

## **Factor V Leiden mutation does not affect coagulopathy or outcome in lethal H1N1 influenza**

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## **Abstract**

Influenza A is a major cause of mortality. Knowledge on coagulation activation in influenza infection is limited. The factor V Leiden (FVL) mutation is possibly subject to positive selection pressure. It is unknown whether this mutation impacts on the outcome of severe influenza. We here determined the effect of lethal influenza on pulmonary and systemic coagulation activation and determined whether FVL mutation alters coagulation activation in and the course of lethal influenza.

Wild-type mice and mice heterozygous or homozygous for FVL were infected intranasally with a lethal dose of H1N1 influenza A. Mice were sacrificed after 48 or 96 hours for determination of coagulation activation, histopathology, pulmonary inflammatory parameters and viral loads or were observed in a survival study.

Extensive local and systemic coagulation activation during lethal influenza was demonstrated by increased lung and plasma levels of thrombin-antithrombin complexes and fibrin degradation products and by pulmonary fibrin deposition. FVL mutation did not influence the procoagulant response, lung histopathology or survival. FVL mice demonstrated elevated viral loads 48 hours after infection.

In conclusion, coagulation is activated locally and systemically during lethal murine influenza A infection. The FVL mutation does not influence coagulation activation, lung inflammation or survival in lethal influenza A.

## **Introduction**

Influenza is a major cause of morbidity and mortality: Seasonal influenza annually causes over 200,000 hospitalizations and approximately 41,000 deaths in the United States [1]. Influenza viruses can be classified as A, B or C. Influenza A is found in humans, other mammals and birds and is the only influenza virus known to have caused pandemics, like the three 20<sup>th</sup> century pandemics and the current influenza pandemic from swine origin [2]. Although the greatest proportion of mortality caused by influenza A infection is due to secondary bacterial pneumonia, the virus itself is also an important cause of community-acquired pneumonia (CAP), causing 5-10% of CAP-cases in various case series [3,4]. As such, influenza infection is a major concern for pulmonologists and intensive care physicians. Cardiovascular complications contribute to influenza-related morbidity and probably also mortality [5].

Severe infection and inflammation have been closely linked to activation of coagulation on the one hand and downregulation of anticoagulant mechanisms and fibrinolysis on the other hand (reviewed in [6]). While much research has been done on coagulation activation during severe bacterial infection, like bacterial pneumonia, peritonitis and sepsis, data on coagulation activation in viral infections are sparse. Recently, systemic coagulation activation was shown in a non-lethal mouse model of influenza A [7]. However, it is unclear to date, to what extent coagulation is activated, in the circulation and locally in the lung, during a more severe influenza A infection and whether this is related to outcome.

The factor V Leiden (FVL) mutation, a missense mutation in FV gene replacing arginine at position 506 with glutamine, which results in resistance of activated FV (FVa) to inactivation by activated protein C (APC) [8], is a major risk factor for venous thrombo-embolism [9]. The high prevalence of this mutation - 4-6% in Caucasians - despite its prothrombotic effects, has prompted

speculation that the mutation might be subject to positive selection pressure [10]. For example, it has been speculated that FVL carriers might benefit a lower rate of intraventricular bleeding during infancy and that heterozygous FVL carrier status might improve embryo implantation via an unknown mechanism [11,12]. An alternative hypothesis for a survival advantage for heterozygous FVL carriers has been driven by the PROWESS trial in which it was suggested that heterozygous FVL carriers in severe sepsis had a survival advantage as compared to non-carriers and by animal studies showing an increased survival for heterozygous FVL mice in murine endotoxaemia as compared to wild-type (WT) mice [13,14]. However, FVL Leiden mice displayed an unaltered mortality in experimental sepsis induced by viable Gram-negative [15] or Gram-positive bacteria [16]. To the best of our knowledge, the impact of the FVL mutation on the outcome of severe viral infection has not been studied to date. Therefore, we here investigated whether carriership for the FVL mutation influences the host response to lethal influenza A infection. For this we infected heterozygous and homozygous FVL mice with a lethal dose of a mouse adapted influenza A strain and compared their responses with regard to coagulation, inflammation, viral load and mortality with those in normal WT mice.

## **Materials and methods**

### **Animals**

FVL mice carrying an R504Q amino acid mutation [17] were backcrossed four times to a C57BL/6J background (N4) whereafter N4 FVL heterozygous mice were intercrossed to obtain WT, heterozygous and homozygous offspring for use in experiments. All mice were bred and maintained in the animal care facility of the Academic Medical Center (University of Amsterdam)

according to institutional guidelines with free access to food and water. Sex- and age-matched (9–11 weeks old) mice were used in all experiments (n = 8 per group per time point, n = 14 for the survival study). Four uninfected WT, heterozygous and homozygous FVL mice were used as controls. All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

### **Experimental infection**

Influenza infection was induced as described previously [7;18]. In brief, mouse-adapted influenza A/PR/8/34 (H1N1, ATCC no. VR-95, Rockville, MD) was grown on LLC-MK2 cells (RIVM, Bilthoven, the Netherlands). Virus was harvested by a freeze/thaw cycle, followed by centrifugation at 680 g for 10 minutes. Titration was performed in LLC-MK2 cells to calculate the median tissue culture infective dose (TCID<sub>50</sub>) of the viral stock. The stock was not contaminated by other respiratory viruses such as influenza B, human parainfluenza virus type 1, 2, 3, 4A and 4B, respiratory syncytial virus A and B, rhinovirus, enterovirus, corona virus or adenovirus, as determined by PCR or cell culture. The stock was stored in aliquots at -80°C. Viral stock aliquots were thawed immediately before use and diluted in phosphate-buffered saline (PBS, pH 7.4). Mice were anaesthetized by inhalation of isoflurane 2% (Abbott Laboratories, Kent, UK) and inoculated intranasally with 200\* TCID<sub>50</sub> influenza A (28,000 viral copies), which has been established to be a lethal dose [18], in a final volume of 50 µL PBS. Mice were euthanized at 48 or 96 hours after infection or observed up to 9 days. On the predefined time points mice were anesthetized with ketamine (Nimatek®, Eurovet Animal Health, Bladel, The Netherlands) and medetomidine (Domitor®, Pfizer Animal Health Care, Capelle a/d IJssel, the Netherlands). Blood was drawn from the vena cava inferior, diluted with citrate (1:5), gently mixed and stored on ice. Blood was centrifuged at 600 g. Plasma was snap frozen in liquid

nitrogen and stored at -80°C until analysis. Lungs were harvested and homogenized at 4°C in 4 volumes of saline using a tissue homogenizer (Pro 200, Pro Scientific Inc., Oxford, CT) and processed as described below.

### **Assays**

For measurements in lung tissue, lung homogenates were diluted 1:2 with lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 1% (v/v) Triton X-100, pH 7.4) with protease inhibitor mix added (AEBSF (4-(2-aminoethyl)benzeensulfonylfluoride), EDTA-NA<sub>2</sub>, Pepstatin and Leupeptin, all from MP Biomedical; concentrations in accordance with the manufacturer's recommendations) and incubated for 30 minutes on ice, followed by centrifugation at 680 g for 10 minutes. Supernatants were stored at -20°C until analysis. Thrombin-antithrombin complexes (TATc) and fibrin degradation products (FDP) were measured in lung homogenates and plasma using ELISA (TATc: Behringwerke AG, Marburg, Germany; Fibrin degradation products; (FDP) [19]. Tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, IL-12p70, monocyte chemotactic protein (MCP)-1, IL-10 and interferon (IFN)- $\gamma$  were measured by cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA). Keratinocyte-derived chemokine (KC) and myeloperoxidase (MPO) were measured by ELISA (R&D systems, Minneapolis, MN and HyCult Biotechnology, Uden, the Netherlands, respectively).

### **Histology and immunohistochemistry**

The right lung was fixed in 10% formalin/PBS at room temperature for 24 hours and embedded in paraffin. Sections of 5  $\mu$ m were cut, stained with haematoxylin and eosin (H&E) and analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, the lung surface was analyzed with respect to the following parameters: bronchitis, interstitial

inflammation, oedema, endothelialitis, pleuritis and thrombus formation. Each parameter was graded on a scale of 0 to 4 (0: absent, 1: mild, 2: moderate, 3: severe, 4: very severe). The total histopathological score was expressed as the sum of the scores for the different parameters, the maximum being 24.

Granulocyte staining was performed using fluorescein isothiocyanate-labeled anti-mouse Ly-6G mAb (Pharmingen, San Diego, CA) as described earlier [20,21] and fibrin(ogen) staining was performed as described [22,23]. Ly-6G and fibrin(ogen) stained slides were photographed with a microscope equipped with a digital camera (Leica CTR500, Leica Microsystems, Wetzlar, Germany). Ten random pictures were taken per slide. Stained areas were analysed with Image Pro Plus (Media Cybernetics, Bethesda, MD) and expressed as percentage of the total surface area. The average of ten pictures was used for analysis.

### **Determination of viral load**

Viral load was determined using real-time qPCR as described [18,24]. In brief, 50  $\mu$ L of lung homogenate was treated with 500  $\mu$ L of Trizol reagent to extract RNA. RNA was resuspended in 10  $\mu$ L DEPC-treated water. cDNA synthesis was performed using 1  $\mu$ L of purified RNA and a random hexamer cDNA-synthesis kit (Applied Biosystems, Foster City, CA). Five out of 25  $\mu$ L of the cDNA-preparation was used for amplification in a real-time qPCR reaction (ABI PRISM 7700 Sequence Detector System). The viral load was calculated using a standard curve of particle counted influenza virus included in the assay run. The following primers were used: forward 5'-GGACTGCAGCGTAGACGCTT-3', reverse 5'-CATCCTGTTGTATATGAGGCCCAT-3' and 5'-CTCAGTTATTCTGCTGGTGCCTTGCC-3' (5'-FAM labelled probe). Viral load was normalized for total RNA as determined by NanoDrop (Spectrophotometer ND-1000, Thermo Scientific, Wilmington, DE).



## **Statistical analysis**

Data are expressed as box-and-whisker diagrams (depicting the smallest observation, lower quartile, median, upper quartile and largest observation) or as medians with interquartile ranges. Differences between groups were determined with Kruskal-Wallis, Mann-Whitney *U* test or log-rank test. Analyses were performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). P-values of less than 0.05 were considered statistically significant.

## **Results**

### **Activation of coagulation**

To determine whether coagulation is activated locally and systemically during murine lethal influenza we determined levels of TATc and FDP in lung homogenates (**Figure 1A and B**) and plasma (**Figure 1C and D**) at baseline and 48 and 96 hours after inoculation with influenza A virus. Infection with a lethal dose of influenza A markedly and significantly increased levels of TATc and FDP in both lung homogenates and plasma at both time points, indicating that coagulation activation during influenza is both a local and a systemic phenomenon. Remarkably, however, both at baseline and during infection, there were no differences in TATc and FDP levels between WT, heterozygous and homozygous FVL mice.

To further substantiate coagulation activation during lethal influenza we performed fibrin(ogen) staining. As compared to baseline, fibrin deposition was seen after 48 (not shown) and 96 hours of infection in all mouse strains (**Figure 2A, B and C**). Again, there were no differences between WT, heterozygous and homozygous FVL mice both after 48 and 96 hours (**Figure 2D**).

## **Pulmonary inflammation**

Influenza infection was associated with pulmonary inflammation as evidenced by the occurrence of bronchitis, interstitial inflammation, oedema and endothelialitis both at 48 hours (not shown) and 96 hours after infection in all mouse strains (**Figure 3A, B and C**). There were no differences in total histopathological scores between WT, heterozygous and homozygous FVL mice after 48 hours and 96 hours (**Figure 3D**). Moreover, there were no differences in the separate scores for bronchitis, interstitial inflammation, oedema and endothelialitis (not shown).

One of the prominent features in lethal influenza is neutrophil influx into the lung both after 48 hours (not shown) and 96 hours (**Figure 4A, B and C**). However, there were no differences in neutrophil influx between WT, heterozygous and homozygous mice as evidenced by equal percentages of positivity in Ly-6G stainings both at 48 hours and 96 hours after infection (**Figure 4D**). In line with their similar histopathology and Ly-6G scores, pulmonary MPO concentrations, indicative for the number of neutrophils in lung tissue, were similar in WT, heterozygous and homozygous FVL mice at both 48 and 96 hours (**Table 1**).

To obtain further insight into the impact of the FVL mutation on lung inflammation, we measured the levels of various cytokines and the chemokine KC in lung homogenates (**Table 1**). At 48 hours after infection homozygous FVL mice had lower pulmonary levels of the pro-inflammatory cytokine TNF- $\alpha$  as compared to WT mice. Heterozygous FVL mice also tended to have lower TNF- $\alpha$  levels than WT mice, however this did not reach statistical significance ( $P = 0.08$ ). After 96 hours differences in TNF- $\alpha$  levels had subsided. After 48 hours of infection heterozygous FVL mice had substantially lower levels of the anti-inflammatory cytokine IL-10 in lung homogenates as compared to WT mice. Homozygous FVL mice also tended to have lower IL-10 levels than WT mice, however this did not reach statistical significance ( $P = 0.07$ ). After 96 hours differences in

IL-10 levels had subsided. At 96 hours after infection the only difference in cytokine levels was a modest increase in IFN- $\gamma$  levels in heterozygous FVL mice as compared to WT mice. Lung KC levels did not differ between groups at either time point.

### **Viral load**

To investigate the influence of the FVL mutation on viral load after influenza A infection, we determined pulmonary viral loads in lungs. After 48 hours, heterozygous and homozygous FVL mice had more than 4-fold more pulmonary viral copies than WT mice (**Figure 5**). Viral copies in heterozygous and homozygous FVL mice were not significantly different. After 96 hours of infection, the differences had subsided.

### **Survival**

To substantiate whether differences in viral load and cytokine levels between WT, heterozygous and homozygous mice at 48 (and 96) hours were associated with an altered mortality we performed a survival study. The infection was associated with 100% lethality within 9 days in all groups. Mortality curves did not differ between WT, heterozygous and homozygous FVL mice (**Figure 6**).

### **Discussion**

Influenza is an important cause of pneumonia, causing 5-10% of all CAP-cases [3,4], and the 7<sup>th</sup> leading cause of mortality in the United States [25]. While severe bacterial pneumonia and sepsis have been closely linked to activation of coagulation and downregulation of anticoagulant

mechanisms (reviewed in [6]), data on coagulation activation in influenza pneumonia are sparse. One clinical study in hospitalized pediatric patients has indicated that severe influenza can be associated with disseminated intravascular coagulation [26]. In addition, mice infected with a non-lethal dose of influenza A displayed a rise in plasma TATc levels from four days after infection, indicating coagulation activation at the systemic level [7]. Interestingly, this study also suggested that endogenous APC may reduce influenza-induced coagulation, since mice with a mutation in their *thrombomodulin* gene, resulting in a minimal capacity for endogenous APC generation, demonstrated increased plasma TATc levels after 4 days as compared to WT mice [7]. We here show that lethal influenza in mice causes both pulmonary and systemic activation of coagulation, as evidenced by increased lung and plasma TATc and FDP levels in influenza A infected animals already from two days after infection, resulting in pulmonary fibrin deposition. Moreover, we demonstrate that the FVL mutation does not impact on either systemic or local coagulation activation, inflammation or on survival during severe influenza.

The FVL mutation results in resistance of FVa to inactivation by APC [8], leading to increased thrombin generation, which presumably accounts for the elevated risk of (mainly venous) thrombotic events in FVL carriers [27]. The FVL mutation, which may be viewed as a gain of function mutation, has been suggested to be subject to positive selection pressure because its prevalence has remained stable over generations despite the thrombotic complications it causes [10]. We here investigated whether the FVL mutation influences the procoagulant response during lethal murine influenza A infection. We were unable to demonstrate an altered local or systemic procoagulant response as evidenced by unchanged lung and plasma TATc and FDP levels and unaltered fibrin deposition in mice that were heterozygous or homozygous for the FVL mutation as compared to WT littermates. Evidence indicates that the FVL mutation results in reduced anticoagulant effects of exogenously administered (A)PC: infusion of recombinant human APC in

patients with severe sepsis failed to prolong the aPTT in heterozygous FVL carriers and resulted in a less pronounced decrease in plasma D-dimer levels in heterozygous FVL carriers than in patients not carrying the FVL mutation [13]. Moreover, homozygous FVL mice administered with lipopolysaccharide followed two hours later by human PC demonstrated higher plasma TATc levels than WT mice treated likewise [13]. Our current data, indicating that the FVL mutation does not influence the procoagulant response to severe influenza, is in accordance with previous studies that reported similar baseline plasma concentrations of biomarkers of coagulation activation in patients with severe sepsis with or without the FVL mutation [13] and similar rises in plasma TATc levels in FVL and WT mice injected intraperitoneally with *Escherichia coli* [15].

It has been suggested that the FVL mutation may impact on acute inflammatory responses in the lung [28]. The current study does not support this notion: relative to WT animals, FVL mice showed a modestly (if at all) altered inflammation in their lungs upon infection with influenza A, as reflected by similar lung histopathology scores, a similar influx of neutrophils to the site of infection and largely similar cytokine and chemokine concentrations in lung homogenates. In accordance, the FVL mutation did not influence baseline plasma IL-6 levels in patients with severe sepsis [13] and the extent of systemic cytokine release did not differ in FVL and WT mice during gram-negative sepsis [15].

Pulmonary viral loads were temporarily increased around 4-fold in both heterozygous and homozygous FVL carriers two days after infection. These differences between FVL and WT mice had disappeared four days post infection. The transiently elevated viral loads in FVL mice are surprising considering that this mutation is not known to impact on antiviral mechanisms and moreover did not influence the inflammatory response to influenza A in a way that might have impaired host defense. In preliminary studies we have found that the administration of recombinant mouse APC transiently reduces viral loads in the same influenza model (unpublished

data). More research is warranted to investigate the mechanisms by which FVL and APC impact on influenza A viral infection.

Notably, our data do not fully exclude a role for the FVL mutation in the susceptibility to human influenza infection. The mouse adapted influenza strain (influenza A/PR/8/34) used here has been strain used extensively in mouse models of influenza and has been found valuable for the characterization of the pathogenesis and immunology of influenza virus infections. Nonetheless, caution is warranted to directly extrapolate results to human influenza. We used a lethal infectious dose to mimic severe human influenza pneumonia, which, like our model, is associated with severe lung pathology [29].

## **Conclusions**

In conclusion, we show that in murine lethal influenza A infection coagulation is activated both in the pulmonary and systemic compartment and that the FVL mutation does not impact on influenza induced coagulation activation. Moreover, we show that although viral load was temporarily enhanced in both heterozygous and homozygous FVL carriers as compared to WT mice, there were no consistent differences in the pulmonary and systemic inflammatory response to the infection and, moreover, no differences in survival. Whether activation of coagulation in lethal influenza A infection contributes to disease outcome or merely is an epiphenomenon and whether the outcome of severe influenza A infection can be influenced by interventions that modulate the procoagulant response remains to be established.

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## References

1. Dushoff J, Plotkin JB, Viboud C, Earn DJ, Simonsen L. Mortality due to influenza in the United States--an annualized regression approach using multiple-cause mortality data. *Am J Epidemiol* 2006; 163: 181-187.
2. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivaller P, Smagala J, De Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD Jr, Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI, Cox NJ. Antigenic and Genetic Characteristics of Swine-Origin 2009 A(H1N1) Influenza Viruses Circulating in Humans. *Science* 2009; 325: 197-201
3. Blanquer J, Blanquer R, Borrás R, Nauffal D, Morales P, Menendez R, Subias I, Herrero L, Redon J, Pascual J. Aetiology of community acquired pneumonia in Valencia, Spain: a multicentre prospective study. *Thorax* 1991; 46: 508-511.
4. Lauderdale TL, Chang FY, Ben RJ, Yin HC, Ni YH, Tsai JW, Cheng SH, Wang JT, Liu YC, Cheng YW, Chen ST, Fung CP, Chuang YC, Cheng HP, Lu DC, Liu CJ, Huang IW, Hung CL, Hsiao CF, Ho M. Etiology of community acquired pneumonia among adult patients requiring hospitalization in Taiwan. *Respir Med* 2005; 99: 1079-1086.
5. Smeeth L, Thomas SL, Hall AJ, Hubbard R, Farrington P, Vallance P. Risk of myocardial infarction and stroke after acute infection or vaccination. *N Engl J Med* 2004; 351: 2611-2618.
6. Schouten M, Wiersinga WJ, Levi M, Van der Poll T. Inflammation, endothelium, and coagulation in sepsis. *J Leukoc Biol* 2008; 83: 536-545.



7. Keller TT, Van der Sluijs KF, De Kruif MD, Gerdes VEA, Meijers JCM, Florquin S, Van der Poll T, Van Gorp ECM, Brandjes DPM, Büller HR, Levi M. Effects on coagulation and fibrinolysis induced by influenza in mice with a reduced capacity to generate activated protein C and a deficiency in plasminogen activator inhibitor type 1. *Circ Res* 2006; 99: 1261-1269.
8. Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, Deronde H, Vandervelden PA, Reitsma PH. Mutation in Blood-Coagulation Factor-V Associated with Resistance to Activated Protein-C. *Nature* 1994; 369: 64-67.
9. Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (activated protein C resistance). *Blood* 1995; 85: 1504-1508.
10. Majerus PW. Human-Genetics - Bad Blood by Mutation. *Nature* 1994; 369: 14-15.
11. Gopel W, Gortner L, Kohlmann T, Schultz C, Moller J. Low prevalence of large intraventricular haemorrhage in very low birthweight infants carrying the factor V Leiden or prothrombin G20210A mutation. *Acta Paediatr* 2001; 90: 1021-1024.
12. Gopel W, Ludwig M, Junge AK, Kohlmann T, Diedrich K, Moller J. Selection pressure for the factor-V-Leiden mutation and embryo implantation. *Lancet* 2001; 358: 1238-1239.
13. Kerlin BA, Yan SB, Isermann BH, Brandt JT, Sood R, Basson BR, Joyce DE, Weiler H, Dhainaut JF. Survival advantage associated with heterozygous factor V Leiden mutation in patients with severe sepsis and in mouse endotoxemia. *Blood* 2003; 102: 3085-3092.
14. Hofstra JJ, Schouten M, Levi M. Thrombophilia and outcome in severe infection and sepsis. *Semin Thromb Hemost* 2007; 33: 604-609.
15. Brüggemann LW, Schoenmakers SH, Groot AP, Reitsma PH, Spek CA. Role of the factor V Leiden mutation in septic peritonitis assessed in factor V Leiden transgenic mice. *Crit Care Med* 2006; 34: 2201-6.
16. Sun H, Wang X, Degen JL, Ginsburg D. Reduced thrombin generation increases host susceptibility to group A streptococcal infection. *Blood* 2008; 113: 1358-64

17. Cui J, Eitzman DT, Westrick RJ, Christie PD, Xu ZJ, Yang AY, Purkayastha AA, Yang TL, Metz AL, Gallagher KP, Tyson JA, Rosenberg RD, Ginsburg D. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. *Blood* 2000; 96: 4222-4226.
18. Van der Sluijs KF, Van Elden LJ, Arens R, Nijhuis M, Schuurman R, Florquin S, Kwakkel J, Akira S, Jansen HM, Lutter R, Van der Poll T. Enhanced viral clearance in interleukin-18 gene-deficient mice after pulmonary infection with influenza A virus. *Immunology* 2005; 114: 112-120.
19. Elms MJ, Bundesen PG, Rowbury D, Goodall S, Wakeham N, Rowell JA, Hillyard CJ, Rylatt DB. Automated-Determination of Cross-Linked Fibrin Derivatives in Plasma. *Blood Coagul Fibrinolysis* 1993; 4: 159-164.
20. Rijneveld AW, Levi M, Florquin S, Speelman P, Carmeliet P, Van der Poll T. Urokinase receptor is necessary for adequate host defense against pneumococcal pneumonia. *J Immunol* 2002; 168: 3507-3511.
21. Knapp S, Wieland CW, Florquin S, Pantophlet R, Dijkshoorn L, Tshimbalanga N, Akira S, Van der Poll, T. Differential roles of CD14 and Toll-like receptors 4 and 2 in murine *Acinetobacter pneumonia*. *Am J Respir Crit Care Med* 2006; 173: 122-129.
22. Rijneveld AW, Weijer S, Florquin S, Esmon CT, Meijers JC, Speelman P, Reitsma PH, Ten Cate H, Van der Poll T. Thrombomodulin mutant mice with a strongly reduced capacity to generate activated protein C have an unaltered pulmonary immune response to respiratory pathogens and lipopolysaccharide. *Blood* 2004; 103: 1702-1709.
23. Weijer S, Schoenmakers SH, Florquin S, Levi M, Vlasuk GP, Rote WE, Reitsma PH, Spek CA, Van der Poll T. Inhibition of the tissue factor/factor VIIa pathway does not influence the inflammatory or antibacterial response to abdominal sepsis induced by *Escherichia coli* in mice. *J Infect Dis* 2004; 189: 2308-2317.
24. Van Elden LJ, Nijhuis M, Schipper P, Schuurman R, Van Loon AM. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. *J Clin Microbiol* 2001; 39: 196-200.

25. Beigel JH. Influenza. *Crit Care Med* 2008; 36: 2660-2666.
26. Watanabe T, Yoshikawa H, Abe Y, Yamazaki S, Uehara Y, Abe T. Renal involvement in children with influenza A virus infection. *Pediatr Nephrol* 2003; 18: 541-544.
27. Koster T, Rosendaal FR, Deronde H, Briët E, Vandenbroucke JP, Bertina RM. Venous Thrombosis Due to Poor Anticoagulant Response to Activated Protein-C - Leiden Thrombophilia Study. *Lancet* 1993; 342: 1503-1506.
28. Russell JA. Genetics of coagulation factors in acute lung injury. *Crit Care Med* 2003; 31: S243-S247.
29. Taubenberger JK, Morens DM. The pathology of influenza virus infections. *Annu Rev Pathol* 2008; 3: 499-522.

**Table 1. Pulmonary myeloperoxidase, cytokine and chemokine levels 48 and 96 hours after induction of lethal influenza A infection.**

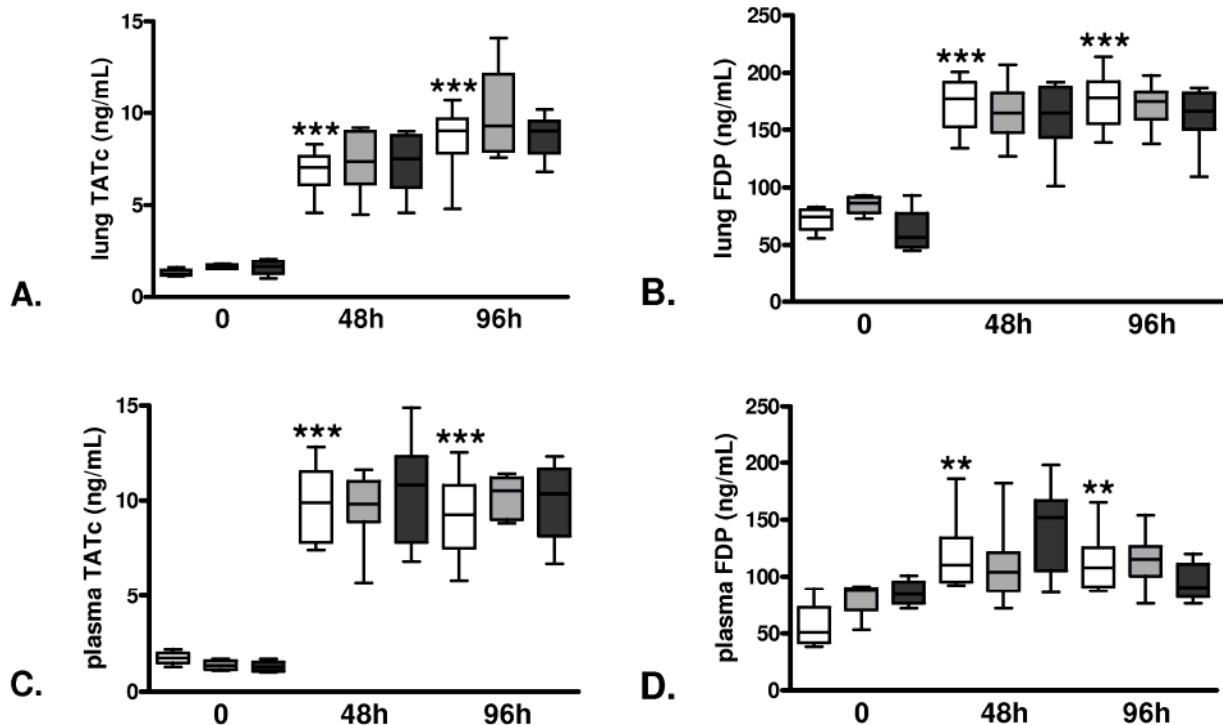
	48 hours			96 hours		
	WT n = 8	heterozygous FVL n = 8	homozygous FVL n = 8	WT n = 8	heterozygous FVL n = 8	homozygous FVL n = 8
MPO (ng/mL)	9.3 (6.7-10)	8.0 (7.1-8.8)	9.1 (7.1-9.8)	14 (12-17)	14 (12-17)	14 (12-15)
TNF- $\alpha$ (pg/mL)	317 (196-423)	183 (171-250)	121 (127-262) *	344 (190-433)	420 (376-577)	503 (352-770)
IL-6 (ng/mL)	2.6 (1.7-6.1)	1.5 (1.2-1.9)	1.9 (1.1-2.2)	1.6 (0.8-2.3)	3.2 (1.7-4.8)	2.0 (1.7-5.3)
IL-12 (pg/mL)	B.D.	B.D.	B.D.	5.0 (5.0-12)	10 (5.0-35)	8.8 (5.0-33)
MCP-1 (ng/mL)	8.9 (5.8-10)	5.4 (5.2-6.4)	5.8 (5.3-7.5)	10 (5.8-10)	10 (10-10)	10 (9.1-10)
IL-10 (pg/mL)	749 (500-1058)	281 (185-335) **	424 (130-882)	244 (210-426)	343 (187-1401)	534 (360-577)
IFN- $\gamma$ (pg/mL)	46 (29-79)	44 (34-50)	24 (14-45)	7.6 (12-17)	31 (16-56) *	14 (9.5-32)
KC (pg/mL)	941 (622-1297)	753 (522-906)	673 (615-1025)	1090 (770-1334)	941 (759-1256)	675 (559-892)

Data are medians (interquartile ranges). \* and \*\* indicate statistical significance as compared to wild type (WT) (P<0.05 and P<0.01 respectively). FVL = Factor V Leiden, MPO = myeloperoxidase. TNF- $\alpha$  = tumor necrosis factor- $\alpha$ , IL = interleukin, MCP-1 = monocyte chemotactic protein, IFN- $\gamma$  = interferon- $\gamma$ , KC = keratinocyte-derived chemokine. B.D. = below detection.

## Figure legends

**Figure 1. Activation of coagulation in lethal influenza A infection.** Levels of **A.C.** thrombin-antithrombin complexes (TATc) and **B.D.** fibrin degradation products (FDP) in **A.B.** lung and **C.D.** plasma at baseline and 48 and 96 hours after induction of lethal influenza A infection in wild type mice (white) and mice heterozygous (light grey) or homozygous (dark grey) for the factor V Leiden mutation. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. \*\* and \*\*\* indicate statistical significance as compared to baseline ( $P < 0.01$  and  $P < 0.001$  respectively, Mann-Whitney U test).

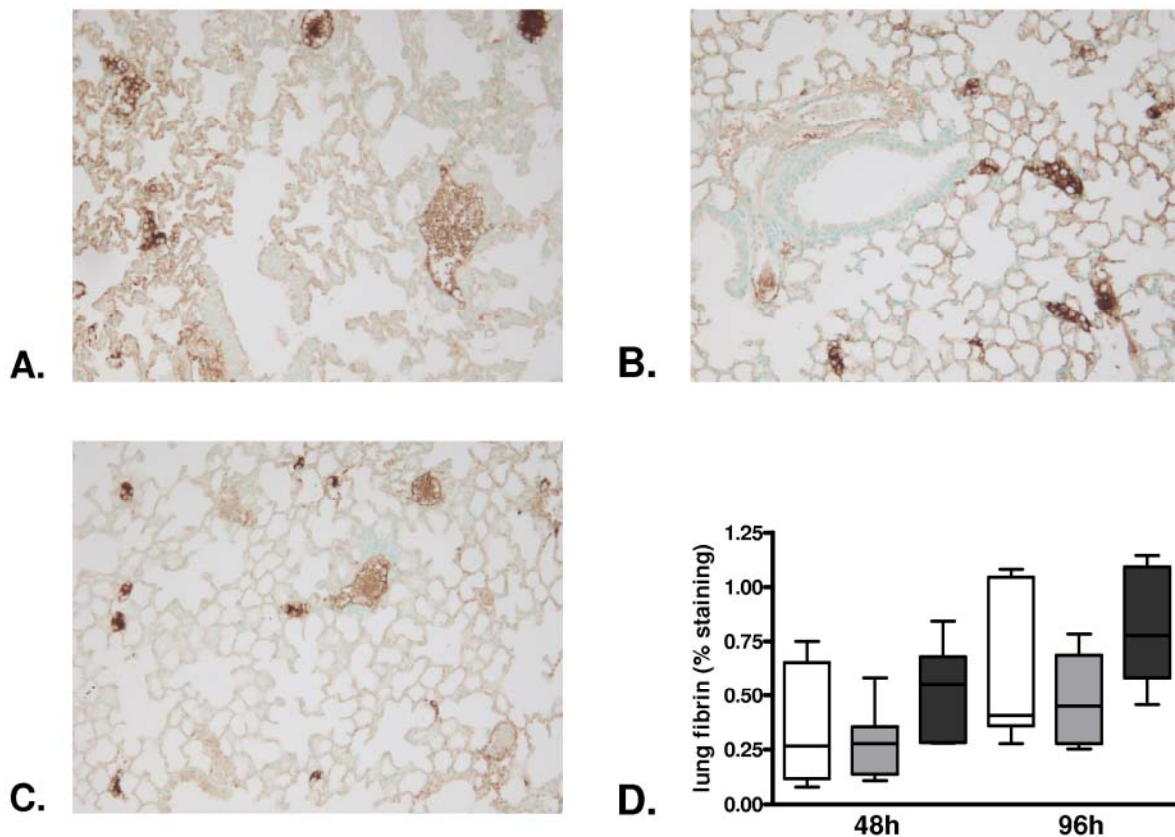
**Figure 1.**



**Figure 2. Pulmonary fibrin deposition in lethal influenza A infection.** Representative slides of lung fibrin staining (brown) 96 hours after induction of lethal influenza A infection in **A.** wild-type

mice, **B.** mice heterozygous and **C.** mice homozygous for the factor V Leiden mutation (original magnification x 100). **D.** Quantitation of pulmonary fibrin 48 and 96 hours after induction of lethal influenza A infection in wild type mice (white) and mice heterozygous (light grey) or homozygous (dark grey) for the factor V Leiden mutation. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. There were no statistical differences between the groups at either time point.

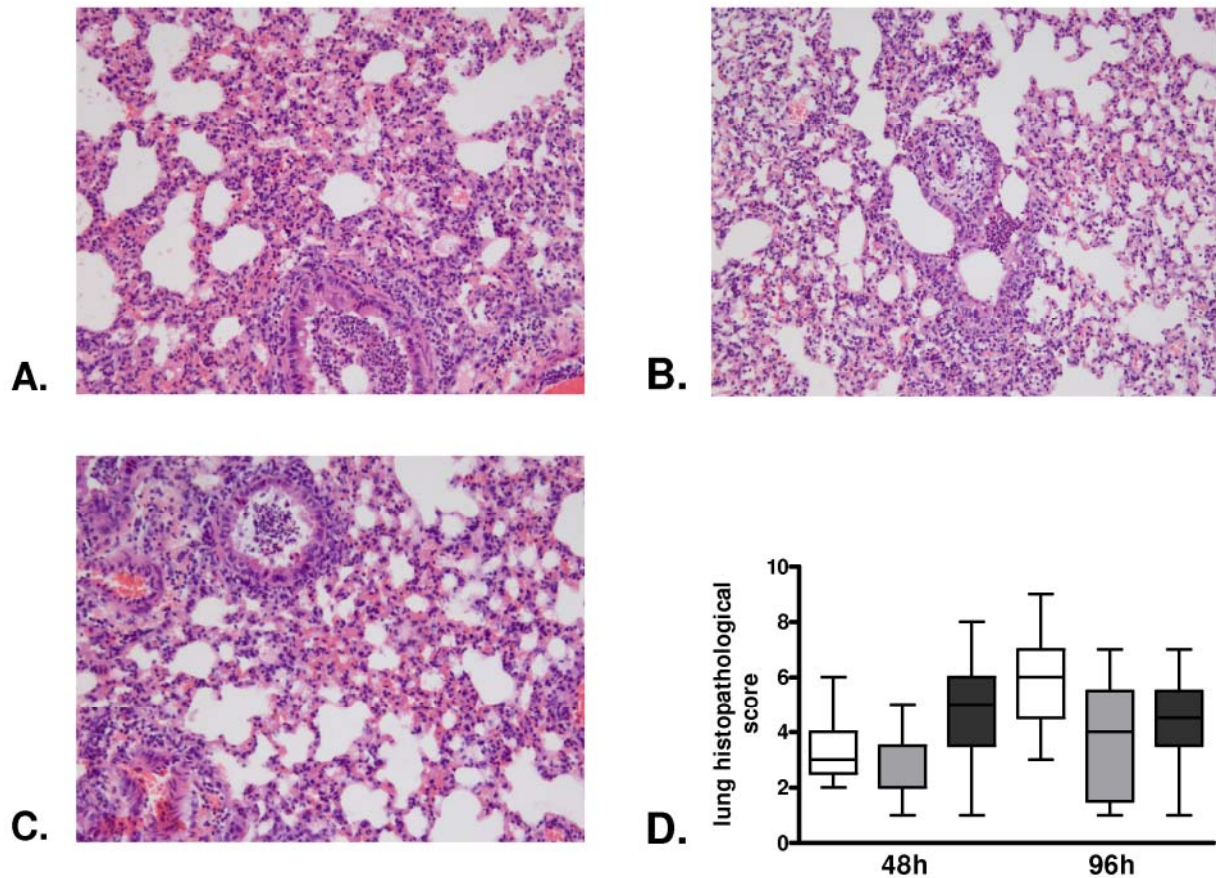
**Figure 2.**



**Figure 3. Lung histopathology in lethal influenza A infection.** Lung haematoxylin and eosin staining 96 hours after induction of lethal influenza A infection in **A.** wild-type mice, **B.** mice heterozygous and **C.** mice homozygous for the factor V Leiden mutation (original magnification x 100). **D.** Total lung pathology score 48 and 96 hours after induction of lethal influenza A infection in wild type mice (white) and mice heterozygous (light grey) or homozygous (dark grey) for the

factor V leiden mutation. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. There were no statistical differences between the groups at either time point.

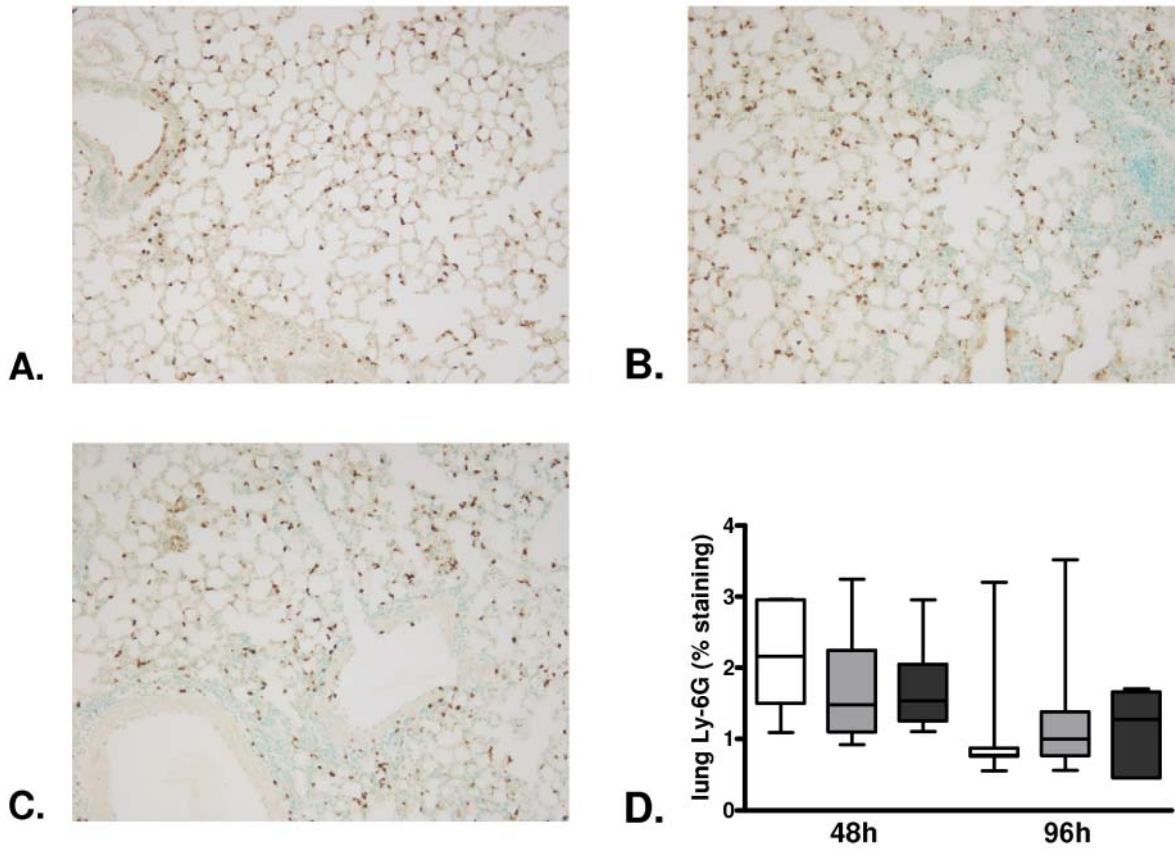
**Figure 3.**



**Figure 4. Pulmonary neutrophil influx in lethal influenza A infection.** Representative slides of lung Ly-6G staining (brown) 96 hours after induction of lethal influenza A infection in **A.** wild-type mice, **B.** mice heterozygous and **C.** mice homozygous for the factor V Leiden mutation (original magnification x 100). **D.** Quantitation of pulmonary Ly-6G 48 and 96 hours after induction of lethal influenza A infection in wild type mice (white), and mice heterozygous (light grey) or homozygous (dark grey) for the factor V leiden mutation. Data are expressed as box-and-

whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. There were no statistical differences between the groups at either time point.

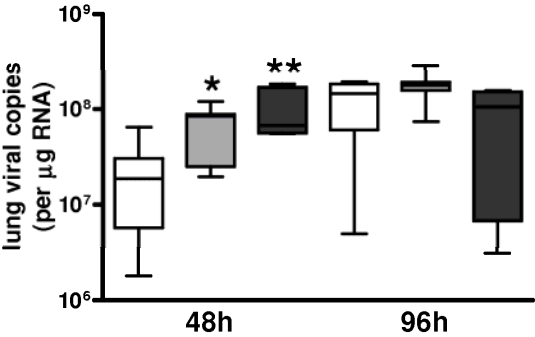
**Figure 4.**



**Figure 5. Pulmonary viral load in lethal influenza A infection.** Lung viral copies 48 and 96 hours after induction of lethal influenza A infection in wild type mice (white) and mice heterozygous (light grey) or homozygous (dark grey) for the factor V Leiden mutation. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation. \* and \*\* indicate statistical significance as compared to wild type mice ( $P < 0.05$  and  $P < 0.01$  respectively, Mann-Whitney U test).



Figure 5.



**Figure 6. Survival in lethal influenza A infection.** Survival of wild type mice (open squares) and mice heterozygous (light grey squares) or homozygous (dark grey squares) for the factor V Leiden mutation in lethal influenza A infection. There were no statistical differences between the groups (P=0.23, log rank test).

Figure 6.

