UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Zentrum für Geburtshilfe, Kinder- und Jugendmedizin
Klinik für Geburtshilfe und Pränatalmedizin

Prof. Dr. med. Kurt Hecher

Identification and Characterization of Virus-Host Interactions Involved in Influenza A Virus Pathogenicity during Pregnancy in Mice

Dissertation

zur Erlangung des Doktorgrades Dr. rer. biol. hum. / PhD
an der Medizinischen Fakultät der Universität Hamburg.

vorgelegt von:

Géraldine Laura Engels
aus Zürich (Schweiz)

Hamburg 2016

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am: 08.09.2016

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg.

Prüfungsausschuss, der/die Vorsitzende: Prof. Dr. Petra Arck

Prüfungsausschuss, zweite/r Gutachter/in: Prof. Dr. Udo Markert

Content

List of figures

List of tables

1. Introduction

Every year, influenza A viruses infect three to five million people and account for approximately 250,000-500,000 deaths worldwide [1]). Influenza A viruses naturally occur in several subtypes which differ in their clinical course, pathogenicity and virulence. Some subtypes are capable of not only causing epidemics but also pandemics, as last seen in the year 2009 [2]. In 2009, it was recognized that influenza viruses poses an increased threat to pregnant women, as unveiled by the increased pathogenicity observed in this group of patients. It was observed that pregnant women infected with the 2009 pandemic H1N1 influenza presented more frequently with severe illness, pneumonia, increased rates of hospitalization, preterm and emergency cesarean delivery and even death [3-7].
Up to date, only little information is available on the molecular and immunological determinants involved in the enhanced pathogenicity observed in pregnant women upon pandemic influenza virus infections.

1.1 Influenza viruses

1.1.1 Classification

The influenza virus is a negative-sense, single-stranded, segmented RNA virus, belonging to the family of *Orthomyxoviridae* [8]. The *Orthomyxoviridae* are currently divided into several genera, including Influenza A, B, C and the recently discovered influenza D [9] viruses as well as tick-transmitted Thogoto and Dhori viruses [10], Isavirus and Quaranjavirus [11, 12]. Influenza A viruses can infect several species, among them humans, swine, horses, seals, birds and water fowl. Influenza B viruses could only be identified in humans and seals, influenza C viruses in humans, swine and dogs [12] and influenza D viruses in cattle and swine [9]. Influenza A and B viruses both consist of eight different gene segments. Instead, influenza C viruses possess seven gene segments of which one encodes for a hemagglutinin-esterase-fusion protein (HEF) which combines the functions of the HA and NA proteins [12]. The convention by which influenza A and B virus strains are named is as follows: genus (type), species from which the virus was isolated (if isolated in humans, human is not mentioned), location of isolation, isolate number, isolation year and - for influenza A viruses – the hemagglutinin (H) and neuraminidase (N) subtypes in brackets [8]. To date 18 hemagglutinin (H1 to H18) and 11 different neuraminidase (N1 to N11) subtypes have been described [13]. Except H17N10 and H18N11 which have only been found in bats so far, all other known subtypes of influenza A viruses have been found among birds [14].

1.1.2 Virion structure

The viral particles of influenza viruses are pleomorphic and differ in size and shape. The main configuration is at spherical shape with 80-120 nm diameter in size but also filamentous virions with a size of 300 nm can be found, especially in fresh clinical isolates [8, 12, 15-17]. The surface of the virion consists of a lipid bilayer membrane derived from the host cell membrane [8, 18]. In this outer layer, the viral envelope, the viral HA, NA and the matrix 2 (M2) ion channel are integrated. Electron

micrographs of influenza A virus´s morphology show the HA and NA glycol surface proteins protruding as spikes from the viral envelope in a 4:1 HA to NA ratio. Inside the envelope the matrix 1 (M1) protein coats the lipid membrane which encloses the virion core. The core of influenza viruses contains all eight viral genome segments in the form of viral ribonucleoprotein complexes (vRNPs) which each consist of a viral RNA segment, the polymerase proteins (polymerase basic 1 [PB1], polymerase basic 2 [PB2], polymerase acids [PA]) and the nucleoprotein (NP). Furthermore, the nuclear export protein (NEP, also called nonstructural protein 2 [NS2]) and the nonstructural protein 1 (NS1) are present inside the virion [8, 19].

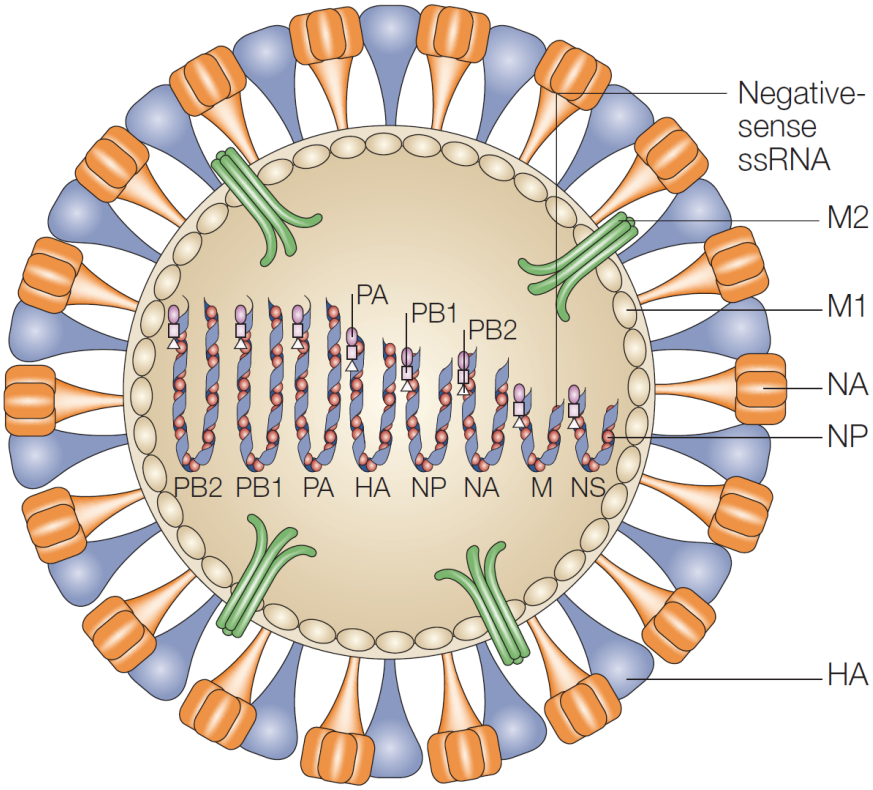


Figure 1: Schematic representation of an influenza virus particle. The lipid bilayer of the virion which forms the envelope, contains the HA, NA and M2 proteins. The M1 protein can be found inside the virion. The vRNP complex contains a vRNA segment encapsidated by the viral NP and associated with the three subunits of the viral polymerase (PB1, PB2 and PA) (modified from [20]).

1.1.3 Genome structure

Influenza A viruses consist of eight negative-sense, single-stranded RNA (vRNA) segments accounting in total for a genome size of approximately 13.6 kb. The eight RNA segments are numbered in order of decreasing length and encode for at least 13 proteins [12, 21]. Every vRNA segment includes noncoding regions of varying lengths at both the 3´- and 5´-ends). These untranslated regions of all segments are highly conserved among all influenza A virus genome

segments. The segmented genome found in influenza A viruses enables *antigenic shift*, in which an influenza A virus strain acquires RNA segments from another influenza subtype [19] (Table 1).

Table 1: Genome structure and gene products of influenza A viruses.

Segment	Size [bp]	Protein	Molecular weight [kDa]	Protein function
1	2341	PB2	80	Polymerase basic protein 2 Component of the vRNP- and polymerase complex, cap recognition
		PB2-S1	55	Inhibition of RIG-I-dependent interferon signaling pathway, interference with viral polymerase activity [22]
2	2341	PB1	90	Polymerase basic protein 1 Component of the vRNP- and polymerase complex, endonuclease activity, elongation, RNA-dependent RNA-polymerase
		PB1-F2	10	Potential virulence factor, regulation of virus replication, pro-apoptotic role in immune cells and viral polymerase activity [23-25]
		PB1-N40	82	Undefined function [24, 26]
3	2233	PA	83	Polymerase acid protein Component of the vRNP- and polymerase complex, protease
		PA-X	29	Role in virus replication, inhibition of host antiviral response [27]
		PA-N155	62	Undefined function [28]
		PA-N182	60	Undefined function [28]
4	1778	HA	77	Hemagglutinin Surface glycoprotein, receptor binding, fusion activity, major antigen
5	1565	NP	55	Nucleoprotein Component of vRNP complex, RNA binding, RNA synthesis, RNA nuclear import

6	1413	NA	56	Neuraminidase Surface glycoprotein, neuraminidase activity
7	1027	M1	28	Matrix protein 1 vRNP and surface glycoprotein interaction, nuclear export, budding
		M2	15	Matrix protein 2 Membrane protein, proton channel activity, viral assembly
		M42	~ 15	Support of efficient virus replication [29]
8	890	NS1	26	Nonstructural protein 1 Multifunctional protein, interferon antagonist, regulation of cellular gene expression
		NS2/NEP	11	Nonstructural protein 2/ nuclear export protein Regulatory protein, nuclear export of vRNPs
		NS3	17	Provides replicative gain-of-function [30]

The HA, NP and NA gene segments encode for the corresponding protein. PB2-S1, M2, M42, NS2/NEP and NS3 proteins are encoded by spliced mRNAs, whereas the PB1-F2 results from a second open reading frame (ORF). PB1-N40, PA-X, PA-N155 and PA-N182 are also encoded via alternative ORFs (modified after [8, 12, 19, 22-25, 27-30]).

1.1.4 Viral replication cycle

The influenza virus replication cycle consists of the following stages: virus attachment, virus entry, synthesis of viral RNA, synthesis of viral proteins, packaging of RNA and assembly of virus, virus budding and release [19, 31] (**Figure 2**).

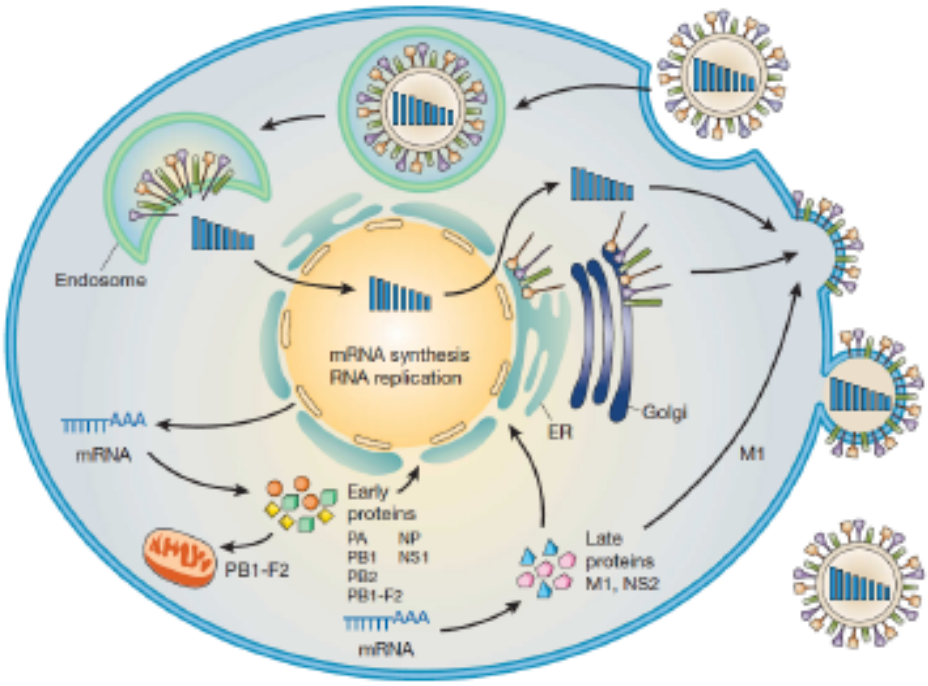


Figure 2: Viral replication cycle of Influenza A viruses. Following receptor-mediated endocytosis, the virus is engulfed by the cell plasma membrane and an endosome is formed. The viral membrane then fuses with the endosomal membrane and the VRNP complexes are transported into the nucleus, where viral replication and transcription takes place. Viral messenger RNAs are exported to the cytoplasm for translation, whereas early viral proteins – that are required for replication and transcription – are transported back to the nucleus. M1 and NS2 proteins enable the nuclear export of newly formed vRNPs. The assembly and budding of progeny virions takes place at the plasma membrane (modified from [31]).

Virus attachment

In order to initiate infection and replication, influenza A viruses bind to the sialic acids expressed on the host´s cells surface [8]. Sialic acids (SA) are found ubiquitous on many cell types and in many animal species. The sialic acids appear in two configurations which are preferentially recognized by influenza viruses: α2,3- or α2,6-linked SA. In the human upper respiratory tract, α2,6-linked SA are predominant, while α2,3-linked SA are more common in human lower respiratory tract and in the avian gastrointestinal tract [19, 32, 33].

Virus entry

Once the influenza virus HA protein attaches to the sialic acid on the host cell, the virus is internalized via receptor-mediated endocytosis. For this, a low pH of the endosomal compartment is crucial in order to allow influenza virus uncoating: First, the acidic environment triggers a conformational change in the HA, exposing a fusion peptide that induces the fusion of the viral envelope with the membrane of the endosome. As a result, a pore opens through which the viral RNPs are released into the host cell cytoplasm [34, 35]. Second, protons from the endosome enter the virus particle via the M2 proton channel which disrupts internal protein-protein interactions and releases the viral RNPs from the viral matrix of the virion into the cellular cytoplasm [8, 19, 36].

Synthesis of viral RNA

Once the RNPs are released into the cell plasma, they are trafficked to the host cell nucleus by viral proteins' nuclear localization signals (NLSs). NLSs induce the import of the RNPs by cellular viral proteins into the host cell nucleus. Transcription and replication of the influenza virus genome then takes place in the nucleus. The viral messenger RNA (mRNA) is then translated by the host cell machinery into viral proteins. The viral RNA dependent RNA polymerase – a vRNP component – uses the negative-sense vRNA as a template to synthesize two positive-sense RNAs: viral mRNA for viral protein synthesis, whereas the complementary RNA (cRNA) is transcribed by the vRNP into more copies of negative-sense, genomic vRNA. Once polyadenylated and capped, mRNA of viral origin can be exported out of the nucleus and translated like host mRNA. Nuclear export of vRNA segments is then mediated by the viral proteins M1 and NEP/NS2 [37]. M1 interacts with both, vRNA and NP, and is thought to conjoin these two components within the RNP complex. M1 is additionally associated with the nuclear export protein NEP, which mediates the M1-vRNP export via nucleoporins into the cytoplasm [8, 19].

Synthesis of viral proteins

The envelope proteins HA, NA and M2 are translated from viral mRNA in ribosomes on the rough endoplasmic reticulum. They are folded in the endoplasmic reticulum and are trafficked to the Golgi apparatus for post-translational modifications. The three proteins show apical sorting signals that direct them to the cell membrane for virion assembly. Although little is known considering the translation and sorting of the non-envelope proteins, M1 is thought to play a role in bringing the vRNP-NEP complex into contact with the envelope-bound HA, NA, and M2 proteins for packaging at the host cell membrane [8, 19].

Packaging of RNA and assembly of virus

Unless the influenza virus particle contains all eight vRNA segments it is not considered to be fully infectious. Packaging appears to be a selective process, by which discrete packaging signals on all vRNA segments assure that a complete genome is incorporated into virus particles [8, 19, 38-41].

Virus budding and release

Initiated by the accumulation of M1 protein at the cytosolic face of the lipid bilayer, the influenza virus budding process takes place at the cell membrane. Following the budding process, the HA molecules continue to tether the virions via sialic acid moieties on the cell surface. They are actively released by the sialidase activity of viral NA. If NA is inactive or absent, or if neuraminidase inhibitors are present, virus particles aggregate at the cell surface and as a result the infectivity is reduced [8, 19, 42, 43].

1.1.5 The natural reservoir of influenza A virus

Influenza is a zoonotic disease infecting humans and animals and being transmitting between these likewise. The natural reservoir of influenza A viruses are wild waterfowl but influenza viruses have also been isolated from other birds and mammalian species such as pigs, horses and sea mammals

(**Figure 3**). The recently detected H18 subtype has so far only been detected in bats [13]. Surveillance programs in the past revealed that avian influenza viruses are pervasively found in aquatic birds reaching from completely asymptomatic to symptomatic systemic infections with central nervous system involvement and frequent death – the latter including viruses of the H5 and H7 subtypes [16]. H5 and H7 subtypes are classified as highly pathogenic influenza A viruses and contain a multibasic cleavage site in the HA. This multibasic cleavage site can be cleaved by ubiquitously appearing proteases [44]. On the other hand, low pathogenic and mammalian influenza A viruses consist of a monobasic cleavage site that is cleaved by cellular proteases found in the respiratory tract or in the gastrointestinal tract in the avian host [16]. In birds, influenza viruses preferentially replicate in the epithelial cells of the gastrointestinal tract and are therefore found in high concentrations in feces [45]. Via the infected fecal material deposited in the water, waterfowl can efficiently transmit influenza viruses to other animals. The asymptomatic appearance of influenza infection in waterfowl is most likely the result of a successful viral adaptation to its host and allows the influenza viruses to circulate without selective pressure [16]. Nevertheless, interspecies transmission has been described in the past and avian influenza outbreaks were recorded in pigs [46], seals [47, 48], whales [49], horses [50] and mink [51]. While avian influenza viruses appear to be in evolutionary stasis in avian hosts, the genetic pool of influenza viruses provides the genetic variability needed to allow the emergence of pandemic influenza viruses in humans and animals. Continuous viral evolution is mainly observed in the surface glycoproteins of influenza viruses but occurs in all eight gene segments. The variability results from following known mechanisms: antigenic drift (point mutation including substitution, deletion, insertion) in the HA and NA protein [12], defective-interfering particles, RNA recombination and antigenic shift (genetic reassortment / point mutation) [16]. Furthermore, the RNA polymerase allows for fast adaption to a new host and environment since it lacks a proofreading function and therefore contributes with its error rate of 10^{-4} bases per replication cycle [16] to the high number of replication errors and antigenic drift observed [52, 53]. Every influenza virus replication cycle leads to a mixed virus population, also referred to as viral quasispecies, of which most are not viable but some have potentially advantageous mutations which can become dominant under a certain selective pressure [16]. Genetic reassortment is an important mechanism for influenza viruses to guarantee rapid diversity. Since the influenza virus genome is segmented, gene segments can be exchanged arbitrarily if a cell is infected simultaneously by two or more influenza virus subtypes [8, 16]. This high genetic diversity allows for the emergence of antigenically new virus subtypes which can, when introduced to an immunologically naïve population, cause the outbreak of a pandemic [31].

Avian influenza viruses are transmitted to humans directly, via other infected birds, mostly poultry, or pigs [54-56]. As pigs can be infected by avian and human influenza viruses, they serve as a so called “mixing vessel”. Once a pig is simultaneously infected by avian and human influenza viruses, reassortment of the viruses is possible. Up to date humans have mainly been infected by the H1, H2 and H3 influenza subtypes [8], whereas avian hosts can be infected with low pathogenic avian influenza viruses (LPAIV) or highly pathogenic avian influenza viruses (HPAIV). LPAIVs generally lead to mild or even asymptomatic infections in chicken and other birds [8, 16] – few mild human infections with LPAIV have been described [57-59]. Since 2013, a LPAIV of the H7N9 subtype has caused severe disease in humans changing the perceived risk of LPAIVs in general [60]. HPAIV on the other

hand can lead to severe avian influenza outbreaks. Here, the H5 and H7 subtypes frequently result in systemic infection in chicken or even death [16, 61]. In the past twenty years, H5N1 and H7N7 infections in humans and other animal species were observed, causing severe disease and frequently death in the case of H5N1 infection [59, 62, 63].

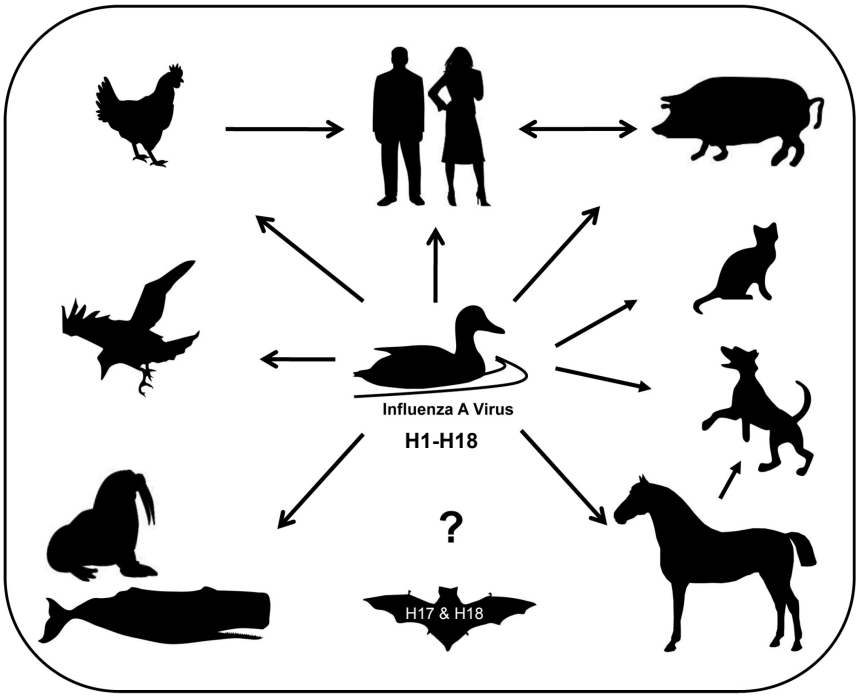


Figure 3: Host range of influenza viruses. The natural reservoir of influenza A viruses are wild waterfowl. Usually, in order to enter the mammalian hosts, avian influenza A viruses are transmitted to domestic birds – such as chickens and ducks – or swine, which serve as a “mixing vessel” as they are susceptible to both avian and human influenza viruses. Both domestic birds and swine can spread influenza A viruses to humans which occasionally causes pandemics, depending on the population´s existing immunity and the virus characteristics. Up to date, no actively replicating viruses have been isolated from bats and it is unclear if bats can transmit influenza viruses to other species (modified from [8, 16, 64, 65]).

1.1.6 Influenza in humans

1.1.6.1 Epidemics

Influenza is an acute, highly contagious respiratory illness affecting humans of all ages [66]. Influenza A viruses are typically transmitted via droplets or aerosol, spreading from person-to-person through sneezing, coughing or contact with contaminated surfaces [67]. Influenza viruses circulate in annual epidemics and recurrent, yet irregular, pandemics [8]. In temperate climate in the Northern hemisphere, seasonal influenza occurs mainly in winter while influenza may appear throughout the year in tropical regions [66]. It is estimated that 5-15 % of the adult population and 20-30 % of children in the Northern hemisphere are affected by influenza every year [1, 68]. The World Health Organization (WHO) estimates that three to five million patients suffer from severe illness every year, resulting in a relevant socioeconomical burden and approximately 250.000 to 500.000 deaths [1, 69].

Up to date, it is still not fully understood why influenza occurrence peaks in winter. Theories include seasonal modifications in melatonin and vitamin D levels, both influencing the host immune competence; behavioral changes such as crowding indoors, imperfect ventilation due to closed windows and environmental factors as temperature, humidity, UV irradiation and upper atmosphere air movement [70-73]. More recent analysis could demonstrate that absolute humidity strongly modulates the airborne survival and transmission of influenza viruses, moreover, that the onset of influenza-related mortality in winter is associated with low absolute humidity levels throughout the USA [74].

1.1.6.2 Pandemics of the 20th century

Influenza pandemics have occurred throughout history. Since 1918 three big pandemics caused high mortality and fatality rates in patients suffering from influenza in the 20th century [16] (**Figure 4**). Pandemic outbreaks are observed when an influenza virus which has not previously circulated in humans and no preexisting immunity is observed, transmits among humans. In 1918 the “Spanish flu” has presumably killed 20-50 million people worldwide, while the “Asian flu” and “Hong Kong flu” showed lower mortality rates.

The 1918 H1N1 influenza virus could not be isolated during the outbreak but genomic sequences from a formalin-fixed, paraffin-embedded sample and one frozen lung sample obtained by *in situ* biopsy of a victim buried in permafrost since 1918, many years later, revealed an avian-like H1N1 virus containing human-like signature amino acids in several proteins as well as a multibasic HA cleavage site which is known to be a characteristic of highly pathogenic avian influenza viruses [31, 73, 75]. Overall, the death rate with 20-50 million fatalities was 5 – 20 times higher than expected [73]. Atypically, the “Spanish flu” showed a high mortality rate among young adults [73]. This observation lacks full explanation to this day. The morbidity rate on the other hand was as expected: children younger than 15 years were the most affected group. In 1918 – due to the fact that antibiotics were not discovered yet – most patients died of bacterial superinfection caused by *Haemophilus influenzae*. Extrapulmonary infection was rarely observed [31, 73, 76].

The “Asian flu” (H2N2) was first detected in 1957 in Southern China, from where it spread. The pandemic was caused by a human/ avian reassortant virus that encountered an immunological naïve human population for the avian PB1, HA and NA [31]. This pandemic lead to approximately one to two million deaths [77, 78].

The „Hong Kong flu“(H3N2) in 1968 replaced the H2N2 with another human/ avian influenza virus reassortant. As in 1957, the PB1, HA and NA gene originated from an avian virus [31]. As a partial immunity against the NA was acquired in the human population through the previous “Asian flu” outbreak [8], the death toll was lower with approximately one million fatalities [78].

In 1977 the “Russian Flu” (H1N1) affected mainly people younger than 25 years of age, nevertheless the disease was considered as mild [79]. Presumably the virus could have escaped from a laboratory since it was identical with the influenza virus causing the epidemic in 1950 [16, 80].

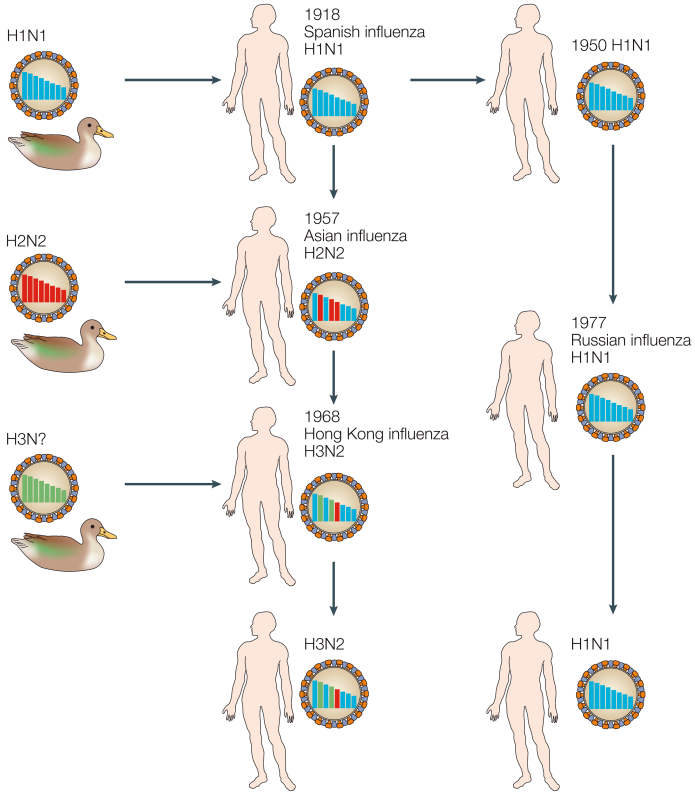


Figure 4: Influenza pandemics of the last 100 years: It is suspected that the1918 Spanish influenza pandemic was caused by an avian H1N1 influenza virus that was transmitted to humans. In 1957, a reassortant H1N1 virus possessing three avian gene segments caused the “Asian flu”. “The Hong Kong flu” in 1968 carried the PB1 and HA genes from an H3 avian virus. The H1N1 virus from 1977 circulating was almost identical to the H1N1 virus spreading in the 1950s suggesting it to be an escaped laboratory strain (modified from [20]).

1.1.6.2.1 The 2009 pH1N1 influenza pandemic

The first pandemic of 21th century was declared in June 2009 by the WHO. By then, 74 countries and territories had reported laboratory confirmed influenza cases [81]. Unlike the seasonal flu, the new virus caused high levels of infections during summer in the Northern hemisphere, and even higher numbers of infections during colder months. The new 2009 pandemic H1N1 (pH1N1) showed increased rates of death and progression into more severe illness. As the virus continued spreading, it showed moderate severity overall [82]. However, younger people, especially pregnant women (detailed description in 1.3) or patients with underlying chronic diseases appeared to be at increased risk to suffer from complications or severe course of illness [82, 83]. Interestingly, those older than 60 years of age represented only about 2% of all 2009 pH1N1 cases, whereas 80% of the cases in Europe appeared in the <30 years old population [84]. This could be partially explained by previously acquired cross reactive antibodies to H1N1 from which the elderly could profit [85, 86]. Nevertheless, the majority of hospitalized patients and fatal cases had underlying medical conditions [81, 87]. Overall 18.500 laboratory-confirmed deaths were reported worldwide from April 2009 to August 2010 but it is estimated that an additional 201.200 respiratory and 83.300 cardiovascular deaths were associated

with the 2009 pH1N1 [88]. However, 80 % of these estimated deaths appeared in patients younger than 65 years of age and 51 % occurred mainly in Southeast Asia and Africa. Overall, Dawood et al. estimated a 15 times higher mortality than the reported laboratory-confirmed 2009 pH1N1 cases as they have included deaths in Africa and Southeast Asia in their estimates [88].

The 2009 pH1N1 virus is a triple-reassortant virus containing gene segments from human, swine and avian influenza A viruses. The virus most likely resulted from a reassortment of recent North American H3N2 and H1N2 swine with Eurasian avian-like swine viruses [2, 31]. The virus contains PB2 and PA genes of the North American avian origin and a PB1 of human H3N2 origin. HA, NP and NS are from swine origin and the NA and M genes emerged from Eurasian avian-like swine origin [31, 89] (**Figure 5**).

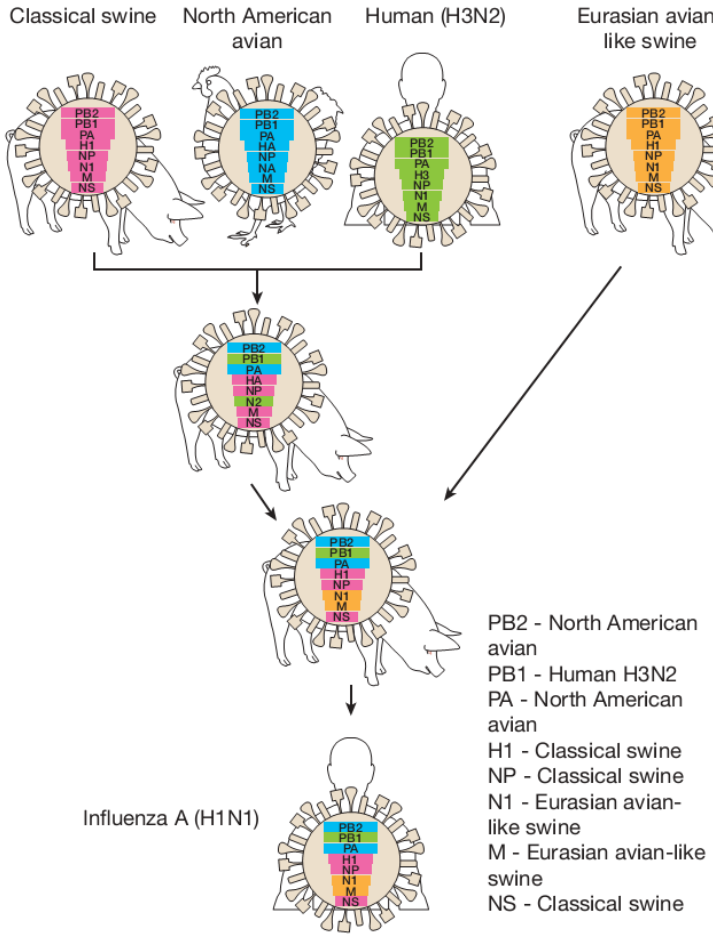


Figure 5: Reassortment of 2009 pH1N1 virus. Host and lineage origins from the 2009 pH1N1 gene segments. The reassortant most likely evolved in swine and was transmitted to humans. The PB2 and PA gene segments originate from North American avian hosts, whereas the PB1 segment emerged from human H3N2 viruses. The HA, NP and NS gene segments originated from swine origin, the NA and M segments were detected in avian-like swine origin (modified from [31]).

1.1.6.3 Clinical signs, pathogenesis and diagnosis of influenza virus infections

Influenza is characterized by sudden onset of disease, following a short incubation time of approximately one to two days, in rare cases up to four days [90]. Patients usually present with high fever [4, 5, 91-96], cough [4, 5, 91-97], malaise [4, 98], myalgia [4, 5, 91, 96] and/or joint pain [98], headache [4, 5, 91, 92, 95, 96], sore throat [4, 5, 91, 92, 94, 95] and rhinorrhea [4, 5, 94, 95]. Children present with comparable clinical signs but additionally often show symptoms of a middle ear infection. The influenza virus, which is usually spread by droplets or aerosols (as described in 1.1.6.1), first comes into contact with the oral mucosa, nasal mucous membrane and the ocular conjunctivae where it infects epithelial cells [12]. Virus replication is highest approximately 48 hours after infection and virus is usually shed for about six days [99].The virus spreads from the upper to the lower respiratory tract, with viremia being rarely observed in H1, H2 or H3 influenza viruses [12]. Acute symptoms and fever can often be detected for 7 to 10 days, whereas the feelings of weakness and fatigue may remain for several weeks [99]. Less common signs of disease and often signs of more severe influenza pathogenicity include gastrointestinal symptoms (nausea, vomiting and/or diarrhea) [4, 5, 91-94], bacterial coinfection [4, 91, 93, 94, 100-102], conjunctivitis [4], cyanosis [97], acute respiratory failure and distress syndrome (ARDS)/ hypoxemia [4, 92, 95], dyspnea/ respiratory distress [4, 5, 91-93, 97] or confusion [90, 94]. The clinical signs and symptoms of influenza are most likely due to the damage at the site of virus replication and to local and systemic release of cytokines and other inflammatory mediators [103, 104].

Influenza related complications

In risk group patients, during pandemics or infection with highly pathogenic avian influenza viruses, severe influenza-related complications are observed. Risk groups suffering from severe influenza and its complications include pregnant women, patients with underlying chronic diseases (such as diabetes, asthma bronchiale, cystic fibrosis, chronic obstructive pulmonary diseases (COPD), cardiovascular diseases [12], immunocompromised patients, the elderly and infants. The most common influenza-related complication is primary viral pneumonia and secondary bacterial pneumonia. Clinically, viral pneumonia is characterized by dyspnea or even cyanosis, as well as prolonged fever. In 4-8 % of older than 60 years old patients, secondary bacterial pneumonia is diagnosed [90, 105]. Even in the era of antibiotics, bacterial superinfections were detected in 34 % of intensive care patients and 55 % of fatal cases of patients infected with the 2009 pH1N1 implicating an increased susceptibility to secondary bacterial infection [106] [107]. The most commonly identified bacteria are *Staphylococcus aureus,* including methicillin-resistant strains*, Streptococcus pneumoniae, Streptococcus pyogenes* and *Haemophilus influenzae [76, 106, 108]*. Further influenza related complications in adults are summarized in Table 2.

Table 2: Complications observed among influenza patients.

System	Symptoms
Neurological complications [4]	Confusion [94] Seizures [94, 101] Encephalitis [92, 94, 101] Encephalopathy [92, 94, 101] Hemiplegia [101] Quadriparesis [94, 101] Acute myelopathy [101] Ataxia [101] Guillain-Barré syndrome [101]
Cardio-vascular complications [4]	Hypotension [92, 109] Heart failure [101] Hypovolemic shock [94, 100]/ toxic shock [4] Pulmonary embolism [92] Myocarditis [92, 94, 101] Pericarditis [92]
Musculo-skeletal complications [4]	Myositis [92, 94, 101] Rhabdomyolysis [92, 94, 101]
Obstetric complications (2009 H1N1)	Abortion/ pregnancy loss [4, 94, 96, 97, 110, 111] Increased risk for cesarean section [5, 6, 102] Preterm labor/ delivery [6, 95, 110-112] Intrauterine growth restriction/ children small for gestational age [6, 95, 110-112] Rarely: placental transmission [110]
Further complications	Increased mortality [4-6, 97, 109, 112] Renal failure [6, 94] Exacerbation of chronic disease (chronic obstructive pulmonary disease [COPD], asthma, congestive heart failure) [4, 93, 94, 100] Multi organ failure [113] laryngotracheitis in young children [114]

Influenza histopathology:

Histopathological observations in humans are usually post-mortem observations. One of the few studies looking at uncomplicated influenza in humans showed trachea-bronchitis, damage of the respiratory epithelium which included vacuolization, edema and absence of cilia to extensive desquamation of epithelial cells. Furthermore, the bronchial biopsies from these patients showed a *lamina propria* with edema and hyperemia and infiltration of lymphocytes and histiocytes [115]. In case

of viral pneumonia, the damage of the alveolar epithelium results in reduced gas exchange function [113], diffuse alveolar damage [93, 94] with hyaline membranes and septal edema [94] due to hyperemia of alveolar capillaries, interstitial edema and leukocyte infiltration [113], alveolar hemorrhage [93, 94], pulmonary vascular congestion [94], capillary thrombosis [113] or pulmonary embolism [93, 94].
The edema and mononuclear infiltration most likely trigger the local influenza symptoms of cough and sore throat, whereas the systemic symptoms like headache, fever, myalgia and malaise are probably a result of cytokine production and, in severe cases, the so called “cytokine storm” [116].

Diagnosis of influenza
As the clinical signs of influenza are similar to respiratory infections caused by other infectious agents, laboratory diagnosis is usually required. Following methods are recommended by the Center of Disease Control (CDC): viral tissue cell culture, rapid cell culture, direct or indirect immunofluorescence, real-time quantitative chain reaction (RT-qPCR), other molecular assays (mainly based on influenza viral RNA detection) and rapid influenza virus diagnostic tests (antigen detection) [117].

1.1.6.4 Immune response towards influenza virus infection

The host immune system responds to influenza virus infection with a complex cascade of reactions. Simultaneously, the immune system initiates the induction of immunological memory to protect against future influenza virus infections.

Innate immune system
The first line of defense is formed by the innate immune system which recognizes virus-infected cells through non-antigen-specific mechanisms [118]. Constituents of the innate immune system such as mucus or salvia are aim to prevent infection of respiratory epithelial cells with influenza virus. In a next step, immune cells are activated to control virus replication [119].

Pattern-recognition receptors (PRRs) are host sensors that recognize viral components, such as viral RNA. The PRRs are expressed on several cell types and consist of toll like receptors (TLRs), retinoic acid inducible gene-I (RIG-I) and the NOD-like receptor family pryin domain containing 3 (NLRP3) protein [120, 121].The signaling to TLRs and RIG-I receptors results in the expression of proinflammatory cytokines and type I interferons [122-124], especially IFN-α and IFN-β [125] which are known to have a strong antiviral activity. Interferons attach to receptors on neighboring cells and inhibit protein synthesis, recruit monocytes/macrophages, T and NK cells and enhance maturation of antigen-presenting cells [118]. IFN-α and IFN-β further induce the expression of interferon stimulated genes (ISGs) via the JAK/STAT signaling pathway [126] and they are known to stimulate dendritic cells (DCs). DCs present antigens to CD4+ and CD8+ T cells and hereby initiate robust adaptive immune responses [127, 128].

DCs then degrade viral proteins and present the respective immuno-peptides (epitopes) at the cell surface where they are recognized by either specific CD8+ cytotoxic T cells (CTL) or CD4+ T helper (Th) cells [129, 130]. Subsets of DCs, so called plasmacytoid DCs (pDC), recognize viral DNA and RNA and secrete interferons, especially IFN- α [131].
As Natural killer cells can detect antibody-bound influenza virus infected cells and lyse these cells, they are considered as important effector cells [132, 133].

Adaptive immune system
The second line of defense is formed by the adaptive immune system which consists of humoral and cellular responses triggered by virus-specific antibodies and T cells [119].

Humoral immunity
Virus-specific antibody responses are induced by influenza virus infection [134, 135]. In this case, the antibodies against the surface glycoproteins HA and NA of the virus are of importance since they correlate with protective immunity [136] as long as they match the virus subtype that is causing the infection [137].

Cellular immunity
Following influenza virus infection CD4+ T cells, CD8+ T cells and regulatory T cells (Tregs) are induced. CD4+ T cells show cytolytic activity towards infected cells [138], T helper (Th) cells on the other hand produce different types of cytokines. Th2 cells produce IL-4 and IL-13 and predominantly promote B cell responses [8], whereas Th1 cells produce IFN-γ and IL-2 and are mainly involved in cellular immune responses. In addition, regulatory T cells (Tregs) and T helper 17 (Th17) cells are involved in regulation of the cellular immune response [119]. CTLs on the other hand recognize and eliminate influenza virus-infected cells and prevent production of progeny virus [139].

1.1.6.5 Treatment and prophylaxis of influenza

Treatment
Additional to the generally applied symptomatic treatment of influenza, antivirals are available. These drugs are not only used to treat influenza but can also be used for disease prevention. Two main groups of influenza antivirals are administered: neuraminidase inhibitors and M2 channel inhibitors (amantadines). The latter block the M2 proton channel which has a critical role in the acidification of the virion upon endosomal uptake leading to the release of vRNPs into the cytoplasm. Amantadine and rimantadine (both amantadines representatives) are only active against influenza A viruses and high levels of resistance (>99%) have been detected in H3N2 and 2009 pandemic H1N1 viruses. As a result, amantadine and rimantadine are not recommended anymore for treatment or chemoprophylaxis [2, 140, 141]. Neuraminidase inhibitors on the other hand, interfere with the enzymatic activity of the NA protein, thereby interrupting efficient release of newly synthesized viruses from infected cells [31]. They show activity against both influenza A and B viruses. Currently, the following neuraminidase inhibitors are available: oral oseltamivir (Tamiflu®), inhaled zanamivir

(Relenza®), and – in the USA - intravenous peramivir (Rapivab®) [2, 140, 141]. Due to the high mutation rate of influenza viruses, new antivirals are urgently needed. Promising candidates are currently under development or already enrolled in clinical trials [31].

Prevention

Vaccines against influenza virus infection, which have been available since the 1960s, are the most important and effective strategy to prevent influenza virus infection. Usually, several influenza virus subtypes are co-circulating every year triggering limited cross-immunity between the different IAV subtypes. Therefore, several influenza virus subtypes are combined in the annual influenza vaccine. In the last years two influenza A viruses (H1N1 and H3N2) and one influenza B virus were included in the vaccine formulation [31, 142]. The current recommendation for the winter 2015/2016 in the Northern hemisphere by the WHO consists of an A/California/7/2009 (H1N1)pdm09-like virus; an A/Switzerland/9715293/2013 (H3N2)-like virus and a B/Phuket/3073/2013-like virus [143]. As influenza viruses constantly evolve by *antigenic drift* [12, 31], vaccine recommendations are updated annually in February at the WHO influenza strain selection meeting where virological, epidemiological, immunological and vaccine-performance information are evaluated and a recommendation is given for the upcoming season [142]. Vaccines are then generally grown in embryonated chicken eggs and after formaldehyde-inactivation and further processing, the so-called split, inactivated or subunit vaccines are administered intramuscularly or subcutaneously [144]. In children and younger adults the vaccine efficacy is around 60-80%, whereas the rate is lower in the elderly – one of the main risk groups for influenza virus infection. The reduced vaccine efficacy is proposed to be due to the decreased immune response in elderly [144, 145] but could additionally be explained by the concurrent intake of non-steroidal anti-inflammatory drugs (NSAIDs, i.e. aspirin, paracetamol, ibuprofen) which have been shown to lower the host defense after vaccination [146]. Furthermore, a recent study could show that influenza vaccines do not only reduce incidence of disease but also reduce the risk of developing influenza-associated pneumonia [147]. Nevertheless, development of improved influenza vaccines is aimed. One approach is live attenuated vaccines which trigger humoral and cellular immune response and function probably superior to inactivated vaccines. Another promising approach is the development of a “universal” vaccine on the basis of the conserved M2 protein, the relatively invariant stalk domain of the HA or multivalent approaches [31, 148].

1.2 The immune system during pregnancy

During pregnancy, the maternal immune system adapts and modulates itself in order to tolerate the foreign, semiallogenic fetus, as 50 % of the genes are from paternal origin. These adaptations include local immune responses [149] but also alternations in the peripheral immune response [150]. Sex hormones, such as progesterone and estradiol, contribute to the maintenance of pregnancy and interact with immune cells in order to induce tolerance [151].

Overall, pregnancy can be characterized by three immunological phases [152]. In the pro-inflammatory phase of the first trimester, blastocysts damage the endometrial tissue in order to implant

and the trophoblast replaces the endothelium and vascular smooth muscles to ensure sufficient blood supply [153]. In the second trimester, the fetus grows and develops and an anti-inflammatory stage is induced. Finally, in the last trimester, the fetus is completely developed and all organs are functional. In this phase, an inflammatory stage is required for parturition where the influx of immune cells in the myometrium is observed [154, 155]. Via this pro-inflammatory environment the uterus contracts, the baby is expulsed and the placenta is rejected [152].

Immune cells during pregnancy

Following implantation, fetal trophoblast cells infiltrate the uterine endometrium leading to the development of the decidua. Local decidual immune cells, including dendritic cells (DC), uterine NK cells and macrophages modulate and control the balance between fetal trophoblast invasion and their tolerance, the latter two also being involved in regulating spiral artery remodeling [156-160]. However, the frequency of these decidual immune cells differs throughout pregnancy [161]. During healthy pregnancy, high numbers of regulatory T cells (Tregs), helper and cytotoxic T lymphocytes can be found in the human decidua [161-163]. Taken together, these immune cells contribute to the acceptance of the fetus, implantation and placentation.

Once the placental circulation is established after 8-12 weeks of pregnancy, the maternal blood is in close contact with the semiallogenic villous trophoblasts. Hereby factors such as Interleukin (IL)-4, syncytiotrophoblast fragments [164] or fetal cells [165, 166] can enter and affect the maternal blood stream.

The innate immune system is activated during pregnancy, shown i.e. by the increased numbers of monocytes and granulocytes and their phenotypical and functional activation [167-169]. On the other hand, DCs, peripheral NK cells and their production of interferon (IFN) γ are reduced during pregnancy [170-172]. Especially the reduction in NK cells is important for healthy pregnancy, as it could be shown in the past that after *in vitro* fertilization no living infants were born when the percentage of maternal peripheral NK cells was above 18 % [173].

In the past the underlying concept of the immune regulation during pregnancy was based on the shift from T helper 1 (Th1: cell-mediated response) towards T helper 2 (Th2: antibody [humoral]-mediated response) resulting in a well-balanced system able to induce cell – mediated and humoral responses [174]. Nevertheless, it is now accepted that the immunological modulations during pregnancy are more complex, as Th17 – a $CD4^+$ subset protecting against extracellular pathogens by promoting inflammation – and Tregs are also involved [175, 176]. Especially Tregs, a specialized $CD4^+$ T cell subpopulation expressing the transcription factor forkhead box P3 (FOXP3), have been shown to be essential for the promotion of immune tolerance during pregnancy [162, 177, 178]. Further insights on the induction of fetomaternal immune tolerance has been provided by Nancy *et al.*, whom could demonstrate that due to epigenetically silencing effector T cells – which can be potentially harmful to the fetus - cannot accumulate within the decidua [179].

Despite these maternal immune changes during pregnancy, most pregnant women feel and are healthy. Furthermore, these immunological adaptations to pregnancy can result in advantages for maternal health, i.e. in the case of cell-mediated autoimmune diseases such as multiple sclerosis or rheumatoid arthritis [102, 180, 181]. On the other hand, one key function of the immune system is to protect and clear the host from foreign antigens and pathogens. By protecting the maternal host, the immune system also prevents damage to the fetus [152]. If required, these immune responses in combination with the pregnancy related adaptations can worsen maternal health when the pregnant women suffer from certain infections such as influenza [182].

1.3 Influenza and Pregnancy

During the last influenza A virus pandemic in the year 2009 it was recognized that especially pregnant women suffered from influenza-related complications such as severe illness, increased rate of hospitalization, pneumonia, preterm and emergency cesarean delivery or even death [6]. In the aftermath of previous pandemics and seasonal influenza outbreaks it became clear that pregnant women generally suffer from increased morbidity and disease severity when infected with influenza viruses during pregnancy [4, 183] (**Figure 6**).

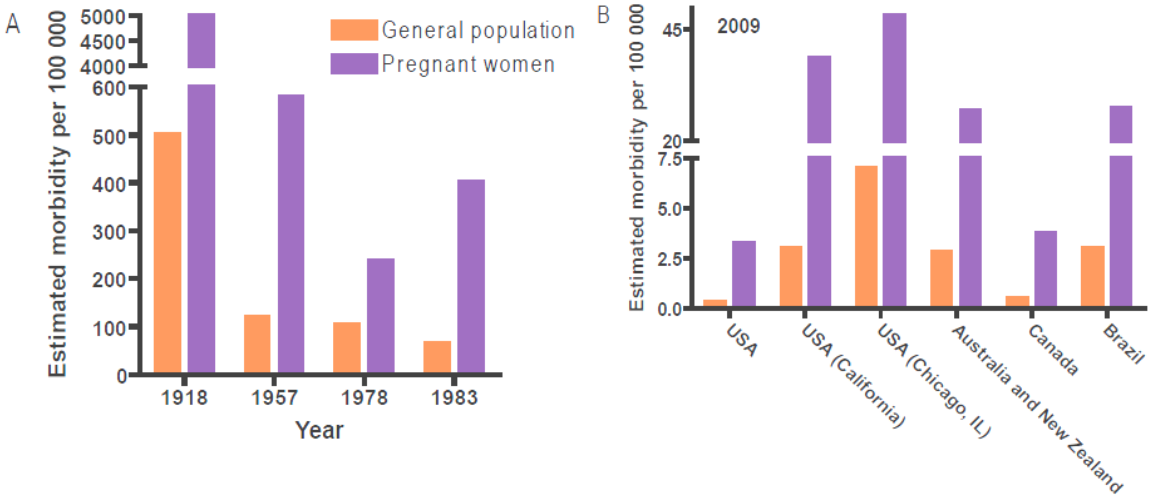


Figure 6: Rates of severe influenza disease among pregnant women. A) Estimated morbidity per 100 000 inhabitants during two pandemic (1918, 1957) and two interpandemic (1978, 1983) years for the general population (orange bars) and pregnant women (purple bars), respectively. B) Morbidity estimated from April to June 2009 in different regions and countries (modified from [183]).

During the last years, influenza prevention, early diagnosis and management of pregnant women infected with influenza have become routine for physicians and health care institutions. The clinical symptoms pregnant women present themselves with to physicians are comparable to the clinical signs observed in the general non-pregnant population [5] (clinical signs summarized in chapter 1.1.6.3). However, the risk of severe influenza complications could not be decreased so far, suggesting that the high incidence of severe influenza cannot be explained by higher incidence of infection alone [102]. As during pregnancy not only immunological alterations are observed (as described in chapter 1.2).

Physiological and anatomical changes as elevation of the diaphragm, increased respiratory rate, hear rate, stroke volume, oxygen consumption and intra-abdominal pressure, decreased chest compliance and a resulting increased aspiration risk, increase the risk of respiratory failure and complicate the therapy of respiratory diseases [102, 184-187]. These observations suggest that pregnant women suffer more severely from seasonal influenza compared to non-pregnant women. Several studies in the past could show that pregnant women had a three to four times increased risk to be hospitalized with acute cardiopulmonary symptoms during seasonal influenza epidemics compared to postpartum women. The pregnant women were mostly in their third trimester of pregnancy when hospitalized [188, 189]. The risk of need for hospitalization and also death was further increased if the pregnant women were suffering from underlying conditions such as asthma and other chronic pulmonary diseases, chronic cardiac disease, diabetes mellitus, chronic renal disease, malignancies, obesity or immunosuppressive disorders [4, 6, 112, 189, 190].

During the 1918 H1N1 pandemic, pregnant women showed a dramatically increased risk of severe disease and death, with an overall fatality rate of 27 %. 50 % of all pregnant patients presented with pneumonia [191]. But also during the H2N2 pandemic in 1957, 20 % of all pregnancy related deaths were due to influenza and 50 % of the women in their reproductive age who died from the pandemic were pregnant [192].

Recently, the 2009 pandemic underlined these observations already made in the past. In 2009 pregnant women were at an increased risk of hospitalization, admission to intensive care units and even death [193]. Data from the United States highlight that pregnant women were four times more likely to be hospitalized than the general population [5] and although only representing 1 % of the American population, accounted for 5 % of all 2009 pH1N1 related deaths [194]. In a systematic review of the 2009 pandemic data available, pregnant women accounted for 6.3 % of hospitalizations, 5.9 % of intensive care admission and 5.7 % of deaths [6].

Highest risk to suffer from severe complications during influenza virus infection appeared to be in the second, but mainly third trimester, were most deaths occurred [194-196].This increased severity is not only observed during influenza virus infection but also when pregnant women are infected with malaria, hepatitis E or herpes simplex. This is most likely due to reduced T cell, NK cell and possibly B-cell activity and increased monocyte, DC, polymorphonuclear-cell activity and increased α-defensin levels [182].

The administration of medication during pregnancy is usually only performed very cautiously and only when absolutely necessary due to the feared risk of potential negative side effects on the embryo or fetus. Up to now, only limited data is available on the effects of antiviral administration during pregnancy but overall, the data from 2009 suggest that women receiving antiviral treatment within 2 days of disease onset are less likely to die and less likely in need of intensive care treatment [194]. Therefore, antiviral treatment was recommended although the licensed antiviral drugs are classified as category C drugs, meaning that no clinical studies have been performed in pregnant women and that animal studies showed either adverse effects or have not been performed yet [102, 193].

Infection with pandemic influenza has been associated with a fivefold increase in perinatal mortality, miscarriages, stillbirths as well as early neonatal illness and death [197, 198]. Additionally, a threefold increased risk for prematurity and complicated birth was observed [199]. Furthermore children from infected mothers were more likely to be born pre-term, via cesarean section, with a low birth weight or small for gestational age [6, 200].

Effects of influenza virus infection during pregnancy on the fetus/newborn

As viremia is infrequently diagnosed during influenza infection, transplacental transmission is rarely observed [201-204]. Transplacental transmission, i.e. virus positive placental tissue and fetal lung cells, has been documented upon HPAIV H5N1 infection where viremia is diagnosed more frequently [193, 205, 206].

Even when no transplacental transmission is observed, the unborn child may be affected nevertheless, especially when the mother is severely ill. Analyses from the past, especially from 1918, showed an increased rate of pregnancy loss and preterm delivery [191, 207]. A study looking at outcomes of infants born to influenza infected mothers in 2009, showed an increased risk of adverse outcomes such as preterm birth, admittance to neonatal intensive care and 25 % were born small for gestational age (compared to 10 % of all US births) [200]. Further adverse outcomes have been proposed following seasonal or pandemic influenza outbreaks but overall the data is limited. Some studies postulate an increased risk of congenital anomalies (cleft lip with or without cleft palate, neural tube and congenital heart defects), as well as adverse outcomes including leukemia, schizophrenia, Parkinson disease and allergic diseases [208-213]. Fever, often examined in influenza patients, increases the risk for adverse infant outcome and may be in fact responsible for some of the adverse outcomes such as congenital heart defects and orofacial clefts, described before [214-216].
It is currently still unclear if and which long term impairments the unborn child may face upon maternal influenza virus infection. However, when considering long lasting consequences for children born with low birth weight, it is know that these children have an increased risk to develop cardiovascular, metabolic and inflammatory diseases later in life [217, 218].

German cases during the 2009 influenza pandemic

In Germany 496 pregnant women were reported with influenza disease to the Robert Koch Institute, of which 27 % were hospitalized compared to 4% non-pregnant women in the same age group (17-49 years) [219]. Despite the bias that in 2009 presumably more pregnant women were hospitalized due to the observed increased risk, 2.6 % of pregnant influenza infected women in Germany suffered from pneumonia, compared to 0.9% in the age-matched reference group[219]. Most likely owing to the overall low number of infected pregnant women in Germany and a highest standard health care system, the Robert Koch Institution reported only two fatal influenza cases in pregnant women in 2009 [97, 219].

Overall, number of pregnant women affected by influenza virus infection and the degree of severity are unknown, as most likely many patients with influenza-like symptoms do not present themselves to

a physician and if they do, the consulted physicians often treat the patients only symptomatically without performing further diagnostic tests [90].

1.3.1 Immune response to influenza virus infection during pregnancy

Several defense mechanisms against infections take action during pregnancy. On one hand, there are mechanical barriers such as the amniotic fluid, fetal membranes, the placenta and maternal decidua which prevent further viral spread. On the other hand, complex immunological defense mechanisms are involved [116]. Influenza viruses can infect the placenta and amniotic fluid [220] but others have suggested that influenza viruses replicate preferentially in the decidua [221].
The number of pDCs, also known as “interferon-producing cells”, is significantly reduced during pregnancy [222], however protein levels of certain TLRs seem to increase throughout pregnancy on pDCs [223]. These observations were associated with increased IL6 and IL12 and tumor necrosis factor (TNF)α levels. The observed changes in pDC phenotype may have an impact on viral clearance [116, 222]. Further, NK cells with their cytotoxic and cytokine-producing functions are present in the maternal decidua and can destroy virus-infected cells via perforin-dependent mechanisms [224].

Cytokines are additional key players in the regulation of intrauterine functions, including parturition and defense against infections and pathogens. A recent study comparing blood cytokine levels of pregnant women to postpartal cytokine levels showed alterations in proinflammatory and chemotactic cytokines. These changes included decreased IFN-γ and monocyte chemotactic protein 1 (MCP-1) as well as increased TNF-α and granulocyte colony-stimulating factor (G-CSF) levels and were most pronounced in the second and third trimester of pregnancy [225]. These changes may have an impact on the response towards influenza infection.

It is known that levels of progesterone and glucocorticoids, which have anti-inflammatory effects, increase during pregnancy [226]. This is one potential explanation for the increased disease severity during the last trimester of pregnancy since prompt immune response is required for the efficient elimination of influenza viruses [5]. When it comes to estrogens, the situation is more complicated. Estrogen appears to have both anti- and proinflammatory effects which can result in differences in disease severity [227, 228].

Conclusively, the understanding of the underlying mechanisms explaining the increased influenza disease severity in pregnant women is limited and needs further evaluation.

1.3.2 Pregnancy and influenza mouse models

The first mouse model used to evaluate the effects of influenza infection during pregnancy was established in the 1970s. *C3H* inbred and *Prince Henry* outbred mice were infected with the A/WSN/33 (H1N1) influenza strain in their first or third week of gestation. The infection of the mice was followed by harmful effects on neonatal growth and development as well as increased maternal mortality [229].

Following the 2009 H1N1 pandemic, further mouse models were established. In one study, *Chan et al.* infected syngenically mated BALB/c mice with wildtype 2009 pH1N1 (A/HK/415742/09) virus or a mutant 2009 pH1N1 influenza virus. The mutant virus contained a mutation at position 222 in the viral HA that was found with increased frequency in patients suffering from severe influenza. The pregnant mice were infected on days 12 to 14 of gestation, which corresponds to the last trimester of mouse pregnancy. The infected pregnant mice showed higher viral lung titers, histological evidence of pneumonia and a significant higher mortality rate compared to non-pregnant infected mice. Proinflammatory cytokines and chemokines in lung homogenates could be detected at higher levels compared to non-pregnant mice, with the exception of IFN-γ. CD3+/CD4+ and CD3+/CD8+ peripheral T lymphocytes and serum antibody levels were lower in pregnant mice [230].
In a comparable study with the same syngenical BALB/c mating model, a significant increased maternal mortality and fetal absorption was observed upon 2009 H1N1 infection. This was not observed when infecting the pregnant mice with seasonal H1N1 influenza virus. The authors could show that pregnant BALB/c mice had higher viral titers in lungs and elevated levels of inflammatory cytokines and chemokines (e.g. IL-1a, IL-6, G-CSF, RANTES (regulated on activation, normal T cell expressed and secreted) and MCP-1) when infected with 2009 pandemic H1N1 virus versus seasonal H1N1 influenza A virus [231].
Furthermore, Marcelin *et al.* proposed, using their syngenically mated *BALB/c* pregnancy model, that the increased mortality rate among pregnant 2009 H1N1 infected mice was due to a reduced regeneration of the respiratory epithelium. They could furthermore show increased levels of pulmonary chemoattractants, macrophages and neutrophils suggesting that the elevated cellular recruitment is a major contributor to severe influenza disease in pregnant mice [232].

1.3.3 Vaccine safety, attitude towards vaccination and vaccine uptake among pregnant women

During the last years, low to moderate seasonal and pandemic influenza vaccination compliance was observed in pregnant women in industrialized countries [233] ranging from 1.7 % - 88.4 % for seasonal influenza and 6.2 % - 85.7 % for pandemic influenza vaccine depending on the country [234]. This wide range in vaccination compliance is observed despite the fact that several studies have shown that the vaccines are safe for both mother and child [235, 236] and that the WHO recommends vaccination of pregnant women with highest priority [237]. It is currently believed that the poor vaccination compliance observed in pregnant women can be improved by protective countermeasures, such as education of the population, especially physicians and family member of pregnant women and offering easy access to vaccination [234]. A recent study published by the US Center for Disease Control strengthens this approach. In this study [238] increased influenza vaccination compliance could be reached by recommendation of vaccination with the vaccine being available (70.5 %), recommendation of vaccination alone resulted in a vaccination rate of 46.3 %. In turn, lowest vaccination rate (16.1 %) was observed in pregnant women receiving no recommendations at all.
An exemplary study performed in Germany could show concerns and misconceptions regarding vaccine safety, low risk perception of disease and poor knowledge related to vaccines present some

of the barriers towards influenza virus vaccine uptake [233]. Furthermore, the risk of influenza vaccine was perceived higher than the risk of disease [239].
Vaccination of pregnant women is of importance since not only the mother but also her child can be protected by the influenza vaccine from infection. It has been shown that vaccination of the mother can protect the unborn fetus from severe influenza related consequences as well as reduce infection of the infant for up to six months of age [6, 235, 236]. Especially in the first six months, young infants are highly susceptible to influenza and develop more severe symptoms and complications if infected [240]. However, breast-fed newborns are protected passively by maternal influenza antibodies acquired by natural infection or vaccination [241-244]. Up to date, influenza vaccination is recommended from an age of 6 months on in the USA [245].
Taken together, these data indicate that vaccination of pregnant women is crucial since it can prevent infection and severe disease outcome of mother and child and further spread of the virus.

1.4 Aim of study

During the last years, around 213 million women were pregnant per year worldwide [246]. All of these women have an increased risk to suffer from severe influenza. Facing this high number of women with the potential of developing severe illness, it is absolutely necessary to understand the underlying virological and immunological determinants involved in the increased disease severity observed.

Following objectives were addressed in this doctoral thesis:

- Establishment of a semi-allogenic mouse infection model in order to study the effects of influenza virus infection during pregnancy (seasonal versus 2009 pandemic infections)
- Evaluation of morbidity parameters and mortality rates comparing non-pregnant and pregnant mice upon 2009 pandemic H1N1 (pH1N1) infection
- Investigation of the effects of 2009 pH1N1 influenza virus infection on the maternal immune response
- Effects of the endocrine triggered epigenetic chemokine-receptor expression changes during pregnancy on infection outcome in infected mice
- Detection of viral mutations appearing in pregnant influenza A virus infected mice and generation of recombinant single-point mutant and multiple-gene reassortant viruses by reverse genetics
- Comparison of disease outcome, pathogenicity and virulence upon infection of non-pregnant mice with single-point mutant and multi-gene reassortant viruses
- Functional characterization of the identified single-point mutant and multi-gene reassortant viruses
- PRINCE cohort: antibody and vaccine compliance determination among pregnant women

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals, solutions and buffers

Name/ substance	Composition/ producer
Agarose	Serva
Calcium chloride	Merck
Chicken erythrocytes with citrate	Lohmann Tierzucht
Citrate buffer	DCS
Cytidine-5`-monophospho-N-acetylneuraminic acid sodium salt (CMP)	Sigma-Aldrich
Ethidiumbromide (EtBr)	Fluka
Ethanol (EtOH)	Merck
Acetic acid, 1 %	J.T.Baker
Glycerol	Invitrogen
Glycogen (Mytilus edulis)	Sigma-Aldrich
Goldner solution 1	0.33 g Ponceau de Xylidine (Sigma-Aldrich) 0.1 g Acid Fuchsin (Sigma-Aldrich) 3 ml acetic acid ad 500 ml Aqua dest.
Goldner solution 2	20.0 g Molybdatophosphoric acid hydrate (Merck) 10.0 g Orange G (Roth GmbH & Co) ad 500 ml Aqua dest.
Goldner solution 3	1.0 g Light Green SF yellowish (Merck)
Hematoxylin	Shandon
Human blood serum with EDTA	PRINCE cohort University Medical Center Hamburg- Eppendorf, Transfusion medicine University Medical Center Hamburg-Eppendorf
Isopropanol (2-propanol)	Fluka
Iodine 0.5 M I2 (1,0 N)	Fluka
Lipofectamin®2000	LifeTechnologies
MassRuler DNA Ladder Mix, 80-10 000 bp	Fermentas / Thermo Scientific
Maxima SYBR Green/ROX qPCR Master Mix	ThermoScientific
Paraffin	DCS
Paraformaldehyd (PFA)	BioChemica

PBS (10x)	26,8 mM KCl (Carl Roth) 17,6 mM KH_2PO_4 (Merck) 1,37 M NaCl (Merck) 51,3 mM $Na_2HPO_4 \cdot 2H_2O$ (Merck) ad. 1 L aqua dest. pH 7.4
PBS-Tween (0.05 %)	0.05 % Tween-20 (Serva) in 1x PBS
RNAlater	QIAGEN
Sodium chloride (NaCl)	Merck
SuperBlock T20 (TBS)	Thermo Scientific
Solvent-free glue	UHU
TaqMan Universal PCR Master Mix	LifeTechnologie
Triton-X-100	Merck
TrueBlue™ Peroxidase Substrate	KPL
Turkey erythrocytes with Alsever	Charles River laboratories
Weigert's iron hematoxylin staining solution (equal ratio of Ferric Hematoxylin solution A and B)	Waldeck GmbH & Co.
Xylene I and II	Greyer

2.1.2 Cell culture media and additives

Name/ substance	**Composition/ producer**
Ampicillin (100 mg/ml)	Serva
Avicel (microcrystalline cellulose)	FMC BioPolymer
Avicel-*Overlay* medium	50 % *Overlay* medium 50 % Avicel solution (2,5% Avicel in 1xPBS)
Bacteria freezing medium	50 % LB-Amp-Medium 50 % Glycerol
Bovine serum albumin, 35 % in DPBS	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich
Dulbecco's PBS (1x)	Sigma-Aldrich
Fetal calf serum (FCS)	Biochrom
Growth medium MDCK cells	MEM

	10 % FCS 1 % L-glutamin 1 % penicillin and streptomycin
Growth medium HEK 293T cells	DMEM 10 % FCS 1 % L-glutamin 1 % penicillin und streptomycin
Infection medium MDCK	MEM 0.2 % BSA 1 % L-glutamin 1 % penicillin and streptomycin
LB-Amp100 agar	LB-Amp100 medium 1.5 % Bacto-Agar (BD Biosciences)
LB-Amp100 medium	10 g/L peptone 5 g/L <east extract 10 g/L NaCl pH 7.5 0.1 mg/ml ampicillin
Minimal Essential medium (MEM)	Sigma-Aldrich
Modified Eagle Medium 2x (2x MEM)	GIBCO
Overlay medium for plaque assay	2x MEM 0.4 % BSA 2 % L-glutamin 2 % penicillin and streptomycin
Penicillin and streptomycin (P/S, 100x)	Sigma-Aldrich
Reduced Serum Medium (Opti-MEM)	GIBCO
Transfection medium HEK 293T	DMEM 10 % FCS 1 % L-glutamin
Trypsin-EDTA (1x)	Sigma-Aldrich

2.1.3 Enzymes and kits

Name/ substance	**Composition/ producer**
α2,3-(N)-sialyltransferase *Pasteurelle multocida* Product Reference: S1951	Sigma-Aldrich
α2,6-(N)-sialyltransferase *Photobacterium damsel*,	Sigma-Aldrich

recombinant, expressed in E.*coli* BL21 Product Reference: S2076	
DNA-Polymerase Phusion	NEB
DNAse I, RNase free	Roche
dNTP-Mix (10 mM each)	QIAGEN
Dual-Luciferase® Reporter Assay System	Promega
Innuprep RNA Mini Kit	Analytik Jena
NucleoBond® Xtra Maxi	Macherey-Nagel
Omniscript RT Kit	QIAGEN
ProcartaPlex Mouse Cytokine & Chemokine Panel 1A	Affymetrix eBiosciences
Progesterone ELISA kit	Cayman chemicals
QIAamp Viral RNA Mini Kit	QIAGEN
QIAprep Spin Miniprep Kit	QIAGEN
QIAquick Gel Extraction Kit	QIAGEN
QIAquick PCR Purification Kit	QIAGEN
rDNAse I Kit	LifeTechnologies
Restriction Enzyme *DpnI*	Fermentas/ Thermo Scientific
Restriction Enzyme *SacI*	NEB / Fermentas
Restriction Enzyme *SmII*	Fermentas
RNeasy Plus Universal Mini Kit	QIAGEN
SuperScript III	Invitrogen
TaqMan Gene Expression Assay for *Cxcl10* (Assay ID Mm99999072_m1)	Thermofisher
TaqMan Gene Expression Assay for *Gapdh* (Assay ID Mm99999915_g1 VIC)	Thermofisher
Trypsin-TPCK	Sigma-Aldrich
TruSeq RNA Sample Preparation Kit v2	Illumina
Vibrio cholerae neuraminidase (VCN)	Sigma-Aldrich
Zytochem-Plus HRP Kit	Zytomed

2.1.4 Primer

Oligonucleotides for sequencing and genotyping of viral DNA, as well as for site-directed mutagenesis were designed using the *Clone Manager 9 Professional Edition* software and verified by an online primer design tool (http://www.genomics.agilent.com/primerDesignProgram.jsp). The oligonucleotides were ordered and synthesized by Sigma-Aldrich. Fw stands for forward, rv for reverse.

Sequencing primer

Name	**Sequence (5'→3')**
HA_601fw	ACTAGTGCTGACCAACAAAG

Site directed mutagenesis primer

Name	**Sequence (5'→3')**
HH15-HA-Q223R_a719g_fw	cccaaagtgagggatcgagaagggagaatg
HH15-HA-Q223R_a719g_rv	cattctcccttctcgatccctcactttggg
HH15_NS-R211K/D54N_g632a_fw	ggagaaactgtgatgagaatgggaaaccttcactacct
HH15_ NS-R211K/D54N_g632a_rv	aggtagtgaaggtttcccattctcatcacagtttctcc

Cloning primer

Name	**Sequence (5'→3')**
pHW-1918-PB2f	*gaagttgggggg* AGCGAAAGCAGG TC
pHW-1918-PB2r	*ccgccgggttatt* AGTAGAAACAAGG TCGTTT
pHW-1918-PB1f	*gaagttgggggg* AGCGAAAGCAGG CAAAC
pHW-1918-PB1r	*ccgccgggttatt* AGTAGAAACAAGG CATTT
pHW-1918-PAf	*gaagttgggggg* AGCGAAAGCAGG TAC
pHW-1918-PAr	*ccgccgggttatt* AGTAGAAACAAGG TACTT
pHW-1918-HAf	*gaagttgggggg* AGCAAAAGCAGG GG
pHW-1918-HAr	*ccgccgggttatt* AGTAGAAACAAGG GTG
PHW-1918-NPf	*gaagttgggggg* AGCAAAAGCAGG GTA
pHW-1918-NPr	*ccgccgggttatt* AGTAGAAACAAGG GTATTTTT
pHW-1918-NAf	*gaagttgggggg* AGCGAAAGCAGG AGT
pHW-1918-NAr	*ccgccgggttatt* AGTAGAAACAAGG AGT
pHW-1918-Mf	*gaagttgggggg* AGCAAAAGCAGG TAG
pHW-1918-Mr	*ccgccgggttatt* AGTAGAAACAAGG TAG
pHW-1918-NSf	*gaagttgggggg* AGCAAAAGCAGG GTG
pHW-1918-NSr	*ccgccgggttatt* AGTAGAAACAAGG GTG

Uni12	AGCGAAAGCAGG

2.1.5 Plasmids and vectors

Name	**Origin**
pHW2000	Empty vector for reverse genetics ([247])
pRL-TK	Luciferase-reporter construct (*Renilla reniformis*) (Promega)
pPol-I-NP-Luc-human	Luciferase-reporter construct (*Photinus Pyralis*) with the luciferase gene (GenBank: AF053462), under the control of the human polymerase I promoter and flanked by the 3´and 5´ non-coding regions of viral NP segment of A/WSN/33 (H1N1) (GenBank: M30746). (T. Wolff, Robert Koch-Institut, Berlin, Deutschland)

2.1.6 Virus stocks

Name	**Origin / description**
A/Hamburg/NY1580/09 (pH1N1) → HH15	Sigrid Baumgarte, Institut für Hygiene und Umwelt, Hamburg, Germany ([248])
A/Sachsen-Anhalt/101/09 (pH1N1)	Dr. Brunhilde Schweiger, Robert-Koch-Institute, Berlin, Germany
A/Solomon Islands/3/06-like (H1N1)	Armin Balliot, Niedersächsisches Landesgesundheitsamt, Hannover, Germany
A/Netherlands/213/03 (H3N2)	Thijs Kuiken/Debby van Riel, Erasmus Medical Center, Rotterdam, Netherlands
H5N1-$HA_{monobasic}$	7+1 reassortant virus of A/PR/8/34 (H1N1) and the HA of A/Vietnam/11/94 (H5N1) without the multibasic HA cleavage site (H5 control). Thijs Kuiken/Debby van Riel, Erasmus Medical Center, Rotterdam, Netherlands
2009 pH1N1-HA_{Q223R}	Single point mutant of recombinant 2009 pH1N1 virus
2009 pH1N1-$NS_{R211K/D54N}$	Single point mutant of recombinant 2009 pH1N1 virus
2009 pH1N1- HA_{Q223R} + $NS_{R211K/D54N}$	Multi-gene reassortant of recombinant 2009 pH1N1 virus
Sendai Virus	Institute of Virology, University of Marburg, Marburg, Germany

2.1.7 Bacteria stocks

Name	Origin
Escherichia coli XL10 gold	Stratagene Genotype: TetrΔ (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F′ proAB lacIqZΔM15 Tn10 (Tetr) Amy Camr]

2.1.8 Cell lines

Name	Description
HEK293T	Human Embryonic Kidney Cells
MDCK	Madin-Darby Canine Kidney Cells
Vero	Adult African green monkey Kidney Cells

2.1.9 Antibodies

Name	Origin	Producer	Application
Anti-NP	Mouse monoclonal	Abcam (ab43821)	1:1000 plaque assay
Anti-Mouse IgG-HRP	Rabbit polyclonal	SouthernBiotech (6170-05)	1:2000 plaque assay
Anti-NPV	Rabbit polyclonal serum raised against A/FPV/Rostock/34 (H7N1)	Kindly provided by H.-D. Klenk, Marburg, Germany	1:2000 immunohistochemistry
Anti-Rabbit-Biotin	Donkey polyclonal	Jackson ImmunoResearch (# 711-066-152)	1:200 immunohistochemistry

2.1.10 Anesthetics, analgetics and additives

Name	Producer
Forene (isofluran 100 %)	Abbvie
Ketamine (100 mg/ml)	WDT
Sodium chloride (0.9 %)	B. Braun Melsungen AG
Sedaxylan (xylazin-hydrochloride, 20 mg/ml)	WDT

2.1.11 Consumables

Consumables were purchased if not indicated otherwise from the following companies: Falcon, Sarstedt, Biozym, Nunc.

Name	**Producer**
Amicon® Ultra-4, PLHK Ultracel-PL Membrane, 100 kDa (UFC8100024)	Millipore
BRAND® disposable Delbrück BLAUBRAND® micropipettes (Na-heparinised) (20µl)	BRAND
Grinding balls (ceramic beads: Ø 0,50-0,75 mm)	Retsch
Grinding balls (glass beads: Ø 0,50-0,75 mm)	Retsch
Lancets, sterile, ACCU-CHEK Softclix XL (21G 0,8 mm)	Roche
Microlance™ 3 Needles (26G 3/8“, 0,45 x 10 mm)	BD
Microlance™ 3 Needles (25G 1“, 0,5 x 25 mm)	BD
Microtiter plates for Tristar LB 941 Mikrowin2000-*Software* v.4.41	Berthold Technologies, Software from Mikrotek Laborsysteme GmbH
Needles (20G 1“, 0,9 x 25 mm)	BD
Omnifix® Syringes (3 ml / Luer Lock Solo)	B. Braun Melsungen AG
Omnifix® Syringes (10 ml / Luer Solo)	B. Braun Melsungen AG
TERUMO® U-100 Insulin Syringes (1 ml / 6 % Luer)	TERUMO Corporation
96-Well Polystyrene Conical Bottom MicroWell™ Plates	Nunc

2.1.12 Equipment

Name	**Producer**
Centrifuge Multifuge 3S-R	Heraeus
Centrifuge Varifuge 3.0R	Heraeus
Centrifuge 5424R	Eppendorf
Centrifuge 5424	Eppendorf

Centrifuge 5427R	Eppendorf
CFX96 Real-Time System	Biorad
CO_2 incubator BBD6220	Thermo Scientific
CO_{2i} incubator HERACELL 150	Thermo Scientific
Digital photo camera SZ-10	Olympus
Drying cabinet 6120	Heraeus
Gel documentation system Gel Doc XR	Bio-Rad
GeneAmp® PCR System 9700 Thermocycler	Applied Biosystems
HiSeq 2500 instrument	Illumina
Infinite 200 PRO NanoQuant	TECAN
Isoflurane inhalation chamber	UNO
Light microscope IM	Zeiss
Luminex 200	BioRad
Magnetic stirrer MR3001	Heidolph
Magnetic stirrer MR80	Heidolph
Microtome HM325	Microm
Microtome SM2010R	Leica
Micro oven R-647	Sharp
Mixer Mill MM400	Retsch
Panoramic viewer software	3DHISTECH Ltd.
Precellys 24 tissue homogenizer	PeQlab
pH meter 766 Calimatic	Knick
Pipettes Eppendorf Reference	Eppendorf
Pipetboy Pipetus	Hirschmann Laborgeräte
Precision balance Extend	Sartorius
Precision scale ED224S	Sartorius
Powerpac Basic	Bio-Rad
Safire 2 plate reader	Tecan
Saftey cabinet Herasafe KS 12	Thermo Scientific
Safety cabinet Herasafe KS 18	Thermo Scientific

Slide Scanner Miramax Midi	Zeiss
StepOnePlus Real-Time PCR system	LifeTechnologie
Spectrophotometer Nanodrop 1000	Peqlab
Shaker Digital MaxQ 6000	Thermo Scientific
Shaking water bath SW-22	Julabo
Surgical scissors	Fine Science Tools
Surgical forceps	Fine Science Tools
Tumbling table WT12	Biometra
Vortex mixer 7-2020	neoLab
Water purification system Milli Q	Millipore

2.1.13 Animals

Embryonated chicken eggs:

For virus propagation fresh, specific pathogen free (SPF) eggs were purchased from Lohmann and incubated in an egg incubator for 10 days at the *Forschungstierhaltung* of the University Medical Center Hamburg-Eppendorf.

Mice:

Animals were kept under constant 12 h light/ dark circles and constant temperature; in ventilated type II L IVC cages (Tecniplast) and received food and water ad libitum. After one week of acclimatisation, eight to ten weeks old female C57BL/6JRccHsd mice (Harlan, Netherlands) or C57BL/6J (Charles River, Germany) were mated with male BALB/c or C57BL/6J mice (Charles River, Germany).

2.2 Methods

All working steps containing infectious material were conducted in class II biosafety cabinets in Bio safety Level 2 or 3 facilities according to the regulations and risk assessment of the *Gentechnikbehörde* of the City of Hamburg.

2.2.1 Cell culture

MDCK or HEK293T cells were kept in a CO_2 incubator at 37 °C, 5 % CO_2 and 95 % relative humidity and split according to the application needed or once they reached 90 – 100 % confluence. For MDCK cells, 10 % FCS, 1 % penicillin and streptomycin and 1 % L-glutamine was added to the MEM cell culture medium, the same applied for the HEK293T cells, with the difference that DMEM was used as cell culture medium. When passaging the cells, they were washed with 1x PBS and incubated with Trypsin-EDTA at 37 °C until the cell layer dispersed. By adding the appropriate cell culture growth medium containing FCS, the Trypsin-EDTA was inactivated and the cells were subcultivated as needed.

2.2.2 Virus growth

Virus stocks used for *in vitro and in vivo* animal experiments were passaged at most two times in order to minimize the occurrence of random mutations appearing due to the missing proofreading capacity of the viral polymerase [52].

All virus stocks were grown in MDCK cells and 1µg/ml TPCK-Trypsin was added as all virus stocks have a monobasic cleavage site. For successful virus propagation MDCK cells were seeded one day previous in a 75 cm^2 cell culture flask. If the cells were 80-90 % confluent, they were washed with PBS and infected with 1.5 ml virus dilution. After 30 min of incubation at 37 °C, the virus inoculum was removed and 6 ml infection medium were added to the flask. After infection, cells were incubated and regularly checked for cytopathic effects (CPE) greater than 50 % (approximately after 24-36 h) or positive hemagglutination with chicken erythrocytes in a HA assay. Cell culture supernatant was then harvested and centrifuged at 1000 g for 5 minutes to remove cells and cell debris. The supernatant was aliquoted in 100 µl aliquots and stored at – 80 °C.

2.2.2.1 Sendai virus growth in embryonated chicken eggs

Sendai virus was grown in for 10 days embryonated and incubated SPF chicken eggs. First, the eggs were checked with a lamp for presence of a viable embryo. Then, eggs were disinfected with iodine and a small hole was drilled into the egg with a hand drill. Next, 200 µl of virus dilution (10^{-3} or 10^{-4}) were injected into the allantois cavity with a syringe (0.5 x 25 mm). The opening was then closed with solvent-free glue. The eggs were incubated at 37 °C for 48 or 72 h and then stored at 4° C over night in order to induce vasoconstriction. The next day, the allantoic fluid was harvested with a single-use

Pasteur pipette and an HA assay was performed as described in the following (2.2.3.). Egg batches with similar HA titers were pooled and the titer was determined by Tissue Culture Infective Dose 50 ($TCID_{50}$) on Vero cells as previously described [249].

2.2.3 Hemagglutination assay

The hemagglutination assay (HA assay) is based on the ability of the viral HA protein to bind red blood cells. The binding of virus to the erythrocytes is mediated by α2,3- and/or α2,6-linked sialic acid on the cells surface as receptor. If no virus is present in the 96-well plate, the erythrocytes sediment to the ground of the plate. The HA assay was performed in 96-well V-bottom shaped (conical) plates. 100 µl sample (i.e. cell culture supernatant from virus growth/ allantoic fluid) was pipetted into the first well of the 96-well plate, followed by a two-fold serial dilution in 1x PBS. Next, 50 µl of 1 % chicken erythrocytes suspension (chicken erythrocytes were diluted in 0.9 % NaCl) were added to each well and the suspension was incubated for 30 minutes at 4 °C. Following the incubation time, agglutination patterns were observed – agglutination causes the red blood cells to not settle at the bottom of the well, giving it a cloudy appearance. If no agglutination occurred, the red blood cells settle at the bottom of the well as a "button" or circle of cells [250].

2.2.4 Virus titer determination by plaque assay

In order to determine the titer of infectious virus particles in a virus suspension, a plaque assay was performed (modified from [251]). MDCK cells were seeded into a 6-well plate and were kept in culture overnight. If the cells had a confluence of 70-80 %, they were washed with PBS and the cells were inoculated with 333 µl of the relevant virus dilution. In case of virus titer determination, a ten-fold dilution in PBS of the unknown sample or virus stock was performed for inoculation. Following inoculation the cells were kept for 30 minutes at 37 °C in the incubator and occasionally carefully tilted to avoid drying-out of the cells. Next, 3 ml of avicel-overlay medium including 1 µg/ml TPCK-Trypsin was added and incubated for 72 h at 37 °C. After 72 h the avicel-overlay medium was removed, the cells were washed with PBS and fixed for at least 30 minutes with 4 % PFA at 4 °C.
The viral plaques were made visible via immunostaining of the viral NP protein. Cells were washed with PBS and permabilized for 30 minutes with 0.3 % Triton-X on a tumbling table. Cells were then incubated with 500 µl per well of the primary antibody, an anti-NP antibody (Abcam), for one hour at room temperature. Plates were then washed three times with PBS-Tween (0.05 %) and 500 µl of the secondary antibody, an anti-mouse IgG-HRP conjugated antibody (SouthernBiotech), were added for another hour. The primary antibody was diluted 1:1000 in superblock, the secondary 1:2000. Following this incubation step, the plates were washed three times with PBS-Tween. The detection of the viral plaques was carried out by applying True-Blue (KPL) which serves as a substrate for the HRP conjugate of the secondary antibody.
With this method viral plaques could be detected in the cell layer. The titer was then calculated by determining the *plaque forming units* per milliliter (p.f.u./ml) in each dilution.

2.2.5 Hemagglutinin inhibition assay

As described under 2.2.3. the HA of influenza viruses binds to the sialic acids found on erythrocytes and hereby causes hemagglutination. If antibodies against influenza are present in the serum of a human or animal, these antibodies can prevent the hemagglutination of the viral HA to the erythrocytes as they bind to the virus instead. In order to determine the level of antibodies a two-fold serial dilution was performed. First of all, serum samples were heat-inactivated to remove residual complement activity (56°C, 30 min). In the first well of the V-bottom shaped 96-well plate, the 1:10 diluted serum was pipetted and subsequently diluted in a two-fold series in PBS. Then, 50 µl of the virus solution (the virus solution was diluted in order to obtain a hemagglutination unit of 4; virus used: A/Sachsen-Anhalt/101/09 [pH1N1]) was added to each well and the 96-well plate was incubated for 30 minutes at room temperature. Last but not least, 1 % chicken erythrocytes were added for 30 minutes at 4 °C and antibody titers were determined. Hemagglutination was observed in all wells where the serum contained no antibodies. Hemagglutination inhibition was observed upon antibody presence. Under these circumstances the erythrocytes are not agglutinated by the virus and sediment to the ground forming dots/buttons [252].

2.2.6 Animal experiments

All animal experiments were conducted according to the guidelines of the animal protection law and the approved protocols by the relevant German authority *(Behörde für Gesundheit und Verbraucherschutz* Hamburg, approval number G124/12).

2.2.6.1 Mating of mice

After a one week of acclimatisation, eight to ten weeks old female C57BL/6JRccHsd mice (Harlan, Netherlands) or C57BL/6J (Charles River) were mated with male BALB/c or C57BL/6J mice (Charles River, Germany).The presence of a vaginal plug in the morning was considered as gestational day (gd) 0.5. Maternal weight was controlled on gd 8.5 and 10.5 in order to confirm pregnancy. Non-pregnant, age-matched mice served as control.

2.2.6.2 Narcosis and euthanasia

Prior to infection (2.2.6.3) mice were sedated with isoflurane, which was administered via placement of mice in an inhalation chamber followed by intraperitoneal anesthesia with a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine ad 200 µl 0.9 % NaCl. Before drawing blood or euthanizing the mice, a short inhalative isoflurane narcosis was administered. If the mice were euthanized, cervical dislocation was performed additionally to the isoflurane narcosis.

2.2.6.3 Infection and survival

Following narcosis, mice were infected with the respective virus concentration - 10^1-10^5 p.f.u. of the 2009 pH1N1 virus strain A/Hamburg/NY1580/09, the 2006 sH1N1 virus strain A/*Solomon* Islands/3/2006-like or the recombinant mutant pH1N1 viruses (pH1N1-HA_{Q223R}, pH1N1-$NS_{R211K/D54N}$ or pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$) - diluted in 50 µl PBS or mock infected with PBS only by pipetting the solution slowly intranasally in both nostrils. Mice were then monitored daily for weight loss and signs of disease until 14 days post infection (p.i.) and euthanized upon >25% weight loss, according to the guidelines of the animal protection law and the approved protocols by the relevant German authority (*Behörde für Gesundheit und Verbraucherschutz Hamburg,* approval number G124/12).
The lethality of the virus stocks was determined by calculating the mouse lethal dose 50 (MLD_{50}) – the virus dose where 50 % of the infected mice succumb to the infection – as established by Reed and Munch [253].

2.2.6.4 Blood and organ harvesting

As mentioned in 2.2.6.2., mice were anesthetized with isoflurane before blood was drawn or mice were euthanized for organ harvesting. Blood was drawn either in the retroorbital plexus with micropipettes (Na-heparinized) or in the *Vena facialis* with the help of lancets. In either way, the blood was collected in an EDTA-coated tube. Whole blood samples were centrifuged and the serum was either used directly for downstream applications or frozen at -80 °C.
For organ harvesting, the thoracic and abdominal cavities were opened and lung, lymph nodes and gut were removed and placed in RNAlater, PBS or 4 % PFA. The organs stored in RNAlater or 4 % PFA were stored for 24 h at 4 °C, the organs stored in PBS were homogenized for later plaque titration.

2.2.6.5 Homogenization of organs

In order to determine the viral titer in an organ, the harvested organ was weighed and then stored in a tube containing 1 ml 1x PBS and glass or ceramic beads. The organ was then homogenized in a mixer mill at 20 Hz for 10 minutes at 4 °C. Following the mixer mill, the tubes were centrifuges for 6000 xg for 5 minutes at 4 °C. The supernatant was then directly used for viral titer determination by plaque assay or stored at -80 °C.

2.2.7 High throughput sequencing

High throughput sequencing was performed in collaboration with the Next Generation Sequencing technology platform headed by Prof. Dr. Adam Grundhoff at the Heinrich Pette Institute.

RNA extraction: RNA was isolated from lungs of non-pregnant and pregnant mice infected with 10^3 p.f.u. of the pH1N1 virus at 3 and 6 d p.i. using the innuprep RNA Mini Kit (Analytik Jena, Germany) according to the manufacturer's protocol.
RNA-seq library construction, sequencing and quality control: Three to four replicates per group were pooled and 1µg of total RNA was used for generating libraries with the Illumina TruSeq RNA Sample Preparation Kit v2 as recommended by the manufacturer. Size and quality of the libraries were assessed using a BioAnalyzer High Sensitivity Chip. Diluted libraries (2nM) and were multiplex-sequenced on the Illumina HiSeq 2500 instrument (2x 100 bp paired end run) with 40-50 million reads per sample.
Expression quantification of ISGs: Reads were aligned to the murine reference transcriptome (UCSC mm10) using Bowtie2 (v2.2.2) [254]. DESeq(Anders and Huber, 2010) was employed to assess differential expression. Full names of genes in alphabetical order: *Cxcl10*: C-X-C motif ligand 10; *Ddx58*: DEAD (Asp-Glu-Ala-Asp) box polypeptide 58; *Eif2ak2*: eukaryotic translation initiation factor 2-alpha kinase 2; *Ifih1*: interferon induced with helicase C domain 1; *Ifit3*:interferon-induced protein with tetratricopeptide repeats 3; *Isg15*: ISG15 ubiquitin-like modifier; *Mx1*: myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse); *Oas1a*: 2'-5' oligoadenylate synthetase 1A; *Oas1b*: 2'-5' oligoadenylate synthetase 1B; *Oas1c*: 2'-5' oligoadenylate synthetase 1C; *Oas1g*: 2'-5' oligoadenylate synthetase 1G; *Socs1*: suppressor of cytokine signaling 1; *Stat1*: signal transducer and activator of transcription 1; *Usp18*: ubiquitin specific peptidase 18.
Variant calling to identify mutations of pH1N1 virus: Sequences of viral RNA in the lung homogenates were compared to the parental pH1N1 strain. Alignment to the reference sequences (accession no.: GU480807.1 (PB2), HQ104924.1 (PB1), HQ104925.1 (PA), HQ104926.1 (HA), HM598305.1 (NP), HQ104927.1 (NA), HQ104928.1 (M), HQ104929.1 (NS)) was performed with the Burrows Wheeler Aligner (v0.7.5a) [255]. The program was parameterized to trim reads from the 3'-end using a quality threshold of 15. Putative PCR duplicates were removed using SAMtools (v0.1.19) [256]. Consecutive calling of variants was conducted with SAMtools and VarScan (v2.3.6) [257]. Bases with Phred quality scores below 30 were not considered for calling variants.
Data Availability: Sequence data for all samples have been submitted to the European Nucleotide Archive (ENA) and are publicly available at http://www.ebi.ac.uk/ena/data/view/PRJEB12200.

2.2.8 Cytokine detection

Cytokine detection in the serum of mice was performed in collaboration with the group of Prof. Dr. Marcus Altfeld at the Heinrich Pette Institute.

Cytokines were determined in supernatants of homogenized lungs and/or collected sera from infected pregnant and non-pregnant mice as well as uninfected control groups at 3 d p.i.. Serum cytokines were captured using the ProcartaPlex Mouse Cytokine & Chemokine Panel 1A (Affymetrix eBiosciences) multiplex immunoassay with magnetic beads following the manufacturer’s instructions. Serum samples were run in duplicate. Measurement of cytokine levels was performed using the BioRad Luminex 200 machine.

2.2.9 Quantification of *Cxcl10* expression in lung by Real-Time-quantitative Polymerase Chain Reaction (RT-qPCR)

RNA isolation from lungs of non-pregnant and pregnant mice infected with 10^3 p.f.u. of pandemic H1N1 at 3 and 4 d p.i. was performed using the RNeasy Plus Universal Mini Kit (QIAGEN) or the innuprep RNA Minikit (analytik Jena), following the manufacturer's protocol. Prior to RNA isolation, lung tissue preserved in RNAlater (QIAGEN) was homogenized using micro packaging vials with ceramic beads (1.4 mm) in the Precellys 24 tissue homogenizer (PeQlab). Following RNA isolation, DNAse digestion was performed in order to minimize the amount of DNA in the sample using the rDNAse I Kit (Lifetechnologies). In a next step, total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and cDNA concentrations were quantified by using the Infinite 200 PRO NanoQuant (TECAN). The gene expression analysis of *Cxcl10* was performed using the StepOnePlus Real-Time PCR System and corresponding software (LifeTechnologie, Germany), using 100ng cDNA. The following TaqMan Gene Expression Assays were purchased: *Cxcl10* (Assay ID Mm99999072_m1) and Gapdh (Assay ID Mm99999915_g1 VIC). The amplifications were performed using 2×TaqMan Universal PCR Master Mix (LifeTechnologie, Germany). The qRT-PCR was conducted with cDNA as template in a final volume of 20 µl. Cycling conditions using a standard two-step qRT-PCR were the following: initial 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 15 s denaturation at 95 °C and 60 s annealing and extension at 60 °C. The gene expression analysis of *Cxcl9* and *Cxcl11* were conducted by the CFX96 Real-Time System (Biorad, Germany) and corresponding software. The following primer pairs were purchased: *Cxcl9* (5'-CCCAAGCCCCAATTGCA-3' and 5'-GCAGGTTTGATCTCCGTTC-3') and *Cxcl11* (5'-GAGAAAGCTTCTGTAATTTACCCGAGTA-3 and 5'-GTCCAGGCACCTTTGTCGT-TTA-3'), *Gapdh* (5'-GGA-TGCAGGGATGATGTTCT-3' and 5'-AACTTTGGCATT-GTGGAAGG-3'). The amplifications were performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific by LifeTechnologies GmbH, Germany), using 5ng cDNA. The qRT-PCR was conducted with cDNA as template in 12 µl reactions. Reactions were performed in 40 cycles using the following protocol: 95°C for 10 min, 15 s denaturation at 95°C and 60 s primer annealing at 60°C and elongation at 72°C. All experiments were performed in triplicates. The amplifications were normalized to the expression of *Gapdh*. Note, for *Cxcl11* at the cDNA concentration used, a signal was only obtained at cylce 38. Relative transcript levels were calculated applying the equation described in [258]. The relative mRNA expression of the target gene from non-pregnant animals at day 3 p.i. was set 1.

2.2.10 Progesterone detection

The basis of this enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical) is the competition between progesterone and progesterone-acetylcholinesterase (AChE) conjugate for a limited number of progesterone-specific rabbit antiserum binding sites. Progesterone levels were detected in serum of non-pregnant and pregnant mice infected with 10^3 p.f.u. of pH1N1 on days 3 and 6 p.i. using the ELISA kit according to the manufacturer's instructions. The plate was read in a Safire 2

plate reader (Tecan) between λ=405 and λ=420 nm. Progesterone concentration was calculated based on a standard curve run simultaneously.

2.2.11 Plasmid generation

Plasmids needed for generation of recombinant pH1N1 viruses were generated as described in "*Charakterisierung der Pathogenität und Transmissibilität von 2009 pandemischen H1N1 Influenza A Viren in Kleintiermodellen*", Dissertation 2013 by Anna Otte and kindly provided by Dr. Otte.

2.2.11.1 Site-directed mutagenesis

The technique of site directed mutagenesis is used for the introduction of a specific mutation into plasmid DNA. The mutation can result in a substitution, insertion or deletion of nucleotides. The desired mutation was inserted by PCR using two complementary primers. The primers were designed (2.1.4.) in order to carry the desired mutations. Two mutations were included in the pHW2000 empty vector: in the HA of 2009 pH1N1, alanine was replaced by glycine at position 719 and in NS of HH15, glycine was replaced by alanine at position 632. Amplification occurred via the DNA-polymerase *Phusion I* which possesses proof-reading function. The parental strand of the plasmid, which does not carry the inserted mutations, is methylated as it originates from *E.coli* bacteria in which CAN always exists in a methylated or hemi-methylated form. Methylated and hemi-methylated plasmids can therefore be digested by the restriction endonuclease *DpnI*. Hereby, the parental plasmids are digested and only the newly generated strains of the plasmid holding the desired mutations is introduced into bacteria. The mutation containing plasmids, carrying an ampicillin resistance, were then transformed into competent *E.coli XL10 gold*.

PCR program: Initial denaturation: 98 °C, 5 min
20x three step cycles:
Denaturation: 98 °C, 30s
Primer hybridization: 55 °C, 1 min
Elongation: 72 °C, 10 min

2.2.11.2 Plasmid DNA amplification

The amplification of the plasmids containing the desired mutations was performed in competent *XL10 E.coli* bacteria. Here, with the desired plasmid transformed bacteria were spread on LB Amp100 agar plates and incubated at 37°C overnight. Successfully transformed and now ampicillin-resistant bacteria formed colonies. The next morning, clones were picked and grown in 3 ml LB media with ampicillin for 18 to 24 h at 37 °C in a shaker at 220 rpm (Thermo Scientific).

2.2.11.3 Plasmid DNA preparation

After cultivating of bacteria carrying the desired plasmids, the plasmid DNA was isolated using either the *QIAprep Spin Miniprep Kit* (QIAGEN) – for isolation of a small amount of plasmid DNA– or the NucleoBond® Xtra Maxi (Macherey-Nagel) for larger amounts of plasmids. The latter was used after sequencing of the plasmids in order to confirm introduction of the desired mutations. Sequencing was performed by the service provider Seqlab, Göttingen.
Cryostocks containing 1 ml of the desired plasmids in LB Amp100 and 1 ml of glycerol were generated for preservation of the plasmids and frozen at -80 °C.

2.2.12 Generation of recombinant mutant pH1N1 viruses

In order to generate recombinant mutant 2009 pH1N1 viruses by reverse genetics, the pHW2000 based 8-plasmid system was used as described by Hoffmann *et al.* [259].
First, HEK293T cells were transfected with the eight plasmids encoding for the different gene segments of pH1N1 (of which the pHW2000-HA and pHW2000-NS included the mutations HA_{Q223R} or $NS_{R211K/D54N}$, respectively). In thses pHW2000 constructs, the viral gene segment is inserted between the viral RNA polymerase I (pol I) promoter, a RNA polymerase II (pol II) promoter and a polyadenylation site. The orientation of the two transcription units allows the synthesis of positive-sense cRNA mediated by the vRDRP and positive sense viral mRNA generated by the cellular polymerase [259].
For transfection, the eight plasmids encoding for the different viral gene segments were pipetted into 250 µl Opti-MEM. Here, 1 µg of the pH1N1 pHW2000 constructs and 2 µg of pHW2000-$HA_{Q223R,}$ pHW2000-$NS_{R211K/D54N}$ or both pHW2000-HA_{Q223R} + $NS_{R211K/D54N}$ were used in three independent approaches. For each transfection, 250 µl Opti-MEM were incubated for 5 min with the transfection reagent Lipofectamin®2000 in a plasmid [µg]: Lipofectamin®2000 [µl] ratio 1:2 ratio, then added to the plasmid mix and incubated at room temperature for 20 min in order to allow the formation of plasmid DNA-liposome complexes. For transfection, $3*10^6$ HEK293T cells were seeded in 3 ml DMEM transfection medium in a 6 cm tissue culture dish. Then, the plasmid mix including Lipofectamin and Opti-MEM was added and the transfected cells incubated for 48 h at 37 °C in a CO_2 incubator. During this incubation time, recombinant viral particles were formed. 500 µl of the resultant supernatant was then pipetted as inoculum on confluent and adherent MDCK cells seeded in 35 mm dishes, 1 µg/ml TPCK-Trypsin was added and the cells were incubated for 72 h. 500 µl of this MDCK supernatant was used to inoculate MDCK cells seeded in a T25 (25 cm^2) flask. In parallel, hemagglutination assays were performed at all stages after transfection and infection in order to confirm virus growth. The generated mutant recombinant viruses were sequenced in order to confirm inclusion of the desired mutations.

2.2.13 Interferon-β promoter assay using the *Dual Luciferase Reporter Assay System*

As the NS1 protein is known to act as an interferon antagonist upon influenza virus infection, it was aimed to determine if the mutation appearing in the NS1 protein during pregnancy affects the interferon antagonistic function of this mutant virus. To address this question, the interferon-β apromotor ctivity in HEK293T cells infected with wildtype 2009 pH1N1 or the recombinant mutant pH1N1-$NS_{R211K/D54N}$ virus was assessed by the *Dual Luciferase Reporter Assay System*. As a negative control, vRNPs were transfected omitting the PB2 subunit. Sendai virus (SeV) served as a positive control as SeV is also a nonsegmented, negative-stranded RNA virus belonging to the Paramyxovirus family [260] which has been shown to trigger a strong interferon-β response upon infection [261].
HEK293T cells were co-transfected with a reporter construct encoding for the firefly luciferase under the control of the interferon-β promotor and the pRL-TK construct as transfection control. In the pRL-TK construct the Renilla luciferase gene is under the control of the constiutively expressed HSV-thymidin kinase promoter. For each transfection, 1 µg of the reporter construct encoding for the firefly luciferase and 1 µg pRL-TK transfection control were incubated with 250 µl Opti-MEM and incubated for 5 min at room temperature. Then, 250 µl Opti-MEM and 2 µl Lipofectamin®2000 per transfection were mixed, added to the plasmid mix and incubated for 20 min at room temperature. For transfection, 10^6 HEK293T cells were seeded in 2.5 ml DMEM transfection medium per 6-well-plate well and the DNA-Opti-MEM mix was slowly added to the cell suspension. Infection of the transfected cells was performed 24 h post transfection with the wildtype 2009 pH1N1, the pH1N1-$NS_{R211K/D54N}$ virus or SeV at different multiplicities of infection (MOI). Lysis of cells was performed at either 6 h or 16 h post infection and luciferase activity was determined using the *Dual-Luciferase Reporter Assay System* (Promega) in the Tristar LB 941 Luminometer (Berthold Technologies). *Firefly* luciferase data was set in relation to *Renilla* luciferase activity and the resulting relative luciferase activity was set 100 %.

2.2.14 HA resialylation assay

The HA resialylation assay described here was established and mainly performed by Carola Dreier at the Heinrich Pette Institute.

The HAs of different influenza viruses bind preferentially to distinct forms of sialic acids. Viruses isolated from avian origin prefer binding to α2,3-linked sialic acids (SA), whereas human viruses prefer α2,6-linked SA. Mutations in the HA protein can potentially alter the binding preference of the virus to SA. Usually, native turkey red blood cells (TRBCs), which are used for this assay, present with α2,3- , α2,6- and α2,8- linked SA and one cannot distinguish the binding preference of the virus to one specific SA. In order to address the question if the mutation in the HA at position 223 (H1 numbering) alters the binding preference, all α2,3- , α2,6- and α2,8- linked SA were removed from the surface of the TRBCs by *Vibrio cholerae* neuraminidase (VCNA) treatment. Then, either α2,3- or α2,6-linked SA were used for resialylation by either an α2,3- or α2,6-(N)-sialyltransferase and by adding CMP. Resialylation was confirmed by hemagglutination (HA assay) of control viruses for which the receptor specificity is known (A/Netherlands/213/03 (H3N2) and H5N1-$HA_{monobasic}$). Finally, viruses of interest,

including the control viruses, were concentrated via Amicon® Ultra Centrifugal Filters (Millipore) according to the manufacturer´s protocol and the actual HA assay was performed with virus dilutions of 2^6 HAU.

2.2.15 PRINCE cohort study

The *Prenatal Identification of Children's Health* (PRINCE) cohort is a prospective study established at the University Medical Center in Hamburg-Eppendorf, Germany since 2011. Women that were included in the cohort had to be 18 years or older and in their 13–15 weeks of gestation. Women with infections (HIV, hepatitis B/C), known drug or alcohol abuse, multiple pregnancies or pregnancies conceived after assisted reproductive technologies (ART) were excluded. Three study visits were scheduled between 13 and 15, 23–25 and 35–37 weeks of gestation. During each visit, data on pregnancy progression, maternal anthropometry and health status and performed fetal ultrasound measurements were documented as described [262].

2.2.16 Histology

Preparation of histological slides

After overnight fixation in 4 % PFA, the harvested, formalin-fixed organs were prepared for histological thin sectioning by generation of paraffin-embedded samples. First, the specimen was dehydrated by immersing it in increasing concentrations of ethanol in order to remove water and formalin (see below). Next, the specimen was cleared from alcohol by xylene which allows infiltration of paraffin and results in embedding in a paraffin block. The paraffin-embedded tissue blocks were then cooled down to -12 °C for 30 minutes before they were 4 µm thick thin sections were generated using the HM325 microtome. The slides were mounted on glass slides and then dried overnight at 37 °C.

Dehydration and infiltration steps:

1. Ethanol 70 %, 1 h
2. Ethanol 80 %, 1 h
3. Ethanol 90 %, 1 h
4. Ethanol 95 %, 1 h
5. Ethanol 100 %, 1 h
6. Ethanol 100 %, 1.5 h
7. Xylene I, 1 h
8. Xylene II, 1 h
9. Paraffin type 3, 58 °C, 1 h
10. Paraffin type 3, 58° C, 1 h
11. Paraffin type 3, 58 °C, 1 h

Before staining, slides must be deparaffinized as remaining paraffin can lead to poor staining.

Deparaffinzation and rehydration steps:

1. Xylene, 3 x 5 min
2. Ethanol 100 %, 5 min
3. Ethanol 70 %, 5 min

4. Ethanol 40 %, 1 min
5. Storage of slides in water

2.2.16.1 Hematoxylin and Eosin staining

This staining is mainly used for demonstrating the nucleus and cytoplasm of the specimen. Hematoxylin stains the basophilic nuclei blue/violet and Eosin stains the eosinophilic cytoplasm reddish.

Procedure:
1. Hematoxylin solution, 4 min
2. ddH_2O, 10 s
3. tap water washing, 4 min
4. Eosin G-solution 1 %, 30 s
5. 3x ddH_2O, 15 s
6. 2x ethanol 70 %, 15 s
7. 2x ethanol 90 %, 15 s
8. 2x ethanol 100 %, 15 s
9. 3x xylene, 5 min

2.2.16.2 Immununohistochemistry

NP staining:

For the immunohistochemical staining, a polyclonal rabbit anti-FPV (H7N1) serum was used. This primary antibody serum is then detected by a secondary anti-rabbit biotin-conjugated antibody. The deparaffinized tissue sections were pre-treated with 0.1 M citrate buffer (pH 6.0). The primary rabbit serum was detected by a biotin-conjugated anti-rabbit antibody (Jackson ImmunoResearch, USA) followed by the application of the Zytochem-Plus HPR kit (Zytomed, Germany) and the DAB-substrate (Dako, Germany). Tissues were counterstained with hematoxylin for histopathological analysis.

NP staining was performed following the standard protocol:

1. Inhibition of endogenous peroxidase: 3 % H_2O_2 in methanol, 15 min
2. 2x washing with PBS, 5 min
3. AVIDIN-BIOTIN block, 15 min
4. 3x washing in PBS, 5 min
5. Protein block, 5 min
6. 2x washing with PBS, 5 min
7. Pre-incubation with 10% donkey serum, 30 min
8. Incubation with primary antibody, 1h 15 min
9. 3x washing in 0.5 % PBS-Tween, 5min
10. 3x washing in PBS, 5 min
11. Incubation with secondary antibody, 25 min

12. 3x washing in 0.5 % PBS-Tween, 5min
13. 3x washing in PBS, 5 min
14. Application of Zytochem-Plus HPR kit, 15 min
15. 2x washing with PBS, 5 min
16. Detection with DAB-substrate
17. Termination of reaction with ddH_2O
18. Counterstaining with hematoxylin, 8 s

Masson-Goldner trichrome staining:

Placental tissue was dissected into histological sections at the midsagittal plane.

Masson-Goldner trichrome staining was performed following standard protocols:

1. Weigert's iron hematoxylin staining solution (equal ratio of Ferric Hematoxylin solution A and B), 8 min
2. Washing of slides with ddH_2O
3. Rinsing of slides with tap water, 10 min
4. Washing of slides with ddH_2O
5. Incubation with Goldner solution 1 (0.33 g Ponceau de Xylidine , 0.1 g Acid Fuchsin and 3 ml acetic acid ad 500 ml ddH_2O), 8 min
6. 2x washing in 1 % ethanoic acid, 30 s
7. Incubation with Goldner solution 2 (20.0 g Molybdatophosphoric acid hydrate and 10.0 g Orange G ad 500 ml Aqua dest.), 9 min (until destaining of the connective tissue)
8. 3x washing in 1 % ethanoic acid, 30 s
9. Incubation with Goldner solution 3 (1.0 g Light Green SF yellowish and 1 ml acetic acid ad 500 ml Aqua dest.), 3 min
10. Washing with 1 % ethanoic acid for 30 sec
11. Ethanol 70 %, 2-3 min
12. Ethanol 96 %, 2-3 min
13. Ethanol 100 %, 2-3 min
14. 2x XEM, 5 min
15. Mounting of slides in media "Eukitt"

Following the histological staining, all slides were scanned by a slide scanner (Mirax Midi, Zeiss) and Pannoramic Viewer software (3DHISTECH Ltd., Hungary, Version 1.15.2) was used to generate the pictographs.

2.2.16.3 Quantification of histology slides

Quantification of inflamed area in HE-stained lungs of non-pregnant infected (*n*=4) and pregnant infected (*n*=5) mice on day 3 p.i. with 10^3 p.f.u. 2009 pH1N1 was performed as follows: the outer margin of the total lung tissue visible on the slide was traced and the total area was calculated by

Pannoramic Viewer, Version 1.15.2.. In a next step, the inflamed area, which was defined as the area of increased lymphocyte infiltration, protein and erythrocyte accumulation was traced and the area measured was set into relation to the total area of the section. Thereafter, the occurrence of viral antigen positive epithelial cells in immunohistochemical stained lung sections of non-pregnant (*n*=3) and pregnant (*n*=5) mice on day 3 p.i. was assessed. Again, the outer margin of the total lung tissue visible on the slide was traced and the total area was calculated by Pannoramic Viewer. Then, the amount of viral antigen positive epithelial cells was assessed (in percentage of total epithelial cells visible in one specific cross section cut) in the bronchi/bronchioli visible in complete cross section cut and the mean was calculated for each slide. In average, 28 bronchi/bronchioli in a mean total area of 60132442.6 μm^2 were screened in non-pregnant infected mice, 26 bronchi/bronchiole in a mean total area of 51238191.8 μm^2 in pregnant infected mice.

2.2.17 Statistics

For the statistical analyses of survival rates, the Gehan-Brelow-Wilcoxon test was used. For the experimental data, mean ± SEM and *p*-values were calculated. Statistical significance between groups was tested using Student's *t*-test (unpaired, two-tailed) for normally distributed data and Mann-Whitney test if normal distribution was not present, as stated in each figure legend. Statistical significance was defined as $p<0.05$ (*$p<0.05$; ** $p<0.01$; *** $p<0.001$). Statistical analyses were performed with GraphPad Prism 5 software.

3 Results

As observed in 2009, especially pregnant women were suffering from severe influenza A virus infection and influenza related complications [6]. Interestingly, this not only holds true for the 2009 pandemic but also for the pandemics of 1918, 1957 and even interpandemic years [4, 183]. Surprisingly, information and knowledge explaining the underlying molecular, virological and immunological determinant are still sparse.

3.1 Establishment of pregnant mouse infection model to study influenza A virus infections

In a first step, two different pregnancy mouse infection models were established. In this first, syngenic mating approach, C57BL/6 females were mated to C57BL/6 males and female mice were infected on day 12.5 of gestation with the 2009 pH1N1 influenza A virus. As mouse pregnancy lasts for 18-20 days, day 12.5 represents the beginning of the third trimester. In order to establish a more natural – allogeneic – mating combination which allows a higher genetic variability, female C57BL/6 were mated to male BALB/c mice.

Pregnant mice were subsequently infected with 10^3 p.f.u. of 2009 pH1N1 (A/Hamburg/NY1580/09) – a sublethal dose (MLD_{50} of 2009 pH1N1: $10^{3.83}$ p.f.u.) of the 2009 pH1N1 for non-pregnant animals – and survival and weight loss were monitored for 14 days in all groups (**Figure 7**). Allogenic mated compared to syngenic mated pregnant mice showed an increased mortality and morbidity (**Figure 7A, B** and **C**), the latter determined by increased and prolonged weight loss [263]. Furthermore, syngenic mated pregnant mice that survived the influenza infection fully recovered within 14 days post infection (Figure 7B). Non-pregnant C57BL/6 females showed no increased mortality upon infection and they fully recovered from influenza-related weight loss after 14 days (Figure 7D and E).

Based on this observation, all further experiments were performed in allogenic mated pregnant mice as this mating model mirrors closer the circumstances in human pregnancy.

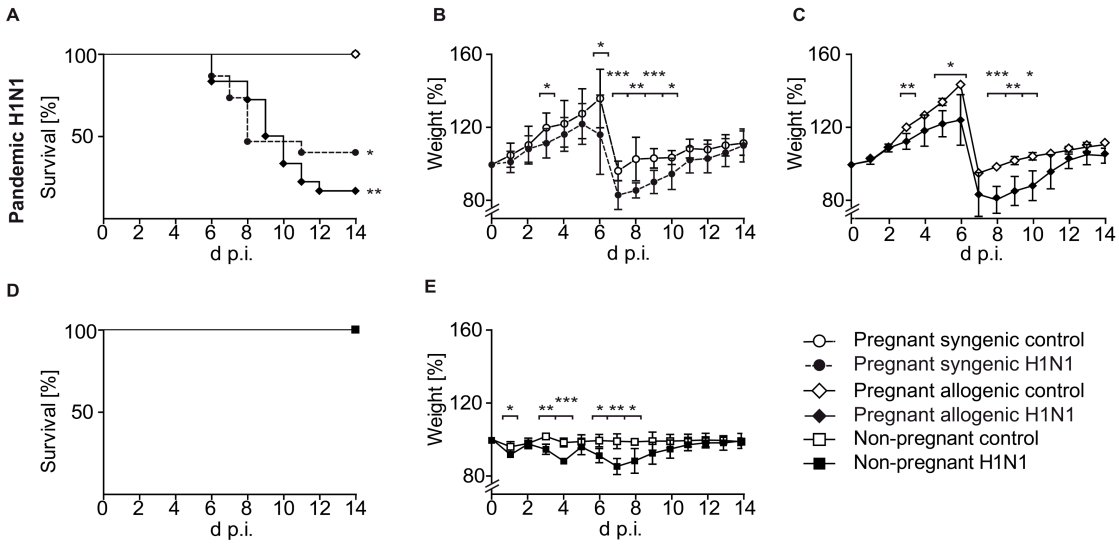


Figure 7: Pathogenicity of 2009 pH1N1 virus infection in syn- and allogenic pregnant and non-pregnant mice. BALB/c-mated, allogenic pregnant C57BL/6 (*n*=18) and C57BL/6-mated, syngenic pregnant C57BL/6 females (*n*=15) were infected with 10^3 p.f.u. of the 2009 pH1N1 virus. Syngenic (*n*=8) and allogenic (*n*=3) pregnant control groups received PBS only. Survival (A) and weight loss in syngenic (B) and allogenic (C) pregnant mice were monitored for 14 d p.i.. Note: the weight loss occurring around 6 d p.i and in PBS controls in (B,C) is associated with birth of offspring. (D) Survival and (E) weight loss in non-pregnant C57BL/6 females (*n*=5) infected with 10^3 p.f.u. of 2009 pH1N1 virus, compared to non-infected non-pregnant control animals. Data are presented as mean and SD. Statistical significance was calculated by Gehan-Brelow-Wilcoxon and Student´s t-test to assess the differences between respective infected pregnant mice and infected non-pregnant mice (**p<0.05, **p<0.01, ***p<0.001*).

3.2 Infection of non-pregnant and pregnant mice with seasonal H1N1

Data from the human population suggest that pregnant women suffer from increased influenza-related morbidity also in interpandemic years [4, 183], although less influenza-related complications are reported [7]. To address if these observations hold true in our mouse model, we infected non-pregnant and pregnant mice with a 2006 seasonal H1N1 (sH1N1) (A/Solomon Islands/3/06-like) strain **Figure 8**). Mice were first infected with 10^3 p.f.u. of the sH1N1 strain but here no relevant weight loss or mortality could be observed in all groups (Figure 8A, B and C). Furthermore, even when increasing the viral dose to 10^5 p.f.u. all mice survived the infection (Figure 8D, E and F).

In the following, experiments were performed with the 2009 pH1N1 as differences between non-pregnant and pregnant mice could not be observed whilst infecting with sH1N1.

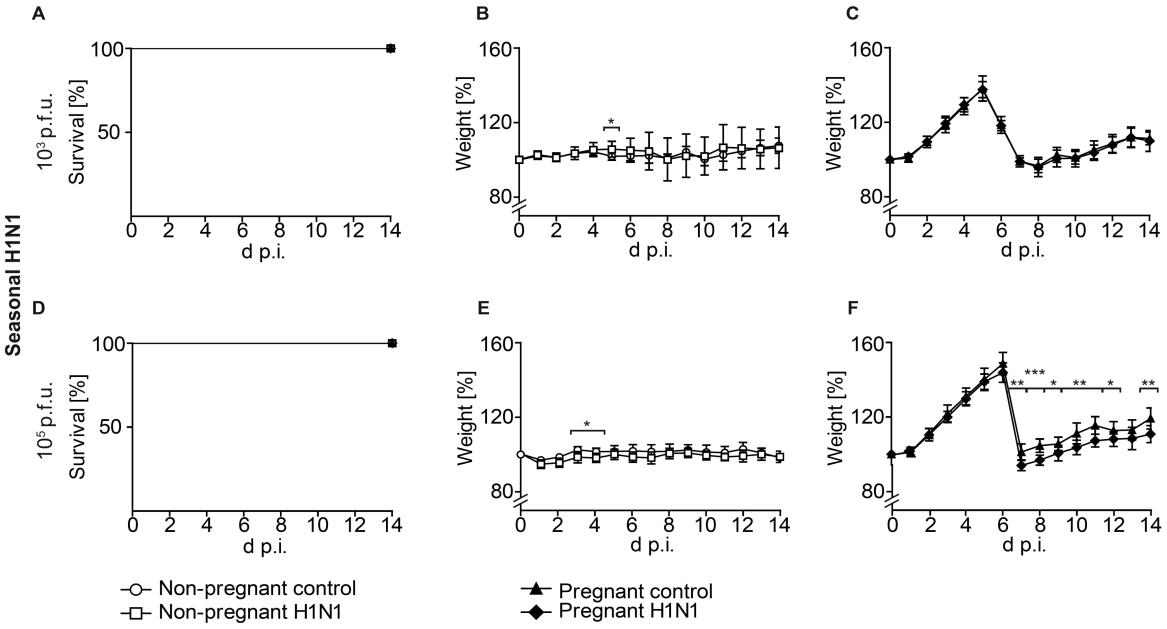


Figure 8: Pathogenicity of seasonal H1N1 virus infection in pregnant and non-pregnant mice with different virus doses. Mice were infected with 10^3 p.f.u. (non-pregnant: *n*=9; pregnant: *n*=7) (A-C) or 10^5 p.f.u. (D-F) of sH1N1 (non-pregnant: *n*=5; pregnant: *n*=9), controls received PBS only (non-pregnant: *n*=5-10, pregnant: *n*=7-9). Survival (A, D) and weight loss (B,C and E,F) were monitored for 14 d p.i.. Data are presented as mean and SD. Statistical significance was calculated by Gehan-Brelow-Wilcoxon and Student´s t-test to assess the differences between respective infected pregnant mice and infected non-pregnant mice (**p<0.05, **p<0.01, ***p<0.001*).

3.3 Infection of non-pregnant and pregnant mice with different doses of 2009 pH1N1 IAV

Following the infection with 10^3 p.f.u. of 2009 pH1N1, we determined the MLD_{50} of the 2009 pH1N1 IAV (A/Hamburg/NY1580/09). Here, non-pregnant and pregnant mice were infected with 10^2 p.f.u. and 10^4 p.f.u. of 2009 pH1N1, as it is necessary to infect mice with a dose where less than 50 % die and a dose were more than 50 % of the infected mice succumb to infection (**Figure 9**). As the virus dose was reduced 10 fold (10^2 p.f.u.) in comparison to the experiments shown in **Figure 7** and **8** the infection became non-lethal also for pregnant mice. Nevertheless, a significant weight loss in non-pregnant and pregnant mice could be detected (**Figure 9A-C**). When applying a 10-times higher dose than 10^3 p.f.u. (10^4 p.f.u.) mortality and weight loss further increased in pregnant mice and non-pregnant mice also succumbed to the infection which had not been observed with lower doses (Figure 9D-F).

In the following, non-pregnant and pregnant mice were infected with 10^3 p.f.u. of 2009 pH1N1 as this dose mirrors most closely the observations made in the human population.

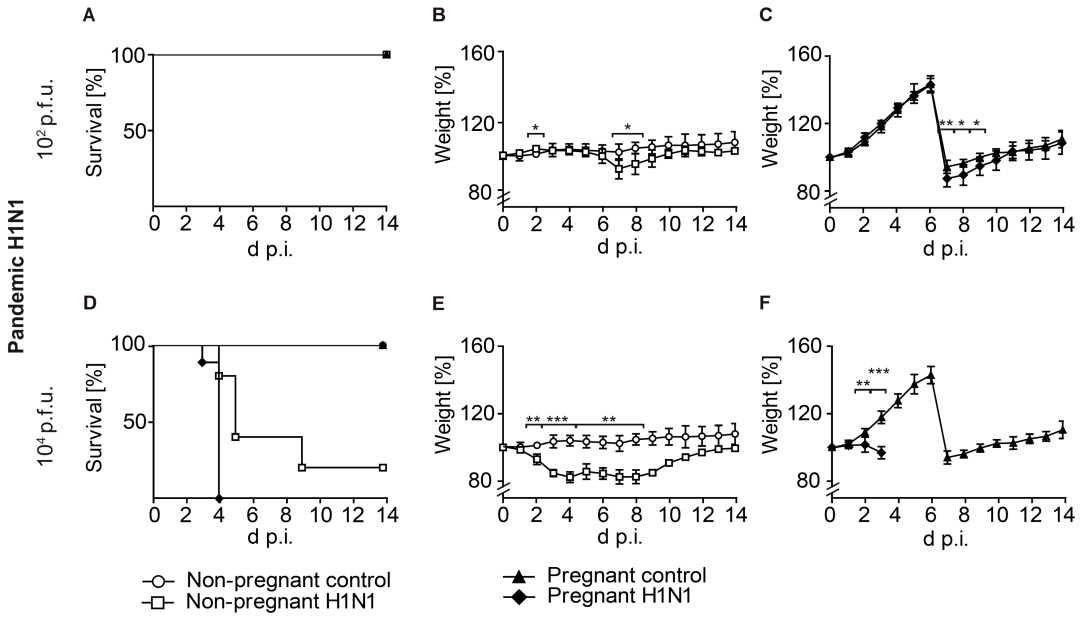


Figure 9: Pathogenicity of 2009 pH1N1 virus infection in pregnant and non-pregnant mice with different virus doses. Non-pregnant or pregnant mice were infected with 10^2 p.f.u. (A-C) or 10^4 p.f.u. (D-F) of pH1N1 (non-pregnant: *n*=5, pregnant: *n*=9) influenza virus. Non-pregnant and pregnant control groups received PBS (non-pregnant: *n*=4, pregnant: *n*=11). Survival (A, D) and weight loss (B,C and E,F) were observed for 14 d p.i.. The statistical significance in the experiments was calculated by Student´s t-test (**p<0.05; **p<0.001, ***p<0.001*).

3.4 Viral titer determination of 2009 pH1N1 influenza A virus in lungs and extrapulmonary organs of infected animals

Virus titers were determined via plaque assay on MDCK cells in supernatants of lung and one extrapulmonary organ homogenates, the gut, harvested on days 3 and 6 p.i of non-pregnant and pregnant mice infected with the 2009 pH1N1 (**Figure 10**). The viral titer in the gut was determined as many patients in 2009 reported gastrointestinal symptoms such as diarrhea [4, 5, 91-94]. Pregnant infected mice showed significant higher viral titers in the lungs 3 d p.i. compared to non-pregnant mice. On 6 d p.i., this difference was no longer observed (**Figure 10A**). The viral titers in the gut showed less pronounced differences. Nevertheless, higher numbers of pregnant compared to non-pregnant mice presented with positive titers (**Figure 10B**).

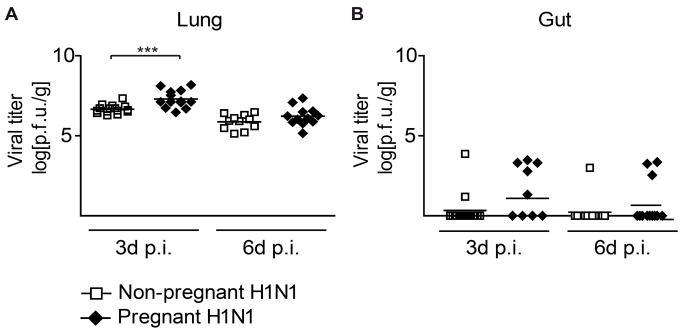


Figure 10: Determination of virus titer in lungs and gut of non-pregnant and pregnant mice upon 2009 pH1N1 virus infection. 2009 pH1N1 virus titers were determined in lung (A) and gut (B) at 3 and 6 d p.i. in non-pregnant (*n*=11-15) and pregnant (*n*=12-14) mice. Statistical significance was calculated by Student´s t-test to assess the differences between respective infected pregnant mice and infected non-pregnant mice (**p<0.05, **p<0.01, ***p<0.001*).

Histology

Histological formalin-fixed, paraffin-embedded sections of lungs harvested 3 d p.i. of non-pregnant and pregnant mice were stained either with hematoxylin and eosin or immunhistochemically against viral antigen (NP staining) for further evaluation. Here, it became visible that the area of inflammation in the murine lung was increased in pregnant compared to non-pregnant infected mice, as well as the number of viral antigen positive epithelial cells (**Figure 11**).

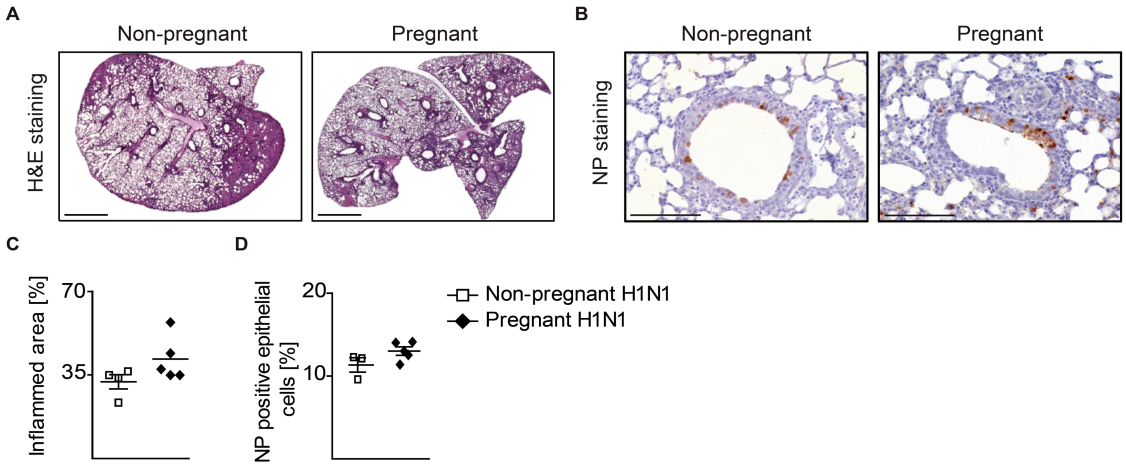


Figure 11: H&E and immunohistochemical staining of lung sections and analysis of inflamed area and viral antigen positive epithelial cells of lungs infected with 2009 pH1N1 of non-pregnant and pregnant mice. Lung sections were stained with H&E (A) (non-pregnant: *n*=4; pregnant: *n*=5) and immunohistochemically (IHC-P) against viral antigen (B). The scale bar represents 2 mm in H&E and 100 µm in viral antigen staining. (C) Inflamed areas (percentage of inflamed area over total lung area) and number of viral antigen positive epithelial cells (D) were assessed. Statistical significance was calculated by Student´s *t*-test to assess the differences between respective infected pregnant mice and infected non-pregnant mice. No statistical significant differences were found.

3.5 Reproductive outcome of 2009 pH1N1 infected pregnant mice

The reproductive outcome was compared to pregnant control animals (**Figure 12**). Overall no differences in placenta morphology or viral NP protein could be detected (**Figure 12A**). The number of total implants (**Figure 12B**), abortion rate (**Figure 12C**), fetal weight (**Figure 12D**) and progesterone concentration (**Figure 12E**) on day 3 and 6 p.i. were unaltered when comparing the infected and control dams. Progesterone was determined as is it is known to be essential for maternal adaptation to pregnancy and for establishment of a protective immune milieu, furthermore, epidemiological studies suggest that reduction in progesterone levels can contribute to increased abortions observed in stressed mice [264]. Gestational length and number of living offspring was not only compared between infected and control dams, but also between syngenically and allogenically mated pregnancies (**Figure 12F** and **G**). Overall no differences were detected here.

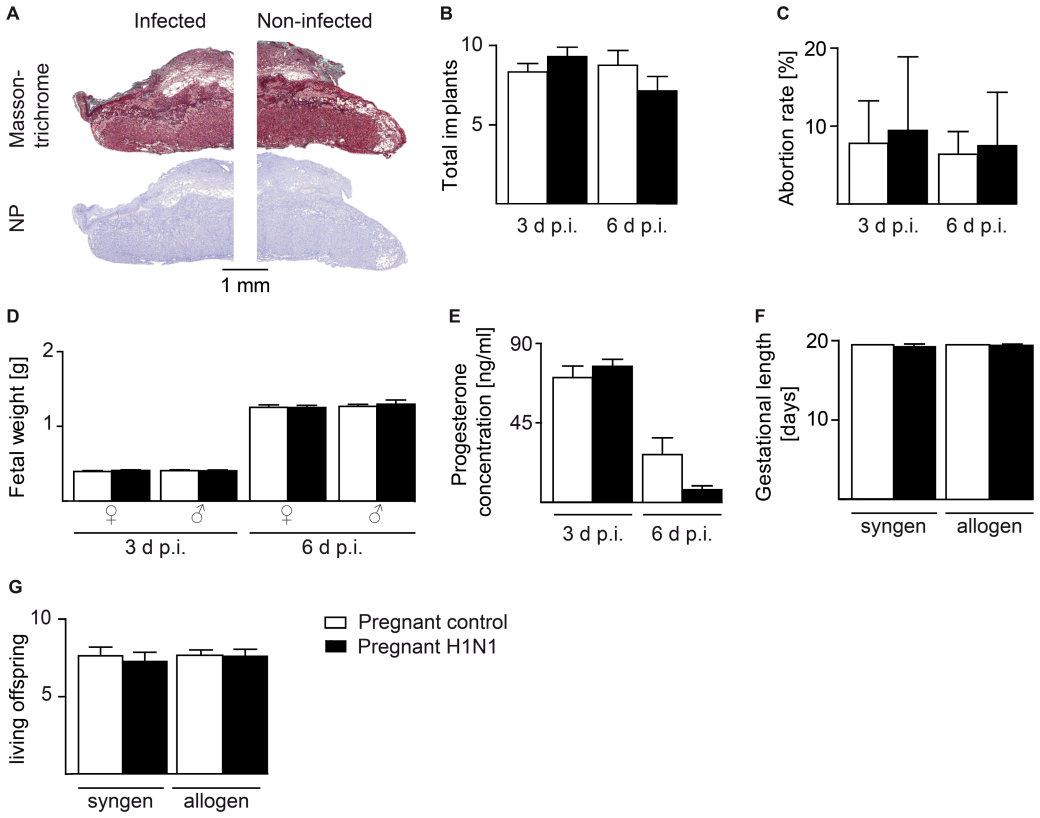
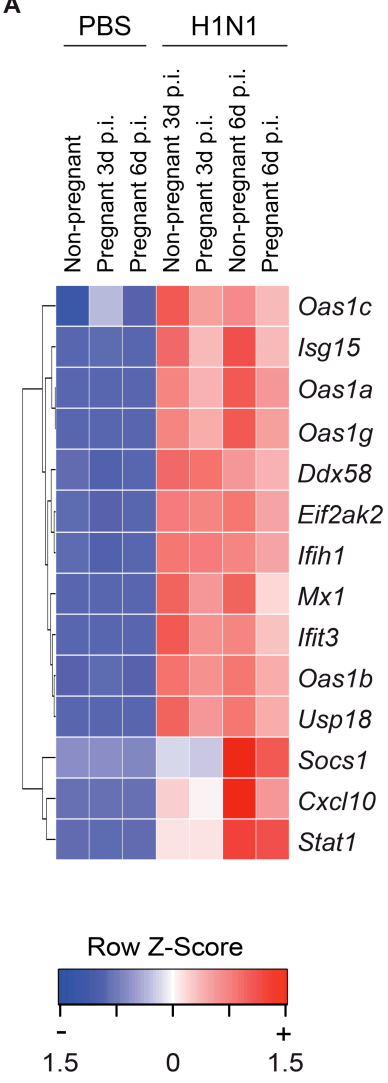


Figure 12: Reproductive outcome of 2009 pH1N1 infected pregnant mice. Pregnant mice were infected with 10^3 p.f.u. of pH1N1 influenza virus, while control groups were inoculated with PBS only. Placentae were stained with Masson-Goldner trichrome staining for morphological analysis and by immunohistochemistry for viral antigen (NP) detection (A). Total number of implants (B), abortion rate (C), fetal weight (D) and maternal serum progesterone levels (E) in pregnant pH1N1 (*n*=8-12) and pregnant controls (*n*=8-9) dams, were assessed 3 and 6 d p.i. Gestational length (F) and number of living offspring (G) in non-infected pregnant (syngenic *n*=8, allogenic *n*= 3) and infected dams (syngenic *n*=12, allogenic *n*=15). Note that the data shown for pH1N1 dams in (F and G) could only be assessed in surviving dams. Data are shown as mean and SEM in (B-G). The statistical significance in the experiments was calculated by Student´s *t*-test, no significant differences between groups could be detected.

3.6 Expression of interferon stimulated genes in 2009 pH1N1 infected pregnant mice

Interferon-stimulated genes (ISGs) are central for efficient first line defence against influenza viruses [265]. Interferons (IFNs), especially type I IFNs, induce an antiviral state in cells and limit the replication and therefor the spread of viruses, including influenza viruses [266]. High throughput sequencing (RNAseq) of selected ISG was therefore performed in lungs of non-pregnant and pregnant control and infected mice on days 3 and 6 p.i (**Figure 13**). As expected, ISG mRNAs known to be important for influenza virus control [267] were expressed at higher levels in infected mice. Moreover, the mRNA expression levels in non-pregnant infected mice were higher compared to pregnant infected mice (**Figure 13A**). When evaluating the log2 fold change data comparable observations were made (**Figure 13B**).

A



B

Gene name	3 d p.i.	6 d p.i.
Oas1c	-0,14	-0,09
Isg15	-0,44	-0,54
Oas1a	-0,24	-0,32
Oas1g	-0,24	-0,36
Ddx58	-0,02	-0,13
Eif2ak2	-0,04	-0,18
Ifih1	-0,04	-0,17
Mx1	-0,29	-0,66
Ifit3	-0,28	-0,34
Oas1b	-0,14	-0,22
Usp18	-0,25	-0,26
Socs1	-0,25	-0,38
Cxcl10	-0,28	-0,68
Stat1	0	-0,07

Figure 13: Expression of selected ISGs in non-pregnant and pregnant mice. Differential expression of murine ISGs in lungs of non-pregnant and pregnant 2009 pH1N1 and control mice was analyzed by RNAseq (A). The color code symbolizes the Z-score of normalized read counts according to the legend shown at the bottom. Log2-fold change of ISGs between pregnant and non-pregnant infected mice (B).

Full names of genes in alphabetical order: *Cxcl10*: C-X-C motif ligand 10; *Ddx58*: DEAD (Asp-Glu-Ala-Asp) box polypeptide 58; *Eif2ak2*: eukaryotic translation initiation factor 2-alpha kinase 2; *Ifih1*: interferon induced with helicase C domain 1; *Ifit3*:interferon-induced protein with tetratricopeptide repeats 3; *Isg15*: ISG15 ubiquitin-like modifier; *Mx1*: myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse); *Oas1a*: 2'-5' oligoadenylate synthetase 1A; *Oas1b*: 2'-5' oligoadenylate synthetase 1B; *Oas1c*: 2'-5' oligoadenylate synthetase 1C; *Oas1g*: 2'-5' oligoadenylate synthetase 1G; *Socs1*: suppressor of cytokine signaling 1; *Stat1*: signal transducer and activator of transcription 1; *Usp18*: ubiquitin specific peptidase 18.

3.7 Inflammatory cytokine expression in 2009 pH1N1 infected pregnant mice

Following the RNAseq data showing reduced selected ISG expression, we determined protein amounts of inflammatory cytokines which are known to be key regulators of viral suppression [118]. We determined the cytokine levels in serum (**Figure 14**) of non-pregnant and pregnant infected mice. Cytokines were generally reduced in pregnant compared to non-pregnant infected mice; IFNγ and IL6 were significantly reduced in serum of infected pregnant mice.

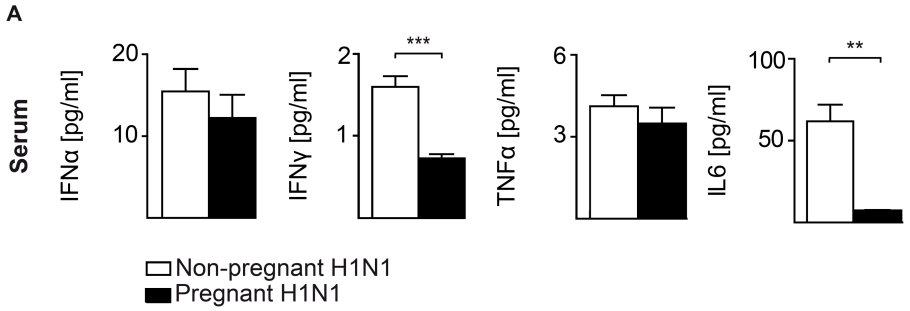


Figure 14: Levels of type I interferons and inflammatory cytokines in 2009 pH1N1 virus infected non-pregnant and pregnant mice. Cytokine concentrations were measured on day 3 p.i. in sera (A) (non-pregnant: *n*=4; pregnant: *n*=5) of mice infected with 10^3 p.f.u. of 2009 pH1N1 influenza virus. All data are presented as mean and SEM. The statistical significance in the experiments was calculated by Student´s *t*-test (**p<0.05, **p<0.01, ***p<0.001*).

3.8 Recruitment of leukocytes to lungs of infected mice

A recently published study [179] could show that in the uterus, due to epigenetic silencing of *Cxcr3* and its ligands, *Cxcl10* and *Cxcl9*, fetal tolerance is granted when recruitment of effector T cells is restricted. Effector T cells are known to attack fetal antigens and have an important role in the clearance of viral infections [268]. I therefore quantified the expression of *Cxcl10* in the lungs of non-pregnant and pregnant infected mice on day 3 p.i.. Hereby the question was addressed whether the observed epigenetic silencing in the uterus is also seen in the lungs of pregnant mice. Following this approach, a reduced expression of *Cxcl10* in the lungs of pregnant infected mice compared to non-pregnant infected mice was detected (**Figure 15**).

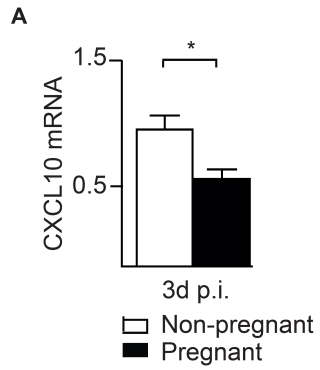


Figure 15: Expression of *Cxcl10* in lungs of non-pregnant and pregnant infected mice. Relative mRNA expression levels of *Cxcl10* were determined on day 3 p.i. in lungs of non-pregnant (*n=25)* and pregnant (*n*=17) infected mice by qRT-PCR. Mean values and SEM are shown, the statistical significance between the different groups was calculated using the Mann-Whitney U-test (**p<0.05*).

3.9 Emergence of viral quasi species in 2009 pH1N1 virus infected pregnant mice

Based on the data presented here and further immunological data (data not shown here; submitted in Engels, Hoffmann, Thieme *et al*.) there is evidence that the maternal immune system fails to mount a sufficient immune response to clear 2009 pH1N1 influenza A virus during pregnancy. To address the question if this failure to mount a sufficient immune response allows for the emergence of novel virus variants – mediated by an altered viral RNA-dependent RNA polymerase (RdRp) fidelity [269] - we sequenced the entire viral RNA genome of viruses isolated from lungs of infected non-pregnant and pregnant mice via RNAseq (**Figure 16**). We further compared the obtained viral sequences to those of the parental 2009 pH1N1 strain which was used for inoculation of the mice and found several synonymous and non-synonymous mutations (**Figure 16A**). Interestingly, two mutations appeared with high frequencies in pregnant infected mice, the frequency of these mutations even increased from day 3 to 6 p.i.. One of the found mutations is located in the HA (HA_{Q223R}) (**Figure 16B**), the other found mutation in the NS gene which encodes for the NS1 and NEP proteins ($NS_{R211K/D54N}$) (**Figure 16C**). All mutations that were detected in viruses isolated from lungs of pregnant mice and respective frequencies of the occurring mutations are depicted in Table 3.

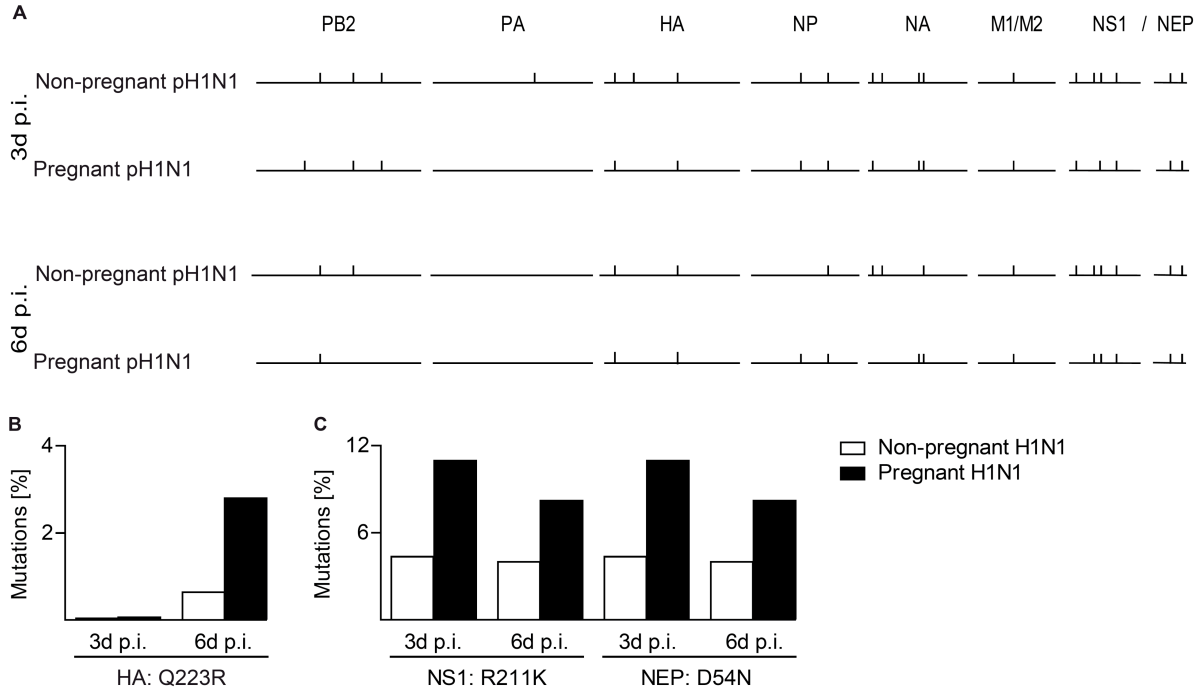


Figure 16: Frequency of viral mutations in viruses isolated from lungs of non-pregnant and pregnant 2009 pH1N1 infected mice. Sequences of viral RNA isolated from lung homogenates of infected mice were compared to the parental 2009 pH1N1 strain by high-throughput sequencing of RNA (RNaseq) (A). Non-synonymous mutations occurring in the eight RNA segments are represented by vertical lines. Non-synonymous mutations with highest frequency among all mutations appear in the HA (B) and the two distinct NS proteins (NS1, NEP) (C) of the virus.

Table 3: **Frequency of nucleotide exchanges during the infection course of non-pregnant and pregnant mice**

Gene	Nucleotide exchanges compared to consensus sequence	Non-pregnant		Pregnant		Amino acid sequence change	
		3d p.i.	6d p.i.	3d p.i.	6d p.i.		
PB2	G491A	-	-	2.99 %	-	PB2	S155N
	C646A	0.58 %	1.84 %	-	1.62 %	PB2	L207I
	G949A	0.10 %	2.27 %	0.11 %	-	PB2	V308I
	T1492C	0.18 %	-	1.57 %	-	PB2	S489P
PB1	T222A	0.06 %	1.09 %	0.04 %	0.18 %	PB1-F2	syn.
PA	A1662G	1.41 %	-	-	-	PA	I554M
	G1674A	0.12 %	2.35 %	0.16 %	-	PA	syn.
HA	G121A	0.04 %	0.03 %	1.31 %	0.03 %	HA	V24I
	T243C	0.01 %	1.81 %	0.01 %	0.05 %	HA	syn.
	G331A	2.32 %	-	-	-	HA	D94N
	A719G	0.04 %	0.64 %	0.06 %	2.80 %	HA	Q223R
NP	C847G	0.04 %	-	0.03 %	1.35 %	NP	L283V
	C1246A	0.03 %	0.03 %	1.09 %	0.04 %	NP	syn.

	G1087A	0.04 %	3.09 %	0.04 %	0.02 %	NP	V363I
NA	G238A	0.06 %	1.54 %	0.08 %	-	NA	V80M
	C376T	1.37 %	0.08 %	-	-	NA	P126S
	G1006A	0.03 %	-	0.07 %	1.16 %	NA	G336S
	G1066A	1.18 %	0.11 %	0.03 %	0.07 %	NA	G356S
M	C500T	7.81 %	3.24 %	4.34 %	1.57 %	M1	T167I
	A856G	1.01 %	-	0.02 %	-	M2	syn.
NS	G62A	0.03 %	1.43 %	0.07 %	-	NS1	R21Q
	G406A	0.01 %	1.57 %	-	0.06 %	NS1	V136I
	T583C	0.25 %	0.07 %	0.10 %	2.17 %	NS1	S195P
						NEP	syn.
	T627C	1.09 %	1.07 %	0.75 %	0.93 %	NEP	M52T
	G632A	4.35 %	4.00 %	10.96 %	8.21 %	NS1	R211K
						NEP	D54N

Frequencies of nucleotide exchanges occurring during infection of non-pregnant and pregnant mice with 10^3 p.f.u. of 2009 pH1N1 influenza virus on days 3 and 6 p.i.. Nucleotide sequences were compared to parental 2009 pH1N1 strain by high-throughput sequencing of RNA (RNaseq).
syn.=synonymus

3.10 Infection of non-pregnant mice with recombinant pH1N1 strains

In order to address whether the HA_{Q223R} or the $NS_{R211K/D54N}$ mutation which appeared with high frequencies in pregnant infected mice (as described under 3.9) influence morbidity and/or mortality, we generated recombinant viruses including these mutations in the 2009 pH1N1 background by reverse genetics. Two single point mutant viruses including either the mutation found in the HA (pH1N1-$HA_{Q223R)}$ or the NS (pH1N1-$NS_{R211K/D54N}$) gene were generated as well as one multi-gene reassortant virus including both (pH1N1- $HA_{Q223R+}NS_{R211K/D54N}$), the HA and NS mutations. Non-pregnant mice (**Figure 17**) were first infected with the defined standard dose of 10^3 p.f.u. to avoid dose dependent differences in virulence and pathogenicity (**Figure 17 A-D**). Remarkably, increased mortality and morbidity were observed in non-pregnant mice upon infection with all the different recombinant pH1N1 virus strains. However, the highest mortality and morbidity rates were observed in mice infected with the multi-gene reassortant virus pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$. In order to determine the MLD_{50} of these recombinant viruses, non-pregnant mice were subsequently infected with 10^1 p.f.u. (Figure 17I and K) and 10^2 (Figure 17 E-H) p.f.u., respectively. The MLD_{50} was calculated according to the method of Reed and Muench [249] as displayed in Table 4.

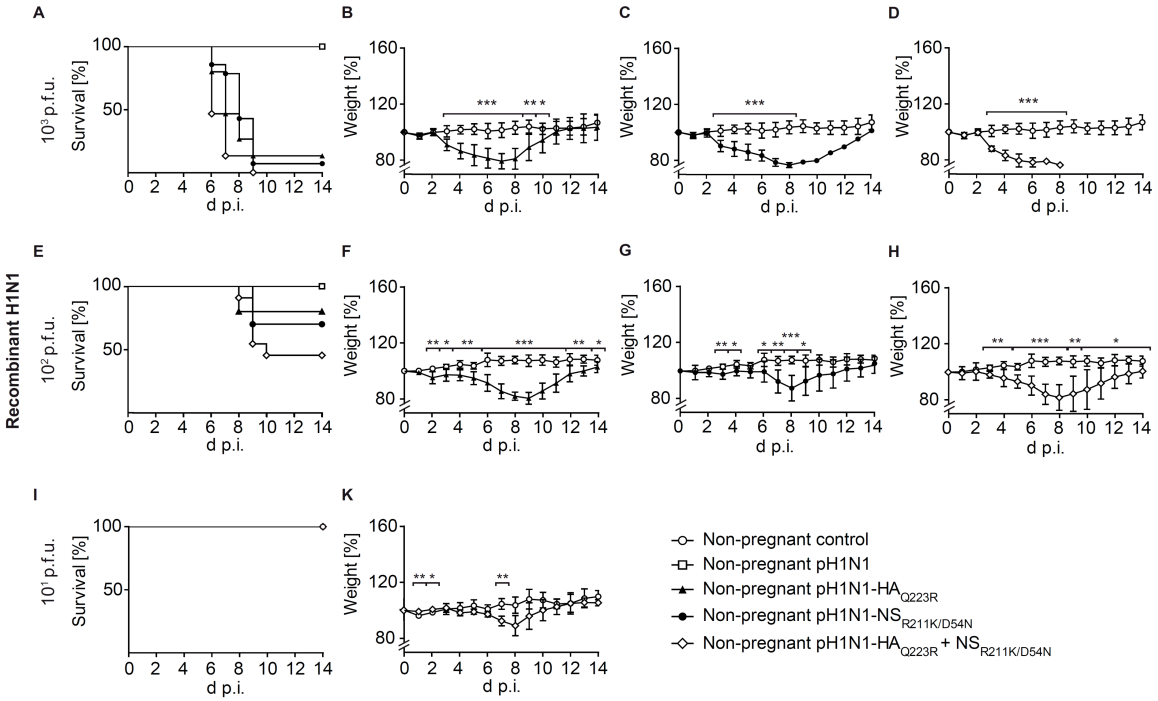


Figure 17: Pathogenicity of non-pregnant mice infected with recombinant H1N1 virus strains at different doses. Survival (A,E,I) and weight loss (B-D, F-H and K) in non-pregnant mice infected with 10^3 p.f.u. (A-D: pH1N1-HA$_{Q223R}$, pH1N1-NS$_{R211K/D54N}$ or pH1N1-HA$_{Q223R}$+NS$_{R211K/D54N}$) (non-pregnant control: *n*=14, non-pregnant pH1N1-HA$_{Q223R}$: *n*=15, pH1N1-NS$_{R211K/D54N}$: *n*=14, pH1N1-HA$_{Q223R}$+NSR$_{211K/D54N}$: *n*=15), 10^2 or 10^1 p.f.u. of recombinant pH1N1 strains (2009 pH1N1: *n*=10; pH1N1-HA$_{Q223R}$: *n*=10; pH1N1-NS$_{R211K/D54N}$: *n*=10; pH1N1- HA$_{Q223R+}$ NS$_{R211K/D54N}$; *n*=5-10). All mice were monitored for 14 d p.i.. Statistical significance of the obtained data was calculated by Student´s *t*-test (**p<0.05, **p<0.01, ***p<0.001*).

Table 4: **MLD$_{50}$ of pH1N1 and recombinant H1N1 in non-pregnant mice**

Virus	Dose for infection [p.f.u.]	Survival [%]	MLD$_{50}$ [p.f.u.]
2009 pH1N1	10^4	60	$10^{3.83}$
	10^3	100	
	10^2	100	
pH1N1 – HA$_{Q223R}$	10^3	13	$10^{2.46}$
	10^2	80	
pH1N1 – NS$_{R211K/D54N}$	10^3	7	$10^{2.34}$
	10^2	70	
pH1N1 – HA$_{Q223R}$ + NS$_{R211K/D54N}$	10^3	0	$10^{1.83}$
	10^2	40	
	10^1	100	

Non-pregnant mice were infected with serial 10-fold virus dilutions (10^1 to 10^4 p.f.u.) of pandemic H1N1 or recombinant pH1N1 mutant viruses (pH1N1-HA$_{Q223R}$, pH1N1-NS$_{R211K/D54N}$ or pH1N1-HA$_{Q223R}$ + NS$_{R211K/D54N}$) and observed for 14 d p.i. for weight loss and survival. The MLD$_{50}$ was calculated as described by Reed and Muench [249].

3.11 Receptor binding specificity of recombinant mutant pH1N1 viruses

In humans, α2,3- linked sialic acids are predominantly found in the lower respiratory tract (bronchioles and alveoli), whereas α2,6- linked sialic acids are mainly expressed in the upper respiratory tract [17]. As described under 2.2.14, depending on their avian or mammalian origin, influenza viruses preferentially either bind α2,3- or α2,6- linked sialic acids, respectively. Moreover, mutations in the HA protein can potentially alter the binding preference of the virus. In order to address whether the observed mutations in HA (pH1N1-HA_{Q223R} and pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$) of the pH1N1 recombinant viruses alter the receptor binding specificity, an HA resialylation assay was performed. We could hereby confirm previously published evidence by Chen et al. [270] that the HA_{Q223R} mutation switches the receptor binding specificity from α2,6- to α2,3- linked sialic acids.

Table 5: HA resialylation assay showing HA titers and specific receptor binding affinities of the wildtype 2009 pH1N1 and recombinant mutant 2009 pH1N1 viruses

	HA titer			
	untreated turkey erythrocytes	**VCNA treated turkey erythrocytes (all sialic acids removed)**	**α2,3-resialylated erythrocytes**	**α2,6-resialylated erythrocytes**
2009 pH1N1	64	0	4	64
pH1N1-HA_{Q223R}	64	0	32	0
pH1N1-HA_{Q223R}+ $NS_{R211K/D54N}$	64	0	8	0
H3N2	64	0	0	128
H5N1-HAmono	64	0	128	0

Table 5 shows one of three representative results. Assays were performed three times independently. Data was kindly generated by Carola Dreier.
VCNA: Vibrio cholerae neuraminidase

3.12 Interferon-β antagonistic function of recombinant mutant 2009 pH1N1 viruses

One mutation found with high frequency in viruses isolated from the lungs of pregnant infected mice was located in the NS gene of the virus encoding for NS1. NS1 is known to have an interferon antagonistic function which can be addressed with an *in vitro* interferon-β promotor assay using the Dual Luciferase® Reporter Assay System (**Figure 18**). In this assay, HEK293T cells were infected with either the wildtype 2009 pH1N1 virus or the pH1N1-$NS_{R211K/D54N}$ at a MOI of 0.1 or 1 or as a positive control Sendai virus (SeV) at MOIs of 0.0001, 0.001 and 0.002, respectively.
The interferon-β activity was assessed by a Dual Luciferase® Reporter Assay. The p125-luc luciferase plasmid contains the full length IFN-β promoter upstream of the firefly luciferase gene. Firefly luciferase levels were normalized against Renilla luciferase [271]. HEK293T cells were lysed 6 (**Figure**

18A) or 16 h p.i. (**Figure 18B**), respectively. SeV generally triggered a stronger interferon-β response than infection with 2009 pH1N1 or mutant recombinant viruses. Furthermore, no differences in interferon-β induction could be detected upon infection with either wildtype 2009 pH1N1 or pH1N1-$NS_{R211K/D54N}$. Nevertheless, these data are preliminary and require further confirmation.

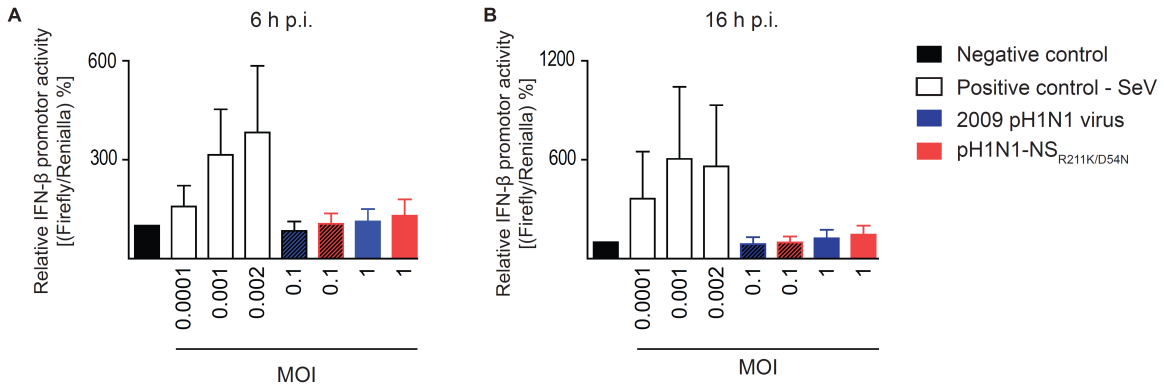


Figure 18: Interferon-β promotor activity upon infection with wildtype 2009 pH1N1 or mutant pH1N1-$NS_{R211K/D54N}$. HEK293T cells were infected at different MOIs with wildtype 2009 pH1N1 or mutant pH1N1-$NS_{R211K/D54N}$ and interferon-β activity was asses by a Dual Luciferase® Reporter Assay. Infection with Sendai virus at different MOIs served as a positive control. Infected cells were lysed 6 h p.i. (A) or 16 h p.i. (B), respectively. Experiments were performed in duplicates in five different independent experiments.Data are shown as mean and SD values. The statistical significance in the experiments was calculated by Student´s *t*-test, no statistical significant differences between wildtype 2009 pH1N1 or mutant pH1N1-$NS_{R211K/D54N}$ groups could be detected.
NC = negative control, PC = positive control.

3.13 Occurrence of influenza virus infections during pregnancy and influenza vaccination uptake among pregnant women in Hamburg, Germany

The Prenatal Identification of Children's Health (PRINCE) cohort is a prospective study that was established in 2011 at the University Medical Center in Hamburg-Eppendorf, Germany and is running until today. Starting in 2013, women (*n*= 138) were asked influenza and influenza vaccination related questions at three defined time points (13 and 15 [1st trimester], 23–25 [2nd trimester], and 35–37 [3rd trimester] weeks of gestation). First, pregnant women were asked if they had suffered from influenza-like illness during the last three months. If confirmed, it was assessed whether affected women consulted a physician or not. The second part of the questions dealt with of influenza vaccination uptake. It was asked whether women got vaccinated against influenza in general (possible answers included: *no; yes, annually; yes, irregularly*) and if confirmed, when the last vaccine had been applied (following answers could be chosen: *this year [when exactly]; in the last 1-2 years, in the last 2-4 years, in the last 5 years or more*). All of the data obtained was self-reported and not validated i.e. by checking the vaccination cards.

Overall, around 20 % of the pregnant women reported influenza-like illness during pregnancy (detailed listing can be found in **Table 6**) of which a majority reported never having been vaccinated against influenza. Of the women reporting no influenza-like illness over 40 % reported that they had received an influenza vaccine in the past. Of all interviewed women (*n*=138), 61 % reported to have received an

influenza vaccine in the past. Of the 39 % of women who had never received an influenza vaccination in their life, 25 % reported influenza-like illness during pregnancy. 12.3 % of the PRINCE cohort got vaccinated during pregnancy against influenza.

Table 6: **Incidence of self-reported influenza-like illness and influenza vaccination uptake in the PRINCE cohort**

	1st trimester of pregnancy	**2nd trimester of pregnancy**	**3rd trimester of pregnancy**
Number of patients with self-reported ILI	*n*=32 **(23.2 %)**	*n*=21 **(15.2 %)**	*n*=28 **(20.3 %)**
	Influenza vaccination: yes, annually: *n*=4 (2.9 %) yes, irregular: *n*=11 (7.9 %) no: *n*=17 (12.3 %)	Influenza vaccination: yes, annually: *n*=2 (1.4 %) yes, irregular: *n*=7 (5 %) no: *n*=12 (8.6 %)	Influenza vaccination: yes, annually: *n*=5 (3.6 %) yes, irregular: *n*=8 (5.7 %) no: *n*=15 (10.8 %)
Number of patients without self-reproted ILI	*n* =106 **(76.8 %)**	*n* =117 **(84.7 %)**	*n* =110 **(79.7 %)**
	Influenza vaccination: yes, annually: *n*=11 (7.9 %) yes, irregular: *n*=25 (18.1 %) no: *n*=70 (50.7 %)	Influenza vaccination: yes, annually: *n*=13 (9.4 %) yes, irregular: *n*=32 (23.1 %) no: *n*=72 (52.1 %)	Influenza vaccination: yes, annually: *n*=15 (10.8 %) yes, irregular: *n*=24 (17.3 %) no: *n*=71 (51.4 %)

Data kindly provided by Dr. Janina Goletzke.
ILI: influenza-like illness

In order to detect the incidence of influenza virus infection and the prevalence of influenza antibodies during pregnancy, an HAI assay (assay was performed in order to detect 2009 pH1N1 as described in 2.2.5) was performed of all women with no history of influenza vaccination in their life. The women who had received a vaccine at some point in their life were excluded, as it is not possible to distinguish with the HAI assay between antibodies acquired through natural infection or vaccination. Sera from the 1st and 3rd trimester of pregnancy was used for the HAI assay. Of the women assessed in the PRINCE cohort, 39 % have never received an influenza vaccination. Of these 39 % (*n*=54) women, 25 % (*n*=13) reported to have suffered from influenza-like illness throughout pregnancy. However, 3.7 % (*n*=2) of pregnant, non-vaccinated women were indeed infected with a 2009 pH1N1 virus during pregnancy (**Figure 19**).

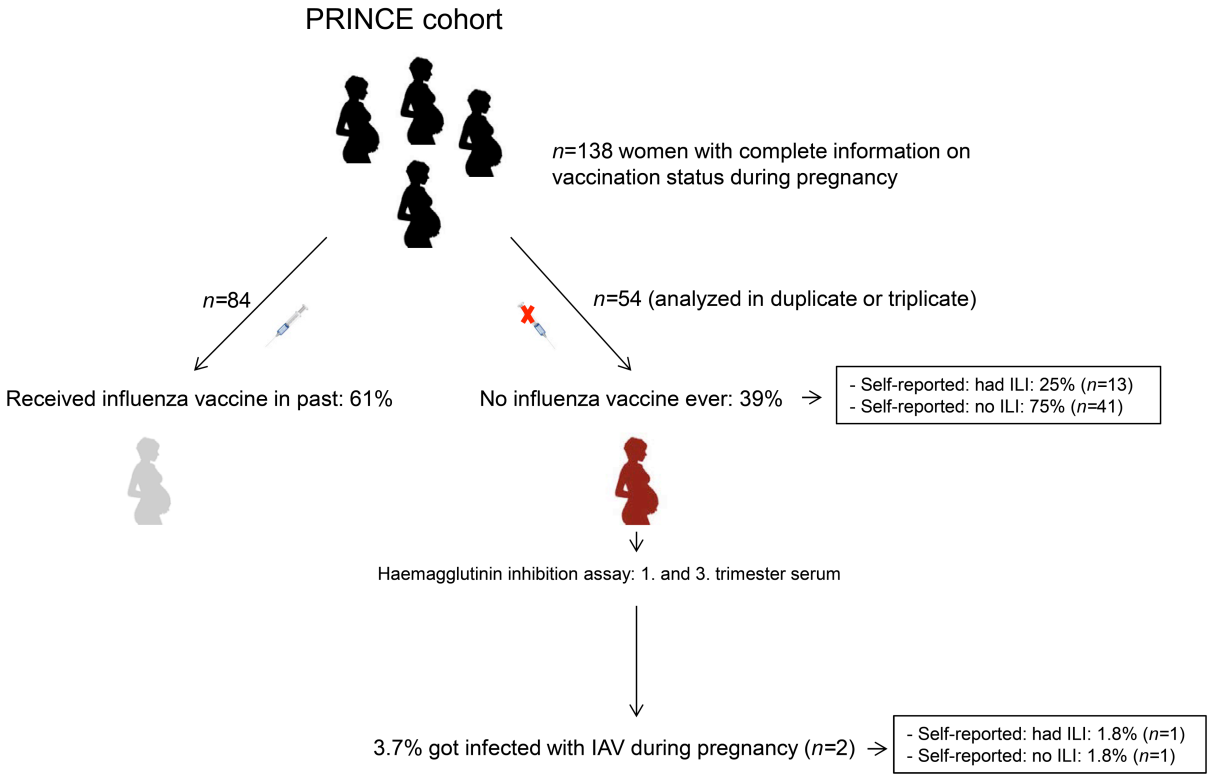


Figure 19: Overview of PRINCE cohort considering influenza vaccine uptake in the past and infection with influenza virus during pregnancy. 138 patients participated in this analysis. 39% had never been vaccinated against influenza in their life. Of these women, 25% reported ILI during pregnancy. HAI assay was performed with sera from 1st and 3rd trimester to cover potential influenza infection during pregnancy.
ILI = influenza-like illness, IAV = influenza A virus.

4 Discussion

Infectious diseases pose a severe threat to women during pregnancy, as recently highlighted during the 2009 H1N1 influenza pandemic. In 2009, pregnant women were recognized to show higher influenza related mortality and morbidity. Following these observations, vaccine recommendations by the World Health Organization (WHO) have been revised and now recommend vaccination of pregnant women with first priority. However, to date, the underlying viral and immunological determinants causally involved in the increased influenza pathogenesis during pregnancy are still largely unknown.

In order to address this lack of knowledge, a pregnant mouse infection model was established. With this model, it was possible to mirror the clinical observations made in the human population upon infection with the 2009 pandemic influenza virus during pregnancy. With the established semi-allogenic mouse infection model it was possible to perform a detailed analysis of the underlying immunological and virological mechanisms explaining the increased disease severity and elevated risk for influenza related complications during pregnancy. Nevertheless, this thesis mainly focuses on the virological mechanisms, the immunological mechanisms were addressed by colleagues and summarized in Engels, Hoffmann, Thieme *et al*.. Following NGS analysis, viral mutations appearing with increased frequency in lungs of pregnant infected mice were detected. Recombinant viruses containing these mutations were generated and non-pregnant mice showed increased morbidity and mortality upon infection with these recombinant viruses. The functional assays performed revealed a switch in receptor binding specificity of the viral HA from an α2,6-linked sialic acid preference towards an α2,3-linked one but no changes in interferon-β induction. Lastly, in a translational, clinical approach, it could be shown that influenza vaccine uptake during pregnancy and in general the infection rate of women, who never been vaccinated against influenza, are low.

Following the 2009 pH1N1 outbreak, the need for appropriate animal models to study the underlying immunological and virological mechanisms explaining the increased disease severity among pregnant women arose. Different authors have addressed these questions by using a syngenic BALB/c mating combination [230-232] and in one study C57BL/6 were mated syngenically [228]. Here, we decided to perform our experiments and establish our mating combinations with C57BL/6 mice, as Otte and Gabriel could show that C57BL/6 mice are suited best to study effects of 2009 pH1N1 infection and pathogenicity in mice [272]. Furthermore, during this thesis not only C57BL/6 females were mated to C57BL/6 males, we also established a more natural, allogenic, mating model by mating C57BL/6 females with BALB/c males which allows higher genetic variability. In regard to reproductive outcome, no differences in gestational length and number of living offspring were detectable between syngenically and allogenically mated control and infected mice (Figure 12F and G), respectively. On the other hand, allogenically mated pregnant mice showed an increased mortality and morbidity upon 2009 pH1N1 infection compared to the syngenically mated pregnant mice (Figure 7). As the allogenically mated pregnant infected mice reflected hereby more precisely the observations seen in the human population, all further experiments were performed with allogenically mated C75BL/6 mice.

DISCUSSION

Data from the human population revealed that especially women in their third trimester were suffering from severe influenza and accompying complications [194-196]. Pregnant mice were therefore infected on gestation day 12.5 that represents the beginning of the third trimester of mouse pregnancy. Other studies found in the literature chose a comparable time point of infection varying from gestation day 12 to 14 [230-232]. Pazos *et al.* in contrast, infected their mice in the second trimester of mouse pregnancy, on gestation day 10 [228].

Here, mice were infected with different doses of 2009 pH1N1 virus. Since 4 days after infection with 10^4 p.f.u of 2009 pH1N1, all pregnant mice had succumbed to the infection and only very small differences in morbidity between non-pregnant and pregnant mice were seen when infecting with 10^2 p.f.u. (Figure 9), 10^3 p.f.u. was chosen for this study. At this sublethal dose of the 2009 pH1N1 virus in non-pregnant mice, pregnant mice showed increased mortality (Figure 7). Therefore, all further experiments were pursued with the dose of 10^3 p.f.u. of 2009 pH1N1.

Nevertheless, infection with 10^3 p.f.u. of 2009 pH1N1 did not influence reproductive outcome (Figure 12). Analysis of placentae by Masson-trichrome staining showed no morphological differences between control and infected pregnant mice. Moreover, no influenza virus antigen could be detected in placentae of infected pregnant mice. These findings match current epidemiological findings in humans from 2009 and 2010, where viral replication in the placenta could not be detected [203, 204]. These findings are further reflected by the unaltered number of total implants days 3 and 6 p.i. in control and infected pregnant mice, as well as in the comparable abortion rate, although the latter appears to be slightly increased in the pregnant infected mice. Fetal weight was similar 3 and 6 days p.i. in offspring of control and infected pregnant mice, which might suggest that no virus passed the placental barrier. These results further reflect observations in humans, where, despite detection of chronic villitis, birth weight of the neonates was within expectations [203]. Pazos *et al.* showed comparable numbers of offspring, suggesting no increased abortion rate in their setting although this group infected pregnant mice earlier in pregnancy (gestation day 10 versus gestation day 12.5 in our setting). Nevertheless, Pazos *et al.* showed that fetal weight was reduced in offspring of infected pregnant mice compared to control mice. This can be explained by the earlier time point of infection during pregnancy, when the fetus is less developed in comparison to 12.5 days of gestation [228]. However, Kim *et al.* observed a mortality rate of 20 % in non-pregnant 2009 pH1N1 infected mice and additionally observed increased abortion rates in 2009 pH1N1 infected pregnant dams. These observations can be explained by the higher infection dose chosen (10^5 egg infectious dose 50, EID_{50}) [231]. We could observe similar effects by infecting non-pregnant and pregnant mice with the high dose 10^4 p.f.u. of 2009 pH1N1 (Figure 9). Here, all pregnant mice and 80 % of non-pregnant mice succumbed to the infection. In a next step, progesterone concentrations in serum of control and infected pregnant mice 3 and 6 days p.i. were compared using an ELISA, as progesterone is known to have anti-inflammatory effects [226]. We could not detect any differences in progesterone levels on day 3 p.i., but on day 6 p.i. the progesterone levels in infected pregnant mice were lower than in control mice. This potentially results in lower occurrences of anti-inflammatory effects which could partially explain the increased disease severity observed during pregnancy.

Prior to this study, only one other publication the compared effects of seasonal and pandemic IAV infection in non-pregnant and pregnant mice [231]. Similar to my observations, all mice survived upon infection with 10^3 p.f.u. of sH1N1 and weight loss curves were similar. Even when non-pregnant and pregnant mice were infected with 100-times more virus (10^5 p.f.u.), all mice survived although slight differences in recovery of mice, measured in terms of weight gain, could be detected (Figure 8). The latter observation matches quite well the epidemiological data in humans where seasonal IAV infections appear to be less severe than infections during pandemic IAV outbreaks [183].

The analysis of the viral titers in lungs on day 3 p.i. upon infection with 2009 pH1N1 showed significant higher lung titers in pregnant infected mice compared to non-pregnant infected mice. These differences were no longer visible on day 6 post infection when titers had decreased in general compared to day 3 p.i. (Figure 10A). The levels of lung titers detected in pregnant mice infected with the 2009 pH1N1 are controversially described in the literature as different assessment time-points, virus strains and infection routes were used. While the study of Chan *et al.* could detect significantly increased viral titers in lungs of infected mice on day 3 p.i. and an rapid reduction of the titers on day 6 p.i. [230], Marcelin *et al*., Kim *et al*. and Pazos *et al.* determined viral titers at different time points (3, 5 and 7 days p.i. respectively) and therefore cannot be directly compared to the data generated in this thesis [228, 231, 232]. Marcelin *et al*. could not detect any significant differences in lungs titers of non-pregnant and pregnant mice [232], whereas Kim *et al*. showed higher lung titers 5 days p.i. in pregnant 2009 pH1N1 infected mice [231]. Similar to these observations, Pazos *et al.* could show increased lung titers in infected pregnant mice on day 7 p.i. but not on day 3 p.i.[228]. The contradictory results can be possibly explained by the different virus strains, routes of infection and doses used. Pazos *et al*. for example, used the common lab strain A/PR/8/1934 (H1N1) for their experiments and mice were infected via an Inhalation Exposure System (mice were aerosol exposed for 30 minutes) [228]. Overall however, it appears that pregnant infected mice show higher lung titers than non-pregnant infected mice.

Despite the observation that many young patients suffered from gastrointestinal symptoms such as diarrhea [4, 5, 91-94], we could not find any data investigating viral titers in the gastrointestinal tracts upon infection of pregnant mice. Although no significant differences were seen between non-pregnant and pregnant infected mice, more pregnant mice showed positive gut titers on both 3 and 6 days p.i. (Figure 10B). One can speculate that this is due to the more severe course of influenza disease in pregnancy allowing viremic spreading of the influenza A virus. However, systemic infection has rarely been observed in the human population and if, then mainly in infections caused by the highly pathogenic H5N1 subtype. HPAIV can replicate efficiently in endothelial cells of the vasculature and perivascular parenchymatous cells which contribute to viral dissemination and systemic infection [20, 193, 201, 202, 205, 206].

When analyzing the histological data (Figure 11), I observed – most likely due to the relatively small number of animals analyzed not statistically significant – an increased area of inflammation, as well as more viral antigen positive epithelial cells in lungs of pregnant infected mice compared to non-pregnant mice. Setting the histological analysis of infected lungs from non-pregnant and pregnant mice in context with published literature, Chan *et al.* and Kim *et al*. consistent with my findings, detected less viral NP positive cells in lungs of infected non-pregnant mice compared to pregnant mice

[230, 231]. Marcelin *et al*. on the other hand could not detect differences in NP staining between non-pregnant and pregnant infected mice [232].

Analyses of influenza virus infected patients in 2009 have shown that viral infections with influenza A viruses lead to the production of specific cytokines such as TNFα, IL1, IL6 and IL10 [273, 274]. Type I and II interferons (IFNs) on the other hand, are antiviral cytokines that are activated early during influenza virus infection and are released by epithelial cells and key immune cells, as peripheral blood mononuclear cells (PBMCs).These cells appear to be important producers of IFN, such as IFNα [275]. Viral sensors such as Toll-like receptors (TLRs) trigger the release of IFNs [276]. This release leads to the induction of a variety of antiviral and immunomodulatory signaling pathways and thereby the transcription of more than 200 IFN-stimulated genes (ISGs) that are involved in several processes such as antiviral and antiproliferative functions and immune modulation [277-279]. In our approach we analyzed selected ISGs by High throughput sequencing (RNAseq) in lungs of non-pregnant and pregnant mice infected with the 2009 pH1N1 virus and corresponding controls (Figure 13). Up to expectations, the levels of ISGs involved in antiviral control were increased in infected animals compared to control mice. Further, ISG mRNA expression levels in non-pregnant mice were higher than in pregnant infected mice on both days 3 and 6 p.i.. This observation was also reflected in the log2-fold change data. Here, biggest fold changes were observed 6 days p.i. for *Mx1* and *Cxcl10*. Mx1, on one hand restricts influenza virus infection, most likely by binding to viral nucleoproteins [280], whereas Cxcl10 attracts activated NK and Th1 cells [281-283] which have an essential role in viral clearance [284]. As 3 to 4 samples were pooled for each group in the NGS analysis, no statistical analysis could be performed. Cytokines were further measured in serum (Figure 14) and lungs (lung data was generated earlier in our group and therefore is not shown here; submitted in Engels, Hoffmann, Thieme *et al*.) of non-pregnant and pregnant infected mice. Here, a significant reduction in protein amounts of IFNγ and IL6 and a non-significant reduction of IFNα and TNFα amounts were found in 2009 pH1N1 infected dams. Contrary to our data, other authors found increased levels of proinflammatory cytokines in lung homogenates potentially due to the higher virus dose used or other influenza virus strains administered [230-232]. Only Pazos *et al.* presented data matching my findings [228]. Data from pregnant women suffering from 2009 pH1N1 influenza A virus infection showed lower levels of IFNα and IFNλ production in PBMCs [279]. Cérbulo-Vázquez *et al.* showed non-significant lower IL6 levels but increased levels in TNFα, IL10 and IL1β in pregnant women upon infection with 2009 pH1N1 compared to healthy (non-infected) non-pregnant women. Unfortunately the authors did not include non-pregnant infected women as a control group in their study making it difficult to decide whether the particular cytokine and chemokine levels were due to infection or pregnancy or both. Overall, cytokine and chemokine levels seem to vary largely among the pregnant infected host, potentially dependent of infection dose and the influenza virus strain causing the influenza virus infection.

A complex immune response is needed to successfully combat influenza virus infection. Among the immune cells activated, effector T cells are known to have a key role in combating viral infections such as influenza virus infection [285, 286]. Precise recruitment of effector T cells to the site of infection is

crucial and is mediated by chemokine gradients that target cells secrete in order to allow homing of effector T cells [287-289]. During influenza virus infection, the recruitment of effector T cells to the site of infection is controlled by the generation of a local milieu triggered by early innate responses and the resulting chemoattractant signals. Here, influenza virus infection triggers rapid expression of high level inflammatory chemokines such as CXCL10 [290]. Effector T cells on the other side are known to attack fetal antigens and can therefore be harmful for the fetus and pregnancy maintenance [268]. A study published by Nancy *et al.* could show that due to epigenetic silencing, the recruitment of effector T cells to the uterus is restricted. In this study, during healthy pregnancy, endocrine triggered epigenetic changes lead to the downregulation of the CXCR3 ligands, *Cxcl9* and *Cxcl10*, which attract effector T cells [179]. I therefore addressed whether a similar downregulation can be observed in the lungs of pregnant infected mice compared to non-pregnant infected mice. I could indeed show a downregulation of *Cxcl10* on day 3 p.i. in lungs of infected dams via qRT-PCR (Figure 15). Other findings in our group (data not shown here; submitted in Engels, Hoffmann, Thieme *et al*.) further show *Cxcl10* downregulation on day 4 p.i. and *Cxcl9* downregulation on days 3 and 4 p.i.. Furthermore, we could also show a reduced recruitment of T cells into the lung (data not shown here; submitted in Engels, Hoffmann, Thieme *et al*.). These findings strengthen the hypothesis that due to endocrine triggered epigenetic silencing of chemokine ligands attracting effector T cells, which are essential for influenza virus control, pregnant women are more prone to suffer from severe influenza related disease as they cannot mount a sufficient immune response towards the infection. Further immunological assays (data not shown here; submitted in Engels, Hoffmann, Thieme *et al*.) could confirm this hypothesis, as it was shown that the up-regulation of costimulatory markers of antigen-presenting cells (alveolar macrophages and dendritic cells) was reduced. Furthermore, B cell response was altered and despite an enhanced frequency of 2009 pH1N1 virus-specific and effector $CD8^+$ T cells in pregnant infected mice compared to non-pregnant infected mice, their capacity to lyse virus infected cells was reduced. However, upon adoptive transfer of virus-specific $CD8^+$ T cells from infected non-pregnant donors, recovery but not survival was improved in pregnant infected mice. Taken together, these data suggest that the less stringent selective pressure during pregnancy results in a failure to mount the required innate and adaptive immune response to clear the virus and hereby facilitates the emergence of novel pH1N1 influenza virus variants.

This inability to mount a sufficient immune response towards influenza virus infection during pregnancy can be further exploited, as the error-prone viral RNA-dependent RNA polymerase that does not possess a proof-reading function allows for the emergence of novel viral mutations [269]. Here, we could show by high-throughput sequencing that several mutations appeared in all viral segment except PB1 of viruses isolated from lungs of 2009 pH1N1 infected non-pregnant and pregnant mice on day 3 and 6 p.i. (Figure 15). Surprisingly and contrary to our expectations, more mutations were found in non-pregnant infected mice compared to pregnant infected mice. Nevertheless, these mutations were only found on day 3 p.i. and they did not increase throughout the time assessed (Table 3). Overall, three mutations were found with increased frequency in pregnant infected mice compared to non-pregnant infected mice that resulted in three amino acid sequence changes: HA_{Q223R} and $NS_{R211K/D54N}$. As RNA of 3-4 lung samples of infected mice was pooled for sequencing, we could not distinguish in how many animals the respective mutations appeared and we

could not perform any statistical analysis. Therefore, pooling of samples should be critically evaluated for future experiments. The mutation in HA at position 223 (H1 numbering) has been described to be located in the receptor binding site of pH1N1 viruses. The receptor binding site consists of three secondary structure elements: the 190-helix, the 130-loop and the 220-loop where the Q223R substitution is located in [291]. Furthermore, the HA_{Q223R} mutation could be detected in clinical samples from Japanese patients at frequencies from 2.39 % to 4.64 % in the first wave of the 2009 pandemic, in the second wave of the pandemic the frequency further declined to 0.47 % to 0.63 %. Here, Yasugi *et al.* suggest that the Q223R variant is competent for human-to-human transmission but that the preference for α2,3-linked sialic acid binding (shown in [270]) results in low transmissibility [292]. In another study performed in China, 17 patients infected with 2009 pH1N1 virus presented with the HA_{Q223R} mutation. Of these 17 patients, 16 had a mild course of infection and one patient was severely affected [293]. In an Indian study, one male pediatric patient presented with the HA_{Q223R} mutation – this patient fully recovered from the infection [294]. This data could suggest that the HA_{Q223R} mutation does not lead to enhanced disease severity compared to patients not presenting with this mutation. Nevertheless, non-pregnant mice infected with a recombinant virus containing the HA_{Q223R} mutation showed a dramatically increased mortality when compared to non-pregnant mice infected with the wildtype 2009 pH1N1 virus. We could confirm with a HA resialylation assay performed (Table 5), that the receptor binding preference change towards α2,3-linked sialic acids from α2,6-linked SA in the mutants pH1N1-HA_{Q223R} (32 HAU) and pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$ (8 HAU) in comparison to the 2009 pH1N1 wildtype virus (4 HAU). As α2,3-linked sialic acids are predominantly found in the lower respiratory tract of the human host [17], it is believed that IAVs containing the HA_{Q223R} mutation replicate predominantly in the lower respiratory tract.

An increased replication in the lower respiratory tract can lead to pneumonia which reflects in general a more severe course of infection which could be observed in our setting with an increased pathogenicity upon mutant pH1N1-HA_{Q223R} or pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$ virus infection (Figure 17) [90]. This is also underlined by the lower MLD_{50} of recombinant pH1N1-HA_{Q223R} (MLD_{50} of $10^{2.46}$) and pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$ (MLD_{50} of $10^{1.83}$) viruses compared to wildtype 2009 pH1N1 virus (MLD_{50} of $10^{3.83}$). Currently, experiments questioning the increased transmissibility of the pH1N1-HA_{Q223R} are planned, as mutations in the HA often lead to changes in transmissibility [295] and could potentially harm other humans in close proximity to pregnant women such as family members or colleagues. For transmission studies, guinea pigs are a well-established animal model. The guinea pig infection model is established in our laboratory at the Heinrich Pette Institute and the respective transmission experiments are planned.

The mutations found in the NS gene on the other hand have not been described in the literature so far. So far it is known that the NS1 protein acts – among other functions – as an interferon antagonist. I therfore infected non-pregnant mice with the recombinant mutant pH1N1-$NS_{R211K/D54N}$ or pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$ viruses at different doses in order to evaluate if the mutations resulted in an altered morbidity and mortality upon infection. Indeed, both morbidity and mortality were increased in non-pregnant mice infected with pH1N1-$NS_{R211K/D54N}$ or pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$ compared to wildtype 2009 pH1N1 virus. Furthermore, morbidity and mortality upon pH1N1-$NS_{R211K/D54N}$ or pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$ virus infection were also increased compared to the pH1N1-HA_{Q223R} virus

infection as reflected in the corresponding survival curves at different doses and the MLD_{50}. Unfortunately, no difference in the interferon-β antagonistic function could be shown between wildtype 2009 pH1N1 and recombinant pH1N1-$NS_{R211K/D54N}$ virus infection of HEK293T cells at both 6 and 16 h p.i.. Probably, the increased morbidity and mortiality upon infection with recombinant pH1N1-$NS_{R211K/D54N}$ or pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$ does not affect interferon-β expression but other players in the interferon pathway. Furthermore, the observed increased morbidity and mortality could also be explained by alteration in the nuclear export function of the NEP.

Overall, the multiple-gene reassortant recombinant virus, pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$, led to the highest morbidity and mortality in mice, which was also clearly reflected by the MLD_{50}. This suggests that the occurrence of several mutations in different gene segments, due to the failure of the maternal immune system to mount a precise and sufficient immune response towards the IAV infection, further burdens the mother.

Overall, the following conclusion can be drawn from the obtained results in our pregnant mouse infection model (as depicted in

Figure 20):

Upon infection with the 2009 pH1N1 influenza A virus the immune system of the pregnant host is altered in which it presents reduced ISG expression, a reduced secretion of type I interferon and inflammatory cytokines, as well as a reduced ability for antigen presentation (data not shown here; submitted in Engels, Hoffmann, Thieme *et al.*). Furthermore, we could demonstrate that the adaptive immune response is impaired since recruitment of CD8 T cells to the lung is reduced (data not shown here; submitted in Engels, Hoffmann, Thieme *et al.*) and Cxcl9 and Cxcl10 expression, which attract effector T cells as previously mentioned [179], is down-regulated. Reduced virus cell lysis capacity was further observed which may contribute to the emergence of a systemic infection. Taken together, the less stringent, physiological, environmental immune pressure induced by the pregnant host, is very likely a driving force for the emergence of viral quasispecies associated with increased virulence. These adaptations to the altered innate and adaptive immune system of the pregnant host in combination account for the higher morbidity and mortality observed in influenza A virus infected pregnant hosts.

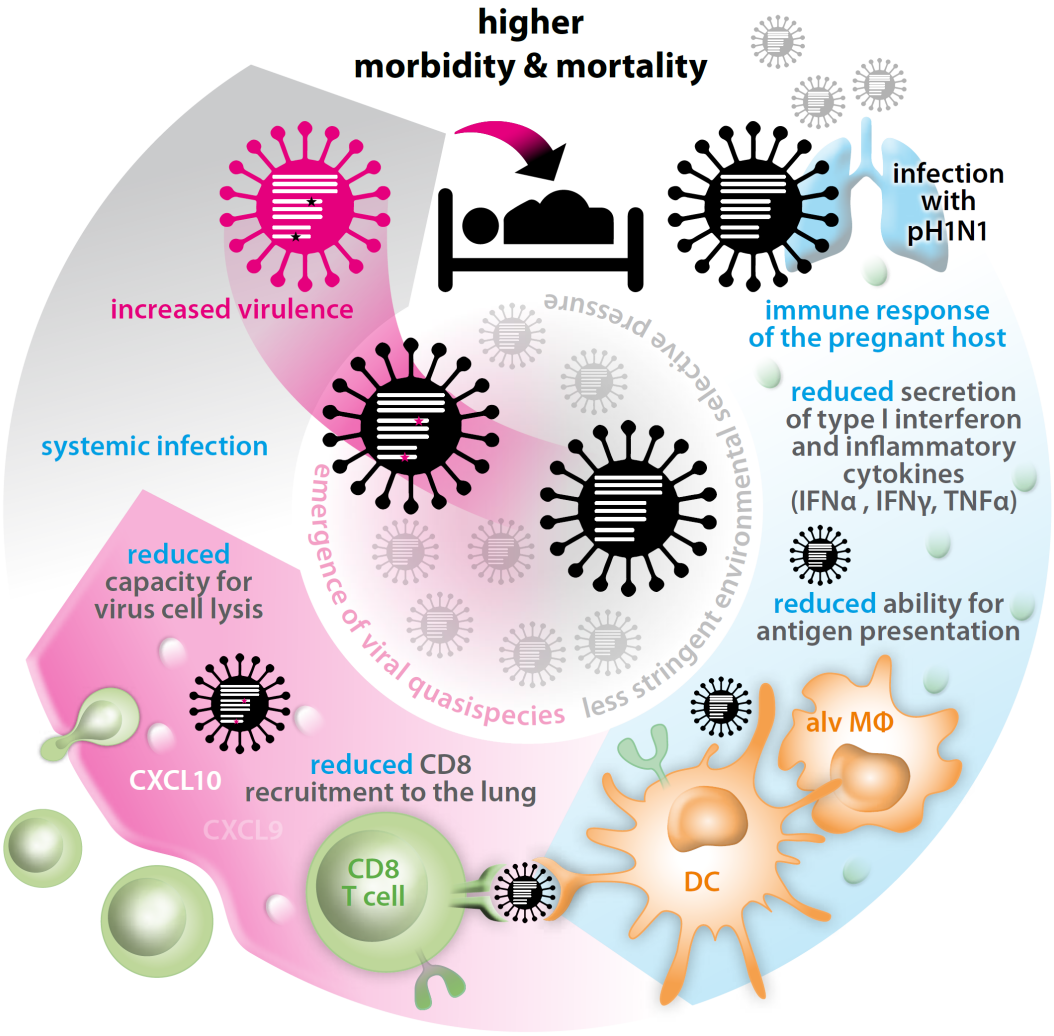


Figure 20: Key features contributing to the increased morbidity and mortality observed upon influenza A virus infection in pregnant mice. Following infection with the 2009 pH1N1 influenza A virus the immune system of the pregnant host is altered in which it presents reduced ISG expression, a reduced secretion of type I interferon and inflammatory cytokines, as well as a reduced ability for antigen presentation. Furthermore, adaptive immune response is impaired since recruitment of CD8 T cells to the lung is reduced and Cxcl9 and Cxcl10 expression, which attract effector T cells, is down-regulated. Reduced virus cell lysis capacity was further observed which may contribute to the emergence of a systemic infection. Taken together, the less stringent, physiological, environmental immune pressure induced by the pregnant host, allows for the emergence of viral quasispecies with an increased virulence. Figure kindly generated by Detlev Riller.
IFN = interferon, alv Mø = alveolare macrophages, DC = dendritic cells, CD = cluster of differentiation, CXCL = C-X-C motif ligand.

The semi-allogenic mouse infection model was successfully established here, this model could be used in the future to assess the pathogenic potential of circulating influenza viruses for the pregnant host. However, our findings in the semi-allogeneic pregnant mouse infection model need validation and translation into clinical findings observed in human pregnancy. The PRINCE cohort, a cohort of pregnant women in the greater Hamburg area, allows us to partially validate our findings in human pregnancy. However, since 2009, no pregnant woman needed hospitalization due to influenza A virus infection at the University Medical Center Hamburg-Eppendorf. In general, information on influenza A

virus infection incidence during pregnancy is scarce in Germany as influenza does not belong to the notifiable diseases. By including questions related to influenza-like illness (ILI) and vaccination uptake against influenza virus into the PRINCE study questionnaire, it was aimed to estimate the occurrence of ILI during pregnancy in pregnant women. Sera from non-vaccinated women were analyzed in order to estimate the number of pregnant women suffering from influenza A virus infection during pregnancy. Several studies in the past have addressed related questions, such as occurrence of influenza A virus antibodies or characterization of humoral immune response towards influenza viruses during pregnancy. Few studies provided and/or collected data on vaccination status of participants [296-298], whilst other studies do not provide any vaccination status related information [299-302]. In our setting, I could observe a seroconversion rate of 3.7 % in pregnant women that never had been vaccinated against influenza. In the literature seroconversion rates largely deviate and range from 15.7 % to 43.6 % in studies covering the 2009 H1N1 pandemic [297-301]. These differences can be partially explained by the varying time points the sera were analyzed: in the study from Mahmud *et al.* describing a seroconversion rate of 15.7 %, sera were taken in August 2009, after the first wave of the pandemic [300]. The high seroconversion rate of 43.6 % described by Honarvar *et al.* reflects sera taken during the 2009 H1N1 pandemic from November 2010 to January 2011 [298], almost one and a half years later. Furthermore, as already mentioned, vaccination status was not considered in every study, resulting in a potentially increased number of false-positive samples, as seroconversion is also observed upon influenza vaccination, not only upon infection with influenza viruses [303]. Moreover, the serum samples were collected in distinct geographical regions which can also influence the seroconversion rate. Interestingly, seroconversion rates from pre-pandemic times are closer to our findings. Tuyishime *et al.* observed a seroconversion rate of 6.6 % in 2002 [296], Mahmud *et al.* one of 7.1 % in 2009 pre-pandemic samples [300]. While analyzing our data, we realized that some of the vaccination status related questions were answered inconsistently, pointing to the limitations of self-reported data which is believed to suffer from reporting or recalling bias [234]. However, studies in past have found that self-reported data is valid in pregnant women [304, 305]. Nevertheless, we observed several times that the vaccination uptake question was answered differently by the patient: i.e., once it was referred to as "*yes, annually*", during the next appointment "*yes, once*" was answered. It was further recognized that no question was included covering the appearance of ILI before pregnancy. This appeared to be important, as the analysis of the data revealed that six out of forty-one women who had never been vaccinated against influenza virus in their life had positive influenza titers although thy did not reported ILI in the first trimester of pregnancy. Overall, 12.3 % of PRINCE cohort women vaccinated during pregnancy against influenza. This number is comparable to another study performed in Germany from 2012 to 2014 were 10.9 % of pregnant women vaccinated against seasonal influenza [239]. The comparison to another German cohort seems the most valid, as vaccine acceptance and attitude towards vaccines can differ largely among countries. In a review by Yuen and Tarrant, they summarize that the vaccine uptake for seasonal influenza varies from 1.7 % to 88.4 % and in the case of pandemic influenza from 6.2 % to 85.7 %. Here, lowest vaccine uptake was observed in Hong Kong for both seasonal and pandemic influenza, the highest rates were detected in the United States. Yuen and Tarrant further emphasize that different studies in various geographical regions revealed that pregnant women are unaware of the increased risk to suffer from severe

influenza and its complications. Furthermore, they were more likely to underestimate the risk of disease to themselves and their child [234]. A recently performed study in Germany confirmed these findings [239]. Yuen and Tarrant emphasize that informing and educating pregnant women about vaccine safety and its benefits for mother and child is crucial [234]. Nevertheless, several studies state independently that the most effective way to increase vaccine uptake is to directly recommend and offer vaccines [238, 306-308]. This point is especially important, as the WHO has changed its vaccine recommendations in 2012, now prioritizing pregnant women among all risk groups [237]. In future, health care providers should improve accessibility to vaccines as this can prevent influenza virus infection in pregnant women and their unborn child efficiently.

In conclusion, as allogenic pregnant mice fail to mount an adequate innate and adaptive immune response, novel, more virulent pH1N1 virus variants occur. Underlined by the low influenza vaccination uptake in the PRINCE cohort, the community's awareness for the risk of influenza virus infection during pregnancy is low and urgently needs to be increased.

5 Summary

During the last influenza pandemic in 2009, it was recognized that pregnant women were more likely to be hospitalized and suffer from severe influenza and its related complications such as pneumonia, preterm delivery, emergency cesarean section and even death. The underlying mechanisms explaining the increased disease severity and risk for influenza related complications in the pregnant host however, remain largely unknown. Here, a semi-allogenic pregnant mouse infection model was established in which pregnant mice showed increased morbidity and mortality upon infection with the 2009 pH1N1 influenza A virus compared to non-pregnant mice mirroring the clinical observations made in human pregnancy. Viral titers in pulmonary and extra-pulmonary organs were increased in pregnant infected mice. Additionally, cytokine responses were dampened and selected interferon-stimulated genes were downregulated in pregnant dams. Cxcl10, which attracts effector T cells that are involved in elimination of influenza viruses, was shown to be downregulated in pregnant infected mice. In addition, next generation sequencing was performed and viral mutations appearing with increased frequency in lungs of pregnant infected mice were identified. Next, recombinant viruses containing these mutations were generated and non-pregnant mice were infected. Upon infection with the recombinant mutant viruses pH1N1-$HA_{Q223R,}$ pH1N1-$NS_{R211K/D54N}$ or pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$ non-pregnant mice showed increased morbidity and mortality suggesting that the newly acquired viral mutations during pregnancy contribute to increased virulence. To further characterize the detected mutations, different *in vitro* assays were performed. Here it was shown that the HA_{Q223R} and HA_{Q223R}+$NS_{R211K/D54N}$ mutations were able to switch the receptor binding specificity, whereas the $NS_{R211K/D54N}$ mutation did not influence the interferon-β induction. In summary, the mouse model used here suggests that the pregnant host physiologically induces a less strict environmental immune pressure, allowing the emergence of viral mutations upon infection with the 2009 pH1N1 influenza A virus. The recombinant viruses containing these mutations showed increased virulence. These findings partially explain the increased severity in pregnant mice upon influenza virus infection. Furthermore, in a translational and clinical approach, it could be shown for the PRINCE cohort that influenza vaccine uptake during pregnancy is low and the infection rate of women, which have never been vaccinated against influenza, is also low during inter-pandemic years.

6 Zusammenfassung

Während der letzten Pandemie im Jahr 2009, wurde deutlich, dass schwangere Frauen im Gegensatz zu Nicht-Schwangeren schwerer an Influenza erkrankten und häufiger hospitalisiert wurden. Es zeigte sich bei infizierten schwangeren Frauen eine Häufung schwerwiegender Komplikationen wie Pneumonie, Frühgeburt, Notfall-Kaiserschnitt und Tod. Die diesen erschwerten Krankheitsverläufen und dem erhöhten Komplikationsrisiko zugrundeliegenden Mechanismen sind bisher nicht bekannt. Daher in dieser Arbeit ein *in vivo* Maus Infektionsmodell etabliert, in dem semi-allogen verpaarte trächtige Mäuse mit Influenza A Viren infiziert wurden. Mit Hilfe dieses Mausmodells konnte eine erhöhte Morbidität und Mortalität in trächtigen, mit 2009 pH1N1 infizierten Mäusen aufgezeigt werden, das die klinischen Beobachtungen in schwangeren Frauen widerspiegelte. Trächtige, infizierte Tiere wiesen hier verglichen mit nicht-trächtigen, infizierten Tieren erhöhte pulmonale und extra-pulmonale Titer auf. Demgegenüber waren Zytokinantwort und die Expression Interferon-stimulierter Gene in trächtigen, infizierten Mäusen reduziert. Mittels Hochdurchsatz-Sequenzierung konnten virale, adaptive Mutationen mit erhöhter Frequenz in Virus, das aus Lungen von trächtigen, infizierten Mäusen isoliert wurde, detektiert werden. Anschließend wurden rekombinante Virusmutanten generiert und mit diesen nicht-trächtige Mäuse infiziert. Die Infektion mit den Mutanten pH1N1-$HA_{Q223R,}$ pH1N1-$NS_{R211K/D54N}$ oder pH1N1-HA_{Q223R}+$NS_{R211K/D54N}$ resultierte in einer erhöhten Morbiditäts- und Mortalitätsrate in nicht-trächtigen, infizierten Mäusen. Weiterhin konnte mittels *in vitro* Versuchen gezeigt werden, dass die Mutationen HA_{Q223R} und HA_{Q223R}+$NS_{R211K/D54N}$ zu einem Wechsel in der Rezeptorbindungsspezifität des viralen HA führen. Die $NS_{R211K/D54N}$ Mutation hatte jedoch keinen Einfluss auf die Interferon-β Induktion. Zusammenfassend konnten wir mit dem hier etablierten Mausmodell zeigen, dass in der trächtigen Maus physiologisch ein weniger stringenter Immundruck vorliegt, was das Entstehen und die Etablierung von viralen Mutationen fördert. Die hier generierten und untersuchten rekombinanten Mutanten zeigten dabei eine erhöhte Virulenz. In der PRINCE Kohorte konnte zudem in einem translationalen und klinischen Ansatz gezeigt werden, dass die Impfrate von Schwangeren gegen Influenza niedrig ist und dass die Infektionsrate von Frauen, die noch nie gegen Influenza geimpft wurden, für interpandemische Zeiten ebenfalls erwartungsgemäss niedrig ist.

7 List of abbreviations

CDC	Center of Disease Control and Prevention
CMP	cytidine-5`-monophospho-N-acetylneuraminic acid sodium salt
CPE	cytopathic effect
cRNA	coding RNA
CTL	cytotoxic T cell
DC	dendritic cell
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
G-CSF	granulocyte-colony stimulating factor
gd	gestation day
HA	hemagglutinin
HA assay	hemagglutination assay
HAI assay	hemagglutination inhibition assay
HEF	haemagglutinin-esterase fusion glycoprotein
HEK293T	human embryonic kidney 293 T cells
HPAIV	highly pathogenic avian influenza viruses
IFN	interferon
IL	interleukin
ISG	interferon-stimulated genes
LPAIV	low pathogenic avian influenza virus
M	matrix protein
MCP-1	monocyte chemoattractant protein-1
MDCK	Madin-Darby canine kidney cells
MLD_{50}	mouse lethal dose 50
MOI	multiplicity of infection
mRNA	messenger RNA
NA	neuraminidase
NK cell	natural killer cell
NLRP3	NLR family pyrin domain containing 3
NLS	nuclear localization signal
NP	nucleoprotein
NS	non-structural protein
NSAIDs	Nonsteroidal anti-inflammatory drugs
ORF	open reading frame
p.f.u.	plaque-forming unit
p.i.	post infection
PA	polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2

LIST OF ABBREVIATIONS

PBMC	peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cells
pH1N1	pandemic H1N1
PPR	Pattern recognition receptors
PRINCE cohort	Prenatal Identification of Children's Health cohort
RANTES	regulated on activation, normal T expressed and secreted
RdRp	RNA-dependent RNA polymerase
RIG-I	retinoic acid-inducible gene 1
RNP	ribonucleoprotein
RT-PCR	Reverse transcription polymerase chain reaction
SA	sialic acid
SeV	Sendai virus
SPF	Specific pathogen free
TH1	T helper cells 1
TH2	T helper cells 2
TLR	toll-like receptors
TNF-α	tumor necrosis factor- α
Tregs	T regulatory cells
VCNA	Vibrio cholerae neuraminidase
vRNA	viral RNA
vRNP	viral ribonucleoprotein
WHO	World Health Organization

8 References

1. WHO. *Fact sheets*. [cited 2016 18.03.2016]; Available from: http://www.who.int/mediacentre/factsheets/fs211/en/.
2. Novel Swine-Origin Influenza, A.V.I.T., et al., *Emergence of a novel swine-origin influenza A (H1N1) virus in humans.* N Engl J Med, 2009. **360**(25): p. 2605-15.
3. CDC. *Flu risk pregnancy*. [cited 2016 18.03.2016]; Available from: http://www.cdc.gov/flu/pdf/freeresources/pregnant/flu-risk-pregnancy-flyer.pdf.
4. Liu, S.L., et al., *Pandemic influenza A(H1N1) 2009 virus in pregnancy.* Rev Med Virol, 2013. **23**(1): p. 3-14.
5. Jamieson, D.J., et al., *H1N1 2009 influenza virus infection during pregnancy in the USA.* Lancet, 2009. **374**(9688): p. 451-8.
6. Mosby, L.G., S.A. Rasmussen, and D.J. Jamieson, *2009 pandemic influenza A (H1N1) in pregnancy: a systematic review of the literature.* Am J Obstet Gynecol, 2011. **205**(1): p. 10-8.
7. Rasmussen, S.A. and D.J. Jamieson, *Influenza and pregnancy in the United States: before, during, and after 2009 H1N1.* Clin Obstet Gynecol, 2012. **55**(2): p. 487-97.
8. Knipe, D.M.u.P.M.H., *Fields Virology*. 5th ed. 2006, Philadelphia: Lippincott Williams & Wilkins.
9. Hause, B.M., et al., *Characterization of a novel influenza virus in cattle and Swine: proposal for a new genus in the Orthomyxoviridae family.* MBio, 2014. **5**(2): p. e00031-14.
10. Briese, T., et al., *Upolu virus and Aransas Bay virus, two presumptive bunyaviruses, are novel members of the family Orthomyxoviridae.* J Virol, 2014. **88**(10): p. 5298-309.
11. Allison, A.B., et al., *Cyclic avian mass mortality in the northeastern United States is associated with a novel orthomyxovirus.* J Virol, 2015. **89**(2): p. 1389-403.
12. Modrow, S., Falke, D., Truyen, U., Schätzl, H., *Molekulare Virologie*. Vol. 3rd. 2003.
13. Tong, S., et al., *New world bats harbor diverse influenza A viruses.* PLoS Pathog, 2013. **9**(10): p. e1003657.
14. CDC. *Transmission of Influenza Viruses from Animals to People*. 2014 17.06.2016]; Available from: http://www.cdc.gov/flu/about/viruses/transmission.htm.
15. Chu, C.M., I.M. Dawson, and W.J. Elford, *Filamentous forms associated with newly isolated influenza virus.* Lancet, 1949. **1**(6554): p. 602.
16. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiol Rev, 1992. **56**(1): p. 152-79.
17. Nayak, D.P., et al., *Influenza virus morphogenesis and budding.* Virus Res, 2009. **143**(2): p. 147-61.
18. Rossman, J.S. and R.A. Lamb, *Influenza virus assembly and budding.* Virology, 2011. **411**(2): p. 229-36.
19. Bouvier, N.M. and P. Palese, *The biology of influenza viruses.* Vaccine, 2008. **26 Suppl 4**: p. D49-53.
20. Horimoto, T. and Y. Kawaoka, *Influenza: lessons from past pandemics, warnings from current incidents.* Nat Rev Microbiol, 2005. **3**(8): p. 591-600.
21. Jagger, B.W., et al., *An overlapping protein-coding region in influenza A virus segment 3 modulates the host response.* Science, 2012. **337**(6091): p. 199-204.
22. Yamayoshi, S., et al., *Identification of a Novel Viral Protein Expressed from the PB2 Segment of Influenza A Virus.* J Virol, 2016. **90**(1): p. 444-56.
23. Zamarin, D., M.B. Ortigoza, and P. Palese, *Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice.* J Virol, 2006. **80**(16): p. 7976-83.
24. Tauber, S., et al., *Behaviour of influenza A viruses differentially expressing segment 2 gene products in vitro and in vivo.* J Gen Virol, 2012. **93**(Pt 4): p. 840-9.
25. McAuley, J.L., K. Zhang, and J.A. McCullers, *The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis.* J Virol, 2010. **84**(1): p. 558-64.
26. Wise, H.M., et al., *A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA.* J Virol, 2009. **83**(16): p. 8021-31.
27. Hayashi, T., L.A. MacDonald, and T. Takimoto, *Influenza A Virus Protein PA-X Contributes to Viral Growth and Suppression of the Host Antiviral and Immune Responses.* J Virol, 2015. **89**(12): p. 6442-52.
28. Muramoto, Y., et al., *Identification of novel influenza A virus proteins translated from PA mRNA.* J Virol, 2013. **87**(5): p. 2455-62.

29. Wise, H.M., et al., *Identification of a novel splice variant form of the influenza A virus M2 ion channel with an antigenically distinct ectodomain.* PLoS Pathog, 2012. **8**(11): p. e1002998.
30. Selman, M., et al., *Adaptive mutation in influenza A virus non-structural gene is linked to host switching and induces a novel protein by alternative splicing.* Emerg Microbes Infect, 2012. **1**(11): p. e42.
31. Neumann, G., T. Noda, and Y. Kawaoka, *Emergence and pandemic potential of swine-origin H1N1 influenza virus.* Nature, 2009. **459**(7249): p. 931-9.
32. Couceiro, J.N., J.C. Paulson, and L.G. Baum, *Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity.* Virus Res, 1993. **29**(2): p. 155-65.
33. Matrosovich, M.N., et al., *Human and avian influenza viruses target different cell types in cultures of human airway epithelium.* Proc Natl Acad Sci U S A, 2004. **101**(13): p. 4620-4.
34. Sieczkarski, S.B. and G.R. Whittaker, *Viral entry.* Curr Top Microbiol Immunol, 2005. **285**: p. 1-23.
35. Stegmann, T., *Membrane fusion mechanisms: the influenza hemagglutinin paradigm and its implications for intracellular fusion.* Traffic, 2000. **1**(8): p. 598-604.
36. Martin, K. and A. Helenius, *Transport of incoming influenza virus nucleocapsids into the nucleus.* J Virol, 1991. **65**(1): p. 232-44.
37. Cros, J.F. and P. Palese, *Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses.* Virus Res, 2003. **95**(1-2): p. 3-12.
38. Bancroft, C.T. and T.G. Parslow, *Evidence for segment-nonspecific packaging of the influenza a virus genome.* J Virol, 2002. **76**(14): p. 7133-9.
39. Duhaut, S.D. and N.J. Dimmock, *Defective segment 1 RNAs that interfere with production of infectious influenza A virus require at least 150 nucleotides of 5' sequence: evidence from a plasmid-driven system.* J Gen Virol, 2002. **83**(Pt 2): p. 403-11.
40. Enami, M., et al., *An influenza virus containing nine different RNA segments.* Virology, 1991. **185**(1): p. 291-8.
41. Fujii, Y., et al., *Selective incorporation of influenza virus RNA segments into virions.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 2002-7.
42. Palese, P. and R.W. Compans, *Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action.* J Gen Virol, 1976. **33**(1): p. 159-63.
43. Palese, P., et al., *Characterization of temperature sensitive influenza virus mutants defective in neuraminidase.* Virology, 1974. **61**(2): p. 397-410.
44. Kawaoka, Y. and R.G. Webster, *Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells.* Proc Natl Acad Sci U S A, 1988. **85**(2): p. 324-8.
45. Webster, R.G., et al., *Intestinal influenza: replication and characterization of influenza viruses in ducks.* Virology, 1978. **84**(2): p. 268-78.
46. Rota, P.A., et al., *Laboratory characterization of a swine influenza virus isolated from a fatal case of human influenza.* J Clin Microbiol, 1989. **27**(6): p. 1413-6.
47. Webster, R.G., et al., *Characterization of an influenza A virus from seals.* Virology, 1981. **113**(2): p. 712-24.
48. Hinshaw, V.S., et al., *Are seals frequently infected with avian influenza viruses?* J Virol, 1984. **51**(3): p. 863-5.
49. Hinshaw, V.S., et al., *Characterization of two influenza A viruses from a pilot whale.* J Virol, 1986. **58**(2): p. 655-6.
50. Guo, Y., et al., *Characterization of a new avian-like influenza A virus from horses in China.* Virology, 1992. **188**(1): p. 245-55.
51. Klingeborn, B., et al., *An avian influenza A virus killing a mammalian species--the mink. Brief report.* Arch Virol, 1985. **86**(3-4): p. 347-51.
52. Drake, J.W., *Rates of spontaneous mutation among RNA viruses.* Proc Natl Acad Sci U S A, 1993. **90**(9): p. 4171-5.
53. Holland, J., et al., *Rapid evolution of RNA genomes.* Science, 1982. **215**(4540): p. 1577-85.
54. Scholtissek, C., *Molecular epidemiology of influenza.* Arch Virol Suppl, 1997. **13**: p. 99-103.
55. Subbarao, K. and J. Katz, *Avian influenza viruses infecting humans.* Cell Mol Life Sci, 2000. **57**(12): p. 1770-84.
56. Ma, W., et al., *Identification of H2N3 influenza A viruses from swine in the United States.* Proc Natl Acad Sci U S A, 2007. **104**(52): p. 20949-54.
57. WHO. *Human animal interface*. 2013; Available from: www.who.int /influenza/ human_animal_interface/ Influenza_Summary_IRA_HA_interface_03July13.pdf.

58. Guo, Y., J. Li, and X. Cheng, *[Discovery of men infected by avian influenza A (H9N2) virus].* Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi, 1999. **13**(2): p. 105-8.
59. Belser, J.A., et al., *Use of animal models to understand the pandemic potential of highly pathogenic avian influenza viruses.* Adv Virus Res, 2009. **73**: p. 55-97.
60. Yu, H., et al., *Human infection with avian influenza A H7N9 virus: an assessment of clinical severity.* Lancet, 2013. **382**(9887): p. 138-45.
61. Jeong, O.M., et al., *Experimental infection of chickens, ducks and quails with the highly pathogenic H5N1 avian influenza virus.* J Vet Sci, 2009. **10**(1): p. 53-60.
62. Subbarao, K., et al., *Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness.* Science, 1998. **279**(5349): p. 393-6.
63. Fouchier, R.A., et al., *Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome.* Proc Natl Acad Sci U S A, 2004. **101**(5): p. 1356-61.
64. Manz, B., M. Schwemmle, and L. Brunotte, *Adaptation of avian influenza A virus polymerase in mammals to overcome the host species barrier.* J Virol, 2013. **87**(13): p. 7200-9.
65. Shi, Y., et al., *Enabling the 'host jump': structural determinants of receptor-binding specificity in influenza A viruses.* Nat Rev Microbiol, 2014. **12**(12): p. 822-31.
66. WHO. *Influenza fact sheet*. [cited 08.04.2016; Available from: http://www.who.int/mediacentre/factsheets/fs211/en/.
67. WHO. *Influenza Infections in Humans*. 2014 [cited 2014; Available from: http://www.who.int/influenza/en/.
68. WHO, c.d. *Communicable diseases, influenza*. 18.03.2016]; Available from: http://www.euro.who.int/en/health-topics/communicable-diseases/influenza/data-and-statistics, .
69. Keech, M., A.J. Scott, and P.J. Ryan, *The impact of influenza and influenza-like illness on productivity and healthcare resource utilization in a working population.* Occup Med (Lond), 1998. **48**(2): p. 85-90.
70. Lofgren, E., et al., *Influenza seasonality: underlying causes and modeling theories.* J Virol, 2007. **81**(11): p. 5429-36.
71. Tamerius, J., et al., *Global influenza seasonality: reconciling patterns across temperate and tropical regions.* Environ Health Perspect, 2011. **119**(4): p. 439-45.
72. Lowen, A.C. and J. Steel, *Roles of humidity and temperature in shaping influenza seasonality.* J Virol, 2014. **88**(14): p. 7692-5.
73. Taubenberger, J.K. and D.M. Morens, *1918 Influenza: the mother of all pandemics.* Emerg Infect Dis, 2006. **12**(1): p. 15-22.
74. Shaman, J., et al., *Absolute humidity and the seasonal onset of influenza in the continental United States.* PLoS Biol, 2010. **8**(2): p. e1000316.
75. Reid, A.H., et al., *Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene.* Proc Natl Acad Sci U S A, 1999. **96**(4): p. 1651-6.
76. Morens, D.M., J.K. Taubenberger, and A.S. Fauci, *Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness.* J Infect Dis, 2008. **198**(7): p. 962-70.
77. Doerr, H., Gerlich, W., *Medizinische Virologie*. 2 ed. 210.
78. Jennings, L.C., et al., *Stockpiling prepandemic influenza vaccines: a new cornerstone of pandemic preparedness plans.* Lancet Infect Dis, 2008. **8**(10): p. 650-8.
79. Kilbourne, E.D., *Influenza pandemics of the 20th century.* Emerg Infect Dis, 2006. **12**(1): p. 9-14.
80. Nakajima, K., U. Desselberger, and P. Palese, *Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950.* Nature, 1978. **274**(5669): p. 334-9.
81. WHO. *Weekly epidemiological record - 24. July 2009, 84th year*. 2009; Available from: http://www.who.int/wer/2009/wer8430/en/.
82. WHO. *Swine Flu - frequently asked questions*. 21.04.2016]; Available from: http://www.who.int/csr/disease/swineflu/frequently_asked_questions/about_disease/en/.
83. Peiris, J.S., W.W. Tu, and H.L. Yen, *A novel H1N1 virus causes the first pandemic of the 21st century.* Eur J Immunol, 2009. **39**(11): p. 2946-54.
84. ECDC. *ECDC Risk assessment 2009 pH1N1 Update 20. July 2009*. 2009; Available from: http://ecdc.europa.eu/en/publications/Publications/TER_ECDC_risk_assessment_2009_influenza_A(H1N1)_pandemic_Update_20%20July_2009.pdf.
85. Hancock, K., et al., *Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus.* N Engl J Med, 2009. **361**(20): p. 1945-52.

86. Verma, N., et al., *Influenza virus H1N1pdm09 infections in the young and old: evidence of greater antibody diversity and affinity for the hemagglutinin globular head domain (HA1 Domain) in the elderly than in young adults and children.* J Virol, 2012. **86**(10): p. 5515-22.
87. CDC. *MMWR - Intensive-Care Patients With Severe Novel Influenza A (H1N1) Virus Infection-Michigan, June 2009*. 2009; Available from: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5827a4.htm.
88. Dawood, F.S., et al., *Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study.* Lancet Infect Dis, 2012. **12**(9): p. 687-95.
89. Garten, R.J., et al., *Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans.* Science, 2009. **325**(5937): p. 197-201.
90. Haas, W., *Influenza - Prävention, Diagnostik, Therapie und öffentliche Gesundheit*. 2009.
91. Ergonul, O., et al., *Predictors of fatality in pandemic influenza A (H1N1) virus infection among adults.* BMC Infect Dis, 2014. **14**: p. 317.
92. *Critical care management of adults with influenza with particular reference to H1N1 (2009)*. 2011, Health Protection Agency.
93. Hui, D.S., N. Lee, and P.K. Chan, *Clinical management of pandemic 2009 influenza A(H1N1) infection.* Chest, 2010. **137**(4): p. 916-25.
94. Writing Committee of the, W.H.O.C.o.C.A.o.P.I., et al., *Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection.* N Engl J Med, 2010. **362**(18): p. 1708-19.
95. Cerbulo-Vazquez, A., et al., *Pregnant women infected with pandemic H1N1pdm2009 influenza virus displayed overproduction of peripheral blood CD69+ lymphocytes and increased levels of serum cytokines.* PLoS One, 2014. **9**(9): p. e107900.
96. Lieberman, R.W., et al., *Seasonal influenza A (H1N1) infection in early pregnancy and second trimester fetal demise.* Emerg Infect Dis, 2011. **17**(1): p. 107-9.
97. Bowkalow, S., et al., *Severe H1N1-infection during pregnancy.* Arch Gynecol Obstet, 2011. **284**(5): p. 1133-5.
98. in *Pregnancy and Pandemic Influenza A (H1N1) 2009: Information for Programme Managers and Clinicians*. 2010: Geneva.
99. Taubenberger, J.K. and D.M. Morens, *The pathology of influenza virus infections.* Annu Rev Pathol, 2008. **3**: p. 499-522.
100. Harper, S.A., et al., *Seasonal influenza in adults and children--diagnosis, treatment, chemoprophylaxis, and institutional outbreak management: clinical practice guidelines of the Infectious Diseases Society of America.* Clin Infect Dis, 2009. **48**(8): p. 1003-32.
101. Punpanich, W. and T. Chotpitayasunondh, *A review on the clinical spectrum and natural history of human influenza.* Int J Infect Dis, 2012. **16**(10): p. e714-23.
102. Memoli, M.J., et al., *Influenza in pregnancy.* Influenza Other Respir Viruses, 2013. **7**(6): p. 1033-9.
103. Hayden, F.G., et al., *Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense.* J Clin Invest, 1998. **101**(3): p. 643-9.
104. Eccles, R., *Understanding the symptoms of the common cold and influenza.* Lancet Infect Dis, 2005. **5**(11): p. 718-25.
105. Lahti, E., et al., *Influenza pneumonia.* Pediatr Infect Dis J, 2006. **25**(2): p. 160-4.
106. Chertow, D.S. and M.J. Memoli, *Bacterial coinfection in influenza: a grand rounds review.* JAMA, 2013. **309**(3): p. 275-82.
107. Gill, J.R., et al., *Pulmonary pathologic findings of fatal 2009 pandemic influenza A/H1N1 viral infections.* Arch Pathol Lab Med, 2010. **134**(2): p. 235-43.
108. Hoffken, G., et al., *[S3-guideline on ambulant acquired pneumonia and deep airway infections].* Pneumologie, 2005. **59**(9): p. 612-64.
109. Dolan, G.P., et al., *The comparative clinical course of pregnant and non-pregnant women hospitalised with influenza A(H1N1)pdm09 infection.* PLoS One, 2012. **7**(8): p. e41638.
110. Yudin, M.H., *Risk management of seasonal influenza during pregnancy: current perspectives.* Int J Womens Health, 2014. **6**: p. 681-9.
111. Steinhoff, M.C. and N.E. MacDonald, *Influenza pandemics--pregnancy, pathogenesis, and perinatal outcomes.* JAMA, 2012. **308**(2): p. 184-5.
112. Karlsson, E.A., et al., *Review on the impact of pregnancy and obesity on influenza virus infection.* Influenza Other Respir Viruses, 2012. **6**(6): p. 449-60.
113. Kuiken, T. and J.K. Taubenberger, *Pathology of human influenza revisited.* Vaccine, 2008. **26 Suppl 4**: p. D59-66.

114. Kash, J.C. and J.K. Taubenberger, *The role of viral, host, and secondary bacterial factors in influenza pathogenesis.* Am J Pathol, 2015. **185**(6): p. 1528-36.
115. Walsh, J.J., et al., *Bronchotracheal response in human influenza. Type A, Asian strain, as studied by light and electron microscopic examination of bronchoscopic biopsies.* Arch Intern Med, 1961. **108**: p. 376-88.
116. Raj, R.S., E.A. Bonney, and M. Phillippe, *Influenza, immune system, and pregnancy.* Reprod Sci, 2014. **21**(12): p. 1434-51.
117. CDC. *Flu diagnostics*. [cited 19.04.2016; Available from: http://www.cdc.gov/flu/professionals/diagnosis/rapidclin.htm#table.
118. Durbin, J.E., et al., *Type I IFN modulates innate and specific antiviral immunity.* J Immunol, 2000. **164**(8): p. 4220-8.
119. Kreijtz, J.H., R.A. Fouchier, and G.F. Rimmelzwaan, *Immune responses to influenza virus infection.* Virus Res, 2011. **162**(1-2): p. 19-30.
120. Pang, I.K. and A. Iwasaki, *Inflammasomes as mediators of immunity against influenza virus.* Trends Immunol, 2011. **32**(1): p. 34-41.
121. Kawai, T. and S. Akira, *Innate immune recognition of viral infection.* Nat Immunol, 2006. **7**(2): p. 131-7.
122. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.* Nature, 2001. **413**(6857): p. 732-8.
123. Heil, F., et al., *Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8.* Science, 2004. **303**(5663): p. 1526-9.
124. Lund, J.M., et al., *Recognition of single-stranded RNA viruses by Toll-like receptor 7.* Proc Natl Acad Sci U S A, 2004. **101**(15): p. 5598-603.
125. Sato, M., et al., *Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7.* FEBS Lett, 1998. **441**(1): p. 106-10.
126. Holzinger, D., et al., *Induction of MxA gene expression by influenza A virus requires type I or type III interferon signaling.* J Virol, 2007. **81**(14): p. 7776-85.
127. GeurtsvanKessel, C.H. and B.N. Lambrecht, *Division of labor between dendritic cell subsets of the lung.* Mucosal Immunol, 2008. **1**(6): p. 442-50.
128. Hintzen, G., et al., *Induction of tolerance to innocuous inhaled antigen relies on a CCR7-dependent dendritic cell-mediated antigen transport to the bronchial lymph node.* J Immunol, 2006. **177**(10): p. 7346-54.
129. GeurtsvanKessel, C.H., et al., *Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus-infected mice.* J Exp Med, 2009. **206**(11): p. 2339-49.
130. GeurtsvanKessel, C.H., et al., *Both conventional and interferon killer dendritic cells have antigen-presenting capacity during influenza virus infection.* PLoS One, 2009. **4**(9): p. e7187.
131. Perussia, B., V. Fanning, and G. Trinchieri, *A leukocyte subset bearing HLA-DR antigens is responsible for in vitro alpha interferon production in response to viruses.* Nat Immun Cell Growth Regul, 1985. **4**(3): p. 120-37.
132. Arnon, T.I., et al., *Recognition of viral hemagglutinins by NKp44 but not by NKp30.* Eur J Immunol, 2001. **31**(9): p. 2680-9.
133. Mandelboim, O., et al., *Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells.* Nature, 2001. **409**(6823): p. 1055-60.
134. Mancini, N., et al., *A potential role for monoclonal antibodies in prophylactic and therapeutic treatment of influenza.* Antiviral Res, 2011. **92**(1): p. 15-26.
135. Potter, C.W. and J.S. Oxford, *Determinants of immunity to influenza infection in man.* Br Med Bull, 1979. **35**(1): p. 69-75.
136. Gerhard, W., *The role of the antibody response in influenza virus infection.* Curr Top Microbiol Immunol, 2001. **260**: p. 171-90.
137. de Jong, J.C., et al., *Mismatch between the 1997/1998 influenza vaccine and the major epidemic A(H3N2) virus strain as the cause of an inadequate vaccine-induced antibody response to this strain in the elderly.* J Med Virol, 2000. **61**(1): p. 94-9.
138. Soghoian, D.Z. and H. Streeck, *Cytolytic CD4(+) T cells in viral immunity.* Expert Rev Vaccines, 2010. **9**(12): p. 1453-63.
139. Metkar, S.S., et al., *Human and mouse granzyme A induce a proinflammatory cytokine response.* Immunity, 2008. **29**(5): p. 720-33.
140. CDC. *Antivirals - summary-clinicans.* 19.04.2016].
141. Poland, G.A., R.M. Jacobson, and I.G. Ovsyannikova, *Influenza virus resistance to antiviral agents: a plea for rational use.* Clin Infect Dis, 2009. **48**(9): p. 1254-6.
142. ECDC. *Seasonal influenza vaccines*. 20.04.2016]; Available from: http://ecdc.europa.eu/en/healthtopics/seasonal_influenza/vaccines/Pages/influenza_vaccination.aspx.

143. WHO. *Vaccine recommendations 2015/2016*. 2015 20.04.2016]; Available from: http://www.who.int/influenza/vaccines/virus/recommendations/2015_16_north/en/.
144. Palese, P., *Making better influenza virus vaccines?* Emerg Infect Dis, 2006. **12**(1): p. 61-5.
145. Sasaki, S., et al., *Limited efficacy of inactivated influenza vaccine in elderly individuals is associated with decreased production of vaccine-specific antibodies.* J Clin Invest, 2011. **121**(8): p. 3109-19.
146. Bancos, S., et al., *Ibuprofen and other widely used non-steroidal anti-inflammatory drugs inhibit antibody production in human cells.* Cell Immunol, 2009. **258**(1): p. 18-28.
147. Grijalva, C.G., et al., *Association Between Hospitalization With Community-Acquired Laboratory-Confirmed Influenza Pneumonia and Prior Receipt of Influenza Vaccination.* JAMA, 2015. **314**(14): p. 1488-97.
148. Pica, N. and P. Palese, *Toward a universal influenza virus vaccine: prospects and challenges.* Annu Rev Med, 2013. **64**: p. 189-202.
149. Trundley, A. and A. Moffett, *Human uterine leukocytes and pregnancy.* Tissue Antigens, 2004. **63**(1): p. 1-12.
150. Veenstra van Nieuwenhoven, A.L., et al., *Endotoxin-induced cytokine production of monocytes of third-trimester pregnant women compared with women in the follicular phase of the menstrual cycle.* Am J Obstet Gynecol, 2003. **188**(4): p. 1073-7.
151. Gabriel, G. and P.C. Arck, *Sex, immunity and influenza.* J Infect Dis, 2014. **209 Suppl 3**: p. S93-9.
152. Mor, G. and I. Cardenas, *The immune system in pregnancy: a unique complexity.* Am J Reprod Immunol, 2010. **63**(6): p. 425-33.
153. Dekel, N., et al., *Inflammation and implantation.* Am J Reprod Immunol, 2010. **63**(1): p. 17-21.
154. Romero, R., et al., *The preterm parturition syndrome.* BJOG, 2006. **113 Suppl 3**: p. 17-42.
155. Romero, R., et al., *Inflammation in preterm and term labour and delivery.* Semin Fetal Neonatal Med, 2006. **11**(5): p. 317-26.
156. Nagamatsu, T. and D.J. Schust, *The immunomodulatory roles of macrophages at the maternal-fetal interface.* Reprod Sci, 2010. **17**(3): p. 209-18.
157. Lash, G.E., S.C. Robson, and J.N. Bulmer, *Review: Functional role of uterine natural killer (uNK) cells in human early pregnancy decidua.* Placenta, 2010. **31 Suppl**: p. S87-92.
158. Shimada, S., et al., *Natural killer, natural killer T, helper and cytotoxic T cells in the decidua from sporadic miscarriage.* Am J Reprod Immunol, 2006. **56**(3): p. 193-200.
159. Ashkar, A.A., J.P. Di Santo, and B.A. Croy, *Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy.* J Exp Med, 2000. **192**(2): p. 259-70.
160. Hanna, J., et al., *Decidual NK cells regulate key developmental processes at the human fetal-maternal interface.* Nat Med, 2006. **12**(9): p. 1065-74.
161. Williams, P.J., et al., *Decidual leucocyte populations in early to late gestation normal human pregnancy.* J Reprod Immunol, 2009. **82**(1): p. 24-31.
162. Aluvihare, V.R., M. Kallikourdis, and A.G. Betz, *Regulatory T cells mediate maternal tolerance to the fetus.* Nat Immunol, 2004. **5**(3): p. 266-71.
163. Bulmer, J.N., D. Pace, and A. Ritson, *Immunoregulatory cells in human decidua: morphology, immunohistochemistry and function.* Reprod Nutr Dev, 1988. **28**(6B): p. 1599-613.
164. Sacks, G.P., et al., *Flow cytometric measurement of intracellular Th1 and Th2 cytokine production by human villous and extravillous cytotrophoblast.* Placenta, 2001. **22**(6): p. 550-9.
165. Bianchi, D.W., et al., *Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum.* Proc Natl Acad Sci U S A, 1996. **93**(2): p. 705-8.
166. Sargent, I.L., *Maternal and fetal immune responses during pregnancy.* Exp Clin Immunogenet, 1993. **10**(2): p. 85-102.
167. Davis, D., R. Kaufmann, and E.J. Moticka, *Nonspecific immunity in pregnancy: monocyte surface Fcgamma receptor expression and function.* J Reprod Immunol, 1998. **40**(2): p. 119-28.
168. Naccasha, N., et al., *Phenotypic and metabolic characteristics of monocytes and granulocytes in normal pregnancy and maternal infection.* Am J Obstet Gynecol, 2001. **185**(5): p. 1118-23.
169. Luppi, P., et al., *Monocytes are progressively activated in the circulation of pregnant women.* J Leukoc Biol, 2002. **72**(5): p. 874-84.
170. Ueda, Y., et al., *Frequencies of dendritic cells (myeloid DC and plasmacytoid DC) and their ratio reduced in pregnant women: comparison with umbilical cord blood and normal healthy adults.* Hum Immunol, 2003. **64**(12): p. 1144-51.
171. Watanabe, M., et al., *Changes in T, B, and NK lymphocyte subsets during and after normal pregnancy.* Am J Reprod Immunol, 1997. **37**(5): p. 368-77.

172. Kuhnert, M., et al., *Changes in lymphocyte subsets during normal pregnancy.* Eur J Obstet Gynecol Reprod Biol, 1998. **76**(2): p. 147-51.
173. Beer, A.E., J.Y. Kwak, and J.E. Ruiz, *Immunophenotypic profiles of peripheral blood lymphocytes in women with recurrent pregnancy losses and in infertile women with multiple failed in vitro fertilization cycles.* Am J Reprod Immunol, 1996. **35**(4): p. 376-82.
174. Wegmann, T.G., et al., *Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon?* Immunol Today, 1993. **14**(7): p. 353-6.
175. Littman, D.R. and A.Y. Rudensky, *Th17 and regulatory T cells in mediating and restraining inflammation.* Cell, 2010. **140**(6): p. 845-58.
176. Saito, S., et al., *Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy.* Am J Reprod Immunol, 2010. **63**(6): p. 601-10.
177. Somerset, D.A., et al., *Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset.* Immunology, 2004. **112**(1): p. 38-43.
178. Sasaki, Y., et al., *Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases.* Mol Hum Reprod, 2004. **10**(5): p. 347-53.
179. Nancy, P., et al., *Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface.* Science, 2012. **336**(6086): p. 1317-21.
180. Ostensen, M., B. Aune, and G. Husby, *Effect of pregnancy and hormonal changes on the activity of rheumatoid arthritis.* Scand J Rheumatol, 1983. **12**(2): p. 69-72.
181. Varner, M.W., *Autoimmune disorders and pregnancy.* Semin Perinatol, 1991. **15**(3): p. 238-50.
182. Kourtis, A.P., J.S. Read, and D.J. Jamieson, *Pregnancy and infection.* N Engl J Med, 2014. **371**(11): p. 1077.
183. Klein, S. *Sex, gender and influenza*. 2010.
184. Goodnight, W.H. and D.E. Soper, *Pneumonia in pregnancy.* Crit Care Med, 2005. **33**(10 Suppl): p. S390-7.
185. Laibl, V.R. and J.S. Sheffield, *Influenza and pneumonia in pregnancy.* Clin Perinatol, 2005. **32**(3): p. 727-38.
186. el-Solh, A.A. and B.J. Grant, *A comparison of severity of illness scoring systems for critically ill obstetric patients.* Chest, 1996. **110**(5): p. 1299-304.
187. Mighty, H.E., *Acute respiratory failure in pregnancy.* Clin Obstet Gynecol, 2010. **53**(2): p. 360-8.
188. Neuzil, K.M., et al., *Impact of influenza on acute cardiopulmonary hospitalizations in pregnant women.* Am J Epidemiol, 1998. **148**(11): p. 1094-102.
189. Cox, S., et al., *Hospitalizations with respiratory illness among pregnant women during influenza season.* Obstet Gynecol, 2006. **107**(6): p. 1315-22.
190. Dodds, L., et al., *Impact of influenza exposure on rates of hospital admissions and physician visits because of respiratory illness among pregnant women.* CMAJ, 2007. **176**(4): p. 463-8.
191. Harris, J.W., *Influenza occuring in pregnant women - a statistical study of thirteen hundred and fifty cases.* JAMA, 1919. **72**(14): p. 978-980.
192. Freeman, D.W. and A. Barno, *Deaths from Asian influenza associated with pregnancy.* Am J Obstet Gynecol, 1959. **78**: p. 1172-5.
193. Rasmussen, S.A., D.J. Jamieson, and T.M. Uyeki, *Effects of influenza on pregnant women and infants.* Am J Obstet Gynecol, 2012. **207**(3 Suppl): p. S3-8.
194. Siston, A.M., et al., *Pandemic 2009 influenza A(H1N1) virus illness among pregnant women in the United States.* JAMA, 2010. **303**(15): p. 1517-25.
195. Louie, J.K., et al., *Severe 2009 H1N1 influenza in pregnant and postpartum women in California.* N Engl J Med, 2010. **362**(1): p. 27-35.
196. Creanga, A.A., et al., *Severity of 2009 pandemic influenza A (H1N1) virus infection in pregnant women.* Obstet Gynecol, 2010. **115**(4): p. 717-26.
197. Michaan, N., et al., *Maternal and neonatal outcome of pregnant women infected with H1N1 influenza virus (swine flu).* J Matern Fetal Neonatal Med, 2012. **25**(2): p. 130-2.
198. Pierce, M., et al., *Perinatal outcomes after maternal 2009/H1N1 infection: national cohort study.* BMJ, 2011. **342**: p. d3214.
199. Yates, L., et al., *Influenza A/H1N1v in pregnancy: an investigation of the characteristics and management of affected women and the relationship to pregnancy outcomes for mother and infant.* Health Technol Assess, 2010. **14**(34): p. 109-82.
200. CDC. *MMWR - Maternal and infant outcomes among severily ill pregnant and postpartum women with 2009 pandemic influenza A*. 2010; Available from: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6035a2.htm.
201. Kanmaz, H.G., et al., *Placental transmission of novel pandemic influenza a virus.* Fetal Pediatr Pathol, 2011. **30**(5): p. 280-5.

202. Irving, W.L., et al., *Influenza virus infection in the second and third trimesters of pregnancy: a clinical and seroepidemiological study.* BJOG, 2000. **107**(10): p. 1282-9.
203. Meijer, W.J., et al., *High rate of chronic villitis in placentas of pregnancies complicated by influenza A/H1N1 infection.* Infect Dis Obstet Gynecol, 2014. **2014**: p. 768380.
204. Bal, A., et al., *Pathology and virology findings in cases of fatal influenza A H1N1 virus infection in 2009-2010.* Histopathology, 2012. **60**(2): p. 326-35.
205. Buchy, P., et al., *Influenza A/H5N1 virus infection in humans in Cambodia.* J Clin Virol, 2007. **39**(3): p. 164-8.
206. Gu, J., et al., *H5N1 infection of the respiratory tract and beyond: a molecular pathology study.* Lancet, 2007. **370**(9593): p. 1137-45.
207. Hardy, J.M., et al., *The effect of Asian influenza on the outcome of pregnancy, Baltimore, 1957-1958.* Am J Public Health Nations Health, 1961. **51**: p. 1182-8.
208. Saxen, L., et al., *Asian influenza during pregnancy and congenital malformations.* Acta Pathol Microbiol Scand, 1960. **49**: p. 114-26.
209. Acs, N., et al., *Pregnancy complications and delivery outcomes of pregnant women with influenza.* J Matern Fetal Neonatal Med, 2006. **19**(3): p. 135-40.
210. Acs, N., et al., *Maternal influenza during pregnancy and risk of congenital abnormalities in offspring.* Birth Defects Res A Clin Mol Teratol, 2005. **73**(12): p. 989-96.
211. Kwan, M.L., et al., *Maternal illness and drug/medication use during the period surrounding pregnancy and risk of childhood leukemia among offspring.* Am J Epidemiol, 2007. **165**(1): p. 27-35.
212. Ebert, T. and M. Kotler, *Prenatal exposure to influenza and the risk of subsequent development of schizophrenia.* Isr Med Assoc J, 2005. **7**(1): p. 35-8.
213. Takahashi, M. and T. Yamada, *A possible role of influenza A virus infection for Parkinson's disease.* Adv Neurol, 2001. **86**: p. 91-104.
214. Edwards, M.J., *Review: Hyperthermia and fever during pregnancy.* Birth Defects Res A Clin Mol Teratol, 2006. **76**(7): p. 507-16.
215. Moretti, M.E., et al., *Maternal hyperthermia and the risk for neural tube defects in offspring: systematic review and meta-analysis.* Epidemiology, 2005. **16**(2): p. 216-9.
216. Oster, M.E., et al., *Associations between maternal fever and influenza and congenital heart defects.* J Pediatr, 2011. **158**(6): p. 990-5.
217. Barker, D.J., et al., *The early origins of chronic heart failure: impaired placental growth and initiation of insulin resistance in childhood.* Eur J Heart Fail, 2010. **12**(8): p. 819-25.
218. Velten, M., et al., *Prenatal inflammation exacerbates hyperoxia-induced functional and structural changes in adult mice.* Am J Physiol Regul Integr Comp Physiol, 2012. **303**(3): p. R279-90.
219. Institut, R.K. *Änderung und Empfehlung zur Impfung der Influenza*. Epid Bull 2010; Available from: https://www.rki.de/DE/Content/Infekt/EpidBull/Archiv/2010/Ausgaben/31_10.pdf?__blob=publicationFile.
220. Yawn, D.H., et al., *Transplacental transfer of influenza virus.* JAMA, 1971. **216**(6): p. 1022-3.
221. Rosztoczy, I., et al., *Replication of influenza virus in organ cultures of human and simian urogenital tissues and human foetal tissues.* Br J Exp Pathol, 1975. **56**(4): p. 322-8.
222. Vanders, R.L., et al., *Plasmacytoid dendritic cells and CD8 T cells from pregnant women show altered phenotype and function following H1N1/09 infection.* J Infect Dis, 2013. **208**(7): p. 1062-70.
223. Young, B.C., et al., *Longitudinal expression of Toll-like receptors on dendritic cells in uncomplicated pregnancy and postpartum.* Am J Obstet Gynecol, 2014. **210**(5): p. 445 e1-6.
224. Uchide, N., et al., *Induction of pro-inflammatory cytokine gene expression and apoptosis in human chorion cells of fetal membranes by influenza virus infection: possible implications for maintenance and interruption of pregnancy during infection.* Med Sci Monit, 2005. **11**(1): p. RA7-16.
225. Kraus, T.A., et al., *Peripheral blood cytokine profiling during pregnancy and post-partum periods.* Am J Reprod Immunol, 2010. **64**(6): p. 411-26.
226. Robinson, D.P. and S.L. Klein, *Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis.* Horm Behav, 2012. **62**(3): p. 263-71.
227. Robinson, D.P., et al., *Elevated 17beta-estradiol protects females from influenza A virus pathogenesis by suppressing inflammatory responses.* PLoS Pathog, 2011. **7**(7): p. e1002149.
228. Pazos, M.A., et al., *Estrogen mediates innate and adaptive immune alterations to influenza infection in pregnant mice.* PLoS One, 2012. **7**(7): p. e40502.
229. Williams, K. and J.S. Mackenzie, *Influenza infections during pregnancy in the mouse.* J Hyg (Lond), 1977. **79**(2): p. 249-57.
230. Chan, K.H., et al., *Wild type and mutant 2009 pandemic influenza A (H1N1) viruses cause more severe disease and higher mortality in pregnant BALB/c mice.* PLoS One, 2010. **5**(10): p. e13757.

231. Kim, H.M., et al., *The 2009 pandemic H1N1 influenza virus is more pathogenic in pregnant mice than seasonal H1N1 influenza virus.* Viral Immunol, 2012. **25**(5): p. 402-10.
232. Marcelin, G., et al., *Fatal outcome of pandemic H1N1 2009 influenza virus infection is associated with immunopathology and impaired lung repair, not enhanced viral burden, in pregnant mice.* J Virol, 2011. **85**(21): p. 11208-19.
233. Bodeker, B., et al., *Cross-sectional study on factors associated with influenza vaccine uptake and pertussis vaccination status among pregnant women in Germany.* Vaccine, 2014. **32**(33): p. 4131-9.
234. Yuen, C.Y. and M. Tarrant, *Determinants of uptake of influenza vaccination among pregnant women - a systematic review.* Vaccine, 2014. **32**(36): p. 4602-13.
235. Zaman, K., et al., *Effectiveness of maternal influenza immunization in mothers and infants.* N Engl J Med, 2008. **359**(15): p. 1555-64.
236. Haberg, S.E., et al., *Risk of fetal death after pandemic influenza virus infection or vaccination.* N Engl J Med, 2013. **368**(4): p. 333-40.
237. Who, *Meeting of the Strategic Advisory Group of Experts on immunization, October 2009--Conclusions and recommendations.* Biologicals, 2010. **38**(1): p. 170-7.
238. CDC. *MMWR - Influenza Vaccination Coverage Among Pregnant Women - United States*. 2013; Available from: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6238a3.htm.
239. Bodeker, B., C. Betsch, and O. Wichmann, *Skewed risk perceptions in pregnant women: the case of influenza vaccination.* BMC Public Health, 2016. **16**(1): p. 1308.
240. Poehling, K.A., et al., *The underrecognized burden of influenza in young children.* N Engl J Med, 2006. **355**(1): p. 31-40.
241. Puck, J.M., et al., *Protection of infants from infection with influenza A virus by transplacentally acquired antibody.* J Infect Dis, 1980. **142**(6): p. 844-9.
242. Puleston, R.L., et al., *Observational study to investigate vertically acquired passive immunity in babies of mothers vaccinated against H1N1v during pregnancy.* Health Technol Assess, 2010. **14**(55): p. 1-82.
243. Reuman, P.D., E.M. Ayoub, and P.A. Small, *Effect of passive maternal antibody on influenza illness in children: a prospective study of influenza A in mother-infant pairs.* Pediatr Infect Dis J, 1987. **6**(4): p. 398-403.
244. Zuccotti, G., et al., *Transplacental antibody transfer following maternal immunization with a pandemic 2009 influenza A(H1N1) MF59-adjuvanted vaccine.* JAMA, 2010. **304**(21): p. 2360-1.
245. CDC. *Flu season 2015-2016*. 2015 06.05.2016]; Available from: http://www.cdc.gov/flu/about/season/flu-season-2015-2016.htm.
246. Sedgh, G., S. Singh, and R. Hussain, *Intended and unintended pregnancies worldwide in 2012 and recent trends.* Stud Fam Plann, 2014. **45**(3): p. 301-14.
247. Hoffmann, E., et al., *Characterization of the influenza A virus gene pool in avian species in southern China: was H6N1 a derivative or a precursor of H5N1?* J Virol, 2000. **74**(14): p. 6309-15.
248. Gabriel, G., et al., *Differential use of importin-α isoforms governs cell tropism and host adaptation of influenza virus.* Nature communications, 2011. **2**: p. 156.
249. Reed LJ, M.H., *A simple method of estimating fifty per cent endpoints.* The American Journal of Hygiene, 1938. **27**(3): p. 493-497.
250. Eisfeld, A.J., G. Neumann, and Y. Kawaoka, *Influenza A virus isolation, culture and identification.* Nat Protoc, 2014. **9**(11): p. 2663-81.
251. Matrosovich, M., et al., *New low-viscosity overlay medium for viral plaque assays.* Virol J, 2006. **3**: p. 63.
252. Hackenberg, A., et al., *Antibody prevalence to the 2009 pandemic influenza A (H1N1) virus in Germany: geographically variable immunity in winter 2010/2011.* Medical Microbiology and Immunology, 2013. **202**(1): p. 87-94.
253. Reed, L.H. and H. Muench, *A simple method of estimating fifty percent endpoints.* American Journal of Hygiene, 1938. **27**: p. 493-97.
254. Langmead, B. and S.L. Salzberg, *Fast gapped-read alignment with Bowtie 2.* Nat Methods, 2012. **9**(4): p. 357-9.
255. Li, H. and R. Durbin, *Fast and accurate short read alignment with Burrows-Wheeler transform.* Bioinformatics, 2009. **25**(14): p. 1754-60.
256. Li, H., et al., *The Sequence Alignment/Map format and SAMtools.* Bioinformatics, 2009. **25**(16): p. 2078-9.
257. Koboldt, D.C., et al., *VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing.* Genome Res, 2012. **22**(3): p. 568-76.
258. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Research, 2001. **29**(9).

259. Hoffmann, E., et al., *A DNA transfection system for generation of influenza A virus from eight plasmids.* Proc Natl Acad Sci U S A, 2000. **97**(11): p. 6108-13.
260. Nakanishi, M. and M. Otsu, *Development of Sendai virus vectors and their potential applications in gene therapy and regenerative medicine.* Curr Gene Ther, 2012. **12**(5): p. 410-6.
261. Strahle, L., D. Garcin, and D. Kolakofsky, *Sendai virus defective-interfering genomes and the activation of interferon-beta.* Virology, 2006. **351**(1): p. 101-111.
262. Diemert, A., et al., *Fetal thymus size in human pregnancies reveals inverse association with regulatory T cell frequencies in cord blood.* J Reprod Immunol, 2016. **113**: p. 76-82.
263. Gabriel, G., et al., *The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18590-5.
264. Arck, P., et al., *Progesterone during pregnancy: endocrine-immune cross talk in mammalian species and the role of stress.* Am J Reprod Immunol, 2007. **58**(3): p. 268-79.
265. Schneider, W.M., M.D. Chevillotte, and C.M. Rice, *Interferon-stimulated genes: a complex web of host defenses.* Annu Rev Immunol, 2014. **32**: p. 513-45.
266. Garcia-Sastre, A. and C.A. Biron, *Type 1 interferons and the virus-host relationship: a lesson in detente.* Science, 2006. **312**(5775): p. 879-82.
267. Iwasaki, A. and P.S. Pillai, *Innate immunity to influenza virus infection.* Nat Rev Immunol, 2014. **14**(5): p. 315-28.
268. Tilburgs, T. and J.L. Strominger, *CD8+ effector T cells at the fetal-maternal interface, balancing fetal tolerance and antiviral immunity.* Am J Reprod Immunol, 2013. **69**(4): p. 395-407.
269. Gabriel, G. and E. Fodor, *Molecular determinants of pathogenicity in the polymerase complex.* Curr Top Microbiol Immunol, 2014. **385**: p. 35-60.
270. Chen, Z., et al., *Generation of live attenuated novel influenza virus A/California/7/09 (H1N1) vaccines with high yield in embryonated chicken eggs.* J Virol, 2010. **84**(1): p. 44-51.
271. Areste, C., M. Mutocheluh, and D.J. Blackbourn, *Identification of caspase-mediated decay of interferon regulatory factor-3, exploited by a Kaposi sarcoma-associated herpesvirus immunoregulatory protein.* J Biol Chem, 2009. **284**(35): p. 23272-85.
272. Otte, A. and G. Gabriel, *2009 pandemic H1N1 influenza A virus strains display differential pathogenicity in C57BL/6J but not BALB/c mice.* Virulence, 2011. **2**(6): p. 563-6.
273. Hasegawa, S., et al., *Serum and cerebrospinal fluid cytokine profile of patients with 2009 pandemic H1N1 influenza virus-associated encephalopathy.* Cytokine, 2011. **54**(2): p. 167-72.
274. Kimura, H., et al., *Cytokine production and signaling pathways in respiratory virus infection.* Front Microbiol, 2013. **4**: p. 276.
275. Khaitov, M.R., et al., *Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells.* Allergy, 2009. **64**(3): p. 375-86.
276. Kawai, T. and S. Akira, *TLR signaling.* Semin Immunol, 2007. **19**(1): p. 24-32.
277. Jewell, N.A., et al., *Lambda interferon is the predominant interferon induced by influenza A virus infection in vivo.* J Virol, 2010. **84**(21): p. 11515-22.
278. Chawla-Sarkar, M., et al., *Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis.* Apoptosis, 2003. **8**(3): p. 237-49.
279. Forbes, R.L., et al., *Pregnant women have attenuated innate interferon responses to 2009 pandemic influenza A virus subtype H1N1.* J Infect Dis, 2012. **206**(5): p. 646-53.
280. Staeheli, P., et al., *Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus.* Cell, 1986. **44**(1): p. 147-58.
281. Bonecchi, R., et al., *Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s.* J Exp Med, 1998. **187**(1): p. 129-34.
282. Inngjerdingen, M., B. Damaj, and A.A. Maghazachi, *Expression and regulation of chemokine receptors in human natural killer cells.* Blood, 2001. **97**(2): p. 367-75.
283. Sallusto, F., et al., *Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes.* J Exp Med, 1998. **187**(6): p. 875-83.
284. Veckman, V., et al., *TNF-alpha and IFN-alpha enhance influenza-A-virus-induced chemokine gene expression in human A549 lung epithelial cells.* Virology, 2006. **345**(1): p. 96-104.
285. Hemann, E.A., et al., *Plasmacytoid Dendritic Cells Require Direct Infection To Sustain the Pulmonary Influenza A Virus-Specific CD8 T Cell Response.* J Virol, 2015. **90**(6): p. 2830-7.
286. Duan, S. and P.G. Thomas, *Balancing Immune Protection and Immune Pathology by CD8(+) T-Cell Responses to Influenza Infection.* Front Immunol, 2016. **7**: p. 25.
287. Griffith, J.W., C.L. Sokol, and A.D. Luster, *Chemokines and chemokine receptors: positioning cells for host defense and immunity.* Annu Rev Immunol, 2014. **32**: p. 659-702.
288. Ward, S.G. and F.M. Marelli-Berg, *Mechanisms of chemokine and antigen-dependent T-lymphocyte navigation.* Biochem J, 2009. **418**(1): p. 13-27.

289. Lim, K., et al., *Neutrophil trails guide influenza-specific CD8(+) T cells in the airways.* Science, 2015. **349**(6252): p. aaa4352.
290. Damjanovic, D., et al., *Immunopathology in influenza virus infection: uncoupling the friend from foe.* Clin Immunol, 2012. **144**(1): p. 57-69.
291. Gamblin, S.J., et al., *The structure and receptor binding properties of the 1918 influenza hemagglutinin.* Science, 2004. **303**(5665): p. 1838-42.
292. Yasugi, M., et al., *Frequency of D222G and Q223R hemagglutinin mutants of pandemic (H1N1) 2009 influenza virus in Japan between 2009 and 2010.* PLoS One, 2012. **7**(2): p. e30946.
293. Wu, C., et al., *Clinical and molecular characteristics of the 2009 pandemic influenza H1N1 infection with severe or fatal disease from 2009 to 2011 in Shenzhen, China.* J Med Virol, 2013. **85**(3): p. 405-12.
294. Potdar, V.A., et al., *Genetic characterization of the influenza A pandemic (H1N1) 2009 virus isolates from India.* PLoS One, 2010. **5**(3): p. e9693.
295. Neumann, G. and Y. Kawaoka, *Transmission of influenza A viruses.* Virology, 2015. **479-480**: p. 234-46.
296. Tuyishime, J.D., et al., *Influenza-like illness during pregnancy: results from a study in the eastern townships, Province of Quebec.* J Obstet Gynaecol Can, 2003. **25**(12): p. 1020-5.
297. Huo, X., et al., *Seroprevalence of pandemic (H1N1) 2009 in pregnant women in China: an observational study.* PLoS One, 2011. **6**(3): p. e17995.
298. Honarvar, B., et al., *Titering of 2009 pandemic H1N1 influenza virus hemagglutinin inhibition antibody in nonvaccinated pregnant women in Shiraz, Southern Iran.* Hum Vaccin Immunother, 2012. **8**(5): p. 604-11.
299. Flahault, A., *First estimation of direct H1N1pdm virulence: From reported non consolidated data from Mauritius and New Caledonia.* PLoS Curr, 2009. **1**: p. RRN1010.
300. Mahmud, S.M., et al., *Estimated cumulative incidence of pandemic (H1N1) influenza among pregnant women during the first wave of the 2009 pandemic.* CMAJ, 2010. **182**(14): p. 1522-4.
301. Dowse, G.K., et al., *Incidence of pandemic (H1N1) 2009 influenza infection in children and pregnant women during the 2009 influenza season in Western Australia - a seroprevalence study.* Med J Aust, 2011. **194**(2): p. 68-72.
302. Tan, S., et al., *Serological responses following influenza A H1N1 2009 infection in adults.* J Infect, 2011. **62**(5): p. 388-93.
303. Ohmit, S.E., et al., *Influenza hemagglutination-inhibition antibody titer as a correlate of vaccine-induced protection.* J Infect Dis, 2011. **204**(12): p. 1879-85.
304. Czeizel, A.E., D. Petik, and P. Vargha, *Validation studies of drug exposures in pregnant women.* Pharmacoepidemiol Drug Saf, 2003. **12**(5): p. 409-16.
305. Natland, S.T., et al., *Maternal recall of breastfeeding duration twenty years after delivery.* BMC Med Res Methodol, 2012. **12**: p. 179.
306. Ahluwalia, I.B., et al., *Correlates of seasonal influenza vaccine coverage among pregnant women in Georgia and Rhode Island.* Obstet Gynecol, 2010. **116**(4): p. 949-55.
307. Shavell, V.I., et al., *Influenza immunization in pregnancy: overcoming patient and health care provider barriers.* Am J Obstet Gynecol, 2012. **207**(3 Suppl): p. S67-74.
308. Yeager, D.P., E.C. Toy, and B. Baker, 3rd, *Influenza vaccination in pregnancy.* Am J Perinatol, 1999. **16**(6): p. 283-6.

9 Acknowledgement

First and foremost, I would like to thank Prof. Dr. Petra Arck and Prof. Dr. Gülsah Gabriel for giving me the possibility to work on the highly relevant and exciting topic of Influenza during pregnancy in a unique collaboration between the UKE and the Heinrich Pette Institute. I would furthermore like to thank Prof. Dr. Petra Arck and Prof. Dr. Gülsah Gabriel for their constant support and encouragement and especially for their care in increasing my scientific skills and abilities.
I would like to thank Prof. Dr. Hans-Willi Mittrücker and Prof. Dr. Gisa Tiegs for being part of my thesis committee and hereby providing fruitful ideas and kind support.
A special thank goes to Dr. Julia Hoffmann and Dr. René Thieme who have started and worked on this project and have introduced me to the topic. Alexandra Hierweger and Susanne Spoerel joined the project more recently; nevertheless I highly appreciate their help and contribution.
I want to thank the whole AG Arck lab – thank you all for your help and the many moments of laughter. Special thanks go to Agnes Wieczorek, Christopher Urbschat and Thomas Andreas for their exceptional technical assistance! I furthermore want to thank Dr. Kristin Thiele and (soon to be Dr.) Ina Stelzer for the fruitful discussions and input considering my thesis.
I also want to thank the whole Gabriel group where I spent most of my time. Thank you for the constant support and constructive discussions – not only work related. I especially want to thank Dr. Swantje Thiele for being such a pleasant person to share the office with. Thank you, Swantje, for listening, explaining me so many things and for proof-reading of my dissertation. I furthermore want to thank Annette Preuß and Gundula Pilnitz-Stolze for their excellent technical help and even more – their warmth and heartiness. A special thank you goes to Carola Dreier for not only having an open ear and supporting me in the lab but also for becoming my friend.
I would also like to thank the following collaborations partners for their help and support: Prof. Dr. Marcus Altfeld (HPI) and his group for their help with the Luminex measurements and the Next Generation Sequencing technology platform headed by Prof. Dr. Adam Grundhoff (HPI). I furthermore want to thank the PRINCE team headed by Dr. Anke Diemert. Especially Dr. Janina Goletzke was a huge help in providing me with information about the pregnant women but always had an open ear for many other things.
Without the financial support of the *Deutsches Zentrum für Infektionsforschung* (DZIF), the *Fonds Nationale de la Recherche Luxembourg* (AFR 6042498) and the *Boehringer Ingelheim Foundation* this thesis would not have been possible.

Finally, I owe my deepest gratitude to my family and my friends for their constant encouragement and unconditional support.
Last but not least I want to thank you, Felix – for your critical questions, your enormous interest and your love. It means everything to me.

10 Curriculum vitae

Omitted for privacy reasons.

11 Publications, oral presentations, posters, scholarships and awards

Publications:

Parts of this PhD thesis have been summarized and have been submitted in the following publication:
Geraldine Engels*, Julia Hoffmann*, René Thieme*, Alexandra Hierweger, Patricia Resa-Infante, Malik Alawi, Adam Grundhoff, Daniela Indenbirken, Michael N. Sirignano, Damián Muzzio, Federico Jensen, Marcus Altfeld, Khalil Karimi, Hans-Willi Mittrücker, Petra Clara Arck# & Gülsah Gabriel#. "Immunity's pregnant pause facing influenza: insights from mouse models". *Cell Host & Microbes, in revision.*
* authors contributed equally, # authors supervised jointly

Debby van Riel, Hans-Willi Mittrücker, **Geraldine Engels**, Karin Klingel, Udo R. Markert and Gülsah Gabriel. „Influenza pathogenicity during pregnancy in women and animal models". *Seminars in Immunopathology 2016.*

Further publications:

Aliz Barakonyi, Eva Miko, Laszlo Szereday, Petra Dora Polgar, Timea Nemeth, Julia Szekeres-Bartho, **Geraldine L. Engels**: Cell death mechanisms and potentially cytotoxic natural immune cells in human pregnancies complicated by preeclampsia*. Reproductive Sciences 2013.*

Carolin Tarnow, **Geraldine L. Engels**, Annika Arendt, Folker Schwalm, Hanna Sediri, Annette Preuss, Peter S. Nelson, Wolfgang Garten, Hans-Dieter Klenk, Gülsah Gabriel and Eva Böttcher-Friebertshäuser: TMPRSS2 is a host factor that is essential for pneumotropism and pathogenicity of H7N9 influenza A virus in mice. *Journal of Virology 2014*

Oral presentations:

Geraldine L. Engels, René Thieme, Julia Hoffmann, Hans-Willi Mittrücker, Khalil Karimi, Gülsah Gabriel, Petra C. Arck:
Influenza a virus infection and pregnancy: reduced ability of leukocyte homing to the lung and high virus mutation rate account for higher morbidity and mortality during pregnancy in mice.
11th Congress of the European and Hungarian Society for Reproductive Immunology, March 2014, Budapest, Hungary

Geraldine Engels, René Thieme, Julia Hoffmann, Hans-Willi Mittrücker, Khalil Karimi, Petra C. Arck, Gülsah Gabriel:
Influenza A virus Infection during Pregnancy: Course and Impact.
Institute of Virology, University of Marburg, May 2014, Marburg, Germany

Geraldine Engels, René Thieme, Julia Hoffmann, Hans-Willi Mittrücker, Khalil Karimi, Petra C. Arck, Gülsah Gabriel:
Influenza and pregnancy: Identification of viral and immunological determinants of disease severity.
HPI Joint Scientific Retreat, October 2014, Hamburg, Germany

Geraldine Engels, Gülsah Gabriel:
Viral Infections in Pregnancy.
HPI 3rd Young Scientist Retreat, October 2014, Ammersbek, Germany

Geraldine L. Engels, Julia Hoffmann, René Thieme, Malik Alawi, Daniela Indenbirken, Adam Grundhoff, Hans-Willi Mittrücker, Gülsah Gabriel, Petra C. Arck: Infection with the 2009 pandemic H1N1 influenza virus during pregnancy promotes viral escape via rapid emergence of influenza virus mutations in mice.
12th Conference of the European Society for Reproductive Immunology, September 2015, Oxford, United Kingdom

Posters:

J. Hoffmann*, **G. Engels***, R. Thieme, K. Hecher, H.-W. Mittrücker, P. Arck and G. Gabriel:
Influenza A virus Infection during Pregnancy: Course and Impact.
23rd Annual Meeting of the Society for Virology, March 2013, Kiel, Germany
*equally contributed

G. Engels*, J. Hoffmann*, R. Thieme, K. Hecher, H.-W. Mittrücker, P. Arck and G. Gabriel:
Influenza A virus Infection during Pregnancy: Course and Impact.
FluResearchNet Meeting, October 2013, Münster, Germany
*equally contributed

J. Hoffmann*, R. Thieme*, **G. Engels***, P. Arck and G. Gabriel:
2009 pandemic H1N1 influenza A virus mortality is associated with an impaired innate and cellular immune response during pregnancy in mice.
4th International Influenza Meeting, September 2014, Münster, Germany
*equally contributed

Scholarships:

Boehringer Ingelheim Fonds Travel grant (2013)

Scholarship of the Fonds National de la Recherche Luxembourg (2013)

Scholarship of the German Center for Infection Research (DZIF) (2014)

Awards:

Young Investigator Award winner in Clinical Research, European Society of Reproductive Immunology, 2015

12 **Affidativ**

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....