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# Reassessment of mitochondrial cyclophilin D as a target for improving cardiac arrest outcomes in the era of therapeutic hypothermia

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Uncertainty exists regarding whether cyclophilin D (CypD), a mitochondrial matrix protein that plays a key role in ischemia-reperfusion injury, can be a pharmacological target for improving outcomes after cardiac arrest (CA), especially when therapeutic hypothermia is used. Using CypD knockout mice (CypD<sup>-/-</sup>), we investigated the effects of loss of CypD on short-term and medium-term outcomes after CA. CypD<sup>-/-</sup> mice or their wild-type (WT) littermates underwent either 5 minute CA followed by resuscitation with and/or without hypothermia at 33°C–34°C (targeted temperature reached within minutes after resuscitation), or a sham procedure. Brain and cardiac injury were assessed using echocardiography, neurological scores, MRI and biomarkers. Seven day survival was compared using Kaplan-Meier estimates. The rate of restoration of spontaneous circulation was significantly higher in CypD<sup>-/-</sup> mice (with shorter cardiac massage duration) than in WT mice ( $P < 0.05$ ). Loss of CypD significantly attenuated CA-induced release of troponin and S100 protein, and limited myocardial dysfunction at 150 minutes after CA. Loss of CypD combined with hypothermia led to the best neurological and MRI scores at 24 hours and highest survival rates at 7 days compared to other groups ( $P < 0.05$ ). In animals successfully resuscitated, loss of CypD had no benefits on day 7 survival while hypothermia was highly protective. Pharmacological inhibition of CypD with cyclosporine A combined with hypothermia provided similar day 7 survival than loss of CypD combined with hypothermia. CypD is a viable target to improve success of cardiopulmonary resuscitation but its inhibition is unlikely to improve long-term outcomes, unless therapeutic hypothermia is associated. (Translational Research 2022; ■■■:■■■-■■■)

**Abbreviations:** ADC = Apparent diffusion coefficient; CI = Confidence intervals; CPR = Cardiopulmonary resuscitation; CsA = Cyclosporine A; CypD = Cyclophilin D; DWI = Diffusion-weighted images; EtCO<sub>2</sub> = End-tidal carbon dioxide concentration; FiO<sub>2</sub> = Inspired fraction of oxygen; HR = Hazard ratio; LV = Left ventricle; MRI = Brain magnetic resonance imaging; PTP =

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Permeability transition pore; ROSC = Restoration of spontaneous circulation; SSF = Surface shortening fraction; WT = Wild-type

## AT A GLANCE COMMENTARY

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### Background

Uncertainty exists regarding whether cyclophilin D (CypD), a mitochondrial matrix protein that plays a key role in ischemia-reperfusion injury, can be a pharmacological target for improving outcomes after cardiac arrest, especially when therapeutic hypothermia is used.

### Translational Significance

Using both genetic and pharmacological approach of CypD inhibition in a murine cardiac arrest model, we found (1) that CypD might be a new target for improving the success rate of cardiopulmonary resuscitation and (2) that pharmacological CypD inhibition is unlikely to improve long-term outcomes unless therapeutic hypothermia is associated. Combining CypD inhibition and therapeutic hypothermia may confer very high survival rate.

## INTRODUCTION

Cardiac arrest (CA) remains a leading avoidable cause of death in western countries.<sup>1</sup> Only one-third of patients with resuscitation attempt is admitted alive to hospital.<sup>1</sup> Even in these patients, the prognosis is poor as a majority of them will subsequently die of the post-CA syndrome that encompasses irreversible brain damage, myocardial dysfunction and systemic ischemia-reperfusion response.<sup>2</sup> Despite huge research efforts, since decades, no new drugs have proven effective in this setting.<sup>3,4</sup> Moreover, to date, only therapeutic hypothermia is recommended to limit the severity of the post-CA syndrome.

Cyclophilin D (CypD)-mediated mitochondrial permeability transition pore (PTP) opening has been demonstrated to play a key role in the pathophysiology of the post-CA syndrome.<sup>5–13</sup> Experimentally, both cyclosporine A (CsA), that is, the reference pharmacological CypD inhibitor, and therapeutic hypothermia, limit the severity of the post-CA syndrome and seem to prevent CypD-mediated PTP opening.<sup>5,6,8–12</sup> Interestingly, our group reported, in a rabbit CA model, that combining these therapeutic strategies did not provide

any additive or synergic short-term (2 hours after CA) cytoprotective effect at the clinical, cellular or molecular levels, suggesting a common underlying mechanism of protection involving the PTP.<sup>10,11</sup> Nevertheless, whether combining hypothermia and CypD-mediated PTP opening inhibition would improve longer-term outcome after CA, which is mainly driven by neurological injury, remains undetermined. Moreover, the pathophysiological role of CypD in favoring post-CA organ failure, including in humans, has only been studied using pharmacological inhibitors.<sup>5,6,8–12,14–16</sup> As these agents lack potency and exhibit substantial off-target effects, either cytoprotective or cytotoxic, the specific contribution of CypD in CA-induced ischemia-reperfusion injury remains unclear.<sup>15–21</sup> For example, CsA binds to extra-mitochondrial cyclophilins, as well as to calcineurin, leading to the activation of numerous signaling pathways unrelated to CypD.<sup>17–19</sup> Another issue with pharmacological PTP inhibitors is that, when given upon reperfusion, their mitochondrial effects might be delayed beyond a narrow therapeutic window, especially in poorly perfused tissues. In the brain, it is even possible that they do not reach their targets because of the blood-brain barrier impermeability to many compounds.<sup>22</sup>

To overcome these limitations and to provide unambiguous data on whether CypD could be a potential viable therapeutic target for improving outcomes after CA in the era of therapeutic hypothermia, we investigated the short and medium-term effects of the complete inhibition of CypD-dependent PTP opening using CypD knockout mice (CypD<sup>-/-</sup>) submitted to CA treated with or without therapeutic hypothermia.

## METHODS

All procedures conformed to the guideline from the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and followed the ARRIVAL guidelines.<sup>23</sup> All experiments were approved by the French Ministry for Teaching and Research (MESR; n DR-2017-07).

**Animals.** CypD<sup>-/-</sup> mice (C57BL/6 x SV129 background) were obtained from the Korsmeyer laboratory (Dana Farber Cancer Institute, Boston, MA, USA).<sup>20,24</sup> Both CypD<sup>-/-</sup> male mice (aged 8–12 weeks and weighting 20–30 g) and their wild-type (WT) littermates were obtained in our laboratory.<sup>25</sup> Mice were group-housed in a climate-controlled animal colony with a 12-hour dark-light cycle with free access to food and water.

**Surgical preparation.** Mice were anesthetized by intraperitoneal (IP) injection of 100 mg/kg ketamine, (Imalgene 1000; Boehringer Ingelheim Animal Health, Lyon, France) and 5 mg/kg xylazine (Rompun; Bayer HealthCare, Puteaux, France) as previously described.<sup>26</sup> Rectal temperature was monitored and maintained at 37°C using a heating pad.

After tracheal intubation using a 22-gauge catheter, animals underwent mechanical ventilation with a rodent ventilator (MouseVent, PhysioSuite, Kent Scientific, Torrington, CT, USA). The tidal volume was 6.5  $\mu$ l/g, the respiratory rate 150/min, and the inspired fraction of oxygen (FiO<sub>2</sub>) 0.4. End-tidal carbon dioxide concentration (EtCO<sub>2</sub>) and pulse oximetry were measured continuously and ventilation was adjusted to keep them within the physiological ranges. An electrocardiogram was recorded continuously through subcutaneous needles.

For drug administration, a microcatheter was inserted into the left internal jugular vein and filled with heparinized 0.9% saline solution. A 5 minute stabilization period was observed before experiments.

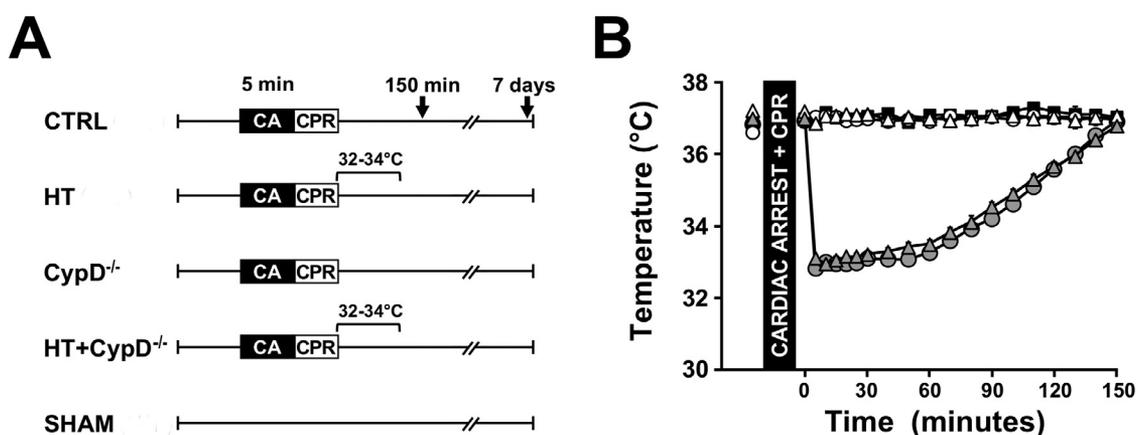
**Cardiac arrest model.** We developed an original murine, asphyxia-induced, non-shockable CA model. Deeply anesthetized mice were paralyzed with an intravenous (IV) injection of 2 mg/kg succinylcholine (Neuraxpharm France, Paris, France). CA was then induced by the withdrawal of mechanical ventilation. CA was determined by echocardiography and defined as biventricular myocardial standstill >10 seconds.

After 5 minutes of untreated CA, cardio-pulmonary resuscitation (CPR) was started with the resumption of

mechanical ventilation (FiO<sub>2</sub>: 100%, respiratory rate: 220/min), chest compressions delivered by finger at a rate of 250–300/min and a single IV bolus of epinephrine (0.1 mg/kg). Restoration of spontaneous circulation (ROSC) was defined as both the return of an organized cardiac rhythm with a heart rate >200/min and an EtCO<sub>2</sub>>15 mmHg, for at least 3 minutes. In the absence of ROSC after 5 minutes, the animal was declared dead. Successful CPR was defined as the restoration of spontaneous breathing allowing weaning from mechanical ventilation; there was no time limit for this weaning. During the first hour of reperfusion, animals received 0.2 ml IV of 2.1% NaHCO<sub>3</sub>.

**Experimental protocol.** Mice were randomly assigned to 5 experimental groups. WT or CypD<sup>-/-</sup> mice underwent 5 minutes CA followed by CPR with or without hypothermia (HT) or a sham procedure (Fig 1, A). Investigators who performed CPR were blinded to the genetic background of the mice. In hypothermic groups, hypothermia was induced at the onset of CPR with a gauze compress moistened with ice water covering the body and maintained at 33°C for 1 hour using a heating pad; animals were then rewarmed to 37°C at a rate of 0.5°C/15 min (Fig 1, B).

Two sets of mice were used in this study: 1 for short-term (150 minutes after CA without awaking from anesthesia) analysis including echocardiography, blood analysis and mitochondrial assays (n = 10–24/group), and another for longer-term (up to 7 days in animals successfully weaned from mechanical ventilation) analysis including neurological assessment at 24 hours and 7 day survival (n = 15–30/group). In the second



**Fig 1.** Experimental protocol Wild-type (WT) or cyclophilin D knockout mice underwent 5 minute cardiac arrest (CA) followed by cardiopulmonary resuscitation (CPR) with or without therapeutic hypothermia; a group of WT mice underwent a sham procedure (panel A). Hypothermia was induced upon CRP and the targeted core temperature of 32°C–34°C maintained for 1 hour before slow rewarming at 37°C (panel B). Analyzes were performed at 150 minutes, 24 hours, and 7 days after CA. CTRL, CA in WT mice (black squares in panel B); HT, hypothermia after CA in WT mice (grey circles); CypD<sup>-/-</sup>, CA in cyclophilin D knockout mice (white triangle); HT+CypD<sup>-/-</sup>, hypothermia after CA in cyclophilin D knockout mice (grey triangle); SHAM, sham-operated WT mice (white circle).

set, animals successfully weaned from mechanical ventilation had their endotracheal tube and catheter removed, their wounds surgically closed, and were placed into a recovery cage for 24 hours before returning to the animal facility. Animals that were unable to drink and/or eat received subcutaneous fluids to prevent dehydration and/or weight loss. CA and CPR data were obtained from both sets.

Because a global genetic modification may have effects on physiology and on disease outcome, an additional subset of mice ( $n = 8$ ) was used to compare the effects of genetic CypD deletion with pharmacological CypD inhibition with a 5 mg/kg IV bolus (given 5 minutes prior CA) of CsA (Sandimmun, Novartis Pharma SAS, Rueil-Malmaison, France) in hypothermia-treated WT mice. This group was added after the other experiments and therefore was not randomized.

### MYOCARDIAL INJURY AND/OR DYSFUNCTION

**Echocardiography.** Left ventricle (LV) surface shortening fraction (SSF) was measured at baseline and 150 minutes after CA by echocardiography using a 13-MHz linear-array transducer (Vivid 7; GE Medical Systems, Milwaukee, WI, USA).

**Biomarkers.** From blood samples collected at 150 minutes after CA, levels of troponin Ic were determined using an ELISA kit (KA4340; Abnova, Taipei City, Taiwan).

### NEUROLOGICAL INJURY AND/OR DYSFUNCTION

**Biomarkers.** From blood samples collected at 150 minutes after ROSC, levels of S100 $\beta$  protein, a validated marker of brain injury, were determined using an ELISA kit (KA0037, Abnova).

**Neurological examination.** For neurological evaluation at 24 hours after CA a validated 12 point score (higher score indicating better neurological function) was used.<sup>27</sup> For animals that died before 24 hours after CA, the score was 0. Assessment was independently performed by an investigator blinded to the group allocation.

**Brain magnetic resonance imaging (MRI).** MRI was acquired at 24 hours after CA. Prior to imaging, mice were slightly anesthetized. T2-weighted images and diffusion-weighted images (DWI) were acquired on a 7T/12 cm magnet (Bruker Biospin, Ettlinger, Germany), as previously described.<sup>28</sup> The field of view was  $20 \times 20$  mm<sup>2</sup> and slice thickness 1.0 mm ( $n = 15$  slices). The apparent diffusion coefficient (ADC) and the T2 parametric maps were reconstructed using the software ParaVision 5.1 (Bruker Biospin). Regions with edema (ie, focal ischemic lesions) were then identified visually

by the presence of DWI hyperintensity and reduced ADC. We developed a simple and easy-to-use 0 to 4 point brain MRI integrity score (higher scores indicating greater brain integrity) based on both T2-weighted and ADC images according to the number and the distribution of ischemic lesions. Briefly, starting at 4 points, 1 point was withdrawn when a single lesion was found and 2 points if  $\geq 2$  lesions were found (an additional point was withdrawn if lesions were bilateral); 1 point was also withdrawn if  $\geq 1$  lesion was visible on  $> 1$  slice. MRI brain images were scored by 2 independent investigators blinded to the group allocation.

### MITOCHONDRIAL ASSAYS

After 150 minutes of reperfusion, the cerebral cortex as well as the heart were harvested and mitochondria were isolated, as previously described.<sup>11</sup> Susceptibility of PTP opening was assessed by the calcium retention capacity (CRC; expressed as moles of CaCl<sub>2</sub> per mg of protein), that is the amount of calcium required to induce permeability transition in isolated mitochondria, with or without in vitro addition of 1  $\mu$ M CsA, as previously described.<sup>11,29</sup>

**Statistical analysis.** Data are expressed as mean  $\pm$  standard error of the mean or number (%). Categorical variables were compared using a 2 sided Chi-square test or Fisher's exact test, as appropriate. Normality of continuous data was assessed using the Kolmogorov-Smirnov test. Continuous data were compared using 1 way ANOVA or by the Kruskal-Wallis test, as appropriate. Time-based measurements within each group were compared using 2 way ANOVA with repeated measures. Time to death was estimated using the Kaplan-Meier method and comparisons made using the Log-rank test. Cox proportional hazards regression was used to assess the independent contribution of hypothermia and CypD deletion and/or inhibition on survival; the results are reported as hazard ratios (HR) with 95% confidence intervals (CI). Statistical analyses were performed using Graphpad Prism 9 software (GraphPad Software, La Jolla, CA, USA). Statistical significance was defined as a value of  $P < 0.05$ .

### RESULTS

Among the 180 mice used in this study, 14 were excluded because of surgical complications or mechanical ventilation issues; results are therefore presented for the remaining 166. A total of 66 mice (SHAM:  $n = 6$ ; CTRL:  $n = 24$ , HT:  $n = 15$ , CypD<sup>-/-</sup>:  $n = 11$ , HT+CypD<sup>-/-</sup>:  $n = 10$ ) were included in the first set of experiments, 92 (SHAM:  $n = 4$ ; CTRL:  $n = 30$ , HT:  $n = 23$ , CypD<sup>-/-</sup>:  $n = 20$ , HT+CypD<sup>-/-</sup>:  $n = 15$ ) in the

second set, and 8 in the additional set using CsA in hypothermia-treated WT mice.

**Cardiopulmonary resuscitation.** The mean duration of asphyxia before CA massage did not differ significantly between groups (Table I). The mean duration of cardiac massage to obtain ROSC was significantly ( $P < 0.05$ ) shorter in the CypD<sup>-/-</sup> and HT+CypD<sup>-/-</sup> groups than in the 2 groups of WT mice (ie, CTRL and HT groups) (Table I). The rate of ROSC was also significantly higher in the 2 groups with CypD<sup>-/-</sup> mice compared to the 2 groups of WT mice, irrespective of the use of hypothermia (Table I).

Among animals used in the second set of experiments, successful weaning from mechanical ventilation was obtained in 11 of 30 (37%) mice in the CTRL group, 13 of 23 (56%) mice in the HT group, 18 of 23 (78%) mice in the CypD<sup>-/-</sup> group, 15 of 15 (100%) mice in the HT+CypD<sup>-/-</sup> group and 7 of 8 (88%) mice in the additional HT+CsA group ( $P < 0.05$  vs the CTRL group for the CypD<sup>-/-</sup>, HT+CypD<sup>-/-</sup> and HT+CsA groups). Time between CA and weaning from mechanical ventilation was significantly shorter in mice treated with hypothermia than in normothermic animals ( $208 \pm 7$  vs  $167 \pm 5$  minutes, respectively;  $P < 0.001$ ).

**Susceptibility to PTP opening.** The mean CRC in mitochondria isolated (150 minutes after CA) from both the heart (Fig 2, A) and brain (Fig 2, B) was significantly lower in normothermic WT animals than in sham animals ( $P < 0.05$ ). The mean CRC (150 minutes after CA) in the brain of hypothermia-treated WT animals was significantly higher than in controls ( $P < 0.05$ ; Fig 2, B); this was significantly higher in both the heart (Fig 2, A) and brain (Fig 2, B) of CypD<sup>-/-</sup> mice compared to sham and WT animals, irrespective of hypothermia ( $P < 0.05$ ). In both brain and heart mitochondria from WT animals, in vitro exposure to CsA significantly increased CRC ( $P < 0.05$ ) to near sham values ( $P = NS$  vs sham animals; Fig 2, C and D). As expected, in both brain and heart mitochondria isolated from CypD<sup>-/-</sup> mice, CRC was not significantly different after in vitro

addition of CsA ( $P = NS$ ) and there was no significant difference ( $P = NS$ ) with sham values.

**Mycocardial injury.** LV systolic function, as assessed by SSF, did not differ significantly between groups at baseline. At 150 minutes after CA, the mean SSF value was significantly lower in controls than in sham animals ( $P < 0.001$ ). The mean SSF was significantly higher in hypothermic and/or CypD<sup>-/-</sup> mice than in controls, without additive effect (Fig 3, A).

The mean level of troponin Ic was significantly ( $P < 0.001$ ) higher in controls than in sham animals (Fig 3, B). It was also higher ( $P < 0.05$ ) in controls than in mice treated with hypothermia and/or in CypD<sup>-/-</sup> mice (Fig 3, B).

**Neurological assessment.** The mean concentration of S100 $\beta$  protein 150 minutes after CA was significantly ( $P < 0.001$ ) higher in controls than in sham mice. It was significantly lower 150 minutes after CA in CypD<sup>-/-</sup> mice, hypothermia-treated CypD<sup>-/-</sup> mice and hypothermia-treated WT mice compared to controls (Fig 4, A).

The groups of CypD<sup>-/-</sup> mice and/or hypothermia-treated WT mice presented, 24 hours after CA, significantly ( $P < 0.05$ ) higher median neurological function score than did controls (Fig 4, B). The group of hypothermia-treated CypD<sup>-/-</sup> mice had the highest median neurological function score ( $P < 0.001$  vs all other CA groups). Brain MRI identified ischemic lesions all control animals vs none in hypothermia-treated CypD<sup>-/-</sup> mice (Fig 4, D). The median brain MRI integrity score at 24 hours after CA was significantly ( $P < 0.05$ ) higher in hypothermia-treated CypD<sup>-/-</sup> mice than in the control animals (Fig 4, D).

## 7 DAY SURVIVAL

There were 2 of 30 (7%) mice that survived 7 days after CA in the control group; 4 of 20 (20%) survived in the CypD<sup>-/-</sup> mice group, 10 of 23 (43%) in the

**Table I.** Cardiopulmonary resuscitation

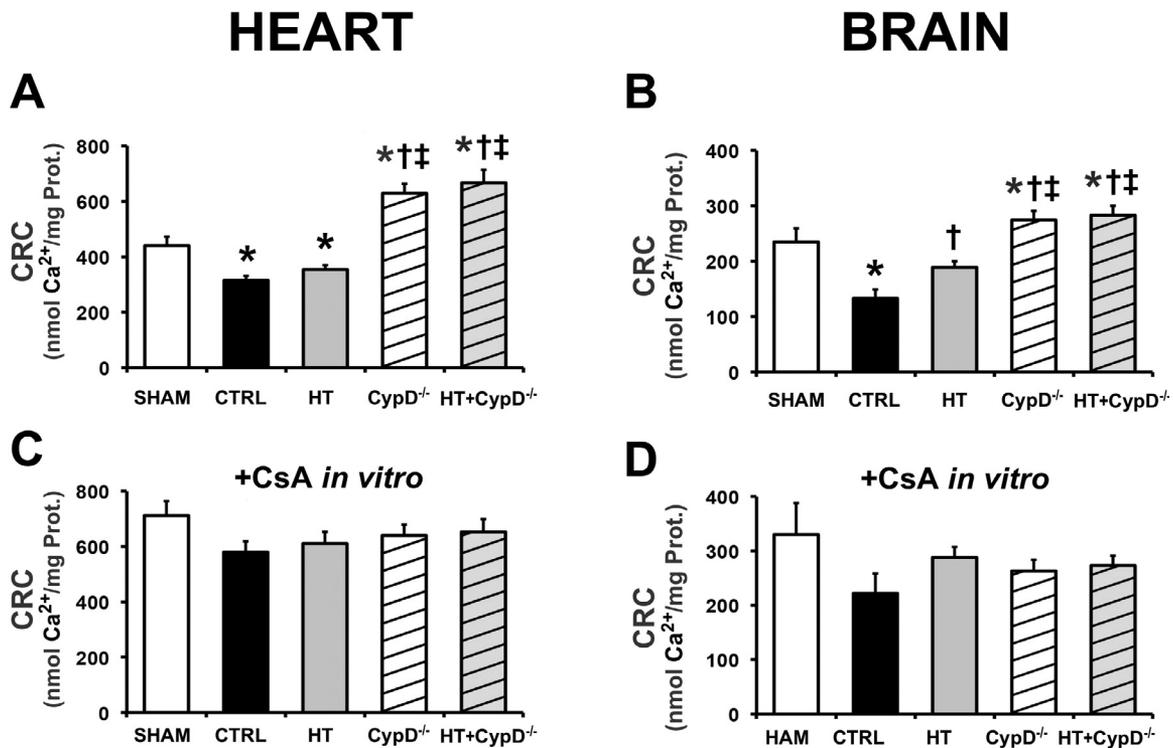
	Duration of asphyxia before cardiac arrest (s)	Duration of heart massage before ROSC (s)	ROSC
CTRL	99 $\pm$ 2	122 $\pm$ 9	39/54 (72)
HT	98 $\pm$ 2	113 $\pm$ 7	0/30/38 (79)
CypD <sup>-/-</sup>	92 $\pm$ 2	78 $\pm$ 4 <sup>*</sup> , <sup>†</sup>	30/31 (97) <sup>*</sup> , <sup>†</sup>
HT+CypD <sup>-/-</sup>	95 $\pm$ 5	82 $\pm$ 4 <sup>*</sup> , <sup>†</sup>	025/25 (100) <sup>*</sup> , <sup>†</sup>

Abbreviations: ROSC, Restoration of spontaneous circulation; CTRL, Controls, cardiac arrest (CA) in wild-type mice; HT, CA in wild-type mice treated with hypothermia; CypD<sup>-/-</sup>, CA in cyclophilin-D knockout mice; HT+CypD<sup>-/-</sup>, CA in cyclophilin-D knockout mice treated with hypothermia.

Data are expressed as mean  $\pm$  SEM or n (%), as appropriate.

<sup>\*</sup> $P < 0.05$  vs CTRL.

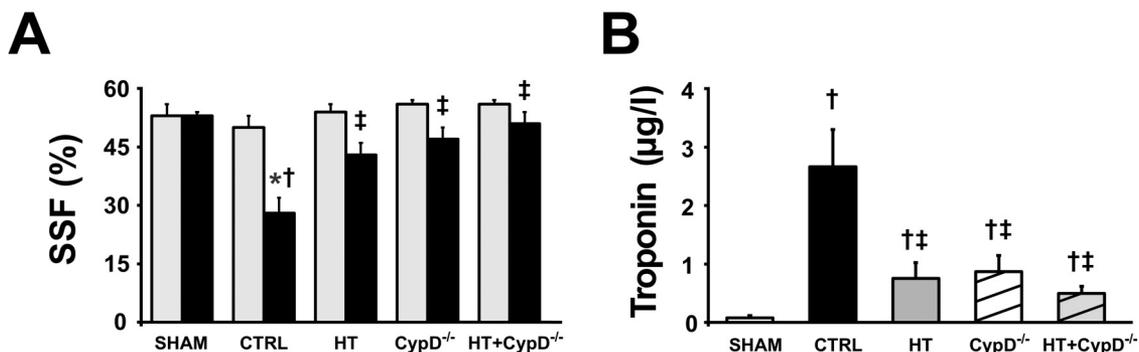
<sup>†</sup> $P < 0.05$  vs HT.



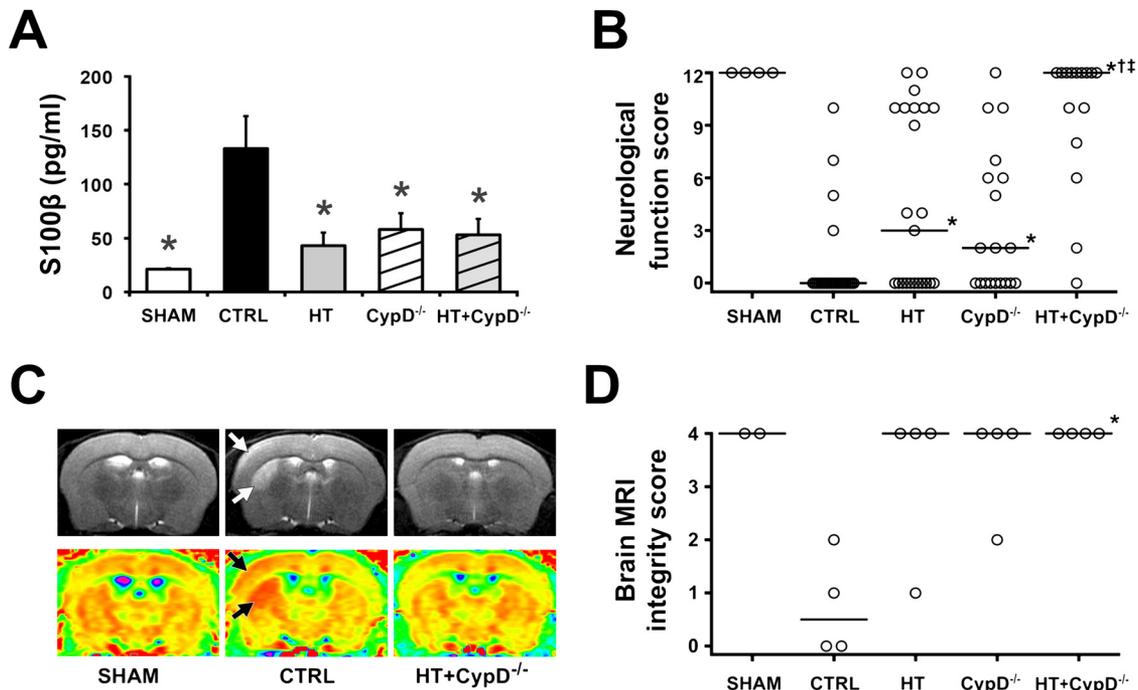
**Fig 2.** Calcium retention capacity Calcium retention capacity (CRC, expressed in nmol Ca<sup>2+</sup>/mg protein) of mitochondria isolated from the heart (panel A) and the brain (panel B) are presented. The effects of *in vitro* addition of cyclosporin A to mitochondria isolated from the heart (panel C) and the brain (panel D) before calcium challenge are also presented. SHAM, sham-operated wild-type animals; CTRL, controls, cardiac arrest (CA) in wild-type mice; HT, hypothermia after CA in wild-type mice; CypD<sup>-/-</sup>, CA in cyclophilin D knockout mice; HT+CypD<sup>-/-</sup>, hypothermia after CA in cyclophilin D knockout mice. Data are expressed as mean  $\pm$  standard error of the mean. \* $P < 0.05$  vs SHAM, †  $P < 0.05$  vs CTRL, ‡  $P < 0.05$  vs HT.

hypothermia-treated WT group, 11 of 15 (73%) in the hypothermia-treated CypD<sup>-/-</sup> mice group (Fig 5). The probability of survival was significantly higher in

hypothermia-treated CypD<sup>-/-</sup> mice compared to hypothermia-treated WT mice ( $P < 0.05$ ) and CypD<sup>-/-</sup> mice ( $P < 0.01$ ; Fig 5). In the additional set of WT



**Fig 3.** Myocardial injury Echocardiographic measurements of the left ventricle surface shortening fraction (SSF) at baseline (grey bars) and 150 minutes after cardiac arrest (CA) are presented in panel A. Blood levels of troponin I<sub>c</sub> (expressed in  $\mu\text{g/l}$ ) measured 150 minutes after CA are presented in panel B. SHAM, sham-operated wild-type animals; CTRL, controls, CA in wild-type mice; HT, hypothermia after CA in wild-type mice; CypD<sup>-/-</sup>, CA in cyclophilin D knockout mice; HT+CypD<sup>-/-</sup>, hypothermia after CA in cyclophilin D knockout mice. Data are expressed as mean  $\pm$  standard error of the mean. \* $P < 0.05$  vs baseline; †  $P < 0.05$  vs SHAM; ‡  $P < 0.05$  vs CTRL.



**Fig 4.** Neurological assessment Levels of S100β protein (expressed in pg/ml) 150 minutes after cardiac arrest (CA) are presented (data expressed mean ± standard error of the mean) for each experimental group in panel A. The neurological function score (higher score indicating better neurological function) was assessed 24 hours after CA (panel B; each circle represents an individual neurological dysfunction score and the thick line the median value of the corresponding group). Panel C shows representatives T2-weighted images (at the top) and ADC images (at the bottom) of brain magnetic resonance imaging (MRI) acquired at 24 hours after CA; arrows indicate areas of ischemia-reperfusion lesions. Brain MRI integrity scores are presented in panel D, each circle represents an individual brain MRI integrity score and the thick line the median value of the corresponding group. For both scores (panels B and D), values of SHAM are presented for information but not included in the statistical analysis. SHAM, sham-operated wild-type animals; CTRL, controls, CA in wild-type mice; HT, hypothermia after CA in wild-type mice; CypD<sup>-/-</sup>, CA in cyclophilin D knockout mice; HT+CypD<sup>-/-</sup>, hypothermia after CA in cyclophilin D knockout mice. \*  $P < 0.05$  vs CTRL; †  $P < 0.05$  vs HT; ††  $P < 0.05$  vs CypD<sup>-/-</sup>.

mice treated with both CsA and hypothermia, 6 of 8 mice (75%) were alive at day 7 after CA (Fig 6). In all groups, all mice alive at day 7 had a normal neurological examination.

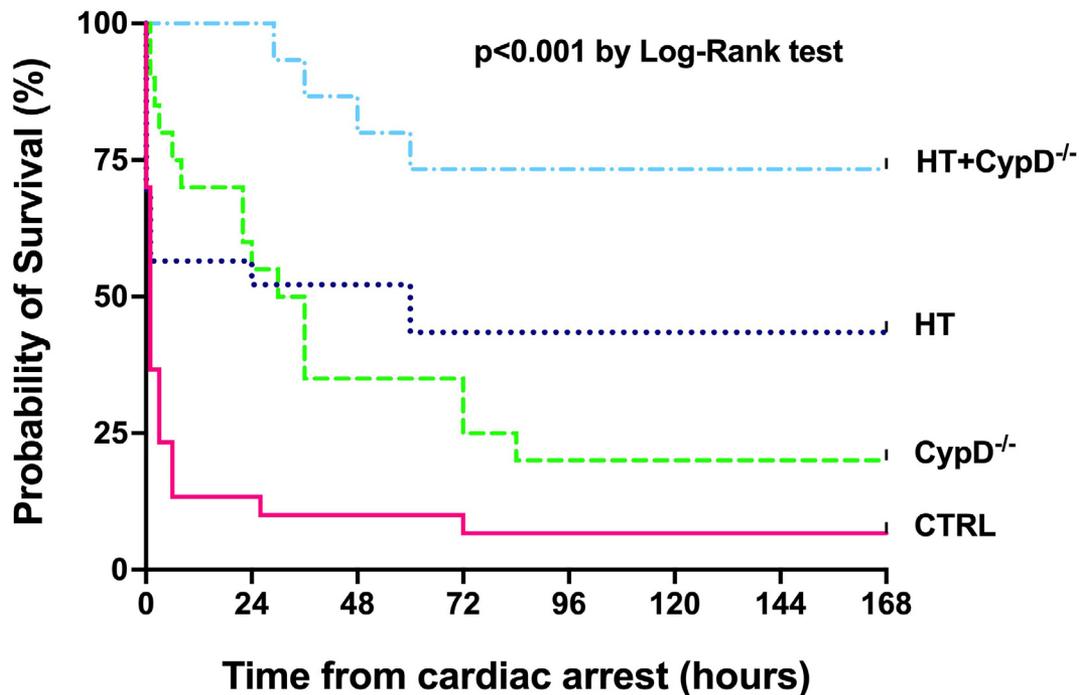
In the Cox regression analysis, both hypothermia (HR 0.45; 95%CI [0.29; 0.69],  $P < 0.01$ ) and deletion and/or inhibition of CypD (HR 0.56; 95%CI [0.37; 0.85],  $P < 0.001$ ) were independently associated with 7 day survival.

To better identify the respective roles of loss of CypD and hypothermia on early and longer-term survival, we analyzed survival only in animals successfully weaned from mechanical ventilation. Seven day survival was significantly ( $P < 0.001$ ) higher in the 2 groups treated with hypothermia (ie, HT, HT+CypD<sup>-/-</sup>) compared to the groups not treated with hypothermia (Fig 7). In the additional set of WT mice treated with both CsA and hypothermia, 6 of 7 (86%) mice that were successfully weaned from mechanical ventilation survived 7 days after CA. There was no

significant difference in 7 day survival among the 3 groups treated with hypothermia ( $P = 0.825$ ) or between the 2 groups not treated with hypothermia ( $P = 0.286$ ) (Fig 6). The cause of death was neurological in all animals that did not survive until day 7 after successful CPR.

## DISCUSSION

In this experimental study, in the absence of therapeutic hypothermia, we found that suppression of CypD-dependent PTP opening increased the rate of ROSC and improved short-term survival but failed to prevent death in animals that survived the first hours after CA. Conversely, therapeutic hypothermia had no significant effect on CPR success but prevented death in animals weaned from the ventilator. Consequently, the combination of suppression of CypD-dependent PTP opening and hypothermia additively and



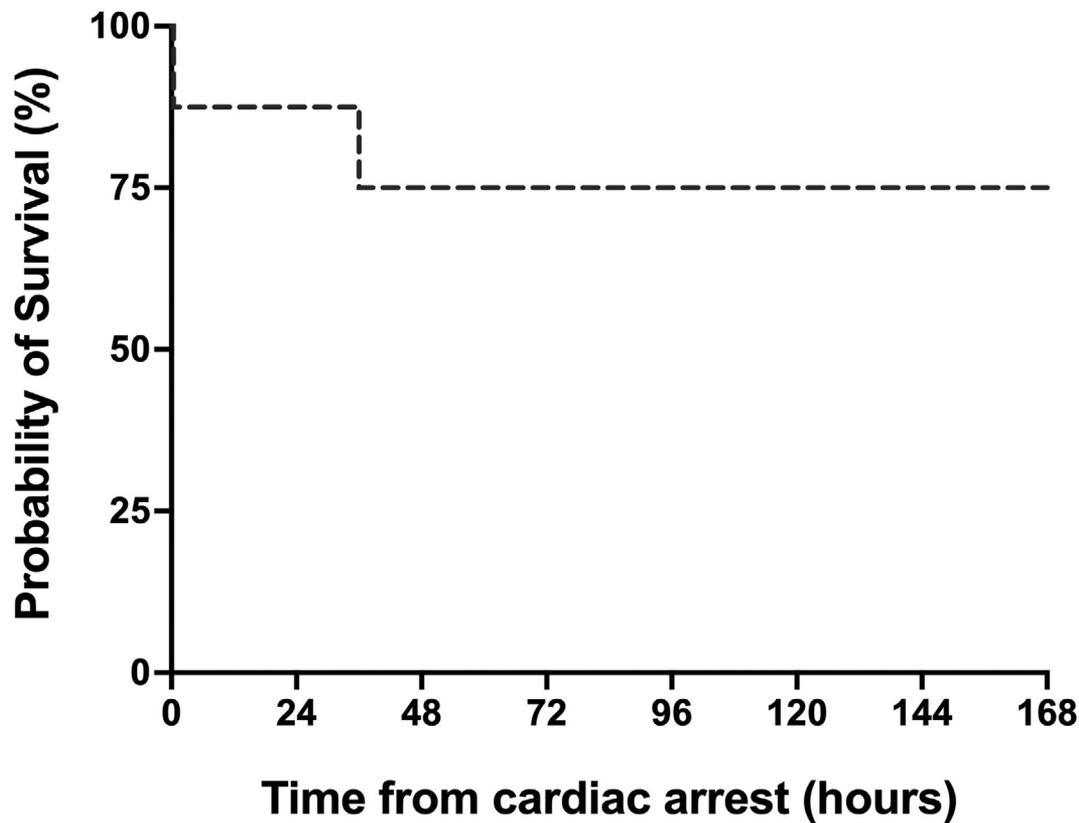
**Fig 5.** Survival after cardiac arrest Kaplan-Meier survival estimates are presented for the 4 experimental groups ( $n = 15-30$ /group) submitted to 5 minutes of cardiac arrest (CA). The 7 day mortality significantly differed among the 4 groups ( $P < 0.001$ , Log-rank test). CTRL, controls, CA in wild-type mice; HT, hypothermia after CA in wild-type mice; CypD<sup>-/-</sup>, CA in cyclophilin D knockout mice; HT+ CypD<sup>-/-</sup>, hypothermia after CA in cyclophilin D knockout mice.

significantly improved 7 day survival after CA in comparison to either hypothermia or loss of CypD alone suggesting partly different mechanisms of protection; this was confirmed using a pharmacological approach.

Although genetic manipulation is an advantage of mouse models, other than the technical difficulties related to surgery and CPR that can be overcome, a limitation of CA models in mice is that it is almost always induced by potassium chloride, which is an uncommon cause of CA.<sup>30</sup> To reproduce a much more clinically relevant scenario, we developed a new model of non-shockable, asphyxia-induced CA in mice. Mortality in controls (around 90%) was close to that observed in non-shockable CA in humans,<sup>14</sup> allowing to assess the effects of interventions on cardiovascular and brain injury using a reasonable number of animals.

As intended, CRC was higher in mitochondria isolated from both heart and brain of CypD<sup>-/-</sup> animals compared to their littermates, indicating a lower susceptibility to PTP opening under stress conditions (eg, calcium overload). In line with previous studies using pharmacological CypD inhibitors, we observed, within the first hours after ROSC, a significant decrease in CA-induced release of troponin and S100 $\beta$  protein and early improvement of cardiovascular dysfunction in

CypD<sup>-/-</sup> mice, confirming the key role of CypD in the pathophysiology of the immediate post-CA syndrome.<sup>10,11</sup> Genetic loss of CypD was also associated with an increase in the rate of CPR success (with shorter duration of cardiac massage). This finding confirms previous observations from our rabbit CA model in which intravenous administration of CsA or its non-immunosuppressive derivative NIM811 at the start of CPR (before epinephrine), significantly increased both the rate of ROSC and 2 hour survival.<sup>8</sup> Thus, genetic or pharmacological approaches to inhibit CypD-dependent PTP opening appear to provide very similar short-term protective effects after CA. To our knowledge, only 1 study reported longer-term (ie, 3 days) benefits of pharmacological PTP inhibition after asphyxial CA in rats.<sup>9</sup> The present study further suggests that CypD-dependent PTP inhibition (without hypothermia) might slightly improve medium-term survival. Nevertheless, although statistically significant, loss of CypD yielded 7 day survival rates as low as 20%, which is far from satisfactory. Moreover, this improvement was almost exclusively explained by the increase in CPR success. Indeed, in animals that survived the first hours after CA, loss of CypD did not prevent death secondary to irreversible brain damage that occurred within 7 days.

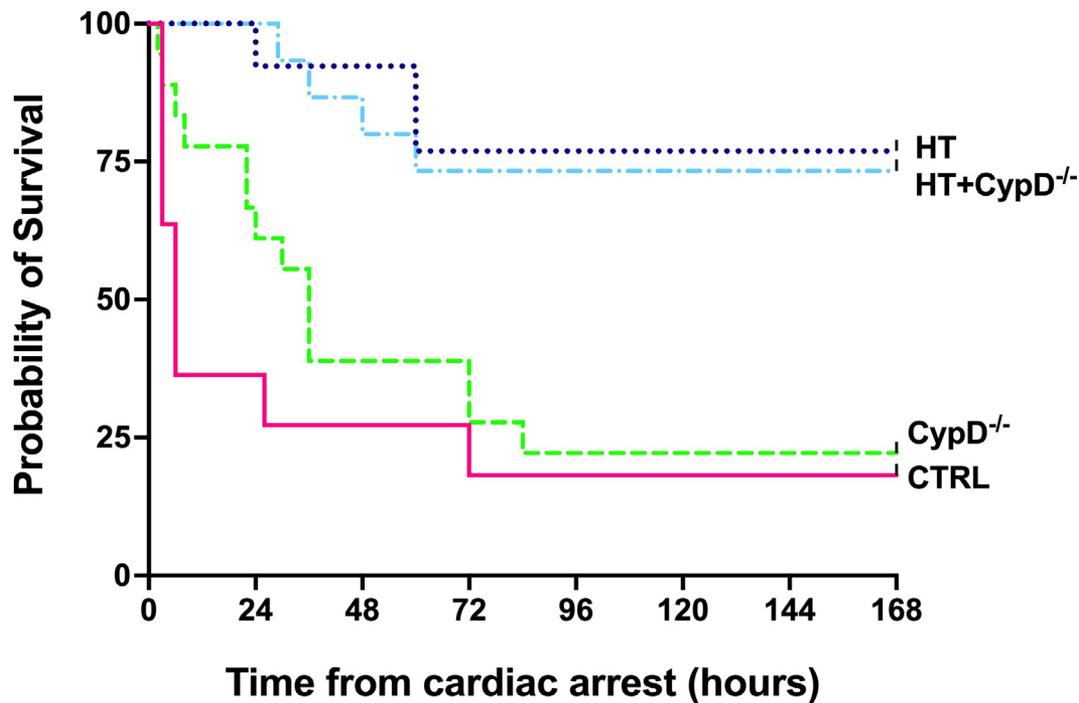


**Fig 6.** Survival in the additional group of mice treated with both hypothermia and cyclosporine. A 7 day cumulative survival rate (dashed line) is presented for the additional subset of wild-type mice treated with both hypothermia and cyclosporine A (n = 8).

The present study confirms the powerful cardioprotective effect of hypothermia when applied very rapidly (ie, within minutes) after CA.<sup>11,27,31</sup> Nevertheless, in mitochondria isolated from the heart of WT mice herein, contrary to that observed in our rabbit model of CA,<sup>8,10,11</sup> CRC was similar after CA, between normothermic and hypothermic animals. This result might be explained by the shorter duration of myocardial ischemia in the mouse model (5 minutes vs 15 minutes in the rabbit model), limiting cell damage in heart and thus the possibility to highlight mitochondrial benefits of hypothermia. However, cardiac contractility was better preserved in the hypothermic WT group, suggesting that the benefits of hypothermia on early post-CA myocardial dysfunction are not related to sustained CypD-dependent mechanisms. The fact that rate of ROSC was different between hypothermia and loss of CypD (or CsA) does not bring into question the assumption that CypD-dependent PTP inhibition during CPR might be involved in the improvement in resectability. Indeed, as hypothermia was applied upon the start of CPR, the decrease in core temperature was delayed by a few minutes, possibly beyond a very narrow window of protection involving CypD.

Concerning the brain, the effects of hypothermia were slightly different than those observed in the heart. In WT animals, hypothermia was associated with an improvement in CRC and lower brain damage as assessed by S100 $\beta$  protein early after CA. This result might be, in part, explained by different effects of hypothermia on brain and heart mitochondria.<sup>32</sup> Clinical neurological benefits of hypothermia were evident as soon as 24 hours after CA; there were better neurological function scores and fewer lesions on brain MRI compared to controls. The use of hypothermia in WT animals was associated with significant increase in neurologically-intact 7 day survival but that remained below 50%. These findings are in line with previous reports and confirm a wider therapeutic window for hypothermia-induced neuroprotection than for cardioprotection.<sup>10,11,33</sup> Interestingly, in animals that were weaned from mechanical ventilation, hypothermia was highly effective to prevent death from neurological cause while loss of CypD did not confer any clinically relevant benefit.

A new and most important finding of this study is that combination of genetic inhibition of CypD-dependent PTP opening and hypothermia conferred a



**Fig 7.** Survival after cardiac arrest in mice successfully weaned from mechanical ventilation Kaplan-Meier survival estimates are presented for the 4 groups (n = 7–15/group) of mice submitted to 5 minutes of cardiac arrest (CA) mice and successfully weaned from mechanical ventilation. The 7 day mortality significantly differed among the 4 groups ( $P < 0.001$ , Log-rank test). CTRL, controls, CA in wild-type mice; HT, hypothermia after CA in wild-type mice; CypD<sup>-/-</sup>, CA in cyclophilin D knockout mice; HT+ CypD<sup>-/-</sup>, hypothermia after CA in cyclophilin D knockout mice.

significantly better medium-term survival after CA than each of these interventions alone. This unanticipated finding was confirmed by a pharmacological approach of CypD inhibition, thus limiting the possibility that the results were due to CypD knockout-induced changes in the physiology of animals unrelated to PTP opening regulation. As discussed above, 1 can reasonably assume that the benefits of CypD inhibition upon reperfusion are related to an improvement in short-term survival, mostly by increasing the rate of ROSC and shortening the duration of CPR. Conversely, CypD inhibition in brain after successfully resuscitated CA seems to be of limited interest when hypothermia is applied early. Thus, the present study highlights that different pathways must be targeted to prevent effectively ischemia-reperfusion injury after CA.

The present study has potential important implications for clinical CA research. Potential benefits of pharmacological CypD inhibition with CsA, given as soon as possible after the start of CPR, were investigated in the randomized CYRUS trial that involved 794 patients.<sup>14</sup> Disappointingly, the severity of post-CA syndrome (primary endpoint) was similar in patients who received or not CsA.<sup>14</sup> To explain these results it was hypothesized that the dose of CsA was

not sufficient or given too late after the start of CPR to protect vital organs against ischemia-reperfusion injury.<sup>34</sup> Even though ancillary studies of CYRUS subsequently showed that CsA may limit the severity of respiratory failure after CA<sup>16</sup> and CA-induced immune dysfunction,<sup>15</sup> the results herein indicate that even a complete inhibition of CypD-dependent PTP opening is unlikely to confer potent long-term neuroprotection in case of successfully resuscitated CA or to reproduce the benefits of hypothermia. Nevertheless, inhibition of CypD-dependent PTP opening might increase the rate of ROSC and short-term survival through mechanisms that remains to be determined. Thus, researchers should better investigate other therapeutic approaches (eg, molar sodium lactate<sup>35</sup>) than trying to optimize pharmacological PTP inhibition for improving outcomes of patients admitted alive to hospital after CA. The increase in the rate of ROSC in CypD<sup>-/-</sup> is a very important finding, because, for many decades, epinephrine has been the only pharmacological option to help to “restart” the heart.<sup>4</sup> Finally, the present study confirms the benefits of therapeutic hypothermia CA especially in times when it is called into question.<sup>36</sup>

In conclusion, complete inhibition of CypD-dependent PTP opening dramatically increased short-term

survival but did not prevent death related to brain injury in case of successfully resuscitated CA, unless it was associated with therapeutic hypothermia. Regarding clinical perspectives, our results indicate that pharmacological inhibition of CypD might a new therapeutic option to investigate for improving CPR success. Conversely, this therapeutic approach should not be longer considered to prevent post-CA syndrome in patients already successfully resuscitated from CA.

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