Septic cardiomyopathy: The value of lactoferrin and CD15 as specific markers to corroborate a definitive diagnosis

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Abstract

Current scientific consensus about the physiopathology in the progression from severe sepsis to septic shock and death focuses on myocardial contractile dysfunction. Nevertheless, objective parameters to establish a pathological correlate of a fatal outcome are lacking; then a cause of death due to sepsis can remain an unsolved problem. We first reviewed all death cases recorded at our institutions during the period from 2007 until 2015. Then, we conducted a retrospective study of a selected autopsy series of people who had received "sepsis" as cause of death. Two pathologists re-examined the heart sections while the most suitable myocardial sample for each case was stained for immunohistochemistry with antibodies targeted for specific inflammatory-related molecules. We used specific antibodies for the following markers: alpha-smooth muscle actin (alpha-SMA); fibronectin; matrix metallopeptidase 9 (MMP-9); intercellular adhesion molecule 1 (ICAM-1); caspase-3; lactoferrin (LF); cluster differentiation 15 (CD15). The statistical significance of differences was assessed using student's *t*-test for unpaired data or non-parametric Mann–Whitney or Wilcoxon tests for skewed variables or one-way analysis of variance and post hoc Scheffe's test for continuous variables and Pearson's χ^2-test for discrete variables. Linear regression analysis was used to determine the presence of a correlation between continuous variables. At our institutions, 2220 deaths have been recorded during the period study. Sepsis accounted as a cause of death for the 20% of total. We finally enrolled 56 cases; of these, only 20 were positive for microbiological analysis. At histological examination, clear inflammation was detectable in the 32% of cases; otherwise, immunohistochemical reaction showed a positive reaction for LF and CD15 in more than a half cases (56%). We still ignore all the underlying mechanisms of sepsis and all its pathophysiological connections with cardiac metabolism; in this sense, we aim to corroborate the diagnostic value of anti-LF and anti-CD15 staining for the post-mortem detection of myocardial inflammation.

Keywords

post-mortem diagnosis, sepsis, septic myocardial dysfunction, septic shock

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Infectious diseases represent one of the greatest concerns about public health worldwide,[1] as they are going to climb soon the rankings of the most common cause of death because of the development of antimicrobial resistance, which has been recently identified as Europe's biggest health

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threat.[2] Similarly, sepsis accounting for more than US$20 billion (5.2%) of total US hospital costs in 2011.[3] The management of this phenomenon in the health-care setting deals also with a critical and very current issue about patient safety and medical liability; most of life-threatening infections registered, in fact, are nosocomial (or health-care associated infection (HAI)).[4]

As the new Consensus definitions for sepsis and septic shock affirm,[3] "further understanding of the biology of sepsis, the availability of new diagnostic approaches, and enhanced collection of data will fuel their continued re-evaluation and revision." Again, "the new definition of sepsis reflects an up-to-date view of pathobiology, particularly regard to what distinguishes sepsis from uncomplicated infection."[3] In 1991, a combined consensus conference of the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) provided for sepsis the definition of a systemic inflammatory response syndrome (SIRS) arising from infection. When sepsis worsens the clinical course and leads to the death of the patient, it usually passes through severe sepsis with organ dysfunction and septic shock, characterized by hypotension and perfusion abnormalities. In 1990, the US Centers for Disease Control and Prevention (CDC) estimated for the previous 20 years a rate of 450,000 cases of septicemia per year, with more than 100,000 deaths; from that moment, it is estimated an increasing by 9% each year through 2000. Nowadays, sepsis is considered the most important cause of morbidity and mortality in intensive care units.[5]

Current scientific consensus about the physiopathology in the progression from severe sepsis to septic shock and death focuses on myocardial contractile dysfunction which directly preludes to the specific acute heart failure characterized by low or normal diastolic pressures. Septic shock is a subset of sepsis in which profound circulatory, cellular and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone.[3] Mechanisms involved in this kind of hemodynamic impairment are functional rather than structural: microcirculatory dysfunction and deregulation of autonomic reflexes;[6] metabolic disorders induced by pro-inflammatory cytokines; deregulation of inflammatory cells and nitric oxide metabolism.[7] Nevertheless, lots of studies have been performed in order to find out if any structural changes happen in the myocardium and above all which ones are specific of a death due to sepsis. Unfortunately, clear answers to these questions are still lacking. Recently, it has been underlined that the effects of the host's immune-inflammatory response focusing on depressant molecules, complement molecules, cellular adhesion molecules, and altered intracellular energetic, dysregulated intracellular calcium fluxes have been called upon in the pathophysiology of myocardial depression in sepsis.

Material and methods

The aim of our study was to define a combination of pathological findings, mainly at histology and immunohistochemistry, with high sensitivity and specificity in the detection of myocardial injury related to lethal sepsis. We searched for different molecules alpha-smooth muscle actin (alpha-SMA); fibronectin; matrix metallopeptidase 9 (MMP-9); intercellular adhesion molecule 1 (ICAM-1); caspase-3; lactoferrin (LF); cluster differentiation 15 (CD15)), as critical factors in mediating myocardial dysfunction in sepsis. The validation of these immunohistochemical diagnostic tools could lead to the better epidemiological representation of the phenomenon and especially to the objective solution about whether a case of death should be related principally to sepsis or to previous, concomitant comorbidities.

We reviewed the causes of death recorded into the medico-legal database of people died at NHS Health Authority n.6 of Vicenza in Italy. We limited the retrospective analysis of the medical records to the year 2014. We assigned each death case to one of the five categories below, dealing with the causes of death reported (primary and secondary, if any) and the significant comorbidities recorded by the pathologist:

1. Sepsis without other comorbidities related to death;
2. Cardiovascular diseases;
3. Cancer;
4. Sepsis as a complication in cardiovascular disease or cancer;
5. Others.

This represented our preliminary epidemiological assessment of the matter related to our territory.

At a later time, we conducted a retrospective study of an autopsy series of people deceased at NHS Health Authority n.6, Vicenza from 2007 to 2015. Inclusion criteria were patients older than 12 years and with a "sepsis" diagnosis as cause of death. Sepsis was clinically defined according to The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) as life-threatening organ dysfunction caused by a dysregulated host response to infection.[3]

Post-mortem samples had been obtained during the routinely autoptic activity of the Unit of Legal Medicine of the S. Bortolo General Hospital in Vicenza were preserved in buffered 10% formalin. Tissue samples for histological evaluation were routinely embedded in paraffin and 5-μm-thick sections were obtained. Microbiologic data were obtained by the in vivo cultures of blood and body fluids (bronchial aspirations or surgical drainage).

For the cases analysis, two pathologists independently re-examined by light microscopy heart sections in hematoxylin and eosin (H&E) staining searching for classic lesions. Then we selected the most suitable myocardial sample for each case and we obtained new sections for immunohistochemistry. We used specific antibodies for the following markers: alpha-SMA; fibronectin; MMP-9; ICAM-1; caspase-3; LF; CD15.

The control group was composed of 25 cases, selected from among traumatic deaths, with negative toxicological analysis, in which no evidence of infections was detected at post-mortem examination. The age of the control group ranged between 20 and 71 years; 18 males and 7 females were included. We prepared immunohistochemical sections with the avidin–biotin technique (Avidin-Biotin Complex Kit, DAKO), as previously described in the literature.[8]

Statistical analysis

Data are expressed as mean values ± standard deviation. The statistical significance of differences was assessed using student's *t*-test for unpaired data or non-parametric Mann–Whitney or Wilcoxon tests for skewed variables or one-way analysis of variance and post hoc Scheffe's test for continuous variables and Pearson's χ^2 test for discrete variables. Linear regression analysis was used to determine the presence of a correlation between continuous variables. A probability of $P < 0.05$ was considered different significantly.

Results

At NHS Health Authority n.6 of Vicenza in Italy, 2220 deaths have been recorded during 2014. The most frequent single category is "Cardiovascular Diseases," accounting for 648 cases, which means almost one third of total. As single cause of death malignancies follows, with 564 cases, that is one quarter of all recorded deaths. Sepsis ranks third. When considered as the only cause of death sepsis includes 363 events; if cases of sepsis occurring as a complication of cardiovascular disease or cancer are aggregated, it counts for 450 deaths, about the 20% of total.

For our autopsy series, we first selected 67 cases. We excluded some cases because they were people younger than 12 years and others because of deep autolysis of the heart specimens. We finally enrolled 56 cases, related to the period from 2007 until 2015. The age of the selected cases ranged from the fourth to the ninth decade, while the age of the control cases ranged from the third to the seventh decade. All autopsies were performed from 24 to 72 h after death.

Of 56 cases, a microbiological analysis was proven positive in 20 (about 35%). It is possible to ascertain a good balancing among the three main categories of pathogens, as mycetes, the most uncommon ones, account for one third of total (7/21). Some cases resulted positive for more than one pathogen. The case proven positive for *Toxoplasma gondii* can be considered as a singularity of our autopsy series.

During the histological review, five principal lesional patterns have been searched for into the myocardium: generalized inflammation (interstitial and septal at the same time); edema or hemorrhage; focal acute inflammation (granulocytic infiltrates); focal lymphocytic infiltrates; septic emboli (SE). All these findings are the usual pathological counterpart of the inflammatory responses, which take place in every kind of tissue, including the myocardium. Among the 56 cases analyzed, 19 resulted negative to any of these criteria, while 10 showed only slight modification considered controversial about the presence or the absence of the specific pattern. The organisms isolated and the occurrence of each lesional pattern are shown in detail in Table 1. Some values are expressed as ranges as we admitted a double interpretation because of the ambiguity of findings.

Table 1. Microbiological analysis and representation of singular lesional patterns.

Pathogens	Cases (number)
Gram positive	363
Gram negative	648
Mycetes	564
Protozoa (*Toxoplasma gondii*)	1

Singular lesional pattern	Cases (number)
Generalized inflammation (GI)	18–24
Edema and/or hemorrhage (E/H)	12–17
Focal granulocytic infiltrates (FGI)	6–7
Septic emboli (SE)	7
Focal lymphocytic infiltrates (FLI)	2

Generalized inflammation was the commonest finding, but it ranged across a considerable variability. The most evident form showed diffuse and large leukocytes infiltrates spread into the myocardium; in these similar cases, we could talk about a "patent leukocytic myocarditis." Anyway, in the same category, we included much milder kinds of inflammation limited to perivascular areas of main septum.

The presence of edema has been evaluated indirectly, referring to the enlargement of inter-septal areas, because it was impossible to find a classic pattern of tissue edema, with accumulation of eosinophilic acellular material in the intercellular matrix. This is the reason why 5 of 17 cases (nearly 1/3) were scheduled as ambiguous findings.

In five cases, focal granulocytic infiltrates (FGI) were found in association with SE, three of which showed also generalized inflammation (GI). Septic microvascular embolism could be observed within the necrosis of the vessel wall. Considering the data from microbiology, a strong and statistically significant association ($P < 0.05$) could be established between the particular feature of septic embolism and the detection of mycetes at the analysis. Furthermore, about correlation between gross histopathology and specific infectious agent, it was possible to find a precise correspondence in the case of toxoplasmosis, as there were numerous small hematoxylinophilic corpses into myocardiocytes sarcoplasm.

In the control group, it was not possible to find granulocytic infiltrates in any case. In the sepsis group, instead, there was only one case of contraction band necrosis, associated with inflammation.

Immunohistochemical assessment

Alpha-actin. The vessels wall showed a clear immunoreactivity both in septal and main vessels and in capillaries (Figure 1). There were no differences between cases and controls in terms of interindividual variability in ratio of immunopositive capillaries per each section. No myofibroblasts proliferation could be detected in vessels wall, neither in association with septic embolism and perivascular inflammation.

Fibronectin. The immunoreactivity pattern in sepsis cases was extremely variable, ranging from a nuclear, a cytoplasmic, and an interstitial distribution. Otherwise, for controls, the only pattern observed was cytoplasmic coloring.

MMP-9. Signal from MMP-9 could depict both intracellular elements and extracellular ones. More in detail intracellular immunoreactivity interested notably the paranuclear zone which corresponds to the endoplasmic reticulum and the Golgi apparatus. Nevertheless, there was no significative difference between sepsis and control groups.

Caspase 3. This target showed a weak and diffused staining at myocardiocytes cytoplasm, without differences between cases and controls.

CD54 (ICAM-1). The immunopositive reaction involved endothelial cells for both cases and controls, without significant discrepancies about intensity and distribution pattern. The only considerable association could be established between a spectacular intensity and the histological evidence of mycetes aggregates in the myocardium (Figure 2).

LF. Our cases were proven immunopositive in 33 of 56 with a significative higher proportion than controls. Intense staining localized especially at perivascular sites. Controls were characterized by almost negative or very weak staining reactions, except for two of them: one had an acute myocardial infarction; the other deceased for an acute pancreatitis. LF immunoreactivity was linked to the degree of inflammation already detected at H&E staining, especially correlating with the pattern of generalized inflammation; furthermore, it revealed the presence of conspicuous infiltrates
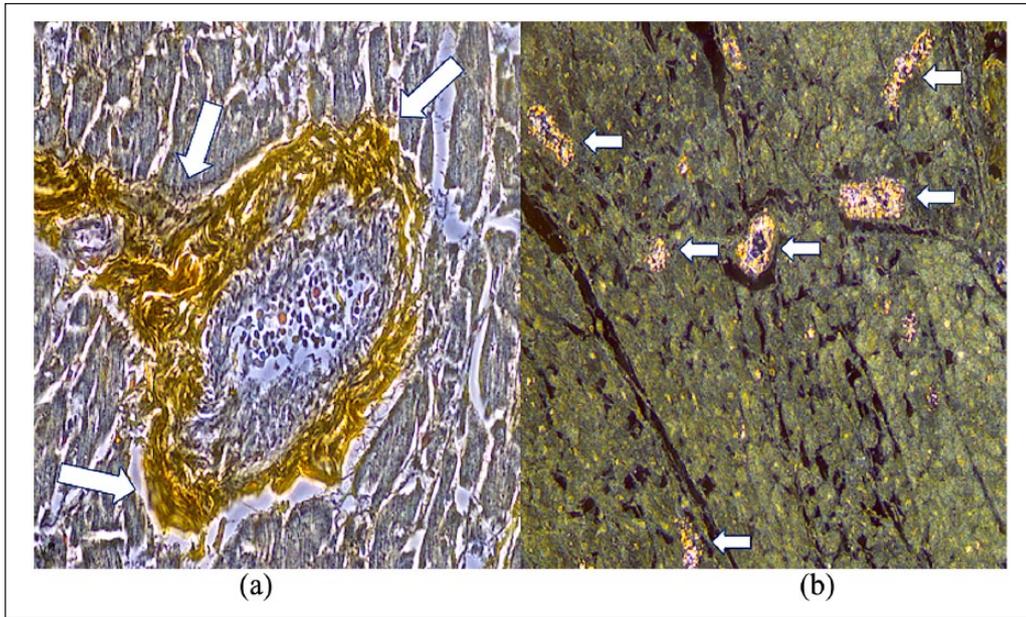
Figure 1. Alpha-actin staining: (a) anti-alpha-actin antibody; (b) immunoreactivity of vessels wall (arrows in (a) and (b)) (H&E 100× and 250×, respectively).
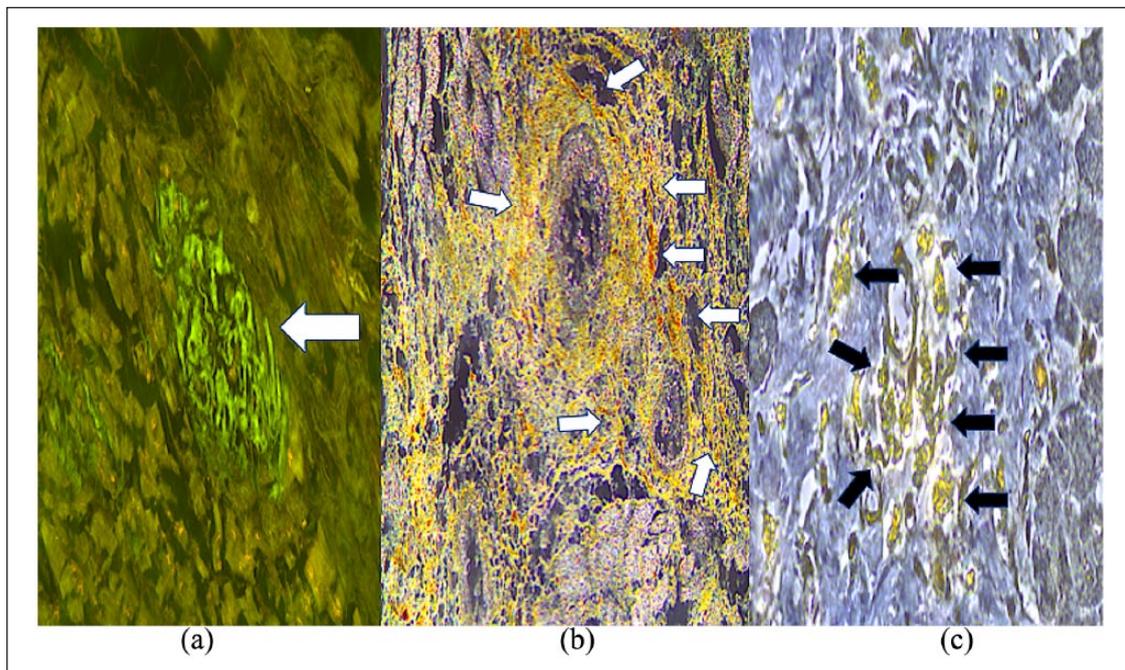

Figure 2. CD54 and lactoferrin stainings: (a) anti-CD54 antibody. Intense positive staining of vascular endothelium around a mycete embolus (arrow) (H&E 25×). Anti-lactoferrin antibody: the number of perivascular granulocytes is markedly increased (arrows) (b). Very intense staining associated with septic embolus (arrows) (c) (H&E 100×).

even in samples that passed unnoticed the common histology (Figure 3).

CD15. The staining obtained was perfectly correspondent to those with anti-LF antibody. Consequently, there were the same associations and the same differences between inflammation at H&E standard preparations and immunoreactivity and also comparing cases and controls. The only difference was a slightly less intense reaction with the anti-CD15 antibody, which offered from the other hand less background staining (Figure 4).
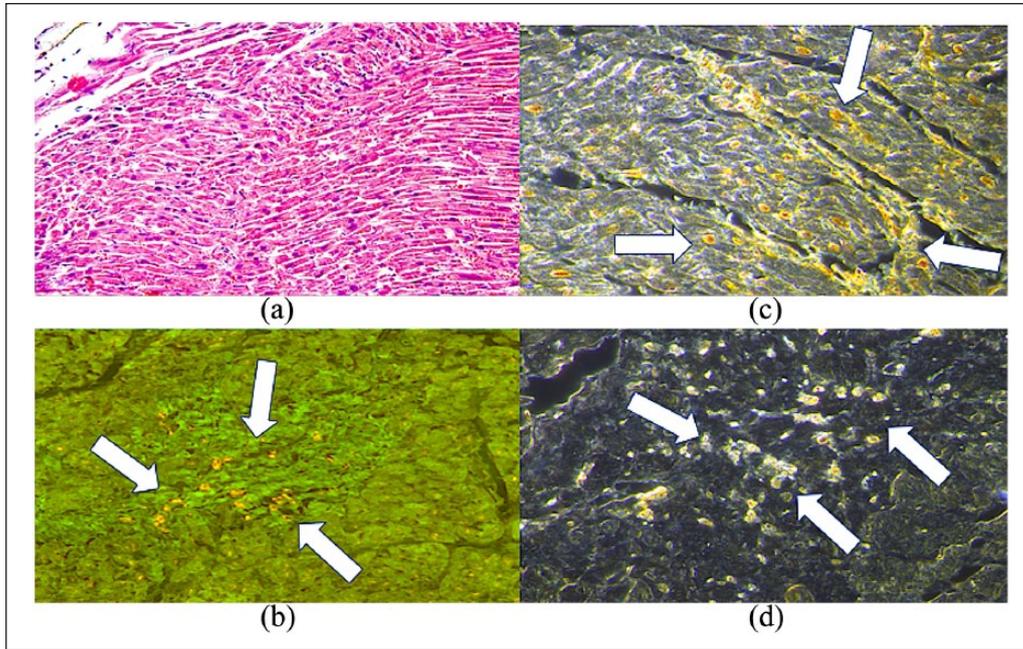
Figure 3. Immunohistochemical essays improve detection of inflammatory infiltrates: (a) myocardium apparently normal (H&E 25×); (b) when processed with neutrophilic granulocyte-specific antibody (anti-lactoferrin antibody), the section shows a microscopic focus (arrows) of myocarditis (dark yellows infiltrate near the center of picture) (25×); (c) analysis by phase contrast (PHACO): diffuse infiltrates (arrows) of uncertain significance in the interstitium (H&E 100×); and (d) immunohistochemical positive reactions (arrows) with evident appearance of an acute inflammatory infiltrate (anti-lactoferrin antibody) (100×).
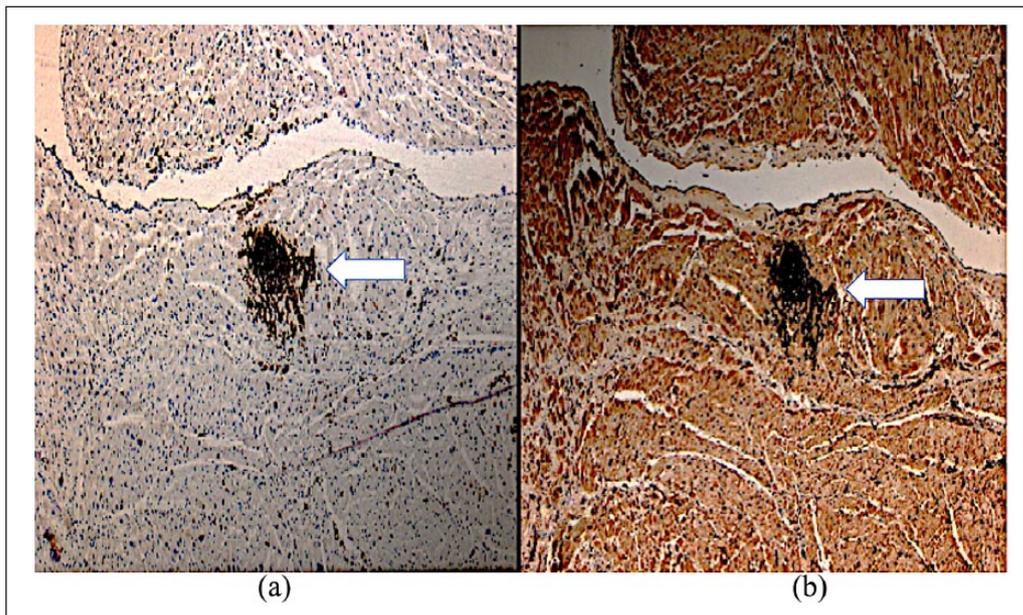

Figure 4. Comparison between CD15 and lactoferrin stainings: (a) anti-CD15 and (b) anti-lactoferrin reactions. Total correspondence (arrows) between the two antibodies immunoreactivity (40×) in the same inflamed area.

Discussion

In the clinical setting, the diagnostic gold standard of sepsis is the laboratory of microbiology as it allows isolating the source of infection and the species of the pathogen organism. Clinicians need reliable tools for an early diagnosis of sepsis rather than for a definite one; they need it especially in order to not delay the necessary antibiotics administration. At the state objective parameters to establish sepsis as cause of

death from the post-mortem examination alone are lacking. When there are not clinical or sufficient circumstancial data about death sepsis can remain unrecognized in pathology casework.

In the clinical setting the definition used for "septic cardiomyopathy" is that of a reversible myocardial depression during septic shock;[6,7] this condition has been described since 1984 and clinical knowledge improved about this kind of dysfunction as new techniques to assess cardiovascular performance has become available. Nevertheless, it was only in the last decade that a focus was pointed on alterations of cardiac cellular phenotype. Although a considerable volume of data has been collected in a recent review about structural changes of heart during severe sepsis,[9] it is still difficult to understand which pathological alteration could work as a specific and sensitive marker. Most of evidence, in fact, comes only from animal models of experimentally induced sepsis.

The pathological records survey conducted in the present study assessed sepsis as the third leading cause of death at our NHS Health Authority; of these, more than the 80% could be ascribed to a HAI (health-care associated infection; data not shown). This fact is consistent with the international landscape. Moreover, the distribution observed in pathogens species is the typical one from a hospitalized cohort, with a plausible prevalence of opportunistic infections. This autopsy series, according to recent research,[10] showed that it was possible to isolate a pathogen organism only in the 35% of cases, and some of them revealed a polymicrobial infection so that it was uncertain which organism caused sepsis and death.

The commonest pathological alteration detected by microscopy with simple H&E staining was generalized inflammation. With routine H&E staining most of cases provided only slight, uncertain evidence, with small infiltrates principally limited to main septum and perivascular areas. A more specific myocarditis pattern could be observed in association with the microbiological evidence of a mycetes-related sepsis. Those cases, in fact, were characterized by septic embolism with fungal material detectable into the lumen of vessels; additionally, they showed an evident infiltrate of polymorphonucleates with intense inflammation of the perivascular areas. As observed elsewhere, our results suggest that mycetes embolism is the specific finding of heart disease in fungal sepsis;

furthermore, in our series, this was the only case which showed an enhanced immunoreactivity with CD54 (ICAM-1).

Dealing with necrosis pattern, as other authors, we observed no association between sepsis and contraction band necrosis or widespread myocytolysis. These findings are in contrast with those made by Schmittinger et al.[11] Probably, this discrepancy is due to the fact that they selected patients from intensive care units treated with prolonged pharmacological cardiovascular support; this may have resulted in a nonspecific stress-induced cardiotoxicity lesional pattern.

We obtained better evidences with immunohistochemical techniques using neutrophils markers, notably LF and CD15. LF is an 80-kDa globular protein belonging to transferring family. In human, it is detectable in various secretory fluids (tears and saliva for instance) but also in the secondary granules of neutrophils. It plays a double immune and antibacterial role as it sequesters iron, which is an essential growing substrate for lots of bacterial species, and it affects outer membrane permeability producing the bacterial cell breakdown.[12] The targeted antibody has been used as a specific immunohistochemical marker for neutrophils. CD15 is an adhesion molecule expressed on membranes of polymorphonucleates. It is specific for this subtype of leukocytes because it is not expressed on lymphocytes.[13] LF had been already validated for the post-mortem diagnosis of sepsis in previous studies. More precisely, the authors described its association with septic lung injury.[14] To the best of our knowledge, CD15 had not been tested yet for a similar purpose, instead. By the way, our results suggest that it can be used in alternative to LF and it could be even preferable, as it provides no background staining. In our series, the addition of these immunohistochemical essays to routine H&E sections proved to be effective in raising the sensitivity of inflammation and infiltrates detection at light microscopy. A generalized inflammatory lesional pattern was hardly recognized in the 32%–42% of cases, while neutrophils immunoreactivity was positive in more than half cases (56%).

We have not obtained satisfactory results with the tested immunohistochemical essays other than LF and CD15. None of them could provide any help in defining a causal relationship between sepsis and death in the specific case examined. Although it is well known that apoptotic cascade

affecting cardiomyocytes may worsen heart performance during sepsis, the staining for caspase-3 used in this study did not show any difference between cases and controls. This is surely due to an intrinsic limit of specificity in the available test.

The alpha isoform of smooth muscle actin is the most commonly used molecular target to mark vascular smooth muscle cells (VSMCs); the antibody targets the N-terminal decapeptide of smooth muscle alpha-actin with no cross-reaction with fibroblast and cardiomyocytes. The lack of positive interstitial staining with alpha-SMA confirms that in case of septic myocardial dysfunction, the tissue inflammation is limited to acute responses without proliferative reactions normally induced by extensive cellular death. This interpretation of the myocardial pathological alterations fits the current conception of septic cardiomyopathy as a fully reversible impairment.

ICAM-1 (CD54) is a transmembrane glycoprotein typically expressed by endothelial and immune system cells. Interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα) enhance its expression in order to mediate leukocyte extravasation. We found no differences between cases and controls with CD54, suggesting that its reliability as a marker for sepsis assessed on pulmonary sections is not extendable to myocardium specimens.[15]

This research remarks the conceptual groove that harmonization and standardization in diagnosing sepsis improve over time as new technologies and markers become available.[3] In this sense, we aim to corroborate the diagnostic value of anti-LF and anti-CD15 staining for the post-mortem detection of myocardial inflammation.

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