com per exemple ajudar-me a trobar la primera substitució de la meva vida a un Institut. **Àlex**, gràcies per aquells tocs d'humor que aportes cada dimarts i dijous, i per les perles de saviesa en forma de lectura que m'has donat. **Laura**, per a tu només puc dir que et recordaré sempre amb un gran amor i afecte, has sigut una inspiració a nivell de forma de ser i de persona, així com a docent. Moltes gràcies per escoltar-me, pel suport a la tesi i sobretot la teva comprensió, que em feia sentir escoltat i recolzat, i per aportar-me la perspectiva que l'experiència t'ha donat, i de la que jo molts cops mancava. **Salut**, gràcies per fer que el laboratori segueixi rutllant amb la mateixa eficàcia de sempre, per aportar sempre un contrapunt fresc en aquest món científic i per venir a petar una mica la xerrada quan estàvem angoixats i ens feia falta (o et feia falta) desconnectar. Us recordaré als quatre amb afecte i sincer i profund agraïment a tots.

Seguidamente, los chorrimil personajes que me he encontrado a lo largo de 4 años en este laboratorio. Supongo que la forma más "lógica" de clasificarlos sería por antigüedad, desde los fósiles del pleistoceno lejano (Xavi, Jaume, Marta y Marc) a los brotes más jóvenes de hierba fresca (Josep, Núria y Laura[2]) así como a los más estacionales y pasajeros.

Old era: **Xavi**. Potser la persona que més m'ha arribat a cuidar a nivell científic i personal del laboratori. Recordo els primers dies de la tesi, que et tornava boig amb els meus daltabaixos d'energia i amb la meva impaciència en general. També recordaré sempre com m'aconsellaves amb tot, i et prenies el teu temps per ajudar-me a arribar on havia d'arribar, així com a generar-me curiositat amb preguntes retòriques i plantejant-me reptes. Mai oblidaré tampoc com ens barallàvem pel menjar, les nostres amanides destructives full atún, queso y nueces o aquells dies a les 6 del matí venint des de Montgat. Ets i seràs una de les persones més importants a la meva vida i t'agraeixo profundament tot el que hem viscut junts i el que has fet per mi durant aquesta tesi. **Jaume**: la persona més curiosa i xerraire que he conegut mai, amb la possible excepció d'en Marc. Gràcies

per tots els moments de discussió científica (i no científica) durant dinars, descansos, esmorzars... Bueno, constant pràcticament. **Marta**: ¡ay Marta, qué voy a hacer contigo! No sé ni por dónde empezar porque diga lo que diga me quedaré corto. El otro gran titán del laboratorio, junto con Xavi, en las peleas de comida. En los momentos de *hate* y de estrés y agobio máximo. Pero también en los momentos de reírnos, de hacer piña y de amor. Creo que eres una magnífica persona, y jamás podré agradecerte suficiente el apoyo interno que has sido para mí, sobre todo en lo personal, pero también en lo científico. Este último año ha sido durillo para los dos, pero siempre has estado para escucharme y darme tu opinión y más que nada, tu apoyo incondicional. Espero que no me faltes nunca. **Marc**, Marcus, IronMarc. La leyenda. El inmutable. El permanente. El árbol perenne entre los caducifolios. Només tinc bones paraules per a tu Marc, i el que més t'agrairé sempre és que ets una gran persona amb la que es pot comptar per a tot i que saps que (si la demanes) et donarà una opinió sincera a la par que comprensiva i sobretot molt ben raonada. Gràcies per tots aquests anys d'aprenentatge al teu costat i, perquè no, d'amistat (eh, Marc Lab xd).

Los transitorios: primero de todo y la más importante con diferencia **Aurora**. Gracias por esos cafés al mediodía y sobre todo por convertirte en una de mis mejores amigas hoy en día. Eres enorme (por dentro obviamente) y siempre guardaré con cariño esos días que pasamos juntos. **Fahad**. The doctor. Él me enseñó que cualquiera puede lograr lo que quiere, si dispone de los recursos adecuados. **Paula**, **Tereza**, **Andrea** (the Craziest), **Steph&Sam** (buen whisky, mejores personas), **Caio** (que esperemos ya haya vuelto de Ibiza), **Carolina**, **Rahul**, **René** (Salsa, tequila, corazón, cerveza muy bueno), **Roberta** (definición de intensa), **Kristoffer** (siempre correcto y elegante), **Sofia** y **Vendy** (the best of the best), **Judit** (Macarra Style), **Maria del Mar** (excelente vino, mejor persona), **Orvokki** (The Silence) y **Alejandra** (está rebueno, ¿eh?). Gracias por amenizar la vida del laboratorio con mil aventuras y anécdotas.

Los brotes verdes: **Josep**. El hombre de pueblo para el pueblo (dame 100 euros en Canetums). Què gran persona ets i com agraeixo que ens hàgim conegut. Tant de bó passem més temps junts en el futur, de debò que ets algú que mereix la pena. **Laura Sarasola**, "the Basque". Creo que eres la definición de la sinceridad y la honradez, y estoy seguro de que llegarás muy lejos en el camino que elijas seguir, ciencia o no. **Laura Gómez**, la mujer que te clava las puñaladas sin saber si es broma o no. Me encanta tu sentido del humor, siempre me pilla desprevenido y adoro tus ironías y puyas. Sigue así

hahahaha. **Núria**. Gracias por ser una persona que me aporta aquello que siempre he echado un poco en falta en el lab: una sinceridad clara y meridiana en formato cáustico bañado en dulzura y cariño (¡¡¡MARC CÁLLATE!!!). A los cuatro: gracias por hacer este último año más llevadero y espero sinceramente que no perdamos el contacto y sigamos haciendo todo lo que hacemos hasta ahora juntos y más aún: bolos, roomscapes, casas rurales, barbacoas o hundir moralmente a Josep. Me apunto a todo.

Los extras: como siempre, hay muchísima gente que pertenece al colectivo científico que no forma parte de nuestro laboratorio. Habría que destacar a los Altafajianos: **Xavi**, Macarena, **Silvia** y Ana. Gracias por todos los momentos compartidos dentro y fuera del laboratorio, en especial Macarena y Ana. **Maca**, ojalá hubiéramos pasado más tiempo juntos, pero siempre fuiste un referente para mí a nivel científico y creo que conectamos y encajamos divinamente desde el minuto uno. Ojalá nuestros caminos se encuentren otra vez. **Ana**, que te voy a decir que no te haya dicho ya, con ese cariño que desarrollamos de forma casi instantánea y que seguimos manteniendo hoy en día. Quiero y deseo que sigamos teniendo esta relación muchísimo tiempo más, porque realmente nos hacemos reír, escuchamos y entendemos hablemos de lo que hablemos. Gracias por todo siempre. También en los pasillos oscuros y lóbregos de Bellvitge encontramos a personajes como **Arturo, Marta Alonso** o **Cecilia Velázquez**. Nuestros encuentros fortuitos siempre serán un recuerdo agradable de esta época, así como los momentos compartidos dentro y fuera de Bellvitge. Una vez más, gracias por todo. Gràcies també pels riures i sobretot pel suport científic a en **Leif,** la **Verónica, l'Hildegard** i **l'Olga,** sense oblidar-nos d'en **Benja, la Bea i l'Esther**, amb els que he compartit riures i llàgrimes, frustracions i alegries, i sobretot més hores que un rellotge als Serveis. Sense vosaltres res d'això hagués sigut possible.

Para terminar, pero no menos importante por ello, todo el conjunto de gente de mi entorno personal, es decir, mis amigos. **Fran** (la roca), **Marta** (gracias por dejarme sin sangre), **Micky** (la voz de la sabiduría), **Noe** (explosiva e implosiva), **Rubén** (el alma), **Aida**, **Chencho**, **Guillem/Héctor/Víctor**, (los héroes que siguen siendo mis amigos después de 26 años de amistad), **Judith** y **Albert** (los Mas Que Finos), **Conchi**, **Collin** y los **Cucos** (mi familia política, a la que quiero tanto como a la mía misma), a **Diana** (¡después de 12 años ya somos Doctores!) y a **Anna Macià** (en la distancia pero en mi pensamiento, también una buena sangradora).

Como nota final, el conjunto de la tesis abarca tantos aspectos de la vida que es complicado agradecer a todo el mundo como corresponde y espero que, si he omitido a alguien por descuido, no me lo tenga en cuenta, ya le invitaré a un *cacaolat* para compensarlo. Gracias a todos aquellos que he mencionado y a los que no, pero que me han permitido llegar al final del viaje con éxito. Así que con esto concluyo este apartado, citando a mi queridísimo amigo Gandalf el Blanco:

"No os diré no lloréis, pues no todas las lágrimas son amargas".