**Supplementary figures and tables:**

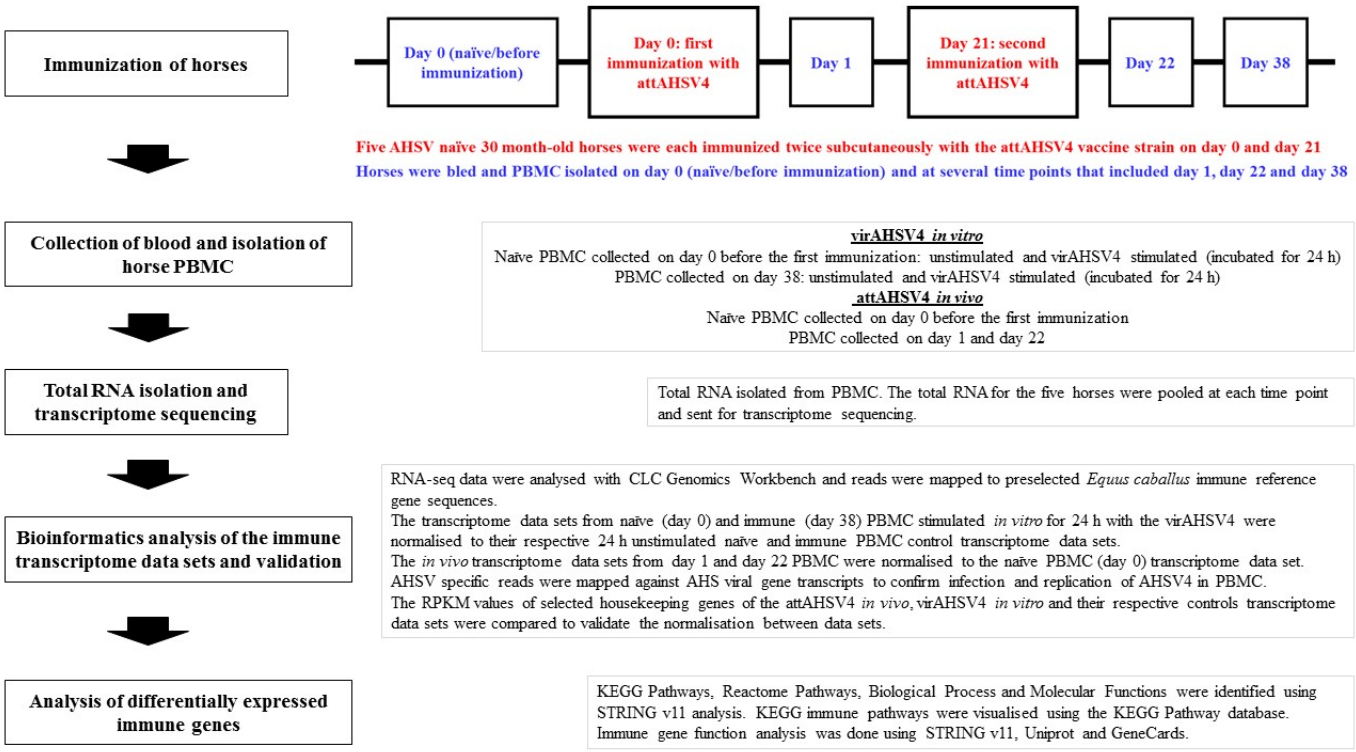


**Fig. S1**. Overview of the experimental design of this study. Flow chart illustrates the immunization of horses, collection of blood and isolation of horse peripheral blood mononuclear cells (PBMC), total RNA isolation and transcriptome sequencing, bioinformatics analysis of the immune transcriptome data sets, validation and the analysis of differentially expressed immune genes.

**Table S1.** The normalized fold change values of some of the major pro-apoptotic genes that were significantly (normalized fold change values ≥ ±1,2 and *P*-values ≤ 0,05) up-regulated (black, positive) and down-regulated (red, negative) during the virulent AHSV4 (vAHSV4) and the attenuated AHSV4 (aAHSV4) primary (prim) and secondary (sec) immune responses. Genes not differentially expressed nor significantly up-regulated or down-regulated are indicated with (-). The gene functions are shown in table.

| Genes | vAHSV4 | | aAHSV4 | | Pro-apoptotic gene functions (www.string-db.org/, www.uniprot.org/ and www.genecards.org/) |
|---|---|---|---|---|---|
| | prim | sec | prim | sec | |
| BIM | 1,6 | 2,8 | 1,5 | 1,2 | Cooperates with pro-apoptotic activator proteins to induce MOMP |
| ERO1A | 1,5 | 3,2 | 1,3 | 1,4 | Involved in ER stress-induced and CHOP-dependent apoptosis by activating the IP3Rs |
| TRAIL | 1,5 | 1,6 | 1,4 | 1,3 | Induces the extrinsic apoptosis pathway upon binding TRAILR |
| APAF1 | 1,6 | 2,3 | 1,3 | 1,2 | Binds to CYCS in cytoplasm to form the apoptosome and activate CASP9 |
| CASP10 | 1,4 | 9,1 | 1,2 | 1,4 | Apoptotic initiator. Activates the apoptotic effectors/executioners |
| ITPR1 | 1,3 | 2,9 | 1,6 | 1,5 | Mediates calcium release from the ER. Involved in ER stress-induced apoptosis |
| ITPR2 | 1,3 | 4,1 | 1,7 | 1,8 | Mediates intracellular calcium release |
| TRAILR | - | 3,9 | 2,4 | 2,1 | Receptor for TNFSF10/TRAIL |
| PML | - | 1,9 | 1,4 | 1,6 | Promotes apoptosis through several pathways |
| TP73 | - | 9,6 | 2,1 | 1,8 | Involved in apoptosis in response to DNA damage |
| ZMAT3 | - | 5,1 | 3,5 | 4,8 | TP53 target gene that contributes to TP53-mediated apoptosis |
| SHISA5 | -1,3 | 1,2 | 2,1 | 1,9 | Induces caspase-dependent apoptosis and plays a role in TP53-dependent apoptosis |
| CASP2 | -1,2 | 9,2 | 1,8 | 1,9 | Functions in stress-induced cell death pathways. Activates pro-apoptotic proteins and inactivates pro-survival proteins |
| FOS | -1,4 | 1,9 | 2,2 | 1,2 | Expression has been associated with apoptosis |
| FOXO3 | -1,2 | 10,6 | 1,5 | 1,6 | Transcription activator that induces apoptosis when survival factors are absent |
| IRE1α[a] | - | 11,3 | 1,7 | 1,8 | Activates the UPR |
| GCN2[a] | - | 1,3 | 1,2 | 1,3 | Activates the ISR |
| ITPR3 | - | 2,2 | 1,3 | 1,6 | Mediates intracellular calcium release |
| PRF1 | - | -1,3 | 1,6 | 1,3 | Forms pores in the plasma membranes of target cells |
| GZMB | -1,3 | -1,5 | 1,5 | 1,6 | Induction of caspase-dependent apoptosis or cleaves caspase substrates directly |
| BAD | - | -4,2 | 1,9 | 1,8 | Binds to and reverses the repressor effects of anti-apoptotic BCL2 and BCL-XL |
| PUMA | - | - | 2,7 | 2,5 | Cooperates with pro-apoptotic activator proteins to induce MOMP |
| ARTS | - | -1,6 | 1,3 | 1,6 | Mitochondrial protein released into the cytoplasm. Promotes apoptosis |
| CASP9 | - | -1,2 | 1,4 | 1,3 | Apoptotic initiator. Activates the apoptotic effectors/executioners |
| MCU | -1,2 | -1,2 | 1,6 | 1,5 | Mediates calcium uptake into mitochondria |
| PERK[a] | 1,3 | 1,6 | - | - | Activates the UPR and the ISR |
| PKR[a] | 1,9 | 6,3 | - | - | Activates the ISR |
| TP53 | - | - | - | 1,3 | Induces cell cycle arrest, senescence, DNA repair or apoptosis |
| BAK1 | - | - | - | 1,2 | Induces MOMP |
| HRK | - | 4,5 | -1,6 | -1,4 | Promotes apoptosis by binding to anti-apoptotic BCL2 and BCL-XL |
| NOXA | -1,4 | 2,2 | - | - | Promotes efflux of pro-apoptotic proteins from the mitochondria |
| FASL | - | 2,7 | - | - | Induces the extrinsic apoptosis pathway upon binding FAS |
| FADD | - | 1,5 | - | - | Apoptotic adaptor molecule that recruits CASP8 and CASP10 |
| CASP6 | - | 2,1 | - | - | Apoptotic effector/executioner. Cleaves specific intracellular protein substrates |
| CASP7 | - | 2,2 | - | - | Apoptotic effector/executioner. Cleaves specific intracellular protein substrates |
| CASP8 | - | 1,4 | - | -1,2 | Apoptotic initiator. Activates the apoptotic effectors/executioners |

| | | | | | |
|---|---|---|---|---|---|
| FAS | - | 1,6 | - | 1,2 | Receptor for TNFSF6/FASL |
| CASP3 | - | 1,3 | - | 1,2 | Apoptotic effector/executioner. Cleaves specific intracellular protein substrates |

[a] The initial phases of both the unfolded protein response (UPR) and the integrated stress response (ISR) are pro-survival to restore cellular homeostasis. However, the UPR and the ISR switch to pro-apoptotic signaling during unresolvable stress conditions.

**Table S2**. The normalized fold change values of the significant up-regulated (black, positive) and down-regulated (red, negative) MHC class I genes during the virulent AHSV4 (vAHSV4) and the attenuated AHSV4 (aAHSV4) primary (prim) and secondary (sec) immune responses. Genes not differentially expressed nor significantly up-regulated or down-regulated are indicated with (-).

| **MHC class I genes** | **vAHSV4** | | **aAHSV4** | |
|---|---|---|---|---|
| | **prim** | **sec** | **prim** | **sec** |
| MHCBI (ecb:100034209) | - | -1,6 | - | 1,2 |
| HLA-B-15 alpha (ecb:100053764) | -1,2 | -1,5 | 1,2 | 1,3 |
| EQMHCB2 (Eqca-1) (ecb:100034210) | - | -1,5 | - | - |
| EQMHCC1 (ecb:100034211) | - | -1,3 | - | - |
| MHCB3 (ecb:100034212) | -1,2 | -1,4 | - | - |
| MHCI-Gogo-OKO alpha (ecb:100049798) | - | -1,4 | - | 1,2 |
| MHCI-saoe alpha (ecb:100050706) | - | -1,3 | -1,3 | 1,2 |
| Non-classical MHC class I (ecb:100051657) | - | -1,6 | 1,2 | 1,3 |
| MHCI-patr-A-2 (ecb:100054114) | -1,3 | - | - | - |
| MHCI-saoe alpha (ecb:100054397) | - | -1,8 | - | 1,2 |
| MHCI-saoe alpha (ecb:100054448) | - | - | -1,3 | 1,2 |
| Eqca-2 (ecb:100056062) | - | -1,7 | - | - |
| EQMCE1 (ecb:100050550) | - | -1,3 | - | - |
| MHCI-Gogo-B*0103 alpha (ecb:100050473) | - | -1,5 | - | - |
| HLA-B-14 alpha (ecb:100051586) | - | -1,6 | - | 1,2 |
| HLA-B-15 alpha (ecb:106782285) | -1,2 | -1,8 | - | 1,2 |
| HLA-B-15 alpha (ecb:100057688) | - | -1,3 | 2,2 | 2,3 |
| HLA-Cw-12 (ecb:100057920) | - | -1,2 | -1,2 | - |
| HLA-B-46 alpha (ecb:100053918) | - | -1,4 | 1,3 | 1,5 |
| HLA-B-40 alpha (ecb:100056182) | - | - | - | - |
| MHCX1 (ecb:100056391) | -1,2 | -1,6 | - | - |

**Table S3**. The total significant up-regulated (black, positive) and down-regulated (red, negative) differentially expressed genes during the attenuated AHSV4 (aAHSV4) primary and secondary immune responses associated with the CD8+ T cell response (Biological Process and Molecular Function). Genes not differentially expressed nor significantly up-regulated or down-regulated in the pathways are indicated with (-). The false discovery rates (FDR) are shown in table.

| STRING v11 analysis | aAHSV4 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Primary immune response | | | | Secondary immune response | | | |
| | Up | FDR | Down | FDR | Up | FDR | Down | FDR |
| **Biological process (total genes in pathway)** | | | | | | | | |
| Antigen processing and presentation of peptide antigen via MHC class I (43) | 13 | 2,09E-05 | - | | 14 | 4,35E-06 | -5 | 1,14E-02 |
| Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent (25) | 7 | 3,70E-03 | -4 | 4,30E-03 | 9 | 1,80E-04 | - | |
| CD8-positive, alpha-beta T cell differentiation (8) | 3 | 4,17E-02 | - | | 3 | 4,01E-02 | - | |
| Positive regulation of CD8-positive, alpha-beta T cell activation (7) | 3 | 3,34E-02 | - | | 3 | 3,17E-02 | - | |
| **Molecular Function (total genes in pathway)** | | | | | | | | |
| TAP binding (7) | 4 | 9,20E-03 | - | | 4 | 8,80E-03 | - | |
| MHC class I protein binding (20) | 5 | 3,14E-02 | - | | 6 | 8,50E-03 | - | |

**Table S4**. The total significant up-regulated (black, positive) and down-regulated (red, negative) differentially expressed genes during the virulent AHSV4 (vAHSV4) primary and secondary immune responses associated with the CD8+ T cell response (Biological Process and Molecular Function). Genes not differentially expressed nor significantly up-regulated or down-regulated in the pathways are indicated with (-). The false discovery rates (FDR) are shown in table.

| STRING v11 analysis | vAHSV4 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Primary immune response | | | | Secondary immune response | | | |
| | Up | FDR | Down | FDR | Up | FDR | Down | FDR |
| **Biological process (total genes in pathway)** | | | | | | | | |
| Antigen processing and presentation of peptide antigen via MHC class I (43) | 8 | 6,22E-05 | -8 | 4,86E-05 | 14 | 8,49E-07 | -12 | 2,20E-07 |
| Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent (25) | 6 | 2,20E-04 | -5 | 1,60E-03 | 7 | 1,60E-03 | -8 | 1,78E-05 |
| CD8-positive, alpha-beta T cell differentiation (8) | - | | - | | - | | - | |
| Positive regulation of CD8-positive, alpha-beta T cell activation (7) | - | | - | | - | | - | |
| **Molecular Function (total genes in pathway)** | | | | | | | | |
| TAP binding (7) | - | | -3 | 9,70E-03 | - | | -5 | 1,40E-04 |
| MHC class I protein binding (20) | - | | - | | - | | -6 | 6,50E-04 |

**Table S5**. The normalized fold change values of some of the main anti-apoptotic and pro-survival genes that were significantly up-regulated (black, positive) and down-regulated (red, negative) during the virulent AHSV4 (vAHSV4) and the attenuated AHSV4 (aAHSV4) primary (prim) and secondary (sec) immune responses. Genes not differentially expressed nor significantly up-regulated or down-regulated are indicated with (-). The gene functions are shown in table.

| Genes | vAHSV4 | | aAHSV4 | | Anti-apoptotic/pro-survival gene functions (www.string-db.org/, www.uniprot.org/ and www.genecards.org/) |
|---|---|---|---|---|---|
| | prim | sec | prim | sec | |
| XIAP | 1,5 | 6,1 | 1,2 | 1,4 | Inhibits the activities of CASP3, CASP7 and CASP9 |
| HIF1A | 1,3 | 1,8 | - | -1,3 | Role in cell survival to maintain homeostasis |
| NAIP | 1,3 | 2,4 | - | -1,2 | Inhibits the activities of CASP3, CASP7 and CASP9 |
| NRF2 | 1,2 | 1,3 | -1,2 | -1,4 | Transcription activator that binds to antioxidant response elements |
| BCL-XL[a] | - | 1,2 | - | 3,2 | Inhibits caspase activation. Regulates VDAC to reduce CYCS and ROS release from the mitochondria. Also anti-inflammatory by interacting with NLRP1 that blocks CASP1 activation, thus preventing cleavage and release of IL-1β |
| BCL2[a] | -1,2 | - | 1,6 | 2,4 | Inhibits caspase activation by preventing CYCS release from the mitochondria. Also anti-inflammatory by interacting with NLRP1 that blocks CASP1 activation, thus preventing cleavage and release of IL-1β |
| BCL2A1 | 1,2 | -1,4 | -1,8 | -1,6 | Inhibits caspase activation by preventing CYCS release from the mitochondria |
| BIRC3 | 1,4 | -1,2 | -1,4 | -1,8 | Suppresses apoptosis by ubiquitinating CASP8 and RIPK1 |
| CSF1 | 2,1 | - | -1,2 | - | Promotes the survival, proliferation and differentiation of mononuclear phagocytes |
| DNAJA1 | 1,4 | -1,8 | -1,4 | -1,7 | Negatively regulates the translocation of BAX from cytoplasm to mitochondria during cell stress |
| FTMT | 1,4 | -1,6 | -2,3 | -2,1 | Stores iron in a non-toxic form |
| RFWD2 | 1,2 | -1,2 | -1,2 | -1,3 | Inhibits TP53-dependent apoptosis |
| SOD2 | 1,5 | -1,5 | -1,3 | -1,2 | Destroys and clears mitochondrial ROS |

[a] Anti-apoptotic or anti-inflammatory functions.

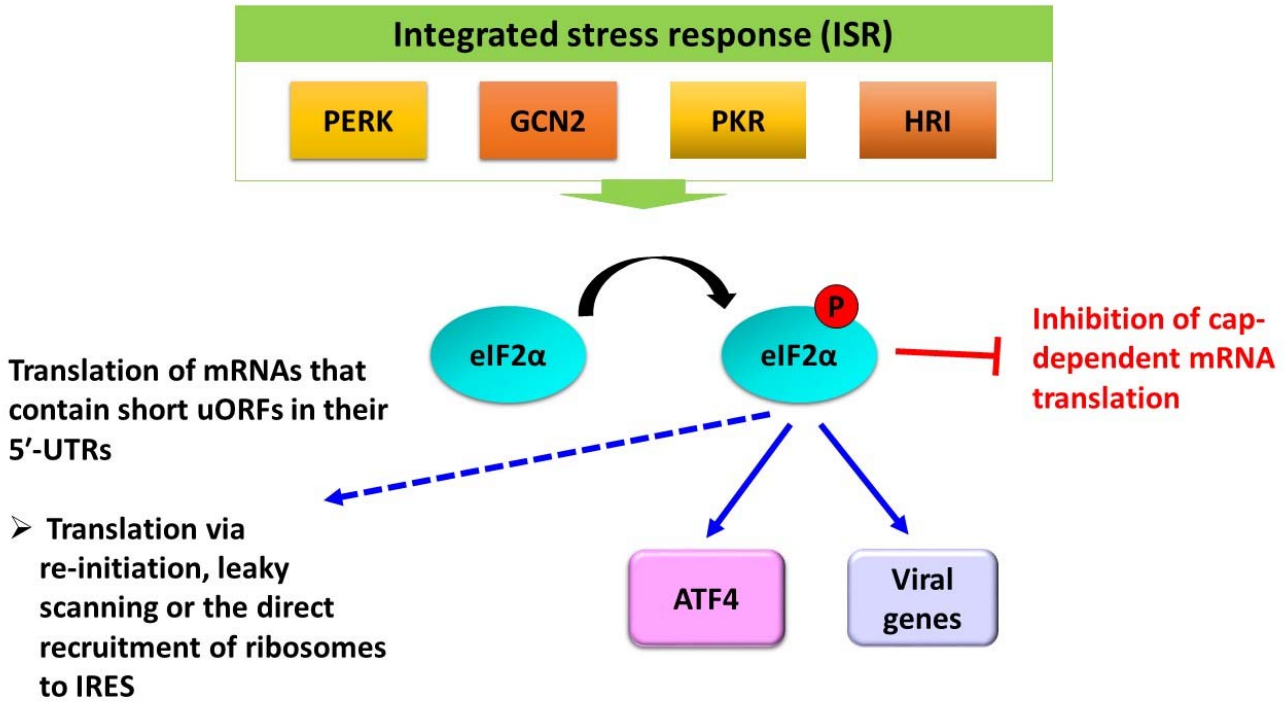


**Fig. S2**. The phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α) at serine 51 by PERK, HRI, PKR and/or GCN2 that leads to a decrease in global mRNA translation and the translation of selected genes (e.g. ATF4) is known as the integrated stress response (ISR) (Grootjans et al., 2016; Pakos-Zebrucka et al., 2016). PERK is activated during endoplasmic reticulum (ER) stress, HRI during heme deprivation, PKR is activated by dsRNA and GCN2 during amino acid starvation (Grootjans et al., 2016; Pakos-Zebrucka et al., 2016; Gong et al., 2017). PERK, HRI, PKR and GCN2 are also activated in response to oxidative stress (Pakos-Zebrucka et al., 2016). The phosphorylation of eIF2α blocks the formation of the ternary complex, which results in the inhibition of global mRNA translation (Grootjans et al., 2016; Pakos-Zebrucka et al., 2016; Gong et al., 2017), specifically cap-dependent mRNA translation (Pakos-Zebrucka et al., 2016) and the preferential translation of selected genes (e.g. ATF4). Inhibition of global mRNA translation reduces the influx of proteins entering the ER (Dufey et al., 2014; Grootjans et al., 2016; Pakos-Zebrucka et al., 2016; Gong et al., 2017). The mRNAs that are preferentially translated during stress conditions when the availability of the ternary complex is limiting contain short upstream open reading frames (uORFs) in their 5'-untranslated regions (5'-UTRs) (Dufey et al., 2014; Pakos-Zebrucka et al., 2016). These mRNAs are typically translated by leaky scanning, re-initiation (Pakos-Zebrucka et al., 2016) or the direct recruitment of ribosomes to mRNAs that have an internal ribosome entry site (IRES) (Pakos-Zebrucka et al., 2016; Gong et al., 2017). Similar to selected host mRNAs (e.g. ATF4) that need the limited availability of the ternary complex to be translated (Dufey et al., 2014; Pakos-Zebrucka et al., 2016), certain viral mRNAs require these stress conditions to be translated. Ebola virus proteins (VP35, VP30 and VP24) are translated by leaky scanning during the ISR that was either induced by PKR or ER stress (Basler, 2015) and the viral mRNAs of picornaviruses and hepatitis C virus are dependent on IRES-mediated translation (García-Sastre, 2017). Similarly, it is likely during the activation of the ISR and the subsequent translation of selected host genes, that certain African horse sickness virus (AHSV) genes that include NS3A and NS4 are translated. The dsRNA segments of the orbivirus genomes are mostly monocistronic, where each of the separate genome segments encode a single protein from one ORF (Belhouchet et al., 2011; Ahasan et al., 2019). Whereas some of the individual genome segments encode two proteins from overlapping ORFs, including genome segment 9 (Seg-9) and segment 10 (Seg-10) of AHSV (Ferreira-Venter et al., 2019) and bluetongue virus (BTV) (Belhouchet et al., 2011; Ferreira-Venter et al., 2019). AHSV NS3 and NS3A are encoded from overlapping ORFs on Seg-10 (Huismans et al., 2004; Ferreira-Venter et al., 2019). AHSV NS4 and VP6 are encoded from overlapping ORFs on

Seg-9 (Zwart et al., 2015). The overlapping genes of the orbiviruses appear to be translated via leaky scanning (Firth, 2008; Ahasan et al., 2019). The locations of AHSV NS3A (Huismans et al., 2004) and AHSV NS4 (Firth, 2008; Zwart et al., 2015) on their respective genes indicate that they might be translated by leaky scanning, similar as BTV NS4 (Firth, 2008; Ratinier et al., 2016). Additionally, an A-rich polypurine sequence, which functions as an IRES, was identified upstream of NS4 in almost all of the orbiviruses that were analysed, including AHSV (Firth, 2008). Demonstrating the possibility that AHSV NS4 requires IRES-mediated translation.

**References**


Ahasan MS, Subramaniam K, Campos Krauer JM, Sayler KA, Loeb JC, Goodfriend OF, Barber HM, Stephenson CJ, Popov VL, Charrel RN, Wisely SM, Waltzek TB, Lednicky JA. Three New orbivirus Species Isolated from Farmed White-Tailed Deer (Odocoileus virginianus) in the United States. Viruses. 2019;12(1):13. doi: 10.3390/v12010013.

Basler CF. Innate immune evasion by filoviruses. Virology. 2015;479-480:122-30. doi: 10.1016/j.virol.2015.03.030. Review.

Belhouchet M, Mohd Jaafar F, Firth AE, Grimes JM, Mertens PP, Attoui H. Detection of a fourth orbivirus non-structural protein. PLoS One. 2011;6(10):e25697. doi: 10.1371/journal.pone.0025697.

Dufey E, Sepúlveda D, Rojas-Rivera D, Hetz C. Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. 1. An overview. Am J Physiol Cell Physiol. 2014;307(7):C582-94. doi: 10.1152/ajpcell.00258.2014. Review.

Ferreira-Venter L, Venter E, Theron J, van Staden V. Targeted mutational analysis to unravel the complexity of African horse sickness virus NS3 function in mammalian cells. Virology. 2019;531:149-161. doi: 10.1016/j.virol.2019.03.005.

Firth AE. Bioinformatic analysis suggests that the orbivirus VP6 cistron encodes an overlapping gene. Virol J. 2008;5:48. doi: 10.1186/1743-422X-5-48.

García-Sastre A. Ten strategies of interferon evasion by viruses. Cell Host Microbe. 2017;22(2):176-184. doi: 10.1016/j.chom.2017.07.012. Review.

Gong J, Wang XZ, Wang T, Chen JJ, Xie XY, Hu H, Yu F, Liu HL, Jiang XY, Fan HD. Molecular signal networks and regulating mechanisms of the unfolded protein response. J Zhejiang Univ Sci B. 2017;18(1):1-14. doi: 10.1631/jzus.B1600043. Review.

Grootjans J, Kaser A, Kaufman RJ, Blumberg RS. The unfolded protein response in immunity and inflammation. Nat Rev Immunol. 2016;16(8):469-84. doi: 10.1038/nri.2016.62. Review.

Huismans H, van Staden V, Fick WC, van Niekerk M, Meiring TL. A comparison of different orbivirus proteins that could affect virulence and pathogenesis. Vet Ital. 2004;40(4):417-25.

Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. The integrated stress response. EMBO Rep. 2016;17(10):1374-1395. Review.

Ratinier M, Shaw AE, Barry G, Gu Q, Di Gialleonardo L, Janowicz A, Varela M, Randall RE, Caporale M, Palmarini M. Bluetongue Virus NS4 Protein Is an Interferon Antagonist and a Determinant of Virus Virulence. J Virol. 2016;90:5427-39. doi: 10.1128/JVI.00422-16.

Zwart L, Potgieter CA, Clift SJ, van Staden V. Characterising Non-Structural Protein NS4 of African Horse Sickness Virus. PLoS One. 2015;10:e0124281. doi: 10.1371/journal.pone.0124281.