

ADVANCEMENTS IN INFLUENZA GLYCOBIOLOGY: IMPACTS IN INFLUENZA  
A VIRUS EVOLUTION, FITNESS, AND VACCINE DEVELOPMENT

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Master of Sciences

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by

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ADVANCEMENTS IN INFLUENZA GLYCOBIOLOGY: IMPACTS IN INFLUENZA  
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## DEDICATION

I could not have gone this far without my family's reassurance and encouragement to pursue my education. My parents would always say, "¡échale ganas!", which roughly translates to, "give it your all". I would also like to specifically say thank you to my older sister, Beatriz, who I always looked up to and would always make me get out of my comfort zone even when at the time I dreaded it. Looking back on those memories, I am happy to have gone through those experiences. Overall, I looked forward to planned or unplanned calls and hangouts with my siblings throughout graduate school. Through my educational journey I am thankful to have my parents and siblings' support.

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Dr. Xiu-Feng Henry Wan

**ABSTRACT**

The *Alphainfluenzavirus influenzae* (FLUAV), is an enveloped, negative sense, single stranded RNA virus and part of the *Orthomyxoviridae* family. The FLUAV is comprised of eight genome segments, with segments HA and NA being the primary targets for host humoral responses and determining the subtypes of the influenza viruses. N-linked glycosylation may occur at the N-X-S/T sequon sites of NA and HA, and potentially impact the structure of HA and NA and thus transmissibility, antigenicity, and/or immunology of the FLUAV. FLUAVs circulating in humans have shown an increase of N-linked glycosylation sites, compared to those circulating in avian. Monitoring the heterogeneity and site occupancy of N-linked glycosylation is important in understanding the natural history of FLUAVs. Advancements in glycomics technology and methods have helped precisely characterize N-linked glycosylation and develop therapeutic targets and broadly protective vaccines for influenza prevention and control.

## CHAPTER 1 Introduction

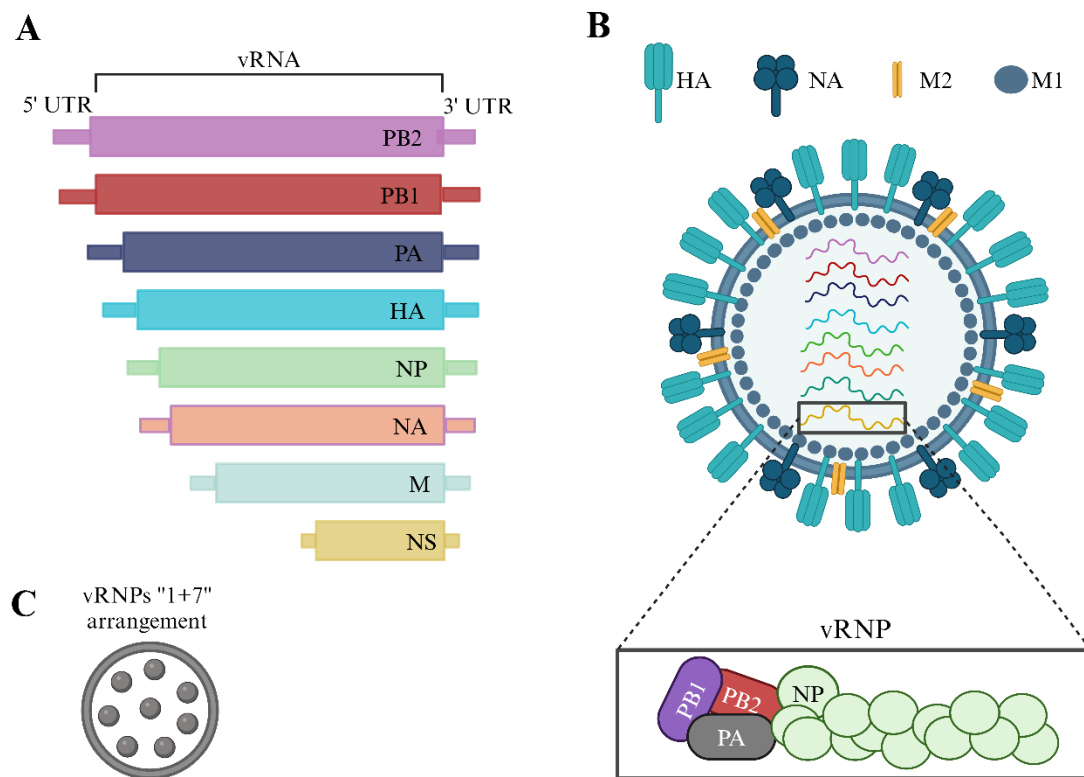
### 1.1 Background of influenza structure and strains

Influenza virus is an enveloped negative-sense single-stranded 80 to 120 nm diameter RNA virus in the Orthomyxoviridae family<sup>1,2</sup>. As of June 2023, the International Committee on Taxonomy of Viruses (ICTV) made changes to the International Code of Virus Classification and Nomenclature (ICVCN) to set a standard nomenclature for virus species<sup>3</sup>. Influenza virus species have a new species name and abbreviations to accommodate to the mandated binomial nomenclature where the first part of the name refers to the genus while the second part is the species<sup>4</sup>. The classification is as follows: influenza A virus is now *Alphainfluenzavirus influenzae* (FLUAV), influenza B virus is now *Betainfluenzavirus influenzae* (FLUBV), influenza C virus is now *Gammainfluenzavirus influenzae* (FLUCV), and influenza D virus is now *Deltainfluenzavirus influenzae* (FLUDV)<sup>3,5,6</sup>. In humans, an influenza infection will occur in either the upper or lower respiratory tract, and the patients, particularly those infected with FLUAV, experience fever, headaches, muscle pain, cough, sore throat, or runny nose<sup>7,8</sup>. In addition to humans, FLUAV has a natural reservoir in wild aquatic birds but infects domestic poultry and a wide range of other mammalian species, including swine, horses, bats, seals, dogs, cats, and ferrets to name a few<sup>9-11</sup>. FLUBV primarily infects the upper respiratory tracts of humans and a few other mammalian hosts, such as seals, ferrets, and swine<sup>11-13</sup>.

FLUAV and FLUBVS are constructed with eight viral genome segments, polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), hemagglutinin (HA), matrix (M), neuraminidase (NA), nucleoprotein (NP), and non-

structural protein (NS) (**Figure 1a**)<sup>12,14</sup>. The main difference between FLUAV and FLUBV genome segment replication is how FLUBV only has splicing for NS to form NS1 and NS2 while FLUAV also has splicing for NS, it also splices M to form M1 and M2<sup>14</sup>. For FLUAV and FLUBV replication, the HA attaches to the sialic acid receptor to let the cell go through endocytosis to enter the cell. Sialic acids are sugars with nine-carbon backbone present on the surface of the glycoproteins and glycolipids of a host's surface<sup>15</sup>. As the endosome becomes acidic via the M2 pumping in protons into the endosome, the virus envelope and endosomal membrane fuse. The low pH also induces a conformational change in HA leading to HA2 fusion peptide to be exposed<sup>16</sup>. The fusion peptide aids in bringing together the viral and endosomal membrane together<sup>17</sup>. The viral RNA dependent RNA polymerase (RdRp) is made up of three viral proteins: PB1, PB2, and PA, with PB2 containing the endonuclease activity. The RdRp along with the one copy of a negative sense vRNA covered in many copies of NP forms a viral ribonucleoprotein (vRNP) (**Figure 1b**)<sup>18</sup>. The vRNPs are released from the M1 membrane into the host cell cytoplasm<sup>7</sup>. The vRNPs enter the nucleus via nuclear localization signals<sup>19</sup>. To begin replication, the vRNP's RdRp transcribes the negative sense vRNA into positive sense mRNA by cap snatching the 5' methylated cap from host mRNA to prime viral transcription<sup>20,21</sup>. The viral mRNA is exported into the cytosol to start translation and produce proteins for replication<sup>22,23</sup>. The proteins needed for replication and translation are transported back into the nucleus<sup>19</sup>. Now replication begins as the viral negative sense vRNA is converted into a positive sense complementary RNA (cRNA) to serve as the template<sup>23</sup>. Each eight complementary cRNAs are used to make a negative sense vRNA which exits the nucleus and into the cytoplasm as a vRNP<sup>24</sup>. In the

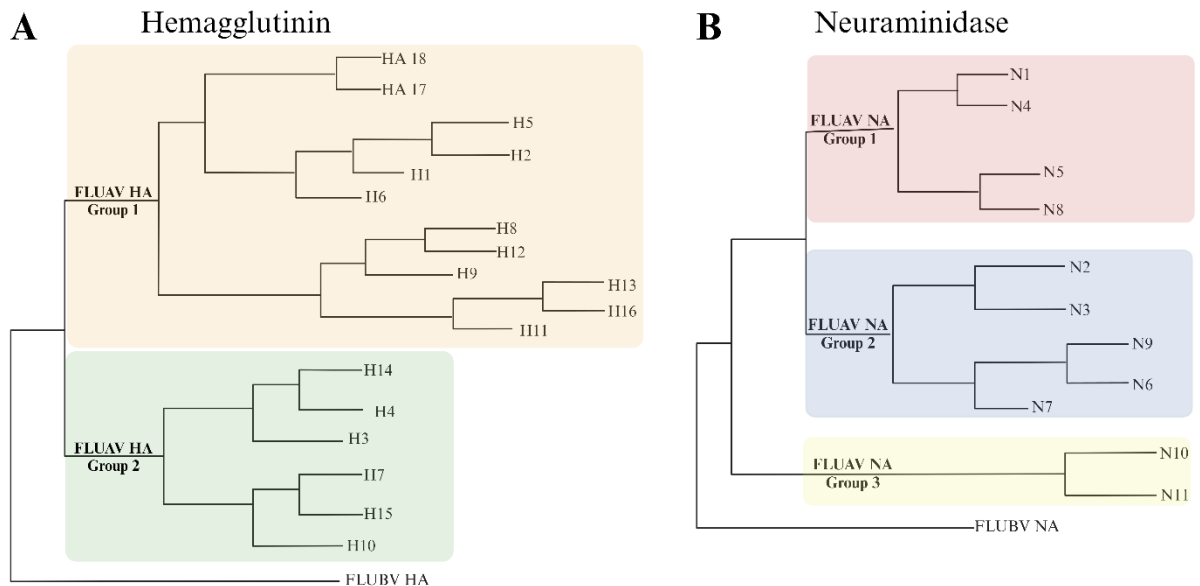
cytoplasm the HA and NA undergo polymerization, glycosylation, and acylation. The HA, NA, and M go to the plasma membrane and begin the budding process, using the host cell membrane to form the envelope<sup>25</sup>. When at least eight distinct vRNP segments meet at the budding site, they are organized into a 1+7 conformation pattern (**Figure 1c**)<sup>26</sup>. The virion buds off and NA cleaves the bonds with sialic acid to let the virion leave the cell<sup>23</sup>.



**Figure 1: FLUAV and FLUBV genome segments and structure characteristics.** (A). Genome organization of the eight negative-sense, single-stranded vRNA gene segments PB2, PB1, PA, HA, NP, NA, M, and NS of FLUAV and FLUBV. (B) Virion structure of FLUAV and FLUBV containing the eight vRNA which are wrapped around many copies of viral NP to make the vRNPs complexes. The lipid bilayer membrane contains HA, NA, and M2. The M1 is located under the membrane. A close up of the viral RNA-dependent RNA polymerase, comprised of PB1, PB2, and PA, attached to the vRNP covered with multiple viral NP are the minimal components needed for replication and translation. (C). A top view cross section of the virion highlighting how the eight vRNP form a "1 + 7" configuration. Created with BioRender.com. Based on a model published in 2018 Dou, Revol, Östbye, Wang and Daniels. *Frontiers in Immunology*.

FLUAV subtypes are determined by the antigenic properties of surface glycoproteins HA and NA<sup>19</sup>. Glycoproteins are a type of protein that have carbohydrate groups like sugars, starch, or cellulose attached to the polypeptide chain<sup>27</sup>. For FLUAVs, 18 HA and 11 NA subtypes have been determined by their serological cross-reactivity<sup>28</sup>. HA can be categorized into group 1 and group 2<sup>29</sup>. Group 1 of HA consists of H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18 while group 2 consists of H3, H4, H7, H10, H14, and H15 (**Figure 2a**)<sup>22</sup>. The 11 NAs are divided into three groups: group 1 contains N1, N4, N5, and N8 subtypes, and Group 2 contains N2, N3, N6, N7, and N9, while group 3 consists of the bat-origin N10 and N11 subtypes (**Figure 2b**)<sup>30-33</sup>. The combinations of HA and NA groups are utilized to name FLUAV subtypes<sup>32</sup>. The FLUAV subtypes documented to cause pandemic or epidemics in humans are H1N1, H2N2, and H3N2, and these viruses are speculated to possibly originate from either swine or avian<sup>34</sup>. The FLUAVs that are currently epidemic in the human population are the FLUAV subtypes H1N1 and H3N2, which have been co-circulating since the 1970s<sup>14,19,35</sup>.

Comparably, FLUBV antigenically distinguishable lineages Yamagata and Victoria have been circulating in humans during the influenza season since the 1980s<sup>22,36</sup>. The low amount of FLUBVS host species and slower mutation rate compared to FLUAVs host species could be the reason why FLUAVs are more diverse than FLUBVs<sup>37</sup>.



**Figure 2: Phylogenetic trees of FLUAV HA and NA.** Influenza B is utilized as the outgroup for the phylogenetic analysis. (A) The 18 HA organized into either group 1 or group 2 based on Phylogenetic similarities. Created with BioRender.com.

## 1.2 The role of HA and NA in FLUAV

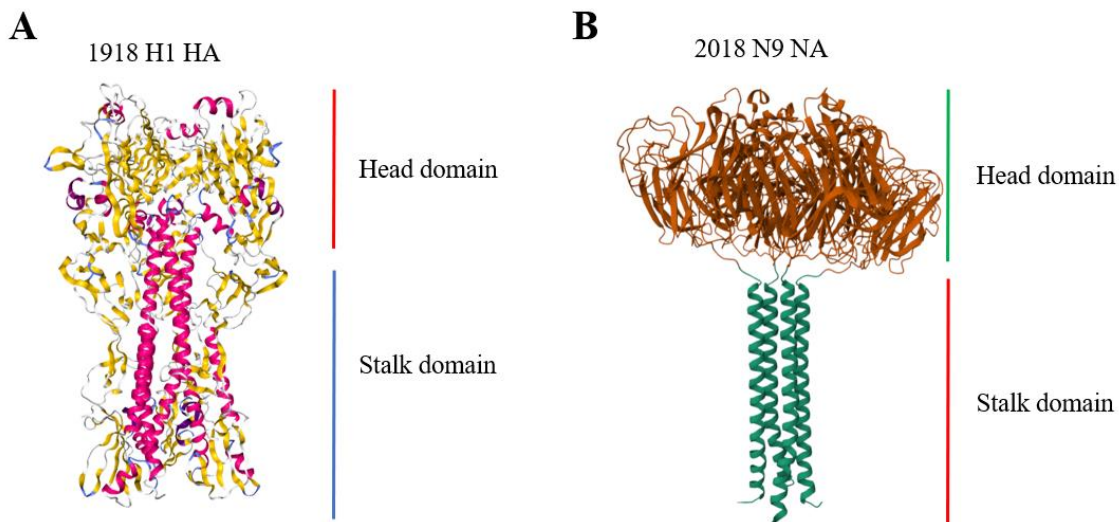
HA and NA are important influenza surface glycoproteins that serve as a prominent immunogenic target for the host immune system<sup>38</sup>. HA is the more abundant envelope protein compared to NA<sup>39</sup>. For the time being, the most characterized protein for FLUAV is HA which consists of a homotrimer of a head and a stalk domain that forms spikes on the viral envelope to bind sialic acid present on the surface of the cell membrane<sup>39</sup>. The most common sialic acid types FLUAV can target are the  $\alpha$ -2,3 linked sialic acid which has a linear conformation and the  $\alpha$ -2,6 linked sialic acid which has a more bent conformation<sup>40,41</sup>. The  $\alpha$ -2,3 linked sialic acid is widely distributed in avian while the  $\alpha$ -2,6 linked sialic acid is mainly in the upper respiratory system in humans<sup>42</sup>. Sialic acid receptors are part of glycoproteins and serve as the primary receptor targets for

FLUAVs<sup>41</sup>. HA binds to the host cells through the salic acid receptors and initiates viral entry into the host cell cytosol via viral and endosomal membrane fusion<sup>43</sup>. This fusion involves both HA domains HA1 and HA2 which are created by proteolysis of HA and linked by disulfide bonds<sup>17</sup>. Subunit HA1 contains the receptor binding domain while HA2 has the fusion peptide<sup>44</sup>. The HA1 of subtype H3N2 and H1N1 seasonal FLUAVs has five antigenic sites, where amino acid substitutes were shown to occur frequently<sup>45-47</sup>. In H3N2, the antigenic sites are categorized as site A, B, C, D, and E with each site containing many epitopes, each of which is composed of approximately 12-15 amino acids, can initiate immunological responses, and interact with the antibody<sup>38,48-50</sup>. Among these five sites of H3N2, sites A and B are the most immunodominant sites of HA and were shown to be one of the major sites having driving antigenic drift of seasonal H3N2 FLUAV in the past five decades<sup>38</sup>.

The NA glycoprotein FLUAV consists of a head domain, stalk domain, a transmembrane (TMD), and a short N-terminal cytoplasmic tail which form a homotetrameric type II membrane protein resembling a mushroom-like shape<sup>51,52</sup>. The head domain contains the enzyme active site, which is also known as the highly conserved catalytic region<sup>53</sup>. The main role of NA in FLUAV is as a sialidase which aids in viral release by cleaving the sialic acid receptor bond between the infected host cell and HA to release the influenza virus<sup>15,44</sup>. If NA is not present in the virus, then the viral particle will not release from the plasma membrane<sup>51</sup>. NA has seven independent antigenic sites which contribute to directing antigenic drift<sup>54,55</sup>. The seven antigenic sites surround the enzyme active site, the highly conserved catalytic region<sup>56,57</sup>.

FLUAV's genetic diversity is due to having an error prone RdRp, as it lacks a proof-reading mechanism, and a high reassortment rate due to their segmented genomes<sup>58,59</sup>. FLUAV Antigenic drift in FLUAV from the error prone RdRp causes mutations in the HA or NA antigenic surface epitopes which are then often selected by host immunological pressures<sup>43</sup>. Antigenic shift is caused by FLUAV reassorting the HA and NA of FLUAVs when they co-infect the same cell to yield a new FLUAV subtype<sup>19</sup>. Antigenic shift in FLUAVs has been responsible for influenza pandemics, such as the 1918 Spanish flu, the 1957 Asian flu, and the 1968 Hong Kong flu while antigenic drift is responsible for seasonal outbreaks in humans<sup>14</sup>. The evolution of HA and NA for FLUAV is shown to have significantly been influenced by immunological pressure from the virus host<sup>19</sup>.

Even as both HA and NA subunits undergo similar antigenic and immunogenic pressure, NA is less inclined to amino acid changes than HA<sup>60</sup>. Antigenic drift is not routinely examined for NA and only a few studies have focused on NA antigenic evolution by using data sets and construction antigenic maps<sup>52,55</sup>. From those studies, it was shown with an antigenic map that there was antigenic stasis in human H1N1 NA for over ten years prior to 2009 influenza season even though there were genetic changes in NA<sup>61</sup>.

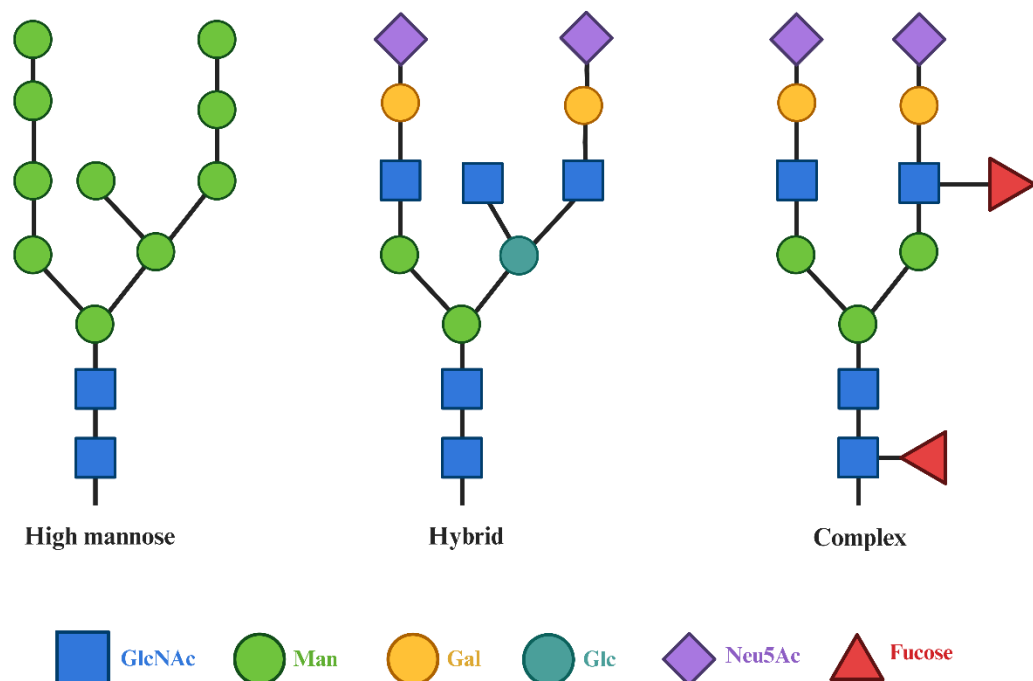


**Figure 3: HA and NA structure.** A.) Head and stalk domain of 1918 H1 HA. Structure from PDB ID: 1RUZ. B.) Head and stalk domain of 2018 N9 NA. Structure from PDB ID: 6CRD. Figures generated on the Protein Data Bank website.

### 1.3 Glycosylation explanation and its role in antigenic drift

Glycosylation is the chemical addition of carbohydrates, also called glycans, to the backbone of a protein via an enzymatic reaction<sup>62</sup>. N-linked and O-linked glycosylation are the most common types of glycosylation in. O-linked glycosylation has not been frequently observed in FLUAVs, but N-linked glycosylation is often detected in both HA and NA of FLUAV<sup>56</sup>. N-linked glycosylation sites are only found in a specific sequence of amino acids called a sequon<sup>63</sup>. The sequon is Asn-X-Ser/Thr where X signifies any possible amino acid except proline and the glycans covalently attached to the asparagine (N)<sup>64</sup>. There are three types of N-Linked glycan types: high mannose, complex, and hybrid (**Figure 4**)<sup>65,66</sup>. High mannose is comprised of only mannose sugars for the glycan type that extend from the N-acetylglucosamine (GlcNAc) core<sup>57</sup>. Complex glycosylation has extensions made from N-acetyl glucosaminyl transferases that expand from the core while hybrid is a mix of the complex and high mannose structures<sup>65,66</sup>. Furtherly, the type

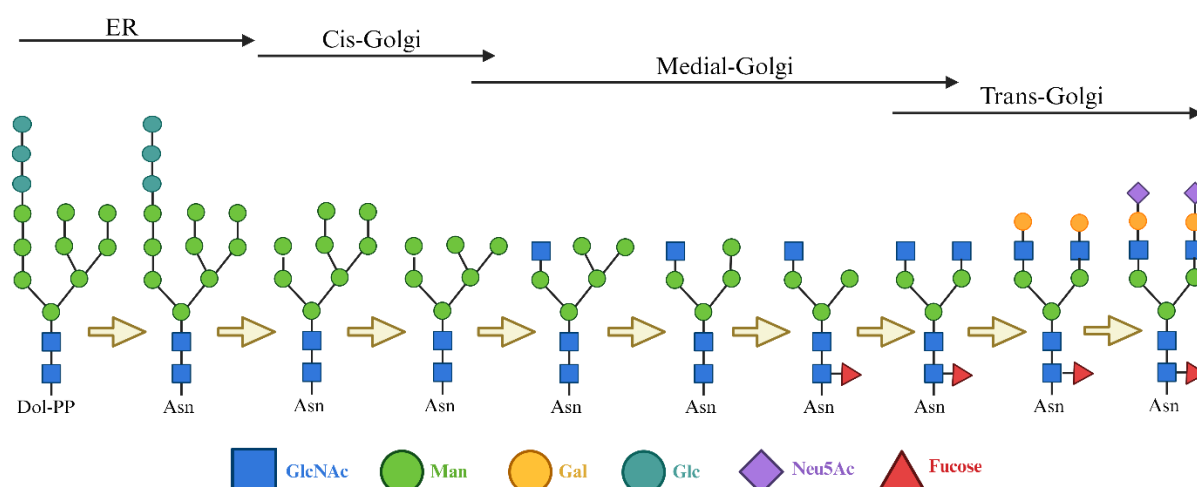
of cell impacts glycan heterogeneity per the cellular glycosylation machinery and the specific conditions that the cell is exposed to<sup>67</sup>. The glycoforms in N-linked glycosylation at a specific site can aid in immune evasion from the host immune system such as masking antigenic sites, also known as epitope masking<sup>68</sup>.



**Figure 4: Types of N-Linked Glycosylation.** Overview of high mannose, hybrid, and complex glycan structures. Created with BioRender.

Both FLUAV HA and NA proteins acquire N-linked glycosylation post translationally<sup>63,69</sup>. But first the glycan structure must be assembled in the endoplasmic reticulum (ER) where glycans are further added, trimmed, and branched out by glycosidases and glycosyltransferases as the glycoproteins move through the Golgi apparatus (**Figure 5**)<sup>44,70</sup>. Specifically, the first phase of N-linked glycosylation is

initiated on the surface of ER or the cytoplasmic site of ER<sup>71</sup>. This assembly starts by the attachment of two GlcNAc and five mannose residues on a dolichol phosphate (Dol-P) carrier molecule (**Figure 5**)<sup>67</sup>. Then, one mannose and three glucose residues are removed with glucosidase I, II and ER  $\alpha$ -mannosidase<sup>72</sup>. From that stage, glycans are either added or subtracted by various glycosidases, such as Endo H, and glycosyltransferases as the structure makes its way through the Golgi Apparatus. *N*-linked glycosylation maturation in the Golgi apparatus is approximately categorized into three steps: branch formation, elongation, and capping<sup>73</sup>. The glycan structure is considered mature once there are sialic acids at the end of each branch<sup>72,74</sup>.



**Figure 5: *N*-linked Glycosylation synthesis:** The general steps for constructing an *N*-linked glycosylation structure in the ER and Golgi apparatus.<sup>71</sup> Created with BioRender.com.

*N*-linked glycosylation in HA can play a role in influencing virus infectivity and host immune responses while *N*-linked glycosylation in NA impacts the structure, activity, specificity, virulence, and thermostability thus affects virus release<sup>75</sup>. The *N*-linked glycosylation on HA and NA proteins help the influenza strain better evade the host immune system by covering epitopes that elicit neutralizing antibodies which could have

the potential to improve viral fitness or help the host immune system recognize the strain<sup>76,77</sup>. Notably, N-linked glycosylation can lessen the effect of antibody-mediated cross neutralization via antigen masking which was observed with the 1918 influenza pandemic strain and the A(H1N1)pdm09 strain with the addition of N-linked glycosylation sites at the 129 and 163 residues<sup>78-80</sup>. The amount, length, and types of N-linked glycosylation on the surface HA strongly impact antigenicity and virus immunogenicity on the immune response<sup>81</sup>. In fact, under-glycosylation of glycoproteins impact HA by causing defects on folding and transport<sup>70</sup>. The addition or deletion of an N-linked glycosylation site in HA and NA are important factors in influenza's ability to escape the host immune responses. For example, adding FLUAV N-linked glycosylation at antibody binding sites of seasonal H3N2 FLUAVs may reduce antibody binding to HA and NA by masking antigenic sites<sup>26</sup>. A prior study showed that changes in N-linked glycosylation facilitates the viral adaptation of seasonal H3N2 viruses to human populations<sup>46,47</sup>. A novel N-linked glycosylation site in HA or NA can be used as a strain marker. For instance, in the 1918 and 2009 influenza pandemics strains where they have a specific 104 amino acid residue with an N-glycosylation site that is one way to track if an influenza reassortment came from 1918 or 2009 influenza pandemic strains<sup>82</sup>. Overall, N-linked glycosylation site mutation in the HA and NA impact FLUAV's virulence, pathogenicity, and sensitivity to neutralizing antibodies.

#### **1.4 Influenza A viruses in swine and avian**

In addition, to humans, FLUAV nationally infects many animals, most commonly avian, swine, equine, canine, and sea mammals<sup>83</sup>. Human contact with domestic animals

is one of the common pathways FLUAV transmission occurs for animals to humans and *vice versa*<sup>84,85</sup>. The evolution and ecology of FLUAVs presence in the swine and poultry populations are less monitored in many countries compared to those in humans<sup>86</sup>. The limited surveillance for virus transmission between swine and poultry is concerning. Swine are the metaphorical ‘mixing vessels’ for avian and swine FLUAV strains since swine have both avian-like alpha 2,3-linked and human-like alpha 2,6-linked sialic acid receptors meaning swine are a host for frequent emergence of novel reassortment avian and swine FLUAVs<sup>87</sup>. These emerging novel reassortment FLUAV could have the potential to be highly infectious, enhance host immune evasion, or have better transmission within swine and even between swine and humans<sup>9,88</sup>. On the topic of transmission, N-linked glycosylation sites in NA are noted to have a part in FLUAV virulence and transmission as deletions or additions of N-linked glycosylation sites on HA and NA can impact cross-species transmission<sup>89,90</sup>. For example, the deletion of an amino acid at 131 on the H5N6 HA head produces a new a N-linked glycosylation site at the 129 residue which lowered the pH threshold for HA and cell membrane fusion and enhanced HA thermal stability to promote the cross species transmission from birds to humans<sup>90</sup>,

Further animal trading across regions, states, and even countries have been shown to facilitate the emergence and spread of a novel FLUAV virus<sup>86,91,92</sup>. The FLUAV subtypes most detected in swine are H1N1, H1N2, H3N1, and H3N2, which can also sporadically spill over to humans<sup>93</sup>. In domestic poultry, subtype H3, H5, H6, H7, H9, and H10 are frequently detected, with subtype H5 and H7 can be switched from a low pathogenic form to a high pathogenic form<sup>90,94,95</sup>. Some of these avian viruses, such as H5, H6, H7,

and H10 caused sporadic infections in humans, although no or only limited transmission of these viruses were observed in humans<sup>9,94</sup>. Subtype H5 and H7 HPAI FLUAV can lead to a high mortality rate in domestic poultry, particularly chickens and turkeys and cause economic repercussions<sup>9,96</sup>. Compared to domestic poultry, particularly chicken and turkey, migratory aquatic birds, such as waterfowl ducks and geese, typically suffer much less or even no mortality after being infected<sup>97</sup>. Of note, until the emergence of the recent A/Goose/Guangdong/1/1996(H5N1)-liked lineage, neither H5 nor H7 HPAIVs were shown to cause deaths in wild aquatic birds<sup>98</sup>. Often these wild aquatic birds can become carriers and spread the virus during migration. Infection from these avian strains to humans, mainly infecting humans in their lower respiratory system containing alpha 2,3-linked sialic acid and a higher mortality rate, is rare but insights into how N-linked glycosylation impact virulence can be used to better understand the adaptable capabilities of cross-species transmission into mammalian/human host<sup>95,99</sup>. For instance, the N-linked glycosylation deletion mutations N27Q and N39Q in H5N1 HA lowers their virulence on human dendritic cells specific ICAM-3 grabbing non-integrin (DC-SIGN) lectin receptors and impacts transmission to humans<sup>99,100</sup>. Swine infected with subtype H5N1 HPAI typically have minor clinical signs, although the HPAI virus may replicate in swine respiratory tissues<sup>101</sup>. In order to infect humans from other humans, these avian or swine viruses need to have an efficient binding efficiency to the human-like alpha 2,6-linked sialic acid receptors<sup>101,102</sup>. These avian or swine origin FLUAVs are typically antigenically different from those in humans and can cause a pandemic strain as the human population has little to no immune protection to these infections<sup>90</sup>. Thus,

pandemic preparedness against these FLUAVs at the animal-human interfaces are critical.

### **1.5 Vaccine designs for influenza**

Seasonal FLUAV circulates across the globe year-round between the northern and southern hemispheres during their respective flu seasons<sup>103</sup>. These influenza epidemics in humans causes millions of deaths, economic losses, and disruptions and shortages of hospital time and resources due to the surge of infections<sup>104,105</sup>. Currently vaccination against influenza is the best way to prevent and control the spread and transmission of influenza with antiviral treatment being utilized to treat serious influenza infection<sup>106</sup>.

Seasonal influenza vaccines currently used are either the trivalent or the quadrivalent vaccine based on the predicted influenza strains circulating in the region to be immunodominant based on antigenic profiling and extensive genetic analysis collected from the last influenza season as the basis of the prediction of next year's potential infectious strains<sup>107,108</sup>. The quadrivalent vaccine was made due to a recommendation in 2012/2013 season of including both FLUBV lineages as the trivalent vaccine, which contained FLUAV H1N1, H3N2, and one FLUBV lineage, had a low antigenic match during influenza season<sup>12</sup>. The quadrivalent vaccine containing the two FLUBV Victoria and Yamagata lineages, with the two FLUAV H1N1 and H3N2 subtypes has coverage of both FLUBV to eliminate the guessing game of which strain is dominant and to reduce the burden of FLUBV infection in hospitals<sup>109</sup>. However, for the 2024/2025 influenza season in the northern hemisphere, the World Health Organization is recommending removing FLUBV Yamagata lineage as it has not been detected circulating in humans

since March 2020. This would make the 2024/2025 influenza shot a trivalent vaccine with FLUVB and two FLUAV strains<sup>110</sup>.

The vaccine development starts six months before the start of that hemisphere's influenza season and mainly utilizes chicken eggs and to a lesser extent cell culture or recombinant-based technology to grow the virus<sup>111</sup>. Within those six months testing, quality control, and clinical testing of the HA antigen's ability to stimulate neutralizing antibodies are conducted<sup>112</sup>. Currently, HA head domain antigenicity is targeted more in vaccine development as the abundance of neutralizing antibodies against the HA head domain after vaccination are considered more effective to protect against FLUAV<sup>113</sup>. However, a shift to consider the more conserved HA stem epitopes for broader protection and possibly longer lasting protection as the HA head domain epitopes do not have broad protection against similar FLUAV strain<sup>114,115</sup>. One thing to note is that the H1 HA stalk has a highly conserved N-linked glycosylation site located above the anchor epitope which has the potential to obstruct antibody targeting for that epitope<sup>114</sup>.

A natural infection of the influenza virus elicits a host immune response against both HA and NA<sup>52</sup>. Antigenic drift and shift impact the antigenicity of the FLUAV HA and NA glycoproteins and how influenza vaccine targets are developed<sup>111</sup>. HA glycoproteins are more immunodominant than NA, but HA has a higher mutation rate which makes it harder to make productive and long-term therapeutics targeting HA<sup>115,116</sup>. Additionally, the loss or gain of a N-linked glycosylation site in HA or NA alters antigenicity and virulence<sup>19,117</sup>. A flaw in the influenza vaccine method is not considering that a N-linked glycosylation site mutation in the circulating virus can cause an antigenic mismatch between the circulating virus and the vaccine. These mutations can happen in the

circulating strains as the process of the influenza vaccine is being developed and in the middle of influenza season. The loss in protection for that influenza season is in part due to the inflexible timeline of the current influenza vaccine method<sup>49,118</sup>. Additionally, when grown in eggs, it has been seen that a mismatch of the vaccine virus and the circulating virus can happen, like in 2016-2017 influenza season where the circulating strain had T160, a glycosylation site addition mutant, while the egg grown strain mutated back to the less dominant mutation K160<sup>119</sup>. Another oversight is FLUAV strains circulating between poultry and swine that can cross-species transmit to humans due to antigenic shift can also lead to the influenza vaccine having less protection that year<sup>18,120,121</sup>.

There are a few hurdles for implementing the influenza vaccine to the public. Aside from the potential of the vaccine not being highly protective against the circulating strain due to mismatch, there is the potential of people having an allergic reaction to the vaccine components, and the supply and demand problems<sup>112,122</sup>. The influenza vaccine component that most people are allergic to is the egg protein from the vaccine virus being grown in chicken eggs<sup>123</sup>. To remedy this other vaccine methods have been developed, such as cell-based vaccines<sup>124</sup>. The cell-based production of a vaccine is faster at producing and responding to circulating influenza strains compared to egg-based flu vaccines as it is not dependent on the supply of eggs available. It also will not contain any egg protein making it safe for people with egg allergies. Additionally, the N-linked glycosylation sites of the cell-based vaccine are less likely to mutate compared to vaccines grown in chicken eggs<sup>119,125</sup>.

The inactivated vaccine Flucelvax is a cell-based influenza vaccine grown in Madin-Darby Canine Kidney (MDCK) cell line and is already on the market<sup>126</sup>. As an inactive vaccine, meaning the virus was killed and cannot cause the disease, Flucelvax has a safe record, and it is not likely for people with dog allergies to have a reaction<sup>127</sup>. Another example is the recombinant HA vaccine FluBlok which is grown in insect cells infected with a baculovirus vector<sup>128</sup>. A recombinant vaccine is a type of vaccine that contains a specific version of a protein, in FluBlok's case it uses the HA glycoprotein as an antigen<sup>126</sup>. When utilizing a baculovirus vector a virus like particle (VLP) which is hollow would be composed of proteins, in this case HA, and would be the antigen presenting peptide<sup>129</sup>.

Utilizing antigens like purified proteins or polysaccharides is safer but less immunogenetically responsive meaning an adjuvant, a component in vaccines that is used to help the immune response detect and create a better immune response to the target component in the vaccine, must be used<sup>130-132</sup>. Other cell types that can be used are plant cells, *E. coli*, or yeast cells<sup>133</sup>. Another vaccine type is mRNA vaccines which are faster to produce and use messenger RNA (mRNA) to make proteins to trigger an immune response making it highly safe as has no risk of causing disease<sup>134,135</sup>. Additionally, subunit vaccines are another type of vaccine which uses a section from the surface of the pathogen instead of the whole pathogen, like the HA or NA subunits of influenza<sup>107,130</sup>.

N-linked glycosylation is an important parameter for influenza subunit vaccines<sup>136</sup>. Especially as the addition of N-linked glycosylation sites in NA and HA sites can increase antigenic drift, mask antigenic sites, and can impact the binding of antibodies to the NA and HA segments<sup>34,61,137,138</sup>. In fact, N-linked glycosylation

mismatch in circulating flu to the vaccines during the 2016-2017 influenza season was due to the eggs used to grow the vaccine which virus reverted to a past mutation which caused a loss of a N-linked glycosylation site and led to the vaccine having poor neutralization<sup>119</sup>. This mismatch was due to egg-adaptive mutations preferring  $\alpha$ 2,3-linked receptors causing the loss in N-linked glycosylation site mutation, the change in virulence, and the switched HA receptor binding specificity in virus vaccine production<sup>119,125</sup>

Compared to fully glycosylated glycoprotein neutralization, vaccines with monoglycosylated HA glycoprotein had a broader range of antigen protection against influenza strains<sup>139</sup>. Monoglycosylated samples induce a broader but stronger immune response for the antigenic epitopes compared to the regular glycosylated site<sup>140</sup>. It has been observed that a vaccine made with monoglycosylated HA (HA<sub>mg</sub>) from avian influenza H5N1 had a better antibody response than the fully glycosylated HA (HA<sub>fg</sub>)<sup>139</sup>. Additionally, the host's dendritic cells more readily engulf the HA<sub>mg</sub> and produced a higher number of cross-reactive antibodies compared to the HA<sub>fg</sub><sup>139,141</sup>. The monoglycosylation works as having a single GlcNAc residue to Asn in N-Linked glycans is enough for the glycoprotein to fold and stabilize<sup>12</sup>. Subunit vaccines and monoglycosylation have mainly been focused on the HA segment of influenza as it is more antigenically reactive but has a higher mutation rate compared to the NA segment<sup>130,139</sup>. Targeting monoglycosylated NA sites from many past pandemic strains and having a mixture of monoglycosylated NA for a broader protection vaccine has the potential to last more than one flu season<sup>142</sup>. The gap in knowledge in N-linked glycosylation evolution is higher for NA compared to HA as HA has been the focus of

FLUAV vaccine development for year<sup>107,108,143-145</sup>. Although, it has been shown that there has been an increase in specific NA N-linked glycosylation sites that are known to contribute to antigenic drift in circulating influenza H3N2 strains which has led to higher escape mutations or lower protection from human monoclonal antibodies<sup>146</sup>. The utilization of a glycosylation database for NA N-linked glycosylation sites and types will be useful for future vaccine development as there is less knowledge about NA evolution<sup>145</sup>.

## **CHAPTER 2: Impact of N-linked Glycosylation on Influenza Evolution and Viral Fitness**

### **2.1 Structural impact of N-linked glycosylation on influenza**

The addition of sugars, also known as glycans, for the N-linked glycosylation of FLUAV's HA and NA surface glycoproteins is a crucial step for N-linked glycosylation impacting the molecular mass, the biophysical properties, folding, structure, and influencing protein targeting of host immune system<sup>90,147,148</sup>. The glycans at N-linked glycosylation sites are crucial for FLUAV cell entry and shielding from antibody recognition which aids in evasion of the host's immune system<sup>149</sup>. The high structural diversity of the glycans at the N-linked glycosylation sites is due to their biosynthesis not being template driven but dependent on host factors like the type of host animal, tissue, cell, and location<sup>149</sup>. The variety of monosaccharide glycans utilized as the building blocks aids in the high complex structural diversity as there are various ways the glycans can attach, bond, be enzymatically removed, and configurate into the positions and linkages within the N-linked glycosylation site<sup>71,149</sup>.

The glycans in FLUAV can increase the HA and NA's folding efficiency by recruiting lectin chaperons, like calnexin and calreticulin, to aid in disulfide bond formations<sup>150</sup>. This function of glycans is important in HA and NA glycoprotein sites that contain a lot of intramolecular disulfide bonds. Calreticulin and calnexin are lectins that bind to new glycoproteins with monoglycosylated N-linked glycans in the endoplasmic reticulum (ER) and are chaperones that promote glycan assembly, quality control, and proper folding of glycoproteins<sup>150,151</sup>. The absence or presence of N-linked glycosylation sites and the types of glycan structures can impact the viral fitness and the FLUAV's ability to grow in certain cells<sup>152</sup>.

FLUAV HA and NA are arranged in a patch wise distribution on the virus surface in a range of 4:1 to 9:1 ratio<sup>99</sup>. This distribution of HA and NA on the surface contributes to FLUAV's motility and entry balance<sup>52</sup>. The N-linked glycosylation of HA and NA is important for virus survival as the balanced ratio of receptor binding activity and virus release is needed<sup>51,153,154</sup>. Having a few amounts of N-linked glycosylation modifications on HA can cause it to bind too tightly to the receptor leading to higher NA activity to release the virus from the cell surface. Alternatively, having too many N-linked glycosylation modifications leads to weak receptor binding and less need for NA activity to release from the cell surface<sup>47</sup>.

HA is dependent on N-linked glycosylation to modulate proper folding, receptor interaction and efficient virus release from cell surface<sup>76,77,138,150</sup>. The N-linked glycosylation of HA impacts the structural maturity and influences the pace of folding and formation of disulfide bonds<sup>155,156</sup>. HA N-linked glycosylation sites in the stalk region are crucial for proper folding and structural confirmation<sup>47</sup>. For instance, the N-linked glycosylation site deletion mutation N27A in HA produces a nonfunctional virus and upon further inspection N-linked glycosylation at N27 is vital for HA folding<sup>90,157</sup>. N-linked glycosylation sites near the head could shield antigenic sites from host cell immune recognition and can contribute to antigenic diversity<sup>60,138,156</sup>. Since not all N-linked glycosylation sites are glycosylated as there are a limited number of glycans, glycosidases, and glycotransferases in a cell means that FLUAV HA has regions that are glycosylated and regions without N-linked glycosylation, this is referred to as site occupancy<sup>62</sup>.

There are places in FLUAV HA where N-linked glycosylation can aid or harm the virus survival, like near the catalytic site and in the head stem region<sup>34,158</sup>. If there is an N-linked glycosylation site too close to the HA cleavage site, the glycans have the potential to interfere with virus entry by preventing protease access at the cleavage site<sup>80</sup>. Also, when deletion mutations of N-linked glycosylation in the HA stem region occur caused faster folding while deletion of the HA head domain inhibited folding<sup>150</sup>. N-linked glycosylation mutations that are not lethal aid in the structural diversity of FLUAV<sup>152</sup>. N-linked glycosylation sites on the HA stem are conserved and shown to be crucial for the development and/or maintenance of HA<sup>159</sup>. N-linked glycosylation is necessary for HA folding, the number and location of the N-linked glycosylation site are important for virulence<sup>34,159-161</sup>.

The N-linked glycosylation of the NA stalk aids in NA head stability<sup>52</sup>. Adding N-linked glycosylation sites to NA increases NA's enzymatic activity while decreasing N-linked glycosylation sites in NA decrease the enzymatic activity lowering virulence, and transmission of FLUAV<sup>147</sup>. For instance, the conserved N-linked glycosylation sites 44-50 and 68-70 on the NA stalk are suspected to aid in protecting the enzymatic cleavage site<sup>68</sup>. Generally, N-linked glycosylation in NA increases its stability and rigidity while reducing entropy<sup>147</sup>. Having a crowded NA surface increases entropy, can lead to a conformational change, and make the protein structure more compact and stable<sup>147,162</sup>. This crowding phenomenon can impact cellular pathways and biological interfacing of the NA glycoprotein as glycans impact stability<sup>162</sup>. Overall, N-linked glycosylation of HA and NA can have positive or negative impact on the FLUAV's

functionality, immunogenicity, folding, stability, and modulation of virus receptor binding<sup>80,147,163,164</sup>.

## **2.2 N-linked glycosylation site mutations impact on virulence and pathogenicity**

The FLUAV binds to glycans on the host cell that terminate in sialic acids while the glycans on the FLUAV are also recognized by the hosts immune cell's glycan binding proteins<sup>41,74,165</sup>. Host cell glycosylation can impact the FLUAV HA's ability to bind to its receptors and interact with neutralizing antibodies<sup>152</sup>. The host immune cells with the glycan binding receptors can capture FLUAV but the amount and place of the N-linked glycosylation site in FLUAV can either promote or prevent immune escape and infection<sup>9</sup>. The importance of N-linked glycosylation recognition mediating FLUAV infection is also influenced by the amount and location of the N-linked glycosylation sites on NA and HA and its impact on glycan masking<sup>137,166,167</sup>. Mutations that add N-linked glycosylation sites near antigenic sites in the HA and NA genome can produce an FLUAV with resistance to antibodies able to bind to masked FLUAV epitopes<sup>152</sup>. For example, N-linked glycosylation at 142, 172, and 177 on Ha can aid in antigenic masking<sup>68</sup>. Additionally, hyperglycosylation near masked epitopes has the potential to have a growth advantage in humans and escape immune pressures<sup>168</sup>. Alternatively, having low-glycosylated NA can elicit a greater immunogenicity than hyperglycosylated NA<sup>169</sup>. Interestingly, unglycosylated NA had similar immunogenicity to the hyperglycosylated NA<sup>169</sup>. Measuring the N-linked glycosylation sites and potential sites of FLUAV and virus host interactions can aid in broadening understanding of FLUAV ecology and pandemic-like strain emergence<sup>87,95,170-173</sup>.

FLUAV N-linked glycosylation is a key component of host range and zoonotic transmission with differences in N-linked glycosylation presence impacting molecular interactions of the virus and host<sup>69,76, 170,171 77</sup>. For example, human FLUAV H1N1 pandemic strain had four N-linked glycosylation sites on the HA's globular head while avian and swine H1N1 HA strains did not have these sites<sup>152</sup>. Glycans of N-linked glycosylation sites can aid or hold back FLUAV transmission between animals<sup>72,80,81,84,174,175</sup>. The type of sialic acid HA prefers to bind to, avian or human, critically impacts FLUAV transmission<sup>14</sup>. In the case of avian influenza like HPAI strains, transmission into humans only happened within direct close contact of avian to human but rarely transmitted from human to human<sup>99</sup>. However, when infected, the infection in the human was in the lower lungs where cells express the  $\alpha$ -2,3 linked sialic acids and was more severe compared to human circulating FLUAV which infect the upper respiratory system containing the  $\alpha$ -2,6 linked sialic acids<sup>149</sup>. The transmission of HPAI H5N1 viruses from avian to humans is a concern as it can cause a potential pandemic in humans<sup>95,97</sup>. Further, H5N1 HA contained five highly conserved N-linked glycosylation sites and two non-conserved sites, 181NVT and 170NST, when circulating in avian and when H5N1 crossed the species barriers to infect humans. The conserved N-linked glycosylation sites in H5N1 HA stayed the same as the strain circulated human populations, but the non-conserved N-linked glycosylation sites had changing N-linked glycosylation patterns to escape from host immune pressures<sup>176,177</sup>. One of these changes in N-linked glycosylation in H5N1 is the lack of a N-linked glycosylation site at the 158 amino acid residue improves binding to the human  $\alpha$ -2,6 linked receptor while enhancing transmission and pathogenicity at the cost of reducing virus replication<sup>178,179</sup>.

In the swine host where both the  $\alpha$ -2,3 linked and the  $\alpha$ -2,6 linked sialic acids are present in the respiratory tract, FLUAV can cocirculate and undergo mutations and reassortments to switch from the avian  $\alpha$ -2,3 to the human  $\alpha$ -2,6 linked sialic acid leading to a possible transmissible strain that is potentially a pandemic-like strain for humans<sup>85,86,180</sup>. For example, the first 21<sup>st</sup> century influenza pandemic was from interspecies reassortments in swine causing the emergence of the 2009 swine flu, known as A(H1N1)pdm09 virus<sup>181</sup>. The A(H1N1)pdm09 virus contained gene segments from three different FLUAV subtypes, the HA subunit from classical H1N1, the M and NA subunit from an Eurasian avian-like H1N1 swine influenza subtype, and the rest of the subunit genes from the triple reassortment internal gene (TRIG) cassette<sup>85,170,182</sup>. Interestingly, A(H1N1)pdm09 virus only had one N-linked glycosylation site, at residue 104, the same site but different residue as the 1918 H1N1 pandemic. This one N-linked glycosylation site in A(H1N1)pdm09 virus disrupted the notion that all influenza viruses pandemics in humans have to have an increase of glycosylation sites. Moreover, human circulating FLUAVs usually spill back into swine leading to more genetic diversity in the HA and NA genes. After the A(H1N1)pdm09 pandemic occurred, it replaced the seasonal H1N1 virus and is now a seasonal human FLUAV cocirculation globally with H3N2, H1N2, and FLUBV<sup>183</sup>. Specifically, when A(H1N1)pdm09 virus crossed back into swine its gene segments, like PA, NP and M recombined and replaced the TRIG counterparts to become part of the dominant circulating strain in swine<sup>181</sup>. These FLUAV with the A(H1N1)pdm09 reassortments had less N-linked glycosylation sites on FLUAV HA and NA compared to pre-2009 influenza seasons<sup>81,177</sup>. Interestingly, compared to seasonal H1N1 before 2009, two N-linked glycosylation sites (142N and 177N) on HA were

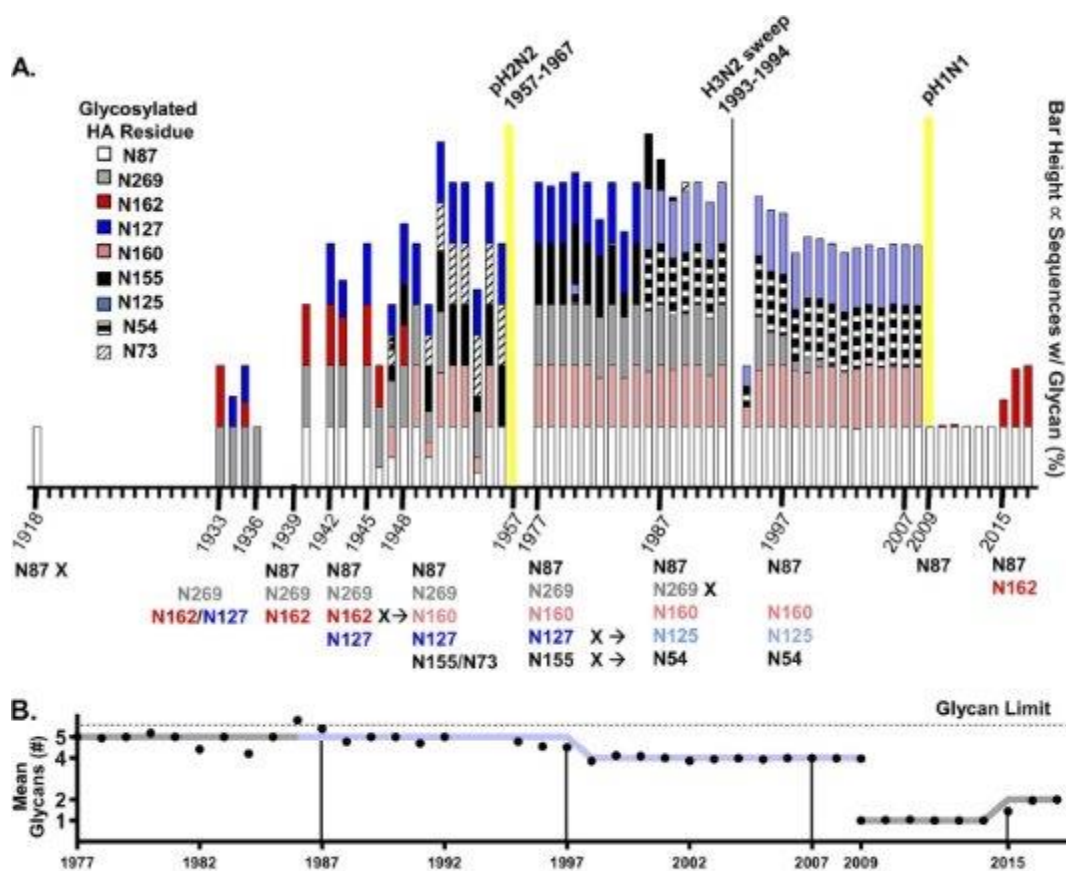
absent from the A(H1N1)pdm09 virus<sup>61,180,182</sup>. Adding these two N-linked glycosylation sites back into A(H1N1)pdm09 virus strains increased virulence and pathogenicity but could reduce the sensitivity to neutralizing antibodies<sup>179</sup>.

How N-linked glycosylation aids the zoonosis, the transmission of the disease to humans from animal host, needs to be better studied to understand the biological constraints on cross species transmission and as a tool for filling in the gaps of zoonotic predictive risk assessment and vaccine design<sup>184</sup>. An infection of FLUAV in a host elicits an immune response to both HA and NA<sup>52</sup>. Having a vaccine with a combination of HA and NA could provide a better protection to FLUAV compared to a vaccine just using HA<sup>52</sup>. Overall, the addition or deletion of an N-linked glycosylation site in HA can impact the pathogenicity and virulence by effecting fusion activation into the host cell or by blocking antigenic sites on FLUAV.

### **2.3 Evolution of N-linked glycosylation sites in FLUAVs**

Glycans are found at the surface or extracellular matrix of eukaryotic, archaea and bacteria<sup>74</sup>. The glycans of FLUAV are located at the surface of their glycoproteins HA and NA giving way to the glycans being a factor in molecular recombination events<sup>149</sup>. The location of the N-linked glycosylation sites for HA and NA is determined by the nucleotide sequence and mutations that add or remove these sites happen at a high frequency due to FLUAV high recombination and mutation rate<sup>152</sup>. Holistically viewing FLUAV N-linked glycosylation mutations and host function can allow one to begin to observe and predict the evolutionary constraints and selective pressures on FLUAV<sup>68,145,185</sup>.

Human FLUAV's have a documented example of linear evolution of adding N-linked glycosylation sites over time, especially in HA<sup>186</sup>. The only exception is the pandemic A(H1N1)pdm09 strain, but circulating strains after the 2009 season do show an increase in N-linked glycosylation sites<sup>187</sup>. **(Figure 6A)** The intervals for adding N-linked glycosylation sites in HA are five years for H1 strains and seven years for H3 strains until the functional limit was reached<sup>76,138</sup>. At the point of reaching the functional limit for the addition of N-linked glycosylation sites, the HA genome had mutations where the N-linked glycosylation sites swapped at 2-fold longer intervals<sup>138</sup>. The NA glycoprotein evolved slower at the nucleotide level than HA<sup>52</sup>. This gradual pace can be seen in the number of N-linked glycosylation sites in NA has been steady in the stem region and head region<sup>56,117,142</sup>. Additionally, there is a divergence between the NA's antigenic and genetic evolution<sup>52</sup>.



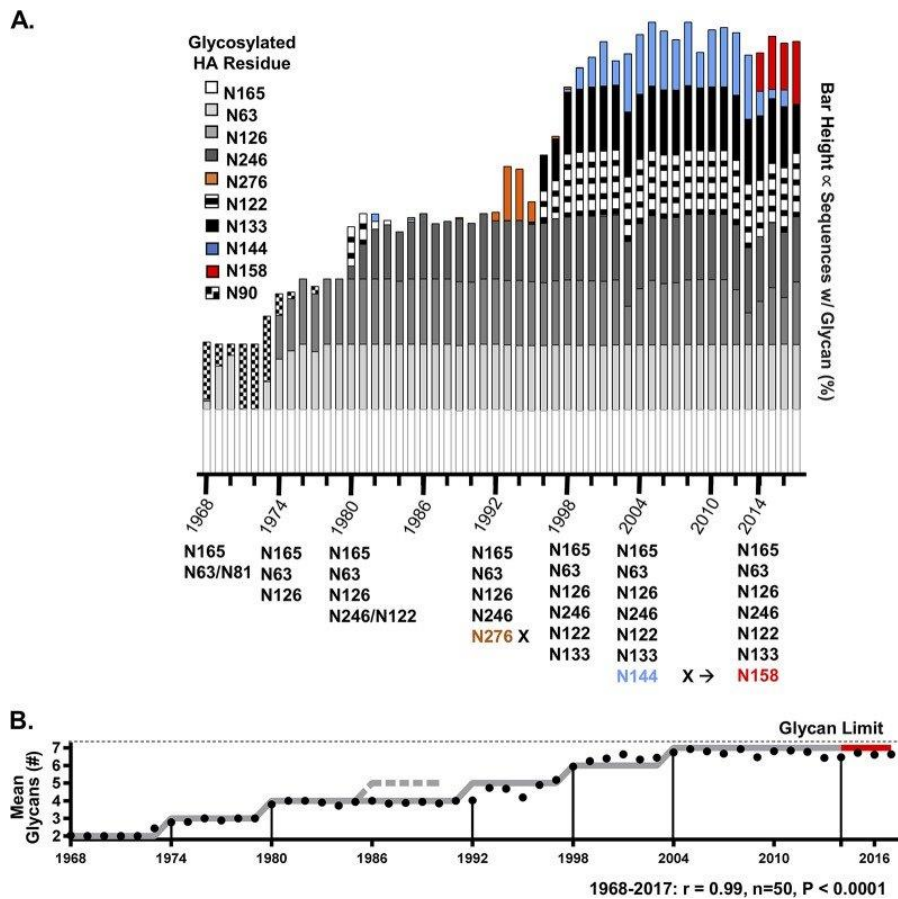
**Figure 6: H1 HA N-linked Glycosylation Evolution from 1918 to 2017:** (A). Timeline of H1 HA glycosylation addition, deletion, and site switching frequencies of certain amino acids N-linked glycosylation sites. Years with no data are left blank and yellow lines correspond to pandemic FLUAV glycosylation emergence. (B). Predictive mean of glycans along the sequences from each year after 1977. Figure from reference<sup>186</sup>. Copyright © 2019 Atzman et al. mBio.

Substitutions in the amino acids in circulating FLUAV were correlated with antigenic epitopes and receptor binding sites evolution<sup>47,145</sup>. It has been suggested that the accumulation of N-linked glycosylation sites aid in immune evasion<sup>38</sup>. The accumulation of N-linked glycosylation sites has been observed in both H3N2 and H1N1 subtypes<sup>34,46,61,170,182</sup>. For example, there were four potential N-linked glycosylation sites in the human HA stalk for 1918 H1N1 which increased to eight potential N-linked

glycosylation sites for the circulating strains, with the new four N-linked glycosylation sites found in the HA globular head (**Figure 6B**)<sup>59,166,178</sup>.

When H3N2 first started circulating in humans it only had six N-linked potential glycosylation sites, two of which were in the HA globular head<sup>46,55</sup>. For decades, the HA head of H3N2 FLUAV subtype has gradually been accruing potential N-linked glycosylation sites, from two sites in 1968 to the seven sites in the 2000 flu season (**Figure 7A**)<sup>47</sup>. Current circulating H3N2 subtypes have 13 potential N-linked glycosylation sites in the HA, four in the stalk domain and 9 in the globular head domain (**Figure 7B**)<sup>145</sup>. Additionally, the amino acid at 165 usually contains a high mannose N-linked glycosylation which is conserved in H3N2 subtypes<sup>47</sup>. With pre-1975 H3 strains, there were N-linked glycosylation sites in the HA stalk region from human strains that contained two or more sites that were not found in avian H3 HA's strains. These N-linked glycosylation sites are nowhere to be seen in the avian H3 HA pre or post 1975<sup>61,72</sup>. This could suggest that the human FLUAV HA strains that have gained N-linked glycosylation sites are more prevalent in circulating populations<sup>174</sup>. Especially when H1 FLUAV strains could be preserving N-linked glycosylation sites in the tip of the HA head domain in humans while eliminating them in avian and swine populations due to selective host pressures<sup>160,172</sup>. Interestingly, as N-linked glycosylation in H1 at the tip of HA head domain is not required for the FLUAV growth in human cells, the H1 lab strains WSN and PR8, which have been grown for years, do not have N-linked glycosylation at the tip of the HA head domain<sup>187</sup>. This suggest that human H1 with more N-linked glycosylation sites on the tip of HA head domain have been only circulating in human populations and not spilling back to avian populations since the 1950s<sup>152</sup>. The

increased rate of N-linked glycosylation sites in the HA head domain of FLUAV could be an evolutionary adaptation for further circulating in a population that has immunities to past strains<sup>80,139,142,172,188</sup>.



**Figure 7: H3 N-linked Glycosylation Evolution from 1968 to 2017.** (A). Timeline of H3 HA glycosylation addition, deletion, and site switching frequencies of certain amino acids N-linked glycosylation sites. Years with no data are left blank (B). Predictive mean of glycans along the sequences from each year after 1968. Figure from reference<sup>186</sup>. Copyright © 2019 Atلمان et al. mBio.

For the other FLUAV subtypes, the 1957 H2N2 pandemic strain only had one N-linked glycosylation site on the HA and NA globular head and has not experienced any change<sup>138</sup>. HPAI H5N1 infections in humans have six N-linked glycosylation sites

available<sup>90,160</sup>. Sequencing of H5 HA isolates from 2004-2020 showed that there were nine N-linked glycosylation sites, two of which were conserved<sup>56,97,189</sup>. The N-linked glycosylation patterns in HPAI H5N1 has not evolved in humans for long<sup>99</sup>. The HA of X31 strain, which contains the six internal gene from PR8 and the surface proteins from H3N2, had seven N-linked glycosylation sites, four in the stem domain, one in the hinge, and two in the globular head<sup>150</sup>. As FLUAV undergo rapid evolutionary changes year to year, monitoring the genetic and antigenic changes in the circulating viruses can aid in prevention and controlling the transmission of FLUAV<sup>183</sup>. As the amount of N-linked glycosylation sites in FLUAV increase, the morbidity, mortality, and viral titers are impacted<sup>16,19,88,164</sup>. This showcases the necessity of routine surveillance for genomic changes which can cause evolutionary changes of FLUAV. Especially, the transmission and antigenic analysis of circulating FLUAV from locally, regionally, urban to rural and vice versa.






## CHAPTER 3: Developments in N-linked Glycosylation Analyses in FLUAVs

### 3.1 Mass spectrometry utilized in characterizing glycan type and sequences.

As N-linked glycosylation has a role in early folding aided by the molecular chaperones calreticulin and calnexin on the FLUAV HA and NA glycoproteins, it is important to assemble a structural analysis of the N-linked glycosylation structure and glycan type composition<sup>72,190–192</sup>. In the past structural analysis and glycoforms composition have been pieced together using a combination of nuclear magnetic resonance, X-ray crystallography, theoretic calculation, and most importantly mass spectrometry (MS)<sup>30,190,193</sup>. Utilizing MS one can analyze released glycan structures, map out glycan site locations, glycan site occupancy, and determine site specific glycan heterogeneity with only a small volume of materials to produce this glycan profile<sup>128,194</sup>.

MS can measure a sample's mass-to-charge ratio ( $m/z$ ), meaning the molecular weight of the ions divided by the number of charges present, and one is able to calculate the exact molecular weight of the sample<sup>195–197</sup>. For large molecules like proteins, which contain many ionic charges, the  $m/z$  ratio is a fraction of the ion mass<sup>198,199</sup>. MS utilizes mass spectrometers containing an ion source to get ionized by the machine, a mass analyzer to sort the ions by  $m/z$  ratio, and a detector to display a mass spectrum chart of the measured ions<sup>195</sup>. It is crucial that the samples are ionized to get detected as they accelerate in the machine<sup>195,198,200</sup>. To make sure the samples do get ionized one has to prepare the sample into a liquid or gaseous phase<sup>201</sup>. The most common way is utilizing liquid or gas chromatography, but other ways are electrospray ionization and capillary electrophoresis<sup>198,202</sup>.

Determining the whole complex structure of a glycan at an N-linked glycosylation site is difficult as glycans have a poor ionization efficiency and can have linear or branched oligosaccharides making it more difficult to characterize than other proteins or peptides<sup>202,203</sup>. Because of this, the field of glycomics and glycoproteomics have not advanced as fast as other -omics fields like genomics and proteomics<sup>198</sup>. Currently, MS is viewed as an important method for virus glycoprotein glycosylation characterization. When MS is coupled with molecular and structural experiments it can lead to further understanding of glycoprotein glycosylation<sup>204</sup>. MS can accurately determine the types of glycans present in a glycosylation site as the monosaccharides comprised in the N-linked glycosylation structure, such as mannose, fructose, glucose, galactose, GlcNAc, and N-Acetylneuraminic acid (Neu5Ac), otherwise called sialic acid, have a known mass and can be profiled accordingly (**Figure 8**)<sup>197,200</sup>. With this information, one can monitor the heterogeneous N-linked glycosylation population for changes in glycans, like a rise of fructose meaning there are more complex or hybrid N-linked glycosylation in FLUAV<sup>204</sup>. As N-linked glycosylation follows a non-template driven process and the glycans attached are reliant on the host's diversity of glycosidase and glycosyltransferases, understanding glycosylation profiles and advancements in glycomics and proteomics matters<sup>198,202,205</sup>.

Monosaccharides	Isotopic Mass
 GlcNAc	203.0794
 Man	162.0528
 Fucose	146.0579
 Gal	162.0528
 Neu5Ac	291.0954

**Figure 8: Glycan isotopic mass.** List of monosaccharides in order from top to bottom with their corresponding weights: GlcNAc, mannose, fructose, galactose, and Neu5Ac. Created with BioRender.

### 3.2 Advancements in methods pertaining to glycosylation.

Even though MS yields accurate mass and gives sequence information of N-linked glycosylation fragments of FLUAV HA and NA, a variety of techniques can aid in acquiring more complete glycomics detail<sup>192,206,207</sup>. A major development in MS analysis are the advancements in liquid chromatography (LC) which is the most prevalent separation technique for glycans<sup>198,208,209</sup>. Moreover, liquid chromatography MS (LC-MS) has an efficient isomeric separation without commonly resorting to using nonvolatile salts making it a compatible system to use<sup>208</sup>. An improvement in the LC-MS system is the addition of high-performance liquid chromatography (HPLC) where higher operational pressure is used compared to LC which uses gravity force for the separation. HPLC has better efficiency as the pressure delivers the mobile phase at a controlled flow rate<sup>201,206</sup>. With this, HPLC can be used to separate purified glycans based on its characteristics and make a map which identifies glycan compositions and utilize a

software to predict structures in FLUAV<sup>201</sup>. Another advancement is matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) assays. The MALDI-TOF-MS is fast, has high throughput, uses low sample consumption, has soft ionization, and automation potential<sup>209,210</sup>. MALDI-TOF-MS can determine the mass of proteins, peptides and glycans by soft ionization causing little fragmentation and the ions of the samples<sup>198,210,211</sup>. Because of the low fragmentation, MALDI-TOF-MS is used for screening complicated mixtures of glycans along with glycan sequencing using exoglycosidic digestion to give glycosidic linkage information<sup>210</sup>. A major aid in N-linked glycosylation characterization was the development of the highly sensitive glycan array<sup>208,212,213</sup>. It allowed for quick screening of virus binding specificity and could be made to express HA<sup>213,214</sup>. It coupled with the high-throughput analysis of glycoproteins, like FLUAV HA and NA, advanced glycomics greatly<sup>212</sup>. Also, advancements in partial or fully deglycosylation reagents, such as Endo H, Endo F3, and PNGase F, have aided in N-linked glycosylation type analysis<sup>72,193,202,215</sup>.

### **3.3 The role of bioinformatics in characterizing potential N-linked glycosylation sites for vaccine design.**

As glycosylation analysis requires many methods to fully characterize it, the high amount of glycomics and glycoproteomic data produced are stored in databases<sup>193,197,199,216</sup>. These databases are made to store sometimes very specific data, general data, data on glycans in specific host and cells, or have a database of databases for others to search for specific information<sup>190,207,217</sup>. One of these databases is a computer simulation using an open-source pipeline of glycans called GlycoSHIELD<sup>207,218,219</sup>. These simulations predict how glycans on static protein

structures interact with therapeutics, its shielding impact, and its influence on the protein structure<sup>218</sup>. Another name for glycan shielding is glycan masking. As glycan masking happens naturally on FLUAV to help escape the immune system, the application of using glycan masking to aid in vaccine design shifts the immune response from specific epitopes to others or add focus on a specific epitope<sup>89,220,221</sup>. Both approaches aid in broadening the immune response and can alter the number of total antibodies elicited<sup>166,218</sup>. Additionally, glycan masking was utilized to develop the Neutralizing Immunogenicity Index (NII) which is utilized to quantitatively differentiate between epitopes with a strong therapeutic response from the weak responses and how they contribute to viral neutralization<sup>166</sup>.

Influenza databases, such as SWISS-PROT, Protein databank (PDB), and GlycoSHIELD to name a few, are a good basis of using genomics to aid in targeting N-linked glycosylation sites for vaccine research<sup>190,192,218,222–224</sup>. The additional benefit is how one can store genomic and proteomic data produced in their lab and share it with the community especially if it is linked to a reference viral database<sup>217</sup>. However, for the data to be useful it must be high quality and labeled<sup>225</sup>. With a glycomics analysis and structural mapping, FLUAV HAs specifically from human pandemic viruses H1N1 and H3N2 had substantial amount of high-mannose N-linked glycans in the HA head compared to the minor amount in avian HA stem domains<sup>194,226</sup>. Certain high mannose N-linked glycosylation sites, especially those near well conserved the receptor binding site (RBS) in the HA head were more prone to mutate and impact antigenic drift<sup>158</sup>. The high mannose glycans can be selectively labeled using a mannose selective lectin and used for detection of the HA glycoprotein in glycan microarrays<sup>158,226</sup>.

In 2014 a group of scientists in the glycobiology community published a standard for the minimum information required for a glycomics experiments (MIRAGE) for LC-MS based data<sup>227</sup>. The MIRAGE requirements are to report the general features, the description of columns used, the mobile phase, the properties of the column run, the pre- and post-run processes, and the outputs obtained<sup>227</sup>. The requirements also state that the ion source conditions along with the software tools utilized for data analysis need to be included<sup>200,227</sup>. Later guidelines were added for reporting the sample preparation and microarray-based data<sup>198</sup>.

Data analysis of structural proteins and RNA of different FLUAV strains has led to researchers focusing their attention from N-linked glycosylation in the HA head domain to the more conserved areas of the HA stalk and NA as a vaccine target<sup>220,228–231</sup>. The N-linked glycosylation on FLUAV HA stem has a role in protein folding and fusion meaning those sites are highly conserved and a prime therapeutic target<sup>168,191,226</sup>. Machine learning can also be used to aid in finding conserved sites and predicting N-linked glycosylation sites in FLUAV from sequences retrieved from the influenza database<sup>207,232,233</sup>. This bioinformatics machine learning data has the potential to be a rapid method for pre-vaccine assessment and utilized to create a universal influenza vaccine design<sup>218,234,235</sup>.

## **CHAPTER 4: Potential Impacts**

### **4.1 In research and industry**

The continued pursuit of understanding FLUAV N-linked glycosylation and sharing the data can aid in better recognition of finding a vaccine target<sup>236</sup>. Additionally, research into N-linked glycosylation's impact on FLUAV evolution, antigenicity, and immunology has led to labs looking into overlooked sites and molecules like the HA stem, the NA glycoprotein, monoclonal antibodies against N-linked glycosylation, and different forms of N-linked glycosylation like monoglycosylated, to try to develop an effective and broadly protective influenza vaccine<sup>188,237,238</sup>. Advancements in technologies, like HPLC, MALDI-TOF-MS, and deglycosylation reagents, such as Endo H, Endo F, and PNGase F, have highly influenced the amount of structural information of glycosylation that can be attained<sup>72,73,198,202</sup>. Additionally, better access and organization of the glycomics databases aids in finding targets, like with glycan masking epitopes, especially if there is a pipeline that makes for quick analysis of sequence data<sup>211,239</sup>. The ease of access to the information gives collaborators better access to share and discuss while also consolidating resources across research and industry<sup>211,233,240,241</sup>. As more methods are being upgraded and made to better research and analyze the N-linked glycosylation of FLUAV in both research and industry the field of glycomics will have better tools for predicting, preparing, and producing therapeutics for when an FLUAV pandemic like strain appears<sup>112,242</sup>.

### **4.2 Benefits for the public**

Advancements in the field of glycomics not only aid in general scientific knowledge in the field but also impacts the public. With access to journals, talks, and science communication events dissemination of the educational knowledge from the

research to the public about glycosylation and the process of targeting therapeutics and vaccines using the knowledge attained from the N-linked glycosylation studies in FLUAV<sup>78,236</sup>. The science literacy of the public is important to foster as development and utilization of a broadly effective and long-term protective Influenza vaccine against seasonal and potential pandemic like influenza strain<sup>242</sup>. As research advances it is important to make sure the public understands the importance of prevention measures for influenza seasonal infection and pandemic-like strains.

## CONCLUSIONS

As FLUAV evolves, it mainly circulates in humans, avian, and swine, and the N-linked glycosylation site addition and swapping of sites leads to a change in immunogenicity and antigenicity of FLUAV. The HA and NA glycoproteins on the surface of the FLUAV envelope are where these antigenic sites are located and where N-linked glycosylation impacts antigenicity. Finding out and knowing the type and glycan site occupationally percent of the N-linked glycosylation on HA and NA can aid in antigenic drift and impact the transmissibility of FLUAV within species and across species, like avian to human. Observing the current influenza vaccine pipeline, there are many efficient ways vaccine development can be improved, and the timeline is fastened to accommodate changes within the influenza season. Utilization of bioinformatics, databases, and improvements in the glycomics field of influenza has helped in understanding N-linked glycosylation function and structure while also aiding in finding influenza vaccine therapeutic targets. With more research and innovation in the field of glycomics it can advance the development of making a long lasting broadly protective influenza vaccine and help monitor for an emerging pandemic-like influenza strain.

## REFERENCES

1. Nichols JE, W. LeDuc J. Chapter 27 - Influenza. In: Barrett ADT, Stanberry LR, eds. *Vaccines for Biodefense and Emerging and Neglected Diseases*. Academic Press; 2009:497-525. doi:<https://doi.org/10.1016/B978-0-12-369408-9.00027-5>
2. Stanley WM. The size of influenza virus. *Journal of Experimental Medicine*. 1944;79(3). doi:10.1084/jem.79.3.267
3. Relich RF, Loeffelholz MJ. Taxonomic Changes for Human Viruses, 2020 to 2022. *J Clin Microbiol*. 2023;61(1). doi:10.1128/jcm.00337-22
4. Zerbini FM, Siddell SG, Mushegian AR, et al. Differentiating between viruses and virus species by writing their names correctly. *Arch Virol*. 2022;167(4). doi:10.1007/s00705-021-05323-4
5. ICTV. ICTV Master Species List 2019 v1 (MSL35). *ICTV Master Species List 2019 v1 [Internet]*. Published online 2020.
6. Zerbini FM, Siddell SG, Lefkowitz EJ, et al. Changes to virus taxonomy and the ICTV Statutes ratified by the International Committee on Taxonomy of Viruses (2023). *Arch Virol*. 2023;168(7). doi:10.1007/s00705-023-05797-4
7. Couch RB. *Orthomyxoviruses*. 4th edition. (Baron S, ed.); 1996.
8. Eccles R. Understanding the symptoms of the common cold and influenza. *Lancet Infectious Diseases*. 2005;5(11). doi:10.1016/S1473-3099(05)70270-X
9. Nelson MI, Vincent AL. Reverse zoonosis of influenza to swine: New perspectives on the human-animal interface. *Trends Microbiol*. 2015;23(3). doi:10.1016/j.tim.2014.12.002
10. Kessler S, Harder TC, Schwemmler M, Ciminski K. Influenza A viruses and zoonotic events—are we creating our own reservoirs? *Viruses*. 2021;13(11). doi:10.3390/v13112250
11. Kuchipudi S V., Nissly RH. Novel flu viruses in bats and cattle: “Pushing the envelope” of influenza infection. *Vet Sci*. 2018;5(3). doi:10.3390/vetsci5030071
12. Wolff T, Veit M. Influenza B, C and D Viruses (Orthomyxoviridae). In: *Encyclopedia of Virology: Volume 1-5, Fourth Edition*. Vol 1-5. ; 2020. doi:10.1016/B978-0-12-809633-8.21505-7
13. Osterhaus ADME, Rimmelzwaan GF, Martina BEE, Bestebroer TM, Fouchier RAM. Influenza B virus in seals. *Science* (1979). 2000;288(5468). doi:10.1126/science.288.5468.1051

14. Dou D, Revol R, Östbye H, Wang H, Daniels R. Influenza A virus cell entry, replication, virion assembly and movement. *Front Immunol.* 2018;9(JUL). doi:10.3389/fimmu.2018.01581
15. Cohen M, Zhang XQ, Senaati HP, et al. Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. *Virology.* 2013;10. doi:10.1186/1743-422X-10-321
16. Fontana J, Steven AC. Influenza virus-mediated membrane fusion: Structural insights from electron microscopy. *Arch Biochem Biophys.* 2015;581. doi:10.1016/j.abb.2015.04.011
17. Serrão VHB, Cook JD, Lee JE. Snapshot of an influenza virus glycoprotein fusion intermediate. *Cell Rep.* 2021;35(7). doi:10.1016/j.celrep.2021.109152
18. Zhang H, Li H, Wang W, et al. A unique feature of swine ANP32A provides susceptibility to avian influenza virus infection in pigs. *PLoS Pathog.* 2020;16(2). doi:10.1371/journal.ppat.1008330
19. Medina RA, García-Sastre A. Influenza A viruses: New research developments. *Nat Rev Microbiol.* 2011;9(8). doi:10.1038/nrmicro2613
20. Sparrer KMJ, Kirchhoff F. Cap snatch prevention: a novel approach to tackle influenza viruses. *Signal Transduct Target Ther.* 2023;8(1). doi:10.1038/s41392-023-01474-9
21. Li L, Dai H, Nguyen AP, Hai R, Gu W. Influenza A virus utilizes noncanonical cap-snatching to diversify its mRNA/ncRNA. *RNA.* 2020;26(9). doi:10.1261/RNA.073866.119
22. Nogales A, Martínez-Sobrido L. Reverse genetics approaches for the development of influenza vaccines. *Int J Mol Sci.* 2017;18(1). doi:10.3390/ijms18010020
23. Li HC, Yang CH, Lo SY. Strategies of Influenza A Virus to Ensure the Translation of Viral mRNAs. *Pathogens.* 2022;11(12). doi:10.3390/pathogens11121521
24. Li X, Gu M, Zheng Q, Gao R, Liu X. Packaging signal of influenza A virus. *Virology.* 2021;18(1). doi:10.1186/s12985-021-01504-4
25. Yang R, Pan M, Guo J, et al. Mapping of the influenza A virus genome RNA structure and interactions reveals essential elements of viral replication. *Cell Rep.* 2024;43(3). doi:10.1016/j.celrep.2024.113833
26. Noda T, Murakami S, Nakatsu S, et al. Importance of the 1+7 configuration of ribonucleoprotein complexes for influenza A virus genome packaging. *Nat Commun.* 2018;9(1). doi:10.1038/s41467-017-02517-w

27. Legaz ME, Pedrosa MM, Vicente C. Chapter 6 Carbohydrates, Polymeric Sugars and their Constituents. In: *Journal of Chromatography Library*. Vol 60. ; 1998. doi:10.1016/S0301-4770(08)60304-8
28. Nachbagauer R, Palese P. Is a Universal Influenza Virus Vaccine Possible? *Annu Rev Med*. 2020;71. doi:10.1146/annurev-med-120617-041310
29. Sánchez-de Prada L, Sanz-Muñoz I, Sun W, et al. Group 1 and group 2 hemagglutinin stalk antibody response according to age. *Front Immunol*. 2023;14. doi:10.3389/fimmu.2023.1194073
30. Zhu X, Yang H, Guo Z, et al. Crystal structures of two subtype N10 neuraminidase-like proteins from bat influenza A viruses reveal a diverged putative active site. *Proc Natl Acad Sci U S A*. 2012;109(46). doi:10.1073/pnas.1212579109
31. Li Q, Sun X, Li Z, et al. Structural and functional characterization of neuraminidase-like molecule N10 derived from bat influenza A virus. *Proc Natl Acad Sci U S A*. 2012;109(46). doi:10.1073/pnas.1211037109
32. Wu Y, Wu Y, Tefsen B, Shi Y, Gao GF. Bat-derived influenza-like viruses H17N10 and H18N11. *Trends Microbiol*. 2014;22(4). doi:10.1016/j.tim.2014.01.010
33. Ellis D, Lederhofer J, Acton OJ, et al. Structure-based design of stabilized recombinant influenza neuraminidase tetramers. *Nat Commun*. 2022;13(1). doi:10.1038/s41467-022-29416-z
34. Sun X, Jayaraman A, Maniprasad P, et al. N-Linked Glycosylation of the Hemagglutinin Protein Influences Virulence and Antigenicity of the 1918 Pandemic and Seasonal H1N1 Influenza A Viruses. *J Virol*. 2013;87(15). doi:10.1128/jvi.00593-13
35. *Encyclopedia of Infection and Immunity*.; 2022. doi:10.1016/c2018-1-04055-x
36. Biere B, Bauer B, Schweiger B. Differentiation of influenza b virus lineages yamagata and victoria by real-time PCR. *J Clin Microbiol*. 2010;48(4). doi:10.1128/JCM.02116-09
37. Liu Y, Tan HX, Koutsakos M, et al. Cross-lineage protection by human antibodies binding the influenza B hemagglutinin. *Nat Commun*. 2019;10(1). doi:10.1038/s41467-018-08165-y
38. Broecker F, Liu STH, Sun W, Krammer F, Simon V, Palese P. Immunodominance of Antigenic Site B in the Hemagglutinin of the

- Current H3N2 Influenza Virus in Humans and Mice. *J Virol.* 2018;92(20). doi:10.1128/jvi.01100-18
39. Samji T. Influenza A: Understanding the viral life cycle. *Yale Journal of Biology and Medicine.* 2009;82(4):153-159.
  40. Baskakov I V. Role of sialylation in prion disease pathogenesis and prion structure. In: *Progress in Molecular Biology and Translational Science.* Vol 175. ; 2020. doi:10.1016/bs.pmbts.2020.07.004
  41. Ghosh S. Sialic acid and biology of life: An introduction. In: *Sialic Acids and Sialoglycoconjugates in the Biology of Life, Health and Disease.* ; 2020. doi:10.1016/b978-0-12-816126-5.00001-9
  42. Zhao C, Pu J. Influence of Host Sialic Acid Receptors Structure on the Host Specificity of Influenza Viruses. *Viruses.* 2022;14(10). doi:10.3390/v14102141
  43. Breen M, Nogales A, Baker SF, Martínez-Sobrido L. Replication-competent influenza a viruses expressing reporter genes. *Viruses.* 2016;8(7). doi:10.3390/v8070179
  44. Mubareka S, Palese P. Influenza virus: The biology of a changing virus. *Birkhauser Advances in Infectious Diseases.* Published online 2011. doi:10.1007/978-3-0346-0279-2\_1
  45. Das SR, Hensley SE, Ince WL, et al. Defining influenza a virus hemagglutinin antigenic drift by sequential monoclonal antibody selection. *Cell Host Microbe.* 2013;13(3). doi:10.1016/j.chom.2013.02.008
  46. Suzuki Y. Positive selection for gains of n-linked glycosylation sites in hemagglutinin during evolution of H3N2human influenza a virus. *Genes Genet Syst.* 2011;86(5). doi:10.1266/ggs.86.287
  47. Vigerust DJ, Ulett KB, Boyd KL, Madsen J, Hawgood S, McCullers JA. N-Linked Glycosylation Attenuates H3N2 Influenza Viruses. *J Virol.* 2007;81(16). doi:10.1128/jvi.00769-07
  48. Aartse A, Mortier D, Mooij P, et al. Primary antibody response after influenza virus infection is first dominated by low-mutated HA-stem antibodies followed by higher-mutated HA-head antibodies. *Front Immunol.* 2022;13. doi:10.3389/fimmu.2022.1026951
  49. Orsi A, Ansaldi F, de Florentiis D, et al. Cross-protection against drifted influenza viruses. *Hum Vaccin Immunother.* 2013;9(3). doi:10.4161/hv.23239

50. Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell*. 1982;31(2 PART 1). doi:10.1016/0092-8674(82)90135-0
51. McAuley JL, Gilbertson BP, Trifkovic S, Brown LE, McKimm-Breschkin JL. Influenza virus neuraminidase structure and functions. *Front Microbiol*. 2019;10(JAN). doi:10.3389/fmicb.2019.00039
52. Creytens S, Pascha MN, Ballegeer M, Saelens X, de Haan CAM. Influenza Neuraminidase Characteristics and Potential as a Vaccine Target. *Front Immunol*. 2021;12. doi:10.3389/fimmu.2021.786617
53. Shtyrya YA, Mochalova L V, Bovin N V. Influenza Virus Neuraminidase: Structure and Function. *Acta Naturae*. 2009;1(2). doi:10.32607/20758251-2009-1-2-26-32
54. Colman PM, Varghese JN, Laver WG. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. *Nature*. 1983;303(5912). doi:10.1038/303041a0
55. Wang Y, Lei R, Nourmohammad A, Wu NC. Antigenic evolution of human influenza H3N2 neuraminidase is constrained by charge balancing. *Elife*. 2021;10. doi:10.7554/eLife.72516
56. Sun S, Wang Q, Zhao F, Chen W, Li Z. Glycosylation site alteration in the evolution of influenza a (H1N1) viruses. *PLoS One*. 2011;6(7). doi:10.1371/journal.pone.0022844
57. Air GM, Els MC, Brown LE, Laver WG, Webster RG. Location of antigenic sites on the three-dimensional structure of the influenza N2 virus neuraminidase. *Virology*. 1985;145(2). doi:10.1016/0042-6822(85)90157-6
58. Cheung PPH, Watson SJ, Choy KT, et al. Generation and characterization of influenza A viruses with altered polymerase fidelity. *Nat Commun*. 2014;5. doi:10.1038/ncomms5794
59. Slaine PD, MacRae C, Kleer M, et al. Adaptive mutations in influenza a/California/07/2009 enhance polymerase activity and infectious virion production. *Viruses*. 2018;10(5). doi:10.3390/v10050272
60. DeDiego ML, Chiem K, Topham DJ. Directed selection of amino acid changes in the influenza hemagglutinin and neuraminidase affecting protein antigenicity. *Vaccine*. 2018;36(43). doi:10.1016/j.vaccine.2018.09.005
61. Sandbulte MR, Westgeest KB, Gao J, et al. Discordant antigenic drift of neuraminidase and hemagglutinin in H1N1 and H3N2 influenza

- viruses. *Proc Natl Acad Sci U S A*. 2011;108(51). doi:10.1073/pnas.1113801108
62. Fan Y, Hu Y, Yan C, et al. Loss and gain of N-linked glycosylation sequons due to single-nucleotide variation in cancer. *Sci Rep*. 2018;8(1). doi:10.1038/s41598-018-22345-2
63. York IA, Stevens J, Alymova I V. Influenza virus N-linked glycosylation and innate immunity. *Biosci Rep*. 2019;39(1). doi:10.1042/BSR20171505
64. Bagdonaite I, Wandall HH. Global aspects of viral glycosylation. *Glycobiology*. 2018;28(7). doi:10.1093/glycob/cwy021
65. Higel F, Seidl A, Sörgel F, Friess W. N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins. *European Journal of Pharmaceutics and Biopharmaceutics*. 2016;100. doi:10.1016/j.ejpb.2016.01.005
66. Yap SSL, Nguyen-Khuong T, Rudd PM, Alonso S. Dengue virus glycosylation: What do we know? *Front Microbiol*. 2017;8(JUL). doi:10.3389/fmicb.2017.01415
67. Goh JB, Ng SK. Impact of host cell line choice on glycan profile. *Crit Rev Biotechnol*. 2018;38(6). doi:10.1080/07388551.2017.1416577
68. Sun S, Wang Q, Zhao F, Chen W, Li Z. Prediction of biological functions on glycosylation site migrations in human influenza H1N1 viruses. *PLoS One*. 2012;7(2). doi:10.1371/journal.pone.0032119
69. Makarava N, Baskakov I V. Role of sialylation of N-linked glycans in prion pathogenesis. *Cell Tissue Res*. 2023;392(1). doi:10.1007/s00441-022-03584-2
70. Gallagher PJ, Henneberry JM, Sambrook JF, Gething MJ. Glycosylation requirements for intracellular transport and function of the hemagglutinin of influenza virus. *J Virol*. 1992;66(12). doi:10.1128/jvi.66.12.7136-7145.1992
71. Hayes MR, Pietruszka J. Synthesis of glycosides by glycosynthases. *Molecules*. 2017;22(9). doi:10.3390/molecules22091434
72. Cao L, Diedrich JK, Ma Y, et al. Global site-specific analysis of glycoprotein N-glycan processing. *Nat Protoc*. 2018;13(6). doi:10.1038/nprot.2018.024
73. Nagae M, Yamaguchi Y, Taniguchi N, Kizuka Y. 3D structure and function of glycosyltransferases involved in N-glycan maturation. *Int J Mol Sci*. 2020;21(2). doi:10.3390/ijms21020437

74. Stanley P, Moremen KW, Lewis NE, Taniguchi N, Aebi M. *N-Glycans.*; 2022.
75. Alymova I V., Cipollo JF, Parsons LM, et al. Aberrant Cellular Glycosylation May Increase the Ability of Influenza Viruses to Escape Host Immune Responses through Modification of the Viral Glycome. *mBio.* 2022;13(2). doi:10.1128/mbio.02983-21
76. Das SR, Hensley SE, David A, et al. Fitness costs limit influenza A virus hemagglutinin glycosylation as an immune evasion strategy. *Proc Natl Acad Sci U S A.* 2011;108(51). doi:10.1073/pnas.1108754108
77. Wu NC, Otwinowski J, Thompson AJ, Nycholat CM, Nourmohammad A, Wilson IA. Major antigenic site B of human influenza H3N2 viruses has an evolving local fitness landscape. *Nat Commun.* 2020;11(1). doi:10.1038/s41467-020-15102-5
78. Tate MD, Job ER, Deng YM, Gunalan V, Maurer-Stroh S, Reading PC. Playing hide and seek: How glycosylation of the influenza virus hemagglutinin can modulate the immune response to infection. *Viruses.* 2014;6(3). doi:10.3390/v6031294
79. Wei CJ, Boyington JC, Dai K, et al. Cross-neutralization of 1918 and 2009 influenza viruses: Role of glycans in viral evolution and vaccine design. *Sci Transl Med.* 2010;2(24). doi:10.1126/scitranslmed.3000799
80. Vigerust DJ, Shepherd VL. Virus glycosylation: role in virulence and immune interactions. *Trends Microbiol.* 2007;15(5). doi:10.1016/j.tim.2007.03.003
81. Chang D, Hackett WE, Zhong L, Wan XF, Zaia J. Measuring Site-specific Glycosylation Similarity between Influenza A Virus Variants with Statistical Certainty. *Molecular and Cellular Proteomics.* 2020;19(9). doi:10.1074/mcp.RA120.002031
82. Jones S, Nelson-Sathi S, Wang Y, et al. Evolutionary, genetic, structural characterization and its functional implications for the influenza A (H1N1) infection outbreak in India from 2009 to 2017. *Sci Rep.* 2019;9(1). doi:10.1038/s41598-019-51097-w
83. Gyles CL. Influenza in animals. *Anim Health Res Rev.* 2010;11(1). doi:10.1017/S1466252310000101
84. Schrauwen EJA, Fouchier RAM. Host adaptation and transmission of influenza A viruses in mammals. *Emerg Microbes Infect.* 2014;3. doi:10.1038/emi.2014.9

85. Khalil AM, Martinez-Sobrido L, Mostafa A. Zoonosis and zoonothroponosis of emerging respiratory viruses. *Front Cell Infect Microbiol.* 2024;13. doi:10.3389/fcimb.2023.1232772
86. Mena I, Nelson MI, Quezada-Monroy F, et al. Origins of the 2009 H1N1 influenza pandemic in swine in Mexico. *Elife.* 2016;5(JUN2016). doi:10.7554/eLife.16777
87. Khanna M, Kumar B, Gupta N, et al. Pandemic swine influenza virus (H1N1): A threatening evolution. *Indian J Microbiol.* 2009;49(4). doi:10.1007/s12088-009-0064-3
88. Olsen CW, Carey S, Hinshaw L, Karasin AI. Virologic and serologic surveillance for human, swine and avian influenza virus infections among pigs in the north-central United States. *Arch Virol.* 2000;145(7). doi:10.1007/s007050070098
89. Wua CY, Lina CW, Tsaia TI, et al. Influenza A surface glycosylation and vaccine design. *Proc Natl Acad Sci U S A.* 2017;114(2). doi:10.1073/pnas.1617174114
90. Sun H, Deng G, Sun H, et al. N-linked glycosylation enhances hemagglutinin stability in avian H5N6 influenza virus to promote adaptation in mammals. *PNAS Nexus.* 2022;1(3). doi:10.1093/pnasnexus/pgac085
91. Jesan T, Menon GI, Sinha S. Epidemiological dynamics of the 2009 influenza A(H1N1)v outbreak in India. *Curr Sci.* 2011;100(7).
92. Pushan SS, Samantaray M, Rajagopalan M, Ramaswamy A. Evolution of Indian Influenza A (H1N1) Hemagglutinin Strains: A Comparative Analysis of the Pandemic Californian HA Strain. *Front Mol Biosci.* 2023;10. doi:10.3389/fmolb.2023.1111869
93. Anderson TK, Chang J, Arendsee ZW, et al. Swine influenza a viruses and the tangled relationship with humans. *Cold Spring Harb Perspect Med.* 2021;11(3). doi:10.1101/cshperspect.a038737
94. Peacock TP, Harvey WT, Sadeyen JR, Reeve R, Iqbal M. The molecular basis of antigenic variation among A(H9N2) avian influenza viruses. *Emerg Microbes Infect.* 2018;7(1). doi:10.1038/s41426-018-0178-y
95. Morens DM, Taubenberger JK, Fauci AS. H7N9 avian influenza A virus and the perpetual challenge of potential human pandemicity. *mBio.* 2013;4(4). doi:10.1128/mBio.00445-13
96. Forrest HL, Webster RG. Perspectives on influenza evolution and the role of research. *Animal health research reviews / Conference of*

- Research Workers in Animal Diseases*. 2010;11(1).  
doi:10.1017/S1466252310000071
97. Skeik N, Jabr FI. Influenza viruses and the evolution of avian influenza virus H5N1. *International Journal of Infectious Diseases*. 2008;12(3). doi:10.1016/j.ijid.2007.07.002
  98. Wan XF. Lessons from Emergence of A/Goose/Guangdong/1996-Like H5N1 Highly Pathogenic Avian Influenza Viruses and Recent Influenza Surveillance Efforts in Southern China. *Zoonoses Public Health*. 2012;59(SUPPL.2). doi:10.1111/j.1863-2378.2012.01497.x
  99. Yang ZS, Huang SW, Wang WH, et al. Identification of important n-linked glycosylation sites in the hemagglutinin protein and their functional impact on DC-SIGN mediated avian influenza H5N1 infection. *Int J Mol Sci*. 2021;22(2). doi:10.3390/ijms22020743
  100. Lin B, Qing X, Liao J, Zhuo K. Role of Protein Glycosylation in Host-Pathogen Interaction. *Cells*. 2020;9(4). doi:10.3390/cells9041022
  101. Nidom CA, Takano R, Yamada S, et al. Influenza a (H5N1) viruses from pigs, Indonesia. *Emerg Infect Dis*. 2010;16(10). doi:10.3201/eid1610.100508
  102. Liang Y. Pathogenicity and virulence of influenza. *Virulence*. 2023;14(1). doi:10.1080/21505594.2023.2223057
  103. Boktor SW, Hafner JW. *Influenza*.; 2024.
  104. Nabakooza G, Owuor DC, de Laurent ZR, et al. Phylogenomic analysis uncovers a 9-year variation of Uganda influenza type-A strains from the WHO-recommended vaccines and other Africa strains. *Sci Rep*. 2023;13(1). doi:10.1038/s41598-023-30667-z
  105. World Health Organization. *Global Influenza Strategy 2019-2030*.; 2019.
  106. Crépey P, Redondo E, Díez-Domingo J, et al. From trivalent to quadrivalent influenza vaccines: Public health and economic burden for different immunization strategies in Spain. *PLoS One*. 2020;15(5). doi:10.1371/journal.pone.0233526
  107. Wong SS, Webby RJ. Traditional and new influenza vaccines. *Clin Microbiol Rev*. 2013;26(3). doi:10.1128/CMR.00097-12
  108. Rockman S, Laurie KL, Parkes S, Wheatley A, Barr IG. New technologies for influenza vaccines. *Microorganisms*. 2020;8(11). doi:10.3390/microorganisms8111745

109. Shasha D, Valinsky L, Hershkowitz Sikron F, et al. Quadrivalent versus trivalent influenza vaccine: clinical outcomes in two influenza seasons, historical cohort study. *Clinical Microbiology and Infection*. 2020;26(1). doi:10.1016/j.cmi.2019.05.003
110. *Recommended Composition of Influenza Virus Vaccines for Use in the 2024-2025 Northern Hemisphere Influenza Season.*; 2024.
111. Moore KA, Ostrowsky JT, Kraigsley AM, et al. A Research and Development (R&D) roadmap for influenza vaccines: Looking toward the future. *Vaccine*. 2021;39(45). doi:10.1016/j.vaccine.2021.08.010
112. World Health Organization. Pandemic Influenza Preparedness and Response: A WHO Guidance Document. *Pandemic Influenza Preparedness and Response: A WHO Guidance Document*. Published online 2009.
113. Myers ML, Gallagher JR, Kim AJ, et al. Commercial influenza vaccines vary in HA-complex structure and in induction of cross-reactive HA antibodies. *Nat Commun*. 2023;14(1). doi:10.1038/s41467-023-37162-z
114. Guthmiller JJ, Han J, Utset HA, et al. Broadly neutralizing antibodies target a haemagglutinin anchor epitope. *Nature*. 2022;602(7896). doi:10.1038/s41586-021-04356-8
115. Zhu W, Wang C, Wang BZ. From variation of influenza viral proteins to vaccine development. *Int J Mol Sci*. 2017;18(7). doi:10.3390/ijms18071554
116. Gao Z, Robinson K, Skowronski DM, De Serres G, Withers SG. Quantification of the total neuraminidase content of recent commercially-available influenza vaccines: Introducing a neuraminidase titration reagent. *Vaccine*. 2020;38(4). doi:10.1016/j.vaccine.2019.11.041
117. Panda A, Elankumaran S, Krishnamurthy S, Huang Z, Samal SK. Loss of N-Linked Glycosylation from the Hemagglutinin- Neuraminidase Protein Alters Virulence of Newcastle Disease Virus. *J Virol*. 2004;78(10). doi:10.1128/jvi.78.10.4965-4975.2004
118. An Y, Parsons LM, Jankowska E, Melnyk D, Joshi M, Cipollo JF. N - Glycosylation of Seasonal Influenza Vaccine Hemagglutinins: Implication for Potency Testing and Immune Processing . *J Virol*. 2019;93(2). doi:10.1128/jvi.01693-18
119. Zost SJ, Parkhouse K, Gumina ME, et al. Contemporary H3N2 influenza viruses have a glycosylation site that alters binding of antibodies

- elicited by egg-adapted vaccine strains. *Proc Natl Acad Sci U S A*. 2017;114(47). doi:10.1073/pnas.1712377114
120. Pascua PNQ, Choi YK. Zoonotic infections with avian influenza A viruses and vaccine preparedness: a game of “mix and match.” *Clin Exp Vaccine Res*. 2014;3(2). doi:10.7774/cevr.2014.3.2.140
  121. Howden KJ, Brockhoff EJ, Caya FD, et al. Special report rapport spécial: An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm. *Canadian Veterinary Journal*. 2009;50(11).
  122. Kelso JM. Administering influenza vaccine to egg-allergic persons. *Expert Rev Vaccines*. 2014;13(8). doi:10.1586/14760584.2014.933079
  123. Chung EY, Huang L, Schneider L. Safety of influenza vaccine administration in egg-allergic patients. *Pediatrics*. 2010;125(5). doi:10.1542/peds.2009-2512
  124. Schwarzer J, Rapp E, Hennig R, et al. Glycan analysis in cell culture-based influenza vaccine production: Influence of host cell line and virus strain on the glycosylation pattern of viral hemagglutinin. *Vaccine*. 2009;27(32). doi:10.1016/j.vaccine.2009.04.076
  125. Raul Ortiz de Lejarazu-Leonardo RO de LLCM. Estimation of Reduction in Influenza Vaccine Effectiveness Due to Egg-Adaptation Changes—Systematic Literature Review and Expert Consensus. *Vaccines (Basel)*. Published online 2021.
  126. Cox MMJ, Izikson R, Post P, Dunkle L. Safety, efficacy, and immunogenicity of Flublok in the prevention of seasonal influenza in adults. *Ther Adv Vaccines*. 2015;3(4). doi:10.1177/2051013615595595
  127. Manini I, Domnich A, Amicizia D, et al. Flucelvax (Optaflu) for seasonal influenza. *Expert Rev Vaccines*. 2015;14(6). doi:10.1586/14760584.2015.1039520
  128. Zhang S, Sherwood RW, Yang Y, et al. Comparative characterization of the glycosylation profiles of an influenza hemagglutinin produced in plant and insect hosts. *Proteomics*. 2012;12(8). doi:10.1002/pmic.201100474
  129. Milián E, Kamen AA. Current and emerging cell culture manufacturing technologies for influenza vaccines. *Biomed Res Int*. 2015;2015. doi:10.1155/2015/504831
  130. Xu Z, Moyle PM. Bioconjugation Approaches to Producing Subunit Vaccines Composed of Protein or Peptide Antigens and Covalently Attached Toll-Like Receptor Ligands. *Bioconjug Chem*. 2018;29(3). doi:10.1021/acs.bioconjchem.7b00478

131. Fox CB BSDMRSVTS. Immunomodulatory and Physical Effects of Oil Composition in Vaccine Adjuvant Emulsions. *Vaccine*. Published online 2011.
132. Vemireddy Sravanthi MCPPSRBSSHMSK. Oleic acid nanoemulsion for nasal vaccination: Impact on adjuvanticity based immune response. *J Drug Deliv Sci Technol*. Published online 2015.
133. Legastelois I, Buffin S, Peubez I, Mignon C, Sodoyer R, Werle B. Non-conventional expression systems for the production of vaccine proteins and immunotherapeutic molecules. *Hum Vaccin Immunother*. 2017;13(4). doi:10.1080/21645515.2016.1260795
134. Wang Y, Zhang Z, Luo J, Han X, Wei Y, Wei X. mRNA vaccine: a potential therapeutic strategy. *Mol Cancer*. 2021;20(1). doi:10.1186/s12943-021-01311-z
135. Zhang G, Tang T, Chen Y, Huang X, Liang T. mRNA vaccines in disease prevention and treatment. *Signal Transduct Target Ther*. 2023;8(1). doi:10.1038/s41392-023-01579-1
136. Lu Y, Landreth S, Liu G, et al. Innate immunomodulator containing adjuvant formulated HA based vaccine protects mice from lethal infection of highly pathogenic avian influenza H5N1 virus. *Vaccine*. 2020;38(10):2387-2395. doi:https://doi.org/10.1016/j.vaccine.2020.01.051
137. Ge P, Ross TM. Evolution of A(H1N1) pdm09 influenza virus masking by glycosylation. *Expert Rev Vaccines*. 2021;20(5). doi:10.1080/14760584.2021.1908135
138. Sealy JE, Peacock TP, Sadeyen JR, et al. Adsorptive mutation and N-linked glycosylation modulate influenza virus antigenicity and fitness. *Emerg Microbes Infect*. 2020;9(1). doi:10.1080/22221751.2020.1850180
139. Chen JR, Yu YH, Tseng YC, et al. Vaccination of monoglycosylated hemagglutinin induces cross-strain protection against influenza virus infections. *Proc Natl Acad Sci U S A*. 2014;111(7). doi:10.1073/pnas.1323954111
140. Wang CC, Chen JR, Tseng YC, et al. Glycans on influenza hemagglutinin affect receptor binding and immune response. *Proc Natl Acad Sci U S A*. 2009;106(43). doi:10.1073/pnas.0909696106
141. Tseng YC, Wu CY, Liu ML, et al. Egg-based influenza split virus vaccine with monoglycosylation induces cross-strain protection against influenza virus infections. *Proc Natl Acad Sci U S A*. 2019;116(10). doi:10.1073/pnas.1819197116

142. Krammer F, Fouchier RAM, Eichelberger MC, et al. NAction! how can neuraminidase-based immunity contribute to better influenza virus vaccines? *mBio*. 2018;9(2). doi:10.1128/mBio.02332-17
143. Sedova ES, Shcherbinin DN, Migunov AI, et al. Recombinant Influenza Vaccines. *Acta Naturae*. 2012;4(4). doi:10.32607/20758251-2012-4-4-17-27
144. Doyle TM, Hashem AM, Li C, et al. Universal anti-neuraminidase antibody inhibiting all influenza A subtypes. *Antiviral Res*. 2013;100(2). doi:10.1016/j.antiviral.2013.09.018
145. Lei R, Tan TJC, Hernandez Garcia A, et al. Prevalence and mechanisms of evolutionary contingency in human influenza H3N2 neuraminidase. *Nat Commun*. 2022;13(1). doi:10.1038/s41467-022-34060-8
146. Wan H, Gao J, Yang H, et al. The neuraminidase of A(H3N2) influenza viruses circulating since 2016 is antigenically distinct from the A/Hong Kong/4801/2014 vaccine strain. *Nat Microbiol*. 2019;4(12). doi:10.1038/s41564-019-0522-6
147. Seitz C, Deveci İ, McCammon JA. Glycosylation and Crowded Membrane Effects on Influenza Neuraminidase Stability and Dynamics. *J Phys Chem Lett*. 2023;14(44):9926-9934. doi:10.1021/acs.jpcclett.3c02524
148. Casalino L, Seitz C, Lederhofer J, et al. Breathing and Tilting: Mesoscale Simulations Illuminate Influenza Glycoprotein Vulnerabilities. *ACS Cent Sci*. 2022;8(12). doi:10.1021/acscentsci.2c00981
149. Pegg CL, Schulz BL, Neely BA, Albery GF, Carlson CJ. Glycosylation and the global virome. *Mol Ecol*. 2023;32(1). doi:10.1111/mec.16731
150. Hebert DN, Zhang JX, Chen W, Foellmer B, Helenius A. The number and location of glycans on influenza hemagglutinin determine folding and association with calnexin and calreticulin. *Journal of Cell Biology*. 1997;139(3). doi:10.1083/jcb.139.3.613
151. Helenius A, Trombetta ES, Hebert DN, Simons JF. Calnexin, calreticulin and the folding of glycoproteins. *Trends Cell Biol*. 1997;7(5). doi:10.1016/S0962-8924(97)01032-5
152. Schulze IT. Effects of glycosylation on the properties and functions of influenza virus hemagglutinin. In: *Journal of Infectious Diseases*. Vol 176. ; 1997. doi:10.1086/514170
153. Samji T. *Influenza A: Understanding the Viral Life Cycle*. 2009.

154. Du R, Cui Q, Chen Z, Zhao X, Lin X, Rong L. Revisiting influenza A virus life cycle from a perspective of genome balance. *Viol Sin.* 2023;38(1). doi:10.1016/j.virs.2022.10.005
155. Bakshi T, Pham D, Kaur R, Sun B. Hidden Relationships between N-Glycosylation and Disulfide Bonds in Individual Proteins. *Int J Mol Sci.* 2022;23(7). doi:10.3390/ijms23073742
156. Daniels R, Kurowski B, Johnson AE, Hebert DN. N-linked glycans direct the cotranslational folding pathway of Influenza hemagglutinin. *Mol Cell.* 2003;11(1). doi:10.1016/S1097-2765(02)00821-3
157. Liao HY, Hsu CH, Wang SC, et al. Differential receptor binding affinities of influenza hemagglutinins on glycan arrays. *J Am Chem Soc.* 2010;132(42). doi:10.1021/ja104657b
158. Wu NC, Wilson IA. A Perspective on the Structural and Functional Constraints for Immune Evasion: Insights from Influenza Virus. *J Mol Biol.* 2017;429(17). doi:10.1016/j.jmb.2017.06.015
159. Alymova I V., York IA, Air GM, et al. Glycosylation changes in the globular head of H3N2 influenza hemagglutinin modulate receptor binding without affecting virus virulence. *Sci Rep.* 2016;6. doi:10.1038/srep36216
160. Deshpande KL, Fried VA, Ando M, Webster RG. Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. *Proc Natl Acad Sci U S A.* 1987;84(1). doi:10.1073/pnas.84.1.36
161. Reading PC, Pickett DL, Tate MD, Whitney PG, Job ER, Brooks AG. Loss of a single N-linked glycan from the hemagglutinin of influenza virus is associated with resistance to collectins and increased virulence in mice. *Respir Res.* 2009;10. doi:10.1186/1465-9921-10-117
162. Löwe M, Kalacheva M, Boersma AJ, Kedrov A. The more the merrier: effects of macromolecular crowding on the structure and dynamics of biological membranes. *FEBS Journal.* 2020;287(23). doi:10.1111/febs.15429
163. Bao D, Xue R, Zhang M, et al. N-Linked Glycosylation Plays an Important Role in Budding of Neuraminidase Protein and Virulence of Influenza Viruses. *J Virol.* 2021;95(3). doi:10.1128/jvi.02042-20
164. Feng T, Zhang J, Chen Z, et al. Glycosylation of viral proteins: Implication in virus–host interaction and virulence. *Virulence.* 2022;13(1). doi:10.1080/21505594.2022.2060464

165. de Vries RP, Smit CH, de Bruin E, et al. Glycan-Dependent Immunogenicity of Recombinant Soluble Trimeric Hemagglutinin. *J Virol.* 2012;86(21). doi:10.1128/jvi.01084-12
166. Martina CE, Crowe JE, Meiler J. Glycan masking in vaccine design: Targets, immunogens and applications. *Front Immunol.* 2023;14. doi:10.3389/fimmu.2023.1126034
167. Li Y, Liu D, Wang Y, Su W, Liu G, Dong W. The Importance of Glycans of Viral and Host Proteins in Enveloped Virus Infection. *Front Immunol.* 2021;12. doi:10.3389/fimmu.2021.638573
168. Abbadi N, Mousa JJ. Broadly Protective Neuraminidase-Based Influenza Vaccines and Monoclonal Antibodies: Target Epitopes and Mechanisms of Action. *Viruses.* 2023;15(1). doi:10.3390/v15010200
169. Yang YL, Chang SH, Gong X, Wu J, Liu B. Expression, purification and characterization of low-glycosylation influenza neuraminidase in  $\alpha$ -1,6-mannosyltransferase defective *Pichia pastoris*. *Mol Biol Rep.* 2012;39(2). doi:10.1007/s11033-011-0809-z
170. Klein EY, Serohijos AWR, Choi JM, Shakhnovich EI, Pekosz A. Influenza a H1N1 pandemic strain evolution - Divergence and the potential for antigenic drift variants. *PLoS One.* 2014;9(4). doi:10.1371/journal.pone.0093632
171. Belser JA, Maines TR, Tumpey TM. Importance of 1918 virus reconstruction to current assessments of pandemic risk. *Virology.* 2018;524. doi:10.1016/j.virol.2018.08.009
172. Tscherne DM, García-Sastre A. Virulence determinants of pandemic influenza viruses. *Journal of Clinical Investigation.* 2011;121(1). doi:10.1172/JCI44947
173. Madhav N, Oppenheim B, Gallivan M, Mulembakani P, Rubin E, Wolfe N. Pandemics: Risks, Impacts, and Mitigation. In: *Disease Control Priorities, Third Edition (Volume 9): Improving Health and Reducing Poverty.* ; 2017. doi:10.1596/978-1-4648-0527-1\_ch17
174. Stray SJ, Pittman LB. Subtype- and antigenic site-specific differences in biophysical influences on evolution of influenza virus hemagglutinin. *Virol J.* 2012;9. doi:10.1186/1743-422X-9-91
175. She YM, Farnsworth A, Li X, Cyr TD. Topological N-glycosylation and site-specific N-glycan sulfation of influenza proteins in the highly expressed H1N1 candidate vaccines. *Sci Rep.* 2017;7(1). doi:10.1038/s41598-017-10714-2

176. Chen W, Zhong Y, Qin Y, Sun S, Li Z. The Evolutionary Pattern of Glycosylation Sites in Influenza Virus (H5N1) Hemagglutinin and Neuraminidase. *PLoS One*. 2012;7(11). doi:10.1371/journal.pone.0049224
177. Chen W, Sun S, Li Z. Two glycosylation sites in H5N1 influenza virus hemagglutinin that affect binding preference by computer-based analysis. *PLoS One*. 2012;7(6). doi:10.1371/journal.pone.0038794
178. Gao R, Gu M, Shi L, et al. N-linked glycosylation at site 158 of the HA protein of H5N6 highly pathogenic avian influenza virus is important for viral biological properties and host immune responses. *Vet Res*. 2021;52(1). doi:10.1186/s13567-020-00879-6
179. Hervé PL, Lorin V, Jouvion G, Da Costa B, Escriou N. Addition of N-glycosylation sites on the globular head of the H5 hemagglutinin induces the escape of highly pathogenic avian influenza A H5N1 viruses from vaccine-induced immunity. *Virology*. 2015;486. doi:10.1016/j.virol.2015.08.033
180. Keenliside J. Pandemic influenza A H1N1 in swine and other animals. *Curr Top Microbiol Immunol*. 2013;370. doi:10.1007/82-2012-301
181. Griffin EF, Tompkins SM. Fitness Determinants of Influenza A Viruses. *Viruses*. 2023;15(9). doi:10.3390/v15091959
182. He W, Zhang W, Yan H, et al. Distribution and evolution of H1N1 influenza A viruses with adamantanes-resistant mutations worldwide from 1918 to 2019. *J Med Virol*. 2021;93(6). doi:10.1002/jmv.26670
183. Owuor DC, de Laurent ZR, Nyawanda BO, et al. Genetic and potential antigenic evolution of influenza A(H1N1)pdm09 viruses circulating in Kenya during 2009–2018 influenza seasons. *Sci Rep*. 2023;13(1). doi:10.1038/s41598-023-49157-3
184. Killingley B, Nguyen-Van-Tam J. Routes of influenza transmission. *Influenza Other Respir Viruses*. 2013;7(SUPPL.2). doi:10.1111/irv.12080
185. Wanzeck K, Boyd KL, McCullers JA. Glycan shielding of the influenza virus hemagglutinin contributes to immunopathology in mice. *Am J Respir Crit Care Med*. 2011;183(6). doi:10.1164/rccm.201007-1184OC
186. Altman MO, Angel M, Košík I, et al. Human influenza a virus hemagglutinin glycan evolution follows a temporal pattern to a glycan limit. *mBio*. 2019;10(2). doi:10.1128/mBio.00204-19
187. Östbye H, Gao J, Martinez MR, Wang H, de Gier JW, Daniels R. N-Linked Glycan Sites on the Influenza A Virus Neuraminidase Head Domain Are

- Required for Efficient Viral Incorporation and Replication. *J Virol.* 2020;94(19). doi:10.1128/jvi.00874-20
188. Du R, Cui Q, Rong L. Flu Universal Vaccines: New Tricks on an Old Virus. *Virol Sin.* 2021;36(1). doi:10.1007/s12250-020-00283-6
189. Scheibner D, Salaheldin AH, Bagato O, et al. Phenotypic effects of mutations observed in the neuraminidase of human origin H5N1 influenza A viruses. *PLoS Pathog.* 2023;19(2). doi:10.1371/journal.ppat.1011135
190. Suga A, Nagae M, Yamaguchi Y. Analysis of protein landscapes around N-glycosylation sites from the PDB repository for understanding the structural basis of N-glycoprotein processing and maturation. *Glycobiology.* 2018;28(10). doi:10.1093/glycob/cwy059
191. Roberts PC, Garten W, Klenk HD. Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. *J Virol.* 1993;67(6). doi:10.1128/jvi.67.6.3048-3060.1993
192. Petrescu AJ, Milac AL, Petrescu SM, Dwek RA, Wormald MR. Statistical analysis of the protein environment of N-glycosylation sites: Implications for occupancy, structure, and folding. *Glycobiology.* 2004;14(2). doi:10.1093/glycob/cwh008
193. Davis SJ, Crispin M. Solutions to the glycosylation problem for low- and high-throughput structural glycoproteomics. In: *Functional and Structural Proteomics of Glycoproteins*. Vol 9789048193554. ; 2011. doi:10.1007/978-90-481-9355-4\_6
194. Cruz E, Cain J, Crossett B, Kayser V. Site-specific glycosylation profile of influenza A (H1N1) hemagglutinin through tandem mass spectrometry. *Hum Vaccin Immunother.* 2018;14(3). doi:10.1080/21645515.2017.1377871
195. Olshina MA, Sharon M. Mass spectrometry: A technique of many faces. *Q Rev Biophys.* 2016;49. doi:10.1017/S0033583516000160
196. Pan KT, Chen CC, Urlaub H, Khoo KH. Adapting Data-Independent Acquisition for Mass Spectrometry-Based Protein Site-Specific N-Glycosylation Analysis. *Anal Chem.* 2017;89(8). doi:10.1021/acs.analchem.6b04996
197. Hackett WE, Zaia J. Calculating glycoprotein similarities from mass spectrometric data. *Molecular and Cellular Proteomics.* 2021;20. doi:10.1074/MCP.R120.002223

198. Ruhaak LR, Xu G, Li Q, Goonatileke E, Lebrilla CB. Mass Spectrometry Approaches to Glycomic and Glycoproteomic Analyses. *Chem Rev.* 2018;118(17). doi:10.1021/acs.chemrev.7b00732
199. Zaia J. Mass spectrometry and glycomics. *OMICS.* 2010;14(4). doi:10.1089/omi.2009.0146
200. Wuhrer M. Glycomics using mass spectrometry. *Glycoconj J.* 2013;30(1). doi:10.1007/s10719-012-9376-3
201. Saito T, Yagi H. High-Performance Liquid Chromatography Mapping Method for the Structural Analysis of N-Glycans at the Molecular, Cellular, and Tissue Levels. In: *Methods in Molecular Biology.* Vol 2556. ; 2022. doi:10.1007/978-1-0716-2635-1\_2
202. Chang D, Zaia J. Methods to improve quantitative glycoprotein coverage from bottom-up LC-MS data. *Mass Spectrom Rev.* 2022;41(6). doi:10.1002/mas.21692
203. She YM, Li X, Cyr TD. Remarkable Structural Diversity of N-Glycan Sulfation on Influenza Vaccines. *Anal Chem.* 2019;91(8). doi:10.1021/acs.analchem.8b05372
204. Hargett AA, Renfrow MB. Glycosylation of viral surface proteins probed by mass spectrometry. *Curr Opin Virol.* 2019;36. doi:10.1016/j.coviro.2019.05.003
205. Chang D, Klein J, Hackett WE, Nalehua MR, Wan XF, Zaia J. Improving Statistical Certainty of Glycosylation Similarity between Influenza A Virus Variants Using Data-Independent Acquisition Mass Spectrometry. *Molecular and Cellular Proteomics.* 2022;21(10). doi:10.1016/j.mcpro.2022.100412
206. Han L, Costello CE. Mass spectrometry of glycans. *Biochemistry (Moscow).* 2013;78(7). doi:10.1134/S0006297913070031
207. Tsai YX, Chang NE, Reuter K, et al. Rapid simulation of glycoprotein structures by grafting and steric exclusion of glycan conformer libraries. *Cell.* 2024;187(5). doi:10.1016/j.cell.2024.01.034
208. Palmigiano A, Messina A, Sturiale L, Garozzo D. Advanced LC-MS Methods for N-Glycan Characterization. *Comprehensive Analytical Chemistry.* 2018;79. doi:10.1016/bs.coac.2017.06.009
209. An Y, Cipollo JF. An unbiased approach for analysis of protein glycosylation and application to influenza vaccine hemagglutinin. *Anal Biochem.* 2011;415(1). doi:10.1016/j.ab.2011.04.018

210. Angeletti S. Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) in clinical microbiology. *J Microbiol Methods*. 2017;138. doi:10.1016/j.mimet.2016.09.003
211. M. Hashem A, M. Doyle T, Van Domselaar G, et al. Recent Developments in Bioinformatics Analyses of Influenza A Virus Surface Glycoproteins and their Biological Relevance. *Curr Bioinform*. 2011;6(4). doi:10.2174/157489311798073007
212. Gulati S, Lasanajak Y, Smith DF, Cummings RD, Air GM. Glycan array analysis of influenza H1N1 binding and release. *Cancer Biomarkers*. 2014;14(1). doi:10.3233/CBM-130376
213. Wu CS, Lee TY, Chou RH, et al. Development of a highly sensitive glycan microarray for quantifying AFP-L3 for early prediction of hepatitis B virus-related hepatocellular carcinoma. *PLoS One*. 2014;9(6). doi:10.1371/journal.pone.0099959
214. Cohen M, Fisher CJ, Huang ML, et al. Capture and characterization of influenza A virus from primary samples using glycan bead arrays. *Virology*. 2016;493. doi:10.1016/j.virol.2016.03.011
215. Tiwold EK, Gyorgypal A, Chundawat SPS. Recent Advances in Biologic Therapeutic N-Glycan Preparation Techniques and Analytical Methods for Facilitating Biomanufacturing Automation. *J Pharm Sci*. 2023;112(6). doi:10.1016/j.xphs.2023.01.012
216. Pang KT, Tay SJ, Wan C, et al. Semi-Automated Glycoproteomic Data Analysis of LC-MS Data Using GlycopeptideGraphMS in Process Development of Monoclonal Antibody Biologics. *Front Chem*. 2021;9. doi:10.3389/fchem.2021.661406
217. Goodacre N, Aljanahi A, Nandakumar S, Mikailov M, Khan AS. A Reference Viral Database (RVDB) To Enhance Bioinformatics Analysis of High-Throughput Sequencing for Novel Virus Detection. *mSphere*. 2018;3(2). doi:10.1128/mspheredirect.00069-18
218. Gecht M, Bülow S von, Penet C, Hummer G, Hanus C, Sikora M. GlycoSHIELD: a versatile pipeline to assess glycan impact on protein structures. *bioRxiv*. 2022;(5).
219. Seitz C, Casalino L, Konecny R, Huber G, Amaro RE, McCammon JA. Multiscale Simulations Examining Glycan Shield Effects on Drug Binding to Influenza Neuraminidase. *Biophys J*. 2020;119(11). doi:10.1016/j.bpj.2020.10.024
220. Liu WC, Jan JT, Huang YJ, Chen TH, Wu SC. Unmasking Stem-Specific Neutralizing Epitopes by Abolishing N-Linked Glycosylation Sites of

- Influenza Virus Hemagglutinin Proteins for Vaccine Design. *J Virol.* 2016;90(19). doi:10.1128/jvi.00880-16
221. Hariharan V, Kane RS. Glycosylation as a tool for rational vaccine design. *Biotechnol Bioeng.* 2020;117(8). doi:10.1002/bit.27361
222. Apweiler R, Hermjakob H, Sharon N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta Gen Subj.* 1999;1473(1). doi:10.1016/S0304-4165(99)00165-8
223. Zhang M, Gaschen B, Blay W, et al. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. *Glycobiology.* 2004;14(12). doi:10.1093/glycob/cwh106
224. Lee HS, Qi Y, Im W. Effects of N-glycosylation on protein conformation and dynamics: Protein Data Bank analysis and molecular dynamics simulation study. *Sci Rep.* 2015;5. doi:10.1038/srep08926
225. Ritsch M, Cassman NA, Saghaei S, Marz M. Navigating the Landscape: A Comprehensive Review of Current Virus Databases. *Viruses.* 2023;15(9). doi:10.3390/v15091834
226. Thompson AJ, Cao L, Ma Y, et al. Human Influenza Virus Hemagglutinins Contain Conserved Oligomannose N-Linked Glycans Allowing Potent Neutralization by Lectins. *Cell Host Microbe.* 2020;27(5). doi:10.1016/j.chom.2020.03.009
227. Campbell MP, Abrahams JL, Rapp E, et al. The minimum information required for a glycomics experiment (MIRAGE) project: LC guidelines. *Glycobiology.* 2019;29(5). doi:10.1093/glycob/cwz009
228. Kim P, Jang YH, Kwon S Bin, Lee CM, Han G, Seong BL. Glycosylation of hemagglutinin and neuraminidase of influenza A virus as signature for ecological spillover and adaptation among influenza reservoirs. *Viruses.* 2018;10(4). doi:10.3390/v10040183
229. Gubareva L, Mohan T. Antivirals Targeting the Neuraminidase. *Cold Spring Harb Perspect Med.* 2022;12(1). doi:10.1101/cshperspect.a038455
230. Yasuhara A, Yamayoshi S, Kiso M, Sakai-Tagawa Y, Okuda M, Kawaoka Y. A broadly protective human monoclonal antibody targeting the sialidase activity of influenza A and B virus neuraminidases. *Nat Commun.* 2022;13(1). doi:10.1038/s41467-022-34521-0
231. Kosik I, Angeletti D, Gibbs JS, et al. Neuraminidase inhibition contributes to influenza A virus neutralization by anti-hemagglutinin

- stem antibodies. *Journal of Experimental Medicine*. 2019;216(2). doi:10.1084/jem.20181624
232. Liu JH, Chang CC, Chen CW, Wong LT, Chu YW. Conservation region finding for influenza A viruses by machine learning methods of N-linked glycosylation sites and B-cell epitopes. *Math Biosci*. 2019;315. doi:10.1016/j.mbs.2019.108217
233. Gorbalenya AE, Lauber C. Bioinformatics of virus taxonomy: foundations and tools for developing sequence-based hierarchical classification. *Curr Opin Virol*. 2022;52. doi:10.1016/j.coviro.2021.11.003
234. Li L, Chang D, Han L, Zhang X, Zaia J, Wan XF. Multi-task learning sparse group lasso: A method for quantifying antigenicity of influenza A(H1N1) virus using mutations and variations in glycosylation of Hemagglutinin. *BMC Bioinformatics*. 2020;21(1). doi:10.1186/s12859-020-3527-5
235. Marz M, Beerenwinkel N, Drosten C, et al. Challenges in RNA virus bioinformatics. *Bioinformatics*. 2014;30(13). doi:10.1093/bioinformatics/btu105
236. Chang D, Zaia J. Why glycosylation matters in building a better flu vaccine. *Molecular and Cellular Proteomics*. 2019;18(12). doi:10.1074/mcp.R119.001491
237. Esmail S, Manolson MF. Advances in understanding N-glycosylation structure, function, and regulation in health and disease. *Eur J Cell Biol*. 2021;100(7-8). doi:10.1016/j.ejcb.2021.151186
238. Zhang H, Wang L, Compans RW, Wang BZ. Universal influenza vaccines, a dream to be realized soon. *Viruses*. 2014;6(5). doi:10.3390/v6051974
239. Hufsky F, Beslic D, Boeckeaerts D, et al. The International Virus Bioinformatics Meeting 2022. *Viruses*. 2022;14(5). doi:10.3390/v14050973
240. Hufsky F, Beerenwinkel N, Meyer IM, et al. The international virus bioinformatics meeting 2020. *Viruses*. 2020;12(12). doi:10.3390/v12121398
241. Pappas N, Roux S, Hölzer M, et al. Virus Bioinformatics. In: Bamford DH, Zuckerman M, eds. *Encyclopedia of Virology (Fourth Edition)*. Fourth Edition. Academic Press; 2021:124-132. doi:https://doi.org/10.1016/B978-0-12-814515-9.00034-5

242. Meeting WHO. 042-Main operational lessons learnt from the WHO Pandemic Influenza A (H1N1) Vaccine Deployment Initiative Report of a WHO Meeting. *World Health Organization*. 2010;(December).