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Comparative Pathology of African Swine Fever in Domestic Pigs and Wild Boar

Insights from Experimental and Natural Infections

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Cover: Illustration of a wild boar (*Sus scrofa scrofa*) opened for sampling by ©2026 Jasmine Stavenow Jerremalm

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Comparative Pathology of African Swine Fever in Domestic Pigs and Wild Boar. Insights from Experimental and Natural Infections

Abstract

African swine fever (ASF), caused by African swine fever virus (ASFV), continues to spread globally, with European wild boar serving as a major reservoir and driver of viral transmission in Europe and Asia. This thesis comparatively evaluates disease dynamics, pathogenesis, macroscopic and histopathologic lesions, and viral antigen distribution in domestic pigs (*Sus scrofa domesticus*) and European wild boar (*Sus scrofa scrofa*) following intranasal inoculation with the highly virulent genotype II strain "Armenia 2007". It contrasts these findings with naturally infected wild boar from field outbreaks. In the experimental infection, wild boar showed shorter incubation periods, earlier onset of clinical signs, earlier viremia, and faster systemic viral spread than domestic pigs. The medial retropharyngeal lymph nodes were key early replication sites in both subspecies, with viral antigen appearing earlier in wild boar, from 3 days post-infection (pi). Despite reaching humane endpoints earlier, day 6 vs. 9 pi, wild boar showed less severe and extensive macroscopic and histopathological lesions and lower levels of viral antigen in tissues, even though viral antigen appeared and increased earlier, and lesions also began and progressed sooner. These results suggest a reduced tolerance to tissue damage rather than a lower pathogenic potential of the virus in wild boar. While naturally infected wild boar also exhibited lesions consistent with ASF, these were often more severe and heterogeneous than those observed in experimental animals. Lesions in naturally infected wild boar reflect a longer disease course and concurrent comorbidities. Overall, these results demonstrate clear differences in host responses between domestic pigs and wild boar and underscore the influence of both host-specific and environmental factors on disease expression and progression. Collectively, the findings provide important insights for improving surveillance, informing control measures, and for vaccine development in both domestic and wild suid populations.

Keywords: African swine fever virus (ASFV), domestic pigs, wild boar, *Sus scrofa*, clinical course, disease outcome, macroscopic and histologic lesions evolution, virus dynamics, virus antigen distribution, natural infection.

Patologiska skillnader vid afrikansk svinpest hos tamgris och vildsvin baserade på experimentella och naturliga infektioner

Sammanfattning

Afrikansk svinpest (ASF), orsakad av afrikanskt svinpestvirus (ASFV), sprids fortsatt globalt, där europeiska vildsvin fungerar som en viktig reservoar och bidrar till virusets spridning i Europa och Asien. Denna avhandling jämför sjukdomsutveckling, patogenes, makroskopiska och histopatologiska förändringar, och virusdistribution hos tamgrisar (*Sus scrofa domesticus*) och europeiska vildsvin (*Sus scrofa scrofa*) efter intranasal inokulation med ASFV genotyp II och nyanserar även resultaten med naturligt infekterade vildsvin från utbrott av ASF. Vid den experimentella infektionen visade vildsvin högre känslighet, med kortare inkubationstid, tidigare kliniska sjukdomstecken, tidigare viremi och snabbare systemisk spridning av virus i vävnader jämfört med tamgrisar. De mediala retropharyngeala lymfknutorna identifierades som viktiga tidiga replikeringsställen i båda underarterna, med virusantigen upptäckt tidigare hos vildsvin (från tre dagar efter infektion). Trots att vildsvinen nådde den humana slutpunkten i försöket tidigare (6 vs. 9 dagar efter infektion), uppvisade de mindre allvarliga och mindre omfattande makroskopiska och histopatologiska förändringar samt lägre nivåer av virusantigen i vävnaderna. Virusantigen dök upp och ökade tidigare, och lesionerna började och utvecklades snabbare, men trots detta var vävnadsskadorna mindre omfattande. Resultaten tyder därför på att skillnaden beror på en lägre tolerans för vävnadsskador hos vildsvinen, snarare än att viruset har en mindre sjukdomsframkallande förmåga hos dem. Naturligt infekterade vildsvin uppvisade generellt lesioner förenliga med ASF. De var ofta mer omfattande och varierande än vid experimentella infektioner, vilket kan spegla ett längre sjukdomsförlopp och saminfektioner, såsom parasitär lunginflammation. Resultaten tydliggör skillnader i sjukdomsrespons mellan tamgrisar och vildsvin och visar hur värd och miljöfaktorer kan påverka hur sjukdomsbilden ser ut. Sammantaget ger studierna viktiga insikter för förbättrad sjukdomsövervakning, effektiva kontrollåtgärder och utveckling av vacciner för både tam- och vildsvinspopulationen.

Keywords: Afrikanskt svinpestvirus (ASFV), tamgrisar, vildsvin, kliniskt förlopp, sjukdomsutgång, makroskopiska och mikroskopiska lesioner, virusdynamik, virusantigendistribution, naturlig infektion.

Preface

The work presented in this thesis was conducted between 2021 and 2026, a period marked by the ongoing global challenges of transboundary animal diseases. In particular, African swine fever (ASF) continued to spread across Europe and into Asia. ASF is a devastating viral disease of pigs with severe socioeconomic consequences. This work is part of a broader effort to improve the understanding of ASF and collaboration between government agencies, universities, and international partners has been crucial.

The detection of ASF in wild boar in central Sweden in 2023 represented the first occurrence of the disease in the country, emphasizing the topical relevance of the research undertaken in this project. Sweden was also officially declared free from ASF in 2024 through effective national disease surveillance and control efforts. Research results presented in this thesis, derived in part from this outbreak, provide valuable insights for enhancing the continued surveillance and management of ASF.

This thesis is also a story about preparedness, response, and reflection. The work began as part of an effort to improve knowledge to strengthen readiness for a disease that, sooner or later, would likely reach Sweden. The unexpected but still anticipated ASF outbreak transformed this preparation into practice. Following the outbreak and successful eradication, the focus of this thesis shifted to learning from the experience and contributing to addressing knowledge gaps.

Dedication

For my family.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Sánchez-Cordón, P. J., Lean, F. Z. X., Batten, C., Steinbach, F., Neimanis, A., Le Potier, M.F., Wikström-Lassa, E., Wynne, F., Strong, R., McCleary, S., Crooke, H., Gavier-Widén, D., Núñez, A. (2024). Comparative evaluation of disease dynamics in wild boar and domestic pigs experimentally inoculated intranasally with the European highly virulent African swine fever virus genotype II strain “Armenia 2007”. Vet Res, 55, 89. doi:10.1186/s13567-024-01343-5

II. Emil Wikström-Lassa, Dolores Gavier-Widén, Fabian Z. X. Lean, Alejandro Núñez, Björnar Ytrehus, Karl Ståhl, Pedro J. Sánchez-Cordón, Aleksija Neimanis (2026). Comparative evaluation of histopathological lesions and viral antigen distribution in domestic pigs and wild boar inoculated intranasally with the highly virulent ASFV genotype II strain "Armenia 2007". Vet Res, 57, 19. doi:10.1186/s13567-025-01701-x

III. Emil Wikström-Lassa, Pedro J. Sánchez-Cordón, Dolores Gavier-Widén. Ulrika Larsson Petterson, Karl Ståhl, Aleksija Neimanis. Pathology of African Swine Fever in naturally infected Wild Boar from Two European outbreaks caused by Genotype II strains. (manuscript)

The contribution of Emil Wikström Lassa to the papers included in this thesis were as follows:

I. Description of contribution to paper I. Methodology, Data collection*, writing-review and editing.

II. Description of contribution to paper II. Conceptualization, Methodology, Data collection, Formal analysis, Writing and preparing original draft. Reviewing and editing. Corresponding author.

III. Description of contribution to paper III. Conceptualization, Funding acquisition, Methodology, Data collection, Formal analysis. Writing and preparing the original draft. Reviewing and editing. Corresponding author.

*Planned data collection was disrupted by the SARS-CoV-2 pandemic

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Abbreviations

APHA	Animal and Plant Health Agency
ASF	African Swine Fever
ASFV	African Swine Fever Virus
DP	Domestic Pig
dpi	Days post infection
FLI	Friedrich-Loeffler-Institute
HE	Haematoxylin and eosin staining
IHC	Immunohistochemistry
INIA	National Institute of Agricultural and Food Research and Technology
KSLA	The Royal Swedish Academy of Agriculture and Forestry
MRPLN	Medial retropharyngeal lymph node
pi	Post infection
SLU	Swedish University of Agricultural Sciences
SVA	Swedish Veterinary Agency
WB	Wild Boar

AI declaration

During the preparation of this work, AI tools were used solely to support the writing process in the last manuscript and the thesis summary (kappa) to enhance readability and language. **Grammarly** (writing assistant software, free version) was used to improve grammar and spelling. Large language model **ChatGPT** (OpenAI, GPT-5 mini, free version) was used as a copy-editing tool, i.e., translation, search for synonyms etc. No conclusions, analyses, or factual content were generated by these tools. All AI-assisted content was carefully reviewed and edited, and the author takes full responsibility for the final version.

1. Introduction

African swine fever (ASF) represents one of the most significant transboundary animal health challenges affecting global pig production. As pork remains a major source of animal protein worldwide, the impacts of ASF extend beyond individual herds, influencing pig industry and trade and having substantial socioeconomic effects on rural economies. In regions with large-scale pig production, including parts of Europe and Asia, outbreaks have strained veterinary services, biosecurity, and disease surveillance. At the same time, the global spread of ASF has intensified research efforts, driving progress in diagnostics, epidemiology, vaccine development, and disease modelling, demonstrating the essential role of scientific research in strengthening preparedness against disease incursions.

1.1 African swine fever

ASF is a serious, contagious, febrile haemorrhagic disease of suids with high lethality, first described by Eustace Montgomery over 100 years ago (Montgomery, 1921). Development of vaccines continues to face challenges (Urbano et al., 2022) and there are no treatments for this viral disease. The outcome of the disease depends on the host, the route of infection and the virulence of the virus, and the disease presentation ranges from subclinical to fatal. ASF was first described in sub-Saharan countries where the virus is maintained in a natural cycle involving wild suids such as bushpigs (*Potamochoerus larvatus*) and warthogs (*Phacochoerus africanus*), along with soft ticks of the genus *Ornithodoros*. Unlike the bushpigs and warthogs in Africa, the domestic pig (*Sus scrofa domesticus*) and European wild boar (*Sus scrofa scrofa*) are highly susceptible to the disease, which typically results in outbreaks with high case fatality rates (Dixon et al., 2019).

1.1.1 African Swine Fever Virus

African swine fever virus (ASFV) is a large, double stranded DNA virus classified as the sole member of the Asfaviridae family, genus Asfivirus (Alonso et al., 2018). ASFV is also the only DNA virus that depends on arthropod vectors for transmission between its natural hosts i.e. bushpigs and warthogs. Partial sequencing of the p72 gene has identified 24 genotypes

(Achenbach et al., 2017; Netherton et al., 2019; Quembo et al., 2018). Within each genotype, strains are categorised as low-, moderate-, or high-virulence (Salguero, 2020).
ASFV targets and replicates in cells of the mononuclear-phagocytic system, macrophages and reticular cells of natural hosts (Urbano et al., 2021). Replication has also been observed in mesenchymal and epithelial cells but is deemed to play a minor role in the pathogenesis. In later stages, the virus can infect additional cell types, including neutrophils, dendritic cells, endothelial, epithelial, and other parenchymal cells, demonstrating its pantropic nature (P. Sánchez-Cordón et al., 2021)

The virus is stable over a broad pH range and is capable of surviving for years under low-temperature conditions (Alonso et al., 2018). ASFV is endemic in some regions of Africa, while in others it occurs as sporadic outbreaks. Between the 1950s and 1980s, ASFV genotype I strains caused outbreaks across Europe, the Caribbean and South America. With the exception of the island of Sardinia. (Laddomada et al., 2019), ASF was eradicated from regions outside of Africa in the 1990s.

The ASFV strains currently circulating and responsible for the ongoing global ASF epidemic derive from a highly virulent genotype II strain originating from southeastern Africa (Mthombeni et al., 2023). It was introduced into Georgia in 2007, from where it spread to Russia, the European Union, Asia, and the Caribbean (Gogin et al., 2013; Ramirez-Medina et al., 2022; Zhang et al., 2023).

1.1.2 Transmission and epidemiology

The epidemiology of African swine fever comprises four main transmission cycles (Fig 1). In the sylvatic cycle, the virus is maintained between the common warthog (*Phacochoerus africanus*), bushpig (*Potamochoerus larvatus*), and soft ticks (*Ornithodoros moubata complex*), although the role of the bushpig remains unclear. In these wild hosts, the virus typically does not cause clinical disease. (Lv et al., 2022). The tick-pig cycle involves transmission between infected soft ticks and domestic pigs, mainly in sub-Saharan Africa. In the domestic cycle, the virus spreads among domestic pigs or via contaminated pig products, independent of wildlife reservoirs. Finally, the wild boar-habitat cycle is characterized by virus persistence within wild boar populations through both direct transmission and indirect exposure via infected carcasses in the environment (Probst et al., 2017). Although these

cycles are largely independent, interactions between them, often driven by human activities, can facilitate spillover and contribute to the geographic spread of the disease (Chenais et al., 2018).

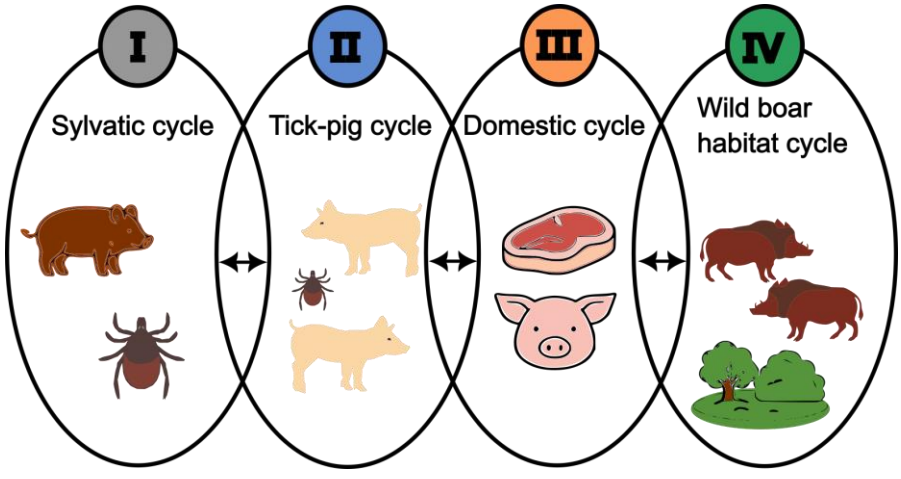

Figure 1. Overview of the four transmission cycles of African swine fever. African swine fever circulates through four main epidemiologic cycles, each involving distinct hosts and transmission pathways:1) in the sylvatic cycle, the virus is maintained between the common warthog (*Phacochoerus africanus*) and soft ticks (*Ornithodoros moubata complex*); 2) in the tick-pig cycle, transmission occurs between soft ticks and domestic pigs (Sus scrofa domesticus); 3) in the domestic cycle, the disease spreads among domestic pigs and via pig-derived products and 4) in the wild boar-habitat cycle, virus transmit among wild boar (*Sus scrofa scrofa*), through both direct transmission and indirect exposure via infected carcasses in the environment.

Epidemiologically, ASF is endemic or causes sporadic outbreaks in much of sub-Saharan Africa and, since 2007, has spread extensively through Europe and Asia, driven largely by human-mediated activities. Spread is associated with movement of infected pork products, pigs, swill feeding, contaminated vehicles, equipment, clothing, and poor biosecurity practices. Wild boar populations facilitate regional persistence and cross-border spread, particularly in Europe, while backyard farming systems with low biosecurity have amplified outbreaks in parts of Eastern Europe and Asia. Overall, ASF epidemiology is shaped by a combination of biological factors (virus stability, multiple hosts) and human behaviour, making control particularly difficult in the absence of a globally available vaccine. (Dixon et al., 2019)

1.1.3 Clinical signs

Clinical presentation of ASF is an outcome of the virulence of the strain involved, host, and route of infection and presents itself in varying forms, peracute, acute, subacute or chronic disease, where the acute presentation is the most common in natural outbreaks (P. Sánchez-Cordón et al., 2021). The majority of knowledge on ASF comes from domestic pigs and, to a lesser extent, from wild boar. Under natural conditions, the incubation period is estimated to range from 4 to 19 days, while experimental infections show shorter periods of 2 to 7 days, with the clinical course usually lasting about a week. The case fatality rate for the highly virulent strains is extremely high, reaching 90–100%. Clinical signs of acute ASF include high fever (40.5–42 °C), loss of appetite, apathy, weakness, recumbency, and abortions at any stage of pregnancy. Affected pigs may also exhibit respiratory distress, nasal and ocular discharges, vomiting, diarrhoea (often blood-stained), neurological signs, and skin changes such as erythema, cyanosis and haemorrhages. Death typically occurs within 3–9 days post-inoculation with highly virulent isolates. A more prolonged disease course is seen with moderately virulent isolates (P. Sánchez-Cordón et al., 2021).

1.1.4 Pathology and pathogenesis

Macroscopic lesions in ASF vary widely, influenced by both the virulence of the virus and the host response. In acute ASF, affected pigs are often in good body condition and may show generalized organ congestion, and yellow to red fluid accumulation in body cavities (hydrothorax, hydropericardium, mild ascites). In light-skinned pigs a mild skin flushing may be observed. The spleen is often markedly enlarged, dark red, and friable, and may display petechiae or small infarcts, while lymph nodes are frequently oedematous and haemorrhagic (Figure 2). The thymus and tonsils may occasionally show petechiae. Cardiorespiratory lesions include lung congestion, alveolar oedema, foamy or blood-stained exudate in the trachea, and petechiae in the heart's epicardium and endocardium. Kidneys usually present subcapsular, cortical and medullary petechiae, occasionally with mild perirenal oedema, and the urinary bladder wall may also display petechial haemorrhages. Gastrointestinal lesions include mild congestion with serosal and mucosal petechiae, hyperaemia, and occasional bloody faecal contents. The liver may show mild hepatomegaly and congestion, with

occasional oedema of the bile duct or gall bladder wall and petechiae on serosal or mucosal surfaces. Overall, these lesions reflect the systemic vascular damage and haemorrhagic tendencies induced by highly virulent ASFV (P. Sánchez-Cordón et al., 2021). Macroscopic lesions of the subacute form are similar to those described in acute ASF but generally are characterised by more severe and extensive haemorrhages as a consequence of a longer disease course. The spleen may be only partially enlarged and not friable and the accumulation of fluid in body cavities is often more severe than in the acute form. Moderate perirenal oedema is also a common feature of subacute ASF, as well as more severe congestive and haemorrhagic changes in the gastrointestinal tract.

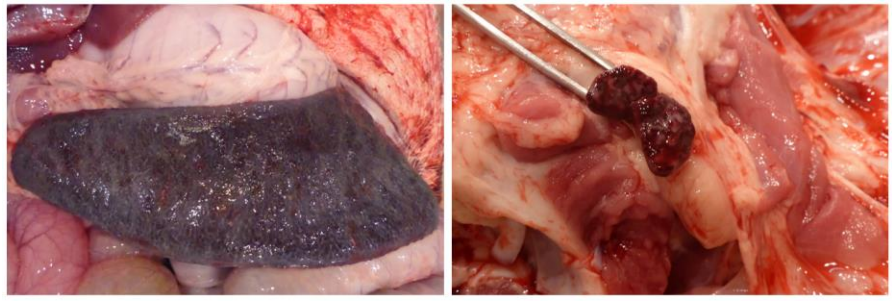

Figure 2. Macroscopic lesions characteristic of acute African swine fever (ASF). Left: enlarged, dark red spleen (Splenomegaly). Right: Enlarged and haemorrhagic lymph node. Photo: Emil Wikström Lassa

Over the past five decades, extensive studies in experimentally infected pigs have clarified key aspects of ASFV replication, dissemination, and the development of characteristic lesions. ASFV primarily targets cells of the monocyte/macrophage lineage (Gomez-Villamandos et al., 2013). Replication occurs in cytoplasmic viral factories, with the full replication cycle completed in about 24 hours (Muñoz-Moreno et al., 2015). Infection induces cytopathic effects, including necrosis and apoptosis of macrophages, causing massive viral release (Gomez-Villamandos et al., 2013). A characteristic feature of ASFV is haemadsorption, in which infected cells bind erythrocytes; a large proportion of circulating virus particles can also attach to red blood cells, facilitating systemic spread and persistence (Borca et al., 1994). Following infection, the virus replicates in tonsils and regional lymph nodes before spreading via blood and lymph to organs rich in mononuclear phagocytic cells, such as spleen, lymph nodes, and bone

marrow (P. Sánchez-Cordón et al., 2021). ASFV is shed in oronasal secretions, urine, and faeces with blood containing the highest viral titres (Bellini et al., 2016; Guinat et al., 2014).

The hallmark histopathological features of ASF include lymphoid depletion and widespread vascular damage. The most consistent microscopic lesion is marked apoptosis and necrosis of lymphocytes in the spleen, lymph nodes, tonsils, and thymus, often accompanied by congestion, oedema and multifocal haemorrhages (Figure 3B). The spleen commonly shows destruction of white pulp architecture with erythrophagocytosis, while lymph nodes exhibit fibrin deposition and extensive haemorrhage (Figure 3A). A key feature of acute ASF is vascular injury, including endothelial activation and increased permeability, leading to disseminated haemorrhages in multiple organs. In the lungs, interstitial and alveolar oedema and congestion are frequent, and kidneys may display glomerular and interstitial haemorrhages (Figure 3C) (P. Sánchez-Cordón et al.). Lymphocyte apoptosis contributes significantly to immunosuppression and disease severity (Carrasco et al., 1996). In subacute or chronic cases, lesions may include persistent lymphoid depletion, vasculitis, and occasional granulomatous inflammation (P. Sánchez-Cordón et al.).

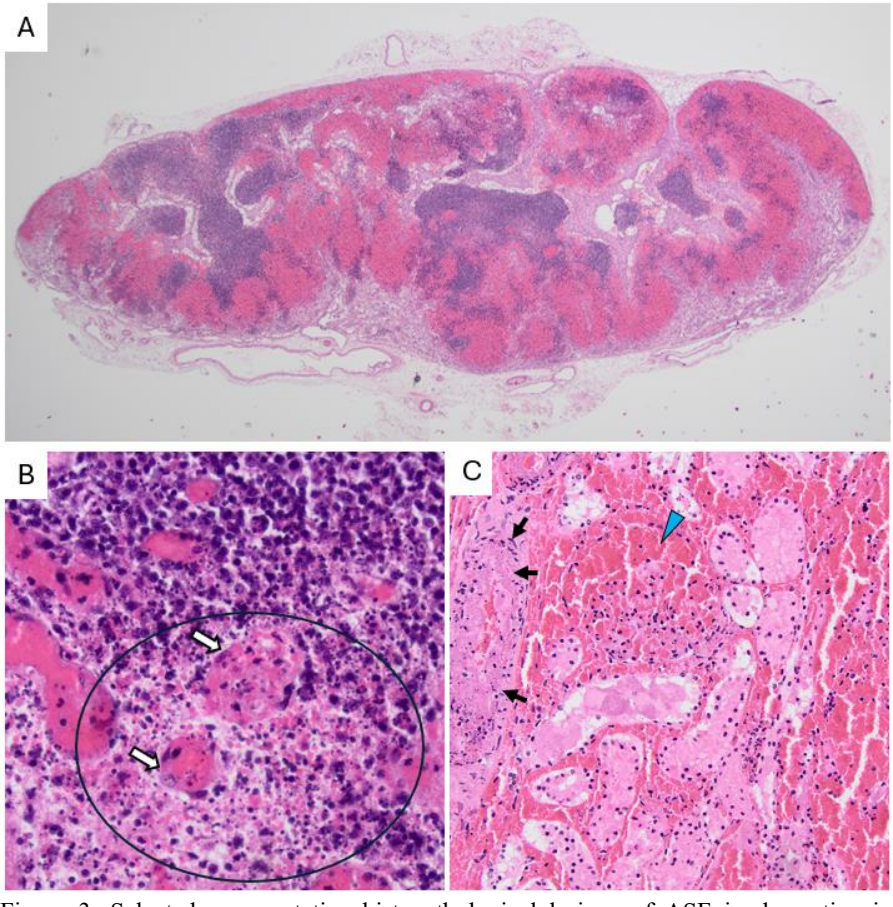

Figure 3: Selected representative histopathological lesions of ASF in domestic pig intranasally infected with ASFV. (A) Extensive haemorrhages in a renal lymph node. (2x). (B) Pyknosis and cell fragmentation (circle) vascular damage and plump endothelial cells (white arrows) In the medial retropharyngeal lymph node (40x). (C) Haemorrhages within glomeruli, blue arrowhead, and in the interstitium. Vasculitis and microthrombi (black arrows) in the kidney (40x). Photos: Emil Wikström Lassa

1.1.5 Vaccine and control measures

Despite decades of research, a safe, effective, and widely deployable vaccine against ASFV is still not available. Recent advances have explored multiple vaccine platforms, with attenuated live-virus vaccines showing the greatest promise to date (Urbano et al., 2022). Gene-deleted ASFV strains have demonstrated protective immunity and improved safety profiles in experimental settings, although issues such as reversion to virulence and

effects on reproductive performance highlight ongoing challenges (Nguyen et al., 2025; Vu et al., 2024). Real-world examples of progress include Vietnam, where live attenuated vaccines have been used in domestic pigs, although field results have been mixed and at times contradictory (Tran et al., 2022). Despite the epidemiological importance of wild boar, most of the available data on the pathology and pathogenesis of the currently circulating ASFV genotype II still derive largely from studies in domestic pigs. Similarly, vaccine trials for ASF have traditionally been conducted almost exclusively in domestic pigs, although in recent years an increasing number of studies have begun to focus on wild boar (Barasona et al., 2019).

There are cautionary examples of the application of domestic animal vaccines to wild animals, with differences in vaccine efficacy and pathogen shedding. Vaccines must therefore be developed in parallel, but specifically for domestic pigs and wild boar respectively (Gavier-Widén et al., 2020).

1.2 Role of the wild boar

1.2.1 Wild boar populations

The European wild boar is a robust mammal with a bristly coat and tusks. It is closely related to domestic pigs because pigs originated from wild boar that were domesticated approximately 9000 years ago (Giuffra et al., 2000). Wild boar are omnivores, feeding on roots, fruits and small animals and live in social groups. They are also known to scavenge (Cukor et al., 2020; Selva et al., 2005). They are now one of the most widespread and abundant large mammals in Europe, thriving in forests and farmland, but also near urban areas. Historical data show that wild boar once inhabited the Scandinavian peninsula. (Rosvold et al., 2010). It is not known exactly when wild boar disapeared, and small, local populations have established in Sweden from time to time, but all of these were eventually eradicated. (Bergqvist et al., 2024) The current Swedish population of wild boar originates from wild boar that escaped enclosures where they were kept for hunting and meat purposes during the 1970s and onwards. They initially were considered an invasive species, but in 1986, a decision was made (Swedish hunting regulation 1987:905 and report 1986/87: JoU 15) to let wild boar roam the Swedish forests once again. Although exact numbers are unavailable, the Swedish wild boar population is estimated at approximately 300,000 individuals, with

the highest densities occurring in southern Sweden. (Augustsson et al., 2024). This area overlaps with Sweden's most intensive areas of pig production.

1.2.2 Wild boar as a reservoir for pathogens

The genetic similarity (Groves, 1981) between the domestic pig (*Sus scrofa domesticus*) and wild boar (*Sus scrofa scrofa*) makes the wild boar an important reservoir of pathogens of pigs like African and classical swine fever (Artois et al., 2002; Lentz et al., 2023). Wild boar are highly susceptible to ASF, usually developing severe clinical disease with fatal outcomes (Pietschmann et al., 2015; Pikalo et al., 2020; Sánchez-Cordón et al., 2019). Wild boar can also carry and transmit other disease-causing agents, including those with zoonotic potential like *Salmonella* spp, *Yersini*a spp, and hepatitis E (Sannö et al., 2014; Wacheck et al., 2009; Widén et al., 2010). A good understanding of the wild boar-pig interface is crucial for management and mitigation of pathogen spillover and spread (Ernholm et al., 2023).

1.3 African swine fever in Sweden and Hungary

Sweden has a national passive disease surveillance program for wildlife that relies on volunteers to report dead or sick animals to the Swedish Veterinary Agency (SVA). Carcasses or samples are submitted to SVA for analysis to follow wildlife health status and identify potential outbreaks of new diseases.

On August 25th, 2023, two dead wild boar were reported from the small town of Fagersta in Sweden. By September 6th, it was confirmed that one of the wild boar was positive for ASFV. A restriction zone was set up and after a thorough search by local hunters in which all carcasses were tested, a core area was established and fenced in. When the number of wild boar was reduced, the remaining wild boar inside the core area were culled. Sweden was declared free from ASF in September 2024. The source of infection was never established. The virus was genotyped as ASFV genotype II.

In Hungary, wild boar are widespread and historically have been a significant part of the country's big game fauna. ASF has persisted in Hungary's wild boar since the first confirmed case caused by an ASFV genotype II strain in Heves County on 21 April 2018 (Authority et al., 2018). Regular detections have been reported in recent years. During this time, no

ASF has been reported in domestic pigs. Both these outbreaks provide an opportunity to learn more about ASF pathology in wild boar.

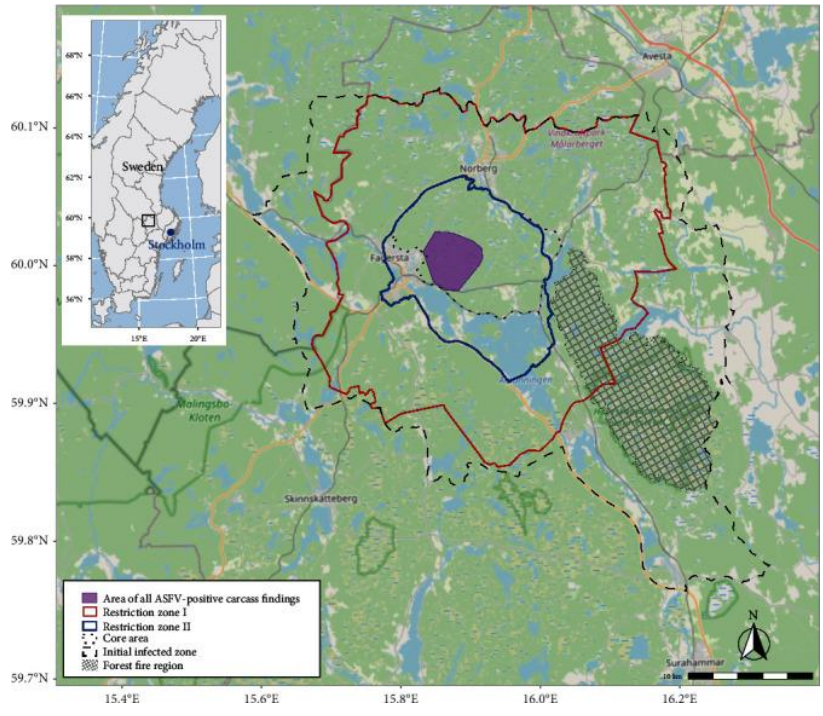

Figure 4. Map indicating the location of the ASF outbreak in Sweden. The dashed line marks the infected zone defined on 7 September 2023, the dotted line marks the core area of the outbreak, and the red and blue lines mark the zones established by the European Commission on 30 November 2023 (red line = restricted zone I, blue line = restricted zone II, fenced off). The purple area marks the area in which all the infected carcasses were found. The crosshatched zone marks an area that was affected by wildfire in 2014 that could not easily be accessed by foot. Figure reproduced from (Chenais et al., 2024), Porcine health management under the terms of Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/4.0/).

1.4 Rationale for this research

Wild boar play a crucial role in the maintenance and spread of ASF across Europe and Asia (Cadenas-Fernández et al., 2022; Chenais et al., 2019; EFSA et al., 2022). Despite this epidemiological significance, most data on the pathology and pathogenesis of the current circulating ASFV genotype II come from studies in domestic pigs. Experimental studies on ASF in wild

boar are limited, particularly those using the oronasal route of infection that mimics natural transmission. Differences in methodology across studies also make it difficult to compare results between domestic pigs and wild boar. Furthermore, the available literature on experimental infections in both wild boar and domestic pigs predominantly describes macroscopic and histopathological findings observed at advanced stages of disease, often at death or at predefined humane endpoints. Such approaches provide only limited insight into early host–virus interactions and the initial phases of disease progression, representing a significant research gap. With a few exceptions, data on histopathological lesions in naturally infected wild boar also remain scarce (Perez et al., 1998; Phan et al., 2024; Sai Balaji et al., 2024; Sehl-Ewert et al., 2022). Obtaining high-quality, representative tissue samples from wild boar carcasses is particularly challenging due to the rapid onset of decomposition and autolysis, as well as the strict biosecurity measures required to contain the virus. Although experimental infection aims to mimic the disease as it occurs in naturally infected animals, free-ranging wild boar are exposed to environmental variability, co-infections, nutritional differences, and periods of stress. Furthermore, in natural infections, it is not possible to control the route of infection, viral dose, or number of exposure events. Recognizing the range of ASF presentations in naturally infected wild boar and understanding how these might differ from outcomes observed in controlled experimental infections is critical for improving ASF surveillance and for strengthening the knowledge base required to support vaccine development for wild boar.

2. Aims

The general aim of this project is to address knowledge gaps in the pathobiology of ASFV Genotype II in wild boar and domestic pigs. Current knowledge is largely based on experimental studies in domestic pigs and is often extrapolated to wild boar, despite indications that disease progression and host responses may differ between the two subspecies and the limited number of comparative ASF studies involving both.

Specific aims:

- To characterize the pathogenesis of African swine fever caused by ASFV genotype II strain "Armenia 2007" in domestic pigs and wild boar, with emphasis on early host–virus interactions using an experimental infection model (study I and II).

- To compare disease susceptibility, clinical progression, and pathological outcomes between domestic pigs and wild boar using a comparative approach and a standardized scoring systems (study I and II).

- To investigate the pathology of naturally infected wild boar from ASF outbreaks in Sweden and Hungary (study III).

- To evaluate how well experimental infection models reflect the natural course of disease in free-ranging wild boar (study III).

3. Materials and Methods

Details of the materials and methods for each study in this thesis are presented in the manuscripts. In this section, an overview of the methodology and additional considerations and limitations are provided.

3.1 Animals

Both pigs and wild boar were studied under experimental conditions in studies I and II, while study III was based on naturally infected free-ranging wild boar from ASF outbreaks in Sweden and Hungary.

An important challenge of the comparative experimental infection (study I and II) was obtaining suitable animals, particularly in the case of wild boar. Nineteen commercial domestic large white/landrace cross pigs, age 10-12 weeks, and 19 farmed wild boar, age 16-18 weeks, were used. The wild boar had good health status and were under veterinary supervision. The age difference was considered because age might impact ASF disease outcomes. There is high lethality of up to 90–100% in naïve young animals, whereas older animals are more likely to survive (Post et al., 2017). However, due to factors such as genetics, diet and living conditions on farms, domestic pigs and wild boar have different growth rates and average daily gains, with pigs growing faster. To balance these differences in animal size, we used domestic pigs that were slightly younger than the wild boar. This modest age difference was not expected to influence disease dynamics or outcomes, as both groups consisted of sexually immature but immunologically mature animals that are highly susceptible to ASFV.

Except for two wild boar shot within the core zone of the Swedish outbreak, the study animals from the Swedish and Hungarian outbreaks (Paper III) were carcasses found during the search and removal phase of the outbreaks.

Because of the opportunistic nature of the cases, no selection criteria based on sex, age, or other factors were applied. Suitability for sampling was the sole criterion. A summary of sampled wild boar naturally infected with ASFV genotype II is presented in Table 1.

Table 1. Summary table of sampled wild boar (*Sus scrofa scrofa*) carcasses naturally infected with highly virulent African swine fever virus (ASFV) genotype II in Hungarian and Swedish outbreaks.

Wild boar ID	Country	Weight (kg)	Sex	Source Carcass/ hunted	Decomposition/autolysis[a]	Body condition[b]
S1	SWE	10	NA	Carcass	fresh	Normal
S2	SWE	20	F	Carcass	Early decay	Good
S3	SWE	11,5	F	Hunted	Fresh	Poor
S4	SWE	30	M	Carcass	Early decay	Normal
S5	SWE	35	F	Carcass	Early decay	Good
S6	SWE	21	M	Hunted	Fresh	Emaciated
H1	HUN	NA	NA	Carcass	Fresh*	NA
H2	HUN	NA	NA	Carcass	Early decay*	NA

(Swe): Sweden; (Hun): Hungary; (M): male; (F): female; [a]Decomposition was categorized following criteria previously described (Chenais et al., 2024) [b]Body condition was assessed based on the degree of protuberance of vertebrae and ribs, as well as amount of internal fat. *Decomposition was based on histopathological evaluation.

3.2 Study design and sampling of tissues

The data from the first two studies (study I and II) came from an *in vivo* experimental infection carried out in UK in 2020, funded through a VetBioNet transnational access grant. The purpose of the animal infection experiment was to provide material and data for comparative assessment of the pathogenic and immunological mechanisms from early infection to terminal disease in wild boar and pigs infected with ASFV genotype II strain "Armenia 2007". The inoculation trial was performed in the BSL3 facilities at the Animal and Plant Health Agency (APHA), Weybridge, UK. The experimental design is summarised in figure 5.

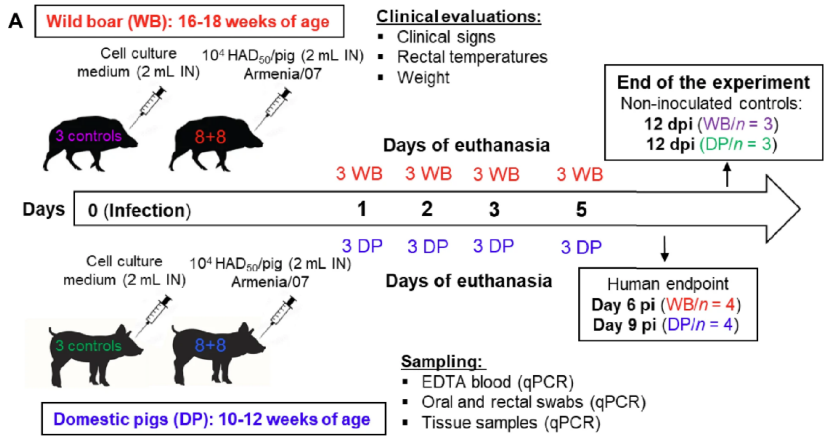

Figure 5. Schematic overview of the experimental design (study I and II). Wild boar and domestic pigs were intranasally inoculated with African swine fever virus genotype II isolate, "Armenia 2007", monitored over multiple days post-infection, and euthanized at predetermined time points for sampling and analysis. The timeline illustrates inoculation, monitoring, and sample collection events for each group. Figure adapted from (Sánchez-Cordón et al., 2024), Veterinary Research under the terms of Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/4.0/)

A total of 38 animals were included: 19 pigs and 19 farmed European wild boar of both sexes. Animals were housed in high-containment facilities and randomly allocated to infected or mock-infected control groups. Following a 7-day acclimatisation period, animals assigned to the infected groups (16 pigs and 16 wild boar) were intranasally inoculated with a highly virulent ASFV genotype II isolate, "Armenia 2007" to mimic natural infection, while control animals (3 pigs and 3 wild boar) were mock-inoculated with cell culture medium. All animals were monitored daily for clinical signs, rectal temperature, and welfare using a predefined scoring assessment system with established humane endpoints.

To characterise disease progression, sequential euthanasia of infected animals was performed on days 1, 2, 3, and 5 post-infection (pi), with three pigs and three wild boar euthanised at each time point. Remaining animals (4 pigs and 4 wild boar) were euthanised at humane endpoints (day 6 pi for wild boar and day 9 pi for pigs). To help evaluate lesions associated with ASF infection, mock-inoculated control animals were culled at the end of the study (day 12 pi).

Blood samples from inoculated and non-inoculated control animals were collected before inoculation (day 0). Following inoculation, blood samples, along with nasal and rectal swabs, were obtained at the scheduled euthanasia time points on days 1, 2, 3, and 5 pi, as well as at the humane endpoint (day 6 pi for wild boar and day 9 pi for domestic pigs). Additional blood samples and swabs were also taken from the remaining infected domestic pigs on day 8 pi. For the mock-inoculated control animals, blood samples and swabs were collected on day 8 pi and at the end of the experiment on day 12 pi.

At euthanasia, full necropsies were conducted and a standardised macroscopic lesion scoring system was applied to systematically document pathology. A range of tissues (including tonsils, lymph nodes, spleen, lungs, and other organs) were collected. Blood, swab eluates and tissue samples were frozen at −80 °C for subsequent detection of the ASFV genome through qPCR (Study I). In Study II, the collected tissues were examined by histopathology and immunohistochemistry to assess lesion development and the progressive systemic spread of viral antigen.

Study III was based on sampling of wild boar during natural ASF outbreaks in Sweden and Hungary. In Sweden, a subset of wild boar was sampled under field conditions or at a temporary field necropsy facility established to maintain biosecurity, while additional animals were transported to a BSL-3 laboratory for full necropsy. All carcasses included from the Swedish outbreak (n= 6) underwent necropsy to briefly document macroscopic pathological findings, assess body condition, and collect tissues for histopathological and virological analyses. To complement the Swedish material, tissue samples from two wild boar collected during the first confirmed ASF outbreak in Hungary in 2018 were included.

3.2.1 Ethical considerations

The experimental design strictly adhered to the principles of 3Rs and was conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, under project licence PF971B5E3 and ethical approval from the Animal and Plant Health Agency (APHA) Animal Welfare and Ethical Review Board Ethics committee.

Replacement: This study necessarily involves the use of animals. No alternative methods can adequately replicate the complex biological interactions that occur within a whole organism following infection with

African swine fever virus (ASFV) and generate the diversity of samples needed.
Reduction: The study design used the minimum sample size needed for the design to be robust enough and have statistical validity (19 WB and 19 DP). The extensive sampling from this experiment generated not only material for this thesis project but also material for immunology and histopathology studies outside of the scope of this thesis project, along with banking of materials for future studies to reduce future need for animal experiments on ASFV genotype II.
Refinement: The animals were handled by well-trained staff, and the number of procedures, data collection, and blood and swab collection was kept to the minimum required for statistical validity. A 10-parameter clinical monitoring scheme requiring clinical scores to be recorded twice daily to closely monitor the progression of clinical signs were applied. Regular monitoring using a clearly defined clinical assessment protocol allowed for rapid euthanasia once predefined humane endpoints were reached, thereby reducing suffering and considering animal welfare.

3.3 Field conditions (study III)

While experimental conditions in studies 1 and 2 were highly controlled, the material for study III was highly influenced by field conditions. Following the first detection of the virus in Sweden, an active carcass search was conducted to determine the extent and core area of the outbreak. Local hunters coordinated by authorities performed searches on foot. Work initially expanded outward from the first detected carcasses, with priority given to wild boar habitats and familiar hunting areas. As operations progressed, efforts shifted to previously unsearched zones and areas with earlier positive cases to ensure full coverage.

Field conditions were characterized by warm autumn temperatures, which frequently resulted in advanced carcass decomposition and extensive maggot infestation, limiting the number of carcasses suitable for sampling. All found carcasses or remains were removed from the forest using strict biosecurity measures, including the use of personal protective equipment and thorough cleaning and disinfection of footwear, vehicles, and equipment. Carcasses were then transported to a preliminary field sampling station by designated patrols, sampled by official veterinarians, and incinerated on-site.

Figure 6. Field conditions during Swedish outbreak of African swine fever 2023. Left: Searching for wild boar carcasses dead from ASFV under field conditions. Right: Field sampling centre set up during the Swedish ASF outbreak. Photo: Erik Ågren (Left) Emil Wikström-Lassa (Right)

3.4 Laboratory diagnostics and data analysis (study III).

Presence of ASFV was determined using real-time PCR at the Swedish Veterinary Agency (SVA) following previously described protocols (Chenais et al., 2024). Samples were also sent for confirmation to the European reference laboratory for ASF (Centro de Investigación en Sanidad Animal (INIA), Valdeolmos, Spain) and to the Friedrich Loeffler Institute (FLI). For wild boar sampled during the Hungarian outbreak ASFV was detected in spleen samples using real-time PCR (Virotype ASFV - Qiagen) at the Veterinary Diagnostic Directorate (VDD), Budapest.

3.4.1 Sequencing

The outbreaks in both countries were caused by highly virulent ASFV genotype II strains, derived from original strains introduced into Georgia from southeastern Africa in 2007 (Authority et al., 2018; Chenais et al., 2024).

3.4.2 Histopathology

During necropsy, tissue samples were collected, fixed in 10% buffered formalin solution and routinely processed for histopathological and

immunohistochemical studies. Paraffin-embedded tissue samples from the collected organs were sectioned at 4 µm and stained using Mayer's haematoxylin and eosin (HE) protocol for light microscopic examination.

3.4.3 Immunohistochemistry

During the doctoral project, an immunohistochemical method was developed at SVA to visualize ASFV in tissues. Despite optimizing a commercial antibody, the results were not sufficiently satisfactory. Consequently, paraffin-embedded tissue sections from the experimental infection (Study II) were sent to APHA for immunohistochemical staining using a locally developed, non-commercial monoclonal antibody targeting the p30/CP204L viral protein. However, optimization of our protocol at SVA continued in parallel and was successfully applied in study III. Thus, for samples obtained from the Swedish and Hungarian outbreaks (study III), the commercial monoclonal primary antibody against VP72 (18BG3, Ingenasa, Madrid, Spain), specific for ASFV, was used. Protocols are described in detail in manuscript of study III.

Cells immunolabelled for viral antigen (study II and III) were tentatively identified based on morphological features, such as cell size and anatomical location. While double immunolabelling may be useful for identifying the types of infected cells, this approach was discussed prior to the study but ultimately ruled out. Based on the experience of the research team, double immunolabelling often results in a reduced number of immunolabelled cells compared with single staining. Because one of the study aims was to examine early pathogenesis, when infected cells may be few, this approach was considered unsuitable. In the absence of complementary techniques, morphological features, location, and size are criteria commonly applied by veterinary pathologists to identify the types of immunolabelled cells.

3.4.4 Evaluation of lesions

For all studies (study I, II and III), previous standardized scoring protocols were used to support the macroscopic, histopathological and immunohistochemical evaluations (P. J. Sánchez-Cordón et al., 2021). This helped to ensure systematic evaluation and generate data for statistical analyses. Macroscopic lesions were scored and individually documented to generate the cumulative lesion scores across the various organs examined in each animal. Similarly, histopathological changes and the presence of cells

immunolabelled for viral antigen were evaluated and scored across the different components of each organ. In brief, the following semi-quantitative scoring system was applied: (0) no histopathologic changes/no presence of immunolabelled cells; (1) minimal histopathologic changes/occasional presence of immunolabelled cells; (2) mild histopathologic changes/mild presence of immunolabelled cells; (3) moderate histopathologic changes/moderate presence of immunolabelled cells; (4) severe histopathologic changes/abundant presence of immunolabelled cells. All tissue sections were examined by a single veterinary pathologist (Emil Wikström Lassa) blinded to the animals' identity and origin.

4. Results and discussion

Intranasal inoculation with the highly virulent ASFV genotype II strain "Armenia 2007" resulted in acute disease in both European wild boar (*Sus scrofa scrofa*) and domestic pigs (*Sus scrofa domesticus*) (Studies I and II). Despite identical experimental conditions, inter-subspecies differences were observed with respect to incubation period as well as the development and severity of lesions. In comparison, wild boar naturally infected with ASFV genotype II in outbreaks displayed features of subacute disease and lesions were more diverse than seen in the experimental infections. This section provides an overview and general discussion of the results, including the main challenges and limitations of the studies. Detailed, in-depth analyses are presented in the respective individual papers.

4.1 Clinical disease course

In the comparative experimental infection between wild boar and domestic pigs (study I and II), wild boar developed clinical disease earlier than domestic pigs, although clinical scores of the respective subspecies were similar at the humane endpoint. In wild boar, rectal temperatures began to rise as early as day 3 pi, followed by the first signs of clinical disease, such as loss of appetite and reduced activity, on day 4 pi (Fig 4). All wild boar reached the humane endpoint at day 6 pi. In contrast, pigs did not display any clinical illness until day 7 pi, when a rise in rectal temperature and systemic signs of disease, similar to those observed in the wild boar, became apparent. Thus, the incubation period until the appearance of clinical signs was approximately 4 days for the wild boar and 7 days for the pigs. Humane endpoint for the pigs was reached at day 9 pi, corresponding to a clinical disease duration of approximately 3 days. This was comparable to the wild boar group, indicating that once clinical signs appeared, the disease duration did not differ between the two subspecies. One of the domestic pigs did not reach the clinical endpoint scores but was euthanised to avoid single housing.

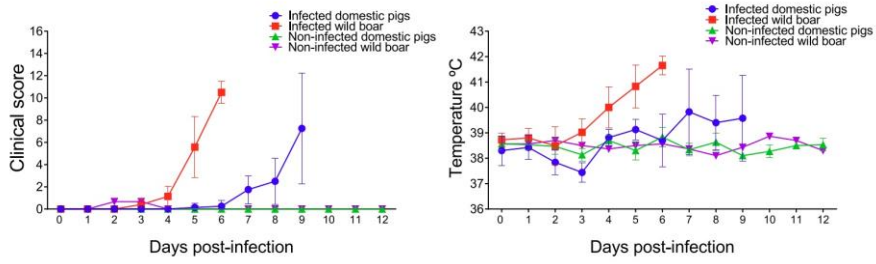

Figure 7. Differences in clinical scores and rectal temperatures at different days after infection. (Mean±SD) between wild boar and domestic pigs after intranasal infection with ASFV genotype II strain 'Armenia 2007'. Figure adapted from (Sánchez-Cordón et al., 2024), Veterinary Research under the terms of Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/4.0/)

4.2 Gross pathology (study I and III)

In the comparative study between wild boar and domestic pigs (study I) the earliest visible macroscopic lesions characteristic of ASF were observed at day 3 pi in both subspecies and included mild lymphadenomegaly and petechiae or larger haemorrhages in the lymph nodes. Petechiae were also observed in the urothelium in one wild boar and one domestic pig, respectively. By day 5 pi, more severe lesions were seen in one wild boar compared to the domestic pigs, including widespread haemorrhagic lymphadenopathy. However, at their respective humane endpoints, wild boar exhibited somewhat less severe macroscopic lesions and overall disease severity than pigs.

Macroscopic findings showed that lymphoid organs in the oronasal region, especially the medial retropharyngeal lymph nodes (MRPLN) and tonsils, play a central role in the early stage of infection. These tissues displayed evident macroscopic changes, including haemorrhages and enlargement, alongside high levels of virus genome. This makes them well-suited as target organs for field sampling to detect early disease, while also avoiding the need to open the carcass and thereby reducing the risk of environmental viral spread.

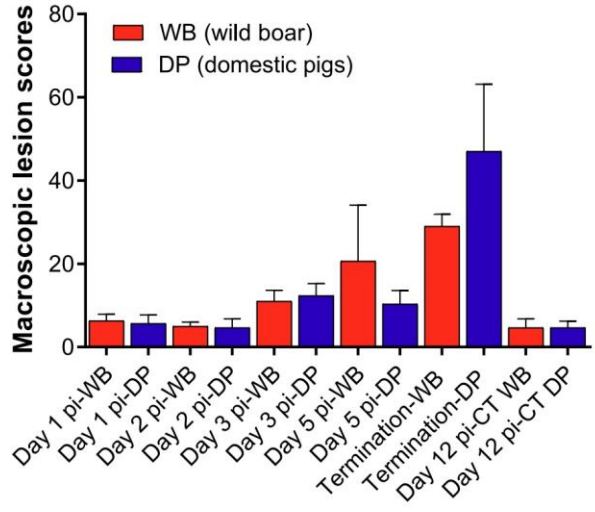

Figure 8. Macroscopic evaluation of lesions. Mean±SD of the cumulative macroscopic scores (y-axis) in each group of domestic pigs or wild boar euthanised on different days after infection. Uninfected animals (CT) euthanised on day 12 pi are also shown (x-axis). Figure adapted from (Sánchez-Cordón et al., 2024), Veterinary Research under the terms of Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/4.0/)

The macroscopic evaluation of the naturally infected wild boar (available from the Swedish outbreak only), revealed more severe and widespread haemorrhagic and congestive changes in the gastrointestinal tract compared with the experimental infections. These lesions are indicative of a subacute presentation of ASF (P. Sánchez-Cordón et al., 2021). The most prominent findings included markedly enlarged, dark-red spleens, as well as enlarged, oedematous, and haemorrhagic lymph nodes. Widespread haemorrhages were observed in multiple organs, including the heart and kidneys. Small amounts of blood-tinged fluid were present in body cavities in some cases.

4.3 Virology and viral distribution

Blood samples from both domestic pigs and wild boar (study I) tested negative for ASFV by qPCR on days 1 and 2 pi. In wild boar, a significant amount of the virus genome was detected in blood samples from one animal euthanised on day 3 pi and from all three euthanised on day 5 pi. In contrast, no virus genome was detected in any of the blood samples from the domestic pigs euthanised on day 3 or 5 pi. While all four wild boar that reached a

humane endpoint on day 6 pi were viraemic, only three of the four domestic pigs were viraemic from day 8 pi. The levels of viremia in the domestic pigs that reached the humane endpoint were high and similar to those detected in wild boar.

Across both experimental infection studies (study I and II), the MRPLN consistently emerged as the earliest and most consistent site of viral replication following intranasal inoculation. In wild boar, ASFV antigen and viral genome were detected in the MRPLN as early as 3 days post-infection (dpi), whereas pigs remained negative until 5 dpi. The submandibular lymph node also showed early involvement in wild boar but exhibited delayed or absent detection in pigs during the early stages of infection. Following early replication in lymph nodes, wild boar exhibited rapid systemic dissemination of the virus to secondary target organs. By 5 dpi, viral antigen and viral genome were detected across multiple organ systems in wild boar, including spleen, lungs, liver, kidneys, and regional draining lymph nodes. In contrast, pigs at the same time point showed limited dissemination, with viral antigen largely restricted to the MRPLN and only sporadically present in the bone marrow.

At the humane endpoint, viral antigen was widely distributed in both subspecies. Monocyte/macrophages were the main targets cells for ASFV, and lymphoid organs consistently harboured large numbers of ASFV-immunolabelled macrophages. As the disease progressed, additional cell type became immunolabelled for viral antigen, including hepatocytes, pneumocytes, endothelial cells, epithelial cells, and reticular cells. Viral genome loads were high at terminal stages in both subspecies.

ASFV DNA was not detected in nasal or rectal swabs prior to day 5 pi, and virus shedding was observed only immediately before the humane endpoint, at moderate titres. These findings indicate that virus excretion during the early phase of the disease is limited. Consequently, transmission via respiratory secretions or faeces is likely to play only a minor role during early and mid-stages of disease. Instead, virus spread in wild populations is more plausibly driven by contact with infectious blood or carcasses, which may retain viable virus for prolonged periods.

Viral antigen was detected by immunohistochemistry in all tissues of sufficient quality for evaluation from wild boar that tested positive by real-time PCR during the Swedish and Hungarian outbreaks.

Lymphoid tissues such as the spleen, tonsil, and lymph nodes exhibited the highest abundance of immunolabeled cells. In contrast to the experimental infections, which showed a more consistent relationship between antigen detection and tissue-specific viral loads, the distribution of antigen in naturally infected wild boar varied markedly between organs and among individuals. Direct comparisons should be made with caution, as the naturally infected animals varied in the degree of autolysis. Despite this, immunohistochemistry remained a useful tool for assessing viral distribution, although autolysis significantly affected the results. Differences in tissue preservation appeared to contribute more to the quantitative variation in immunolabelled cells than actual differences in viral load among the less well-preserved individuals.

4.4 Histopathology

In study II, the histopathological lesions followed the temporal course of viral replication. Prior to detectable viral antigen, both subspecies showed only minimal and nonspecific histopathological changes. In wild boar, characteristic ASF-associated lesions such as haemorrhages, lymphoid depletion, karyorrhexis, and vascular damage appeared earlier, particularly in lymphoid tissues of the oronasal region.

At 5 dpi, wild boar exhibited virus-associated lesions in the MRPLN and submandibular lymph nodes, whereas pigs showed no distinct virus-associated pathology at this stage. However, at the humane endpoint, pigs consistently displayed more severe and extensive microscopic lesions across multiple organ systems, including severe lymphoid depletion, pulmonary inflammation and oedema, hepatocellular necrosis, renal haemorrhages, vasculitis, and cutaneous vascular lesions. Wild boar showed qualitatively similar lesions, but with reduced severity and more limited tissue involvement.

The histopathological scoring confirmed that although lesion development occurred earlier in wild boar, cumulative severity of lesions at humane endpoint was greater in pigs (Table 2).

Table 2. Summary of histopathological and virus antigen scores in pigs and wild boar euthanised on different days after intranasal infection with ASFV genotype II strain 'Armenia 2007'.

Domestic pigs (DP)				Wild Boar (WB)			
ID	IHC	HP	Euthanasia	ID	IHC	HP	Euthanasia
DP25	0	26	1 dpi	WB44	0	17	1 dpi
DP26	0	17	1 dpi	WB45	0	25	1 dpi
DP27	0	36	1 dpi	WB46	0	19	1 dpi
DP28	0	21	2 dpi	WB47	0	20	2 dpi
DP29	0	22	2 dpi	WB48	0	24	2 dpi
DP30	0	17	2 dpi	WB49	0	16	2 dpi
DP31	0	29	3 dpi	WB50	1	21	3 dpi
DP32	0	23	3 dpi	WB51	7	25	3 dpi
DP33	0	26	3 dpi	WB52	1	20	3 dpi
DP34	3	33	5 dpi	WB53	43	49	5 dpi
DP35	4	17	5 dpi	WB54	11	23	5 dpi
DP36	1	25	5 dpi	WB55	10	21	5 dpi
DP37	153	155	9 dpi	WB56	99	85	6 dpi
DP38	0	22	9 dpi*	WB57	87	84	6 dpi
DP39	153	180	9 dpi	WB58	38	54	6 dpi
DP40	170	182	9 dpi	WB59	91	74	6 dpi
DP22	0	13	12 dpi (control)	WB41	0	18	12 dpi (control)
DP23	0	10	12 dpi (control)	WB42	0	10	12 dpi (control)
DP24	0	21	12 dpi (control)	WB43	0	15	12 dpi (control)

(ID): Animal identification; (IHC): Virus antigen scores; (HP): Histopathological lesion scores; (dpi): days post infection; *Did not reach clinical endpoint (euthanised to prevent single housing). Figure reproduced from (Wikström-Lassa et al., 2026), Veterinary Research under the terms of Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/4.0/)

Despite differences in age, sex, and carcass decomposition, no remarkable differences were observed in key organs, particularly lymphoid tissues, among the naturally infected wild boar from the two outbreaks. The main lesions consisted of haemorrhages and lymphoid depletion, accompanied by pyknosis and cell fragmentation within lymphoid tissues. The naturally infected animals also displayed features indicative of subacute form of ASF, including more severe lymphoid depletion and haemorrhages.

Compared with the lesions exhibited in the experimental infections, the histopathological findings in tissue samples from naturally infected wild boar

carcasses (study III) were somewhat more heterogeneous and, in some cases, more severe. In the experimental infections, the animals were euthanised as early as they reached a humane endpoint, in accordance with ethical requirements The more severe and heterogeneous lesions observed in the wild boar likely reflect a longer disease course in field cases. Additionally, some of the lesions observed in naturally infected wild boar were not directly attributed to ASFV infection. Additional factors, such as comorbidities, secondary infections, and trauma, likely contributed to the more heterogeneous disease presentation seen in wild animals compared with the controlled conditions of experimental infections. An illustrative example was the pneumonia observed in several wild boar. Microscopic evaluation revealed a high burden of lung parasites, such as *Metastrongylus* spp., which are commonly reported in wild boar and are sometimes associated with verminous pneumonia (da Silva et al., 2013; Spieler et al., 2021). These findings suggest that, in these cases, pneumonia was likely parasite-related rather than ASFV-induced. The ASFV scoring system used in studies II and III includes a wide range of tissue alterations. As a result, comorbidities, such as parasitic pneumonias or other co-infections, may increase the overall lesion scores and potentially hinder the interpretation of findings, since not all observed lesions would be directly attributable to ASFV.

4.5 Study limitations

Extrapolating from a single experimental infection on how wild boar respond to ASF compared with pigs is subject to important limitations. The main constraint in both the experimental studies (study I and II) and the natural infection study (study III) was the small sample size. Given the small number of animals studied, some of the observed differences may simply reflect characteristics of the specific individuals included. Furthermore, the experimental conditions in studies I and II, such as the inoculated virus dose, the route of infection, and the controlled laboratory environment, may not accurately reflect natural exposure scenarios, limiting the extent to which these results can be generalised. Without replication across multiple populations and contexts, conclusions about consistent biological differences between the two subspecies remain tentative.

4.6 Conclusions

The combined findings from the experimental infection with ASFV (study I and II) suggest that wild boar have a shorter incubation period and are less tolerant to tissue damage before reaching the humane endpoint than pigs. Moreover, they displayed an earlier viremia, presence of viral antigen, and tissue lesions. The mechanisms for this, however, remain unclear.

These studies also show that lymphoid tissues within the oronasal tract, including the medial retropharyngeal and submandibular lymph nodes, are important organs in early infection, exhibiting early detection of virus genome, viral antigen and tissue damage. Avoiding the need to open the carcass and thereby reducing the risk of environmental viral spread would also make these lymph nodes an excellent alternative for ASF positivity testing in the field.

In naïve populations, infection with highly virulent ASFV isolates generally results in an acute form of the disease (Sánchez-Cordón, Vidaña et al.). However, one notable finding in our study of natural infection (study III) was the presence of lesions suggestive of subacute disease. Similar observations have been reported in another study of naturally infected wild boar (Sehl-Ewert, Deutschmann et al. 2022). Our findings included extensive haemorrhages in multiple lymph nodes, marked congestive and haemorrhagic changes in the intestines, and more severe lymphoid depletion than typically observed in the acute form of disease. However, the lesions observed showed considerable overlap between acute and subacute patterns, which may indicate a relatively prolonged course of disease in naturally infected wild boar. This may be explained by a lower initial infectious dose of ASFV under natural conditions compared with experimental infections. It also illustrates how disease progression in naturally infected animals can be more complex than in controlled experimental settings. These findings are relevant for understanding the natural course of the disease, informing surveillance strategies, and improving the interpretation of pathological findings from wild boar in ASF outbreak areas.

Differences in disease response between wild boar and pigs have also been documented in other viral infections. For example, in experimental infections with highly pathogenic Porcine Reproductive and Respiratory Syndrome Virus (HP-PRRSV), several wild boar succumbed to the disease, whereas none of the domestic pigs died (Do et al., 2015). Differences have also been demonstrated in a comparative experimental infection with the

moderately virulent ASFV strain "Estonia 2014," carried out in specific-pathogen-free (SPF) pigs and commercial farm pigs. This study showed that baseline innate immune activity and hygienic status can influence disease severity (Radulovic et al., 2022). In our study III, naturally infected wild boar consistently showed more severe and heterogeneous lesions than animals infected under controlled experimental conditions (studies I and II). Although the contribution of genetic, immunological, and virological factors remains unclear, these findings collectively highlight that prior immune experience is a major determinant shaping disease progression and clinical presentation.

We advocate the use of scoring protocols because they facilitate standardization, enable robust statistical analyses, and support meaningful comparisons across studies. However, their application in naturally infected animals is constrained by substantial biological variability and the presence of confounding comorbidities. In our study, elevated scores, particularly in the lungs, were largely attributable to parasitic pneumonia rather than ASFV-specific lesions. This highlights the need to supplement our evaluation protocols with additional criteria so they can reliably identify and assess lesions arising from comorbidities of infectious, metabolic, traumatic, or toxic origin, which may exacerbate the severity of ASFV-specific lesions in naturally infected animals. Furthermore, this also underscores the importance of using both histopathology and immunohistochemistry to determine whether lesions are specifically associated with ASFV infection.

5. Concluding remarks and future perspectives

The recognition of the wild boar's importance as both a reservoir and a susceptible host has evolved considerably. In the 1990s, wild boar were largely regarded as incidental bystanders, considered unimportant in the epidemiology of ASF and unable to sustain the virus in areas where domestic pigs were disease-free (Pérez et al., 1998). Today, they are understood to be key players in maintaining the disease (Lentz et al., 2023). The challenges associated with experimental infections in wild boar, combined with the limited opportunities to sample naturally infected wild boar and their close genetic similarity to domestic pigs, have constrained our understanding of ASF pathology in wild boar, leaving much of it inferred from studies in domestic pigs. Overall, the studies in this thesis provide valuable data on early ASFV infection dynamics under conditions that closely resemble natural exposure in both domestic pigs and wild boar, and they offer new insights into ASF pathology under true natural infection in wild boar. Identifying the MRPLN as an early replication site and demonstrating the heightened susceptibility of wild boar have important implications for surveillance, diagnosis, and vaccine development. Future studies should investigate the immunological or genetic determinants underlying these inter-subspecies differences and evaluate whether similar patterns occur with less virulent or emerging ASFV variants. Using field carcasses and tissue samples of naturally infected wild boar, we were also able to provide a more nuanced picture of the disease and test the scoring system and its limitations under real field conditions.

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Popular science summary

African swine fever (ASF) is one of the most devastating infectious diseases affecting pigs worldwide. Caused by a highly virulent virus, it infects both domestic pigs and wild boar and often leads to high fever, haemorrhages, multi-organ failure, and death. As there is no curative treatment and no widely available commercial vaccine, control depends entirely on strict biosecurity measures, rapid detection, and culling of infected animals, making ASF a major economic and animal welfare concern. Over recent decades, the disease has spread across large parts of Europe and Asia, with wild boar playing a key role in maintaining and transmitting the virus.

Much of our detailed understanding of how ASF causes disease comes from experimental studies in domestic pigs. The research presented in this thesis, largely based on a comparative approach between wild boar and domestic pigs, aimed to expand knowledge of the disease in wild boar and demonstrated that disease progression may differ between the two subspecies. In this experimental infection, wild boar showed signs of illness earlier than domestic pigs. They exhibited lethargy, loss of appetite, and other characteristic symptoms as early as four days after infection with the virus. Domestic pigs showed similar signs, but some days later, from day seven post-infection. Tissue changes and viral replication were also detected earlier in wild boar than in domestic pigs. Despite this delayed onset, domestic pigs developed more severe tissue damage before reaching the humane endpoint. Taken together with the fact that wild boar tolerated less tissue damage before reaching the humane endpoint, this indicates that they were more susceptible and less resistant to ASFV. Post-mortem examinations revealed extensive lesions in organs such as the spleen, liver, and lymph nodes, whereas corresponding changes in wild boar were generally less pronounced. In both subspecies, infection typically began in lymphoid tissues located in the head, around the nose and mouth, reflecting early interaction with the immune system at these sites. These tissues serve as primary targets for ASF virus replication before the virus spreads to multiple organs.

Although experimental models provide essential insights, they do not fully capture the complexity of natural infection in the field. Wild boar live in dynamic environments with fluctuating food availability, parasitic burdens, concurrent infections, and environmental stressors, all of which can

influence disease expression. A unique opportunity to study naturally infected animals arose in September 2023, when the first case of ASF in Sweden was confirmed after the virus was detected in a dead wild boar found in the forest. During the outbreak, samples were collected from multiple wild boar, allowing detailed pathological investigations and comparisons with findings from a European outbreak that occurred in Hungary in 2018.

Examinations of these naturally infected wild boar showed changes consistent with acute to subacute ASF. The spleen and lymph nodes were severely affected, with extensive haemorrhages and marked depletion of immune cells. Compared with the experimentally infected animals, the naturally infected wild boar often displayed more varied and sometimes more severe tissue changes. A major contributing factor was the presence of other diseases or infections, especially parasites in the lungs, which caused changes unrelated to ASF. These findings highlight the importance of combining conventional microscopic examination with specific techniques that identify the virus in tissues, such as immunohistochemistry, to accurately distinguish ASF-associated changes from those caused by other diseases.

Overall, naturally infected wild boar developed the same typical disease and changes described in experimental settings, although the disease picture in the field was more complex and difficult to assess. Understanding what happens to wild boar during natural infection is crucial for improving surveillance, refining diagnostic approaches, and guiding the development of vaccines and control strategies.

Populärvetenskaplig sammanfattning

Afrikansk svinpest (ASF) är en av de mest förödande infektionssjukdomarna som drabbar grisar världen över. Sjukdomen orsakas av afrikanskt svinpestvirus (ASFV) och drabbar både tamgrisar och vildsvin och leder till hög feber, blödningar, organsvikt och död. Sjukdomen går inte att bota och eftersom det saknas både behandling och ett allmänt tillgängligt kommersiellt vaccin är bekämpningen helt beroende av strikta smittskyddsrutiner, snabb upptäckt och avlivning av smittade djur, vilket gör ASF till ett stort problem både ekonomiskt och ur djurvälfärdssynpunkt. Under de senaste decennierna har sjukdomen spridits över stora delar av Europa och Asien, där vildsvin spelar en central roll för att upprätthålla och sprida viruset.

Mycket av vår förståelse av sjukdomen bygger på experimentella studier på tamgrisar. Forskningen som presenteras i denna avhandling, som till stor del baseras på en jämförande studie mellan vildsvin och tamgrisar, syftade till att öka kunskapen om sjukdomen hos vildsvin och visade att sjukdomsförloppet kan skilja sig mellan de två underarterna. I den genomförda experimentella infektionen utvecklade vildsvin sjukdomssymtom tidigare än hos tamgrisar och uppvisade slöhet, nedsatt aptit och andra karakteristiska symtom redan fyra dagar efter att de hade infekterats med viruset. Tamgrisarna visade liknande symtom senare, först sju dagar efter infektionstillfället. Vävnadsskada och uppförökning av viruset hos djuren påvisades också tidigare hos vildsvin jämfört med tamgrisar. Trots det senare insjuknandet utvecklade tamgrisarna mer omfattande vävnadsskador innan de nådde slutpunkten i försöket. Sammantaget tyder detta på att vildsvinen i försöket hade lägre motståndskraft mot viruset. Obduktioner visade omfattande skador i organ såsom mjälte, lever och lymfknutor hos tamgrisar, medan motsvarande förändringar hos vildsvin generellt var mindre uttalade. Hos båda arterna började infektionen typiskt i lymfoida vävnader nära huvudet, vilket speglar en tidig interaktion med immunförsvaret. Dessa vävnader fungerar som första mål för viruset innan det sprids vidare till flera organ.

Även om experimentella modeller ger viktiga insikter, speglar de inte fullt ut komplexiteten vid naturlig smitta i fält. Vildsvin lever i dynamiska miljöer med varierande födotillgång, parasitbörda, samtidiga infektioner och miljörelaterade stressfaktorer, vilket alla kan påverka hur sjukdomen tar sig

uttryck. En möjlighet att studera naturligt infekterade djur uppstod i september 2023, då det första fallet av ASF i Sverige bekräftades efter att viruset påvisats i ett dött vildsvin som hittats i skogen. Under utbrottet samlades prover in från flera vildsvin, vilket möjliggjorde detaljerade patologiska undersökningar och jämförelser med fynd från ett annat europeiskt utbrott i Ungern 2018.

Undersökningar av dessa naturligt infekterade vildsvin visade organskador karakteristiska för ASF. Mjälte och lymfknutor var kraftigt påverkade med omfattande blödningar och en tydlig förlust av immunceller. Jämfört med de experimentellt infekterade djuren uppvisade de naturligt infekterade vildsvinen ofta mer varierande och ibland mer uttalade patologiska förändringar. En viktig bidragande faktor var förekomsten av samtidiga sjukdomstillstånd, särskilt parasitära lunginfektioner, som orsakade inflammatoriska förändringar utan koppling till ASF. Dessa resultat understryker vikten av att kombinera konventionell histopatologi med virusspecifika metoder såsom immunohistokemi för att korrekt kunna skilja ASF-relaterade skador från förändringar orsakade av andra sjukdomar.

Sammanfattningsvis utvecklade naturligt infekterade vildsvin samma grundläggande sjukdomsprocesser som beskrivits i experimentella studier, men den kliniska och patologiska bilden i fält var mer komplex. Ökad förståelse för vad som sker hos vildsvin vid naturlig infektion är också en viktig del för att förbättra övervakning, förfina diagnostiska metoder och vägleda utvecklingen av vacciner och effektiva bekämpningsstrategier.

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Veterinary Research

RESEARCH ARTICLE

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Comparative evaluation of disease dynamics in wild boar and domestic pigs experimentally inoculated intranasally with the European highly virulent African swine fever virus genotype II strain "Armenia 2007"

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Abstract

Since the reintroduction of African swine fever virus (ASFV) in Europe in 2007 and its subsequent spread to Asia, wild boar has played a crucial role in maintaining and disseminating the virus. There are significant gaps in the knowledge regarding infection dynamics and disease pathogenesis in domestic pigs and wild boar, particularly at the early infection stage. We aimed to compare domestic pigs and wild boar infected intranasally to mimic natural infection with one of the original highly virulent genotype II ASFV isolates (Armenia 2007). The study involved euthanising three domestic pigs and three wild boar on days 1, 2, 3, and 5 post-infection, while four domestic pigs and four wild boar were monitored until they reached a humane endpoint. The parameters assessed included clinical signs, macroscopic lesions, viremia levels, tissue viral load, and virus shedding in nasal and rectal swabs from day 1 post-infection. Compared with domestic pigs, wild boar were more susceptible to ASFV, with a shorter incubation period and earlier onset of clinical signs. While wild boar reached a humane endpoint earlier than domestic pigs did, the macroscopic lesions were comparatively less severe. In addition, wild boar had earlier viremia, and the virus was also detected earlier in tissues. The medial retropharyngeal lymph nodes were identified as key portals for ASFV infection in both subspecies. No viral genome was detected in nasal or rectal swabs until shortly before reaching the humane endpoint in both domestic pigs and wild boar, suggesting limited virus shedding in acute infections.

Keywords African swine fever virus (ASFV), domestic pigs, wild boar, clinical course, disease outcome, macroscopic lesion evolution, virus dynamics

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Introduction

African swine fever (ASF) has remained one of the most important threats to the global pig industry in recent decades, with more than 50 countries across Europe, Asia, the Caribbean and Africa being affected [1]. The causative agent, African swine fever virus (ASFV), is a large and complex double-stranded DNA arbovirus, the sole member of the *Asfarviridae* family within the genus *Asfivirus* [2]. Currently, there are 24 distinct genotypes [3]. The disease affects both domestic and wild suids of various breeds and ages, resulting in substantial lethality rates exceeding 90% among susceptible animals. Notably, soft ticks from the *Ornithodoros* genus, including *O. moubata* and *O. erraticus*, have been highlighted as reservoirs and carriers of ASFV [4]. Additionally, transmission routes, including direct contact, contact with wild boar and aerosols, are important, particularly in the context of the disease situation, which has continued since 2007 [5]. Efforts to develop effective ASF vaccines face significant challenges, exemplified by a recent live attenuated vaccine surprisingly approved for commercialisation and use in Vietnam [6], albeit with many doubts about its efficacy and safety in the field.

Significant progress has been made in controlling ASF within domestic pig populations, primarily through robust biosecurity measures. However, the challenge posed by wild boar as notable reservoirs in Europe and Asia persists, as they threaten the food chain, hinder eradication efforts and contribute to the geographical spread of the disease. In vivo studies have shown that wild boar are more susceptible to ASFV infection than are domestic pigs [7–9]. For instance, oral and intramuscular experimental infections of adult wild boar with genotype II isolates from the Caucasus region (Armenia in 2008 and Chechen Republic in 2009) resulted in acute disease, with 100% lethality in less than 10 days, without a serological response [7, 10]. While limited shedding of ASFV through nasal discharge or faeces has been reported, efficient transmission to in-contact wild boar is evident, leading to acute disease in the recipients. Furthermore, differences in disease outcomes in wild boar infected with lower-virulence ASFV variants in Europe [11] also increase the complexity of our understanding of ASFV pathobiology in wild boar.

Since the incursion of genotype II in the Caucasus region in 2007, most studies have focused primarily on assessing the impact of ASFV strains belonging to genotype II in domestic pigs [8, 9, 12–15]. More contemporaneous genotype II strains, such as those from Estonia 2014 or Belgium 2018/1, have been used in the experimental infection of both domestic pigs and wild boar [9, 16–19]. In contrast, the original isolates obtained in the Caucasus region after the reintroduction of the disease in Europe have been investigated in only a few in vivo studies in wild boar [7, 8, 10, 20]. The diversity of genotype II ASFV strains has evolved over time due to viral mutations and recombination. However, the lack of experimental data on the ancestral isolates of genotype II ASFV limits our understanding of the changes in infection dynamics. Furthermore, extensive research on the pathogenic mechanisms of the disease has only been performed using high- and moderate-virulence ASFV isolates of genotype I, which emerged after the introduction of the ASFV to the Iberian Peninsula during the 1950s and [21]. In contrast, the understanding of disease pathogenesis and the resulting immune response against genotype II strains remains limited in domestic pigs and is even less explored in wild boar. Currently, there is only one published comparative study that examines the infection kinetics in wild boar and domestic pigs following oronasal infection with a moderately virulent genotype II isolate [17].

In both domestic pigs (*Sus scrofa domesticus*) and wild boar (*Sus scrofa spp.*), in vivo experimental results have shown variations in the outcomes and severity of clinical disease following infections with the prevailing ASFV genotype II strains from Europe. These variations, often exceeding expected biological differences, may be attributed to differences in experimental conditions. For instance, the intramuscular route has been widely employed for experimental in vivo ASFV research due to its ability to reproduce clinical disease. However, it bypasses the mucosal surfaces and innate defence mechanisms encountered during natural infection. Experimental oronasal infections more accurately represent natural infection and early clinical disease dynamics and pathogenic mechanisms [22].

The objective of this study was to evaluate and compare the pathogenesis of ASFV in domestic pigs and wild boar starting at 24 h post-intranasal infection using Armenia 2007, one of the original highly virulent genotype II isolates that emerged after disease reintroduction in the Caucasus region. This study sought to understand the initial disease dynamics in both subspecies. Specifically, we compared various aspects, including disease progression, macroscopic lesions, viremia levels, virus shedding, and virus load within target organs. This knowledge provides valuable information to aid in early diagnosis and to contribute to the evaluation and development of new vaccines essential for effectively preventing and combating the spread of ASF.

Materials and methods

Virus

The highly virulent ASFV genotype II isolate used (Armenia 2007) was kindly provided by the EU reference laboratory for ASF (CISA-INIA/CSIC, Valdeolmos, Madrid Spain). Stocks for inoculation were grown in primary porcine blood peripheral monocytes, and viral titres were determined as the amount of virus causing haemadsorption in 50% of inoculated cultures (HAD_{50}/mL).

In vivo experimental design

In vivo experiments were carried out in containment facilities at the Animal and Plant Health Agency (APHA, Weybridge, UK). These experiments were reviewed by the APHA Animal Welfare and Ethical Review Board and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under project licence PF971B5E3.

Nineteen commercial domestic Large White/Landrace cross pigs of both sexes, aged 10–12 weeks at the commencement of the experiment, were used alongside nineteen farmed European wild boar of both sexes, aged 16–18 weeks, and sourced from a commercial provider (Figure 1A). Following a 7-day acclimatisation period, a total of thirty-eight animals (19 domestic pigs and 19 wild boar) were randomly allocated into four groups consisting of 8 animals each (either wild boar or domestic pigs), which were assigned to the infected groups. Two additional groups consisting of either 3 domestic pigs or 3 wild boar were assigned as mock-inoculated controls. Animals were assigned to groups with similar average weights and similar distributions of males and females. To prevent potential aggression, domestic pigs and wild boar were housed separately. As described in Figure 1A, the animals were euthanised at predetermined time points following infection prior to inoculation.

To mimic natural infection, all animals were sedated with Stresnil and then intranasally inoculated with a dose of 10^4 HAD_{50}/pig of the virulent ASFV isolate "Armenia 2007". One millilitre of inoculum was administered per nostril using intranasal mucosal atomisation devices (MAD Intranasal, Teleflex). To ensure thorough administration of the inoculum, the animals' heads were maintained in an upright position for 1 min post-inoculation. On the day of inoculation, the inoculum was titrated to verify the administered dose. The mock-inoculated controls were housed separately and subjected to mock infection using identical procedures, but RPMI cell culture medium (Gibco) was used in place of the viral inoculum.

The day of experimental infection was designated day 0. All animals were weighed before the experimental inoculation (day 0). Throughout the experiment, rectal temperatures were measured once daily, and clinical signs were monitored daily using a previously described scoring system [23]. The sum of the points was recorded as the clinical score, which was also used to establish humane endpoints before commencing the experiment. On days 1, 2, 3, and 5 post-infection (pi), six animals that had been randomly assigned beforehand (comprising 3 domestic pigs and 3 wild boar) were sedated and euthanised by the administration of barbiturate (Figure 1A). The remaining inoculated animals (4 pigs and 4 wild boar) were euthanised and necropsied upon reaching predetermined humane endpoints on day 6 (all remaining wild boar) and day 9 pi (all remaining domestic pigs). Mock-inoculated control animals ($n=6$) were euthanised at the conclusion of the experiment (day 12 pi).

Sampling and macroscopic evaluation of lesions

Blood samples were collected into vacutainers containing EDTA (BD biosciences) from the anterior vena cava of all animals (inoculated and non-inoculated) before inoculation (day 0). Following inoculation, blood samples, along with nasal and rectal swabs, were obtained at the scheduled euthanasia time points on days 1, 2, 3, and 5 pi, as well as at the humane endpoint (day 6 pi for wild boar and day 9 pi for domestic pigs). Additional blood samples and swabs were also taken from the remaining infected domestic pigs on day 8 pi. For the mock-inoculated control animals, blood samples and swabs were collected on day 8 pi and at the end of the experiment on day 12 pi. Blood samples and swabs were frozen at −80 °C for subsequent detection of the ASFV genome by quantitative PCR (qPCR).

All euthanised animals underwent necropsies, during which macroscopic evaluations were conducted in accordance with a previously established macroscopic scoring protocol [24]. Macroscopic lesions were scored and individually documented, representing the cumulative score observed across various organs in the examined animals.

Additionally, tissue samples were collected from the palatine tonsils, lungs (right cranial lobe), spleen, medial retropharyngeal, tracheobronchial, and gastrohepatic lymph nodes, and these samples were frozen at −80 °C for subsequent detection of the ASFV genome through qPCR.

Quantification of viral DNA levels in blood, tissues and nasal swabs

DNA was extracted in duplicate from 100 µL of EDTA blood, tissue homogenate, or swab eluate using the MagVET extraction kit on the KingFisher Flex automated

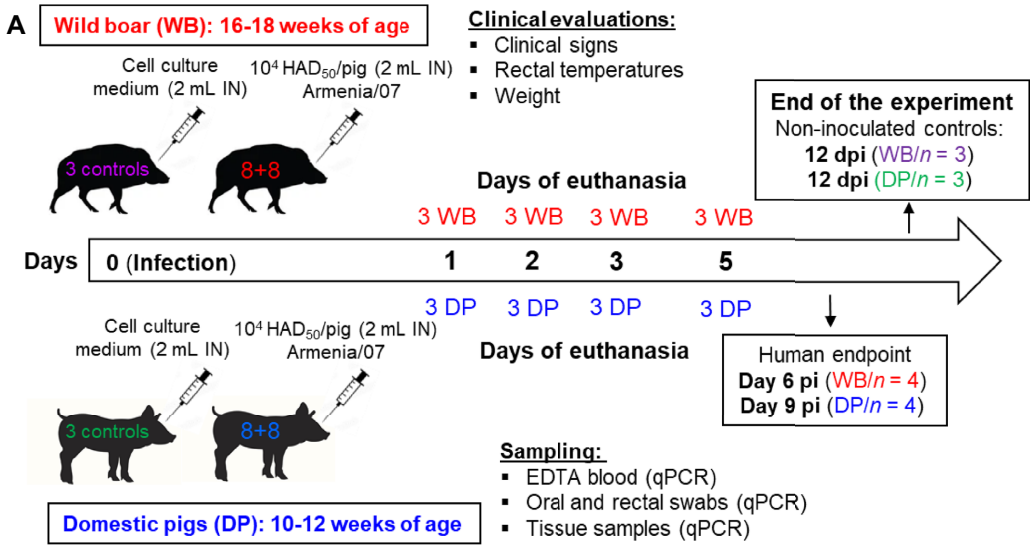

Figure 1 Experimental design, survival rate and weight assessment. A Infection and euthanasia schedule. Nineteen commercial domestic Large White/Landrace cross pigs (DPs) of both sexes, aged 10–12 weeks at the commencement of the experiment, were used alongside nineteen farmed European wild boar (WBs) of both sexes, aged 16–18 weeks. Animals were randomly allocated into four groups consisting of 8 animals (either wild boar or domestic pigs) that were intranasally inoculated (IN) with a dose of 10^4 HAD_{50}/pig of the highly virulent ASFV genotype II isolate ASFV isolate "Armenia 2007" (1 mL per nostril). Two additional groups consisting of 3 domestic pigs or 3 wild boar were used as mock-inoculated controls and received the same volume of cell culture medium via the intranasal route. On days 1, 2, 3, and 5 post-infection (pi), six animals that had been randomly assigned beforehand (comprising 3 domestic pigs and 3 wild boar) were euthanised. The remaining inoculated animals (4 pigs and 4 wild boar) were euthanised and necropsied upon reaching predetermined humane endpoints on day 6 (all remaining wild boar) and day 9 pi (all remaining domestic pigs). Mock-inoculated control animals were euthanised at the conclusion of the experiment (day 12 pi). **B** Mortality of infected wild boar ($n = 4$) and infected domestic pigs ($n = 4$) that were kept alive until a humane endpoint was reached. Mock-infected animals are also shown. **C** Comparative weight assessment between infected and noninfected animals.

extraction system (ThermoFisher, Paisley, UK). Positive and negative controls were included in the extraction, and DNA was eluted with 80 µL of elution buffer. DNA was stored at + 4 °C prior to analysis. qPCR was performed in duplicate on an Applied Biosystems 7500 Fast instrument using the King et al. assay targeting ASFV VP72 [25], and genome copies per mL or mg were determined by comparison to an ASFV VP72 plasmid standard [25].

Statistical analysis

All the statistical analyses and data visualisations were performed with GraphPad Prism Version 7.0 (GraphPad

Software, La Jolla, CA, USA). The statistical tests applied to each dataset are indicated in the figure legends.

Results

Clinical findings

Wild boar and domestic pigs were euthanised at predetermined time points: 1, 2, 3, and 5 pi (Figure 1A). At 5 pi, four animals from each subspecies were retained until they reached humane clinical endpoints. All wild boar reached their humane endpoint on day 6 pi, whereas for domestic pigs, this endpoint was reached on day 9 pi (Figure 1B).

Throughout the study, both the infected domestic pig and wild boar groups exhibited a minimal to modest increase in mean weights compared to their pre-infection weights, but this increase was markedly lower than the weight gain of the uninfected animals (Figure 1C). Specifically, the weight of the infected domestic pigs increased less than 10% over a 9-day period, whereas that of the uninfected pigs increased from 20 to 40% by the end of the experiment. The weight of infected wild boar, which were on average 9 kg lighter than those of domestic pigs at the start of the study, remained relatively stable at approximately 15%, while the weight of uninfected boar increased by 20% to 30% by the end of the experiment. In summary, while no significant weight gain was observed among the infected domestic pigs and wild boar, the infected animals exhibited less weight gain than did the uninfected animals (the individual weights of the infected and uninfected animals are shown in Additional file 1).

Compared with domestic pigs, wild boar developed clinical disease earlier, although their clinical scores were comparable at the humane endpoint (Figure 2A). Mild and nonspecific signs were observed starting on day 4 pi in 5 out of 7 infected wild boar (Figure 2B); however, no signs were detected in domestic pigs (Figure 2C) at this time point ($p \leq 0.01$; Figure 2A). These signs included a reduction in liveliness, apathy, and leftover food after feeding. Subsequently, on day 5 pi, the clinical scores increased significantly, with signs such as lethargy, weakness, recumbency, difficulty or unwillingness to stand, lack of coordination or a stiff gait, loss of appetite, increased respiratory rate, ocular discharge, and soft faeces becoming evident. The four wild boar that were not euthanised at predefined time points reached the predetermined clinical endpoint on day 6 pi and were euthanised with high clinical scores.

In contrast, none of the domestic pigs euthanised up to day 5 pi exhibited notable clinical signs (Figure 2C). With the exception of one pig (#38), the remaining inoculated animals that survived until day 9 pi (#37, #39, and #40) exhibited a significant increase in clinical scores starting from day 7 pi, with clinical signs similar to those described for the infected wild boar. Starting at 8 pi, these animals also exhibited erythematous pinnae, kyphosis, and tremors, with the highest scores (a maximum of 11) recorded on 9 pi. Pig #38 did not reach the clinical endpoint scores but was euthanised to avoid single housing. The mock-inoculated domestic pigs and wild boar remained healthy and displayed no clinical signs throughout the study period (Additional file 2).

As part of clinical monitoring, rectal temperatures were recorded to assess disease development. Wild boar exhibited pyrexia earlier than domestic pigs after virus inoculation (Figure 2D). Among the infected wild boar, the mean rectal temperature began to rise on day 3 pi, with a significant increase compared to pre-inoculation values observed by day 4 pi ($p \leq 0.0001$). The temperatures peaked on day 6 pi, averaging 41.7 °C (Figure 2D). Notably, starting at 4 pi, three of the seven remaining infected wild boar developed hyperthermia (>40.5 °C) (Figure 2E). The temperatures of all four wild boar that survived until day 6 (animals #56, #57, #58, and #59) exceeded 41.5 °C from day 5 pi, with peaks reaching 41.9 °C (Figure 2E).

In the case of the infected domestic pigs, the mean temperature significantly increased compared to the pre-inoculation value ($p \leq 0.01$) from day 7 pi and remained elevated until day 9 pi, when the humane endpoint was reached (Figure 2D). The average temperatures ranged between 39.4 and 39.8 °C. Three of the four domestic pigs that survived until day 9 pi (animals #37, #39, and #40) exhibited hyperthermia (> 40.5 °C), with the highest temperature reaching 41.4 °C (Figure 2F). Pig #38 remained normothermic. The mock-infected animals maintained temperatures ranging between 38 and 39 °C, which is within the expected range (Additional file 2).

In summary, the incubation period was shorter in infected wild boar (4 days) than in infected domestic pigs (7 days). Infected wild boar exhibited higher temperatures in the early stages after infection, while domestic pigs only developed elevated temperatures starting from day 7 pi, with rectal temperatures notably higher in the wild boar. Clinical disease was observed in the infected wild boar from day 4 pi, with the most significant differences noted on day 6 pi. In contrast, domestic pigs developed clinical disease from day 7 pi, with the most significant differences observed on day 9 pi. The duration of clinical courses to the humane endpoint was similar between the groups of infected animals that were not euthanised at the established time points (3 days). However, while all four infected wild boar reached high clinical scores leading to the humane endpoint, only three out of the four infected domestic pigs reached the clinical endpoint.

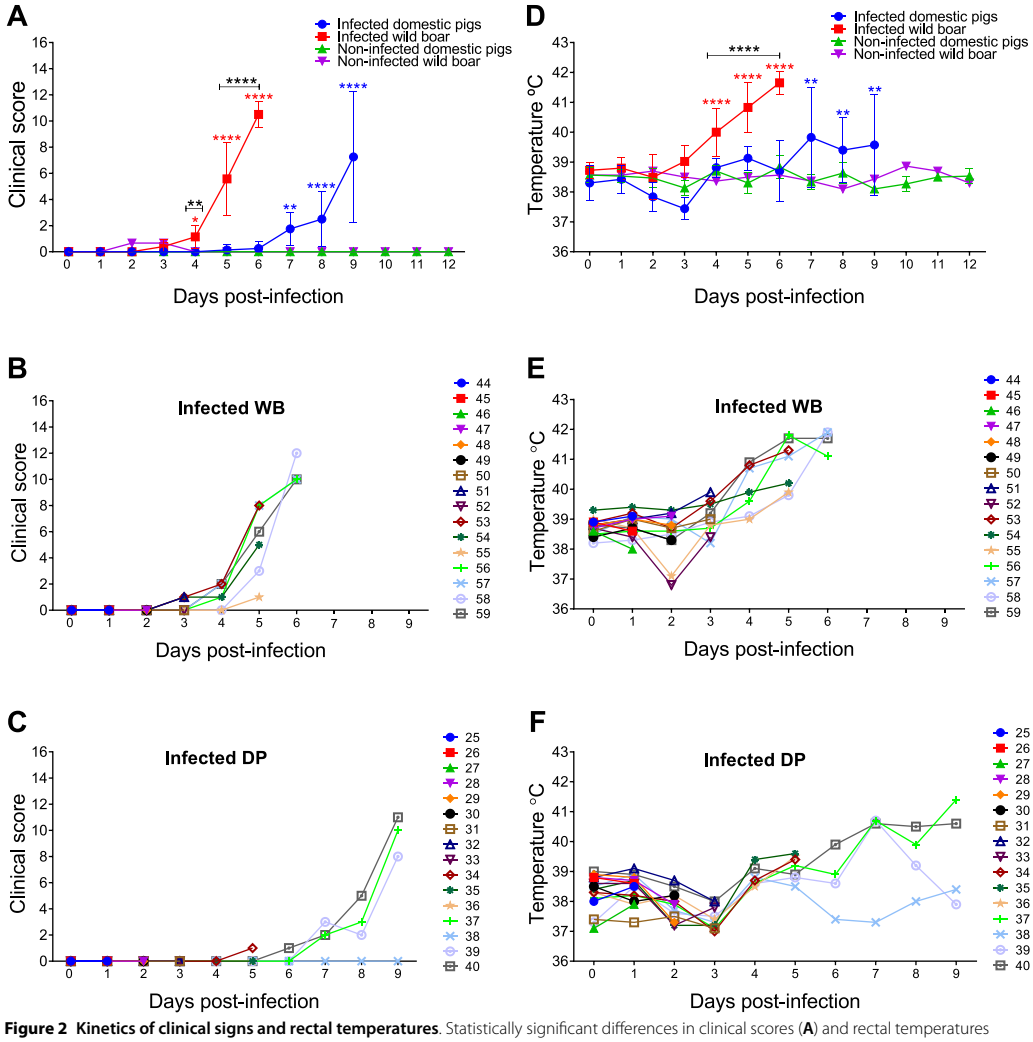

Figure 2 Kinetics of clinical signs and rectal temperatures. Statistically significant differences in clinical scores (**A**) and rectal temperatures (**D**) at different days after infection (mean ± SD) between the experimental groups were assessed by two-way ANOVA. Black asterisks indicate statistically significant differences between infected wild boar and infected domestic pigs. Statistically significant differences in rectal temperatures and clinical scores (mean ± SD) within each experimental group at different days after infection compared to pre-infection values were assessed by one-way ANOVA. Statistical differences are represented by red (infected boar group) and blue (infected domestic pig group) asterisks. Individual kinetics of clinical scores (**B**, **C**) and rectal temperatures (**E**, **F**) in infected wild boar (WBs) and domestic pigs (DPs). Day post-infection (x-axis); temperature and clinical score (y-axis); variables of significance (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$).

Macroscopic pathology

Starting at day 3 pi, both infected domestic pigs and infected wild boar exhibited a small increase in macroscopic scores compared to those of noninfected animals (Figure 3A). These scores were significantly different from those of the control animals on day 5 pi and at the end of the study (day 6 pi) in the infected wild boar group. In the infected domestic pig group, these differences were observed only at the end of the study (day 9 pi). However, the difference was not statistically significant; the macroscopic scores were slightly greater in the wild boar group than in the domestic pig group

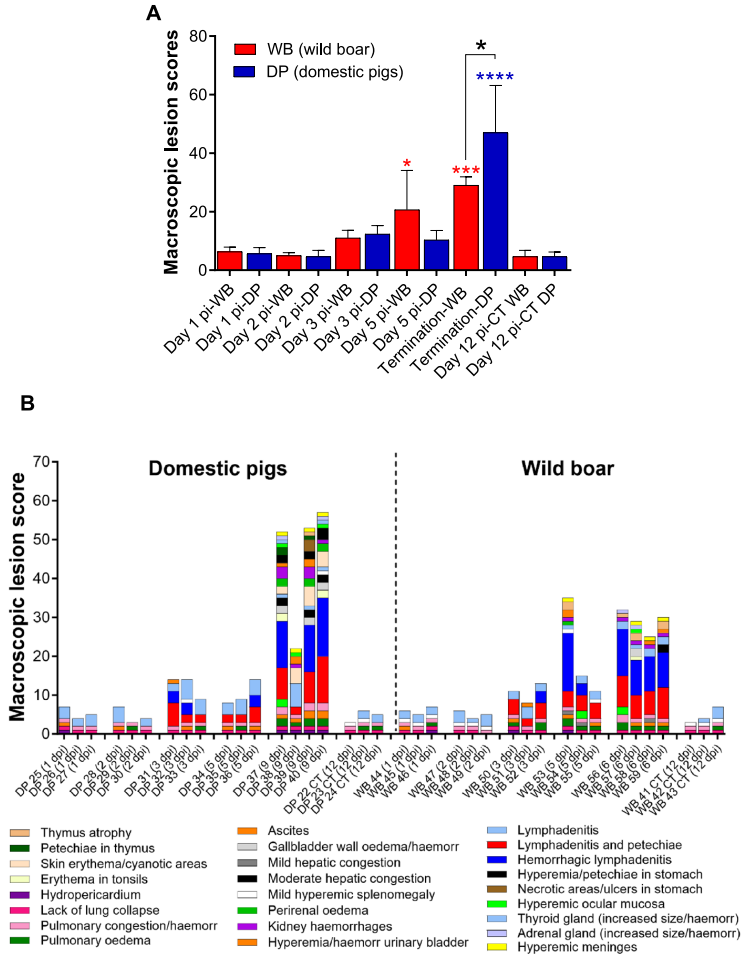

Figure 3 Macroscopic evaluation of lesions. A Mean ± SD of the cumulative macroscopic scores (y-axis) in each group of domestic pigs or wild boar euthanised on different days after infection. Uninfected animals (CT) euthanised on day 12 pi are also shown (x-axis). Statistical analysis was performed by one-way ANOVA. Black asterisks indicate statistically significant differences between the two groups of infected animals (wild boar and domestic pigs) euthanised on different dates, while red or blue asterisks indicate statistically significant differences with respect to the uninfected wild boar or domestic pig group; significant variables (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$); **B** Macroscopic scoring of lesions. Macroscopic lesions were scored and documented individually, representing the cumulative score observed in the different organs of the infected animals examined; macroscopic lesion score (y-axis); (x-axis): individual representation of animals evaluated in each experimental group and euthanised on different dates after infection. Uninfected animals (CT) euthanised on day 12 pi are also shown.

on day 5 pi. However, on the day of termination at the clinical endpoint, the pathology scores of domestic pigs were significantly greater than those of wild boar ($P < 0.05$).

The mock-inoculated domestic pigs and wild boar had only mild and nonspecific macroscopic changes that were attributed to the euthanasia procedures, including a lack of pulmonary collapse, lung congestion, alveolar oedema, splenomegaly, and occasional lymphadenopathy, mainly in the submandibular and retropharyngeal lymph nodes (Figure 3B). Both domestic pigs and wild boar euthanised on days 1 and 2 pi displayed nonspecific macroscopic findings similar to those of the control animals, although some animals (domestic pigs #25 and #28 and wild boar

#44 and #46) also had mild hydropericardium and ascites (Figure 4A).

Along with the mild ascites and hydropericardium observed in some animals, all domestic pigs and wild boar euthanised on day 3 pi displayed mild lymphadenomegaly with petechiae in the submandibular, medial retropharyngeal, gastrohepatic, renal, and ileocecal lymph nodes. In addition, two domestic pigs (#31 and #32) and one wild boar (#52) also had haemorrhagic medial retropharyngeal and sub-lumbar lymph nodes. Domestic pig #31 and wild boar #51 also showed petechiation on the urothelium (Figure 4B).

In domestic pigs euthanised on day 5 pi, the macroscopic lesions were quite similar to those described in domestic pigs on day 3 pi. In contrast, the lesions were much more severe in the wild boar, especially in animal #53 (Figure 5) and, to a lesser extent, in animal #54. Wild boar #54 exhibited hepatic and splenic congestion and lymphadenomegaly with petechiae (only the gastrohepatic lymph node had severe haemorrhages). In contrast, wild boar #53 displayed a wider range of lesions, including hydropericardium, pulmonary congestion, alveolar oedema, ascites, thymic atrophy, hepatic congestion (Figure 5G), perirenal oedema, petechiae in the renal cortex and urinary bladder, meningeal hyperaemia, and severe generalised haemorrhagic lymph nodes (retropharyngeal, gastrohepatic, renal, mediastinal, and sub-lumbar; Figures 5A, C, E, G, I). The four wild boar that reached a humane endpoint on day 6 pi (#56, #57, #58, and #59) all displayed macroscopic findings and lesion scores similar to those described for wild boar #53, which was euthanised on day 5 pi. Wild boar #57 also showed hyperaemia of the ocular mucosa, erythematous palatine tonsils, and gallbladder wall edema with petechiae on the mucosa.

Three of the four domestic pigs that reached the humane endpoint on day 9 pi (#37, #39, and #40) had a wider range of lesions, with higher scores than those observed in wild boar euthanised on day 6 pi, and in some instances lesions were only present in the domestic pigs but not in the wild boar (Figure 6). These included severe erythematous palatine tonsils (Figure 6A), hyperaemic ocular mucosa (Figure 6C), cutaneous erythema, and cyanosis in various areas, such as the pinnae (Figure 6E), snout, periorbital region, neck, ventral chest, dorsolateral skin areas, abdomen, scrotal sac, perianal area, tail, and distal limbs. These skin lesions were not observed in any of the wild boar on day 6 pi. Lesions such as hydropericardium, pulmonary congestion, alveolar oedema, hepatic congestion (Figure 6G), gallbladder wall oedema (arrow, Figure 6G), ascites (Figure 6M), perirenal oedema (arrow, Figure 6i), renal haemorrhages (arrowhead, Figure 6I; Figure 6N), hyperaemia, and petechiae in the urinary bladder (Figure 6K) or meningeal hyperaemia were also more severe and more frequently detected in domestic pigs. In addition, there was gastric mucosal congestion, petechiae and ecchymoses, as well as scattered necrotic areas in one pig (#39) (Figure 6O). For lymphoid organs such as the spleen, lymph nodes, and thymus, the severity of lesions was equivalent to that observed in wild boar, with mild to moderate splenomegaly with congestion and thymic atrophy in some animals, as well as generalised, severe haemorrhagic lymphadenitis. The fourth domestic pig, #38, displayed only mild lesions, such as cutaneous erythema and cyanosis, hyperaemic ocular mucosa, hyperaemia, and petechiae in the urinary bladder; ascites; meningeal hyperaemia; and mild lymphadenomegaly with oedema but without severe haemorrhagic changes.

Viral load in blood samples and nasal and rectal swabs

Blood samples from both domestic pigs and wild boar tested negative for ASFV by qPCR before virus inoculation and on days 1 and 2 pi (Figure 7A). In wild boar, a significant amount of the virus genome was detected in blood samples from one animal ($n = 1/3$) euthanised on day 3 pi (#51; 1.53×10^5 copies/mL) and from all three animals ($n = 3/3$) euthanised on day 5 pi (up to 1.38×10^9 copies/mL, #53). These two animals (#51 and #53) also exhibited the highest clinical scores and temperatures among all animals euthanised on days 3 and 5 pi (Figures 2B and E). Wild boar that reached a humane endpoint on day 6 pi (#56, #57, #58, and #59) tested positive for the virus, showing higher viral loads (up to 1.13×10^{10} copies/mL). In contrast, no virus genome was detected in any of the blood samples from the domestic pigs euthanised on day 3 or 5 pi. Among the domestic pigs that reached a humane endpoint on day 9 pi, only three (#37, #39, and #40) were viraemic from day 8 pi. The levels of viremia in the domestic pigs were high (up to 1.56×10^{10} copies/mL in #40 on day 9 pi) and similar to those detected in wild boar at the humane endpoint.

The level of virus shedding was evaluated by nasal and rectal swabs. No viral genome was detected in any of the swabs taken from virus-inoculated wild boar or domestic pigs euthanised up to day 5 pi (Figures 7B and C). However, high viral loads were detected in nasal swabs taken from the four wild boar euthanised on day 6 pi (up to 5.93×10^5 copies/mL) and in 3 of the 4 domestic pigs euthanised at 9 dpi (up to 2.94×10^5 copies/mL), except for the domestic pig #38, which tested negative (Figure 7B). In the rectal swabs, the virus

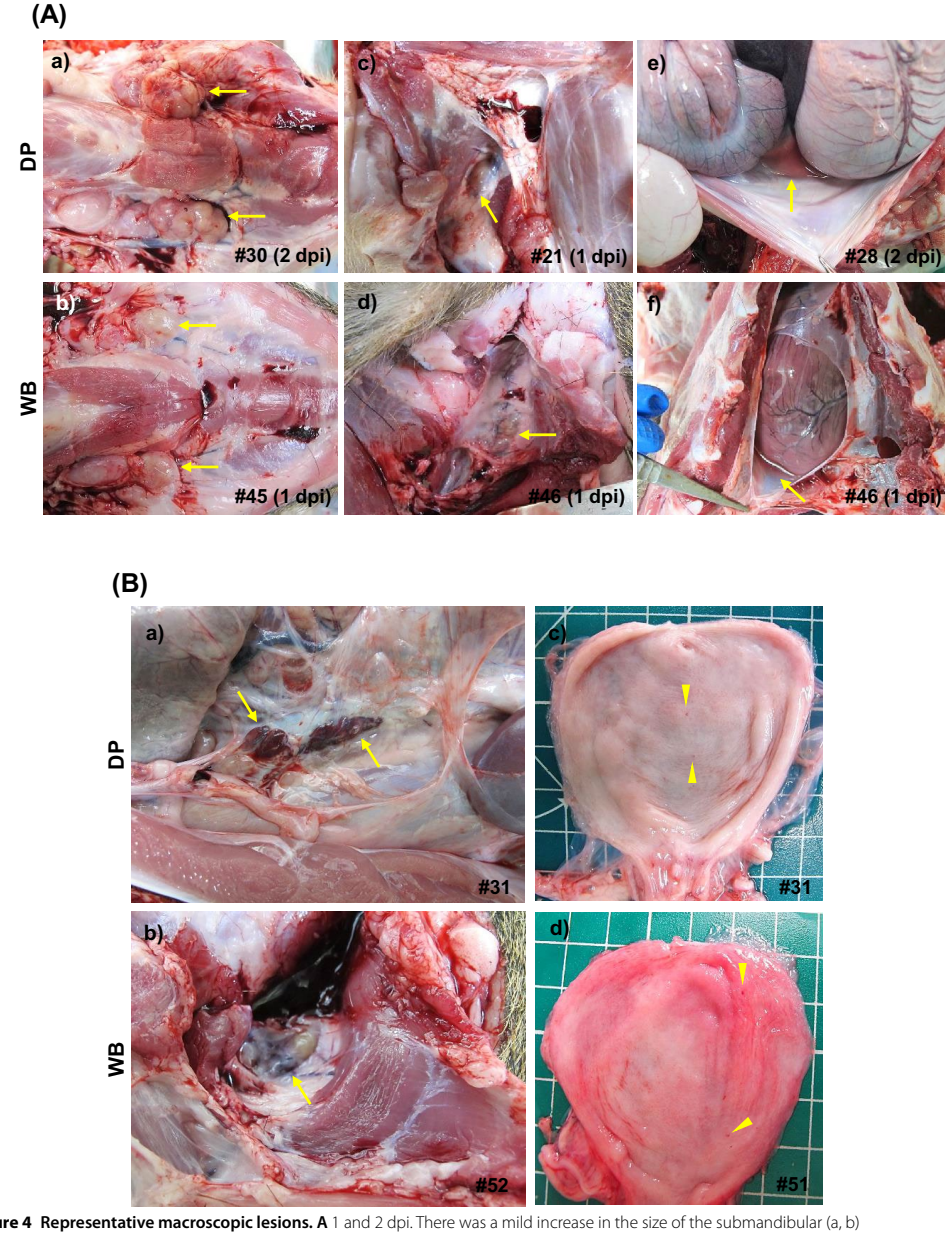

Figure 4 Representative macroscopic lesions. A 1 and 2 dpi. There was a mild increase in the size of the submandibular (a, b) and retropharyngeal (c, d) lymph nodes; mild ascites (e) and mild hydropericardium (f); **B** 3 dpi. Haemorrhagic sublumbar (a) and medial retropharyngeal (b) lymph nodes; petechial haemorrhages on the urothelium (c, d). DP: domestic pig; WB: wild boar.

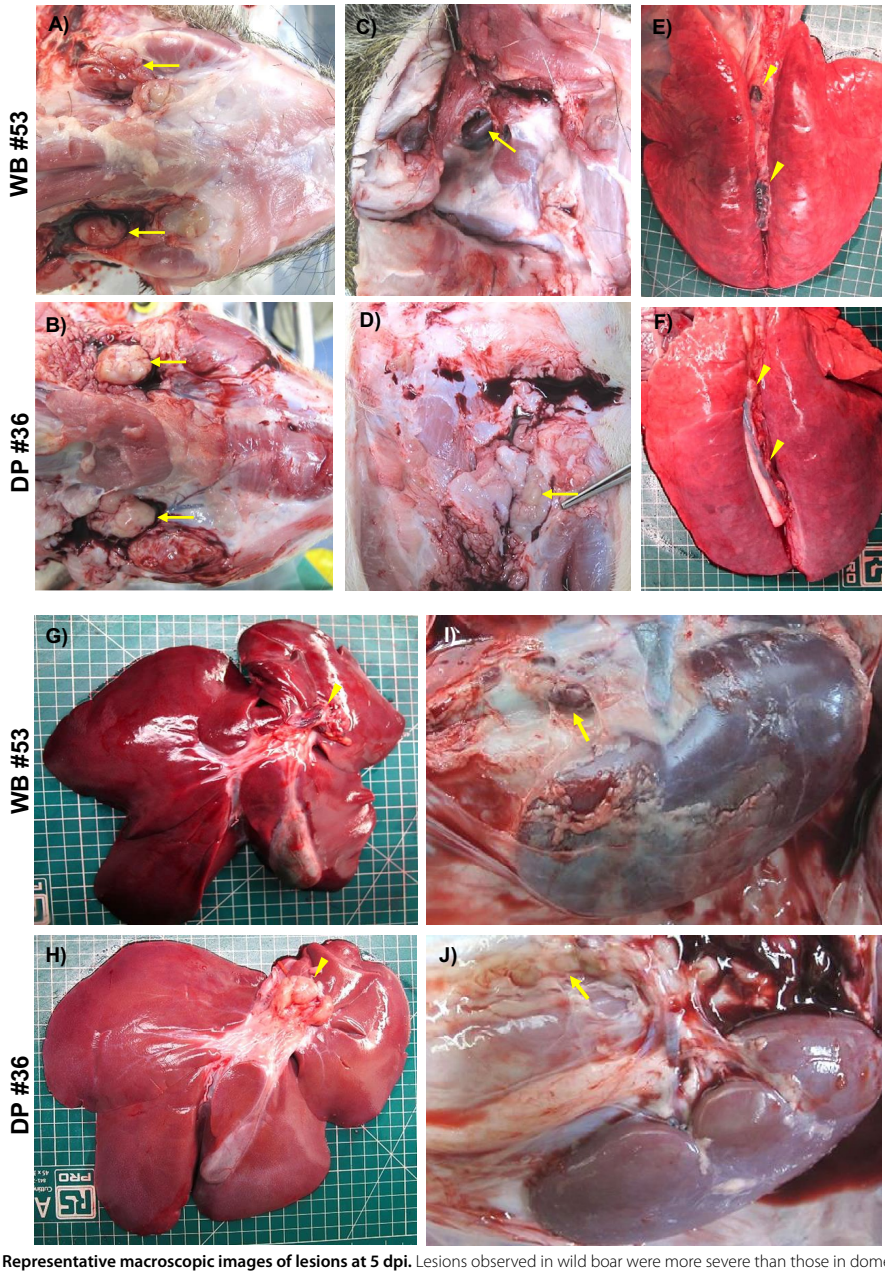

Figure 5 Representative macroscopic images of lesions at 5 dpi. Lesions observed in wild boar were more severe than those in domestic pigs. Note how the submandibular (**A**), retropharyngeal (**C**), mediastinal (**E**), gastrohepatic (**G**) and renal (**I**) lymph nodes of wild boar showed haemorrhagic lesions (from petechiae to severe diffuse haemorrhages) compared to those of domestic pigs, which showed only mild to moderate lymphadenopathy (**B**, **D**, **F**, **H**, **J**). Additionally, moderate hepatic congestion was observed in wild boar (**G**) compared to domestic pigs (**H**). DP: domestic pig; WB: wild boar.

genome was detected in only 3 of the 4 wild boar and domestic pigs euthanised on days 6 and 9 pi, respectively (Figure 7C). An exception was wild boar #58, which also had the lowest virus loads in blood and nasal swabs. Both swabs of domestic pig #38 tested negative. At the time of termination, virus genome loads in nasal and rectal swabs were comparable between wild boar and domestic pigs that tested positive, ranging between 10^5 and 10^6 copies/mL. Blood samples and swabs (nasal and rectal) collected on 8 and 12 pi from mock-inoculated control animals tested negative for the virus genome.

Viral load in tissue samples

Viral loads in the palatine tonsils, right cranial lobe of the lungs, spleen, medial retropharyngeal, tracheobronchial, and gastrohepatic lymph nodes were assessed using qPCR. The virus genome was detected earlier in the wild boar than in domestic pigs, with detection as early as day 2 pi in one wild boar, while domestic pigs only tested virus-positive by day 5 pi (Figure 7D).

In the wild boar, the viral genome was detected in the spleen of one animal (#47) on day 2 pi, with a viral load of 10^6 copies/mg. At 3 dpi, all three wild boar were positive for the virus genome in at least one tissue, with consistent detection of the virus in the medial retropharyngeal lymph node (MRPLN) in all three animals. Animal #51 had the highest viral load, approximately 10^8 copies/mg. Similarly, on day 5 pi, the virus genome was detected in all tested tissues from wild boar, particularly with high viral loads in the spleen (10^8 to 10^9 copies/mg) of MRPLN (10^9 to 10^{10} copies/mg). In contrast, in domestic pigs, the virus was first detected at 5 pi and was detected only in the MRPLN (10^5 to 10^7 copies/mg) at that time.

In both wild boar and domestic pigs euthanised on day 6 and 9 pi, respectively, the viral genome was detected in all tested tissues. The MRPLN, spleen, and lung had the highest viral loads (10^8 to 10^{10} copies/mg). However, in wild boar, the viral loads detected in the palatine tonsils (10^5 to 10^7 copies/mg), gastrohepatic lymph nodes (10^6 to 10^9 copies/mg), and tracheobronchial lymph nodes (10^5 to 10^8 copies/mg) were generally 10^1 to 10^3 times lower than those detected in domestic pigs, with the most noteworthy difference observed in the tonsils.

Discussion

The present in vivo experimental findings demonstrated that wild boar were considerably more susceptible to infection than domestic pigs following intranasal exposure to the highly virulent isolate Armenia 2007, as characterised by an earlier onset of viremia and detection of the virus in tissues, a shorter incubation period and higher body temperatures and clinical scores. This finding concurs with previous studies that pointed to a greater susceptibility of wild boar after oronasal infection [7–9]. Only one study to date, with a similar experimental approach, has studied the kinetics of disease in both subspecies after oronasal infection. However, although that study used a moderately virulent Baltic isolate, it also revealed a greater susceptibility of wild boar to ASFV [17].

In line with our study, previous research has consistently shown that wild boar develop clinical signs shortly after oronasal infection. Highly virulent strains from the Caucasus region, typically within 2 to 5 days post-infection [7, 8] and between 3 and 4 days post-infection following oronasal infection of wild boar with highly virulent isolates such as Belgium 2018/1 [18], or moderately virulent Baltic isolates such as Estonia 2014 [9, 17]. Unexpectedly, the results also revealed a lower weight gain in infected wild boar than in uninfected wild boar.

The period between the onset of clinical signs and the humane endpoint remained consistent at 3 days for both groups of infected animals that had to be euthanised on day 6 and 9 pi. While all four infected wild boar necessitated euthanasia due to high clinical scores, only three out of four infected domestic pigs reached this predefined endpoint. Previous research [18] showed that oronasal infection of 1- to 2-year-old wild boar with the highly virulent Belgium 2018/1 isolate resulted in deaths between 8 and 10 days post-infection, leading to slightly longer clinical courses, possibly due to increased resistance in older animals [26]. Conversely, and in line with our results, younger wild boar at 9 weeks of age that were oronasally infected with highly virulent strains from the Caucasus region survived for 5 to 9 days [7, 10]. For domestic pigs, experiments with weanlings (8 to 10 weeks old) infected intranasally with the highly virulent Georgia 2007 isolate [27] or oronasally with the highly virulent Belgium 2018/1 isolate [19] showed incubation periods of 4 to 5 days, and survival times (8 to 10 days post-infection) were consistent with our study. However, variations were observed when, in the same experiment [19], 50% of 18-week-old domestic pigs inoculated intranasally with Belgium 2018/1 either died between day 7 and 14 pi or recovered. On the other hand, limited research has indicated that the age of wild boar does not affect the early onset of clinical signs following infection [28].

For domestic pigs, experiments with intranasally infected weanlings (8 to 10 weeks old) with the highly virulent Georgia 2007 isolate [27] or oronasally infected with the highly virulent Belgium 2018/1 isolate [19] showed incubation periods of 4 to 5 days, and survival times (8 to 10 days post-infection) were consistent with our study.

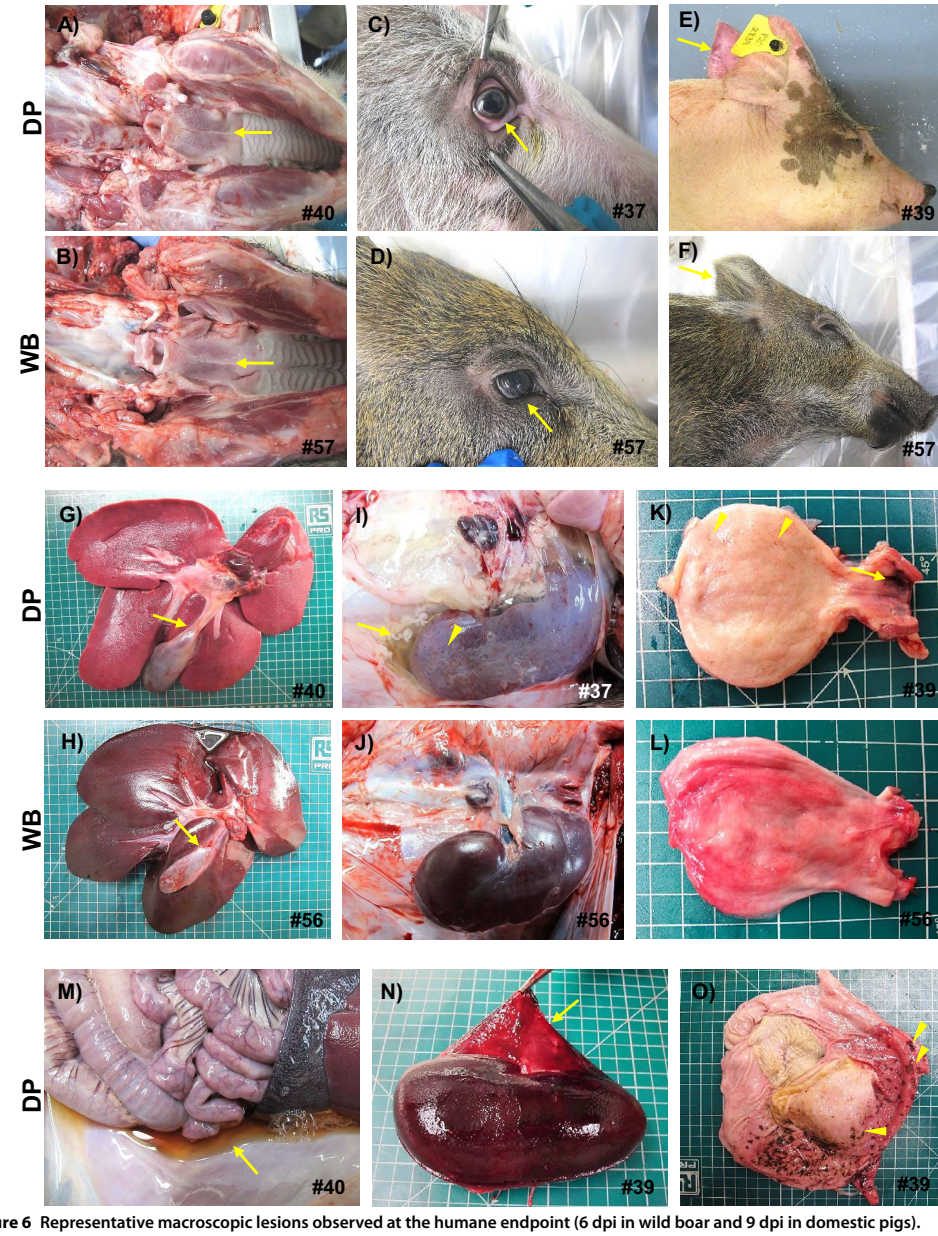

Figure 6 Representative macroscopic lesions observed at the humane endpoint (6 dpi in wild boar and 9 dpi in domestic pigs). Domestic pigs had a wider range of lesions and greater severity than did wild boar. Note the differences between domestic pigs and wild boar in terms of erythema on palatine tonsils (**A, B**), ocular mucosal hyperaemia (**C, D**) or cutaneous erythema on pinnae (**E, F**). Lesions such as hepatic congestion (G, H), gallbladder wall oedema (arrow; **G, H**), perirenal oedema (arrow; **I, J**), renal haemorrhages (arrowheads; **I, J**) and haemorrhages in the urinary bladder (**K, L**) were also more severe and more frequently detected in domestic pigs. Domestic pigs also had severe ascites with abundant amber fluid (**M**), severe renal interstitial haemorrhages involving the renal capsule (arrow; **N**) and congested gastric mucosa with petechiae and ecchymosis, as well as scattered necrotic areas (arrowheads; **O**).

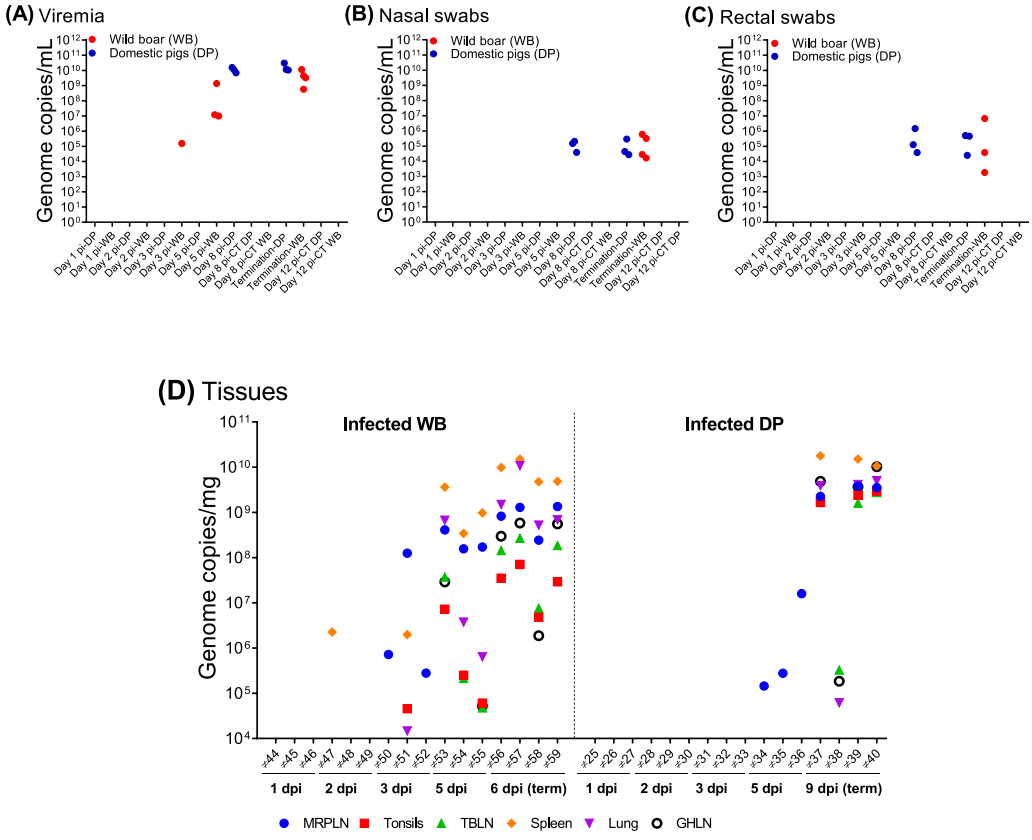

Figure 7 Viral genome copies in blood samples (A), nasal swabs (B) and rectal swabs (C) taken from infected domestic pigs and wild boar. The individual values obtained are shown. Viral genome copies were determined by qPCR and are represented as the total number of genome copies per millilitre (y-axis); (x-axis): group of domestic pigs or wild boar euthanised or sampled on different dates after infection. Uninfected animals (CT) euthanised on day 12 pi are also shown; **(D)** virus genome copies in tissue samples taken from infected domestic pigs and wild boar; (y-axis): genome copies per milligram; (x-axis): individual identification of each domestic pig (DP) or wild boar (WB) euthanised on different dates after infection; (term): termination date after reaching the humane endpoint; MRPLN: medial retropharyngeal lymph node; TBLN: tracheobronchial lymph node; GHLN: gastrohepatic lymph node.

The available literature describing macroscopic changes observed in experimental infections with isolates belonging to genotype II of wild boar and/or domestic pigs usually describes findings in advanced stages of the disease in animals, including at death or at the humane endpoint. This provides limited information that neither provides information on disease progression nor reflects the spectrum of disease severity, which can be quite diverse in field infections [7–10, 16–19, 27]. To address this gap in knowledge about host–virus interactions and disease dynamics in the critical period immediately after infection, our experimental approach involved sequential euthanasia of animals beginning on day 1 pi. During this process, we systematically assessed the animals using established macroscopic evaluation protocols [24, 29] to provide more precise information on affected organs, lesion types and severity to establish differences between animals and experimental groups in the early stages after infection. Such protocols could also be useful and easy-to-use tools for the early detection of disease during outbreaks, requiring only minimal training from veterinarians.

Notable macroscopic lesions were detected as early as day 3 pi, and both infected domestic pigs and wild boar showed characteristic lesions associated with acute ASF [24, 29]. These included lymphadenomegaly with

petechiae in the submandibular, medial retropharyngeal, gastrohepatic, renal, and ileocecal lymph nodes. The medial retropharyngeal and sublumbar lymph nodes exhibited diffuse haemorrhages in some patients. Previous studies have primarily focused on the gastrohepatic and renal lymph nodes, where haemorrhagic lesions were observed in wild boar at different stages, ranging from as early as day 4 pi in experiments with scheduled sacrifices [17] to days 6–7 pi, when animals reached a humane endpoint [8, 16, 18]. Other lymph nodes, such as the medial retropharyngeal lymph node (MRPLN), had not been assessed in prior studies. In our investigation, the MRPLNs consistently exhibited high viral loads along with macroscopic lesions. Given that lymphoid tissues such as tonsils and the MRPLN are responsible for immune surveillance of the oronasal environment, the gross lesions detected imply an early role in virus–cell interactions, the host immune response and virus transmission.

There were significant differences in macroscopic lesions on day 5 pi. Wild boar displayed more severe and extensive changes, while in domestic pigs, lesions were milder. In contrast, domestic pigs that reached the humane endpoint exhibited markedly greater macroscopic scores than did wild boar euthanised at the same clinical endpoint, with a greater number of affected organs and more severe and extensive haemorrhagic lesions. Accordingly, while wild boar develop severe disease more rapidly and reach the clinical endpoint earlier, they develop less severe macroscopic lesions, underscoring their greater susceptibility to ASFV.

The greater susceptibility of wild boar to early virus replication and spread could be related to their virological profile. While none of the infected domestic pigs euthanised up to day 5 pi showed viremia, one wild boar (#51) exhibited viremia on day 3 pi. In previous studies, oronasal infections with high-dose highly virulent strains originating from the Caucasus region [7, 10] or with moderately virulent isolates originating from the Baltic region [9, 16, 17] resulted in viremia at 4 to 5 days pi in wild boar. In oronasal infections of domestic pigs with the moderately virulent Baltic isolates viremia developed by 3 to 4 days pi [9, 16, 17], whereas with the highly virulent isolates Belgium 2018/1 or Georgia 2007 viremia developed by 6 to 7 days pi [19, 27], the latter more in line with our results. In our study, both wild boar and domestic pigs consistently showed the presence of the virus genome in the MRPLN on 3 and 5 pi, respectively, which underlines the role of the MRPLN in early virus replication following intranasal infection before viremia and demonstrates differences in virus replication kinetics between the two subspecies, with a delay in domestic pigs. However, notably, the tonsils did not serve as a main organ for viral genome detection on day 3 in wild boar or day 5 in domestic pigs, despite this organ being conventionally recognised as one of the initial key sites for ASFV replication and diagnostic sampling [30, 31].

As mentioned, the haemorrhagic MRPLN exhibited high viral loads at early stages after infection, demonstrating its importance, together with tonsils, for the early detection of ASFV during post-mortem examinations. Conversely, in lymph nodes with less severe haemorrhages, such as tracheobronchial or gastrohepatic lymph nodes, the early presence of the viral genome was not detected. Earlier studies on domestic pigs infected with highly virulent genotype I isolates suggested that the presence of haemorrhagic lesions in lymph nodes at 3 dpi was not a result of viral replication in endothelial cells but rather the consequent activation and disruption of endothelial cells mediated by cytokines [21, 32]. A similar pathogenic mechanism may explain the presence of haemorrhagic lesions in our animals before the detection of viral antigens in the lymph nodes, although further studies are required to confirm this hypothesis.

The viral genome remained undetected in nasal and rectal swabs from euthanised wild boar and domestic pigs up to day 5 pi. However, in the wild boar, moderate levels of the viral genome were detected on the day of termination (day 6 pi) and on days 8 and 9 pi in domestic pigs. This indicates limited virus shedding until shortly before death, coinciding with the peak viremia. These results are consistent with previous reports where low viral genome loads were detected in oral and faecal swabs from infected wild boar [7, 10, 16] and domestic pigs [9] between 3 and 5 pi, with higher genome loads detected in most infected animals from 6 pi onwards. Given that only a low viral dose is required to infect an animal [8], low to moderate virus shedding, as detected in our study, may suggest the potential for disease transmission through nasal secretions and faecal excretions, although only efficiently in the later stages of the disease. This means that in earlier stages of infection, transmission probably occurs more efficiently through contact with contaminated blood or tissues, biological materials where the virus is able to maintain its infectivity for several months, especially at low temperatures [33]. In this regard, wild boar carcasses persisting in the environment have been identified as a source of infection where the virus can persist for several months, and together with cannibalism in wild boar, this is considered to play a substantial role in the ASF epidemic. [34].

In conclusion, our results demonstrate that wild boar are more susceptible to ASFV than are domestic pigs. The wild boar exhibited shorter incubation periods, earlier onset of clinical signs and hyperthermia, and more rapid development of severe and extensive haemorrhagic lesions. Moreover, wild boar displayed earlier viremia and an earlier presence of the virus genome in target organs. Notably, lymphoid tissues within the oronasal tract, including the medial retropharyngeal lymph nodes, were identified as key portals for ASFV infection and the establishment of systemic infection and disease following intranasal exposure. Recognising these different host responses in wild boar and domestic pigs is critical for designing effective control strategies such as vaccine development and understanding the dissemination of ASFV in different host populations. Further research is needed to uncover the genetic and immunological factors contributing to these differences.

Supplementary Information

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Additional file 1. Individual weights of infected (A, B) and noninfected (C, D) domestic pigs (DP) and wild boar (WB). Day post-infection (x-axis); Weight (y-axis).

Additional file 2. Individual kinetics of clinical scores (A, B) and rectal temperatures (C, D) in uninfected domestic pigs (DP) and uninfected wild boar (WB) used as controls. Day post-infection (x-axis); temperature and clinical score (y-axis).

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Author's contributions
Conceptualisation: PJS-C, CB, FS, A. Neimanis, M-FLP, HC, DG-W, A. Núñez; methodology: PJS-C, FZXL, CB, FW, RS, SM, EW-L, A. Neimanis; formal analysis: PJS-C, FZXL; funding acquisition: PJS-C, DG-W, A. Núñez; writing-original draft preparation: PJS-C, FZXL; writing-review and editing: all authors. All the authors have read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request. The dataset supporting the conclusions of this article is included within the article (and its supplementary information files).

Declarations

Ethics approval and consent to participate
These experiments were reviewed by the APHA Animal Welfare and Ethical Review Board and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under project licence PF971B5E3. Animal experiments were carried out in containment facilities at the Animal and Plant Health Agency (APHA, Weybridge, UK).

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

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Comparative evaluation of histopathological lesions and viral antigen distribution in domestic pigs and wild boar inoculated intranasally with the highly virulent ASFV genotype II strain "Armenia 2007"

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Abstract

African swine fever (ASF) is a devastating viral disease that affects domestic pigs (*Sus scrofa domesticus*) and European wild boar (*Sus scrofa scrofa*). A previous study indicated that wild boar were more susceptible to ASF than pigs, with shorter incubation periods and earlier onset of the disease. This follow-up study aims to compare the pathogenesis and progression of the disease between the two subspecies during the early and late stages of infection following intranasal inoculation with the highly virulent genotype II strain 'Armenia 2007'. Histopathological changes and viral antigen distribution by immunohistochemistry (IHC) were assessed over time following intranasal inoculation. Viral antigen and histological changes were detected earlier in wild boar than in pigs. In wild boar, the medial retropharyngeal (MRPLN) and submandibular lymph nodes were among the earliest sites of virus replication from 3 days post-infection (dpi), with widespread dissemination occurring by 5 dpi. In pigs, the viral antigen was first detected in the MRPLN at 5 dpi. At the humane endpoint, which occurred at 6 dpi in wild boar and 9 dpi in pigs, virus antigen and histopathological scores were lower in wild boar than in pigs, even though the appearance and increase of viral antigen in tissues, onset and development of lesions and humane endpoint occurred earlier in the former. The lower severity and lesser extent of lesions in wild boar suggest lower tolerance of tissue damage prior to reaching the humane endpoint, demonstrating their greater susceptibility to and lower resistance against ASFV.

Keywords African swine fever virus, domestic pigs, wild boar, *Sus scrofa*, histopathology, virus antigen distribution, immunohistochemistry

†Pedro J. Sánchez-Cordón and Aleksija Neimanis have contributed equally as last authors.

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Introduction

African swine fever (ASF), caused by African swine fever virus (ASFV), which is the sole member of the Asfarviridae family [1, 2], is typically a fatal haemorrhagic disease that affects pigs (all *Sus scrofa*) and many other *Sus* species [3, 4]. ASF is currently one of the biggest threats to the global pig industry due to its high mortality rate and the high resistance of the virus, which allows it to spread easily [5–7]. The absence of treatments or globally licensed commercial vaccines [8] also makes ASF a significant animal welfare problem in both domestic pigs (*Sus scrofa domesticus*), hereafter referred to as pigs, and wild boar (*Sus scrofa scrofa*.).

The ASFV strains currently circulating in European, Asian and Caribbean countries are derived from the highly virulent genotype II strains that entered Georgia in 2007 from south-eastern Africa [9]. Despite the important role of wild boar as reservoirs and mediating the spread of the virus in Europe and Asia [10, 11], most of the data on the pathology, pathogenesis and host-virus interactions related to ASFV infection are derived from pigs [12–18] and, to a lesser extent, from wild boar [4, 15, 19–24]. As such, the knowledge of ASF for wild boar generally is inferred from the pig. Experimental data comparing these two subspecies are limited, and frequently are constrained by differences in experimental design, including age of the animals, doses, route of inoculation, virulence of the strains used or clinicopathological parameters evaluated. In vivo studies have shown that wild boar might be more susceptible to infection with virulent genotype II strains than pigs following oronasal infection [15, 18, 23]. However, these studies did not carry out sequential, predetermined culling of both pigs and wild boar at different times after infection, as required for accurate and rigorous comparative studies of pathology and pathogenesis. To date, only two studies, including our previous study, have used this experimental approach with highly virulent [25] or moderately virulent isolates [26], which also indicated that wild boar were more susceptible to ASFV than pigs.

Direct translation of the pathobiology of ASF in pigs to wild boar may therefore be problematic and represents a clear research gap. Elucidating the differences between pigs and wild boar regarding pathogenesis and mechanisms of immune response to the ASFV is essential to better understand the epidemiology and dynamics of the disease. This knowledge subsequently is needed for the development of reliable challenge models for vaccines or therapeutic assessments in these subspecies. Routine histopathological evaluations, in combination with immunohistochemical techniques used for the detection of viral antigens, can provide critical information for understanding disease dynamics and host-virus interactions.

Our previous study on clinical, gross pathological and virological findings following experimental intranasal inoculation with a highly virulent genotype II strain (Armenia 2007) suggested that wild boar had a more rapid disease progression. This included a shorter incubation period, earlier viremia and onset of clinical signs, and more rapid development of macroscopic haemorrhagic lesions than pigs [25]. The aim of this study is to further describe these differences in order to improve the understanding of disease mechanisms at the tissue and cellular level. To do this, we characterise, quantify and compare the histopathological changes and virus antigen distribution in pigs and wild boar using a standardised microscopic scoring protocol to describe disease progression from early stages of infection to humane endpoint.

Materials and methods

Experimental design

Details of the experimental design (animals, virus strain, dose and route of inoculation), the sequential predetermined culling of pigs and wild boar, the clinical and macroscopic evaluations performed as well as the sampling and quantification of viral DNA by qPCR in blood, swabs (rectal and nasal) and tissues have been described previously [25]. Briefly, thirty-eight animals (19 pigs aged 10–12 weeks and 19 wild boar aged 16–18 weeks) were randomly allocated into four groups consisting of 8 animals each (either wild boar or pigs), which were assigned as the infected groups. Two further groups, consisting of either 3 pigs or 3 wild boar, were assigned as non-infected controls. Before inoculation, the animals were assigned to predetermined time points at which they would be euthanised following infection. The animals were sedated and then intranasally inoculated with the ASFV virulent isolate "Armenia 2007" (genotype II). On days 1, 2, 3, and 5 post-infection (dpi), six animals (3 pigs and 3 wild boar) were sedated and euthanised each day. The remaining inoculated animals (4 pigs and 4 wild boar) were euthanised and examined by necropsy upon reaching the predetermined humane endpoint. This corresponded to 6 dpi for all remaining wild boar and 9 dpi for all remaining pigs. Pig DP38 did not reach the humane endpoint but was euthanised with its stablemates to prevent single housing. The control animals ($n=6$) were euthanised at the conclusion of the experiment (12 dpi). Necropsy was performed on all animals, and macroscopic lesions were assessed following a standardised scoring system [27].

Histopathological and immunohistochemical evaluations

During necropsies, an extensive suite of tissue samples from each animal was collected, fixed for 7 days in 10% buffered formalin solution, routinely processed, and embedded in paraffin blocks. To follow disease

progression, organs from different systems and their regional lymph nodes (LNs) were grouped according to their location as follows: (a) Oronasal tract (nasal mucosa, palatine and pharyngeal tonsils, retropharyngeal and submandibular LN); (b) Lower respiratory tract (trachea, right cranial and caudal lung lobes, tracheobronchial LN); (c) Hepatobiliary tract (liver, gallbladder, gastrohepatic LN); (d) Intestinal tract (distal ileum, ileocaecal valve, colon, ileocaecal LN); (e) Urinary tract (kidney, urinary bladder, renal LN); (f) Other lymphoid organs (spleen, thymus, bone marrow) and g) Integument (skin). Serial tissue sections obtained from paraffin blocks were stained with haematoxylin and eosin (HE) and also used for immunohistochemical detection of ASFV antigen using a monoclonal antibody against p30/CP204L virus protein (kindly provided by Dr Linda Dixon, The Pirbright Institute, Pirbright, UK) following previously described protocols [27]. The sections were evaluated microscopically by a veterinary pathologist who was blinded to sample identity and group assignment, using previously documented histopathological and immunohistochemical scoring systems [27]. In brief, histopathological changes and the presence of cells immunolabelled for viral antigen were evaluated in different components of each organ, based on pathological evaluation criteria combined with a semi-quantitative scoring system: (0) no histopathologic changes/no presence of immunolabelled cells; (1) minimal histopathologic changes/occasional presence of immunolabelled cells; (2) mild histopathologic changes/mild presence of immunolabelled cells; (3) moderate histopathologic changes/moderate presence of immunolabelled cells; (4) severe histopathologic changes/abundant presence of immunolabelled cells. Morphological features including cell size, as well as anatomical location were the criteria applied to tentatively identify the types of cells that were immunolabelled.

Statistical analysis

Statistical analyses and data visualisation were performed with GraphPad Prism version 9 (GraphPad Software La Jolla, CA, USA). The differences in total scores between the experimental groups with respect to histopathological changes and cells immunolabelled for viral antigen, as well as the differences between the experimental groups in the various organs evaluated, were tested for statistical significance using an unpaired *t*-test.

Results

Trends in the evolution of virus antigen and histopathologic lesion scores over time

Immunohistochemical evaluations detected cells immunolabelled against ASF-p30 antigen as early as 3 dpi in wild boar tissues (Figure 1A), whereas in pigs, the viral antigen was not detected until 5 dpi (Figure 1A). Wild boar showed higher virus antigen scores than pigs at 5 dpi (Figure 1B). At the clinical endpoint, virus antigen scores were higher in pigs compared to wild boar, but not statistically significant (Figure 1B; $p=0.37$). A single pig DP38 was euthanised at 9 dpi to avoid single housing following euthanasia of its three pen mates that had reached humane endpoint. No immunolabelled cells or significant histopathologic alterations were observed in any of the organs in pig DP38.

Histopathological lesion scores of inoculated pigs and wild boar were virtually the same from 1 to 3 dpi, showing a variety of mild and non-specific findings similar to those observed in control animals (Figure 1C). These consisted mainly of interstitial and alveolar oedema in the lungs, together with mild hyperaemia and occasional small haemorrhages observed in the medulla of the lymph nodes (gastrohepatic, tracheobronchial, medial retropharyngeal and renal), which in the absence of viral antigen, could be attributed to the euthanasia procedure. At 5 dpi, wild boar exhibited a slightly higher, albeit non-significant, histopathological

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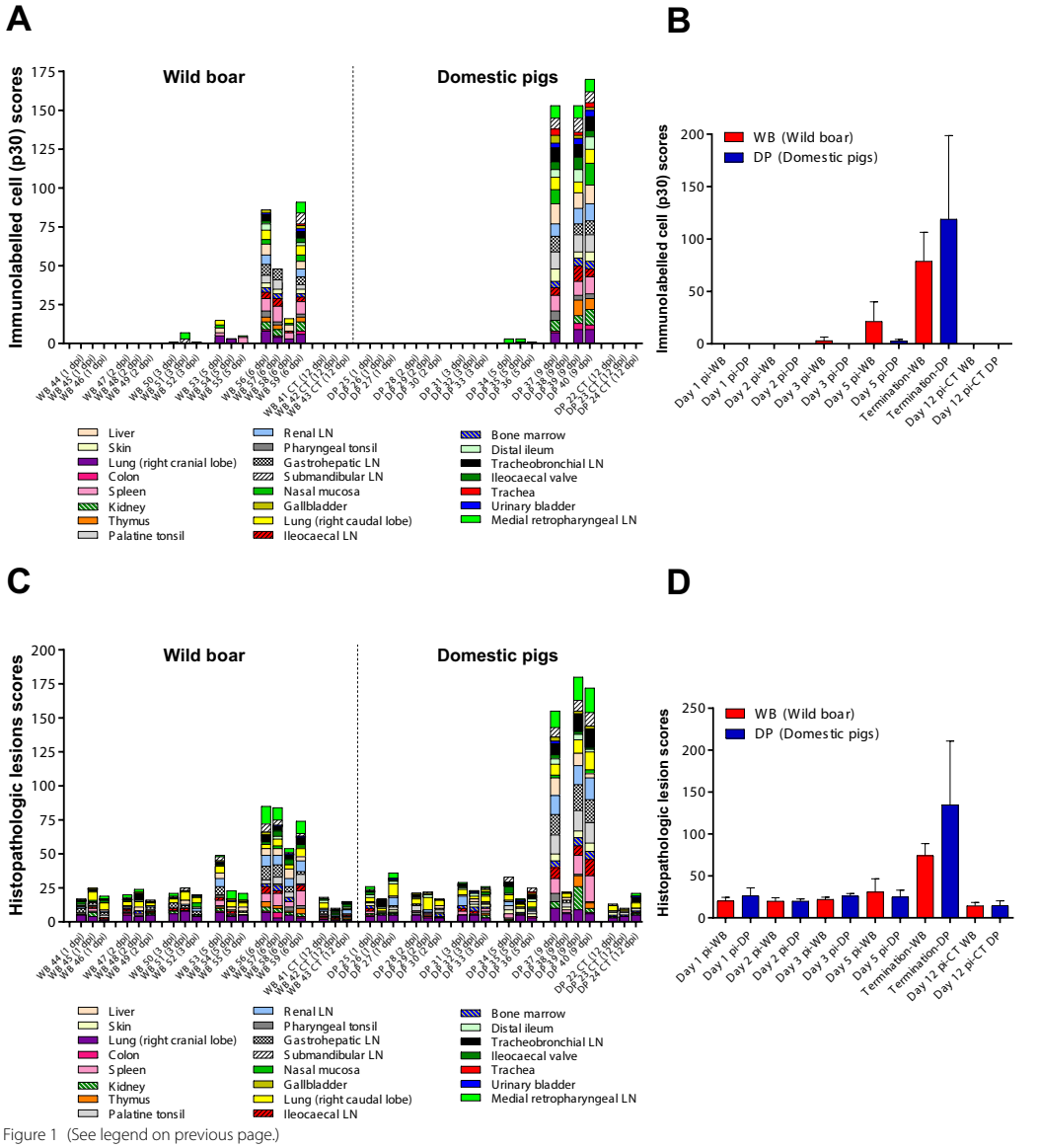
Figure 1 Trends in the evolution of virus antigen and histopathologic change scores in wild boar (*Sus scrofa scrofa*) and domestic pigs (*Sus scrofa domesticus*) intranasally inoculated with African Swine Fever Virus genotype II "Armenia 2007" strain. Virus antigen (**A**) and histopathologic changes (**C**) were scored and documented per individual. A semiquantitative assessment of cells immunolabelled for viral antigen (protein p30/CP204L) and histopathological changes severity was performed in different histological structures of the organs, which were evaluated as follows: (0) no presence of immunolabelled cells/no histopathologic changes; (1) occasional presence of immunolabelled cells/minimal histopathologic changes; (2) mild presence of immunolabelled cells/mild histopathologic changes; (3) moderate presence of immunolabelled cells/moderate histopathologic changes; (4) abundant presence of immunolabelled cells/severe histopathologic changes. Each multicoloured bar represents the cumulative score observed in the different organs of the examined animals and the Immunolabelled cell/histopathologic scores for each organ are represented within the bars as different colours. Immunolabelled cell/histopathologic scores are shown on the y-axis and individual animals evaluated in each experimental group and euthanised on different dates after infection are shown on the x-axis. Uninfected animals (CT) euthanised on 12 dpi are also shown. (**B**, **D**) Mean ± SD of the cumulative virus antigen/histopathologic change scores (y-axis) in each group of pigs or wild boar euthanised on different days after infection (x-axis). Uninfected animals (CT) euthanised on 12 dpi are also shown (x-axis). Statistical analysis was performed using an unpaired t-test. No significant differences were observed.



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score compared to pigs (Figure 1D) and pathological changes were associated with the presence of viral antigen in wild boar. Only the groups of animals that were euthanised at the humane endpoint showed a marked increase in histopathological scores compared to the uninfected control groups. Scores were substantially higher, but not significantly (Figure 1d; $p = 0.16$) in pigs (euthanised at 9 dpi) compared to wild boar (euthanised at 6 dpi). In summary, the number of organs with histopathological lesions and the severity of these lesions increased as the experiment progressed. Lesions became apparent following viral replication and coincided with antigen detection.

Dynamics of viral antigen distribution and appearance of histopathological lesions

The dynamics of the virus antigen and the histopathologic change scores in each of the organs evaluated are plotted over time in Figures 2 and 3. The total scores for immunolabelled cells and histopathological lesions are presented for each individual (Additional file 1).

Early stage of infection (1–5 dpi)

The medial retropharyngeal lymph node (MRPLN), located in the oronasal tract, was the first organ in which cells immunolabelled against ASFV-specific antigen were detected. This occurred at 3 dpi in all three wild boar and 5 dpi for the three pigs (Figure 2). Immunolabelled cells morphologically consistent with macrophages were mainly observed in the medullary area, but in wild boar WB51, these cells were also observed in the interfollicular areas and occasionally within lymphoid follicles (Figure 4A). Additionally at 3 dpi, wild boar WB51 also exhibited immunolabelling in the submandibular lymph node, with virus antigen detected in macrophages within the medullary and interfollicular areas. In comparison, none of the pigs euthanised at 3 dpi showed any immunolabelled cells anywhere (Figure 4A).

At 5 dpi, in addition to the MRPLN in all three wild boar, viral antigen was detected in the other organs of the oronasal tract (Figure 2). Wild boar WB53 showed immunolabelled macrophages in the medullary and interfollicular areas of the submandibular LN (Figure 4B), immunolabelled intravascular monocytes i.e. -large in size with abundant cytoplasm and an indented or bean-shaped nucleus—in the palatine tonsil, occasional immunolabelled macrophages in the lamina propria of the pharyngeal tonsil as well as intravascular immunolabelled monocytes and macrophages infiltrating the lamina propria of the nasal mucosa (Figure 4C, black arrows). However, the MRPLN was the only organ in the oronasal tract that showed immunolabelled cells in the three pigs euthanised at 5 dpi (Figures 2 and 4B). The bone marrow was the only other location, and only in pig DP35, where some immunolabelled cells (myeloid cells) were observed in pigs culled at 5 dpi. The same immunostaining pattern was also observed in the bone marrow of wild boar WB53 at 5 dpi (Figure 4C, red arrows). The bone marrow showed no histopathological alterations in either animal.

No conspicuous histopathological changes were observed in any of the evaluated organs of the oronasal tract until 5 dpi (Figure 3), when mild to moderate alterations such as pyknotic and fragmented cell nuclei (karyorrhexis) were observed in medullary and interfollicular areas of the MRPLN of wild boar WB54 and WB55 (Figure 4D). In addition, mild karyorrhexis was observed in the medullary, interfollicular areas and lymphoid follicles of the submandibular LN in wild boar WB53 and pig DP36, despite the absence of viral antigen in the latter, as well as moderate, multifocal haemorrhages in the medulla of pig DP34 (Figure 4D). On 5 dpi, no conspicuous lesions were observed in the palatine tonsils, pharyngeal tonsils or nasal mucosa in either pigs or wild boar.

Viral antigen was also detected in other tissues at 5 dpi in some wild boar, but in none of the pigs (Figure 2). These included the lower respiratory tract (right cranial and caudal lung lobes and tracheobronchial LN), hepatobiliary tract (liver and gastrohepatic LN), urinary tract (kidney and renal LN) and other lymphoid organs (spleen). No viral antigen was detected in any of the other organs evaluated up to 5 dpi in the wild boar.

In the lung, there was a moderate amount of immunolabelled interstitial macrophages (Figure 5A, black arrows) and some alveolar macrophages (Figure 5A, green arrow) in the cranial lung lobe of wild boar WB53 and WB54. In wild boar WB53, immunolabelled cells morphologically consistent with type II pneumocytes were also occasionally observed (Figure 5A, red arrows). In comparison, there were fewer immunolabelled cells in the caudal lung lobe, and antigen was only detected in macrophages. Occasionally, immunolabelled macrophages were also found in the interfollicular areas and within the lymphoid follicles in the tracheobronchial LN of wild boar WB53. Up to 5 dpi, both pigs and wild boar only showed nonspecific lung lesions similar to those described in the control groups, such as moderate, diffuse congestion, moderate interstitial and alveolar oedema and mild peribronchial mononuclear infiltrates. Moderate to severe medullary haemorrhages observed in the tracheobronchial LN of pig DP26 on 1 dpi and in wild

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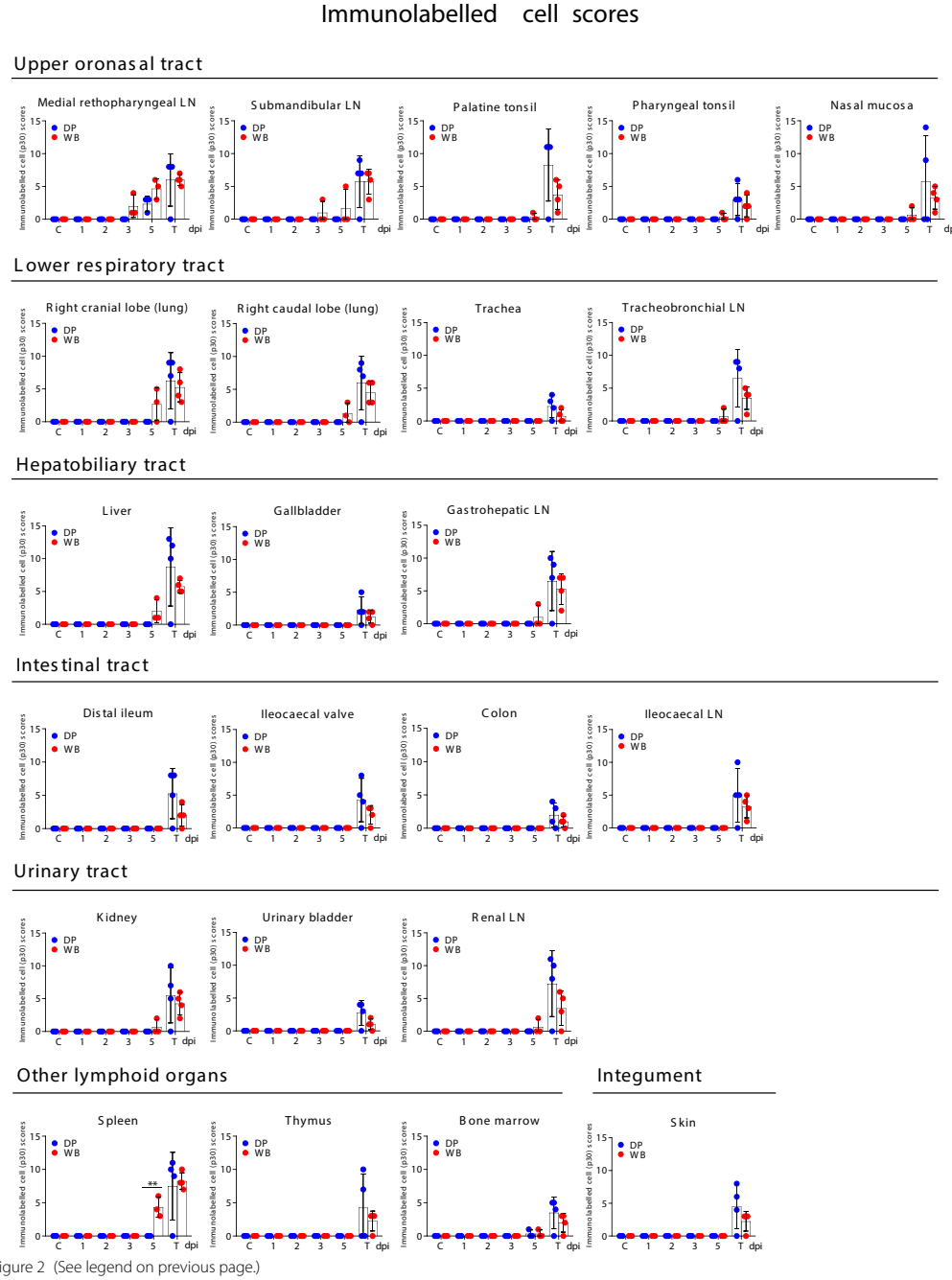
Figure 2 Comparative evaluation of the dynamics of virus antigen scores (mean ± SD) in each of the examined organs in wild boar (*Sus scrofa scrofa*) and domestic pigs (*Sus scrofa domesticus*) intranasally inoculated with African Swine Fever Virus genotype II "Armenia 2007" strain. Each animal is represented by a dot within its respective group: Domestic pigs (DP); Wild boar (WB). Statistical analysis was performed using an unpaired t-test. Black asterisks indicate statistically significant differences between the two groups of infected animals (wild boar and domestic pigs) euthanised on different dates; significant variables (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). Score of immunolabelled cells (y-axis); day-post infection (dpi); uninfected control groups (C); lymph node (LN); groups of infected animals euthanised at humane endpoint (T): 6 dpi for WB and 9 dpi for DP.



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boar WB48 on 2 dpi were considered to be nonspecific lesions or artefacts caused by tissue processing.

All three wild boar euthanised at 5 dpi showed intravascular immunolabelled monocytes within the liver (Figure 2). These were observed mainly in the sinusoids, but also in the portal and central veins of WB53, in which immunolabelled hepatocytes (Figure 5B, red arrows) and Kupffer cells (Figure 5B, black arrow) also could be detected. In this animal, a small number of immunolabelled macrophages were also seen in the gastrohepatic LN, in the medullary areas and occasionally in the interfollicular areas (Figure 5B). However, while no conspicuous histopathological findings were observed in the livers up to 5 dpi, the gastrohepatic LN in wild boar WB50 euthanised at 3 dpi showed moderate medullary haemorrhages (Figure 5B). Haemorrhages could also be observed affecting medullary and interfollicular areas in wild boar WB53 euthanised at 5 dpi (Figure 5B).

In the urinary tract, wild boar WB53 was the first animal to show occasional immunolabelled mononuclear interstitial infiltrates (Figure 5C) and intravascular monocytes in the kidney at 5 dpi. In addition, the renal LN of this animal showed occasional immunolabelled cells in the medullary and interfollicular areas (Figure 5C). However, while kidneys from wild boar and pigs euthanised up to 5 dpi showed no histopathological alterations, severe, diffuse haemorrhages involving the medullary and interfollicular areas were observed in the renal LN of pig DP31 at 3 dpi and in wild boar WB53 at 5 dpi (Figure 5C).

As for other lymphoid organs, immunolabelled macrophages were detected in the spleen of the three wild boars euthanised at 5 dpi, but not in any of the pigs euthanised on the same dpi (significant differences in viral antigen score, $p=0.008$). These macrophages were mainly found in the red pulp (Figure 5D), with occasional viral antigen also observed in the ellipsoids. In wild boar, immunolabelled macrophages were also occasionally detected within the lymphoid follicles of WB54 and WB55. The spleen of both pigs and wild boar euthanised up to 5 dpi showed no histopathological changes that differed from those observed in the spleen of control animals.

Humane endpoints (6 dpi for wild boar and 9 dpi for pigs)

One pig, DP38, did not reach the humane endpoint but was euthanised to avoid anxiety and stress associated with single housing. No viral antigen or histopathological changes were observed in this animal; therefore, it is not included in the results below. For all other pigs and wild boar at the humane endpoint, viral antigen and histopathological lesions were widespread in many organ systems.

A common pattern was seen in the lymph nodes (medial retropharyngeal, submandibular, tracheobronchial, gastrohepatic, ileocaecal and renal) assessed in each of the organ tracts in the four boars, as well as in the three pigs (DP37, DP39 and DP40) that reached the humane endpoint. All of them showed a large number of macrophages immunolabelled for viral antigen. These were observed mainly in the medullary and interfollicular areas, but also occasionally within the lymphoid follicles. Many of these macrophages had an increased amount of cytoplasm containing phagocytised, immunolabelled cell debris (tingible bodies; regarded to represent remains of phagocytised apoptotic cells). In addition, immunolabelled intravascular monocytes, endothelial cells and reticular cells were occasionally observed (Figures 6C, 7C and 8A, C). Viral antigen scores were usually higher in pig than in wild boar (Figure 2, Additional file 1). Multifocal and diffuse haemorrhages in the medulla and interfollicular areas, together with mild to focally severe lymphoid depletion, were the most notable histopathological lesions in these lymph nodes in both pigs and wild boar. This was accompanied by features of cell death (karyorrhexis) in the interfollicular areas and lymphoid follicles. The overall severity was slightly lower in wild boar (Figures 6C, 7C and 8A, C). Among pigs, the lesions were particularly severe in DP39 and DP40, while WB56 showed the most prominent changes among wild boar.

In the pharyngeal and palatine tonsils, immunolabelled cells could be seen in the three pigs that reached the humane endpoint at 9 dpi (pig DP37, DP39, and DP40) (Figure 2). These cells were mainly macrophages and occasional lymphocytes that infiltrated epithelium of the crypts, the diffuse lymphoid tissue surrounding the crypts, the interfollicular areas and the lamina propria.

(See figure on next page.)

Figure 3 Comparative evaluation of the dynamics of histopathological change scores (mean ± SD) in each of the examined organs in wild boar (*Sus scrofa scrofa*) and domestic pigs (*Sus scrofa domesticus*) intranasally inoculated with African Swine Fever Virus genotype II "Armenia 2007" strain. Each animal is represented by a dot within its respective group: domestic pigs (DP); wild boar (WB). Statistical analysis was performed using an unpaired *t*-test. Black asterisks indicate statistically significant differences between the two groups of infected animals (wild boar and domestic pigs) euthanised on different dates; significant variables (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001). Score of immunolabelled cells (y-axis); day-post infection (dpi); uninfected control groups (C); lymph node (LN); groups of infected animals euthanised at humane endpoint (T): 6 dpi for WB and 9 dpi for DP.

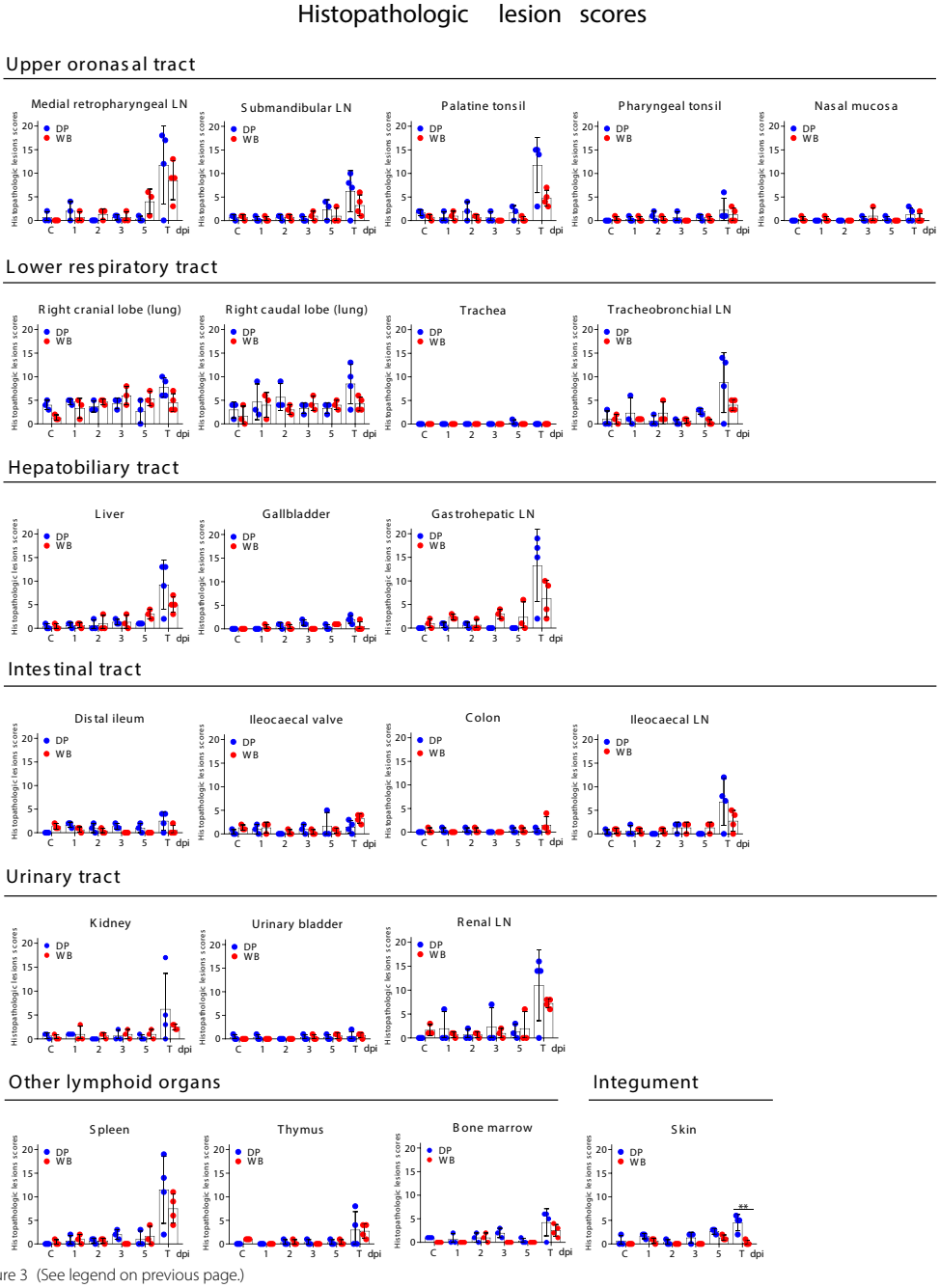

Figure 3 (See legend on previous page.)

Antigen-positive cells within the lymphoid follicles were scarce (Figure 6A). Pig DP40 also had immunolabelled epithelial cells in the epithelium of the pharyngeal tonsil (Figure 6A, black arrows). Wild boar euthanised at the humane endpoint (6 dpi) showed a similar pattern in both tonsils, but with lower virus antigen scores (Figure 2). Mild lymphoid depletion, together with moderate amount of pyknotic cells and cell fragmentation in interfollicular areas and lymphoid follicles, were the most obvious histopathological lesions in the pharyngeal tonsil of pig DP37. In contrast, the palatine tonsil of pigs DP37, DP39 and DP40 showed severe lymphoid depletion in the interfollicular areas and mild lymphoid depletion in the lymphoid follicles, together with cell fragmentation (Figure 6A, HE staining, black circle) and tingible body macrophages. The latter were especially abundant within the lymphoid follicles (Figure 6A, red arrows). The epithelium of the crypts also showed moderate to severe infiltrates of mononuclear cells displaying severe pyknosis and cell fragmentation (Figure 6A, HE staining, white circle). These lesions, although less severe, were also present in wild boar at the humane endpoint. Like the tonsils, the nasal mucosa did not begin to show evidence of viral antigen in pigs until 9 dpi, with large numbers of immunolabelled macrophages mainly in the mucosa, submucosa (Figure 6B, red arrows) and perichondrium. Occasionally, immunolabelled epithelial cells could also be seen in the respiratory epithelium (Figure 6B, black arrow) along with occasional labelled endothelial cells (Figure 6B, green arrows). The wild boar group euthanised at 6 dpi showed a scant number of immunolabelled macrophages in the mucosa and interfollicular areas of the nasal mucosa (Figure 6B, red arrows). Apart from mild karyorrhexis in lymphoid tissue in pig DP37, pig DP40 and wild boar WB57, no other specific histopathological lesions were observed in the nasal mucosa.

In the lower respiratory tract of pigs DP37, DP39 and DP40, there was an abundance of interstitial (Figure 7A, green arrows) and intravascular (Figure 7A, black arrows) immunolabelled macrophages in both the cranial and caudal lobes, as well as fewer immunolabelled alveolar macrophages (Figure 7A, red arrows) and occasional immunolabelled pneumocytes. Additionally, numerous macrophages within the bronchial-associated lymphoid tissue (BALT) were immunolabelled in pig DP39. All four wild boar showed a similar immunostaining pattern, but without the presence of immunolabelled intra-BALT macrophages. Histopathological evaluation of pigs revealed moderate alveolar septal thickening in the lungs, along with mild to moderate mononuclear cell infiltrates consisting mainly of macrophages and lymphocytes (Figure 7B, yellow arrows), oedema (Figure 7B, black arrows), cell debris (Figure 7B, white circle) and fibrin deposits (Figure 7B, green arrows) in the alveolar lumen. This was accompanied by bronchiolar oedema, sloughed epithelium and mononuclear cells (bronchiolitis; Figure 7B, red arrows), vascular hypertrophy as well as pyknotic cells and karyorrhexis in the BALT. The latter finding was particularly notable in the caudal lobe. However, in wild boar the lesions were less severe, with only diffuse, mild congestion and alveolar oedema (Figure 7B, WB59) together with minimal vascular endothelial hypertrophy.

In the trachea, all three pigs and two of the four wild boar, WB57 and WB59, that reached the humane endpoint showed immunolabelled cells. These consisted mainly of macrophages infiltrating the mucosa and occasionally the epithelium (Figure 7C). In the trachea, no lesions were found throughout the experiment, neither in pigs nor wild boar.

In the case of pigs, viral antigen was detected in organs of the hepatobiliary tract only in the animals that reached the humane endpoint (Figure 2). Their livers displayed a large number of hepatocytes (Figure 7D, black arrows) and interstitial macrophages in the portal spaces (Figure 7D, red arrows) immunolabelled for viral antigen. Immunolabelled Kupffer cells (Figure 7D, green arrow) and intravascular monocytes (Figure 7D, blue arrow) were also present. In wild boar, the same type of

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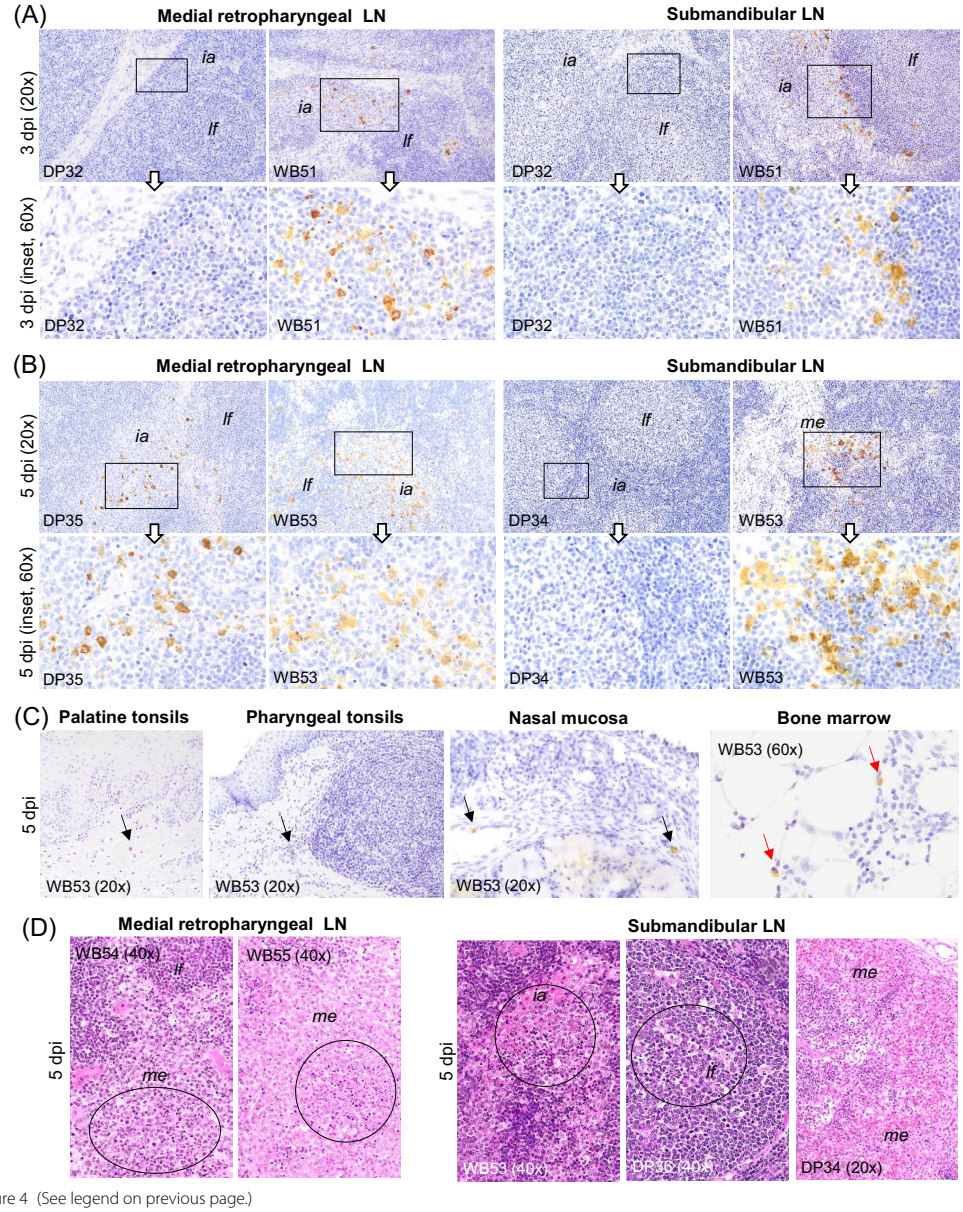
Figure 4 Histopathologic changes and presence of cells immunolabelled for ASFV-specific antigen (P30). Organs taken from wild boar (*Sus scrofa scrofa*) and domestic pigs (*Sus scrofa domesticus*) intranasally inoculated with African Swine Fever Virus genotype II "Armenia 2007" strain euthanised between 1 and 5 dpi. (**A**, **B**) IHC. Representative images of cells, primarily macrophages, immunolabelled for viral antigen in the medial retropharyngeal (MRPLN) and submandibular lymph nodes at 3 and 5 dpi. **A** Note that viral antigen was only present in the WB at 3 dpi. **B** MRPLN was also the first organ in which viral antigen was detected in the DP at 5 dpi. **C** IHC. Intravascular monocytes and infiltrating macrophages immunolabelled for the virus in tonsils (palatine and pharyngeal) and nasal mucosa (black arrows) at 5 dpi. Immunolabelled myeloid cells (red arrows) were also observed in the bone marrow of a wild boar at 5 dpi. **D** HE staining. Characteristic pyknotic and fragmented cell nuclei (karyorrhexis, indicated by circles) observed in the medulla and interfollicular areas of the MRPLN in wild boar. Karyorrhexis (circles) was also observed in the medulla, interfollicular areas and lymphoid follicles of the submandibular lymph node in wild boar and pigs, together with multifocal haemorrhages in the medulla of DP34. Immunohistochemistry against P30 protein (IHC); Haematoxylin–eosin staining (HE); Original magnification (number x); interfollicular areas (*ia*); lymphoid follicle (*lf*); medullary area (*me*); Wild boar (WB); Domestic pig (DP); White arrows indicate histopathological details of the selected areas within the boxes at higher magnification.



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cells were immunolabelled, but they were less abundant. Extensive liver damage was also evident in the euthanised pigs at the humane endpoint. Frequently centred in the portal areas or interlobular septa, the lesions were characterised by mild to moderate interstitial mononuclear infiltrates that exhibited karyorrhexis (Figure 7D, HE staining, red arrowheads). In addition, foci of hypereosinophilic hepatocytes with nuclear karyorrhexis and karyolysis (necrosis) accompanied by mononuclear cell infiltration were observed (Figure 7D, HE staining,

black circles). An increase in intravascular leukocytes in the hepatic sinusoids (sinusoidal leukocytosis) was also noted, along with the presence of enlarged Kupffer cells. These findings were more prominent in pigs DP37 and DP40. In comparison, hepatic lesions were limited to diffuse, mild to moderate liver congestion in all four wild boar, along with mild leucocytosis only observed in wild boar WB58.

In the gallbladder, Pig DP37 showed a moderate amount of immunolabelled macrophages in the interfollicular areas of the lymphoid tissue of the submucosa (Figure 7E, black arrow). Occasional macrophages in the lamina propria (Figure 7E, red arrow) and intravascular monocytes were also seen in pig DP37, DP39 and DP40. Positive intravascular monocytes could also be found in wild boar WB56, WB57 and WB59. Pig DP40 and wild boar WB56 and WB59 also showed occasionally immunolabelled endothelial cells (Figure 7E, blue arrow). Histological examination revealed minimal to mild mononuclear infiltrates in the lamina propria of the gallbladder of pigs DP37, DP39, DP40 and wild boar WB56, with wild boar WB57 also showing mild, submucosal oedema.

In the distal ileum, ileocaecal valve and the colon of the three pigs that reached the humane endpoint, there were abundant immunolabelled macrophages, most frequently observed in the interfollicular areas, followed by the lamina propria. In contrast immunolabelled macrophages were only occasionally observed in the lamina propria in wild boar (Figure 8B). Microscopically, minimal lesions restricted to the lamina propria and characterised by the presence of mild hyperaemia and mild mononuclear infiltrate were observed in the distal ileum, ileocaecal valve and colon only in some pigs and wild boar.

In the urinary tract of all three pigs, immunolabelled cells were mainly observed in the renal cortex. These were identified as capillary endothelial-like cells within the glomeruli (Figure 8C, black arrow), interstitial macrophages (Figure 8C, red arrow), intravascular monocytes (Figure 8C, green arrow), and epithelial cells of the renal ducts (Figure 8C, blue arrow). There were fewer labelled cells in the medulla and pelvic area. In all four wild boar, the same type of cells were immunolabelled and evenly distributed throughout the kidney but observed less frequently. Histopathological changes of the kidney were mild in all four wild boar as well as in pigs DP37 and DP40, which showed mild diffuse congestion and occasional small, interstitial mononuclear infiltrates in the renal cortex. The exception was pig DP39, which showed severe, diffuse congestion together with severe, multifocal haemorrhages in the renal cortex (Figure 8C, HE staining, black arrowhead), medulla and pelvic area, accompanied by vasculitis and microthrombi mainly in the cortex (Figure 8C, HE staining, white arrowhead). In addition to moderate interstitial mononuclear infiltrates with pyknotic cells and moderate karyorrhexis, tubular nephrosis was also observed in the renal cortex in this animal (Figure 8C, HE staining, yellow arrowhead). In the urinary bladder, small numbers of immunolabelled macrophages in the lamina propria and occasional endothelial cells were observed in all animals reaching the humane endpoint except for wild boar WB58. However, no specific lesions were observed in any of the animals throughout the experiment.

Regarding other lymphoid organs, which are considered main targets for ASFV, the spleen contained abundant immunolabelled macrophages. These were observed mainly in the red pulp, but also occasionally in lymphoid follicles (Figure 8D, red arrows), periarteriolar lymphoid sheaths and ellipsoids in both the pigs and the wild boar that reached the humane endpoint. In pig DP37, there were also occasional immunolabelled lymphocytes (Figure 8D, black arrowhead) and endothelial cells (Figure 8D, blue arrow). In pigs, histopathological changes included moderate to severe karyorrhexis in the red pulp, lymphoid follicles and periarteriolar sheaths, accompanied by mild to severe lymphoid depletion of the white

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Figure 5 Histopathologic changes and presence of cells immunolabelled for ASFV-specific antigen (P30). Organs taken from wild boar (*Sus scrofa scrofa*) and domestic pigs (*Sus scrofa domesticus*) intranasally inoculated with African Swine Fever Virus genotype II 'Armenia 2007' strain euthanised between 1 and 5 dpi. **A** Right cranial lung lobe, IHC. At 5 dpi, immunolabelled cells were only present in wild boar. Note the presence of immunolabelled interstitial macrophages (black arrows) and alveolar macrophages (green arrow). Immunolabelled pneumocytes were also occasionally observed (red arrows). **B** Liver and gastrohepatic lymph node, IHC. At 5 dpi, immunolabelled cells were visible only in wild boar. Note the presence of immunolabelled hepatocytes (red arrows) and Kupffer cells (black arrows) in the liver, as well as the occasional presence of immunolabelled macrophages in the medullary areas of the gastrohepatic lymph node; Gastrohepatic lymph node, HE staining. Medullary haemorrhages (WB50, 3 dpi); Haemorrhages affecting medullary areas (WB53, 5 dpi). **C** Kidney, IHC. Immunolabelled interstitial macrophage (WB53, 5 dpi). In the same animal, the renal lymph node showed occasional immunolabelled cells (IHC) as well as diffuse haemorrhages (HE staining) in the medullary and interfollicular areas. **D** Spleen, IHC, 5 dpi. Immunolabelled macrophages were present in the spleens of the three wild boars euthanised at 5 dpi, mainly in the red pulp, but not in any of the pigs euthanised on the same day. Immunohistochemistry against P30 protein (IHC); Haematoxylin–eosin staining (HE); Original magnification (number x); interfollicular areas (*ia*); lymphoid follicle (*lf*); medullary area (*me*); splenic red pulp *(rp)*; Wild boar (WB); Domestic pig (DP); White arrows indicate histopathological details of the selected areas within the boxes at higher magnification.

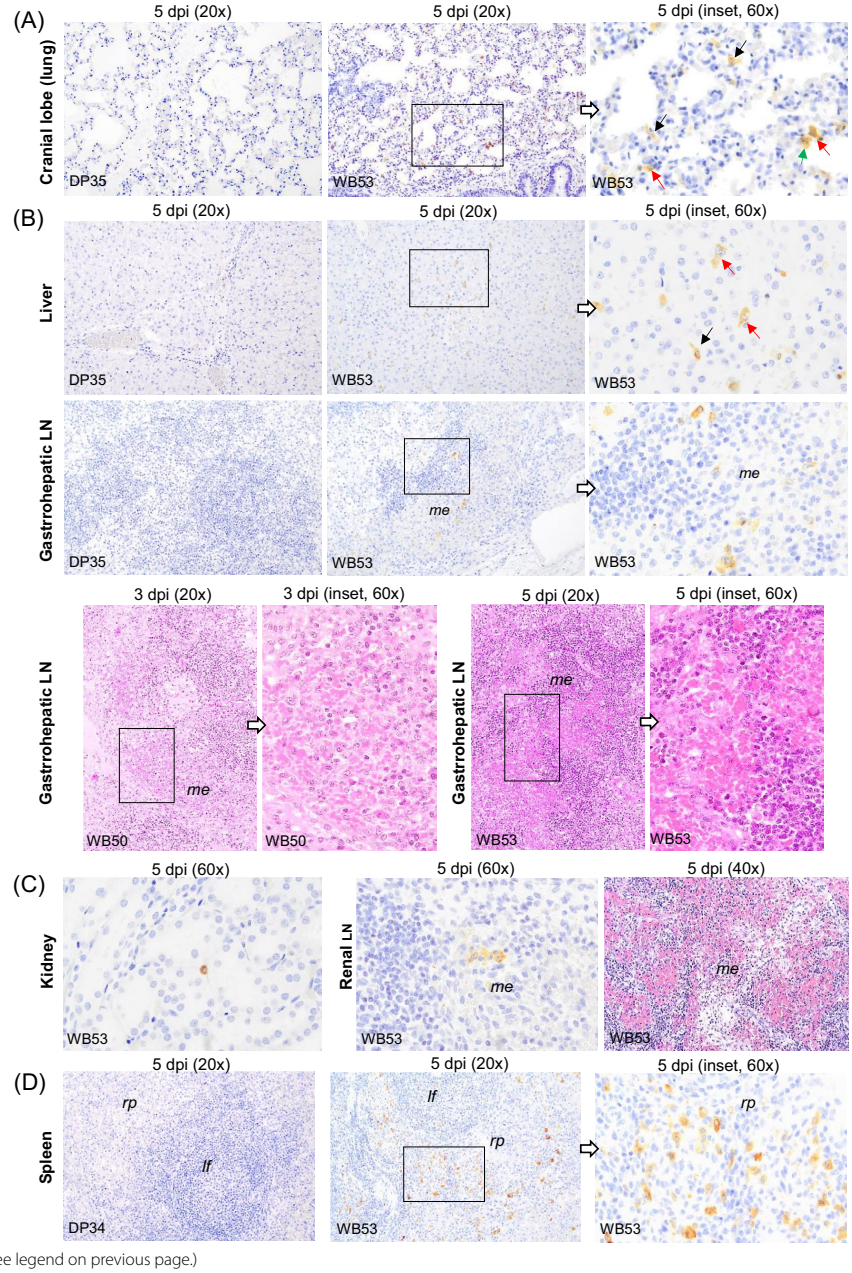

Figure 5 (See legend on previous page.)

pulp (Figure 8D, HE staining). In addition, there was engorgement of the red pulp and mild haemorrhages within the lymphoid follicles and the periarteriolar lymphoid sheaths. Wild boar showed a similar lesion pattern but with a slightly lesser severity.

In the thymus (Figure 8E), immunolabelled macrophages (red arrows), tingible body macrophages (red arrowheads) and occasional lymphocytes (black arrowheads) were evenly distributed in the cortex, medulla and the corticomedullar junction, being especially numerous in pig DP39, and generally more abundant in pigs compared to wild boar. Occasionally, antigen was also detected in stellate cells, consistent with reticuloepithelial cells, in the corticomedullary junction and medulla (blue arrowhead). Microscopically, the lesions observed in pigs consisted of mild lymphoid depletion, moderate karyorrhexis and a high number of tingible body macrophages (Figure 8E, HE staining, red arrowheads). Thymuses of the wild boar assessed at humane endpoint also showed similar lesions, but to a lesser extent.

In the bone marrow in pigs, there was a moderate number of immunopositive cells, primarily myeloid cells (Figure 8F). A similar pattern was observed in wild boar, but with lower numbers of labelled cells. However, no specific lesions were evident in any of the animals evaluated, apart from the presence of occasional cell debris and a mild increase in apoptotic or cloud-like nuclei within megakaryocytes in pigs and, to a lesser degree, in wild boar.

Finally, a moderate number of immunolabelled macrophages was observed in the dermis of the dorsal skin samples taken from pigs, which were more prominent in the perivascular mononuclear infiltrates (Figure 8F, red arrows). Intravascular monocytes and capillary endothelial cells (Figure 8F, green arrows) stained for viral antigen were also detected. The wild boar showed only occasionally immunolabelled cells, mainly macrophages. Along with moderate perivascular mononuclear infiltrates, primarily consisting of lymphocytes and occasional macrophages, mild vasculitis was also observed in pigs. This lesion was especially noticeable in pig DP40 (Figure 8F, HE staining). In comparison, wild boar skin showed no specific lesions, and histopathological scores differed significantly ($p = 0.033$).

Discussion

Comparative histopathological evaluations of tissue samples collected from pigs and wild boar inoculated intranasally with the highly virulent ASFV genotype II strain, "Armenia 2007", revealed differences in the occurrence and distribution of viral antigen, as well as in the progression of lesions at tissue level between the two subspecies.

In wild boar, detection, increase and dissemination of viral antigen occurred earlier than in pigs. The organs of the oronasal tract, particularly the MRPLN and submandibular lymph node, were shown to be the main locations for virus replication as early as 3 dpi. Quantity of viral antigen increased in subsequent days, which was associated with local virus proliferation. The MRPLN was also the first location in which the viral antigen was detected in pigs, albeit at 5 dpi. Interestingly, despite the route of infection and the high dose used, viral antigen was only occasionally visualised in the nasal mucosa of a wild boar at 5 dpi. It was not observed in the tonsils of wild boar nor pigs at 3 dpi and was only sporadically observed at this site in one wild boar at 5 dpi. Viral antigen was also detected at 5 dpi in the lungs of wild boar and occasionally in the tracheobronchial LN, but not in pigs.

These results highlight the importance not only of MRPLN in early virus replication after intranasal infection prior to viraemia, as has been suggested previously [25, 28], but also of the submandibular LN. The role of the latter as a primary, albeit minor, target organ for virus replication has also been reported previously [28–31]. Our results, however, contrast with other studies in which the tonsils are considered to be the usual route of entry and replication of ASFV [29–33]. They instead

(See figure on next page.)

Figure 6 Histopathologic changes and presence of cells immunolabelled for ASFV-specific antigen (P30). Organs taken from infected animals euthanised at humane endpoint (6 dpi for wild boar and 9 dpi for pigs). **A** Palatine and pharyngeal tonsil, IHC. Cells immunolabelled for viral antigen, mainly macrophages and occasional lymphocytes, infiltrating the crypt epithelium, the diffuse lymphoid tissue surrounding the crypts, the interfollicular areas and the lamina propria. Observe the scarcity of viral antigen within the lymphoid follicles. The pharyngeal tonsil also showed immunolabelled epithelial cells in the epithelium (black arrows); Palatine tonsil, HE staining. Lymphoid depletion in the interfollicular areas, cell fragmentation (oval circle) and tingible body macrophages within the lymphoid follicles (red arrows). Note the infiltrates of mononuclear cells displaying severe pyknosis and cell fragmentation in the crypt epithelium (white circle). **B** Nasal mucosa, IHC. Immunolabelled macrophages in mucosa and submucosa. Note the occasional presence of immunolabelled epithelial cells in the respiratory epithelium (black arrow). Occasional immunolabelled macrophages were also observed in the mucosa and interfollicular areas of the nasal mucosa in wild boar (red arrow). **C** Submandibular lymph node, IHC. Marked presence of immunolabelled macrophages in the medullary and interfollicular areas. Note the presence of phagocytised immunolabelled cell debris (tingible bodies, red arrows) as well as intravascular monocytes (black arrow) and endothelial cells (green arrow) immunostained; HE staining. Areas with lymphoid depletion accompanied by abundant pyknotic cells, karyorrhexis (circle) and macrophages showing abundant cytoplasm containing phagocytised cell debris (tingible body macrophages, red arrows). Immunohistochemistry against P30 protein (IHC); Haematoxylin–eosin staining (HE); Original magnification (number x); interfollicular areas (*ia*); lymphoid follicle (*lf*); epithelium (*ep*); crypt epithelium (*cr*); mucosa (*mu*), submucosa (*smu*); Wild boar (WB); Domestic pig (DP); White arrows indicate histopathological details of the selected areas within the boxes at higher magnification.

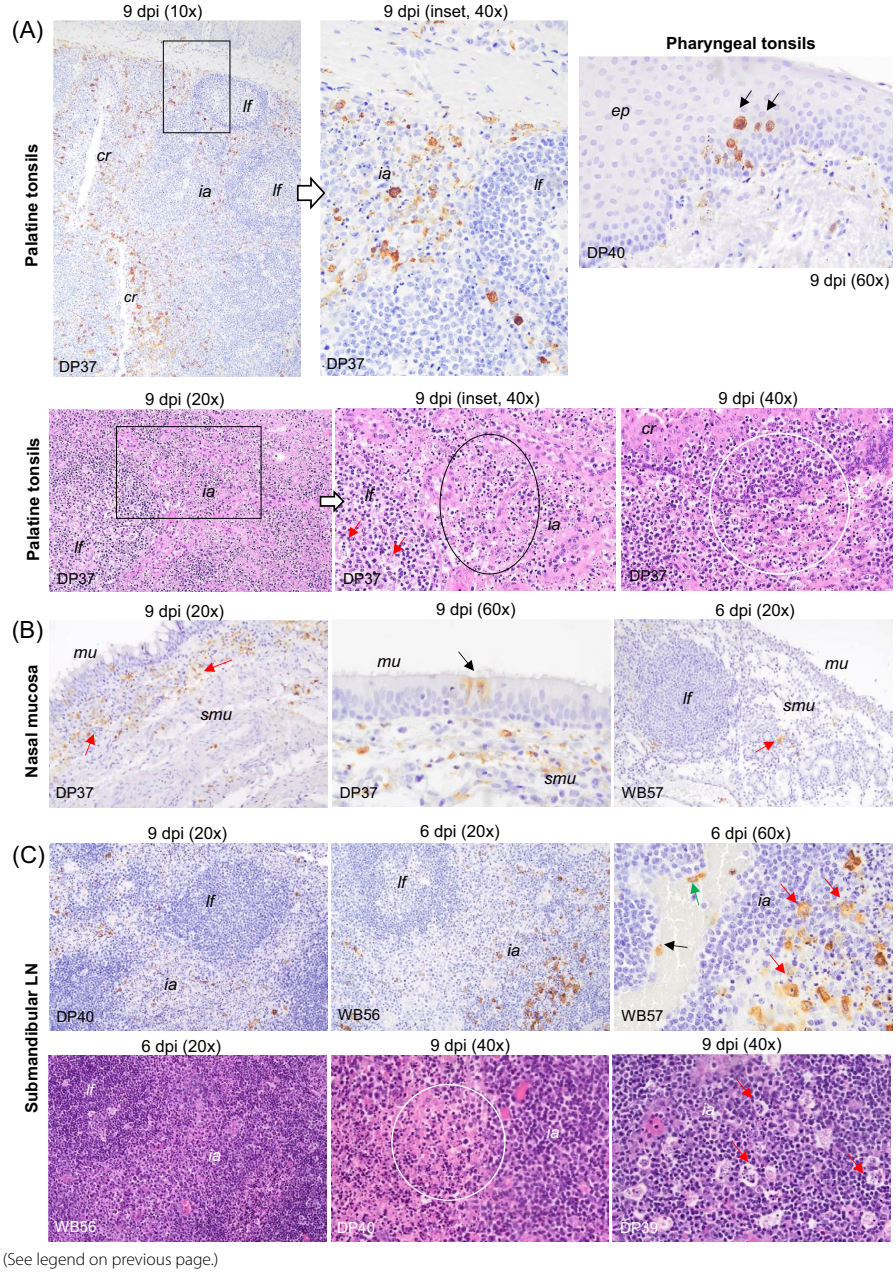

Figure 6 (See legend on previous page.)

support the hypothesis that after intranasal inoculation, ASFV can replicate in cephalic lymph nodes without first passing through the tonsils [28]. In addition, the nasal mucosa, lungs and tracheobronchial lymph nodes do not appear to play a significant role in the initial entry and proliferation of the virus following intranasal inoculation,

which is consistent with other studies [25, 28]. These findings could explain why, in the current experimental infection [25] as well as in previous experiments [18, 23, 24], the viral genome was either undetectable or present at very low levels up to 5 dpi in oronasal swabs taken from wild boar and pigs that were infected oronasally with high and moderate virulence genotype II isolates. Our results also support studies suggesting that the potential for virus transmission through nasal secretions would not be efficient in early stages of infection [34], a fact that should be considered when using oronasal samples for diagnostic sampling.

Similarly, no viral antigen was detected in any of the intestinal organs or regional lymph nodes that were examined up to 5 dpi, despite the presence of mucosa-associated lymphoid tissue in these organs in both wild boar and pigs. These results also suggest that the intestinal tract may play a minor role as a portal of entry and replication for ASFV. This in turn would explain the undetectable or low levels of viral genome detected in faecal swabs taken from experimentally infected wild boar and pigs in the present [25] and previous studies [18, 23, 24].

Although none of the infected pigs euthanised up to 5 dpi became viraemic, moderate levels of viraemia were detected in one infected wild boar euthanised at 3 dpi, and higher levels in infected wild boar euthanised at 5 dpi [25]. High viraemia levels occurred concurrently with the appearance of viral antigen in the spleens of these wild boar, but not in the pigs. Both viraemia and the presence of the virus in the spleen are considered to be signs of the virus spreading throughout the body, which was deferred in pigs. Therefore, a generalised spread of the virus was evident in wild boar, but not in pigs, at 5 dpi when the viral antigen was detected by immunohistochemistry in multiple organ systems. This is consistent with the detection of viral genome in multiple tissues in our previous study [25]. In wild boar, viral antigen was not only detected in the oronasal tract, but also in the respiratory, hepatobiliary and urinary tracts. The spleen, lung and liver are organs with high resident macrophage populations and are highly susceptible to ASFV infection [35–37]. Virus replication mainly occurs in these macrophage populations, and secondarily in other cell types such as hepatocytes, endothelial cells, epithelial cells and reticular cells [27]. Replication in these organs would contribute to increased viraemia and highlights the role of these organs as secondary sites of virus replication following virus spread.

Our findings show that following intranasal inoculation of ASFV, initial replication occurs in the lymph nodes of the upper oronasal tract prior to viraemia, particularly in the medial retropharyngeal and submandibular lymph nodes. The efferent lymphatic vessels from both lymph nodes converge in the tracheal duct, which then discharges into the brachiocephalic vein, thus returning lymph to the bloodstream [38]. The virus therefore could use this pathway to reach secondary replication sites such as the spleen, liver and lungs, either within monocytes, attached to the surface of erythrocytes or freely within the bloodstream as proposed previously [12, 28, 39]. Replication of the virus in these secondary organs concurrently with replication in the primary sites mentioned would contribute to the onset and increase of viremia.

Histopathological lesions and their severity increased as the experiment progressed. However, microscopic changes, predominantly haemorrhagic lesions and characteristic findings of lymphoid tissue destruction such as pyknosis and cellular fragmentation of mononuclear cells [12, 13, 39], only emerged or became evident after detection of viral antigen. Thus, cell destruction was first observed in the MRPLN and to a lesser extent in the

(See figure on next page.)

Figure 7 Histopathologic changes and presence of cells immunolabelled for ASFV-specific antigen (P30). Organs taken from infected animals euthanised at humane endpoint (6 dpi for wild boar and 9 dpi for pigs). **A** Lung caudal lobe, IHC. Abundant Interstitial (green arrows), intravascular (black arrows) and alveolar (red arrows) macrophages immunolabelled; **B** Lung caudal lobe, HE staining. Alveolar septal thickening along with mononuclear cell infiltrates consisting mainly of macrophages and lymphocytes (yellow arrows), oedema (black arrows), cell debris (white circle) and fibrin deposits (green arrows) in the alveolar lumen. Note also the presence of inflammatory cells in the bronchiolar lumen (bronchiolitis, red arrow); **C** Trachea and tracheobronchial lymph node, IHC. Macrophages infiltrating the mucosa and the epithelium of the trachea; Tracheobronchial lymph node, IHC. Immunolabelled macrophages in the medullary and interfollicular areas; HE staining. Note the presence of karyorrhexis and haemorrhages (asterisk) within lymphoid follicles of pigs compared to mild lymphoid tissue depletion in wild boar; **D** Liver, IHC. Abundant immunolabelled hepatocytes (black arrows) and interstitial macrophages in the portal spaces (red arrows). Occasional stained Kupffer cells (green arrow) and intravascular monocytes (blue arrows) were also observed; HE staining. Liver damage was characterised by the presence of interstitial mononuclear infiltrates in the portal spaces and interlobular septa areas, which exhibited karyorrhexis (red arrowheads), and multifocal necrotic foci of hepatocytes with mononuclear cell infiltration (circles); **E** Gallbladder, IHC. Occasional immunolabelled macrophages in the interfollicular areas of the lymphoid tissue (black arrow) and the lamina propria (red arrow). Occasionally immunolabelled endothelial cells (blue arrow). Immunohistochemistry against P30 protein (IHC); Haematoxylin–eosin staining (HE); Original magnification (number x); interfollicular areas (*ia*); lymphoid follicle (*lf*); medullary area (*me*), epithelium (*ep*); lamina propria (*lp*), portal space (*ps*); bronchus-associated lymphoid tissue (*balt*); Wild boar (WB); Domestic pig (DP); White arrows indicate histopathological details of the selected areas within the boxes at higher magnification.

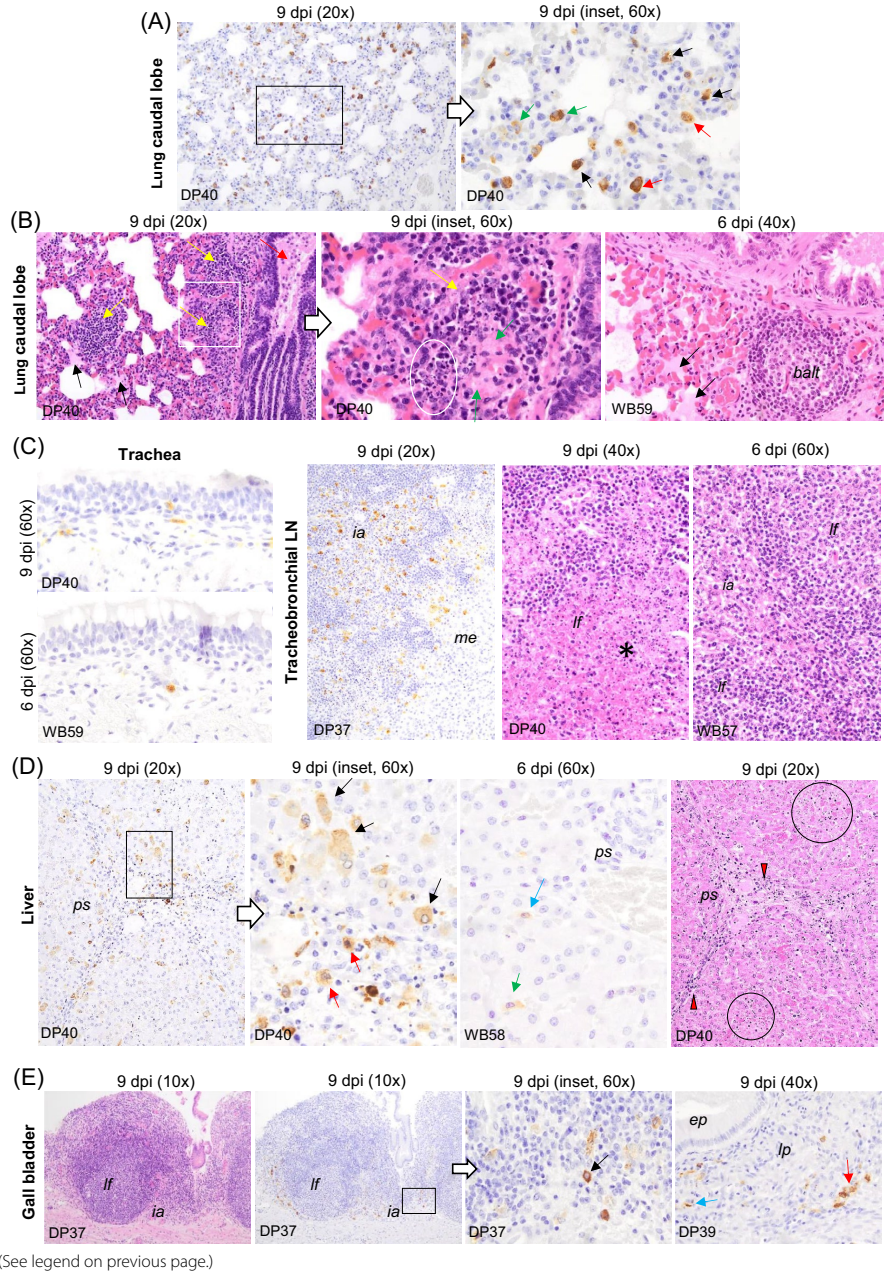

Figure 7 (See legend on previous page.)

submandibular LN of wild boar at 5 dpi in parallel with an increased presence of infected macrophages. Therefore, the replication of the virus in these target cells may induce their destruction, as well as that of bystander cells such as lymphocytes. This occurs via indirect mechanisms induced by proinflammatory cytokines secreted by

infected macrophages, resulting in an initial mild tissue injury in lymphoid tissue [35, 40–42]. Notably, haemorrhagic lesions in the submandibular LN of pigs euthanised at 5 dpi occurred in the absence of infected cells, but infected cells were observed in the nearby MRPLN. Such haemorrhages could have originated from the activation and disruption of endothelial cells, caused by an increase in intravascular cellular debris or increased vascular permeability induced by chemical mediators generated by other injured organs [12, 43].

Both wild boar and pigs that reached the humane endpoint at 6 and 9 dpi, respectively, exhibited a notable increase in the number of cells immunolabelled against viral antigen, as well as in the presence and severity of histopathological lesions in all tested organs. In addition, virus antigen and histopathological scores at the human endpoint were generally higher in pigs than in wild boar in the studied organs, even though the appearance and increase of immunolabelled cells and the appearance and progression of lesions occurred later in pigs. Thus, the lower severity and extent of lesions in wild boar were evidence of their lower tolerance of tissue damage prior to reaching the humane endpoint. This demonstrates their greater susceptibility to and lower resistance against the ASFV, characteristics previously noted in other studies [18, 23, 25, 26].

The mechanisms underlying these differences, which likely involve both viral and host factors, constitute a huge knowledge gap. Wild boar exhibit higher levels of genetic diversity than domestic pigs [44, 45], although differentiation between the two subspecies relies on only a few genetic markers [46]. It has been suggested that the innate immune response is key to controlling levels of ASFV replication and pathogenesis in different infected hosts. ASFV may be more effective in evading innate responses in domestic pigs and wild boars compared to wild African suids, leading to disease. Conversely, host genetic factors, as is likely the case in wild African suids, may reduce the activation of potentially harmful responses, thereby controlling the replication of the virus and reducing the appearance of clinical signs and tissue damage [47]. Other studies have pointed to differences in T-cell responses that may explain some of the differences in ASF progression in wild boar and pigs, although the authors did not elucidate any mechanism of protection or resistance against ASFV [48, 49]. The influence of genetic, immunological and virological factors on host susceptibility and resistance is a question that will need to be addressed in future studies. The higher susceptibility to and lower tolerance of tissue damage in wild boar compared to pigs should also be taken into account during vaccine trials, in order to avoid inaccurate assessments resulting from the occurrence of only moderate pathology in wild boar. This emphasises the need to improve our understanding of ASF in wild boar to develop vaccines that are specifically designed to control and eradicate the disease in wild boar. Comorbidities, including parasitic diseases, nutritional discrepancies and environmental stressors present in field conditions, should also be considered as factors that will make this task difficult.

To conclude, we demonstrated that viral antigen in tissues and histological lesions were detected earlier in wild boar than in pigs after intranasal inoculation with ASFV, with MRPLN and submandibular lymph nodes being among the earliest sites for virus replication. Virus antigen and histopathological scores at the human endpoint were lower in wild boar than in pigs, even though the appearance and increase of viral antigen in tissues,

(See figure on next page.)

Figure 8 Histopathologic changes and presence of cells immunolabelled for ASFV-specific antigen (P30). Organs from infected animals euthanised at humane endpoint (6 dpi for wild boar and 9 dpi for pigs). **A** Gastrohepatic lymph node, IHC. Immunolabelled macrophages in medulla and interfollicular areas; HE staining. Haemorrhages (asterisk) in medullary areas; **B** Ileocaecal valve and colon, IHC. Interfollicular areas and lamina propria with immunolabelled macrophages in pigs. In wild boar immunostained cells were scarce; **C** Kidney, IHC. Capillary endothelial cells within glomeruli (black arrow), interstitial macrophages (red arrow), intravascular monocytes (green arrow), and epithelial cells of the renal ducts (blue arrow) immunolabelled; HE staining. Haemorrhages cortex (black arrowhead), vasculitis and microthrombi (white arrowhead). Tubular nephrosis and interstitial mononuclear infiltrates also observed (yellow arrowhead); Renal LN, IHC and HE staining. Immunolabelled macrophages in medullary and interfollicular areas. Haemorrhages (asterisk) and lymphoid depletion in lymphoid follicle; **D** Spleen, IHC. Abundant immunolabelled macrophages in red pulp with occasional presence within lymphoid follicles (red arrows). Immunolabelled lymphocytes (black arrowhead) and endothelial cells (blue arrow) also observed; HE staining. Lymphoid depletion affecting lymphoid follicles and periarteriolar sheaths; **E** Thymus, IHC. Macrophages (red arrows), tingible body macrophages (red arrowheads) and occasional lymphocytes (black arrowheads) immunolabelled in cortex and medulla. Occasionally stellate cells consistent with reticuloepithelial cells (blue arrowhead); HE staining. Lymphoid depletion, Karyorrhexis and tingible body macrophages in cortex (red arrowheads); **F** Bone marrow, IHC. Immunostained cells, mainly myeloid cells; Skin, IHC. Perivascular mononuclear infiltrates with numerous immunolabelled macrophages in pigs (red arrows) and Capillary endothelial cells (green arrows); HE staining. Perivascular mononuclear infiltrates and vasculitis. Immunohistochemistry against P30 protein (IHC); Haematoxylin–eosin staining (HE); Original magnification (number x); interfollicular areas (*ia*); lymphoid follicle (*lf*); medullary area (*me*); lamina propria (*lp*); glomeruli (*gl*), red pulp (*rp*), periarteriolar lymphoid sheaths (*ps*), cortex (*co*); Wild boar (WB); Domestic pig (DP).

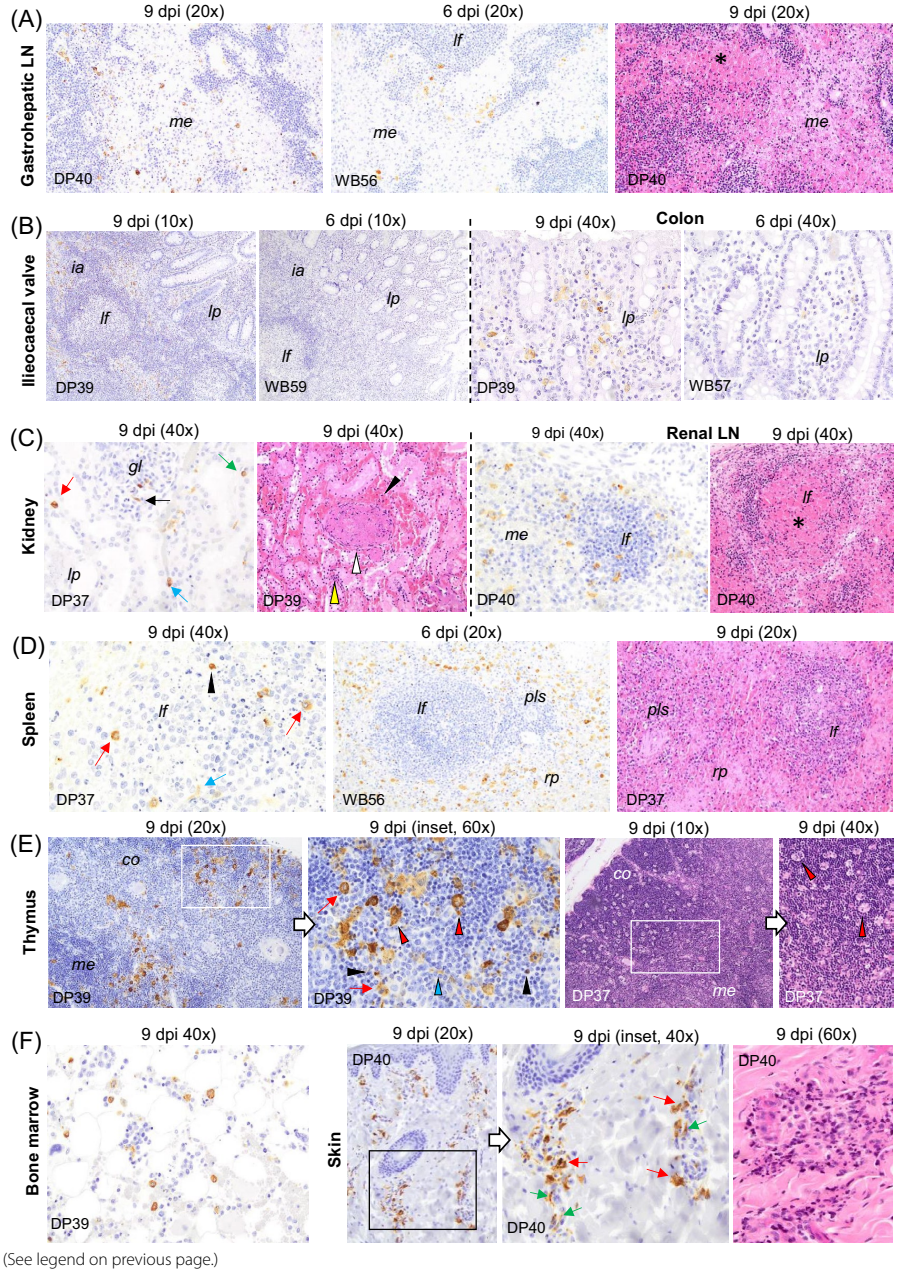

Figure 8 (See legend on previous page.)

the progression of lesions and the humane endpoint occurred earlier in wild boar. Thus, the lower tolerance of tissue damage prior to reaching the humane endpoint demonstrated the higher susceptibility and lower resistance of wild boar to ASFV. The mechanisms behind these differences remain unclear. These findings should

be considered if ASF candidate vaccines intended to wild boar are evaluated in pig models, as surrogates for wild boar. The results from this study also provide information on co-localisation of the virus with lesions at the cellular and tissue level and complement previously published data on macroscopic lesions and virus presence in the same animals.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13567-025-01701-x.

Additional file 1 Summary of histopathological and virus antigen scores in pigs and wild boar.

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Authors' contributions
Conceptualisation: PJS-C, ANe., DG-W, ANu., FZXL; methodology: EW-L, PJS-C, ANe., ANu., FZXL; formal analysis: PJS-C, EW-L, ANe; funding acquisition: PJS-C, DG-W, ANe., ANu., KS; writing-original draft preparation: EW-L; writing-review and editing: PJS-C, ANe., DG-W, ANu., FZXL, KS, BY. All authors read and approved the final manuscript.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The animal experiment was carried out in containment facilities at the Animal and Plant Health Agency (APHA, Weybridge, UK) in 2020. This experiment was reviewed by the APHA Animal and Plant Welfare and Ethical Review Board and was conducted in accordance with the UK Animals (Science Procedures) act 1986 under project licence PF971B5E3 [25].

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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This thesis investigates the pathogenesis and disease dynamics of African swine fever virus genotype II in domestic pigs and wild boar following intranasal infection. By integrating clinical observations with macroscopic, histopathological, and immunohistochemical analyses, the work demonstrates earlier viral replication, lesion development, and disease onset in wild boar compared to pigs, highlighting species-specific differences in susceptibility and tissue tolerance relevant to ASF epidemiology and control. It also examines the pathology of naturally infected wild boar to enhance disease surveillance.

Emil Wikström Lassa received his postgraduate education at the Department of Animal Biosciences. His veterinary degree was obtained at the Swedish University of Agricultural Sciences (SLU), Uppsala.

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