

## Mapping genetic changes in the cAMP-signaling cascade in human atria

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## ARTICLE INFO

## Keywords:

Atrial fibrillation

Heart failure

Right atria

Left atria

cAMP-signaling cascade

β-Adrenergic pathway

## ABSTRACT

**Aim:** To obtain a quantitative expression profile of the main genes involved in the cAMP-signaling cascade in human control atria and in different cardiac pathologies.

**Methods and results:** Expression of 48 target genes playing a relevant role in the cAMP-signaling cascade was assessed by RT-qPCR. 113 samples were obtained from right atrial appendages (RAA) of patients in sinus rhythm (SR) with or without atrium dilation, paroxysmal atrial fibrillation (AF), persistent AF or heart failure (HF); and left atrial appendages (LAA) from patients in SR or with AF. Our results show that right and left atrial appendages in donor hearts or from SR patients have similar expression values except for *AC7* and *PDE2A*. Despite the enormous chamber-dependent variability in the gene-expression changes between pathologies, several distinguishable patterns could be identified. *PDE8A*, *PI3Kγ* and *EPAC2* were upregulated in AF. Different phosphodiesterase (PDE) families showed specific pathology-dependent changes.

**Conclusion:** By comparing mRNA-expression patterns of the cAMP-signaling cascade related genes in right and left atrial appendages of human hearts and across different pathologies, we show that 1) gene expression is not significantly affected by cardioplegic solution content, 2) it is appropriate to use SR atrial samples as controls, and 3) many genes in the cAMP-signaling cascade are affected in AF and HF but only few of them appear to be chamber (right or left) specific.

**Topic:** Genetic changes in human diseased atria.

**Translational perspective:** The cyclic AMP signaling pathway is important for atrial function. However, expression patterns of the genes involved in the atria of healthy and diseased hearts are still unclear. We give here a general overview of how different pathologies affect the expression of key genes in the cAMP signaling pathway in human right and left atria appendages. Our study may help identifying new genes of interest as potential therapeutic targets or clinical biomarkers for these pathologies and could serve as a guide in future gene therapy studies.

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## 1. Introduction

The  $\beta$ -adrenergic receptor ( $\beta$ -AR)/cyclic adenosine monophosphate (cAMP) pathway is a major pathway involved in the sympathetic regulation of heart function. It regulates cardiac contractility, relaxation and heart rate.  $\beta$ -AR activation by noradrenaline or adrenaline triggers a sequence of signaling events which starts by G-protein activation, stimulation of different adenylyl cyclase (AC) isoforms which synthesize cAMP leading to protein-kinase-A (PKA) mediated phosphorylation of several key proteins involved in excitation-contraction (EC) coupling, such as L-type  $\text{Ca}^{2+}$  channels (LTCC), phospholamban (PLB), ryanodine receptors type-2 (RyR2) and troponin-I (TnI) [1]. A-kinase anchoring proteins (AKAPs) bind PKA [2] and phosphatases (PP2 and PP1), which act as signaling scaffolds to promote phosphorylation of target proteins. cAMP has also PKA-independent targets in the heart, such as channels for cyclic nucleotides transport across the membranes (MRP) [3–5] or the small G-protein exchange-factor Epac (Exchange Protein directly Activated by cAMP) [6] involved in hypertrophic growth [7,8], which in turn activates calcium/calmodulin-dependent kinase type-II $\delta$  (CaMKII $\delta$ ). The levels of cAMP and thus the degree of PKA and Epac activation are finely regulated by cyclic nucleotide phosphodiesterases (PDEs). There are 5 families (PDE1-4 and PDE8) of cardiac PDEs degrading cAMP [9]. Other proteins also play an important role in EC coupling by modulating  $\beta$ -AR desensitization ( $\beta$ -arrestins) or activating phosphorylation after an intracellular calcium increase (CaMKII $\delta$ ) (Fig. 5).

One important question is whether differences in neurohumoral regulation between right and left heart chambers are limited to electrophysiological and phenotypic features [10–12] or extend to genetics, and how it can differentially affect left and right atria. During the last years, many publications have highlighted the importance to study genetic differences between cardiac cavities. Phillips and colleagues [13] reported no difference in the expression level of more than 600 proteins between the RV and the LV from pig and rabbit including contractile/structural proteins and oxidative phosphorylation components. No plasma membrane voltage-gated ion channel was analyzed, but a few major  $\text{Ca}^{2+}$  handling proteins (SERCA2, RyR2) and signal transduction components ( $\text{Ca}^{2+}$ /CaMKII $\delta$ , PKA type-I and type-II) showed no variation in their expression level [13]. A recent single-cell resolution analysis also showed similar nuclear transcriptional patterns when comparing right *versus* left atria from dead donors non-failing patients [14]. However, only limited information about  $\beta$ -AR/cAMP pathway genes and their expression in human healthy and diseased atria is available.

Several electrophysiological studies have shown the importance of the  $\beta$ -AR/cAMP pathway in the development and maintenance of many cardiovascular diseases such as heart failure (HF) and atrial fibrillation (AF). However, the relationship between electrophysiological changes in cellular signaling and changes in  $\beta$ -AR/cAMP pathway regulatory genes has mainly been investigated for individual targets in specific diseases and heart regions.

Unbiased genetic studies in the human heart considering both interchamber differences and pathological re-modeling are extremely important to characterize new potential therapeutic targets before bringing them into deeper mechanistic and translational studies as well as to better understand adaptation or maladaptation during disease. Recently, gene therapy technology has begun to be tested to treat ventricular pathologies. However, expression of the tested genes and their disease driven changes have not always been studied in the human tissues, or only in a very small number of samples with variable reference genes, leading to contradictory results. Here, we aimed to study gene expression changes that occur in the human atrium with various pathologies at right and left chambers in order to gain a general understanding of the remodeling of the  $\beta$ -AR/cAMP pathway promoted by different pathologies. We studied the expression of 48 target mRNAs in 113 appendage tissue samples from control donor patients, patients

being in sinus rhythm (SR) with or without atrial dilation, having different forms of AF, or suffering from HF. Our data reveal that many genes involved in the  $\beta$ -AR/cAMP pathway are affected in AF and HF but only few changes are right or left atrial specific.

## 2. Methods

### 2.1. Human atrial-tissue samples

A total of 113 human atrial appendage-tissue samples were collected from a total of 97 patients undergoing open-heart surgery either at the Institut Hospitalier Jacques Cartier, Massy, France, at the Cardiac Surgery Department, Hospital de la Santa Creu i Sant Pau/Universitat Autònoma de Barcelona, Spain, at the Cardiology and Pneumology Department, Georg-August-Universität Göttingen, Germany, at the Cardiac Surgery Department, University Hospital Essen, Germany, at the University Medical Center Hamburg-Eppendorf, Germany, or at the Department of Pharmacology and Pharmacotherapy, University of Szeged, Hungary. 81 specimens of right (RAA) and left (LAA) atrial appendage tissues were obtained from 80 patients in sinus rhythm with (SRd) or without (SRnd) atrial dilation, or with paroxysmal atrial fibrillation (pAF) or long-standing persistent atrial fibrillation (cAF), subjected to atrial cannulation for extracorporeal circulation. 32 right and left atrial appendage tissues were collected from 8 control donors (Ctl) and 9 end-stage heart failure dilated atria (HF) from explanted human hearts, respectively, at the time of the transplantation. All samples were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Adipose tissue was removed before the cryopreservation of the sample. The study was conducted in accordance with the Declaration of Helsinki principles, and approved by the Ethical Committees of all included institutions. Informed consent was obtained from each patient. Details regarding the clinical characteristics of the patients and their medication are shown in Tables 1 and 2.

### 2.2. RNA isolation and cDNA synthesis

76 snap-frozen tissue samples were weighed and placed in pre-cooled tubes containing TRIzol® reagent (Invitrogen, Life Technologies, France) and rapidly subjected to automated grinding in a Bertin Precellys 24 (Bertin Technologies, France). Total RNA extraction was carried out using standard procedure according to the manufacturer's instructions. RNA concentration and purity were evaluated by optical density (Biophotometer, Eppendorf, BioServ, France) and the integrity of the RNA samples were analyzed on a Bioanalyzer 2100 with the RNA6000 Nano Labchip Kit (Agilent Technologies, Santa Clara, CA, USA). The RNA-integrity number (RIN) was calculated by the instrument software. First strand cDNA synthesis was performed from 1- $\mu\text{g}$  of total RNA with random primers and MultiScribe™ Reverse Transcriptase according to the provided protocol (Applied Biosystems, Life Technologies, France). To minimize intergroup variations, samples of each experimental group were processed simultaneously.

Total RNA content, 260 nm/280 nm optical-density (OD) ratio and RNA-integrity number (RIN) values were obtained from right and left atrial appendages without or with pathological conditions. RIN values were ranged between 5.5 and 8.5, with a mean value of  $6.5 \pm 0.3$  for RAA SRnd,  $7.0 \pm 0.2$  for RAA SRd,  $6.4 \pm 0.3$  for RAA pAF,  $6.6 \pm 0.1$  for RAA cAF,  $7.5 \pm 0.6$  for LAA SR,  $6.5 \pm 0.3$  for LAA AF,  $6.9 \pm 0.4$  for RAA Ctl,  $7.0 \pm 0.4$  for LAA Ctl,  $7.0 \pm 0.5$  for RAA HF and  $7.1 \pm 0.3$  for LAA HF. There were no differences in RNA content between the groups. The quality control parameters for human total RNA samples were similar regardless of human tissue-sample collection, with no difference between the groups.

### 2.3. Real-time qPCR and quantification

Real-time PCR assays were performed using TaqMan® 384-well

**Table 1**

Clinical characteristics of patients/samples used for gene expression experiments.

Patients (n)	SRnd	SRd		pAF	cAF		Ctl	HF n = 9	
	RAA, n = 8	RAA, n = 8	LAA, n = 5	RAA, n = 8	RAA, n = 8	LAA, n = 7	RAA-LAA, n = 8	RAA, n = 8	LAA, n = 8
Female gender	3 (38%)	4 (50%)	2 (40%)	2 (25%)	3 (38%)	2 (29%)	3 (38%)	3 (38%)	3 (38%)
Age (years)	69.5 ± 2.9 <sup>#</sup>	69.8 ± 3.1 <sup>#</sup>	64.4 ± 3.3 <sup>#</sup>	76.0 ± 4.0 <sup>#</sup>	74.3 ± 2.3 <sup>#</sup>	71.6 ± 3.8 <sup>#</sup>	44 ± 4.7	63.0 ± 3.4 <sup>#</sup>	61.9 ± 3.7 <sup>#</sup>
Smoking	2 (25%)	2 (25%)	1 (20%)	1 (13%)	3 (38%)	2 (29%)	N/A	N/A	N/A
Hypertension	5 (62%)	4 (50%)	3 (60%)	4 (50%)	5 (62%)	4 (57%)	4 (50%)	8 (100%)	8 (100%)
CAD	5 (62%)	3 (37%)	1 (20%)	2 (25%)	2 (25%)	1 (14%)	0 (0%)	0 (0%)	0 (0%)
AVD/MVD	1 (13%)	4 (50%)	3 (60%)	3 (38%)	4 (50%)	4 (57%)	0 (0%)	0 (0%)	0 (0%)
CAD + AVD/MVD	2 (25%)	1 (13%)	1 (20%)	3 (38%)	2 (25%)	2 (29%)	0 (0%)	0 (0%)	0 (0%)
Transplantation	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	8 (100%)	8 (100%)	8 (100%)
LAD (mm)	39.2 ± 1.4	44.3 ± 0.9*	46.8 ± 0.6*	43.8 ± 2.7	46.0 ± 3.9*	53.0 ± 6.7*	N/A	N/A	N/A
LVEF (%)	61.4 ± 2.3	62.7 ± 2.0	49.4 ± 0.4	57.9 ± 2.6	62.8 ± 2.6	64.0 ± 5.2	N/A	25.5 ± 1.5*	29.5 ± 5.5*
Digitalis	0 (0%)	0 (0%)	0 (0%)	1 (13%)	0 (0%)	1 (14%)	N/A	N/A	N/A
ACE inhibitors	3 (38%)	4 (50%)	4 (80%)	3 (38%)	4 (50%)	5 (71%)	4 (50%)	N/A	N/A
AT <sub>1</sub> blockers	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	N/A	N/A	N/A
Beta-blockers	5 (63%)	4 (50%)	5 (100%)	4 (50%)	5 (63%)	5 (71%)	N/A	N/A	N/A
Ca <sup>2+</sup> -antagonists	0 (0%)	2 (25%)	0 (0%)	1 (13%)	3 (38%)	2 (29%)	0 (0%)	N/A	N/A
Diuretics	1 (13%)	2 (25%)	3 (60%)	3 (38%)	5 (63%)	4 (57%)	4 (50%)	N/A	N/A
Nitrates	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	N/A	N/A
Statins	5 (63%)	2 (25%)	4 (50%)	2 (25%)	1 (13%)	2 (29%)	0 (0%)	N/A	N/A
Amiodaron	0 (0%)	0 (0%)	0 (0%)	6 (75%)	2 (25%)	2 (29%)	0 (0%)	N/A	N/A

Values are presented as mean ± SEM or number of patients (%). SRnd: Sinus Rhythm without atrium dilation; SRd: Sinus Rhythm with atrium dilation; pAF: Paroxysmal Atrial Fibrillation; cAF: Chronic Atrial Fibrillation; HF: Heart Failure; Ctl: Control donors; ACE: angiotensin-converting enzyme; AT: angiotensin receptor; AVD/MVD: aortic/mitral valve disease; CAD: coronary artery disease; LAD, left atrial diameter; LVEF, left ventricular ejection fraction; N/A: not available. \* indicates  $p < 0.05$  vs. SRnd, <sup>#</sup> indicates  $p < 0.05$  vs. Ctl. CAD, AVD/MVD, and CAD + AVD/MVD reflect the indications for cardiac surgery (bypass surgery, valve surgery or both).

microfluidic card technology from Applied Biosystems (TaqMan® Array Card or TAC, Life Technologies, France) and the TaqMan® Human Endogenous Control Panel. These TAC were designed to study the expression of 48 target genes: *ABCC4* (*MRP4*), *ABCC5* (*MRP5*), *ADCY2* (*AC2*), *ADCY4* (*AC4*), *ADCY5* (*AC5*), *ADCY6* (*AC6*), *ADCY7* (*AC7*), *ADCY9* (*AC9*), *AKAP5*, *AKAP6*, *AKAP7*, *AKAP9*, *AKAP13*, *ARRB1* (*Arrestin b1*), *ARRB2* (*Arrestin b2*), *AURKAIP1* (*AKIP*), *CAMK2D* (*CaMKIID*), *NPPA* (*ANP*), *NPPB* (*BNP*), *PDE1A*, *PDE1C*, *PDE2A*, *PDE3A*, *PDE3B*, *PDE4A*, *PDE4B*, *PDE4D*, *PDE8A*, *PIK3CG* (*PI3Kγ*), *PKIA*, *PKIB*, *PPP1CA* (*PP1A*), *PPP1R1A* (*IPP1*), *PPP1R2* (*IPP2*), *PPP2CA* (*PP2CA*), *PRKACA* (*PKACA*), *PRKACB* (*PKACB*), *PRKARIA* (*RIA*), *PRKAR2A* (*RIIA*), *PRKAR2B* (*RIIB*), *RAPGEF3* (*EPAC1*), *RAPGEF4* (*EPAC2*), *ADRB1* (*β1-AR*), *ADRB2* (*β2-AR*), *GNAI1* (*Gi1*), *GNAI2* (*Gi2*), *GNAI3* (*Gi3*), *GNAS* (*Gs*) (Supplemental Table 1) relative to 5 reference genes (*POLR2A*, *YWHAZ*, *GAPDH*, *IPO8*, *PPIA*) recently validated for a study using right and left cardiac cavities from different human tissue cohorts [15]. Each PCR reaction was performed on 4-ng of cDNA in a volume of 1-μl. The thermal cycling conditions for PCR amplification on TAC were 10-min at 94.5 °C, followed by 40 cycles of 30-s at 97 °C and 1 min at 59.7 °C, on an ABI-Prism 7900HT Sequence Detection Instrument (Applied Biosystems, Life Technologies, France). Each TaqMan® assay was previously validated by Applied Biosystems and the efficiency of amplification was certified to be superior to 90% by the supplier. For each group, an average Ct value was calculated. The determination of the relative gene-expression ratio was achieved using the ΔΔCt method and normalized by the geometric mean of a set of stable housekeeping genes.

## 2.4. Western blot analysis

37 snap frozen human atrial appendage-tissue samples were homogenized in RIPA lysis buffer (NaCl 150 mM, Triton 1%, SDS 0.1%, SOD 0.5%, Tris 50 mM, Protease- and Phosphatase-Inhibitor Cocktail (Roche)) using a homogeniser (MICCRA D-1). After three homogenization steps for 20 s each, always followed by cooling of the samples in liquid nitrogen, samples were incubated on ice for 1-h. After centrifugation (12,000 rpm, 5-min, 4 °C) supernatants were stored at −20 °C until usage. Protein quantification was performed using BCA Protein Assay (Pierce BCA Protein Assay Kit, Thermo Scientific, #23227) and

50-μg of total protein were loaded on 8% SDS gels for SDS polyacrylamide gel electrophoresis. Then, proteins were transferred to nitrocellulose membrane (Amersham, #106000 02) using a tank blot system. For immunoblot analysis, the following antibodies were used: PDE2A (Fabgennix 101AP, rabbit polyclonal antibody, dilution 1:750 in 3% milk, sample cooking 70 °C 10-min), PDE8A (Santa Cruz Biotechnology sc-17,232, goat polyclonal antibody, dilution 1:500 in 3% BSA, sample cooking 70 °C 10-min), EPAC2 (Cell signaling #4156, mouse monoclonal antibody, dilution 1:250 in 3% BSA, sample cooking 55 °C 30-min), PIK3γ (kindly provided by Dr. Emilio Hirsch, mouse polyclonal antibody, dilution 1:100 in 3% BSA, 70 °C 10-min), GAPDH (HyTest #5G4 6C5, mouse monoclonal antibody, dilution 1:160,000 in 5% milk). For quantification, band densitometry analysis was done using ImageJ software.

## 2.5. Statistical analysis

Results are expressed as mean ± SEM. Statistical significance was evaluated using a Student's *t*-test and ANOVA followed by Tukey's test was used for comparison of multiple effects. A difference was considered statistically significant when  $p < 0.05$ .

## 3. Results

Using RT-qPCR, we simultaneously evaluated the expression of 49 target genes and normalized it to the appropriate reference genes [15]. In total, 113 human atrial appendage-tissue samples (Tables 1 and 2) from right and left atria controls or with distinct cardiovascular diseases were analyzed. Western Blot was used to confirm protein changes for specific genes in 37 samples.

### 3.1. Validation of the control groups

Working with human cardiac samples entails intrinsic subject heterogeneity. For instance, SR atrial appendage samples obtained from patients undergoing cardiac surgery due to cardiovascular diseases, which do not affect the atrium or the heart rhythm, were snap-frozen immediately after resection from the patient. In contrast, Ctl atrial appendage samples derived from donor hearts not suitable for

**Table 2**

Clinical characteristics of patients/samples used for protein expression experiments.

Patients (n)	SRnd	SR	pAF		cAF	
	RAA, n = 8	LAA, n = 6	RAA, n = 8	LAA, n = 3	RAA, n = 6	LAA, n = 6
Female gender	1 (38%)	1 (38%)	1 (25%)	3 (100%)	0 (0%)	1 (29%)
Age (years)	64.6 ± 4.2	60.8 ± 8.1	65.3 ± 4.8	61.7 ± 3.3	71.5 ± 3.1	69.8 ± 1.8
Smoking	3 (37%)	1 (17%)	3 (38%)	1 (33%)	3 (50%)	2 (33%)
Hypertension	5 (62%)	2 (33%)	5 (62%)	2 (67%)	5 (83%)	5 (83%)
CAD	5 (62%)	0 (0%)	2 (25%)	1 (33%)	2 (33%)	2 (33%)
AVD/MVD	2 (25%)	6 (100%)	5 (62%)	2 (67%)	3 (50%)	3 (50%)
CAD + AVD/MVD	1 (13%)	0 (0%)	1 (13%)	0 (0%)	1 (17%)	1 (17%)
Transplantation	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
LAD (mm)	39.6 ± 1.9	44.2 ± 3.3	49.7 ± 4.5*	52.5 ± 17.5	47.0 ± 4.5*	52.8 ± 6.2
LVEF (%)	55.9 ± 2.9	48.3 ± 4.7	53.5 ± 2.4	60.3 ± 0.3	60.3 ± 0.4	54.5 ± 4.1
Digitalis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ACE inhibitors	6 (75%)	4 (67%)	3 (38%)	2 (67%)	4 (67%)	5 (83%)
AT <sub>1</sub> blockers	0 (0%)	0 (0%)	1 (12%)	0 (0%)	1 (17%)	0 (0%)
Beta-blockers	4 (50%)	4 (67%)	7 (87%)	3 (100%)	5 (83%)	5 (83%)
Ca <sup>2+</sup> -antagonists	1 (12%)	0 (0%)	0 (0%)	0 (0%)	1 (17%)	3 (50%)
Diuretics	0 (0%)	1 (17%)	1 (12%)	0 (0%)	2 (33%)	4 (67%)
Nitrates	1 (12%)	0 (0%)	0 (0%)	2 (67%)	0 (0%)	0 (0%)
Statins	3 (37%)	2 (33%)	1 (12%)	0 (0%)	3 (50%)	5 (83%)
Amiodaron	0 (0%)	0 (0%)	2 (25%)	0 (0%)	0 (0%)	0 (0%)

Values are presented as mean ± SEM or number of patients (%). SRnd: Sinus Rhythm without atrium dilation; SRd: Sinus Rhythm with atrium dilation; pAF: Paroxysmal Atrial Fibrillation; cAF: Chronic Atrial Fibrillation; HF: Heart Failure; Ctl: Control donors; ACE: angiotensin-converting enzyme; AT: angiotensin receptor; AVD/MVD: aortic/mitral valve disease; CAD: coronary artery disease; LAD, left atrial diameter; LVEF, left ventricular ejection fraction; N/A: not available. \* indicates  $p < 0.05$  vs. SRnd. CAD, AVD/MVD, and CAD + AVD/MVD reflect the indications for cardiac surgery (bypass surgery, valve surgery or both).

transplantation due to logistical reasons were usually perfused with, and stored in, a cardioplegic solution, histidine-tryptophan-ketoglutarat Custodiol® solution. We first tested the possibility that differences might exist between the cardioplegia treated or not treated samples under such conditions. We found comparable expression values between SR and Ctl atrial groups (Supplemental Table 2). These results suggest that 1) cAMP-signaling cascade related genes are not affected by the content of this cardioplegic solution, and that 2) it is appropriate to use diseased (SR) samples as potential controls.

### 3.2. Chamber-specific gene expression patterns in atria of the control donors human hearts

All tested genes were expressed in the right and the left atrial appendages. Furthermore, most of the genes showed similar gene expression values in the two chambers in the Ctl and SR samples (Fig. 1). The only significant differences were observed in *AC7* and *PDE2A*, which tended to a higher expression in the left atrium when comparing right and left atrial appendages from SR patients. Western blotting experiments for *PDE2A* confirmed this tendency in protein expression.

### 3.3. Gene expression changes in human atrial fibrillation

Upregulation of the  $\beta$ -AR/cAMP pathway and increased phosphorylation of key proteins in EC coupling are the hallmarks of AF. Accordingly, many genes involved into the  $\beta$ -AR/cAMP pathway are affected by AF (Fig. 2). Previous studies described gene expression changes in AF on *PP1* [16] and *BNP* [17,18] and no change on *IPP1*, *IPP2* [19], *PP2CA* [16,19] and *CaMKIID* [20]. By using a validated set of reference genes [15], we found a downregulation of *AC9*, *AKAP9*, *AKIP*, *PDE3A*, *PDE4B*, *IPP2* and *PKACA* when comparing right and left atrial appendages in SR versus AF (Fig. 2A). *AKAP5* and *Epac1* also showed a tendency to diminution (Supplemental Table 2). *PDE8A*, *PI3K $\gamma$*  and *EPAC2* were upregulated (Fig. 2A), with *Arrestin b2* and *PDE1A* showing a clear trend augmentation in AF (Supplemental Table 2). We confirmed the upregulation of *PDE8A*, *PI3K $\gamma$*  and *EPAC2* at the protein level using immunoblots (Fig. 2B). In contrast, *MRP4*, *MRP5*, *AC2*, *AC4*, *AC5*, *AC6*, *AC7*, *AKAP6*, *AKAP7*, *AKAP13*, *Arrestin b1*, *BNP*, *CaMKIID*, *PDE1C*, *PDE2A*, *PDE3B*, *PDE4A*, *PDE4D*, *PKIA*, *PKIB*, *PP1A*, *PP2CA*, *IPP1*, *PKACB*, *RIA*, *RIIA*, *RIIB*,  $\beta$ 1-AR,  $\beta$ 2-AR, *Gi1*, *Gi2*, *Gi3* and *Gs* showed similar gene-expression patterns in all groups.

Interestingly, some genes appeared to be regulated in a chamber-specific manner. While *BNP*, *PDE8A*, *PP2CA* and *EPAC2* expression increased in the RAA with pAF or cAF (Fig. 2C), *PI3K $\gamma$*  expression increased and *MRP5* and *PKIA* expression decreased only in the LAA with AF (Fig. 2D).

### 3.4. Atrial heart failure-related gene-expression patterns

Although HF is extensively studied in the ventricle, less is known about whether and how HF affects the atria. Fig. 3A shows that HF strongly affects atrial cAMP-pathway gene expression. *MRP4*, *MRP5*, *Arrestin b2*, *PDE4B*, *PDE4D* and *PKIB* are downregulated, while *AC2*, *AC5*, *AC6*, *AC9*, *AKAP9*, *AKIP*, *PDE1C*, *PDE3B*, *PKIA*, *PKIB*, *PP2CA*, *PKACA*, *PKACB*, *RIA*, *RIIB*, *EPAC1* and *CaMKIID* are upregulated when comparing right and left atrial appendages together in Ctl versus HF.

Although *MRP4*, *PKACB* and *PDE1C* expression tends to change in both atrial appendages in HF compared to Ctl, *MRP4* downregulation and *PKACB* upregulation seem to be more specific to the RAA (Fig. 3B), and *PDE1C* is upregulated particularly in the LAA (Fig. 3C).

### 3.5. Gene-expression changes due to $\beta$ -blocker treatment

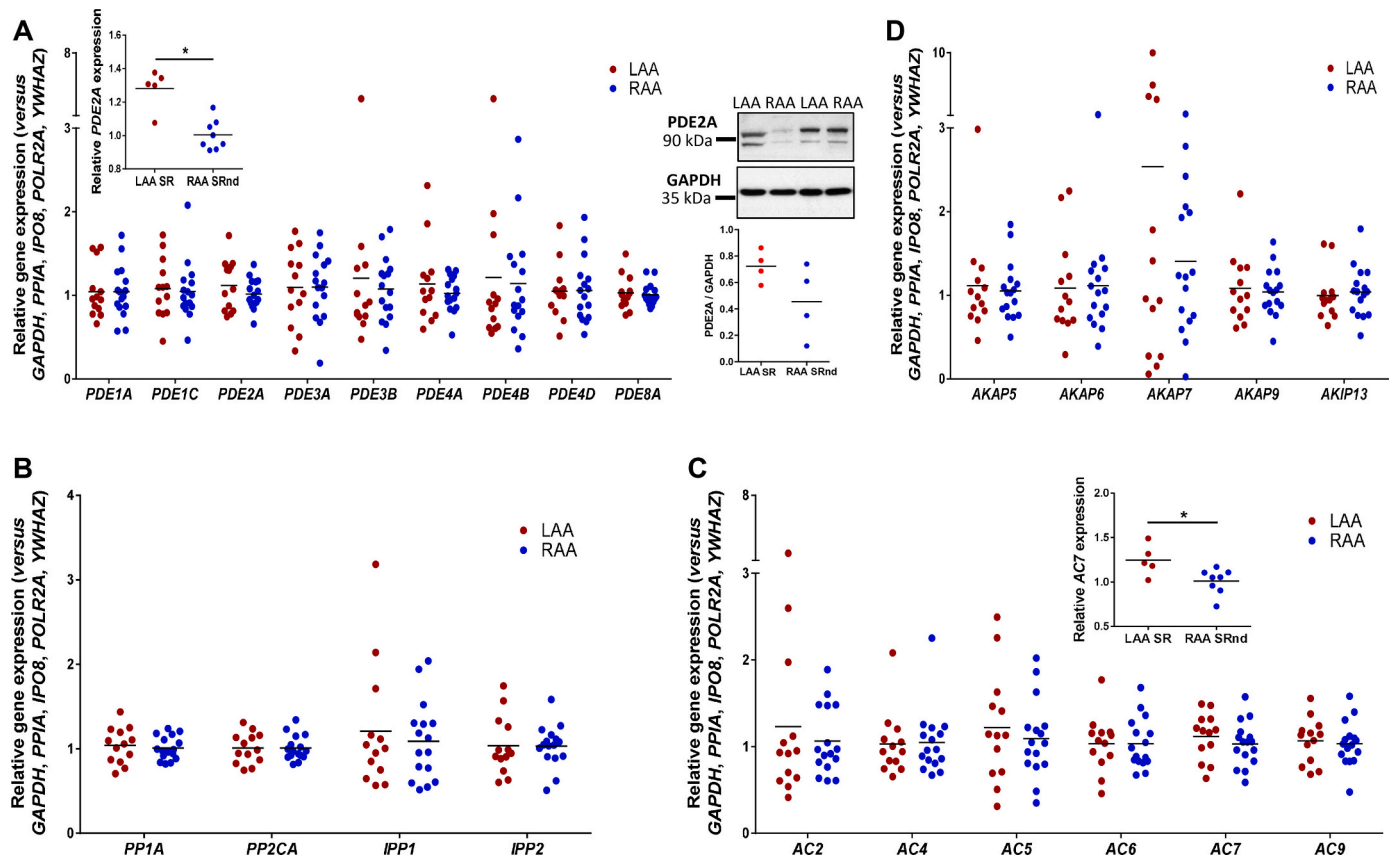
The above results suggest that genetic remodeling of cAMP pathway in AF and HF patients might contribute to the altered sympathetic regulation of atrial function observed in these patients. To explore this further, we examined whether treatment of patients with  $\beta$ -blockers had any impact on their gene expression level. For this, samples from all groups of patients (see Table 1) were separated according to the absence or presence of AF, the absence or presence of atrial dilation and the absence or presence of  $\beta$ -blockers in their medication. Only few alterations in gene expression were found to be associated with  $\beta$ -blocker treatments (Fig. 4). Importantly, treatment of AF-patients with  $\beta$ -blockers did not seem to promote a reverse remodeling.

## 4. Discussion

The present study is to our knowledge the first to compare mRNA-expression pattern of cAMP-cascade related genes in right and left atrial appendages of control human heart tissues and in hearts with different pathologies. The results are summarized in Fig. 5.

HF and AF are intrinsically associated with increased endogenous neurohormonal activation, which induces several changes in the heart and vascular system to maintain cardiovascular homeostasis. At the same time, chronic neurohormonal activation is known to be one of the most important mechanisms underlying pathological remodeling and promoting changes in gene expression of several signaling pathways.





**Fig. 1.** Plot showing an overview of the results on the gene expression values for the different phosphodiesterases (PDEs, A), phosphatases (PPs, B) adenylyl cyclases (ACs, C) and A-kinases-anchoring proteins (AKAPs, D) within the non-cardiac disease atrial cavities (LAA: dilated and non-dilated left atrial appendages,  $n = 13$ ; RAA: dilated and non-dilated right atrial appendages,  $n = 16$ ). Separated small graphs in A and C panels show the only significant differences in gene expression between the samples from right and left non-cardiac disease atrial appendages (LAA SR: left atria,  $n = 5$ ; RAA SRnd: non-dilated right atria,  $n = 8$ ) as well as its corresponding protein expression (LAA SR  $n = 4$ , RAA SRnd  $n = 4$ ). Right chambers values are indicated in blue, left chambers values are indicated in red. \* indicates  $p \leq 0.05$  versus LAA in tissue from patients in sinus rhythm without atrial dilation (SRnd). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

While changes occurring at the level of  $\beta$ -AR [21–23] have been extensively studied, only a few studies focussed on gene expression changes in the cAMP pathway.

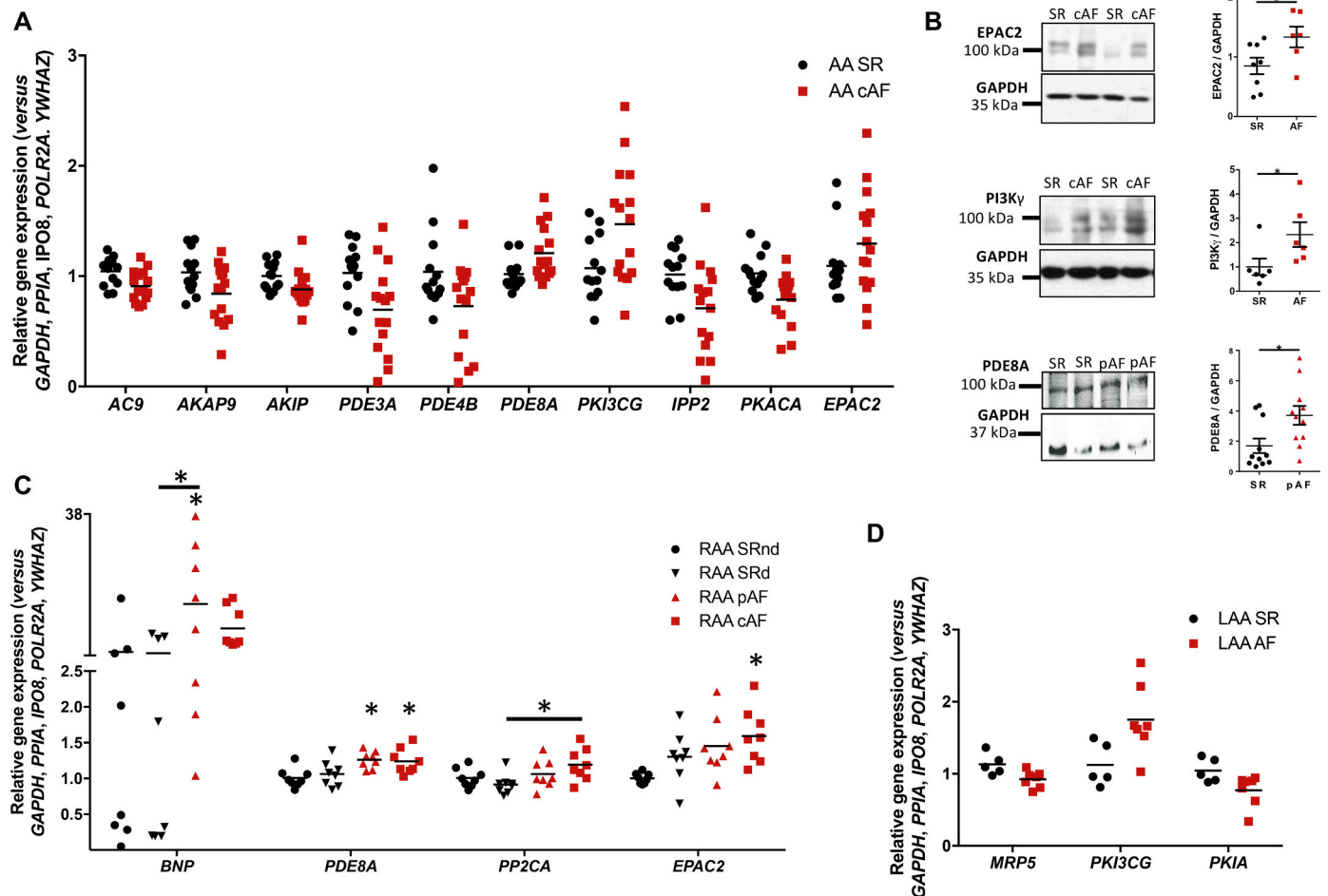
In contrast to previous work in ventricular samples describing gene changes due to exposure to cardioplegic solutions [24], we found similar expression levels between SR patients and donor Ctl individuals. This suggests that gene expression is less sensitive to cardioplegic solution in the atria than in the ventricles. However, many studies do not specify the kind of cardioplegic solution used. One major limitation of studies using human is the selection of appropriate controls. Atrial appendage tissue from patients being in sinus rhythm at time of tissue collection is frequently used as control group for studies dealing with AF or HF [25,26]. Our results suggest that atrial appendage samples from SR patients match those obtained from donor Ctl individuals, validating the use of atrial samples from SR patients as putative controls. This is important considering the very limited availability of atria from healthy individuals. Also, the absence of differences between non dilated and dilated SR in our set of genes made possible to combine these two groups as controls versus different pathologies. However, this may not necessarily apply to other genes outside the cAMP-pathway.

We found that the right and left atrial appendages in control hearts had similar gene expression values for all studied genes with the exception of AC7 and PDE2A, which showed particularly higher abundance in left atria. However,  $\beta$ 1-AR and  $\beta$ 2-AR expression and density were previously found to be unchanged in AF [21,27] but, opposite to our results, higher in left versus right atrium [22]. Although similar gene expression profile between RAA and LAA was already described [14], it

is important to note that PDE4, AKAP7, IPP1, AC2 and AC5 showed highly-variable expression patterns. Even if human samples are difficult to obtain, using low number of samples, especially in studies involving highly variable genes, could lead to erroneous conclusions.

Despite the enormous variability in gene-expression profiles among specific pathologies, several distinguishable patterns could be identified. Expression of AC4, AC7, AKAP5, AKAP6, AKAP7, AKAP13, *Arrestin b1*, PDE1A, PDE2A, PDE4A, PP1A, IPP1, RIIA,  $\beta$ 1-AR,  $\beta$ 2-AR, Gi1, Gi2, Gi3 and Gs remain unaltered during pathological remodeling in both atrial appendages. MRP5, PDE4B and PP2CA by contrast have similar expression changes in both pathologies. Expression of AC9, AKAP9, AKIP, PKIA and PKACA also change in all pathologies but in opposite direction: their expression increases in HF but is reduced in AF. The different PDE isoforms appeared regulated in a pathology-specific manner. For instance, PDE3A and PDE8A expression was modified in AF, but PDE1C, PDE3B and PDE4D expression was modified in HF. PDE8A expression increased in AF together with EPAC2 and PI3K $\gamma$  but not CaMKIID. On the other hand, EPAC1 and CaMKIID gene expression was upregulated in HF, together with AC2, AC6, AC5, RIA, RIIA and PKACB while MRP4, *Arrestin b2* and PKIB gene expression was downregulated.

We already reported reduced PDE4 and total PDE (IBMX-inhibited PDEs) activity in AF [25]. Here we confirm that PDE4B is downregulated in AF at the gene-expression level. Furthermore, PDE3A, one of the major isoforms in controlling cAMP-hydrolytic activity in human atria, is also downregulated in AF. Of note, the IBMX-insensitive PDE8A was upregulated in AF, particularly in the RAA.



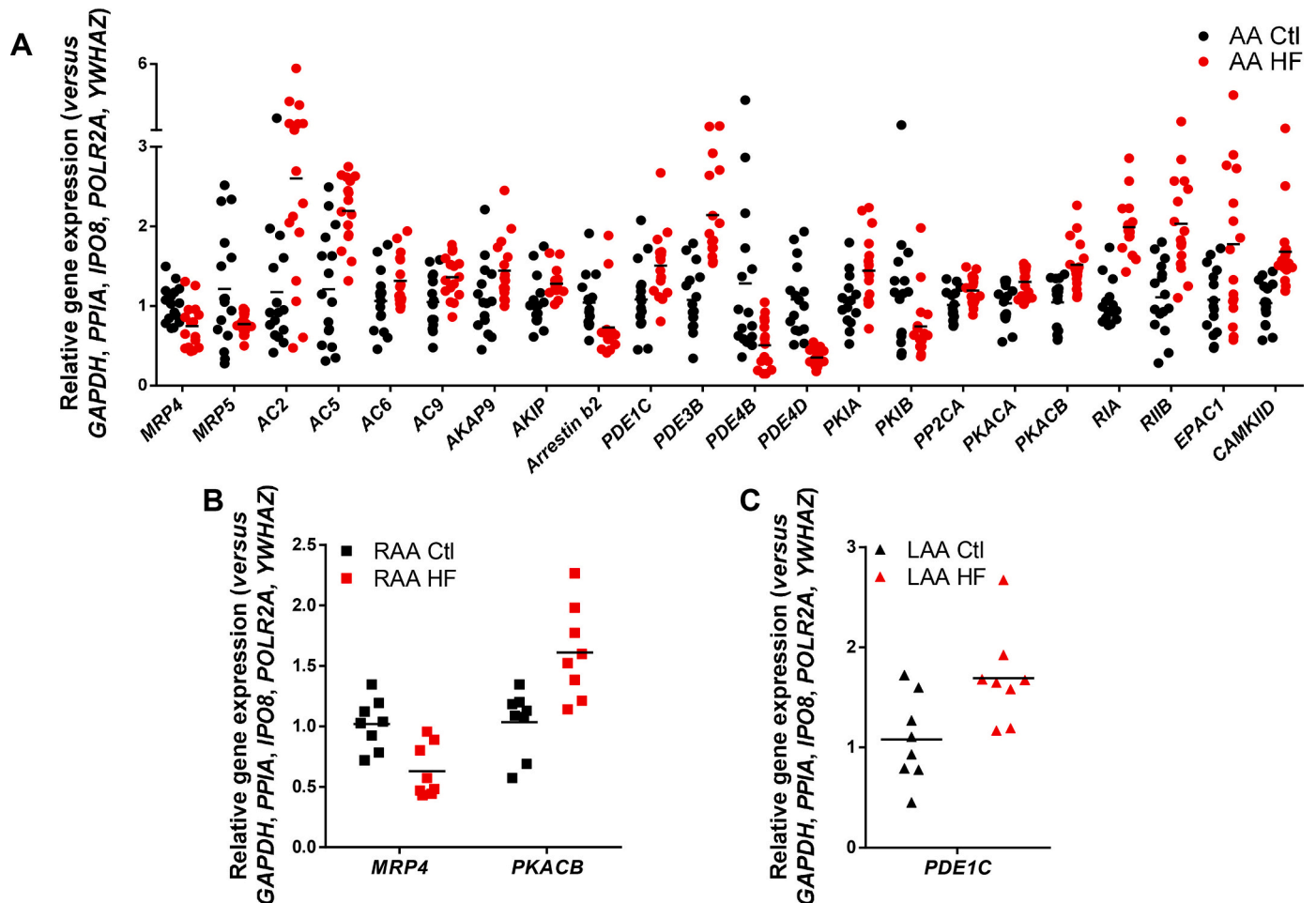
**Fig. 2.** Relative quantification of gene expression in atrial appendage samples from patients in sinus rhythm (SR) or with atrial fibrillation (AF). (A, C, D) Plot of the mean and individual expression values only for the genes whose expression levels were significantly different ( $p \leq 0.05$ ) between SR ( $n = 13$ ) and AF ( $n = 15$ ) in both right and left atrial appendage samples (AA); or between right atrial appendage (RAA) samples from SR patients without atrial dilation (SRnd,  $n = 8$ ), SR patients with atrial dilation (SRd,  $n = 8$ ), patients with paroxysmal AF (pAF,  $n = 8$ ) and patients with persistent-chronic AF (cAF,  $n = 8$ ) (C); or between left atrial appendage samples (LAA) from SRnd ( $n = 5$ ) and cAF ( $n = 7$ ) patients (D). The data are normalized to the validated set of reference genes. (B) Representative immunoblots of EPAC2 and PDE8A in right atrial samples and of PI3K $\gamma$  in left atrial samples from patients in SR ( $n = 8, 6$  and  $11$ , respectively) and AF ( $n = 6, 6$  and  $11$ , respectively). The GAPDH levels were used as internal control. Values from SR patients are indicated in black, values from AF patients in red. \* indicates  $p \leq 0.05$  versus SR. Panels B and C show the genes with chamber-specific differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

No change in *PDE1A* as well as an increase in *PDE1C* was observed in HF. Although *PDE2* was previously found to be upregulated in the ventricles of HF patients [28], we found it stable in both atrial appendages and all pathologies. The results about expression levels of phosphatases are inconsistent. *PPIA* mRNA levels were previously found to be unchanged in AF [19], in agreement with our results, but also downregulated in another study [16]. Both *IPP1* and *IPP2* were found to be unchanged in AF [19], in agreement with our results. However, *PP2CA* levels were reported to be unaltered in AF [16], while we found an increased expression in cAF versus SRd. Similar discrepancies exist for *PPIA* [29,30], which we found unaltered. Several factors can be responsible for such large variability among studies: intrinsic genetic differences among patients, underlying pathology or state of the pathology, medical treatments, age or sex differences. Also, various HF models and underlying aetiologies display different mRNA expression profiles [31]. Variability may also be caused by the use of a single and/or inappropriate reference gene which could change the conclusions [15]. One example is *MRP5*. The multidrug resistant protein 4 (*MRP4*) and 5 (*MRP5*) act as cyclic nucleotides efflux pump regulating cytosolic cAMP and cGMP levels, respectively. *MRP5* gene expression was previously found to be downregulated in atria compared to ventricle and

upregulated in ischemic cardiomyopathy [32]. Unfortunately, this study used  $\beta$ -actin as housekeeping gene, which was found to be one of the most variable genes in human heart samples [15]. Our results based on the use of a validated set of 5 reference genes [15] show reduced *MRP4* and *MRP5* gene expression levels in HF. By contrast, only *MRP5* expression was reduced in AF, yet only in the left atrial appendage. Although some reports have evidenced the functional role of *MRP4* in ventricular myocytes function [33], to date, no heart disease or atrial myocyte dysfunction has been linked to altered *MRP4* gene expression.

EPAC shows a distinctive expression pattern, with very high levels of *EPAC1* in HF and a tendency for downregulation in AF, while *EPAC2* shows a strongly increased expression. On the other hand, although some studies found an increased protein expression or activity of CaMKII $\delta$  in cAF [26], we and others [34] found no change in its gene expression level or even a tendency for a decrease. By contrast, we found an increased expression of *CaMKIID* in the atrial appendages of patients with HF.

Of particular interest is the increased expression of *PI3K $\gamma$*  in AF. *PI3K $\gamma$*  regulates *PDE3* and *PDE4* activity in mouse ventricle [35] and acts as a PKA-anchoring protein for *PDE3B*, enhancing its activity [36]. Indeed, *PI3K $\gamma$*  links  $\beta_2$ -AR signaling to *PDE4A*, *PDE4B* and *PDE3A*,



**Fig. 3.** Relative quantification of gene expression in atrial appendage samples from control donor patients (Ctl) or with end-stage heart failure (HF). (A–C) Plot of the mean and individual expression values only for the genes whose expression levels were significantly different ( $p \leq 0.05$ ) between atrial appendage samples (AA) from Ctl ( $n = 16$ ) and HF ( $n = 16$ ) patients (A); between right atrial appendage (RAA) samples from Ctl ( $n = 8$ ) and HF ( $n = 8$ ) patients (B); or between left atrial appendage samples (LAA) from Ctl ( $n = 8$ ) and HF ( $n = 8$ ) patients (C). The data are normalized to the validated set of reference genes. Values from Ctl patients are indicated in black, values from HF patients are indicated in red. Panels B and C show the genes with chamber-specific differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

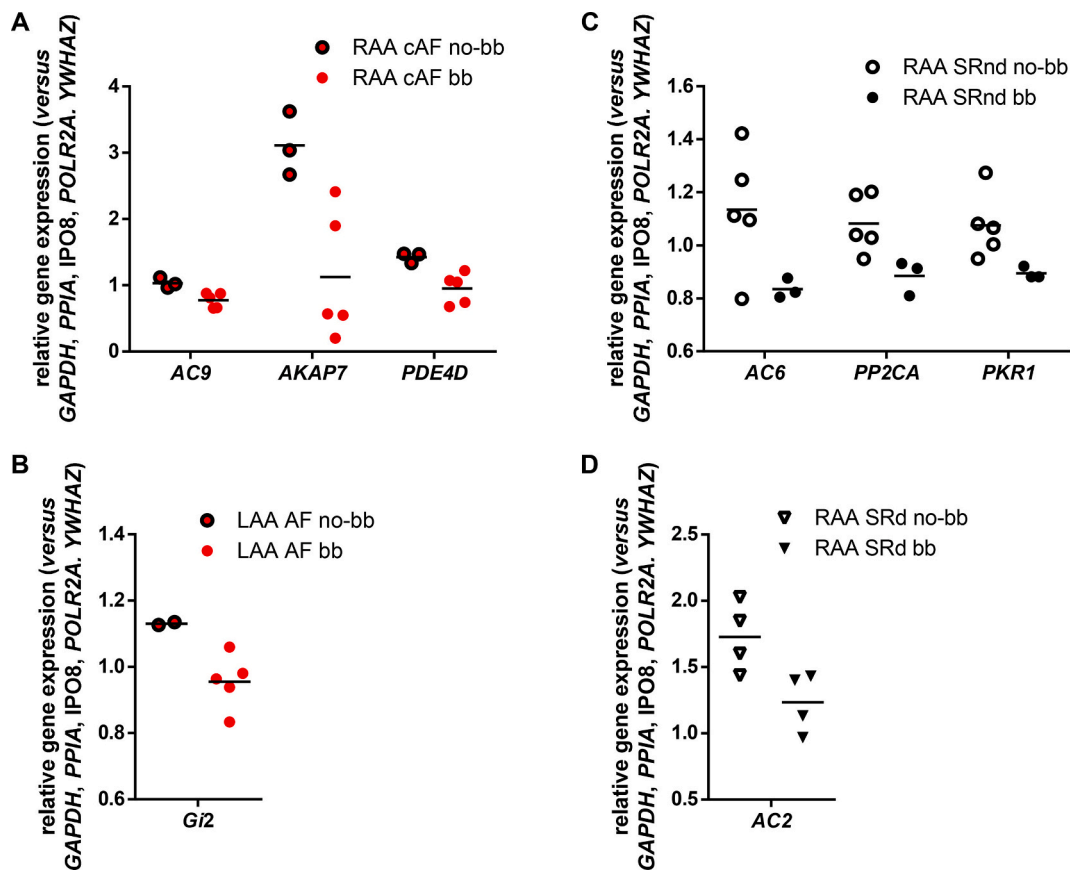
limiting  $\beta_2$ -AR-induced cAMP elevation and PKA-dependent phosphorylation of LTCC and PLB, preventing spontaneous arrhythmogenic  $\text{Ca}^{2+}$  releases in ventricles [35]. Our results demonstrating  $PI3K\gamma$  upregulation suggest a potential role of this molecule in the AF-related remodeling of LTCCs. On the other hand,  $PI3K\gamma$  upregulation may be a compensatory mechanism in order to limit arrhythmogenic  $\text{Ca}^{2+}$  abnormalities linked to AF, the reduction of  $PDE3A$  and  $PDE4B$  expression observed here or the downregulation of  $PKACA$  expression.

Unexpectedly, *BNP* but not *ANP* was upregulated in pAF samples from RAA. Previous studies already reported an increase of *BNP* levels in AF [18] or *ANP* levels in HF [37]. Importantly, and in line with other authors [24], we found a highly variable natriuretic peptides (NP) expression, especially in donor Ctl individuals, probably because some non-failing hearts might have some pathology that made them unsuitable for transplantation and/or subjected to strong medication cocktails until organs extraction. On the other hand, it is well known that plasma levels of NP change quickly depending on the clinical conditions, interventions and treatments of the patient but the relationship between transcriptional and plasma level changes in NP expression and its different metabolites is not clear.

*AKAP9* binds to potassium channels (*KCNQ1*) and was shown to be linked to long-QT syndrome [38] so it was expected to change its expression in pathological conditions. While *AKAP9* expression increased in HF in both atrial appendages, it is decreased in cAF.

Although *AKAP5* binds to LTCC [39], this anchoring protein seems to be unchanged in AF. *AKAP13* was reported to be upregulated during hypertrophy [40] as well as in the transverse aortic constriction mouse model [41], but its expression was unchanged between our pathological groups or between right and left atrial appendages, as previously shown [42]. It was also shown that *AKAP6* might induce myocyte hypertrophy by regulating calcineurin [43] although we found no change in its expression. Our results on *AKAP6* are in agreement with the study of Zakhary and colleagues [44] pointing towards a decreased PKA-RII autophosphorylation as a reason for limiting AKAP/PKA-RII interaction and affecting PKA targets. But these authors also described decreased PKA-RII and PKA-R1 protein levels in human samples from patients with dilated cardiomyopathy. No change in *RIIA* and the *PKACA* subunit, as well as increased *RIA* expression in HF was also previously reported [45]. We found *PKIA*, *PKACA*, *RIA*, *RIIB* and *PKACB* upregulation and *PKIB* downregulation in HF, and no change in gene expression for *RIIA*.

In agreement with previous studies [21,22,46–48], no expression changes were observed in  $\beta_1$ -AR,  $\beta_2$ -AR, *Gs*, *Gi1*, *Gi2* and *Gi3* genes between chambers or in pathologies (Supplemental Table 2). However,  $\beta_1$ -AR gene expression showed a tendency to decrease in atrial appendage samples from HF patients compared to Ctl. Furthermore, separation of the patients according to the absence or presence of  $\beta$ -adrenergic receptor blocker treatment revealed very few alterations in



**Fig. 4.** Relative gene expression changes in response to  $\beta$ -blocker treatment. (A–C) Plot of the mean and individual expression values only for the genes whose expression levels were significantly different ( $p \leq 0.05$ ) between patients with (bb) or without  $\beta$ -blockers treatment (no-bb): A in right atrial appendage samples (RAA) from patients with persistent atrial fibrillation (cAF); B in left atrial appendage samples (LAA) from patients with atrial fibrillation (AF); C in RA samples from patients in sinus rhythm without atrial dilation (SRnd); and D in RAA samples from patients in sinus rhythm with atrial dilation (SRd). Values from SR patients are indicated in black, values from AF patients in red.

the expression of genes linked to cAMP pathway and, by comparing the effects of  $\beta$ -blocker treatment and the effects of AF on gene expression, none of those changes seemed associated to a reverse remodeling.

## 5. Limitations

Despite the statistically significant differences in gene expression between specific groups, the number of tissue samples for some comparisons were relatively limited (LAA-SR  $n = 5$ ). In addition, only a few right and left atrial appendage samples were paired. On the other hand, patients of the Ctl group were younger than the other groups of patients. Thus, age could be a confounding factor on the interpretation of the data, especially when comparing Ctl and HF. Furthermore, some information regarding medication of the Ctl and HF groups is missing making it not possible to check if gene expression could change because of the treatment of the patients in these groups. No comorbidities promoted differences that could change the results of the study.

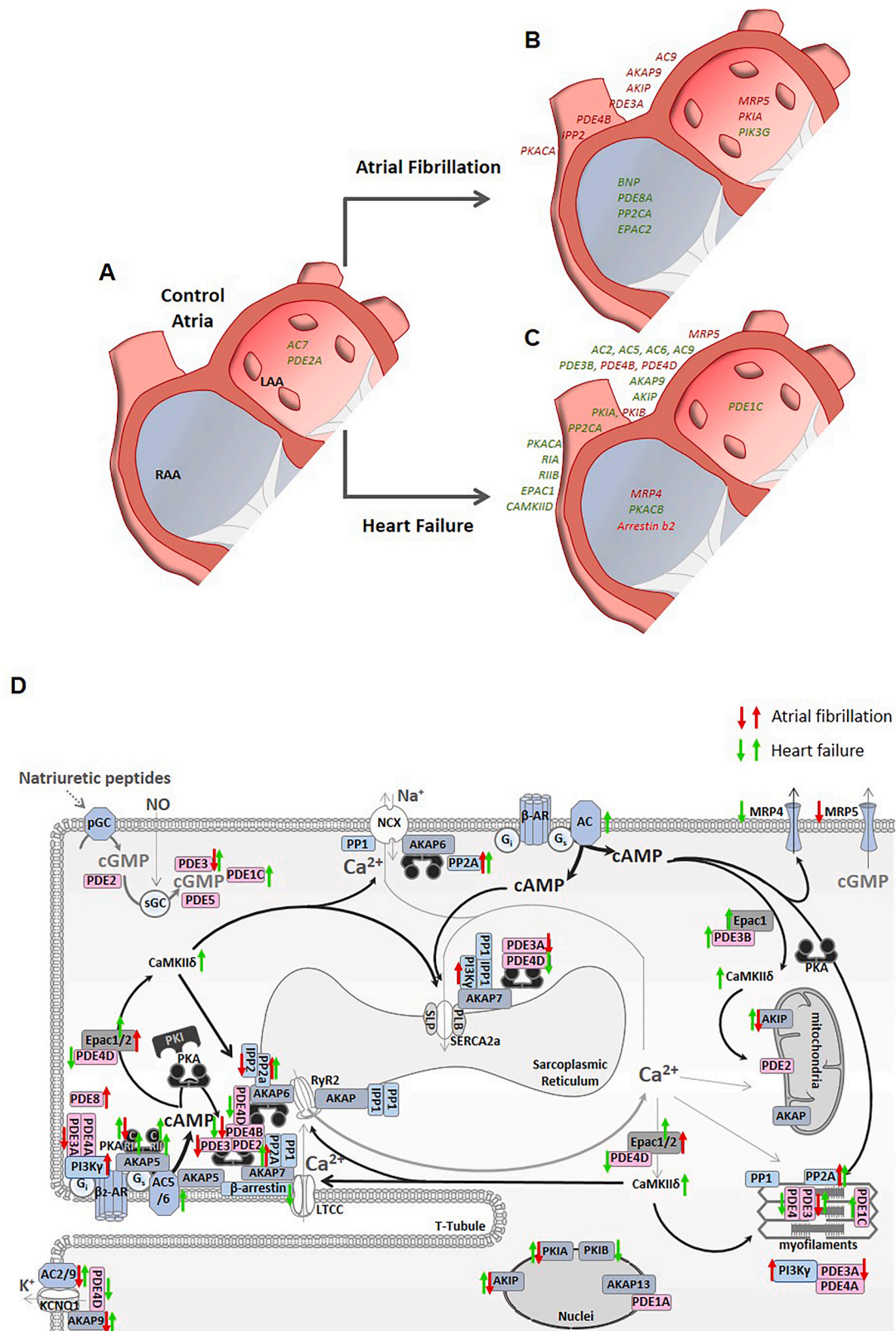
Due to better accessibility we were able to use tissue only from right and left atrial appendages. Despite evidence for a potential role of these regions for AF pathophysiology, the atria are highly heterogeneous and other regions (e.g. left posterior free wall or the pulmonary veins) could be considered more important in the evolution and maintenance of AF [49]; these issues should be considered when translating our results to other atrial regions and interpreting their potentials roles in promoting AF or HF. In addition, experiments were performed in human whole tissue samples. Thus, the results are based not only on cardiomyocyte mRNA content, but also include many other cell types like fibroblasts, endothelial and immune cells. A nuclear but not whole cell

transcriptional analysis from a recent single-cell resolution study revealed that ADCY4, ADCY7, ADCY9, AKAP9, ARRB1, ARRB2, PRKAR2B, RAPGEF3, RAPGEF4, PDE1A, PDE1C, PDE2A, PDE3B, PDE4B and PDE8A are also relatively highly expressed in endothelial, fibroblast, macrophages, pericytes and/or neuronal cells [14]. So, it is important to keep this in mind even if cardiomyocytes account for the majority of heart mass and are the more common cell type in our samples without fat. Nevertheless, cell-type heterogeneity does not change the main conclusion of our results and the possible functional consequences of the detected differences.

## 6. Conclusions

In the present manuscript, we studied the remodeling of the cAMP-signaling cascade induced by different pathologies in human atrial appendages. Our data indicate that cAMP-signaling cascade related genes of atrial SR samples are not affected by cardioplegic solution content, providing a potential alternative for control donor individuals and serving as appropriate controls. Our data also reveal that in the control hearts, only AC7 and PDE2A expression is different between atrial chambers, being higher in the left versus right atrial appendages. Furthermore, many genes in the cAMP pathway are affected in AF and HF, but only few changes were also chamber-specific. Of particular interest is the increased expression of *PI3K $\gamma$* , *EPAC2* and *PDE8A* in AF. By comparing mRNA-expression patterns of the cAMP-signaling cascade related genes in RAA and LAA of human hearts and across different pathologies, our study may help identifying new genes of interest as potential therapeutic targets or clinical biomarkers for these





**Fig 5.** Graphical summary of the differences in gene expression between control right and left atrial appendages (A) or the differences within each atrial chamber induced by atrial fibrillation (B) or heart failure (C). Genes that are downregulated are indicated in red, and those upregulated in green. (D) Schematic representation of the changes in gene expression during atrial fibrillation and heart failure in human atrial myocytes. Only the genes involved in cAMP-signaling cascade studied here are represented by their respective transcribed proteins, enzymes or receptors with respect to their role in regulating excitation-contraction coupling. Up and down arrows indicate up- or downregulation in atrial fibrillation (in red) or heart failure (in green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pathologies.

## Funding

This work was supported by a Postdoctoral Lefoulon Delalande grant from the Institut de France to CEM, by the Deutsche Forschungsgemeinschaft (grant number ES 569/2-1) and Marie Curie IEF grant (grant number PIEF-GA-2012-331241) to CEM, by the Deutsche Forschungsgemeinschaft (grant number Ma 1982/5-1) to LSM, by the Deutsche Forschungsgemeinschaft (grant number Do 769/4-1) to DD, by the Nemzeti Kutatási, Fejlesztési és Innovációs Hivatal (grant number GINOP-2.3.2-15-2016-00040) to IB, by the National Institutes of Health (grant numbers R01-HL131517, R01-HL136389, and R01-HL089598) to DD, and by a grant from Agence Nationale de la Recherche (ANR-16-ECVD-0007-01) to RF. UMR-S1180 is a member of the Laboratory of Excellence in Research on Medication and Innovative Therapeutics supported by the Agence Nationale de la Recherche (ANR-10-LABX-33) under the program “Investissements d’Avenir” (ANR-11-IDEX-0003-01).

## Declaration of competing interest

None declared.

## Acknowledgments

We would like to acknowledge Priscilla Ponien for her contribution to the RT-qPCR experiments. We also thank Claudine Deloménie (transcriptomic platform of UMS IPSIT) for her expertise in molecular biology. A special acknowledgement for Ramona Nagel and Bettina Mausa for their contribution to the western blotting experiments.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yjmcc.2021.02.006>.

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