ABSTRACT

Title of Thesis:	DEVELOPING A BROADLY PROTECTIVE MRNA INFLUENZA VACCINE: A REVIEW
Team Name:	Team MUTATE
Mentor:	Dr. Stephanie Yarwood, Department of Environmental Science and Technology

Current influenza vaccines are limited in their efficacy due to antigenic drift of the hemagglutinin target; advances in mRNA vaccines in response to the COVID-19 pandemic may provide a new direction for influenza vaccine development. Existing literature shows that mRNA vaccines have higher efficacy in preventing illness, hospitalizations, and death. We evaluated eleven influenza A viral proteins as potential targets for an mRNA vaccine under the following criteria: degree of conservation, ability to elicit a robust immune response, and ability to prevent illness and death. We recommend future researchers direct their efforts towards developing an annually administered tri-sequence mRNA vaccine targeting hemagglutinin head (HA1), the matrix 2 ectodomain (M2e), and nucleoprotein (NP). Development of a highly effective influenza mRNA vaccine would be significant for prevention of disease burden worldwide

DEVELOPING A BROADLY PROTECTIVE MRNA INFLUENZA VACCINE: A REVIEW

by

Wellington Acquah, Cameron Amini, Saharsh Buddula, Michelle Chen, Navya Chintala, Quinn Dang, Noa Ferziger, Grace Hollis, Devin Jameison, Jyostna Jayaram, Joseph Anthony Manus, Jacob Rosenberg, Julia Zhiteneva

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Chapter 1: Introduction

Influenza is a contagious respiratory viral illness that impacts millions of people globally every year. Young children, adults over 65, pregnant women, and individuals with chronic illnesses are especially susceptible to adverse effects (CDC, 2021a). Influenza-related complications are responsible for 250,000 - 500,000 deaths annually, according to the World Health Organization (2018). In the 2019-2020 influenza season, the CDC estimated 38 million influenza illnesses, 18 million medical visits, and 22,000 deaths. In that season, they estimated that the influenza vaccine prevented 7.52 million illnesses, 3.69 million medical visits, and 6,300 deaths (CDC, 2019).

Although the current influenza vaccine provides benefits for public health, it is associated with a multitude of problems. The rapid rate of viral mutation leads to numerous subtypes, thus posing an obstacle to vaccine effectiveness since current influenza vaccines are highly subtype-specific. The subtype of influenza and sequence of the viral protein used in the vaccine is based on an annual prediction made by the WHO Global Influenza Surveillance and Response System (GISRS). As circulating subtypes develop and evolve around the world over time, the GISRS predicts the direction of evolution with varying accuracy, which means that the effectiveness of the vaccine varies by season (Table 1). For example, the 2013-2014 and 2014-2015 influenza seasons both had an estimated 30 million illnesses. However, the estimated number of cases prevented was drastically different: 7.5 million in 2013-2014 and 1.4 million in 2014-2015. Similar trends can be observed in medical visits, hospitalizations, and deaths (Table 1). Generally, flu vaccines are 40% to 60% effective (CDC, 2021b).

Table 1. The Efficacy of the Influenza Vaccine on Annual Disease Burden, as Estimated by the CDC

Year	Estimated Illnesses	Prevented Illnesses	Estimated Deaths	Prevented Deaths
2013-2014	30 million	7.5 million	38,000	12,000
2014-2015	30 million	1.4 million	51,000	3,700
2015-2016	24 million	5.3 million	23,000	5,900
2016-2017	29 million	5.3 million	38,000	5,200
2017-2018	45 million	6.2 million	61,000	5,700
2018-2019	36 million	4.4 million	34,000	3,500
2019-2020	38 million	7.5 million	22,000	6,300

Note: The efficacy of the influenza vaccine, as estimated by the CDC. The wide range of estimated and prevented illnesses throughout time bears testament to the variety of prevalent influenza strains and the varying efficacy of the influenza vaccine throughout time. Adapted from "Past Seasons Estimated Influenza Disease Burden Averted by Vaccination" by Centers of Disease Control and Prevention, 2020.

Out of all four types of influenza virus (A, B, C, and D), influenza A is the biggest concern; it has the most vectors of transmission, ranging from birds to swine to humans, and is responsible for all past influenza pandemics and 75% of seasonal infections (Nyirenda et al., 2016). Unlike influenza A, influenza B is only derived from humans, which means that the risk of development of a new viral influenza B subtype is relatively low compared to that of influenza A. Though symptoms are more pronounced in influenza A, the influenza A and B viruses have similar symptoms of fever, chills, fatigue, and cough. H1N1 and H3N2 are the two main subtypes of the influenza A virus responsible for worldwide spread and included in the flu

vaccine, along with two other influenza B subtypes. H1N1 has been dominant since the 1918 pandemic and was most recently responsible for the 2009 swine flu pandemic (CDC, 2019). In contrast, the H3N2 subtype has been around since 1968 (CDC, 2019).

The current influenza vaccine targets the head of the hemagglutinin protein present on the virus surface. This protein mutates quickly, causing new subtypes to emerge that existing influenza antibodies do not recognize, preventing viral clearance. A broadly protective vaccine could target more conserved proteins and thus protect against a wider range of subtypes. This improved vaccine could reduce the burden of influenza illness with its increased efficacy.

mRNA Vaccines

Messenger RNA (mRNA) vaccines have been researched for many years, but the COVID-19 emergency research funding accelerated mRNA vaccine optimization and utilization. The production, approval, and widespread distribution of the Pfizer and Moderna mRNA COVID-19 vaccines offers valuable insights for future improvements in viral vaccine technology, including future influenza vaccines. mRNA vaccines have the potential to be superior compared to the traditional vaccine technologies because of accelerated development and cost effectiveness. mRNA vaccines can be produced in a matter of weeks, which allows for the rapid inclusion of additional subtypes or other vaccine modifications to keep up with a changing virus (Mascola & Fauci, 2020). In addition, mRNA vaccines do not contain infectious components and do not pose the risk of integrating into the host genome (Kowalzok et al., 2021). mRNA vaccines are safer compared to live-attenuated vaccines (Tombácz et al., 2021) that have the potential to revert back to their pathogenic form and cause harm (Reichmuth et al., 2016). The plasticity of mRNA vaccines could be a feasible countervail to the rapid mutation rate of the influenza virus. A proposed mRNA vaccine would still be administered yearly, as the current

influenza vaccine, but would enable a faster response to detection of new strains. Lipid nanoparticles encapsulate the mRNA, protecting it from immune system degradation and allowing it to reach the targeted cells. Gene-based vaccines, such as those containing mRNA, induce high levels of CD4+ and CD8+ T-cell responses, generating a powerful cell mediated response in addition to a high level humoral (antibody) response (Tombácz et al., 2021). The effectiveness of the Pfizer and Moderna mRNA COVID-19 vaccines is over 90%, compared to the flu vaccine at 40% to 60% (Zheng et al., 2021). For these reasons, formulating a more broadly protective influenza vaccine using mRNA technology may result in a more effective vaccine.

Given the rapid advancement of mRNA vaccines over the last two years, it is feasible that this technology could be applied to develop a broadly protective mRNA influenza vaccine. Our goal was to evaluate and recommend influenza proteins that can be used in a future tri-sequence mRNA vaccine, with the hopes of a yearly administered influenza vaccine with higher efficacy than existing influenza vaccines. mRNA vaccines have shown promise in increased efficacy, especially when compared to the low effectiveness of existing influenza vaccines. This review compares the efficacy of potential influenza type A surface protein targets, synthesizing research over the last decade. We compared conservation and the immune responses of individual protein targets and in combinations, with the goal of recommending the most suitable target gene for the development of a mRNA vaccine. The results summarize details of eleven influenza type A proteins.

Chapter 2: Methods

This systematic review includes peer-reviewed primary literature identified through several database searches using a set of standard search terms (Table 2). We focused on recent papers written in English within the last ten years but expanded to papers from the last fifty years in reference to protein structures and proteins with less research. To ensure that all research came from well-established journals, any journal we included had to have an impact factor.

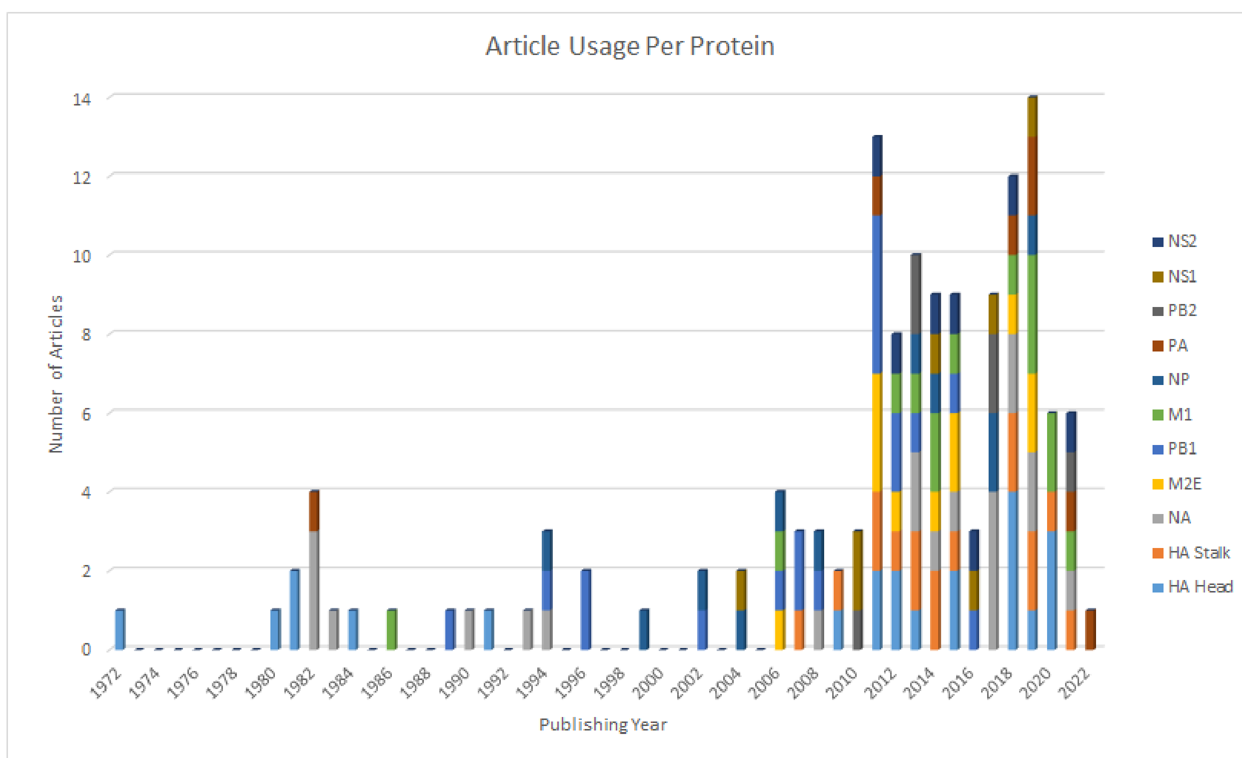

Figure 1: An overview of the articles that were examined over the course of this research. There was a focus on papers within the past 10 years, but for certain proteins that had little published research within the past 10 years, we expanded the criteria out to 30 years.

Based on our literature search, we organized eleven investigated influenza type A virus proteins into three categories: primary, secondary, and tertiary. The classification of the proteins was based on conservation, immune-related response (IRR), and potential for protein to be used in vaccine development based on prior research. IRR is a broad category that refers to markers measuring the immune response such as: Ig antibody titers, inhibition assays, peripheral blood

mononuclear response (PMBC), absorbance, and Interferon-gamma (IFN-γ). Conservation of the amino acid sequence of the protein structure is crucial to having a vaccine-induced antibody capable of recognizing multiple viral subtypes. The protein is only effective as a vaccine target if it can induce an immune response and trigger antibody production.

Table 2. Protein Names, Search Terms, and Databases Used. This table outlines the set of search terms that were used to find literature on each given protein. In addition, the protein names and their variations are listed, as well as the databases that were searched.

Protein Names and Variation	Search Terms	Databases
"PA" "polymerase acidic subunit"	[protein name]	PubMed
"HA" "hemagglutinin"	[protein name] "influenza"	Science Direct
"M2e" "matrix protein 2"	[protein name] "influenza vaccine"	Google Scholar
"M1" "matrix protein 1" "M1 matrix protein"	[protein name] "flu"	ClinicalTrials.gov
"NS2" "NEP" "nuclear export protein"	[protein name] "flu vaccine"	
"NA" "neuraminidase"	[protein name] "universal flu vaccine"	
"PB1" "polymerase basic 1"	[protein name] "function"	
"MVA-NP+M1 vaccine"	[protein name] "structure"	
"PB2" "polymerase basic 2"	[protein name] "conserved"	
	[protein name] "based immunity influenza vaccine"	
	[protein name] "antibodies influenza virus"	
	[protein name] "universal vaccine"	
	[protein name] "broadly protective"	

Chapter 3: Results

The majority of published research focused on primary proteins, hemagglutinin and neuraminidase, while tertiary proteins were the focus of the fewest research studies. The following sections list the eleven proteins and describe their structure and level of amino acid conservation. Each section also describes the current knowledge concerning IRR and morbidity and mortality.

Proteins

Protein	Size	Quantified degree of conservation	Immune related response (IFN-γ and % elicited)	Individual immune related response in combination	Types of vaccines/delivery methods studied	subtypes studied	immunogenicity
Hemagglutinin head (HA1)	327AA (H1N1) (Zhirnov et al., 2002); 55 kDa (Sriwilaijaroen & Suzuki, 2012)	Amino acids: HA198, −134, −138, −153, −183, and b−195 (2%) are conserved among 13 different strains (Nobusawa et al., 1991)	Multiple sites studied → receptor binding site seems most promising due to high conservation, antibody CH65 neutralizes infectivity of 83% H1N1 strains (Whittle et al., 2011)	N/A	Live-attenuated mRNA DNA Inactivated	H1N1, H3N2	2017-2018 seasonal vaccine had effectiveness against medically attended ARI of 36% (Flannery et al., 2018) 2019-2020 seasonal vaccine effectiveness against any influenza illness was 39% (Tenforde et al., 2020)
Hemagglutinin stalk (HA2)	222 AA (H1N1)4; 25 kDa8	AAs 1–11 (5%) are conserved[6]	Chimeric HA immunogens shows promise. Two cHA constructs used H8 and H5 HA head domains in conjunction with the	N/A	Inactivated DNA mRNA	H5N1, H10N8	statistically significant increase in mean titer (60,221 to 72,637) (Park et al, 2018)
Neuraminidase (NA)	200 kDa10,11; 469 AA12	AAs 222-230 (2%) (N2 based) are conserved[13]	Low levels of broadly protective titers in humans[14,15]	N/A	Live-attenuated Inactivated Recombinant protein Recombinant subunit VLP Nanoparticles DNA	IAV: H1N1 A/PR/8/34; H3N2 A/Hong Kong/1/68; H1N1 A/USSR/90/77; H3N2 A/Bangkok/1/79; H1N1 A/Taiwan/1/86; H3N2 A/Leningrad/360/86; H1N1 A/Texas/36/91; H3N2 A/Beijing/353/89; H3N2; H1N2 A/Panama/2007/99; H1N1 A/New Caledonia/20/99; H1N1 A/WS/33; H1N1 A/Brevig Mission/1/18; H2N2	IFN-γ: >100 SFC/10^6 cells (Assarsson, et al. 2008) NI Antibody Titers: Br/07 as high as 1280, Ca/09 as high as 2560 (after 2nd dose). Compared to <5 for no vaccine. (Sultana, et al. 2014) Relative Fluorescent Units (RFU): Br/07 as high as 1.06(10^6), Ca/09 as high as 1.07(10^6). Compared to 3.41(10^4) and 3.81(10^4) respectively. (Wan, et al. 2017)

Table 3. List of Primary Proteins and Associated Values

Size	Quantified degree of conservation	Individual immune related response (IFN- and % elicited)	Immune related response in combination	Types of vaccines/delivery methods studied	subtypes studied	immunogenicity
23 AA; 2.5 kDa (Mezhenskaya et al. 2019)	39% (AAs 1-9) nearly identical among all strains. 38% (5 AAs) in the M2e region are highly conserved (Deng et al. 2015)	Very low due to small size (Feng et al. 2006)	Can be combined with HA, NP, and other proteins, and other viruses as carriers (Deng et al. 2015) Fourfold rise in antibodies from M2e-flagellin vaccine (Turley et al. 2011) M2e-NA nanoparticle vaccine has IFN of ~10^6 (Wang et al. 2019)	Recombinant protein Recombinant VLP Nanoparticles Inactivated DNA	H3N2, H1N1, H7N9, H9N2, H5N2	~120 (1x10^6) splenocytes for IFNgamma
757 AA; 87 kDa (UniProt 2020)	Overall sequence is 84% conserved across all outbreak strains (Powell et al. 2007)	7.6-fold increase in IFN-gamma secretion (Goodman et al. 2011)	Can be combined with NP, M1, NS1, and PA to elicit greater response (Goodman et al. 2011, Wang et al. 2015) NP+PB1+M1 DNA + rVV vaccines show 100% survival in mice (Wang et al. 2015)	DNA Recombinant vaccinia virus Live-attenuated Inactivated	IAV: H1N1 A/PR/8/34; H3N2 A/Hong Kong/1/68; H1N1 A/USSR/90/77; H3N2 A/Bangkok/1/79; H1N1 A/Taiwan/1/86; H3N2 A/Leningrad/360/86; H1N1 A/Texas/36/91; H3N2 A/Beijing/353/89;	400-800 PFU (vs. over 10,000 for the negative control) (Kosik et al, 2012) IFN-γ of >100 SFC/10^6 cells, where 100 is the cutoff for a significant response (Assarsson et al. 2008)
252 AA; 28 kDa24 (UniProt 2020)	Nearly 67%25 of AAs are conserved, including key AAs: 76-78, 91-105, 181-193 (Hom et al 2019, Das et al 2012, Svancarova et al 2018)	100ug DNA + 100ug M1 gave 100%; IFN of ~320 pg/ml; 80% loss in body weight26 (Fen Liu et al., 2020) 5 vaccinations with M1 and 50ug DNA 5/20 (lethal dose challenge); IFN of ~130 spots/10^6 cells; ~32% loss in body weight	IFN of DNA +rVV ~10^5; 100% of mice survived (10 mice); ~85% loss in body weight28 (Wang et al., 2015) MVA-NP+m1/MVA-NP+m1 generated IFN-γ ELISpot response of 200 SFU/million cells (9 chickens) 29 (Boyd et al. 2013) IFN ~ 1200 SFC per 10^6 PMBC (tested on 6 people) source 30 and (Folgetti 2019, Antrobus 233-238) Phase I trial produced IFN median of 1443 SFC/10^6 PMBC (Antrobus et al. 233-238).	DNA (by itself) Recombinant vaccinia virus (in combination) Modified vaccinia virus (in combination)	H1N1, H3N2, H2N2, H5N1, H6N1, H7N7, H9N2	3-7-fold increase in IFN-γ-secreting cells/million cells (all above 100 SFU/10^6 cells) compared to baseline (Antrobus et al, 2014) NP+M1 vaccine induced significant IFN-γ-secreting cells/million cells (Boyd et al, 2013)
			Combination of ChAdOx1 NP+M1; ELISpots greatest increase from baseline ~ 1,268 SFUs/10⁶ (15 people) (Antrobus 668-674).			
498 AA; 53 kDA31 (48) (Uniprot, 2021)	59% of AAs are highly conserved32 (Kukol and Hughes, 2014)	92.9-98% immunogenicity in human epitopes of influenza A virus33 (Isakova-Sivak et. al, 2017)	Combination NP/PB1/M1 vaccines provided complete or partial protection in mice. Of the three antigens, NP-based vaccines exhibited the greatest protective effect23 (Wang et. al, 2015)	DNA23 (Wang et. al, 2015)	H7N9, sars-COV-9 (Aleru and Barber, 2020)	NP antigen averaged 1552 SFUs/10^6 PBMCs across the animals in the pNP vaccine group compared to 32 SFUs/10^6 PBMCs across the untreated rabbits (Yung et al, 2019)

Table 4. List of Secondary Proteins

Protein	Size	Quantified degree of conservation	Individual immune related response (IFN- and % elicited)	Immune related response in combination	Types of vaccines/delivery methods studied	subtypes studied	immunogenicity
Nonstructural 1 (NS1)	281 AA; 26 kDa[41]	conservation across over 95% of influenza A	containing mutated NS1 proteins elicited	N/A	Cell-based (vero cells) Live attenuated	H1N1	3B: https://pubmed.ncbi.nlm.nih.gov/1938127
Nonstructural 2 (NS2) --> Nuclear Export Protein (NEP)	121 AA; 14 kDa (Uniprot)	93.4% AA conservation across all influenza A strains (Paterson & Fodor, 2012)	N/A	N/A	N/A	H1N1 (Nogales et al., 2016)	HAI: 32 and 128 FFU/mL (Nogales et al., 2016)
Polymerase acidic (PA)	716 AA; 83 kDa34 (UniProt, 2021)	85-93% conserved in swine strains (influenza A) and 91-94% conserved in avian strains (influenza A) 94-98% in human strains (El Hefnawi et al., 2011)	Viral titers in early viral replication were lower than wild-type; CEN activity inhibited by BXA in influenza A and B and antiviral potency; EC90 (90% maximal effective concentration) values of 0.79 ± 0.13, 0.46 ± 0.1, 0.79 ± 0.12 and 0.98 ± 0.20, 0.64 ± 0.30 (Noshi et. al, 2018)	PA acts cohesively with PB1 and PB2 in transcription and viral replication	Cell-based	H1N1, H3N2, H9N2	EC90 (90% maximal effective concentration) values of 0.79 ± 0.13, 0.46 ± 0.1, 0.79 ± 0.12 and 0.98 ± 0.20, 0.64 ± 0.30; lgEID50/mL ± SD values of 4.73± 0.49, 5.57± 1.27, 7.88± 1.01 (Noshi et al., 2018)
Polymerase basic 2 (PB2)	759 AA; 86	42 total AAs are 100% conserved, but scattered throughout PB2	100% survival rate with VX-787 with an efficacy quotient of	Some residues (1–269 and 580–683 segments) can be combined	Live-attenuated	H1N1	vaccination with PB2-deficient viruses decreased viral repliation (Uraki et al, 2017)

Table 5. List of Tertiary Proteins

Primary Proteins

Hemagglutinin

Hemagglutinin is a glycoprotein. The sequence codes for a precursor protein (HA0) (Sriwilaijaroen & Suzuki, 2012). This precursor protein is cleaved by cellular proteases, causing the formation of two disulfide-linked subunits (HA1 and HA2) (Das et al., 2018). The HA1 subunit makes up the globular head of the hemagglutinin protein, while the HA2 subunit makes up the core of the protein and anchors it to the viral membrane. HA1 aids in the attachment of the virus to the host cell through an interaction between the HA1 subunit and sialic-acid receptors on the membrane of the host cell (Boonstra et al., 2018). Changes in pH cause conformational changes in the HA2 subunit which promotes the process of viral and host membrane fusion (Das et al., 2018).

Epitopes within the globular head of the hemagglutinin protein are the current targets for influenza vaccines. These epitopes are divided into distinct antigenic sites: Sa, Sb, Ca1, Ca2, and Cb for H1 influenza viruses (Gerhard et al., 1981). For H3 viruses, these sites are labeled: A, B, C, D, and E (Webster & Laver, 1980; Skehel et al., 1984). Due to high mutation rates, these regions vary between different subtypes of influenza A, resulting in vaccines with low cross-protection (Krammer & Palese, 2015). Some amino acids are conserved including HA198, −134, −138, −153, −183, and b−195 (2%). These were found to be conserved among 13 different subtypes (Nobusawa et al., 1991) (Table 3).

More conserved epitopes on the hemagglutinin head could serve as targets for a more broadly protective influenza vaccine. One of these regions is the receptor binding site (Watanabe et al., 2019; Wilson et al., 1981). The receptor binding site is structurally constrained due to its requirement for the attachment of the virus to the host cell (Schmidt et al., 2015). The conserved

amino acid residues of the receptor binding site are Y98, H183, E190, W153, and L194 (Wilson et al., 1981).

The lateral patch, located on the side of the H1-subtype hemagglutinin head, is another conserved region. One antibody clonal lineage, CL6649, recognizes the lateral patch (Raymond et al., 2018), but the immune response of this site has yet to be determined.

Multiple antibodies have been found that target the receptor-binding site: antibodies CH65, CH67, 641 I-9, H252, and 5J8 mimic sialic acid (the traditional substrate of the site) (Schmidt et al., 2015). The monoclonal antibody CH65 neutralized the infectivity of 30 out of 36 subtypes of H1N1 tested (Whittle et. al., 2011) (Table 3). The antibody 5J8, targeting an epitope between the receptor-binding site and the Ca2 antigenic site on the hemagglutinin head, broadly neutralized all tested H1N1 subtypes from 1918 to 1977 (Krause et al., 2011). Antibody S139/1, targeting the receptor binding site, neutralized 35 different hemagglutinin molecules amongst 13 subtypes (Lee et al., 2012; Yoshida et al., 2009).

As the current target of the annual influenza vaccine, much information exists regarding the morbidity and mortality of using the hemagglutinin head. One method used to measure morbidity is to measure the percentage of patients with medically attended acute respiratory illness (ARI). The 2017-2018 seasonal influenza vaccine had an effectiveness against medically attended ARI of 36% (Flannery et al., 2018) (Table 3). A study using the influenza vaccine of the 2019-2020 season found that vaccine effectiveness against any influenza illness was 39% (Tenforde et al., 2020) (Table 3).

The hemagglutinin stalk is an elongated trimer composed of 55 amino acid long alpha-helices (Lu et al., 2018, Table 3). Each monomer contains an α-helix at the N-terminus and C-terminus, which are connected by a flexible loop region (Lu et al., 2018).

In contrast to the head, the hemagglutinin stalk is more conserved (Kirkpatrick et al, 2018) and has been the focus of broadly protective influenza vaccine development (Krammer & Palese, 2013). The stalk only changed by three amino acids in H1 and H3 subtypes versus a 2.1% change per variant in the head domain over a single time period (Han & Marasco, 2011). The stalk's high conservation is in part due to its function mediating virus fusion with the host cell membrane (Sano et. al, 2021).

Despite the stalk's high conservation, it does not elicit high immune response as the head and fewer antibodies can target the stalk region. Monoclonal antibodies from mice and humans can bind to group 1 and group 2 HAs, causing some neutralization (Tan et al., 2014). Other monoclonal antibodies prevent endosomal and viral membranes from fusing together, disrupting the release of their genetic material (Tan et al., 2014). Furthermore, antibodies FI6v3 (Corti et al., 2011), CR9114 (Dreyfus et al., 2012), and CR6261 (Ekiert et al., 2009) induce protective immunity against multiple influenza subtypes. Additionally, one study found that infection by the H3N2 virus induced stalk-specific broadly neutralizing antibodies were sufficient to neutralize the virus (Margine et al., 2013). Following vaccination with the subtype H5N1, humans were also found to broadly produce neutralizing stalk-specific antibodies (Ellebedy et al., 2014). However, these antibodies were less effective than antibodies that target the head.

Alongside antibody response to the head following infection, some influenza subtypes also induce stalk-specific antibody response. In some studies, the head of an avian influenza subtype is combined with the stalk of a human influenza subtype (Krammer & Palese, 2019). Combination of two virus variant components are promising in targeting proteins that lead to low levels of cell-mediated immune response. This allows the immune system to recognize the stalk from this combination due to the unfamiliarity of the head, thereby triggering antibody

production in response to the stalk. An additional concern with concentrated immune system effort on the HA stalk is that evolutionary pressure can cause escape shift mutations. An amino acid mutation at the A338V position induced resistance of the HA stalk to monoclonal antibodies (Park et al., 2020). This could be a challenge in manufacturing an HA stalk mRNA based vaccine.

IRR against the HA stalk can be quantified with hemagglutinin inhibition assays, IgA and IgH antibody titers, and ELISA anti-stalk antibody assays. In human subjects, when IgA and IgH antibodies were produced, HAI titers of >40 were sharply associated with protection (Christensen et al., 2019). In the ELISA assay with human trials, anti-HA stalk antibody titers increased in 64% of subjects and a statistically significant increase in mean titers was observed to 72,637 after 8 weeks. (Wohlbold et al., 2015)

One method used to measure morbidity was through mouse models. Using the H10N8 subtype, treatment of mice with 5 mg/kg of anti-HA head, anti-HA stalk, and anti-NA MAbs antibodies along with viral infection resulted in no weight loss or death. Mice exposed to an anti-H6 specific MAb with viral infection quickly lost weight and died by the ninth day. At 0.5 mg/kg, anti-N8 MAb 2E11 antibodies were still protective and prevented weight loss or mortality (Wohlbold et al., 2015).

Neuraminidase

Neuraminidase (NA), a homotetrameric protein, is approximately 50 kDa each polypeptide chain (Colman, P. M. 1994; Blok, J. et al., 1982) (Table 3). There are approximately 40 to 50 NA proteins anchored to the average sized viral membrane via a short hydrophobic tail (McAuley J et. al 2019). The four NA protein units form a long central pocket that acts as the

active site for severing bound hemagglutinin (HA) from its receptor (Colman, P. M. 1994; Varghese, J. N., et al. 1983).

Neuraminidase functions in tandem with hemagglutinin to facilitate virus infection in the host. Hemagglutinin binds the virus to sialic acid protein receptors and NA frees the virus from the host cell surface allowing the virus to move from the upper airway towards epithelial cells in the lungs with a higher density of sialic acid receptors. The exact mechanism by which this cleavage occurs is not entirely understood, but the role bears substantial implications for inhibitor possibilities since sialic acid receptors are localized to surface mucins. Neuraminidase also acts to sever the sialic acid residues from the newly formed virus, allowing them to exit the host cell (McAuley J et. al 2019; Benton, Donald J., et al. 2017).

Neuraminidase conserved epitopes include the active site (Colman, 1994; Stadlbauer, et al. 2019), the NA head (Wohlbold, T. J., et al. 2017; Air, G. M. et al. 1990), and a region of the cytoplasmic tail (McAuley J et. al 2019). The most conserved region in all Influenza A Virus subtypes is close to 100% conserved and consists of an M-N-P-N-Q-K sequence found in the cytoplasmic tail (Blok, Air 1982). The second most conserved region in the N2-based subtype was found between amino acids 222 and 230; amino acids I-L-R-T-Q-E-S-E/S-C have > 99% conservation, with exception to E229 which has 94.5% conservation (Doyle, et al., 2013). Antibodies against neuraminidase provide protection against viral strains of the same N-subtype, but not necessarily against viral strains of different N-subtypes (Wohlbold, T. J., et al. 2015). However, highly conserved regions have reportedly induced broad protection, but with low effectiveness (Krammer, F., et al. 2018). More conserved epitopes that are exposed on the protein's surface are needed to gain heterosubtypic immunity.

IRR of neuraminidase has been measured in multiple ways. IFN-γ ELISPOT SFC (spot-forming cells) showed a few NA amino acid sequences with significant values above 100 SFC/10^6, hinting at their potential for an immunogenic vaccine (Assarsson, et al. 2008; Table 3). NA antibodies significantly increased after vaccination (Sultana, et al. 2014; Table 3) as did relative fluorescent units (Measurement of fluorescence commonly used to analyze electrophoresis methods) which measured NA activity and likely corresponds with an increase in antibody response (Wan, et al. 2017; Table 3). Additionally, Neuraminidase Inhibition (NI) titers significantly increased after both one and two doses (Wan, et al. 2017; Table 3).

Currently, reports show that humans commonly present with low titer levels of broadly protective NA antibodies. Some recent influenza A strains had lower titers than historically dominant strains, and elderly patients presented higher titers than younger individuals (Rajendran, M., et al. 2017). A future vaccine could increase induction of these broadly protective NA antibodies. Current influenza vaccines induce antibody production against HA; however, natural infections produce antibodies against both HA and NA (Chen, Y.Q., et al. 2018).

Laninamivir, Oseltamivir, Peramivir, and Zanamavir are NA inhibitors available to take close to the time of infection, which reduce the infectivity of the influenza virus (McKimm-Breschkin, J. L. 2013). These inhibitors raise hopes for a potential long-acting, broadly protective NA vaccine. If different inhibitors are able to recognize the NA protein and work effectively, that hints at potential conserved epitopes that can be targets in the formation of a successful vaccine. A theoretical NA vaccine may be very effective if it can increase the already existing broadly protective antibodies in humans. However, there are not many clinical trials examining NA by itself to confirm or reject this belief.

Neuraminidase seems to provide lower protection from infection but higher morbidity and mortality protection. Johansson et al. (1993) took female Balb/c mice that were given either a whole virus (WV) vaccine or an NA vaccine and compared them to unvaccinated mice. The mice were subsequently infected with the H3N2 influenza A virus. In all cases, the NA vaccine did not prevent infection but significantly reduced morbidity and mortality, whereas the WV vaccine prevented infection in most cases (Table 3). Unvaccinated mice lost between 16 and 28% of their weight and had a mortality rate of 30 to 35%, while mice with the NA vaccine only lost 4 to 10% of their weight and no deaths (Table 3). Skarlupka et al. (2021) created a vaccine based on a computationally generated NA antigen. Mice that received this vaccine and were challenged with H1N1 lost 14 to 17% of their body weight in less than one week, while unvaccinated mice lost over 20% of their body weight in the same time period (Table 3).

Secondary Proteins

Matrix 2 Ectodomain (M2e)

The Matrix 2 (M2) protein is a tetrameric, proton-selective ion channel allowing for the escape of the influenza viral genome as well as the initiation of several priming events essential to virus assembly and budding (Saelens, 2019). Structurally, M2 consists of an N-terminal ectodomain, a transmembrane domain, and a C-terminal cytoplasmic domain (Cho et al. 2015). M2e refers to the ectodomain of M2, a potential target for a broadly protective influenza vaccine. Little is known about the role of M2e, but it is presumably responsible for controlling the N-out, C-in orientation of M2 (Saelens, 2019).

Unlike HA and NA, M2e is small, ~2.5 kDa, and less subject to genetic drift. It consists of only 23 amino acid residues, of which the first nine are almost completely conserved among all human and avian influenza A viruses (Bakkouri et al. 2011, Table 3). The genome segments

coding for M2e also code for important proliferative signals for other influenza proteins, thus contributing to the high conservation of M2e (Saelens, 2019). Conservation of M2e may also be attributed to the fact that M2e-specific antibody responses are rarely induced following infection, so there is a low natural immune pressure directed against M2e (Deng et al, 2005).

A study by De Filette et al. (2006) examined the effect of different M2e constructs on immune responses. The investigators created virus-like particles (VLPs) by fusing one, two, or three copies of the M2e coding sequence to a hepatitis B virus core sequence. When administered in mice, the VLP containing three copies of M2e elicited an M2e-specific IgG1 titer of 1.0×10^7, 21 times higher than IgG1 titer levels from mice vaccinated with only one copy of M2e. These results suggest that multiple copies of M2e may induce a stronger host immune response.

As M2e cannot consistently generate an immune response; it is typically coupled with another protein, or an adjuvant, to induce an adequate immune response (Mezhenskaya et al. 2018). Multiple types of vaccines, such as protein, DNA, VLPs, and vectored vaccines are capable of producing M2e-specific antibody responses in animal models.

A study by Stepanova et al. (2018) created recombinant proteins consisting of either one viral protein (four tandem copies of M2e) or two viral proteins (M2e and HA2) fused with flagellin, an adjuvant. After three immunizations, mice treated with four copies of M2e (4-M2e) had an anti-M2e IgG titer of 4.1×10^6, while the mice treated with the M2e-HA construct had a titer of 1.5×10^6 (Stepanova et al. 2018). These vaccines demonstrated efficacy in protection against H3N2, H2N2, and H5N1. In another study, researchers replaced the transmembrane and cytosolic tail sequences of M2e with the $NSP4_{98\text{-}135}$ fragment from rotavirus (Andersson et al.

2012). The M2e-NSP4 vaccine construct induced an IgG1 titer of nearly 1.0×10^5 in immunized mice and significantly reduced the viral load in their lungs (Andersson et al. 2012).

Hervé et al. (2014) immunized mice intranasally with a recombinant vaccine created by a fusion of the C-terminus of NA to either one or three copies of M2e (N-M2e or N-3M2e). 80% of N-3M2e immunized mice survived 13 days post-infection, compared to only 30% of unvaccinated mice. In another experiment, investigators created a DNA vaccine composed of HA from H1N1 with M2e and infected 20 mice with the H5N2 subtype. 100% of mice vaccinated with M2e-HA survived 20 days post-infection without significant weight loss (Park et al. 2011). All of the mice in the unvaccinated control group experienced significant weight loss and died only 6 days post-infection (Park et al. 2011). Turley et al. found that only 1 in 16 people immunized against influenza A with a M2e-flagellin vaccine experienced severe systemic reactogenicity, while the rest of the participants experienced systemic symptoms which resolved within 12-18 hours post administration (2011).

Polymerase Basic 1 (PB1)

In influenza A, the heterotrimeric RNA polymerase consists of polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acidic (PA) subunits. The PB1 protein consists of 757 amino acids and has a mass of 87 kDa (UniProt 2021, Table 3). The active site of the polymerase that catalyzes viral RNA synthesis is contained within the PB1 protein (Nakagawa et al. 1996, Toyoda et al. 1996, Reuther et al. 2011). Functions of PB1 during RNA replication and transcription include recognition and binding of promoters and origins, binding nucleoside triphosphates, initiation and elongation, post-transcriptional modification of the poly-A tail, and read-through of the poly-A signals on the mRNAs (Poch et al. 1989, Muller et al. 1994, Nakagawa et al. 1996).

Four conserved sequences of epitopes have been identified on the PB1 protein (Wu et al. 2012). Across 75 H1N1 strains from human, swine, and avian vectors, the protein has an average pairwise conservation of 96% (Warren et al. 2013). PB1 is the most conserved segment (98.1% conserved) across human influenza A viruses (El Hefnawi et al. 2011). Across all outbreak strains, the overall sequence is 84% conserved (Powell et al. 2007, Table 3).

Many studies indicate that PB1 may be an important component for a more broadly-protective influenza vaccine. The protein contains one CD4+ stimulating epitope and three CD8+ stimulating epitopes (Wu et al. 2012). As described by Assarsson et al, PB1 epitopes elicited positive responses in peripheral blood mononuclear cells (PBMCs) using IFN-γ ELISPOT assays. Specifically, seven PB1 peptides were found to elicit a response of a maximum of over 100 SFC/10^6 (Assarsson et al. 2008, Table 3).

PB1 has had some success when used on its own, including stimulating an immune response via a DNA vaccine in mice (Kosik et al. 2012). PB1 can be successfully expressed in vivo and then isolated (Toyoda et al. 1996, Honda et al. 2002, Zamarin et al. 2006). Kosik et al. found that the PB1 gene can also be used in a DNA vaccine to provide protective immunity from influenza A (Kosik et al. 2012, Table 3). PB1 contains both a significant number of epitopes recognized by CD4+ and CD8+ T- cells, and the most epitopes presented by MHC classes I and II (Assarsson et al. 2008). PB1 induces CD4+ and CD8+ T-cell responses in response to both influenza A and influenza B viruses (Assarsson et al. 2008, Uddback et al. 2016). At this time, no research on PB1 with mRNA vaccines has been conducted.

Ichihashi et al. found that vaccination with PB1 increased survival rates, decreased illness severity, and delayed the onset of weight loss in mice (Ichihashi et al. 2011). Goodman et al. discovered that when combined with other proteins, PB1 vaccinations in mice decreased viral

load in the lungs and also delayed mortality (Goodman et al. 2011, Table 3). An NP-based vaccine with inserted PB1, M1, NS1, and PA epitopes reduced morbidity and mortality in mice (Goodman et al. 2011). Compared to the control vaccination with an empty plasmid, vaccination with this combination of proteins generated a 7.6-fold increase in IFNγ-secreting cells and a 2.9-fold increase in overall T-cell response (as measured by $IFN\gamma^{+}$ and $IFN\gamma^{+}TNF\alpha^{+}$ cells) (Table 3). When combined with HA and NA, there was a 9.1-fold increase in T-cell response compared to the control (Goodman et al. 2011). Wang et al. found that it produced a strong immune response in combination with NP and M1 (2015).

Vaccines using PB1 in combination with other protein targets have had some success in decreasing morbidity and mortality in mice. One study found that although such a vaccine (in this case a DNA vaccine) did not ultimately prevent death, it did delay death by 1-2 days (Goodman et al. 2011). In another study, mice immunized with a vaccine containing a PB1 plasmid (in combination with other protein targets) exhibited a 100% survival rate. Mice receiving a saline injection did not exhibit this same survival rate (Kosik et al. 2012, Table 3). Similarly, mice immunized with an intranasal vaccine containing $PA_{130\text{-}138}$, $PB1_{430\text{-}438}$, and $PB2_{549\text{-}557}$ survived (although with weight loss), while mice receiving other combinations did not survive (Ichihashi et al. 2011). Wang et al. found a survival rate of 80% in mice immunized with a vaccine containing PB1 as a target (2015). Though PB1 does not confer complete protection on its own, the fact that it reduced morbidity in these studies, which used various influenza subtypes, indicates that it is a promising target for a more broadly protective influenza vaccine.

Matrix 1 Protein

The matrix 1 protein (M1) is multifunctional but primarily a structural protein. M1 plays roles in the virus infectious cycle and morphology (Uniprot 2020, Hom et al. 2019, Das et al.

2012, Švančarová et al., 2018). M1 is located under the viral lipid bilayer forming a coat to aid in maintaining structural integrity. Furthermore, M1 associates with the ribonucleoprotein and the cytoplasmic domains of hemagglutinin and neuraminidase to form the matrix layer (Švančarová et al., 2018). The structure can be divided into two major domains, the N-terminal and C-terminal domains, connected by a protease-sensitive loop (Das et al. 2012, Švančarová et al., 2018, Seltzer et al. 2020). The N-terminal domain mediates protein-protein contact with NP while the C-terminal domain allows for binding to vRNA and NP (Švančarová et al., 2018).

Due to the M1's functional roles in the virus structure and life cycle, much of the protein sequence is conserved (Hom et al. 2019, Table 3). Within M1 lies several key amino acid sequences. 91-105 serves as a nuclear localization signal (Das et al. 2012, Švančarová et al., 2018). M1 forms a complex with vRNPs which can associate with NEP and be exported from the nucleus of a cell into the plasma membrane (Hom et al. 2019, Das et al. 2012). Additionally, the arginine triplet located on 76-78 plays a role in intracellular localization of the protein. Lastly, 181-193 control the oligomerization pattern that the protein adopts - spherical or filamentous (Uniprot 2020, Das et al. 2012, Švančarová et al., 2018).

Due to M1 being an internal protein, not many vaccines targeting it alone have been produced. However, one such vaccine involves a DNA prime-subunit protein boost strategy (Fen Liu et al. 2020, Table 3). When tested on mice, the vaccine protected against the lethal homologous H9N2 subtype and showed some cross protection against the heterosubtypic PR8 H1N1. The vaccine induced nearly equal levels of IgG2a and IgG antibodies – approximately 14 antibody titer – which can potentially lead to a balanced Th1/Th2 immune response (Liu et al., 2020).

M1 can be combined with other internally conserved proteins, such as NP and PB1, to induce protective immune response. Researchers combined DNA plasmid with recombinant vaccinia virus (rVV) to express NP, PB1, and M1 yielding IgG titer levels of ~10^5 (Wang et al., 2015, Table 3). Additionally, a modified vaccinia virus Ankara (MVA) was with the adenovirus to express a fusion construct of NP and M1 generating an IFN-γ response of 200 SFU/million cells (Boyd et al., 2013, Table 3). Similarly, researchers constructed a novel recombinant simian adenovirus ChAdOx1 composed of NP and M1 which moved into clinical trials. It was administered to fifteen healthy humans (Antrobus et al., 2014b, Table 3).

Researchers further tested the previously mentioned MVA-NP+M1 vaccine in clinical studies as an adjuvant. It succeeded in phase I clinical trials leading to an increase in CD8 T-cell responses (Folegatti et al, 2019, Table 3, and Masopust et al. 2006). However, phase IIb trials ended after the first season due to researchers not meeting their desired reduction in viral shedding (Swayze et al 2019, Vaccitech 2021).

The Lui et al. (2020) DNA prime-subunit vaccine fared well against HN92 subtype leading to 100% survival rate (Table 3). Against the lethal PR8 H1N1 subtype only 50% of the mice survived (Liu et al., 2020). Likewise, the DNA plasmid with rVV was effective (Wang et al., 2015, Table 3). Both the MVA-NP+M1 and the ChAdOx1 vaccine moved into clinical trials producing only mild to moderate systemic and local side effects. However, three individuals vaccinated with the max dosage of ChAdOx1 developed fevers (Folegatti et al. 2019, Antrobus et al. 233-238, Antrobus et al., 2014b).

Nucleoprotein (NP)

Nucleoprotein (NP) encloses viral RNA in order for the virus to become infectious. It is the most abundantly expressed protein during the course of infection, and functions in viral RNA synthesis, metabolism regulation, and translation (Hu et al, 2017). However, its primary function is to encapsulate the virus genome and allow for RNA transcription, replication, and packaging (Protella and Digard, 2002). Although it is known that NP plays a major role in transcription and forms the ribonucleoprotein complex responsible for it, the specific role of NP in this mechanism is still not clear (Turrell, Lyall, and Tiley, 2013). The NP binds with high affinity and no sequence specificity, which allows it to be universally useful for many viruses (Baudin et al., 1994).

Structurally, the protein has distinct head and body domains with intricate designs for the binding site at the protein cleft (Ye et al., 2006, Ng et al., 2008). The NP molecule inserts its tail loop to bind NPP through a salt bridge and other nuclear signals in order to import polymerase complexes and contribute to the infectious nature of the protein. The high flexibility of this portion of the molecule confers high plasticity, which in turn allows the NP range of oligomers and structures (Ye et al., 2006, Ng et al., 2008). This can be useful in vitro for RNA binding and other intermolecular interactions. NP has a mass of 56 kDa (Zhirnov et. al, 1999).

The NP sequence is highly conserved because it is broadly required for viral genome replicability and host adaptability. In a large-scale study of 4,430 amino acid sequences and 5,094 NP sequences in influenza A, researchers found six highly variable sites and many highly conserved sites on NP, with 59% of amino acids highly conserved (Kukol and Hughes, 2014, Table 3). The high conservation of NP makes it a good potential vaccine target and also contributes to the reproducibility of the protein. One study suggests that NP is required for

replication of genomes for any virus. It is therefore a large part of any virus's infection cycle and contributes to the morbidity of influenza A.

Many studies suggest that NP is a significant part in many infectious disease patients. A study conducted about ECD-specific IgA discovered that most COVID-19 patients generated a distinct response against NP, which suggests that using NP in future assays could result in more accurate results. The r value for this assay was 0.6689, with a p value of <0.0001, when conducted on patients infected with the COVID-19 virus (Aleru and Barber, 2020). Another study showed that mice immunized with a virus containing NP from the master donor virus (MDV) produced higher antibody titers, with the geometric mean titre (GMT) increasing from 2.5 to 10 in vaccinated subjects (Isakova-Sivak et al., 2016). These studies suggest that the nature of conservation for this protein is wide, as it is able to appear in viruses of many different varieties. This also suggested that NP’s ability to elicit an immune response in organisms is high and could contribute to vaccine creation more efficiently. There were no quantifiable numbers found for NP as it relates to influenza.

In response to stimulation with nucleoprotein peptide pools, multiple 10- to 12-week-old rabbits averaged 1,552 SFUs (spot forming units) per 106 (PBMCs)—a larger antigen count than the untreated rabbits, which averaged 32 SFUs/106 PBMCs. This suggests that vaccination with NP peptides produces a strong immune response from these rabbits and increases their survival rate (Yung et al., 2019).

Tertiary Proteins

NS1

Polymerase Acidic Subunit (PA)

Polymerase acidic subunit (PA), a 716 amino acid residue (UniProt, 2021,Table 3), functions in transcription and replication of the viral genome, acting cohesively with PB1 and PB2—other essential subunits of polymerase. PA is also an essential component of viral RNA packaging. In terms of conservation, PA is highly conserved in the influenza A strain. According to a study in which the conserved regions of various protein subtypes within influenza A were tested, PA had a conservation percentage between 85-93% in swine strains, 91-94% in avian strains, and 94-98% in human strains (El Hefnawi et al., 2011).

A combination influenza virus containing the PA subunit provides a glimpse into what can be achieved when internal proteins are targeted. A study done by Xie et al. (2019) employed a DNA+TTV based influenza vaccine using a PAPB1M1 protein combination. After exposing mice infected with H1N1 to the combination vaccine, mice morbidity and mortality was measured through body weight loss percent and percent survival. In terms of morbidity, it was found that the mice experienced an overall 18% weight loss eight days post-infection when inoculated with the PA combination vaccine, compared to 28% in the control group (Xie et al., 2019). As for mice mortality, the control group had a 100% mortality rate ten days after exposure to the H1N1 virus, while the vaccinated group maintained a 0% mortality rate throughout the testing period of 14 days (Xie et al., 2019).

The IRR of the PA combination vaccine was also measured using IFN-γ ELISpot assays providing CD8+ T-cell percentage values for cells that were IFN-γ+/TNF-α+, IFN-γ+, or TNF-α+ (Xie et al., 2019)(Table 3). Although not eliciting a completely stronger immune response in comparison to the control, the DNA+TTV vaccine achieved a broad cellular response as more peptides such as PA were able to elicit discernible IFN-γ induction through the immune cells.

Uddbäck et al. (2022) used an adenovirus vector with a PA insertion to stimulate mice. They found that CD8+ memory T-cells were stimulated via a specific PA epitope, namely PA224-233. Depending on the mouse strain, these PA-specific memory CD8+ T-cells confer either partial or full protection during viral challenge (Uddbäck et. al, 2022).

Polymerase Basic Protein 2 (PB2)

Influenza A polymerase basic protein 2 (PB2) is a part of the trimeric RNA polymerase proteins responsible for the replication of viral RNA genome. It consists of about 759 amino acid residues (Patel et al., 2017, Table 3) and is responsible for recognizing capped RNA in generating transcription primers. Residues 318-482 are involved in the cap-snatching; they allow viruses to recognize methylated guanosine and bind to the host RNA. PA protein then cleaves the RNA, leaving 10-13 primer residues to start transcription (Patel et al., 2017).

PB2 is highly flexible - it rotates about 70 degrees to relay cleaved host mRNA to PB1, and adopts a transcription pre-activation state where the cap-binding site blocks mRNA binding (Ma et al. 2017). Conserved regions are scattered all around the PB2. They include residues of the N-terminal (1-37); mGTP region cap binding domain. PB2's C-terminal, however, is found on the peripheral section of the influenza virus. It has been found that, in mice and humans, $CD8^+$ T cell responses are focused on internal proteins like PB2 (Schmidt and Lapuente, 2021). A study from Ma et al. revealed the selectivity and weak binding affinity of PB2's C-terminal. They determined that PB2's cap-binding site could be inhibited using azaindole derivative (VX-787), to immobilize the virus. They extended its N and C terminals and folded them into a new domain called mid-linker. because PB2's cap binding affinity to VX-787 was very weak (approximately 56 nM) (Ma et al., 2017). Thus, PB2 binding domains are not as highly conserved as other proteins found inside the virus.

A PB2-based vaccine has a high immune response in host cells that could express PB2. Uraki et al. (2013) were able to generate two PB2-KO (PB2 knockout) influenza viruses and inject them into mice. These flu viruses had their PB2 proteins replaced with HA proteins. Lacking active PB2 proteins, these influenza A viruses (PB2-KO) only replicate in the presence of PB2 protein-expressing cells. The result yielded mice that developed virus-specific antibodies and achieved a bivalent influenza immunity; antibodies were able to recognize PB2 activity, and inactivate the virus. IRR of the vaccine doubled upon immunizing mice with more PB2-KO virus. An IgG absorbance value of 0.4 was measured against H1N1 and H5N1 subtypes. Thus, mice are protected from lethal challenges of the virus. A similar experiment done on a human host would not produce the same IRR effect because humans are unable to express PB2 proteins. This also proves that antibodies are unable to recognize integral proteins such as PB2.

Recent studies have also determined PB2's effect on morbidity and mortality. Solórzano et al. (2010), mutated PB1 and PB2 of a live attenuated vaccine and inoculated 12 DBA/2 mice with the vaccine, while a mock group of mice were inoculated with PBS. Both mice groups were introduced to the H1N1 virus, and their weight and mortality were recorded for 14 days. DBA/2 mice showed no signs of morbidity or weight loss 13 days after vaccination, while mock mice experienced about 20% weight loss and some deaths after 5 days.

Nonstructural Protein 1 (NS1)

Of the eight segments that comprise the influenza A genome, the smallest is the NS (nonstructural) gene, which encodes an mRNA that is spliced into nonstructural protein 1 (NS1) and nuclear export protein (NEP), also known as nonstructural protein 2 (NS2) (Nogales et al., 2016). NEP is the least abundant of these two proteins, accounting for less than 20% of the produced transcripts (Robb et al., 2010). Both NS1 and NEP are multifunctional conserved

proteins, with roles including, but not limited to, regulation of viral synthesis. Out of the two NS1 is more well-studied..

NS1 facilitates viral replication by inhibiting the interferon binding response (Marc et al. 2014). Additionally, NS1 can regulate the synthesis of NEP within a host cell, which implies that it can be used to control the occurrence and rate of viral protein export post-infection (Killip et al. 2017). While the IFN protein binding interference mechanism is not fully understood, NS1's ability to negate interferons makes it promising for mRNA vaccine development. The physical structure of NS1 also has yet to be fully confirmed, although it is believed to be a dimer (Zhang et al 2019).

NS1 IRR is seen through analysis of antibodies and interferon titers specific to NS1 and immune response to mutant NS1. These antibodies include mucosal immunoglobulins, notably G2 (IgG2), G1 (IgG1), A (IgA), 1 (Ig1), and 2 (Ig2). A study conducted by Ferko et al. (2004) involved NS1 mutants encoding dysfunctional RNA binding domains. Despite the replication mutation, high titers of IgG2 and IgA exceeding those of the normal virus were produced. (Ferko et al. 2004, table 3). Replication-deficient NS1 mutants were able to yield high Ig1 but low IgA. Moreover, cytokine production and high CD8 T-cell counts were observed in mice immunized with mutant NS1. (Ferko et al. 2004).

Nuclear Export Protein (NEP)

Nuclear export protein (NEP), also known as nonstructural protein 2 (NS2), has a critical role in the transportation of the viral ribonucleoproteins (vRNPs), which are the newly-synthesized gene segments, out of the parent virus into cytoplasm for incorporation into the budding daughter virion (Akarsu et al., 2011). The protein assists with the regulation of viral synthesis, associating with the matrix protein 1 (M1) to inhibit polymerase activity (Nogales et

al., 2014). Akarsu et al. (2011) showed that NEP also contributes to virion shape because of these interactions with M1.

While 93.4% of the NEP sequence is conserved across subtypes of influenza A (Paterson & Fodor, 2012, Table 3), research on use of this protein in vaccines is limited, likely because of its small size and its location inside the influenza virus rather than on the surface. Zhang et al. (2015) showed that a NEP mutation promoted growth of an influenza A strain without affecting its virulence, making it an appropriate donor strain for a cell-based influenza vaccine. When combined in a potential vaccine with M1, NEP elicits 32 HAI (hemagglutination inhibition) titers at 0.01 MLD_{50} (50% mouse lethal dose) and 128 HAI titers at 0.1 MLD_{50}, which is similar to the HAI titers generated against PR8 H1N1 live-attenuated vaccine and shows efficient immune response (Nogales et al., 2016, Table 3). Additionally, Akarsu et al. (2011) found that multiple mutations in the residues on NEP that interact with M1 created an attenuated virus for possible use in attenuated influenza vaccines. Though cellular IRRs have not been extensively studied for NEP, one study found cross-reactive CD8+ T-cells that recognized NEP after stimulation with NA and HA (Reber et. al, 2018).

Chapter 4: Discussion

Based on our analysis of the conservation of each protein across influenza A subtypes, its immunogenicity both alone and in combination, and its in vivo morbidity and mortality, we recommend that a combination of HA1, NP, and M2e would be the most effective direction for a future influenza A mRNA vaccine. An mRNA vaccine would be optimal for adjusting the yearly vaccine according to new strains. A three-protein sequence vaccine construct would likely increase immune response, but we have limited the vaccine to three proteins to minimize risks of unattributed adverse effects. Incorporation of multiple mRNA sequences allows for the translation and production of multiple proteins inside an inoculated cell, as opposed to a single protein. The results of in vivo experiments using multiple proteins, such as the M1-NP-PB1 DNA recombinant vaccinia virus, support our hypothesis (Wang et al., 2015). The recombinant vaccine constructs investigated in this review showed that multi-target vaccines are able to produce anti-protein IgG antibodies unique to each protein utilized (Wang et al., 2015). This broadens the scope of the immune response, leaving the body better equipped to recognize and eliminate the influenza A virus upon host infection. Targeting multiple proteins, including two with highly conserved sequences, should maintain vaccine effectiveness against new subtypes of influenza A.

Other proteins lacked sufficient evidence to support their use in a more broadly protective vaccine. While the primary proteins HA2 and NA have been extensively researched, they were deficient in work specifically on our criteria and in benefits that were not already covered by other proteins. For example, HA2 lacked research in combination with other proteins, which is critical for the feasibility of our proposed vaccine. The NA literature revealed high antigenic drift like HA1, but a poorer IRR response, as it acts later in the viral life cycle (McAuley et al. 2019;

Table 3). The secondary proteins PB1 and M1 had other specific downsides, such as low abundance in the cell for PB1 and unpromising conservation, morbidity, and mortality values for M1 (Russell, Trapnell, & Bloom, 2018; Table 3). Lastly, all four tertiary proteins—PA, PB2, NS1, and NEP—had little to no vaccine research. PA and PB2 are both internal proteins with low presence in the virus (Russell, Trapnell, & Bloom, 2018). NS1 and NEP not only exist in low amounts inside the virion, but also have scattered unclear purposes in the virus life cycle (Killip et al. 2017; Akarsu et al., 2011).

Recommendations

The first protein, HA1, is the most well-researched and those findings have been foundational to research into other proteins. The investigation of HA1 in mRNA vaccines is already underway (Petsch et al., 2012). The greatest benefit of HA1 is its degree of immunogenicity. The current standard flu vaccine targeting HA1 has been shown to increase antibody titer levels 3- to 4-fold and yields high antibody titer levels after vaccination (Cowling et al., 2020). However, the comparatively low capacity of HA1 to prevent illness is a weakness. When scientists predict flu subtypes with a high degree of accuracy, HA1-targeting vaccines, the main type currently used by the public, are still only 40-60% effective, due in part to genetic drift (CDC, 2021). The high immunogenicity and volume of research qualify HA1 for inclusion, but its lesser ability to prevent illness warrants the inclusion of two other protein targets.

The second recommended protein target is the M2e. Like HA1, M2e is a viral surface protein, and it has shown positive results in combination with HA1. A trial challenging mice with the avian flu showed a 100% survival rate (compared to 67% effectiveness with just HA1 or 32% effectiveness with just M2e) when mice were vaccinated with a combination HA1-M2e DNA vaccine and lower weight loss levels as well (Park et al., 2011). Additionally, M2e would

make the proposed vaccine more resistant to genetic drift as well, as 10 amino acids (39%) of its short chain are conserved across influenza A subtypes. (Bakkouri et al. 2011, Table 3)

Since HA1 and M2e have relatively low degrees of conservation, the proposed mRNA vaccine should include a highly conserved protein target in order to optimize resistance to genetic drift. NP would be the best target to include for this purpose. Not only does NP have 293 amino acids (59%) conserved in influenza A, but it is also highly abundant in the cell, both of which contribute to antibody recognition of the virus after being exposed to the components of the vaccine (Kukol and Hughes, 2014).

Limitations and Future Directions

Although comprehensive, this review may inadvertently exclude research not indexed in Google Scholar, PubMed, NCBI, ScienceDirect, and ClinicalTrials.gov or not appearing in the peer review literature due to proprietary development. Additionally, because studies used a wide variety of different units (geometric titers, IgG or IgA titers, SFU, SFC, PFU, etc.) in IRR tests, standardizing our collected data for quantitative analysis was infeasible. These limitations warrant further investigation of each of the targets using the same experimental conditions (subtype, dose, adjuvant) and measurements to allow for quantitative comparison of immunogenic potential. Finally, mRNA vaccination technology is a new development with limited studies and lacks ample influenza-specific literature. We suggest further investigation of our recommended combined HA1-M2e-NP vaccine beginning with in vitro cell-based trials and graduating to animal trials in case of promising results. Success in preclinical trials may warrant graduation to clinical trials.

Chapter 5: Equity

The SARS-CoV-2 (COVID-19) pandemic has changed daily life since December 2019. This section addresses some of the scientific and sociological lessons learned from the COVID-19 pandemic that could be used to improve influenza research and to mitigate future influenza outbreaks. Acknowledging the devastating effects that viral infections can have on the global population and potential long term impacts on the human body has focused attention on vaccine development, anti-viral treatments, and a more equitable healthcare system.

Modeling, Contact Tracing

COVID-19 shed light on disease modeling. Many countries adopted social distancing measures to slow the spread of the virus. Modeling disease transmission is crucial to saving lives and minimizing the economic and social consequences of the pandemic, as predicting the propagation pattern will give insight into the virus's behavior. Mathematical modeling of biological systems allows experts to simulate virus spread and create 'what if' scenarios to predict the future. A system of differential equations is used with real data to estimate unknown parameters, such as the R0 value, which indicates how contagious an infectious disease is (McLean, 2010). Knowing these parameters helps scientists and officials gauge how effective social distancing policies are and what actions need to be taken to slow the spread.

Contact tracing is integral to disease modeling. As lockdown measures are lifted and case numbers fall, the de-escalation of physical distancing must still be monitored. Contact tracing enables officials to track disease spread through conventional methods and mobile applications (Kleinman & Merkel, 2020). Upon testing positive, the patient is asked to provide the information of people they have recently seen so that their contacts can isolate and prevent further spread of the virus. Data from contact tracing methods further helps understand

transmission patterns. Implementing contact tracing measures and using better modeling techniques could mean better prediction of future influenza patterns (Hassan et al., 2021).

Testing Optimization

Three types of COVID-19 tests are available: antibody, molecular, and antigen, all of which analyze a patient's nasal, throat, or saliva sample. Antibody tests detect the presence of IgM and IgG antibodies that work to expel the virus and prevent re-infection (Ejazi et al., 2021). These tests cannot screen for active COVID-19 cases, as antibodies appear about a week after infection.

Molecular tests look for viral RNA sequences present in a sample. RT-PCR is the gold-standard for COVID-19 testing (Everitt et al., 2022). Using primers that match specific viral genome sequences, a patient's sample goes through a series of heating and cooling cycles allowing for primer attachment and sequence amplification. This process yields highly accurate results but is time consuming and limited to commercial or central laboratories due to complex user steps and expensive equipment required (Everitt et al, 2022).

Antigen tests offer the quickest test-to-result time, but with less accuracy. These tests detect the presence of viral proteins in the body and can return a result in as little as 15 minutes. Most rapid tests are processed through an automated analysis machine. A number of instrument-free rapid tests are available at pharmacies for potentially COVID-positive individuals to be tested and diagnosed at home, without exposing people outside of their residence to the virus. However, over-the-counter tests can be prohibitively expensive and often low in supply (Everitt et al., 2022).

Contact tracing is critical to initiating self-quarantine and curbing viral spread, but is only an effective preventative measure if diagnostic tests are available and accessible (Everitt et al.,

2022). Despite multiple testing options, American healthcare infrastructure was not equipped to deal with the enormous testing demand at multiple points throughout the pandemic. The vast majority of COVID-19 tests are processed in large, centralized laboratories. Staffing and supply shortages combined with high testing volumes and sample shipping times overwhelmed these facilities, resulting in multi-day wait periods for both molecular and rapid tests. Delays in test results hindered efficient contact tracing and quarantining procedures, propagating viral spread.

These problems can be alleviated by point-of-care (PoC) testing. PoC tests are diagnostic procedures performed in the presence of a patient without the need to send a sample to a laboratory. The World Health Organization's generalized guidelines for PoC infectious disease diagnostics are described by the acronym ASSURED - affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to those in need. At-home COVID-19 tests meet the user-friendly, rapid, and equipment-free criteria, but fail to deliver in the other areas of ASSURED (Everitt et al., 2022).

The pitfalls associated with laboratory test processing and lack of equitable PoC testing are not unique to COVID-19. At-home tests for influenza currently do not exist, restricting testing to only those who have the time, money, and means to go to a physician's office or urgent care. Total reliance on laboratories could overwhelm healthcare infrastructure in the case of a future influenza pandemic. Research efforts into isothermal nucleic acid amplification, RT-LAMP, and RT-RPA for COVID-19 testing can be directed towards influenza as well to create PoC influenza diagnostic options.

Treatments

Drug repurposing is a strategy used to identify if existing drugs have multiple functions. Creating new drugs from scratch is a time consuming and expensive process, an ineffective

solution in a pandemic setting. Drugs developed from a repurposing approach can quickly enter the clinical trial phase, accelerating the development timeline.

Broad-spectrum antiviral agents (BSAAs) that have been determined safe in humans are ideal candidates for this purpose. Remdesivir, a viral RNA-dependent RNA polymerase inhibitor, was used in preclinical studies against Middle East respiratory syndrome (MERS-CoV), as well as in a controlled trial against the Ebola virus (Singh et al., 2020). Chloroquine and hydroxychloroquine showed promising in vitro data for antimalarial studies, and can enhance the antiviral activity of Remdesivir. Monoclonal antibodies against the interleukin-6 receptor are helpful in reducing inflammatory response and improving organ function in cancer, rheumatoid arthritis, and thus potentially COVID-19 patients (Chakraborty et al., 2020).

Pfizer's Paxlovid was approved by the FDA for use as a COVID-19 antiviral pill in December 2021. It was developed and approved for emergency use within two years of COVID-19 emergence due to Pfizer's prior investigations into SARS in 2003. Their research showed that a particular viral protein, the protease, plays a large role in host cell integration. This insight from SARS behavior successfully translated to COVID-19 - Paxlovid is a protease inhibitor, and showed an 89% reduction in hospitalization and serious illness when administered (Mahase, 2021).

In silico drug repurposing uses disease and pharmacological data from a database of disease phenotypes and predicts molecular targets on the disease, offering compounds that can disable said target (Mirabelli et al., 2021). Data such as drug-target interactions, gene expression, protein networks, clinical trial reports, and electronic health records have become accessible in standardized forms through the development of databases. Such databases could be used in the future as influenza changes and as new viruses develop to "recycle" existing treatments.

Numerous drug repurposing trials are already in progress for influenza treatment, including Nitazoxanide, a drug used to treat parasitic infection and chronic hepatitis that also inhibits HA maturation and transport in the influenza virus. Nitazoxanide is in phase III clinical trials for influenza, and, in 2022, is being evaluated for potential effectiveness against COVID-19 (Pizzorno et al 2019) (Rossignol et al. 2022).

Battling Distribution Speeds Versus Mutation Rates

As of March 9, 2022, only 65.2% of the U.S. population has been vaccinated for COVID-19 since the FDA approval of the Moderna vaccine December 18, 2020 (Mayo Clinic, 2022). Vaccine rollout began slowly, at a rate of 0.49 doses per day in February 2021 and mostly plateaued by May 2021 (Diesel et al. 2021). A major concern for public health professionals has been the vaccination rate's ability to keep up with the spread of COVID-19 and its subsequent capacity to mitigate the spread of illness. There continue to be spikes in rates of infection in the US population as recent as January 2022, when infection rates were reaching over 1.4 million people per day (Allen et al. 2022).

This concern is heightened by the emergence of new variants of the constantly evolving COVID-19 virus. New variants such as Delta and Omicron continue to arise "at a furious rate," according to the Los Alamos National Laboratory, who, by April 30, 2020, had already identified 14 concerning Spike protein variations, the protein targeted by the currently available COVID-19 vaccines. New variants, as one would expect according to the basic proceedings of natural selection, spread and infect faster than those before them, constantly increasing in ability to persist. This is a shared problem amongst infectious diseases, including influenza, that has yet to be solved. Dr. Anthony Fauci, the president's chief medical advisor, has addressed the topic of variants as a global problem, reporting: "Viruses that love to mutate don't mutate unless they

replicate. If you can prevent them from replicating either by vaccination or public health measures, then you will diminish the potential of their mutating."

Efforts to accelerate vaccine distribution against the tide of variants are in progress, and include the transformation of stadiums and amusement parks into large-scale vaccination centers across the country. There are a plethora of barriers to vaccine distribution, from public distrust inherent to hasty vaccine development to the expensive storage demands of the vaccines, which do not generalize to the influenza vaccine. There is still much to be learned about resolving obstacles to mass vaccination against viruses like COVID-19 or the flu.

Messenger RNA (mRNA) Vaccines

Possibly the biggest scientific breakthrough as a result of COVID-19 research and development of a mRNA vaccine that is approved and distributed to the public. While mRNA vaccines had been the focus of research for many years previous to the pandemic, it was only because of the pandemic that the widespread production of an mRNA vaccine was expedited and achieved. The fast production, high rate of efficacy, and the approval of the mRNA COVID-19 vaccine paves the way for future improvements on viral research, including, but not limited to, influenza.

Accelerated Vaccine Development Timeline

With the onset of COVID-19, the process surrounding vaccine development has morphed greatly to accommodate society's dire need for a vaccine. The usual timeline for vaccine development takes around 12-15 years given the multiple phases that must be completed to release the vaccine to the public (Krammer, 2020). During this period, there is a thorough exploratory stage encompassing two or more years of research , followed by clinical trials during which the vaccine is tested repeatedly amongst small groups, and eventually larger populations

of people (CDC, 2014). The trials, having been categorized into 3 separate phases, usually take anywhere from 7 to 11 years to conclude (Marino et al., 2022). Once the trials are complete, the vaccine must undergo a rigorous review and approval process, in order to be accepted and released into the general public, which may take upwards of one year. In addition to the development and trial process, the vaccine is subject to a manufacturing review process in which the means of manufacturing, labeling, and licensing the vaccine is done. Following the release of the vaccine, the side-effects and unintended instances must be recorded (CDC, 2021).

The COVID-19 pandemic has accelerated this process immensely, to the point where the development procedure has been cut down to only 12-18 months(Sanicas et al., 2020) . The usual research process entails only six months of investigation and experimentation. On the spectrum of clinical trials, Testing phases are combined and overlapped to streamline and shorten the process to release the vaccine into the public. Implementing this method can shorten the time needed for clinical trials to only about 12-14 months rather than the usual multi-year process (CDC, 2021). However, compared to the regular vaccine timeline, the timetable for the accelerated pandemic vaccine includes a lengthier follow-up segment, where side-effects are meticulously registered.

Despite the accelerated vaccine development timeline followed during pandemics such as COVID-19, the level of standards and efficacy remains the same. Pharmaceutical companies must still adhere to the stringent quality control measures and regulations that are in place during the development of a vaccine that does not occur during a pandemic. Any side-effects and unexpected results must be reported from the get-go, with more emphasis placed on them, in an effort to uphold the standards (FDA, 2020). Similar to a non-pandemic vaccine, the FDA still proceeds with stringent oversight of the manufacturing and production process of the vaccine

which continues the length of the license given to the manufacturer (FDA, 2020). Hence, regardless of the presence of a pandemic and a much shortened timeline, the quality of the vaccine being produced and distributed to the public always remains constant in that only once several necessary safety checkpoints have been passed, can an individual receive their vaccine.

The COVID-19 pandemic has also greatly altered the landscape of entities involved in vaccine production. In the race to create a safe and effective vaccine for COVID-19, smaller companies such as startups have become involved in the development process rather than a playing field filled with large pharmaceuticals and incorporations (National Institutes of Health (NIH), 2020). The wealth of knowledge spread has also been immense such that pharmaceutical companies are partnering with one another and sharing manufacturing processes to help speed-up the vaccine development process. These elements are ones that have been vast improvements in the field of vaccine development, and are sure to be continued in the future with the development of subsequent vaccines.

Thus far, the BioNTech-Pfizer, Johnson & Johnson, and Moderna NIAID vaccines are the main vaccines approved for individuals in the United States. However, given the dire need for vaccination, the FDA was able to provide emergency but not full approval until mid- 2021. Pfizer vaccine was fully approved by the FDA in August 2021, for individuals 16 and older (CDC, 2022). Later on in January of 2022, the Moderna vaccine was fully approved by the FDA 18 and older. In addition to the release of the vaccines to the general public, it was further recommended that a booster dose should be administered given that the effectiveness of the vaccine is reduced over time. This was recommended for children ages 12 and up, and for those who are 5 months departed from having completed their original vaccination dose(s) (CDC, 2022).

The multidimensional acceleration of vaccine development enabled by the necessity of the COVID-19 pandemic will allow a delay in the influenza vaccine development timeline that will be based on more accurate predictions of subtypes and allow for more immediate adjustment to detection of new subtypes of influenza.

Scientific Communication and Collaboration in the Wake of COVID-19

The COVID-19 pandemic has led to vast improvements in scientific communication that can now prepare the world for future pandemics and navigating other viruses such as influenza. From the start of the pandemic, the remarkable development of mRNA vaccines with unprecedented speed was possible due to these levels of communication. The genetics of SARS-CoV-2 was shared to other countries shortly after scientists in Wuhan were able to sequence the virus. This helped lead to the development of mRNA vaccines to help quell the worldwide virus outbreak. Having scientists from around the globe share information with each other can help quell large scale outbreaks before they can have the chance to reach pandemic levels (Matta, 2020).

Further, constant communication was something that governments sought to do. The White House's COVID-19 task force would constantly update the Trump and Biden administration on the newest information about the virus and then provide this update to the public. The general spread of this information could help provide the most up to date information on COVID-19 to the general public so they were kept informed and knew the proper safety precautions they should take. Being able to do this with other respiratory viruses can help in keeping the general public informed with new breakthroughs and the most up to date information with public health crises.

Disease Inequity and Unequal Distribution of Resources

One of the biggest takeaways of the COVID-19 pandemic is the display of disproportionate burden of disease on a massive scale. New figures even early on in the pandemic showed the number of cases and deaths by ethnicity and economic status. Although socioeconomic determinants of health are well-established, COVID-19 showed just how badly certain socioeconomic groups were affected by the virus due to their large infection numbers and unfavorable outcomes. People of lower economic status are less likely to be able to afford a healthy diet, live in a neighborhood where it is safe to exercise outside, or own a gym membership and they are more likely to live in neighborhoods with a high density of fast-food establishments (CDC, 2020). A combination of factors lead to a higher likelihood of hypertension, heart disease, and diabetes. This is in conjunction with the fact that these groups have a lack of access to healthcare and treatment. These comorbidities, in the case of viruses like COVID-19 and the flu, increase the likelihood of severe illness and therefore number of deaths (CDC, 2020). Even when testing and vaccines became more widely available, these groups were the last to be vaccinated (and many remain unvaccinated) and get tested/at home test kits.

This is in addition to the fact that "essential" workers (people with jobs that cannot be done from home) in sectors like construction, agriculture, and grocery stores are often undercompensated for their services, especially with the increased risk since 2020 (The Lancet, 2020). Increased exposure to COVID-19 and, pre-pandemic, the flu, increased the comparative frequency of cases, that confounds risk based on socioeconomic status and therefore worsens the burden of disease in this segment of the population. Growing efforts to improve access to better healthcare in low-income communities is key to lowering the burden of disease from the flu.

Vaccine Hesitancy Among Minority Populations

Vaccine hesitancy has generally been the highest amongst minority populations, and this trend has continued into the age of COVID-19 vaccines. Within the United States, vaccine hesitancy was the greatest within African-American and Hispanic populations, in comparison to their caucasian counterparts (Ngyuen et al., 2022). Specifically in terms of vaccination rates, African-American populations were less likely to be vaccinated than white populations, and these rates were increased in groups with lower socioeconomic status and a smaller degree of education (Ngyuen et al., 2022).

Although part of this hesitancy could lie within general uncertainty regarding the safety of the vaccine itself, or even having a more recent COVID-19 infection, a large element of hesitancy has been distrust and wariness of doctors and the medical system within the United States by African-American populations. This mistrust in part originates from the Tuskegee syphilis study, where African-American men with syphilis were not given treatment in the form of penicillin, which was approved to treat the disease in years prior (Scharff et al., 2010). In addition, the unethical harvesting and preservation of HeLa cells from Henrietta Lacks sheds more light to the atrocities committed by the medical system, further contributing to the foundation of distrust that many groups have about the medical field in the United States (Wolinetz & Collins, 2020). Apart from direct unethical acts committed by the U.S medical system, a significant portion of distrust also lies in the fact that many minority groups, including African Americans, are much less represented in clinical and disease trials. This could be attributed to how the studies are designed, along with a lower degree of health literacy. Continuation of current efforts and new initiatives to build trust in the public health system, especially among minority populations is imperative for the success of future flu vaccines.

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