Diagnostic and Prognostic Utility of Circulating Cytochrome *c* in Acute Myocardial Infarction

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Rationale: **In contrast to cardiomyocyte necrosis, which can be quantified by cardiac troponin, functional cardiomyocyte impairment, including mitochondrial dysfunction, has escaped clinical recognition in acute myocardial infarction (AMI) patients.**

Objective: **To investigate the diagnostic accuracy for AMI and prognostic prediction of in-hospital mortality of cytochrome *c*.**

Methods and Results: **We prospectively assessed cytochrome *c* serum levels at hospital presentation in 2 cohorts: a diagnostic cohort of patients presenting with suspected AMI and a prognostic cohort of definite AMI patients. Diagnostic accuracy for AMI was the primary diagnostic end point, and prognostic prediction of in-hospital mortality was the primary prognostic end point. Serum cytochrome *c* had no diagnostic utility for AMI (area under the receiver-operating characteristics curve 0.51; 95% confidence intervals 0.44–0.58; *P*=0.76). Among 753 AMI patients in the prognostic cohort, cytochrome *c* was detectable in 280 (37%) patients. These patients had higher in-hospital mortality than patients with nondetectable cytochrome *c* (6% versus 1%; *P*<0.001). This result was mainly driven by the high mortality rate observed in ST-segment–elevation AMI patients with detectable cytochrome *c,* as compared with those with nondetectable cytochrome *c* (11% versus 1%; *P*<0.001). At multivariable analysis, cytochrome *c* remained a significant independent predictor of in-hospital mortality (odds ratio 3.0; 95% confidence interval 1.9–5.7; *P*<0.001), even after adjustment for major clinical confounders (odds ratio 4.01; 95% confidence interval 1.20–13.38; *P*=0.02).**

Conclusions: **Cytochrome *c* serum concentrations do not have diagnostic but substantial prognostic utility in AMI. (*Circ Res*. 2016;119:1339-1346. DOI: 10.1161/CIRCRESAHA.116.309792.)**

Key Words: acute myocardial infarction ■ cardiac troponin ■ cytochrome *c* ■ mitochondrial dysfunction ■ prognosis

Cardiac troponins (cTn) are commonly used as diagnostic and prognostic biomarkers[1,2] and to evaluate the extent of myocardial necrosis in patients with acute myocardial infarction (AMI). In particular, an incremental relationship between risk of death and cTn levels has been demonstrated.[1,2]

In addition to the extent of myocardial necrosis, as reflected by cTn levels, several functional changes may occur during the early phase of AMI, contributing to myocardial cell dysfunction and ventricular impairment, thus increasing morbidity and mortality. Among them, multiple lines of evidence suggest that mitochondria have a pivotal role in the pathogenesis of cell dysfunction.[3,4] Indeed, the contraction of the heart is an energy-dependent process requiring large amounts of adenosine triphosphate generated by mitochondrial oxidative metabolism.[3,4] During ischemia and reperfusion, oxygen-dependent mitochondrial processes are impaired, and this may adversely affect cellular

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Nonstandard Abbreviations and Acronyms	
AMI	acute myocardial infarction
cTn	cardiac troponins
hs	high-sensitivity
mtDNA	mitochondrial DNA
NSTEMI	non–ST-segment–elevation myocardial infarction
STEMI	ST-segment–elevation acute myocardial infarction

homeostasis and cardiomyocyte contraction.[3,4] However, the clinical relevance of mitochondrial dysfunction in the setting of AMI is still unclear. This is mainly because of the lack of accurate markers able to assess mitochondrial function.

Cytochrome *c* is a water-soluble protein located in the inner mitochondrial membrane with an important role in mitochondrial energy metabolism.[5] Under physiological conditions, it localizes within the mitochondria, and it is not detectable in the blood of healthy human subjects.[5] Experimental models demonstrated that prolonged ischemia and reperfusion may predispose to mitochondrial dysfunction[3,4] and cause a release of cytochrome *c* into the cytosol.[3–5] Moreover, elevated levels of circulating cytochrome *c* have been reported in association with cardiac arrest, chemotherapy, fulminant hepatitis, systemic inflammatory response syndrome, and influenza-associated encephalopathy.[6–10] All these conditions are characterized by critical mitochondria dysfunction and release in the blood of cytochrome *c*, whose circulating levels have been demonstrated to be inversely related to survival outcomes. Preliminary human studies showed that circulating cytochrome *c* is also measurable in the blood of patients with ST-segment–elevation acute myocardial infarction (STEMI).[11,12] Moreover, its serum levels are associated with electrocardiographic and angiographic signs of impaired myocardial reperfusion, extension of infarct size, and 1-year mortality.[11,12] Thus, cytochrome *c* in AMI patients seems to be a potential biomarker of mitochondrial dysfunction that may provide diagnostic and prognostic information complementary to that provided by cTn.

The aim of this study was to better characterize the diagnostic and the prognostic utility of serum concentrations of cytochrome *c* in AMI.

Methods

Population of the Diagnostic Cohort

Patients presenting to the Emergency Department of the University Hospital of Basel, Switzerland, with symptoms suggestive of AMI were recruited (Online Data Supplement). Patients with kidney failure requiring dialysis were excluded. For this analysis, also patients with STEMI or with an unknown diagnosis after adjudication and at least 1 elevated high-sensitivity (hs)-cTnT level possibly indicating AMI were excluded.

Population of the Prognostic Cohort

Patients with definite AMI (STEMI and non-STEMI [NSTEMI]) admitted to the Centro Cardiologico Monzino, Milan, Italy, were prospectively considered eligible for enrollment. The only exclusion criterion was lack of informed consent. The study was performed according to the principles of the Declaration of Helsinki; the ethics committee of the Institute approved the study; and all patients gave written consent to use part of their blood for scientific purposes.

Study Protocol

In all patients, peripheral venous blood was drawn at hospital admission to measure hs-cTnT (diagnostic cohort), cTnI (prognostic cohort), serum cytochrome *c*, and standard biochemical parameters. Moreover, in the prognostic cohort, we measured circulating cell–free mitochondrial DNA (mtDNA), a biomarker that has been associated with survival in critically ill patients.[13]

In-hospital outcomes and 1-year mortality were assessed for all patients included in the prognostic cohort. Patient follow-up was obtained through regularly scheduled outpatient visits or by telephone contacts performed by dedicated medical personnel.

Diagnostic accuracy for AMI was the primary end point in the diagnostic cohort, and prognostic prediction for in-hospital mortality was the primary end point in the prognostic cohort. The following secondary end points were considered in the prognostic cohort: (1) the combination of in-hospital mortality, acute pulmonary edema requiring mechanical ventilation, and cardiogenic shock requiring intra-aortic balloon pump; and (2) 1-year mortality.

Laboratory Assays

In the diagnostic cohort, hs-cTnT (ng/L) was measured by an electrochemiluminescence immunoassay (ECLIA, Roche Elecsys 2010 hs-cTnT).

In the prognostic cohort, cTnI (ng/mL) was measured by a 2-site immune-enzymatic (sandwich) chemiluminescent technique, using the UniCell DXI 800 (Beckman Coulter, Fullerton, CA).

In both the diagnostic and prognostic cohorts, cytochrome *c* (ng/mL) was measured in the serum at hospital admission by ELISA, using a commercially available enzyme-linked immunosorbent assay (Quantikine; R&D System Inc, Minneapolis, MI), as previously described (Online Data Supplement).[12] The lowest detection limit was 0.08 ng/mL. Circulating levels of mtDNA were measured in the serum, as reported by Nakahira et al.[14]

Statistical Analysis

Detailed statistical analysis section is given in the Online Data Supplement.

Results

Diagnostic Cohort

From March 26, 2008, to July 8, 2009, a total of 418 patients presenting with symptoms suggestive of AMI were recruited (Online Figure I and Table I).

The diagnostic accuracy of hs-cTnT at admission in the diagnosis of AMI, as quantified by the area under the curve, was 0.94 (95% confidence intervals 0.92–0.97), which was significantly higher than that of cytochrome *c* at admission (area under the curve 0.51; 95% confidence interval 0.44–0.58; $P<0.001$ for comparison). Moreover, the combination of the 2 markers did not improve the diagnostic accuracy compared with hs-cTnT alone (area under the curve 0.94; 95% confidence interval 0.92–0.97; $P=0.10$ for comparison; Online Figure II). hs-cTnT plus cytochrome *c* provided a net reclassification improvement of 4.2% ($P=0.18$) and an integrated discrimination improvement of 1% ($P<0.001$) in AMI diagnosis, when compared with hs-cTnT alone.

Prognostic Cohort

From June 1, 2010, to September 30, 2012, a total of 753 patients with a diagnosis of definite AMI (316 STEMI and 437

Table. Clinical Characteristics of Patients Included in the Prognostic Cohort

Variable	Detectable Cytochrome *c* (n=280)	Nondetectable Cytochrome *c* (n=473)	*P* Value
Age, y	66±13	67±13	0.23
Male sex, n (%)	200 (71)	344 (73)	0.70
STEMI, n (%)	128 (45)	188 (40)	0.11
Body mass index, kg/m^2	26.7±4.5	26.7±4.3	0.96
Diabetes mellitus, n (%)	60 (21)	112 (24)	0.47
Hypertension, n (%)	175 (62)	305 (64)	0.58
Dyslipidemia, n (%)	139 (50)	247 (52)	0.51
Smokers, n (%)	143 (51)	267 (56)	0.15
Prior myocardial infarction, n (%)	79 (28)	119 (25)	0.35
Prior CABG, n (%)	38 (14)	51 (11)	0.25
Prior PCI, n (%)	69 (25)	115 (24)	0.91
Left ventricular ejection fraction,* %	51±13	52±12	0.23
Time-to-treatment, h	3.5 (2–10)	4 (2–10)	0.93†
TIMI risk score	4.3±2.3	4.2±2.2	0.55
In-hospital treatment, n (%)			
PCI	223 (80)	356 (75)	0.16
CABG	11 (4)	8 (6)	0.23
Medical therapy	46 (16)	89 (19)	0.40
Infarct related artery, n (%)			0.90
Left anterior descending	86 (33)	156 (36)	
Right coronary artery	64 (25)	94 (22)	
Left circumflex	60 (23)	98 (23)	
Bypass graft	12 (5)	10 (5)	
Left main	7 (3)	20 (2)	
Laboratory values			
Blood glucose, mg/dL	130 (110–172)	128 (108–169)	0.48†
Serum creatinine, mg/dL	0.9 (0.8–1.1)	0.9 (0.8–1.1)	0.64†
eGFR, mL/min per 1.73 m^2	82±27	85±31	0.41
Baseline troponin I, ng/mL	0.8 (0.2–3.3)	0.6 (0.1–2.1)	0.018†
Peak troponin I, ng/mL	6.2 (1.4–45.6)	6·1 (1.2–32.3)	0.26†
Hemoglobin, g/dL	13.5±2	13.5±2	1.0
Total cholesterol, mg/dL	181±49	181±46	0.82
HDL, mg/dL	39±11	40±12	0.36
LDL, mg/dL	118±45	115±42	0.33
Triglycerides, mg/dL	125±85	130±78	0.45
hs-CRP, ng/mL	3.2 (1.3–10.7)	3.8 (1.4–14.7)	0.30†
Medication at hospital admission, n (%)			
Aspirin	202 (72)	330 (70)	0.49
ACE/ARB	117 (42)	208 (44)	0.55
Beta-blockers	105 (37)	175 (37)	0.89
Statins	95 (34)	162 (34)	0.92
Warfarin	12 (4)	21 (4)	0.92
Medication at hospital discharge, n (%)			
Aspirin	255 (96)	451 (96)	0.82
ACE/ARB	163 (61)	292 (64)	0.51
Beta-blockers	193 (72)	333 (72)	0.97
Statins	234 (88)	407 (89)	0.74

ACE indicates angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; CABG, coronary artery bypass graft; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; LVEF, left ventricular ejection fraction; PCI, percutaneous coronary intervention; STEMI, ST-segment–elevation myocardial infarction; and TIMI, Thrombolysis In Myocardial Infarction.

*LVEF was measured by echocardiography at hospital admission in all patients (before or soon after primary PCI in STEMI).

†The *P* value was calculated by Kruskall–Wallis test.

NSTEMI) were consecutively enrolled (Online Figure III). At hospital admission, cytochrome *c* was detectable in the blood of 280 (37%) patients. Table shows the demographic and clinical characteristics of patients with detectable and nondetectable cytochrome *c*. These 2 groups were comparable for all evaluated variables. The only observed significant difference between AMI patients with detectable cytochrome *c* and those without detectable cytochrome *c* was in mtDNA levels (290 [142–490] copies/μL versus 210 [22–362] copies/μL; *P*=0.003). Clinical characteristics of the 2 groups were also similar when STEMI and NSTEMI patients were analyzed separately (Online Table II).

In-hospital mortality and combined end point rates were significantly higher in patients with detectable cytochrome *c* than in those without detectable cytochrome *c* (Online Table III). These results were mainly driven by the high rates observed in STEMI patients with detectable cytochrome *c* as compared with those with nondetectable cytochrome *c*. A significant gradient of increasing incidence of in-hospital mortality and of the combined end point was observed going from patients with nondetectable cytochrome *c* to those with detectable cytochrome *c* below the median value and ending with those with a detectable cytochrome *c* above the median value. Again, this trend was particularly evident in STEMI patients (Figure 1).

The correlations between cytochrome *c* and cTnI, hs-C-reactive protein, and mtDNA are reported in the Online Table IV, while their distribution in patients who died versus those who survived is shown in the Online Figure IV.

Cumulative 1-year mortality was higher in patients with detectable cytochrome *c* than in those without detectable cytochrome *c* (Online Table III). The Kaplan–Meier curves for

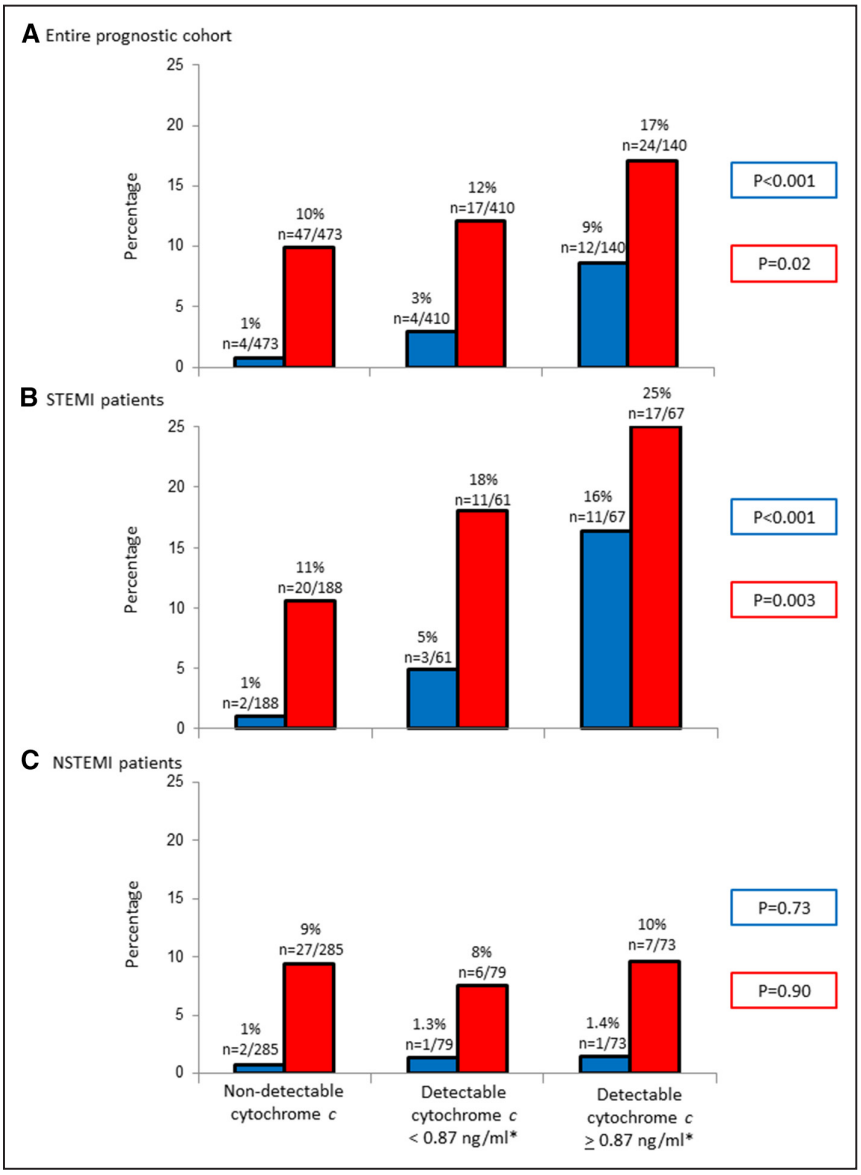

Figure 1. In-hospital mortality (blue bars) and combined end point (red bars) rates in the entire prognostic cohort (A), in ST-segment–elevation myocardial infarction (STEMI) patients (B), and in non-STEMI (NSTEMI) patients (C), grouped according to serum cytochrome *c* value (nondetectable vs detectable below the median value vs detectable above the median value). *Median value refers to that of the prognostic cohort. The *P* values within the blue boxes refer to *P* for trend for in-hospital mortality, while the *P* values within the red boxes refer to *P* for trend for the combined end point.

1-year mortality in the entire prognostic cohort, as well as in STEMI and NSTEMI patients analyzed separately, are shown in Figure 2. Again, the prognostic impact of cytochrome *c* was observed in STEMI patients only, and it was limited to the first 30 days, as demonstrated by the landmark analysis (Online Figure V).

At multivariable analysis, cytochrome *c* remained a significant independent predictor of in-hospital mortality, even after adjustment for major recognized determinants of mortality in AMI (Online Table V). When cytochrome *c* and cTnI at hospital admission were considered together in the entire population, a complementary predictive value on mortality was found (Figure 3).

Discussion

The main finding of this study was that, despite its lack of utility for diagnosis, cytochrome *c* at hospital admission is strongly associated with in-hospital mortality in AMI. The prognostic power of cytochrome *c* is particularly evident in STEMI patients, and it was independent of clinical variables that are known to affect AMI outcome.

The mechanisms involved in ventricular impairment during the early phase of AMI are not completely elucidated. Growing evidence suggests that functional processes may be implicated in addition to the extent of myocardial necrosis. Although myocardial necrosis may be estimated by enzymatic release,[1,2] the amount of functional impairment cannot be easily assessed in clinical practice. Ischemia and reperfusion phenomena cause intracellular calcium overload and generation of reactive oxygen species,[4] which predispose to mitochondrial dysfunction, and may contribute, in addition to cell necrosis, to myocardial contractility impairment.[3,4] Apart from the underlying mechanism, impairment of mitochondrial function is followed by cytochrome *c* release which, therefore, can be considered a marker of mitochondrial dysfunction.[5–10]

In our study, we measured circulating cytochrome *c* at hospital admission in patients with suspected and definite

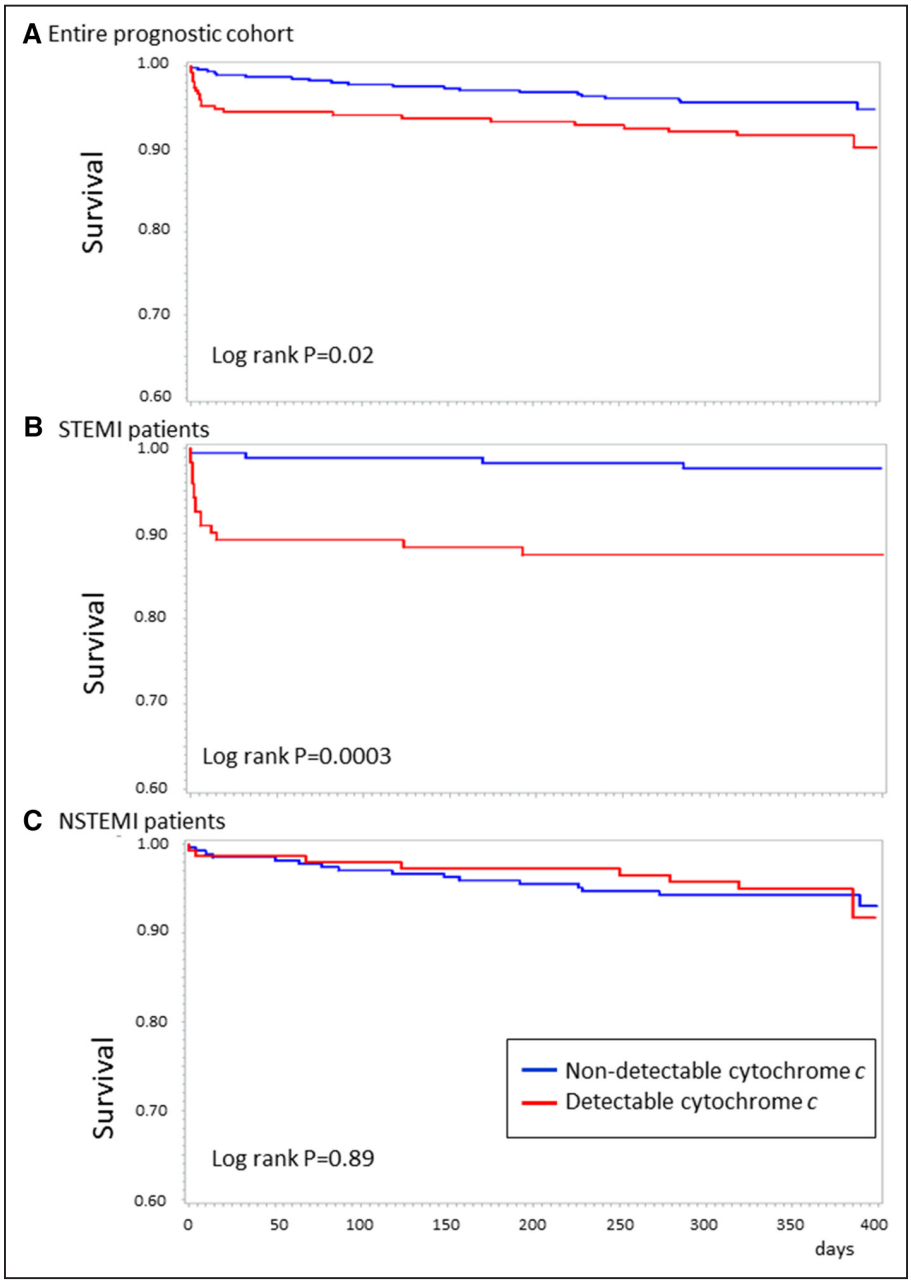

Figure 2. Kaplan–Meier curves according to serum cytochrome *c* detection at hospital admission in the entire prognostic cohort (A), in ST-segment–elevation myocardial infarction (STEMI) patients (B), and in non-STEMI (NSTEMI) patients (C). *P* value =Log-rank test.

AMI to characterize its diagnostic and prognostic utility. Although it has no diagnostic accuracy, we found a striking association between the levels of this protein and in-hospital cardiac mortality and morbidity in patients with AMI. Notably, we observed a 10-fold higher in-hospital mortality in STEMI patients when cytochrome *c* was detected, despite similar baseline risk profile, drug treatment, and mechanical reperfusion rate between patients with or without detectable cytochrome *c*. In NSTEMI patients in whom cytochrome *c* was detected, a 2-fold higher in-hospital mortality rate was observed. However, the mortality difference between them and NSTEMI patients without detectable cytochrome *c* did not reach statistical significance. Although we cannot deduce from our study the reasons behind this, we can speculate that the low number of in-hospital events and the smaller ischemic involvement, both characterizing NSTEMI, may explain this result.

Several findings of our study indicate that cytochrome *c* detection in the blood may be a marker of acute left ventricle impairment beyond that because of myocardial necrosis. First, unlike cTn, cytochrome *c* did not help to diagnose AMI in patients presenting with chest pain. Notably, cytochrome *c* was detected in 66% of patients without AMI included in the diagnostic cohort, and no correlation was found between cytochrome *c* and either admission or peak cTnI in the prognostic cohort. Second, cytochrome *c* predicted in-hospital mortality independently of cTnI value at admission. Third, despite similar left ventricular ejection fraction at admission and cTnI peak value, death was in most cases because of hemodynamic complications, as shown by a higher incidence

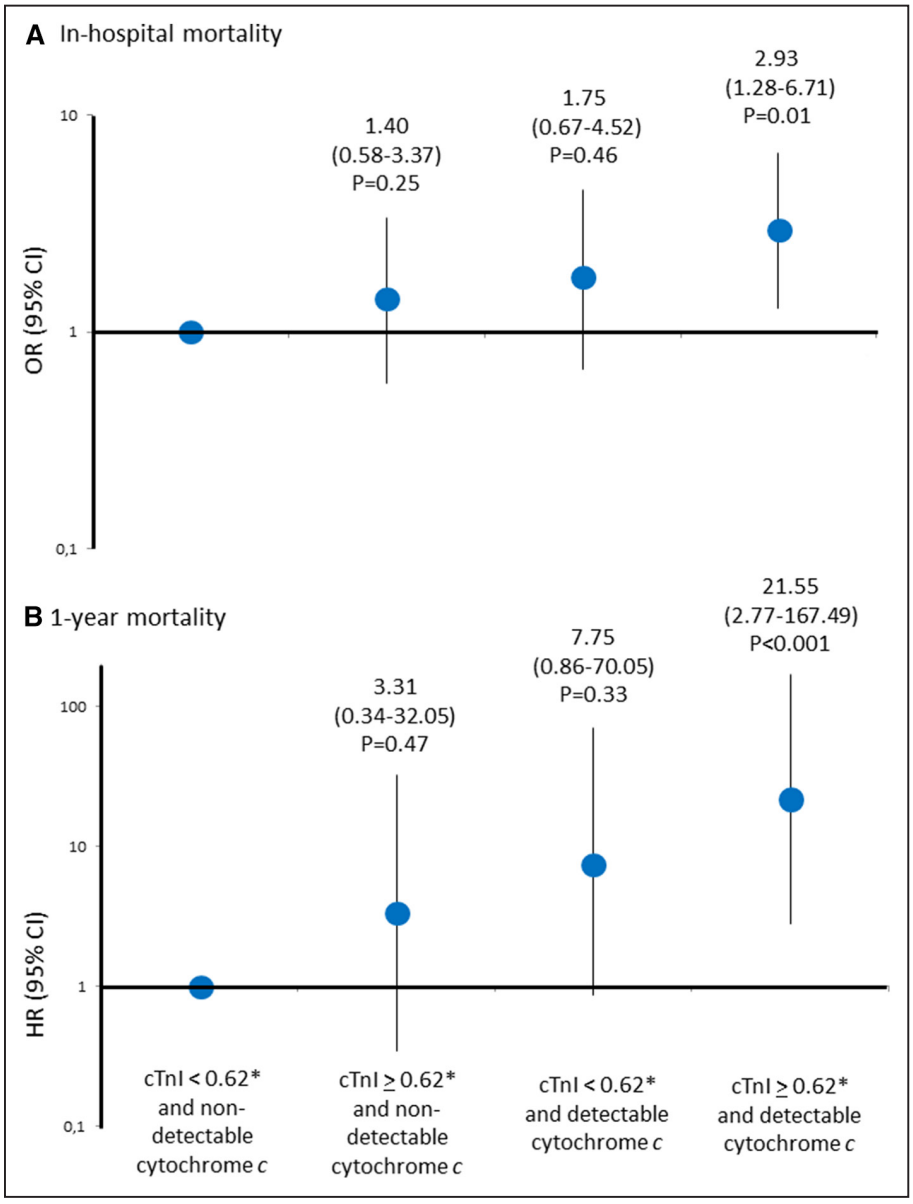

Figure 3. In-hospital mortality odds ratio ([OR] and 95% confidence intervals [CI]; A) and 1-year mortality hazard ratio ([HR]; and 95% CI; B) in the entire prognostic cohort, grouped according to cardiac troponin I (cTnI) value (<or ≥ median value) and cytochrome *c* at hospital admission. *cTnI median value of the entire prognostic cohort. OR, *P* value =logistic regression analysis; HR, *P* value =Cox regression analysis.

of cardiogenic shock and acute pulmonary edema in patients with detectable cytochrome *c*. Finally, the prognostic relevance of cytochrome *c* was evident in the early phase of AMI because the survival curves of STEMI patients with or without detectable cytochrome *c* showed a parallel trend after 30 days. Therefore, combining the measurement of cTn and cytochrome *c* at hospital admission may provide independent and complementary prognostic information, considerably improving early risk stratification of AMI patients.

How cytochrome *c* reaches the bloodstream is not well understood and cannot be inferred from our data. Similarly, the mechanisms underlying the association between cytochrome *c* and a worse outcome in the clinical setting of AMI remain unclear. Several studies showed that the release of cytochrome *c* outside the cell occurs without concomitant release of larger molecules, such as lactate dehydrogenase, which is considered a marker of cell necrosis.[7] This suggests that in parallel with myocardial necrosis, cardiac cells that are still viable may be only functionally impaired because of mitochondrial dysfunction.

Based on these considerations, our study suggests that circulating cytochrome *c* may be a biomarker of mitochondrial dysfunction and may be used to prognosticate survival in AMI patients. Similar findings were obtained in animal studies and preliminary clinical reports performed in small series of patients with other critical conditions. Radhakrishnan et al[15] reported a rise in plasma cytochrome *c* levels in an experimental model of cardiac arrest, with higher levels in the rats that did not survive. Serum cytochrome *c* showed a similar predictive value of poor prognosis in patients with systemic inflammatory response syndrome, causing multiorgan failure,[7] influenza-associated encephalopathy,[9] and fulminant hepatitis.[8] Thus, unlike cTn, cytochrome *c* is not a specific marker of cardiac cell injury; instead, it seems to be a sensitive marker of mitochondrial dysfunction occurring in organs that are rich in mitochondria and have a high

metabolic rate with propensity to ischemia and reperfusion injury. Indeed, we cannot exclude that cytochrome *c* increase is caused by systemic hypoperfusion in organs other than the heart. Notably, we found significantly higher mtDNA levels at hospital admission in AMI patients with detectable cytochrome *c*, supporting the hypothesis of cytochrome *c* release from the mitochondria. However, mtDNA did not correlate with cytochrome *c* and, differently from other critical settings,[13,14] it was not associated with mortality. The lack of an mtDNA prognostic relevance in our study might be explained by the different clinical setting (AMI) and the early mtDNA assessment (soon after AMI onset), when compared with previous reports.[13,14] Future studies are warranted to specifically investigate the role of mtDNA in AMI patients.

Our findings may have some relevant clinical implications. Cytochrome *c* measurement at admission may be used to identify high-risk patients who cannot be recognized only on the basis of the current clinical variables. Indeed, novel therapeutic strategies in addition to standard pharmacological and interventional treatments could be used for improving the outcome of AMI patients who have detectable cytochrome *c*. In particular, cardioprotective pharmacological and mechanical therapies supporting ventricular contractility without increasing oxygen consumption, such as levosimendan or intra-aortic counterpulsation, and preserving mitochondrial integrity and function, like cyclosporine A, metformin, and other manipulating mitochondrial agents, should be investigated.

In interpreting our data, some limitations should be considered. First, the diagnostic cohort was relatively small. Second, the global applicability of our prognostic findings remains uncertain, and it should be validated in a larger multicenter study. Third, our data generate hypotheses only because they do not provide evidence to support direct mechanisms underlying the connection between cytochrome *c* and mortality nor myocardial dysfunction. In particular, the serial assessment of ventricular function after AMI could help to clarify the link between cytochrome *c* and acute cardiac functional impairment. Moreover, experimental data are required in suitably designed models to explore the specific mechanism(s) inducing cytochrome *c* release, as well as its biological meaning. Finally, we did not serially evaluate cytochrome *c* levels in our AMI patients. Because most of them underwent emergency or urgent percutaneous coronary revascularization, we cannot exclude that cytochrome *c* measurement performed also after the procedure, instead of at-hospital admission only, could have increased its prognostic potential by incorporating mitochondrial damage associated with mechanical reperfusion injury. Indeed, a further increase of circulating cytochrome *c* has been shown in STEMI patients undergoing primary percutaneous intervention.[12] Of note, its peak value was associated with impaired myocardial reperfusion and 1-year mortality.[11,12] Nevertheless, cytochrome *c* measurement at hospital admission allowed us to distinguish 2 groups of patients with a similar risk profile but with a strikingly different prognosis.

In conclusion, our study demonstrated that circulating cytochrome *c* detected at hospital admission does not have diagnostic but substantial prognostic utility in AMI, particularly in STEMI. Therefore, cytochrome *c* could be considered a potential biomarker of mitochondrial dysfunction that may integrate markers of necrosis to predict patients' survival.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Impaired mitochondrial function in ischemic myocardium plays a critical role in cardiac dysfunction post acute myocardial infarction (AMI).
- Cytochrome *c* is a mitochondrial protein that can be found in the blood of critically ill patients.

What New Information Does This Article Contribute?

- Detection of cytochrome *c* in the blood at hospital admission is associated with higher in-hospital mortality in patients with AMI.

In patients with a bout of AMI, cell necrosis and stunning lead to cardiac remodeling. The former can be accurately detected by measuring plasma levels of troponins, while the currently available markers cannot accurately assess the latter. We show that detection of circulating cytochrome *c*, a marker for mitochondrial damage, was associated with increased in-hospital mortality, independent of other risk factors, drug treatment, mechanical reperfusion rate, and peak troponin values. Circulating cytochrome *c* is associated with poor prognosis in other critically ill settings. However, to our knowledge, this is the first study demonstrating the independent prognostic value of circulating cytochrome *c* in AMI. Thus, detection of cytochrome *c* might complement troponin assays in evaluation of prognosis in patients with AMI. Future studies are warranted to confirm our findings and elucidate the potential mechanisms underlying the association between circulating cytochrome *c* and a worse outcome in patients with AMI.