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Determination of antibody-levels in hematopoietic stem cell transplant recipients during follow-up of 180 days after influenza vaccination

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AFFIDAVIT

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1. Introduction

Influenza viruses circulate in all parts of the world and cause an acute respiratory infectious disease called influenza like-illness (colloquial *the flu*) [1], [2]. Most infected individuals will present with a sudden onset of fever and coughing, which typically subsides after two weeks. Some individuals, however, have a higher risk for serious complications associated with morbidity (e.g. due to the underlying diseases, bacterial pneumonia) and even an increased mortality. In particular, children under the age of 2, adults above the age of 65, pregnant women, and individuals with chronic medical conditions (e.g. diabetes mellitus and chronic obstructive pulmonary disorder (COPD)) are considered high-risk groups [3]. An additional high-risk group includes individuals who have undergone hematopoietic stem cell transplantation (HSCT). HSCT is used as a curative treatment for leukemia and other hematological diseases. The procedure includes whole-body irradiation and intensive chemotherapy, which leads to a phase of severe immunosuppression until the transplanted hematopoietic system is engrafted and functional. Due to the severe immunosuppression, the mortality rate of HSCT recipients with untreated Influenza infection is up to 30% [4]–[6]. A previous study conducted on 286 HSCT patients showed that 44% were hospitalized, 33% developed lower respiratory tract disease, 12% required mechanical ventilation, and 6% died due to pandemic A/H1N1 influenza infection [7].

Vaccination is the number one strategy for prevention of influenza infection, although it has been shown that humoral immune responses are lower in high risk patients compared to healthy individuals [4]. Due to the lower immunity, two doses of influenza vaccines are administered to patients after HSCT at the University Hospital Basel as a standard of care, whereas healthy individuals only receive one dose. Also, HSCT patients in the Geneva University Hospital received two doses of the AS03-adjuvanted split influenza H1N1/A/09 vaccine according to Mohty et al. [5]. In this project, the influenza-specific antibody levels against five influenza strains of 53 allogeneic HSCT recipients and 25 healthy controls (HC) at five time points after vaccination were measured. Two methods were used and compared in this study: one of them, the hemagglutination inhibition assay, optimized to quantify influenza-specific antibody titers, has been recently accepted for publication in JOVE (reference, Kaufmann et al. in press). The

second method used is an enzyme-linked immunosorbent assay (ELISA) to measure influenza specific IgG levels.

Our hypotheses are: (i) HSCT recipients shortly after transplantation show significantly lower antibody titers compared to recipients with a longer time span since transplantation and (ii) a second vaccine shot increases the antibody titers significantly.
The specific aims of this study were: (i) to improve the methods in a high throughput setting and (ii) to quantify the progression of antibody (Ab) production after vaccination in HSCT recipients and also healthy controls.
As an outlook, we aim to predict individual risks of HSCT patients for influenza by assessing immunological and clinical parameters pre- and post-vaccination, which has the potential to optimize post-transplant management of patients and improve overall outcomes of transplantation. A paper by Hollenstein, Linnik et al. with clinically relevant characteristics of the HSCT recipients is in process.

1.1. Influenza viruses

The influenza viruses are genera of the family of the *Orthomyxoviridae* and are separated into influenza viruses A, B, C and D [8]–[10]. These viruses cause influenza infection in vertebrates, including humans, birds, pigs, seals and horses [8], [11].

1.1.1. Virion structure and organization

Influenza viruses are characterized by segmented, negative-strand RNA genomes (8 segments for influenza A and B viruses, and 7 segments for influenza C viruses). The influenza A and B virus genomes contain about 14000 bases and code for 11 proteins each. The viral RNA (vRNA) segments are coated with nucleoproteins (NP) and hetero-trimeric RNA-dependent RNA polymerase proteins (PB1, PB2 and PA). These four proteins form the ribonucleoprotein (RNP) complex and the nuclear export protein (NEP; also called nonstructural protein 2, NS2) are covered by a matrix of M1 proteins. The virion core is completed by the lipid envelope, which contains integral membrane proteins. The envelopes of influenza A and B viruses contain the two proteins hemagglutinin (HA) and neuraminidase (NA) (**Fig. 1**). Influenza A viruses additionally have a third type of integral membrane protein called M2, which is an ion channel. Instead of M2, the influenza B envelope has the two proteins NB and BM2.

Influenza C virions are structurally distinct from those of influenza A and B; however, Influenza C is compositionally similar with a glycoprotein-studded lipid envelope overlying a protein matrix and the RNP complex. Instead of three (influenza A) and four (influenza B) membrane proteins, only two proteins are present at influenza C viruses: One major surface glycoprotein, the hemagglutinin-esterase-fusion (HEF) protein, which combines the functions of the hemagglutinin and the neuraminidase and a minor envelope protein (CM2), which functions as an ion channel [11], [12].

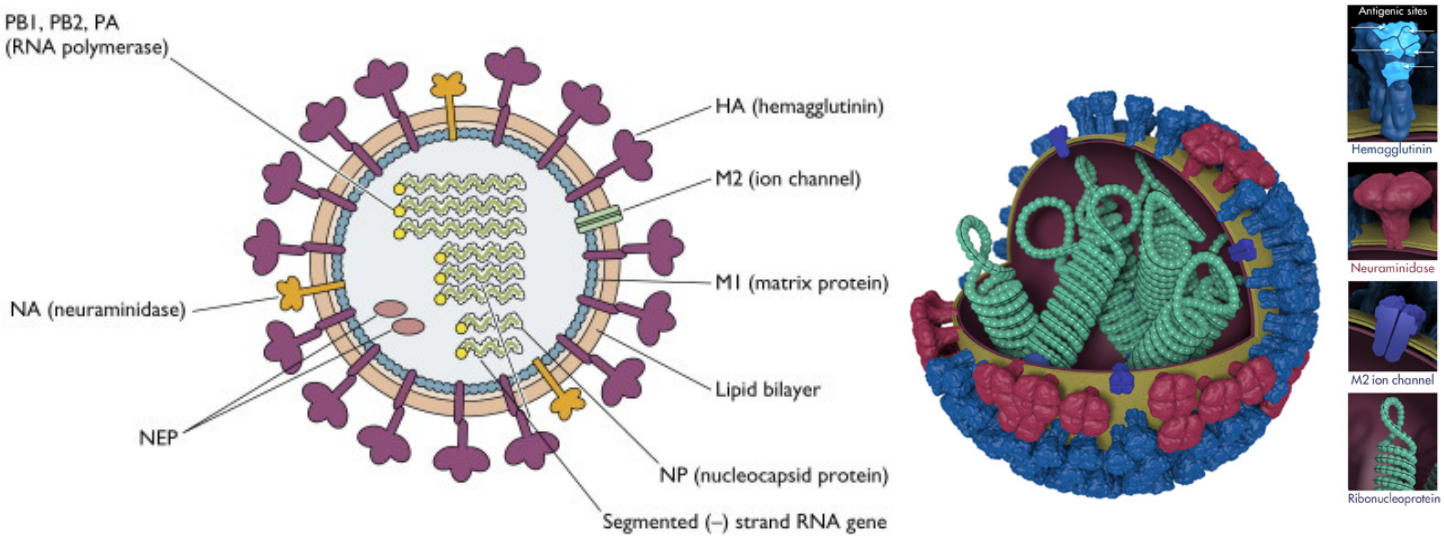


Fig. 1. Structure of an influenza A virion. The enveloped influenza A virion consists of three membrane proteins (HA, NA, M2), a matrix protein (M1) below the lipid bilayer, a ribonucleoprotein complex (viral RNA segments coated with nucleoproteins and the heterotrimeric RNA polymerase (PB1, PB2 and PA)) and the NEP/NS2 protein. Influenza A and B viruses are spherical or filamentous in shape and structurally very similar. Influenza C viruses however form long cordlike structures, but the composition is similar to the others. The major surface glycoprotein in influenza C viruses combines the functions of hemagglutinin and neuraminidase and is called hemagglutinin-esterase-fusion (HEF) protein. Figures adapted from [12] (left) and [13] (right).

The eight different negative-sense, single-stranded viral RNA segments (HA, NA, M, NP, PA, PB1, PB2 and NS) of influenza A viruses encode 11 proteins (HA, NA, NP, M1, M2, NS1, NEP, PA, PB1, PB1-F2, PB2). The genome of influenza C viruses consists of just seven segments, due to the combined hemagglutinin and neuraminidase functions of the HEF protein [11], [14].

1.1.2. Replication cycle

Influenza is typically transmitted from infected mammals through aerosols, which contain influenza virus particles, by sneezes and coughs. The virus particles may also be present in nasal secretions and saliva, as well as in feces and blood, albeit at lower concentrations, of the infected mammals. Individuals are infected due to contact with these virus-containing bodily fluids or contaminated surfaces.

At human body temperature, influenza viruses can remain infectious for approximately one week [15]. Outside the body, the viability of the virus depends on the temperature and several other factors, such as exposure to sunlight, relative humidity, salinity, and pH. In general, human influenza viruses remain infectious for less than 8-12 hours on porous surfaces at room temperature. At 4°C, some viruses survived for at least 30-40 days [16], [17]. Influenza A viruses are susceptible to a wide variety of disinfectants and detergents, and can be heat inactivated at 60-70°C. These effects have not been examined extensively with influenza B and C viruses, but it is assumed to be similar [16], [18], [19].

In infected animals, the influenza viruses recognize sialic (N-acetylneuraminic) acid on the host epithelial cells surface in the lung and throat with the HA glycoprotein (HEF protein in case of influenza C). Sialic acids are nine-carbon acidic monosaccharides, which are ubiquitous on many cell types and in many animal species. The sialic acids can form α-2,3- or α-2,6-linkages, which result in unique steric configurations of the terminal sialic acid. This region is recognized and bound by HA spikes on the surface of influenza viruses, which have a preferential specificity for one of the two different linkages. In human tracheal epithelial cells, α-2,6-linkages are predominant, but α-2,3-linkages are also present. Alternatively, in the gut epithelium of ducks, sialic acids with terminal α-2,3-linkages are more common. This means humans can be infected by avian influenza viruses, though with less efficiency than by human strains [11].

The influenza virus is imported into the cell by endocytosis after the HA protein (HEF protein in case of influenza C) is attached to the sialic acid (**Fig. 2**, Step 1). Inside the endosome, the pH value decreases, making it acidic, which is crucial for the viral uncoating. The low pH triggers a conformational change in the HA, exposing a fusion peptide that induces the fusion of the viral envelope with the endosomal membrane. Additionally hydrogen ions from the endosome are pumped into the virus particle via

the ion channel. Due to the internal acidification of the virion, protein-protein interactions are detached, which allows the viral RNPs to be released into the cytoplasm and guided to the nucleus of the host cell (Step 2). In the nucleus, the RNA-dependent RNA polymerase uses the negative-sense vRNA as a template to synthesize positive-sense complementary RNA (cRNA). The positive-sense cRNA is used in two ways: (i) the cRNA is used as mRNA (capped, polyadenylated messenger RNA), exported into the cytoplasm and translated to viral proteins by using the host cells ribosomes (Steps 3a and 4) or (ii) the cRNA is used as template for the RNA-dependent RNA polymerase to synthesize more copies of negative-sense, genomic vRNA that form the genomes of progeny viruses (Step 3b). The newly synthesized viral envelope proteins HA, NA and M2 (in case of influenza A viruses) are secreted through the Golgi apparatus onto the cell surface (Step 5b). The matrix protein M1, the nuclear export protein NEP, the nonstructural regulatory protein NS1 and the nucleoprotein NP are transported back into the nucleus to bind nascent vRNAs (Step 5a). NEP mediates the M1-RNP complex export via nucleoporins into the cytoplasm. At the host cells membrane, a new influenza virion is packed, due to accumulation of M1 at the cytoplasmic side of the lipid bilayer. The envelope contains the integral membrane proteins HA, NA and M2 (Step 6). To release the virus particles from the host cell, the enzyme neuraminidase (NA) plays a key role. When budding is completed, HA spikes still bind the virions to the sialic acids on the cell surface. The neuraminidase enzymatically cleaves terminal sialic acid residues on the surface of the infected cell to release the progeny virus (Step 7). The cleavage of virions budding from the infected cells is a critical step in the replication cycle to infect new cells – this is also were oseltamivir, a so-called neuroaminidase inhibitor, acts to reduce the amount of released viral particles. The NA also cleaves sialic acid residues from the virus envelope itself to prevent aggregation of viruses. After many new virions are produced and cleaved, the host cell dies and the virions infect other cells [11], [20]–[22].

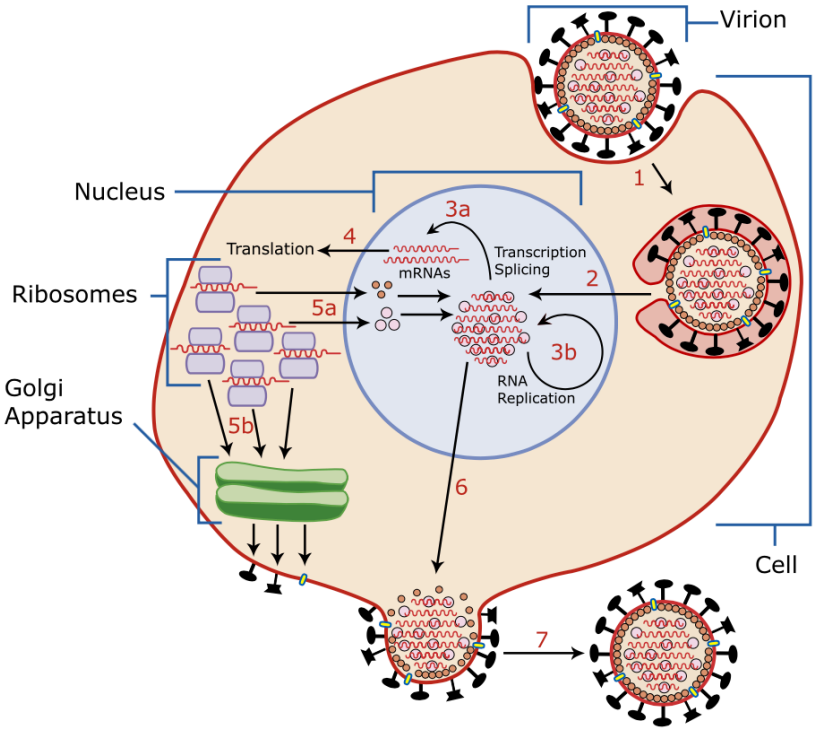


Fig. 2. Scheme of influenza A virus replication cycle. The influenza virion attaches to the sialic acid on the surface of the host cell via HA and enters the cytoplasm by endocytosis (1). The acidic pH inside the endosome triggers the fusion of the viral envelope with the endosomal membrane, M1 dissociates from the nucleocapsid and vRNPs are translocated into the nucleus of the host cell (2). In the nucleus, the viral RNA-dependent RNA polymerase transcribes (3a) and replicates (3b) the vRNAs. Newly synthesized mRNAs are exported to the cytoplasm, where they are translated by using the host cells ribosomes (4). The viral envelope proteins HA, NA and M2 undergo posttranslational processing at the Golgi apparatus and are secreted to the cell membrane (5b). NP, M1, NS1 and NEP/NS2 reenter the nucleus where they bind newly synthesized vRNAs and form nucleocapsids (5a). The nucleocapsids migrate into the cytoplasm in a NEP-mediated process and assemble to complete influenza virions with the cell membrane where HA, NA and M2 are present as integral membrane proteins (6). NA cleaves sialic acid residues of cellular receptors on the surface to release the progeny virions from the host cell (7). Figure adapted from [23].

1.1.3. Antigenic variability

Influenza viruses undergo constant genomic adaptation [11], [16], [24]–[26]. The persistent evolution of the virus is the primary reason why influenza leads to annual epidemics and occasional pandemics in humans. The variability results from accumulation of genetic changes in the eight RNA segments, which can occur by a number of different mechanisms, including point mutations, gene reassortment (RNA

recombination) and defective-interfering particles. Although alterations can occur in each of the eight gene segments of both, type A and type B influenza viruses, changes in the surface glycoproteins HA and NA have the biggest impacts related to the immune system [26]. In particular, HA is the major antibody binding site on the virus surface and therefore also a key component of vaccines – towards HA a strong evolutionary selection pressure is active. Two processes are responsible for these changes in the envelope proteins of influenza A viruses:

One process is called *"antigenic drift"*, which is based on point mutations within the antibody-binding sites in HA, NA or both [27]. These small genetic changes usually produce viruses, which are closely related to one another and share the same antigenic properties. It is possible that an immune system exposed to a similar virus will recognize it and respond through a process known as cross-protection - but small genetic changes can accumulate over time and result in viruses that are antigenically different [24]. If this occurs, the immune responses against the former HA and NA may no longer be protective, because the immune system cannot recognize those viruses anymore (**Fig. 3**) [16], [24]. Antigenic drift occurs in both, influenza A and B viruses [25]. A lack of the proofreading function of the RNA-dependent RNA polymerase contributes to replication errors on the order of 1 in 10^4 bases, which leads to these point mutations. There are five antibody-binding sites (antigenic domains A-E) found on the HA1 protein [26], [27].

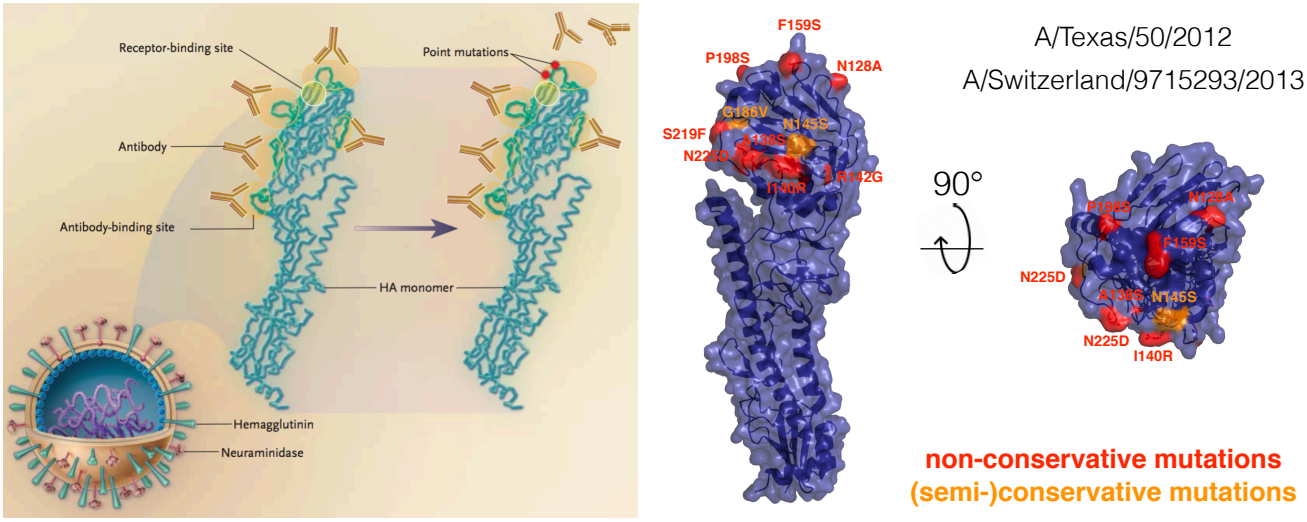


Fig. 3. Antigenic drift shown at the HA1 subunit of the glycoprotein hemagglutinin. Antigenic drift occurs in both, influenza A and B viruses. The gradual changes by point mutations within the antibody-binding sites in HA and NA results in the inability of antibodies to neutralize the mutant virus. Left part of figure adapted from [27]; right part from [Kaufmann et al. in press].

The second process is called *"antigenic shift"* (**Fig. 4**). An antigenic shift is a process of genetic reassortment, which leads to major changes in the influenza virus genome. When two or more different influenza A virus strains infect the same cell, gene segments from both viruses may be packed into a single, novel virion [16]. This major reassortment results in a completely new influenza A virus containing new proteins, which have emerged from an animal population. The antibody-binding sites are significantly different and therefore most infected animals do not have any pre-existing adaptive immunity to the virus [24]. For this reason, antigenic shift is often responsible for worldwide influenza pandemics with millions of infected people [25]. In 2009 for example, a H1N1 virus with a new combination of genes emerged to infect people and quickly spread, causing a pandemic [8], [24].

Antigenic shift happens only occasionally, while influenza viruses are changing by antigenic drift on a constant rate due to natural adaptation within every infected host on an annual basis. Both processes occur within influenza A viruses, whereas influenza B viruses change only by the more gradual process of antigenic drift, because there is no known influenza B virus gene pool except in humans [24], [26]. Only influenza A viruses are known to cause pandemics [2].

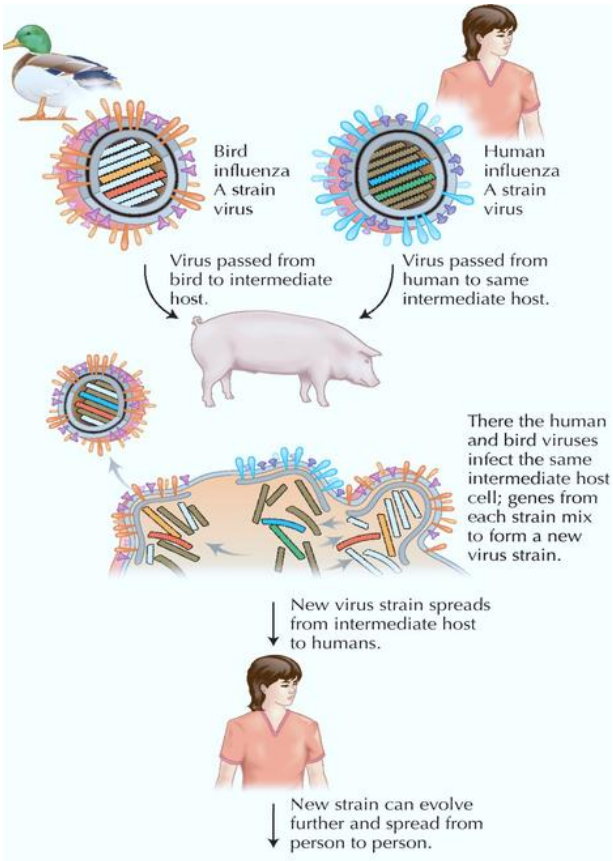


Fig. 4. Scheme of the genetic reassortment-process of antigenic shift. Two different influenza A virus strains, one from birds, one from humans, are passed to an intermediate host (pig). One cell of the host is infected by both virus strains and genes from each strain are mixed to form a novel virus strain. The new virus spreads from the intermediate host back to humans. The new strain can evolve further and spread from person to person, causing an epidemic and even a pandemic. Figure adapted from [28].

1.1.4. Nomenclature

"A revision of the system of nomenclature for influenza viruses: a WHO Memorandum" [29] was accepted in 1979 and published in 1980 in the Bulletin of the World Health Organization (WHO) after a convention reconsidered the old system of nomenclature for influenza viruses decided in 1971. This renewed system of nomenclature uses the following internationally accepted components:

- The antigenic type (e.g. A, B, C, D)
- The host of origin (e.g. swine, equine, chicken, etc. For human-origin viruses no host of origin designation is given.)
- Geographical origin (e.g. Brisbane, California, Switzerland, etc.)
- Strain number (e.g. 02, 50, etc.)
- Year of isolation (e.g. 57, 2012, etc.)
- For influenza A viruses the hemagglutinin and neuraminidase antigen description in parentheses (e.g. (H1N1), (H2N3), etc.)

For example:

- A/duck/Alberta/35/76 (H1N1) for an influenza A virus strain from duck origin
- B/Massachusetts/02/2012 for an influenza B virus strain from human origin

Influenza A viruses are divided into subtypes based on the 18 different hemagglutinin subtypes (H1-H18) and 11 different neuraminidase subtypes (N1-N11). Within these subtypes they can be further broken down into different strains. Currently circulating subtypes of influenza A viruses in humans are H1N1 and H3N2 strains [8], [30].

Influenza B viruses, however, are divided into lineages and strains instead of subtypes. The currently circulating influenza B viruses belong to one of the two lineages: B/Yamagata and B/Victoria [8].

1.2. Human influenza

The disease caused by influenza viruses is also known as "flu" and is an acute respiratory infection of mild to severe illness. Infections by influenza C viruses are detected much less frequently and usually cause mild infections [2]. The symptoms of influenza are characterized by a sudden onset of fever, usually dry cough, headache,

muscle and joint pain, and severe malaise. Although less common, a sore throat and runny nose may also be present. The World Health Organization (WHO) definition for an influenza-like illness only includes sudden onset of fever and coughing [31]. Not all of the symptoms have to occur in infected individuals [32]. Most people recover within one to two weeks without requiring any medical treatment. The cough and tiredness may persist longer than fever and the other symptoms [2], [33]. In addition, secondary bacterial or viral infections, so called super-infections, can exacerbate or prolong the symptoms [16].

Influenza can also cause severe illness or even death if people at high risk get infected. Children under 2 years, adults above 65 years, pregnant women and people who have specific medical conditions such as lung diseases, diabetes, cancer, chronic kidney, lung or heart problems, immunosuppressed people (people with HIV infection, solid organ or stem cell transplant recipients) are seen as high risk groups. Also health-care workers are included, due to the increased exposure to influenza viruses [2], [3], [33], [34].

Because of the transmission through the air by droplets and small particles excreted when infected individuals cough or sneeze, the virus can easily be passed from person to person [33]. Another way the virus can spread is by contact with contaminated surfaces [34], especially in crowded areas like schools, nursing homes, and other public places the transmission of the influenza virus can be rapid [2]. Influenza leads to seasonal epidemics in winter, because the cold and dry weather enables the virus to survive longer outside the body. The virus enters the body through the upper respiratory tract and it then takes between one to four days to develop symptoms, which is known as "incubation period" [32], [33]. On the basis of symptoms alone, it is difficult to distinguish influenza from respiratory illnesses caused by other pathogens. Therefore, several laboratory-based influenza tests for diagnostics were developed. These assays mainly include the detection of influenza antigens and detection of viral nucleic acids by polymerase chain reaction (PCR) or isothermal amplification [33], [34]. Serological tests and virus cultures are outdated for routine diagnostics due to labor intensiveness, high costs, long turnaround times and low diagnostic accuracy. After confirmation of influenza virus infection, antiviral drugs can be used to reduce severe complications. In most cases, neuraminidase inhibitors such as oseltamivir are used; however, to be effective, these drugs have be administered within 24-48 hours after the onset of symptoms [2].

1.3. Influenza vaccines

The WHO recommends annual vaccination for prevention of influenza infection in high-risk patients. Among the elderly people (aged more than 65 years), the influenza vaccine may be less effective in preventing illness compared to healthy adults, but vaccination reduces severity and incidence of complications and deaths. The vaccine is most effective when the circulating viruses are well matched with the vaccine viruses. Among healthy adults, the influenza vaccine provides protection, even when circulating viruses may not exactly match the vaccine viruses [2]. A previous study [34] showed that there has to be at least 85% consensus between the vaccine strains and the circulating viruses to successfully induce protection. Twice a year, the WHO updates the recommendations on the composition of the influenza vaccine. There are separate recommendations for use in Northern and Southern hemispheres [35]. Due to the constant evolution of influenza viruses (see 1.1.3), the circulating strains in humans have to be monitored continuously. Based on these records, the compositions of the vaccines are established [32], [35], [36]. A common form of influenza vaccine is a trivalent inactivated vaccine composed of two different influenza A subtypes, generally one H1N1 and one H3N2 strain, and one influenza B strain of either yamagata or victoria lineage. Since the 2013/2014 influenza season, there is also a quadrivalent inactivated vaccine available where strains of both influenza B lineages are included [32], [34], [35]. Inactivated vaccines are available in three different types: whole virus vaccines, split virus vaccines and subunit vaccines. For producing whole virus vaccines, live viruses are grown in chicken embryos (egg-based) or cell cultures (cell-based), inactivated with formaldehyde, purified and concentrated to 15 µg doses of hemagglutinin [34], [37]. Also, the recombination technology is used for the production of influenza vaccines (recombinant flu vaccines) [38]. In split virus vaccines, the inactivated viruses were additionally treated with a detergent to dissociate the viral envelope proteins. In subunit vaccines, the surface glycoproteins HA and NA have been further purified by removal of other viral components [34], [37]. All three types of inactivated vaccines are generally injected intramuscularly and show similar immunogenicity. Many inactivated vaccines predominantly induce an IgG response against strain specific hemagglutinins and neuraminidases [34]. Some types of inactivated vaccines are coupled with adjuvants, such as Alum, AS03 or MF59, to enhance the influenza specific immune response due to the relatively little immunogenic impact [34], [37], [39].

Besides inactivated influenza vaccines, there are also licensed live virus vaccines. These attenuated live virus vaccines are based on the concept of imitating a natural influenza infection to induce both humoral and cellular immune responses. The attenuated virus strains are sensitive to temperature and adapted to grow at 25°C, which is the temperature of the nasal passage. They are not able to grow in the lower respiratory tract at a temperature of 35°C [34], [37]. Attenuated live virus vaccines are administered intranasally and data was published that the induced humoral response lasts longer. This form of vaccination is especially effective in children but is not recommended in immunosuppressed patient groups such as HSCT recipients and HIV infected individuals [34], similar to other live vaccines such as measles and yellow fever.

Relating to vaccine effectiveness, the consensus between the vaccine strains and the circulating viruses plays a very important role. Even when the recommended vaccine strains and circulating influenza viruses are well matched, the vaccine effectiveness may vary depending on virus type or subtype. A study from 2016 [40] shows a vaccine effectiveness of 33% against H3N2 viruses, compared with 61% against H1N1 and 54% against influenza B viruses. A reason for the decreased effectiveness against influenza H3N2 may be that circulating H3N2 viruses undergo more frequent antigenic changes compared to H1N1 and influenza B viruses. A second reason could be that egg-adapted changes (changes in the virus when grown in eggs) in influenza H3N2 viruses tend to be more varied compared with changes in other influenza viruses, when growing in eggs for vaccine production [36]. In addition to how well matched the vaccine strains are with circulating viruses, the ability of influenza vaccines to prevent illness also depends on characteristics of the vaccine recipient, like age and immunocompetence [35], [36].

1.4. Immune responses to influenza

During an influenza infection, both innate and adaptive immune responses are stimulated [41]. Influenza viruses infect epithelial cells in the human respiratory mucosa, rapidly producing large amounts of virus particles [42].

1.4.1. Innate immune responses

The innate immune system forms the first line of defense during the early phase of infection. Physical barriers composed of mucins and collectins are formed as the initial response to prevent the infection of respiratory epithelial cells [34], [43]. If epithelial

cells are infected, pattern recognition receptors (PPRs) trigger the production of chemokines and cytokines [34]. This induces the recruitment of immune cells of the innate immune system such as macrophages, neutrophils and natural killer (NK) cells to the site of infection [41]. Type I interferons (IFN-α/β) are very important cytokines with several antiviral functions. They induce an antiviral state when binding to neighboring cells by promoting the production of intracellular antiviral proteins via the expression of multiple interferon stimulated genes such as Mx1, IFIT-1, etc. that inhibit viral protein synthesis (**Fig. 5**, A). Also, the recently discovered Type III interferon, Interferon lambda, plays an important role in the innate immune defense against influenza [44]. Additionally, type I interferons recruit macrophages, NK cells and T cells and enhance antigen-presenting cell (APC) maturation and the expression of major histocompatibility complex (MHC) class I and II molecules on their surfaces. This leads to increased antigen presentation which is important for the mechanisms of the adaptive immune system [41]. However, influenza viruses are able to decrease the efficacy of IFN-α/β with the help of NS1 protein, which acts as an antagonist [34].

The recruited macrophages, neutrophils and NK cells are producing additional cytokines, chemokines and other antiviral proteins to promote adaptive immune responses by upregulating the MHC machinery. Furthermore, macrophages, neutrophils and NK cells help to limit the viral replication of influenza viruses.

NK cells are able to recognize reduced expression of MHC class I in infected cells and destroy them by apoptosis (**Fig. 5**, B) [34], [41]. In addition, NK cells also recognize antibody-bound infected cells and lyse them in a process called antibody-dependent cellular cytotoxicity (ADCC) [43].

The recruited alveolar macrophages are producing interleukins (IL-6 and IL-12) and the tumor necrosis factor alpha (TNF-α) as pro-inflammatory cytokines [45], [46]. Macrophages are also killing infected cells by phagocytosis and thus, they are very important for limiting the spread of new infections. A study in pigs showed that a massive decrease of alveolar macrophages leads to impaired cytotoxic CD8+ T-cells and reduced antibody titers [47].

Dendritic cells (DCs) are another type of immune cells involved in the responses to influenza. These immune cells also act as a messenger between the innate and the adaptive immune systems by presenting antigens to T cells, which can happen via two pathways. If influenza viruses infect DCs themselves, the viral antigens are processed and presented by MHC class I molecules to cytotoxic T lymphocytes (CTLs). Another

mechanism involves the presentation via MHC class II molecules after active phagocytosis of virus particles and infected epithelial cells to CD4+ T helper cells [48], [49].

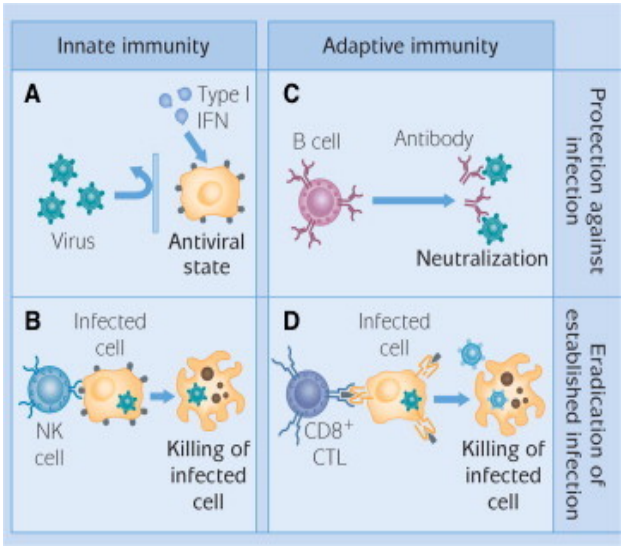


Fig. 5. Immune responses to influenza virus infections. During the early phase of infection the innate immune system is stimulated. Released type I IFNs induce an antiviral state when binding to cells (A). Infected cells are killed by NK cells to prevent the rapid spread of virus particles (B). As part of the immune response pathways also the adaptive immune system gets activated. Specific antibodies produced by B cells neutralize virus particles (C) and CTLs destroy virus-infected cells (D). Figure edited [41].

1.4.2. Adaptive immune responses

The second line of defense is built by the adaptive immune system, which is essential for eliminating the viral infection completely and for developing immunological memory [41], [43]. It consists of humoral immunity, including influenza virus specific antibodies and cellular immunity, mediated by T cells such as CD4+ T helper cells, cytotoxic CD8+ T cells and regulatory T cells [43].

Antigenic influenza virus peptides are processed and presented on the surface of professional antigen-presenting cells (APCs), especially dendritic cells, via MHC class II molecules. The CD4 receptors on the surface of CD4+ T cells are able to dock to these MHC class II molecules. Due to the recognition of the viral peptides by the T cell receptor (TCR), the CD4+ cells are activated and differentiated into T-helper subsets called Th1 and Th2 [50]. While Th1 helper cells trigger the activation of macrophages and CD8+ T cells by producing IFNγ and IL-2, Th2 type cells are responsible for activation and production of antibodies from B cells due to the production of IL-4, IL-5 and IL-13 [51]–[54].

Antigenic influenza virus peptides presented on MHC class I molecules are recognized by the TCR of CD8+ T cells, assisted by their CD8 molecules. Subsequently, the CD8+ T cells are activated and differentiate into cytotoxic CD8+ T cells (CTLs). The CTLs are then recruited to the sites of infection to eliminate infected cells and prevent further production of virus particles (**Fig. 5**, D) [55].

Activated B cells induce the humoral immunity of the adaptive immune system. The B cells can be activated during influenza virus infection or after influenza vaccination and produce influenza specific antibodies [56]. Specific antibodies targeting the surface glycoproteins HA and NA are of importance. In a previous study, it was shown that antibodies against HA primarily correlate with immune protection, due to the prevention of viral attachment to host cells and blocking the receptor mediated endocytosis [57]. The HA specific antibodies are binding to the trimeric globular head of the surface glycoprotein and have a neutralizing impact (**Fig. 5**, C). This sterilizing immunity is strain dependent and fails to work against other subtypes [58]. In a publication of Whittle et al., antibodies directed against the HA receptor-binding pocket, which can neutralize antigenically diverse influenza viruses of the same subtype, are described [59]. Compared to the variable globular head of the HA protein, the HA stem region is highly conserved, because it is physically masked for the immune system. Even so, there is a small amount of antibodies targeting this region during influenza virus infection. Some of these antibodies show a broad neutralizing capacity by recognizing and binding HA molecules from different subtypes [60]–[62]. Antibodies targeting the surface protein NA do not have a neutralizing impact, but limit the spread of infection, due to the inhibition of virus release [63]. Furthermore, antibodies targeting the third envelope protein M2 and the ribonucleoproteins have been previously identified [64], [65]. These non-neutralizing antibodies could be associated with the mechanism of antibody-dependent cellular cytotoxicity (ADCC) [66].

As response to the primary infection, IgM, IgA and IgG subtypes of antibodies are produced, whereas IgM is not produced as response to a secondary infection [67]. IgA are secretory antibodies located at the mucosal surface, while IgG subtypes are circulating in the blood, from where they transfer to the airways and lungs. The priming of B-cells to produce influenza-specific IgG with high avidity takes about one month upon primary encounter with an antigen – in a re-encounter, much more rapid IgG can be produced via Plasmablasts B-cells. The memory based IgG immune response takes only about 7 days for high titer production [41].

1.5. Allogeneic hematopoietic stem cell transplantation

The transplantation of multipotent hematopoietic stem cells is called hematopoietic stem cell transplantation (HSCT). The transplanted stem cells are normally derived from bone marrow, peripheral blood or also umbilical cord blood [68], [69]. In contrast to autologous HSCT (auto-HSCT), where the patient's own stem cells are transplanted, the stem cells of allogeneic HSCT (allo-HSCT) arise from a related or unrelated person (healthy donor). For allogeneic HSCT, it is mandatory that the human leukocyte antigen (HLA) types of both, the donor and recipient, are matched to a high degree. The survival of the recipients after allo-HSCT depends on HLA-matching, the graft-versus-host (GvH) response and the development of a graft-versus-leukemia effect [70]. However, in recent years, mismatched or haploidentical donors are used, because of the development of better prevention strategies against GvH disease (GvHD) [71]. Allogeneic HSCT is used for treatment of a variety of malignant and non-malignant conditions, like lymphoma, leukemia, immune-deficiency illnesses, congenital metabolic defects and hemoglobinopathies [70]. In 2012, 68146 cases of HSCT (53% autologous and 47% allogeneic) in 77 countries were reported. 16433 donors of the allo-HSCT recipients were unrelated, whereas 15493 donors where related to the recipients. Compared with 2006, the total HSCT cases were increased by 46%, with an increase of 57% in allo-HSCT and 38% in auto-HSCT cases. Prior to the HSCT procedure, whole body irradiation and intensive chemotherapy is performed in order to remove the "old" hematopoietic system including e.g. leukemic cells. After the "new" hematopoietic system is established, there is a period of severe immunosuppression due to pancytopenia for about 2-6 weeks until the "new" cells are engrafted and start to proliferate. In this phase, the risk for infectious diseases is highest [72].

1.5.1. Influenza infections in HSCT recipients

Morbidity and mortality rates in HSCT recipients after influenza infections are increased, compared to people who are not belonging to a high risk group, as shown in several studies. In a retrospective study over 12 seasons (1989-2002), 62 (1,3%) of 4797 HSCT recipients were diagnosed with influenza. 18 of these 62 influenza cases developed pneumonia (29%) and among them, the mortality was 5/18 (28%) [6]. During 1997 to 2000, 3,5% of allo-HSCT recipients got infected with influenza and 15,3% of the infected patients died in 37 European centers [34]. Another study from

Ljungman et al. was performed with 286 HSCT recipients (222 allo-HSCT and 64 auto-HSCT), who got infected with pandemic A/H1N1 strains in 2009. They showed that 43,7% were hospitalized, 32,5% developed lower respiratory tract disease, 11,5% required mechanical ventilation and 6,3% died from the influenza infection or its complications [7].

Influenza vaccination in HSCT patients show impaired antibody responses in comparison to immunocompetent individuals [34]. Therefore, various strategies have been evaluated to improve vaccine outcomes e.g. booster dosage, higher antigen concentration. In particular, a second dose of influenza vaccine (booster-strategy) resulted in better seroprotection rates post-vaccine compared to a single dose regimen [5], [73]–[77].

2. Materials and methods

In this chapter, all chemicals, reagents, and materials used are listed. Additionally, the principles and workflows of the hemagglutination inhibition assay (HI assay) and the enzyme-linked immunosorbent assay (ELISA) are described. Furthermore, the serum collection and the statistical analysis are declared. The study protocols were approved through the local ethical review board (www.EKNZ.ch) and written informed consent was obtained from all participants. The method to determine influenza-specific antibody titers with the HI assay were recently published (reference, Kaufmann et al. JOVE in press)

2.1. Chemicals, reagents and materials

Table 1 contains an alphabetical list of all chemicals and reagents used.

Table 1: List of chemicals and reagents

Hemagglutination inhibition assay		
name	*company*	*product number*
25 ml Disposable Multichannel Pipette Reservoirs	Integra	4312
8-well PCR tubes	Brand GMBH	781332
96-well microtiter plate, U-shaped	TPP	92097
96-well microtiter plate, V-shaped	Corning Costar	3897
Aqua ad iniect. Steril	Bichsel AG	1000004

Chicken RBC (10%)	Cedarlane	CLC8800
Cholera filtrate (RDE)	Sigma-Aldrich	C8772
Dulbecco's PBS	Sigma-Aldrich	D8537
Eppendorf Multichannel pipette, 12-channel, 10-100 µl	Sigma-Aldrich	Z683949
Eppendorf Multichannel pipette, 8-channel, 10-100 µl	Sigma-Aldrich	Z683930
Guinea Pig RBC (10%)	Cedarlane	CLC1800
Influenza Anti-A/California/7/09 HA serum	NIBSC	14/134
Influenza Anti-A/Switzerland/9715293/13-like HA serum	NIBSC	14/272
Influenza Anti-A/Texas/50/2012-Like HA serum	NIBSC	13/178
Influenza Anti-B/Brisbane/60/2008-HA serum	NIBSC	13/254
Influenza Anti-B/Massachusetts/02/2012 HA serum	NIBSC	13/182
Influenza antigen A/California/7/09 (H1N1)(NYMC-X181)	NIBSC	12/168
Influenza antigen A/Switzerland/9715293/2013 (NIB88)	NIBSC	14/254
Influenza antigen A/Texas/50/2012 (H3N2)(NYMCX-223)	NIBSC	13/112
Influenza antigen B/Brisbane/60/08	NIBSC	13/234
Influenza antigen B/Massachusetts/02/2012	NIBSC	13/134
Serum-Tubes	S-Monovette	01.1601.100
Single Donor Human RBC, Type 0	Innovative Research	IPLA-WB3
Turkey RBC (10%)	Cedarlane	CLC1180
ELISA		
name	*company*	*product number*
Bovine Serum Albumin	Sigma-Aldrich	A7030
Dulbecco's PBS	Sigma-Aldrich	D8537
F96 Maxisorp Nunc-Immuno Plate	Thermo Fisher Scientific	442404
H_2SO_4 95-97%	Merck	100731
Influenza antigen A/California/7/09 (H1N1)(NYMC-X181)	NIBSC	12/168
Influenza antigen A/Switzerland/9715293/2013 (NIB88)	NIBSC	14/254
Influenza antigen A/Texas/50/2012 (H3N2)(NYMCX-223)	NIBSC	13/112
Influenza antigen B/Brisbane/60/08	NIBSC	13/234

Influenza antigen B/Massachusetts/02/2012	NIBSC	13/134
Microtiter 96 Deep Well Plate	Thermo Fisher Scientific	95040450
PBS Dulbecco powder	Biochrom	L182
Polyclonal Rabbit Anti-Human IgG/HRP	Dako	P0214
TMB substrate Reagent Set	BD Biosciences	555214
Tween 20	Sigma-Aldrich	P7949

2.2. Serum collection and vaccine regimen

Blood samples from the HSCT recipients and healthy controls (HC) were directly collected in S-Monovette serum tubes. The first serum sample of each person was taken at the same day when the trivalent influenza vaccine injection (Agrippal, Novartis, 2014/2015) was given (day 0). The trivalent vaccine contained 15 µg HA of the influenza strains A/California/7/09 (H1N1), A/Texas/50/2012 (H3N2) and B/Massachusetts/02/2012, respectively. The other blood samples were taken at day 7, day 30, day 60 and day 180 after vaccination. The HSCT patients received a second dose of the trivalent influenza vaccine at day 30, whereas the HC only received a single influenza vaccine.

The uncentrifuged blood samples are only storable at room temperature for a maximum of 24 hours. To obtain the serum, the serum tube was centrifuged at 1200 g for 10 minutes at room temperature. After that, the serum was aliquoted to 3 cryo vials (about 500 µl each) and frozen at -75°C. Subsequent analyses were performed batchwise for each patient to reduce variability within the same patient.

2.3. Hemagglutination inhibition assay

For a better understanding of the hemagglutination inhibition (HI) assay, this chapter is divided into the subchapters: principle of the assay, HA titration, and performance of the HI assay.

2.3.1. Principle of the assay

In solution, erythrocytes (red blood cells; RBCs) settle down at the bottom of a microtiter well and occur as a compact button (**Fig. 6**, A). The glycoprotein hemagglutinin (HA), located on the envelope of influenza viruses, has the ability to

clump erythrocytes together ("agglutinate"). This process is also called hemagglutination [78]. The lattice structure of interconnected RBCs and virus particles prevents the RBCs to settle down as a compact button on the bottom of a well. The agglutinated cells can be seen as a diffuse reddish solution instead (**Fig. 6**, B). This interaction can be used to detect antibodies against that particular virus. For example, if influenza virus particles are added to serum, where anti-influenza antibodies are present, the antibodies will bind to the virus particles. When adding RBCs to the well afterwards, the RBCs will settle down as a compact button, because the antibody-virus interaction prevents attachment of the virus to the RBCs (**Fig. 6**, C). This means hemagglutination is inhibited, which is also featured in the assay's name. It is also possible to quantify the antibodies in the serum by preparing serial dilutions and adding the same amount of virus, respectively, before adding the RBCs. The antibody levels are indicated as titers. The highest dilution of serum antibodies that inhibits hemagglutination is called the hemagglutination inhibition titer (HI titer) of the serum [25], [79].

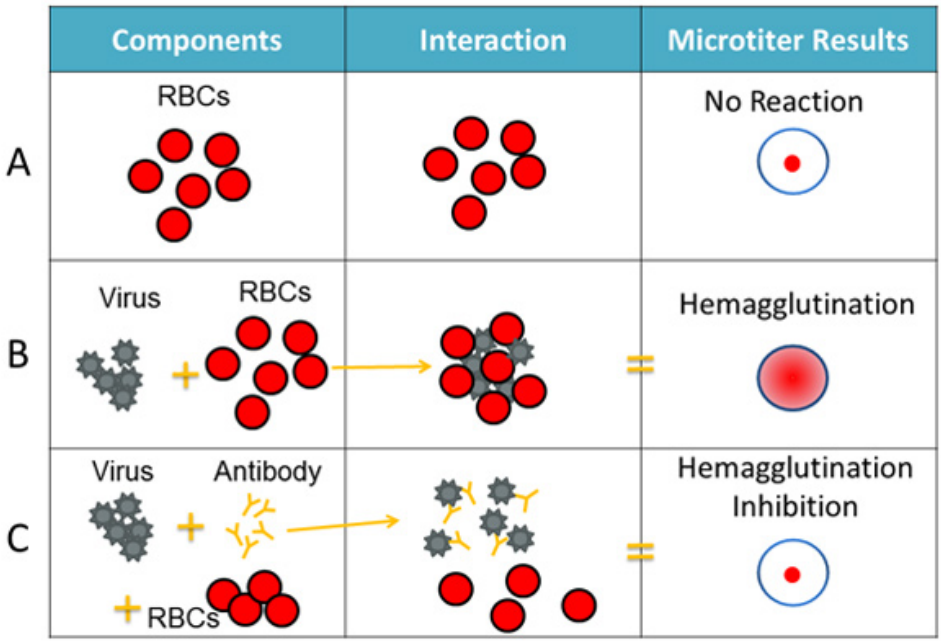


Fig. 6. Principle of hemagglutination and hemagglutination inhibition. When in solution red blood cells (RBCs) settle down at the bottom of a well and occur as a compact button (A). Adding virus particles with hemagglutinin (HA) on their surface leads to the agglutination of the cells (hemagglutination). This can be seen as a diffuse reddish solution (B). When antibodies against the particular virus are present, the hemagglutination is inhibited, due to the binding of the antibodies to the virus particles (hemagglutination inhibition). The RBCs then settle as a compact button (C). Figure adapted from [13].

To make the HI assays comparable among themselves, the same amount of virus particles (antigens) has to be used when adding to dilutions of different serum samples. To quantify the virus particles a HA titration (HA assay) is performed and the amount is declared as HA units (*see 2.3.2* HA titration). According to the WHO, the standard of the HI assay is 4 HA units of antigen added to twofold dilutions of antisera [25]. Depending on the antigen, different species of RBCs were used for the assay. After consulting NIBSC, chicken RBCs were used with Influenza antigen A/California/7/09 (H1N1), turkey RBCs were used with the two Influenza B strains (B/Brisbane/60/08 and B/Massachusetts/02/2012) and guinea pig RBCs were recommended to use with Influenza antigen A/Switzerland/9715293/2013 and Influenza antigen A/Texas/50/2012 (both H3N2) as it is listed in Table 2.

Table 2: Influenza antigens and corresponding species of RBCs

Influenza antigen	A/California/ 7/09 (H1N1)	A/Switzerland/ 9715293/2013 (H3N2)	A/Texas/50 /2012 (H3N2)	B/Brisbane/ 60/08	B/Massachusetts /02/2012
RBC species	Chicken	Guinea pig		Turkey	

The different species of RBCs were used in different types of 96-well microtiter plates and the incubation time as well as the appearance of the non-agglutinated cells differentiated (Table 3). V-shaped 96-well microtiter plates were chosen when using chicken or turkey RBCs. The incubation time was 30 minutes at 25°C and the settled cells appeared as a button [25]. For the readout the plate was tilted 90 degrees for 25 seconds and the results were marked immediately, while the plate was still in the tilted position. The non-agglutinated, settled cells flow down. When hemagglutination occurs, no button can be observed, or the button remains constant and when the cells flow just half the way down, it was marked as half-agglutinated (**Fig. 7**; avian patterns). Tilting the plate is crucial for the differentiation of avian patterns, because all of the three different types of agglutination patterns (completely agglutinated, partially agglutinated and non-agglutinated) can occur as a button when not tilted. The reason why even the completely agglutinated cells can occur as a button, is a slightly higher RBC concentration of 0,75% used for the assay, compared to the WHO protocol [25]. Lower concentrations were tested, but a RBC concentration of 0,75% exposed as the optimum for distinguishing the agglutination patterns and to avoid mistakes at the readout (see

Fig. 15).

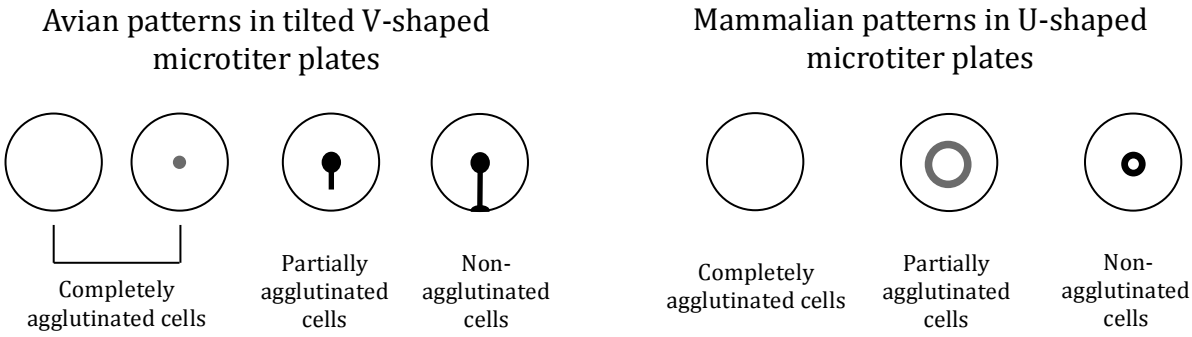


Fig. 7. Agglutination patterns of avian and mammalian RBCs. The readout of the assay is more pretentious when using avian RBCs from chicken or turkey. The results should be marked very fast after 25 seconds of tilting the plate, because afterwards also the remained buttons of the agglutinated cells slowly start to flow. For the readout of the agglutination patterns of mammalian RBCs (guinea pig or human RBCs), tilting the plate is not necessary. The non-agglutinated cells are seen as a halo. If the circle of the halo appears bigger, it was marked as half agglutinated.

When using mammalian RBCs like guinea pig or human RBCs, U-shaped 96-well microtiter plates were chosen. The used RBC concentration was 1% and the incubation time at 25°C was 1 hour (Table 3). When hemagglutination occurs, the agglutinated cells don't settle down whereas non-agglutinated cells appear as a halo at the bottom of the well (**Fig. 7**; mammalian patterns). The halo of the partially agglutinated cells is less intense and has a bigger diameter.

Table 3: Assay conditions with different species of RBCs

RBC species	Chicken	Turkey	Guinea pig	Human type 0
Concentration of RBCs (v/v)	0,75%	0,75%	1%	1%
Type of microtiter plate	V-Bottom	V-Bottom	U-Bottom	U-Bottom
Incubation time, 25°C	30 min	30 min	1 hour	1 hour
Appearance of non-agglutinated cells	button*	button*	halo	halo

* flows when tilted

2.3.2. HA titration

The HA titration (also HA assay) was performed with every of the five antigens and the corresponding RBC species to determine the proper dilution factor of the antigen (4 HA units) used for the hemagglutination inhibition assay. The RBCs were used within two weeks to avoid the loss of erythrocytes due to hemolysis. The HA titration was repeated every time new RBCs arrived, because the concentration and the condition of RBCs may slightly differentiate (biological material).

The first step was diluting the RBC stock solutions (10%, v/v) with PBS to get the proper concentrations for avian and mammalian RBCs of 0,75% and 1%, respectively. Then 25 µl of PBS were added to wells 1 to 12 of each used row by using a multichannel pipette. Afterwards 25 µl of antigen were added to the first well of the antigen-rows, which were arranged in duplicates. No antigen was added to the control-rows. The next step was a two-fold serial dilution by transferring 25 µl from the first well of the antigen-rows to successive wells, also by using a multichannel pipette. The final 25 µl of the last wells were discarded. Then again 25 µl of PBS were added to wells 1 to 12 of each used row to a total volume of 50 µl per well. The final step of the HA titration was adding 50 µl of the RBC suspension to each used well. After the incubation at 25°C the results were marked (**Fig. 8**).

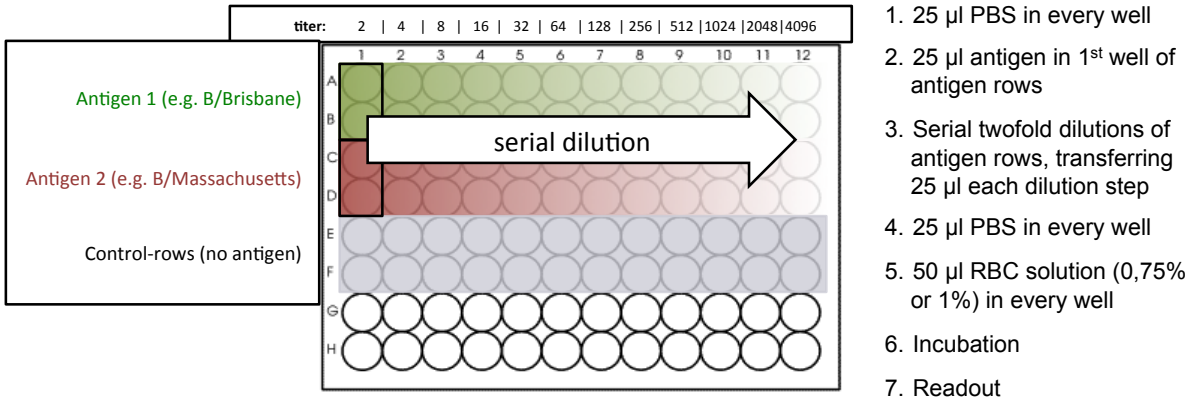


Fig. 8. Stepwise manual of the HA titration. The HA titration was performed to determine the titer of 4 HA units. Therefor the use of the appropriate type of microtiter plate, RBC species and concentration and incubation time has to be considered.

For the readout the results were marked at a printed scheme of the 96-well plate as shown in **Fig. 9**. The highest dilution of virus that still causes complete hemagglutination

is defined as the HA titration end point and the HA titer is the reciprocal of this dilution. For example, if a virus causes complete hemagglutination up to the well with the 1:256 dilution, the HA titer is 256 [80].

The HA titer contains one unit of hemagglutination. A "unit" of hemagglutination is an operational unit dependent on the method used for HA titration and is not a measure of an absolute amount of virus. Therefore, an HA unit is defined as the amount of virus needed to agglutinate an equal volume of a standardized red blood cell suspension [25]. For the HI assay 4 HA units of virus or antigen are added to twofold dilutions of antisera. Since 25 µl of antigen is added to each well at the HI assay, a virus dilution of 4 HA units per 25 µl is needed. Hence, 4 HA units can be calculated by dividing the HA titer (which is based on 25 µl) by 4, or multiplying the dilution by 4. For example the HA titer is 256, 1 HA unit corresponds to the titer 256 and 4 HA units are contained in a titer of 64. To prepare the antigen for the HI assay, the antigen has to be diluted 1:64 in this case (**Fig. 9**).

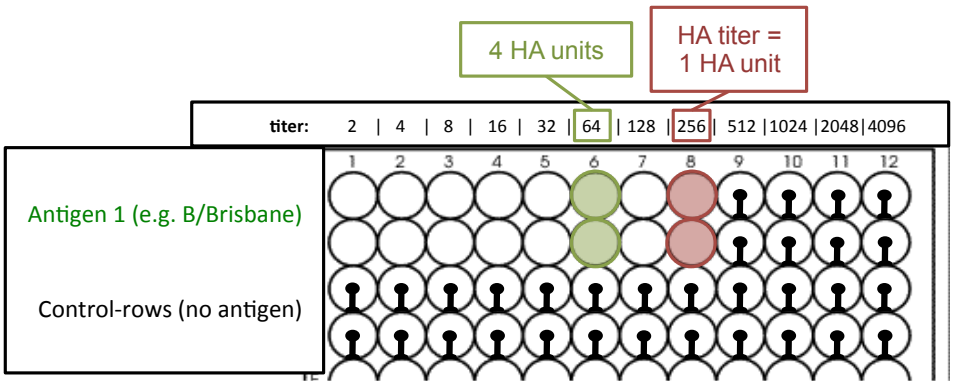


Fig. 9. Readout of the HA titration with avian RBCs to determine the titer of 4 HA units. The last well where complete hemagglutination occurs is the HA titer and contains 1 HA unit. Because of the twofold dilutions of the antigen, two wells ahead of the HA titration end point, the titer corresponds to 4 HA units (4 HA units equals the HA titer divided by 4). In this example the HA titer is 256 and 4 HA units equals a titer of 64. Therefore the antigen has to be diluted 1:64 when used for the HI assay.

2.3.3. Performance of the HI assay

After the determination of the correct antigen dilution of 4 HA units per 25 µl, the serum samples were prepared. First, the frozen serum samples of each time point of every person were thawed at room temperature. Aliquots of 20 µl of each thawed serum

sample were added to the wells of 8-well PCR tubes. The PCR tubes were arranged in groups of 10 wells, so that all five time points of two persons are in one column (**Fig. 10**). The big advantage of using PCR tubes is, that for the following steps a multichannel pipette can be used, which saves a lot of time when measuring a huge amount of serum samples. The aliquoted serum samples in the PCR tubes were stored at -75°C. One day before the serum samples were used for the HI assay, cholera filtrate, which contains the enzyme neuraminidase, was added. The cholera filtrate removes nonspecific inhibitors and is also called receptor-destroying enzyme (RDE). 60 µl of the RDE solution were added to each of the 20 µl serum aliquots, respectively (3 vol. of RDE to 1 vol. of serum) by using the multichannel pipette and briefly vortexed. For the readout of the assay, it must be considered that the serum samples are diluted 1:4 after adding RDE. The samples were incubated overnight at 37°C using a thermocycler (Biometra T3) and heated the next morning at 56°C for 30 minutes to inactivate the RDE, also by using the thermocycler. Afterwards, the tubes were stored at 4°C until they were used for the assay. The respective anti-sera were also treated with RDE the same way before used for the assay.

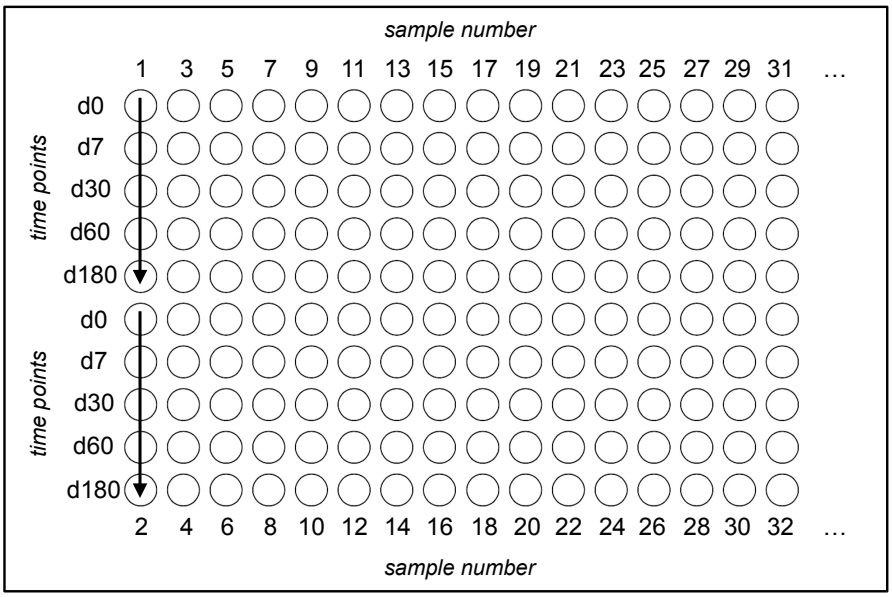
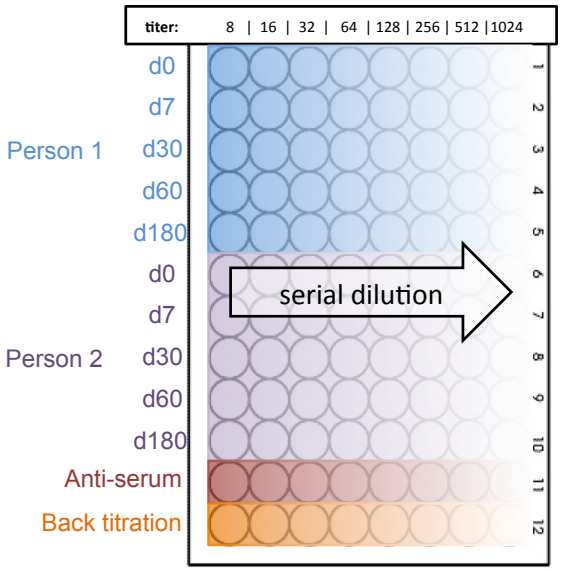


Fig. 10. Arrangement of the aliquoted serum samples in PCR tubes. All five time points of two persons are in one column. The serum samples are later also added in this order to the 96-well microtiter plate for the HI assay. Hence, a multichannel pipette can be used, which saves a lot of time. The 20 µl aliquots of the serum samples in the PCR tubes are stored at -75°C.

Before performing the HI assay, the amount of antigen needed was calculated according to the number of plates used and the proper dilution of 4 HA units was prepared. The amount of RBCs needed was also calculated and diluted to the correct concentration. PBS was used for diluting the antigens and the RBCs and it was prepared in falcon tubes.
Afterwards the 96-well plates were labeled and 25 µl of PBS were added to every well except for the first well of the "back titration" row (12th row, **Fig. 11**). A back titration was performed to check if the used antigen dilution equals 4 HA units. An antigen titer of 4 HA units is indicated if hemagglutination occurs in the first three wells of the back titration row. If an antigen does not have an HA titer of 4, it must be adjusted accordingly by adding more antigen to increase units or by diluting to decrease units. It is acceptable to have complete hemagglutination in two or three wells, which represents 2 or 4 HA units, respectively.
The next step was adding 25 µl of the RDE-treated serum samples to the first wells of rows 1 to 10 on each plate, using the multichannel pipette. 25 µl of the appropriate anti-serum were added to the first well of the 11th row as a positive control. 50 µl of the diluted antigen were added to the first well of the back titration row (12th row). The next step was serial twofold dilutions by transferring 25 µl from the first well of each row (1-12) to successive wells, also by using a multichannel pipette. The samples were well mixed before transferring to the next wells. 25 µl of the respective last wells were discarded. Then, 25 µl of the antigen solution were added to each well of rows 1 to 11 (serum samples and anti-serum) and 25 µl of PBS instead of antigen were added to each well of the back titration row (12th row). After carefully tapping the plates, the plates were incubated at room temperature for 30 minutes. Afterwards 50 µl of the RBC-solution is added to every well and carefully mixed by tapping. The plates were incubated again at room temperature for the appropriate timespan according to the RBCs being used [25], [80]. Then, the agglutination patterns were marked on a hardcopy of the plate scheme and later the HI titers of every time point of each person were transferred to a computer-based table.



1. 25 µl PBS in every well, except for 1st well of back titration row
2. 25 µl RDE-treated serum in 1st well of rows 1 - 11
3. 50 µl of diluted antigen in 1st well of back titration row
4. Serial twofold dilutions of every row, transferring 25 µl each dilution step, discarding 25 µl of the last wells
5. 25 µl diluted antigen to every well of rows 1 - 11
6. 25 µl PBS to back titration row
7. Tapping plate and incubation at 25°C for 30 min
8. 50 µl RBC solution (0,75% or 1%) in every well
9. Tapping plate and incubation
10. Readout

Fig. 11. Stepwise manual of the HI assay. Five time points of two people could be measured on one plate. The HI titer range was from 8 to 1024. An anti-serum of the used antigen served as positive control and a back titration was performed to check if the antigen dilution equals 4 HA units. For almost every step a multichannel pipette could be used, which saves a lot of time and reduces the risk of adding components to wrong wells.

For the readout, it is important to consider that the RDE-treated sera were already diluted 1:4 and after the serial dilution step the sera in the first wells are diluted 1:8, which is an HI titer of 8. If an antigen-antibody reaction occurred, hemagglutination of the RBCs was inhibited. The HI titer is the reciprocal of the last dilution of (anti-) serum that completely inhibits hemagglutination. For example, if a serum sample inhibits hemagglutination up to the 4th well (1:64 dilution), the HI titer is 64 (**Fig. 12**, 2nd row). Partially agglutinated wells were marked as "1/2 – agglutinated". For example, if a serum sample completely inhibits hemagglutination up to the 3rd well (1:32 dilution) and the 4th well (1:64 dilution) is partially agglutinated, the HI titer is set 45 (**Fig. 12**, 1st row), which is a 0,5 step on a base-2 logarithmic scale between these two titers. In contrast to the WHO-protocol [25], in which just the completely agglutinated wells were considered, we included the partially agglutinated wells to our results to be more accurate in distinguishing the different antibody-levels. In **Fig. 12** the readout of one plate is shown.

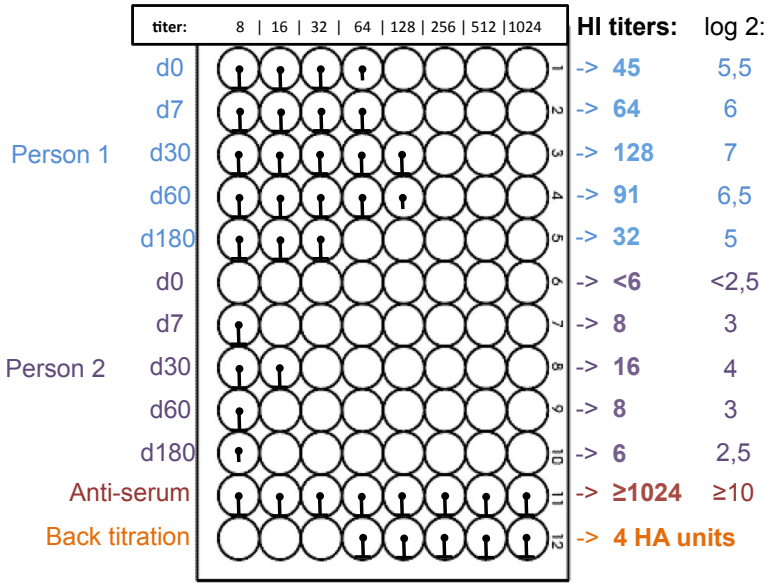


Fig. 12. Illustration of the agglutination patterns of one plate. If an antigen-antibody reaction occurs, hemagglutination of the RBCs is inhibited. The HI titer is the reciprocal of the last dilution of (anti-) serum that still inhibits hemagglutination. The non-agglutinated cells are seen as a flowed button, due to the use of avian RBCs. Person 1 has higher HI titers than person 2. If the RBCs are partially agglutinated in one well, it was marked as "half-agglutinated" and the HI titer then is half a step on a base-2 logarithmic scale less then the titer of this well.

2.4. Statistical analysis

For this research study, the HI titers of 25 healthy controls and 53 allogeneic HSCT recipients at five time points were measured, to assess vaccine-induced antibody responses. Baseline characteristics of HSCT patients and healthy controls were shown in Table 4. For the data analysis titers below 6 (titers ≤4) were assigned a value of 2 and geometric mean titers (GMTs) with 95% CI were computed, like it was done in several publications before [1], [5], [81]–[83]. For calculations all titers were log2-transformed and due to the Shapiro-Wilk normality test, they are not normally distributed. Therefore non-parametric tests were used to compare two groups, such as the Mann-Whitney U test (if samples are not related to each other) and the Wilcoxon signed-rank test (if samples are related). To determine the progress of the antibody titers over a time period, not only the GMTs prior and after vaccination were examined. Conventional criteria to assess influenza vaccine efficacy are GMT ratios, seroprotection rates (defined as a post-vaccination HI titer more than 1:40) and the rates of seroconversion. Seroconversion is defined as the percentage of people with either a pre-vaccination HI

titer < 1:10 and a post-vaccination HI titer > 1:40 or a pre- vaccination HI titer > 1:10 and a minimum four-fold rise in post-vaccination HI antibody titer [5], [84], [85].
The creation of the graphs and the calculations were done with the software Prism (GraphPad, version 6.0h) and Excel (Microsoft, version 14.5.9).

Table 4: Baseline characteristics of patients and controls

	HSCT recipients	**Healthy Controls**
Number of people, n (%)	**53 (100)**	**25 (100)**
Age at vaccination, median (range), years	55 (22-72)	36 (22-65)
<40 years, n (%)	6 (11,3)	16 (64)
40-49 years, n (%)	16 (30,2)	5 (20)
50-59 years, n (%)	12 (22,6)	3 (12)
≥60 years, n (%)	19 (35,8)	1 (4)
Female, n (%)	25 (47,2)	14 (56)
Male, n (%)	28 (52,8)	11 (44)
Transplantation-to-vaccination interval, median (range), years	4,1 (0,7-25,1)	
<5 years, n (%)	29 (54,7)	
≥5 years, n (%)	24 (45,3)	

2.5. Enzyme-linked immunosorbent assay

To measure the immunoglobulin G (IgG) levels against the different influenza strains prior and after the vaccination, an indirect ELISA was performed. The principle of the assay is shown in **Fig. 13**. Therefore, the same five inactivated antigens that were used for the HI assay (see Table 2) also were used in the ELISA to coat the F96 Maxisorp Nunc-Immuno plates. The antigens were diluted with PBS to a concentration of 0,5 µg/ml and 60 µl were added to each well. The caps were wrapped in aluminum foil and the plates were incubated overnight at 4°C. The next morning the plates were washed three times each with PBS + 0,05% Tween. For blocking 100 µl per well of PBS + 5% BSA were added and incubated at room temperature for 2 hours on the shaker. After second time washing with PBS + 0,05% Tween (three times), 100 µl/well of the serum samples (1:5000 in PBS + 0,5% BSA) were added to the plates. The dilution of the serum was performed in three steps (1:10, 1:10 and 1:50), using U-shaped 96 well plates and a 96 deep-well plate for the last dilution step. Measuring duplicates of 100 µl each at 5 plates (5 antigens), altogether 1 ml of each serum sample was used. For each antigen a serum

with high antibody titer (1024) was used as reference. Twofold dilutions of this serum were added to generate a standard curve. The sample pattern on the plate is shown in **Fig. 14**. The plates were incubated again at room temperature for 2 hours on the shaker and washed for the third time with PBS + 0,05% Tween (three times). Afterwards 100 µl per well of the detection antibody, which was a polyclonal rabbit anti-human IgG with HRP (diluted 1:6000 in PBS + 0,5% BSA), were added and incubated at room temperature for 1 hour on the shaker. The plates were washed three times with PBS + 0,05% Tween and a fourth time with just PBS to avoid bubbles. 100 µl per well of TMB substrate (TMB has to be at room temperature) were added and after 17 minutes on the shaker the reaction was stopped by adding 50 µl/well of 2N H_2SO_4. The read out was performed with a microplate reader (synergy H1 from biotek, Switzerland) at 450 nm.

There is no reference serum, with a known concentration of specific IgGs against different influenza viruses available. The common method to quantify these serum IgG-levels is to use a sample with a high antibody titer (against the used influenza strain) as reference serum pool and generate a standard curve by serial diluting the serum. To get actual values, which can be used for the data analysis, arbitrary units (AU) are introduced [86], [87]. The absorbances of the highest dilutions of the reference sera (1:5000, same as samples) were set as 1024 AU (each had a HI titer of 1024). By two-fold serial diluting the reference sera, the ranges of AU reached from 1024 AU to 8 AU. The standard curves were generated with the software Prism (GraphPad, version 6.0), by plotting the measured mean absorbances of the standard against the fixed log10-transformed AU values. A four-parameter logistic (4PL) curve fit was used to interpolate the mean absorbances of the measured serum samples to the standard curves. The interpolated values were computed as log10-values, which had to be transformed to AU using the formula (x=10^x).

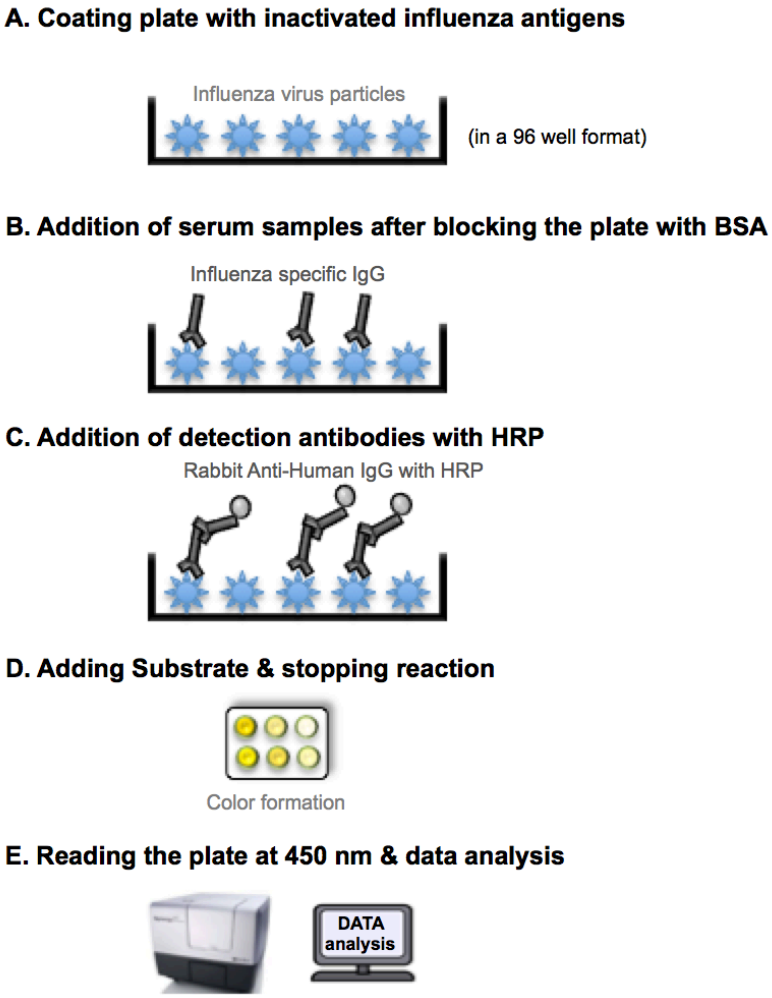


Fig. 13. Principle of the ELISA used to quantify the IgG-levels against different influenza strains. The 96-well maxisorp nunc-immuno plates were coated with inactivated influenza virus particles with a concentration of 0,5 µg/ml. After blocking with BSA, a 1:5000 dilution of the serum samples were added. The detection antibody used, was a polyclonal rabbit anti-human IgG with HRP, which was added at a 1:6000 dilution. TMB was added as substrate and after 17 minutes the reaction was stopped with 2N H_2SO_4. The read out was performed with a microplate reader at 450nm. Figure edited [88].

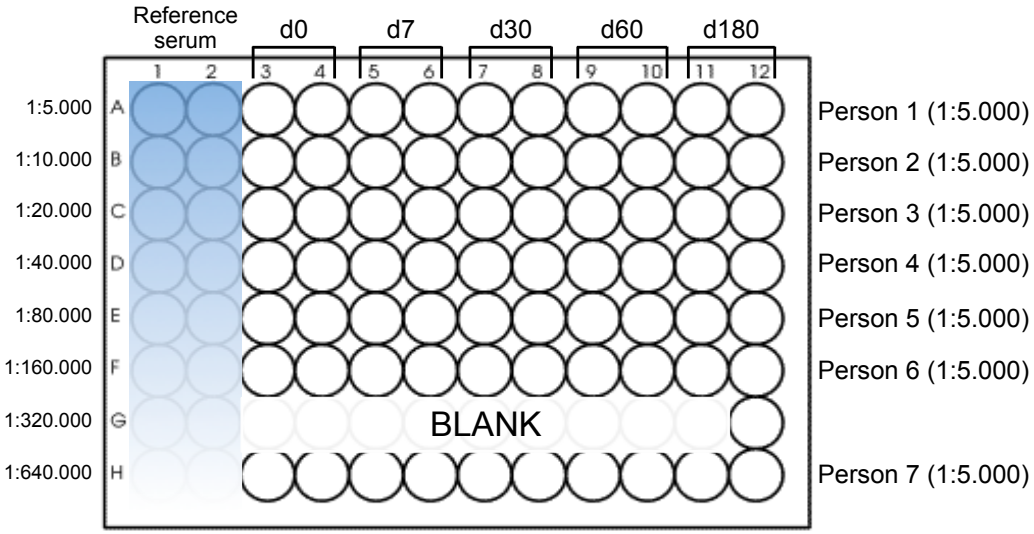


Fig. 14. Scheme of ELISA plates. On each plate all five time points of seven persons could be measured in duplicates. The serum samples were diluted 1:5000 in PBS + 0,5% BSA. For each antigen a serum with a high HI titer (1024) was used as reference. Twofold dilutions of this serum were added to generate a standard curve.

3. Results

Before measuring antibody titers in serum samples, the assay itself had to be improved. To determine the optimal amount of red blood cells used for the assay, a HA titration (see 2.3.2) with different RBC concentrations was performed. In

Fig. 15 a HA titration-plate with turkey RBCs and influenza B/Brisbane and B/Massachusetts antigens is shown. The used RBC concentrations were 0,5%, 0,75% and 1%. At a concentration of 0,5%, it cannot be clearly distinguished between agglutinated and non-agglutinated patterns (**Fig. 15**). The settled, non-agglutinated cells are not flowing down after tilting the plate due to the low amount of red blood cells. A turkey RBC concentration of 0,75% leads to a button of settled cells, which flows down, if the cells are not agglutinated. When hemagglutination occurs however, the agglutinated erythrocytes are seen as a diffuse reddish solution. Hence, 0,75% of turkey RBCs were later used for the assay. When using a concentration of 1%, some RBCs can settle and flow after tilting even at agglutinated wells, because of the high amount of RBCs.

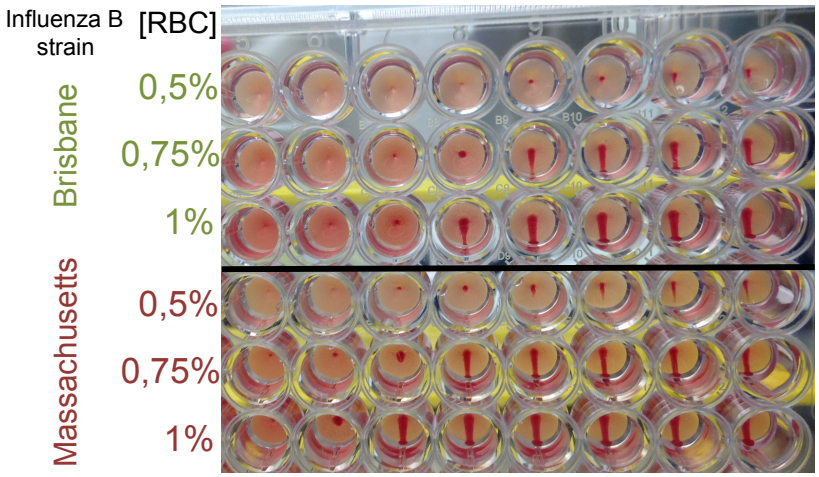


Fig. 15. HA titration to determine the optimum concentration of turkey RBCs for the assay. A RBC concentration of 0,5% is not enough to clearly differ agglutinated patterns from non-agglutinated once. A turkey RBC concentration of 0,75% was exposed to be the optimum amount for use at the assay.

The same trials were also performed with chicken and guinea pig RBCs, using influenza viruses A/H1N1 and A/H3N2, respectively. The best chicken RBC concentration to use, relating to the read out of the assay, was 0,75%. For guinea pig RBCs, 1% was highlighted as the optimum concentration to work with.

3.1. Reproducibility

To determine an important characteristic of the assay, the reproducibility, the antibody titers against influenza B/Brisbane and A/California of 44 different serum samples were measured at two different days. The p-value of the log2-transformed antibody titers was 0,8633, based on the Wilcoxon matched-pairs signed rank test (**Fig. 16**). The maximum deviation within a pair was 1 titer step.

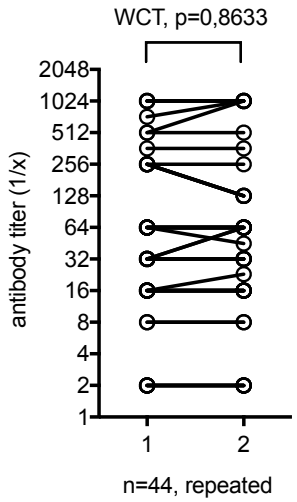


Fig. 16. Reproducibility of the HI assay. The antibody titers against two influenza viruses (B/Brisbane and A/California) of 44 different serum samples were measured at two different days. The p-value of the log2-transformed antibody titers was 0,8633 and the highest deviation within a pair was 1 titer step.

3.2. HA titration

The HA titration was performed with every of the five antigens and the appropriate RBC species to determine the proper dilution factor of the antigen used for the hemagglutination inhibition assay. Titers equal to 4 HA units (*see 2.3.2*) are defined as the proper antigen dilution factor for use at the HI assay. With dilution factors between 1/45 and 1/16, the usage of influenza B/Massachusetts antigen was higher, compared to the other antigens (**Fig. 17**). The lowest amount used for the HI assay was from influenza A/California (H1N1) antigen, with dilution factors between 1/181 and 1/128 (**Fig. 17**).

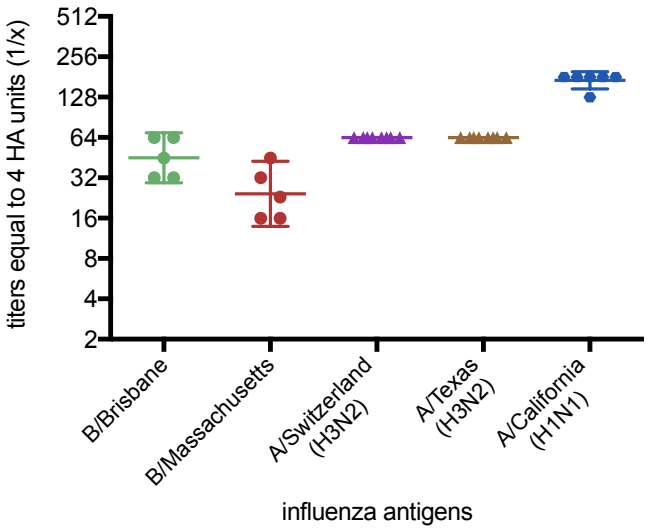


Fig. 17. Antigen titers equal to 4 HA units of all five antigens to determine the proper dilution factors. The results show geometric mean titers equal to 4 HA units with 95% CI. The dilution factor was lowest at influenza A/California antigen (1/170) and highest at influenza B/Massachusetts antigen (1/24).

The geometric mean titers (GMTs) equal to 4 HA units of the HA titrations are shown in **Table 5**.

Table 5: GMT equal to 4 HA units of all 5 antigens

Antigens	Proper dilution factor (1/x)
Brisbane	45 (n=5)
Massachusetts	24 (n=5)
Switzerland	64 (n=8)
Texas	64 (n=8)
California	170 (n=6)

3.3. Cross-reactivity

To check if antibodies against a specific influenza strain can also bind to other influenza viruses or strains and further inhibit hemagglutination, we performed cross-reactivity assays. Antibody titers of each antiserum against all five influenza viruses were determined (**Fig. 18**). The antiserum against B/Brisbane with specific antibodies against the HA glycoprotein, has an antibody titer of 1448 to the influenza B/Brisbane antigen. It also shows cross-reactivity with the influenza B/Massachusetts strain, with an antibody titer of 181. However, no cross-reactivity between anti-Massachusetts antibodies and B/Brisbane antigens was observed. The anti-Massachusetts antibodies

only bind to B/Massachusetts antigens and inhibit hemagglutination up to an antibody titer of 724. The anti-Switzerland serum has an antibody titer of 1448 to the influenza A/Switzerland antigen and shows high cross-reactivity with the second H3N2 subtype, A/Texas, with an antibody titer of 1024. The cross-reactivity between the two H3N2 strains is observed in both directions, because anti-Texas antibodies can also bind A/Switzerland antigens, with a titer of 1024. The anti-California (H1N1) serum does not show high cross-reactivity with other strains and has an antibody titer of 724 to A/California antigens.

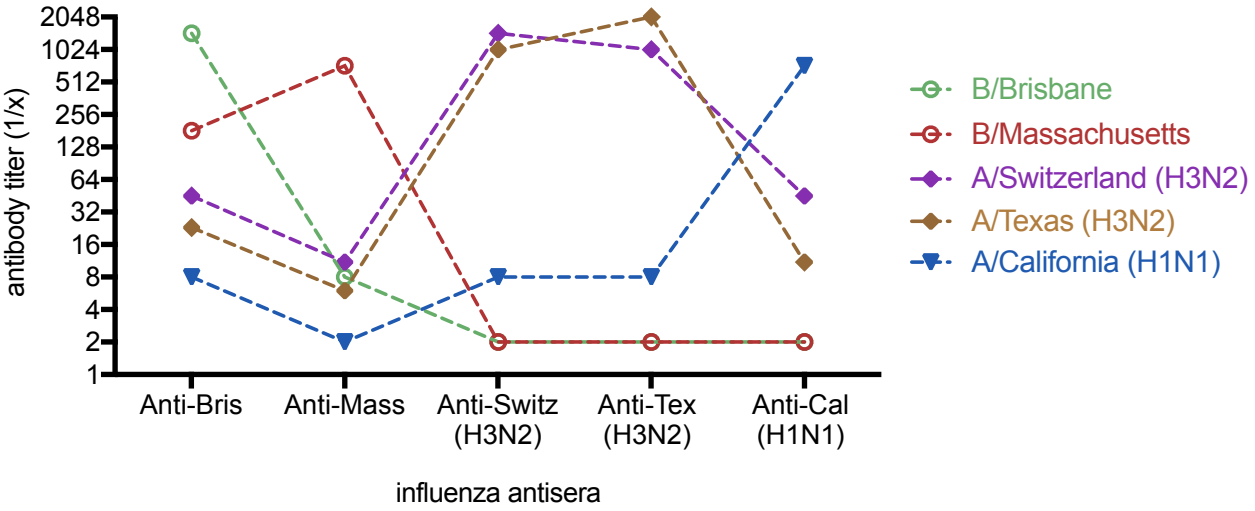


Fig. 18. Cross-reactivity assay of specific antisera to the five different influenza antigens. Cross-reactivity between anti-Brisbane antibodies and B/Massachusetts antigens was observed, with a HI titer of 181 (red circle at "Anti-Bris"-column). However, there was no cross-reactivity of anti-Massachusetts antibodies with other antigens ("Anti-Mass"-column). A high cross-reactivity between the two H3N2 strains was observed. The anti-Switzerland antiserum showed a HI titer of 1024 to the Texas strain (brown rhombus at "Anti-Switz"-column) and the anti-Texas antiserum had also a HI titer of 1024 to the Switzerland strain (purple rhombus at "Anti-Tex"-column). The anti-California antiserum didn't show high cross-reactivity with other strains ("Anti-Cal"-column).

3.4. Geometric mean titers (GMTs) to different influenza strains

Antibody levels of 53 HSCT recipients and 25 healthy controls (HC) against five influenza viruses and at five time points were measured (**Fig. 19**). The first time point (d0) reflects administration of the vaccine. The other time points were at day 7, day 30,

day 60 and day 180 after the vaccination. All HSCT patients received a second dose of the vaccine, 30 days (d30) after the first dose was administered, whereas HC only received a single vaccine dose. The trivalent vaccine contained 15 µg HA of the influenza strains A/California (H1N1), A/Texas (H3N2) and B/Massachusetts (Yamagata lineage), respectively. The strains B/Brisbane (Victoria lineage) and A/Switzerland (H3N2) were not included in the vaccine. The antibody levels were displayed as geometric mean titers with 95% CI.

In general, antibody titers to the three viruses, which were part of the vaccine, were higher than titers to the strains, which were not included in the vaccine (**Fig. 19**). Comparison of the graphs also shows, that HSCT patients showed higher baseline GMTs to influenza A strains compared to HC. Interestingly, HC showed higher baseline GMTs against Influenza B viruses compared to HSCT patients. Overall, we observed a significant increase in GMTs from d0 to d30 for all viruses in both groups, although the rate of seroprotection and seroconversion was variable between different viruses and groups. For HSCT recipients and HC the antibody titers to B/Brisbane (**Fig. 19**) were significantly lower than the seroprotection threshold.

Healthy controls showed at d0 and d7 significantly higher GMTs to B/Massachusetts compared to HSCT patients ($p=0.0019$ and $p=0.0061$, respectively). The antibody titers to B/Massachusetts of the HC were above the seroprotection threshold (>1:40) at all five time-points. The antibody titers of the HSCT recipients in contrast, are below this threshold at d0 and d7 and above at d30, d60 and d180. HSCT patients show significantly higher GMTs to A/Switzerland compared to HC at d30 to d180 ($p=0.0138$, $p=0.0012$, and $p=0.0066$, respectively). However, both groups remained below the seroprotection threshold through the observation period. The progression of the antibody levels of HSCT patients and HC to A/Texas looks very similar to A/Switzerland, just with higher GMTs to A/Texas (**Fig. 19**). We also noted significantly higher GMTs of HSCT patients at d30, d60 and d180 compared to HC ($p=0.0154$, $p=0.0033$, and $p=0.0195$, respectively). The GMTs to A/California did not show a significant difference between HSCT recipients and HC (**Fig. 19**).

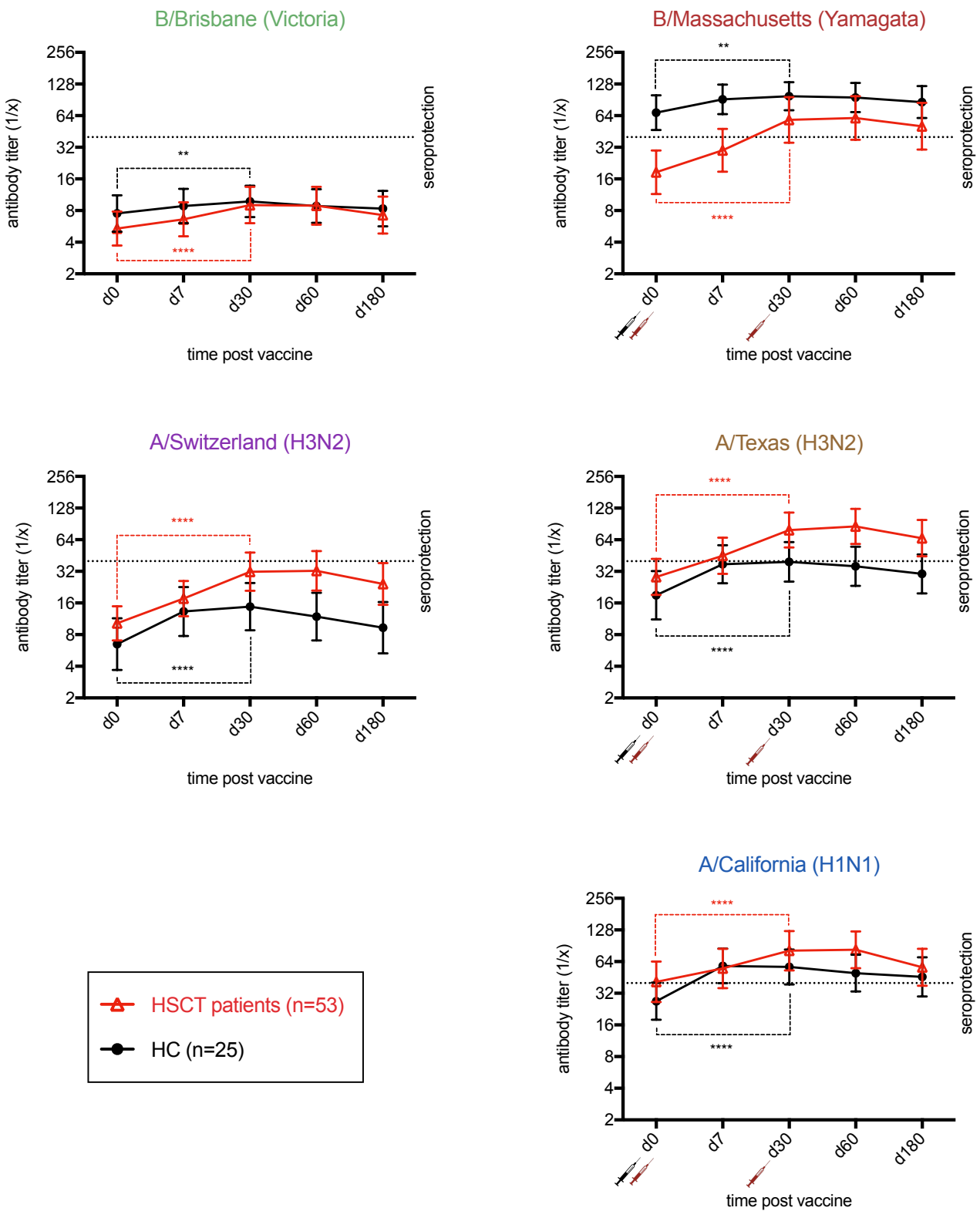


Fig. 19. Geometric mean titers (GMTs) with 95%CI of HSCT patients and HC at five time points to five influenza antigens. The time points of the vaccines were displayed with syringe symbols below the graphs, but only at the viruses, which were contained in the trivalent vaccine. Black syringes belong to healthy controls, red syringes to HSCT recipients. The seroprotection threshold is set at an antibody titer of 1/40.

3.5. Seroprotection rates against different influenza strains

Baseline seroprotection rates (pre-vaccination; seroprotection already at d0) and vaccine-induced seroprotection rates (post-vaccination; seroprotection initially after vaccination at d30 or d60) of HC and HSCT patients against five different influenza viruses were determined (**Fig. 20**). "Overall" seroprotection rates combine pre- and post-vaccination rates and show the percentage of seroprotected people at d30 or d60, regardless of whether seroprotection was reached due to vaccination or even before. Seroprotection rates of HC and HSCT patients were generally higher against viral antigens contained in the vaccine (**Fig. 20**; B/Massachusetts, A/California and A/Texas) compared to viral antigens not in the vaccine (B/Brisbane, A/Switzerland). Also, the pre-vaccination seroprotection rates were higher against viruses in the vaccine. HSCT patients reach a relatively high seroprotection rate to A/Switzerland of 59% (with a 40% increase pre- to post-vaccination.

For HSCT recipients and HC, the seroprotection rates against B/Brisbane were very low. The seroprotection threshold to B/Brisbane was not reached by a single individual of the HC group at any time point. 9% of the HSCT recipients already showed seroprotective titers to B/Brisbane before the first vaccine shot and another 9% of patients reached seroprotection level after vaccination for the first time (**Fig. 20**).

64% of the HC and 42% of the HSCT recipients were seroprotected against B/Massachusetts at d0. 16% of the HC and 26% of patients further reached the seroprotection threshold post-vaccination.

36% of HC and 51% of the HSCT recipients were seroprotected against A/California at d0. 32% of HC and 28% of patients further reached the seroprotection threshold within d7 to d180.

The seroprotection rate of the HC group against A/Switzerland was also low, with overall 8% (4% pre-vaccination and 4% post-vaccination). The seroprotection rates against A/Texas were 52% (24% pre- and 28% post-vaccination) in HC and 79% (32% and 47%) in HSCT patients, respectively.

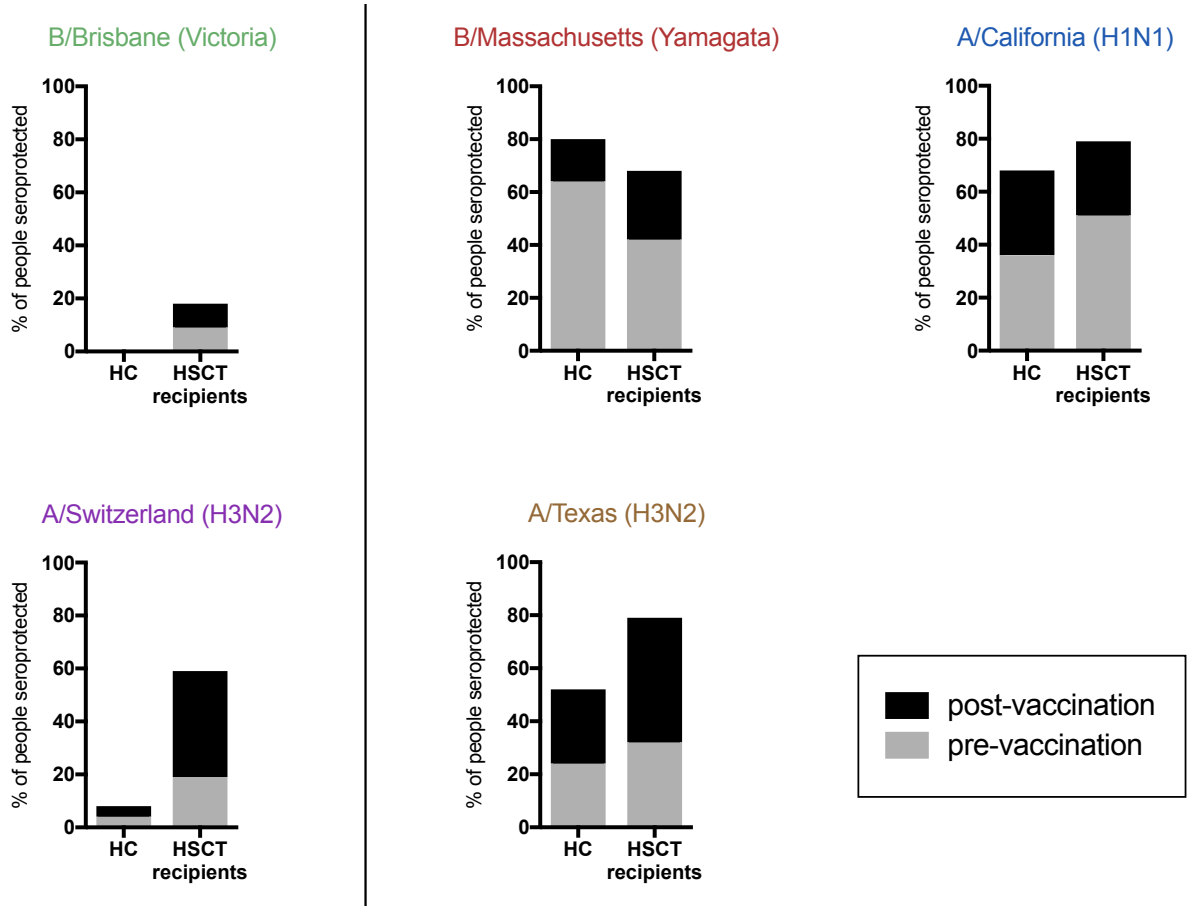


Fig. 20. Seroprotection rates of HC and HSCT patients against five influenza viruses. "Overall" seroprotection rates are composed of post-vaccination and pre-vaccination rates. Seroprotection rates are generally higher against viral antigens contained in the vaccine.

As HSCT patients received a booster vaccine dose, we determined how many of them reached a seroprotective titer at day 60 for the first time (**Fig. 21**). About 20% of the HSCT recipients who attained seroprotection due to vaccination (post-vaccination group) reached the seroprotection threshold only after the second vaccine shot for the first time (at d60). The exact rates are 20% at B/Brisbane, 14% at B/Massachusetts, 24% at A/Switzerland, 24% at A/Texas and 20% at A/California.

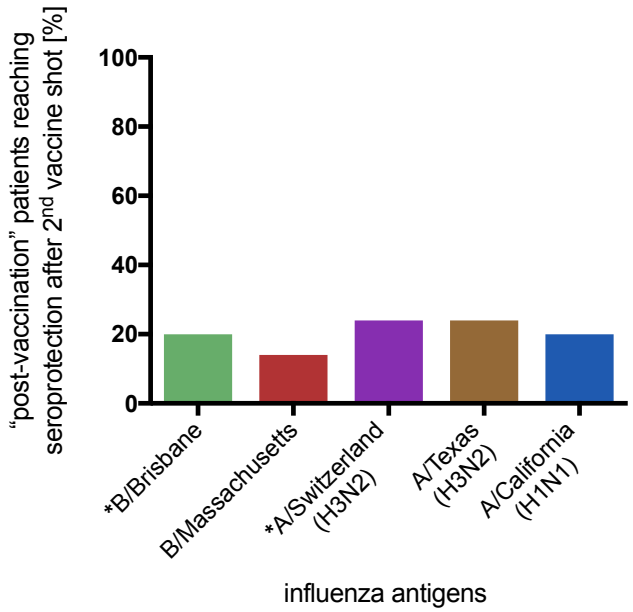


Fig. 21. Percentage of HSCT patients who reached seroprotective titers due to the booster vaccine dose administered at d30. About 20% of the post-vaccination patients (vaccine-induced seroprotection) reached seroprotection at d60 for the first time (30 days after the booster vaccine shot). *Antigens were not part of the trivalent vaccine.

3.6. Titer progressions 30 days post-vaccination

To visualize the average increase in antibody titers 30 days after the first vaccine injection, the logarithm of the titer-ratio d30/d0 of each person was calculated and plotted on a graph (**Fig. 22**; "d30/d0 ratio"). The average increase in antibody titers was slightly higher in HSCT patients compared to the HC group to all tested antigens, except for A/California. The titer increase was lowest to B/Brisbane (mean log (d30/d0) of 0,35 in HC and 0,74 in patients), which was not part of the trivalent vaccine. The titer increase 30 days after the first vaccination in HSCT recipients to B/Massachusetts (1,65 log titer steps) was significantly higher than the increase in the HC group (0,52 log titer steps).

To determine if the second vaccine dose, administered at d30, led to another increase in antibody titers of HSCT patients, a logarithmic titer-ratio of d60/d30 was calculated for each HSCT patient and also each HC person (**Fig. 22**; "d60/d30 ratio"). In contrast to the first vaccine shot, no significant increase in GMTs of patients could be observed 30 days after the second vaccination (see **Fig. 19**). The antibody titers remained almost the same from d30 to d60 in HSCT recipients, regarding to the titer-ratio (**Fig. 22**). However, there was a significant decrease in the d60/d30 titer-ratio of HC to A/Switzerland. The

d60/d30 titer-ratios of HC to B/Brisbane, B/Massachusetts, A/Texas and A/California were also decreased, but due to the Wilcoxon test not significantly (p=0,13, p=0,75, p=0,09 and p=0,06, respectively).

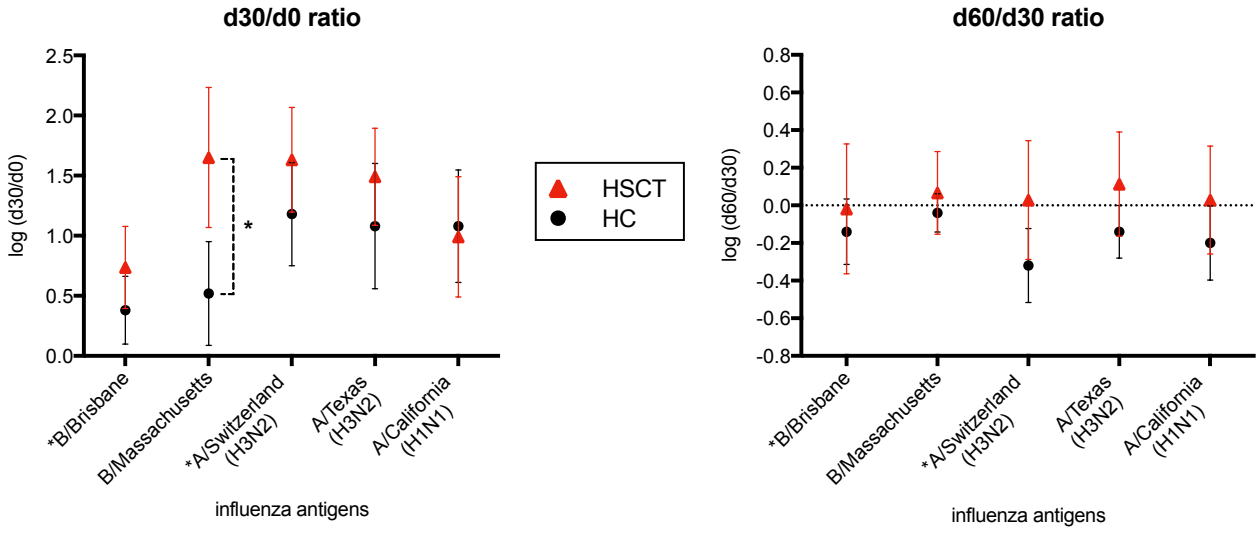


Fig. 22. Titer progressions of HC and HSCT recipients 30 days post vaccination. The logarithms of the titer-ratios d30/d0 and d60/d30 were calculated for each person and plotted as medians including the 5-95 percentiles. The increase in antibody titers 30 days post-vaccination in patients was higher compared to HC, to most antigens. There was no significant increase of antibody titers in patients 30 days after the second vaccination. Whereas, a significant decrease in HI titers of HC to A/Switzerland, A/Texas and A/California at d60 compared to d30, could be observed.

3.7. Seroconversion

Seroconversion is defined as the percentage of people with either a pre-vaccination HI titer < 1:10 and a post-vaccination HI titer > 1:40 or a pre- vaccination HI titer > 1:10 and a minimum four-fold rise in post-vaccination HI antibody titer. In general, we observed that HSCT patients seroconverted more often compared to HC (**Fig. 23**). The seroconversion rates to the B/Brisbane antigen, which was not included in the vaccine, were low. No seroconversion was observed in the HC group and 8% of the HSCT patients underwent seroconversion. 12% of HC and 34% of HSCT recipients underwent seroconversion to the B/Massachusetts antigen. Seroconversion to A/Switzerland, which was also not part of the vaccine, was observed in 4% of the HC and 38% of the HSCT patients. Slightly higher rates were seen to A/Texas: 12% of HC and 45% of

patients underwent seroconversion. Seroconversion to A/California was observed in 16% of the HC group and 25% of the patients group.

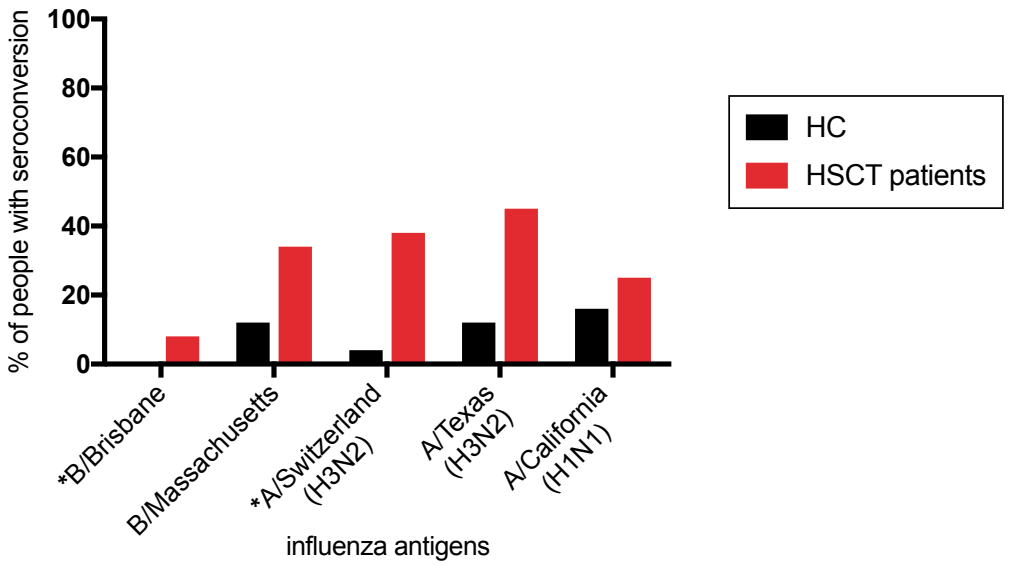


Fig. 23. Seroconversion rates of HC and patients to five influenza viruses after vaccination. Seroconversion is defined as the percentage of people with either a pre-vaccination HI titer < 1:10 and a post-vaccination HI titer > 1:40 or a pre- vaccination HI titer > 1:10 and a minimum four-fold rise in post-vaccination HI antibody titer. *Antigens were not part of the trivalent vaccine.

3.8. Reverse cumulative distribution of antibody titers

The reverse cumulative distribution of antibody titers also shows that the titers to antigens included in the trivalent vaccine are in general higher than titers to antigens not included in the vaccine (Fig. 24). Furthermore, we observed that the first vaccine dose has a clear impact on the antibody titers of both, HSCT patients and healthy controls. Conversely, the second vaccine shot of HSCT recipients seems to have no obvious impact. Baseline antibody titers to B/Brisbane and A/Switzerland are noticeably lower than those to the other three antigens.

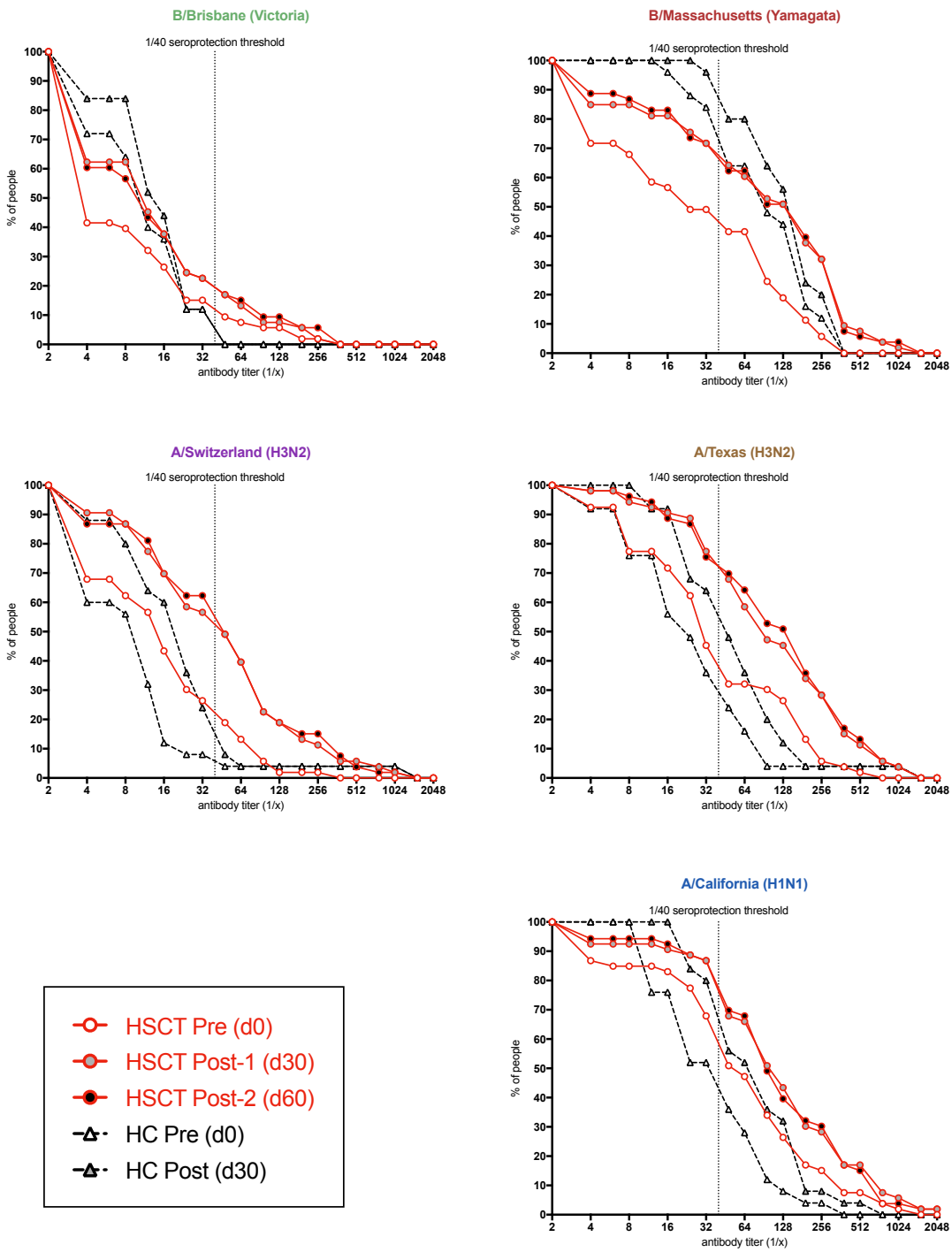


Fig. 24. Reverse cumulative distribution of antibody titers to five antigens in HSCT recipients and HC. The reverse distribution curves represent the distribution of individual antibody levels in HSCT recipients (red) and HC (black) pre- and post-vaccination.

3.9. Impact of time post-HSCT on GMTs

Next, we checked if the time after HSCT had an impact on the antibody titers. At a five-year threshold, significant differences could be observed (**Fig. 25**). HSCT recipients who

had the transplantation more than 5 years prior to the vaccination showed significantly higher GMTs compared to patients who had the transplantation within the last 5 years. We could observe this for all five viruses used in the HI assay at every time point, with the exception of A/California at d0 (p=0.051). The progressions of titers of both groups looked very similar at all used antigens, due to the consistent gap between the titers over the entire timespan. The differences in antibody titers between the two groups are highest to B/Massachusetts.

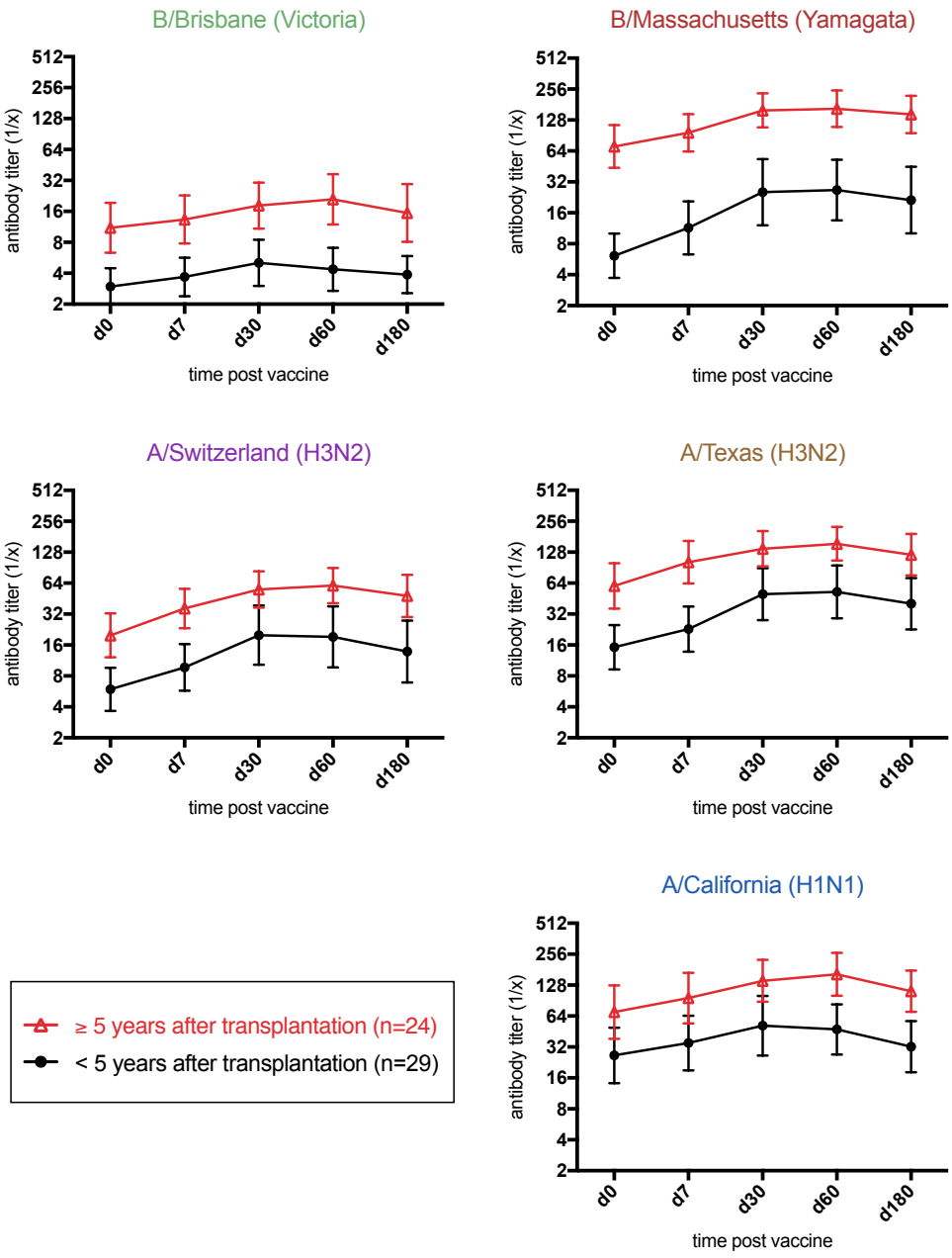


Fig. 25. Impact of the timespan between HSCT and first vaccination (d0) on GMTs to five influenza viruses. Five years post-HSCT seems to be a significant threshold relating to antibody titers. People with a timespan of at least five years post-HSCT have higher antibody titers than people with a timespan less than five years (pre- and post-vaccination).

We also observed significant differences in seroprotection rates (% of people with a HI titer above 40) between patients with less and more than five years post-transplantation (**Table 6**). The overall seroprotection rates are higher in ≥ 5 years post-transplantation patients to all five antigens. The baseline seroprotection rates (d0; pre-vaccination) were higher in these patients. The post-vaccination rates, where the seroprotection level was reached after vaccination for the first time, were higher in this group to B/Brisbane and A/Switzerland, were higher in the other group to B/Massachusetts and almost equal to A/Texas and A/California.

Table 6: Seroprotection rates of HSCT patients < and ≥ 5 years post-transplantation.

time post-HSCT	sero-protection	B/Brisbane (Victoria)	B/Massachusetts (Yamagata)	A/Switzerland (H3N2)	A/Texas (H3N2)	A/California (H1N1)
<5 years (n=29)	pre-vaccination	7%	10%	7%	17%	45%
	post-vaccination	7%	35%	34%	48%	28%
	overall	**14%**	**45%**	**41%**	**65%**	**73%**
≥5 years (n=24)	pre-vaccination	12,5%	79%	33%	50%	58%
	post-vaccination	12,5%	17%	46%	46%	29%
	overall	**25%**	**96%**	**79%**	**96%**	**87%**

3.10. Impact of age on antibody titers of HSCT patients

We correlated the age of the HSCT patients with HI titers at d0 and d30 to different influenza viruses (**Fig. 26**). The age groups 20-29 years, 30-39 years and 70-79 years were left out, because there were not enough patients in these groups (3 in each) for proper analysis. We only observed a significant difference in antibody titers to A/California at d0 ($p=0.0226$, Kruskal-Wallis testing) between the age groups.

We observed that the antibody titers of the oldest group (60-69 years) were lowest at both time points and to each antigen compared to other age groups. However, this effect was not statistically significant.

Gender did not impact the HI titers at d0 and d30 to the five antigens (data not shown).

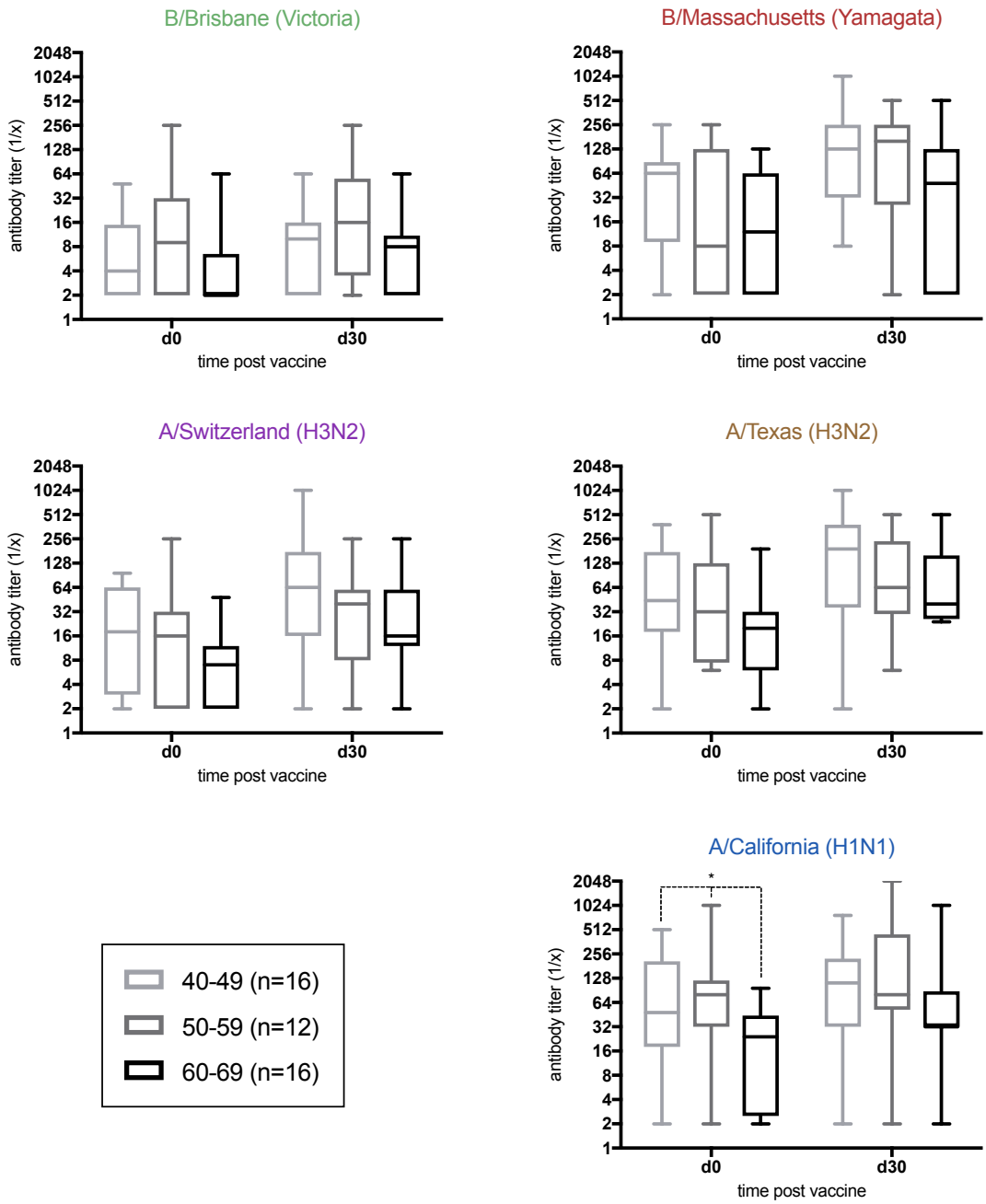


Fig. 26. Impact of age on antibody titers of the HSCT recipients. The titers of different age groups at d0 and d30 were shown in box-and-whiskers plots with 5-95 percentiles. There are no significant differences between the age groups due to the Kruskal-Wallis test, except in antibody titers to A/California at d0 (p=0,0226).

3.11. Impact of graft-versus-host disease on antibody titers of HSCT patients

Graft-versus-host diseases (GvHD) of HSCT recipients were classified from “no” to “mild” (58% of HSCT patients) and “moderate” to “severe” (42% of patients) [89]. The antibody

titers of both groups to five antigens were tested for significant differences, but no such differences could be observed (**Fig. 27**).

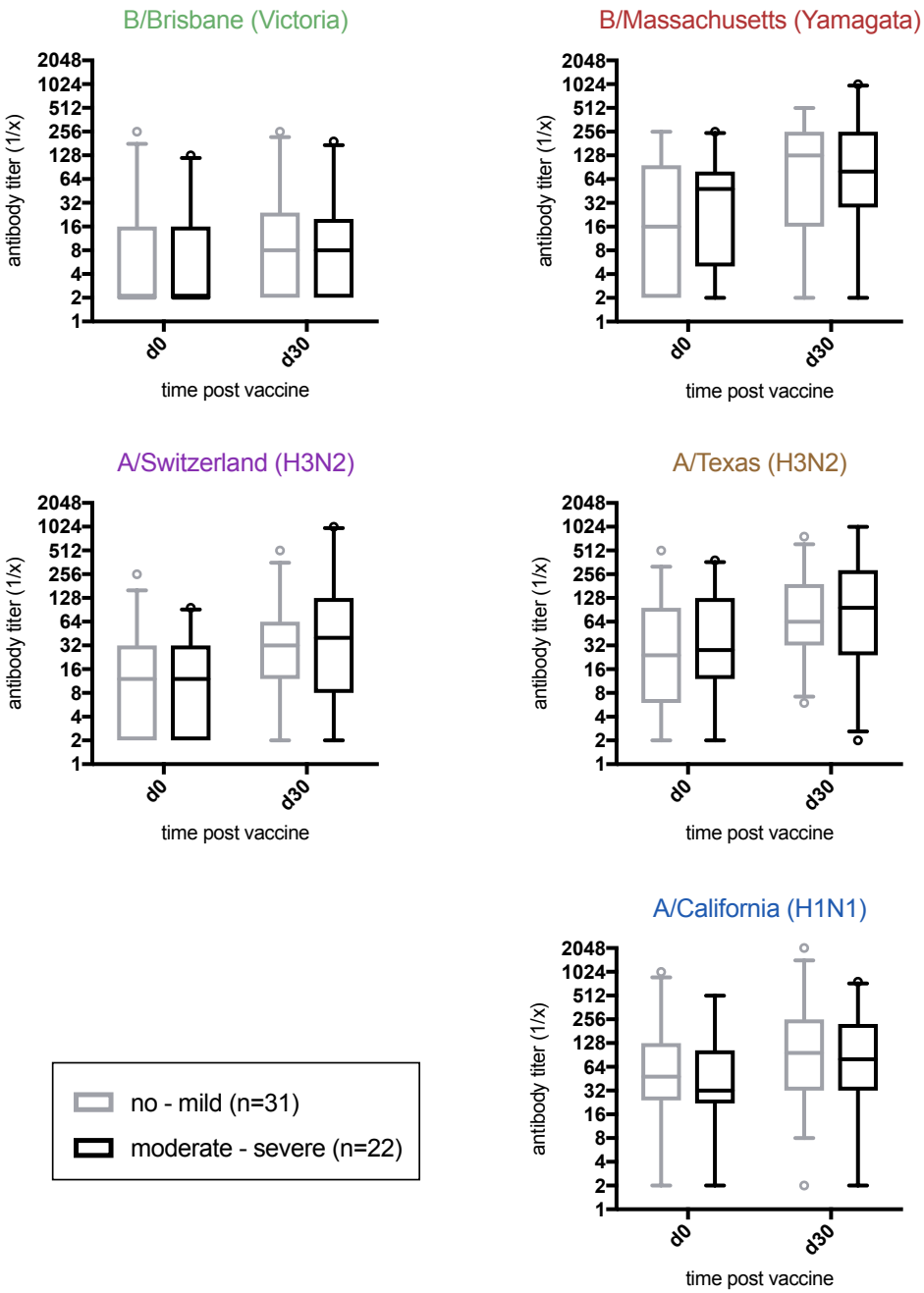


Fig. 27. Impact of GvHD responses on antibody titers of HSCT recipients. The titers were shown in box-and-whiskers plots with 5-95 percentiles. There were no significant differences between the two groups regarding the Mann-Whitney test .

3.12. Antibody titers of patients undergoing influenza infection

During the follow up of 180 days, three HSCT patients were tested positive with influenza B virus infections and another four patients showed positive results for the H3N2 influenza A virus-subtype. The influenza-virus subtype (e.g. H3N2) which caused the infection was determined via PCR (polymerase chain reaction) assay, but it's not possible to define the specific strain of this subtype (e.g. Switzerland or Texas) with this method. **Fig. 28** shows the antibody titers of the infected patients. The influenza subtype, towards which a patient showed a positive result, is highlighted in color, whereas the other subtypes are colored grey. The time point of the PCR assay is also indicated as dashed line. It is noticeable that all infected patients were tested positive between day 60 (January/February) and day 180 (May/June). It is also noticeable that the antibody titers to the subtypes, which caused the infections, are obviously increased at d180 compared to d60. An increase of two or more titer steps can be seen at each infected patient. The antibody titers to the other subtypes in contrast remain on the same level or are even decreased at d180. All three influenza B cases seem to be influenza B/Massachusetts infections, due to the titer progression. Three of the four influenza A cases show increased titers to both H3N2 strains, A/Switzerland and A/Texas, which may be explained with the high cross-reactivity between them (see **Fig. 18**). Four of the seven infected patients reached the seroprotection threshold before getting infected. Two patients had antibody titers below the detection limit before the infection (**Fig. 28**; "Influenza B - 3 " and "Influenza A (H3N2) – 4"). The HSCT recipient with the influenza B infection had then, after the positive test, an antibody titer of 32 to B/Massachusetts (d180). The patient with influenza A (H3N2) - infection had a titer of 11 to A/Texas and remained under the detection limit to A/Switzerland after the positive test, at d180.

These results demonstrate that influenza infections between d60 and d180 after the first vaccination (d0) can be indicated with an atypical increase of antibody titer to the infection subtype.

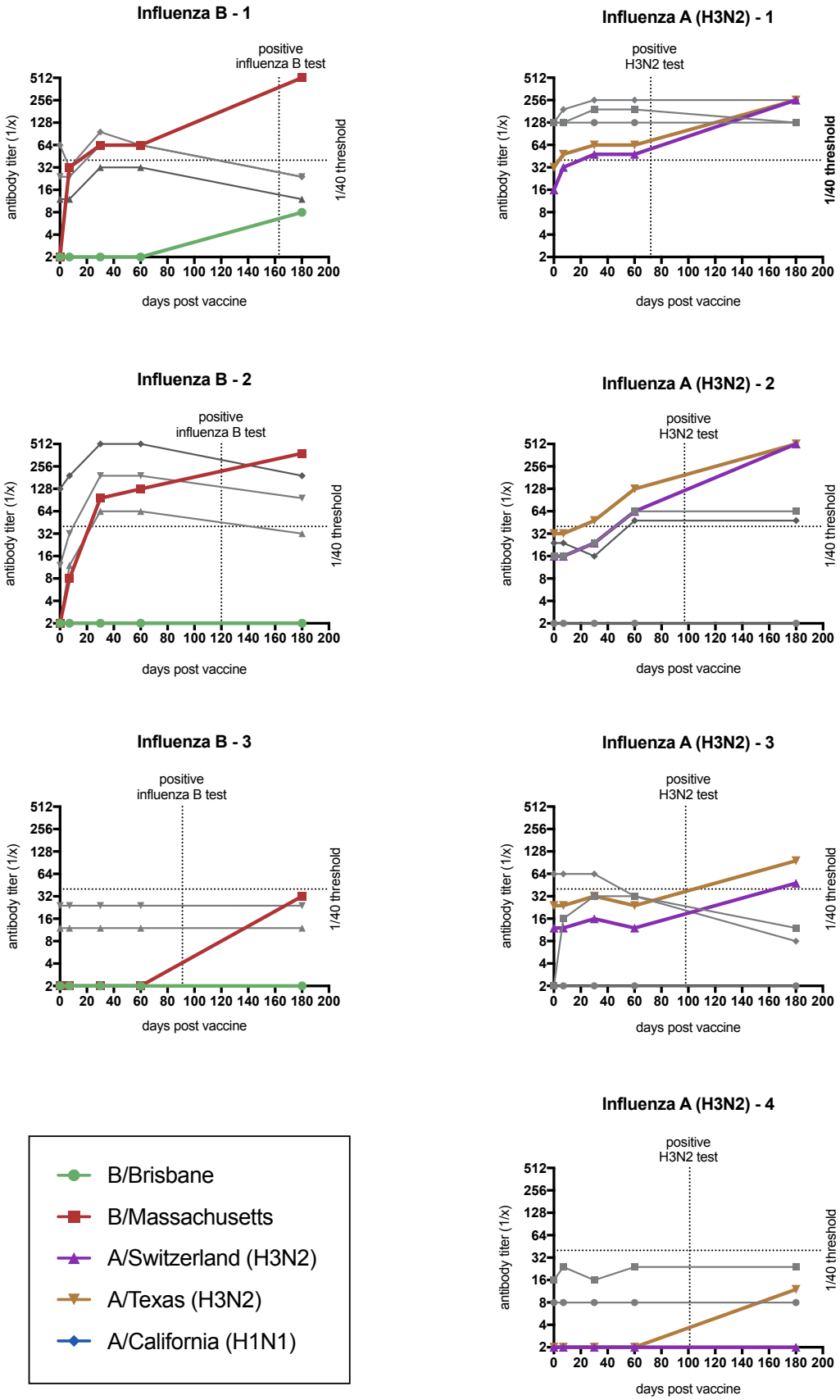


Fig. 28. Antibody titers of people undergoing influenza infection. The influenza subtypes, which caused the infections, are highlighted in colors. The time point of the PCR assay is also indicated as dashed line. An increased HI titer after the PCR assay can be observed at each influenza case.

3.13. ELISA vs. HI assay

We performed an indirect enzyme-linked immunosorbent assay (ELISA) to measure the immunoglobulin G (IgG) levels of 14 HSCT recipients and 7 healthy controls against the five different influenza viruses, prior and after the vaccination. We used samples with high antibody titers as reference sera for the ELISA because there are no reference sera with known concentrations of specific IgGs against different influenza viruses available,. We measured serial diluted reference sera to generate standard curves simultaneously to the serum samples. We introduced arbitrary units to obtain actual values that can be used for the data analysis. The absorbance values of the highest dilutions of the reference sera were set as 1024 AU (each had a HI titer of 1024). By twofold serial diluting the reference sera, the ranges of AU reached from 1024 AU to 8 AU. The standard curves were generated by plotting the measured mean absorbances of the reference sera against the fixed log10-transformed AU values. We used a four-parameter logistic (4PL) curve fit to interpolate the mean absorbances of the measured serum samples of patients and HC to the standard curves. We computed the interpolated values as log10-values, which had to be transformed to AU using the formula (x=10^x). The arbitrary units were used as indicator for serum IgG levels.

The geometric mean arbitrary units of patients and HC to the five influenza viruses were plotted and are shown in **Fig. 29**. Also the antibody titers of these serum samples are shown for comparison. Even if just IgG levels are measured with the ELISA and not all Ig-classes that bind to the virus particles like in the HI assay, similarities in the curve progressions can be seen, especially to B/Massachusetts and A/California (and except for B/Brisbane). There is also an increase in IgG levels after the first vaccination (d0 to d30) and this increase is also higher in HSCT recipients (except for A/California), like it was observed with the HI assay. It is also noticeable, that the curves of A/Switzerland and A/Texas look very similar when measured with the same method, which can be based on the observed cross-reactivity between these H3N2 strains.

Cross-reactivity between B/Brisbane and B/Massachusetts at the HI assay was low and just in one direction: antibodies against B/Brisbane can bind to B/Massachusetts antigens, but not the other way round. The IgG levels measured with the ELISA however, show similar curve progressions to B/Brisbane and B/Massachusetts.

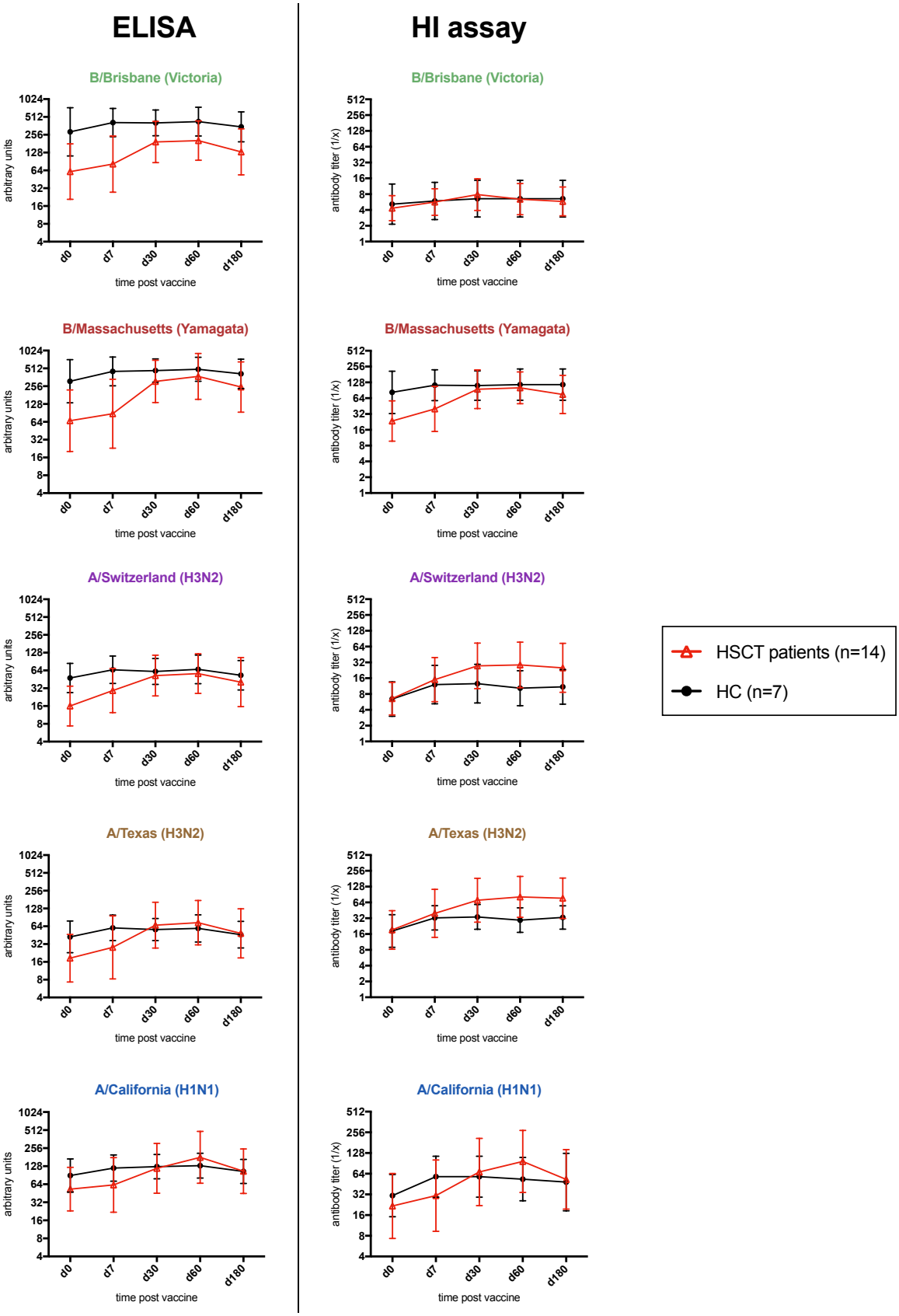


Fig. 29. Geometric mean AU and GMTs with 95%CI of 14 patients and 7 HC to five influenza viruses. The arbitrary units were obtained by measuring the serum IgG levels via ELISA and interpolating the absorbances to standard curves. Except for B/Brisbane, similarities in the curve progressions of the 2 methods can be observed.

We plotted the arbitrary units of each measured person against their antibody titers to perform regression analysis to determine if the arbitrary units (IgG level in serum against a specific influenza virus) correlate with the antibody titers (all classes of Igs in the serum against a specific influenza virus), Arbitrary units and antibody titers were both plotted logarithmically to insert the regression line (**Fig. 30**). The correlation between AU and HI titers was worst to B/Brisbane with R^2 = 0,21. That can also be observed when comparing the curve progressions at **Fig. 29**. Regression analysis of AU and antibody titers to B/Massachusetts resulted in R^2 = 0,49. The best correlations could be observed at A/Switzerland (R^2 = 0,92) and A/Texas (R^2 = 0,82). A good correlation was also determined between AU and antibody titers to A/California with R^2 = 0,68.

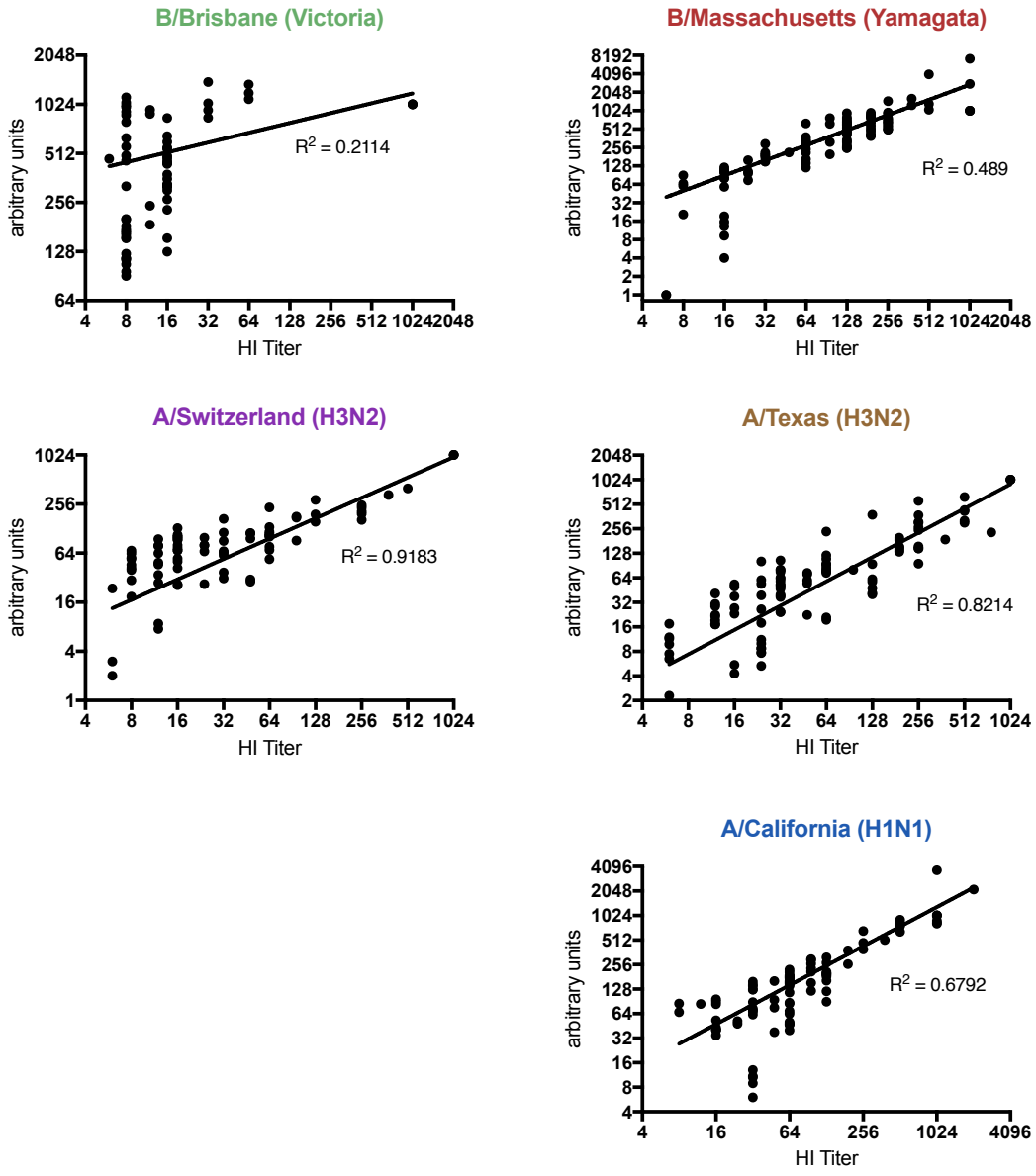


Fig. 30. Regression analysis of arbitrary units and antibody titers. Logarithmic arbitrary units and antibody titers of all measured persons were plotted and a regression line was computed.

4. Discussion

Influenza infections in hematopoietic stem cell transplantation (HSCT) recipients are associated with high morbidity and mortality. Therefore, preventing these infections is of tremendeous importance for HSCT patients. A key strategy for prevention is vaccination; however, the efficacy of vaccination in HSCT patients depends on a variet of factors e.g. state of immunosuppression, age of the patient, type of vaccine, genetic polymorphisms and others [90], [91]. Patients with a potentially reduced vaccine efficacy need to be identified for a specific risk assessment and eventual adaptation of the vaccine strategy. The immunological assessment of influenza specific humoral immunity correlates with the clinical risk for influenza infection. The measurement of influenza-specific IgG is therefore an important step in the evaluation of particular risks (personalized vaccination) [41], [92]. Our aim was to optimize the hemagglutination inhibition assay for a high throughput setting in a vaccine study. In addition, we aimed to characterize the progression of antibody production after vaccination in HSCT patients and healthy controls.

The "Applied Microbiology Research" group previously performed a prospective observational study including 53 adult allogeneic HSCT recipients (at least one year post-transplantation), receiving two doses of a trivalent influenza vaccine prior the 2014/2015 - influenza season. The second dose was administered 30 days after the first dose was injected and the trivalent vaccine contained HA glycoproteins of the influenza strains A/California/7/09 (H1N1), A/Texas/50/2012 (H3N2) and B/Massachusetts/02/2012. Additionally, 25 healthy controls received one dose of the trivalent vaccine at day 0. As part of my master thesis, we determined the antibody titers against the three influenza viruses included in the vaccine and two additional strains (B/Brisbane/60/08 and A/Switzerland/9715293/2013 (H3N2)) were prior the first vaccine injection at day 0 and day 7, day 30, day 60 and day 180 post-vaccination.

The quantification of virus-specific antibody titers can be performed with various immunological methods, including solid-phase [93] or bead-based [94] ELISA assays, the HI assay [25] and neutralizing assays [95]. ELISA-based methods allow the screening of relatively large amounts of serum samples against various antigens. Also, pathogen-specific Immunoglobulin (Ig)M and IgG can be separately explored. Although the

characteristics of an antigen, *e.g.*, the linear amino acid sequence or virus-like particle may influence the binding of antibodies, the spectrum of potential epitopes is very broad and does not provide information on whether an antibody response has functional relevance. In contrast, the neutralization assay determines the potential of antibodies to functionally inhibit the infection of cells and therefore reflects the neutralization potential. However, this method is labor intensive, requires culturing of specific cell lines and live viruses, and is therefore time-consuming, expensive, and requires special equipment (reference, Kaufmann et al. in press). For these reasons, we utilized the hemagglutination inhibition assay to determine vaccine induced humoral immunity. Hemagglutination is a characteristic effect of some viruses leading to the agglutination of erythrocytes. The inhibition of this effect with patient sera allows the measurement of inhibitory antibody concentrations, which reflects a neutralizing effect. Before measuring real case serum samples, the hemagglutination inhibition assay had to be optimized and adapted to a high throughput situation. Altogether 1950 single antibody titers had to be measured (25+53=78 people * 5 time points * 5 viruses = 1950 samples) for our study. Therefore, the WHO HI assay protocol [25] was slightly modified:

- We modified the protocol by using PCR tube strips for serum preparations. This modification helped to significantly reduce the workload and to increase the throughput of the assay.
- We used a slightly higher RBC concentration than in the WHO protocol (0,75% instead of 0,5% for avian RBCs and 1% instead of 0,75% for mammalian RBCs), to achieve a good visual read out. When using the concentrations of the WHO-protocol, it couldn't be clearly distinguished between agglutinated and non-agglutinated patterns (**Fig. 15**). A reason for that could be, that there are small differences in the concentrations of the RBC stock solutions. We received our RBC stock solutions, which were listed as a 10% solution, form a company and in the WHO-protocol the RBC stock solutions were prepared by themselves. Therefore it's recommended to check the read out of the assay with different RBC-concentrations to determine the optimum concentration for the different RBC species.
- At the HA titration, which was performed to determine the proper influenza antigen concentration needed for the HI assay, we used just half the volume of the antigen-PBS-mixture compared to the WHO protocol (50 µl instead of 100 µl). We also started at a titer of 1/2 instead of undiluted antigen, which means we

just used 25 µl of the pure antigen solutions, compared to 100 µl, to save resources and money. Because we used the same amount of RBC suspension as in the WHO protocol (50µl), the proper antigen concentration of 4 HA units/25 µl was calculated by dividing the HA titer by 4 instead of 8 as in the WHO protocol.

- In contrast to the WHO-protocol, in which just the completely agglutinated wells were considered at the read out, we also included partially agglutinated wells to our results to be more accurate in distinguishing the different antibody-levels.
- Back titration and positive control were included in the antibody measurement plate to serve as a proper internal control and to monitor the aging of erythrocytes.

The hemagglutination is strongly dependent on the particular species of erythrocytes (avian or mammalian) [25]. The optimal species of RBC should be tested before antibody titers to a particular virus strain are determined. The same RBC species should be used throughout the assay. Another critical aspect of the assay is the hemolysis of the erythrocytes as they age over time. For these reasons, we performed the HA titration regularly and if we detected advanced hemolysis, we used a new batch of erythrocytes.

To compare antibody titers to different influenza strains, it is important to consider if they are cross-reactive and how strong this effect might be. Therefore, we tested the cross-reactivity for all five influenza viruses used in this study. The results indicated a one-way cross-reactivity within the two influenza B lineages, Victoria and Yamagata. Anti-B/Brisbane (Victoria) antibodies can also bind to B/Massachusetts (Yamagata) antigens and showed a HI titer of 181 (**Fig. 18**). This cross-reactive effect is quite distinct compared to the HI titer of the specific B/Massachusetts antiserum (724), with just two log2 titer steps below. However, we could not detect cross-reactivity of anti-B/Massachusetts (Yam) antibodies with the B/Brisbane (Vic) antigen (HI titer of 8). The WHO published similar results in the "Manual for the laboratory diagnosis and virological surveillance of influenza" [80]. The WHO also determined cross-reactivity between B/Victoria antiserum with B/Yamagata antigens. The HI titer was about three log2 titer steps lower than the HI titer of the specific B/Yamagata antiserum (40 compared to 320). There was also no cross-reactivity in the other direction (B/Yamagata antiserum with B/Victoria antigen) observed (HI titer <10).

Cross-reactivity in both directions could be seen between the H3N2 strains A/Texas and A/Switzerland. The A/Texas antiserum had a HI titer of 1024 to the A/Switzerland antigen. To compare, the A/Switzerland antiserum had a HI titer of 1448 to its specific antigen. In return, anti-A/Switzerland antibodies can also bind to A/Texas antigens (HI titer of 1024). The A/Texas antiserum showed a HI titer of 2048 to its appropriate antigen. These cross-reactivity effects should be considered when comparing GMTs between different viruses.

The geometric mean titers were noticeably higher to strains included in the vaccine, compared to strains not included (**Fig. 19**). The cross-reactive effects observed at the cross-reactivity assay could also be seen when measuring the serum samples of HSCT patients and healthy controls. GMTs to B/Brisbane are lowest compared to all other, which could be explained as B/Brisbane antigens were not included in the trivalent vaccine and therefore no vaccine-specific immunity could be generated. Additionally, there is no cross-reactivity from anti-B/Massachusetts antibodies, which are present in high levels. In contrast the GMT to A/Switzerland was visibly higher, although A/Switzerland antigen was also not in the vaccine. Most likely, the significant cross-reactivity between anti-A/Texas antibodies and the A/Switzerland antigen is the reason for this. In addition, the titer progressions to A/Switzerland and A/Texas look very similar, with just lower GMT levels at A/Switzerland. Nevertheless, in the 2014/2015-influenza season an important miss-match between the vaccine and circulating influenza strain was noted. The main circulating influenza virus strain responsible for most of the infections in Switzerland this season was A/Switzerland. However, the trivalent vaccine included the A/Texas and not the A/Switzerland strain. Considering the cross-reactivity observed with our assay, even antibodies against A/Texas should have shown a protective impact against the A/Switzerland strain. Several aspects may explain these effects: (i) Although a cross-reactivity between the two strains are observed, overall the A/Switzerland titers induced by A/Texas were significantly lower, which can also be seen at the lower seroprotection rates; (ii) the cross-reactivity assay was performed with antiserum reagents prepared in sheep. Furthermore, the HI assay just reflects a neutralizing ability of the antibodies, due to the hemagglutination inhibition.

Interestingly, the GMTs of HSCT patients to A/Switzerland and A/Texas were significantly higher than those of HC at d30 to d180 post-vaccination, but not at d0 and

d7 (**Fig. 19**). That indicates that the vaccine response was "better" in HSCT recipients than in HC. It seems that also to B/Massachusetts the vaccine response worked better in patients than in HC. The baseline antibody titers at d0 and also d7 were significantly higher in HC, but from d30 to d180 post-vaccination, no significant differences are observed anymore due to the strong titer increase of HSCT patients. Furthermore the level of significance regarding the titer increase from d0 to d30 is higher in patients than in HC ($p<0.0001$ vs. 0.0098). This can also be seen when comparing the log (d30/d0) GMT ratios (**Fig. 22**). The ratio is significantly higher at HSCT patients to B/Massachusetts and seems to be also higher to A/Switzerland and A/Texas, compared to the healthy controls. Also the seroconversion rates are higher in patients compared to healthy controls to B/Brisbane (8% vs. 0%), B/Massachusetts (34% vs. 12%), A/Switzerland (38% vs. 4%), A/Texas (45% vs. 12%) and A/California (25% vs. 16%)(**Fig. 23**). Although somewhat speculative, the reason for this effect could be that the immune system of the HSCT recipients can be more efficiently stimulated due to repeated vaccine exposures and therefore shows a higher dynamic change – however this hypothesis has to be specifically assessed with different experiments.

Upon examination of the seroprotection rates (**Fig. 20**), it is noticeable that baseline seroprotection rates were quite high against B/Massachusetts (64% in HC and 42% in patients), A/Texas (24% in HC and 32% in patients) and A/California (36% in HC and 51% in patients). In a publication of Ambati et al. [4], similar baseline seroprotection rates against the antigens B (71,8% in donors and 64,7% in patients), H3 (24,8% in donors and 33,6% in patients) and H1 (17,9% in donors and 18,5% in patients) were observed. In another publication of Mohty et al. [5], baseline seroprotection rates against A/California were a bit lower in controls (14,8%) and patients (6,6%). We also determined the overall seroprotection rates after vaccination during the follow-up for B/Massachusetts (80% in HC and 68% in patients), A/Texas (52% in HC and 79% in patients) and A/California (68% in HC and 79% in patients). Ambati et al. also published seroprotection rates during follow-up of HSCT patients to the antigens B (77,1%), H3 (45%) and H1 (28,2%), but the study design of this study was quite different to ours, with just 51,6% of HSCT patients receiving influenza vaccination and also much earlier (at a median of 193 days after transplantation). In the study of Mohty et al., overall post-vaccination seroprotection rates against A/California of 87% in HC and 84% in HSCT patients were determined.

Our hypothesis that a second vaccine shot increases the antibody titers of HSCT recipients significantly could not be confirmed (see **Fig. 19** and **Fig. 22**). The study was not specifically designed to answer this question, but no obvious increase could be observed after the booster administration. While the GMTs of HSCT patients stay constant from d30 to d60, the GMTs of HC are slightly decreased (**Fig. 19** and **Fig. 22**). It could be that the booster helped to stabilize the vaccine-induced titer of the first dosage – however to properly address this, a randomized clinical trial with two different vaccine regiments would be needed.

We also observed that about 20% of HSCT patients who received seroprotection through vaccination (post-vaccination group), did reach the seroprotection threshold for the first time after the second vaccine dose (**Fig. 21**). In a study of de Lavallade et al. [73], the seroprotection rate of allogeneic stem cell transplant recipients at day 21 post-vaccination was 46% and after a second dose seroprotection rates increased to 73%. Many other publications indicate that two doses of adjuvants containing pandemic vaccine resulted in better seroprotection rates than a single dose [5], [74]–[77], [96].

Our second alternative hypothesis that HSCT recipients with a shorter time post-HSCT show significantly lower antibody titers compared to recipients with a longer time span could be confirmed. At five-years significant differences could be observed for all five antigens and all time points, except for A/California at d0 ($p=0.0511$) (**Fig. 25**). The importance of "time after transplantation" may reflect that the immune cells of patients with a longer post-HSCT time span were more often exposed to different influenza viruses before d0, due to prior vaccinations or natural infections than those of patients with a shorter time span. We also observed significant differences between seroprotection rates for the two groups (**Table 6**).

In other publications, the transplant-to-vaccination interval was also determined as powerful predictor of antibody responses in HSCT recipients [5], [97]. In the study of Issa et al. [97], seroprotection rates to A/California were 37% in patients who received the pH1N1 vaccine less than 6 months after HSCT and 50% in those who received the vaccine between 6 months and one year after HSCT. The seroprotection rate was 38% to A/California for patient who received a vaccination one to two years after HSCT and 69% in those who did so two years or later after HSCT.

We also tested if age (**Fig. 26**), gender or graft-versus-host disease (**Fig. 27**) of HSCT patients had significant impact on the antibody titers, but no such impact could be observed. Graft-versus-host diseases (GvHD) can be divided into four grades based on the severity of symptoms [98], [99]. We pooled cases of "no" and "mild" GvH-diseases in one group and "moderate" and "severe" cases in a second group. No significant differences in GMTs could be observed. Regarding seroprotection, Issa et al. [97] published that age ≥60 years, gender, race/ethnicity, type of HSCT, presence or absence of GvHD, type of immunosuppression, previous seasonal influenza vaccination, history of influenza-like illness did not influence the rate of achieving a seroprotective titer against A/California. In a study of Mohty et al. [5], vaccine responses to A/California were poor in HSCT recipients with ongoing graft-versus-host disease.

An interesting subset of patients had PCR confirmed natural infection with Influenza despite having received the vaccine. The long follow-up of 180 days allowed to identify those patients based on significant titer changes (**Fig. 28**). Three HSCT patients (5,7%) were tested positive for an influenza B infection and another four patients (7,5%) showed positive results for the H3N2 influenza A-subtype. All infected patients were tested positive between day 60 (January/February) and day 180 (May/June). Interestingly, the antibody titers to the subtypes, which caused the infections, are obviously increased due to the infection, with an increase of two or more titer steps. This increase can be observed at each infected patient and the antibody level prior the infection seems to have no big influence. The antibody titers to the other subtypes in contrast remain on the same level or are even decreased at d180. That indicates that a real infection boosts the antibody production in a greater extend compared to an antibody response after vaccination.

In the last part of my master's thesis project, we designed an indirect ELISA to measure influenza specific IgG levels. We used the same five influenza antigens as in the HI assay. Influenza specific IgGs in the human serum samples can bind to the antigens and were detected with detection antibodies, which were polyclonal rabbit anti-human IgGs with HRP. A disadvantage of this method is, that there are no reference sera with known concentrations of specific IgGs against different influenza viruses available. That's why we used serum samples with high antibody titers (1024) as reference sera for quantification. When taking a look at the progressions of arbitrary units, interestingly at

baseline (d0) the geometric mean arbitrary units (AU) of healthy controls are higher compared to those of patients to all five antigens (**Fig. 29**). Conversely, the increase during the first 30 days post-vaccination is obviously higher in patients. Regarding the cross-reactivity, the curve progressions to B/Brisbane and B/Massachusetts look very similar, which was not the case at the HI assay. A reason could be that there is maybe a cross-reactivity of B/Massachusetts IgGs, which can bind to the HA antigen of the influenza B/Brisbane virus but not at a proper epitope to inhibit hemagglutination. The curve progressions to the influenza A viruses look also very similar. It also seems that the geometric mean AU to the influenza B viruses are higher than those to influenza A viruses. When comparing the GMTs and geometric mean AU it is noticeable that there are similarities especially at B/Massachusetts and A/California. Regarding to the performed regression analysis, good correlations between antibody titers and arbitrary units was determined at A/Switzerland, A/Texas and also A/California. B/Massachusetts also showed a good correlation except of view outliers (**Fig. 30**). The correlation was worst at B/Brisbane, which may be because of the above-described hypothetical cross-reactivity between anti-B/Massachusetts IgGs and the B/Brisbane antigen. The influenza specific indirect ELISA needs further optimization, but it worked well to have a first approach of strain specific IgG quantifications.

Conclusions:

In conclusion, we successfully optimized and adapted the HI assay to a high throughput situation. This was a crucial step in order to measure a large number of samples. A two-way cross-reactivity between the two H3N2 strains A/Switzerland and A/Texas was observed, whereas only Anti-B/Brisbane (Victoria) antibodies can bind to B/Massachusetts (Yamagata) antigens and not the other way round, due to the HI assay. The cross-reactivity between A/Switzerland and A/Texas was also seen at serum samples. Furthermore, a significant increase in GMTs 30 days post-vaccination could be observed to all antigens and both groups, HSCT recipients and healthy controls. The seroprotection rates were high in both groups against the three viruses included in the trivalent vaccine. A second vaccine dose for HSCT patients seems to stabilize the antibody concentrations and about 20% of HSCT patients who received seroprotection after vaccination, did only reach the seroprotection threshold for the first time after the second vaccine shot. The seroconversion rates were higher in HSCT recipients compared to those of HC at all five antigens. We also showed that HSCT recipients with a shorter

time post-HSCT show significantly lower antibody titers and seroprotection rates compared to recipients with a longer time span, especially at a five-year threshold. No significant impact of age, gender and the presence of GvHD could be found. We observed that antibody titers to a subtype, which caused an infection, are increased by two or more titer steps, independent of the antibody titer prior the natural infection.

Future projects will include another follow-up study with further HSCT recipients during the influenza season 2015/2016. The aim is to better understand influenza vaccine efficacy in HSCT recipients and to develop personalized vaccine algorithms based on the patients "risk profile".

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