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Antibodies against 1940s era a/H1N1 influenza strains a/Weiss/43 and a/FM/1/47 and heterotypic responses after seasonal vaccination of an elderly Spanish population

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Abstract

Background and methods: Elderly people have experienced several influenza natural infections and seasonal vaccinations during their lives. The aim of this work was to evaluate in an elderly Spanish population the presence of antibodies (Abs) against some 1940s era A/H1N1 influenza viruses and some new influenza viruses. We also evaluated the homologous and heterotypic responses after seasonal influenza vaccination. We collected pre- and post-vaccination serum samples from 174 elderly people (≥65 years) who were vaccinated with seasonal influenza vaccines during the 2006–2007, 2008–2009, 2009–2010, and 2010–2011 northern hemisphere influenza campaigns. The presence of Abs against the 1940s era A/Weiss/43 and A/FM/1/47 strains of the A/H1N1 influenza virus was evaluated by using hemagglutination inhibition assays.

Results: Pre-vaccination Abs against the A/Weiss/43 and A/FM/1/47 strains were present at protective titres (≥1/40) in 43.7% and 20.1% of the study population respectively. Seasonal influenza vaccination induced heterotypic seroconversion against A/Weiss/43 in 16.1% of the individuals and against A/FM/1/47 in 13.2% of the individuals. The seroprotection rate for the study population after seasonal vaccination was 63.2% against A/Weiss/43 and 31.0% against A/FM/1/47. The heterotypic response did not satisfy the European Medicament Agency criteria for people aged ≥60 years.

Conclusions: A moderate percentage of elderly people had Abs against the 1940s era A/Weiss/43 and A/FM/1/47 strains of the A/H1N1 influenza subtype. Seasonal influenza vaccination induced a low but significant heterotypic response against both 1940s era influenza strains, reaching a high seroprotection rate for the A/Weiss/43 strain. Seasonal influenza vaccination can increase, within certain limitations, the Abs titres against old influenza strains not included in the composition of the vaccine itself.

Background

Influenza A/H1N1 subtype viruses have been circulating intermittently for 82 of the 100 years since the 1918 Spanish Influenza pandemic [1]. This subtype has been extinguished two times during its existence. The first time was in 1957 at the emergence of the A/H2N2 pandemic subtype [2], after which it re-emerged in 1977 as the A/USSR/90/1977 strain [3]. The second time was in 2009 after the emergence of the A/H1N1pdm09 pandemic subtype [4]. The drift experienced by A/H1N1 subtypes since 1918 has caused slight antigenic and genetic differences among the viruses circulating between the first decades of the twentieth century and the first decade of the twenty-first century [5].

It is likely that people born before 2009 have been in contact with different strains of A/H1N1 during their life. Thus, while elderly people have had more experiences with A/H1N1 viruses and can still have protective antibodies (Abs) against the different strains, it is probable that younger people

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are not protected against the 1940s era influenza strains. The ability of the Abs present in elderly people to protect other age groups against possible re-emergence of older strains is uncertain. It is probable that within 30–40 years there will be no persons with Abs against the 1940s era influenza strains such as A/Weiss/43 or A/FM/1/47. Thus, the existing herd immunity will be gradually phased out. This may pose a risk of re-emergence of any of these viruses [2, 6].

Vaccination is still the best method to avoid influenza and to limit the severity of these infections [7]. Vulnerability and immune-senescence processes [8, 9] make elderly people one of the main targets for seasonal influenza vaccination [10]. Although the elderly population is not the age group with the highest prevalence of flu infection, they usually register the highest rates of mortality and of clinical complications [11]. Because of that, it is of special interest to identify the particular requirements of elderly people for increasing the efficacy of seasonal influenza vaccination in this age group.

One of the most relevant aspects of elderly susceptibility to influenza infection and associated morbidity is the high number of contacts with different influenza viruses that they have experienced by natural infections and vaccinations during their life. Elderly people have been in contact with very old influenza viruses, such as those circulating during the 1940 decade, or even others phylogenetically close to the 1918 Spanish Flu virus. It is probable that these people currently have Abs against the 1940s era influenza viruses. On the other hand, current seasonal vaccines could help to increase the titre of pre-existing Abs by means of heterotypic responses, as has been shown in other studies against other influenza viruses [12]. These heterotypic responses are based on antigenic and genetic homology between the different types and subtypes of influenza viruses [13, 14] and are extremely important for the design of universal influenza vaccines.

The ability of current seasonal influenza vaccines to increase Abs against influenza viruses that are not specifically targeted for the current expected strains is not well known. Recent history has taught us that the re-emergence of certain subtypes of influenza, especially A/H1N1, is not frequent, but it presents significant public health problems when it happens [3]. Thus, it is of high importance to know what the Ab levels are to older influenza strains and how current vaccines can help maintain them at a high level. The aim of this study was to describe in a ≥ 65-year-old population the presence of pre-vaccination Abs against two different 1940s era A/H1N1 strains, A/Weiss/43 and A/FM/1/47, and also to assess the heterologous response against those strains after seasonal influenza vaccination.

Methods

Patient recruitment

Pre- and post-vaccination sera were analysed from 174 healthy individuals ≥65-years who were recruited in vaccination programs run by primary health care centres during the Influenza Vaccine Campaigns (IVCs) of 2006–2007 ($n_1 = 45$), 2008–2009 ($n_2 = 43$), 2009–2010 ($n_3 = 43$), and 2010–2011 ($n_4 = 43$). The sera were obtained by clinicians of the Influenza Sentinel Surveillance Network of Castile and Leon (Spain). The samples were stored at − 20 °C before sending to the Valladolid National Influenza Centre for analysis. Pre-vaccination sera were sampled immediately before influenza vaccination, and post-vaccination sera were obtained 28 days after seasonal vaccination. The administered trivalent influenza seasonal vaccines contained the A and B influenza strains recommended by the World Health Organization (WHO) for the northern hemisphere in each IVC. Thus, the A/H1N1 and A/H1N1pdm09 vaccine components were administered: in winter 2006–2007 the A/New Caledonia/20/99 strain, in winters 2008–2009 and 2009–2010 the A/Brisbane/59/2007 strain, and the A/California/07/2009 strain in winter 2010–2011 [15–18].

Informed consent was obtained, and the recruitment of the patients was done following Spanish Organic Law for Data Protection, patient's rights and obligations for clinical documents (BOE n°298 of 14th December of 1999, Law 41/2002). This research was performed according to the Declaration of Helsinki.

Influenza a/H1N1 viruses used for serological assays

The viruses that we selected for analysis (Table 1) included two of the most representative influenza A/H1N1 strains, A/Weiss/43 and A/FM/1/47 [19, 20], that circulated during the 1940 decade. It also included the strains A/New Caledonia/20/99 (A/H1N1 subtype), A/Brisbane/59/2007 (A/H1N1

Table 1 Influenza A/H1N1 and A/H1N1pdm09 strains included in the study

Viruses tested			
Subtype	Strain	Origin	Type of Ab evaluation
A/H1N1	A/Weiss/43	WHO Collaborating Centre Francis Crick Institute, London, UK	Heterotypic
A/H1N1	A/FM/1/47	WHO Collaborating Centre Francis Crick Institute, London, UK	Heterotypic
A/H1N1	A/New Caledonia/20/99	WHO Collaborating Centre Francis Crick Institute, London, UK	Homologous/Heterotypic
A/H1N1	A/Brisbane/59/2007	WHO Influenza Reagent Kit for Identification of Influenza Isolates	Homologous/Heterotypic
A/H1N1pdm09	A/California/07/2009	GSK, Brentford, UK	Homologous/Heterotypic

WHO World Health Organization

subtype), and A/California/07/2009 (A/H1N1pdm09 subtype). The presence of antibodies and the heterologous responses after seasonal vaccination were evaluated against both the 1940s era influenza A/H1N1 strains, i.e., A/Weiss/43 and A/FM/1/47. Also, the presence of antibodies and the homologous responses after vaccination were evaluated against the strains A/New Caledonia/20/99 in the IVC 2006–2007, A/Brisbane/59/2007 in the 2008–2009 and 2009–2010 IVCs, and A/California/07/2009 in the 2010–2011 IVC. The heterologous responses in those seasons in which the strains were not included in the seasonal vaccine were also evaluated.

Hemagglutination inhibition assay

The presence of anti-hemagglutination (HA) Abs was analysed in pre- and post-vaccination sera by the hemagglutination inhibition assay (HIA). Following the protocol published by WHO and the Influenza Surveillance Network for the surveillance of influenza viruses and vaccine efficacy [21, 22], nonspecific inhibitors of the HIA were remove by combining 100 μl of serum with 300 μl of receptor destroying enzyme (RDE, Denka Seiken, Japan). The RDE-serum combination was incubated at 37 °C in a water bath for 18 h and then inactivated at 56 °C for 1 h. Serial double dilutions of 50 μl of each serum sample were performed in 96-V-microwells plates. After that, 50 μl of a standard containing 4 haemagglutinin units was added to each well and the plates were incubated for 30 min at room temperature. Hens erythrocytes (0.75%, 50 μl) were added and incubated for another 30 min. The Ab titre was determined as the highest dilution that caused complete hemagglutination inhibition.

Phylogenetic analysis

We performed a phylogenetic analysis of the HA gene of old A/H1N1 strains, vaccine strains, and A/H1N1pdm09 subtype (A/Weiss/43-EPI_ISL_66107; A/FM/1/47-EPI_ISL_69263; A/New Caledonia/20/99-EPI_ISL_22227; A/Brisbane/59/2007-EPI_ISL_154502; A/California/07/2009-EPI_ISL_227813). In this analysis it was also included the A/South Carolina/1/18 strain of 1918 Spanish Influenza virus (EPI_ISL_1213), A/PR/8/34 strain (EPI_ISL_14962) and also the A/USSR/90/1977 strain (EPI_ISL_243351). Because HIAs identify only specific epitopes of the globular head domain of haemagglutinin proteins [23], the genetic analysis was only performed for the haemagglutinin 1 (HA1) subunit. HA1 DNA sequences were aligned using the ClustalW algorithm of Bioedit 7.2.3 software. The best model for the phylogenetic analysis was predicted using the Best-Fit tool of Mega 5.2 software (MegaSoftware, Tempe, AZ, USA). The general time reversible model, with gamma-distributed rates, produced the highest Bayesian information criterion score. Thus, we used this model for constructing a phylogenetic tree based on the aligned DNA sequences of the HA1 gene subunit. The reproducibility of the phylogenetic tree was guaranteed by a bootstrap analysis of 1000 replications. We also constructed a distance matrix for the HA1 subunit, using the maximum composite likelihood algorithm. The results of this matrix were inversely transformed and expressed as a percentage of genetic homology (% of similarity/100).

Statistical analysis

The results were analysed using the classic serological European Medicament Agency (EMA) criteria for the evaluation of vaccine efficacy [24]. The criteria establish different parameters for analysing the vaccine efficacy in people ≥60 years. The criteria included a seroprotection rate (SPR) ≥60%, a seroconversion rate (SCR) ≥30%, and a geometric mean titre (GMT) increase ≥2.0. The GMT increase was calculated as the rate between post- and pre-vaccination serum GMT [25]. Negative results obtained in the HIA were assumed to be half of the detection value (1/10) for the calculation of the GMT. For this study, a titre ≥1/40 was considered to be protective [25]. Although some studies suggest that higher protective titres may be used for evaluating seroprotection in ≥65 years [26], the current consensus maintains that 1/40 is a protective titre; therefore we decided to comply with that criterion. Seroconversion was defined as a titre increase of at least four-fold between the pre- and post-vaccination sera. Additionally, seroconversion was considered to have occurred in negative pre-vaccination titres that reached ≥1/40 after vaccination. Different statistical parametric and non-parametric tests were used, such as the Bonferroni test and McNemar test, using SPSSV20 (IBM, Armonk, NY, USA). Statistical significance was taken at the $p < 0.05$ value.

Results

Population characteristics

The mean age of the study population was 75.9 years (95% confidence interval [CI95%]:74.9–77.0), and 57.5% were males ($n = 100$). The mean age of individuals recruited during the 2006–2007 IVC was 74.7 years (CI95%:72.9–77.0), 79.2 during the 2008–2009 IVC (CI95%:76.7–81.4), 74.8 in the 2009–2010 IVC (CI95%:72.7–76.8), and 75.3 in the 2010–2011 IVC (CI95%:72.8–77.7). The mean age was significantly higher in 2008–09 IVC than in the rest of IVCs (Bonferroni = 3.678; $p = 0.013$). The percentage of men in each IVC was 64.4% ($n_1 = 29$) in the 2006–2007 IVC, 46.5% ($n_2 = 20$) in the 2008–2009 IVC, 62.8% ($n_3 = 27$) in the 2009–2010 IVC, and 55.8% ($n_4 = 24$) in the 2010–2011 IVC.

Presence of pre-vaccination antibodies against 1940s era a/H1N1 viruses and against new a/H1N1 viruses

Within the entire study population of elderly subjects, pre-vaccination Abs against either or both of the 1940s era influenza strains, A/Weiss/43 and A/FM/1/47, were present

in 89.7% of the individuals. Among these, 58.6% had pre-vaccination Abs against both of the 1940s era influenza strains, 20.7% against only the A/Weiss/43 strain, and 10.3% against only the A/FM/1/47 strain. Among the subjects, 88.5% had pre-vaccination Abs against the A/H1N1 subtype strains A/Brisbane/59/2007 and A/New Caledonia/20/99, and 20.7% against the A/H1N1pdm09 subtype.

The percentages of individuals having protective pre-vaccination Abs during the full study period and in each IVC are described in Fig. 1. Of all the study population individuals who expressed Abs prior to seasonal vaccinations, 43.7% had protective Ab titres (≥1/40) against A/Weiss/43, 20.1% against A/FM/1/47, 39.1% against A/H1N1 A/New Caledonia/20/99 and the A/Brisbane/59/2007 vaccine strains, and 3.4% against the A/H1N1pdm09 subtype. Before vaccinations in each IVC, almost all subjects had protective Ab titres against all of the viral strains. The only exception was against the A/H1N1pdm09 subtype during the 2006–2007 IVC.

Abs response after seasonal vaccination

After seasonal vaccination, 97.1% of the entire study population had Abs against the A/Weiss/43 or A/FM/1/47 strains, though not all of the titres were sufficiently high to provide protection. Among the subjects, 74.1% had Abs against both 1940s era A/H1N1 strains, 17.2% against only the A/Weiss/43 strain, and 5.8% against only the A/FM/1/47 strain. Most individuals, 98.9%, had Abs against the A/H1N1 subtype strains A/New Caledonia/20/99 and A/Brisbane/59/2007 and 70.1% against the A/H1N1pdm09 subtype. After seasonal vaccination, 63.2% of the study population had protective levels of Abs against the A/Weiss/43 strain, 31.0% against the A/FM/1/47 strain, 73.0% against the A/H1N1 subtype strains A/New Caledonia/20/99 and A/Brisbane/59/2007, and 35.6% against the A/H1N1pdm09 subtype.

The 2006–2007, 2008–2009, and 2009–2010 IVCs volunteers were vaccinated against A/New Caledonia/20/99 and A/Brisbane/59/2007 strains (A/H1N1 subtypes). These vaccinations induced a significant heterotypic seroconversion against the A/Weiss/43 strain (McNemar, $p < 0.05$) (Table 2). The 2010–2011 IVC volunteers was vaccinated against the A/California/07/2009 (A/H1N1pdm09 subtype), which induced a significant heterotypic seroconversion against the A/FM/1/47 strain (McNemar; $p < 0.05$). During the 2006–07, 2008–09, and 2009–10 IVCs, vaccination with the A/H1N1 strains also induced significant heterotypic seroconversion against A/H1N1pdm09 (McNemar; $p < 0.05$). Finally, vaccination with the A/H1N1pdm09 subtype during the 2010–2011 IVC also induced heterotypic seroconversion against the A/Brisbane/59/2007 strain of the A/H1N1 subtype (McNemar; $p < 0.05$).

These data show that seasonal vaccination induced a significant homologous seroconversion against A/H1N1 vaccine strains in all IVCs vaccinated against this subtype (McNemar; $p < 0.05$). Also, seasonal vaccination induced a significant homologous seroconversion against the A/H1N1pdm09 subtype in the 2010–11 IVC (McNemar; $p < 0.05$). The number of seroconversions and the SCR induced by influenza seasonal vaccination in the whole study period and in each IVC are summarized in Table 2.

Heterotypic and homologous responses to seasonal vaccination according to EMA requirements

Analysis of the seasonal influenza vaccination efficacy for the whole study period and each IVC was assessed by applying the classical EMA criteria for people ≥60 years. The pre- and

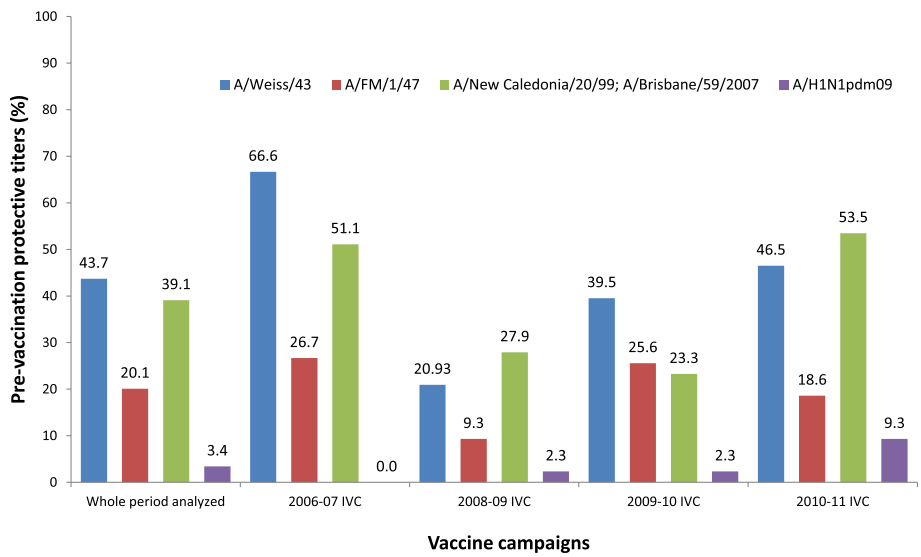

Fig. 1 Percentage of individuals showing pre-vaccination protective Abs against 1940s era A/H1N1 strains, A/H1N1 vaccine strains, and the A/H1N1pdm09 subtype during the whole study period and in each IVC (influenza vaccine campaign)

Table 2 Number of seroconversions and seroconversion rate against 1940s era A/H1N1 strains, A/H1N1 vaccine strains, and A/H1N1pdm09 subtype

	A/Weiss/43		A/FM/1/47		A/H1N1		A/H1N1pdm09	
Vaccinated cohorts (strain included in seasonal vaccine)	SCn[a]	SCR[b]	SCn	SCR	SCn	SCR	SCn	SCR
Whole period analysed (*N* = 174)	28	16.1	23	13.2	59	33.9	55	31.6
2006–2007 (A/New Caledonia/20/99) (n1 = 45)	11	24.4	5	11.1	19	42.2	6	13.3
2008–2009 (A/Brisbane/59/2007) (n2 = 43)	6	14.0	2	4.7	26	60.5	6	14
2009–2010 (A/Brisbane/59/2007) (n3 = 43)	7	16.4	3	7.0	7	16.3	9	20.9
2010–2011 (A/California/07/2009) (n4 = 43)	4	9.3	13	30.2	7	16.3	34	79.1

[a]Number of seroconversions
[b]Seroconversion rate

post-vaccination GMT, GMT increase, SPR, and SCR values are described in Table 3. Within the entire study population, the SPR was higher than 60% against the A/Weiss/43 strain (63.2%) and against the A/H1N1subtype strains A/New Caledonia/20/99 and A/Brisbane/59/2007 (73.0%). The SCR after seasonal vaccination was higher than 30% against the A/H1N1subtype strains A/New Caledonia/20/99 and A/Brisbane/59/2007 (33.9%) and against the A/H1N1pdm09 subtype (31.6%). The GMT increase was higher than 2.0 against all viruses except the A/FM/1/47 strain.

For each IVC, the SPR was higher than 60% against the A/Weiss/43 strain in 2006–2007 and 2009–2010 IVCs

Table 3 Pre- and post-vaccination GMT values, GMT increase, seroprotection, and seroconversion rates in each IVC against all analysed A/H1N1 and A/H1N1pdm09 viruses

			Vaccinated cohorts			
Strain/Subtype	Input	Whole period analyzed	2006-2007	2008-2009	2009-2010	2010-2011
	Vaccine strain		A/New Caledonia/20/99	A/Birsbane/59/2007	A/Birsbane/59/2007	A/California/07/2009
A/Weiss/43						
	Pre-vaccine GMT[a](CI95%)	18.3 (13.5-23.1)	38.4 (23.6-63.3)	8.2 (5.1-13.7)	18.7 (11.0-31.4)	19.0 (10.2-32.9)
	Post-vaccine GMT(CI95%)	44.2 (33.6-52.8)	83.8 (50.8-143.1)	24.5 (16.6-37.3)	45.8 (28.8-73.7)	39.4 (21.0-68.0)
	GMT increase	2.4	2.2	3.0	2.4	2.1
	SPR[b]	63.2	84.4	46.5	65.1	55.8
	SCR[c]	16.1	24.4	14.0	16.3	9.3
A/FM/1/47						
	Pre-vaccine GMT(CI95%)	7.8 (6.3-9.8)	7.8 (4.9-13.0)	3.4 (2.2-5.1)	12.5 (8.7-18.1)	11.3 (7.6-16.7)
	Post-vaccine GMT(CI95%)	13.6 (10.6-17.4)	20.5(13.7-31.0)	4.6 (2.9-7.1)	15.3 (10.4-22.6)	23.1 (13.6-37.0)
	GMT increase	1.7	2.6	1.4	1.2	2.0
	SPR	31.0	35.6	14	30.2	44.2
	SCR	13.2	11.1	4.7	7	30.2
A/New Caledonia/20/99						
A/Brisbane/59/2007						
	Pre-vaccine GMT(CI95%)	20.9 (16.6-25.1)	34.5 (27.6-51.2)	13.6 (8.3-21.1)	13.2 (8.6-19.8)	29.8 (21.7-39.8)
	Post-vaccine GMT(CI95%)	63.8 (53.1-77.6)	112.3(69.7-145.8)	103.5 (72.3-147.4)	27.8 (19.5-37.4)	50.1 (37.9-66.7)
	GMT increase	3.1	3.3	7.6	2.1	1.7
	SPR	73.0	82.2	83.7	58.1	67.4
	SCR	33.9	42.2	60.5	16.3	16.3
A/H1N1pdm09						
	Pre-vaccine GMT(CI95%)	1.8 (1.5-2.1)	1.5 (1.2-1.9)	1.6 (1.1-2.3)	1.9 (1.3-2.6)	2.4 (1.7-3.7)
	Post-vaccine GMT(CI95%)	12.7 (9.5-17.2)	5.4 (3.4-8.7)	7.5 (4.8-11.2)	7.2 (4.2-11.9)	93.0 (54.3-150.0)
	GMT increase	7.1	3.6	4.7	3.8	38.8
	SPR	35.6	13.3	20.9	23.3	86
	SCR	31.6	13.3	14	20.9	79.1

[a]Geometric mean titers; [b]Seroprotection rate; [c]Seroconversion rate

(84.4% and 65.1% respectively), but the SCR was lower than 30% in all IVCs. The GMT increase against the A/Weiss/43 strain was higher than 2.0 in all IVCs except 2010–2011. The SCR for the A/FM/1/47 strain after seasonal vaccination was higher than 30% only in the 2010–2011 IVC (30.2%), but the SPR was not ≥60% in any of the IVCs analysed. The GMT increase against A/FM/1/47 strain was higher than 2.0 in 2006–2007 IVC (2.6) and 2010–2011 IVC (2.0). The SPR was higher than 60% for the A/H1N1subtype strains A/New Caledonia/20/99 and A/Brisbane/59/2007 in all IVCs except for the volunteers of the 2009–2010 IVC (58.1%) that was vaccinated against the A/Brisbane/59/2007 strain. The SCR for the A/H1N1subtype strains A/New Caledonia/20/99 and A/Brisbane/59/2007 was higher than 30% in the 2006–2007 IVC (42.2%) with vaccination against A/New Caledonia/20/99 strain, and in the 2008–2009 IVC (60.5%) with vaccination against the A/Brisbane/59/2007 strain. The GMT increase was higher than 2.0 against both A/H1N1 strains in all IVCs analysed excepting 2010–2011. Both SPR and SCR were higher than 60% and 30% respectively for the A/H1N1pdm09 subtype only in the 2010–11 IVC (86.0% and 79.1% respectively). GMT increase was higher than 2.0 against A/H1N1pdm09 subtype in all IVCs analysed.

Phylogenetic analysis

The homology (% of similarity/100) between the different A/H1N1 and A/H1N1pdm09 strains analysed in this work is described in Table 4. The mean genetic homology between the HA1 subunit of the HA gene between all viruses was 0.833 (83.3%, CI95%: 0.795–0.867). The highest homology was between the A/New Caledonia/20/1999 and A/Brisbane/59/2007 strains (96.7%), and the lowest was between the A/Brisbane/59/2007 and A/California/07/2009 strains (64.2%). The phylogenetic tree constructed using the DNA sequences of the HA genes is shown in Fig. 2.

Discussion

The year 2018 marks 100 years since the Great Pandemic of 1918 caused by the Spanish Flu virus. This pandemic affected over 500 million people and caused the death of over 50 million, representing a mortality rate over 2.5% [1]. The large period of circulation of A/H1N1 subtypes since their emergence has caused a moderate intrasubtypic antigenic and genetic drift. The A/H1N1 viruses phylogenetically close to those circulating in 1918 are slightly different from the A/H1N1 viruses circulating during the first decades of this century [5]. This makes it likely that very young people will not be protected against the 1940s era viruses. Thus, all of the protection available for younger individuals against these strains of influenza resides in the antibodies present in older individuals.

Our data show that before vaccination, a high proportion of the elderly population had Abs that recognized the 1940s era A/H1N1 strains. Many of these Abs where found at protective titres. The detection of these Abs after a prolonged time lapse without any documented circulation of these strains is relevant. The results of our work demonstrate that serologic protection against influenza viruses can persists for many years. Consistent with other reports [27, 28], our data suggest that past infections could protect a large part of the population throughout their lives.

The origin of these Abs is uncertain. The mean age of the populations analysed was high, so it is possible that these Abs could have been induced by multiple exposures of the elderly study population with A/H1N1 viruses since their childhood, some of them even with Spanish influenza. On the other hand, because elderly people are a risk group with a specific recommendation for seasonal influenza vaccination [10], they are likely to have been vaccinated several times during their lives. These vaccinations could have induced previous heterotypic or cross-immune reactions against viruses not included in the vaccine composition, but that are phylogenetically related to the vaccine strains. Because we do not know the previous vaccination history of the elderly people in the study population, it is difficult to ascertain with certainty the origin of the observed Abs.

The phylogenetic analysis conducted in our work showed that the mean genetic homology between the HA1 subunits

Table 4 Similarity values expressed as a percentage of genetic homology (% of similarity/100) between A/H1N1 and A/H1N1pdm09 viruses

Influenza A/H1N1 strains	A/South Carolina/1/18	A/PR/8/34	A/Weiss/43	A/FM/1/1947	A/USSR/90/1977	A/New Caledonia /20/1999	A/Brisbane /59/2007	A/California /07/2009
A/South Carolina/1/18	1.000	–	–	–	–	–	–	–
A/PR/8/34	0.891	1.000	–	–	–	–	–	–
A/Weiss/43	0.873	0.929	1.000	–	–	–	–	–
A/FM/1/1947	0.864	0.912	0.934	1.000	–	–	–	–
A/USSR/90/1977	0.840	0.891	0.916	0.952	1.000	–	–	–
A/New Caledonia/20/1999	0.796	0.844	0.860	0.886	0.913	1.000	–	–
A/Brisbane/59/2007	0.780	0.824	0.841	0.864	0.888	0.967	1.000	–
A/California/07/2009	0.771	0.711	0.704	0.709	0.679	0.651	0.642	1.000

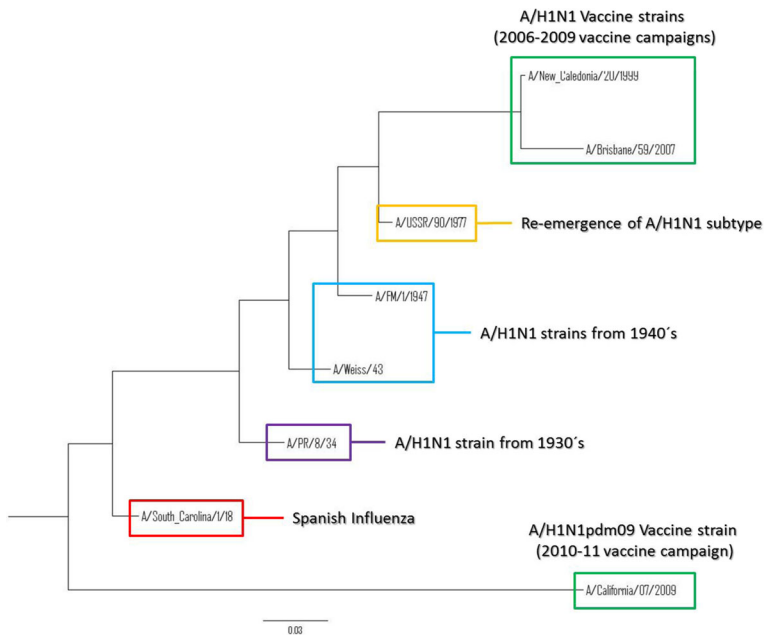

Fig. 2 Phylogenetic tree of the A/H1N1 and A/H1N1pdm09 strains

of the A/H1N1 strains that we analysed is high (> 80%). Influenza A type viruses share specific antigenic epitopes that can be recognized by a wide range of Abs [13, 14]. Cross-immune reactions between different influenza subtypes and strains are frequent and have been documented by different authors [14, 29]. These types of results are the reason for the high interest and hope for a universal influenza vaccine [30, 31]. Our data also showed the presence of pre-vaccination Abs against the A/H1N1pdm09 subtype in a small percentage of the elderly people before the emergence of the 2009 pandemic. This issue was documented previously with different percentages among different countries [32–34]. It was probably responsible for the lower incidence of A/H1N1pdm09 subtype in elderly people compared to younger groups during the 2009 pandemic [34, 35].

The lower percentage of individuals having pre-vaccination protective Abs against both of the 1940s era A/H1N1 influenza strains, i.e., A/Weiss/43 and A/FM/1/47, in the 2008–09 IVC is surprising. The population recruited during 2008–09 IVC had a significantly higher mean age than the rest of the groups, so these people were born 10 to 15 years before the circulation of A/Weiss/43 and A/FM/1/47 strains. It is probable that the 2008–09 IVC was primed with older strains than those circulated during the 1940 decade, and this population seems to have generated a lower humoral response against the A/H1N1 viruses that circulated later. This issue can be caused by the so called "Original Antigenic Sin" [36–38], and it marks the importance of the first contacts with influenza viruses in a person's life. Unfortunately, a weakness of our work is that we were not able to analyse the presence of heterotypic Abs against other A/H1N1 strains older than those circulated during the 1940 decade, e.g., A/PR/8/34, so this issue cannot be tested with certainty.

Despite the high genetic homology present between both old A/H1N1 strains (93.4%), a lower percentage of individuals showed pre-vaccination Abs against the A/FM/1/47 strain than the A/Weiss/43 strain in all IVCs. This was probably caused by events that occurred during the emergence of the A/FM/1/47 strain. According to some authors, this strain emerged because of a more pronounced intrasubtypic drift than in previous years [20]. However, other authors suggest that this strain was separated from the strains circulating during the early years of the 1940 decade and did not produce epidemics until 1947 [19]. The spread of this strain throughout the world was fast during 1947, and it produced an unusually high number of cases but without an increase in the mortality rates of previous epidemics [20]. Repeated infections with minor variants of the same influenza subtype decreased the humoral response against the new strains that were phylogenetically close [39]. While this effect has not been well documented, some authors suggest that this phenomenon induced a lower immune response against influenza viruses that were different from the virus that primed each individual [40–42]. The lower response was likely to be harmful for the host. More people than in a normal epidemic were infected during 1947, and the humoral response in them was lower than in a normal epidemic due to the phylogenetic proximity of the strain to previously circulated strains. This hypothesis is consistent with our data, explaining the lower percentage of people with pre-vaccination protective Abs against the A/FM/1/47 strain.

After influenza seasonal vaccination, 63.2% of elderly people showed protective Abs against the A/Weiss/43 strain, 31.0% against A/FM/1/47 strain, 73.0% against the A/H1N1subtype strains A/New Caledonia/20/99 and A/Brisbane/59/2007, and 35.6% against the A/H1N1pdm09 subtype. Our results showed that seasonal vaccination induced a low but significant heterotypic response against the A/Weiss/43 strain when the elderly people were vaccinated against the A/New Caledonia/20/99 and A/Brisbane/59/2007 strains. Thus, this heterotypic response against the A/Weiss/43 strain was higher when this population was vaccinated with the A/New Caledonia/20/99 strain. Vaccination with A/H1N1 strains did not induce a significant heterotypic response against the A/FM/1/47 virus, while vaccination with the A/H1N1pdm09 subtype induced a moderate and significant heterotypic response against the 1947 strain.

As previously described, genetic homology between the 1940s era A/H1N1 strains is high, 93.4%, and it is also high between the 1940s era strains and the A/H1N1 vaccine strains included in seasonal vaccines, 84–87%. However, vaccination with the A/H1N1pdm09 subtype induced a higher than expected heterotypic response to the A/FM/1/47 strain, 30.2%, compared to the closely related A/Weiss/43 strain, 9.3%. This surprising observation lead us to hypothesize that despite the close genetic homology between the A/FM/1/47 and A/Weiss/43 strains, the epitopes formed by A/H1N1pdm09 subtype were more similar to those formed by the A/FM/1/47 strain than the A/Weiss/43 strain. This has been documented in other influenza viruses before [43]. Further research is needed to discover the cause of the divergent heterotypic responses of the A/H1N1pdm09 subtype vaccine in the production of Abs to the A/FM/1/47 and A/Weiss/43 epitopes.

Influenza seasonal vaccination induced a significant homologous response against A/H1N1 vaccine strains in all IVCs in which these subtypes were used. Also, we observed a significant heterotypic response against A/H1N1 subtypes when elderly people were vaccinated against A/H1N1pdm09 (2010–2011 IVC). Seasonal vaccination against A/H1N1 vaccine strains also induced a significant heterotypic response against the A/H1N1pdm09 subtype in all IVC (from 2006 till 2009). This heterotypic response was observed in at least 20% of the individuals the 2009–2010 IVCs. The results of our study demonstrate that seasonal vaccination with an A/H1N1 subtype seroprotected a low-to-moderate percentage of the elderly population against the 2009 pandemic virus before its emergence. These kinds of heterotypic responses after vaccination, and those induced by natural infections, may be responsible for the lower incidence of A/H1N1pdm09 subtype in the elderly population during the 2009 pandemic [34, 35].

The homologous response to influenza A/H1N1 strains was moderate, but sufficient to achieve high seroprotection rates in those older than 65 years. However, in the 2009–2010 IVC, there was a very low seroconversion rate, only 16.3%. In that IVC, the new pandemic subtype A/H1N1pdm09 emerged and was very actively circulated in Spain during the 2009–2010 IVC. We hypothesize that a certain percentage of the individuals vaccinated during that campaign were already infected or were in contact with this subtype during their window period. This could have negatively affected the humoral response. Further research is needed to clarify the true reasons for the low homologous response to the subtype A/H1N1 this IVC. The homologous response was very high in the season vaccinated with the A/H1N1pdm09 subtype. Our data show the complexity of the response to vaccination, which is influenced not only by the strains introduced in the vaccine, but also by the characteristics of the viruses, the target populations, the scheduling of the vaccination programs, and probably many other variables.

According to classical EMA criteria for vaccine efficacy evaluation in individuals ≥60-years old [24], the seasonal influenza vaccine was not effective in inducing a heterotypic humoral response against any of the 1940s era A/H1N1 strains that we analysed. The heterotypic response against the A/Weiss/43 strain was limited in most of the IVCs. However, the moderate percentage of individuals with pre-vaccination protective titres was likely responsible for the 48–85% of the elderly population that was seroprotected after vaccination against a subtype that had not been in circulation for more than 60 years. This issue has interesting implications for the protection of the population against re-emerging viruses. Despite the fact that there is only a low risk of re-emergence of old A/H1N1 strains, we are not exempt from biological incidents like the one that triggered the emergence of the A/USS/90/1977 strain during 1977 [3]. The Abs observed in a large percentage of the elderly population represent a moderate-to-high herd immunity that may protect other populations in the case of re-emergence of any of the cited A/H1N1 strains [44, 45]. More research is needed to understand the seasonal vaccine responses to old influenza strains in adults and children and the natural presence of antibodies in naïve people.

One of the limitations of this study is that the methodology did not allow the evaluation of the presence of heterotypic Abs against the HA2 subunit of haemagglutinin, which is the most conserved part of this protein. To know more about the nature of the heterotypic reactions observed in this work, it will be necessary to extend this study using other methodologies such as microneutralization or prior adsorption of sera with seasonal viruses before testing them against older viruses. It will also be instructive to perform these experiments with other old strains from other decades such as A/PR/8/34 or A/USSR/90/1977. For a wider view of seroprotection, these studies need to be conducted with adults in other age groups and with children that never have been in contact with the 1940s era A/

H1N1 strains. The results of our study show that seasonal vaccines can increase the Ab titres against already extinct strains. This may provide a better understanding of the cross-immunity phenomena that is necessary to achieve a truly universal vaccine against influenza.

Conclusions

In summary, seasonal influenza vaccination is useful in elderly people for strengthening seroprotection and increasing the Ab titres against older A/H1N1 influenza viruses. However, we do not know the exact role of immune memory in the presence of these Abs before vaccination. Protective Abs are present in a moderate percentage of the elderly population, and they may contribute to herd immunity that can prevent the re-emergence of extinguished influenza viruses. The results of our study provide data regarding cross-immune responses between different influenza viruses and how seasonal vaccines can be useful against other viruses not included in the vaccine composition. Our results also support the need for increasing the seasonal influenza vaccine coverage in the elderly.

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Availability of data and materials
The genetic data analysed during the current study are available in the Global Initiative on Sharing Avian Influenza Data (GISAID) database, weblink: https://www.gisaid.org/.

Authors' contributions
IS, SR and ROL designed the study. IS and SR performed the experiments. IS, SR and ST wrote the manuscript. IS, SR, ST, JME and ROL revised the manuscript. All authors approved the final version of this manuscript.

Ethical approval and consent to participate
Informed consent was obtained, and the recruitment of the patients was done following Spanish Organic Law for Data Protection, patient's rights and obligations for clinical documents (BOE n°298 of 14th December of 1999, Law 41/2002). This performance of this research followed the Declaration of Helsinki.

Competing interest
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

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