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Transient Blood Release of Synthetic SARS-Cov-2 Spike Protein after mRNA-Based COVID-19 Vaccination Possibly Contributes to ACE2 Dysfunction Leading to Rare Cases of Myocarditis

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Abstract

Myocarditis has been recognized as a possible rare complication of COVID-19 mRNA vaccination. It concerns between 0.3 and five vaccinated people per 100,000 in the general population, with increased incidence in adolescent and young adult men. Most often, cases of myocarditis have been reported in the days following the second dose of vaccine mainly in younger male patients. This complication of vaccination usually resolves within days or weeks. However, the pathophysiological events responsible for the increase in frequency of myocarditis after COVID-19 vaccination remain unclear. Recent reports have highlighted that free spike proteins circulating in patients' blood at high levels appear to play a major role in myocarditis. Here, we review the most recent data that partly lift the veil on the molecular mechanisms of the induction of myocarditis following mRNA-based COVID-19 vaccination. We hypothesize that a mechanism of molecular mimicry of the viral spike triggers transient dysregulation of angiotensin-converting enzyme 2, leading to increased soluble angiotensin II binding to the transmembrane receptor angiotensin II type I receptor, similar to what is observed during SARS-CoV-2 infection. We suggest to standardize the management of suspected cases of mRNA-based COVID-19 vaccine-induced myocarditis, including the monitoring of angiotensin II and spike antigenemia.

Keywords: COVID-19 vaccine; SARS-CoV-2 spike; Myocarditis; ACE2; Renin-angiotensin system

Introduction

The coronavirus disease 2019 (COVID-19) pandemic is caused by a Sarbecovirus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Most infected individuals have mild symptoms before they recover, but SARS-CoV-2 infection has sometimes resulted, mostly in the first two years of the pandemic, in a severe acute respiratory illness requiring mechanical ventilation and a mortality of around 1% of SARS-CoV-2 infected patients is observed [1-4]. In 2020, SARS-CoV-2 was spreading very rapidly across the world to the point of being declared a pandemic by the World Health Organization (WHO), Faced with this emerging disease, creating an effective vaccine as quickly as possible was considered the most suitable strategy to reduce the incidence and disease severity of COVID-19. Under these extreme emergency conditions to found solutions, several vaccine candidates, including the mRNA-based COVID-19 vaccines from Pfizer and Moderna, have been produced at an unprecedented high speed shortening all the steps of vaccine development until these vaccines were approved by

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the health authorities and put on the market. The mRNA-based COVID-19 vaccines were made possible thanks to the knowledge and opitimizations made since a French team first published a scientific paper reporting that intravenous or subcutaneous injections of liposome-entrapped synthetic nonreplicating mRNA can trigger immune responses against the encoded antigen, and thus could be used as a vaccine [5]. Then, numerous works contributed to the improvement of this concept up to human clinical trials (mostly in cancer patients) which were driven primarily by companies such as Acuitas, BioNTech, CureVac and Moderna in association with academic teams in Europe, USA and Canada [6]. What is unprecedented, however, is the number of mRNA-based vaccine injections into humans since this strategy was approved in 2020 to respond to the COVID-19 pandemic. With the hindsight we have today on vaccination, large-scale vaccination does not seem to have had a drastic effect on the spread of COVID-19 worldwide during the three years that saw successive waves of infections with viruses showing genetic variations from the original Wuhan strain. According to the World Health Organization, as of September 21, 2023, there was 770,778,396 confirmed cases of SARS-CoV-2 infection and 6,958,499 deaths reported worldwide (WH0 Coronavirus COVID-19 dashboard; accessed on September 27, 2023). However, the WHO considered that this mass vaccination, in particular with the mRNA-based COVID-19 vaccines which have been the most used, saved lives by reducing the severity of the disease. Globally, vaccination against SARS-CoV-2 has been recognized a major tool in preventing COVID-19-related hospitalization and mortality rate in the general population. The vaccine effectiveness regarding the COVID-19 mortality was estimated 68% in the first dose and 92% in the second dose [7]. Anti-SARS-CoV-2 vaccination was also reported highly effective against COVID-19 mortality among older adults [8]. It is worth noting that a study aimed to assess the effect of COVID-19 vaccines in women (1,813,947 women) before or during pregnancy (knowing that pregnant women with COVID-19 are considered at high risk of severe disease and death) recently reported that anti-SARS-CoV-2 vaccination (mainly with an mRNA-based COVID-19 vaccine) reduces the risk of infection by 61% and the risk of hospital admission by 94% [9].The WHO also concluded that the benefit-risk balance of vaccination is highly positive despite the existence of some side effects including confirmed cases of pericarditis (inflammation of the heart's outer lining, a fluid-filled sac named pericardium that encases the muscular body of the heart and the roots of the aorta, superior vena cavae, pulmonary trunk and pulmonary veins) and myocarditis (inflammation of the heart muscle) following mRNA-based COVID-19 vaccinations [10-13].

Acute myocarditis is commonly associated with viral infections [14-15], including SARS-CoV-2 infections. Myocarditis is believed to be cause by direct invasion of the heart muscle by the virus, virus-induced cytokine storm, and/or reduced blood flow to parts of the heart. The focal/diffuse degrees of myocardial inflammation determine the severity of symptoms in patients with myocarditis and the fulminant myocarditis cases predominantly progress with elevated serum cardiac troponins [16-20]. A detailed analysis of temporal variations of excess cardiovascular mortality (observed deaths versus expected deaths predicted by the negative binomial log-linear regression model) during the COVID-19 pandemic found an excess cardiovascular death percentage (5.7% and 4.0% in men and women, respectively) in the COVID-19 era [21]. Cardiovascular complications have been described in many COVID-19 patients and myocarditis (from subclinical myocardial injury to fulminant lethal myocarditis) has been proposed to account for a small fraction of cardiac injury among patients infected with SARS-CoV-2 [22-31] (**Figure 1**).

Recently, it has been reported that SARS-CoV-2 infects coronary vessels, inducing pro-atherogenic inflammatory responses that could trigger acute cardiovascular complications and increase the long-term cardiovascular risk [35]. Since their approval by the Food and Drug Administration (FDA, USA), several billion doses of this type of vaccine have been injected to humans in the world over the last three years which saw successive and concomitant outbreaks of SARS-CoV-2 variants. According to the WHO, up to 13,505,089,801 vaccine doses had been administered as of September 19, 2023 (WH0 Coronavirus COVID-19 dashboard; accessed on 27 September 2023), a large majority of which were mRNA-based COVID-19 vaccines. With the rapid rollout of COVID-19 vaccinations, numerous associated and suspected adverse events have been reported nationally and worldwide but, most of the time their occurrence remains unpredictable. Among these adverse effects, many publications described confirmed cases of pericarditis and myocarditis following mRNA-based COVID-19 vaccinations [10-13,28,30,36-41]. In response to the earliest surveillance alerts regarding the COVID-19 vaccines, the WHO vaccine safety committee noted in the mid-2021 that myocarditis and pericarditis following vaccination with mRNA-based COVID-19 vaccines required further investigation of cases. The central question being to clarify whether these adverse effects are the direct consequence of vaccination or coincidental. Even today, the pathophysiological events which could lead to the increase in frequency of myocarditis after COVID-19 vaccination remain unclear. Although the expression of the vaccine spike proteins should theoretically be membrane-bound on cells having received mRNA-based COVID-19 vaccine, several recent papers reported surprising evidence suggesting that free spike proteins circulating in the blood of patients at high levels could be associated with the induction of rare cases of post-vaccination myocarditis [42-47]. This opens up new perspectives to try to understand the molecular crosstalk underlying this pathological process and to explain

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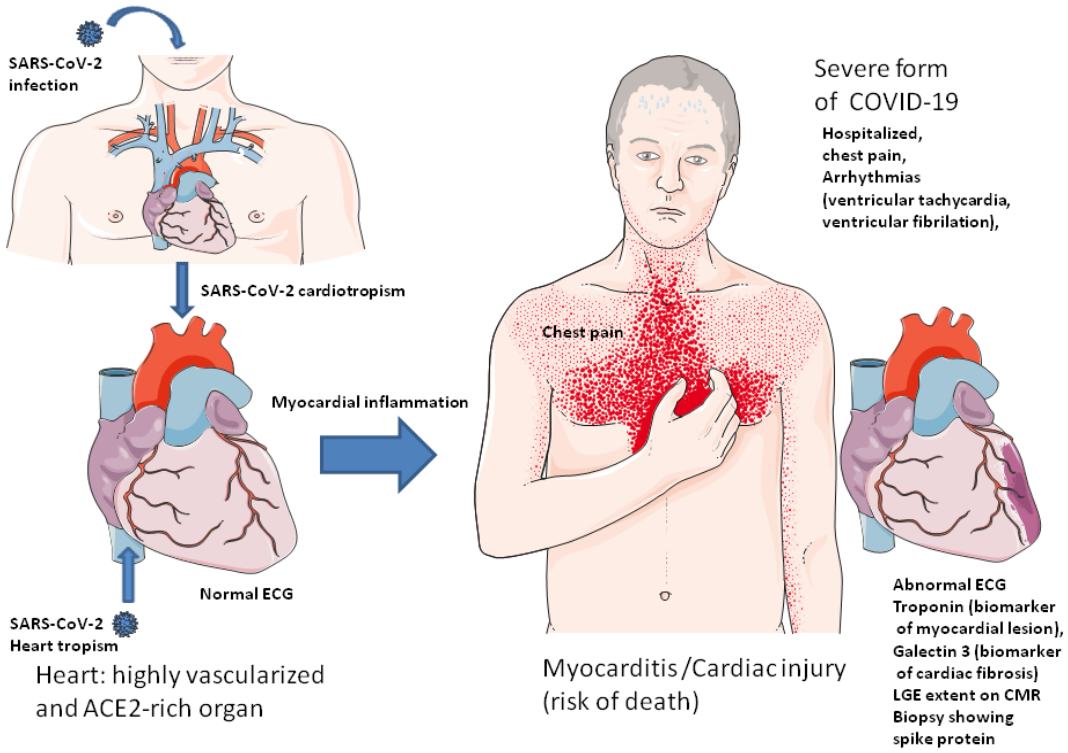

Figure 1: Schematic representation of myocarditis in patients with COVID-19. Clinical presentation: Most frequently associated with raised troponin blood level, abnormal electrocardiogram (ECG), abnormal echocardiography, late gadolinium enhancement (LGE) involving subepicardial regions, more than 1 abnormality in cardiac magnetic resonance (CMR). Since 2009, a CMR-based diagnosis of myocarditis has been supported by the Lake Louise criteria (LLC), targeting three aspects of myocardial inflammation: oedema, hyperaemia and necrosis and/or fibrosis. Most of the cases of COVID-19-related myocarditis were diagnosed based on CMR findings, with endomyocardial biopsy performed in very few cases because it requires a specific expertise and it is not performed in stable patients. However, endomyocardial biopsy (EMB) remains the gold standard for the diagnosis of myocarditis with identification of SARS-CoV-2 in the myocardium. Beside the identification of myocardial damage, a fundamental step in the diagnostic of myocarditis is the exclusion of obstructive coronary artery disease, especially when the clinical presentation resembles to an acute coronary syndrome. Myocarditis remain uncommon in COVID-19 patients. Since failed human hearts have a higher percentage of ACE2-expressing cardiomyocytes, patients with heart failure are likely more susceptible to myocarditis [32-34].

why it remains a relatively rare undesirable effect (between 0.3 and five cases per 100,000 people who received a dose of vaccine) whereas, in theory, it should be a quite frequent adverse effect if we postulate that the each vaccinated individual receives the same quantity of vaccine as the other vaccinated people and that the adverse effect is strictly a consequence of injection of mRNA-based COVID-19 vaccine. We must therefore seek another explanation for these rare cases of post-vaccine myocarditis. The purpose of this review is to discuss molecular models that could account for post-vaccine myocarditis.

The Pfizer/BioNTech's BNT162b2 and Moderna's mRNA-1273 vaccines and post-vaccine myocarditis

Following mass vaccination using the Pfizer/BioNTech BNT162b2 (Comirnaty® ; 30 µg RNA per injection) or the Moderna mRNA-1273 (SpikeVax®; 100 µg RNA per injection) mRNA-based COVID-19 vaccines [14,48], caregivers and epidemiologists observed a small but significant increase in the frequency of acute myocarditis and acute coronary syndrome associated with rare but sometimes fatal cardiovascular complications [49-51]. Numerous studies worldwide reported similar observations regarding the adverse effects of mRNA-based COVID-19 vaccines. These detrimental effects of the vaccine are characterized by the inflammation of the myocardium resulting in myocyte death and tissue necrosis [10,11]. The clinical course is generally mild (yet mild cases are likely under-diagnosed) with rare cases of left ventricular dysfunction, heart failure and arrhythmias [28]. A study of an Israeli cohort of patients who suffer from myocarditis symptoms following administration of the Pfizer/BioNTech BNT162b2 vaccine (n=304 patients; symptoms were mild in 95% of vaccine recipients), found an increased risk of myocarditis in young adult males after injection of the second dose of vaccine [12]. A large scale retrospective Danish study [13] reviewed data from five million residents over the age of twelve, four million of whom had received mRNA-based COVID-19 vaccines (either the Pfizer/BioNTech BNT162b2 or the Moderna mRNA-1273 vaccines), and reported that the incidence of myocarditis

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after mRNA-based COVID-19 vaccines was of 1.4/100,000 vaccinated individuals. With the Moderna mRNA-1273 vaccine, myocarditis or myopericarditis was highlighted with an overall incidence of 4.2/100,000 (76% male subjects; 86% had received a second dose of vaccine). The risk was substantially higher after the second dose of vaccine. A British study [38] analyzed the incidence of deaths from myocarditis, pericarditis and cardiac arrhythmias following administration of mRNA-based COVID-19 vaccines (Pfizer/BioNTech BNT162b2, n = 16,993,389; Moderna mRNA-1273, n = 1,006,191) and reported an increased risk of myocarditis associated with the first dose of the Pfizer/BioNTech BNT162b2 vaccine and the first and second doses of the Moderna mRNA-1273 vaccine over the 1–28 day post-vaccination period. The incidence of myocarditis was estimated as an extra 0.1/100,000 event per person vaccinated with the Pfizer/BioNTech BNT162b2 and 0.6/100,000 event per person vaccinated with the Moderna mRNA-1273. An increased risk of cardiac arrhythmias was also evidenced after a second dose of the Moderna mRNA-1273 vaccine. A study of a cohort in Hong Kong [52] analyzed 160 patients with myocarditis and 1,533 without, linking health care records to vaccination records. The BNT162b2 mRNA vaccine was reported to be associated with 20 cases of myocarditis, leading the authors to conclude that patients who were vaccinated with the Pfizer/BioNTech BNT162b2 vaccine had a three-fold increased risk of developing myocarditis compared to unvaccinated subjects. The highest incidence of myocarditis after vaccination with mRNA-based COVID-19 vaccines has occurred within three to four days after the second vaccination in young males. From the 27.3 million Pfizer-BioNTech (BNT162b2) doses administered in Australia to 2 January 2022, there have been 415 reports of likely myocarditis and 735 reports of likely pericarditis and that from the 1.8 million Moderna (mRNA-1273) doses administered in Australia to 2 January 2022, there have been 40 reports of likely myocarditis and 52 reports of likely pericarditis (COVID-19 vaccine weekly safety report - 06-01-2022 | Therapeutic Goods Administration (TGA); accessed on 7.1.2022). However, it is unclear how many of these cases are a direct consequence of the vaccine versus coincidental. An elegant single-centre retrospective analysis of all patients presenting to St Vincent's Hospital, Sydney, Australia with suspected COVID-19 vaccine-related myocarditis (9 suspected cases) and pericarditis (97 suspected cases) was recently published [53]. These authors used *the Brighton Collaboration Case Definition of Myocarditis and Pericarditis* [20] to categorize patients into groups based on diagnostic certainty and cardiac magnetic resonance imaging findings were reviewed against updated Lake Louise Criteria (LLC, which target three aspects of myocardial inflammation: edema, hyperemia, and necrosis and/or fibrosis) [16,18] for diagnosing patients with suspected myocarditis (**Table 1**). They confirmed 10 cases of possible or probable myocarditis and pericarditis of which 80% had electrocardiogram abnormalities and one patient had multisystem inflammatory syndrome following vaccination with severely impaired left ventricular ejection fraction. Another study in Canada, reported that among 19,740,741 doses of COVID-19 mRNA administered, there were 297 reports of myocarditis or pericarditis [54].

Table 1: Diagnostic criteria for myocarditis.

Diagnostic criteria for myocarditis	**CDC***	**Brighton Collaboration**
	criteria	**criteria**
	Level 1 (confirmed)	**Level 1 (definitive)**
	Symptoms consistent with myocarditis and at least one of:	Symptoms consistent with myocarditis and at least one of:
	Abnormal histopathology	Abnormal histopathology
	OR	**OR**
	Elevated troponin **AND** abnormal CMR	Elevated troponin **AND** abnormal CMR
		OR
		Elevated troponin **AND** abnormal TTE
	Level 2 (probable)	**Level 2 (probable)**
	Symptoms consistent with myocarditis and at least one of:	Symptoms consistent with myocarditis and at least one of:
	Elevated troponin	Elevated troponin **OR** CKMB
	OR	**OR**
	Abnormal ECG	Abnormal ECG
	OR	**OR**
	Abnormal TTE	Abnormal TTE
	OR	
	Abnormal CMR	
		Level 3 (possible case)
		Symptoms consistent with myocarditis
		AND
		Enlarged heart on CXR **OR** non-specific ECG abnormalities

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	Diagnostic target	Lake Louise criteria (LLC)
CMR diagnostic criteria for myocarditis	Myocardial edema	T2-weighted imaging, increased Bright signal intensity
	Myocardial injury	Increased global early gadolinium enhancement ratio between myocardium and skeletal muscle.
	Hyperemia	At least one focal lesion with non-ischemic regional distribution on late gadolinium enhancement
	Myocardial necrosis	Pericardial effusion; Systolic left ventricular wall motion abnormality

Abbreviations: .CDC: Center for Disease Control, CMR: Cardiac magnetic resonance imaging; TTE: transthoracic echocardiogram; ECG: electrocardiogram; CKMB: creatine kinase myocardial band; CXR: chest X-ray; LLC: Lake Louise criteria. If two Lake Louise criteria are positive, CMR is considered indicative of active myocardial inflammation. Parametric mapping with CMR permits the routine spatial visualization and quantification of changes in myocardial composition based on changes in T1, T2, and T2(star) relaxation times and extracellular volume (ECV). The clinical recommendations for CMR mapping can be found in the publication by Messroghli and colleagues [55].

CDC advocates myocarditis and pericarditis screening for patients who develop acute chest pain, shortness of breath, or palpitations, particularly in adolescents and young adults palpitations within 7 days of receiving the COVID-19 mRNA vaccine. Younger children who have myocarditis or pericarditis may have non-specific symptoms such as irritability, vomiting, poor feeding, tachypnea (fast breathing), or lethargy (CDC last reviewed October 10, 2023; https://www.cdc.gov/vaccines/covid-19/clinical-considerations/myocarditis.html; accessed on November 12, 2023). A recent multinational analysis (in total, 183,559,462 doses of Pfizer-BioNTech BNT162b2, and 36,178,442 doses of Moderna mRNA-1273) by the Global Vaccine Data Network (GVDN), confirmed pre-established safety signals for the mRNA-based COVID-19 vaccines with a significant increase in frequency of some adverse effects such as myocarditis and pericarditis [56]. Risks were assessed using observed versus expected (OE) ratios with lower bound of the 95% confidence interval (LBCI) greater than 1.5 and the authors found that the OE ratios for myocarditis and pericarditis following Pfizer-BioNTech BNT162b2, and Moderna mRNA-1273, were significantly increased with LBCIs > 1.5. Despite the potential adverse effects of vaccination, it has been globally recognized that its benefits far outweigh the risks in the management of COVID-19. Most often, the prognosis of the rare cases of post-COVID-19 vaccine myocarditis appears to be favorable, with full recovery in both males and females and most benefit-risk studies regarding COVID-19 vaccine concluded on the clear benefits of COVID-19 mRNA vaccination with respect to myocarditis. A paper published in Nature Reviews Cardiology by Heymans and Cooper [39,40] stated : "The risk of acute myocarditis associated with COVID-19 mRNA vaccination has garnered intense (social) media attention. However, myocarditis after COVID-19 mRNA vaccination is rare and usually resolves within days or weeks. Moreover, the risks of hospitalization and death associated with COVID-19 are greater than the risk associated with COVID-19 vaccination. Therefore, COVID-19 vaccination should be recommended in adolescents and adults". This paper highlights that in mRNA-based COVID-19 vaccine-associated myocarditis, up to 90% of patients will functionally recover, usually after a chest pain syndrome. A recently reported multinational (Denmark, Finland, Norway and Sweden) cohort (12,271,861 person-years) study concluded that" a booster dose of vaccine was associated with increased risk of myocarditis within 28 days of vaccination in 12- to 39-year-old males with incident rates of 0.86 per 100,000 vaccinated individuals for the BioNTech/Pfizer vaccine and 1.95 per 100,000 for the Moderna vaccine; No deaths occurred within 30 days of vaccine-related myocarditis cases in this study" [57]. Currently, the overall incidence of myocarditis after injection of mRNA-based COVID-19 vaccines is estimated to be between 0.3 and five cases per 100,000 people but with significant differences according to age and gender. Nonetheless, despite a extremely low absolute risk, these detrimental effects of mRNA-based COVID-19 vaccines cannot be ignored, particularly as they concern young populations who have a low risk of death from SARS-CoV-2 infection in the absence of known comorbidity factors.

Shedding light on the manufacture of mRNA-based COVID-19 vaccines

Vaccine manufacturers use a large panel of routine strategies to design their vaccines, including the traditional live attenuated virus (mutant virus), the inactivated virus (inactivated by chemical or physical treatments), with or without adjuvant such as aluminum salts, the use of viral vector vaccines expressing a recombinant envelope protein of the target infectious pathogen, and the production of recombinant envelope proteins or epitope-based synthetic polypeptides delivered by polymers, or liposome nanoparticles [58,59]. Alongside the most classic vaccine approaches, another path using next-generation mRNA technology vaccines [59], the "mRNA-based vaccine strategy", was recently made possible and rapidly gained attention, because it theoretically present several advantageous: (1) it is easy to design; (2) it involves short development and production cycles; (3) it presents great flexibility in term of manipulating the coding sequence; (4) it makes it possible to design multiple mRNAs in a single vaccine dose; and, (5) it can be produced at a low cost. The

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Table 2: Brief summary of the main studies intended to estimate the number of cases of myo-carditis associated with anti-SARS-CoV-2 vaccination. Given the heterogeneity of the studies' evaluation criteria, the main conclusions of the studies are summarized in this table, without interpretation on the part of the authors. Please refer to the main text of this article for the references. Mevorach *et al*., 2021 [12]; Husby *et al*., 2021 [13]; Patone *et al*., 2022 [38]; Lai *et al*., 2022 [52]; Buchan *et al*., 2022 [54]; Faksova *et al*., 2024 [56]; Hvidd *et al*., 2024 [57]. OE: estimated ratio of total observed versus total expected events; IRRs: Incidence rate ratios.

Study	Year	Country	Number of vaccinated subjects	Vaccine			Criteria of analysis	Results
				BNT162b2	mRNA -1273	ChadOx1		
Mevorach et al	2021	Israel	5,442,696	yes	no	no	Incidence after the first and second vaccine doses (21 days apart)	1.76 per 100,000 persons
							Rate ratio 30 days after the second dose as compared with unvaccinated persons	2.35 (95% CI, 1.10 to 5.02)
Husby et al	2021	Danish	4,931,775	yes	yes	yes	Rate ratios of myocarditis or myopericarditis within 28 days after vaccination	BNT162b2 : 1.48 (0.93 to 2.36)
								mRNA-1273 : 6.25 (2.83 to 13.82)
Patone et al	2022	UK	38,615,491	yes	yes	yes	Incidence rate ratio 1-28 days after second dose	BNT162b2 : 0.95
								mRNA -1273: 1.46 (1.09,1.98)
								ChadOx1: 0.95 (0.94,0.96)
Lai et al	2022	Hong Kong	1.693	yes	no	no	Adjusted Odds ratios compared to unvaccinated persons	OR: 3.57 (1.93-6.6)
Buchan et al	2022	Canada	19,740,741	yes	yes	no	Rate of myocarditis after second dose	BNT162b2: 5.92 per 1 000 000
								mRNA -1273: 29.95 per 1 000 000
Faksova et al	2024	International	99,069,901	yes	yes	yes	Aggregated OE ratios by 2nd dose, cardiovascular condiitons on a period 0-42 days	BNT162b2 : OE ratios:2.86
								mRNA -1273: OE ratios: 6.10
								ChadOx1: OE ratios: 1.31
Hvidd et al	2024	International	8,900,000	yes	yes	no	Incidence rate ratios (IRRs) of myocarditis comparing vaccination schedules on young men after second dose	BNT162b2 : IRR : 2.08
								mRNA -1273: IRR8.89 (2.26 -35.01)

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objective of such vaccines was to safely induce immunity limiting the pandemic and reducing the frequency of the severe forms of COVID-19. These apparent advantages of next-generation mRNA technology vaccines lead to an unprecedented rapid development and approval of mRNA-based COVID-19 vaccines resulting in the mass production of full-length SARS-CoV-2 spike protein in the vaccinated subjects [48,60-61]. BNT162b2 by Pfizer-BioNTech and mRNA-1273 by Moderna were planned for use in mass-immunization programs to curb the pandemic. This strategy led to Professors Katalin Kariko and Drew Weissman, who developed the technology behind mRNA COVID-19 vaccine, to recently be awarded the 2023 Nobel Prize in Physiology or Medicine. The Pfizer/BioNTech BNT162b2 mRNA vaccine is a cationic lipid nanoparticle (LNP)-encapsulated, nucleoside-modified RNA vaccine (codon-optimized modified spike mRNA), that encodes a SARS-CoV-2 full-length spike protein stabilized in prefusion conformation [14,62]. The production process of the mRNA-based COVID-19 vaccines is quite easy to set up, with a simpler manufacturing process compared to other "more traditional" vaccines, and it theoretically offers several advantages including rapid response and rapid adaptability, enhanced physical stability, and safety. However, this production process involves a number of steps requiring extreme rigor to ensure the presence of intact RNA in the batches [63,64] and an acceptable purity of samples intended for human vaccination. The European Medicines Agency (EMA) guidelines on the quality of clinical aspects associated with RNA vaccines stipulates that DNA should be no more than 0.033% of the total nucleic acids in vaccine doses (Rolling Review Critical Report: https://factreview.gr/wp-content/uploads/2023/07/Rolling-Review-Report-Quality-COVID-19-mRNA-Vaccine-BioNTech.pdf, page 74).). As there are 30 µg of spike mRNA per vaccine dose of 0.3 mL the maximal quantity of residual DNA tolerated per dose of vaccine is 10 ng.

The manufacturing process of the mRNA-based COVID-19 vaccines includes:

1. Isolation of SARS-CoV-2 and the extraction of its RNA genome. Synthesis by reverse transcription (RT) of a double stranded (ds) DNA template of the gene coding for the spike protein. A synthetic DNA sequence encoding the viral spike protein is inserted into a bacterial plasmid (7,824 base pairs for the Pfizer BNT162b2 mRNA vaccine and 6,777 base pairs for the Moderna mRNA 1273 vaccine) that contains a bacterial origin (ori) of replication and a kanamycin resistant (aminoglycoside phosphotransferase Neo/Kan) gene [65]. Notably, the wild type spike sequence (NCBI accession: NC_045512) is found to be 45.3% identical to the BNT162b2 vaccine only and the GC content is 37.3% for the wild type and 56.9% for the BNT162b2 vaccine. Eight small ORFs were found to overlap the Pfizer BNT162b2 mRNA vaccines compared to eleven overlapping ORFs in the wild type [66].

2. The recombinant bacterial plasmid containing a double stranded DNA copy of the gene coding for the spike protein as well as a DNA-dependent RNA-polymerase promoter and a kanamycin-resistance selection gene are stored at -150°C until use. The, plasmid is then transfected into an *Escherichia coli* (*E. coli*) bacteria that has been made competent for DNA uptake (the construct includes missense codons leading to two major changes in the S2 spike protein sequence with K986P and V987P substitution aimed at stabilizing the protein, the proline is a very rigid amino acid forming a bend aimed at improving the stability of the spike protein by preventing the conformational change of the pre-fusion into the post-fusion structure) [67,68].

3. *E.coli* colonies are grown at 37°C for 24 hours on Petri dishes filled with solid medium. During this process the plasmid is transmitted to daughter bacteria of the *E.coli* colonies when the bacteria divide (bacteria multiply every 20 minutes). To avoid event of plasmid loss its maintenance is enforced by selection with a kanamycin antibiotic added to the growth medium. Bacteria are then grown into flasks filled with medium and then moved into a large fermenter that contains up to 300 liters of a nutrient broth where they are grown for four days. After amplification in bacteria serving as a master cell bank [69] , bacteria are chemically broken down and the plasmid DNA is purified from bacterial debris. The products were tested for purity and gene sequence control. Each one liter batch of plasmid DNA is intended to finally produce about one million doses of the vaccine.

4. The ring-shaped plasmid (7,824 base pairs) is linearized through the action of a restriction enzyme releasing the sequence encoding the synthetic spike. Highly concentrated linearized DNA templates are added to the reaction mixture aimed to produce the mRNA vaccine. The cell-free *in vitro* transcription of DNA into RNA is achieved using a T7 RNA polymerase to generate a synthetic mRNA with a 5' cap. The sequence of mRNA is 4100-4300 nucleotides long with a 5'cap [70]. At this stage, the synthetic nucleoside *N-methyl-pseudouridine* (mψU) is incorporated into the artificial RNA instead of the natural uridine nucleoside to further increase RNA stability, to enhance translation efficiency in host cells, and to remove alternative start codons - avoid overlapping ORFs - and internal ribosome entry sites, thus preventing non-specific recognition by ribosomal complexes [71]. Moreover, such modifications avoid recognition by immune receptors TLR-7 and TLR-8. It was recently reported that incorporation of mψU into mRNA can result in +1 ribosomal frameshifting *in vitro*

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and that cellular immunity to +1 frameshifted products from vaccine mRNA translation occurs after vaccination [72]. The addition of a 5′ cap structure is a critical part of this production step that has been improved by new technology suitable for large-scale production [73,74]. A poly(A) tail is needed for efficient translation of mRNA vaccines and is also a critical part during manufacture [75].

5. *In vitro* transcription is followed by several steps of mRNA purification, including the removal of DNA and dsRNA (a critical step of DNA cleavage and degradation by using DNase I treatment), which could lead to an excessive innate immune response by dsRNA sensing [76] and mRNA is filtered and frozen. Analysis of mRNA requires diverse techniques such as RT-qPCR (usually targeting a sequence of 69 base pairs long), capillary and gel electrophoresis, high-pressure liquid chromatography (HPLC) and immunoblotting. The FDA (USA) recommends manufacturers to limit amount of residual DNA in the final product to be below 10 ng/dose and the size of DNA to be below the size of a functional gene [77].

6. The thawed mRNA is mixed with water. In a separate process, the oily lipids are mixed with ethanol, and mRNA and lipids (including phospholipids, cholesterol, cationic lipids and polyethylene glycol lipids that are mixed together) are mixed to create lipid nanoparticles [78]. When the lipids come into contact with the mRNA, electric charge pulls them together in a nanosecond. The mRNA is enveloped in several layers of clinically translatable lipid nanoparticles (multilayer liposomes), forming an oily protective vaccine particle. Liposomes or lipid nanoparticles (LNPs) which facilitate the mRNA cytosolic transport, are known to function as adjuvants and can also modulate the immune response [79,80]. The newly made vaccine is filtered to remove the ethanol, concentrated and filtered again to remove any impurities, and finally sterilized. Machines inject 0.45ml of a concentrated vaccine solution into vials, enough for six doses after dilution. The vials are sealed with foil and capped with purple lids and stored at -70°C. After further quality testing, the vials from the same batch are ready to ship.

7. Packaged vaccine doses (preserved at low temperature), are shipped and processed for the market. The mRNA-based COVID-19 vaccine is administered by injection into the deltoid muscle leading to capture of mRNA by muscle cells. The lipid nanoparticles protect RNA (a usually fragile molecule) from RNase-dependent degradation and facilitate cellular uptake by lipid fusion with lipids of cell membrane. Spike coding mRNA is released into target cell cytoplasm (**Figure 2**).

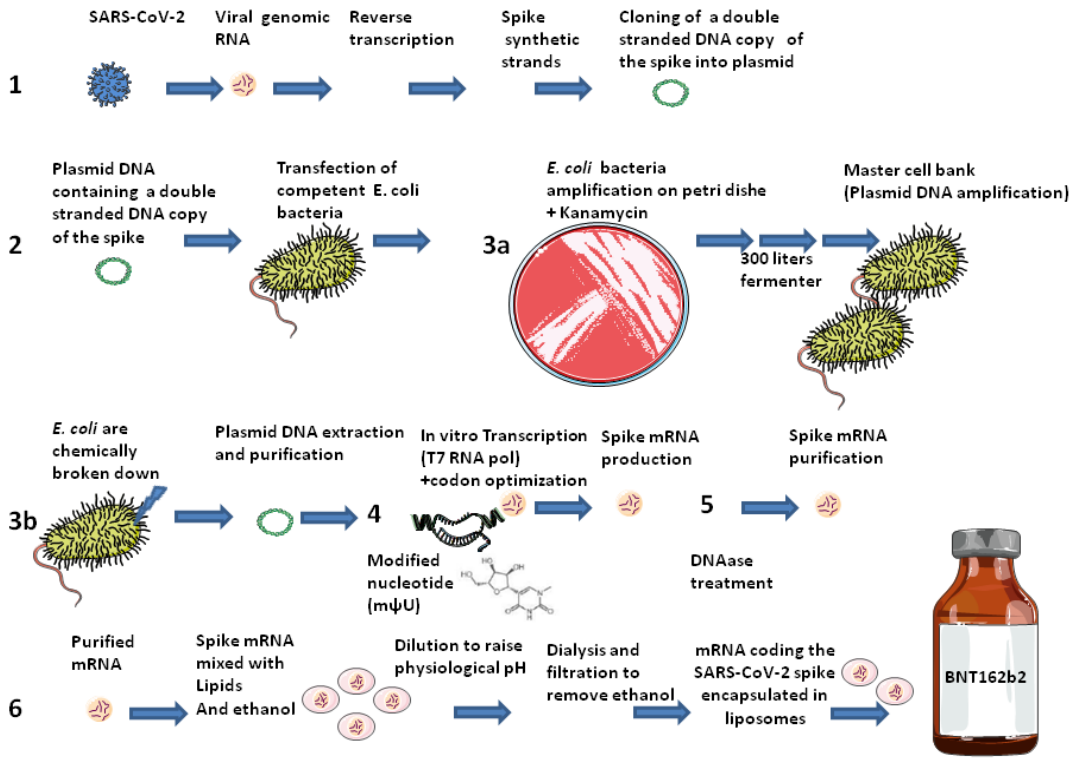

Figure 2: Schematic representation of the main steps in the production process of mRNA-based COVID-19 vaccine mRNAs: the Pfizer BNT162b2 mRNA vaccine is a lipid nanoparticle-encapsulated, nucleoside-modified RNA vaccine that encodes a SARS-CoV-2 full-length spike protein stabilized in prefusion conformation (see the main text for details).

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This vaccine strategy is expected to limit vaccine diffusion near the site of injection and local lymph nodes and is considered safe, a requirement for its marketing authorization. However controversial results about the safety of the mRNA-based COVID-19 vaccine have increasingly been reported by different teams [81,82]. Although Pfizer scrupulously respects the tolerance of 10 ng of residual DNA per 30 µg of mRNA, there was a concern of some academic scientists who claimed that that the robustness of the DNAase I digestion step does not destroy all DNA compounds and that a fraction of the nucleic acid contained in some batches of the Pfizer BNT162b2 are indeed traces of non-degraded plasmid DNA [83]. It was also hypothesized that the recombinant DNA found in mRNA vaccines can be introduced into the cells, favored by cationic lipid nanoparticles, at the time of vaccine administration just as with the mRNA itself, leading to possible integration [84], a hypothesis that remains the subject of fierce debate [85]. According to the vaccine company experts, the mRNA vaccines are analyzed using a range of time-consuming and costly methods to ensure product safety. Recently Gunter and colleagues [86] described a streamlined method to analyze mRNA vaccines using long read nanopore sequencing (VAX-seq). According to these authors, VAX-seq can comprehensively measure sequence, length, integrity, nucleoside modifications, and purity of mRNA vaccines. Currently, there is also a concern that the vaccine could activate the endogenous LINE-1 reverse transcriptase, leading to possible reverse transcription of the mRNA vaccine into DNA with a risk of integration into the host genome [87], Surprisingly, deep sequencing experiments were recently claimed to provide evidence of heterogeneity in the purity of the mRNA-based COVID-19 vaccine batches and traces of DNA including residual full vector matrix [65].

Biodistribution and persistence of COVID-19 mRNA vaccines

The biodistribution and duration of persistence of the COVID-19 mRNA vaccines may be important for understanding of the adverse side-effects of the vaccine. After injection into the deltoid muscle, the vaccine spike mRNA nanoparticles are expected to be captured and endocytosed by muscle cells. The cellular endocytosis of the mRNA is expected to take about one minute and the translation into spike protein is expected to take about two minutes (as calculated considering a translation speed of 10 amino acids per second and a protein made up of 1273 amino acids) [88,89]. This newly synthesized vaccine spike protein is expected to be delivered at the cell surface in a prefusion conformation, as previously reported by Wrapp and colleagues [62], and to activate an anti-spike immune response including the induction of neutralizing antibodies [90-92]. Using a cellular model, Fertig and colleagues [93] observed that the endolysosomal compartments of most vaccine-treated cells were enriched with electron dense, multilayered lipid structures as compared to controls, suggesting the successful endocytosis of LNPs, followed by the endosomal-mediated disintegration of LNPs, translation into spike protein and synthesis of spike-like structures clustering on isolated protrusions of the plasma membrane of some cells after 12 hours incubation with the Pfizer/BioNTech BNT162b2 vaccine (10 µg mRNA per 1 × 10^6 cells). The biodistribution and persistence of mRNA vaccines after intramuscular injection, has been studied in rodents and non-human primates. These studies indicated that intramuscular injection leads to an initial accumulation of the vaccine spike protein at the injection site within hours of the vaccine injection [93,94]. LNPs are then rapidly transported to proximal lymph nodes by passive draining as well as actively carried by antigen-presenting cells and neutrophils, while the remaining unprocessed LNPs reach systemic circulation [93,95-96]. This indicates that nanoparticles containing the mRNA coding for the virus spike can also be picked up by the immune cells in the proximal lymph node. A study by Krauson and colleagues [97] found that COVID-19 mRNA vaccines elicit antigen-specific germinal center B cell responses only in draining lymph nodes but not in mediastinal lymph nodes. Using a specific RT-qPCR based assays to detect COVID-19 mRNA vaccine these authors found the presence of spike mRNA in the axillary lymph nodes in the majority of patients who died within 30 days of vaccination. Vaccine was also detected in the myocardium in a subset of patients within 30 days of death. It was reported that a single intramuscular immunization with the Pfizer/BioNTech BNT162b2 vaccine (containing the branched-tail ionizable lipid ALC-0315 that includes a tertiary amine, branched tails and ester linkers biodegradable structure) can activate dendritic cells, monocytes and macrophages in the draining lymph nodes to produce interferons [98]. Another ionizable lipid SM102 (sharing structural features with ALC-0315) used in the Moderna mRNA-1273 vaccine LNPs was found to activate IL-1 RA cytokine production [99]. Moreover, it was also reported that among patients with histologically confirmed myocarditis after mRNA-based COVID-19 vaccine, anti- IL-1 RA antibody were found in 75% of patients younger than 21 years of age as compared to 11% of patients 21 years of age or older and no detectable in controls [100]. It has also been found that exosomes with a spike protein on their surface are induced by the Pfizer/BioNTech BNT162b2 vaccine and circulate prior to the development of specific antibodies. Circulating exosomes expressing spike proteins were found on day 14 after vaccination, followed by anti-spike antibodies being detected 14 days after the second dose [101,102].The beneficial effect of vaccination is usually determined by monitoring the specific anti-spike antibody responses [103], although this represents only part of the immune response against the virus. In a murine animal model, the intramuscular administration of LNPs containing ionizable lipid and mRNA encoding the spike of SARS-CoV-2, the titers of antibodies against SARS-CoV-2 was

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increased tenfold with respect to the vaccine encoding for the unadjuvanted antigen [104].

The mRNA-based COVID-19 vaccines, consist of nonreplicating mRNA and are expected to be naturally degraded after translation through intracellular RNAase activities within the cytosol. They should therefore be rapidly eliminated from the injection site. The half-life of this foreign mRNA in the vaccinated subject is unknown, theoretically from a few hours to ten days based on general data for cellular mRNA [105,106], but vaccine-associated synthetic mRNA has been reported to persist in systemic circulation for at least two weeks [93] and full-length or fragments of SARS-CoV-2 spike mRNA vaccine sequences have been found in blood up to four weeks after COVID-19 vaccination [107]. In another study, both the mRNA vaccine and spike protein were found in germinal centers in a lymph node up to eight weeks post-injection in some cases [108]. Thus, in contrast to disrupted germinal centers in lymph nodes observed during SARS-CoV-2 infection, the Pfizer/BioNTech BNT162b2 vaccine stimulates robust germinal centers containing vaccine mRNA and vaccine spike antigen. Moreover, mRNA COVID-19 vaccine was also claimed to be detectable in human breast milk [109]. In a study by Ogata and colleagues [42], 11 of 13 participants exhibited spike antigen in their plasma after a first injection of the Moderna mRNA 1273 COVID-19 vaccine, whereas nucleocapsid concentrations were insignificant in all participants, indicating that the detected spike originated from the vaccination and not from a natural infection. This study suggested that the spike protein leaves the site of the COVID-19 vaccine injection and enters the bloodstream, accumulating in other parts of the body (**Figure 3**).

This was confirmed by Brogna and colleagues [43] using mass spectrometry examination of biological samples. In vaccinated subjects (n = 20), these authors evidenced the presence of the spike protein translated from the mRNA vaccine (distinguishable from the wild-type protein due to specific amino acid variations introduced to maintain the protein in a prefusion state) in 50% of vaccinated individuals while this was not the case in unvaccinated individual (n = 20).

Previous hypothesis proposed in an attempt to explain the increased risk of post mRNA-based COVID-19 vaccines myocarditis

It has been hypothesized that the rare adverse side effects reported in young subjects after injection of mRNA-based COVID-19 vaccines (e.g., myocarditis and multisystem inflammatory syndrome), could be the unintended

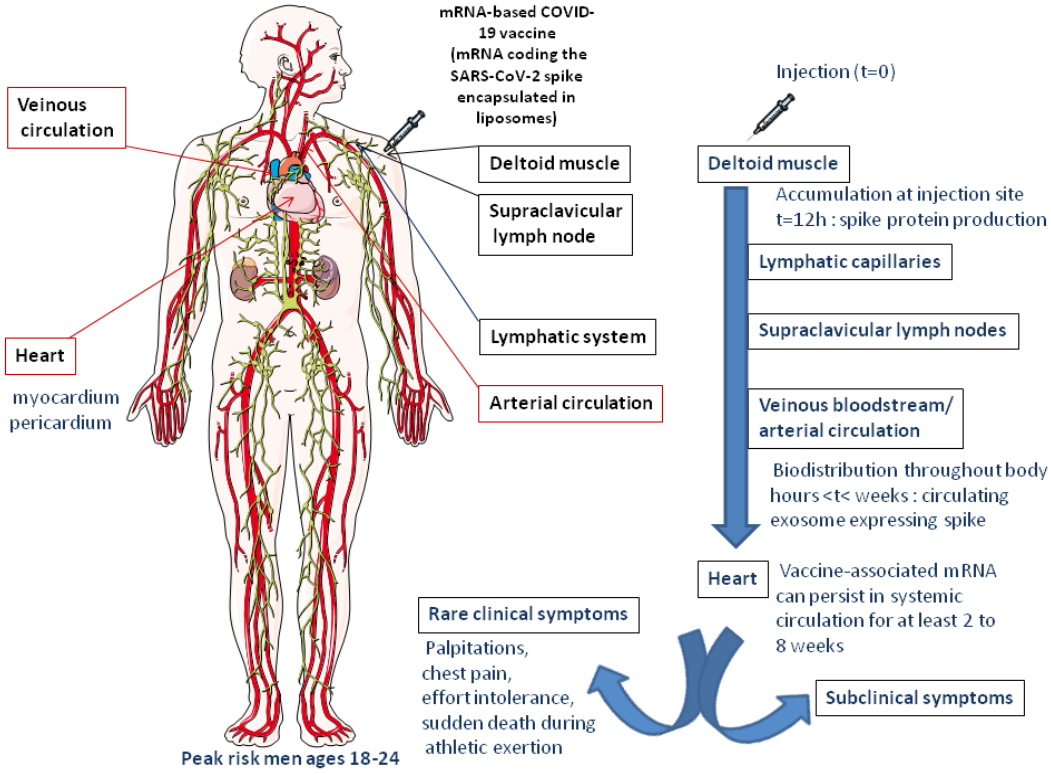

Figure 3: Hypothetical model accounting for the possible route taken by vaccine-derived compounds (mRNA coding for the viral spike and/or spike protein) which can be found in the blood circulation and the heart after injection of mRNA-based COVID-19 vaccine in the upper arm (deltoid muscle). Men aged between 18 and 24 are at higher risk of developing myocarditis following mRNA-based COVID-19 vaccination. If undetected, there is a risk of sudden death. When detected, exercises must be prohibited due to effort intolerance and this adverse effect could be related to the role played by ACE2 in the physiological adaptation of the heart muscle to exercise [110,111].

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consequence of an inappropriate use of the vaccine such as at the time when the antiviral immune response is already highly stressed during a low-noise infection in the process of healing, leading to exacerbated cytotoxic T cell response killing cells and triggering tissue damage [13,112-116]. The potential implication of lipid nanoparticles present in the vaccine and epigenetic modifications which occur with inoculations with vaccines, have also been suggested as being possibly involved in the pathogenesis of myocarditis associated with mRNA-based COVID-19 vaccines [79,80,117-119]. Myocarditis was also suggested to be associated with a specific immune genetic background such as genetic variants in genes encoding HLA, desmosomal, cyctoskeletal or sarcomeric proteins [120]. Stress response, hypertensive response, and/or alcohol consumption known to affect the expression of angiotensin-converting enzyme 2 (ACE2) have also been suggested as being responsible for the adverse effects of mRNA-based COVID-19 vaccines [121]. A French retrospective analysis reported that among 21,909 subjects who had received at least one dose of the Pfizer/BioNTech BNT162b2 COVID-19 vaccine, 8,121 people (37.1%) exhibited high blood pressure after the first injection [122]. Another report investigating post-injection adverse effects of mRNA-based COVID-19 vaccines among a population of 1,870 subjects who had received one or two doses of COVID-19 vaccine indicated that 153 subjects (8%) showed an increase in blood pressure values after vaccination, with higher frequency of blood pressure alterations at the second or booster dose [123]. Similarly, the prevalence of abnormalities or increases in blood pressure in a meta-analysis including 357,387 subjects was 3.2% and that of stage III hypertension was 0.6%, mainly in elderly and frail people [124]. Gundry [125] who used the PULS Cardiac Test (Predictive Health Diagnostics Co., Irvine, CA) a clinically utilized measurement of multiple protein biomarkers including IL-16 (proinflammatory cytokine), soluble FAS (inducer of apoptosis) and HGF (T cells chemotaxis) to monitor the increased in endothelial tissues inflammation among a cohort of 566 patients who received mRNA-based COVID-19 vaccine. Dramatic changes in the PULS score (from 11% to 25%) were observed with patients immunized using the Pfizer/BioNTech BNT162b2 and Moderna mRNA 1273 COVID-19 vaccines, and these changes persisted for at least two-and-a-half months after the second dose of vaccine. The author used the results to calculate a five-year risk score (percentage of risk) for new acute coronary syndrome and concluded that the administration of mRNA-based COVID-19 vaccines was associated with an increase in endothelial inflammatory processes, thrombosis and acute coronary syndrome risk. Attention has also been drawn to cases of multisystem inflammatory syndrome that have been reported after administration to children of doses of mRNA-based COVID-19 vaccines [36,126]. This pathophysiological process was linked to an abnormal activation of the immune response. The clinical symptoms affecting these children were found to be associated with elevated anti-AT1R, anti-endothelin receptor, anti-α1 adrenergic receptor, anti-β1 adrenergic receptor, anti-β2 adrenergic receptor and anti-muscarinic cholinergic receptor-2/3/4 autoantibodies [127]. Recently, myocarditis following mRNA-based COVID-19 vaccination has been linked to autoantibodies against endogenous interleukin-1 receptor antagonist (IL-1RA) the function of which is to inhibit interleukin-1 signaling and inflammation [99]. Since development of ACE2 autoantibodies after SARS-CoV-2 infection was described [128,129], it can be speculated that the synthetic spike could also induce this type of autoantibodies. However, a recent study found that anti-ACE2 IgG levels in COVID-19 patients are too low to impair the regulatory activity of ACE2 [130]. It remains to be verified that the vaccination does not induce more ACE2 autoantibodies than SARS-CoV-2 infection. Already on the basis of these observations it can be considered that the adverse effects observed after mRNA-based COVID-19 vaccination may have a multifactorial origin with strong variations between individuals.

Possible role of circulating spike protein in post-mRNA-based COVID-19 vaccines associated myocarditis

Soon after the discovery of the first cases of COVID-19 and the characterization of the SARS-CoV-2, it was demonstrated that the virus receptor was the ACE2 molecule [131-133]. A critical step in the SARS-CoV-2 infection cycle is the binding of the homotrimeric viral spike protein to the peptidase domain of ACE2 [134-136]. Once bound to ACE2, SARS-CoV-2 down-regulates the cellular expression of the ACE2 gene and ACE2 protein and the unopposed action of Angiotensin II (Ang II) was deemed responsible for worsening the outcome of COVID-19 [137]. *In vitro*, SARS-CoV-2 decreases ACE2 methylation and ACE2 lysine 31 hypermethylation decreases binding to SARS-CoV-2 spike protein [138,139]. From the start of the pandemic, we and others warned against considering ACE2 as a simple receptor, knowing the major role this molecule plays in the renin-angiotensin system (RAS) pathway [29,140-142]. More precisely, we hypothesized that by attaching to ACE2 the virus was likely to influence the balance regulating the production of angiotensin II, thus modulating blood pressure, coagulation and inflammatory processes [29,143-145]. We were subsequently able to demonstrate that the presence of the virus in patients can induce an overproduction of angiotensin II [146]. Similar results regarding increased angiotensin II were reported by others [147,148]. On the molecular level, we postulate that the pathophysiological dysfunction observed in COVID-19 mainly involves the angiotensin II/ transmembrane receptor angiotensin II type I receptor (AT1R)/Hypoxia-inducible factor-1 (HIF-1) axis [149]. As this physiopathological process initiated by viral

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infection is dependent on the interaction between the spike of the virus and ACE2, it was very likely that the spike expressed as part of vaccine could induce the same adverse effects as the virus itself. Although the expression of the vaccine spike proteins should theoretically be membrane-bound on cells having received the mRNA-based COVID-19 vaccine, we recently speculated that after vaccination a significant quantity of synthetic spike proteins could transiently be released into the blood circulation (which may involve either the enzymatic cleavage of its membrane forms, transcription of truncated forms without membrane anchoring capacity, or release of free spike after cellular destruction due to activation of the immune system) and thus lead to transient adverse effects of the vaccine by a mechanism of molecular mimicry [150]. The fact that symptomatic hypertension (malaise, headache, tingling in the mouth, diaphoresis and increased blood pressure) subsequent to vaccination with the Pfizer/BioNTech BNT162b2 was reported [151,152] is not surprising and likely supported our hypothesis. In addition, the recent demonstration that free synthetic spike proteins circulate in the blood of recently vaccinated patients [43,45-47], confirms our hypothesis.

Using a mouse model, Rhea and colleagues [153] had previously shown that intravenous injection of the radioiodinated S1 (I-S1) spike protein can cross the blood–brain barrier in male mice and was also taken up by several organs. Thus when a huge quantity of mRNA encapsulated in LNPs is injected, LNPs, exosomes expressing the spike and free spike, can pass into the lymphatic capillaries then axillary/supraclavicular lymph nodes and it is possible that these compounds can enter the venous bloodstream. Thus, these components are likely to diffuse into different organs and therefore to cross the heart many times. Besides, LNPs are likely to fuse with cardiomyocytes where spike mRNA can be translated into spike proteins. Of course, it appeared quite logical to think that the interaction between the circulating vaccine spike and ACE2 expressed on the endothelium could be at the origin of vaccine-induced myocarditis. However, previously there was a lack of experimental evidence to support this hypothesis. According to a work recently published by Yonker and colleagues [45], all post-vaccine myocarditis subjects (n = 16) and age-matched vaccinated controls (n=45) showed similar rise in anti-spike antibodies and anti-spike T-cell response after vaccination (with either the Pfizer/BioNTech BNT162b2 or Moderna mRNA 1273 COVID-19 vaccine). However, one major significant difference between both groups was the high level of circulating full-length spike protein (33.9±22.4 pg/mL), in the plasma of myocarditis patients in association with slight elevations in cardiac troponin T, C-reactive protein and cytokine production [45-47]. This is the first evidence of a direct correlation between myocarditis and the presence of circulating spikes that remains detectable for up to three weeks after vaccination, suggesting that the spike protein translated from the mRNA vaccine may be the causal agent of myocarditis. This is consistent with the work by Krauson and colleagues [95] who detected spike mRNA vaccine in the myocardium of vaccinated patients who died within the 4 weeks after vaccination. The cardiac ventricles of the deceased people in which vaccine was detected had healing myocardial injury and had more myocardial macrophages than controls in which vaccine was not detected.

Conclusion

The relationship between mRNA-based COVID-19 vaccination and the low risk of cardiac injury in young males is currently well established [154]. However, until recently, the exact mechanism linking COVID-19 mRNA innoculation and myocarditis remained speculative. The elegant study by Yonker and colleagues [45] bridges the gap in the model that we previously proposed of a direct effect of the vaccine spike on ACE2 inducing a process of microthrombosis and inflammation that can lead to myocarditis, due to a molecular mimicry mechanism. When a large amount of free spike protein circulates in the bloodstream, it may damage the cardiac pericytes or endothelium by acting on ACE2, reducing nitric oxide production and activating the production of inflammatory molecules in some ways similar to that which has been observed with SARS-CoV-2 infection which has been shown to trigger acute myocardial infarction [155-157]. It can be emphasized that a Danish study of 5,119 patients diagnosed with COVID-19 estimated that the incidence rate of acute myocardial infarction was five times higher during the 14 days after COVID-19 diagnosis [158]. A recent self-controlled case-series study conducted using data from 5.1 million children in England reported no increased risks of adverse events 1-42 days following mRNA-based COVID-19 vaccination in the 5-11 year olds group and, 3 and 5 additional cases of myocarditis per million following a first and second dose of the Pfizer/BioNTech BNT162b2 respectively, in the 12-17 year olds group [159]. Alternatively, it could act by potential molecular mimicry between spike proteins and endogenous antigens that elicit cardiac targeted autoantibodies [160,161]. For the model to be credible, it must be admitted that the circulating rate of mRNA nanoparticles and/or spike proteins will vary depending on the individual who receive the vaccine (to the extent that only a small proportion of individuals who receive the vaccine experience these adverse effects) which then suggests that the dose of vaccine will likely contain variable quantities of intact mRNA, depending on the batch available to clinical staff or, that the process of capturing vaccine mRNA by the cells of the vaccinated person varies from one individual to another. The mRNA integrity directly impacts the effectiveness of the mRNA-based COVID-19 vaccines. The mRNA transcription can be abortive (smaller fragments), mRNA can possibly be fragmented by RNases or hydrolyzed, spike species can derive from cryptic mRNA transcription

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start sites. It could be considered that the quality of the vaccine could vary depending on the more or less perfect method of storage of vaccine vials (e.g. if the number of -70°C freezers and storage spaces were limited) in the facility managing the vaccination process. Obviously, if this is the case, then the abnormal dose delivery of mRNA is simply a technical problem that might be solved by a better production control by the manufacturer and then a better storage in vaccination centers. The main source of concern is the prolonged existence of mRNA that evades destruction, the mechanism of which is still debated [46,86]. As above mentioned, foreign mRNA in the vaccinated subject was predicted to persist in an active form during a period ranging from a few hours to ten days [104,105], but vaccine-associated synthetic mRNA was reported to persist in systemic circulation for at least two to four weeks [93,97] and full-length SARS-CoV-2 spike mRNA vaccine sequences were found in the blood up to four to eight weeks post-injection in some cases [97,106-107]. It was recently reported that both the Pfizer/BioNTech BNT162b2 and Moderna mRNA 1273 COVID-19 vaccines induce specific dysfunction of cardiomyocytes after 48h of spike expression, and that both the cardiac ryanodine receptor (RyR2) impairment and sustained protein kinase A (PKA) activation may likely increase the risk of acute cardiac events [162]. It has also been recently claimed that some batches of COVID-19 mRNA vaccines contain excessive amounts of residual bacterial DNA, including full-length spike plasmid matrix (7,824 bp) and fragments of synthetic spike DNA, packaged in the LNPs [83,88]. If correct, DNA contamination may be prothrombotic, particularly for fragments with high GC contents [163,164]. It has also been suggested that getting the plasmids out of the *E. coli*, may result in residual bacterial endotoxin, in the vaccines. The debate has not yet been settled on the purity of samples during the manufacturing process of mRNA COVID-19 vaccines. Very recently, a publication described methods aimed to evaluate residual DNA impurities in mRNA-based COVID-19 vaccine Comirnaty® [165]. In all batches tested, it was found that the measured DNA value increased considerably after treatment with Triton-X-100 (expected to induce the release of the residual DNA bound in the lipid nanoparticles), with these values ranging from 360 to 534 times the permissible DNA limit (or 3600 to 5340 ng DNA per dose).

Another question recently addressed by Bozkurt and colleagues [37] is "why circulating spike protein levels remained elevated despite adequate levels and functionality of anti-spike antibodies". This author suggested hypothetical explanations such as vaccine overdose, the possible role of anti-idiotype, and prolonged existence of mRNA. Recently, Japanese radiologists published a study [166] on myocardial 18Fluorine-fluorodeoxyglucose (18F-FDG) uptake on images in 700 asymptomatic vaccinated subjects (The majority of the vaccinated individuals,77.6%, received the Pfizer/BioNTech BNT162b2 vaccine followed by 21% who received the Moderna mRNA-1273 mRNA vaccine). The 700 subjects underwent positron emission tomography (PET)/computed tomography (CT) within a period of 1–180 days after their second vaccination and increased 18F-FDG uptake (a marker of myocardial inflammation of diverse origin including viral myocarditis) was found compared with the unvaccinated group (n = 303). However, this increase was not seen in subjects who underwent imaging more than 180 days after vaccination. These results are corroborated by the data reported in the Krauson's studies. Krauson and colleagues [97] detected spike mRNA vaccine in the axillary lymph nodes and myocardium from recently vaccinated deceased patients. Vaccine was detected in the majority of patients dying within 4 weeks of vaccination, but not in patients dying more than 4 weeks from vaccination.(**Figure 4**).

Recently, two papers hypothesized the integration of residual DNA from the mRNA-based COVID-19 as a possible inducer of lymphomas in recently vaccinated individuals [167,168]. Integrated foreign DNA, could theoretically lead to long term stable spike production. However, these authors do not provided proof of a DNA sequence originating from residual DNA found in the mRNA-based COVID-19 vaccine preparation which would be integrated into the genome of lymphomatous cells. Indeed, the risk for recombination, integration and translation of this foreign DNA after delivery into cells should be of extremely low frequency and is quite

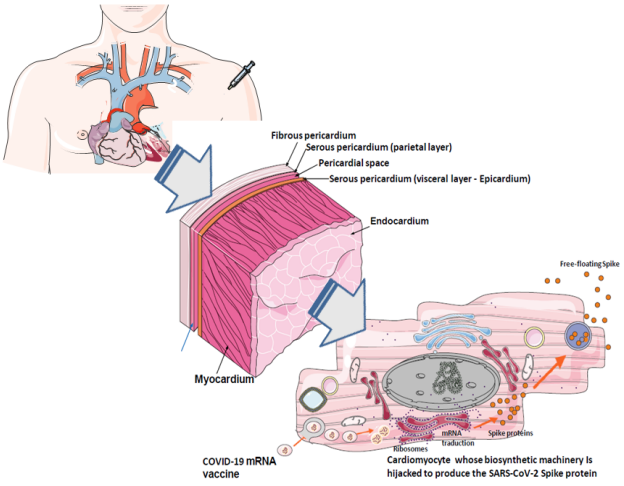

Figure 4: Schematic mechanism of action of the mRNA-based COVID-19 vaccine associated myocarditis. The spike mRNA vaccine was detected in the myocardium from recently vaccinated deceased patients [97]. The LNPs packaging of the mRNA-based COVID-19 vaccine allows nucleoside-modified RNA vaccine to be stably delivered and enter cardiomyocytes after LNPs enter the general bloodstream. Once inside a cardiomyocyte, this mRNA forms a complex with initiation factors and the small subunit of the ribosome, where elongation of the spike polypeptide chain starts. The S proteins first assemble to form homotrimers into the cytoplasm and then migrate to the cell surface to protrude with a native-like conformation. Cardiomyocytes expressing the spike protein can possibly be targets for a specific cytotoxic T cells anti-spike immune response and destroyed.

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improbable [169]. However, from a purely molecular point of view, this debate is not totally closed. In DNA gene therapy, delivery of closed-end linear duplex DNA have shown prolonged transgene expression but non-integrating gene transfer remains unanswered [170,171]. Circular plasmid DNA located in the cytosol could find their way into the nucleus and may be involved in chromosomal integration through crossover events during nuclear envelope membrane reformation at telophase [172]. Comparing transfection of cells with expression vectors in the forms of circular plasmid, unmodified linear DNA with thioester loops, and streptavidin-conjugated blocked-end linear DNA showed that all the forms of linear DNA resulted in a high fraction of the cells being transfected (between 10 and 20% of cells). and about 1-10% of the transiently transfected cells stably expressed a transgene [173]. The circular plasmid DNA has a lower rate of integration than all the linear forms, which show comparable integration frequencies regardless of the configuration of the DNA ends. Although the potential risk for residual DNA coding the viral spike in the mRNA-based COVID-19 vaccine to integrate into the human genome after transfection of cells and to be translated into protein is extremely low, in theory these improbable issues cannot be completely ruled out today. Whatever the conclusions of this important debate, we now know with certainty that the vaccine spike circulates longer than initially assumed, and that it is directly associated with the increased frequency of myocarditis in young men. Recently Barmada and colleagues [174] investigated a cohort of patients who developed myocarditis and/or pericarditis with elevated troponin and C-reactive protein levels. The patients showed cardiac imaging abnormalities shortly after COVID-19 mRNA vaccination, late gadolinium enhancement, and elevations in circulating interleukins (IL-1β, IL-1RA, and IL-15), chemokines (CCL4, CXCL1, and CXCL10) and matrix metalloproteases (MMP1, MMP8, MMP9 and TIMP1). Their immune responses analysis indicated expansion of activated cytotoxic T cells, NK cells in the heart and inflammatory and profibrotic monocytes. The precise molecular mechanisms by which the COVID-19 mRNA vaccines lead to myocardial injury and myocarditis remain to be characterized but we can reasonably hypothesize that it is a process which passes through the soluble angiotensin II binding to the transmembrane receptor angiotensin II type I receptor (AT1R; expressed in the arterioles and several organs including heart), and the intracellular factor hypoxia-inducible factor 1 alpha (HIF-1α). (**Figure 5**).

The prevalence of post-vaccine myocarditis in young men could be associated with the level of expression of ACE2 which varies according to sex and age [175]. A negative association was reported between age and soluble ACE2 plasma concentrations in people above the age of 55 [176]. It is also known that polymorphisms in ACE2 gene 5′ upstream regions might influence ACE2 expression. Differences of more than 1% in minor allele frequency in

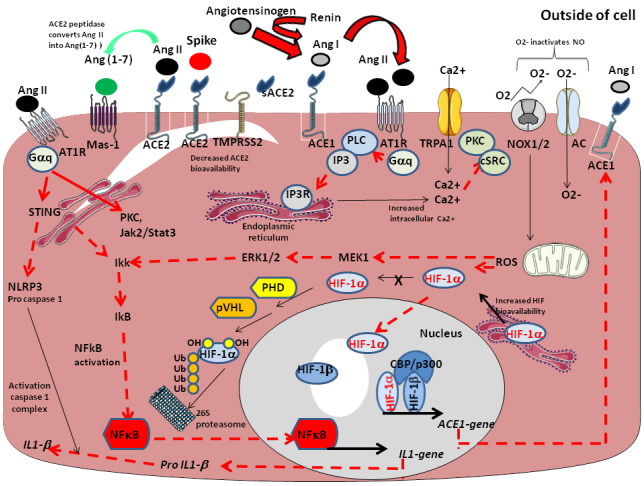

Figure 5: Potential interaction of COVID-19 mRNA vaccine/spike with the renin-angiotensin system (RAS). A free floating spike can be released from cells expressing the COVID-19 mRNA vaccine, leading to a massive interaction with the cell surface ACE2 receptor. As a result, an increased angiotensin II (Ang II) level is likely to lead to an overactivation of AT1R signaling which initiates activation of PKC and c-Src that is required for superoxide production by NADPH oxidases (NOX1 and NOX2). NOX2 also stimulates the production of reactive oxygen species (ROS) by mitochondria. The consequence is an inhibition of the HIF-1 hydroxylation by the prolyl hydroxylase PHD pathway and of its polyubiquitinylation by the ubiquitin ligase usually leading to HIF-1 proteosomal degradation (a mechanism contributing to cell homeostasis). After ROS production, HIF-1α translocates to the cell nucleus where it forms heterodimers with the HIF-β subunit and binds to the HRE element in the promoter of hypoxia-inducible genes and recruits histone acetyltransferases CREB Binding Protein (CBP)/p300. Thus, it up-regulates ACE1 which contributes to Ang II production and abnormal functioning of the RAS pathway. HIF-1α nuclear translocation also activates the transient receptor potential ankyrin 1 (TRPA1) gene expression which leads to increase in intracellular Ca^{2+} and cell injury as well as expression of several genes involved in the control of cell viability and proliferation among others [149]. In parallel, Ang II triggers an increase in cytoplasmic Ca^{2+} that induces NOX5 to generate H_2O_2. HIF also contributes to the down regulation of the ACE2 gene and activation of ADAM17 which leads to cleavage of the ACE2 protein and release of soluble ACE2 (sACE2). AT1R signaling activates a number of signaling pathways, such as G protein–mediated (Gq and Gi), Janus kinase/signal transducers and activators of transcription, extracellular signal regulated kinase (ERK), NF-κB, NLRP3 procaspase 1 pathways leading to induction of pro-inflammatory cytokines.

the 10Kb region upstream to ACE2 analyzed using data from the 1,000 Genomes project, found 57 polymorphisms [29,177] which could explain why not all young men are affected by vaccination in the same way. In addition, there is a known polymorphism of ACE2 and dozen of human ACE2 variants have been characterized, which could impact the affinity for the viral spike or could modify the ACE2 protein stability [178-181]. These individual variations may be highly important in the adverse effects of vaccination, since the expression of ACE2 in the heart is known to be higher than in the lungs. ACE2 is found in the endothelial

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cells of coronary arteries, arterioles, venules, and capillaries as well as vascular smooth muscle cells, and is strongly expressed by cardiac fibroblasts, cardiomyocytes, and cardiac pericytes [182,183]. Myocarditis has been recognized as a complication of COVID-19 mRNA vaccination and remains as issues of concern. A follow-up surveillance study of young adults for at least three months after the onset of vaccine-induced myocarditis concluded that while 81% of patients were considered recovered, 54% of those who received follow-up cardiac MRIs still exhibited cardiac abnormalities and 26% were still prescribed daily medications related to myocarditis [184]. Important gaps remain regarding the risk factors. Improved understanding of the risk factors and pathologic mechanisms resulting in myocarditis secondary to mRNA-based COVID-19 vaccinations may help predict future risk and facilitate the development of a new generation of vaccine that mitigates this risk. A recently reported follow-up surveillance study in Canada (over 105 million COVID-19 vaccine doses) confirmed that the incidence of myocarditis following mRNA-based COVID-19 vaccination varies significantly by age and sex [185]. These authors found that the incidence of myocarditis following a second dose of either mRNA-based COVID-19 vaccine was less than 2.0 per 100,000 doses in males and females under 12 years of age. However, the incidence was increasing in males 12 to 17 and 18 to 29 years of age to between 1.3 to 39.0 cases and 2.9 to 15.7 cases per 100,000 doses, respectively. The medical management of COVID-19 mRNA vaccine-associated myocarditis mainly relies on corticosteroids to challenge the progression of non-specific immune system activation [186,187]. Beta-blockers are often employed in treatment of acute myocarditis, even in uncomplicated disease, presumably by virtue of the perceived protection they provide against arrhythmic events [15]. In a recent study by Yu and colleagues [188], a subset of adolescent patients diagnosed with COVID-19 mRNA vaccine–associated myocarditis were found to show impairment of left ventricular and right ventricular myocardial deformation and persistence of late gadolinium enhancement with up to 1 year of follow-up while global systolic ventricular function appeared to be preserved. According to McDonald and colleagues [185], the management of post-vaccine myocarditis (and/or pericarditis) should follow established guidelines. These authors recommend that "patients with chest pain should be treated with nonsteroidal anti-inflammatory drugs and/or colchicine provided there are no contraindications. Complications such as left ventricular systolic dysfunction and arrhythmia should be treated with guideline-directed heart failure therapy. The role of immunosuppression in severe presentations is uncertain. Selective use of corticosteroids for a short duration may be considered, balancing the risk and potential benefits of immunosuppression. Rarely, escalation of immunosuppressive therapies may be considered in fulminant disease along with mechanical circulatory support at experienced centres".

The long-term effects of mRNA-based COVID-19 vaccine-associated myocarditis is still not fully understood. As we move forward into the next long-term phase of the pandemic with booster vaccinations [189], routine monitoring of angiotensin II and spike antigenemia following mRNA-based COVID-19 vaccines and/or patients with myocarditis should be performed. If spike antigenemia is detected, administration of recombinant soluble ACE2 (hrsACE2) or anti-spike antibodies [147,190-194] could potentially be required to prevent or reverse post-vaccinal myocarditis.

Authors' contributions

CAD and LCJ contributed to the conceptualization of the review. CAD wrote the original draft of the manuscript. LCJ reviewed the manuscript. The two authors agreed with the manuscript submitted for publication.

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Data Availability Statement

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Conflicts of interest

CAD declares a link of interest with the Sanofi and Merck pharmaceutical companies. LCJ declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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