UNIVERSITY OF MEDICINE AND PHARMACY "CAROL DAVILA" BUCHAREST

DOCTORAL SCHOOL

FIELD OF MEDICINE

MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL PROFILE OF CELLULAR POPULATIONS INVOLVED IN THE DYNAMICS OF THE IMMUNE RESPONSE IN MYOCARDITIS

SUMMARY OF THE DOCTORAL THESIS

Thesis Supervisor:

Assoc. Prof. Dr. MIHAIL CONSTANTIN CEAUȘU

PhD Student:

OANA NEAGU

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Introduction

The presented study addresses the complexity and challenges related to the diagnosis and treatment of myocarditis, a common but often underdiagnosed condition due to nonspecific and incomplete diagnostic criteria. Myocarditis can have various causes (viral, bacterial, autoimmune, toxic), and the diagnosis is generally made by exclusion, supported by clinical, paraclinical, and imaging evaluations. Current treatment is limited to alleviating symptoms without addressing the underlying cause of the disease.

Although the Dallas histological criteria are considered a standard, they are incomplete and fail to provide a precise diagnosis. The mentioned study aims to improve these criteria by detailed analysis of myocarditis cases, including immunohistochemical testing to correlate morphopathological and immunohistochemical aspects of inflammatory cells with myocardial lesions. The ultimate goal is to facilitate the correct diagnosis of myocarditis subtypes and thus contribute to improving the treatment of this condition.

I. GENERAL PART

1. Myocarditis - General Characteristics

1.1. Clinical Picture and Diagnosis Confirmation

Recent research has demonstrated the effectiveness of combining cardiac magnetic resonance imaging (MRI) with endomyocardial biopsy for the diagnosis of myocarditis. Biopsies taken from areas with increased contrast signal in magnetic resonance imaging (MRI) images allowed the identification of active acute or chronic myocarditis. In cases where access to the contrast-enhanced areas was not possible, biopsies were taken from the right ventricular septum or interventricular septum. The results indicate a strong correlation between MRI findings and the presence of predominantly macrophagic inflammation accompanied by myocardial cell damage [1]. In cases where the biopsy was not taken from the contrast-enhanced areas, active myocarditis was detected in only one case, with the rest of the patients showing signs of myocarditis in remission or dilated cardiomyopathy. Therefore, the diagnosis of myocarditis is a complex process that involves an algorithm that integrates clinical, paraclinical, and imaging information, aiming to exclude other rapidly treatable pathologies and to orient towards possible etiology and appropriate therapy. Specialized cardiology centers use various and costly resources, require qualified personnel, and the main challenge is establishing the pathogenesis and etiology for effective treatment [2]. The difficulty of this process is accentuated by the diversity of agents causing myocardial inflammation and their sometimes contradictory pathogenic mechanisms.

1.2. Immune cells in myocardial inflammation

The immunohistochemical examination of endomyocardial biopsies (EMB) has become the gold standard for diagnosing myocarditis and idiopathic dilated cardiomyopathy (IDC) according to the 2013 guidelines of the European Society of Cardiology. A threshold of ≥14 leukocytes per mm², including up to 4 monocytes and ≥7 CD3+ T lymphocytes per mm², is recommended (Caforio, Pankuweit, Arbustini, Basso, Gimeno-Blanes, Felix, Fu, Heliö et al.,

2013). However, criteria based solely on CD3+ T cells and macrophages underestimate inflammation, as other cell types and adhesion molecules are also relevant for prognosis. Recent literature describes findings from biopsied myocarditis cases, highlighting the presence of lymphohistiocytic infiltrates and a specific population of PD-1, CD8, and granzyme-B positive T lymphocytes [4].

1.3. Pathophysiological mechanisms in myocarditis

The most studied immune response in myocarditis is that induced by a viral infection. To explain the dynamics of inflammation, the process is divided into three main stages. In the first stage, viral replication and damage to cardiac muscle fibers occur, influenced by the virus's tropism and the host's susceptibility. In the second stage, nonspecific inflammatory cells such as neutrophils, macrophages, and NK cells are attracted to the affected tissue to eliminate the virus with minimal impact on cardiac tissue [5]. The third stage, which varies in duration, depends on the efficiency of the immune response; if the virus is not completely eliminated, it may remain latent, leading to long-term changes and cardiac remodeling, affecting ventricular ejection fraction [6]. The pathogenesis mechanisms have been validated mainly in common viral infections such as those caused by Coxsackie B, Parvovirus B19, and HHV6 [7] [8].

2. Relationships Between Immune Cells and Inflammatory Molecules in Myocarditis

IL-6 plays a crucial role in the cytokine release syndrome and the severity of myocarditis, being associated with inflammation and cardiac lesions, including in COVID-19. Studies have shown a significant correlation between serum IL-6 levels and the severity of myocarditis, suggesting that IL-6 could be a potential therapeutic target [9]. However, IL-6 has a complex role, being protective in acute inflammation but potentially harmful in chronic diseases. Future research is essential to clarify its use in the treatment of myocarditis [10].

Cardiac resident macrophages play a crucial role in maintaining tissue homeostasis and the development of coronary arteries. Following an acute ischemic event, they are rapidly replaced by monocyte-derived macrophages that promote inflammation by secreting pro-inflammatory

cytokines such as IL-1, TNF, and IL-6. The CD163 protein present on mature macrophages is associated with the inflammatory response and tissue healing, and its plasma levels increase in various inflammatory disorders [11]. These changes indicate a possible role of CD163 and macrophages in regulating the systemic inflammatory response and cytokine release syndrome.

Neutrophils are well known for their role in cardiac inflammation and damage following an ischemic injury, but the role of monocytes and macrophages in this process remains debated. In cases of lethal myocarditis, 46% present lympho-monocytic inflammation, 8% neutrophilic inflammation, and 46% mixed lymphomonocytic and neutrophilic inflammation. Myocyte necrosis, rare in incidental myocarditis (31%), is very common in lethal myocarditis (84.6%), occurring predominantly in the anterior wall of the left ventricle. Myocarditis is often asymptomatic and occurs in 50% of cases, characterized by pure or mixed lymphomonocytic infiltrates. Neutrophilic myocarditis, although rare, is potentially lethal, highlighting the severity of this early form of inflammation [12].

ORIGINAL PART

3. Study I: Characteristics of Cardiac Inflammation in Myocarditis Associated with Sepsis

3.1 Working Hypotheses and Study Objectives

Neutrophilic myocarditis is often a consequence of severe bacteremia and may be related to skin infections, trauma, or intravenous drug use. It is rarely detected clinically before death and is frequently associated with endocarditis, suggesting that it is underdiagnosed. Positive airway pressure (PAP) ventilation and an inadequate immune response in sepsis may contribute to the development of this condition, which can lead to multiple organ failure. The study aims to characterize cases of neutrophilic myocarditis at autopsy, assess the prevalence of bacterial myocarditis, analyze associated factors, and explore the relationship between bacteremia, sepsis, and myocarditis.

3.2. Materials and methods

This retrospective study, conducted at the National Institute of Forensic Medicine, evaluated myocarditis cases identified in medico-legal autopsies over a five-year period (2014-2019). Of the 11,660 cases examined, 22 cases of myocarditis with acute neutrophilic inflammation and a history of sepsis, confirmed histologically, were included in the study. The autopsies were performed according to the European protocol, with the detailed collection and analysis of tissue samples, including cardiac samples from the left ventricle or interventricular septum. The diagnosis of acute myocarditis was established according to the Dallas criteria, and myocardial inflammation was quantified through microscopic evaluation of tissue sections.

3.3. Results

Over a five-year period, 22 cases (0.18%) were histologically confirmed with neutrophilic myocarditis. The majority of cases involved adults, with a mean age of 41.5 years, and slightly more men than women were affected. The analyzed cases were associated with various conditions such as trauma, severe burns, drug overdoses, dialysis, prosthetic heart

valves, and severe infections. Prior hospitalization was common in 16 out of 22 cases, and positive pressure ventilation was used in nearly half of them. Pulmonary and renal infections were frequently encountered, and post-mortem analysis revealed the presence of antibiotic-resistant pathogens. Microscopic evaluation of cardiac sections revealed a mixed inflammatory infiltrate, dominated by neutrophils, with fewer macrophages and lymphocytes. In most cases, the inflammation was mild, with small dispersed foci, but in five cases, it was more severe, occupying up to 40% of the section. In 36% of cases, myocarditis was associated with bacterial colonies or fungal elements, and septic microemboli were identified in half of these. Myocardial fibrosis and myocyte hypertrophy were observed in 80% of cases, more pronounced in cases with severe inflammation.

3.4. Discussions

Acute neutrophilic inflammation of the myocardium is frequently associated with severe bacteremia; however, in the present study, less than 10% of cases presented with endocarditis, suggesting that bacteremia is the primary cause and not dissemination from a valvular infection. Myocarditis associated with sepsis was predominantly linked to severe injuries, such as trauma or burns, rather than an immunocompromised status. The majority of cases showed small foci of inflammation scattered throughout the myocardium, often associated with septic emboli, supporting the hypothesis of bacterial seeding. The main pathogens identified were Staphylococcus aureus (MRSA), Klebsiella pneumoniae, Acinetobacter baumannii, and Pseudomonas aeruginosa, with fungal infections being rare but significant. Histologically, myocarditis manifested as small foci of inflammation, with bacterial or fungal colonies detected in 36% of cases, and was associated with septic emboli and arteriosclerosis.

3.5. Conclusions

The study suggests that neutrophilic myocarditis is most likely caused by bacterial seeding of the myocardium. Even if blood cultures are sterile, the risk of myocarditis persists, highlighting the importance of precautions in the use of invasive ventilation and maintaining a sterile environment for critically ill patients.

4. Study II: Quantification of Immune Cells and IL-6 in Cardiac Inflammation Using Immunohistochemical Techniques

4.1 Working Hypotheses and Study Objectives

Myocarditis, including subclinical forms, can be a crucial factor in sudden death, especially in individuals without prior cardiac history or those who abuse drugs, contributing to malignant arrhythmias or acute heart failure. Lymphocytic inflammation, specific to viral myocarditis, although usually self-limiting, can become lethal in the presence of arrhythmias. Macrophages and elevated levels of IL-6 are associated with severe inflammation and myocardial necrosis, suggesting that IL-6 could be a marker of severity and a potential therapeutic target. The study aims to correlate histological findings with the causes of death and analyze the histological and immunohistochemical characteristics of myocarditis, assessing myocardial inflammation and necrosis using specific markers

4.2 Materials and methods

This retrospective and observational study analyzed 77 autopsy cases from the National Institute of Forensic Medicine 'Mina Minovici' Bucharest between 2014 and 2022, focusing on histologically confirmed myocarditis. Cases were selected based on the presence of myocarditis, excluding those with advanced autolytic changes. The histopathological analysis included the evaluation of myocardial inflammation, necrosis, and cardiac remodeling, using a scoring system to classify the severity of inflammation and necrosis. The study employed statistical tests such as Mann-Whitney U and Spearman's correlation coefficient to analyze the collected data.

In this study, 26 out of the 77 myocarditis cases were evaluated immunohistochemically using the markers CD3, CD163, and IL-6. CD3 was used to identify and quantify T lymphocytes, CD163 for macrophages, and IL-6 to assess the presence and number of pro-inflammatory cells. IL-6, a cytokine essential in myocardial inflammation, was detected in the cytoplasm of various immune cells. Neutrophils and eosinophils, cells involved in the nonspecific immune response,

were morphologically identified and quantified. This detailed evaluation provided a better understanding of the severity and inflammatory characteristics in myocarditis.

4.3. Results

4.3.1. Demographic Aspects of the Studied Group

The study analyzed 77 cases of myocarditis, of which 65% were men and 35% women, with a mean age of 52.62 years. Approximately 25% of cases were classified as borderline myocarditis. Men were slightly younger than women at the time of death. Severe myocarditis was frequently associated with severe burns and sepsis, and pneumonia/bronchopneumonia was diagnosed in 40% of cases, mainly affecting younger patients. Patients with burns showed significantly greater severity of myocardial necrosis compared to those without burns, highlighting a strong association between nosocomial infections, sepsis with a cutaneous/mucosal point of origin, and major cardiac dysfunction at the time of death. Drug abuse, frequently encountered in forensic pathology, is associated with multiple chronic and opportunistic infections, complicating the patients' health condition. In the studied group, 14.29% of cases (n=11) involved drug addicts, who were significantly younger than non-users, with a mean age of 31.09 years.

4.3.2. Histopathological Aspects of the Myocardium in the Studied Group

The inflammatory infiltrate in myocarditis varies depending on the distribution and density of inflammatory cells, being classified on a scale from 0 to 4, where 0 represents insignificant inflammation, and 4 represents massive inflammation. The majority of cases had minimal severity inflammation (40.26%). A strong correlation was identified between the severity of inflammation, analyzed quantitatively per section, and the number of inflammatory foci per section, thus validating the system proposed in the study with that known in the literature [13].

The study highlighted a strong correlation between the severity of inflammation and the severity of necrosis in myocarditis, showing that severe inflammation is associated with

extensive necrosis ($p \leq 0.001$). Additionally, cases with thrombi or emboli presented significantly more severe inflammation than those without these vascular complications. The presence of microbial colonies in myocarditis foci was also associated with marked or extensive inflammation ($p \leq 0.001$). These findings underscore the important role of vascular and infectious complications in exacerbating myocardial inflammation, highlighting the need for careful diagnosis and management of these conditions in the context of myocarditis.

The results indicate an association between sudden death and minimal severity myocardial inflammation ($p=0.003$). Furthermore, according to the Dallas criteria, the majority of these cases were classified as borderline myocarditis (42.1%). Histologically, arteriosclerosis-type changes (84.2%) and interstitial fibrosis of at least grade 1 (94.7%) were detected. In contrast, non-sudden deaths presented inflammation of all grades, with a predominance of moderate and severe severity.

The study analyzed the presence of T lymphocytes in post-mortem myocarditis, observing that in 15.4% of cases, CD3 was absent, and the inflammation was dominated by neutrophils, often associated with pneumonia. There is a strong correlation between the decrease in CD3 levels and the increase in the presence of neutrophils, emboli/thrombi, and microbial colonies ($p \leq 0.001$, $p=0.013$, $p=0.021$). Additionally, the severity of inflammation and myocardial necrosis was inversely proportional to the CD3 level. In the context of certain medical conditions, such as pneumonia, subacute infarction, endocarditis, and drug abuse, the CD3 level was lower, indicating a different local immune response.

Macrophages, essential in the immune response, were identified using the CD163 marker in myocarditis cases, with values ranging from 10% to 70%, and an average of 31.92%. Women showed slightly higher levels of CD163 than men. In cases with pulmonary infections and prolonged hospitalization, macrophages dominated the inflammation. CD163 levels were lower in the presence of microemboli and infarction but increased in bacterial or fungal myocarditis. An inverse correlation was observed between CD163 levels and neutrophils, suggesting different roles for these cells in the immune response ($p=0.020$). The study's results indicate that patients with severe myocarditis had significantly higher levels of CD163 ($p=0.040$), suggesting a potential role for this marker in the pathogenesis of severe forms of

myocarditis. Conversely, patients who suffered sudden death had lower levels of CD163, with T lymphocytes dominating the inflammation ($p=0.049$).

The study evaluated IL-6 expression in cardiac tissue, observing that its levels increased with the severity of inflammation ($p=0.028$). In borderline myocarditis cases, IL-6 expression was minimal, while in severe myocarditis, expression was significantly higher, suggesting a potential therapeutic role for anti-IL-6 molecules in managing severe myocarditis. IL-6 expression in myocarditis is predominantly associated with macrophages and neutrophils. A moderate inverse correlation was observed between the number of T lymphocytes and IL-6 levels, indicating that myocarditis with a dominant lymphocyte population has lower levels of IL-6 ($p=0.005$). These results suggest that IL-6 blocking treatments, such as tocilizumab, may be less effective in borderline or mild myocarditis cases, where lymphocytes are the predominant inflammatory cells.

4.4. Discussions and Conclusions

The study analyzed cases of myocarditis observed microscopically, predominantly diagnosed in men in their fifth or sixth decade of life, often with an altered immune response due to chronic conditions or prolonged hospitalization. A younger subgroup, consisting of drug users, frequently presented with severe pulmonary infections and neutrophilic myocarditis. Sudden death observed in this cohort was associated with mild myocardial inflammation, mainly lymphocytic. Inflammation was evaluated using quantitative methods, and immunohistochemical techniques helped identify the dominant inflammatory cells, such as T lymphocytes and macrophages. Myocarditis with neutrophil-rich infiltrates and severe necrosis was frequently associated with bacterial or fungal infections.

The retrospective study highlights that the CD3 marker, specific to T lymphocytes, is not always essential in the post-mortem diagnosis of myocarditis, as many cases reflect complications related to sepsis, endocarditis, or a compromised immune status. In contrast, the CD163 marker, specific to macrophages, has significant diagnostic and prognostic importance, with higher levels correlated with severe myocarditis. Additionally, the cytokine IL-6 was identified as an indicator of the severity of inflammation and myocardial necrosis, suggesting its potential as a marker for assessing disease severity and guiding therapeutic interventions.

5. Study III: Etiology of Myocarditis and the Pattern of Myocardial Inflammation in Sudden Cardiac Deaths in the Pediatric Population

5.1. Working Hypotheses and Study Objectives

The study aimed to identify the etiology, particularly viral, which is common among the young population. Viral myocarditis manifests histologically through subtle inflammatory infiltrates, predominantly of T lymphocytes, especially CD4+. The objectives included a detailed examination of inflammation and myocardial lesions, correlating them with clinical data, and identifying the etiological agent through histopathological, immunohistochemical, and microbiological analyses of cardiac tissue.

5.2 Materials and Methods

A total of 813 pediatric autopsies were investigated over 17 years, confirming 23 cases of acute myocarditis as the primary cause of death. The examinations included detailed histological analyses, immunohistochemistry for phenotyping the inflammatory infiltrate, and real-time PCR for detecting common viral genotypes. The diagnosis of myocarditis was established according to the guidelines of the European Society of Cardiology. Clinical and pathological data were centralized to identify links between histological, genetic, and infectious causes, highlighting the utility of PCR in diagnosing latent viral infections associated with heart disease.

5.3 Results

The majority of children who died from myocarditis were under 2 years old, with a median age of 1.77 years, and a male-to-female ratio of 1.4:1. Nearly half of the deaths occurred in the autumn and winter seasons, but without a significant connection to the viral season. Viral myocarditis was predominant, accounting for 70% of cases, with Enteroviruses and Parvovirus B19 being the most frequently identified. Other identified causes included Streptococcus pyogenes and hypersensitivity myocarditis, while in four cases, the etiology remained unknown.

Typical prodromal symptoms included vomiting, respiratory distress, and lethargy, appearing on average three days before death.

Immunohistochemistry applied in six cases of myocarditis used specific markers to identify the type and extent of inflammation. The markers CD45, CD3, CD4, and CD8 showed the predominance of T helper lymphocytes (CD4+) in viral myocarditis, with cytotoxic T lymphocytes (CD8+) detected only in isolated cases. In idiopathic myocarditis cases, the extended analysis of B lymphocytes (CD20 and CD79) did not reveal significant results. In diffuse forms of myocarditis, CD68-positive macrophages were the predominant cells, confirming the presence of a lymphoid and histiocytic infiltrate in all cases.

5.4. Discussions and Conclusions

The histological analysis of myocarditis cases associated with sudden death in children provides essential information about the etiology and prevalence of this pathology. The retrospective study, using PCR and immunohistochemistry techniques, highlighted that infection with Parvovirus B19 is frequently associated with a diffuse inflammatory infiltrate and fulminant myocarditis. In the case of Enteroviruses, death occurred in the neonatal period, within the first days of life (median age of 8 days). Macrophages predominated in cases where viral genetic material was not detected, underscoring the importance of cellular phenotyping. The diagnosis of viral myocarditis requires the integration of clinical, pathological, and microbiological data, and viral infections remain the primary cause of myocardial inflammation in children. Acute myocarditis accounts for approximately 3% of all non-violent sudden deaths in the pediatric population.

6. Final Conclusions and Personal Contributions

In the first study, neutrophilic myocarditis, previously insufficiently documented, is addressed by highlighting and quantifying myocardial inflammation and lesions. The results identify the link between neutrophil-dominated cardiac inflammation and the septic state, indicating that neutrophilic myocarditis is likely caused by bacterial seeding of the myocardium, often associated with trauma or burns. I also emphasized the importance of integrating the clinical picture into the histological evaluation, which improves diagnostic accuracy and allows the identification of pathophysiological mechanisms and associations with clinical impact. These contributions can influence future approaches in diagnosis and treatment, especially in cases of sepsis and polymicrobial infections.

The second study succeeded in achieving the research objectives by identifying significant correlations between cardiac inflammation and various clinical conditions, such as arteriosclerosis, drug use, and infections. The study demonstrated the advantages of using immunohistochemistry and quantitative analyses of myocardial inflammation and necrosis to better understand the pathogenesis and dynamics of inflammatory cells in myocarditis. Personal contributions include proposing a subclassification of active myocarditis, developing scoring systems for quantifying the severity of inflammation and necrosis, and integrating immunohistochemistry techniques for identifying inflammatory cell populations. These approaches have allowed the identification of relevant correlations that influence the evolution of myocarditis, thus providing a basis for personalized and adaptable diagnostic and treatment strategies for critical cases.

The study on cases of sudden pediatric death associated with myocarditis highlighted that the etiology of cardiac inflammation is predominantly viral, with Enteroviruses and Parvovirus B19 being the main causes. By using advanced molecular biology techniques such as PCR and RT-PCR, the study was able to identify patterns of inflammation and correlate morphological and epidemiological data. Immunohistochemistry was essential for the precise definition of the cell types involved in inflammation, underscoring the role of T lymphocytes and macrophages in the inflammatory response to viral myocardial infection. Personal contributions of the research include detailed documentation of myocarditis cases in sudden

pediatric death, the development of inflammation models, the execution of a semi-quantitative analysis, and the integration of immunohistochemistry and molecular biology techniques for an in-depth documentation of myocarditis pathogenesis.

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