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Identification of optimal reference genes for transcriptomic analyses in normal and diseased human heart

Cristina E. Molina[1,2,3], Eric Jacquet[4], Prishila Ponien[4], Christian Muñoz-Guijosa[5], Istvan Baczkó[6], Lars S. Maier[7], Patrick Donzeau-Gouge[8], Dobromir Dobrev[9], Rodolphe Fischmeister[1]*, and Anne Garnier[1]

[1]Inserm, UMR-S 1180, University of Paris-Sud, Université Paris-Saclay, Châtenay-Malabry, France; [2]Institute of Pharmacology and Toxicology, Heart Research Center Göttingen, University Medical Center, Georg-August University Göttingen, Germany; [3]German Center for Cardiovascular Research (DZHK), Partner Site Göttingen, Germany; [4]Institut de Chimie des Substances Naturelles, UPR 2301, CNRS, University of Paris-Sud, Université Paris-Saclay, Gif-sur-Yvette, France; [5]Cardiac Surgery Department, Hospital de la Santa Creu i Sant Pau, Spain; [6]Department Pharmacology and Pharmacotherapy, University of Szeged, Hungary; [7]Department Internal Medicine II, University Heart Center, University Hospital Regensburg, Regensburg, Germany; [8]Institut Hospitalier Jacques Cartier, F-91300 Massy, France; and [9]West German Heart and Vascular Center, Institute of Pharmacology, University Duisburg-Essen, Essen, Germany

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Aims Quantitative real-time RT-PCR (RT-qPCR) has become the method of choice for mRNA quantification, but requires an accurate normalization based on the use of reference genes showing invariant expression across various pathological conditions. Only few data exist on appropriate reference genes for the human heart. The objective of this study was to determine a set of suitable reference genes in human atrial and ventricular tissues, from right and left cavities in control and in cardiac diseases.

Methods and results We assessed the expression of 16 reference genes (*ACTB, B2M, GAPDH, GUSB, HMBS, HPRT1, IPO8, PGK1, POLR2A, PPIA, RPLP0, TBP, TFRC, UBC, YWHAZ, 18S*) in tissues from: right and left ventricles from healthy controls and heart failure (HF) patients; right-atrial tissue from patients in sinus rhythm with (SRd) or without (SRnd) atrial dilatation, patients with paroxysmal (pAF) or chronic (cAF) atrial fibrillation or with HF; and left-atrial tissue from patients in SR or cAF. Consensual analysis (by geNorm and Normfinder algorithms, BestKeeper software tool and comparative delta-Ct method) of the variability scores obtained for each reference gene expression shows that the most stably expressed genes are: *GAPDH, GUSB*, *IPO8*, *POLR2A*, and *YWHAZ when comparing either right and left ventricle or ventricle from healthy controls and HF patients; GAPDH, IPO8, POLR2A, PPIA*, and *RPLP0* when comparing either right and left atrium or right atria from all pathological groups. *ACTB, TBP, TFRC*, and *18S* genes were identified as the least stable.

Conclusions The overall most stable reference genes across different heart cavities and disease conditions were *GAPDH, IPO8, POLR2A* and *PPIA*. *YWHAZ* or *GUSB* could be added to this set for some specific experiments. This study should provide useful guidelines for reference gene selection in RT-qPCR studies in human heart.

Keywords Human heart • Heart failure • Atrial fibrillation • Quantitative real-time polymerase chain reaction • Reference genes • Ventricle • Atrium

1. Introduction

The four chambers of a healthy heart are characterized by specific genetic, anatomic, metabolic, physiologic, and electric features. For example, right (RV) and left ventricles (LVs) have different mass, volume, morphology and pressures.[1,2] Morphological differences also exist between the right and left atrium.[3] Additionally, there are chamber-specific differences in electrophysiological properties[4,5] including different action potential parameters due to specific expression patterns and function of ion channels.[6,7] Accordingly, regional heterogeneity between left and right heart chambers also exists for the expression of many genes,[6,8–13] microRNAs,[14] as well as frequently used housekeeping genes.[15]

* Corresponding author. Tel: +33 1 46 83 57 71; fax: +33 1 46 83 54 75, E-mail: rodolphe.fischmeister@inserm.fr

Atrial fibrillation (AF) and heart failure (HF) are common cardiac disorders associated with significant morbidity, mortality, and a growing public health cost.[16–18] AF- and HF-related remodelling processes are associated with profound genetic, structural and functional alterations[19–22] which differ between left and right heart chambers.[23–25] Although AF is considered to predominantly occur in the left atrium, recent data by Karapinar *et al.*[26] showed profound changes in the electrical properties in the right atrium of paroxysmal atrial fibrillation (pAF) patients pointing to specific remodelling in the right atrium of these patients. β-adrenoceptor (β-AR) blockers are a well-established therapy for HF, but the β-AR response may differ between left and right ventricles.[5]

Although there is clear evidence that altered gene transcription contributes to the pathological cardiac remodelling leading to AF or HF, the precise mechanisms of altered gene transcription are not completely understood. In this context, gene expression profiling of the human heart has become an essential step to study in-depth the molecular regulatory networks that underlie chamber-specific differences between healthy and diseased hearts. Quantitative real-time polymerase chain reaction (RT-qPCR) is the most sensitive and accurate technique to simultaneously amplify and quantify gene expression by measuring the increment of fluorescence in each PCR cycle. However, PCR readouts need an accurate normalization with internal reference genes whose expression has to be stable[27] and independent of the experimental groups or remodelling processes. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 18S ribosomal RNA (*18S*), β-actin (*ACTB*) or β2-microglobulin (*B2M*) have been used extensively as reference genes in RT-qPCR for heart samples.[28–31] Yet, a number of studies have demonstrated that the expression of some of these classically used housekeeping genes vary significantly according to the species, experimental model, disease condition, tissue type, etc.[30,32–35] In human heart, a recent study used human HF samples to select stable housekeeping genes for normalizing gene expression data between HF left and right ventricles,[36] but no optimal set of reference genes has been identified so far for normalizing gene expression data across right and left atria or for comparing the different cardiac cavities in healthy and diseased states.

The aim of this study was to assess the expression of 16 classical genes used as housekeeping genes (*ACTB*, *B2M*, *GAPDH*, β-D-glucuronidase (*GUSB*), hydroxymethylbilane synthase (*HMBS*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), importin 8 (*IPO8*), phosphoglycerate kinase 1 (*PGK1*), RNA polymerase II subunit A (*POLR2A*), peptidylprolyl isomerase A (*PPIA*), ribosomal protein lateral stalk subunit P0 (*RPLP0*), TATA box binding protein (*TBP*), transferrin receptor (*TFRC*), ubiquitin C (*UBC*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) and 18S ribosomal RNA (*18S*)) and to select a set of reference genes that can be reliably used for normalization of RT-qPCR experiments in atrial and ventricular tissues from left and right cavities of healthy subjects or patients with AF or HF. A combined analysis of the results using GeNorm[35] and NormFinder[37] algorithms, the BestKeeper[38] software tool and the comparative Delta-Ct method[39] was performed to strengthen and validate the selection of the top-ranking reference genes in order to propose an optimal set of reference genes across the different heart cavities and cardiac diseases.

2. Methods

2.1 Selection of candidate reference genes

For this study, we used the TaqMan Human Endogenous Control Arrays (Applied Biosystems, Life Technologies, France) which contain commonly used housekeeping genes that exhibit minimal differential expression across many different tissues: *ACTB, B2M, GAPDH, GUSB, HMBS, HPRT1, IPO8, PGK1, POLR2A, PPIA, RPLP0, TBP, TFRC, UBC, YWHAZ, 18S*. Each array includes three replicates per gene and per sample.

2.2 Human tissue samples used for selection of reference genes

A total of 44 human heart tissue samples were collected from a total of 36 patients undergoing cardiac 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 Abt. Kardiologie und Pneumologie, Georg-August-Universitaet Göttingen, Germany, or at the Department of Pharmacology and Pharmacotherapy, University of Szeged, Hungary. 28 specimens of atrial appendages were obtained from 28 patients subjected to atrial cannulation for extracorporal circulation. Permission to use atrial tissue samples that would normally be discarded during cardiac surgery was obtained from each patient. Healthy control (CT) and failing (HF) RV and LV tissues were collected respectively from 4 healthy and 4 failing explanted human hearts at the time of the extraction. All samples were immediately frozen in liquid nitrogen and stored at -80 °C. Details regarding the clinical characteristics of the patients and their treatments are shown in *Table 1*.

The study was conducted in accordance with the Declaration of Helsinki principles, and approved by the Ethical Committees of our institutions. All protocols for obtaining human cardiac tissue were approved by the Ethical Committees and informed consent was obtained before cardiac surgery.

2.3 Human tissue samples used for validation of the preselected reference genes

A selection of the above described human heart samples was used to measure expression of two target genes, protein phosphatase 2 catalytic subunit alpha isoform (*PPP2CA*) in atrial samples and adenylate cyclase type V (*ADCY5*) in ventricular samples, and validate the proposed set of reference genes. *Table 1* summarizes the clinical characteristics of the subset of patients used in this study. Thus, a total of 15 atrial samples from patients subjected to atrial cannulation during surgery and 7 ventricular samples from explanted human hearts were used for this part of the study.

2.4 RNA isolation and cDNA synthesis

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 (OD) (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 2 μg 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.

Table 1 Clinical characteristics of patients

		Age media ± S.E.M.	Gender (*n*)		Surgery (*n*)					Treatment regimens (*n*)				
			F	M	AVS	MVS	CABG	CTr	CD	β-blockers	Calcium antagonists	ACE inhibitors	Diuretics	Statins
RA	**SRnd***	64 ± 5.8	1	3	1	0	4	0	0	2	0	3	0	1
	SRd*	69 ± 6.5	3	1	1	1	1	0	0	2	1	3	1	1
	pAF*	78 ± 3.6	0	4	2	0	3	0	0	3	0	1	1	1
	cAF*	73 ± 3.1	0	4	2	1	2	0	0	2	1	2	3	0
	HF	71 ± 5.6	0	4	1	2	2	0	0	2	0	2	2	3
LA	**SRnd**	69 ± 3.8	0	4	2	0	1	0	0	1	1	2	2	1
	AFc	69 ± 2	2	2	2	4	1	0	0	3	0	2	3	0
RV* and LV	**CT***	44 ± 4.6	2	2	0	0	0	0	4	0	0	0	0	0
	HF*	59 ± 3.8	2	2	0	0	0	4	0	3	0	4	4	2

AVS, aortic valve surgery; MVS, mitral valve surgery; CABG, coronary artery bypass graft; CTr, cardiac transplantation; CD, cerebral death. Some included patients were submitted to valve surgery and coronary artery bypass graft surgery. RA, right atrium; LA, left atrium; RV, right ventricle; and LV, left ventricle. SRnd, Sinus Rhythm without atrium dilatation; SRd, Sinus Rhythm with atrium dilatation; pAF, paroxysmal atrial fibrillation; cAF, chronic atrial fibrillation; HF, Heart Failure and CT, Healthy controls. *indicate the samples that were used to measure expression of two target genes.

Table 2 TaqMan assays used for RT-qPCR amplification

Gene symbol	Gene name	Applied Biosystems assay	TaqMan probe exons	PCR product size (bp)
ACTB	*β*-actin	Hs99999903_m1	1	171
POLR2A	RNA polymerase II subunit A	Hs00172187_m1	1-2	61
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	Hs00237047_m1	1-2	70
PGK1	phosphoglycerate kinase 1	Hs99999906_m1	4-5	75
PPIA	peptidylprolyl isomerase A (cyclophilin A)	Hs99999904_m1	4	98
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1	3	122
IPO8	importin 8	Hs00183533_m1	16-17	71
HMBS	hydroxymethylbilane synthase	Hs00609297_m1	1-2	64
HPRT1	hypoxanthine phosphoribosyltransferase 1	Hs99999909_m1	6-7	100
GUSB	*β*-D-glucuronidase	Hs99999908_m1	11-12	81
B2M	*β*-2-microglobulin	Hs99999907_m1	2-3	75
RPLP0	ribosomal protein lateral stalk subunit P0	Hs99999902_m1	3	105
TBP	TATA box binding protein	Hs99999910_m1	7	127
TFRC	transferrin receptor	Hs99999911_m1	14	105
UBC	ubiquitin C	Hs00824723_m1	1-2	71
18S	eukaryotic 18S ribosomal RNA	Hs99999901_s1	1	187
PPP2CA	protein phosphatase 2, catalytic subunit, alpha isozyme	Hs00427260_m1	6-7	77
ADCY5	Adenylate cyclase type V	Hs00766287_m1	16-17	72

2.5 Real-time qPCR and quantification

Real-time PCR assays were performed using TaqMan 384-well 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 stability of 16 potential reference genes (*Table 2*). As mentioned earlier, the 16 reference genes were chosen by their common use as endogenous control genes. 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 a 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. Experiments were performed in triplicate for each sample.

2.6 Evaluation of candidate reference genes

Reference gene expression variability was evaluated by using a combined analysis of geNorm, Normfinder, BestKeeper and Delta-Ct method. In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to reach a define threshold.

Table 3 Quality control of RNA samples

		n	RNA content (μg/mg)	260 nm/280 nm OD ratio		RIN	
				Mean	SD	Mean	SD
RA	SRnd	4	0.49 ± 0.01	1.70	0.13	6.9	0.81
	SRd	4	0.55 ± 0.06	1.63	0.16	7.2	0.29
	pAF	4	0.37 ± 0.05	1.57	0.11	6.8	0.54
	cAF	4	0.44 ± 0.05	1.67	0.14	6.9	0.28
	HF	4	0.52 ± 0.16	1.66	0.18	7.5	0.15
LA							
	SR	4	0.43 ± 0.09	1.68	0.07	6.7	0.61
	cAF	4	0.43 ± 0.10	1.61	0.13	6.7	0.80
RV							
	CT	4	0.45 ± 0.22	1.58	0.19	7.1	0.49
	HF	4	0.65 ± 0.06	1.72	0.09	7.6	0.85
LV							
	CT	4	0.71 ± 0.04	1.54	0.06	6.9	1.43
	HF	4	0.70 ± 0.04	1.72	0.09	7.6	0.80

RA, right atrium; LA, left atrium; RV, right ventricle; and LV, left ventricle. SRnd, sinus rhythm without atrium dilatation; SRd, sinus rhythm with atrium dilatation; pAF, paroxysmal atrial fibrillation; cAF, chronic atrial fibrillation; HF, heart failure; and CT, Healthy controls.

Ct levels are inversely proportional to the amount of target nucleic acid in the sample. Ct is specific to the expression of one gene whereas Delta Ct shows the difference of expression between two genes. This ΔCt approach can be used to study the stability of candidate housekeeping genes by comparing the relative expression of 'pairs of genes' within each sample.[39] GeNorm algorithm ranks the candidate reference genes by an expression stability measurement called M-value which is the average pairwise variation of a particular gene with all the other reference genes.[35] The most stable genes are the ones with the lowest M-value. NormFinder algorithm also ranks the set of candidate normalization genes according to their expression stability and it can also take information of samples grouping to define intra- and inter-group variations for each candidate reference gene. The result is an optimum rank of reference genes.[37] BestKeeper determines the most stably expressed genes based on the coefficient of correlation to the BestKeeper Index, which is the geometric mean of the candidate reference gene Ct values. BestKeeper also calculates the standard deviation (SD) so that the best reference genes are those with the lowest SD and coefficient of variation (CV) based on the Ct values of all candidate reference genes.[38] Following these four analyses, each candidate reference gene obtained a specific ranking value. A consensual analysis was finally performed by the calculation of the geometric mean of the four ranking values for each gene leading to a consensus variability score for each reference gene.[40]

PPP2CA and *ADCY5* were selected to confirm the appropriate reference genes pool by measuring their relative expression levels. TaqMan Array Cards were also used to perform real-time PCR experiments under the same experimental conditions and with the subset of samples indicated in *Table 1*. For each group, an average Ct was calculated. The average Ct of the control groups, Sinus Rhythm without atrium dilatation (SRnd) and CT, was used as calibrator for the atria and ventricles, respectively. The determination of the relative gene expression ratio was achieved using the ΔΔCt method, i.e. it is calculated by referring to the calibrator and normalized by a housekeeping gene or the geometric mean of a set of stable housekeeping genes.

2.7 Statistical analysis

Results are expressed as mean ± SEM. For statistical evaluation of two sets of data Mann-Whitney test was used. Kruskal-Wallis test was used for multiple comparisons to evaluate the significance between groups. A difference was considered statistically significant when $P < 0.05$.

3. Results

3.1 Total RNA sample concentration and quality

Total RNA content, 260/280 nm OD ratio and RIN obtained in the four human cardiac cavities under normal or pathological conditions are summarized in *Table 3*. There were no differences in RNA content between the groups neither in the atria nor in the ventricles, or between atria and ventricles. The quality control parameters for human total RNA samples were similar regardless of human heart tissue sample collection, with no difference between the groups. The 260/280 nm OD ratios appeared relatively low (range of 1.5–1.7), but the RIN values were consistent and comparable within different heart cavities and cardiac diseases, with an overall average of 7.1.

3.2 Selection of candidate reference genes

Using RT-qPCR, we evaluated simultaneously the expression of 16 reference gene candidates on the 44 human heart tissue samples (*Table 1*). *Figure 1* illustrates the variation in candidate reference gene abundance across all samples. For sake of clarity, the reference genes are ordered by their respective abundance. This first analysis showed as expected the dominance of *18S* rRNA transcripts compared with the other reference genes. The other reference genes were separated into two groups according to their abundance: (i) *ACTB, B2M, GAPDH, PGK1, PPIA, RPLP0*, and *UBC*, with a median Ct value from 17 to 21; (ii) *GUSB, HMBS, HPRT1, IPO8, POLR2A, TBP, TFRC*, and *YWHAZ* with a median Ct value from 23 to 27. We found that some genes had a high variability in expression, e.g. *18S, ACTB, B2M, TBP*, and *TFRC*, whereas others were more stable, e.g. *HMBS* and *POLR2A*. So, based on their relative abundance and steady expression across all the samples, a pre-selection of candidate reference genes could be considered.

We then performed a combined statistical analysis of the candidate reference gene variability by taking into account the results from the geNorm and NormFinder algorithms, the comparative Delta-Ct method and the BestKeeper software tool. The overall rank order of the most stable reference genes is shown in *Table 4* for different comparisons across heart cavities and disease conditions. We summarized these results using a gene oblong chart to illustrate more easily the set of five reference genes that is the most suitable for normalizing gene expression data according to the considered heart cavity and cardiac disease (*Figure 2*).

Concerning the right atrium, a first analysis including 20 patients in SR or AF and HF brought out the following set of five reference genes: *POLR2A, GAPDH, PPIA, IPO8, RPLP0*. This set of reference genes varied according to the progression from SR [SRnd or sinus rhythm with atrium (SRd)] to pAF and chronic atrial fibrillation (cAF), respectively, or depending on the type of cardiac pathology (AF vs. HF). The optimal set of reference genes for comparison of RA-SRnd vs. SRd appeared to be *RPLP0, PPIA, POLR2A, B2M*, and *HMBS*. *RPLP0*, together with *PGK1, GAPDH, IPO8*, and *HMBS* appear to represent a good choice as reference genes when comparing SRnd with pAF samples. In contrast, when the comparison includes cAF samples, *RPLP0* is no more a reliable option; so, the best selection of reference genes includes *POLR2A, PPIA, GAPDH,*

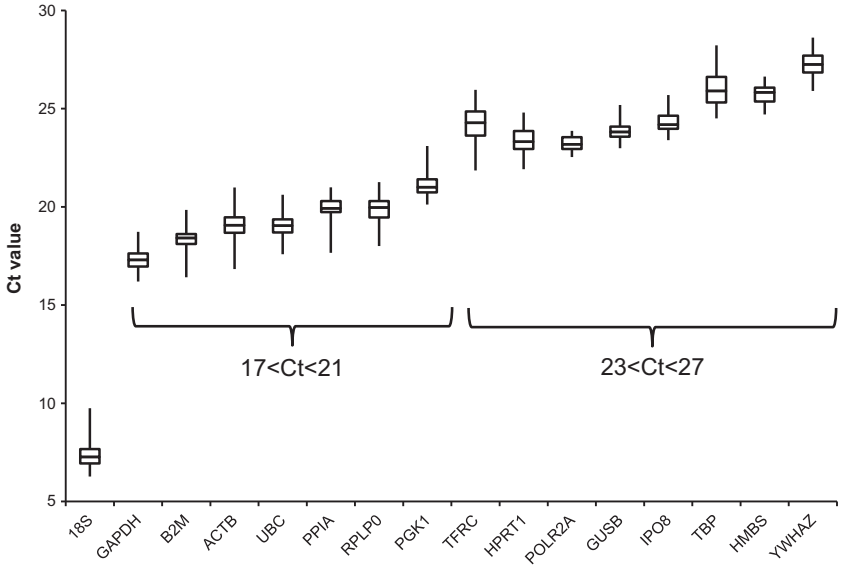

Figure 1 Variation in candidate reference gene expression in all tissue heart samples. Box-and-whisker plots showed the Ct values of each reference gene analyzed in a total of 44 heart tissues from 36 patients. Boxes indicated median (Q2) and quartiles first and third (Q1 and Q3) and whiskers corresponded to the minimum and maximum values.

HPRT1, and *PGK1*. Based on these results, we propose *GAPDH, IPO8, POLR2A, PPIA*, and *RPLP0* as an optimal set of reference genes to perform all the comparisons in the right atrium.

Regarding gene expression studies in all heart cavities, the results point to one common reference gene, *POLR2A*, among the top five for the RA/LA, RV/LV and A/V comparisons. *GAPDH*, *GUSB*, *IPO8*, and *PPIA* can be added to *POLR2A* which should warrant a minimal set of three to four reference genes for normalization of the RT-qPCR data.

GUSB appears as an appropriate reference gene when comparing ventricles in normal and pathological states. *IPO8*, *PGK1*, *HMBS*, and *POLR2A* can be added to *GUSB* when comparing left and right ventricles. For studies of HF in LV samples, the same genes can be used but *YWHAZ* could be preferred over *POLR2A*. However, to elucidate changes in gene expression related to HF in RV, *GAPDH, POLR2A*, *GUSB*, *HPRT1*, and *PPIA* represent the best set of reference genes. Altogether, a minimum of three reference genes among *GAPDH*, *GUSB*, *IPO8*, *POLR2A* and *YWHAZ* would provide a useful set to compare normal and failing ventricular cavities.

Among the 16 reference genes studied for their expression stability in healthy and pathological human heart cavities, our data also show that four genes should not be used for normalization: *18S*, *TBP*, *TFRC*, and *ACTB*. Indeed, in each comparison presented in *Table 4*, the two least stable genes belong to this set.

Finally, for a study comparing all cardiac chambers under normal and pathological conditions, a good set of reference genes should be: *GAPDH, IPO8, POLR2A*, *PPIA*, and *YWHAZ* or *GUSB*. Depending on the groups or conditions compared, this set of reference genes should lead to a minimal set of two to four reference genes to be used for normalization of RT-qPCR data.

3.3 Validation of the set of reference genes

We then tested our normalization strategy by comparing RT-qPCR data normalized to either *18S* or to our final set of reference genes (*GAPDH, IPO8, POLR2A*, *PPIA*, and *YWHAZ*) for two genes of interest: *PPP2CA* in the right atrium from SR (SRnd or SRd), pAF and cAF patients; *ADCY5* in the right ventricle from CTs and HF patients (*Figure 3*).

A large and statistically significant difference in *PPP2CA* gene expression was found between cAF and the other groups when using *18S* as reference gene for normalization (*Figure 3A*) but not when using the set of reference genes for normalization (*Figure 3B*). Since earlier studies using α-actin as reference gene for the RT-qPCR experiments showed no change in *PPP2CA* gene expression in cAF,[41,42] this validates the choice of the set of reference genes from our study when comparing human atrial tissues.

Similarly, when comparing HF vs. CT ventricular samples, a significant increase in the expression level of *ADCY5* was found in HF when normalization was made with the selected set of reference genes (*Figure 3D*) but the expression appeared unchanged between CT and HF samples when normalization was based only on *18S* (*Figure 3C*). A similar absence of variation of *ADCY5* mRNA between CT and HF was found in a pig model when normalizing to the expression of *18S*.[43] In another study in dog heart, a decrease in *ADCY5* expression was observed in HF when normalizing to the expression of *ACTB*,[44] one of least stable reference gene identified in our study. In contrast, an elevation of *ADCY5* mRNA levels was found by Northern analysis in human ventricular samples from patients with HF when normalizing with GAPDH,[45] one of the best stable reference gene selected in this study. This validates the choice of the set of reference genes from our study when comparing human ventricular samples.

4. Discussion

In this study, we provide a detailed analysis of reference genes in cardiac tissues from different regions (right and left atria and ventricles) of the heart of patients with different pathological conditions. We show that

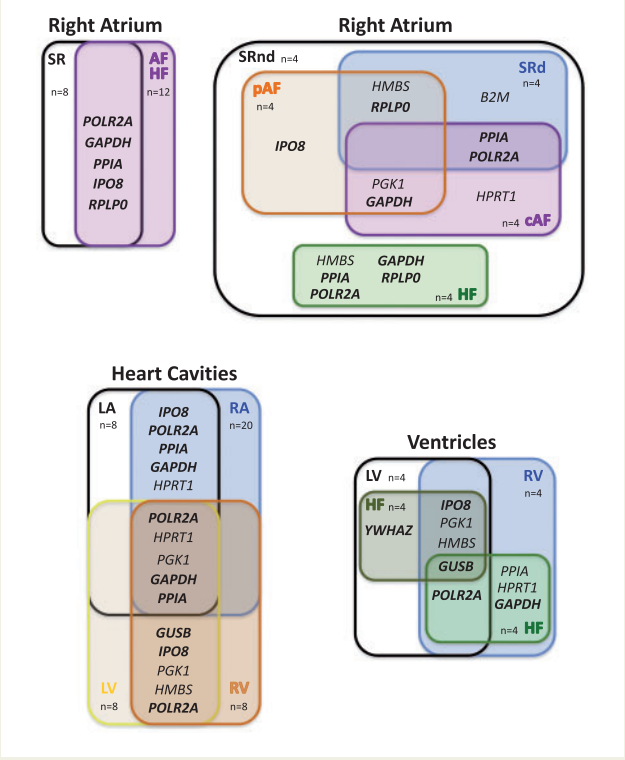

Figure 2 Consensual statistical analysis of variation in candidate reference gene expression in different heart cavities and health conditions. Gene oblong chart illustrating an integrated model of the best gene selection in different human heart cavities and diseases. Gene names indicated in bold are part of the most appropriate selection of 5 reference genes for all comparisons.

there is not one universal gene being appropriate to obtain a correct normalization for all types of human cardiac tissues. Based on a combined statistical analysis, we propose a set of most stable reference genes to be used when comparing RT-qPCR data obtained from healthy or diseased human cardiac tissues.

A considerable number of studies used *18S* or *ACTB* genes as internal controls for RT-qPCR analysis in heart samples. As shown earlier,[46] we found using three different algorithms (GeNorm, NormFinder, and Delta-Ct) that these genes, along with *TBP* and *TFRC*, are the two genes with the least stable expression in all our groups of samples (*Table 4*). Accordingly, using one of these single commonly used reference genes to compare the level of expression of two genes of interest, *PPP2CA* and *ADCY5*, in healthy and diseased cardiac tissues led to misleading results, while on the contrary, using the geometric mean of our set of reference genes for normalization of the RT-qPCR data led to results that were consistent with the literature (*Figure 3*).

The choice of appropriate reference genes is, thus, of crucial importance when comparing RT-qPCR data from mRNA collected from different human heart samples. Although this issue has been addressed in a number of earlier studies, none of them provided a thorough and systematic analysis as performed here. We analysed 16 reference gene candidates and compared their level of expression and degree of stability in the four heart chambers and in control and diseased states. We found that *GAPDH*, *IPO8*, *POLR2A*, *PPIA*, and *YWHAZ or GUSB* provide an optimal set of genes to be used to normalize data in human heart tissue samples. *PPIA* has been shown earlier as an appropriate internal control gene in studies with atrial,[47] right ventricular[15,47] and LV samples from healthy donors[15,48] and from HF patients.[47,49] Martino *et al.*[47] also confirmed our findings about the use of *HPRT1* as a reference gene in human atria and the use of *GAPDH* in right ventricle. These results are also in accordance with the study of Svobodová *et al.*[50] performed in pig heart. Veseneti *et al.*[51] showed that *HMBS*, *HPRT1* and *YWHAZ* are reliable reference genes when working with right or ischemic LV samples. However, together with Koppelkamm *et al.*,[46] they identified *TBP* as an appropriate reference gene when studying LV samples, while our data indicate that expression of this gene is highly variable (*Table 4*). However, *TBP* may be suitable as a reference gene only in combination with *HMBS* and *HPRT1*, and only in the remote region of the left ventricle in rats.[51] On the other hand, Koppelkamm *et al.* recommended the use of *TBP* in combination of at least three other reference genes, including *HMBS*, in post mortem cardiac muscle tissue with RIN mean values of 3.9.[46] Other studies,[49,52] predominantly performed in left ventricles, showed that *GAPDH* is not a good reference gene. Our study shows that *GAPDH* expression is more stable in right than LV samples, and it can also be used as a reference gene in healthy and diseased atria and for comparison between atrial and ventricular tissues (*Figure 2*, Table *4*). This is in agreement with recent works showing that *GAPDH* has the highest expression stability between the tested genes in both HF and healthy heart samples in right and left ventricle.[36,53] *POLR2A* was ranked top three by the geometric consensus method for most atrial and RV studies, while *IPO8* and *GUSB* were the most preserved reference genes in LV samples. This highlights the importance of choosing the most appropriate internal reference gene when studying chamber-specific differences in gene expression. To the best of our knowledge, *IPO8* or *GUSB* have never been used as reference genes in human heart samples.

An important aspect to take into consideration is the quality of the mRNA samples. It was shown earlier that the RIN values are lower and the variation in the RT-qPCR much larger in human than in rodent heart samples,[49] possibly due to differences in harvesting times or a consequence of the type and duration of the diseased condition. Thus, RNA integrity was carefully checked in our samples and the RIN values were consistent and comparable within the different heart cavities and cardiac diseases. Another important aspect is the method of analysis used to evaluate the variability in gene expression. Indeed, we compared the expression variability for the 16 reference gene candidates using four different methods of analysis (geNorm, Normfinder, BestKeeper and comparative delta-Ct) and found that each method could give a different ranking of the 16 genes (*Table 4*). This shows that using a single method of analysis to evaluate the relative expression of a gene cannot be recommended because this could lead to false-positive or false-negative results. We propose to use at least three methods of analysis and to calculate a geometric mean of the rankings as used here.

In conclusion, when performing RT-qPCR analysis on different samples obtained from different cardiac tissues and/or diseased states, we recommend (i) to use a normalization based on a set of three to five stable reference genes rather than on a single gene, and (ii) to use at least three methods of analysis of gene variability and calculate a geometric mean of the rankings to select the most robust set of reference genes. We validated this strategy in human heart using samples obtained from four cardiac chambers and up to five different pathophysiological conditions. Based on our combined results, we recommend the use of *GAPDH*, *IPO8*, *POLR2A*, *PPIA*, and *YWHAZ* or *GUSB* as reference genes for RT-qPCR when performing studies with human heart samples.

Table 4 Analysis of reference gene expression variability

	Genorm		Normfinder		Delta-Ct		BestKeeper		Consensus	
	Genes	Stability value	Genes	Stability value	Genes	SD	Genes	SD	Genes	Geometric mean of ranking values
RA SR/AF-HF (*n* = 8/12)	POLR2A	0.241	POLR2A	0.136	POLR2A	0.38	HMBS	0.17	**POLR2A**	1.50
	GAPDH	0.241	RPLP0	0.196	IPO8	0.41	PPIA	0.22	**GAPDH**	3.13
	IPO8	0.251	IPO8	0.197	PPIA	0.41	B2M	0.28	**PPIA**	3.60
	PGK1	0.257	PPIA	0.223	GAPDH	0.42	GAPDH	0.28	**IPO8**	3.66
	HPRT1	0.261	HPRT1	0.241	RPLP0	0.42	POLR2A	0.30	**RPLP0**	5.61
	UBC	0.276	GAPDH	0.245	HPRT1	0.43	GUSB	0.31	HMBS	6.04
	PPIA	0.290	PGK1	0.262	PGK1	0.44	UBC	0.32	PGK1	6.29
	B2M	0.302	UBC	0.269	B2M	0.44	PGK1	0.35	B2M	6.45
	RPLP0	0.314	B2M	0.270	UBC	0.44	ACTB	0.36	HPRT1	6.51
	GUSB	0.325	GUSB	0.277	GUSB	0.45	IPO8	0.36	UBC	7.42
	HMBS	0.333	HMBS	0.314	HMBS	0.47	RPLP0	0.42	GUSB	8.80
	YWHAZ	0.358	YWHAZ	0.415	YWHAZ	0.54	HPRT1	0.46	ACTB	11.86
	ACTB	0.384	ACTB	0.459	ACTB	0.57	TFRC	0.52	YWHAZ	12.47
	18S	0.422	18S	0.580	18S	0.68	YWHAZ	0.59	18S	14.24
	TFRC	0.457	TFRC	0.618	TFRC	0.71	18S	0.62	TFRC	14.47
	TBP	0.499	TBP	0.725	TBP	0.80	TBP	0.73	TBP	16.00
RA SRnd/SRd (*n* = 4/4)	POLR2A	0.073	RPLP0	0.083	RPLP0	0.30	HMBS	0.15	**RPLP0**	1.73
	RPLP0	0.073	PPIA	0.133	PPIA	0.32	PPIA	0.24	**PPIA**	2.21
	PPIA	0.153	POLR2A	0.139	POLR2A	0.32	B2M	0.25	**POLR2A**	2.82
	B2M	0.173	TFRC	0.157	B2M	0.33	ACTB	0.27	**B2M**	4.12
	IPO8	0.189	GAPDH	0.157	GAPDH	0.33	GUSB	0.28	**HMBS**	5.48
	GAPDH	0.198	B2M	0.173	UBC	0.34	UBC	0.30	GAPDH	6.22
	TRFC	0.217	UBC	0.174	IPO8	0.34	IPO8	0.33	UBC	6.70
	UBC	0.226	IPO8	0.185	TFRC	0.35	POLR2A	0.33	IPO8	6.88
	HMBS	0.237	HPRT1	0.223	HPRT1	0.37	GAPDH	0.34	TFRC	7.05
	HPRT1	0.253	HMBS	0.275	HMBS	0.39	RPLP0	0.34	GUSB	9.64
	PGK1	0.266	PGK1	0.290	PGK1	0.40	TFRC	0.36	ACTB	10.05
	GUSB	0.279	GUSB	0.295	GUSB	0.41	PGK1	0.38	HPRT1	10.32
	ACTB	0.306	YWHAZ	0.436	YWHAZ	0.52	18S	0.49	PGK1	11.24
	YWHAZ	0.334	ACTB	0.458	ACTB	0.53	HPRT1	0.49	YWHAZ	13.73
	18S	0.365	18S	0.523	18S	0.59	YWHAZ	0.61	18S	14.47
	TBP	0.412	TBP	0.697	TBP	0.74	TBP	0.71	TBP	16.00
RA SRnd/pAF (*n* = 4/4)	IPO8	0.082	PGK1	0.102	RPLP0	0.34	HMBS	0.16	**RPLP0**	2.06
	RPLP0	0.082	RPLP0	0.105	GAPDH	0.34	GUSB	0.19	**PGK1**	3.20
	POLR2A	0.123	GAPDH	0.118	PGK1	0.35	B2M	0.21	**GAPDH**	3.46
	GAPDH	0.161	IPO8	0.120	IPO8	0.35	PPIA	0.23	**IPO8**	3.64
	PGK1	0.190	POLR2A	0.150	POLR2A	0.36	ACTB	0.24	**HMBS**	4.60
	B2M	0.210	B2M	0.182	B2M	0.37	GAPDH	0.28	B2M	5.05
	PPIA	0.234	HPRT1	0.228	HMBS	0.40	PGK1	0.28	POLR2A	5.23
	HMBS	0.248	HMBS	0.230	HPRT1	0.41	POLR2A	0.34	GUSB	6.50
	GUSB	0.261	UBC	0.261	GUSB	0.42	RPLP0	0.34	PPIA	7.27
	UBC	0.275	PPIA	0.275	PPIA	0.42	UBC	0.34	UBC	9.43
	HPRT1	0.286	GUSB	0.276	UBC	0.42	18S	0.37	HPRT1	9.64
	TFRC	0.315	YWHAZ	0.432	TFRC	0.53	IPO8	0.37	ACTB	11.40
	YWHAZ	0.341	18S	0.433	YWHAZ	0.54	TFRC	0.37	TFRC	12.47
	18S	0.364	TFRC	0.444	18S	0.54	HPRT1	0.47	YWHAZ	13.21
	ACTB	0.388	ACTB	0.529	ACTB	0.59	YWHAZ	0.56	18S	13.49
	TBP	0.458	TBP	0.918	TBP	0.95	TBP	0.85	TBP	16.00
RA SRnd/cAF (*n* = 4/4)	GAPDH	0.138	POLR2A	0.101	POLR2A	0.37	HMBS	0.15	**POLR2A**	2.14
	PPIA	0.138	HPRT1	0.140	PPIA	0.37	UBC	0.20	**PPIA**	2.34
	POLR2A	0.195	PGK1	0.153	HPRT1	0.38	PPIA	0.21	**GAPDH**	3.60
	HPRT1	0.217	IPO8	0.168	PGK1	0.39	GAPDH	0.25	**HPRT1**	4.03

Continued

Table 4 *Continued*

	Genorm		Normfinder		Delta-Ct		BestKeeper		Consensus	
	Genes	Stability value	Genes	Stability value	Genes	SD	Genes	SD	Genes	Geometric mean of ranking values
	IPO8	0.225	PPIA	0.168	IPO8	0.39	B2M	0.26	**PGK1**	4.90
	PGK1	0.235	RPLP0	0.225	GAPDH	0.40	GUSB	0.26	HMBS	5.20
	UBC	0.249	GAPDH	0.229	UBC	0.41	POLR2A	0.30	UBC	5.29
	RPLP0	0.264	UBC	0.237	RPLP0	0.43	ACTB	0.34	IPO8	5.62
	HMBS	0.283	HMBS	0.309	HMBS	0.45	PGK1	0.34	RPLP0	8.24
	B2M	0.300	B2M	0.321	B2M	0.46	IPO8	0.35	B2M	8.80
	GUSB	0.314	GUSB	0.321	GUSB	0.47	HPRT1	0.39	GUSB	9.03
	ACTB	0.341	ACTB	0.453	ACTB	0.56	RPLP0	0.42	ACTB	11.17
	TFRC	0.371	YWHAZ	0.483	YWHAZ	0.59	TFRC	0.43	TFRC	13.49
	YWHAZ	0.399	TFRC	0.495	TFRC	0.60	TBP	0.61	YWHAZ	13.73
	TBP	0.439	TBP	0.647	TBP	0.72	YWHAZ	0.63	TBP	14.74
	18S	0.490	18S	0.790	18S	0.85	18S	0.83	18S	16.00
RA SRnd/HF (*n* = 4/4)	HMBS	0.134	PPIA	0.077	PPIA	0.34	HMBS	0.16	**HMBS**	1.41
	PPIA	0.134	HMBS	0.126	HMBS	0.35	GAPDH	0.19	**PPIA**	1.50
	POLR2A	0.211	RPLP0	0.168	POLR2A	0.36	UBC	0.20	**POLR2A**	3.83
	GAPDH	0.230	POLR2A	0.173	RPLP0	0.37	PGK1	0.22	**GAPDH**	3.94
	PGK1	0.240	B2M	0.198	GAPDH	0.37	PPIA	0.22	**RPLP0**	5.73
	IPO8	0.247	GAPDH	0.211	B2M	0.38	POLR2A	0.24	PGK1	6.16
	UBC	0.258	IPO8	0.221	IPO8	0.39	IPO8	0.26	IPO8	6.74
	HPRT1	0.267	HPRT1	0.272	PGK1	0.41	18S	0.27	UBC	6.77
	RPLP0	0.278	PGK1	0.288	HPRT1	0.42	GUSB	0.29	B2M	7.58
	B2M	0.287	UBC	0.291	UBC	0.42	RPLP0	0.29	HPRT1	9.30
	18S	0.298	18S	0.291	18S	0.43	B2M	0.31	18S	10.16
	GUSB	0.316	GUSB	0.342	GUSB	0.46	ACTB	0.33	GUSB	11.17
	ACTB	0.344	ACTB	0.464	ACTB	0.55	HPRT1	0.34	ACTB	12.74
	YWHAZ	0.375	YWHAZ	0.501	YWHAZ	0.59	TBP	0.44	YWHAZ	14.24
	TBP	0.407	TBP	0.546	TBP	0.63	YWHAZ	0.55	TBP	14.74
	TFRC	0.451	TFRC	0.700	TFRC	0.76	TFRC	0.60	TFRC	16.00
RA/LA (*n* = 20/8)	POLR2A	0.216	IPO8	0.185	IPO8	0.42	HMBS	0.21	**IPO8**	2.38
	PPIA	0.216	HPRT1	0.227	GAPDH	0.44	PPIA	0.28	**POLR2A**	2.45
	GAPDH	0.245	POLR2A	0.236	POLR2A	0.44	B2M	0.30	**PPIA**	2.51
	IPO8	0.269	GAPDH	0.239	PPIA	0.44	POLR2A	0.31	**GAPDH**	3.31
	HPRT1	0.288	PPIA	0.242	HPRT1	0.44	GAPDH	0.33	**HPRT1**	4.95
	PGK1	0.310	RPLP0	0.270	GUSB	0.47	GUSB	0.37	HMBS	6.71
	UBC	0.325	GUSB	0.273	PGK1	0.47	IPO8	0.41	GUSB	6.90
	B2M	0.342	PGK1	0.281	RPLP0	0.48	UBC	0.41	B2M	7.00
	GUSB	0.354	UBC	0.320	UBC	0.49	ACTB	0.42	PGK1	7.61
	RPLP0	0.367	B2M	0.331	B2M	0.50	PGK1	0.47	UBC	7.94
	YWHAZ	0.387	YWHAZ	0.410	YWHAZ	0.56	HPRT1	0.50	RPLP0	8.52
	HMBS	0.409	ACTB	0.434	ACTB	0.58	RPLP0	0.50	ACTB	11.39
	ACTB	0.429	HMBS	0.471	HMBS	0.59	TFRC	0.56	YWHAZ	11.68
	TFRC	0.460	18S	0.570	18S	0.68	YWHAZ	0.61	TFRC	14.23
	18S	0.487	TFRC	0.582	TFRC	0.69	18S	0.75	18S	14.49
	TBP	0.534	TBP	0.787	TBP	0.86	TBP	0.83	TBP	16.00
RV/LV (*n* = 8/8)	IPO8	0.233	GUSB	0.150	GUSB	0.47	POLR2A	0.27	**GUSB**	2.06
	PGK1	0.233	YWHAZ	0.262	IPO8	0.50	HMBS	0.40	**IPO8**	2.71
	HMBS	0.266	IPO8	0.267	PGK1	0.51	GUSB	0.45	**PGK1**	2.91
	GAPDH	0.288	PGK1	0.308	YWHAZ	0.52	18S	0.47	**HMBS**	3.81
	HPRT1	0.311	POLR2A	0.327	HMBS	0.53	GAPDH	0.47	**POLR2A**	3.96
	GUSB	0.347	PPIA	0.334	HPRT1	0.54	B2M	0.53	YWHAZ	5.26
	POLR2A	0.368	HMBS	0.339	POLR2A	0.55	IPO8	0.53	GAPDH	5.98
	YWHAZ	0.393	HPRT1	0.351	GAPDH	0.56	PGK1	0.53	HPRT1	7.00

Continued

Table 4 *Continued*

	Genorm		Normfinder		Delta-Ct		BestKeeper		Consensus	
	Genes	Stability value	Genes	Stability value	Genes	SD	Genes	SD	Genes	Geometric mean of ranking values
	18S	0.414	RPLP0	0.371	PPIA	0.56	PPIA	0.53	PPIA	8.30
	UBC	0.432	GAPDH	0.373	RPLP0	0.58	HPRT1	0.54	18S	8.59
	PPIA	0.455	18S	0.461	18S	0.62	UBC	0.56	RPLP0	10.89
	RPLP0	0.470	UBC	0.484	UBC	0.63	YWHAZ	0.56	UBC	11.22
	TFRC	0.499	TFRC	0.603	TFRC	0.73	RPLP0	0.65	B2M	11.77
	B2M	0.534	B2M	0.627	B2M	0.76	ACTB	0.74	TFRC	13.69
	TBP	0.566	TBP	0.665	TBP	0.78	TBP	0.76	TBP	15.00
	ACTB	0.611	ACTB	0.838	ACTB	0.92	TFRC	0.83	ACTB	15.47
A/V (*n* = 28/16)	HPRT1	0.302	POLR2A	0.264	HPRT1	0.52	POLR2A	0.31	**POLR2A**	1.68
	PGK1	0.302	HPRT1	0.270	POLR2A	0.52	HMBS	0.35	**HPRT1**	2.11
	GAPDH	0.309	PGK1	0.289	GAPDH	0.53	B2M	0.38	**PGK1**	3.22
	POLR2A	0.351	GAPDH	0.294	PGK1	0.53	PPIA	0.39	**GAPDH**	3.66
	IPO8	0.369	PPIA	0.297	PPIA	0.54	GAPDH	0.40	**PPIA**	5.32
	UBC	0.391	RPLP0	0.307	IPO8	0.55	GUSB	0.43	HMBS	5.80
	HMBS	0.413	IPO8	0.330	RPLP0	0.55	IPO8	0.45	IPO8	6.19
	PPIA	0.428	UBC	0.408	UBC	0.60	UBC	0.48	UBC	7.44
	RPLP0	0.447	HMBS	0.431	HMBS	0.61	PGK1	0.50	B2M	7.95
	GUSB	0.471	GUSB	0.454	GUSB	0.63	HPRT1	0.53	RPLP0	8.21
	B2M	0.488	B2M	0.467	B2M	0.64	ACTB	0.54	GUSB	8.80
	YWHAZ	0.503	YWHAZ	0.473	YWHAZ	0.64	RPLP0	0.55	YWHAZ	12.24
	18S	0.530	18S	0.561	18S	0.71	YWHAZ	0.60	ACTB	13.18
	ACTB	0.558	ACTB	0.610	ACTB	0.75	18S	0.67	18S	13.24
	TFRC	0.592	TFRC	0.731	TFRC	0.84	TFRC	0.77	TFRC	15.00
	TBP	0.625	TBP	0.744	TBP	0.85	TBP	0.81	TBP	16.00
RV CT/HF (*n* = 4/4)	GAPDH	0.185	GUSB	0.176	GAPDH	0.47	POLR2A	0.28	**GAPDH**	1.97
	HPRT1	0.185	PPIA	0.195	GUSB	0.49	HPRT1	0.38	**POLR2A**	3.13
	HMBS	0.203	GAPDH	0.211	PPIA	0.50	UBC	0.38	**GUSB**	3.22
	PGK1	0.226	POLR2A	0.252	POLR2A	0.51	HMBS	0.39	**HPRT1**	3.25
	IPO8	0.239	RPLP0	0.274	PGK1	0.52	GAPDH	0.42	**PPIA**	4.53
	POLR2A	0.270	IPO8	0.312	IPO8	0.52	GUSB	0.50	HMBS	5.73
	UBC	0.304	PGK1	0.329	HPRT1	0.52	PPIA	0.53	PGK1	6.12
	18S	0.329	HPRT1	0.347	RPLP0	0.53	18S	0.54	IPO8	6.34
	GUSB	0.362	YWHAZ	0.360	HMBS	0.54	IPO8	0.55	UBC	7.10
	PPIA	0.389	HMBS	0.367	YWHAZ	0.58	PGK1	0.58	RPLP0	8.52
	RPLP0	0.411	UBC	0.471	UBC	0.62	B2M	0.60	18S	9.80
	YWHAZ	0.434	18S	0.555	18S	0.67	RPLP0	0.62	YWHAZ	10.89
	TFRC	0.475	TFRC	0.679	TFRC	0.80	YWHAZ	0.73	TFRC	13.24
	TBP	0.525	TBP	0.715	TBP	0.82	TFRC	0.82	B2M	13.88
	B2M	0.573	B2M	0.771	B2M	0.88	ACTB	0.85	TBP	14.48
	ACTB	0.623	ACTB	0.889	ACTB	0.97	TBP	0.93	ACTB	15.74
LV CT/HF (*n* = 4/4)	GUSB	0.169	YWHAZ	0.089	YWHAZ	0.45	POLR2A	0.19	**YWHAZ**	1.19
	YWHAZ	0.169	GUSB	0.129	GUSB	0.45	HMBS	0.38	**GUSB**	2.00
	IPO8	0.223	IPO8	0.206	IPO8	0.46	YWHAZ	0.38	**IPO8**	3.71
	PGK1	0.257	PGK1	0.307	PGK1	0.51	GUSB	0.39	**PGK1**	4.23
	HMBS	0.289	HMBS	0.334	HMBS	0.53	18S	0.41	**HMBS**	4.40
	18S	0.323	18S	0.361	18S	0.55	IPO8	0.41	POLR2A	4.74
	POLR2A	0.349	HPRT1	0.379	HPRT1	0.56	PGK1	0.41	18S	6.00
	B2M	0.377	POLR2A	0.408	B2M	0.58	B2M	0.47	B2M	8.24
	GAPDH	0.405	B2M	0.411	POLR2A	0.58	GAPDH	0.47	HPRT1	8.76
	HPRT1	0.424	PPIA	0.441	GAPDH	0.62	PPIA	0.52	GAPDH	9.93
	UBC	0.448	RPLP0	0.469	PPIA	0.62	TBP	0.60	PPIA	10.94
	RPLP0	0.479	GAPDH	0.484	RPLP0	0.63	HPRT1	0.62	RPLP0	12.42
	PPIA	0.501	UBC	0.517	UBC	0.66	ACTB	0.66	UBC	12.70

Continued

Table 4 *Continued*

Genorm		Normfinder		Delta-Ct		BestKeeper		Consensus	
Genes	Stability value	Genes	Stability value	Genes	SD	Genes	SD	Genes	Geometric mean of ranking values
TFRC	0.522	TFRC	0.545	TFRC	0.69	UBC	0.67	TBP	13.88
TBP	0.550	TBP	0.628	TBP	0.74	RPLP0	0.69	TFRC	14.48
ACTB	0.597	ACTB	0.844	ACTB	0.93	TFRC	0.79	ACTB	15.19

A, atrium; V, ventricle; RA, right atrium; LA, left atrium; RV, right ventricle and LV, left ventricle. SR, sinus rhythm, SRnd, sinus rhythm without atrium dilatation; SRd, sinus rhythm with atrium dilatation; AF, atrial fibrillation; pAF, paroxysmal atrial fibrillation; cAF, permanent atrial fibrillation; HF, heart failure; CT, healthy controls.

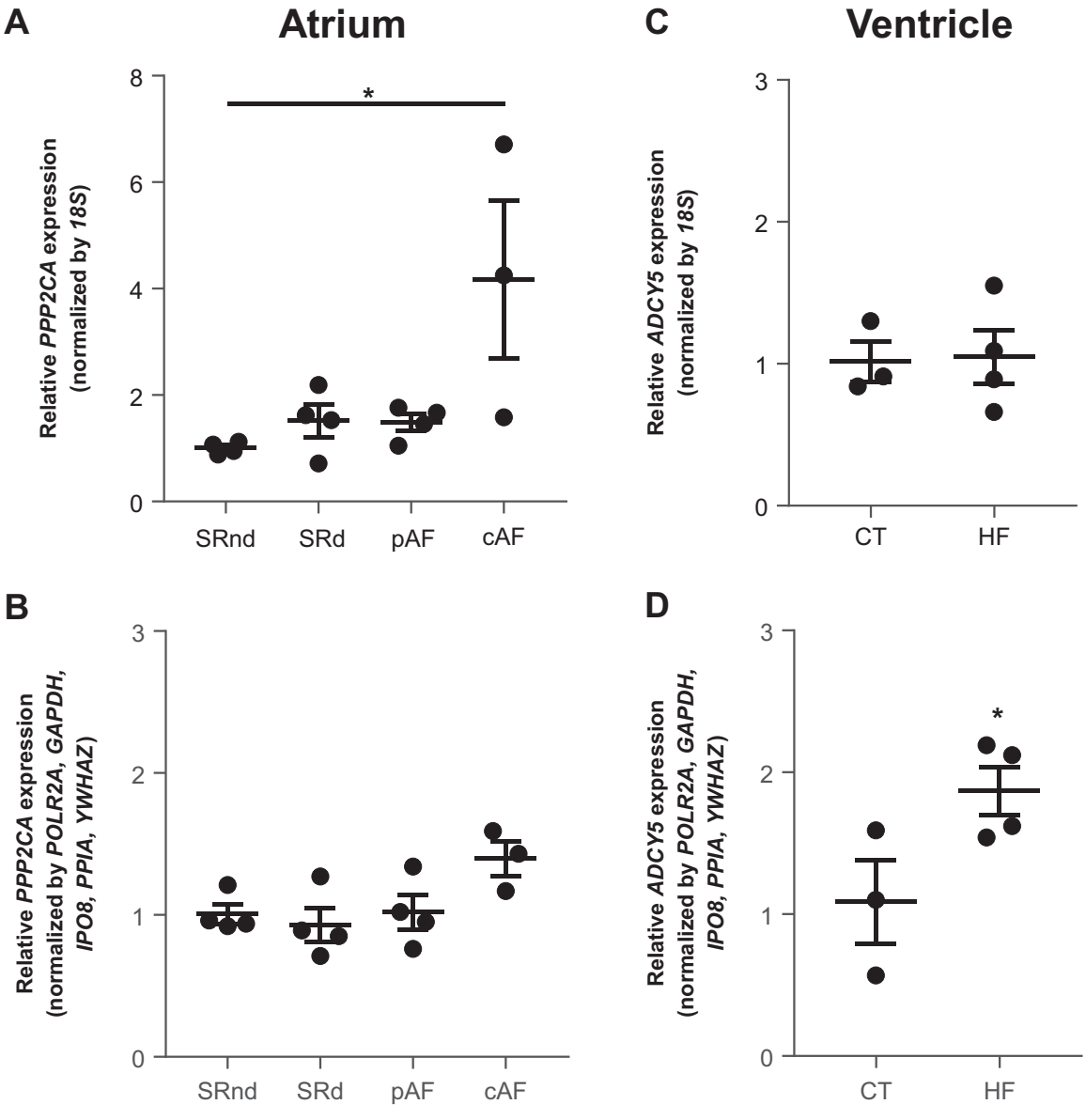

Figure 3 Relative quantification of *PPP2CA* and *ADCY5* expression in normal and diseased right atrial and ventricle samples, respectively. Plot of the mean ± SEM gene expression ratio of *PPP2CA* and *ADCY5* normalized to *18S* (*A, C*), or to the set of 5 reference genes (*GAPDH*, *IPO8*, *POLR2A*, *PPIA*, and *YWHAZ*) (*B, D*) to illustrate the variability in *PPP2CA* and *ADCY5* expression in different heart diseases depending on the selection of the reference gene. SRnd, atrial samples from patients in sinus rhythm without atria dilatation; SRd, atrial samples from patients in sinus rhythm with atria dilatation; pAF, atrial samples from patients with paroxysmal atrial fibrillation; cAF, atrial samples from patients with chronic atrial fibrillation; CT, right ventricle samples from healthy controls; HF, right ventricle samples from patients with heart failure. *$P \leq 0.05$ for comparisons as indicated.

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Corrigendum

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The publisher wishes to inform readers that the last name of Alessandra Giannella was spelled incorrectly when she was cited in the published paper. The online paper has now been corrected.