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HUMORAL IMMUNE RESPONSE AFTER SEASONAL INFLUENZA VACCINATION

Presentada por Laura Sánchez de Prada para optar al grado de Doctora internacional por la Universidad de Valladolid

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A vosotros

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“Science and everyday life cannot and should not be separated”

Rosalind Franklin

“Science is not only a disciple of reason but, also, one of romance and passion”

Stephen Hawking

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ABBREVIATION LIST

- IAV: influenza A virus
- IBV: influenza B virus
- ICV: influenza C virus
- IDV: influenza D virus
- ECDC: European Centre for Disease Prevention and Control
- WHO: World Health Organization
- HPAI: highly pathogenic avian influenza
- LPAI: low pathogenic avian influenza
- HA: haemagglutinin
- NA: neuraminidase
- RNPv: viral ribonucleoprotein
- NP: nucleoprotein
- PB1: basic polymerase 1
- PB2: basic polymerase 2
- PA: acid polymerase
- M: matrix
- NS1: non-structural protein
- IFN-I : type I interferon
- RBS: receptor binding site
- mRNA: messenger RNA
- GISRS: Global Influenza Surveillance and Response System
- NICs: national influenza centres
- WHOCCs: WHO collaborating centres
- ERLs: essential regulatory laboratories
- H5RefLabs: H5 reference laboratories
- ERLI-Net: European reference laboratory network for human influenza
- ScVGE: Sistema Centinela de Vigilancia de Gripe en España (Spanish Influenza Sentinel Surveillance System)
- CNE: centro nacional de epidemiología (national epidemiology centre)

- RCSCyL: Red Centinela Sanitaria de Castilla y Leon (Sanitary Sentinel Network of Castile and Leon)
- PVIG: programa de vigilancia de la gripe (influenza surveillance program)
- cHAs: chimeric haemagglutinins
- TLRs: toll-like receptors
- RLRs: RIG-I-like receptors and the
- NLRP3: NOD-like receptor and pyrin domain containing 3
- NK: natural killer
- TNF-α: tumoral necrosis factor alpha
- EMA: European medicines agency
- HAI/HI: haemagglutination inhibition assay
- SRH: single radial haemolysis test
- MN: microneutralization assay
- ELLA: enzyme-linked lectin assay
- IVC: influenza vaccine campaigns
- ID: immunodominance
- ELISA: enzyme-linked immunosorbent assay
- OD: optical density
- AUC: area under the curve
- GMT: geometric mean of antibody titres (GMT)
- SPR: seroprotection rate
- FI: fold-induction increase
- GMTi: geometric mean titre increase
- SCR: seroconversion rate
- FIR: fold induction rate
- DI: dominance index
- GMFR: geometric mean fold rise

ABSTRACT

Introduction: Seasonal vaccination provides short-lasting and strain specific protection. Different factors influence efficacy and effectiveness and are related to the vaccine and the receptor. Those include adjuvants and strains contained, and age and sex of the receptor. The major target of antibodies after vaccination and infection is the hemagglutinin (HA) head with a limited number of immunodominant classically defined antigenic sites (Sb, Sa, Cb, Ca1 and Ca2) leading to strain-specific protection. In contrast, the HA stalk is subdominant and more conserved, and anti-stalk antibodies are cross-reactive among strains of the same phylogenetic group.

Objective: To evaluate the humoral response after seasonal influenza vaccination considering vaccine composition, age and sex; and determine specific humoral responses against classically defined antigenic sites of the HA head and the stalk domain of influenza A virus.

Methods: A total of 4,818 patients were recruited yearly during 28 seasons (1990-2018) from the Sentinel Surveillance Network of Castile and Leon (Spain). Three retrospective and two prospective designs were used. Serological analyses were conducted in samples obtained before and 28 days after seasonal vaccination by the National Influenza Centre of Valladolid (Spain) and the Mount Sinai Hospital of New York (US). Antibody levels against the HA head and stalk were measured by hemagglutination inhibition assay (HAI) and ELISAs. This research was approved by an Ethics Committee(PI 21-2314). Statistical analysis was performed taking significance at $p<0.05$.

Results:

Manuscript 1: Higher humoral response was found in the elderly against the A(H3N2) subtype with the adjuvanted influenza vaccine (FI 3.4, SCR 46%) compared to non-adjuvanted vaccine (FI 2.7, SCR 38.8%). However, the non-adjuvanted vaccine induced a better response against A(H1N1)pdm09 (FI 4.5, SCR 57.3%) compared to the adjuvanted one (FI 3.2, SCR 45.8%) in the same group.

Manuscript2: Higher humoral response in terms of SCR against classical influenza A (H1N1), A(H1N1)pdm09 subtype and B/Victoria lineage (40.6%, 52.4% and 23.2%) were found in elderly women compared to elderly men (30.2%, 42.0% and 18.9%); and in FI (3.7 vs. 3.0) against A(H1N1)pdm09 in the same comparison.

Manuscript3: Significant heterotypic humoral responses were found between both influenza B lineages, but always lower than the homotypic response. Young adults showed higher homotypic (GMTi 3.2, SCR 41.6%) and heterotypic responses (GMTi 1.7, SCR 18.6%) with B/Victoria vaccine compared to the elderly while similar responses were found with B/Yamagata vaccine.

Manuscript4: Antibodies before vaccination were significantly reduced against all antigenic sites in the elderly and only against Sb and Ca2 in young adults compared to the Wt. Response to vaccination was reduced against all viruses compared to the Wt for the adjuvanted vaccine and only against Sb and Ca2 for the non-adjuvanted vaccine. The strongest reduction was observed against Sb followed by Ca2.

Manuscript5: All age groups elicited a significant increase of anti-stalk antibodies after seasonal influenza vaccination except for ≥ 80-year-old cohort. Additionally, < 65-year-old vaccinees had higher titers against phylogenetic group 1 HAs vs. group 2. Similarly, <50-year-old showed higher increase of anti-stalk antibody titers (GMFR 1.69) compared to ≥ 80-year-old (GMFR 1.08) for group 1.

Conclusions: Age and sex play a role in vaccine responses with higher responses in elderly women compared to men against A(H1N1)pdm09. Better responses against this subtype were found with non-adjuvanted vaccines, while adjuvanted vaccines responded better against the A(H3N2) subtype in the elderly. However, seasonal vaccination can boost the induction of cross-reactive anti-stalk antibodies against phylogenetic groups 1 and 2 of HAs, with higher responses in younger populations. The immunodominance hierarchy of antigenic sites of HA head of the A(H1)pdm09 viruses is dominated by Sb followed by Ca2, but age and adjuvants can broaden responses towards subdominant epitopes. Finally, vaccination with a trivalent influenza vaccine provides cross-reactive protection against the B/lineage not contained.

RESUMEN EN CASTELLANO

Introducción: La protección tras la vacunación antigripal es limitada y específica de cepa. Diferentes factores influyen en la eficacia y efectividad relacionados con la vacuna y con el receptor incluyendo: adyuvantes y composición; y edad y sexo.

La principal diana de los anticuerpos tras la vacunación es la cabeza de la hemaglutinina (HA), inmunodominante y con capacidad de unión a un número limitado de sitios antigénicos(Sb, Sa, Cb, Ca1 y Ca2). En cambio, los anticuerpos frente al tallo de la HA, mucho más conservado y subdominante, presentan reactividad cruzada entre diferentes cepas del mismo grupo filogenético.

Objetivo: Evaluar la respuesta humoral antigripal tras la vacunación considerando la composición de la vacuna, la edad y el sexo de los receptores; y, determinar las respuestas específicas frente a los sitios antigénicos clásicos de la cabeza y al tallo de la HA del virus de la gripe A.

Métodos: Se reclutaron 4.818 pacientes durante 28 temporadas de gripe(1990-2018) por la Red Centinela de Vigilancia de Castilla y León(España). Se utilizaron tres diseños retrospectivos y dos prospectivos. Los análisis serológicos fueron realizados por el Centro Nacional de Gripe de Valladolid(España) y el Hospital Mount Sinai de Nueva York(EE.UU.), en muestras obtenidas antes y 28 días post-vacunación. Se detectaron anticuerpos frente a la cabeza y frente al tallo de la HA mediante ensayos de inhibición de la hemaglutinación (RIH) y ELISAs. Esta investigación fue aprobada por un Comité ético(PI 21-2314). Se realizó el análisis estadístico considerando significante $p<0,05$.

Resultados

Manuscrito 1: Se observó mayor respuesta en ancianos frente a A(H3N2) con vacuna adyuvada (RIC 3,4; 46%) en comparación con vacuna no adyuvada (RIC 2,7; TSC 38,8%). Sin embargo, la no adyuvada indujo una mejor respuesta frente a A(H1N1)pdm09 (RIC 4,5;TSC 57,3%) que la adyuvada (RIC 3,2;TSC 45,8%) en el mismo grupo etario.

Manuscrito 2: Se encontró mayor respuesta frente a A(H1N1) clásico, A(H1N1)pdm09 y el linaje B/Victoria (TSC: 40,6%, 52,4% y 23,2%) en mujeres ancianas comparadas con hombres ancianos (TSC: 30,2%, 42,0% y 18,9%).También frente a A(H1N1)pdm09 en la misma comparación (RIC:3,7 vs. 3,0).

Manuscrito 3: Se encontraron respuestas humorales heterotípicas frente a ambos linajes de gripe B, pero siempre inferiores a la homóloga. Los adultos mostraron respuestas homólogas (RIC:3,2;TSC:41,6%) y heterotípicas (RIC:1,7;TSC:18,6%) mayores con la vacuna de B/Victoria que los ancianos y similares con la vacuna B/Yamagata.

Manuscrito 4: Los anticuerpos pre-vacunales se redujeron frente a todos los epítopos en ancianos y frente a Sb y Ca2 en adultos vs. Wt. La respuesta a la vacunación adyuvada se redujo frente a todos virus vs. Wt y sólo frente a Sb y Ca2 con vacuna no adyuvada. La mayor reducción se observó frente a Sb>Ca2.

Manuscrito 5: Todos los grupos etarios aumentaron los anticuerpos frente al tallo tras la vacunación, excepto ≥ 80 años. Además, los < 65 años presentaron títulos mayores frente al grupo filogenético 1 que al 2. Igualmente, los < 50 años mostraron un aumento mayor de anticuerpos frente al tallo del grupo 1 (RIC:1,69) en comparación con los ≥ 80 años (RIC:1,08).

Conclusiones: La edad y el sexo juegan un papel clave en las respuestas vacunales, siendo mayores en mujeres > 65años que en hombres de la misma edad frente a A(H1N1)pdm09. Se observaron mejores respuestas a este subtipo con vacunas no adyuvadas, y frente a A(H3N2) con vacunas adyuvadas en ancianos . Sin embargo, la vacuna estacional puede inducir anticuerpos anti-tallo de los grupos filogenéticos 1 y ,2 con mayores respuestas en jóvenes. La inmunodominancia de los epítopos de la cabeza de la HA de los virus A(H1)pdm09 está dominada por Sb>Ca2; pudiendo ampliarse hacia epítopos subdominantes por la edad y los adyuvantes. Por último, la vacunación con una vacuna trivalente proporciona protección cruzada contra el linaje B no contenido en ella.

1. INTRODUCTION

1. INFLUENZA VIRUSES

1.1. Discovery and classification

Influenza viruses were discovered as we know them over the 20th century. The viral nature of influenza virus was initially established in 1931 in swine by Shope [1]. However, it was in 1933 when influenza A virus was isolated for the first time in humans by Smith et al. [2]. Subsequently, influenza B virus was isolated by Francis and influenza C virus by Taylor in 1939 and 1951, respectively [3,4]. Finally, in 2011 a virus initially considered as C-like but later classified as type D, was isolated in cattle and swine [5].

Influenza viruses belong to Orthomyxoviridae family. The "myxovirus" designation its related to its affinity to mucin, a mucoprotein which is present in the mucus of secretions, in some epithelial receptors, in the membrane of erythrocytes and in the serum. That affinity is responsible for their ability to agglutinate erythrocytes [6,7]. Currently, 9 different genera conform the Orthomyxoviridae family. Genera *Alphainfluenzavirus, Betainfluenzavirus, Gammainfluenzavirus* and *Deltainfluenzavirus*. correspond to classical influenza A (IAV), influenza B (IBV), influenza C (ICV) and influenza D (IDV) viruses, respectively. While genus *Isavirus, Mykissvirus, Quaranjavirus, Sadinavirus* and *Thogotovirus*, are conformed by viruses associated with fish and arthropods (Table 1) [5,8].

Table 1. *Orthomyxoviridae* family classification *(Adapted from:* International Committee on Taxonomy of Viruses)[8]

FAMILY	GENERA	SPECIES
Orthomyxoviridae	*Alphainfluenzavirus*	*Alphainfluenzavirus influenzae- influenza A virus*
	Betainfluenzavirus	*Betainfluenzavirus influenzae- influenza B virus*
	Deltainfluenzavirus	*Deltainfluenzavirus influenzae- influenza C virus*
	Gammainfluenzavirus	*Gammainfluenzavirus influenzae- influenza D virus*
	Isavirus	*Isavirus salaris*
	Mykissvirus	*Mykissvirus tructae*
	Quaranjavirus	*Quaranjavirus araguariense*
		Quaranjavirus chadense
		Quaranjavirus johnstonense
		Quaranjavirus quaranfilense
		Quaranjavirus tyulekense
		Quaranjavirus wellfleetense
	Sardinovirus	*Sardinovirus pilchardi*

Thogotovirus	*Thogotovirus bourbonense*
	Thogotovirus dhoriense
	Thogotovirus josense
	Thogotovirus ozense
	Thogotovirus sinuense
	Thogotovirus thailandense
	Thogotovirus thogotoense
	Thogotovirus upoluense

Influenza viruses infect a wide host range of animals including humans, with special importance of avian and swine (Figure 1). IAV is a significant public health concern as they affect the greatest number of hosts with often breaches of the species barrier. Aquatic birds pose the greatest threat as they travel vast distances and migrate from different areas of the globe to others. IBV and ICV infect almost exclusively humans with sporadic cases in seals, horses, dogs and pigs the first ones; and dromedaries camels, dogs and pigs the second ones. Finally, IDV includes cattle as its natural reservoir and amplification host with periodic spillover to other mammalian species, including swine. In addition, there is increasing evidence that it might have the potential to infect humans [9,10].

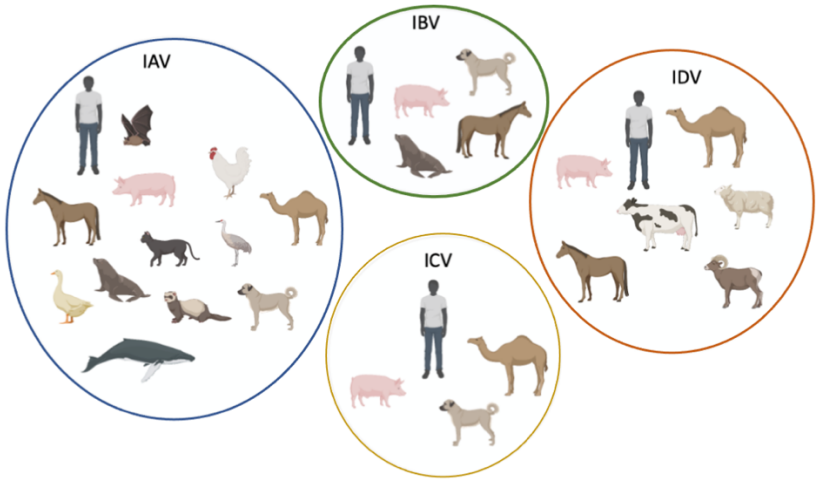


Figure 1. Summary of the breadth of hosts of influenza viruses. Created with Biorender by Laura Sánchez-de Prada.

Standard international nomenclature to designate influenza viruses indicates how and where were they identified and follows the next order:

- Type of influenza virus (A, B, C or D)
- Host of origin (not indicated in the case of human origin)
- Geographical place of isolation or detection
- Identification number of the strain of the laboratory where isolated
- Year of isolation

In the case of IAV the antigenic subtype in parentheses follows the strain designation. It includes the haemagglutinin and neuraminidase present in the virion. For example, human strain A/Puerto Rico/8/1934 (H1N1) would be a type A virus, isolated in Puerto Rico in 1934 and identified as number 8 strain in the laboratory. An example of a non-human strain would be A/chicken/Hong Kong/156/97 (H5N1). On the other hand, for IBV, as antigenic subtypes cannot be determined, nomenclature only describes the strain as indicated above. For example, B/Shanghai/361/2002 [11,12].

1.2. Epidemiology and history

The explosive nature of influenza epidemics has made these events to be recorded with increasing accuracy since 1170 [13–16]. Pandemics however, initially restricted to continents, it is not until the development of shipping and transports, that became a global phenomenon [17].

For an influenza pandemic to take place, certain conditions must be met. According to the European Centre for Disease Prevention and Control (ECDC) an influenza pandemic involves the rapid distribution of a new human influenza virus around the globe. This occurs when a new strain with the ability to infect humans arises, against which most of the population lacks protective immunity and efficient human transmission is present. A pandemic influenza is therefore much more severe in terms of morbidity and mortality compared to annual seasonal epidemics [18].

The internationally accepted definition of pandemic as it is described in the epidemiology dictionary is much more straightforward and familiar: “an epidemic occurring worldwide, or over a very wide area, crossing international boundaries and usually affecting a large number of people” [19]. The World Health Organisation (WHO), however, developed more technical requirements for the definition of pandemic

following the emergence of the new subtype A(H1N1)pmd09 responsible for the 2009 pandemic. That is the emergence of a new influenza A virus genetically different from circulating influenza A viruses and that meets three characteristics, regardless of the severity of the disease: ability to infect humans, cause of illness in humans, and easy transmission between them. In addition, a timeline of phases were defined that are required to meet before declaring a pandemic (Table 2) [6].

Table 2. WHO pandemic influenza phases. Adapted from Doshi P.[20].

PHASES	DESCRIPTION
PHASE 1	No animal influenza virus circulating among animals has been reported to cause infection in humans.
PHASE 2	An animal influenza virus circulating in domesticated or wild animals is known to have caused infection in humans and is therefore considered a specific potential pandemic threat.
PHASE 3	An animal or human-animal influenza reassortant virus has caused sporadic cases or small clusters of disease in people but has not resulted in human-to-human transmission sufficient to sustain community-level outbreaks.
PHASE 4	Human-to-human transmission of an animal or human-animal influenza reassortant virus able to sustain community-level outbreaks has been verified.
PANDEMIC	
PHASE 5	The same identified virus has caused sustained community level outbreaks in two or more countries in one WHO region.
PHASE 6	In addition to the criteria defined in Phase 5, the same virus has caused sustained community level outbreaks in at least one other country in another WHO region.
Post-peak	Levels of pandemic influenza in most countries with adequate surveillance have dropped below peak levels.
Possible new wave	Level of pandemic influenza activity in most countries with adequate surveillance rising again.
Post-pandemic	Levels of influenza activity have returned to the levels seen for seasonal influenza in most countries with adequate surveillance.

According to this classification a pandemic would be considered from phase 5 onwards, where the emergent influenza virus would be effectively transmitting between humans in at least two countries in the same WHO region. In addition, if it were phase 6, active human-to-human transmission would have to have been demonstrated in at least one other country in a different WHO region. In the past 120 years, there have been 6 outbreaks that have reached pandemic magnitude caused by influenza viruses (Figure 2) [21]. However, despite a new influenza pandemic being expected, in December 2019, a new coronavirus, SARS-CoV2, emerged in Wuhan (China) with a spectrum of manifestations ranging from asymptomatic cases to severe respiratory illness and death [22]. It spread worldwide and a pandemic situation was declared on March 11th 2020 [23]; and, as of May 3rd 2023, three years after its emergence, more than 765 million cases and almost 7 million people deaths have been reported [24] and the pandemic was declared to be over a day later [25].

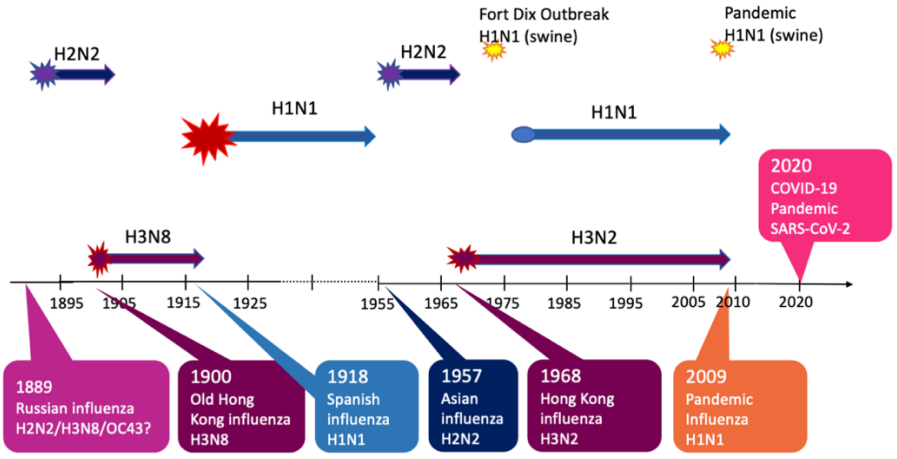


Figure 2. History of recorded influenza pandemics. Adapted from [26]

1.3. Last pandemics of the XIX, XX and XXI centuries

- The 1889 Russian pandemic

The last pandemic of the 19th century, commonly known as "the Russian flu", was the first pandemic to occur in a world that was already highly interconnected. At that time, the 19 largest European countries had more railroads than even today, and

transatlantic travel took less than 6 days [27]. The rapid progression demonstrates that land transport, even if slower and in much smaller flows, was sufficient to spread the virus throughout Europe and the United States in about 4 months and in 6 months got back to Asia, where it emerged [28]. In fact, it got from St. Petersburg to the United States in only 70 days [27,29].

Attack rates would have been around 60% in individuals aged 1-60 years and lower in the elderly. However, influenza-associated mortality was J-shaped with high rates in children over 20 years of age (Figure 3) [29]. This means that the mortality curve is low to moderate in children, very low in adults, and very high in elderly.

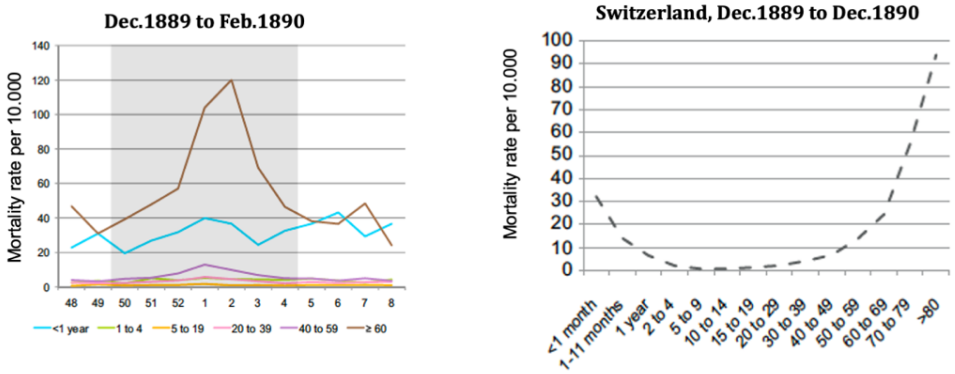


Figure 3. Age-specific mortality rate per week and mortality rate per age with the typical "J" shape during the 1889 pandemic in Switzerland. Taken from: Valtat S et al. Vaccine. 2020;29 (2011):B6–10 [29]

The 1889 pandemic began in the late spring of 1889, peaking in northern Europe and the United States in late 1889 and early 1890 [30]. The term "wave" came into common usage in the aftermath of this pandemic. It was initially employed to designate the four major post-pandemic seasonal influenza mortality peaks that would have befallen many cities between 1890 and 1894 [31].

It is interesting that, although initially this was considered an influenza pandemic probably caused by an A(H3N8)or A(H2N2) virus, recent doubts have raised, and some more recent authors considered it may have been caused by human coronavirus OC43

[32–34]. However, some authors have specified using stored serum samples that the mortality observed during the Spanish flu in young people notes that the most probable explanation was that this pandemic was caused by A(H3N8) subtype [35].

- The 1918 “Spanish flu”

The first information on the unfairly nicknamed “Spanish flu” was published by Spanish media, which did not take part in the First World War (1914-1918) and lacked censorship. Other countries preferred not to inform an already war-weary population so as not to undermine the morale of their troops and nations. The name also comes from the fact that it did not even spare the Spanish royal family. This pandemic is considered as the most devastating in history. It is estimated that one third of the world's population was infected and more than 2.5% died [36]. In absolute numbers, almost 500 million people were infected worldwide and around 50-100 million died [37].

The first medical reports of “severe” flu cases appeared in March 1918 in military camps in Kansas. From there, the virus spread across the United States and into Europe via shiploads of military troops arriving at battlefields in France. Unusual influenza activity had previously been reported in the United States and several European countries prior to the first wave in the spring of 1918. Therefore, it is thought possible that the predecessor of this virus was previously circulating among humans and became more virulent and/or transmissible over time [38].

The first pandemic wave started in the spring of 1918, followed by successively more fatal second and third waves in the autumn and winter of 1918 and 1919 respectively. The first two waves emerged at a time of year that is not usually favourable for the spread of the virus. The second wave caused simultaneous outbreaks in both hemispheres from September to November, in fact, the time periods between waves were so brief as to be virtually undetectable in some places (Figure 4) [30].

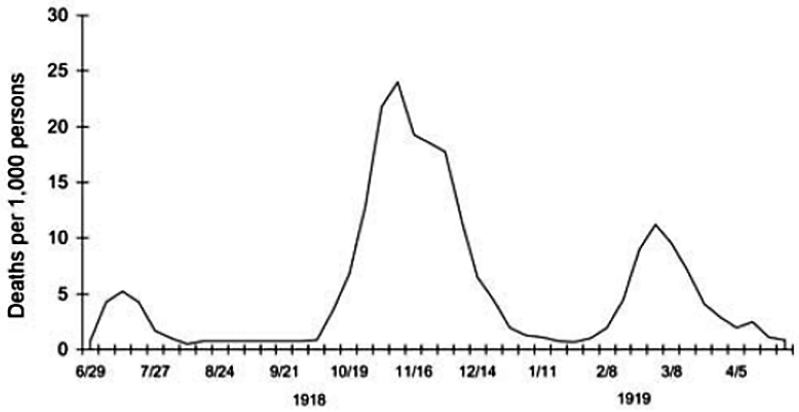


Figure 4. Three pandemic waves in London in 1918-1919: weekly combined influenza and pneumonia mortality. Taken from: Taubenberger JK. y Morens DM. Em.Inf.Dis. Vol. 12, No. 1, Jan 2006 [30].

Although attack rates do not differ from those of other pandemics, and ranged between 20-60%, this one is particularly characterized by morbidity and mortality in young adults [17,39]. The demographic pattern of mortality caused by this pandemic is far from the usual J-shaped patterns, with mortality peaks at the extremes of life, and shows a prominent mortality peak in young people between 20-40 years of age [40]. This W-shaped pattern has not been observed in any other pandemic. Influenza and pneumonia mortality rates in that age group were more than 20 times the normal rates compared to previous years, and an exorbitant number of cases but a much lower mortality rate was observed among children. Another peculiarity of this pandemic is that the absolute risk of death from influenza was highest in those under 65 years of age and accounted for more than 99% of the excess mortality from influenza-related deaths [30]. The causes of this peak appear to be due to an unexplained high incidence of secondary bacterial pneumonia and empyema (Figure 5) [40].

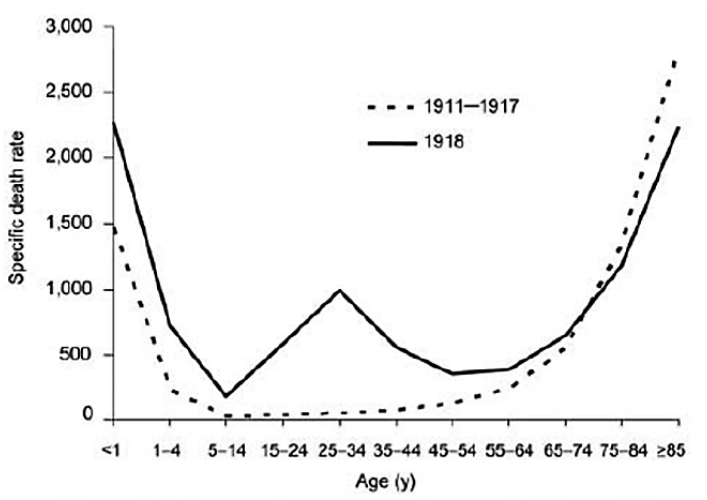


Figure 5. "U-" and "W-" shaped combined influenza and pneumonia mortality by age/100,000 persons in each age group in the United States, 1911-1918. Taken from: Taubenberger JK. y Morens DM. Em.Inf.Dis. Vol. 12, No. 1, Jan 2006 [30].

During the pandemic, the clinical spectrum of the disease varied widely. In the first spring wave, despite the large numbers of young adults affected by the outbreak, the disease had a mild presentation and mortality rates were not particularly high. The second wave began in August and, by the end of 1918, had affected pretty much all countries with unprecedented virulence. Patients typically presented with high fever, cyanosis, and pulmonary oedema. In 5% of fatal cases, death occurred within three days of symptom onset, although in most cases it took 7-10 days from the onset of clinical symptoms to death [38]. It is believed that the "famous" cytokine storm that affected patients at the beginning of the COVID-19 pandemic, had occurred before in those influenza cases [41–47].

In 1918, influenza was assumed to be a bacterial infection and, in fact, *Haemophilus influenzae* was described as the cause of the disease before the influenza virus was isolated and identified. Actually, they were not as far off track as in the second wave, death occurred not as a direct consequence of influenza virus but from bacterial pneumonia. The changes produced by the virus at the level of the respiratory tract and the consequent dysfunction of the immune system, were responsible for the susceptibility to secondary bacterial superinfection [48]. Both the alteration of the immune system in terms of reduced capacity for bacterial elimination and the

amplification of the inflammatory cascade had a severe impact on the course of the disease [49]. The most frequently isolated bacteria in addition to *Haemophilus influenzae* were *Streptococcus pneumoniae, Streptococcus pyogenes* and *Staphylococcus aureus*, all of which are associated with an increased risk of influenza mortality [48].

The reasons for such high mortality rates are not yet fully elucidated, however, recent genetic analysis of the reconstructed 1918 strain suggests that the virus itself was more virulent and had a propensity to cause viral pneumonia and trigger a pattern consistent with a "cytokine storm" [50–52].

The virus responsible for the 1918 pandemic was an A(H1N1) strain. Genetic studies had shown it resulted from a recombination between a new avian influenza strain with human and swine influenza strains that had been circulating in previous years. Another unique feature of the 1918 virus was its ability to infect humans and pigs simultaneously [52].

- The 1957 Asian flu

The 1957 Asian influenza emerged in Guizhou, China, in February of that year and spread rapidly through the country. The epidemic reached Hong Kong in April and the causative agent was isolated in Japan in May of the same year, reaching pandemic proportions in November of that year [39]. This pandemic had a first peak incidence in October 1957, followed by a second wave in January 1958, both with excess mortality.

Compared to the 1918 influenza virus, the overall mortality caused by this pandemic can be considered moderate, having caused at least one million deaths. The highest mortality rates were observed at the extremes of life, and the group of children aged 5-19 years was among the most affected with attack rates of 50% [53–55]. Most deaths were caused by bacterial pneumonia secondary to viral pneumonia, mainly caused by *S. pneumoniae, H. influenzae* and *S. aureus* [49].

The virus responsible for this pandemic was an A(H2N2) virus which, and according to phylogenetic studies, was a direct descendant of the 1918 A(H1N1)

pandemic virus that arose by reassortment with a virus of avian A(H2N2) origin. These reassortments are estimated to have taken place in the years leading up to the pandemic in which the original 1918 virus acquired the 3 avian H2, N2 and PB1 segments and retained the other 5 gene segments of the original 1918 A(H1N1) strain [21,56,57]. This virus circulated for only 11 years after its arrival and was supplanted by the H3 subtype.

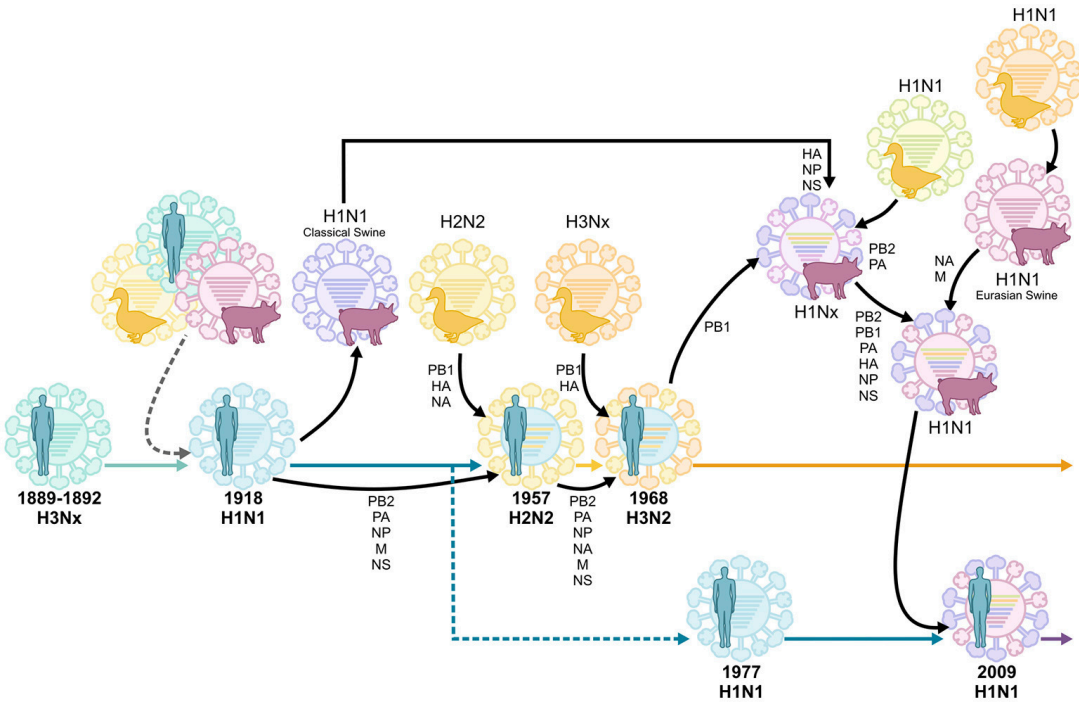


Figure 6. Diagram of the possible evolution of influenza viruses causing pandemics in the 20th century. Taken from: Short et al. Front. Cell. Infect. Microbiol. 8:343 [38].

- The 1968 Hong kong flu

The Hong Kong flu pandemic or also called "Mao flu" was responsible for between 1-4 million global deaths [58]. It first emerged in Hong Kong in July 1968 and appears to have spread more slowly compared to previous pandemics, reaching the United States in December 1968/January 1969 and Europe a year later [39]. Although it is considered to have been milder than the 1957 Asian flu, as well as the pandemic with the lowest excess mortality; the virus caused a broader spectrum of disease. Mortality patterns differed greatly from place to place, being very high in some countries and in others no different from mortality rates caused by seasonal influenza. Still, what is

characteristic of this pandemic is that the highest mortality rate was recorded in children. Both viruses shared the Neuraminidase N2 which may have moderated the severity of the older population due to some cross-immunity [59,60].

The virus that caused this pandemic was an A(H3N2), which arose from recombination of previously circulating human A(H2N2) that acquired the H3 and PB1 gene segments from another avian influenza virus [61]. Phylogenetic analyses suggest that these genetic recombinations between human and avian genes took place between 1966 and 1968 in some intermediate host that could have been a pig or a human [62].

Since 1968 the A(H3N2) virus has co-circulated in humans in the form of seasonal influenza and despite its mildness compared to other pandemics, the burden of disease and mortality caused by its antigenic drift is very high [21,61].

- The 1977 Russian flu

The pandemic caused in 1977 by the reintroduction of an A(H1N1) virus is known as Russian flu or "Red flu", because the first detected outbreak occurred in November 1977 in the Soviet Union. Although this strain of the virus was later acknowledged to have been detected for the first time in China in May of the same year [59,61]. This pandemic was considered as such in a technical sense, as it appeared unexpectedly and spread rapidly around the world causing moderate infections with typical flu-like symptoms, which were almost exclusively restricted to children under 25 years of age with mortality rates reaching 50% [55].

This A(H1N1) virus attracted particular attention when it was antigenically and molecularly characterized because of its similarity to those isolated in the 1950s in all 8 gene segments and which had disappeared by itself in 1957 and after which A(H2N2) and since 1968 A(H3N2) had circulated. Furthermore, this explained that most of the population over 20 years of age had antibodies against viruses of this type [63]. This led to all sorts of speculation about its origin, as it was virtually impossible for a virus to have remained intact and unaffected by antigenic drift in any host for 20 years; or for such drift to have resulted in such a similar virus. Subsequently, the recombinant nature

of the strain was discovered when it was shown that the strain of this virus, called A/USSR/90/77, had 7 gene segments very similar to those of the A/FW/1/50 virus strain and one segment (M) also very similar to A/FM/1/47 [64].

To date, the precise origin of this strain remains unknown, but the most widely accepted possibilities are an accidental laboratory escape, or a virus derived from an A(H1N1) vaccine trial [61,65]. From 1977 onwards this virus circulated seasonally along with A(H3N2) for more than 30 years until the arrival of the 2009 pandemic [38].

- The 2009 pandemic influenza

The 2009 influenza pandemic was the first to occur in the era of genomics with more than 2,000 publicly available sequences from the 2009-2010 infections alone. That provided an unprecedented opportunity for the scientific community to study the pandemic in real time and understand the mechanisms by which it happened [66].

This pandemic, also known as "swine flu", was caused by a new strain of A(H1N1) influenza, dubbed A(H1N1)pdm09, which radically changed the circulation dynamics of the pre-existing seasonal strains by replacing the classical A(H1N1) strain. The virus was first detected in Mexico in April 2009 and spread rapidly to other countries reaching pandemic alert phase 6 declared by the WHO on 11 June. It struck in two waves, the first peaking in spring and spreading throughout the summer, leading to a second, larger wave that peaked in early autumn [67].

It is estimated that there were between 43 and 88 million cases. Some estimates put the number of deaths associated with the pandemic virus at 200,000 of respiratory origin and 83,300 of cardiovascular origin in the first 12 months of the pandemic alone, which the WHO declared to be over by 10 August 2010. Eighty per cent of these deaths were in the population under 65 years of age [68,69].

This pandemic, while not causing a much higher net mortality than seasonal influenza, had a high mortality among children, young adults, and pregnant women, as well as in patients with respiratory pathologies or obesity. Those comorbidities

appeared to be associated with the development of severe viral pneumonia often associated with fulminant acute respiratory distress syndrome [70,71].

Phylogenetic studies place the origin of the virus in a four-way recombination of gene segments derived from American avian (PB2 and PA), A(H1N1) swine classical (HA, NP and NS) and Eurasian (NA and M) viruses and human A(H3N2) lineage (PB1) (Figure 7). In addition, studies have shown that this virus, although undetected, was circulating in humans for 3 months prior to its first identification and may have been circulating in mammals for years before [39,67,72].

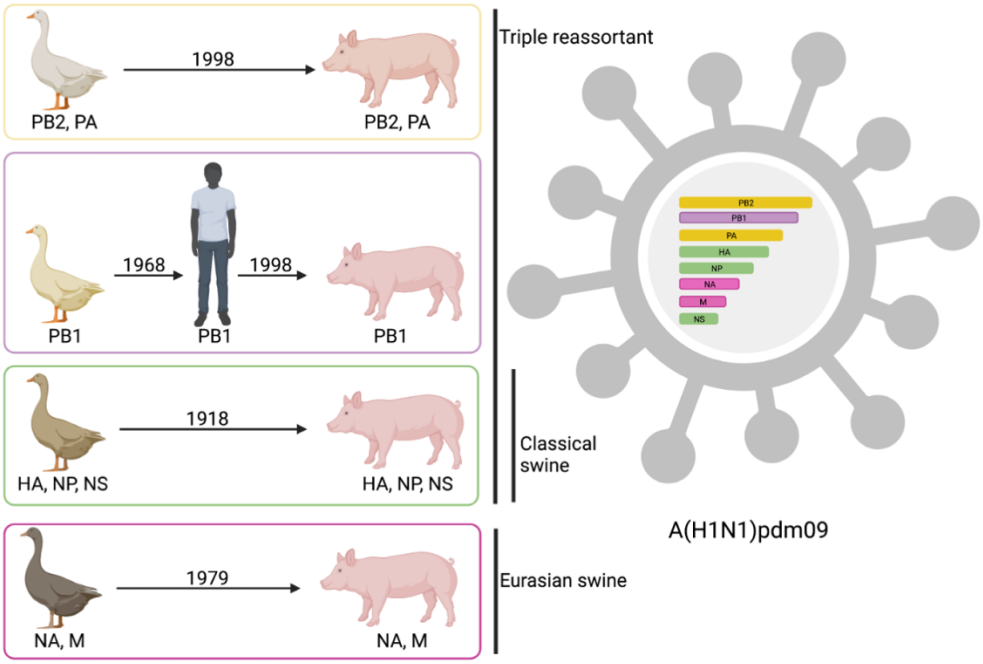


Figure 7. Genetic origin and provenance of virus emerging from a quadruple recombination in 2009. Created with Biorender by Laura Sánchez-de Prada.

- Other influenza viruses with pandemic potential

Avian influenza A viruses rarely infect people. Humans can become infected through contact with infected animals or environments, although these viruses have not yet acquired the capacity for sustained human-to-human transmission.

The main reservoir of these viruses is wild waterfowl and therefore difficult to eradicate. Infections caused are generally mild in birds, but the range of

symptomatology is variable. Viruses with the ability to cause disease resulting in high mortality rates in infected poultry are referred to as highly pathogenic avian influenza (HPAI) viruses while those causing no signs or mild disease in infected poultry (such as ruffled feathers and a drop in egg production) are referred to as low pathogenic avian influenza (LPAI) viruses. Only some avian influenza A(H5) and A(H7) viruses are classified as HPAI A viruses, while most A(H5) and A(H7) viruses circulating among birds are LPAI A viruses.

Five subtypes of avian influenza A viruses are known to have caused human infections (H5, H6, H7, H9, and H10 viruses). The most frequently identified subtypes of avian influenza A viruses that have caused human infections are H5, H7 and H9 viruses. Specifically, A(H5N1) and A(H7N9) viruses have caused the majority of avian influenza A virus infections reported in people, with HPAI A(H5N6) and LPAI A(H9N2) viruses also causing human infections in recent years. Human infections with other subtypes, such as A(H6N1), A(H10N3), A(H10N7), and A(H10N8), have been detected in small numbers of people A(H5N1) and A(H7N9) are considered HPAI, both with case fatality rates higher than seasonal influenza [73,74].

- A(H5) viruses

HPAI A(H5N1) virus was first detected in 1996 in geese in China and in 1997 in humans in an avian outbreak in Hong Kong and since then, it has been routinely circulating in farmed and wild birds in more than 50 countries. Infections have been reported in more than 880 people with approximately 50% case fatality proportion with Egypt, Indonesia and Vietnam grouping most cases. Recently, from 2021 ten cases have been reported in different countries, including 2 cases in Spain in autumn 2022 [75] .Of the few avian influenza viruses that has crossed the species barrier to infect humans, Asian HPAI A(H5N1) has caused the highest number of cases and deaths, as well as the highest severity of disease [76,77].

Other pathogenic A(H5) viruses detected have been HPAI A(H5N6) and HPAI A(H5N8). A(H5N6) infections have been reported in more than 60 people since 2014

and one case was reported in Laos in 2021. And A(H5N8) virus infections were reported in a small number of asymptomatic people in Russia in 2020 [77].

Infection in human hosts manifests after an incubation period of about 2-7 days, with typical uncomplicated flu-like symptoms and progresses to the lower respiratory tract. Uncommon occurrences such as fever and diarrhea precede pneumonia. In addition, severe pneumonia, acute respiratory distress syndrome, multi-organ failure, encephalitis and septic shock have been reported. A characteristic symptom of avian influenza and rare in seasonal influenza is conjunctivitis. It has a case fatality rate of 60% [78,79].

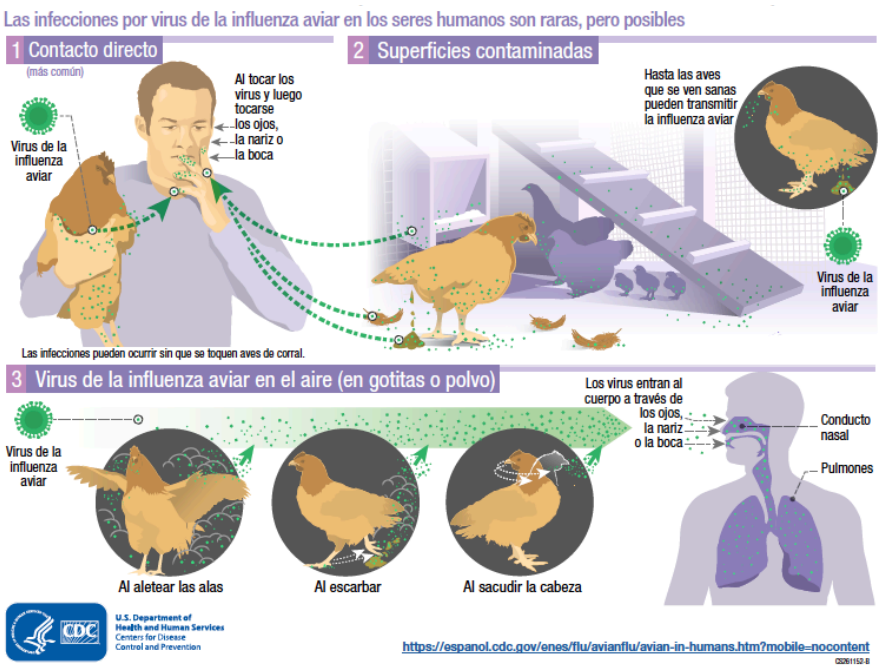


Figure 8. How infected poultry can transmit avian influenza to humans. Taken from: https://www.cdc.gov/flu/avianflu/avian-in-humans.htm [80].

- A(H7) viruses

The most frequently identified A(H7) viruses associated with human infections are avian influenza A(H7N9) viruses, which were first detected in China in February 2013 and have a higher transmissibility and incidence than A(H5N1) viruses. It has been detected in more than 1500 people in China, particularly during epidemics from 2013-

2017. While human infections with HPAI A(H7N9) viruses are uncommon, they have resulted in severe respiratory illness and death in approximately 40% of reported cases.

In addition to HPAI (H7N9) viruses, human infections with A(H7N2), A(H7N3), A(H7N4), and A(H7N7) viruses have been reported and have primarily caused mild to moderate illness with symptoms that included conjunctivitis and/or upper respiratory tract symptoms [74,78]. A(H7N3) and A(H7N7) are considered as HPAI, although A(H7N3) virus infections have been reported in a small number of people with conjunctivitis since 2004 in Canada and in other countries. On the other hand, HPAI A(H7N7) virus infections have been reported in more than 90 people since the first human infection detected in the U.S. in 1959. And even though, most infections have been associated with conjunctivitis; with A(H7N7) severe cases have been reported, including one death, most of them associated with exposures during widespread poultry outbreaks in the Netherlands in 2003 [77].

H7 viruses cause infections after an incubation period of 1-10 days, longer than that of seasonal influenza. As mentioned before, conjunctivitis is also characteristic of this type of virus. The usual symptoms start as a normal flu-like illness with cough and progress to lower respiratory tract involvement including symptoms such as dyspnea. Other typical symptoms are gastrointestinal with diarrhea, vomiting or abdominal pain. Complications can occur in cases of pneumonia, hypoxemia, multi-organ failure, septic shock. [73]

1.4. Influenza virus structure

- Virion structure

All influenza viruses are enveloped negative-sense single-stranded RNA viruses with a segmented genome located inside the viral particle. IAV and IBV virions are pleomorphic and range in size from 100-200 nm. They are usually spherical in shape, although filiform forms have been observed up to 1000nm. The lipid envelope comes from the host they infect and is enriched with lipids and cholesterol. It behaves as a fluid mosaic and is relatively unstable and can be easily inactivated by heat, desiccation, extreme pH and detergents [81]. The lipid envelope contains 3 transmembrane proteins

that sit as projections on the outside, about 500/virion. Those are the haemagglutinin (HA), Neuraminidase (NA), and membrane protein M2 (BM2 for IBV) which acts as an ion channel. The HA and NA are tightly anchored to membrane lipids in a ratio of approximately 4:1, with HA accounting for 80% of envelope proteins. The HA is projected as cylindrical spicules constituting a trimer, and the NA, the second most abundant protein accounting for 17% of the membrane proteins, forms mushroom-shaped spicules composed of tetramers. The matrix protein M1, which confers stability to the viral particle and plays an important role in the binding of the viral ribonucleoprotein (RNPv), is located underneath the envelope [82].

In the case of IAV and IBV, the core of the virions is formed by 8 RNPv complexes, which would be 7 in the case of ICV and IDV. Each complex consists of a segment of viral RNA that forms a pseudo-circular helical hairpin coated by nucleoprotein (NP) and associated with the viral RNA polymerase RNA-dependent RNA complex. This complex is formed by the interaction of three proteins: basic polymerase 1 (PB1), basic polymerase 2 (PB2) and acid polymerase (PA) [17,83].

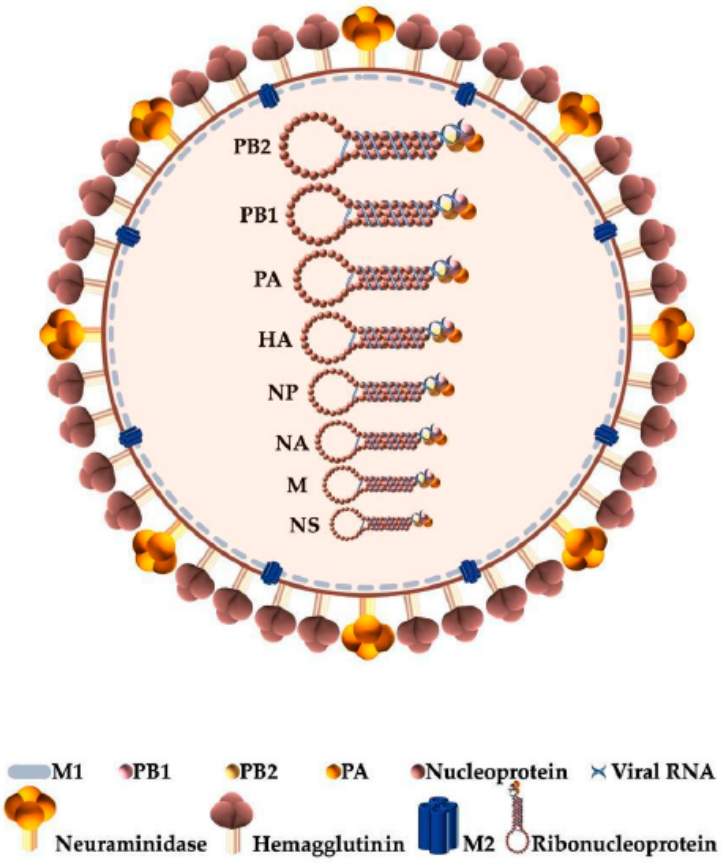


Figure 9. Influenza A virion particle. Taken from: Rosário-Ferreira et al. Int. J. Mol. Sci. 2020, 21, 1511 [84].

- Viral Genome

One of the defining characteristics of the influenza virus genome is its segmented nature. The numbering of these 8 segments, in the case of IAV and IBV, is determined by the length of the segment, from longest to shortest; or named after the main protein it encodes. Thus, from 1 to 8 we have PB2 (basic polymerase 2), PB1 (basic polymerase 1), PA (acid polymerase), HA (haemagglutinin), NP (nucleoprotein), NA (neuraminidase), M (matrix) and NS1 (non-structural protein) respectively. Each segment itself constitutes a replication unit that encodes for at least one of the essential proteins, but in addition, alternative splicing mechanisms or different open reading frames can encode for more specific proteins, fundamentally in M and NS segments, as described in Figure 10 [85].

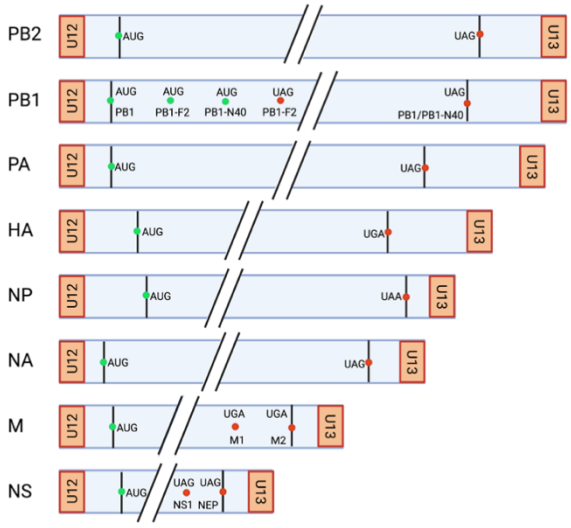


Figure 10. Structure of the influenza virus genome. Created with Biorender by Laura Sánchez-de Prada.

The viral RNA polymerase complex is responsible for the transcription and replication of viral RNA in infected cells. It consists of the interaction of three protein subunits PB1, PB2 and PA that form a heterotrimeric complex with a compact globular structure whose active centre is located in a cavity formed by PB1 and the N-terminal end of PB2 [86].

The NP plays a key role in viral replication. Although it is a structural monomeric protein lacking enzymatic activity, it is the most abundant protein in infected cells as it is a critical component of the RNPv complex. It is involved in RNA packaging, regulation of RNPv nuclear trafficking, and viral RNA transcription and replication [87].

The M gene codes for the M1 and M2 proteins. M1 is the most abundant protein and is found below the lipid membrane interacting with the transmembrane proteins HA, NA and M2 located on the outer side. It also interacts with the RNPv core on the inner side stabilising the virion structure. It plays an important role not only in assembly but also in virus budding and release, affecting virus morphology. The M2 protein, however, is an integral membrane protein which is found in smaller proportion and, like HA and NA, is anchored to the viral envelope. It acts as a viroporin, forming a tetramer that constitutes a proton ion channel. It conducts protons into the viral particle to

acidify the interior and destabilise the M1-bound RNPv complex and promote decapsidation in the cytoplasm of the infected cell [88].

The NS1 is considered a virulence factor as it is able to affect the host immune response. This protein consists of four structural regions including two globular domains, a binding domain, and a disordered “tail”. NS1 has a multitude of strategies to inhibit the host immune response due to its ability to establish interactions between proteins or with RNA. It interferes with both nuclear and cytoplasmic pathways in the infected cells. It hinders type I interferon (IFN-I) production involved in the primary host immune response to control infection. Other functions include attenuation of host gene expression, and it has also been related to increased pathogenicity and virulence [84].

- Immunogenic proteins

The most immunogenic virus proteins are those located on the surface of the virus, HA and NA. There are 18 HA and 11 NA subtypes in influenza A viruses in nature. Influenza B viruses, on the other hand, lack subtypes, but are divided into two antigenically distinguishable lineages (Yamagata and Victoria).

HAs are subdivided into two groups: group 1, which includes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18; and group 2 includes H3, H4, H7, H10, H14, and H15. Similarly to HA, NA has also been classified into two groups, group 1 and group 2, based on primary sequences. N1, N4, N5, and N8 belong to group 1 and N2, N3, N6, N7, and N6 belong to group 2. N10 and N11, are genetically far away from NA molecules found on canonical influenza A viruses(N1-N9) have been proposed to be named group 3 (Figure 11a). Of these, 16 HA and 11 NA subtypes circulate in waterfowl worldwide, and 2 HA and 2 NA subtypes are found in bats [89]. Transmission of avian viruses to mammals requires a number of changes that favour interspecies transmission, including changes in both replication temperature (from 40°C in birds to 37°C in mammals) and sialic acid receptor specificity (α-2.3 to α-2.6). Therefore, animals that possess dual specificity for both sialic acid receptors such as pigs and domestic fowl can act as intermediate hosts for interspecies transmission (Figure 11b) [90] .

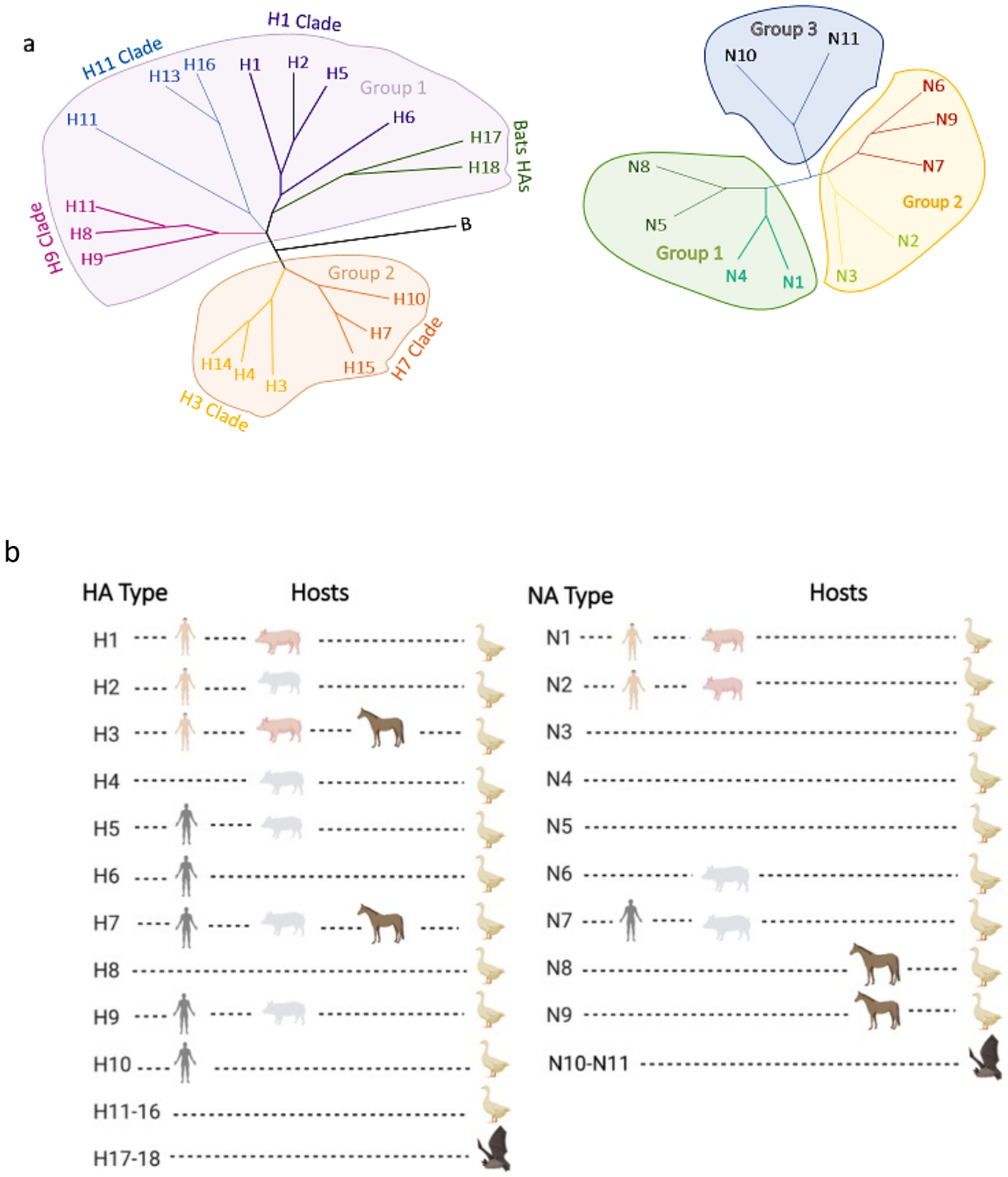


Figure 11. In (a) Phylogenetic tree of Influenza HAs and NAs. In (b) Influenza hosts by HA and NA.

The host immune response is driven by HA and NA, being the HA four times more abundant than NA. Their distribution is not entirely random, with mostly abundant clusters of HA most commonly present, followed by individual NA surrounded by more HAs, and finally, some local clusters of NA. The glycoproteins are closely

packed but irregularly distributed on the surface. The HA is more elongated and has a bilobar "peanut" shape and the NA has a thinner and longer stalk and ends in a bulbar "mushroom" head (Figure 12) [91].

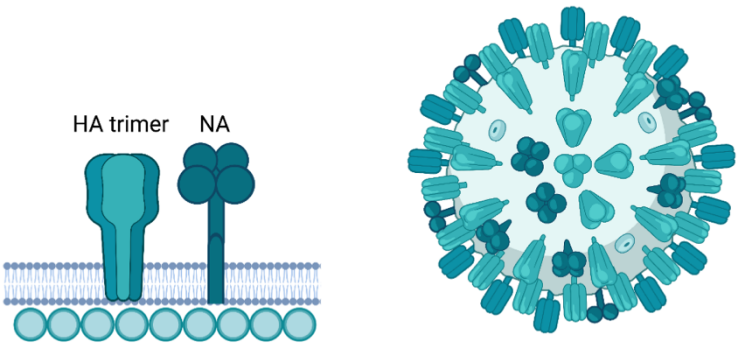


Figure 12. Structure and arrangement of HA and NA on the viral particle. Created with Biorender by Laura Sánchez-de Prada.

- The Hemagglutinin (HA)

It is the most important glycoprotein component accounting for 25% of the virus proteins. It is named after its ability to agglutinate red blood cells (haemagglutination). It plays two main roles in the early stages of the infectious cycle. It participates in the adsorption of the virus to the sialic acid of the mucoprotein receptors of epithelial cells, as well as in the fusion of viral and endosomal membranes for virus entry and decapsidation [17].

The HA is a type I integral membrane protein forming an homotrimer. Its three-dimensional structure is divided into a globular head on top of a stalk that is embedded in the viral envelope. The globular head contains the three sialic acid receptor binding sites (RBS), one per monomer, by which it binds to the epithelial cells. The stem domain contains the fusion peptide at its N-terminal end by which it binds to the endosomal membrane. The HA is initially synthesised as a precursor, the HA0, which is trimerized in the Golgi apparatus and transported to the cell surface. It needs to be cleaved into the two active polypeptide chains, HA1 and HA2, which are linked by disulphide bridges. That proteolytic cleavage is carried out by proteases that are secreted by the non-

ciliated cells of the human respiratory epithelium and also in the gastrointestinal tract of birds. The globular head consists of only one part of HA1 and the stalk domain of both HA1 and HA2 (Figure 13) [92,93].

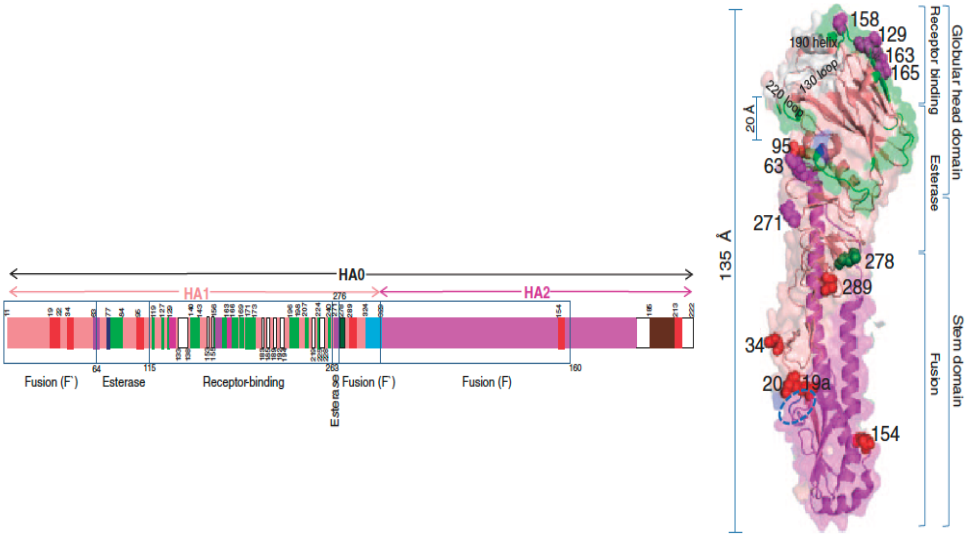


Figure 13. HA coding gene segment and HA monomer structure. Taken from: Sriwilaijaroen, N. y Suzuki, Y.226 Proc. Jpn. Acad., Ser. B 88 (2012)[93].

The first of HA's functions is binding to the terminal sialic acid of host cellular receptor glycoproteins and glycolipids via the RBS. It is located at the top of the HA forming a receptor recognition and binding pocket, which is a conserved region of all influenza subtypes despite antigenic variation. The sialic acid can have either α-2,6 or α-2,3 bonds with the terminal sugar of the glycoproteins and glycolipids. The first one (α-2,6), is the human receptor located on epithelial cells of the human respiratory tract and is therefore recognised by human viruses. The second (α-2,3), is the avian receptor located in the gastrointestinal tract of birds and recognised by avian viruses. Both receptors are recognised by swine viruses. In addition, actually, it seems that both types of receptors can be found in humans. This affinity for one or the other type of linkage is important because it determines interspecies transmission. However, this tropism is a preference, but is by no means an absolute specificity and can change in the case of a large virus inoculum or by a few mutations in the HA gene [83,94]. Also, this tropism can be artificially changed by several passages in cell and egg culture.

The second main function of HA is fusion with the lipid membrane of the

endosome in the infected cell. At neutral pH the N-terminal region of HA2, known as the fusion peptide, is hidden inside the HA molecule. The acidic pH of the endosome triggers an irreversible conformational change to the active form that allows membrane fusion. This also determines the species restriction as the fusion pH of HA varies depending on whether the virus is human or avian [92,95].

- HA Immunodominance

The term immunodominance was first coined in 1966 [96]. It defines the strong tendency of the immune system to respond to complex antigens in a hierarchical manner so that "immunodominant" antigens potentially suppress responses to "subdominant" antigens [97]. This concept can be applied to humoral response towards the HA. As the most abundant component on the surface of the virion, it stimulates the production of neutralising antibodies by the immune system that prevent further HA binding to cellular receptors. Those antibodies are produced against both the head and the stem. However, as the globular head is more exposed, the response against it is immunodominant over the response against the stem.

Thanks to the characterisation of escape mutants, five antigenic domains located in the HA head responsible for the neutralising humoral response were identified. They are called Sa and Sb, which are located at the distal end of each monomer, Ca1 and Ca2, which are located between adjacent monomers halfway up the globular head, and Cb, which is located at the base of the head. Three of them are located surrounding the RBS, and they are Sb, Sa and Ca2 (Figure 14) [93,97,98].

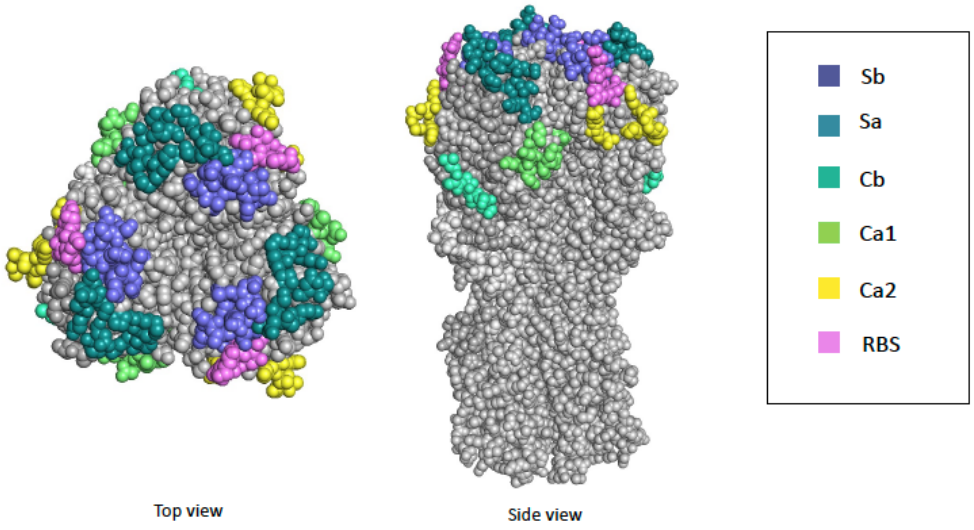


Figure 14. Top and side view of the HA homotrimer. Crystal structure modelled with PyMOL (The PyMOL Molecular Graphics System, Version 2.5.1, Schrödinger, LLC) by Laura Sánchez-de Prada.

- The Neuraminidase (NA)

NA is one of the main surface antigens of the influenza virus accounting for 10-20% of the virion surface glycoproteins. It is less immunodominant than HA as it is found in smaller proportion and relatively masked by its presence [99]. This less immunodominance make this protein to be less prone to change and evolve than HA. It is a type II transmembrane protein that forms a tetramer of four identical units, and it is folded into four domains: the cytoplasmic tail, the transmembrane region, the stalk and the catalytic head (figure 15) [92].

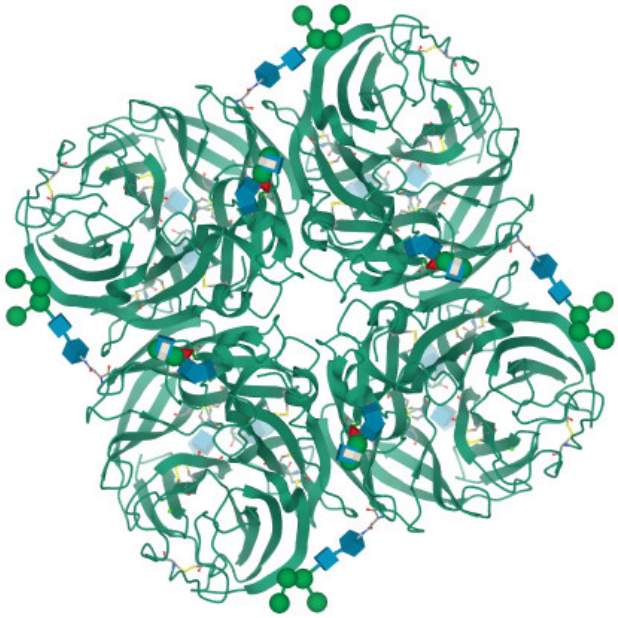


Figure 15. Tetrameric structure of the neuraminidase. Taken from: https://www.rcsb.org/structure/1nn2 (PDB DOI: 10.2210/pdb1NN2/pdb)

Although its main function is cleaving the sialic acid terminals of infected cells to release the new generation of virions, it is involved in several functions [100–102]. Through its sialidase action, it eases the invasion of respiratory tract cells by the virus by dissolving sialic acids from the mucin covering them [103,104]. It also prevents virions from aggregating at the cellular exit site and reinfecting the same cell, thus allowing the infection to spread to new cells [102].

1.5. Antigenic variation and viral evolution

RNA viruses, and especially influenza viruses, have been recognized as highly mutable. DNA viruses are much more stable and replicated with greater fidelity compared to RNA viruses. This is due, in part, to their shorter replication times and the error-prone nature of RNA polymerases that lack effective proofreading mechanisms of the DNA polymerases. In addition to stepwise evolution due to the accumulation of point mutations, RNA viruses own reassortment and recombination mechanisms that enable large-scale evolutionary jumps [105].

The influenza virus has two main mechanisms of antigenic variation known as antigenic drift and antigenic shift (Figure 16). Both mechanisms have evolved to evade the immune response, which also interferes with the response to vaccination [106].

- Antigenic drift

It is responsible for the annual influenza epidemics. It is caused by small amino acid changes in the major epitopes of the two key surface proteins, HA and NA. The epitopes of these proteins progressively accumulate mutations. Thus, if a mutation allows the virus to escape immune control and gives it an advantage in viral fitness, that virus has the opportunity to emerge as a new epidemic strain and replace the previous circulating strain. Strains emerged are very similar to the previous one, but with differential characteristics that turn them into escape mutants. Those cannot be neutralised by the previous specific antibodies of the population and so they spread easily [90,107,108].

- Antigenic shift

In contrast to antigenic drift, antigenic shift involves a drastic change in the antigenicity of circulating HA and NA by acquisition or exchange of these genes. That reassortment or replacement takes place in cells infected with different human and animal viruses, and the resulting virus encodes completely different proteins to which the human population lacks immunity. Typically, the proteins of pandemic viruses are derived from antigenically distinct animal strains acquired by human strains by replacement. Influenza pandemics arise when a new virus is produced to which the immunologically naïve human population is susceptible [85,88,89].

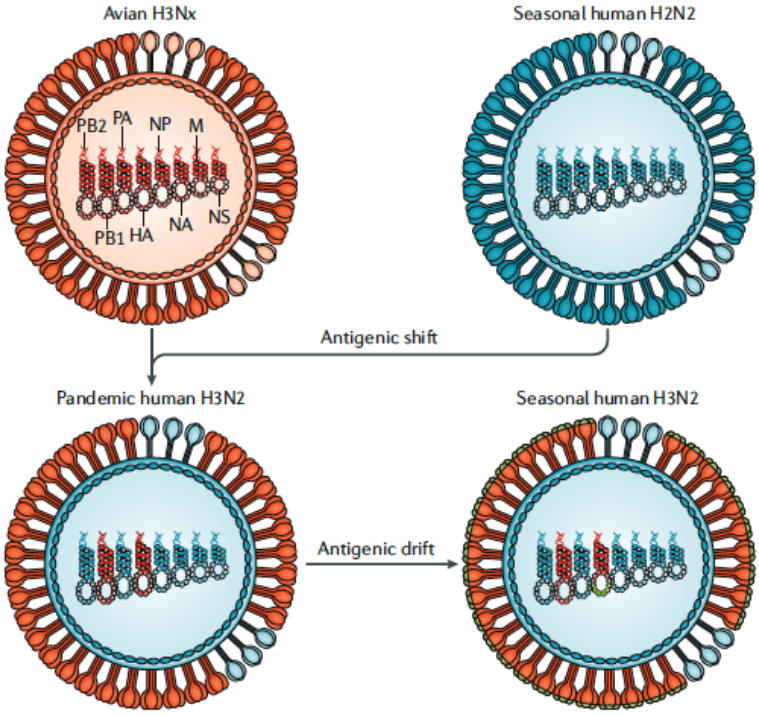


Figure 16. Antigenic drift and antigenic shift. Taken from Krammer F. et al. Nat. Rev: Dis. Prim. 2018 4:3[109]

1.6. Life cycle of influenza virus

Different phases can be described in the life cycle of the virus as it infects hosts (figure 17):

1. adsorption, entry and decapsidation of the virus
2. mRNA synthesis and viral RNA replication
3. post-transcriptional processing of viral mRNA
4. translation and post-translational processing of viral proteins
5. assembly and release of virions

Early in the cycle, the influenza virus uses sialic acids on the surface of epithelial cells as receptors. HA binds to those α-2,6- or α-2,3-linked sialic acids, depending on the species and type of virus. The former present in the epithelial cells of the human upper respiratory tract, and the latter present the intestinal tract of birds. Once bound to the receptor, the virus is internalised by endocytosis. The acidic pH of the endosome activates the M2 protein, which forms ion channels that introduces protons from the

endosome into the viral particle, weakening the union between the RNP and the M1 protein. In addition, the conformational change of the HA is triggered. HA cleaves exposing the peptide that merges the viral envelope and the endosome membrane. The eight vRNPs are inserted into the cytoplasm and transported to the nucleus by NP nuclear transport signals. The virus gene segments are never found as naked RNA, but always enveloped by the NP protein. Once in the nucleus, transcription and replication of viral RNA takes place through the RNA polymerase RNA-dependent RNA complex associated with these segments. This results in 3 types of molecules: i) positive-stranded complementary RNA, that serves as a template to generate more vRNA; ii) small negative-sense RNAs that are thought to be responsible for regulating the switch from transcription to replication; and iii) mRNAs that are exported to the cytoplasm for subsequent translation into viral proteins.

The newly synthesised proteins components of the viral polymerase complex (PB1, PB2 and PA) and NP are transported back to the nucleus to increase the rate of RNA synthesis. And vRNPs along with HA, NA and M2 proteins are exported to the cytoplasm with the help of M1 and NEP. HA and NA are modified in the endoplasmic reticulum and Golgi apparatus, where they are folded into oligomers, an HA trimer and an NA tetramer. Once structured, they are inserted into the host plasmatic membrane and virions are formed from the host plasmatic membrane containing the vRNPs. The sialidase activity of the NA cleaves the sialic acids preventing the virions from reattaching to the same membrane through the HA and they are released, promoting viral shedding. The viral products induce a proinflammatory reaction responsible for the recruitment of innate and adaptive immunity that clears and eliminates the virus [67,81,83,109]

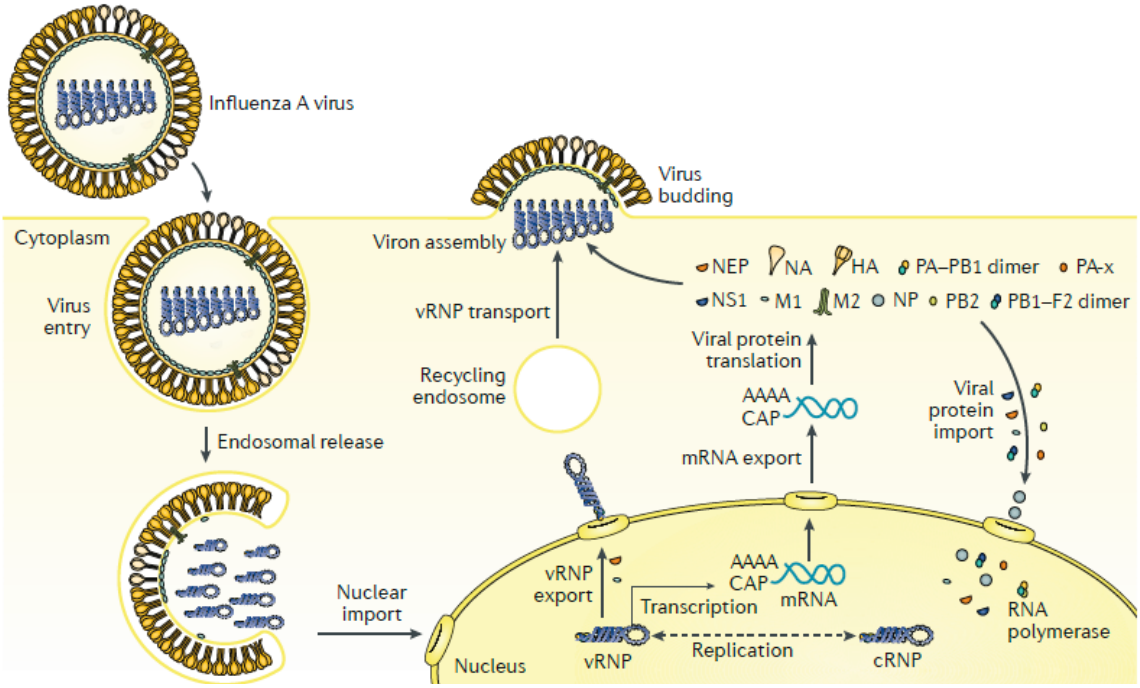


Figure 17. Life cycle of influenza virus. Taken from Krammer F. et al. Nat. Rev: Dis. Prim. 2018 4:3 [109].

2. IMMUNE RESPONSE TO INFLUENZA VIRUS

Infection is transmitted through both respiratory secretions and fomites after an incubation period of 1-4 days. This short incubation period means that outbreaks can occur in particularly vulnerable populations as in the case of pandemics. Influenza virus infection and viral replication takes place mainly in respiratory epithelial cells. The virus is rarely isolated outside the respiratory tract although infection causes signs and symptoms in several organs and is associated with marked constitutional manifestations. This lack of systemic circulation is associated with the preference for trypsin enzyme to activate HA protein, that is widely present in the respiratory tract, but not in other organs. Viremia is exceptionally observed with seasonal viruses but has been more commonly detected in infections caused by avian A(H5N1) viruses [110].

Influenza virus infection is a systemic syndrome that starts in the upper respiratory tract and initiates both a humoral and cell-mediated immune response.

2.1. Innate immune response

It is the first line of defence against infection. It aims to prevent infection in the respiratory epithelium and limit cell replication. Activation of the response occurs via three types of receptors: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and the NOD-like receptor and pyrin domain containing 3 (NLRP3) protein [17] . Receptor binding stimulates the secretion of pro-inflammatory cytokines and type I interferon (IFN-I) and triggers signalling cascades that initiate the recruitment of immune cells involved in both innate and adaptive immunity. Innate immunity involves macrophages, dendritic cells and Natural Killer (NK) cells [6,111].

Macrophages are activated and phagocyte virus-infected cells, limiting viral spread, but in turn secrete Tumoral necrosis factor-alpha (TNF-α) which contributes to the pathogenesis of the infection. Dendritic cells, in turn, present antigens from the respiratory tract and travel through the lymphatic system where they present them to T cells (both LT CD8+ and LT CD4+) to activate them and initiate the adaptive response. Finally, NK cells directly phagocytose viruses surrounded by antibodies [139].

2.2. Adaptative immune response

After the attack of innate immunity, the adaptive response of the immune system is activated and plays an important role in viral clearance, host recovery and the establishment of immunological memory. Adaptive immunity is comprised of humoral (specific Acs) and cellular (T-lymphocytes) responses [112].

- Humoral immune response

It is based on the production of specific antibodies by B-lymphocytes. The main isotypes are IgA, IgM and IgG. IgA is localised in mucosal membranes and provides local protection in the respiratory tract. IgM is produced at primo-infection and IgG in serum confers long-term protection. These Abs are directed against the two surface proteins of the virus, HA and NA. Abs against the HA head neutralise the virus by preventing its binding and entry into the cell. Abs are strain-specific and somewhat less subtype-specific, which explains the susceptibility to infection by new strains of the same

subtype that arise due to antigenic drift of. In contrast to the variability of the head, there are Abs against the HA stem, which are formed at lower titres after infection. This region is much more conserved and much less affected by antigenic drift, so that anti-stem Abs recognise molecules of different subtypes and have a higher neutralisation capacity [111,113,114].

Anti-NA Abs limit the release of new viral particles by interfering with the spread of the virus to new cells. Those Abs are produced in lower titres; however, they are known to be protective against disease and are a correlate of protection independent of anti-HA Abs. Current vaccines carry a non-standardised amount of NA in their formulation, and Abs are induced against it, however, the fact that they are co-formulated with HA makes the immunodominant response to be mainly against HA. NA could thus become a target for the design of new NA-based vaccines [115].

- Cellular immune response

Cell-mediated immunity plays an important role in disease progression control. In contrast to humoral immunity, which generally produces more strain-specific responses through abs; cell-mediated immunity is characterised by greater cross-reactivity and is able to protect against infection by different subtypes (heterotypic immunity) and even variants of antigenic drift. This is because T cells, the main components of cell-mediated immunity, target the most conserved epitopes of external proteins as well as the internal proteins of the virus. T cells recognise viral peptides bound to the two major histocompatibility complex (MHC) expressed on the surface of infected cells or antigen-presenting cells (APCs). Through binding to TLRs and co-stimulatory signals and cytokines, naïve T cells are activated and proliferate, differentiating into effector and memory T cells. Effector cells contribute to viral clearance through recognition and elimination of virus-infected cells (through CD8+ cells), as well as secretion of pro-inflammatory and antiviral cytokines (through CD4+ cells). Memory T cells are ready to be reactivated in the event of subsequent encounters with the virus, in which case they would differentiate and proliferate again to perform their functions.

Within T-cells we differentiate between different types. CD4+ T cells, which bind to MHC class II and are subtype-specific, have different functions. On the one hand, they are helper cells that promote the formation of B-lymphocytes (follicular T-lymphocytes) by maintaining the germinal centre and the production of specific high-affinity abs. Also, they support CD8+ priming, expansion and proliferation, which is important for the establishment of a memory cell pool; and, like them, they have some cytotoxic activity. In addition, both CD4+ and CD8+ have an important role as a source of both anti- and pro-inflammatory cytokines. On the other hand, CD8+ T cells play an important role in the case of heterosubtypic immunity as, through MHC-I, they recognise epitopes located on antigens of M1 internal virus proteins, NPs and polymerases. Furthermore, their efficiency in virus clearance with subsequent host recovery via cytokines and elimination of infected cells is noteworthy [112,116].

Finally, there are also NK cells that lack virus specificity, increase their activity in early stages of infection and are associated with a decrease in viral load [117].

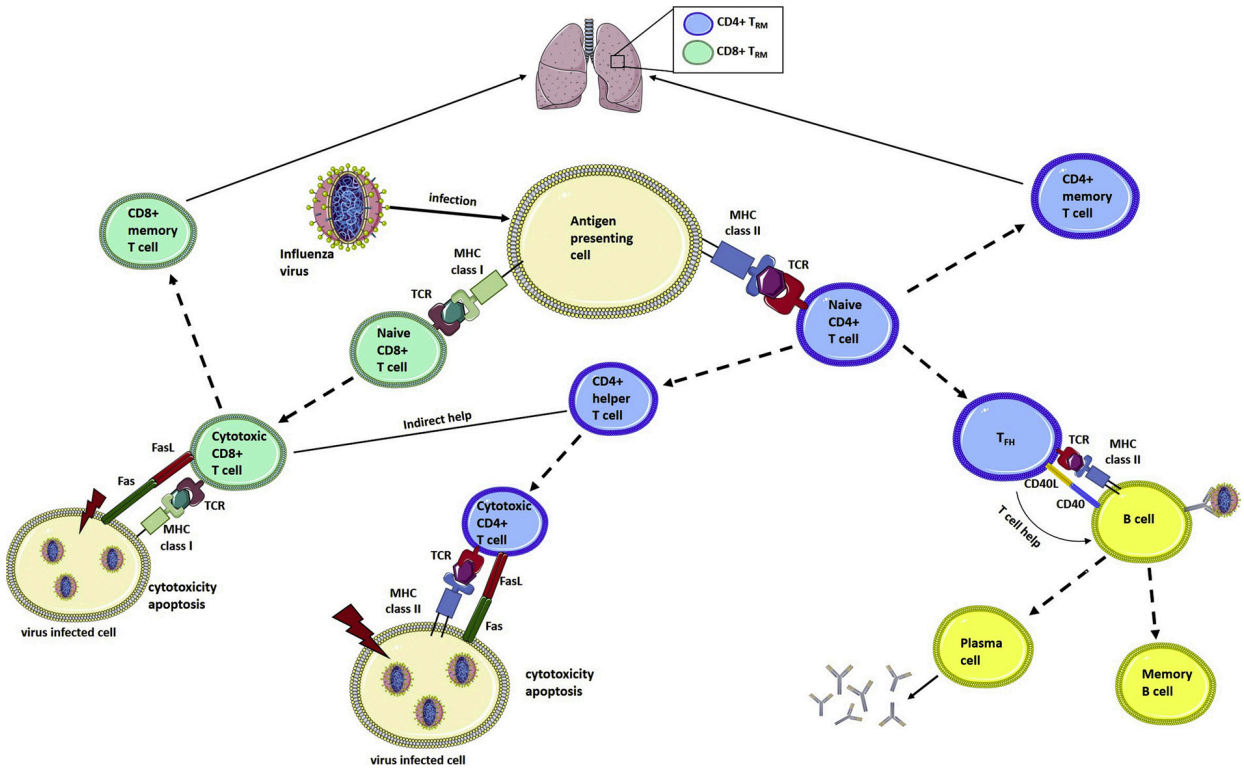


Figure 18. Representation of humoral and cellular immune response against influenza virus . Taken from J.M. Jansen, et al. J.Clin.Vir.119 (2019) 44–52.[116]

2.3. Original antigenic sin

The phenomenon of original antigenic sin (OAS) was first described in 1960 by Sir Thomas Francis for the influenza virus [118]. According to this doctrine, the development of an immune response to a pathogen or antigen (Ag) is modulated by the first contact with that pathogen or related antigen [119]. In other words, the first contact with the influenza virus, which usually occurs in the first years of life, conditions the response we will have in subsequent exposures, either through infection or vaccination. This theory explains that the Ab response generated by B cells after exposure will always be greater against that strain or genetically related viruses at the expense of the generation of de novo Abs [120]. Subsequently, "negative interference" was described in relation to vaccination [121], a hypothesis that describes that those specific Abs produced in the first infection interfere with the induction of an equivalent amount of Abs against newly encountered strains. The OAS, associated with a negative connotation due to negative interference, has also been called antigenic imprinting, which emphasises the importance of first exposure, or antigenic seniority, which emphasises the hierarchical nature of the Ab response to influenza viruses, thus avoiding such connotations. Finally, all of these OAS-like phenomena cannot be categorised as negative or positive but are context-dependent and important to consider in the development of influenza vaccines. In this way, OAS can be used to our advantage to induce a stronger response to conserved epitopes to which there is pre-existing immunity, such as the stalk domain. However, it may be detrimental in the case of vaccine boosters against conserved but non-protective epitopes, at the expense of generating protective responses against epitopes that have undergone antigenic drift [119].

3. EPIDEMIOLOGY OF INFLUENZA VIRUS

3.1. Circulation and incidence

Influenza viruses cause seasonal epidemics affecting 5-20% of the population. They are responsible for 3 to 5 million severe cases and 294,000 to 650,000 deaths annually. That mortality rate is around 1%, although it varies depending on the strain,

the age and characteristics of the affected population.

The incubation period is usually 2 days, but can range from 1 to 4 days, and it is easily transmissible in settings such as schools and nursing homes. Peak viral shedding occurs between days 1-3, lasting up to 2 weeks in children who usually have higher viral loads in the upper respiratory tract; and it can last for months in particularly immunocompromised patients. Clinically, it is characterised by the sudden onset of fever, dry cough, muscle and joint pain, headache and sore throat, severe malaise and abundant nasal discharge.

Seasonal influenza usually has a U-shaped epidemic curve, with attack rates being highest at the extremes of life, i.e. children and the elderly. However, mortality rates are usually highest in those over 65 years of age. Influenza morbidity and mortality is affected, in addition to age, by the presence of chronic diseases and by the type and subtype of influenza virus (Figure 19) [122–125].

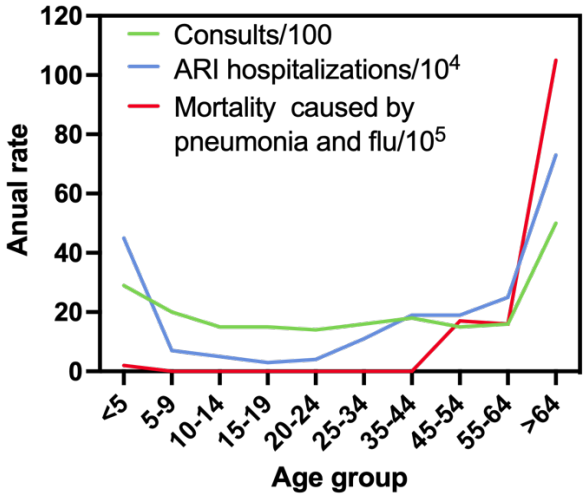


Figure 19. Typical epidemic influenza curve. The graph shows rates of illness requiring medical care (green, rate per 100), acute respiratory illness (ARI) hospitalisations (blue, rate per 10,000) and pneumonia- and influenza-associated mortality (N&G) (red, rate per 100,000) by age. Adapted from: Treanor JJ. Chapter 165, Influenza Virus. Infectious Diseases. Elsevier 7th ed. 2012. Edited by Mandell, Douglas and Dohlin (p2272-96)[122]

Influenza viruses present a marked seasonality in the temperate zones of the northern and southern hemispheres, appearing as seasonal epidemics in the winter

months. November to February and May to October in the northern and southern hemispheres respectively. On the other hand, in tropical and subtropical areas, this seasonality disappears just as it does climatologically, and epidemics can coincide with the rainy season, other areas have semi-annual epidemics or even circulate throughout the year without a marked of peak incidence. Seasonal waves usually last between 6 and 15 weeks, being the average about 11 weeks [126–128]. In our country, the period of highest influenza activity starts in December and lasts until February-March, which could be extended until May in some cases, or even maintain some circulation during the summer season.

The influenza surveillance period in the northern hemisphere lasts from week 40 to week 20 of the following year. In Spain, there is a surge of cases above the baseline threshold in the first week of January, which increases reaching the epidemic peak in early February, with rates of 250-300 cases per 100,000 persons per week depending on the season (Figure 20). From an epidemiological point of view, influenza A viruses are mainly responsible for seasonal epidemics, either in co-dominance or alone, and sometimes accompanied by influenza B viruses. However, every two to three years the epidemic is mostly caused by one of the two influenza B lineages [129,130].

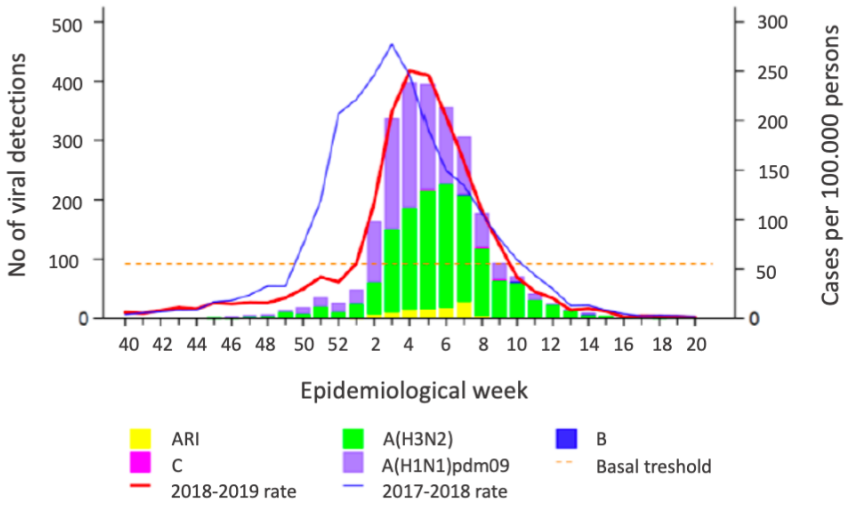


Figure 20. Weekly incidence rate of influenza and number of viral detections. Season 2018-2019. Taken from: CNE.ISCIII. Sentinel Influenza Surveillance System in Spain[111].

3.2. Influenza surveillance

3.2.1. The WHO global surveillance network and the European Network for human influenza: The National Influenza Centres

The WHO Global Influenza Surveillance Network has been implemented through the Global Influenza Surveillance and Response System (GISRS) since 1952. This network, that is the oldest in the World, is formed by 158 reference laboratories in 127 WHO member states [131]:

- 148 National Influenza Centres (NICs) in charge of surveillance and monitoring in 127 countries
- 7 WHO Collaborating Centres (WHOCCs), international centres of excellence on influenza.
- 4 Essential Regulatory Laboratories (ERLs) in charge of surveillance and vaccine development
- 13 H5 Reference Laboratories (H5RefLabs) monitor the interrelationship of human and animal viruses and assist in the detection of new viruses.

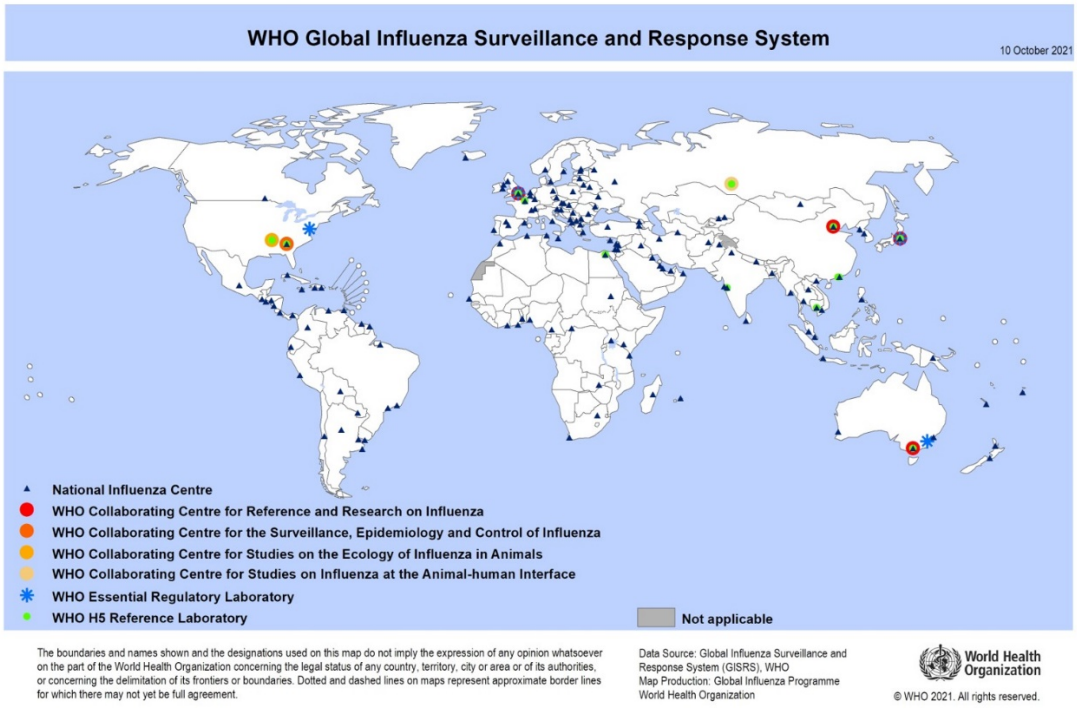


Figure 21. Network of laboratories belonging to the WHO Global Influenza Surveillance Network year 2021 [131].

In addition, within Europe, the NICs are also part of the ECDC's European Reference Laboratory Network for Human Influenza (ERLI-Net), which was founded in 2003 and works in collaboration with the WHO. The tasks performed by the NICs include [132]:

1. Direct detection of influenza A and B viruses using molecular techniques.
2. Culture of influenza viruses, essential for vaccine formulation
3. Determination types and subtypes of seasonal influenza, as well as viruses with pandemic potential (such as influenza A/H5 subtypes and other variants).
4. Antigenic characterisation.
5. Genetic characterisation.
6. Storage of clinical specimens and virus isolates.
7. Shipment of virus isolates and/or clinical specimens to the WHO CC in London.
8. Antiviral susceptibility monitoring.
9. Participation in external quality exercises.
10. Electronic reporting of national and international epidemiological and virological data.

The mission of these laboratory networks is the epidemiological surveillance of influenza viruses with the aim of reducing morbidity and mortality caused by seasonal epidemics, as well as the preparation for a possible influenza pandemic through the PIP (Pandemic Influenza Preparedness Framework) that establishes the sharing of viruses with potential pandemic potential among member states, as well as access to vaccines and other benefits among them [133,134].

The tasks performed by the NICs are the basis for the periodic review and update of annual influenza vaccines due to the constant evolving nature of influenza viruses.

Thus, twice a year, once per hemisphere, WHO organises a consultation with a group of experts who analyse GISRS surveillance data and suggest the vaccine composition recommendations for the following season. Those are given to national vaccine regulatory agencies and pharmaceutical companies to develop and produce influenza vaccines for the Northern and Southern hemispheres each year.

3.2.2. Influenza Sentinel Surveillance System in Spain (ScVGE)

Influenza surveillance began in 1908, when it was added to the list of notifiable diseases. Subsequently, at the beginning of the 1990s, networks of sentinel doctors began to operate in some Autonomous Regions, supported by laboratories that allowed individualised reporting of cases. At present, the ScVGE is made up of [135]:

- 16 networks of sentinel physicians and paediatricians, one in each Autonomous region (Galicia and Murcia do not have influenza sentinel surveillance networks) and in the two Autonomous Cities, coordinated at the national level by the National Epidemiology Centre (CNE)
- 20 laboratories with influenza virus detection and isolation capacity. Three of them are WHO National Influenza Centres, Valladolid, Madrid and Barcelona.
- Administrative units and Public Health institutes, coordinators of the regional sentinel surveillance networks.

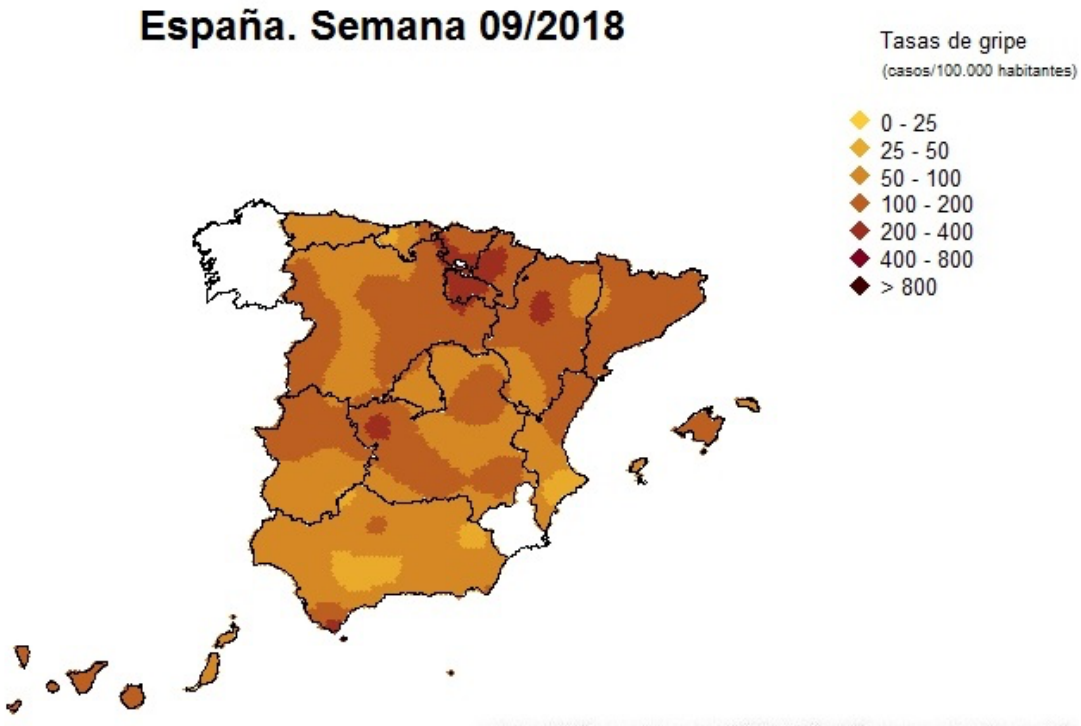


Figure 22. Influenza intensity graph for week 9 of 2018. The autonomous regions in white do not have sentinel systems. Source: National Epidemiology Centre[136]

The main objective of influenza surveillance is to describe the epidemiological and virological aspects of influenza activity in Spain, with the aim of taking measures in order to reduce the burden of influenza-associated disease and to guide disease prevention and control.

The detailed specific objectives are [137]:

1. Describing the evolution of influenza activity (seasonal or pandemic) in Spain and its Autonomous regions by age groups and influenza virus types/subtypes/lineages.

2. Detection of the beginning of the influenza epidemic.

3. Detection and characterisation of influenza viruses circulating in Spain each season, as well as the emergence of new influenza A subtypes.

4. Susceptibility determination of circulating strains to antivirals.

5. Isolation of strains that may be selected by the WHO for the composition of the influenza vaccine for the following season.

6. Determine of the degree of similarity between circulating influenza strains and vaccine strains.

7. Characterize the severity of epidemics/pandemics and identify groups at risk for the occurrence of clinically severe forms of the disease.

8. Provide data to understand the burden of influenza disease and its impact on the population.

9. Contribution to the knowledge of factors related to influenza epidemics and the natural history of the disease.

10. Participation in the exchange of influenza surveillance information at national and European level.

11. Guidance to local and national health authorities in the formulation of measures aimed at influenza prevention and control, including

recommendations for influenza vaccination.

12. Provide information on the effectiveness of influenza vaccination in seasonal or pandemic influenza epidemics.

13. Contribute to national influenza pandemic preparedness and response plans, through surveillance and information provided by other studies.

3.2.3. Sentinel Surveillance Network of Castile and Leon

The objective Sanitary Sentinel Network of Castile and Leon (RCSCyL) is public health surveillance and epidemiological research thanks to the voluntary and active collaboration of health professionals from the health system. This network is made up not only of primary care physicians, pediatricians, and nurses, but also of health professionals and technicians from the Directorate General of Public Health, the Territorial Services of Health, Consumer Affairs and Social Welfare and the Regional Health Management, as well as external collaborators who participate actively in some way [138]. Influenza surveillance is coordinated by the Directorate General of Public Health by means of the Health Sentinel Network of Castile and Leon. The National Influenza Centre of Valladolid act as the reference laboratory of this network. It originally started in 1989 when sentinel doctors together with the Valladolid NIC carried out a multiannual project called "Epidemiological surveillance of influenza in the population of Castile and Leon " [6]. The doctors from the sentinel network are randomly selected, so the samples are representative of the existing population profiles[139].

The epidemiological surveillance of influenza virus is traditionally divided into two periods based on the circulation patterns of the virus:

1. Period of surveillance:

   From epidemiological week 40 to week 20 of the following year

2. Period of non-surveillance:

   Also known as inter-epidemic period. Between weeks 21 and 39 of the same year, where usually there are no influenza cases diagnosed or just some sporadic cases occur. However, 2022 has been a particular year where influenza

has circulated out of season probably due to COVID-19 pandemic (figure 23) [140].

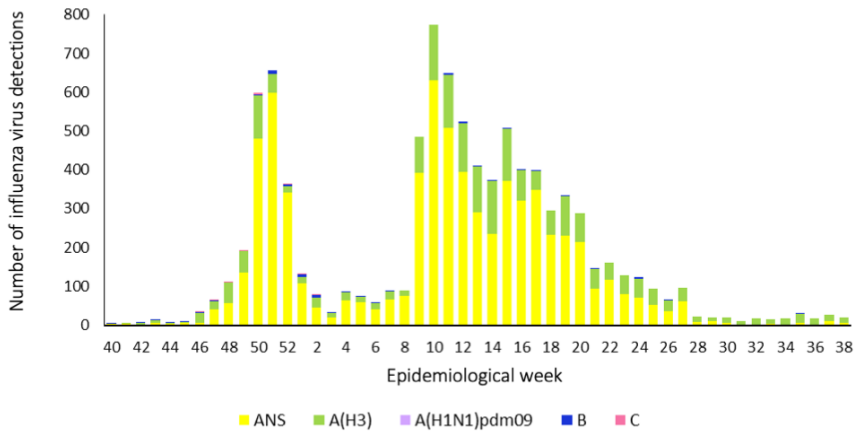


Figure 23. Influenza cases in 2022. Obtained from: https://vgripe.isciii.es/inicio.do[140]

Once a week, the information collected by the Valladolid NIC and the RCSCyL is transmitted to the National Epidemiological Surveillance Network of the Carlos III Health Institute. The aim is to prepare weekly epidemiological reports on influenza in Spain, which include the following points [135]:

1. General information about Sentinel systems
2. Virological surveillance data
3. Information about influenza outbreaks
4. Surveillance of severe diagnosed cases
5. Influenza related mortality
6. International influenza surveillance

Once the influenza season is over, the ScVGE prepares an annual report with all the available information covering the points mentioned above [141]. Likewise, the RCSCyL prepares its own epidemiological reports that can be consulted through the PVIG (Programa de Vigilancia de la Gripe-Influenza Surveillance Program). Those reports include specific information on number of cases, incidence rates, virological information and data, isolation rate, number of cases and cumulative incidence rate segregated by age [142]. In both reports, information is reported weekly during the surveillance period

and bimonthly during the inter-epidemic period [6].

Globally, influenza epidemiological information from all over the world is uploaded weekly to FluNet (figure 24). This platform collects information on the number of detections, indicating the main circulating types and subtypes, their level of circulation, etc., so that the information can be updated on weekly basis. This information is of free access and available to the public thanks to the reports of the NICs, as part of the GISRS [143].

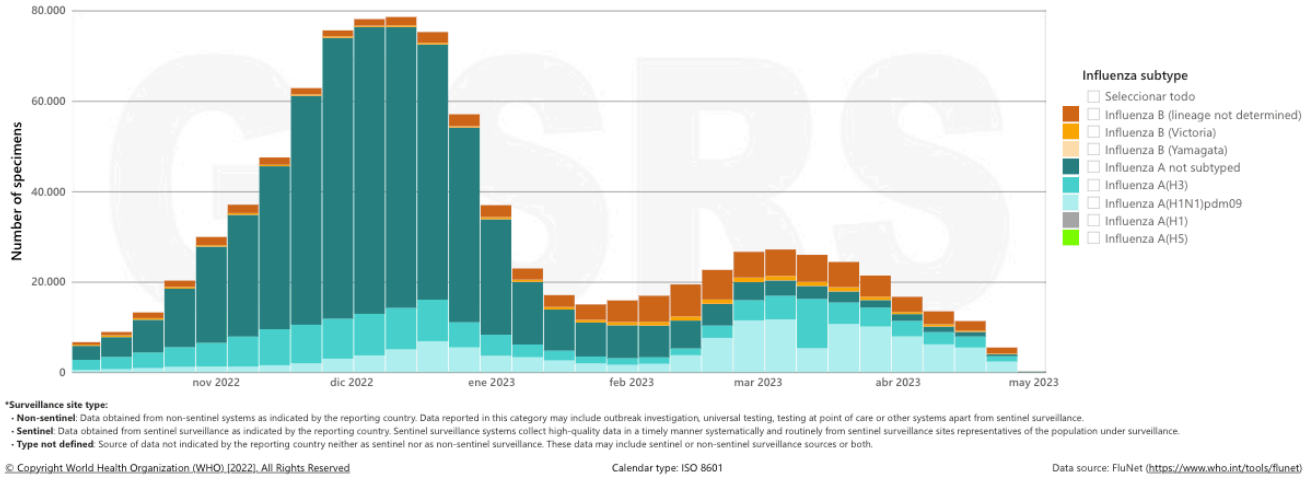


Figure 24. Real time data of influenza cases in FluNet. Available in: https://www.who.int/tools/flunet/flunet-summary [143]

4. INFLUENZA VACCINES

4.1. History of vaccination

The history of vaccines began with Edward Jenner in 1798. He realised that contact with the cowpox virus provided protection against human smallpox virus and conducted what could pass for an 18th century clinical trial. He showed his results to the world, culminating in the eradication of smallpox in 1978. Even so, it would be 80 years later when the next breakthrough in vaccine development came thanks to Louis Pasteur. Whether intentionally or by accident, he realised that it was possible to attenuate bacteria if they were exposed to adverse conditions, although his reasoning for this was flawed. In the last decade of the 19th century, is when vaccine development has advanced the most, and different methods have been developed, including

complete inactivation of bacteria, antitoxins, toxoid vaccines, and protein conjugation with capsular polysaccharides. Regarding viruses, cell culture, attenuation by passage in other living organisms or in cell lines was a major revolution. Finally, the introduction of genetic engineering has allowed bacteria, yeast, animal cells and insects to become substrates for the production of immunogenic proteins such as baculovirus vectors [144].

The first influenza vaccines against influenza A were monovalent and were developed thanks to the work of Thomas Francis, Jonas Salk, Wilson Smith and Macfarlane Burnet in 1934. However, they did not become of wider use until 1940, when they were administered to soldiers in World War II. In 1942, the first bivalent vaccine against influenza A and B was produced after the discovery of influenza B virus [145].

Later on, antigenic drift was discovered, and since 1973 the WHO has been making annual recommendations on the vaccine composition. It was not until 1978 that a vaccine including, for the first time, two strains of influenza A (H1N1 and H3N2) in addition to a strain of influenza B in the composition was designed. This resulted in the annual trivalent vaccine used until recent years. In addition, there is also now, since the recommendation of WHO on 2014, a quadrivalent vaccine including two influenza A strains (H1N1 and H3N2) and two influenza B strains (Victoria and Yamagata lineage) is the preferred formulation [146].

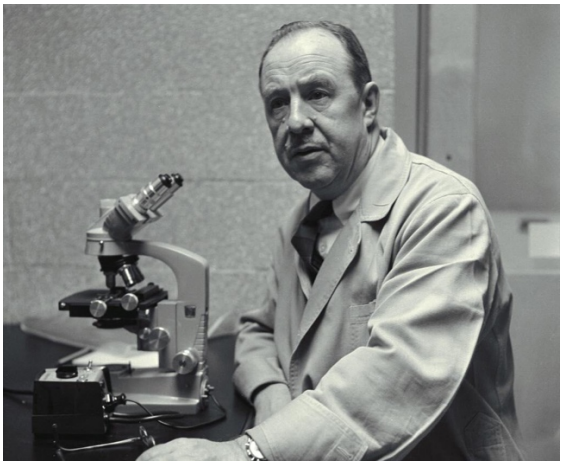


Figure 25. Thomas Francis Jr.

4.2. Vaccine production

As discussed previously, influenza vaccine viruses are selected each year at a biannual meeting of WHO experts based on surveillance data on viruses that have been circulating and predictions of those most likely to circulate in the coming season. The degree of similarity between the viruses available for the vaccine and the circulating viruses is also important, as well as the similarity to those circulating later in the season for which the vaccine is to be produced. Another important factor is that the virus selected is actually available, i.e. that it can be used for vaccine production and that it meets the requirements just mentioned. Historically, viruses for vaccine production were isolated and produced in embryonated chicken eggs, but nowadays cell culture and other new vaccine platforms are gaining visibility and usefulness. Regardless of the production method, viruses must be tested and available in time for both Northern and Southern hemisphere vaccine campaigns. For this reason, some pharmaceutical companies start culturing viruses of various vaccine-susceptible strains before the vaccine composition recommendation has been made, as the process of manufacturing large quantities of influenza vaccine takes at least 6 months [147].

There are currently three methods for the production of influenza vaccines by the pharmaceutical industry: embryonated egg culture, cell culture and recombinant vaccines. However, in the next years, other vaccine designs used during COVID-19 pandemic will appear for influenza vaccines, such as mRNA and vector-based vaccines, among others.

4.2.1. Vaccines produced in embryonated eggs

It is the most common method of vaccine manufacture, still accounting for 88% of production in 2018 and has been in use for over 70 years. They are used to produce both inactivated and live attenuated (nasal spray) vaccines. The production process starts with the transfer of vaccine candidate virus strains grown in embryonated chicken eggs from GISRS reference centres. These strains are re-cultured in embryonated eggs to multiply the virus until sufficient stock is obtained to manufacture the required quantities of vaccine. For the inactivated vaccine, the viruses are deactivated, and the

viral antigen is purified. For the attenuated vaccine, the viral strains are initially live and use a slightly different process that weakens the virus [148,149].

The main advantages of this method are the excellent production capacity with 1.5 billion doses per year at a cost that allows global access to the vaccine. The disadvantages include a slow production time of about 6-8 months, which can be delayed by late issuance of recommendations or because the recommended strains are not as easily cultured, and the stock is smaller than initially expected. In addition, this production time could be sufficient for circulating strains to drift resulting in a mismatch. On the other hand, culturing a human virus in avian tissue in which mutations can accumulate can potentially change the antigenicity of the strain. Therefore, antigenicity controls of the strains produced for the vaccine must be carried out to verify that they do indeed produce the appropriate response, as well as the relevant quality controls by the FDA, which gives the go-ahead for their manufacture [148,149]. Additionally, the dependence of this kind of vaccines on the egg production may be a problem in the case of an "animal pandemic" of an avian influenza virus, such as the current circulation of A(H5N1) in wild birds and farms. The mortality of this viruses in poultry and the culling in farms due to animal outbreaks may jeopardize the use of eggs for human vaccines productions or, at least, limits their use.

4.2.2. Vaccines produced in cell culture

This type of production is used to make inactivated vaccines. Cell culture-based production was approved in 2012 by the FDA. Until 2016, the process started with the contribution of viruses grown from embryonated eggs by the Influenza Reference Centres or the FDA to the pharmaceutical companies. But, since that year, the process has been authorised to be carried out from the candidate strains for the vaccine already obtained by cell culture by the reference centres. The 2019/2020 season was the first in which the four strains of the vaccine produced by cell culture actually came from cell culture, making it egg-free. The production process continues with the inoculation of the candidate strains into cell cultures of mammalian cells, where they are replicated and then the fluid is extracted from the cells and the viral antigen is purified. Finally, the FDA performs the quality and safety checks and is responsible for approval [148].

This production system appears to overcome the limitations of embryonated egg-based vaccines. Production in a cell culture bioreactor is more flexible and scalable and is not affected by the lack of eggs. In addition, they can be used in egg-allergic population, and it has been observed that vaccines produced in this way, result in a moderately higher vaccine efficacy in ≧65 years possibly due to a lower amount of avian adaptive mutations [149].

4.2.3. Recombinant vaccines

They were approved in 2013. These recombinant vaccines do not require a sample of candidate vaccine strains for their production, instead, they are created synthetically. The RNA is directly obtained from wild strains in order to "manufacture" the HA. This RNA is combined with a vector, frequently a baculovirus, which is a virus capable of multiplying in insect cells. Thus, when the baculovirus with these instructions infects the cell line, large quantities of HA begin to be produced. These HA antigens are collected, purified and formulated in the form of rosette trimers to form the recombinant flu vaccine.

In addition to having the same advantages as cell culture, two very important ones are added. It is not dependent on the time required to select candidate vaccine strains and neither on the need of the virus growth in large quantities [148–150].

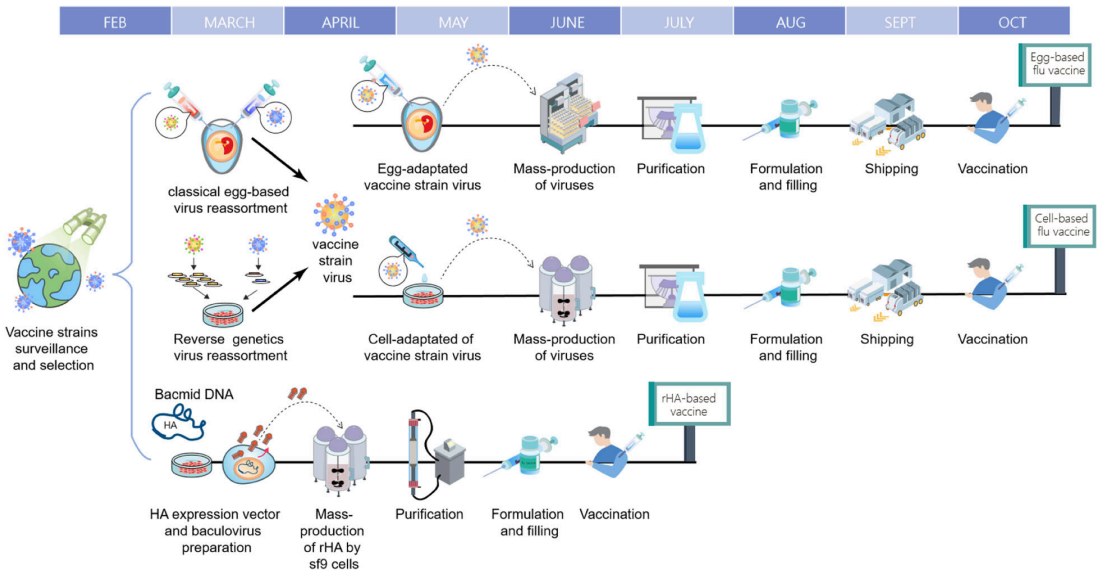


Figure 26. Manufacturing methods of influenza vaccines. Taken from Chen et al. J. Biomed. Sci. 2020; 27(1) 1-11.[149]

4.3. Type of vaccines

4.3.1. Inactivated influenza vaccines

This type of vaccine is obtained from embryonated egg culture, and the viruses are then chemically inactivated with formalin or beta-propiolactone (BLP) [151]. Both trivalent (A(H3) and A(H1N1)pdm09 subtypes and one influenza B lineage) and tetravalent (A(H3) and A(H1N1)pdm09 subtypes and both influenza B lineages) formulations are currently available, which include a standard amount of 15 µg of antigen of each influenza subtype and lineage. In addition, there is an FDA-approved high-dose vaccine (High Dose FluZone - Sanofi Pasteur) that includes 60 µg of antigen of each subtype and lineage included, specially designed for individuals aged 65 years and older to provide greater protection and is also available in both formulations [152,153].

There are five different types of inactivated influenza vaccines according to their antigenic composition and production system [6,154]:

1. Whole virus vaccine

They are purified suspensions of inactivated whole virions. They contain, in addition to HA and NA proteins, other lipid and polysaccharide structures that facilitate antigen recognition and promote cellular and humoral response. It is one of the vaccine types with the highest reactogenicity. This kind of vaccines are not being used now.

2. Split-virus vaccines

They are purified suspensions of virions fractionated by the action of detergents, so they only contain HA, NA proteins and parts of NP and M proteins. Although they are capable of inducing both humoral and cellular immunity, the immunization is focused on humoral response.

3. Sub-unit vaccines

They are vaccines containing only the surface antigens, HA and NA, linked by their lipophilic ends. They are obtained by subdivision and differential zonal purification. They are less reactogenic than whole and split-virus vaccines.

4. Vaccines with antigenic subunits carried in virosomes or liposomes.

These vaccines are obtained by intercalating the HA and NA protein into liposomal membranes that simulate the structure of a virion. This enhances the immune response by presenting the antigen in a more effective way. This results in increased stimulation of different T-cell subpopulations following fusion of the virosome with the macrophage plasma membrane. As the addition of large amounts of protein to these virosomes is not necessary, secondary reactions are less intense.

5. Vaccines carrying adjuvants.

The use of adjuvants such as MF59 (an oleo-aqueous emulsion of squalene with two surfactants) increase and prolong the immunogenicity of the response by enhancing the effect on T-cells. They are particularly recommended for immunocompromised and people over 65, although they have more side effects than non-adjuvanted vaccines.

4.3.2. Attenuated influenza vaccines

These vaccines produce a controlled and safety infection in the individual that is responsible for the immune response. Such vaccines are commonly produced by genetic reassortment of the HA and NA protein genes from the proposed wild-type vaccine strains, with genes from a donor strain attenuated by restriction. The strains commonly used as donors of the low-temperature growth genes are strain A/Ann Arbor/6/60 (subtype A/H2N2) and strain B/Ann Arbor/1/66 [6,17].

The viruses thus obtained can grow and multiply in the upper respiratory tract due to their lower temperature. However, they cannot grow in other organs at 36-38°C, as their ability to grow at physiological temperature is restricted in. This type of vaccines have many advantages. Those include the simulation of a natural infection by stimulating the mucosal response, and the limitation of the carrier status by preventing multiplication at the mucosal level, thus reducing the spread of the virus[6,17].

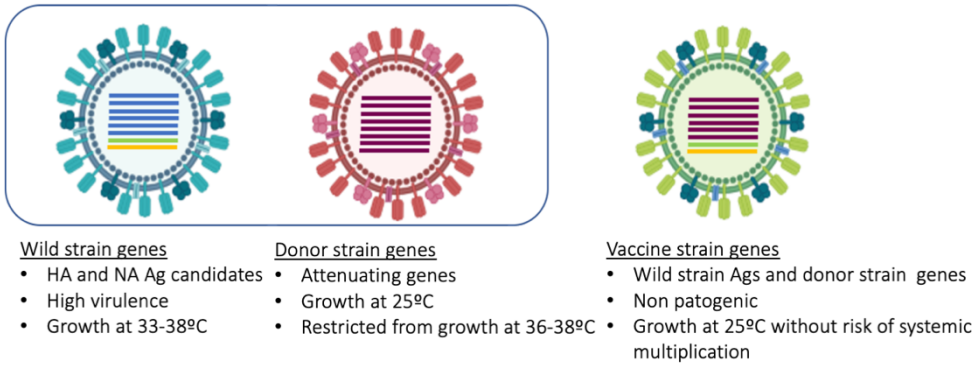


Figure 27. Modified from Ortiz de Lejarazu R. y Tamames S. Enf. Infecc. Microbiol.Clin. 2015;33:480-90.[155]

4.3.3. mRNA influenza vaccines

There is no doubt that influenza vaccines are one of the most effective interventions in terms of preventing infection and reducing severity. However, many problems arise with the production system of influenza vaccines **[156]**:

- Over 80% of production relies on embryonated eggs, which can be compromised in the event of a pandemic which would require an increased production or an outbreak that wipes out the hens laying those eggs.
- Production takes 6-8 months.
- Some viruses are unable to multiply in eggs and tend to undergo antigenic changes to adapt their growth in avian tissues, which may compromise the efficacy of the vaccine.

Until the SARS-CoV2 pandemic, these vaccines were not developed due to conservation and stability issues as -80ºC are required. But the pandemic emergency led to a rapid progress in their commercialisation [157]. This would bring a substantial change in the development of new vaccines using this technology. Two types of mRNA vaccines can now be considered:

1. Non-replicating mRNA: The first study demonstrating the efficacy of mRNA vaccines was published in 1993 [158], but it was not until 20 years later that the first study demonstrating protection against influenza virus after using an mRNA vaccine was published [159]. They are based on the introduction of full-length mRNAs with modified or unmodified nucleosides encapsulated in lipid

nanoparticles as a carriers and coding for an HA. Thus high levels of protein are produced after translation. It is important , though, to use a good carrier and a high dose [160,161].

2. Self-amplifying mRNA: RNA molecules from modified alphavirus genomes are used, as alphaviruses produce large amounts of RNA during their replication cycle. This way, they contain the RNA for the cellular replication system and the information of the antigen in question that we want to introduce, instead of the alphavirus protein-coding genes. Thus, with a small dose, a large amount of immunogen is produced over an extended period of time, due to the intracellular replication of this RNA [160,161].

Influenza mRNA vaccines offer certain advantages over other vaccines. Those include: a good safety profile, controllable immune production, the absence of anti-vector immunity allowing repeated administration, and most importantly, rapid large-scale production without the need for the use of embryonated eggs or complex cell culture systems [126].

4.3.4. Universal influenza vaccines

One of the objectives of the WHO's Influenza Global Strategy 2019-2030 is to promote research and innovation for improved diagnosis and vaccines, as well as treatment of influenza. In addition, there is another document called "WHO preferred product characteristics for influenza vaccines" which describes the characteristics or requirements that a universal vaccine should meet. The WHO's working definitions of a universal vaccine would include a broader spectrum of protection and longer duration of immunity than currently conferred by existing vaccines. A universal influenza vaccine could be a completely new vaccine or an improved version of those already in use. Ideally, it would be a vaccine including a first dose of vaccine for priming and subsequent boosting doses that would induce a lifelong protection without the need for constant boosters. However, it is considered that this will take longer to develop [162].

Currently, several vaccines are in development with the aim of boosting or elongating the immune response. Those employ new antigens and adjuvants that gradually increase the strain-specific nature of current vaccines to include all strains of a

subtype, different subtypes, or all subtypes of a clade/lineage (influenza A clade 1 or clade 2 or influenza B Victoria or Yamagata lineages) with the ultimate goal of creating a truly universal vaccine that produces long-lasting immunity against all influenza A and B viruses [149].

The immune response to influenza vaccines is usually narrow and strain specific. Neutralising antibodies induced by seasonal vaccination focus on the distal part of the membrane of the immunodominant globular head of the HA. This area of the HA has great plasticity and is mainly responsible for antigenic drift, which makes it necessary to reformulate vaccines every year. However, novel vaccine designs are focused on redirect that response to conserved epitopes that are shared between different influenza viruses. This cross-protection can be classified as heterologous protection (within the same subtype) or heterosubtypic protection (including several subtypes-same clade). One of the most developed strategies on universal influenza vaccine design is targeting the stem or stalk of the HA and also, although less developed the M2 protein [163]:

- Stalk based influenza vaccines

Because the stalk is a much more conserved region, antibodies against it show cross-reactivity between the different HA subtypes. The induction of antibodies against the stalk by vaccination follows two design strategies [163]:

- Chimeric HAs (cHAs): These are combinations of H1 (clade 1) or H3 (clade 2) stalks with exotic globular head domains, mainly of avian origin. These HAs fold similarly to natural HAs and viruses expressing these HAs can be cultured without problems and achieving high titres. Sequential exposure to these HAs with the same stalk domain but different head domains specifically induces antibodies against the stalk, yet only produces a primary immune response against the corresponding HA head.

- Headless HAs: These are constructs that completely lack the immunodominant globular head of the HA. However, this affects the

way the stalk folds, which at the time affects antibodies that bind only to conformational epitopes and do not recognise properly these structures.

- M2 based influenza vaccines

The M2 protein is a well-conserved protein among influenza A viruses; one of the first universal vaccine targets was the M2 ion channel ectodomain. Antibodies against this region, while not directly neutralising the virus, can prevent viral shedding, as well as killing infected cells by NK cells and macrophages [164].

4.4. Preferential vaccine administration groups

The vaccine development sector is one of the most rapidly developing, especially due to the emergency situation created by the SARS-CoV2 pandemic. This has led to a rethinking of vaccine policies around the world. In Spain, for example, the vaccination schedule, previously divided into paediatric and adult, has been updated to a single lifelong schedule [165,166]. In the case of the influenza vaccination recommendation in our country, it focus on groups at high risk of suffering complications in the event of the disease, as well as people in contact with them [167]. These groups are described below:

1. Elderly people, preferably from 65 years of age. Special emphasis will be placed on those living in closed institutions.
2. People under 65 years of age who are at high risk of complications from influenza disease.
3. Children (aged 6 months and older) and adults with chronic cardiovascular, neurological or respiratory diseases, including bronchopulmonary dysplasia, cystic fibrosis and asthma.
4. Children (6 months and older) and adults with:
   o diabetes mellitus
   o morbid obesity (body mass index ≥40 in adults, ≥35 in adolescents or ≥3 SD in childhood)
   o chronic kidney disease and nephrotic syndrome
   o haemoglobinopathies and anaemias

o haemophilia, other coagulation disorders and chronic bleeding disorders, as well as recipients of blood products and multiple transfusions

o asplenia or severe splenic dysfunction

o chronic liver disease, including chronic alcoholism

o severe neuromuscular diseases

o immunosuppression (including primary immunodeficiencies and that caused by HIV infection, drugs-including eculizumab treatment, in transplant recipients and complement deficiency)

o cancer and haematological malignancies

o cochlear implantation or awaiting cochlear implantation

o cerebrospinal fluid fistula

o celiac disease

o chronic inflammatory disease

o disorders and diseases that lead to cognitive dysfunction: Down's syndrome, dementia and others.

In this group, special emphasis will be placed on those persons who require regular medical follow-up or who have been hospitalised in the previous year.

5. Children between 6 months and 18 years of age, who receive prolonged treatment with acetylsalicylic acid, due to the possibility of developing Reye's syndrome after influenza.
6. Persons of any age (≥6 months) institutionalised for a prolonged period of time.
7. Pregnant women in any trimester of pregnancy and postpartum women (up to 6 months after delivery and who have not been vaccinated during pregnancy).
8. Children between 6 months and 2 years of age with a history of prematurity of less than 32 weeks of gestation.
9. Persons who can transmit influenza to those who are at high risk of complications:

   o Staff of health centres, services and establishments, both in primary care and specialised and hospital care, public and private, as well as pharmacy

staff. Special emphasis will be placed on staff who have ongoing contact with patients in some of the high-risk groups described above.

o People working in geriatric institutions or in care centres for the chronically ill, especially those who have continuous contact with vulnerable people.

o Trainees in health care institutions.

o Persons providing home care to high-risk or elderly patients (as defined in sections 1 and 2).

o Persons living at home, including minors from the age of 6 months, with others who belong to some of the high-risk groups, due to their special clinical condition (mentioned in point 2).

10. Other groups in which vaccination is recommended:

o Persons working in essential public services, with special emphasis on the following subgroups:

- State security forces and corps, with national, regional or local dependence.
- Firefighters.
- Civil protection services.
- Persons working in the health emergency services.
- Staff of penitentiary institutions and other internment centres by judicial decision (including immigrant reception centres).

o Persons with direct occupational exposure to domestic fowl or pigs in poultry or pig farms and also to wild birds. The aim is to reduce the chance of concomitant infection with human and avian or porcine viruses thus reducing the possibility of recombination or genetic exchange between the two viruses.

For vaccination of the paediatric population, 0.5 ml doses should be administered from 6 months of age (regardless of the vaccine administered). In children under 9 years of age being vaccinated for the first time, two doses of vaccine shall be administered with a minimum interval between doses of 4 weeks. In subsequent seasons, a single dose should be administered.

In Spain, influenza vaccination is carried out by the Autonomous Communities, which decide which vaccines should be administered to each population group. In

Castilla y León in the year 2020/2021 the campaign started on 13 October 2020 and 3 types of vaccines were administered [134]:

- VAXIGRIP TETRA, tetravalent split-virus influenza vaccine; intended for population at risk aged 6 months to 59 years and general population aged 60-64 years.
- CHIROMAS, trivalent influenza vaccine with enhanced immunogenicity (adjuvanted with MF59C.1); intended for the population aged 65 years and older. Not for use under 65 years of age.
- FLUZONE HD, tetravalent split-virus influenza vaccine with high antigenic load; intended for residents of geriatric institutions, with priority for those aged 75 years and older. Not for use in population under 65 years of age.

This system of vaccinating groups at risk contrasts with universal vaccination policies for population-wide coverage in countries such as the USA and Canada. However, in the former, certain groups are prioritised over others in the event of vaccine shortages [168,169].

Figure 28. Influenza vaccination campaign for 2020-2021 influenza season in Castile and Leon [170].

4.5. Vaccine efficacy and effectiveness

The ability of influenza vaccines to prevent the disease has been evaluated in numerous clinical trials with very different designs and populations. Such trials are aimed at evaluating efficacy and effectiveness. It is important to remark differences between the two of them.

Vaccine efficacy is defined as the ability of a vaccine to prevent a given disease in individuals who have received it, with special emphasis on the level of disease reduction induced by the vaccine under ideal conditions [171]. That is, efficacy is measured by means of randomised controlled clinical trials in which an influenza vaccine is administered to one group and a placebo or another known vaccine to another and the number of cases of disease at a given time is compared. In practice, correlates of protection have been established that allow that clinical target to be translated into a serological target which reliably predicts vaccine efficacy. Those correlates are estimated by the detection of haemagglutination inhibiting Abs, accepting that a titre equal to 1/40 provides a protective efficacy that can reach up to 50% in healthy adults [172–174]. Furthermore, these assays are used by the European Medicines Agency (EMA) for the initial evaluation of the vaccine [175,176].

On the other hand, vaccine effectiveness refers to the protection of vaccinated individuals against influenza in their usual conditions of use in clinical practice, which are far from optimal. It is therefore measured by observational epidemiological studies, measuring the burden of influenza disease in vaccinated versus unvaccinated individuals. These may be randomised community trials, cohort studies or case-control studies. In addition, effectiveness should only be assessed when the vaccine has been shown in a controlled clinical trial to be effective [171,175].

4.5.1. Serological techniques for vaccine efficacy assessment

Vaccine efficacy is measured by different serological techniques but two have been traditionally used: the haemagglutination inhibition assay (HAI) and the single radial haemolysis test (SRH). Those methods allow a fairly reliable estimation of the humoral immunogenic power of vaccines. In addition, there are more complex

complementary serological methods to improve this estimation, which focus on measuring Abs against other proteins of the virus different from HA. Those assays include the microneutralization (MN) assay, which tests for the presence of neutralising Abs against all virus proteins [175,177,178]; and the Enzyme-Linked Lectin Assay (ELLA), which measures the presence of Abs against NA [179–182].

- Haemagglutination Inhibition (HAI). It is the WHO reference method for detecting and quantifying Abs against influenza virus HA in serological samples, both human and animal, and therefore the most widely used (figure 28). It is based on the erythrocyte binding capacity of influenza viruses and an anti-HA Ab titre of 1/40 is considered as protective [175,178,183]. This technique is used to evaluate the humoral response to vaccination, to perform serological efficacy studies, as well as sero-epidemiological studies to evaluate the susceptibility of different populations to influenza [174,184]. In addition, it is the technique for antigenically characterising influenza viruses by using antisera from previously infected animals.
- Single Radial Haemolysis (SRH). It takes the second place in assessing the serological efficacy of seasonal vaccines. It is based on passive haemolysis of red blood cells induced by the Ag-Ab complex and mediated by the complement. It detects IgG Abs against influenza viruses but is not exclusive to them. In this technique, an area of haemolysis equal to or greater than 25 mm^2 is accepted as protective [178,185,186].

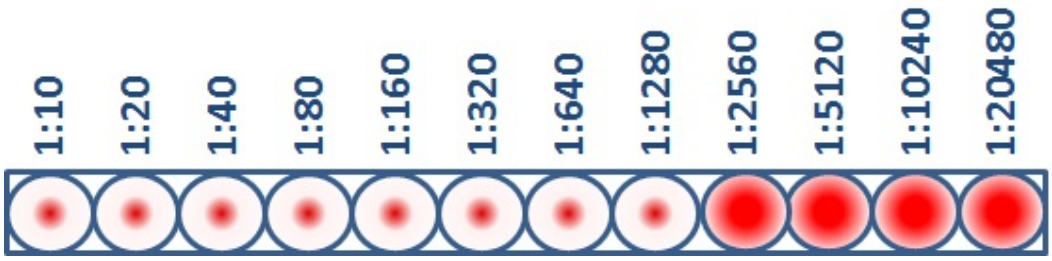


Figure 29. HAI titration. The virus titre is 1280, which means the highest Ab dilution that inhibited haemagglutination was a dilution of 1280. Taken from: https://espanol.cdc.gov/flu/about/professionals/antigenic.htm [187].

4.5.2. Parameters for vaccine efficacy evaluation

The parameters calculated on the basis of the HAI results to measure vaccine efficacy are as follows:

- Geometric mean of antibody titres (GMTs): since Ab titres are values that follow a geometric progression and a non-normal distribution with some right skewness, the arithmetic mean is not a suitable method for the calculation of central tendency values. This causes the use of the arithmetic mean to overestimate the mean value of the Abs score. To avoid this, a logarithmic transformation of those titres is performed to obtain a normal Gaussian distribution, being the central tendency value the arithmetic mean of that transformation.
- Seroprotection Rate (SPR): Represents the percentage of the population tested that possesses anti-HA Abs at titres considered protective, i.e. titres ≥1:40 which is the value considered to be the correlate of serological protection against influenza virus. Studies have shown that an anti-HA titre of 1:40 can prevent at least 50% of influenza infections [172–174].
- Fold-induction(FI) or GMT increase (GMTi): measures the increase in Ab titres experienced by a population in response to influenza vaccination and is defined as the division between post-vaccination GMTs and pre-vaccination GMTs.

  FI or GMTi= post GMTs/Pre GMTs

- Seroconversion Rate (SCR): Represents the percentage of the population that after influenza vaccination shows an increase of at least four times the pre-vaccination serum titre or a FI=4.

In order for new influenza vaccines to be approved, they must be analysed by Randomised clinical trials (RCTs) comparing with other old influenza vaccines. However, for the approval of annual composition of seasonal influenza vaccines, these must be only being analysed in terms of immunogenicity. They must meet a series of vaccine efficacy criteria required by the EMA and issued by the Committee for Proprietary Medicinal Products (CPMP) [175,176,178], based on the parameters described above. The CPMP criteria are applied according to two different age groups: adults between 18

and 60 years, and those over 60 years and are described in table 3. For a vaccine to be considered effective, it must meet at least one of the three requirements. These criteria are not defined for other age groups that also receive influenza vaccines, such as the paediatric population.

Table 3. Criteria used by the by the Committee for Proprietary Medicinal Products (CPMP) for influenza vaccine efficacy assessment and required by the European Medicines Agency (EMA) for commercialization [178].

EMA CRITERIA	18-59 years old	≥ 60 years old
Seroprotection rate	≥ 70%	≥ 60%
Seroconversion rate	≥ 40%	≥ 30%
Fold induction	≥ 2.5	≥ 2.0

4.5.3. Factors influencing vaccine effectiveness

Vaccine effectiveness is conditioned by a series of characteristics of both the subject receiving the vaccine and the vaccine itself [188,189].

- Vaccine characteristics
    - Matching of vaccine strains to circulating virus strains. The ability of influenza viruses to undergo constant antigenic drift requires adequate virological and epidemiological surveillance to correctly select the vaccine strains for each season. Even so, the time it takes to produce the vaccine may mean that these strains do not match.
    - Amount of antigen included in the vaccine: standard dose versus high dose.
    - Vaccine type: live attenuated, fractionated inactivated or subunit vaccine.
    - Presence of immune-enhancing elements such as adjuvants.
    - Routes of administration: subcutaneous, intradermal or intranasal.
    - Origin of the vaccine virus, whether obtained from embryonated egg culture or cell lines, may have a different impact on the protection conferred by the vaccine.

- Subject characteristics

The particularities of each individual are very important to consider when assessing vaccine effectiveness and should be considered when designing vaccination campaigns. Today, medicine is increasingly moving towards personalised medicine and therefore there is still much to be studied in terms of the factors that affect response to vaccination.

It is well known that as one ages, the immune system also ages. This mechanism is known as immunosenescence. This involves a reduction in the generation of Abs and their affinity, as well as a decrease in T helper lymphocytes that limits the B and T lymphocyte response to pathogens, contributing to an increased susceptibility to infection and a reduced response to vaccination [190–193].

Biological sex is also a characteristic to consider. Studies show that antibody production in women is higher, and some authors suggest that half a dose of influenza vaccine generates the same response in a woman as a full dose in a man [194,195]. Other individual factors that condition the response include chronic and/or autoimmune diseases, as well as a history of previous exposure to different influenza viruses[188].

In general, for any public health intervention, efficacy always tends to outweigh effectiveness. However, it is accepted that studies assessing serological parameters of protection may result in an overestimation of vaccine efficacy and, therefore, not be as far from the effectiveness values [196] Therefore, despite the moderate effectiveness of influenza vaccines [197], annual influenza vaccination campaigns remain the most effective measure to fight the economic, health and social burden that influenza disease represents [198].

2.RATIONALE

Seasonal influenza poses an important socioeconomic burden [109]. According to the World Health Organization (WHO), influenza epidemics affect 10–20% of the global population; and cause approximately three to five million severe cases and 290,000–650,000 respiratory deaths every year [123,125]. Additionally, the economic impact has been estimated to be of 6–14 billion euros in the European Union and 87.1 billion dollars in the United States alone [199,200]. The best strategy to address influenza epidemics is through annual vaccination campaigns. However, according to the Centers for Disease Control and Prevention, the effectiveness of the current vaccines is moderate ranging from 20 to 70% depending on the season [201]. Influenza vaccines need to be updated annually due to viral evolution. Many aspects can influence vaccine efficacy and effectiveness. In order to evaluate the humoral response to influenza vaccination different factors were selected to be analysed using the serological data of 22 consecutive influenza seasons from 1996 to 2018. Some related to vaccine nature included type of vaccine regarding the presence of adjuvants and vaccine composition and others related to individuals included sex and age of the receptors of the vaccine were selected.

There are many studies that compare the effect of adjuvants in vaccine response in the elderly as a risk group that is the main target of adjuvanted vaccines. Those studies often are limited in time and patients [202,203]. By using a 22-year-old database it would be easier to avoid confusion factors due to individual responses. Thus, evaluate responses to vaccination using adjuvanted and not adjuvanted vaccines in the elderly and non-adjuvanted in the adults as controls of a response without the impairment of immunosenescence.

Biological sex became another interesting factor in vaccine responses. As we know, clinical trials through history have been often designed to include male patients. Sex differences in influenza pathology are complex and include immunological, hormonal, behavioural, and genetic factors, among others [204]. So, it was logical to study responses taking sex into account which could lead to interesting results for future vaccine strategies.

As potential pandemic viruses, influenza A viruses have been profoundly studied while influenza B viruses have been often neglected, although interest is raising in the latest years[205]. Proof of this is that in Spain trivalent vaccines that include only one of the B lineages were in use until 2019/2020 season despite the existence of tetravalent vaccines in the market. However, as influenza B lineages represent an important burden and co-circulate with A viruses, it made sense to evaluate vaccine responses in the context of sex and age.

In the context of constant antigenic drive of the HA head of influenza A viruses, we considered to perform an immunodominance study to evaluate which of the classically defined antigenic sites weighted more in the humoral response to vaccination against influenza A (H1N1) virus. This could only be performed with samples of one season but could give us a global vision on which antigenic sites were essential in vaccine response and if age and type of vaccine could be modulating factors.

We considered performing these studies would give us a global vision of vaccine responses that would ultimately help in designing vaccine strategies to better protect the population.

Finally, the main problematic with current influenza vaccines is the short-lasting strain-specific protection. In fact, novel influenza vaccine approaches focus on increasing the breadth and duration of protection, with the aim of designing an "universal" influenza vaccine[206]. Some of the most advanced vaccine candidates target conserved epitopes of the HA protein, such as the subdominant stalk domain to provide long-lasting protection against different strains and subtypes of the virus [207–209]. Anti-stalk antibodies are elicited most effectively after natural infection or vaccination with antigenically diverse strains. Several different antigenic strains have circulated in humans (H1N1, H2N2, H3N2,H1N1pdm09) [210] and previous findings suggest that these antibodies tend to increase with age [211–214] and are influenced by first childhood influenza exposure [215,216] In this context, we considered appropriate to investigate the pre-existing levels of this antibodies according to age and the responses elicited after seasonal influenza vaccination against both phylogenetic HA groups.

3. HYPOTHESIS

The immune response to different strains and specific epitopes of the viruses contained in influenza vaccines can be influenced by different factors including presence of adjuvants, age and biological sex, which could be useful to guide the design of future vaccines and influenza vaccine campaigns.

4. OBJECTIVES

General objectives

1. To evaluate the presence of specific humoral immune responses and the humoral response after seasonal influenza vaccination against influenza A and influenza B virus.

Specific objectives

1. To evaluate the influence of the presence of adjuvants in relation to age in humoral responses against the HA of influenza A viruses
2. To assess the importance of age and biological sex in humoral vaccine response against influenza A and B viruses
3. To evaluate homologous and heterologous vaccine response after seasonal influenza vaccination against influenza B viruses
4. To determine immunodominance against different epitopes of the HA head of the A(H1N1)pdm09 (cepa A/Michigan/45/2015) (Sb, Sa, Cb, Ca1, Ca2) subtype before and after influenza seasonal vaccination
5. To determine the presence of abs against the HA stalk domain of phylogenetic groups 1 and 2 before and after seasonal influenza vaccination

5. METHODS

1. STUDY DESIGN

To evaluate the aforementioned objectives we performed different observational descriptive studies. Three studies were retrospective and two of them were prospective. All of them adhered to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines [217]. A total of 4,818 patients were recruited yearly during 28 seasons (1990-2018) by the physicians from the vaccination programs of the Sentinel Surveillance Network of Castile and Leon (Spain), run by primary healthcare centres during Influenza Vaccine Campaigns (IVC). Vaccines were administered following the WHO vaccine composition recommendations for the Northern hemisphere[218]. Based on records available patients included in retrospective studies were organized as described in Figure 30.

Informed consent was obtained, and the recruitment of patients was performed following Spanish Organic Law for Data Protection, patient's rights and obligations for clinical documents (BOE n 298 of 14th December 1999, Law 41/2002). This research was performed according to the Declaration of Helsinki and was yearly approved by the Ethics Committee of East-Valladolid health area under the code PI 21-2314.

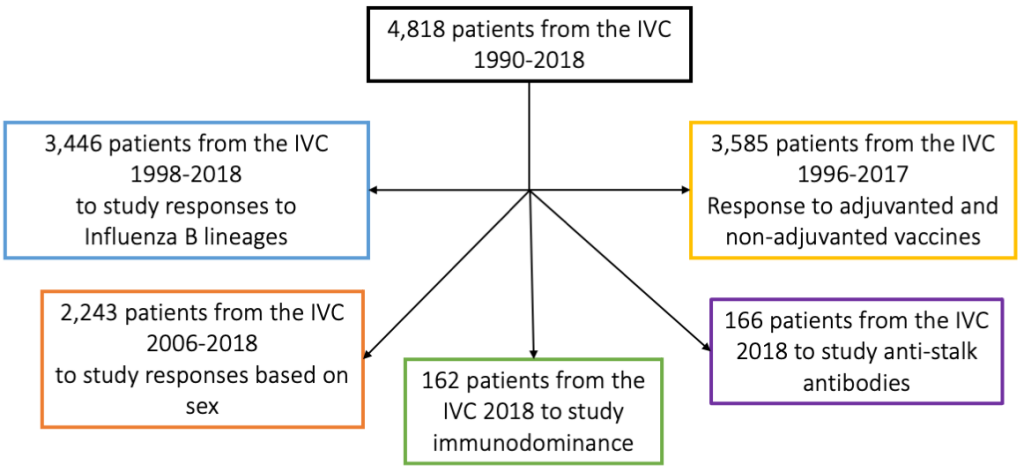


Figure 30. Diagram of population of study selection based on objectives.

2. SAMPLE COLLECTION

A first serum sample were obtained right before influenza vaccination, and a second one was obtained at least 28 days after the flu shot to ensure a correct immunization. These samples were obtained by the physicians involved in the RCSCyL through the annual programme of influenza surveillance in Castilla y León. These blood samples were obtained in 8ml Vacutainer tubes specially designed for serum. Samples were then sent to the National Influenza Centre of Valladolid for their analysis to assess vaccine immunogenicity and for sero-epidemiological surveillance, as a part of the WHO Global Influenza Surveillance and Response System (GISRS). Samples were received in the lab, revised and centrifuged during 10´ at 2.500 rpm. An aliquot of every serum sample was stored at -20ºC for preservation. In addition, samples from the 2018/2019 season were sent to the Mount Sinai Hospital to perform immunodominance (ID) studies and determine the presence of stalk antibodies.

3. LABORATORY TESTING

To assess antibody presence before and after vaccination in serum samples, HAI was performed in all samples against the viruses included in the vaccine each year. In addition, to assess immunodominance a panel of viruses was used, and serological assays were performed again through HAI in triplicates. Likewise, the assessment of neutralization capacity of stalk antibodies required two reassortant viruses and was performed through ELISA.

- HAI: The presence of antibodies in serum samples was analysed following the WHO and the Influenza Surveillance Network for the surveillance of influenza viruses and vaccine efficacy protocol[178]. Beforehand to the HAI, 100µl of each sample was treated with 300µl of RDE (Receptor Destroying Enzyme; Denka Seiken, Tokyo, Japan) to eliminate non-specific inhibitors. This solution was incubated overnight (12-18h) at 37ºC in a water bath and then deactivated with 300µl of 2.5% sodium citrate solution and diluted in 300µl PBS to a work concentration of 1/10. Before performing HAI, each virus was titered and standardized to 4 hemagglutination units (4HU) after thawing. Two-fold dilutions of 50µl of each serum in 50µl of HA buffer

were conducted in 96-V-microwell plates, and then 50µl of the virus was incorporated and incubated for 30 minutes at room temperature. Finally, 50µl of hen erythrocytes at 0.75% were added and incubated at 4ºC for another 45 minutes. The HAI titre was defined as the highest dilution at which hemagglutination inhibition occurred. Negative and positive controls were used in each plate. For the immunodominance study, Mount Sinai´s hospital protocol was followed and differed in volumes. The virus was standardized at 8HU/50µl (4HU/25µl). Two-fold dilutions of 25µl of the serum samples in 25 µl of HA buffer were performed, and 25 µl of the virus were used. Additionally, 50 µl of turkey (instead of hen) erythrocytes at 0.5% were employed.

- Panel of virus to assess ID. A panel of five recombinant viruses were generated: H1-ΔSa, H1-ΔSb, H1-ΔCa1, H1-ΔCa2, H1-ΔCb, were the classically defined H1 antigenic sites (Sb, Sa, Cb, Ca1 and Ca2) had been partially substituted with heterologous antigenic sites from either H5 or H13 HAs. Specifically, each mutant virus contained 5 or more amino acid substitutions within one antigenic site while the other four sites remained intact. The methods and description of the generation of these viruses have been previously published [98]. Viruses were kindly donated by Peter Palese. Viruses were cultured in allantoid fluid in 10-day-old embryonated chicken eggs and then tittered to confirm the growth of each virus before freezing at -80ºC. To ensure the viruses had not suffered egg-adaptative mutations, they were sequenced before their use.

Figure 31 . Culture of viruses in allantoid fluid in 10-day-old embryonated chicken eggs at Mount Sinai Hospital.

- Reassortant viruses to quantify the levels of the stalk-specific antibodies: Two reassortant viruses were used: a cH6/1N5 and a cH14/3N5. The first one had an Ha stalk derived from an H1N1 virus (A/California/04/09) containing an exotic H6 head domain (H6N1 virus A/mallard/Sweden/ 81/02) and an exotic N5 (H12N5 virus A/mallard/Sweden/86/03) and the rest of the segments derived from a PR8. HA head domains were from wild bird origin and hence no specific antibodies should be present in the patients' serum samples. The methods and description of the generation of this virus in insect cells by using a baculovirus expression system have been previously published [219–222]. The second virus had an HA stalk derived from an H3N2 virus (A/Hong Kong/4801/2014) combined with an exotic H14 head domain (A/mallard/Gurjev/263/1982) and an exotic N5 (H12N5 virus A/mallard/Sweden/86/03). To rescue the cH14/3, HA and NA sequences were obtained by gBlock DNA synthesis via IDT and cloned into pDZ plasmids by in-Fusion cloning (Takara) to obtain pDZ-cH14/3 and pDZ-N5 rescue plasmids. HEK 293T cells were transfected with pDZ-cH14/3, pDZ-N5 and pRS-6Seg PR8 [223] for 48 hours. Cells and supernatants were harvested and briefly homogenized by several syringe strokes. Two hundred microliters of the homogenized cell and supernatant mixture were injected into each 8-day old embryonated chicken egg, which was incubated at 37ºC for 2 days. Eggs were then cooled at 4ºC overnight. Allantoid fluids were harvested, and the rescue of virus was determined by HA assay. Virus was plaque-purified on MDCK cells and further amplified in 10-day old embryonated chicken eggs. Viral RNA extraction was performed using QIAamp Viral RNA mini kit. The HA and NA DNA segments were amplified using SuperScript™ III one-Step RT-PCR System with Platinum™ Taq DNA polymerase. The sequences of the HA and NA segments were confirmed by Sanger sequencing (Genewiz).

  Viruses were cultured in allantoid fluid in 10-days-old embryonated chicken eggs and then HAI was performed to confirm the growth of each virus before purification by ultracentrifugation in sucrose gradient. To do so, 5mL of 30% sucrose was added at the bottom of an ultracentrifuge tube and the virus is added slowly on top of it and centrifuge at 25,000 rpm and 4ºC for 2 hours. Then the supernatant was aspirated, and the pellet resuspended in PBS and centrifuged again at 25,000 rpm and 4ºC for 1,5 hours. Finally, the supernatant was aspirated and resuspended in

the desired volume and the virus extracted measured.

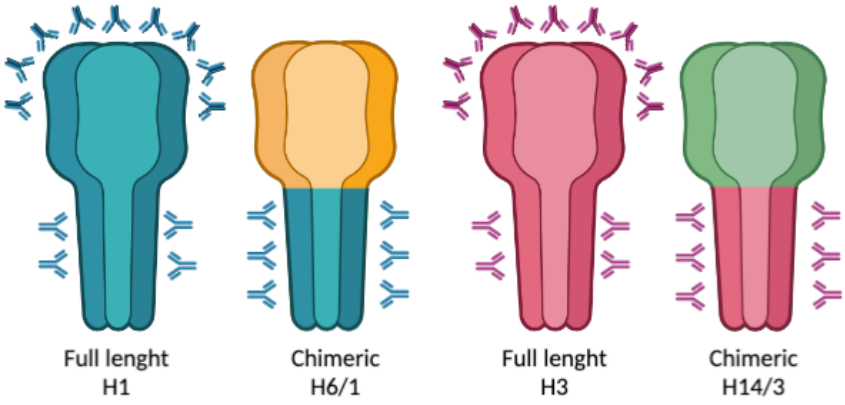


Figure 32 . Chimeric construct of reassortant viruses. Created with Biorender by Laura Sánchez-de Prada.

- Enzyme-linked Immunosorbent Assay (ELISA). Antibodies in human serum were measured as described before [224]. Flat-bottom 96-well plates (Immulon 4 HBX; Thermo Fisher Scientific) were coated with 100 µL/well of recombinant protein in phosphate-buffered saline solution (PBS; pH 7.4; Gibco, NY, USA) at a concentration of 5 mcg/mL each for cH6/1 and cH14/3 and incubated at 4ºC overnight. Next, plates were washed 3 times with washing buffer (PBS containing 0.1% Tween-20; Fisher Scientific). Plates were incubated 1.5 hours at room temperature with 220 µL/well blocking solution (washing buffer containing 0.5% non-fat powdered milk, Boston BioProducts, and 3% goat serum, GIBCO). Blocking solution was removed and 100 µL of two-fold-diluted serum samples starting from 1:800 initial dilution was added to each well and incubated for 1.5 hours at room temperature. Plates were then washed 4 times with washing buffer and 50 µL of a peroxidase-conjugated antihuman IgG (Fc-specific) monoclonal antibody (Sigma) was added at a final concentration of 1:20,000 in blocking solution. After washing, 100 µL of peroxidase substrate (3,30,5,50-Tetramethylbenzidine, TMB, Rockland) was added and incubated in the dark at room temperature for 30 min. The reaction was stopped with 4 N H2SO4 solution (ThermoFisher Scientific). The absorbance was measured at 450 nm with a plate spectrophotometer (Synergy H1 hybrid multimode

microplate reader, Biotek). Optical density (OD) for each well was calculated by subtracting the average background plus three standard deviations and area under the curve (AUC) was computed.

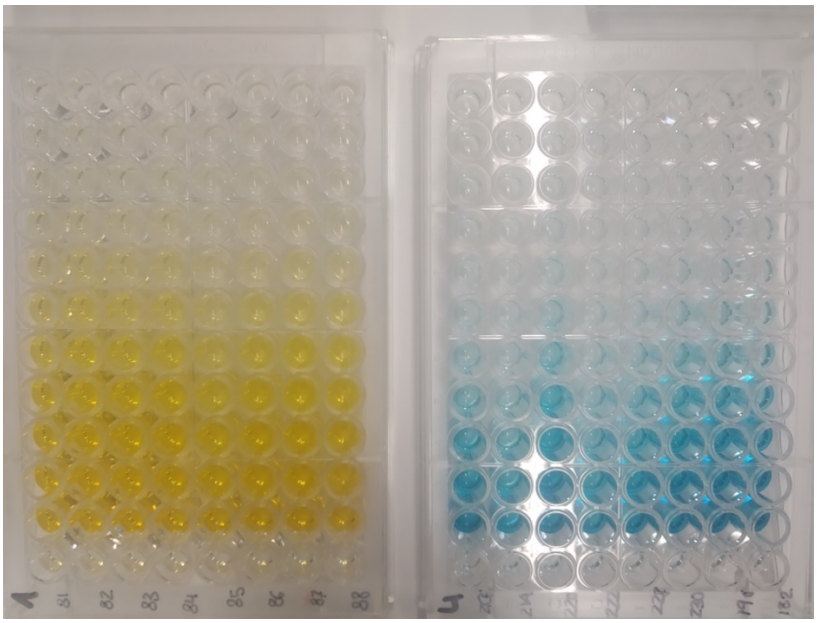


Figure 33 . ELISA plates before and after stopping the reaction with 4N H2SO4 solution.

4. STATISTICAL ANALYSIS

Population characteristics were compared using the chi-square test for categorical variables Mann-Whitney U-test for continuous variables. For HAI analysis, different parameters were calculated. Negative results in HAI were assumed as half of the detection threshold (1/5). The GMT and CI95% were computed by taking the exponent (log10) of the mean and of the lower and upper limits of the CI95% of the log10-transformed values. Fold-induction (FI) or GMT increase (GMTi) was calculated dividing the post-/pre-vaccine GMT. Seroprotection was fixed as antibodies titers to be equal or higher than 1/40. For that, Seroprotection rate (SPR) was calculated as percentage of individuals with antibody titres ≥ 1/40 and, seroconversion rate (SCR), as percentage of individuals showing at least a four-FI of pre-vaccination titres. To represent ID, we calculated two parameters: Fold induction rate (FIR) as "(FI mutant virus/FI Wt H1)x100", and HAI dominance index (DI) as the reduction of HAI titres before and after vaccination of mutant viruses compared its respective Wt H1(GMT Wt H1/GMT mutant virus)[98]. Geometric mean fold rise (GMFR) of anti-stalk antibodies

was computed by calculating the fold rise as the ratio between days 0 and 28 and then by taking the exponent (log10) of the mean fold rise and of the lower and upper limits of the CI 95% of the log10-transformed titres . For quantitative variables, comparisons were computed by repeated measures one-way Bonferroni´s ANOVA for multiple comparisons test with the Geisser-Greenhouse correction, the Brown-Forsythe and Welch ANOVA test adjusted by controlling the False Discovery Rate (FDR) with the two-stage linear procedure of Benjamini, Krieger and Yekutieli for multiple comparisons, the Wilcoxon matched pairs signed rank test and Student-T test when appropriate. For categorical variables was computed by McNemar's test with the continuity correction and by chi-square when appropriate. All reported p-values are based on two-tailed tests computed by SPSS vs 26-29 (IBM, Armonk, NY, USA) and GraphPad Prism vs 8-9 (GraphPad, San Diego, CA, USA), and taking statistical significance at the $p<0.05$ value.

6. RESULTS

MANUSCRIPT 1: ADJUVANTED INFLUENZA VACCINES ELICITS HIGHER ANTIBODY RESPONSES AGAINST THE A(H3N2) SUBTYPE THAN NON-ADJUVANTED VACCINES

Authors: Laura Sánchez de Prada, Iván Sanz Muñoz, Javier Castrodeza Sanz, Raúl Ortiz de Lejarazu Leonardo & José María Eiros Bouza

Journal: Vaccines **Date:** 25th November 2020

IMPACT FACTOR (JCR 2021)	QUARTILE	RANK	CATEGORY
4.961	Q2	59/139	Medicine, Research & Experimental

Reference: Sánchez de Prada L, Sanz Muñoz I, Castrodeza Sanz J, Ortiz de Lejarazu Leonardo R, Eiros Bouza JM. Adjuvanted Influenza Vaccines Elicits Higher Antibody Responses against the A(H3N2) Subtype than Non-Adjuvanted Vaccines. Vaccines (Basel). 2020 Nov 25;8(4):704

DOI: 10.3390/vaccines8040704

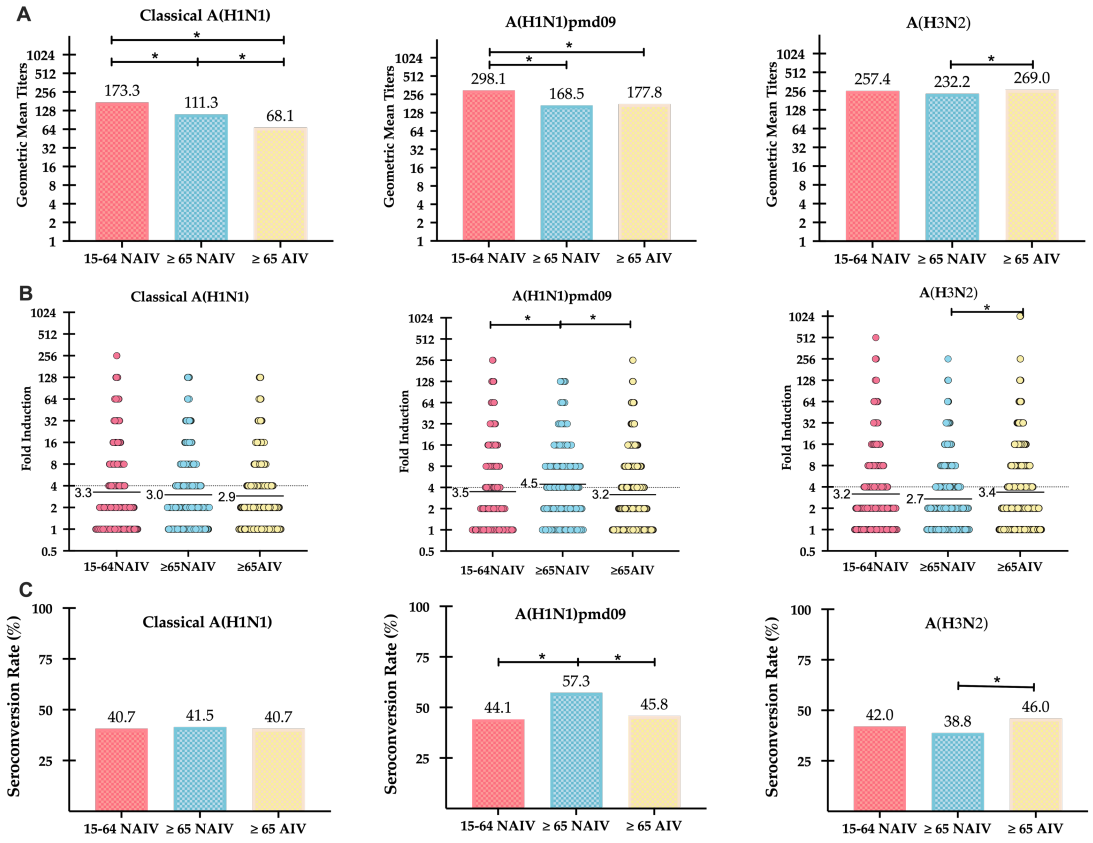
Abstract:

Background: Vaccination is the best approach to prevent influenza infections so far. Serological studies on the effect of different vaccine types are important to address vaccination campaigns and protect our population. In our study, we compared the serological response against influenza A subtypes using the non-adjuvanted influenza vaccine (NAIV) in adults and the elderly and the adjuvanted influenza vaccine (AIV) in the elderly.

Methods: We performed a retrospective analysis by hemagglutination inhibition assay (HI) of serum samples right before and 28 days after seasonal influenza vaccination during the 1996–2017 seasons.

Conclusions: The AIV presents better performance against the A(H3N2) subtype in the elderly whereas the NAIV induces a better response against A(H1N1)pdm09 in the same group

Figure 2 has been corrected and a request submitted to the journal.



MANUSCRIPT 2: DO VACCINES NEED A GENDER PERSPECTIVE? INFLUENZA SAYS YES!
Authors: Laura Sánchez-de Prada, Raúl Ortiz de Lejarazu-Leonardo, Javier Castrodeza-Sanz, Eduardo Tamayo-Gómez, José María Eiros-Bouza & Iván Sanz-Muñoz

Journal: Frontiers in immunology **Date:** 5th July 2021

IMPACT FACTOR (JCR 2021)	QUARTILE	RANK	CATEGORY
8.787	Q1	35/162	Immunology

Reference: Sánchez-de Prada L, Ortiz de Lejarazu-Leonardo R, Castrodeza-Sanz J, Tamayo-Gómez E, Eiros-Bouza JM, Sanz-Muñoz I. Do Vaccines Need a Gender Perspective? Influenza Says Yes! Front Immunol. 2021 Jul 5;12:715688.

DOI: 10.3389/fimmu.2021.715688

Abstract:

Background: Sex differences in immune responses are well known. However, the humoral response in males and females in the case of influenza vaccination is yet to be characterized since studies have shown uneven results.

Methods: A retrospective study was conducted in 2,243 individuals (46.9% males) divided by age (15–64 and ≥65 years old). A serological analysis was performed by hemagglutination inhibition assay (HI) just before and 28 days after annual vaccination against seasonal influenza viruses in people vaccinated during the 2006–2018 seasons. A comparison of the humoral responses against influenza A and B viruses contained in the vaccine, between male and female individuals in young adults and elderly was conducted.

Results: Significative higher humoral response against classical influenza A (H1N1), A (H1N1)pdm09 subtype and B/Victoria lineage in terms of seroconversion rate were found in elderly women. No significant differences were found in the case of A(H3N2) subtype.

Conclusions: Elderly women seem to display a greater humoral response against classical A(H1N1), pandemic A(H1N1)pmd09 and B/Victoria lineage than elderly men. Sex dimorphism does not affect young adults.

MANUSCRIPT 3: INFLUENZA B LINEAGES HAVE MORE IN COMMON THAN MEETS THE EYE. TRIVALENT INFLUENZA VACCINES TRIGGER HETEROTYPIC ANTIBODIES AGAINST BOTH INFLUENZA B VIRUSES

Authors: Laura Sánchez-de Prada, Silvia Rojo-Rello, Marta Domínguez-Gil, Eduardo Tamayo-Gómez, Raúl Ortiz de Lejarazu-Leonardo, José María Eiros, & Iván Sanz-Muñoz

Journal: Frontiers in Microbiology **Date:** 11th November 2021

IMPACT FACTOR (JCR 2021)	QUARTILE	RANK	CATEGORY
6.064	Q1	34/137	Microbiology

Reference: Sánchez-de Prada L, Rojo-Rello S, Domínguez-Gil M, Tamayo-Gómez E, Ortiz de Lejarazu-Leonardo R, Eiros JM, Sanz-Muñoz I. Influenza B Lineages Have More in Common Than Meets the Eye. Trivalent Influenza Vaccines Trigger Heterotypic Antibodies Against Both Influenza B Viruses. Front Microbiol. 2021 Nov 11;12:737216

DOI: 10.3389/fmicb.2021.737216

Abstract:

Influenza B is accountable for an important burden during flu epidemics, causing special impact in children and the elderly. Vaccination is the best approach to address influenza infections. However, one of the main problems of this virus is that two different lineages circulate together, Victoria and Yamagata; and trivalent vaccines, that only contain one of these lineages, are still in use. For that reason, if during an epidemic, the lineage not included in the vaccine predominates, a mismatch would occur, and the vaccine effectiveness will be very poor. In this work, we evaluated the cross-protection given by the trivalent Influenza vaccine and compared serological profiles based on age, sex, and the type of vaccine used. We performed a retrospective analysis of serum samples obtained before and after seasonal influenza vaccination during 20 seasons (1998– 2018). The results showed that heterotypic reactivity between both influenza B lineages is common, but always lower than the homologous response. Age is a relevant factor for this cross-reactivity between both lineages, while the sex and the type of vaccine not. Vaccination with trivalent influenza vaccines elicits cross-reactive antibodies against both lineages, however, this response might not be enough to provide an appropriate serological protection in case of mismatch.

MANUSCRIPT 4: IMMUNODOMINANCE HIERARCHY AFTER SEASONAL INFLUENZA VACCINATION

Authors: Laura Sánchez-de Prada, Iván Sanz-Muñoz, Raúl Ortiz de Lejarazu, José María Eiros, Adolfo García-Sastre & Teresa Aydillo

Journal: Emerging Microbes & Infection **Date:** 4th November 2022

IMPACT FACTOR (JCR 2021)	QUARTILE	RANK	CATEGORY
19.568	Q1 (D1)	9/162	Immunology

Reference: Sánchez-de Prada L, Sanz-Muñoz I, de Lejarazu RO, Eiros JM, García-Sastre A, Aydillo T. Immunodominance hierarchy after seasonal influenza vaccination. Emerg Microbes Infect. 2022 Dec;11(1):2670-2679.

DOI: 10.1080/22221751.2022.2135460

Abstract:

Current influenza vaccines elicit humoral immune responses against the haemagglutinin (HA) protein of influenza viruses. Different antigenic sites have been identified in the HA head as the main target of haemagglutination inhibition (HAI) antibodies (Sb, Sa, Cb, Ca1 and Ca2). To determine immunodominance (ID) of each site, we performed HAI assays against a panel of mutant viruses, each one lacking one of the classically defined antigenic sites and compared it to wild type (Wt). Agglutinating antibodies were measured before and after vaccination in two different regimens: Quadrivalent Influenza Vaccine (QIV) in young adults; or Adjuvanted Trivalent influenza Vaccine (ATIV) in elderly. Our results showed abs before vaccination were significantly reduced against all antigenic sites in the elderly and only against Sb and Ca2 in young adults compared to the Wt. Humoral response to vaccination was significantly reduced against all viruses compared to the Wt for the ATIV and only against Sb and Ca2 for the QIV. The strongest reduction was observed in all cases against Sb followed by Ca2. We concluded that ID profile was clearly dominated by Sb followed by Ca2. Additionally, the antibody response evolved with age, increasing the response towards less immunodominant epitopes of HA head. Adjuvants can positively influence ID hierarchy broadening responses towards multiple antigenic sites of HA head.

MANUSCRIPT 5: GROUP 1 AND GROUP 2 HEMAGGLUTININ STALK ANTIBODY RESPONSE 1 ACCORDING TO AGE

Authors: Laura Sánchez-de Prada, Iván Sanz-Muñoz, Weina Sun, Peter Palese, Raúl Ortiz de Lejarazu, José María Eiros, Adolfo García-Sastre & Teresa Aydillo

Journal: Frontiers in immunology **Date:** 29th May 2023

IMPACT FACTOR (JCR 2021)	QUARTILE	RANK	CATEGORY
8.787	Q1	35/162	Immunology

Reference: Sánchez-de Prada L, Sanz-Muñoz I, Sun W, Palese P, Ortiz de Lejarazu-Leonardo R, , Eiros JM, , García-Sastre A, Aydillo T. Group 1 and group 2 hemagglutinin stalk antibody response 1 according to age. Front Immunol. 2023. May 29;14:1194073

DOI: 10.3389/fimmu.2023.1194073

Abstract:

Objective: Antibodies elicited by seasonal Influenza vaccines mainly target the head of the hemagglutinin (HA). However, antibodies against the stalk domain are cross-reactive and have proven to play a role in reducing influenza disease severity. We investigated the induction of HA stalk-specific antibodies after seasonal influenza vaccination considering the age of the cohorts.

Methods: A total of 166 individuals were recruited during the 2018 influenza vaccine campaign (IVC) and divided in groups: <50(n=14), 50-64(n=34), 65-79(n=61) and ≥ 80 (n=57) years old. Stalk specific antibodies were quantified by ELISA at day 0 and day 28 using recombinant viruses (cH6/1 and cH14/3) containing an HA head domain (H6 or H14) from wild bird origin with a stalk domain from human H1 or H3, respectively. The Geometric mean titer (GMT) and the Fold rise (GMFR) were calculated, and differences assessed using ANOVA adjusted by the False Discovery Rate (FDR) and the Wilcoxon tests ($p<0.05$).

Results: All age groups elicited some levels of increase of anti-stalk antibodies after receiving the influenza vaccine except for ≥ 80-year-old cohort. Additionally, < 65-year-old vaccinees had higher group 1 antibody titers versus group 2 before and after vaccination. Similarly, vaccinees within the <50-year-old showed higher increase of anti-stalk antibody titers when compared to older individuals(≥ 80 years old), especially for

group 1 anti-stalk antibodies.

Conclusion: Seasonal influenza vaccine can induce cross-reactive anti-stalk antibodies against group 1 and group 2 HAs. However, low responses were observed in older groups, highlighting the impact of immunosenescence in adequate humoral immune responses.

7. DISCUSSION

In this work we intended to provide a global vision of the factors affecting humoral responses using a huge database from 28 influenza seasons. Additionally, we tried to dig further in humoral responses to influenza A viruses by studying the immunodominance of different antigenic sites of the A(H1N1)pdm09 subtype; and responses against the stalk domain of both group 1 and group 2 HA viruses in the 2018 season.

Regarding the first, our results show the following paradigms:

i) In the elderly, adjuvanted influenza vaccines induce a higher humoral response against influenza A(H3N2) subtype than standard dose vaccines (non-adjuvanted), while these non-adjuvanted vaccines induce a higher response against influenza A(H1N1)pdm09 subtype in this group. This is important because higher influenza-associated mortality has been found in population ≥ 65 years old in seasons dominated by A(H3N2) in many global and country-specific studies [125,225,226].

ii) Elderly women present better humoral responses than elderly men to influenza vaccination, especially for the A(H1N1)pdm09 subtype, while no differences are found in adult population. These results imply that there are sex differences on how vaccines work in the elderly that should be taken into account to equalize protection in both sexes.

iii) Vaccination with trivalent vaccines containing a B/Victoria lineage trigger significantly higher homologous, but also heterotypic responses to B/Victoria and B/Yamagata lineage in adults, respectively, compared to the elderly. While similar responses are found in both age groups with B/Yamagata vaccines. This may be caused by the genetic and antigenic homology of some epitopes of the haemagglutinin that triggers the development of antibodies that can recognize both B lineages. In this regard, the separation of both influenza B lineages during the 1970s may have impacted this homology, because both lineages descends from a common ancestor, the B/Lee/40 strain[227,228]. Our data reveals that trivalent vaccines induce heterologous responses that may help to induce antibodies against the other strain. And although some authors suggest that the B/Yamagata lineage has become extinct [229], vaccination with trivalent but

better with quadrivalent vaccines could help this to become a reality.

iv) The strongest reduction was observed in all cases against Sb followed by Ca2 when compared to the Wt, therefore those are most immunodominant sites. In the elderly, antibody levels were significantly reduced against all viruses compared to the Wt before vaccination while in adults only against Sb and Ca2. Additionally, the breadth of responses was wider against subdominant antigenic sites with the adjuvanted vaccine, while focused on the immunodominant Sb and Ca2 with non-adjuvanted vaccine. This recalls the importance of targeting the appropriate antigenic sites for future vaccine designs and the use of adjuvants to increase the breadth of protection. Also, these results may be useful for the design of future monoclonal antibodies that can be useful for treating the flu.

v) Pre-existing HA stalk immunity against phylogenetic group 1 is higher in younger populations and seasonal influenza vaccines can moderately boost cross-reactive antibody responses against the stalk domain of both group 1 and group 2 HA viruses. Thus, despite the moderate response of actual vaccines, is not high enough to provide long-lasting, non-strain-specific protection.

As previously mentioned, factors affecting humoral responses to vaccination involve individual factors as biological sex and age, but also vaccine factors like the presence of adjuvants or multiple influenza strains, subtypes, and lineages. First of all, biological sex, so popular these days, have been neglected for so long that a systematic review found no clear conclusion could be extracted regarding the effect of sex on the immunogenicity and effectiveness of seasonal influenza vaccine, and insisted in the need of higher quality evidence [230]. Women have been described to have more adverse events after seasonal influenza vaccination due to the effects of sex hormones on immune cells, genetic factors, and microbiota; but also higher antibody responses towards vaccination due to the same factors [231]. Many studies agree that older females have better responses than older males to seasonal vaccination including lower hospitalization and mortality rates [232] and higher HAI titers [194,233]. However, responses in adults do not account for sex in terms of efficacy of humoral response [234]. Actually, these findings are not restricted to humoral responses, but also involve

expression levels of genes related to T, NK and B cells after vaccination [235]. Here, the term immunosenescence acquires its full sense, as it impairs antibody avidity and B and T cell responses to vaccination as the age increases [190,191]. Age plays a role as a factor affecting humoral responses and here, we could be looking at a more intense declination of immune responses in males. However, this is not the only aspect involving age as a factor. It has been described how first encounters with influenza virus, whether by infection or vaccination, shape the long-lived subtype-specific responses, while subsequent exposures can determine the magnitude of these responses[224]. This phenomenon, commonly known as "original antigenic sin" or "antigenic seniority" [120]. In the assessment of anti-stalk antibodies, we represented antibody levels based on the birth year against both phylogenetic groups. Elderly population (≥ 65 years old), independently of the season studied, would have quite confidently been imprinted with a phylogenetic group 1 HAs virus (as born before 1953). However, our analysis by birth year, did not show a pattern according to the likely first exposure to either HA group of viruses in our age groups. However, the group of 50-65 years old who could have first encountered A(H2N2) had the higher pre-existing immunity while the elderly (≥ 65 years old) showed unexpected results with lower baseline anti-stalk antibody levels against this group and similar to those against HA group 2 levels which could be explained by immunosenescence in the elderly population. Regarding differences between both phylogenetic groups, on one hand, exposures to more divergent strains are more likely to trigger cross-reactive antibodies against more conserved epitopes, such as the stalk domain [236]. On the other hand , immune imprinting induces immune memory to both conserved and variable sites on different influenza virus antigens [216,237]. Additionally, antibody responses to A(H3N2) antigens have been described to be lower in magnitude compared to those induced by A(H1N1) antigens as a result of preferentially driven immune response towards previously encountered antigens in polyvalent seasonal influenza vaccines [238–240]. This leads to the concept of immunodominance, which describes the strong tendency of the immune response to respond in a hierarchical manner, with higher involvement of "immunodominant" antigens that potentially suppress responses to "subdominant" antigens[97]. The study of immunodominance hierarchy against influenza A(H1N1)pdm09 virus shows that inside the same domain of the HA, meaning

the head, we can find 5 different antigenic sites (Sa, Sb, Cb, Ca1 and Ca2) that display different immune dominance. Sb and Ca2 appeared to be the most immunodominant sites. However, age understood as exposure to multiple and diverse influenza viruses, could enhance the breadth of humoral responses. This could take place maybe in response to an allosteric effect of competition of antibodies for the same antigenic site[241]. In addition, our findings suggest adjuvants increase the breadth of protection by increasing antibody response towards all antigenic sites instead of restricted to dominant ones (Sb and Ca2). Similar results showed that adjuvanted vaccines could expand the antibody repertoire and increase avidity against HA head sites [242]. Conversely, we should bear in mind that immune dominance is not only found between different domains or antigenic sites of the same protein, but also to a subtype level [239]. Thus, the role of adjuvants in overcoming immunodominance, could explain why higher responses with adjuvanted vaccines against influenza A(H3N2) subtypes were found, while higher responses against A(H1N1)pdm09 with non-adjuvanted vaccines were observed, when assessing responses to different vaccine types in the elderly [243].

Regarding Influenza B viruses, our results showed higher homologous and heterologous responses when using vaccines containing B/Victoria lineages in adults. Previous studies have shown that B/Victoria viruses undergo faster rates of lineages turnover with evolution acting predominantly in the immunodominant HA protein, in contrast to B/Yamagata lineages with slower antigenic mutation rates that are more focused on the NA protein [244]. In addition, it has also been described that B/Victoria lineages may induce a broader immune response and present subtype immunodominance when formulated in quadrivalent vaccines [239,245]. Regarding the higher HAI levels against B/Yamagata lineage before and after vaccination, it has been suggested that the ether extraction exposes immunodominant conserved epitopes that are naturally inaccessible in the native protein [239]. This theory should be further studied but would explain why responses are not higher when the HAI is done by using native virus instead of ether extracted virus.

8. CONCLUSIONS

The main conclusions of these studies are:

1. Seasonal adjuvanted influenza vaccine induces higher antibody responses against the A(H3N2) subtype, whereas the non-adjuvanted influenza vaccine induces a better humoral response against A(H1N1)pdm09 in the elderly.

2. Elderly women display a greater humoral response against pandemic A(H1N1)pdm09 than elderly men. Sex dimorphism does not affect responses in young adults.

3. Vaccination with a trivalent influenza vaccine provides cross-reactive protection against the B/lineage not contained in the vaccine. However, that heterotypic response produced is considerably low compared to the homotypic response.

4. Immunodominance profile of classical antigenic sites of the HA head of influenza A(H1)pdm09 subtype viruses is clearly dominated by Sb antigen followed by Ca2.

5. Adjuvants can help overcome immunodominance hierarchy by broadening responses towards subdominant antigenic sites of HA head but also subtypes and strains.

6. Seasonal influenza vaccine can boost the induction of cross-reactive anti-stalk antibodies against phylogenetic groups 1 and 2 of HAs, with higher responses in younger populations.

7. Immunosenescence affects vaccine humoral responses against all A and B influenza viruses tested. However, the decline of pre-existing antibodies affects especially to the influenza A(H1) subtype viruses, probably due to being the first encounter with influenza viruses in life of the elderly population studied.

8. Immunodominance, imprinting and previous exposures are factors influencing preferential responses to some subtypes or strains over others and can be influenced by immunosenescence and adjuvants. This should be considered for future vaccine designs.

9. REFERENCES

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10. APPENDIX

APPENDIX 1

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COMITÉ DE ÉTICA DE LA INVESTIGACIÓN CON MEDICAMENTOS
ÁREA DE SALUD VALLADOLID

Valladolid a 3 de junio de 2021

En la reunión del CEIm ÁREA DE SALUD VALLADOLID ESTE del 3 de junio de 2021, se procedió a la evaluación de los aspectos éticos del siguiente proyecto de investigación.

PI 21-2314	VIGILANCIA VIROLÓGICA Y SEROEPIDEMIOLOGICA DE LA GRIPE EN LA POBLACIÓN CASTELLANO-LEONESA	I.P.: IVÁN SANZ MUÑOZ EQUIPO: JOSE MARÍA EIROS BOUZA, LAURA SANCHEZ PRADA, DIANA PEREZ SAN JOSE, RAUL ORTIZ DE LEJARAZU CENTRO NACIONAL DE GRIPE

A continuación, les señalo los acuerdos tomados por el CEIm ÁREA DE SALUD VALLADOLID ESTE en relación a dicho Proyecto de Investigación:

Considerando que el Proyecto contempla los Convenios y Normas establecidos en la legislación española en el ámbito de la investigación biomédica, la protección de datos de carácter personal y la bioética, se hace constar el **informe favorable** y la **aceptación** del Comité de Ética de la Investigación con Medicamentos Área de Salud Valladolid Este.

Un cordial saludo.

[redacted]

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APPENDIX 2

Abstracts in International congresses

- STUDY OF THE HUMORAL RESPONSE OF FRACTIONATED AND ADJUVANTED INFLUENZA VACCINES IN THE ELDERLY.

  Laura Sánchez, Iván Sanz, Sonia Tamames, Ana López, Silvia Rojo, Raúl Ortiz de Lejarazu.

  XXIX EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (ECCMID). AMSTERDAM 13-16 APRIL 2019

  Oral presentation

- STUDY OF SEROPROTECTION OF ADULTS AND ELDERLY AGAINST INFLUENZA A VIRUSES DURING 28 CONSECUTIVE INFLUENZA SEASONS.

  Laura Sánchez, Iván Sanz, Ana López, Silvia Rojo, Raúl Ortiz de Lejarazu.

  XXIX EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (ECCMID). AMSTERDAM 13-16 APRIL 2019

  Poster presentation

- STUDY OF SEROPROTECTION OF ADULTS AND ELDERLY AGAINST INFLUENZA B VIRUSES DURING 24 CONSECUTIVE INFLUENZA SEASONS.

  Laura Sánchez, Iván Sanz, Ana López, Silvia Rojo, Raúl Ortiz de Lejarazu.

  XXIX EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (ECCMID). AMSTERDAM 13-16 APRIL 2019

  Poster presentation

- INFLUENCE OF ADJUVANTS AFTER INFLUENZA VACCINATION IN THE IMMUNODOMINANCE HIERARCHY.

  Laura Sánchez De Prada, Iván Sanz, Raúl Ortiz De Lejarazu, Jose María Eiros, Adolfo Garcia-Sastre, Teresa Aydillo.

  8TH EUROPEAN SCIENTIFIC WORKING GROUP OF INFLUENZA (ESWI) INFLUENZA CONFERENCE. 4-7 DECEMBER 2021

  Oral presentation

- STUDY OF THE HUMORAL RESPONSE AGAINST ADJUVANTED AND NON-ADJUVANTED INFLUENZA VACCINE IN THE ELDERLY BY AGE GROUPS.

Laura Sánchez De Prada, Iván Sanz, Sonia Tamames, Ana Lopez, Jose Manuel Méndez-Legaza, Silvia Rojo, Raúl Ortiz De Lejarazu, Jose María Eiros.

XXX EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (ECCMID). BOOK OF ABSTRACTS (COVID PANDEMIC 2020).

Poster presentation

- STUDY OF THE SEROLOGICAL EFFICACY OF INFLUENZA VACCINE ALONG 28 CONSECUTIVE SEASONS.

Laura Sánchez De Prada, Iván Sanz, Sonia Tamames, Ana Lopez, Jose Manuel Méndez-Legaza, Silvia Rojo, Raúl Ortiz De Lejarazu, Jose María Eiros.

XXX EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (ECCMID). BOOK OF ABSTRACTS (COVID PANDEMIC 2020)

Poster presentation

- DOES VACCINES NEEDS A GENDER PERSPECTIVE? INFLUENZA SAYS YES!

Laura Sánchez De Prada, Iván Sanz, Sonia Tamames, Ana Lopez, Silvia Rojo, Raúl Ortiz De Lejarazu, Jose María Eiros.

XXX EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (ECCMID). BOOK OF ABSTRACTS (COVID PANDEMIC 2020)

Poster presentation

- IMMUNODOMINANCE HIERARCHY AFTER SEASONAL INFLUENZA VACCINATION.

Laura Sánchez De Prada, Iván Sanz, Raúl Ortiz De Lejarazu, Jose María Eiros, Adolfo Garcia-Sastre, Teresa Aydillo

XXX EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (ECCMID). BOOK OF ABSTRACTS (COVID PANDEMIC 2020)

Poster presentation

- NEURAMINIDASE ANTIBODY RESPONSE IN A POPULATION VACCINATED WITH SPLIT AND ADJUVANT INFLUENZA VACCINES

Jose Manuel Méndez Legaza, Iván Sanz, Raúl Ortiz De Lejarazu, Laura Sánchez De Prada.

XXX EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (ECCMID). BOOK OF ABSTRACTS (COVID PANDEMIC 2020)

Poster presentation

- COMPARISON OF HUMORAL RESPONSE AGAINST BOTH HAEMAGGLUTININ AND NEURAMINIDASE AFTER SEASONAL INFLUENZA VACCINATION.

Jose Manuel Méndez Legaza, Iván Sanz, Raúl Ortiz De Lejarazu, Laura Sánchez De Prada.

XXX EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES (ECCMID). BOOK OF ABSTRACTS (COVID PANDEMIC 2020)

Poster presentation

- THE DETERMINED RESPONSE TO VACCINATION WITH B/LINEAGES IN TRIVALENT INFLUENZA VACCINES

Laura Sánchez De Prada, Diana Peréz San José, Silvia Rojo Rello, Raúl Ortiz De Lejarazu Leonardo, José María Eiros Bouza, Iván Sanz Muñoz.

XXXI EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES ((ECCMID)). ONLINE CONGRESS 9-12 JULY 2021.

Poster presentation

- STALK ANTIBODIES ELICITED AFTER SEASONAL INFLUENZA VACCINATION

Laura Sánchez De Prada, Iván Sanz, Raúl Ortiz De Lejarazu, Jose María Eiros, Adolfo Garcia-Sastre, Teresa Aydillo

XXXIII EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES ((ECCMID)). COPENHAGUE 15-18 APRIL 2023

Poster presentation. Top rated poster 2023.

Abstracts in national congresses

- ESTUDIO DE SEROPROTECCIÓN FRENTE A VIRUS DE LA GRIPE A EN ADULTOS Y MAYORES DE 65 AÑOS DURANTE 28 TEMPORADAS CONSECUTIVAS

Laura Sánchez, Iván Sanz, Sonia Tamames, Ana López, Silvia Rojo, Raúl Ortiz de Lejarazu.

XXIII CONGRESO NACIONAL DE ENFERMEDADES INFECCIOSAS Y MICROBIOLOGÍA CLÍNICA (SEIMC). MADRID 23-25 MAY 2019.

Poster presentation

- ESTUDIO DE SEROPROTECCIÓN FRENTE AL VIRUS DE LA GRIPE B EN ADULTOS Y MAYORES DE 65 AÑOS DURANTE 24 TEMPORADAS CONSECUTIVAS

Laura Sánchez, Iván Sanz, Sonia Tamames, Ana López, Silvia Rojo, Raúl Ortiz de Lejarazu.

XXIII CONGRESO NACIONAL DE ENFERMEDADES INFECCIOSAS Y MICROBIOLOGÍA CLÍNICA (SEIMC). MADRID 23-25 MAY 2019.

Poster presentation

- ESTUDIO DE LA EFICACIA SEROLÓGICA DE LA VACUNA DE GRIPE DURANTE 28 TEMPORADAS.

Laura Sánchez De Prada, Iván Sanz Muñoz, Jose Méndez Legaza, Sonia Belén Paredes Gómez, Elena Cantón Benito, Ana María Martínez García, Raúl Ortiz De Lejarazu Leonardo, Silvia Rojo Rello, José María Eiros Bouza.

XXIV CONGRESO NACIONAL DE ENFERMEDADES INFECCIOSAS Y MICROBIOLOGÍA CLÍNICA (SEIMC). SEPTEMBER 2020

Poster presentation

- IMPORTANCIA DEL SEXO EN LA RESPUESTA VACUNAL FRENTE A GRIPE.

Laura Sánchez De Prada, Iván Sanz Muñoz, Jose Méndez Legaza, Sonia Belén Paredes Gómez, Elena Cantón Benito, Ana María Martínez García, Raúl Ortiz De Lejarazu Leonardo, Silvia Rojo Rello, José María Eiros Bouza

XXIV CONGRESO NACIONAL DE ENFERMEDADES INFECCIOSAS Y MICROBIOLOGÍA CLÍNICA (SEIMC). SEPTEMBER 2020

Poster presentation

- ESTUDIO DE LA INMUDOMINANCIA DE LOS EPÍTOPOS DE LA CABEZA DE LA HEMAGLUTININA TRAS LA VACUNACIÓN FRENTE A GRIPE CON VACUNA ADYUVADA Y SIN ADYUVANTE

Laura Sánchez De Prada, Iván Sanz Muñoz, Raúl Ortiz De Lejarazu Leonardo, José María Eiros Bouza, Adolfo García Sastre, Teresa Aydillo Gómez.

XXIV CONGRESO NACIONAL DE ENFERMEDADES INFECCIOSAS Y MICROBIOLOGÍA CLÍNICA (SEIMC). SEPTEMBER 2020

Poster presentation

- ESTUDIO COMPARATIVO ENTRE LA RESPUESTA SEROLÓGICA FRENTE A HEMAGLUTININA Y NEURAMINIDASA DE VIRUS DE LA GRIPE.

Jose Manuel Méndez Legaza, Iván Sanz Muñoz, Laura Sánchez De Prada, Raúl Ortiz De Lejarazu Leonardo.

XXIV CONGRESO NACIONAL DE ENFERMEDADES INFECCIOSAS Y MICROBIOLOGÍA CLÍNICA (SEIMC). SEPTEMBER 2020

Poster presentation