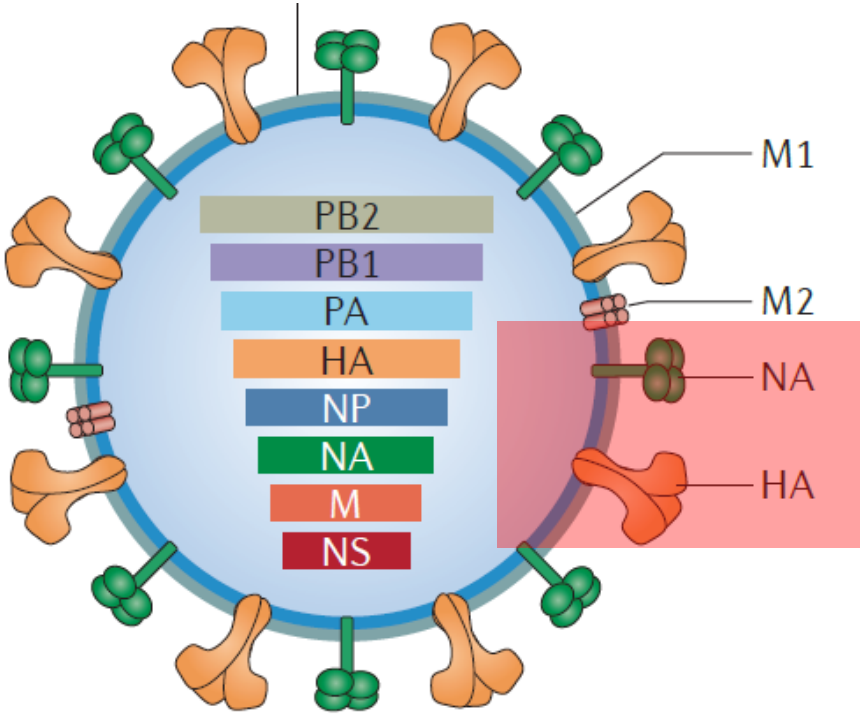
Marcatori molecolari di aumentata patogenicità
(mutazioni e riassortimenti)

FOCUS ON:
INFLUENZA 2017-2018

Piralla Antonio, PhD

Influenza A – genome and structure



Shi et al. Nature, 2014

Table 1: Influenza A virus genes and proteins

Gene ID	Segment	Protein name	Protein function
1	Polymerase B2 (PB2)	Polymerase B2 (PB2)	Internal protein, virus replication
2	Polymerase B1 (PB1)	Polymerase B1 (PB1)	Internal protein, virus replication
		PB1-F2	Mitochondrial targeting and apoptosis
3	Polymerase A (PA)	Polymerase A (PA)	Internal protein, virus replication
4	Hemagglutinin (HA)	Hemagglutinin (HA)	Surface glycoprotein, viral attachment, antigenic determinant, subtype specific (H1 through H16)
5	Nucleoprotein (NP)	Nucleoprotein (NP)	Nucleocapsid protein, RNA coating, nuclear targeting, RNA transcription, type (A,B,C) specific
6	Neuraminidase (NA)	Neuraminidase (NA)	Surface glycoprotein, antigenic determinant, viral release from host cells, subtype specific (N1 through N9)
7	Matrix (M)	Matrix 1 (M1)	Membrane protein stability, type (A,B,C) specific
		Matrix 2 (M2)	Membrane protein, viral uncoating, type (A,B,C) specific
8	Non-structural (NS)	Non-structural 1 (NS1)	Internal proteins
		Non-structural 2 (NS2)	Regulation of virus life cycle, especially mRNA transcription and localization of viral ribonucleic proteins

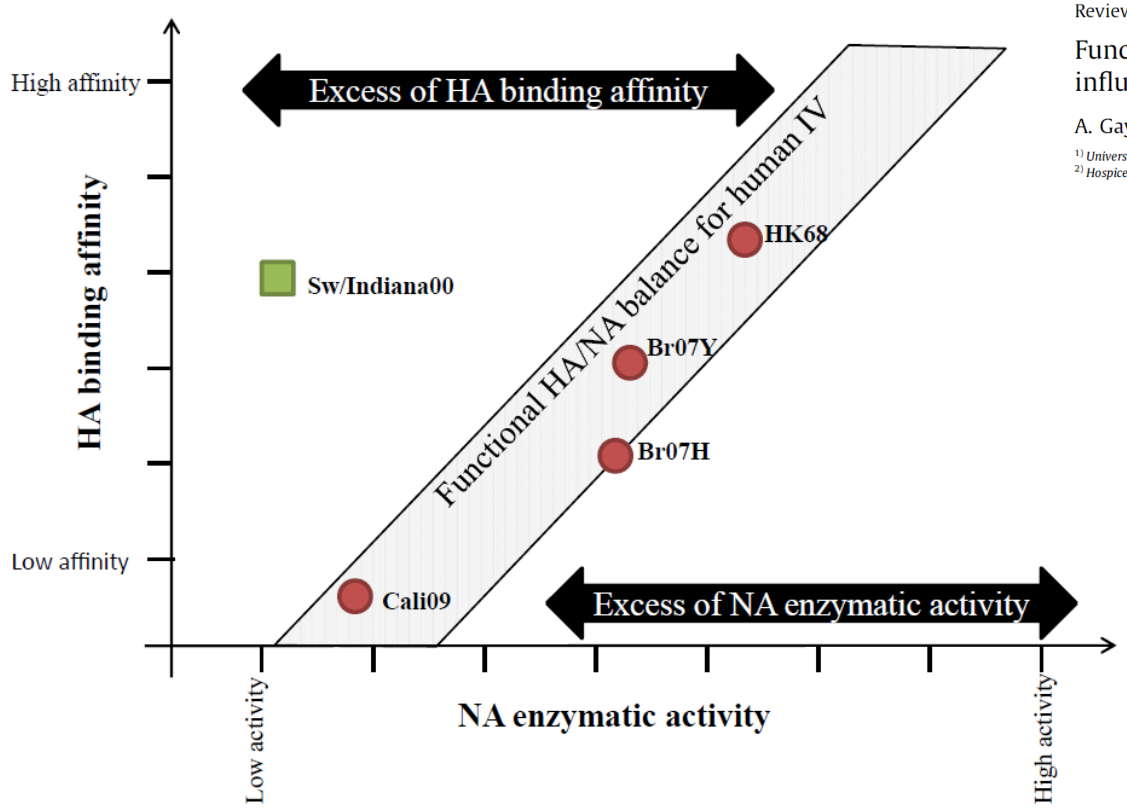
Adapted from Lamb and Krug, 2001.[2]

The FluA contains two major transmembrane glycoproteins (**HA** and **NA**). The antigenic and genetic diversity of these two glycoproteins are used to determine the IAV subtype. **At present, 18 hemagglutinin subtypes (H1–H18) and 11 neuraminidase subtypes (N1–N11) are recognized.**

Key concepts on HA and NA

- These two glycoproteins recognize the same host cell molecule: the sialic acid (SA) (generic term for the N- or O substituted derivatives of neuraminic acid) and have a complementary role in the replication cycle.
- The **HA** initiates the virus entry by binding to the SA and the **NA** facilitates the virion release from infected cells through its sialidase activity.
- **These two glycoproteins are also linked to infectivity, transmissibility, virulence, host specificity and resistance to antiviral treatment.**
- **The equilibrium between the HA binding affinity and the NA enzymatic activity, also called the HA/NA functional balance, has to be optimal for good viral fitness (i.e. an efficient replication and transmission).**

HA/NA balance



Clinical Microbiology and Infection 22 (2016) 975–983

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

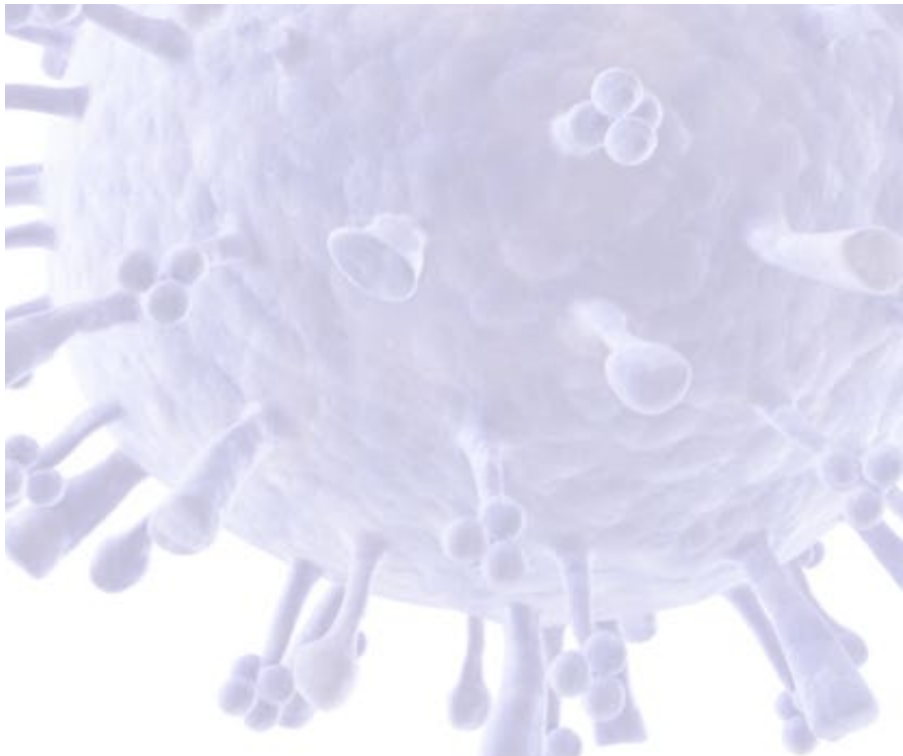
journal homepage: www.clinicalmicrobiologyandinfection.com

Review

Functional balance between neuraminidase and haemagglutinin in influenza viruses

A. Gaymard [1, 2, 3], N. Le Briand [1, 3], E. Frobert [1, 2], B. Lina [1, 2], V. Escuret [1, 2, *]

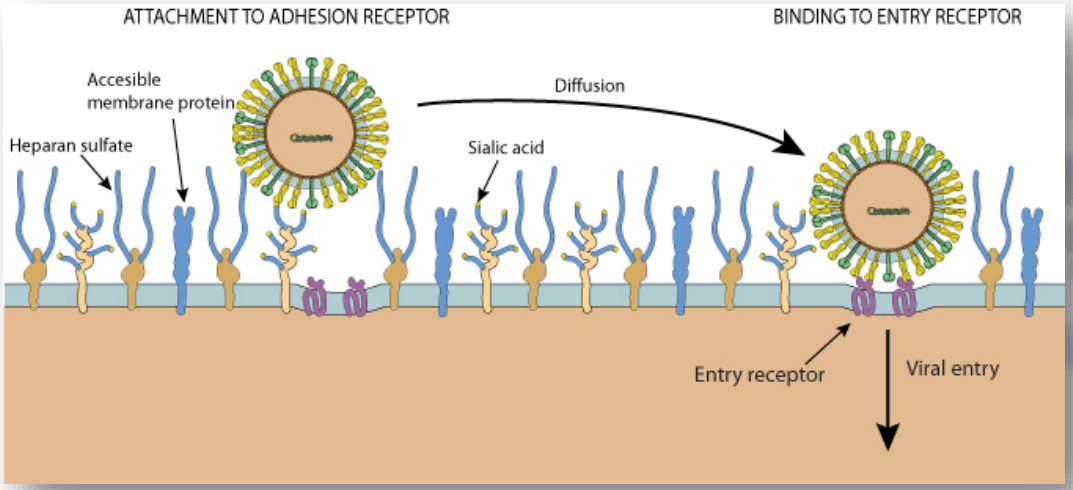
[1] Université Lyon, Université Lyon 1, Faculté de Médecine Lyon Est, CIRI Inserm U1111, équipe Virpath, Lyon, France
[2] Hospices Civils de Lyon, Centre National de Référence virus influenzae France Sud, Laboratoire de Virologie, Groupement Hospitalier Nord, Lyon, France

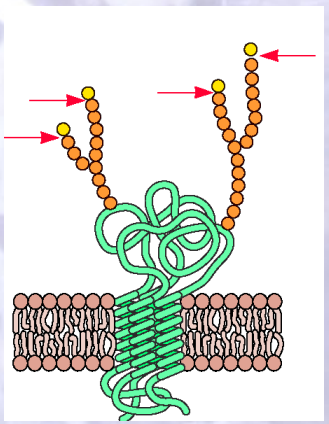


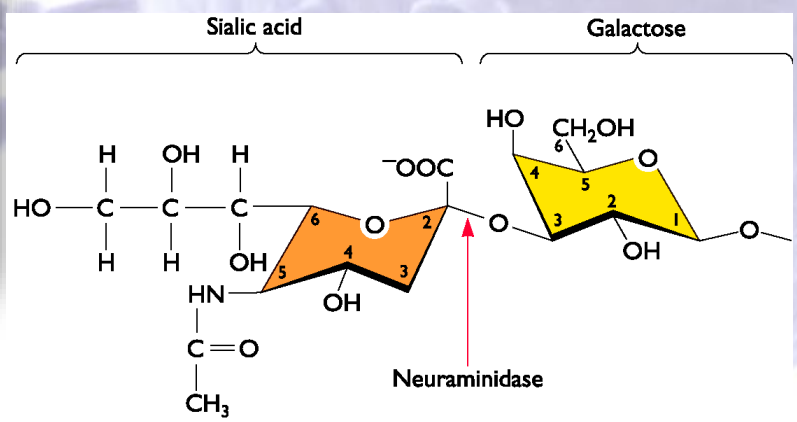
The balance between HA affinity and NA activity as a critical factor in **host adaptation**.

HA role on receptor attachment – sialic acid

- ✓ Binds to cell surface carbohydrate - sialic acid
- ✓ Ubiquitous receptor
- ✓ Can be present as part of glycoprotein or glycolipid







HA has an important role in determining host tropism, as it binds to host cell receptors that contain terminal α-2,6-linked or α-2,3-linked sialic acid (α-2,6-SA or α-2,3-SA) moieties.

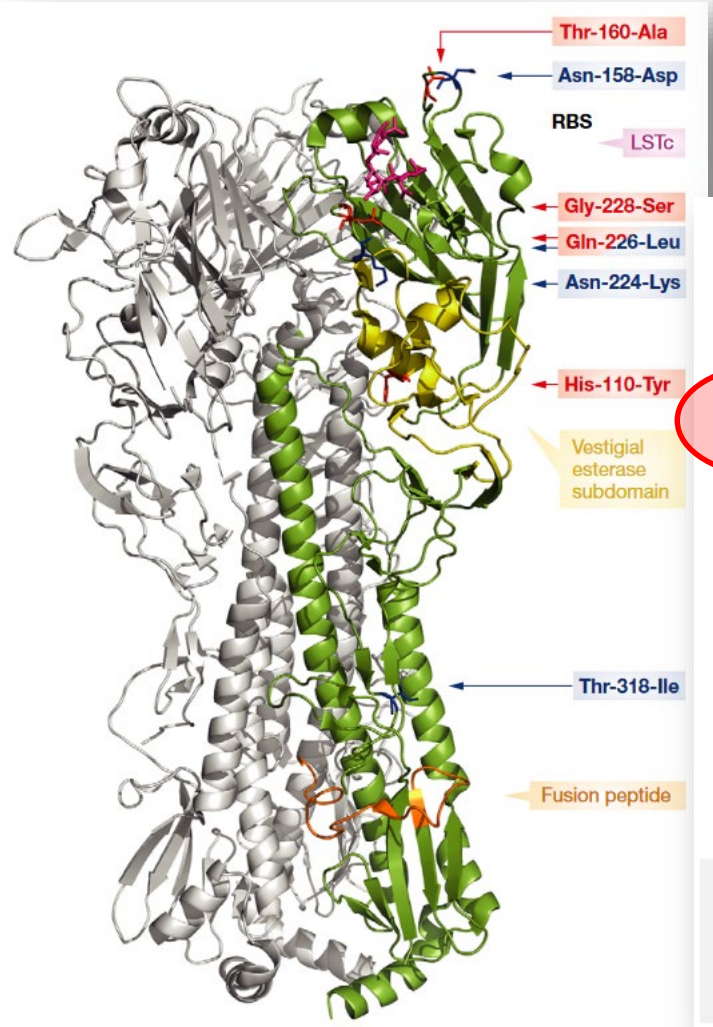
Published online: March 25, 2014

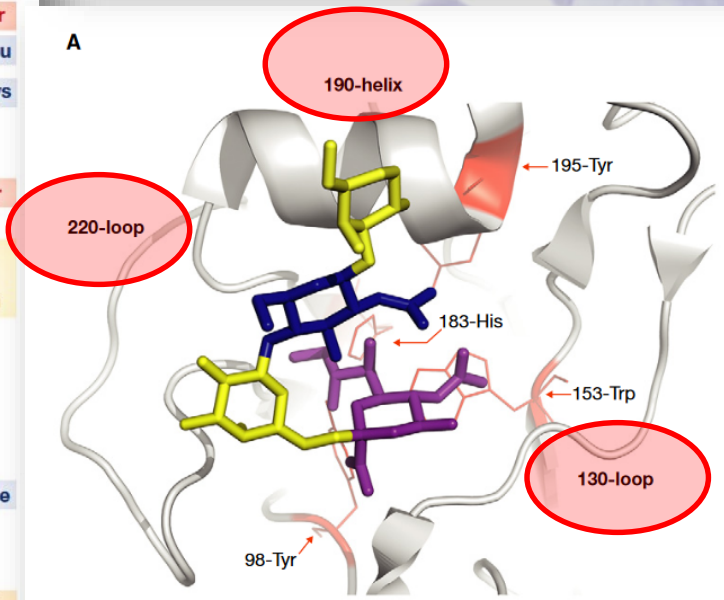
THE EMBO JOURNAL

Review

Role of receptor binding specificity in influenza A virus transmission and pathogenesis

Miranda de Graaf & Ron A M Fouchier*





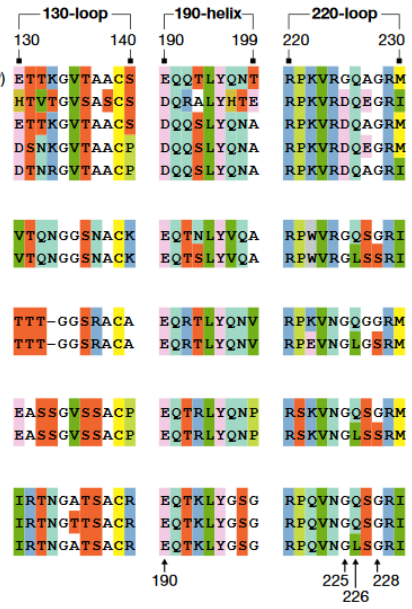
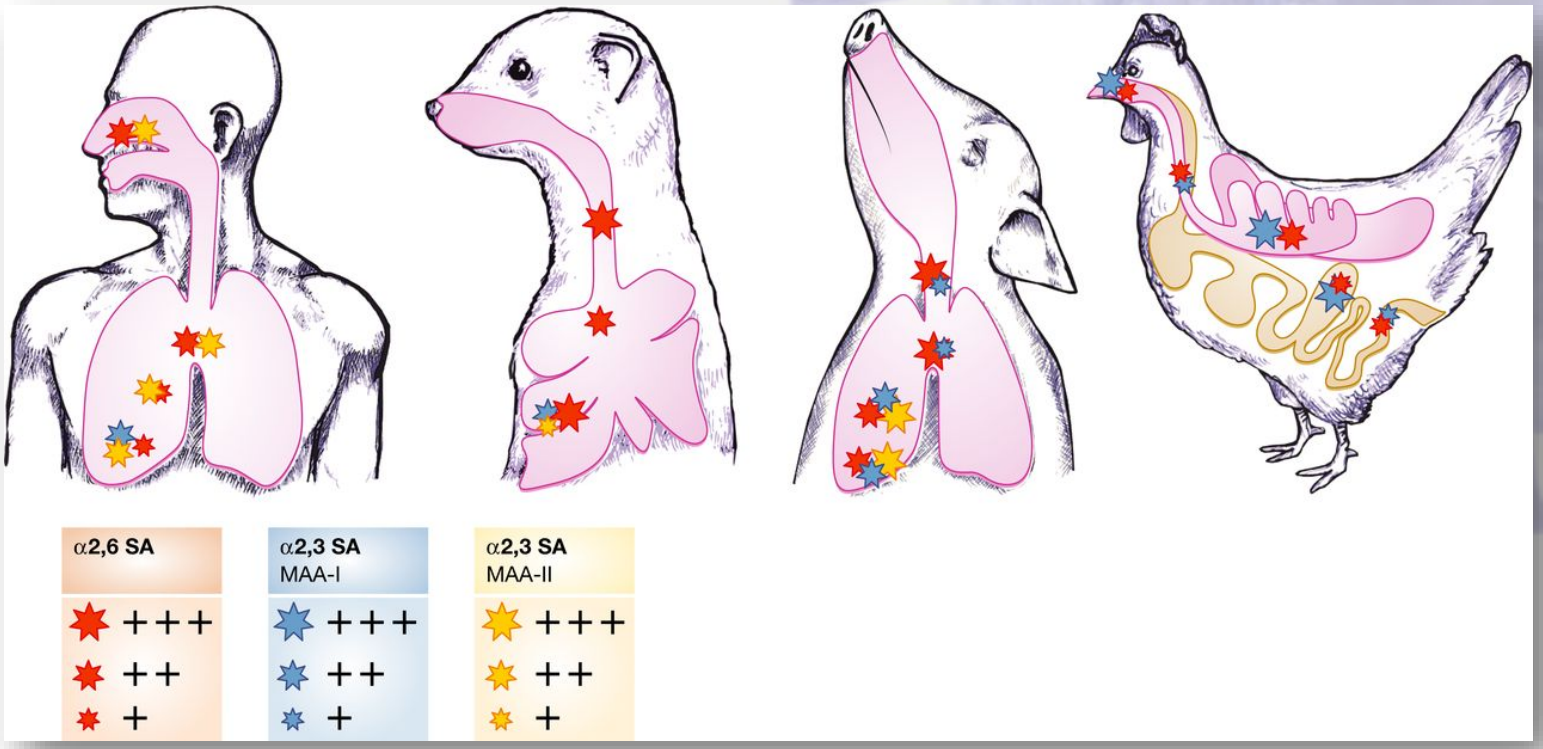


Figure 5. Cartoon representation of a human influenza hemagglutinin (database code 2YP4) receptor binding site with the 130-loop, 190-helix, and 220-loop and the conserved residues, 98-Tyr, 153-Trp, 183-His, and 195-Tyr in complex with the human receptor analog LSTc in *cis* conformation (A). Amino acid alignment of the 130-loop, 190-helix, and 220-loop of human (Hu), avian (Av), pig (Pi) influenza viruses, and an airborne transmissible H5N1 virus (Tr) (B). The sequences are derived from the following virus strains: A/Teal/NL/10/2000 (CY060178), A/Memphis/7/2001 (CY020149), A/SC/1/1918 (F116575), A/Mallard/SW/50/2000 (CY060308), A/HK/01/1968 (CY112249), A/Mallard/NL/5/1999 (CY064950), A/NL/056H1/1960 (CY077786), A/Indo/5/2005 (CY116646), airborne transmissible A/Indo/05/2005 (CY116686), A/Mallard/12/2000 (GU053030), A/NL/219/2003 (AY338459) and A/Shanghai/02/2013 (KF021597).

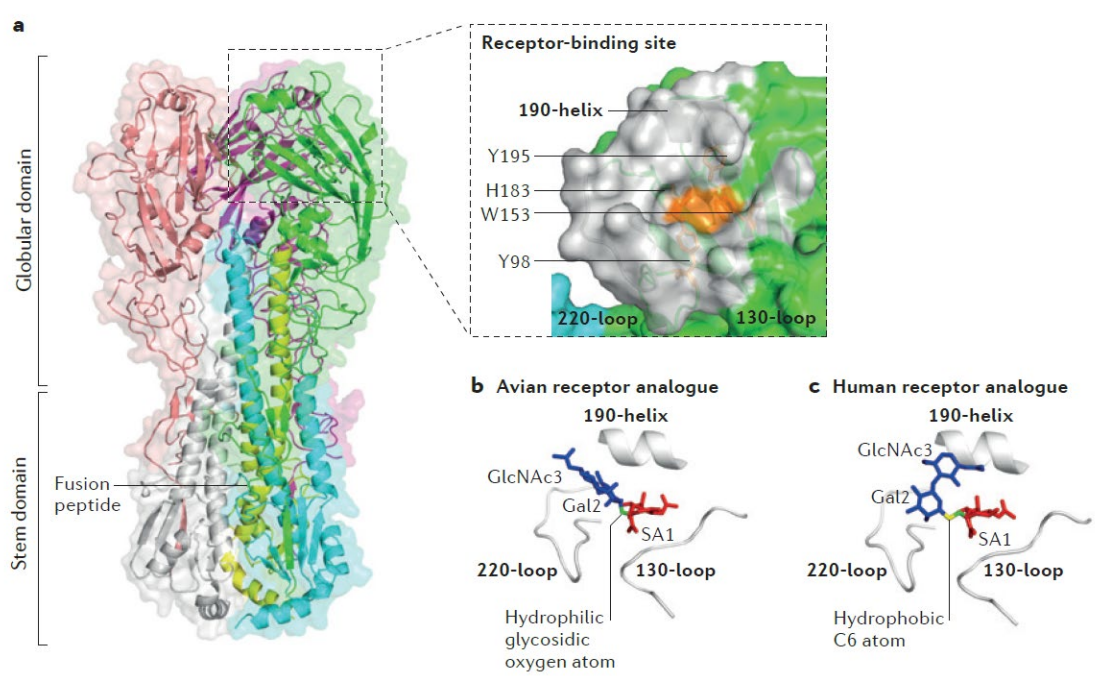
Receptor specificity involved on pathogenicity

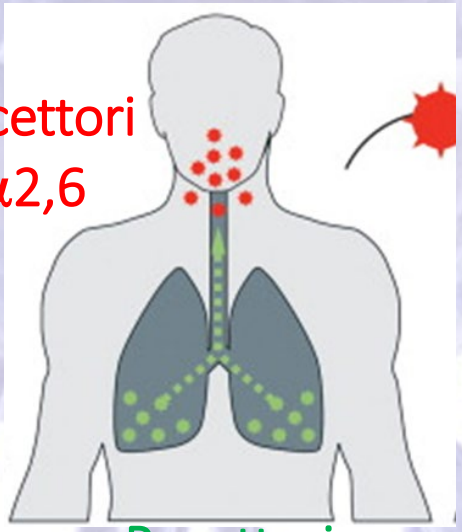
- The HA specificity for a 2,3 or a 2,6 SA depends on the HA origin.
- The HA of human-adapted influenza preferentially recognizes receptors with a terminal a 2,6 SA whereas avian influenza preferentially recognizes receptors with a terminal a 2,3 SA.



Receptor specificity involved on pathogenicity

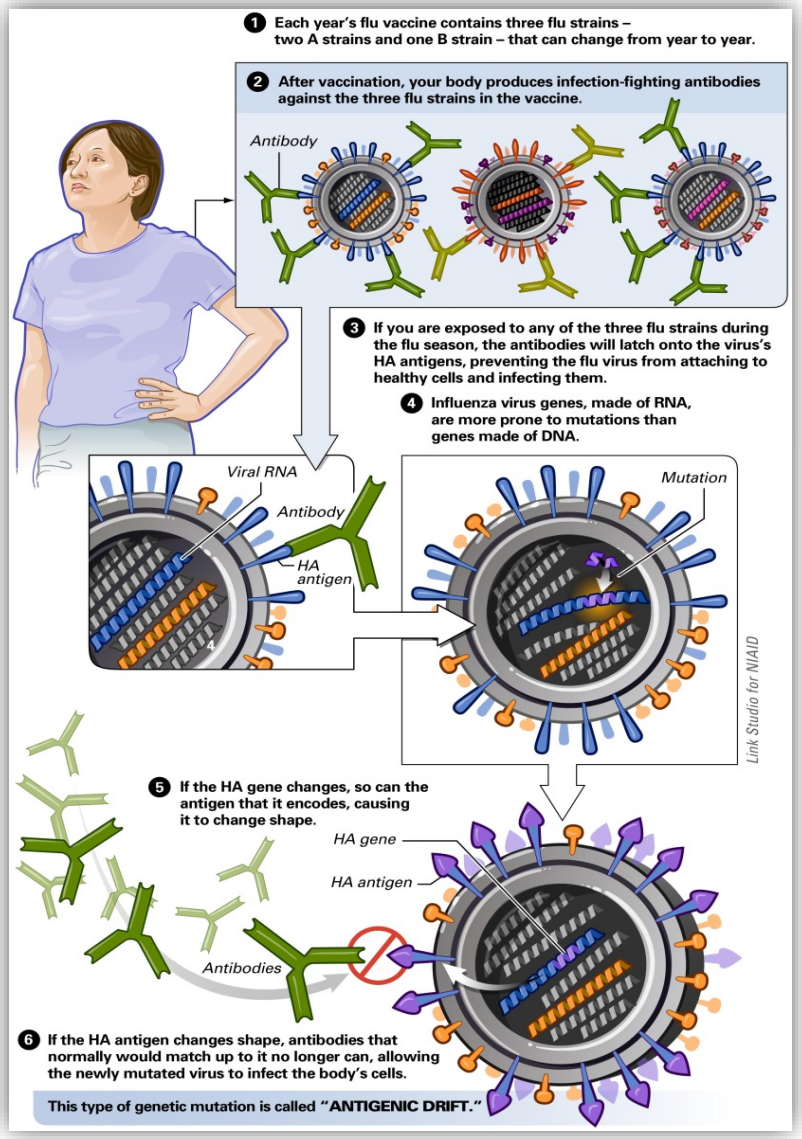
- The predominance of the a 2,6 SA in the upper respiratory tract of humans is likely to contribute to the limited avian to human transmission.
- It has also been suggested that mucus in the human airway is rich in soluble a 2,3 SA, which can trap the avian viruses and inhibit replication and spread.

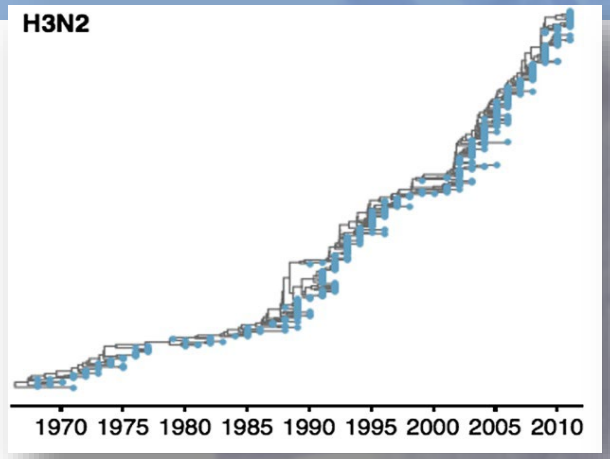


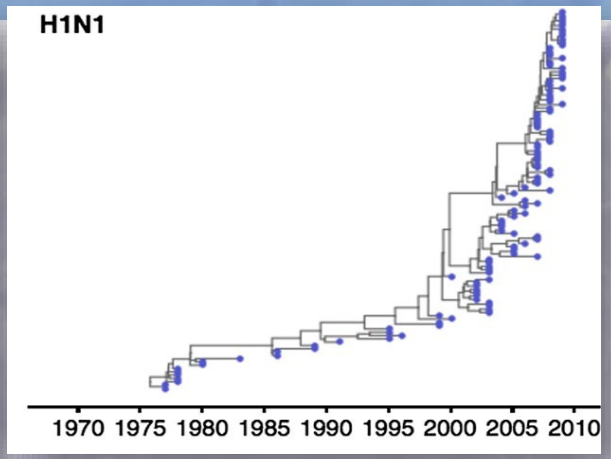


Shi et al. Nature Reviews, 2014

Evolution – Genetic drift



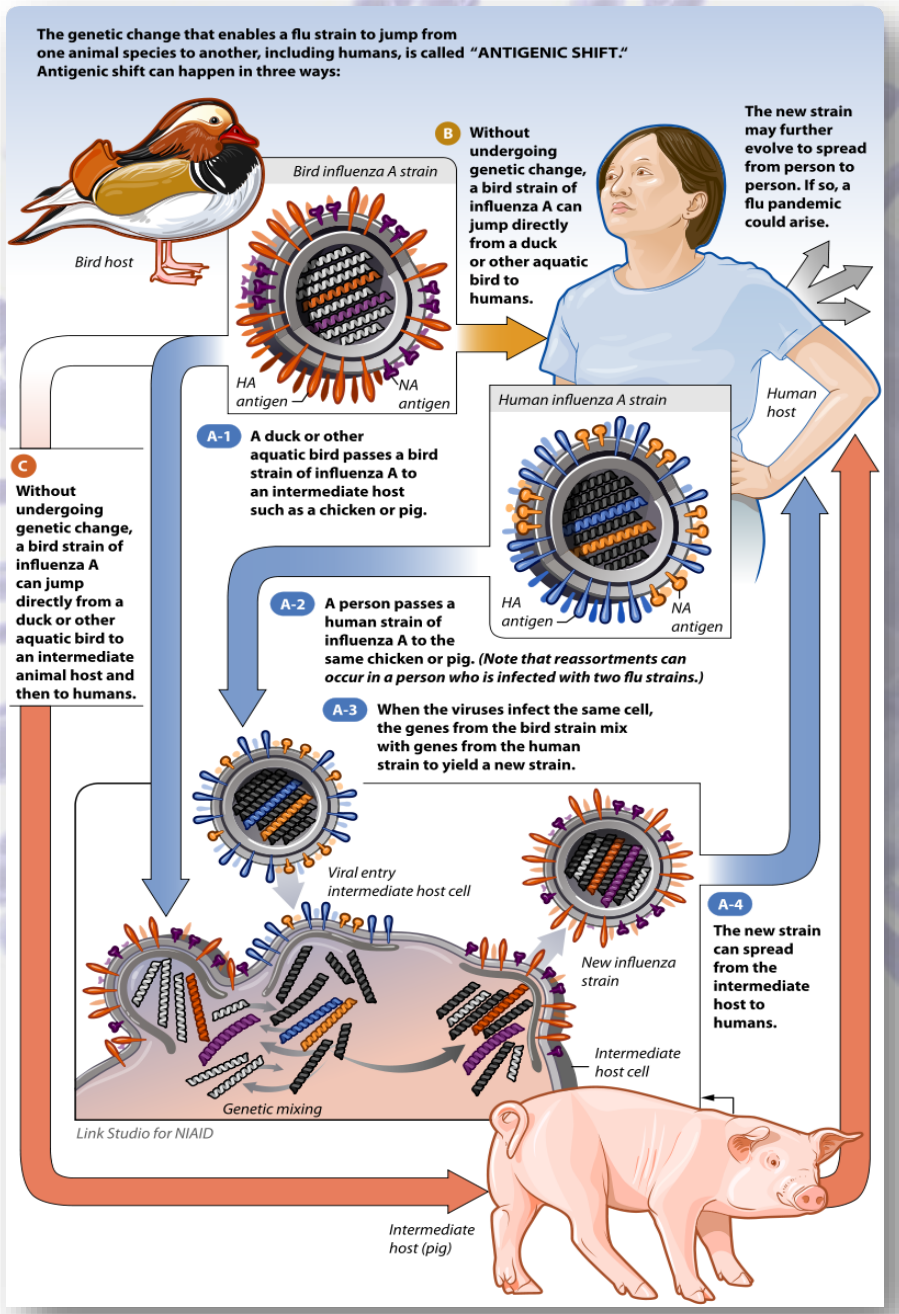




- ✓ These are small changes in the genes of influenza viruses that happen continually over time as the virus replicates.
- ✓ But these small genetic changes can accumulate over time and result in viruses that are antigenically different (further away on the phylogenetic tree).
- ✓ Small changes allow evasion of the immune response the antibody response can be escaped and infection can occur

Evolution - Genetic shift

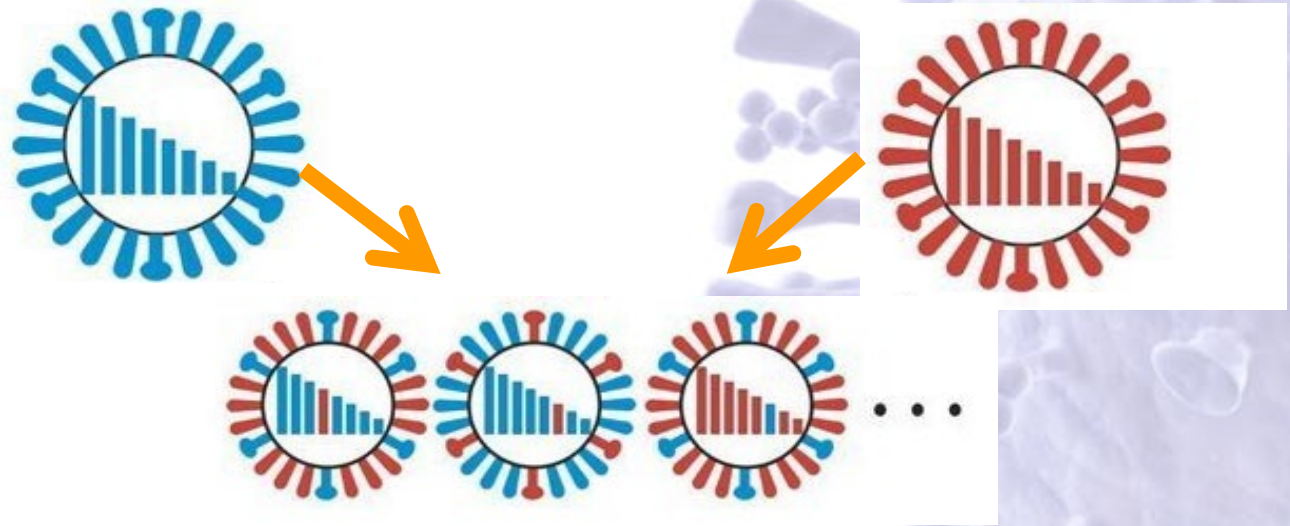
- ✓ Antigenic shift is an abrupt, major change in the influenza A viruses, resulting in new hemagglutinin and/or new hemagglutinin and neuraminidase proteins in influenza viruses that infect humans.
- ✓ Shift results in a new influenza A subtype or a virus with a hemagglutinin or a hemagglutinin and neuraminidase combination that has emerged from an animal population that is so different from the same subtype in humans that most people do not have immunity to the new (e.g. novel) virus.



Biogenesis pandemic virus

Virus non umano

Virus umano



Virus riassortenti

Functional match HA/NA

Host specificity

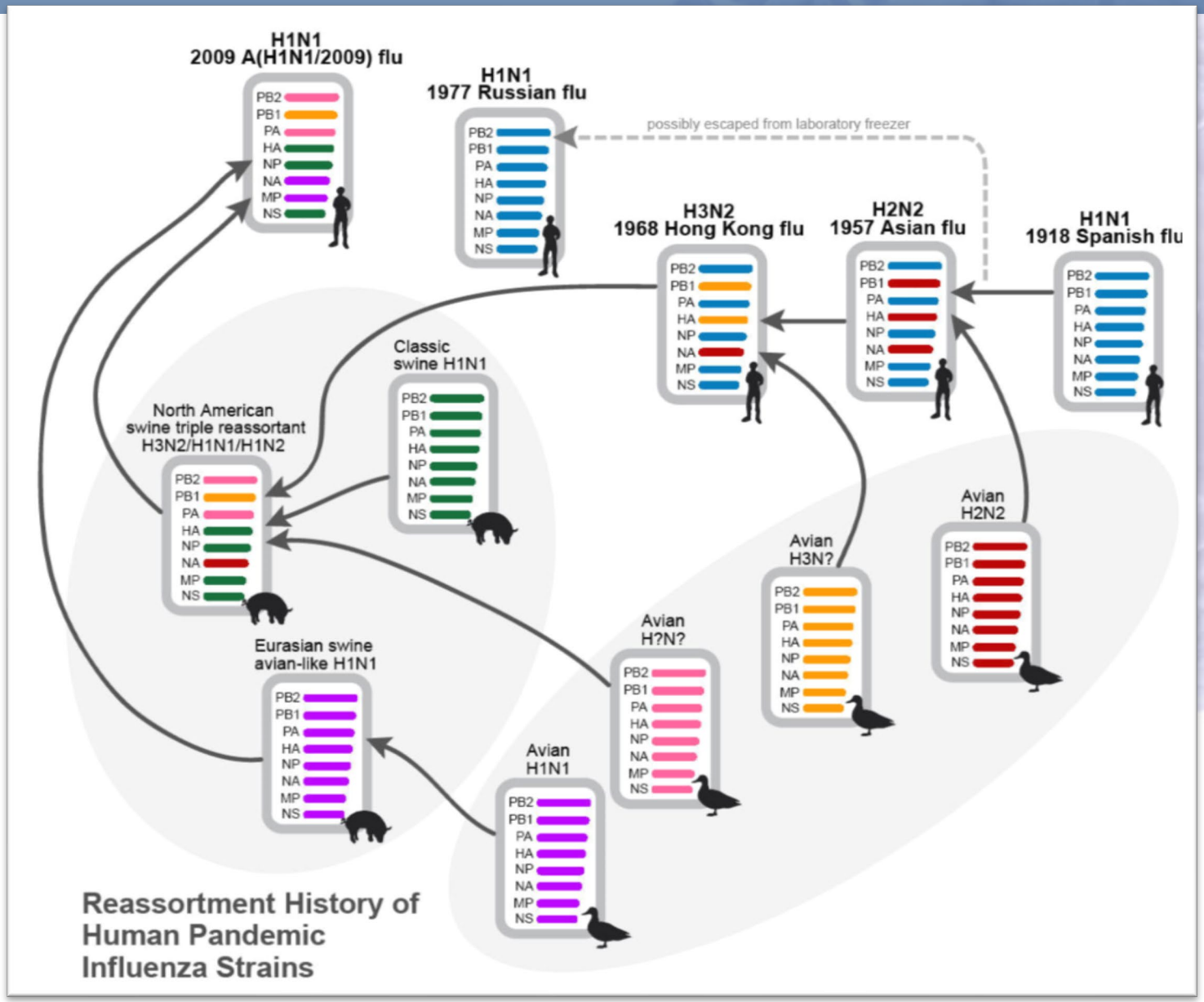
$2^8 = 256$ Possibile Combination!

Polymerase complex PB1, PB2, PA

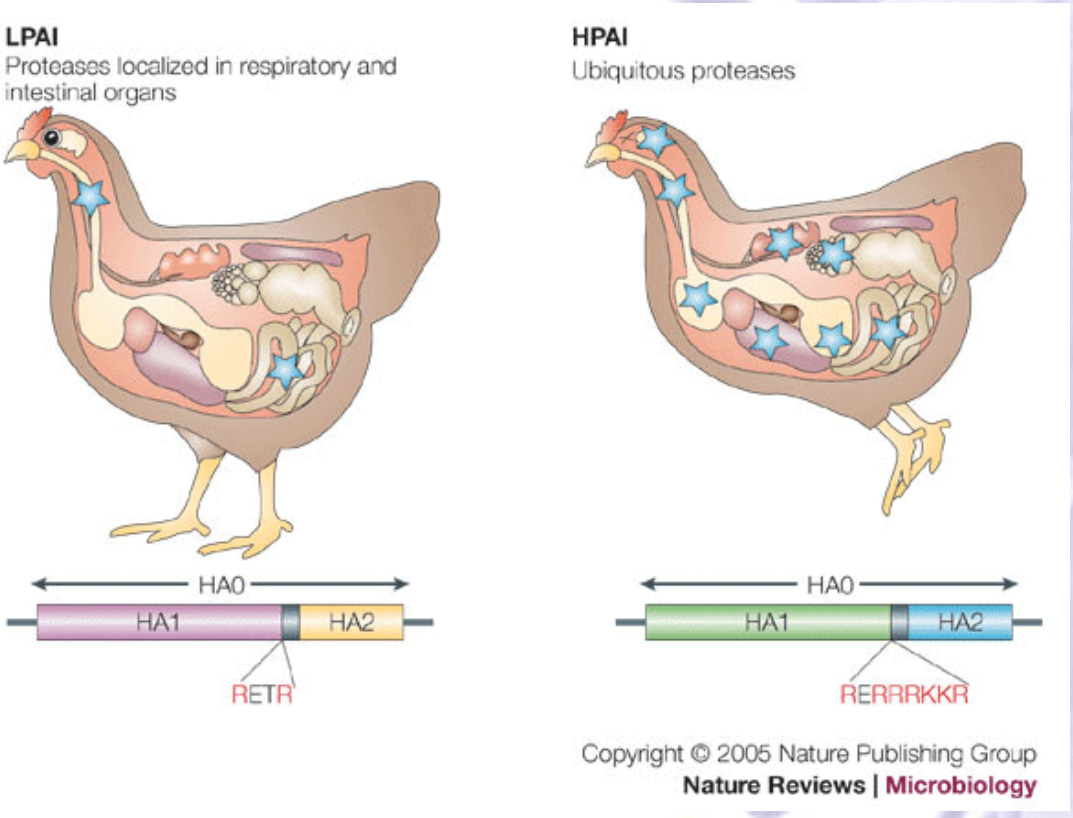
Efficency of replication

Human may accelerate the process?

History of pandemic influenza strains



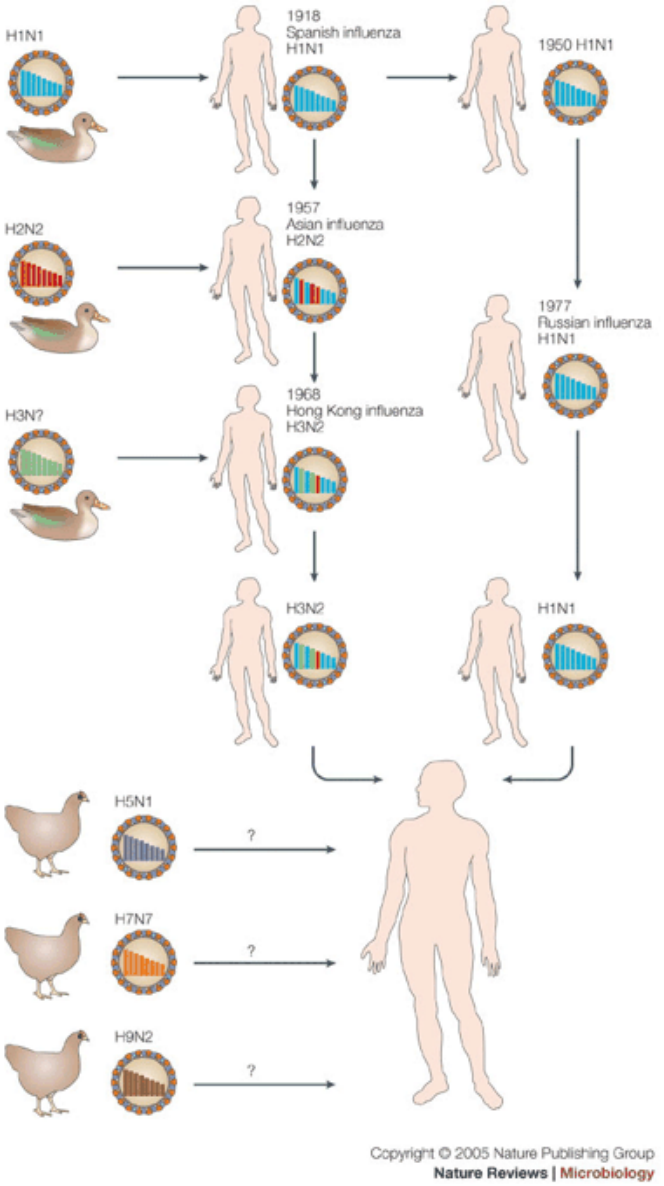
Cleavage site



Horimoto T, Kawaoka Y. Nat Rev Microbiol. 2005 Aug;3(8):591-600.

The HAs of LPAI viruses possess a single basic residue at the **cleavage site** and are usually cleaved by **proteases found in only a limited number of organs**, whereas the HAs of HPAI viruses possess a series of basic amino acids at the cleavage site, which are cleaved **by proteases present in a range of different host cells**.

Cleavage site



	Cleavage site
Avian isolates	
Avirulent strain (H5)	P Q – – – – R E T R ↓ G
Avirulent strain (H7)	P E X P – – – K X R G
Virulent strain (H5)	P Q – – R K R K K R G
Virulent strain (H7)	P E P S K K R K K R G
Human isolates: pandemic strains	
1918 Spanish flu (H1N1)	P S – – – – I Q S R G
1957 Asian flu (H2N2)	P Q – – – – I E S R G
1968 Hong Kong flu (H3N2)	P E – – – – K Q T R G
1977 Russian flu (H1N1)	P S – – – – I Q S R G
Human isolates: avian strains from humans	
1997 Hong Kong (H5N1)	P Q R E R R R K K R G
1999 Hong Kong (H9N2)	P Q – – – – R S S R G
2003 the Netherlands (H7N7)	P E I P – K R R R R G
2004 Asian (H5N1)	P Q R E (R) R R K K R G

Horimoto T, Kawaoka Y. Nat Rev Microbiol. 2005 Aug;3(8):591-600.

Fundamental question in influenza research (2013): can H5-HA-possessing viruses support transmission in mammals?

- None of the recipient ferrets died after airborne infection with the mutant A/H5N1 viruses.
- Four amino acid substitutions in the host receptor-binding protein hemagglutinin, and one in the polymerase complex protein basic polymerase 2, were consistently present in airborne-transmitted viruses.

Receptor specificity involved on pathogenicity

TABLE 1. Critical amino acids for the receptor binding specificity of the influenza HA[a]

Viral HA	Amino acid position (H3 numbering)					
	77	138	186	190	194	225
A/South Carolina/1/1918	D	A	P	D	L	D
A/New York/1/1918	D	A	P	D	L	**G**
Avian H1 consensus	D	A	P	**E**	L	**G**

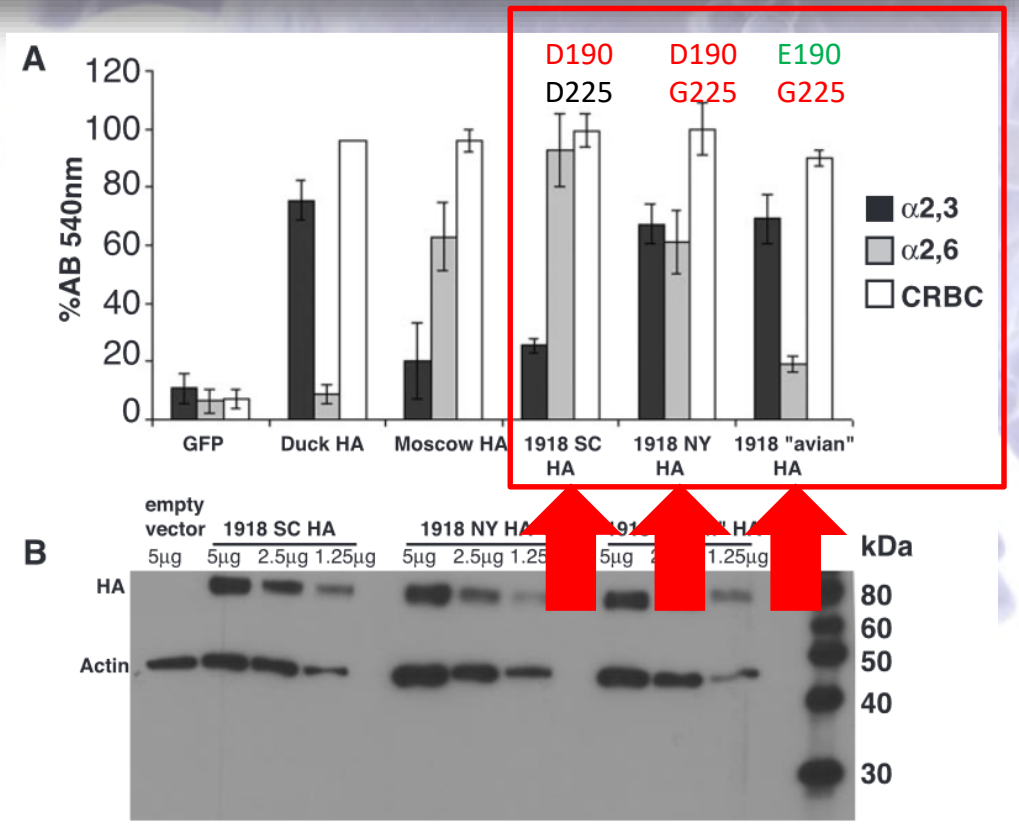
[a] The avian consensus sequence of the H1 HA was determined by comparing human and avian HA sequences (14). The above amino acids are conserved in most avian H1 HAs. Boldface type indicates a change from the A/South Carolina/1/18 HA sequence.

JOURNAL OF VIROLOGY, Sept. 2005, p. 11533–11536 Vol. 79, No. 17
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.17.11533–11536.2005

A Single Amino Acid Substitution in 1918 Influenza Virus Hemagglutinin Changes Receptor Binding Specificity

Laurel Glaser,[1] James Stevens,[2] Dmitriy Zamarin,[1] Ian A. Wilson,[3] Adolfo García-Sastre,[1] Terrence M. Tumpey,[5] Christopher F. Basler,[1] Jeffery K. Taubenberger,[4] and Peter Palese[1]*

The HA of the 1918 A(H1N1) IV had the E190D substitution, that conferred a double affinity for a2,3 and a2,6 SA and is likely to have participated in crossing the species barrier from avian reservoir to humans. The G222D (H1 numbering) substitution allowed to restrict the HA specificity to a2,6 SA.

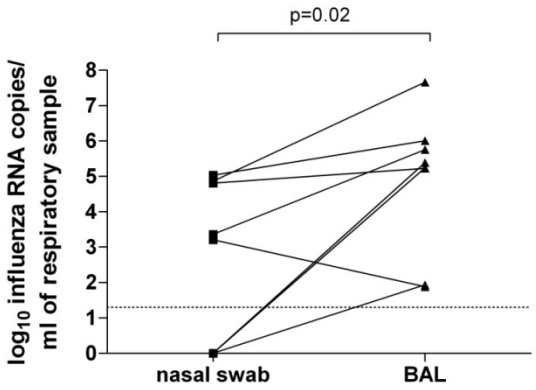


ORIGINAL ARTICLE VIROLOGY

Severe outcome of influenza A/H1N1/09v infection associated with 222G/N polymorphisms in the haemagglutinin: a multicentre study

F. Baldanti[1], G. Campanini[1], A. Piralla[1], F. Rovida[1], A. Braschi[2], F. Mojoli[2], G. Iotti[3], M. Belliato[3], P.G. Conaldi[4], A. Arcadipane[5], E. Pariani[6], A. Zanetti[6], L. Minoli[7] and V. Emmi[3]

1) *Molecular Virology Unit,* 2) *Intensive Care Unit I,* 3) *Intensive Care Unit II, Fondazione IRCCS Policlinico San Matteo, Pavia,* 4) *Institute of Microbiology and Virology,* 5) *Intensive Care Unit, ISSMET, Palermo,* 6) *Dipartimento di Sanità Pubblica-Microbiologia-Virologia, Università degli Studi di Milano, Milan and* 7) *Institute of Infectious Diseases, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Pavia, Italy*



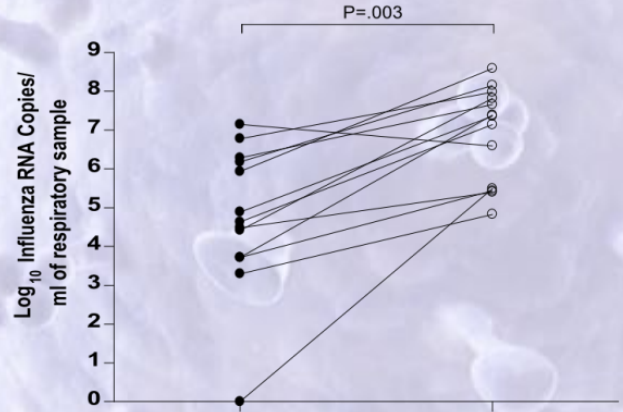
Patient no	Sample date	ILI	NS	BAL
1	27/10/2009	severe	222E	222E
2	05/11/2009	severe	222D/**N**	222D/**G/N**
3	09/11/2009	severe	222D	222D/**G/N**
	16/11/2009		222D	222D/**N**
4	25/11/2009	severe	222E/D	222E/D
5	6/11/2009	severe	ND	222D
6	21/11/2009	moderate	ND	222D/**G**
7	5/11/2009	moderate	222D	222D
8	18/11/2009	severe	222D	222D/**G**
	21/11/2009		222D	222D
9	18/11/2009	severe	222E	222E/**G**

OPEN ACCESS Freely available online PLoS one

Segregation of Virulent Influenza A(H1N1) Variants in the Lower Respiratory Tract of Critically Ill Patients during the 2010–2011 Seasonal Epidemic

Antonio Piralla[1], Elena Pariani[2], Francesca Rovida[1], Giulia Campanini[1], Alba Muzzi[3], Vincenzo Emmi[4], Giorgio A. Iotti[5], Antonio Pesenti[6], Pier Giulio Conaldi[7], Alessandro Zanetti[2], Fausto Baldanti[1]* and the Severe Influenza A Task Force

B



C

	NS	paired samples (n=13)[a]	BAL
A/Pavia/1/2011	222D		**222N**
A/Pavia/15/2011	222D		**222N**
A/Pavia/18/2011	222D		222E
A/Pavia/21/2011	222D		222E
A/Pavia/74/2011	222D		222E
A/Pavia/82/2011	222D		222E
A/Pavia/113/2011	222D		222E
A/Pavia/132/2011	222D		**222G**
A/Pavia/189/2011	222D		**222G**
A/Palermo/1/2011	222D		222E
A/Palermo/2/2011	222D		**222G**
A/Palermo/4/2011	nd[b]		222E
A/Palermo/5/2011	nd[b]		**222G**

URTI, upper respiratory tract; LRTI, lower respiratory tract; ARDS, Acute respiratory syndrome; NS, nasal swab; BAL, bronchoalveolar lavage.

[a] Two patients with 222N (A/Pavia/59/2011) and 222G (A/Pavia/127/2011) in the BAL were excluded from analysis due to imparied samples or nasal swab unavailable.

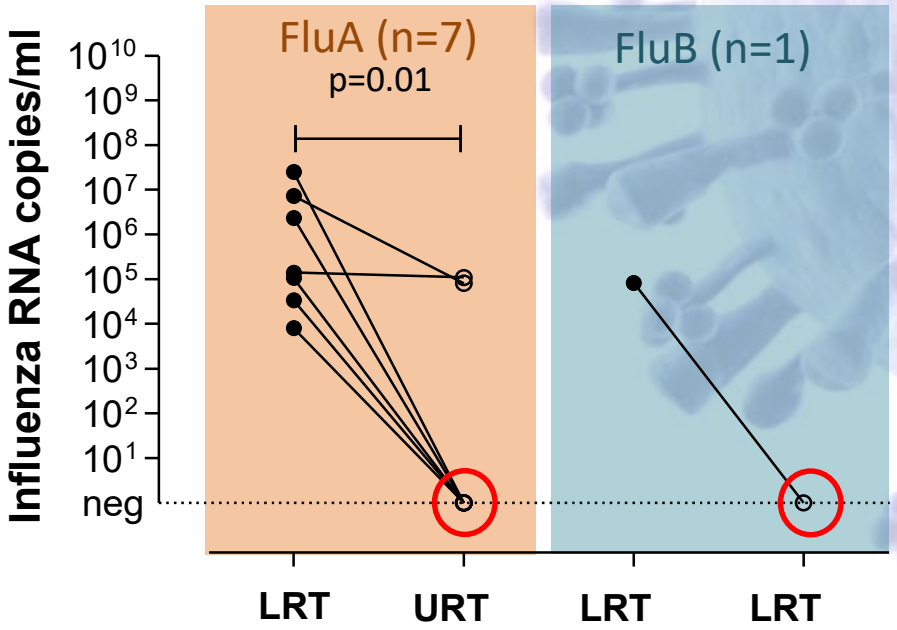
[b] nd, not done due to low viral load

Severe cases of influenza 2017-18 season

58 A/H1N1pdm09 cases on ICUs in Lombardy

Overall, mutations (G/N/A) at codon 222 were observed in **5/58 (8.6%)** influenza A/H1N1pdm09 strains.

- **3/9 (33.3%)** A/H1N1pdm09 strains detected in LRT carried D222G/N
- **2/49 (4.1%)** A/H1N1pdm09 strains detected in URT carried D222G/N



In **5/7 (71.4%)** A/H1N1pdm09 ICU cases the URT sample was negative with at least viral load >10^4 RNA copies/ml in LRT samples

Detection of haemagglutinin D222 polymorphisms in influenza A(H1N1)pdm09-infected patients by ultra-deep pyrosequencing

M. Selleri[1], A. Piralla[2], G. Rozera[1], E. Giombini[1], B. Bartolini[1], I. Abbate[1], G. Campanini[2], F. Rovida[2], L. Dossena[2], M. R. Capobianchi[1] and F. Baldanti[2]

Clin Microbiol Infect. 2013 Jul;19(7):668-73.

TABLE 1. Quasi-species haemagglutinin diversity and position 222 polymorphisms in 28 clinical samples from patients with severe (group A) and moderate–mild (group B) clinical presentations of influenza A(H1N1)pdm09 infection

Group	Patient ID	Sample type	Viral load[a] (log_{10} copies/mL)	Position 222 variant detected: Sanger	UDPS[b]	Frequency of variant estimated by UDPS (%)	Coverage[c]	Diversity[d]
A	1	BAL	6.01	E	E/D/G	98.93/0.80/0.27	7349	3.27
	2	BAL	5.75	D/N	D/N/G/E/A	34.03/43.82/19.06/2.28/0.65	4466	41.76
	3	BAL	7.11	D	D	100	16 100	0.99
	4	NS	5.44	D	D	100	11 340	1.38
	5	NS	4.37	D/G	D/G/N	65.79/33.18/1.00	14 231	19.29
	6	NS	5.47	E	E/D	99.63/0.37	7310	0.63
	7	NS	6.84	D	D	100	16 205	1.93
	8	NS	6.44	D	D	100	8615	0.93
	Median (IQR)		5.88 (5.45–6.74)					1.66 (0.95–15.29)
B	9	NS	4.86	D	D	100	21 257	1.06
		BAL	7.66	D	D	100	9651	0.57
	10	NS	8.24	D	D	100	15 446	0.69
	11	NS	6.67	E	E/D	99.57/0.43	14 176	0.86
	12	NS	5.84	D	D	100	13 118	1.22
	13	NS	6.74	D	D	100	15 881	0.29
	14	NS	7.07	D	D	100	12 416	0.52
	15	NS	6.86	E	E/D	99.60/0.40	5545	0.50
	16	NS	8.16	D	D	100	16 425	0.92
	17	NS	6.21	D	D	100	13 762	1.47
	18	NS	6.00	D	D	100	9020	4.06
	19	NS	5.62	E	E/D	99.58/0.42	16 045	2.62
	20	NS	8.17	E	E/D	99.37/0.63	15 806	0.41
	21	NS	8.32	D	D	100	19 536	0.44
	22	NS	8.61	D	D	100	13 802	0.84
	23	NS	7.58	E	E/D	99.39/0.61	15 791	0.41
	24	NS	7.36	E	E/D	99.34/0.66	18 026	0.29
	25	NS	7.26	D	D	100	11 170	0.42
	26	NS	8.92	E	E/D	99.34/0.66	22 042	18.10
	27	NS	8.92	D	D	100	5931	0.69
	Median (IQR)		7.31 (6.33–8.22)					0.69 (0.43–1.18)
p (A vs. B)[e]			0.01					0.02

BAL, bronchoalveolar lavage; IQR, interquartile range; NS, nasal swab; UDPS, ultra-deep pyrosequencing.
[a]Viral load also represents the number of cDNA copies subjected to UDPS, as there was 1 mL of starting material, and all of the extracted RNA was reverse transcribed and sequenced.
[b]The order of the variants is in accordance with their relative frequency in each patient.
[c]Number of complete reads obtained from each sample by UDPS.
[d]Diversity was calculated by the use of DNA distance (Phylip package), and is expressed as mean substitutions/site × 10^{-4}.
[e]Calculated by Mann–Whitney *U*-test.

TABLE 2. Frequency of non-D222 substitutions detected by ultra-deep pyrosequencing in the patients with severe (group A) and moderate–mild (group B) clinical presentations; the substitutions that have never been reported in the literature are in bold

	Group A (n = 8)			Group B (n = 20)		
	n[a]	Frequency (%)	Patient no.	n[a]	Frequency (%)	Patient no.
Q188stop	–	–	–	1	0.78	11
D196G	1	0.84	4	–	–	–
V199A	–	–	–	1	95.47	18
S203I[b]	1	0.27	1	–	–	–
S203R	–	–	–	2	0.33; 0.38	19; 17
R205G	1	0.33	1	–	–	–
R205K	2	94.36; 99.64	2; 6	1	99.53	16
P218L	1	0.31	2	–	–	–
V220M	1	0.22	5	–	–	–
R221K	1	0.58	8	–	–	–
E235K	1	0.29	1	–	–	–
E235G	2	0.39; 0.27	1; 5	–	–	–
K239Q	1	0.28	5	–	–	–
T241K				1	0.68	9 (NS)
V249M	1	0.27	3	–	–	–
M257I	–	–	–	1	0.52	18
E258K	1	0.40	2	–	–	–
G264S	1	0.36	2	–	–	–
T270A	1	0.29	5	–	–	–
P271L	–	–	–	1	0.35	19
V272A	1	0.29	5	–	–	–
V272I	1	1.97	7	–	–	–
N276D	1	0.38	5	–	–	–
T277I	1	0.31	1	–	–	–

NS, nasal swab.
[a]n, number of patients showing the indicated substitution.
[b]The frequency of S203T, present as a majority variant in all of the samples, has been omitted.

RESEARCH ARTICLE

Frequency of respiratory virus infections and next-generation analysis of influenza A/H1N1pdm09 dynamics in the lower respiratory tract of patients admitted to the ICU

Antonio Piralla[1], Francesca Rovida[1], Alessia Girello[1], Marta Premoli[1], Francesco Mojoli[2], Mirko Belliato[3], Antonio Braschi[2], Giorgio Iotti[2,3], Elena Pariani[4], Laura Bubba[4,5], Alessandro R. Zanetti[4], Fausto Baldanti[1,6]*

pper and lower respiratory tract samples and 3 lower respiratory samples analyzed wit

			es/ml)	No. reads	222 polymorphisms		No. variants
					NGS (%)	Sanger	
				NA	-	-	-
				7566	D (34.59), N (38.53), G (26.88)	D/N/G	30
				3264	D (100)	D	7
		BAL	$1.9x10^5$	4725	D (100)	D	17
3	A/Pavia/42/2015	NS	$1.8x10^2$	ND LVL	-	-	-
		BAL	$1.2x10^8$	5593	D (38.98), N (58.1) A (2.91)	D/N	12
4	A/Pavia/171/2015	NS	$5.2x10^5$	6493	D (100)	D	8
		BAL	$6.4x10^8$	5993	D (100)	D	16
5	A/Pavia/180/2015	NS	$1.7x10^8$	5155	D (100)	D	11
		BAL	$2.9x10^5$	6463	D (100)	D	11
6	A/Pavia/24/2015	NS	$7.1x10^3$	ND, LVL	-	-	-
		Brasp	$1.3x10^6$	7969	D (61.06), N (21.23), G (17.71)	D/N/G	21
7	A/Pavia/160/2015	NS	negative	NA	-	D	-
		BAL	$5.3x10^5$	7092	D (100)	D	8
8	A/Pavia/271/2015	NS	$1.0x10^9$	6818	D (100)	D	4
		Brasp	$2.7x10^9$	6847	D (100)	D	16
9	A/Pavia/247/2015	NS	$4.5x10^2$	ND, LVL	-	-	-
		Brasp	$9.0x10^4$	4798	D (100)	D	3
10	A/Pavia/55/2015	NS	$1.1x10^4$	NA	-	-	-
		Brasp	$1.7x10^6$	5473	D (34.04), N (65.96)	D/N	18
11	A/Pavia/25/2015	BAL	$5.3x10^4$	428	D (100)	D	2
12	A/Pavia/196/2015	BAL	$2.8x10^6$	4895	D (100)	D	7
13	A/Pavia/267/2015	BAL	$1.8x10^2$	ND, LVL	-	-	-

NS, nasal swab; BAL, bronchoalveolar lavage; Brasp, broncho aspirate; NA, not applicable, ND, not done; LVL, low viral load

RESEARCH ARTICLE

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Alessandro R. Zanetti[4], Fausto Baldanti[1]

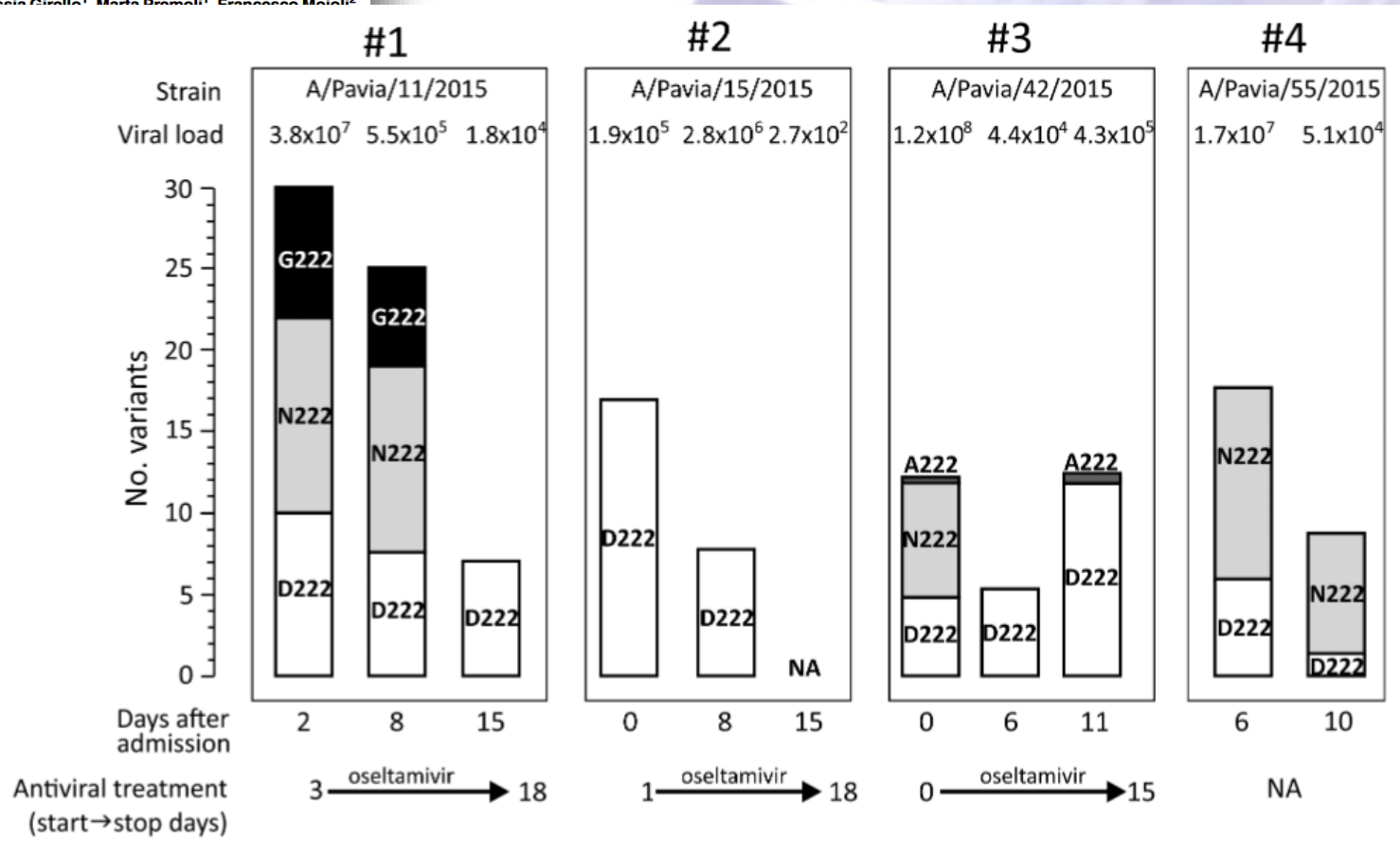


Fig 4. Frequencies of 222 polymorphisms are displayed as a stacked histogram for four patients with sequential lower respiratory tract samples. The number of variants observed and the corresponding viral load are reported above each histogram, while the time after admission and the antiviral treatment period are reported below each histogram. NA, not available.

Polymerase mutation (Avian E627 *vs* human K627)

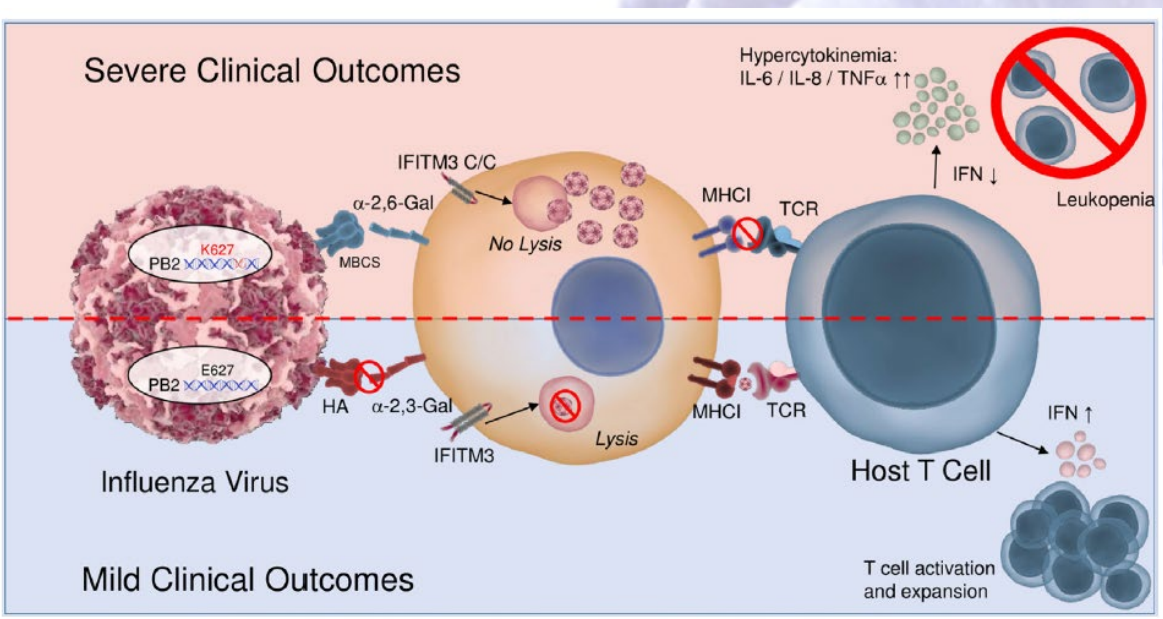
- Avian influenza A virus polymerases almost universally contain a glutamic acid (E) residue at position 627 of PB2, whereas this residue is frequently mutated to lysine (K) in mammal-adapted polymerases.
- It has been shown that changing the glutamic acid residue at position 627 of PB2 to lysine (E627K) restores activity of avian polymerases in mammalian cells.
- Although adaptive mutations have been demonstrated to enhance the activity of avian influenza virus polymerases in mammalian cells, there is disparity in the literature regarding the mechanism through which these mutations enhance polymerase activity.

Host Susceptibility Factors

- In recent years there has been an increasing focus on how host genetic factors can lead to changes in resistance or susceptibility to influenza A viruses.
- In recent studies, the single nucleotide polymorphism (SNP) rs12252-C allele of interferon (IFN)-induced transmembrane (IFITM) has been shown to shorten IFITM3, **leading to decreased inhibition of virus replication in vitro.**
- A single-nucleotide polymorphism (SNP) in the IFITM3 gene, *rs12252-C*, has been shown to strongly correlate to worsened disease progression, as this SNP leads to a truncated splice-variant that affects the protein's ability to localize to the membrane (Yang X et al., *PLoS One* (2015) 10(5):e0124985.; Zhang et al., *Nat Commun* (2013) 4:1418.

Conclusions

- Human and avian influenza viruses are in constant evolution but this evolution may be driven according to specific roles.
- HA and NA are key proteins for virus evolution and adaptation.
- Influenza variants with mutations conferring HA specificity for a α2,3 SA **(LRT-specificity)** have been detected in LRT of patients hospitalized in ICU.
- Host genome implication on virus pathogenicity is still debated.



Acknowledgments

Fondazione IRCCS Policlinico San Matteo

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