# **Supplemental File to Ms. No. EURJHF-22-535R1 “Myocarditis Follow-ing COVID-19 Vaccine: Incidence, Presentation, Diagnosis, Patho-physiology, Therapy, and Outcomes put into Perspective»**

1. **Pathology of Myocarditis following COVID-19 vs COVID-19 Vaccine**

Cardiotropic viruses, such as coxsackieviruses (**Figure 6a in main document**), parvovirus B19 and some herpes viruses including human herpesvirus 6 and Epstein-Barr virus have long been known to induce myocarditis.1,2 Only recently, it has become apparent that infections with coronaviruses including both SARS-CoV and SARS-CoV-2 (**Figure 6b** and **6c in main document**) may induce myocarditis.3

A variety of pathological mechanisms have been implicated in the induction of myocarditis in the course of SARS virus infections (see also below 2.).3-6 Our current understanding is mainly based on findings on EMB and heart tissue samples obtained at autopsy. Immunohistochemical staining of myocardial tissue of patients who died of SARS-CoV infection revealed a significant amount of CD68+ macrophages, whereas infiltration by CD3+ T cells was minimal.7 Similar observations were reported among patients with SARS-CoV-2 infection.3,8,9 Whereas lymphocytic myocarditis was present in only 14% (n=3) of patients who died with COVID-19, interstitial macrophage infiltration was noted in 86% (n=18) of cases.10

In EMB samples of patients with SARS-CoV-2 infection cardiac CD68+ macrophages were found to upregulate C1q and CD163. Moreover, deep phenotyping of EMB tissue from COVID-19 patients revealed a substantial upregulation of MAPK-associated pathways and of the complement system.9 Despite the fact that in electron microscopy studies SARS-CoV-2 viruses were visualized in endothelial cells,3 cardiomyocytes11 and macrophages12 mostly in the early viremic phase of the infection, it is not clear whether SARS-CoV-2 can directly induce cardiac damage and, subsequently inflammation. A direct virus-mediated injury of any type was not substantiated in 40 hearts of patients who died of COVID-19. Indeed, all hearts exhibited evidence of both, pre-existing chronic and acute damage, but only one heart showed signs of myocarditis.13

As stated above, myocarditis associated with mRNA COVID-19 vaccines has been seen especially in young men.14,15 Maladaptive immune and inflammatory responses, delayed hypersensitivity, molecular mimicry and eosinophilic inflammation have been suggested as possible mechanisms.16 However, in most cases, EMB was not performed because of rapid clinical improvement and, thus a conclusive causality between vaccine receipt and development of myocardial inflammation remains uncertain.16 Interestingly, in those cases where EMB-derived histological data were available, different types of myocarditis have been reported. In two cases with fulminant myocarditis which developed 2 weeks after BNT162b2 mRNA vaccination an increased number of macrophages, T-cells, eosinophils, and B cells was found. However, a direct causal relationship was not established as no testing for viral genomes or autoantibodies in the tissue specimens had been performed.17 Histopathological observations in a case of fatal fulminant necrotizing myocarditis in a 57-year-old woman were interpreted as an extremely rare idiosyncratic hypersensitivity reaction following the first dose of the BNT162b2 mRNA vaccine (Pfizer-BioNTechR)18. On the other hand, in EMB-derived specimens of two other BNT162b2 mRNA vaccinated patients, myocyte necrosis, lymphocytes and macrophages, but no eosinophils, consistent with lymphocytic myocarditis, had been observed.19,20 The absence of anti-SARS CoV-2 Spike protein antibodies in one of these patients20 does not support the hypothesis of cross reactivity of antibodies against SARS-CoV-2 Spike protein with myocardial antigens (i.e. molecular mimicry). Therefore, acute myocarditis is rarely diagnosed in heart tissue following mRNA vaccination. In 97 male patients with clinically suspected myocarditis following mRNA vaccination, 7 cases with acute lymphocytic myocarditis (mean age: 34.1 years), one with acute eosinophilic myocarditis (28 years of age) and 25 with a mild healing or chronic lymphocytic myocarditis (mean age: 33.1 years), were diagnosed based on EMB (K. Klingel, unpublished data 2022). In patients with myocarditis following vaccination consistently more macrophages than T cells were observed, similar to findings in patients with COVID-19 (**Figure 6d** and **6e in main document**). It has to be stressed that, so far, no criteria exist which clearly demonstrate that in these cases myocarditis is primarily induced by a mRNA COVID-19 vaccine and not by other preexisting conditions including typical cardiotropic viruses, systemic immune-mediated diseases, toxic drugs or genetically determined autoimmune processes.21

1. **Pathophysiology of Vaccine-related Myocarditis**

It is important to note that the 4 most widely used vaccines in the West licensed by the regulatory authorities, i.e. BioNTech/PfizeRr (BTN162b2/Comirnaty; EMA: 21.12Ambas.20), ModernaR (mRNA-1273/ Spikevax; EMA: 6.1.21), as well as the adenoviral vector-based vaccines of AstraZenecaR (AZD1222/ChAdOx1-S/Vaxzevria; EMA: 29. 1.21) and JanssenR (Ad26.COV2.S; EMA: 11.3.21), encode very similar forms of the SARS-CoV-2 Spike glycoprotein, which mediates virus binding to the host cell membrane and entry into host cells via angiotensin converting enzyme 2 (ACE2) and transmembrane serin protease 2 (TMPRSS2), respectively.22 However, vaccines based on mRNA and adenoviral vectors have different molecular delivery mechanisms. The mRNA vaccines encode a modified fusion-stabilized Spike RNA sequence encapsulated in lipid nanoparticles that are taken up by cells after intramuscular injection. The mRNA cargo is released into the cytosol and the protein translation process occurs in the rough endoplasmic reticulum (ER), resulting in membrane-anchored Spikes. After post-transductional modifications in the ER and Golgi apparatus, the glycosylated Spike proteins are transported in vesicles to the outer cell membrane. On the other hand, adenoviral vector-based vaccines deliver the Spike sequence as a codon-optimized DNA sequence instead of RNA. These vaccines are based on replication-incompetent adenoviruses. After transduction of the host cell, the double-stranded DNA is released and enters the nucleus where genes become transcribed by means of the host cell transcription machinery. The resulting mRNA molecule is subject to post-transcriptional RNA modifications and splicing, as endogenous primary transcripts. The proposed mechanisms by which SARS-CoV-2 vaccines could induce myocarditis involve activation of both innate and adaptive immune responses against the SARS-CoV-2 Spike glycoprotein, but also the recognition of the mRNA itself as an antigen by the immune system.15 As, similar to viral myocarditis, SARS-CoV-2 postvaccine myocarditis occurs predominantly in young men, sex hormones may play a role in genetically susceptible individuals (**Figure 7 in main document**).23,24 Indeed, viral myocarditis has been associated with genetic variants in genes encoding for different HLA factors, but also for structural proteins of the heart.25

After vaccination, follicular T cells activate the transcription factor T-bet eliciting Th1 responses.26 T-bet plays an indirect role in the activation of protective neutralizing antibodies after infections with influenza A or lymphocytic choriomeningitis virus (LCMV)27 and in the development of autoimmune and cardiovascular conditions, including myocarditis.28 In addition, T-bet represses the inhibitory receptor PD-1 in LCMV infection,29 being PD-1 protective against inflammation and in experimental autoimmune myocarditis.30 Single cell RNA-sequencing (scRNA-seq) analysis revealed that CD8+ T cells have low expression of exhaustion-linked genes (PDCD1, Tim-3, CD160, LAG3, CD244 and CTLA4) in individuals after mRNA vaccination. Activated HLA-DR+CD38+CD4+ T cells and cytotoxic molecules are specifically activated after mRNA vaccination, but not in asymptomatic or mildly symptomatic convalescent individuals.31 In addition, studies of the antigenic specificity of these cells towards Spike proteins revealed that the mRNA vaccine promotes Th1 and Th17 responses, the latter being characteristic in the development of myocarditis.32,33

After vaccination, B cell activation triggers the production of specific IgG antibodies to the Spike glycoprotein that prevent SARS-CoV-2 binding to the host cell via its receptor ACE-2. Cross-reactivity between antibodies to SARS-CoV-2 Spike glycoprotein and cardiac autoantigens has been described34. Molecular mimicry between the Spike glycoprotein and structurally identical protein sequences, including myosin heavy chain, may trigger an autoimmune response to cardiac antigens and potentially induce myocardial inflammation.34 scRNA-seq analysis revealed a reduction in IgA+ memory B cells following vaccination.35 Autoantibodies against interleukin-10 (IL-10) and interferon γ have been detected in COVID-19 patients with severe symptoms, and previous reports indicate a cardioprotective effect of these cytokines in humans and rodents.36,37 IgM autoantibodies against several common antigens, including troponin C1 (TNNC1) and IL-1 receptor, were elevated after vaccination and after SARS-Cov-2 infection, which is to be expected given the presence of cardiac injury and inflammation present in both disease scenarios38.

Although the above-mentioned pathophysiological mechanisms seem to be most likely responsible for the development of myocarditis in susceptible individuals, it is possible that other mechanisms are being activated as well. It was assumed that one of the most relevant causes might be a cross-reactivity between antigens that recognize the S protein of the virus and cardiac self-antigens; however, recent data seems to disprove this and suggests innate immunity as a possible cause of myocarditis after vaccination.39

To induce efficient immunity, a vaccine must combine pathogen-specific immunogenic epitopes with a non-specific adjuvant activating antigen-presenting cells, which in turn promotes expansion of pathogen-specific T-cells. This adjuvant effect may also promote expansion of potentially heart-reactive T cells in susceptible individuals and explains why certain vaccines are associated with an increased, albeit small incidence of myocarditis or pericarditis. In fact, mRNA can serve both as vehicle encoding viral protein structures, as well as immunostimulant activating the innate immune system. Single-stranded RNA (ssRNA) as well as double-stranded RNA (dsRNA) activates antigen-presenting cells through binding to endosomal Toll-like receptors (TLR3 and TLR7) and to inflammasome proteins such as melanoma differentiation-associated protein 5 (MDA5), retinoic acid-inducible gene **I** (RIG-I), Nucleotide Binding Oligomerization Domain Containing 2 (NOD2) and double-stranded RNA dependent protein kinase (PKR) in the cytosol. These mechanisms act as a double-edged sword, as a prerequisite for priming and expansion of the SARS-CoV2 specific T cell response, as well as triggers for the expansion of potential heart reactive autoimmune T cells.40 The more efficient and heart specific an autoimmune T cell response is, the more pronounced its impact on cardiac function. In the presence of already existing heart-specific autoimmunity, vaccination can boost self -reactive T cell responses and aggravate preexisting autoimmune heart disease. Such patients are at risk for the development of fulminant and potentially fatal myocarditis. Accordingly, several case reports describe patients with fulminant myocarditis, or distinct myocarditis phenotypes, such as giant cell myocarditis.18,41,42

If this hypothesis would be confirmed, the formation of new antigens from the haptenic activity of some components of BNT162b2 mRNA (Pfizer-BioNTechR) toward cardiomyocyte macromolecules would be indicated and a likely positive response to steroids, be suggested.

EMB may also contribute by using Western Blot to clarify if the spike protein that has been demonstrated to cause cardiac myocyte toxicity43 may have a role in inducing myocardial damage and then inflammation.

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