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BRIEF COMMUNICATION

Housekeeping Proteins Exhibit a High Level of Expression Variability Within Control Group and Between Ischemic Human Heart Biopsies

Anne-Sophie Colombe , PhD*; Pascale Gerbaud, PhD*; Jean-Pierre Benitah , PhD; Guillaume Pidoux , PhD

BACKGROUND: Human cardiac biopsies are widely used in clinical and fundamental research to decipher molecular events that characterize cardiac physiological and pathophysiological states. One of the main approaches relies on the analysis of semiquantitative immunoblots that reveals alterations in protein expression levels occurring in diseased hearts. To maintain semiquantitative results, expression level of target proteins must be standardized. The expression of HKP (housekeeping proteins) is commonly used to this purpose.

METHODS AND RESULTS: We evaluated the stability of HKP expression (actin, β -tubulin, GAPDH, vinculin, and calsequestrin) and total protein staining within control (coefficient of variation) and comparatively with ischemic human heart biopsies (*P* value). All HKP exhibited a high level of intragroup (ie, actin, β -tubulin, and GAPDH) and/or intergroup variability (ie, GAPDH, vinculin, and calsequestrin). Among all, we found total protein staining to exhibit the highest degree of stability within and between groups, which makes this reference the best to study protein expression level in human biopsies from ischemic hearts and age-matched controls. In addition, we illustrated that using an inappropriate reference protein marker misleads interpretation on SERCA2 (sarco/endoplasmic reticulum Ca^{2+} ATPase) and cMyBPC (cardiac myosin binding protein-C) expression level after myocardial infarction.

CONCLUSIONS: These reemphasize the need to standardize the level of protein expression with total protein staining in comparative immunoblot studies on human samples from control and diseased hearts.

Key Words: control groups ■ humans biopsies ■ immunoblot ■ myocardial infarction ■ staining and labeling ■ standardization

The heart is an adaptative and autorhythmic organ, which by contracting in a synchronous and homogeneous manner ensures the supply of an oxygen- and nutrient-rich blood flow to peripheral organs. Cardiac injuries and stresses impair heart function leading to cardiac diseases with severe consequences. Ischemic heart diseases are the major cause of death in industrialized countries. A prolonged cardiac ischemia characterizes myocardial infarction (MI), which occurs after blood clot, coronary artery constriction, or atherosclerosis and leads to irreversible cardiomyocyte death. MI affects cardiac function

and promotes the onset of heart failure. Cardiovascular studies aim at uncovering molecular events regulating cardiac function, which are impaired in pathologies. Often, these investigations are performed on cellular or animal models that attempt to mimic human physiological and pathophysiological conditions. However, observations achieved on these models must be challenged on human samples from healthy and of pathological origin. Unfortunately, such investigations often fail because of disparities between in vitro models, animal pathophysiology and human heterogeneity.¹ In addition, biases in experimental design and analyses

Correspondence to: Guillaume Pidoux, PhD, Inserm UMR-S1180, Faculté de Pharmacie, Université Paris-Saclay, 5 Rue JB Clément, 92296 Châtenay-Malabry, France. Email: guillaume.pidoux@inserm.fr

*A.-S. Colombe and P. Gerbaud contributed equally.

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should be considered. Immunoblots are commonly used to quantify variations of TP expression level between physiological and pathological conditions. TP signal needs to be standardized by the expression level of an internal reference, which will assure the semiquantitative comparison between studied groups. The expression levels of HKP (housekeeping proteins) or a loading control are used as internal references in immunoblot assays. An internal reference should be expressed equally within all samples and between all studied groups. Interestingly, it has been reported, in many cells and tissues, including the heart, that the expression of HKP transcripts fluctuate under experimental treatments and pathological conditions.² Therefore, selecting the suitable HKP can become arduous and an inappropriate choice will lead to misleading results in TP expression levels in healthy and diseased human hearts.

In this study, we investigated the variability of HKP expression level (ie, actin, GAPDH, β -tubulin, vinculin, and calsequestrin), and fluorescent TPS within human left ventricles biopsies from control and ischemic hearts but also between both groups. In addition, we also examined consequences of the normalization by these HKPs and TPS on sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2) and cardiac myosin binding protein-C (cMyBPC) expression level in physiological condition and after MI. Finally, we provided strong evidence on TPS to be the best internal reference in comparative immunoblots studies that include human samples from control and diseased hearts.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Human Samples

Human left ventricular heart tissues were obtained from the cardiovascular biobank of Bichat Hospital (BB-0033-00029), with approval by the Inserm Institutional Review Board and after informed patient written consent. Cardiac biopsies were collected from age-matched healthy male patients without cardiovascular diseases (eg, controls; $n=9$; 63 ± 6 years old) and from male patients with MI ($n=7$; 57 ± 7 years old) that went for cardiac transplantation surgery.

Protein Sample Preparation and Immunoblotting

Cardiac biopsy extracts were prepared as previously described.³ For immunoblots, signals from TP and TPS have been detected within the same linear

dynamic range to avoid membrane and signal saturation. Protein concentration was estimated using Bradford assay. Protein samples from control and MI biopsy extracts (20 μg) were resolved by SDS-PAGE. Total proteins were stained with Revert 700 (Li-Cor) and imaged with iBright FL1000 (ThermoScientific). Next, membranes were immunoblotted with antibodies to actin (0.8 $\mu\text{g}/\text{mL}$, A2066, Merck-Millipore), β -tubulin (0.5 $\mu\text{g}/\text{mL}$, 32-2600, ThermoScientific), GAPDH (1 $\mu\text{g}/\text{mL}$, G8795, Merck-Millipore), vinculin (1 $\mu\text{g}/\text{mL}$, V9131, Sigma), calsequestrin (0.8 $\mu\text{g}/\text{mL}$, PA1-913, ThermoScientific), SERCA2 (1 $\mu\text{g}/\text{mL}$, sc-59724, Santa-Cruz Biotechnology), and cMyBPC (1 $\mu\text{g}/\text{mL}$, sc-50115, Santa-Cruz Biotechnology). After incubation with appropriate HRP-conjugated secondary antibody (ThermoScientific), blots were revealed using Supersignal West Pico+ substrate (ThermoScientific) and recorded with the multi-exposure mode of the iBright FL1000.

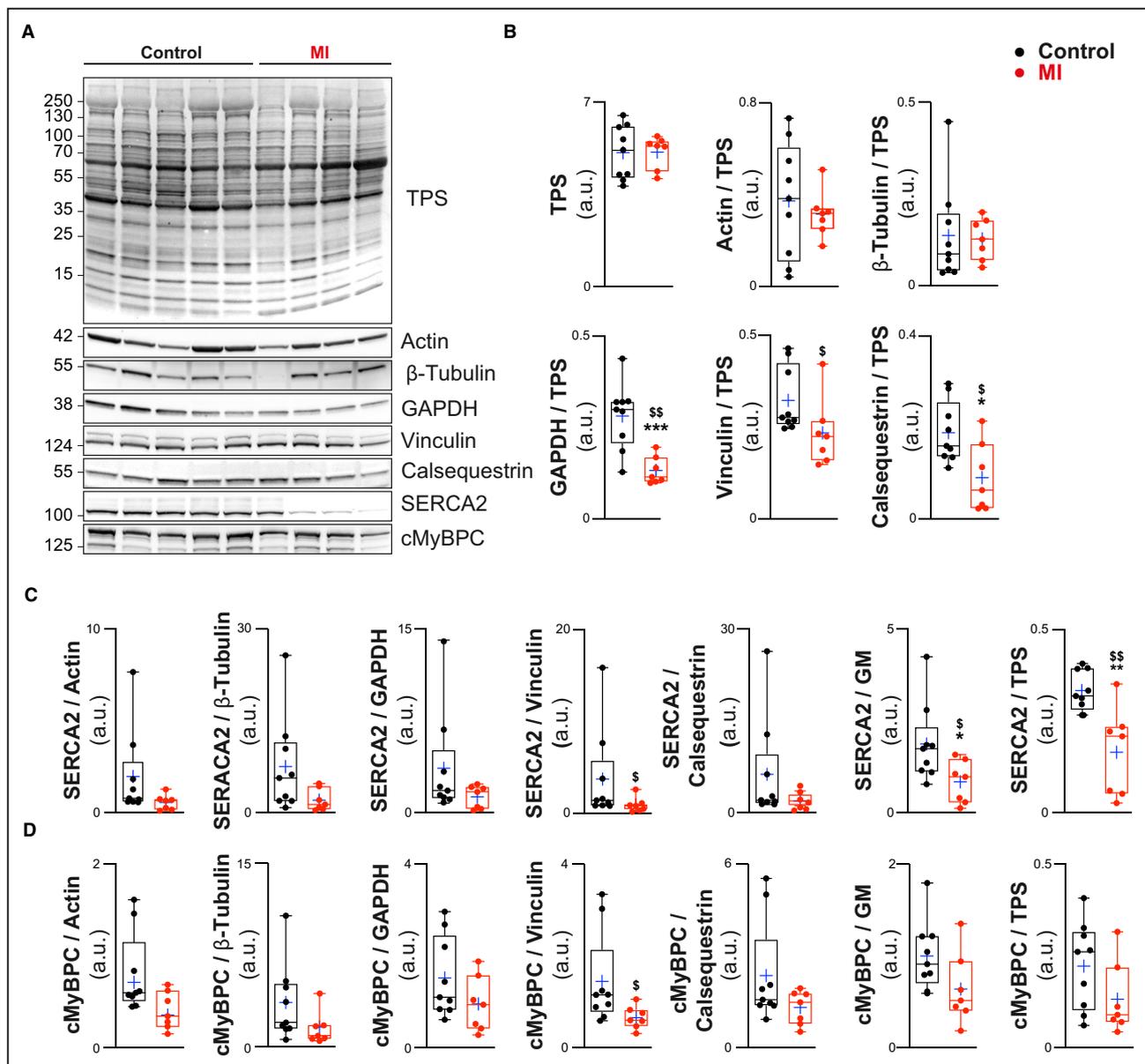
Statistical Analysis

All statistical analyses were performed in GraphPad Prism 8 (GraphPad Software). Grubbs' tests (alpha of 1%) were performed to identify outliers. Gaussian distribution was tested with normality Shapiro-Wilk test. In intergroup comparison studies, nonparametric Mann-Whitney *U*-test and *F*-test were achieved. Thereafter, groups with a normal distribution were subjected to parametric tests (ie, unpaired *t*-test for equal variances and unpaired *t*-test with Welch correction for unequal variances). Statistical significance was assumed with $P<0.05$; 95% CI for the difference between means was reported to provide an estimate of the true difference in the population means. The intragroup variability studies were assessed by calculating the coefficient of variation (VC%), which indicates the data set spread as a proportion of its mean. As previously reported, we set VC% threshold limit to 25%.⁴ Geometric mean (GM) normalization has been performed by using quantification of actin, β -tubulin, and vinculin.

RESULTS

HKP Expression Is Imbalanced Within Human Control Group

Figure 1A showed the expression level of 5 HKP and TPS of male biopsies from MI and age-matched controls. First, the intragroup variability of HKP and TPS were investigated by assessing the VC% within control group (Figure 1A, 1B and 2A). All HKP showed a VC% above the acceptable 25% threshold limit, indicating a high level of expression variability within the human healthy group. In addition, this intragroup inconstancy was also observed within MI biopsies (Figure 1A, 1B



and 2A, VC% >25%). In contrast, TPS turned out to be the most stable with the lowest level of variation within each group (VC% <25% within both groups). Although acceptable, these levels of VC% in TPS signal can be attributed to inherent fluctuations in protein quantification steps (eg, dilution step, linearity error).

Interestingly, TPS also revealed some differences in the expression of specific proteins between samples, which caution on the use of one defined protein as an internal reference. These differences reflect the interindividual variability but also the specificity in biological responses. Nevertheless, TPS appears to be the most

A	TPS	Actin/TPS	β -Tubulin/TPS	GAPDH/TPS	Vinculin/TPS	Calsequestrin/TPS
Median	5.13 / 5.33	0.38 / 0.32	0.08 / 0.13	0.30 / 0.11	0.27 / 0.22	0.16 / 0.06
Range	2.69 / 1.60	0.69 / 0.33	0.41 / 0.15	0.31 / 0.09	0.22 / 0.27	0.18 / 0.19
VC%	20.2 / 12.1	67.7 / 32.4	97.1 / 44.5	32.1 / 28.3	27.5 / 39.5	35.4 / 84.1
Mean	5.09 / 5.11	0.37 / 0.32	0.14 / 0.13	0.28 / 0.13	0.32 / 0.23	0.19 / 0.09
SD	$\pm 1.03 / \pm 0.61$	$\pm 0.25 / \pm 0.10$	$\pm 0.13 / \pm 0.06$	$\pm 0.09 / \pm 0.04$	$\pm 0.09 / \pm 0.09$	$\pm 0.07 / \pm 0.08$
N. distrib. (p <):	Y _(0.24^{n.s.}) /Y _(0.06^{n.s.})	Y _(0.6^{n.s.}) /Y _(0.5^{n.s.})	N _(0.011[*]) /Y _(0.7^{n.s.})	Y _(0.6^{n.s.}) /Y _(0.2^{n.s.})	N _(0.01[*]) /Y _(0.1^{n.s.})	Y _(0.9^{n.s.}) /Y _(0.9^{n.s.})
F-test (p <):	0.23 ^{n.s.}	0.040 [*]	0.053 ^{n.s.}	0.046 [*]	0.88 ^{n.s.}	0.72 ^{n.s.}
U-test (p <):	0.99 ^{n.s.}	0.68 ^{n.s.}	0.60 ^{n.s.}	0.0021 ^{ss}	0.02 ^s	0.04 ^s
t-test (p <):	0.96 ^{n.s.}	-	-	-	-	0.015 [*]
Welch's t-test (p <):	-	0.56 ^{n.s.}	-	0.0009 ^{***}	-	-

B	SERCA2/Actin	SERCA2/ β -Tubulin	SERCA2/GAPDH	SERCA2/Vinculin	SERCA2/Calsequestrin	SERCA2/GM	SERCA2/TPS
Median	0.77 / 0.64	5.59 / 1.25	1.80 / 1.68	1.44 / 0.83	1.99 / 1.64	1.73 / 0.96	0.32 / 0.21
Range	7.10 / 1.17	24.9 / 4.39	13.3 / 2.16	15.0 / 2.43	25.0 / 4.03	3.47 / 1.46	0.14 / 0.32
VC%	120 / 76.2	106 / 91.1	119 / 73.5	133 / 88.9	134 / 80.8	57.3 / 69.9	16.7 / 73.2
Mean	1.96 / 0.54	7.46 / 1.97	3.63 / 1.28	3.80 / 0.92	6.24 / 1.87	1.86 / 0.83	0.33 / 0.17
SD	$\pm 2.36 / \pm 0.41$	$\pm 7.91 / \pm 1.79$	$\pm 4.33 / \pm 0.94$	$\pm 5.05 / \pm 0.82$	$\pm 8.38 / \pm 1.51$	$\pm 1.06 / \pm 0.58$	$\pm 0.06 / \pm 0.12$
N. distrib. (p <):	N _(0.0007^{***}) /Y _(0.4^{n.s.})	N _(0.02[*]) /Y _(0.8^{n.s.})	N _(0.001[*]) /Y _(0.1^{n.s.})	N _(0.0006^{***}) /Y _(0.06^{n.s.})	N _(0.0006^{***}) /Y _(0.4^{n.s.})	Y _(0.07^{n.s.}) /Y _(0.4^{n.s.})	Y _(0.2^{n.s.}) /Y _(0.3^{n.s.})
F-test (p <):	0.004 ^{***}	0.002 ^{**}	0.0015 ^{**}	0.0003 ^{***}	0.0005 ^{***}	0.15 ^{n.s.}	0.047 [*]
U-test (p <):	0.090 ^{n.s.}	0.055 ^{n.s.}	0.25 ^{n.s.}	0.04 ^s	0.21 ^{n.s.}	0.02 ^s	0.005 ^{ss}
t-test (p <):	-	-	-	-	-	0.04 [*]	-
Welch's t-test (p <):	-	-	-	-	-	-	0.009 [*]

C	MyBPC/Actin	MyBPC/ β -Tubulin	MyBPC/GAPDH	MyBPC/Vinculin	MyBPC/Calsequestrin	MyBPC/GM	MyBPC/TPS
Median	0.59 / 0.34	1.99 / 0.92	1.09 / 0.93	1.14 / 0.59	1.55 / 1.47	0.90 / 0.51	0.26 / 0.09
Range	1.16 / 0.53	10.1 / 3.87	3.35 / 1.60	2.76 / 0.74	4.60 / 1.43	1.20 / 1.16	0.35 / 0.27
VC%	55.1 / 51.5	88.6 / 88.7	57.7 / 64.2	70.5 / 37.7	72.0 / 42.9	37.8 / 61.4	57.3 / 75.1
Mean	0.79 / 0.39	3.55 / 1.52	1.51 / 0.94	1.44 / 0.65	2.35 / 1.30	0.99 / 0.63	0.22 / 0.13
SD	$\pm 0.43 / \pm 0.20$	$\pm 3.15 / \pm 1.35$	$\pm 0.87 / \pm 0.60$	$\pm 1.01 / \pm 0.24$	$\pm 1.69 / \pm 0.56$	$\pm 0.37 / \pm 0.39$	$\pm 0.12 / \pm 0.10$
N. distrib. (p <):	N _(0.0006^{***}) /Y _(0.6^{n.s.})	N _(0.03[*]) /N _(0.01[*])	Y _(0.1^{n.s.}) /Y _(0.5^{n.s.})	N _(0.0005^{***}) /Y _(0.98^{n.s.})	N _(0.0003^{***}) /Y _(0.37^{n.s.})	Y _(0.26^{n.s.}) /Y _(0.46^{n.s.})	Y _(0.67^{n.s.}) /Y _(0.08^{n.s.})
F-test (p <):	0.083 ^{n.s.}	0.053 ^{n.s.}	0.38 ^{n.s.}	0.003 ^{**}	0.015 [*]	0.89 ^{n.s.}	0.66 ^{n.s.}
U-test (p <):	0.071 ^{n.s.}	0.11 ^{n.s.}	0.21 ^{n.s.}	0.016 ^s	0.30 ^{n.s.}	0.09 ^{n.s.}	0.14 ^{n.s.}
t-test (p <):	-	-	0.16 ^{n.s.}	-	-	0.08 ^{n.s.}	0.13 ^{n.s.}
Welch's t-test (p <):	-	-	-	-	-	-	-

Figure 2. Descriptive statistics.

A through C, The descriptive statistics of Figure 1B through 1D are compiled, respectively. GM indicates geometric mean; N. distrib., normal distribution (with Y for yes and N for no); TPS, total protein staining; and VC%, coefficient of variation. *P* values are indicated as **P*<0.05, ***P*<0.01, ****P*<0.001 n.s. for non-significant for parametric tests, and ^s*P*<0.05, ^{ss}*P*<0.01 for nonparametric tests.

stable internal reference for studying TP expression in healthy and diseased cardiac biopsies. Of note, VC% of HKP without TPS standardization showed similar intragroup variabilities.

Inconstancy of HKP Expression Between Control and Ischemic Heart Biopsies

HKP intergroup stability was assessed by significant differences (*P* value) between healthy and MI biopsies (Figure 1A, 1B and 2A). Unlike other HKPs and TPS, β -tubulin and vinculin expressions within control group did not follow a normal distribution. Surprisingly, variances of actin, GAPDH, and in a lesser extent β -tubulin, differed between healthy and MI group, indicating for these HKP that control and MI groups were sampled from distinct populations. In addition, mean of GAPDH and calsequestrin expression were significantly reduced after MI with a 95% CI for the difference between the means of [0.0707–0.2275] and [0.0220–0.1749], respectively. A similar significant diminution

in the mean-rank distribution was also observed for GAPDH, vinculin, and calsequestrin after MI (Figure 1A, 1B and 2A). In contrast, TPS showed the highest level of intergroup stability (Figure 1A, 1B and 2A). Their large intra- and intergroup variabilities emphasize to caution the use of these HKP as internal references in comparative studies of TP expression between cardiac healthy and pathological conditions.

HKP Normalization Misleads SERCA2 and cMyBPC Expression Between Healthy and MI Heart Biopsies

We next investigated the influence of HKP normalization on SERCA2 and cMyBPC expression level that are well characterized under cardiac healthy and pathophysiological conditions (Figure 1A, 1C, 1D and Figure 2B, 2C). First, we revealed that SERCA2/HKP and cMyBPC/HKP failed to follow a normal distribution in healthy group (excepted for cMyBPC/GAPDH; Figure 2B and 2C), unlike standardization with the GM

and TPS. No difference in SERCA2 expression was observed after actin, β -tubulin, GAPDH and calsequestrin normalization, while significant diminutions of the mean-rank distribution in MI compared with control group were observed after vinculin, GM and TPS normalization (Figures 1C, 1D, 2B, and 2C). In addition, a significant reduction in the mean value of SERCA2 was found in MI after GM and TPS standardization with a 95% CI for the difference between means of [0.0132–1.4568] and [0.0702–0.2636], respectively. With respect to cMyBPC expression, only a significant diminution in the mean-rank distribution was found after vinculin normalization in MI compared with control. Therefore, using an inappropriate internal reference to normalize TP expression in healthy and diseased cardiac tissues may lead to misleading interpretations.

DISCUSSION

In immunoblot analysis, an internal reference is necessary to quantify TP expression level. An internal reference protein must codistribute with TP and be expressed consistently within and between all samples from each studied group. Here, we emphasized the need to select an appropriate stable internal reference, when studying and comparing the level of protein expression from control and pathophysiological human heart samples. Furthermore, our study also highlights intragroup and intergroup variabilities in the protein expression level of several HKP that may bias later semiquantitative analyses.

Our intragroup comparison revealed TPS to remain remarkably stable among samples within control and ischemic human heart biopsies but also between both groups, whereas HKP exhibited a higher degree of intragroup variability within the same samples. Therefore, we should de facto exclude these HKP as potential internal references in immunoblot experiments, which could otherwise lead to misinterpretations on TP expressions. We believe these inconstancies to reflect principally the individual variability from genetic component, but we cannot exclude dependence upon the location of the biopsies or other factors such as sex, age, and medications. Inclusion of more biopsies may attenuate this variability. However, this can quickly become limiting with healthy human heart samples for obvious reasons and without any guarantee of success especially with groups of high VC%. In addition, our intergroup analyses revealed GAPDH, vinculin, and calsequestrin to be downregulated in human hearts after MI indicating that these are inappropriate internal reference proteins. Considering these high levels of HKP variability, we recommend designing preliminary studies to identify appropriate reference protein markers before to compare control with any diseased human hearts biopsies. Interestingly, variability of housekeeping genes has been reported in comparative transcriptomic studies between normal and diseased heart biopsies.² In addition,

HKP inconstancy has been evoked in various cellular model and organs including the heart.⁵ Actin, β -tubulin, and GAPDH exhibit inconstancy at the mRNA and protein levels in heart after MI.^{5,6} Expression of calsequestrin has been reported to remain stable across healthy and ischemic hearts.⁷ This discrepancy with our observation relies on inappropriate immunoblots standardization performed previously. This includes absence of normalization or the use of inconstant HKP (eg, GAPDH).⁷ However, a decrease in calsequestrin expression fits well with the sarcoplasmic reticulum network disorganization reported by others in heart failure. This includes a decrease in sarcoplasmic reticulum surface area and volume density leading to impaired calcium release and contractile strength dysfunction.⁸ Furthermore, our data are consistent with the decrease in calsequestrin-associated proteins (ie, RyR2, triadin, junctin) observed in MI and heart failure.^{9,10} Lastly, the vinculin diminution in MI supports its disorganization observed by others in cardiac ischemic samples.¹¹

Among all, we revealed TPS to be the best to compare human biopsies from ischemic hearts and age-matched control, which displayed the highest intra- and intergroup stability. TPS offers additional benefits for immunoblot studies including the guarantee of samples integrity, transfer efficacy, and loading homogeneity. Interestingly, TPS revealed loading disparities, which reflect fluctuations in protein assay measurements. These may arise from the differences in amino acids composition between samples, the dilution steps, and the linearity errors induced by standard curve extrapolation. Therefore, we do not recommend normalizing TP expression with the estimated concentration of protein loaded on gels. TPS has emerged as a recommended standard method for immunoblots normalization.^{12,13} Many visible and fluorescent TPS exist. Although exhibiting an affordable cost of use, visible TPS lack of reproducibility, dynamic range, and may affect antibody detections. Therefore, fluorescent TPS provides the perfect alternative to overcome limits of visible staining (eg, digital acquisition, large linear dynamic range) and offers the solution to normalize immunoblot studies. Unlike permanent TPS that can interfere with immunoblotting; we preferred reversible staining solutions, which limit to affect biological or immunological properties of the immobilized proteins. Interestingly, normalization of target proteins with GM offers a possible alternative to TPS. However, compared with the 1-shot revelation of TPS, it requires revealing the expression of ≥ 3 HKP.

In addition, we illustrated herein that using an inappropriate reference protein marker misleads interpretation on SERCA2 and cMyBPC expression after MI. We reported a diminution of SERCA2 expression in human myocardial biopsies when signals were standardized with TPS and GM but not with HKP. The intra- and intergroup variabilities explain these discrepancies.

Our observations are consistent with SERCA2 down-regulation previously reported at the mRNA and protein levels in various models of diseased hearts. This contributes to calcium homeostasis deficiency and contractile dysfunction in failing hearts.¹⁴ Interestingly, we found no changes in cMyBPC expression after MI when standardized with TPS. This supports previous findings, in which total cMyBPC remains constant, while its phosphorylation state diminishes after MI.¹⁵

In conclusion, the characterization at the molecular level of cardiovascular diseases relies on the expression of protein of interest achieved by semiquantitative immunoblots, which require appropriate standardization. Our results clearly indicated that choosing an inappropriate HKP for normalization leads to misleading interpretations. We showed that TPS is the most stable and powerful standard for comparing TP expression levels in control human biopsies and diseased hearts. TPS revealed a high level of HKP inconstancy within control but also between ischemic heart samples. Although we focused on the comparison between human heart samples from healthy and MI biopsies, we cannot rule out that HKP intra- and intergroup variability exist in other cardiac pathologies. Therefore, to overcome these variabilities, we strongly recommend the use of a reversible fluorescent TPS, to normalize the expression of TP in all immunoblot studies.

ARTICLE INFORMATION

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Affiliation

INSERM, UMR-S 1180, Signalisation et Physiopathologie Cardiovasculaire, Université Paris-Saclay, Châtenay-Malabry, France

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Disclosures

None.

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