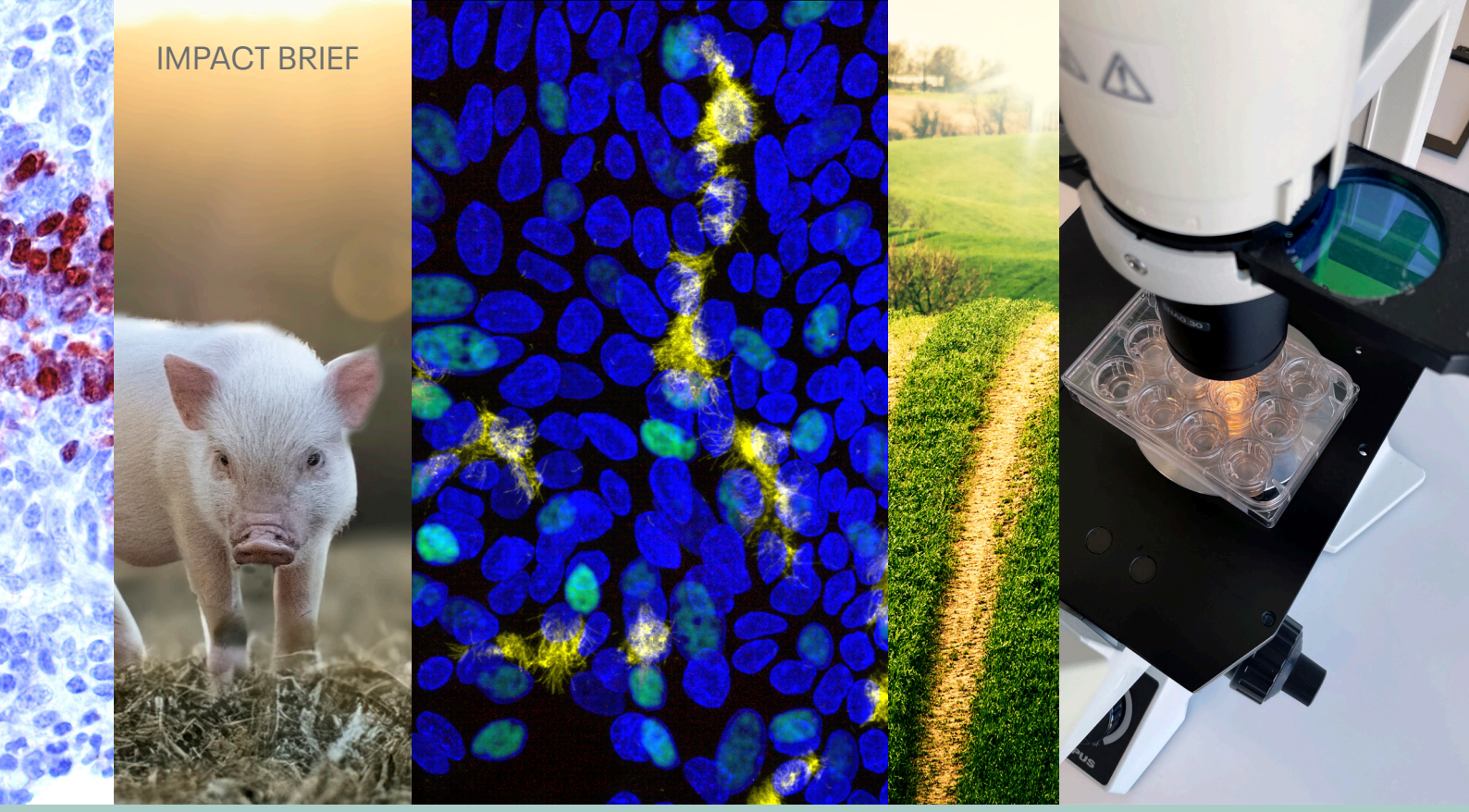
IMPACT BRIEF



FluZooMark

Viral and host factors of zoonotic and pandemic influenza A viruses

FluZooMark

Identifying the viral and host genetic markers that enable cross species transmission of swine influenza A virus.

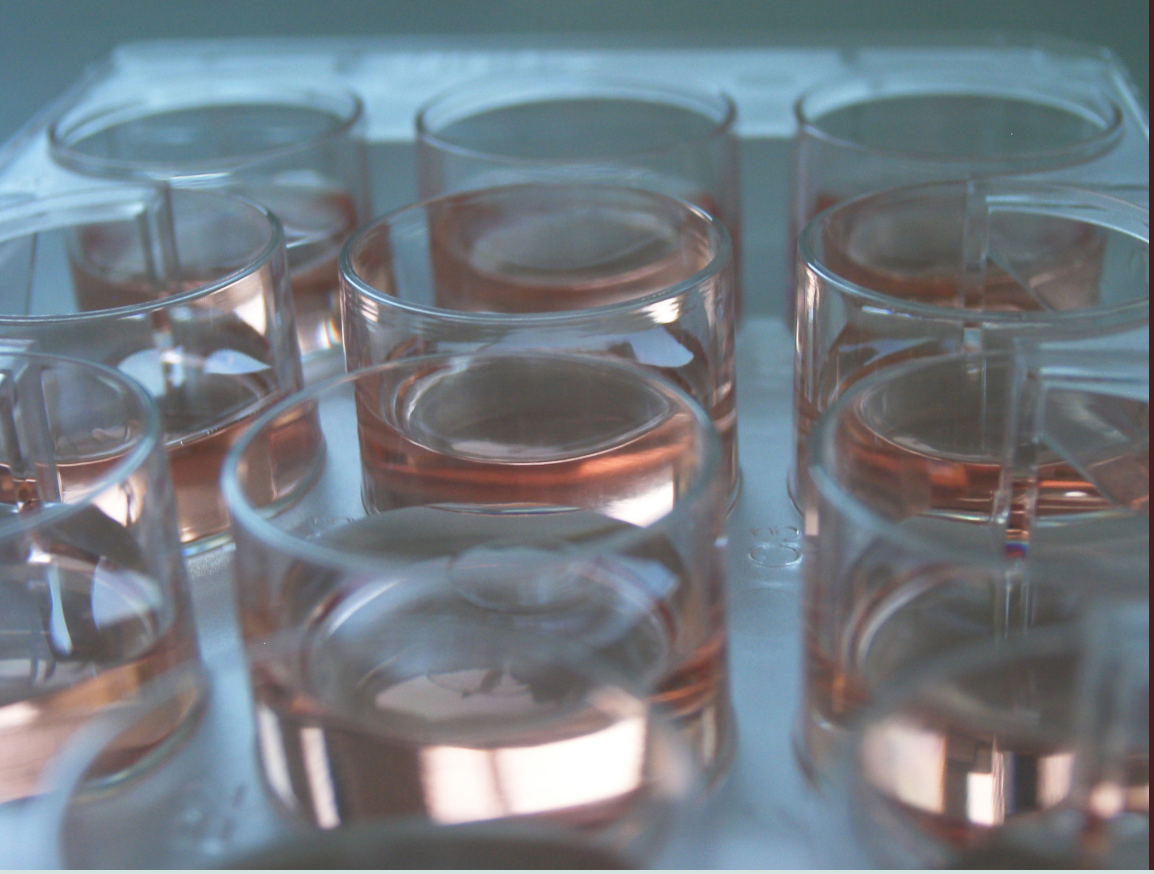
All previous human influenza pandemics have been linked to viruses of animal origin. The most recent pandemic in 2009 emerged in pigs, which have since become a key reservoir where influenza A viruses can diversify and potentially adapt to infect humans. Yet, the viral and host factors enabling these cross species transmissions remain unknown.

FluZooMark, a research project funded by the Novo Nordic foundation (2020-26), addressed this critical knowledge gap by identifying both host and viral genetic markers that enable influenza A viruses to cross the species barrier between pigs and humans. Changes in the virus genome were discovered using whole genome sequencing and then evaluated in vitro and in ferrets and pigs.

The project also showed how influenza A viruses evolve, exchange gene segments (reassortment), and infect different tissues. By combining advanced imaging with molecular methods, the project provided insights into virus–host interactions and how innate immune responses can support or limit infection.

FluZooMark has advanced understanding of how novel influenza A viruses emerge at the human–animal interface. By embedding this knowledge in a One Health framework, the project has produced actionable indicators that enhance influenza surveillance and risk assessment. These outcomes strengthen preparedness and support evidence-based strategies to reduce the impact of future zoonotic spill-over events and influenza pandemics.

> *Identifying viral and host markers associated with influenza virus species jumps allows for earlier detection of the zoonotic and pandemic potential of emerging strains.*
>
> *Lars Erik Larsen, Project leader*



Through the training of 7 PhD students, 13 postdoctoral researchers, and the involvement of more than 40 undergraduate students, the project has made a major contribution to educating the next generation of scientists and has thereby significantly strengthened Denmark's pandemic preparedness.

The Problem & Why it Matters

With around 30 million pigs produced every year and thousands of people in close daily contact with them, Denmark provides a unique interface for virus exchange. Swine influenza A virus in Denmark is enzootic, with more than 90% of Danish herds testing positive. As herds have grown larger and new susceptible pigs are born weekly, short lived influenza outbreaks have transitioned into a constant, self sustaining enzootic disease.

This continuous circulation of influenza in pigs creates ideal conditions for influenza viruses to mix, adapt, and reinvent themselves. Reassortment events, including exchanges with human seasonal influenza strains, are becoming increasingly common. In Denmark alone, more than 22 Danish genotypes are circulating, some of which contain genes of human origin. Globally, more than 200 influenza genotypes have been detected, many of which exhibit zoonotic potential. One such genotype already made the jump in 2009, giving rise to the 2009 H1N1 influenza pandemic, and there is no indication to suggest this occurrence was a one time event.

Despite this high risk environment, we still lack clear insight into the key factors that allow influenza viruses to cross between pigs and humans. Without this knowledge, early warning becomes guesswork, and as viral diversity grows, the likelihood of a new successful spillover increases.

The Key Questions to Address

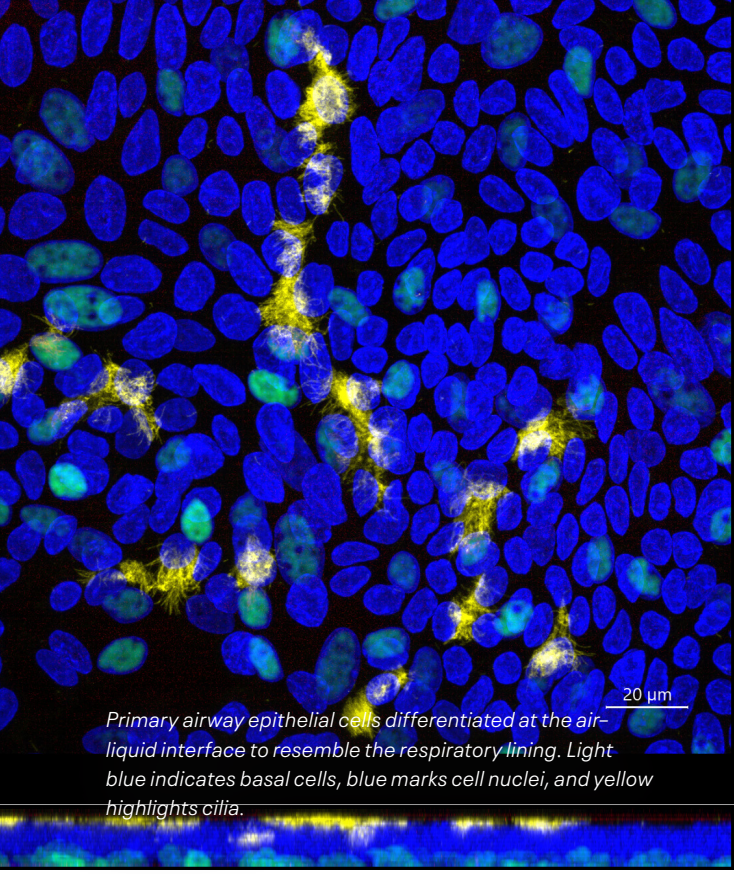
What enables a swine influenza A virus to become a human health threat, and how can we stop it? A pandemic begins long before the first detection in humans. The crucial transition occurs when a virus moves from limited human exposure to efficient and sustained replication in humans.

FluZooMark was established to understand what allows a swine influenza strain to advance past this key threshold. Our goal was to identify viral and host factors that increase the risk of a swine influenza virus progressing from exposure to sustained replication in humans. To do this, FluZooMark focused on two key marker types:

Viral markers: Viral genetic features were analyzed using whole genome sequencing and phylogenetics to pinpoint mutations and protein regions linked to swine to human transmissions.

Host markers: Antiviral immunogenetic and other host factors, including proteases involved in viral activation and mucosal barriers, were investigated to understand how host susceptibility shapes viral adaptation and spillover.

By combining these perspectives, FluZooMark targeted the exact tipping point where a virus shifts from a rare spillover event to a potential human health threat — the moment where prevention is still possible.



Primary airway epithelial cells differentiated at the air-liquid interface to resemble the respiratory lining. Light blue indicates basal cells, blue marks cell nuclei, and yellow highlights cilia.

Key Results & Breakthroughs

Over five years, FluZooMark has delivered a series of high impact discoveries that have advanced our understanding of how influenza A viruses evolve in pigs, adapt to new hosts, and occasionally spill over to humans. Collectively, our findings have significantly advanced the understanding of the viral and host determinants that are most critical during the early stages of pandemic development.

Viral Insights — What Makes a Swine influenza Virus a Human Threat?

Discovery of viral genetic markers linked to zoonotic transmission

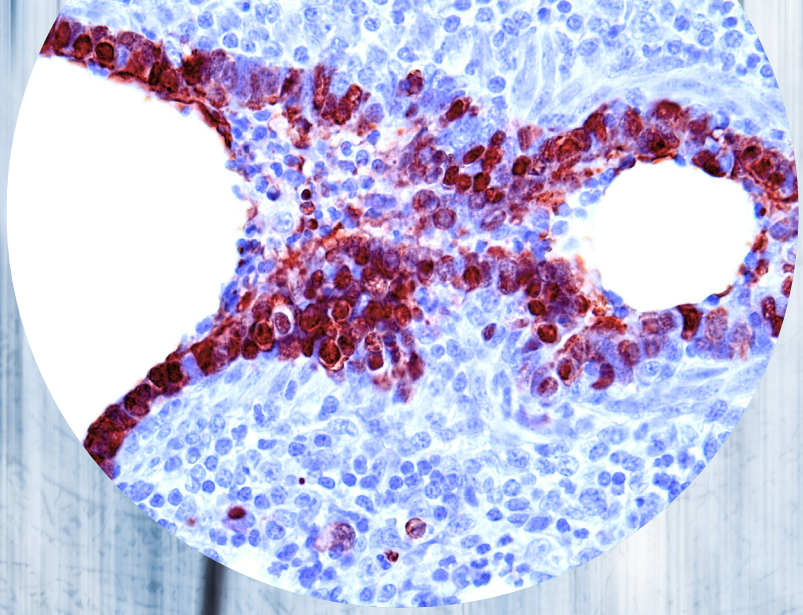
Our genomic analyses identified specific mutations and protein regions that enable certain swine influenza A viruses to cross the species barrier. These features provide early genetic indicators of zoonotic potential within complex, reassorted virus populations.

Distinct H1pdm09 lineages evolving exclusively in Danish pigs

We identified Danish exclusive H1pdm09Nx clusters defined by lineage-specific mutations that led to rapid diversification and swine adaptation following their emergence in 2011–2013. These lineages now form a viral reservoir in pigs, which must be considered in future pandemic preparedness efforts.

Our Approach

FluZooMark was structured around six interconnected work packages (WPs) forming a comprehensive pipeline to understand, test and develop targets to prevent swine-to human influenza transmission. By integrating expertise across disciplines, the project generated knowledge and tools to strengthen pandemic preparedness and support the development of improved vaccines.



Immunohistochemical staining of influenza (red). Marked positive staining of bronchiolar epithelial cells and scant staining of alveolar macrophages or pneumocytes.

Repeated human-to-swine spillover without sustained establishment

Despite repeated introductions of human H1pdm09 viruses into pigs after 2014, none of these human-adapted strains achieved sustained circulation in Danish pigs. This highlights the virological and ecological barriers that limit establishment of human influenza viruses in pigs.

Insights into transmission dynamics across hosts

Establishment of transmission chains in pigs using both human- and swine-adapted influenza A strain identified genetic signatures that are critical for cross-species transmission and early replication in new hosts.

Several ferret studies were conducted during the project, using the gold-standard model for human airborne influenza transmission.

By investigating the transmission of a human seasonal H1N1pdm09 and two swine adapted reassorted influenza A strains in the ferret model, it was revealed that all three viruses were able to infect the ferrets and transmit via direct contact.

However, only the human virus was capable of aerosol transmission, demonstrating the ferret model's capability as a robust model for the predictions of human-to-human airborne transmission.

Host Insights — Why Some Hosts Enable Spillover

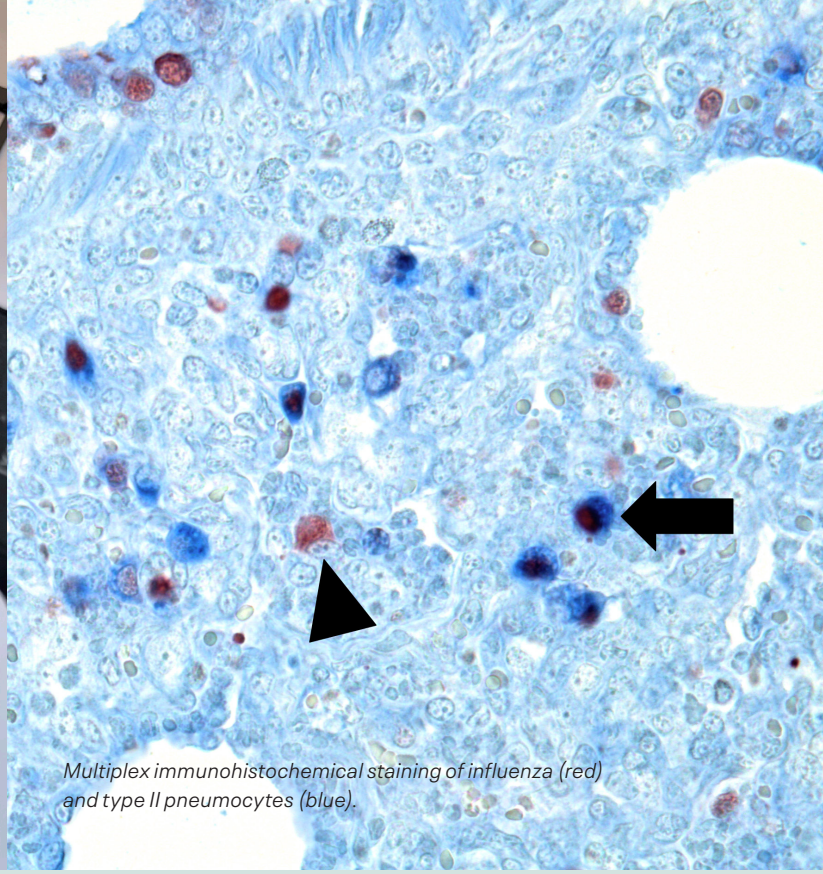
Mucosal environment shapes initial infection success

Early infection stages were shaped by host responses in the nasal mucosa, where barrier properties and local immune activation influenced viral entry and initial replication. In this context, we demonstrated that these early mucosal responses contributed to determining whether infection becomes successfully established, thereby affecting the transmission potential by modulating viral load and early viral expansion at the primary site of infection.

Innate immune responses depend on host adaptation and shape permissiveness

The kinetics and magnitude of antiviral innate immune responses, particularly interferon mediated pathways, varied according to the degree of viral host adaptation. Early differences in innate immune activation were associated with variation in viral replication dynamics and infection outcomes, indicating that innate immunity contributed to shaping host permissiveness.

In vitro cell culture.



Multiplex immunohistochemical staining of influenza (red) and type II pneumocytes (blue).

Epigenetic regulation of antiviral responses and disease severity

Pulmonary microRNA expression was associated with disease severity during influenza A virus infection, with distinct site-specific miRNA profiles observed in mild and moderate disease. These miRNAs were linked to the regulation of host processes involved in antiviral responses and control of the viral life cycle, indicating that epigenetic mechanisms contributed to shaping infection outcomes.

Post-translational modifications in immune regulation

Phosphoproteomic profiling revealed strain-specific differences in phosphorylation patterns of host proteins, resulting in strain-specific regulation of antiviral signalling pathways during infection. Further, our data suggest a potential role for the Hippo pathway as an underexplored regulator in respiratory virus infection and host adaptation.

Tissue-specific protease dynamics shape host response to infection

Protease expression and activity varied across tissues in the respiratory tract during H1N1 infection, resulting in distinct cleavage patterns. In this context, we identified and quantified Legumain as a previously unrecognized protease with potential relevance for infection processes.

Inflammatory state modifies susceptibility

Chronic inflammation induced by a high fat diet altered antiviral immune responses, including the expression of interferons and interferon stimulated genes, both before and after influenza virus infection. In this context, we demonstrated that these immune alterations influenced infection dynamics and disease severity.

Breakthrough findings in receptor and tissue tropism

Our studies showed that pigs express the avian-type receptor in their nasal muco¬sa and express another sialic acid (Neu5Gc) in their trachea. While both human and swine-adapted viruses preferentially bind to the human-type receptor, viral load in the trachea was unexpectedly low, showing that receptors alone cannot explain species barriers or that the receptor hypothesis is more complicated than first anticipated. Other host factors clearly play a critical role.

Ancestral-sequence DNA vaccine enhances immune responses

Five-week-old piglets were vaccinated with DNA constructs encoding swine ancestral or human pandemic (2009) HA and NA, then challenged with a recent zoonotic H1N1 isolate. Following challenge, both DNA vaccines induced neutralizing and NA-inhibiting antibodies and showed effective immune recall. The ancestral vaccine improved viral clearance in the lungs but did not significantly reduce viral shedding in nasal swabs compared to mock-vaccinated controls. These findings indicate that ancestral antigen design can enhance immune responses, but additional strategies will be required to effectively limit transmission and support countermeasures against emerging zoonotic variants.

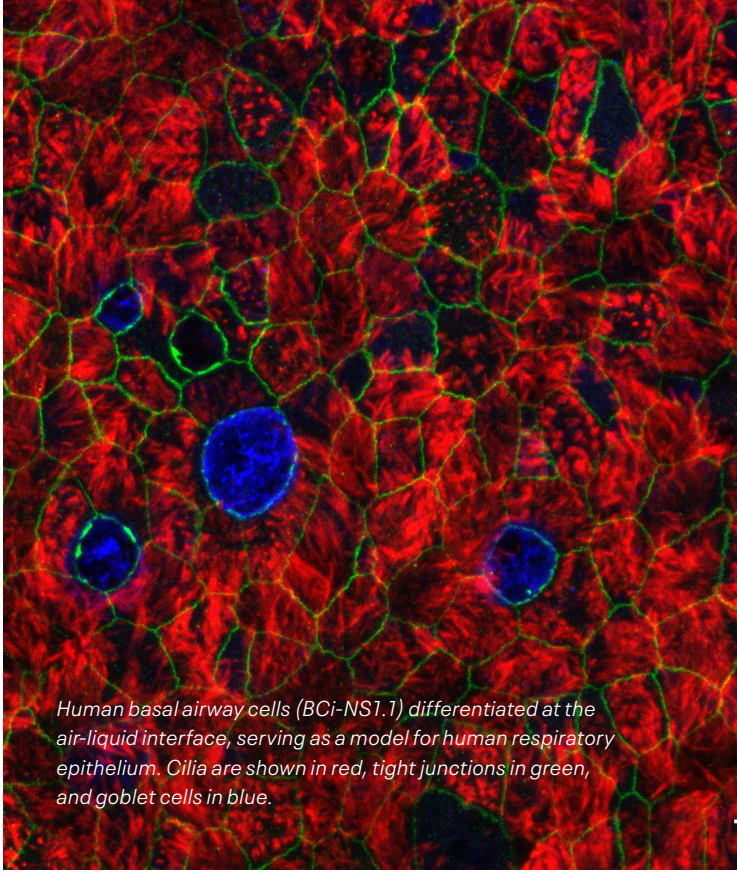
Cross-Species Transmission — Evidence from Real Cases

Two human infections with an unusual NS gene

Two human swine-derived influenza infections were identified in Denmark in 2021. The viruses were carrying an atypical NS gene segment and were like recently identified swine influenza viruses circulating in Danish pigs. The cases demonstrate the ongoing potential for swine influenza virus to cross the host barrier.

Experimental infections of ferrets revealed that the virus resembling the 2nd zoonotic case (H1pdmN1av-2) was able to transmit between ferrets by direct contact but not via aerosol, whereas the H1pdmN1av-1 virus, which had a different NS segment, was efficiently transmitted both by direct contact and aerosols between ferrets.

Cross-reactivity tests between the two strains, using both representative human sera and ferret antisera raised against seasonal influenza vaccines, indicated very low population immunity in the event of human-to-human transmission.



Human basal airway cells (BCi-NS1.1) differentiated at the air-liquid interface, serving as a model for human respiratory epithelium. Cilia are shown in red, tight junctions in green, and goblet cells in blue.

Side-steps — jump of avian influenza virus into cows

Influenza A receptors in cows

In March 2024, a Highly Pathogenic Avian Influenza (HPAIV) H5N1 surprisingly spilled over to US dairy cattle with sustained cattle-to-cattle transmission. Extremely high viral titers were reported in the milk. Using methods developed in FluZooMark, we were the first to demonstrate that the bovine mammary glands expressed both the receptors used by human and avian influenza viruses, providing a rationale for the high viral titers found in the milk. This study also underlines that methods developed in FluZooMark can be immediately applied when new pandemic threats evolve.

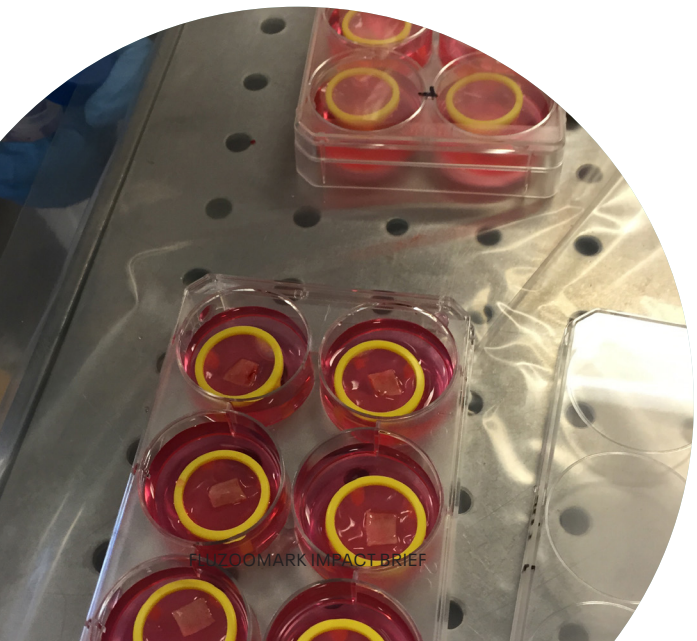
Impact & Dissemination

FluZooMark has delivered actionable markers that improve early detection of high risk influenza strains, and through peer-reviewed international publications, we have substantially enhanced the global understanding of the fundamental mechanisms underlying cross-species transmission.

Through the training of 7 PhD students, 13 postdoctoral researchers, and the involvement of more than 40 undergraduate students, the project has made a major contribution to educating the next generation of scientists and has thereby significantly strengthened Denmark's pandemic preparedness.

Importantly, our unique viral–host datasets, experimental evidence, and One Health framework directly support improved surveillance, more accurate risk assessment, and future vaccine development.

The project has placed a strong emphasis on dissemination of scientific results and basic knowledge about the challenges of the animal-human interface through social media and the production of several podcasts targeting both scientists and the public. Furthermore, the project partners have received broad attention, particularly during and in the wake of the COVID-19 pandemic.



Nasal tissue explants cultured under air–liquid interface conditions to mimic the respiratory epithelium.

Conclusion, Perspectives and Next Steps

Our multi-layered data demonstrate that successful swine-to-human transmission of influenza viruses depends on a combination of viral mutations, host susceptibility, and the biological environment, particularly within the upper respiratory tract. Throughout the project, we expanded and refined our scientific toolbox, enabling more detailed interrogation and validation of emerging hypotheses. Collectively, our findings underscore that cross-species transmission is a highly complex process, driven not by single adaptive events but by intricate interactions between multiple viral and host factors.

Together with key studies from the global research community, our results highlight the central role of interactions between specific host cell proteins and viral proteins, including PA, PA-X, PB1, and NS1, in facilitating host adaptation. In addition, our data reinforces the importance of adaptive changes in viral regulatory proteins, such as NS2, in shaping overall viral fitness. We have identified several conserved and variable motifs within these proteins that represent promising candidate markers for further investigation.

The next step will therefore be to systematically elucidate the functional significance of these candidate markers and other fundamental determinants of viral replication and host response within infected cells, including the warning signals induced during infection. Advancing this knowledge will support more accurate risk assessments of emerging swine influenza strains and aid in the identification of novel targets for antiviral intervention and pandemic preparedness.

Overall, the six-year Challenge Grant from the Novo Nordisk Foundation provided the time and resources needed to establish a unique interdisciplinary research collaboration. This collaboration led to new discoveries that now form the foundation for our future research on viruses with pandemic potential.

Graphic Design: Karen Krarup

Project Partners

University of Copenhagen
Lars Erik Larsen
Professor, Phd, DVM

Statens Serum Institut
Ramona Trebbien
Senior Scientist, PhD, DVM

St. Jude Children's Research Hospital
Richard J. Webby
Director, PhD

Technical University of Denmark - DTU
Kerstin Skovgaard
Senior Researcher, PhD

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