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Chapter 1

Introduction and outline of this thesis
The early history of influenza epidemics

Epidemics of febrile acute respiratory tract infection (ARTI) have been known throughout recorded history as ‘febris catarrhalis epidemica’, referring to descriptions by Hippocrates in 412 BC [1]. Since the late 15th century, these epidemics were characterized as mild self-limiting illnesses of the upper respiratory tract which could progress to severe bronchitis or pleuritis with old age predisposing towards mortality [2]. In the 18th century, the French word ‘la grippe’ and Italian word ‘influenza’ became widely adopted in Europe. The etymology of ‘influenza’ lies with the description ‘influenza di catarro’, referring to medieval beliefs that unfavourable astrological or miasmic influences resulted in these disease outbreaks [3].

Accurate descriptions of influenza epidemics appeared in the 18th century. The rapid spread among all layers of society prompted Grant (1782) and Johnson (1789) to postulate airborne or contact transmission [1, 4]. Medics remained divided on the role of contagionism, until the germ theory was proven by Snow (1855), Pasteur (1876) and Koch (1890). A large influenza epidemic in 1889 received global media attention and was the first recognized pandemic in a modern connected world (Figure 1) [5]. Expeditious studies by Pfeiffer at the laboratory of Koch theorized that a bacterium *Haemophilus influenzae* was the etiologic agent. Ironically, the assumption did not fulfil Koch’s postulates and a ‘contagium vivum fluidum’ was not considered since the 1889 pandemic predated the discovery of viruses in plants (Ivanovsky, 1892; Beijerinck, 1898), animals (Loeffler, 1898) and humans (Reed, 1901).

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**Figure 1.** The 1889 influenza pandemic media reports predated modern photojournalism.
Source: adapted from Le Petit Parisien, Dimanche 12 Janvier 1890. Described in [5].
Virus discovery

The bacterium *H. influenzae* was discredited as the cause of human influenza infections by clinical and comparative pathology studies in the wake of the 1918 influenza pandemic\(^6\). Accumulating data indicated that an unknown agent caused influenza infection and a wide range of pneumonic bacterial co-infections\(^6-8\). High mortality rates during the 1918 pandemic renewed the expeditious pursuit of a causative agent\(^9,10\). The search for a viral agent was fuelled by leading medical journals stating ‘in the course of evolutionary processes there suddenly is liberated a form of infectious agent against which large numbers of people offer little or no resistance and which is transmitted readily from person to person’\(^11\).

Berkefeld virus filters (Figure 2) were important to affirm that a ‘contagium vivum fluidum’ transferred influenza infection among birds, mammals and humans. Filter passing agents transferred fowl plague among chickens (1901, Centanni and Lode), 1918 pandemic influenza among humans (1918, Selter and Nicolle), and swine or human influenza among pigs and ferrets (1931, Shope; 1933, Smith)\(^12-16\). The work by Smith (1935) and Dochez (1936) provided clear evidence of a viral etiology by showing that virus filtrates of human influenza infection specimens could serially be cultivated in embryonated eggs or chick embryo tissue and cause influenza infection in ferrets and human volunteers\(^17-18\). The discovery of virus culture and hemagglutinin inhibition intensified scientific studies on influenza virus characteristics, clinical manifestations, epidemiology, vaccine development and host immunity\(^18,19\).

Figure 2. Berkefeld ‘bacterial water’ or ‘virus’ filter.
Source: adapted from \(^20\).
Virus characteristics

The virus family Orthomyxoviridae consists of enveloped viruses with a single-stranded negative sense segmented RNA genome and include Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus, Thogotovirus and Quaranjavirus. Human influenza infection is mainly caused by influenza A and B viruses. Influenza A viruses have the unique capability to exchange gene segments with avian and mammalian strains which allow new subtypes to emerge against which humans have no pre-existing immunity. The pandemic potential of a new influenza A virus is determined by its tropism for 2,6-linked sialic acid (SA receptor) expressed in the human respiratory tract required for efficient human-to-human transmission.

Influenza A virus has a 13kb genome with 8 segments which encode 14 proteins (Figure 3) [21–23]. Virus subtypes are based on 18 hemagglutinin (HA) and 11 neuraminidase (NA) surface glycoproteins which mediate sialic acid receptor binding or cleavage. Matrix protein (M1) provides envelope rigidity and aids assembly. Matrix transmembrane proteins (M2, M42) allow proton influx to uncoat virus particles during entry. Nucleoprotein (NP) forms structure complexes with viral RNA and polymerase enzymes (PB1, PB2, PA) modulate transcription and “cap snatching” of host cell mRNA. The nuclear export protein (NEP/NS2) mediates host cell transport of ribonucleoprotein complexes. PB1-F2 proteins mediate host cell apoptosis and nonstructural protein 1 (NS1) disrupts type I interferon antiviral signaling and antigen presentation. PB1-N40 and PA-X act as negative virulence regulators.

Figure 3. Influenza virus structure and genome segments.
Source: adapted from [24].
Laboratory diagnostics

Conventional influenza virus laboratory diagnostics include cell culture, antigen detection or serum hemagglutination inhibition tests (HI). Trypsin-based cell culture has low sensitivity and speed but is useful for virus subtyping and phenotypic antiviral susceptibility testing. Antigen detection by immunofluorescence or enzyme immunoassay (EIA) is fast but the sensitivity may be limited. Serum antibody detection by complement fixation test, enzyme-linked immunosorbent assay (ELISA) or HI (Figure 4) is useful to confirm recent influenza infection but not for rapid diagnosis.

Polymerase chain reaction (PCR) tests provide sensitive and specific detection of a wide range of respiratory viruses. Real-time PCR developed at Leiden University Medical Center provide a 100 to 1000 fold higher sensitivity compared to cell culture [25]. Sampling and logistical support may enable rapid diagnostic results to improve clinical management and outbreak control in health care settings. New molecular assays may also improve the detection of antiviral drug-resistance genes and virus strain subtyping [26].

Figure 4. Serum HI tests confirm seroconversion against influenza A (H1N1) virus.
Source: adapted from [27].
Clinical spectrum

Influenza virus attachment and replication occurs in ciliated epithelial and goblet cells in the human airway tracts\textsuperscript{[28]}. After a 2-day incubation period, most cases develop a 5-7 day mild and self-limiting upper respiratory tract infection (URTI) with fever, malaise and myalgia (Figure 5)\textsuperscript{[29]}. Molecular studies confirm that virus excretion peaks on day 2 and decreases steadily until day 8 or 9 of illness\textsuperscript{[30-32]}. Proinflammatory cytokines (eg IL-6) are associated with the development of systemic symptoms including fever and other mild systemic symptoms\textsuperscript{[33]}

Influenza infection may be complicated by different types of pneumonia\textsuperscript{[34-37]}. Rare severe primary viral pneumonia with an acute onset <5 days is poorly defined\textsuperscript{[34, 35]}. Mixed or secondary bacterial and fungal co-infections can emerge after virus-associated host cell apoptosis of ciliated bronchial epithelial cells\textsuperscript{[34, 36, 37]}. Other complications include viral lower respiratory tract infection (LRTI), acute respiratory distress syndrome (ARDS), non-pulmonary organ involvement (e.g. brain, kidneys) and cardiovascular events\textsuperscript{[38, 39]}

Figure 5. The natural course of uncomplicated influenza ARTI.

Source: adapted from\textsuperscript{[29]}

Influenza pandemics and seasonal epidemics

Influenza A virus pandemics are the result of antigenic shift and occur ~3 times each century. Recorded pandemics include 1889 H3N8, 1918 H1N1, 1957 H2N2, 1968 H3N2 and 2009 H1N1 (Figure 6)\textsuperscript{[40–43]}. High mortality rates during the pre-antibiotic era compared to later pandemics (1957 onwards) underscore the importance of antibiotics and developed health care systems. Extreme mortality rates during the 1918 pandemic (Figure 7) remain enigmatic and were probably due to a low pre-existing immunity in humans and high virulence\textsuperscript{[44,45]}. Current seasonal epidemics are caused by A/H1N1pdm09, A/H3N2, B/Yamagata and B/Victoria viruses. Annual recurrence is caused by frequent antigenic variation and host immune evasion due to mutations acquired during replication with a low-fidelity RNA polymerase. Excess morbidity and mortality is a hallmark of influenza epidemiology and supports annual virus surveillance and vaccination of high-risk patients\textsuperscript{[46]}.

\textbf{Figure 6.} Influenza pandemics and epidemics.
Source: adapted from \textsuperscript{[43]}

\textbf{Figure 7.} Extreme mortality rates during the 1918 pandemic.
Source: adapted from \textsuperscript{[44]}.
Virus surveillance

In 1947, the World Health Organisation (WHO) launched global influenza surveillance plans 'to collect and share information on epidemics, strain type and vaccine composition' [47]. National surveillance networks weekly assess influenza-like illness reported by primary health care providers to monitor virus activity and to collect specimens for virus surveillance (Figure 8) [48]. The WHO now promotes hospital-based surveillance to assess clinical relevant virus changes to improve prevention and control measures [49–51]. Annual surveillance is important for the genetic and antigenic characterization of circulating influenza viruses to detect new antigenic clusters and subtypes with pandemic potential. Antigenic clusters that are genetically divergent at key epitopes are selected for vaccine composition (Figure 9) [52]. Molecular tools are increasingly used to monitor virus epidemiology, genetic divergence, antiviral drug-resistance and virulence genes [53].

Figure 8. The Dutch epidemiology of influenza-like illness during the 2014/2015 season.
Source: adapted from [48].

Figure 9. Influenza virus sequence divergence (A) and vaccine strain selection (B, C).
Source: adapted from [52].
Antiviral treatment

Three classes of influenza antiviral agents exist. Adamantanes (amantadine and rimantadine) are M2 channel blocking agents that are now obsolete due to poor effectiveness and common resistance. Neuraminidase inhibitors (NAIs: oseltamivir, peramivir, zanamivir) are effective agents that block the NA enzyme and prevent host cell viral release (Figure 10)\(^\text{[54, 55]}\). A new viral RNA polymerase inhibitor (favipiravir) is not yet approved but is a promising candidate for influenza combination therapy in patients who are most at risk\(^\text{[56, 57]}\).

NAIs initiated <48 hours of onset reduce symptoms by ~1 day in healthy adults\(^\text{[58]}\). NAIs lower the risk of LRTI in healthy adults but the efficacy is low as the numbers needed to treat are high\(^\text{[59, 60]}\). Non-randomized observational studies show that a 5-day NAI treatment lowers the risk of LRTI and mortality significantly in hospitalized and immunocompromised patients\(^\text{[61–68]}\). Severe influenza pneumonia is associated with higher virus levels compared to mild infection and ratifies an extended NAI treatment duration\(^\text{[69]}\).

Figure 10. Schematic view and electron micrographs showing continued viral replication during absence of NAIs (A) and halted viral replication during presence of NAIs (B).

Source: adapted from \(^\text{[54, 55]}\)

Antiviral resistance and permissive mutations

Influenza resistance mutations can emerge during antiviral treatment\cite{70}. Oseltamivir and zanamivir resistance mutations may encode highly reduced inhibition (HRI) (>100 fold) or reduced inhibition (RI) (10-100 fold) IC\textsubscript{50} over wild-type virus (Table 1)\cite{71}. NAI resistance mutations modify the NA active site involved in cleavage of sialic acid binding structures. Fluorometric assays can measure virus NA activity and inhibition by oseltamivir carboxylate and zanamivir to determine IC\textsubscript{50} fold-changes and phenotypic susceptibility\cite{72}. NA gene active site modifications were deemed unlikely to emerge due to compromised viral fitness. In 2007, NA gene H274Y mutated oseltamivir-resistant A/Brisbane/59/07 (H1N1) virus emerged and circulated dominantly (Figure 11A)\cite{73}. Emergence of permissive mutations restored deficient NA folding, surface expression and sialic acid affinity and accommodated the resistance mutation (Figure 11B)\cite{74-76}. Oseltamivir-resistant A (H1N1) virus became extinct in 2009 and was replaced by wildtype A(H1N1)pdm09 virus.

Table 1. Emergence of influenza A virus NAI resistance mutations in the clinical setting.

<table>
<thead>
<tr>
<th>Influenza subtype</th>
<th>NA mutation</th>
<th>Phenotype in NA inhibition assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Oseltamivir</td>
</tr>
<tr>
<td>A(H1N1)</td>
<td>H274Y</td>
<td>HRI</td>
</tr>
<tr>
<td></td>
<td>Q136K</td>
<td>S</td>
</tr>
<tr>
<td>A(H1N1)pdm09</td>
<td>N294S</td>
<td>HRI</td>
</tr>
<tr>
<td></td>
<td>H274Y</td>
<td>HRI</td>
</tr>
<tr>
<td></td>
<td>I222R</td>
<td>RI</td>
</tr>
<tr>
<td>A(H3N2)</td>
<td>N294S</td>
<td>HRI</td>
</tr>
<tr>
<td></td>
<td>R292K</td>
<td>HRI</td>
</tr>
<tr>
<td></td>
<td>Q136K</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>E119V</td>
<td>HRI</td>
</tr>
</tbody>
</table>

HRI, highly induced inhibition; RI, reduced inhibition; S, sensitive. Source: adapted from\cite{71}.

Figure 11. Emergence of influenza antiviral resistance (A) and A(H1N1) virus permissive mutations (B).

Source: adapted from\cite{73,76}.
**High-risk patients**

Influenza-infected high-risk patients are more prone to develop severe complications and adverse outcomes\[^{77}\]. High-risk patients include the elderly, aged ≥65 years (Figure 12) and patients with pre-existing chronic cardiac or pulmonary conditions, cardiovascular disease, diabetes mellitus, renal disease, immunosuppression, and obesity (Table 2)\[^{78, 79}\]. During the 2009 H1N1 pandemic, extreme obesity (BMI ≥40) was recognized as a new independent risk factor for the development of influenza complications\[^{80}\]. Elderly people aged ≥65 years are at risk to develop influenza complications due to frequent co-morbidities and a functional decline of the adaptive immune system (immunosenescence). The annual influenza mortality rate is estimated at a minimum of 1 in 1500 elderly persons\[^{81}\]. Nursing home residents are particularly at risk to develop associated morbidity and mortality\[^{82}\]. Frequent severe influenza outbreaks in nursing homes demand effective preventive and control measures and early diagnostics to guide early clinical management\[^{83}\].

![Figure 12. Age distribution of seasonal human influenza mortality in the human population. Source: adapted from \[^{78}\].](image)

### Table 2. Increased mortality in high-risk patients with influenza A (H1N1)pdm09 virus infection.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nonsevere outcome, no. (%) of patients</th>
<th>Death</th>
<th>RR (95% CI) *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 1171</td>
<td>n = 72</td>
<td></td>
</tr>
<tr>
<td>Pre-existing heart disease</td>
<td>80/1049 (7.6)</td>
<td>16/65 (24.6)</td>
<td>3.5 (2.1–5.9)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>82/1047 (7.8)</td>
<td>13/65 (20.0)</td>
<td>2.7 (1.5–4.7)</td>
</tr>
<tr>
<td>Renal disease</td>
<td>51/1044 (4.9)</td>
<td>7/64 (10.9)</td>
<td>2.2 (1.1–4.7)</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>93/1041 (8/9)</td>
<td>17/64 (26.6)</td>
<td>3.3 (2.0–5.5)</td>
</tr>
<tr>
<td>Lung disease (including asthma)</td>
<td>309/738 (41.9)</td>
<td>35/52 (57.7)</td>
<td>1.8 (1.1–3.1)</td>
</tr>
</tbody>
</table>

Source: adapted from \[^{79}\].
Vaccination

Influenza viruses frequently mutate and evolve into phylogenetic and antigenic variants \[^{84}\]. Annual subtype dominance varies but A (H3N2) viruses appear more common due to more common mutations and antigenic variation (Figure 13 A, B). Multiple observational studies show that vaccines lower the risk of influenza infection, hospitalization and mortality in high-risk patients but confirmatory randomized controlled trials are lacking \[^{85-89}\]. Vaccines lower the risk of laboratory confirmed influenza in community-dwelling elderly \[^{87-89}\], and of pneumonia and associated mortality in institutionalized elderly \[^{90}\]. Serum HI titers ≥40 after vaccination provide ~50% (up to 70%) clinical protection against homologous virus infection and are traditionally considered seroprotective (Table 3) \[^{91, 92}\].

![Image of influenza virus evolution and genetic changes](source: adapted from \[^{84}\].)

**Table 3.** Relationship between serum HI titers and influenza virus infection in volunteers.

<table>
<thead>
<tr>
<th>Titre of serum HI antibody to A/Scotland/74 (H3N2) virus</th>
<th>≤10</th>
<th>20-30</th>
<th>40-60</th>
<th>80-120</th>
<th>160</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of volunteers</td>
<td>19</td>
<td>9</td>
<td>7</td>
<td>16</td>
<td>24</td>
<td>75</td>
</tr>
<tr>
<td>Number of infected *</td>
<td>14</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>Infection (%)</td>
<td>74</td>
<td>33</td>
<td>29</td>
<td>6</td>
<td>4</td>
<td>28</td>
</tr>
</tbody>
</table>

* Infection proved by virus isolation 3 days after infection and/or a fourfold or greater rise in serum HI antibody.

Source: adapted from \[^{92}\].
Host immune responses

Innate and adaptive immune responses are important for influenza infection control (Figure 14, 15)[93, 94]. Host Toll-like receptors, RIG-I receptors and NLRP3 recognize virus pathogen-associated molecular patterns (PAMPs) and activate interferon (IFN) responses[93, 95]. Infected epithelial cells produce CCL2 that attracts monocytes, natural killer (NK) cells and memory T-cells. T cells, macrophages, NK cells, neutrophils limit viral spread by destruction of infected cells or phagocytosis and produce proinflammatory cytokines (e.g. IL-6, TNF-α). Adaptive immune responses and memory development result from antigen presentation by dendritic cells[93, 96]. Plasma B cells produce virus-specific antibodies whereas T cells regulate immunity and virus-infected cell killing. The precise role of innate and adaptive host immune responses in determining symptoms and viral clearance is unclear.

Figure 14. Induction of influenza humoral and cell-mediated adaptive immunity.
Source: adapted from [93].

Figure 15. Host immune responses during influenza virus infection.
Source: adapted from [94].
Hematology-oncology patients with influenza

Influenza-infected hematology-oncology patients are more likely to develop LRTI compared to other immunocompromised patients\(^\text{[97]}\). Risk factors for viral LRTI include age, lack of (early) antiviral treatment, profound lymphopenia and hematopoietic stem cell transplantation (HSCT) donor mismatch\(^\text{[98,64-66]}\). Severe immunodeficiency is associated with high level prolonged virus excretion and with frequent development of resistant virus during antiviral treatment (Figure 16)\(^{[99-103]}\). Hematology-oncology patients develop a wide clinical spectrum ranging from mild to severe virus-associated symptoms\(^{[104,105]}\). Adverse outcomes often occur early after HSCT\(^{[98]}\). Immune monitoring studies are awaited to elucidate the role of host immune responses in determining virus-associated symptoms and viral clearance among an expanding numbers of HSCT patients (Figure 17)\(^{[106,107]}\).

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**Figure 16. Influenza virus levels in severe (♦) vs moderate (○) immunodeficient patients.**
Source: adapted from\(^{[99]}\).

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**Figure 17. Rising numbers of hematology-oncology patients in Europe from 1998 to 2013.**
Source: adapted from\(^{[106]}\).
Outline of this thesis

This thesis focuses on a variety of subjects related to the molecular diagnosis and clinical consequences of seasonal and pandemic influenza virus infections in a wide range of patients. The main objectives were (1) to evaluate influenza real-time PCR diagnostic methods and clinical aspects of seasonal and pandemic influenza virus infection, (2) to investigate the applicability and accuracy of mass spectrometry-based molecular techniques and real-time PCR to detect influenza virus resistance and virulence genes, and (3) to correlate the role of different host immune responses with virus-associated symptoms and viral clearance in immunocompromised patients with prolonged influenza virus excretion.

The studies described in this thesis are presented in the following chapters:

Chapter 2 describes the relative incidence of respiratory virus infections in children presenting to the hospital and virus-specific clinical correlations in young children.

In chapter 3, two children with fatal influenza virus-associated pneumonia, encephalopathy and multiple organ failure are described and we performed molecular and pathological studies to confirm or refute virus dissemination and replication in other organs.

Chapter 4 is a study that compared influenza immunoassay and PCR methods on nursing homes specimens and evaluated the efficacy of Public Health Service outbreak team support.

Chapter 5 describes the clinical manifestation and antiviral resistance development in immunocompromised patients with ≥14 days prolonged influenza virus excretion.

In chapter 6, a phylogenetic relationship was performed among oseltamivir-resistant influenza A (H1N1) viruses in a patient cluster to assess nosocomial virus transmission.

Chapter 7 describes the detection of influenza virus resistance and virulence markers in routine clinical specimens using mass spectrometry-based comparative sequence analysis.

Chapter 8 describes the role of host immune responses in determining influenza virus-associated symptoms and viral clearance in hematology-oncology patients.
References


