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# THE INFLAMMASOME IN ACUTE MYOCARDITIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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## ABSTRACT

### THE INFLAMMASOME IN ACUTE MYOCARDITIS

By Harsha Ramanujam Kannan, BS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2013.

Sponsor: Antonio Abbate, MD, PhD, Department of Internal Medicine

Acute myocarditis is an acute inflammatory syndrome characterized by myocardial damage and dysfunction often due to a viral infection followed by a variable development over time. There are currently no specific treatments and standard treatments for heart failure are generally applied. The inflammasome is a recently identified macromolecular structure that occupies a central role in the amplification of the inflammatory response and promotion of cell death during acute and chronic infections. We hypothesized the formation of the inflammasome in acute myocarditis. To investigate, samples of patients were collected from the *Cardiomyopathy Registry in Trieste*, with 12 cases of biopsy-proven myocarditis and 11 cases of autopsy-proven myocarditis stained for major components of the inflammasome through immunofluorescence; 10 of the 12 (83.3%) biopsy cases and 8 of the 11 (72.7%) autopsy cases presented formation of the inflammasome in a variety of cells including resident cells (i.e. cardiomyocytes, endothelial cells, fibroblasts) and infiltrating cells (i.e. leukocytes) while varying in intensity and distribution. Control samples of 5 subjects not presenting with any acute cardiac events showed no formation of the inflammasome. While further studies should look to elucidate the correlation of inflammasome-formation and progression of disease, this finding paves the way for further insight into the pathophysiology of acute myocarditis.

## INTRODUCTION

### ACUTE MYOCARDITIS

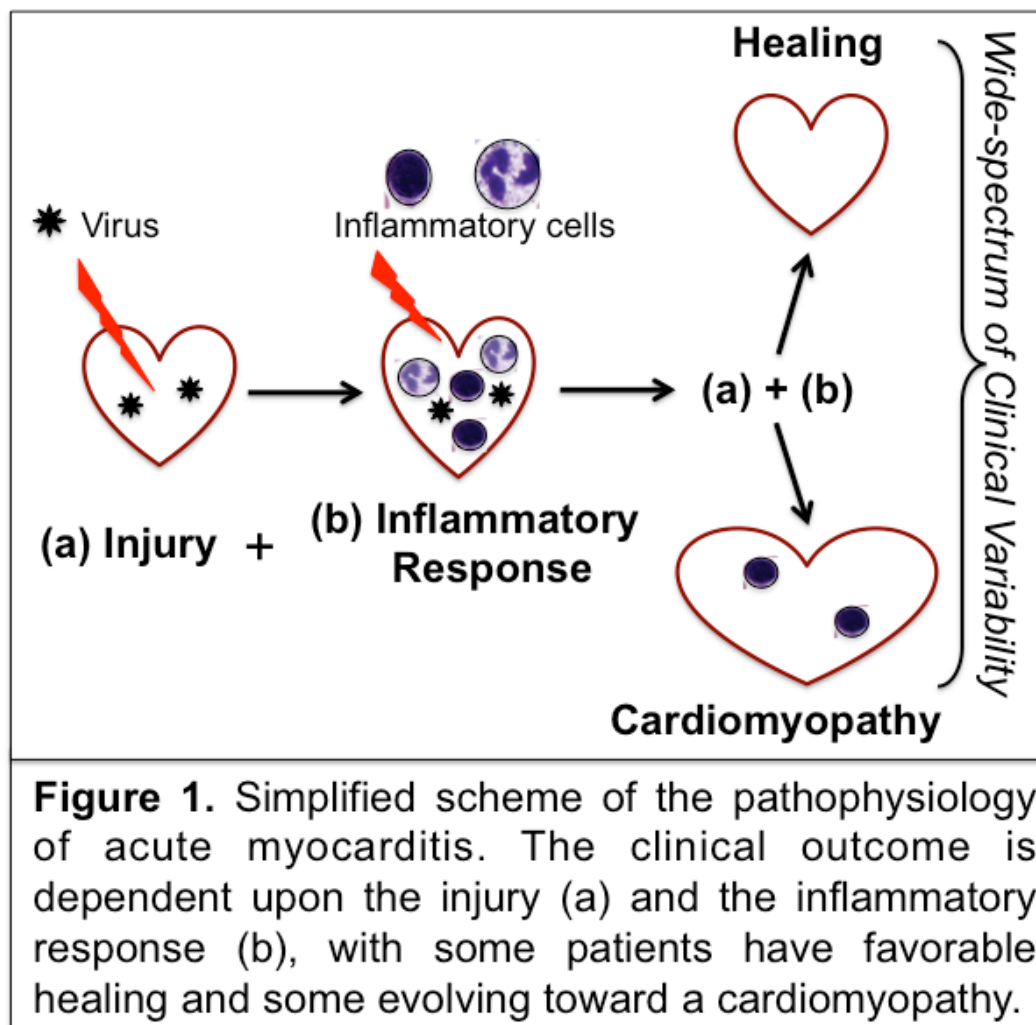
Acute myocarditis is an inflammatory disorder of the heart muscle.<sup>1</sup> Such definition covers a long list of clinical conditions in which the heart muscle (myocardium) is *primarily* involved in an acute inflammatory response that is not secondary to myocardial ischemia or toxic injury.<sup>1-3</sup> Acute myocarditis refers both to the pathologic condition and the clinical manifestation (disease).

The causes for this inflammatory response are many and vary from infectious (primarily viral, but also bacterial) to non-infectious (including 'allergic' and autoimmune).<sup>1-3</sup> The variability in etiology is paralleled by a large variability in the clinical presentation, ranging from asymptomatic to cardiogenic shock and sudden death.<sup>1-3</sup>

What is common to all these conditions is the presence of (a) myocardial injury + (b) an ensuing inflammatory response. The manifestation and severity of the disease is dependent on (a) and (b) and involves the intensity and nature of the injury and the response (Figure 1).<sup>1-4</sup>

As such, acute myocarditis involves both cardiomyocyte dysfunction as a result of the initial virally mediated event as well as tissue injury due to subsequent immune

response. The immune-mediated response in reaction to the viral component leads to myocardial inflammation, necrotic events, and ventricular dysfunction.

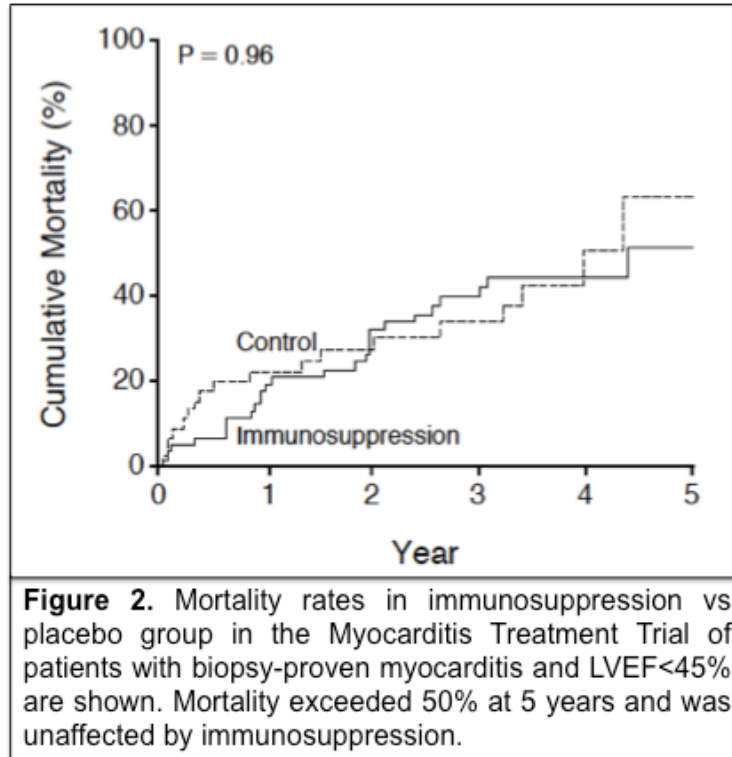


The exact incidence and prevalence of myocarditis is unknown.<sup>1-3</sup> It is often under-recognized or mis-diagnosed.<sup>1-3</sup> This is consequential of the fact that many affected individuals may be asymptomatic at onset while others may be for the entire duration of the disease. It is estimated that 10% of patients with unexplained idiopathic cardiomyopathy, 50% with end-stage heart failure, and approximately



25% of all heart transplantations are due to myocarditis.<sup>5-7</sup> Myocarditis is also found at autopsy in 5-20% of cases of sudden unexplained deaths in young adults.<sup>8-9</sup> Finally, acute myocarditis mimics acute myocardial infarction and pericarditis, and while the literature is full of reports, the true incidence is unknown due to lack of systematic studies.

The natural history of acute myocarditis also shows a wide-spectrum of clinical variability.<sup>1-4</sup> Of patients with acute myocarditis, nearly 50% experience an improvement, whereas the rest will experience worsening cardiomyopathy and heart failure.<sup>1-4,11-12</sup> The Myocarditis Treatment Trial published in 1995 included 111 patients with biopsy-proven myocarditis and depressed left ventricular ejection fraction (LVEF, mean 25%).<sup>13</sup> Patients had paired echocardiograms over 6 and 12 months and were then followed for 5 years for survival. While there was a mean improvement in LVEF of approximately 10%, most patients continued to have depressed LVEF, and the 1-year and 5-year mortality rates were a remarkable 20% and 56% respectively (Figure 2).<sup>13</sup> A more recent prospective longitudinal study enrolling all-comers with biopsy-proven acute myocarditis published in 2012 showed an approximate 5-year mortality of 20%, with patients with lower LVEF being more likely to die.<sup>14</sup> These data show that despite improvements in the treatment of cardiomyopathy, patients with acute myocarditis are at greatly increased risk of death, especially if LV function is depressed.



Acute myocarditis is usually suspected on the basis of the clinical presentation but the diagnosis requires either confirmation with an endomyocardial biopsy (EMB) providing tissue for histologic assessment or with cardiac magnetic resonance which may show a characteristic pattern. Often the diagnosis is also supported by pertinent negative findings excluding other causes (i.e. ischemia). Pathological observation of acute myocarditis is defined by a leukocytic infiltrate associated with cardiomyocyte necrosis (in the absence of ischemia or toxic injury). Cardiotropic virus detection can be performed by PCR techniques and *in situ hybridization* to help identify cell types replicating viral genomes.

At present day, there are no specific treatments for acute myocarditis, and thus standard treatments for heart failure are generally applied. The Myocarditis Treatment Trial randomized patients to immunosuppressive therapy with

prednisone with cyclosporine or azathioprine for 6 months but failed to show an improvement in LVEF or survival (Figure 2).<sup>13</sup> A recent meta-analysis of clinical trials showed similar results on outcomes despite a small effect on LVEF.<sup>15</sup> Immunosuppressive treatment sees a more liberal use in Europe but is reserved to few specific cases in the USA.<sup>1-4</sup> Antiviral treatment revolves around interferon- $\beta$  and interferon (IFN)- $\alpha$ 2 therapy, which in animal studies has shown to protect cardiomyocytes against injury and decrease inflammatory cell infiltrates. However, only IFN- $\beta$  showed effective clearance of the viral load. Initial studies with treatment with IFN- $\beta$  in selected patients with myocardial enteroviral or adenoviral persistence and LV dysfunction has shown to eliminate the viral genomes in those patients exhibiting these conditions and lead to improved LV function.<sup>3</sup>

Specific risk stratification tools are also lacking in acute myocarditis. The commonly explored markers of immunopathology have failed to show a clear prediction in patients with biopsy-proven myocarditis.<sup>9</sup> Similarly novel methods of detection have shown frequent presence of viral genome in biopsies<sup>16</sup> but have failed to link a specific virus or the persistence of genome over time with prognosis.<sup>17-18</sup> In some, but not all studies, lack of viral genome and presence of autoantibodies predicted response to treatment.<sup>19-20</sup>

The pathophysiologic mechanisms leading to successful healing and *restitution ad integrum* in some cases, or with impaired healing, fibrosis, and cardiomyopathy in others, are poorly understood.<sup>1-4,20-21</sup> The initial injury often involves an infection

by a virus or intracellular bacterium. These cardiotrophic infectious pathogens determine an initial injury by replication within the cardiomyocytes. The cellular injury induces an inflammatory response characterized by an acute infiltrate of leukocytes to the heart primarily consisting of lymphocytes. Cytotoxic lymphocytes as well as cytotoxic antibodies are involved in the progression of the injury, whereas regulatory lymphocytes and neutralizing antibodies appear to be involved in protection <sup>4,20-21</sup>. These factors, however, seem to poorly explain and poorly predict outcome and treatments directed specifically at lymphocytes (prednisone, cyclosporine, azathioprine) or autoantibodies (plasma derived immunoglobulins) have been disappointing.

In conclusion, acute myocarditis is a rather common disease, with an unfavorable outcome, no specific therapies, poorly understood pathophysiologic mechanisms, and paucity of clinically useful predictors. It follows that there is an urgent need for further research in this area. In this study, we addressed the role of a new pathophysiologic mechanism with potential implications for diagnosis, prognostication, and therapy.

## THE INFLAMMASOME

The inflammasome is a recently identified macromolecular structure activated during cellular and tissue injury that amplifies the inflammatory response.<sup>22-23</sup> During infection or injury, pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) are sensed and lead to the triggering of the inflammasome. This step has been shown to represent an essential process in the initiation of the local and systemic inflammatory response in virtually all forms of infectious or sterile tissue injury. Viral infections are known to activate the inflammasome and the outcome of the infection is often determined by this interaction leading to either death of the infected cell (pyroptosis) and virus, or survival of both cell and virus.<sup>24</sup> Hence, while viruses are inducers of the inflammasome *per se*, viral proteins may inhibit the inflammasome (“evasion”).<sup>24</sup> It has been recently shown that during sterile ischemic injury to the heart the inflammasome promotes further damage.<sup>25</sup> Others have shown that the same occurs in other cardiomyopathies.<sup>26-28</sup> Yet there has been no report investigating the inflammasome in acute myocarditis. Understanding the pathophysiology of the inflammasome formation in the heart during acute myocarditis may provide clues into the clinical variability of acute myocarditis.

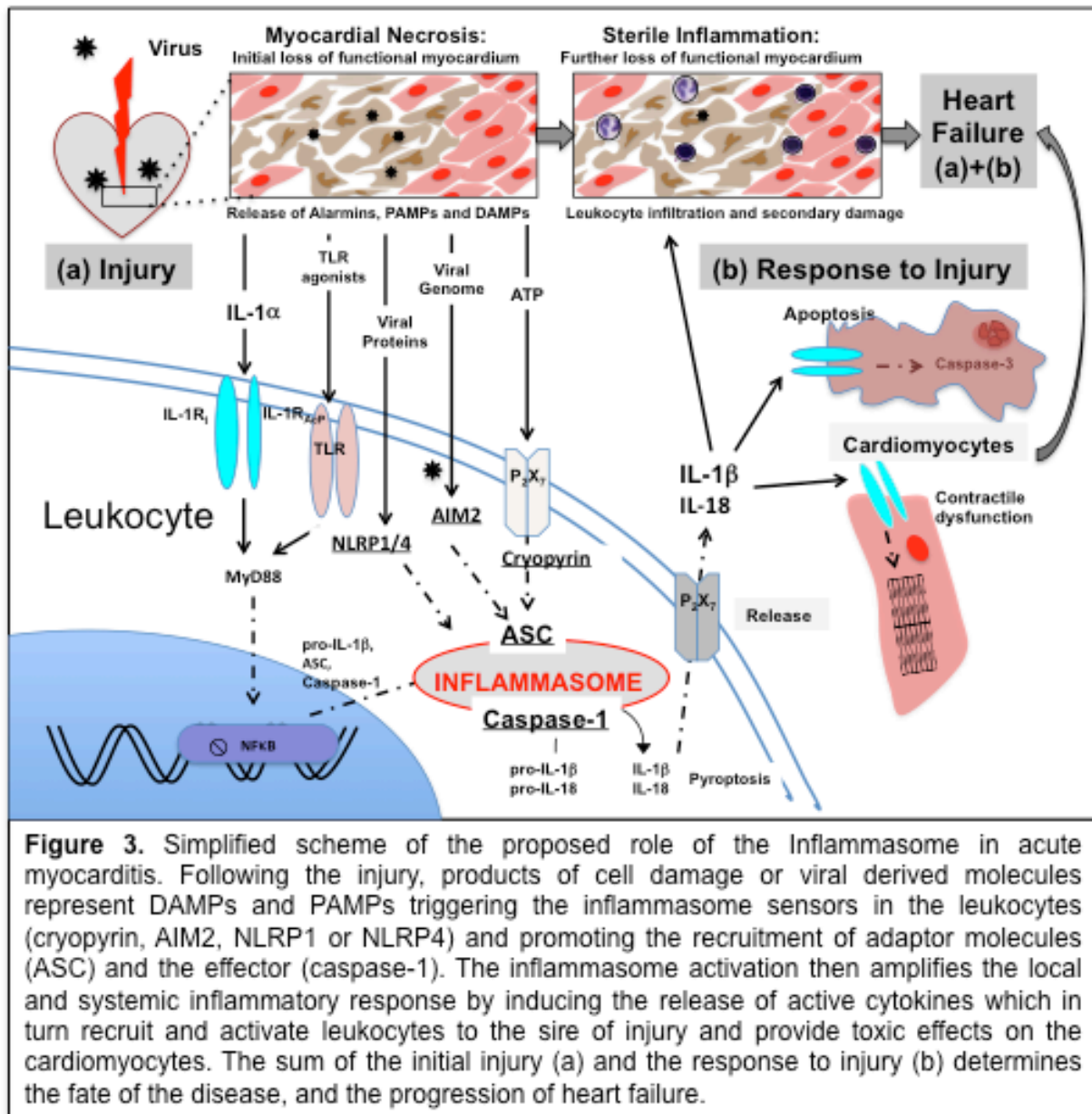
The structure and function of the components of the inflammasome can be summarized with the presence of a sensor (that directly or indirectly binds PAMPs or DAMPs), an adaptor (scaffold), and an effector (enzyme).<sup>22-23</sup> While there are several interchangeable sensors, caspase-1 is the common effector protein, and

defines the inflammasome. Caspase-1 (also called interleukin-1 $\beta$  converting enzyme) leads to the maturation of key pro-inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-18 (IL-18), and induces cell death by pyroptosis.<sup>29</sup> The apoptosis-associated speck-like protein containing a caspase-1 recruiting domain (ASC) is the most common adaptor serving as a scaffold for the inflammasome specks (visible at light microscopy)(Figures 3-4).<sup>22-23,30</sup> Many of the effects of the inflammasome are mediated by the release of active IL-1 $\beta$  and IL-18.

Recent pharmaceutical developments in this area have further stimulated the interest in the inflammasome, IL-1 $\beta$ , and IL-18 in many clinical conditions ranging from rare hereditary diseases related to mutations in inflammasome genes to more common illnesses like rheumatoid arthritis, gout, and diabetes.<sup>31-32</sup> These disparate conditions have in common the property of being “auto-inflammatory”.<sup>33</sup> Great interest in the inflammasome is also evident in the infectious disease literature especially inherent to virus and bacteria.<sup>24,34</sup>

*We therefore hypothesize that the viral infection or the ensuing cellular destruction seen during acute myocarditis leads to the formation and activation of the inflammasome (Figure 3). Identifying a role of the inflammasome would provide an opportunity to explore inflammasome-related markers for diagnostic or prognostic purposes. Furthermore inhibitors of IL-1 $\beta$  and IL-18 are available and ready to be tested, while novel inhibitors are being developed. Recent clinical studies have*

been completed, with 3 pilot clinical trials showing a favorable effect of anakinra (an IL-1 blocker) in patients with acute myocardial infarction or heart failure.<sup>35-37</sup>



## APPROACH

In this study, we investigated the presence of inflammasome in tissue samples obtained through autopsy or endomyocardial biopsy in patients with acute myocarditis. These experiments are made possible by the existence of a unique, well-designed and maintained Cardiomyopathy Registry held in Trieste, Italy, in which all patients with acute myocarditis are identified and followed prospectively, and in which heart specimens derived from endomyocardial biopsies or autopsies are stored for future studies.<sup>10,38-42</sup>

The *Cardiomyopathy Registry in Trieste* was started on January 1, 1981 and it is still current. Between January 1981 and December 2012, the Registry included 98 patients with biopsy-proven myocarditis (or autopsy-proven myocarditis if the patient deceased before the biopsy). All patients underwent clinical evaluation with laboratory testing, ECG, ECG Holter, echocardiography, coronary angiogram and endomyocardial biopsy (with classical pathology analyses). All patients received standard heart failure treatments according to guidelines, and were followed after 6 and 12 months; those with heart failure symptoms or evidence of abnormal heart dimensions or function were followed every 6 months up to 48 months. Endomyocardial biopsies were obtained from the left and right ventricles. Samples were stored in formalin and then embedded in paraffin for histology and immunopathology, or snap frozen with liquid nitrogen for identification of viral genome. Mean age of the patients is 37, with 70% being males. The mortality



rates at 2 and 5 years were 11% and 17%, respectively. Approximately half of the patients presented with heart failure, and these were more likely to have depressed LVEF, with a much lower 5-year transplant-free survival (73%).

We determined the presence of inflammasome aggregates in the different cell types (cardiomyocytes, leukocytes, fibroblasts, and endothelial cells).

## SELECTION OF THE CASES

As explained earlier, acute myocarditis is a heterogeneous syndrome with wide variability in the modality of onset, severity of illness, cause and prognosis. In order to select a more homogeneous population for this study, we included 12 cases of patients with acute myocarditis according to the Dallas criteria.<sup>43</sup> We excluded patients with a longer duration of symptoms as well as asymptomatic patients, as this would limit our insight into the natural history of the disease. We excluded patients with eosinophilic myocarditis, giant-cell myocarditis, sarcoidosis, HIV cardiomyopathy, fungal, and protozoal myocarditis, since all these conditions are uniquely characterized by either a mechanism of disease, a specific and rather effective therapeutic strategy, or additional morbidities. This provided a rather homogenous cohort of cases with viral or autoimmune myocarditis of recent onset. Also included were 11 patients diagnosed at autopsy (fatal cases) for comparison.

## SELECTION OF THE CONTROLS

In order to evaluate the specificity of our findings, we included controls in which, according to our hypothesis, staining was not expected to present formation of the inflammasome. Five subjects were enrolled as controls and presented with chronic cardiac disease but without acute myocarditis or other acute cardiac event who died and underwent autopsy.

## PROCESSING OF THE SAMPLES

The cases were identified in the registry, and the histological slides were prepared in Trieste, Italy, before being shipped to our laboratory at Virginia Commonwealth University. The slides were identified with a progressive number and not with the identity of the subject. The team in Trieste coded the samples in a way that the investigators in Virginia could not distinguish the identity of the cases.

## IDENTIFICATION OF THE INFLAMMASOME IN THE HEART

We employed immunofluorescence staining using antibodies raised against ASC (Sigma Aldrich, PRS2287, rabbit polyclonal) and caspase-1 (Sigma Aldrich, C4851, rabbit polyclonal; Novus Biological, 14F468, mouse monoclonal). The rationale for using ASC or caspase-1 as main markers for the inflammasome is that although multiple sensors may be involved, the effector caspase-1 and adaptor protein ASC are by far the most common components of the inflammasome. Based on our hypothesis, we expected to see inflammasome

“specks” as peri-nuclear cytoplasmatic aggregates in the majority of heart samples with differing degrees of intensity and presence in different cell types including leukocytes, endothelial cells, fibroblasts and cardiomyocytes.

To characterize the cellular pattern we used a double staining technique with antibodies against S100A4 (Sigma Aldrich, 7493P1, goat polyclonal) and CD31 (Cell Signaling, #3528, mouse monoclonal) to recognize fibroblasts and endothelial cells respectively, plus cardiac actin (Sigma Aldrich, A9357, mouse monoclonal) as a marker to denote cardiomyocytes.

## STAINING OF THE SAMPLES

Staining of samples occurred over three days, the first of which involved rehydration of samples, antigen retrieval, and application of primary antibody for ASC or caspase-1. Samples were taken through three changes of xylene, 5 minutes each, two changes of 100% ethanol, and changes through 95% and 70% ethanol for a minute each before being placed in running tap water to remove paraffin embedding and rehydrate. Antigen retrieval was performed using a steamer with samples placed in 0.01M citrate buffer pH 6.0 for 20 minutes. Blocking to minimize non-specific binding used 1% normal donkey serum in PBS for 15 minutes. Application of primary antibody diluted 1:50 (ASC or caspase-1) in PBS was left to incubate overnight in 4°C.

Second day involved washing samples of the primary before application of fluorescence-conjugated secondary antibody diluted 1:100 in PBS incubated for four hours at room temperature. The second primary antibody, marking cell type, was diluted 1:100 in PBS, applied, and left to incubate overnight (4°C). The secondary antibody of a different fluorescence wavelength was applied and left to incubate for four hours on the third day (RT).

Autofluorescence due to lipofuscin-like pigments is seen in the heart and central nervous system.<sup>45</sup> The sarcomere may also be a source of autofluorescence.<sup>46</sup> To account for this and ensure distinction of positive staining for only ASC and caspase-1, we used a solution of 1% Sudan Black in 70% Ethanol for five minutes following the application of the final secondary antibody, quenching the nonspecific fluorescence without compromising the signal of the specific antibodies.<sup>47</sup> Nuclear staining was done with 4',6-diamidino-2-phenylindole (DAPI) 1:1000 for 5 minutes and samples were cover slipped with a tris-glycerol mounting media.

## ASSESSMENT OF THE INFLAMMASOME IN THE HEART

All the final images of samples were acquired with an IX70 microscope and cellSens Dimension digital imaging software (both Olympus) using a 40x objective (400x magnification). Filter sets from the software for each separate fluorescence-conjugated antibody were layered to render a color composite image.

Observation of “specks” was counted and categorized by cell type (cardiomyocyte, non-cardiomyocyte residents, and leukocytes) per field of view (40x).

## STATISTICAL ANALYSIS

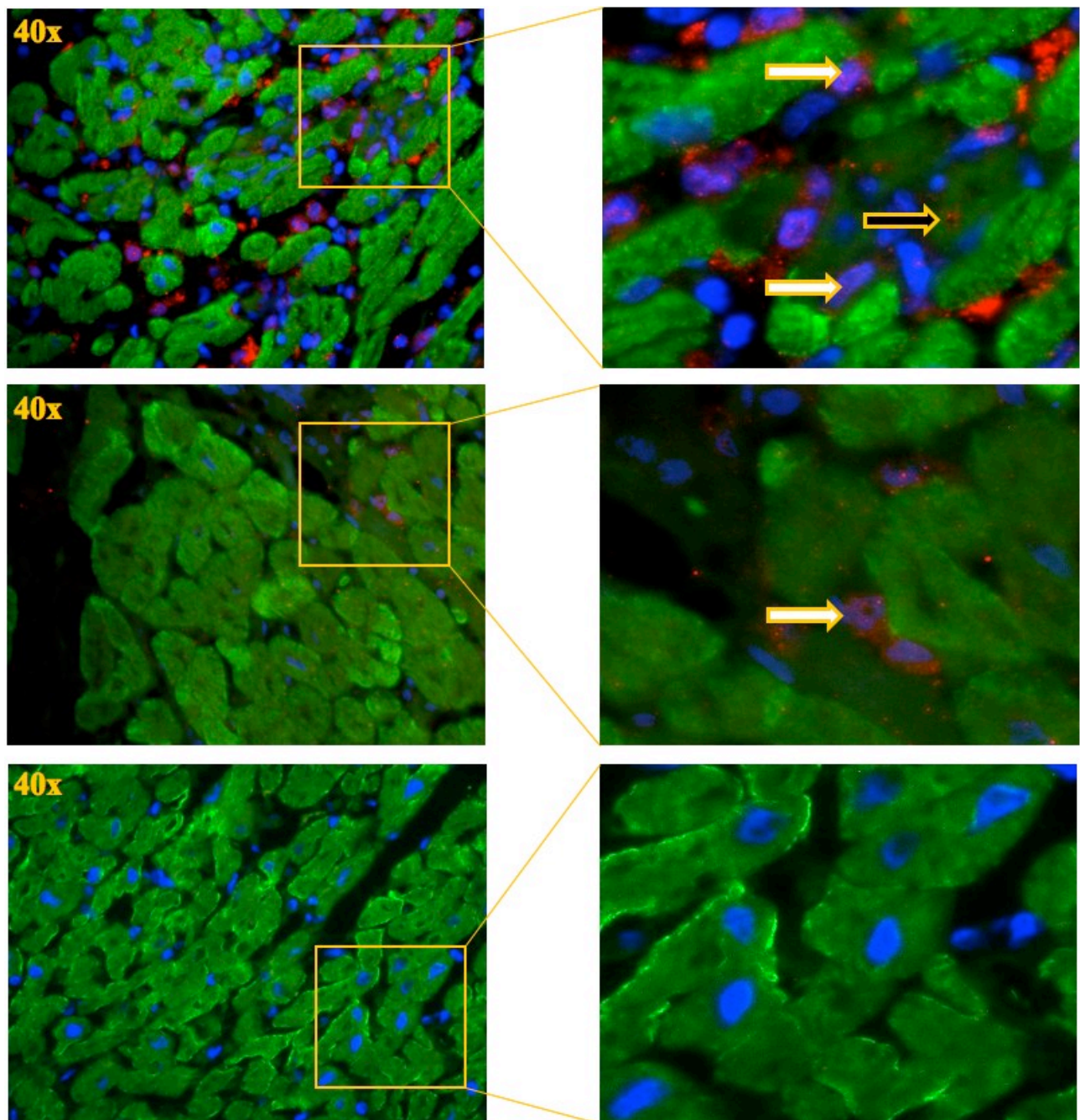
All data are presented as median and interquartile range; non-parametric tests were used because of a potential deviation from a Gaussian distribution. The SPSS statistical package 19.0 was used. The Mann-Whitney test was used to compare continuous variables between 2 different groups (i.e. cases vs controls), the Wilcoxon test was used to compare paired variables within one given group (i.e. necrotic region vs remote region of the heart), the Spearman rank test was used to define correlation between two variables (i.e. inflammasome specks and left ventricular ejection fraction). For each measurement, the effect we were interested in detecting was 2SDs from control. Therefore, a sample size of 6 in each group had a 80% power to detect an effect size of  $\delta=|\mu_1-\mu_2|/\sigma=2$  using an estimate from a t-test (2-sided,  $\alpha=0.05$ ). Multiple comparisons reduces the accuracy; as such we maintained a target  $P<0.05$  for this phase with the understanding that validation will be required.

## RESULTS

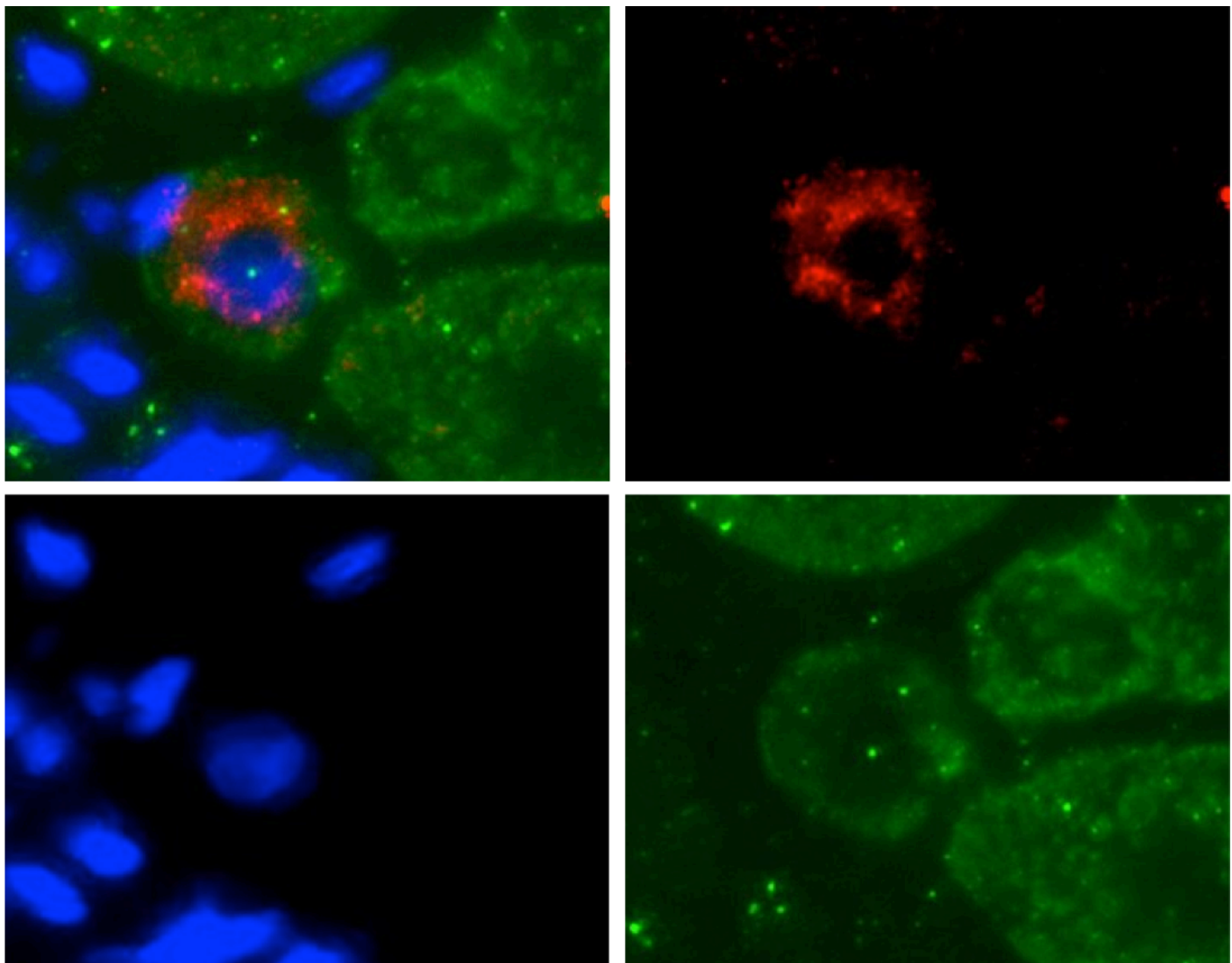
In this preliminary phase we studied 12 patients with biopsy-documented acute myocarditis deriving, 11 cases in which the diagnosis of fatal acute myocarditis was made post-mortem, and 5 cases of patients affected by chronic heart disease but not of acute cardiac issues selected at autopsy, also in Trieste.

Formation of the inflammasome was detected using immunofluorescence for ASC, the scaffold protein that appears as an aggregate, speck(s), during inflammasome formation (Figure 4-7). Confirmation was made by colocalization of ASC and effector caspase-1, definitive of the inflammasome (Figure 8-10).

The inflammasome was detected in the heart in 10 of 12 cases (83.3%) of biopsy-proven myocarditis as well as in 8 of 11 cases (72.7%) of post-mortem diagnosis. Five cases of non-myocarditis samples showed no presence of the inflammasome. In all but 3 cases of post-mortem diagnosis and 2 cases of biopsy-proven myocarditis, few inflammasome specks (median 2, range 1-5) per high power field (HPF, x40) were seen, and mostly in cardiomyocytes, endothelial cells, or fibroblasts. Two cases of fatal myocarditis showed intense staining for the inflammasome (30-40 per HPF), mostly in the leukocytes (Figure 11).

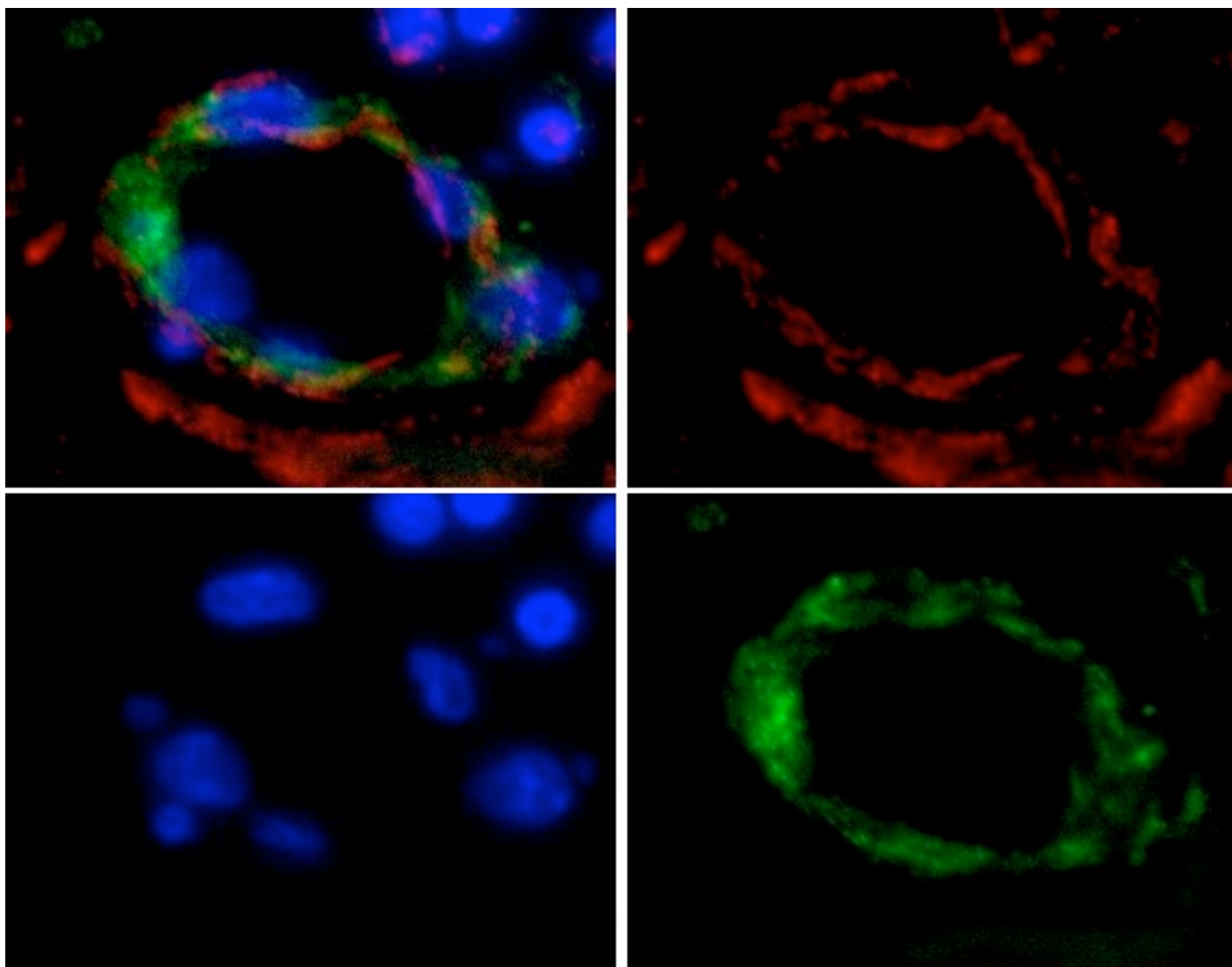


**Figure 4. ASC-cardiac actin co-staining in immunofluorescence.** ASC (red) conjugated with Alexa Fluor 594, cardiac actin (green) with Alexa Fluor 488, and nuclear staining (blue) with DAPI. White arrows point to ASC-positive infiltrating cells (e.g. leukocytes). Black arrow points to ASC-positive cardiomyocyte. Bottom panel illustrates staining of a control sample with no acute cardiac events.

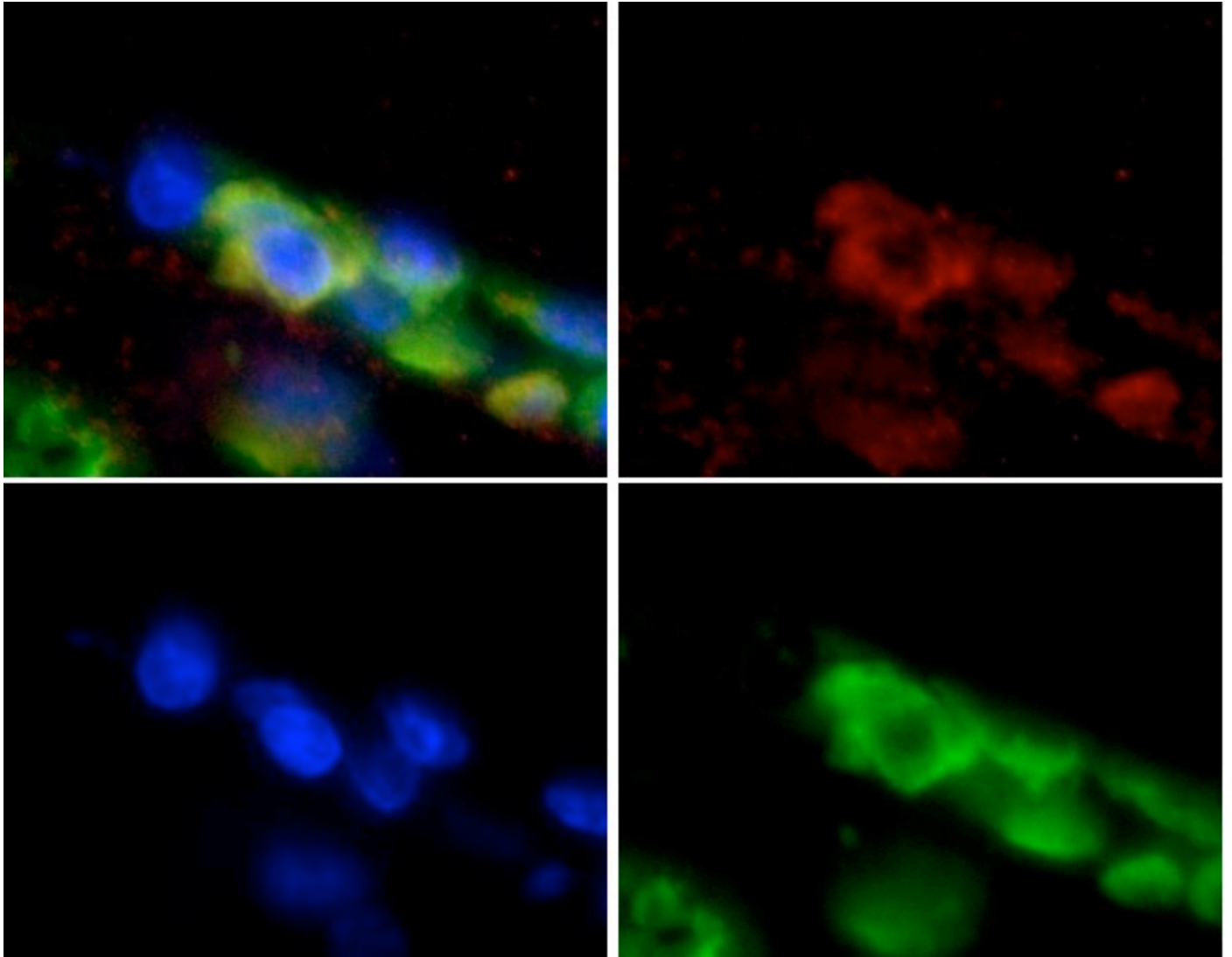


**Figure 5. ASC-cardiac actin co-staining in cardiomyocytes.** Panels shows a cardiomyocyte positive for ASC (red) and cardiac actin staining (green), with nuclear staining by DAPI (blue).



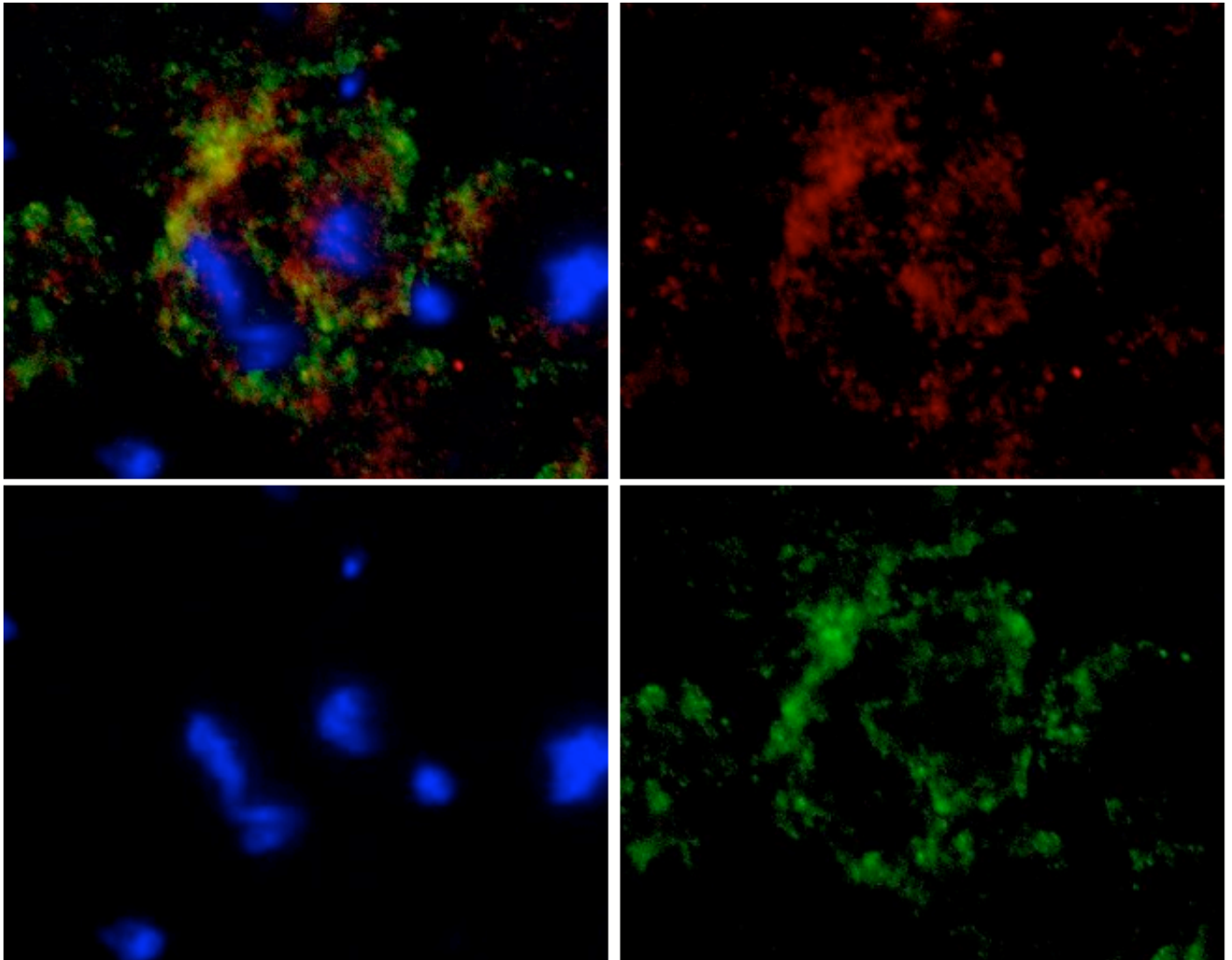


**Figure 6. ASC-caveolin co-staining in endothelial cells.** Panels show endothelial cells positive for ASC (red) and caveolin (green), with nuclear staining by DAPI (blue).

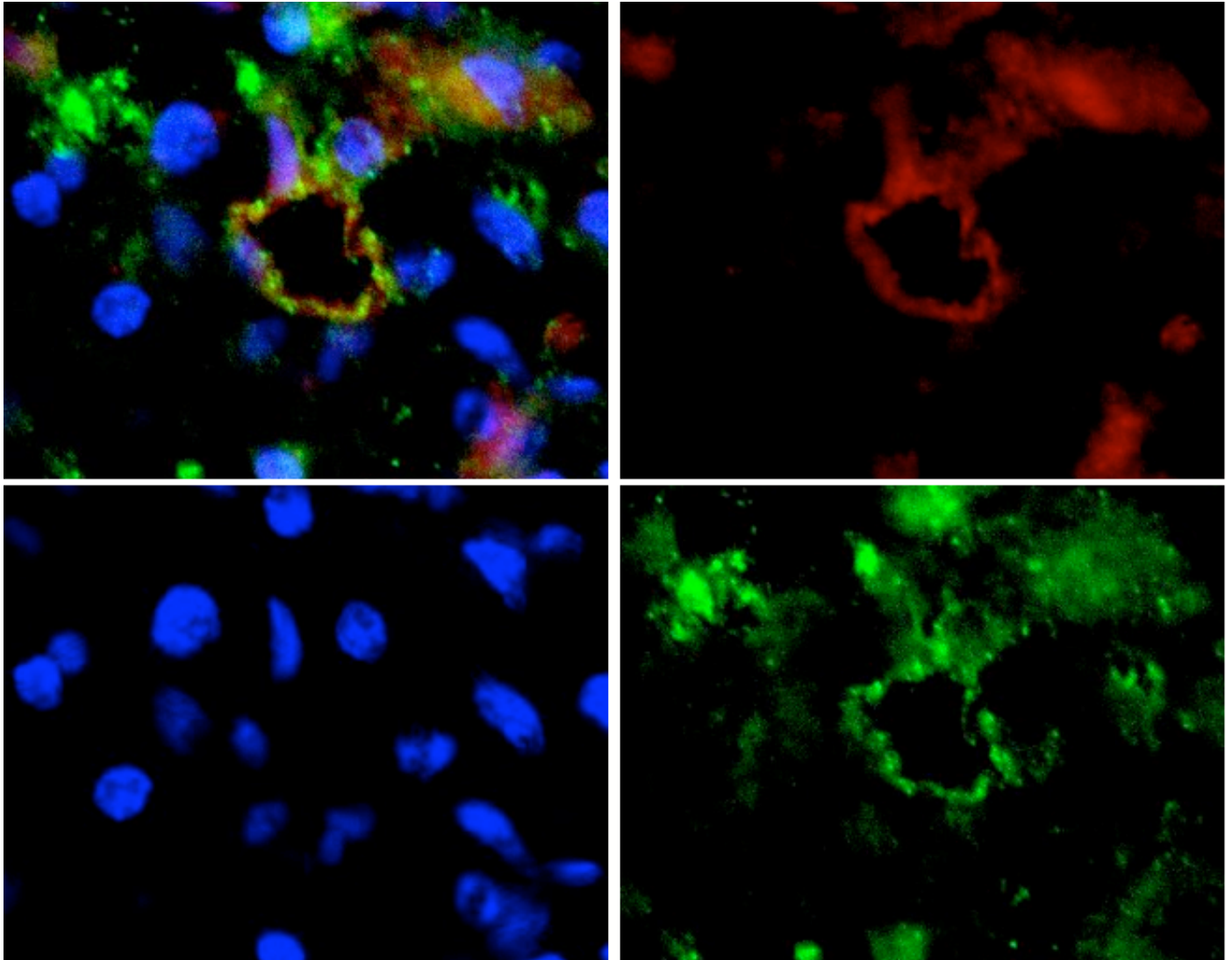


**Figure 7. ASC-S100A4 co-staining in fibroblasts.** Panels shows fibroblasts positive for ASC (red) and S100A4 (green), with nuclear staining by DAPI (blue).

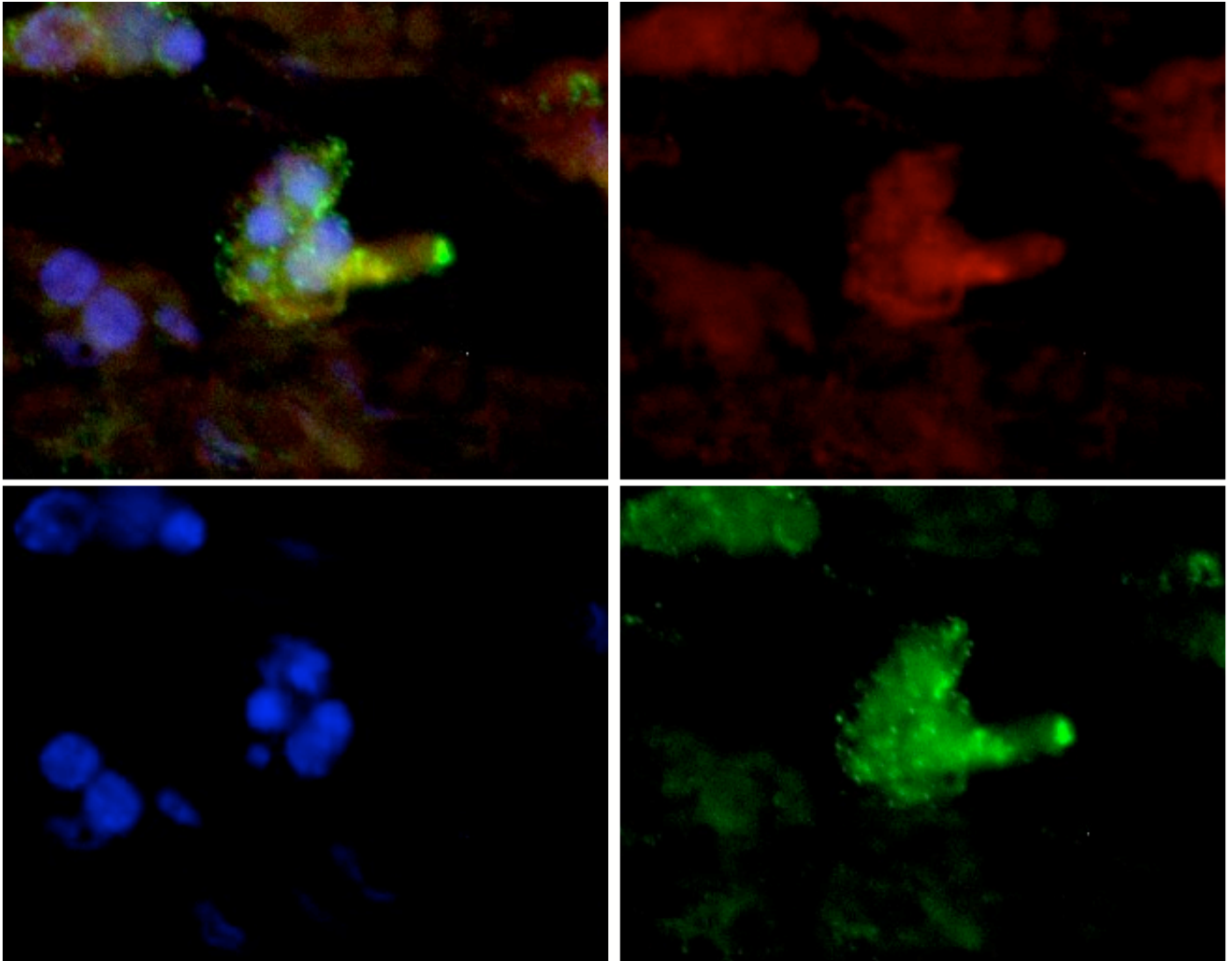
We used co-localization of ASC and caspase-1 to determine the presence of aggregates. The specific antibody used for caspase-1, however, recognized both the pro-form and cleaved form of caspase-1; hence observation of pro-caspase-1 was seen also to be constitutive in endothelial cells and leukocytes, independent of ASC expression.



**Figure 8. ASC-caspase-1 co-staining.** Panels show co-localization (yellow) of ASC (red) and caspase-1 (green), with nuclear staining by DAPI (blue).



**Figure 9. ASC-caspase-1 co-staining.** Panels show co-localization (yellow) of ASC (red) and caspase-1 (green), with nuclear staining by DAPI (blue).



**Figure 10. ASC-caspase-1 co-staining.** Panels show co-localization (yellow) of ASC (red) and caspase-1 (green), with nuclear staining by DAPI (blue).

# ASC-Positive Cells/Field

Biopsy Autopsy Non-myocarditis controls

Myocarditis

**Figure 11.** Quantitative assessment of total number of ASC+ cells per field of image (40x magnification). Averages were taken across 5 images collected from each sample and plotted. ASC+ cell count per field (40x magnification) expressed as median and inter-quartile range per cell category divided by biopsy and autopsy samples, and control samples.

## DISCUSSION

Acute myocarditis remains a poorly understood pathology, with minimal elucidation of the mechanism of action and subsequent deficiency of specific therapies leading to a critical outcome. Herein we present a potential new therapeutic target in the form of the macromolecular complex inflammasome.

Myocarditis can be infectious in origin or non-infectious. Viral infection and subsequent activation of the inflammasome has been studied in previous years.<sup>50</sup> Antiviral immunity has been linked to three distinct inflammasomes: the NLRP3 inflammasome, RIG-1 inflammasome, and AIM2 inflammasome. These variants all involved the adaptor protein apoptosis-associated speck-like protein containing a CARD, or ASC, as a link between the sensor and pro-form of caspase-1 that ultimately leads to cleavage of pro-inflammatory cytokines IL-1 $\beta$  and IL-18. The active forms of these cytokines are coupled to observation of infection, inflammation, and autoimmune processes.<sup>50</sup> It followed that the likelihood of inflammasome formation in acute myocarditis with viral origin was high, a phenomenon previously left unstudied.

Samples collected biopsy or autopsy of patients confirmed to have myocarditis were delivered from the *Cardiomyopathy Registry in Trieste*. Tissues were embedded in paraffin for preservation. Due to the restrictive nature of sample arrangement, most appropriate approach was to utilize immunofluorescence

staining. Previous studies have demonstrated this approach to be a viable and reliable method of assessment.<sup>25</sup>

Upon staining, the presence of the scaffold protein ASC was seen in varying degrees of both intensity and distribution (Figure 4) in cardiomyocyte, fibroblast, and endothelial cells (Figure 5-7) as well as in non-resident infiltrating cells (i.e. leukocytes). As an instrumental component of the inflammasome complex, ASC+ staining suggested the presence of the inflammasome in the cases of myocarditis and not in controls.

ASC+ staining in different cell types demonstrated the presence of the inflammasome in a wide array of cells. Using caspase-1 staining we confirmed the presence of another essential component of the inflammasome necessary for pro-inflammatory cytokine cleavage.

To confirm the incidence of the inflammasome, co-staining for ASC and caspase-1 was conducted for observation of the aggregates in relation to each other – co-localization indicated formation of the aggregates (Figure 8-10).

For comparison that the observations seen were unique to those with myocarditis, samples from patients with no acute cardiac issues were stained for ASC. Negative staining in these control subjects indicated that ASC (and the inflammasome) are not expressed in the heart, in absence of injury (Figure 11).



What remains is observation of the progression of myocarditis and end-stage result in linkage with inflammasome presence and activity.

The pathophysiologic role of the inflammasome in acute myocarditis is however unclear. Further studies are needed to determine whether the intensity of the inflammasome shows correlation to severity of illness at presentation and/or with progression to the cardiomyopathy. If the intensity of the inflammasome should correlate only with the severity of illness initially but remain inconclusive for prediction or is not determinant of clinical progression, inflammasome-targeted strategies could be directed selectively towards patients with hemodynamically unstable acute myocarditis with a high risk of death acutely but potentially favorable recovery with survival (fulminant myocarditis). If the inflammasome intensity should not correlate with the severity of the disease or should relate to even a milder form of the disease, this could suggest that the inflammasome is a protective phenomenon (i.e. virus clearance).<sup>24,34</sup> In this event, it suggests the use of inflammasome-related therapies (i.e. IL-1 $\beta$ /IL-18) to favor healing. If further findings suggest the inflammasome in myocarditis to follow the current paradigm – that is the inflammasome as an adverse remodeling agent in the heart – it follows that classifying the inflammasome (i.e. particular sensor: NLRP3 vs RIG-1 vs AIM2) will be greatly beneficial in adding another target of therapy in addition to currently existing modes of treatment.<sup>35-37</sup>

Still it stands that inhibition of the inflammasome may present itself as a potentially “double-edged sword.” If inhibition of inflammasome should lead to a weakened myocardium following injury, it stands that inflammasome disruption will not be an effective method of treatment, and perhaps inhibition of the products of the inflammasome, i.e. Interleukin-1 $\beta$  may be preferable.<sup>51</sup> In experimental acute myocardial infarction, selective blockade of IL-1 $\beta$  protects the heart from apoptosis and ameliorates remodeling without intervening in formation of the active inflammasome, inhibiting tissue inflammatory response, or disturbing infarct healing following AMI.<sup>51</sup> Its relevance here is the suggestion that IL-1 $\beta$  blockade does not impair tissue response in such conditions as microbial infections.

## CONCLUSIONS

This study shows for the first time the presence of the inflammasome in the heart of patients with myocarditis. This study identifies a potential new pathophysiologic mechanism in acute myocarditis opening the way to improved understanding and potential improved prognostic stratification and treatment of the disease.

The inflammasome was seen in the heart in 78% of samples with an intensity ranging from mild to intense. The pathophysiologic role of the inflammasome formation in the heart during acute myocarditis is however unknown. The next step will be to accurately quantify the intensity of the formation of the inflammasome and analyze for the presence of statistical correlations with the clinical characteristics of the patients or the outcomes.

As of 2013, acute myocarditis remains a poorly understood disease with unacceptably high morbidity and mortality rates. Identifying an effective and safe anti-inflammatory therapy for acute myocarditis represents, therefore, an *urgent unmet medical need*. Therapies directed at IL-1 $\beta$  and IL-18 (products of the inflammasome) are already being tested in clinical trials for non-cardiac as well as cardiac diseases (other than myocarditis), and novel specific inflammasome inhibitors (small molecules) are being developed.

We can foresee 3 main possible outcomes: 1) the intensity of the inflammasome correlates with the severity of illness at presentation and with progression to the cardiomyopathy – this would support studying inflammasome-targeted treatment; 2) the intensity of the inflammasome correlates with the severity of illness initially but does not predict clinical progression – this would support studying inflammasome-targeted strategies in selected patients with hemodynamically unstable acute myocarditis who have a high risk of death acutely yet if they survive have a more favorable recovery; 3) the inflammasome does not correlate with the severity of the disease or even associates with a milder form of the disease – this would suggest a protective role of the inflammasome (i.e. virus clearance)<sup>24,34</sup> and suggest the use of inflammasome-related therapies (i.e. IL-1 $\beta$ /IL-18) to favor healing.

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