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First reported case of fragile foal syndrome type 1 in the Thoroughbred caused by *PLOD1* c.2032G>A

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Summary

Background: Warmblood Fragile Foal Syndrome type 1 (WFFS) is an autosomal recessive disorder reported previously only in warmbloods and thought to be caused by a variant in the gene *procollagen-lysine,2-oxoglutarate 5-dioxygenase 1* (*PLOD1, c.2032G>A, p.Gly678Arg*). Given the presentation of this Thoroughbred case, we hypothesised that a similar genetic mechanism caused this phenotype.

Objectives: To describe the pathological and genetic findings on a foal presenting to a veterinary practice in the UK with skin lesions similar to other Ehlers-Danlos Syndromes, including those documented for warmbloods with WFFS.

Study design: A single case report describing a genetic investigation.

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Authorship

The methodology for genetic investigation was designed by R.R. Bellone and A.M. de Mestre. A.F. Foote performed the post-mortem examination. J.M. Roach extracted DNA and A.F. Foote, A.M. de Mestre and J.M. Roach contributed to the preparation of figures. Genetic data collection and analysis were performed by A.S. Grillos, R.R. Bellone, N.B. Kingsley, and M.J. Mienaltowski. A.S. Grillos and R.R. Bellone drafted the manuscript. All authors contributed to the revision and final version of the article.

Authors' declarations of interest

A.S. Grillos, N.B. Kingsley, and R.R. Bellone are affiliated with the UC Davis Veterinary Genetics Laboratory, which provides genetic diagnostic tests in horses and other species.

Ethical animal research

Ethical approval was granted by the Royal Veterinary College's Clinical Research Ethical Review Board (URN 2017 1660-3).

Informed consent

Informed consent was obtained for the use of tissue and access to clinical records in this study.

Methods: A Thoroughbred foal presenting as a dystocia was euthanised for multiple skin lesions and developmental abnormalities. DNA extracted from the foal was tested for the *PLOD1* variant (*c.2032G>A, p.Gly678Arg)* using the commercially available assay. To confirm causality and further interrogate potential novel causes of Ehlers-Danlos Syndrome, 1799 functional candidate genes, including *PLOD1*, were analysed using whole genome sequencing data generated from DNA extracted from the foal's muscle. These data were compared to 34 control samples from at least 11 other breeds. Variants were prioritised for further evaluation based on predicted impact on protein function.

Results: Post-mortem evaluation concluded that this foal suffered from a condition of collagen dysplasia. The foal was homozygous for the *c.2032G>A PLOD1* variant. Only two other missense variants identified from whole genome sequencing data were also computationally predicted to be deleterious to protein function, (*NPHP3 c.1253T>C, p.Leu418Pro*, *EPDR1 c.154G>C, p.Glu52Gln*). Neither of these genes have been linked to similar phenotypes, or Ehlers-Danlos Syndrome in humans or other species and thus further investigation of these variants as the cause of EDS was not warranted.

Main limitations: This study is a single case report in the Thoroughbred with no additional cases from this breed yet identified to replicate this finding.

Conclusions: Given the clinical presentation similar to WFFS, homozygosity for the *PLOD1* variant, and absence of another more plausible causal variant from the WGS experiment, we conclude that *PLOD1 c.2032G>A* is the likely cause of this foal's condition. This is the first documented evidence of fragile foal syndrome caused by the *PLOD1* variant in a breed outside of warmbloods, the Thoroughbred. We therefore recommend a change in the name of this disorder to fragile foal syndrome type 1 (FFS) and utilisation of genetic testing in Thoroughbreds to avoid producing affected foals.

Keywords

horse; Warmblood Fragile Foal Syndrome Type 1 (WFFS); Fragile Foal Syndrome Type I (FFS) genetics; Thoroughbred; *PLOD1*

Introduction

Warmblood Fragile Foal Syndrome type 1 (WFFS) was previously characterised as an autosomal recessive condition thought to be caused by a variant in the *procollagen-lysine, 2-oxoglutarate 5-dioxygenase1* gene (*PLOD1 c.2032G>A, p.Gly678Arg*) [1]. Foals born with this condition have collagen dysplasia and typically present with an open abdomen and extensive cutaneous lesions, very thin skin, abnormal flexibility of the distal joints, flexed carpal and tarsal joints, deformed spinal cord, pulmonary fetal lung dystelectasis, and intracranial haemorrhage [1,2]. Histologically, keratosis-like thickening of the skin in some areas and empty hair follicles have also been reported [1,2]. This condition has only been reported, thus far, in warmblood horses, with the initial discovery based on a single candidate gene approach utilising only two clinical cases and reported in a patent application [3]. To date, only eighteen cases have been described in the literature despite the high carrier frequency; as high as 17%, that has been reported in several warmblood breeds [1-5].

A similar condition in humans exists and is known as Ehlers-Danlos Syndrome (EDS). EDS is a heterogenous group of connective tissue disorders with mutations in 20 genes being identified as causal [6]. Skin hyperextensibility, joint hypermobility, and widened atrophic scars are the most common reported symptoms of this syndrome in humans [7]. There have been 13 subtypes of EDS described, in which all include the basic clinical findings of joint hypermobility, skin hyperextensibility, and tissue fragility. One of the genes known to be involved in human EDS is *PLOD1*, and it is responsible specifically for Kyphoscoliotic EDS. *PLOD1* functions as a post-translational modifying enzyme in collagen biosynthesis [8]. In humans, over 30 different mutations in *PLOD1* have been reported, of which skin abnormalities such as hyperextensibility and fragility are almost universally described [9].

Another EDS-like syndrome known in the horse is hereditary equine regional dermal asthenia (HERDA). HERDA is also a collagen disorder that has been identified in the Quarter Horse, and is characterised by seromas, haematomas, ulcerations along the dorsum, and hyperextensible skin [10]. Unlike WFFS, clinical signs of HERDA are usually not present at birth, besides occasionally a loose mane or increased joint flexibility [10].

Previous research by members of this research team, identified a low allele frequency of the *PLOD1* variant in adult Thoroughbreds (1.2%) [11]. However, of the 716 adult Thoroughbreds tested for the variant, none were homozygous, suggesting that homozygosity for the *PLOD1* variant may result in a similar fragile foal phenotype in the Thoroughbred population [11]. Most recently, investigating the distribution of the *PLOD1* variant in over 4000 horses, it was determined that the *PLOD1* variant was found to be present in 21 breeds - most of which were warmbloods with the exception of low allele frequency in the Thoroughbred (1.19%), Haflinger (2.08%), American Sport Pony (4.17%), and Knabstrupper (3.26%) [5].

A Thoroughbred foal presenting to a veterinary practice in the UK was identified as having an EDS clinical presentation similar to the condition reported for warmbloods. Given the presence of this allele outside of warmblood breeds, particularly in the Thoroughbred, and the similarity in phenotype of this case, we hypothesised that this foal's condition was caused by homozygosity for the *PLOD1* variant (*c.2032G>A)*. However, since the initial discovery of the *PLOD1* variant in association with WFFS was based on a single candidate gene sequencing approach, and the fact that very few WFFS affected foals have been reported in the literature, we also decided to investigate if this was the causal allele or if other novel variants in collagen synthesis genes could be the cause of an EDS in the Thoroughbred horse.

Materials and Methods

Case Presentation

A 5-year-old maiden Thoroughbred mare was presented to an attending veterinary surgeon in the UK at gestation day 278 days with premature mammary development. Monitoring of sequential blood samples over the subsequent 4 weeks revealed a premature rise in serum progesterone levels. Transrectal ultrasonography noted an unusual multi-compartmentalised cystic structure located subcutaneously on the fetal neck, which measured more than 10 cm long with an increase in size noted during sequential ultrasonographic examinations (Figure

1). There was no apparent placental separation at delivery. The mare foaled spontaneously at 309 days of gestation. The foal presented anteriorly in a ventral (dorso-pubic) position, and the mare was immediately transported and admitted to a veterinary hospital where she was anaesthetised. A live foal was then delivered by a controlled vaginal delivery. During extraction, the foal's skin was fragile and tore very easily from the forelimbs. A large haematoma was noted on the foal's neck, and a spinal scoliosis was present such that it was agreed to euthanise the foal immediately following delivery (Figure 2).

Pathologic and Histologic Examination

A full postmortem examination of the foal and placental tissues was performed specialist in equine pathology. Gross lesions were recorded and photographed. Skin directly associated with gross lesions as well as skin with a normal gross appearance were sampled and fixed in 10% neutral buffered formalin. Tissue was paraffin-embedded and 4 μm tissue sections stained with haematoxylin and eosin.

Genetic Testing and Whole Genome Sequencing

Genomic DNA was obtained from gluteal muscle tissue collected from the foal, using a Qiagen DNeasy Blood and Tissue Kit (Qiagen). The quality and quantity of the genomic DNA was assessed using spectrophotometry (DeNovix Inc.). Isolated DNA was genotyped for the two known EDS-like syndromes in the horse namely WFFS (*PLOD1 c.2032G>A*) and HERDA (*PPIB* c115G>A) using the commercially available assays routinely performed at the UC Davis Veterinary Genetics Laboratory, and appropriate positive and negative controls were run with each assay.

Finally, to rule out a novel cause of an EDS syndrome in this Thoroughbred, genes involved in collagen synthesis, extracellular matrix, or skeletal development were evaluated for potential deleterious variants. Whole genome sequencing was performed using DNA isolated from fetal muscle on the Illumina NovaSeq platform (Illumina) with 2 × 150 bp paired end reads and an average insert size of approximately 400 base pairs. The sequencing was carried out at the DNA Technologies and Expression Analysis Core at the UC Davis Genome Center, supported by NIH Shared Instrumentation Grant 1S10OD010786-01. Sequencing data were processed utilising the HTStream pipeline [12] and were aligned to the reference assembly, EquCab3.0 using Burrows-Wheeler Aligner (BWA) [13]. Variants were called utilising the variant callers FreeBayes [14] and Samtools [15], and annotated with SnpEff [16]. To identify a list of candidate genes for further investigation, known protein-coding genes with function in collagen synthesis, extracellular matrix, or skeletal development across tissues were identified by combining tab-delimited table files from NCBI Entrez Gene Ensembl’s annotation for the EquCab 3.0 genome using “vlookup” command in Microsoft Excel. Genes were selected via queries for GO term names and GO term name definitions with the following contents in order of priority: collagen, extracellular matrix, glycosaminoglycan, basement membrane, and skeletal. Entries for GO term names and GO term name definitions were combined for each queried term and redundant entries were removed such that the entries with highest priority remained and lower priority redundancies were removed, producing the master list of genes that was queried for potential deleterious variants that would explain this case phenotype. In total, 1799 out of 21,129 protein-coding

genes were identified as candidates for further investigation by this approach. Variants in these candidate genes were prioritised for further evaluation by first filtering for those variants homozygous for the alternate allele in the case sample, that were either homozygous reference or heterozygous in 34 controls from 11 other breeds (the breed designation for one sample was unknown), whose data was generated for other projects in the UC Davis Veterinary Genetics Laboratory. Variants annotated by SNPeff as having a moderate effect on protein function were further considered. These variants were then evaluated using PredictSNP, a consensus classifier that predicts the functional consequence using the combined score from six different prediction algorithms [17].

Prioritised variants were validated by Sanger sequencing in both the case sample and from DNA isolated from the horse used to establish the reference genome. Primers were designed using the Primer3 design tool [18]. A DNA fragment including the SNP site was amplified using a polymerase chain reaction (PCR) protocol with 10 ng DNA, 2 μl of PCR buffer with MgCl2, 1 mmol/L dNTPs, 0.8 μl of 5 mmol/L forward and reverse primers and 0.2 μl FastStart Taq DNA Polymerase (Roche Applied Science). For the *EPDR1* variant, 4 μl of GC-Rich Buffer (Roche Applied Science) was additionally added, as the region of interest was rich in guanine and cytosine nucleotides.

Prior to Sanger sequencing, the PCR products were visualised on a 1% ethidium bromide (EtBr) agarose gel to confirm product size. Amplification products were purified with the EdgeBio Quickstep 2 PCR purification kit (EdgeBio) and sequenced using BigDye Terminator v1.1 and the ABI 3730 Genetic Analyzer (Applied Biosystems Inc at ThermoFisher Scientific) Sequencing data were analysed using Sequencher v5.4 (Gene Codes).

Results

Clinical and Pathological Findings

Postmortem examination of the foal revealed a 15 cm diameter partially open intradermal skin defect filled with fibrinous material and lined by organising granulation tissue in the dorsal cranial neck. In the left caudal inguinal region, there was a 4-5 cm diameter raised mass adjacent to the penile urethra, identified as a haematoma (Figure 2). Over the right carpus and proximal metacarpus, the skin was separated at the level of the deep dermis/subcutaneous fascia, with minimal haemorrhage. Overlying the right medial elbow, linear splits of the skin were present, and another small tear of the skin was present over the left tuber coxa. Overall, there were multiple areas of the cutaneous wounds and defects separating at the deep dermis/subcutaneous level, strongly suggestive of collagen dysplasia (Figure 2).

The maxillary and frontal bones were slightly deviated right, and the caudal thoracic vertebrae were deviated and convex on the left side consistent with scoliosis. Both distal hindlimbs had laxity and were hyperextensible. Both carpal joints were mildly contracted with inability to fully extend. There were no abnormalities detected in the appendicular joints, except for mild haemarthrosis in the elbow and shoulder joints.

There were no abnormalities present in the alimentary system, with mild liver congestion. The lungs were partially aerated and in the cardiovascular system, there were localised subendocardial haemorrhages in both the left and right ventricles. The mitral valve leaflets had marginal thickening of the edge of the valve cusps. All organs associated with the urogenital, endocrine, lymphatic, and nervous systems were normal. The placenta was submitted separately and appeared normal, with only a region of congestion at the tip of the gravid horn. The placental tissues did not tear easily, and the umbilical cord was also grossly unremarkable.

Histological Findings

Sections of the haematoma in the neck (Figure 3A) confirmed the central deposits of fibrin (asterisk) surrounded by a zone of organising granulation tissue, with areas of ongoing acute haemorrhage and mild infiltrates of haemosiderophages (not shown). Sections of skin from adjacent unaffected regions of the neck and other regions revealed relatively normal cutaneous architecture, without significantly discernible depletion of collagen fibres at the light microscopic level and only marginal fibre disarray/wavy fibres and clefting in the deep dermis (arrows, Figure 3B).

Genetic Testing

The affected foal was homozygous for the *PLOD1* variant (*c.2032G>A, p.Gly678Arg)*, known to cause WFFS. Additionally, the foal was homozygous for the normal allele at the *PPIB* locus (N/N) and thus did not have the variant that causes HERDA, the only other known mutation causing an EDS like skin condition in horses.

To exclude other variants in functional candidate genes, including genes in collagen synthesis, extracellular matrix, or skeletal development that could cause an EDS condition in Thoroughbreds, whole genome sequencing data from 1799 candidates were genes was analysed. Across the 35 samples evaluated, average depth of coverage was 23 reads per sample. Under a recessive model, a total of 5,810 variants were identified in our analysis (Table S1). None of these variants were predicted to have a high impact on protein function, according to the SNPEff annotation. However, 39 variants were identified as having a moderate impact on protein function and these were investigated further (Table S2). These included one in-frame duplication and 38 missense variants. PredictSNP analysis of the missense variants determined that 89.7% of these missense variants (35 variants) were neutral to protein function with accuracy estimates between 60%-83% (Table S2). Three of these missense variants were predicted to be deleterious to protein function: the known *PLOD1* variant (*ENSECAT00000024861.2, c.2032G>A, p.Gly678Arg*), as well as a variant in *nephrocystin 3* (*NPHP3*, *ENSECAT00000045634.2, c.1253T>C, p.Leu418Pro*) that was predicted to be deleterious with 87% accuracy, and a variant in *ependymin related 1* (*EPDR1*, *ENSECAT00000019264.2, c.154G>C, p.Glu52Gln*), predicted to be deleterious with 72% accuracy (Table S2). This variant was also heterozygous in 4 out of the 34 controls (1 Paint, 1 Thoroughbred, 1 Appaloosa, 1 Tennessee Walking Horse). An additional trinucleotide duplication was identified in *NHS actin remodeling regulator* (*NHS*, *ENSECAT00000034377.2, c.3953_3955dupGCA, p.Ser1318dup*), which would increase the number of serine residues in this region from eight to nine. It has been noted that this

gene contains a naturally high serine residue content [19]. This same region of the identified *NHS* mutation was compared across 16 different species using Clustal Omega analysis, the number of serine residues in this region were found to be variable among the species examined, ranging from 2-11 (Figure 4).

Sanger sequencing of the *NPHP3 (c.1253T>C)*, *EPDR1 (c.154G>C)*, and *NHS (c.3953_3955dupGCA)* variants confirmed homozygosity of the alternate allele for the three variants evaluated in the affected foal under investigation. Further, the reference horse genotyped heterozygous for the *EPDR1* variant (*c.154G>C),* and genotyped homozygous for the reference allele for the two other variants evaluated, as reported in EquCab3.0.

Discussion

The foal in this case was confirmed homozygous for the *PLOD1* variant (*c.2032G>A p.Gly678Arg)* both by commercially available genotyping and by whole genome sequencing analysis. Given that this variant has previously been associated with WFFS type 1 in warmblood horses [1-3] and no other variants in the remaining 1798 candidate genes investigated explain an EDS, the *PLOD1* variant is strongly supported as the cause for this condition. The phenotypic presentation of this case closely aligned with gross and histological findings previously reported in the literature for affected warmblood foals [1-3]. Furthermore, in humans this same *PLOD1* variant *(p.Gly678Arg)* has been shown to cause extensible, fragile skin and joint laxity at birth, further supporting this as the causal variant [9]. Given the high incidence reported in some warmblood breeds [5] and the low frequency with which WFFS cases have been reported (only 19 cases including this one), homozygosity for this *PLOD1* variant (*c.2032G>A*) may more frequently result in the death of the foal during gestation than previously documented (4/18 cases in the literature) [2]. Investigating embryonic loss in connection with homozygosity for the PLOD1 variant is needed.

Similarly, clinical observations of the pregnancy prior to delivery of a foal with WFFS has not, to our knowledge, previously been reported. This case presented a month prior to delivery with premature mammary development, a premature rise in progesterone in the absence of any obvious placental separation and identification of an abnormal structure on the fetal neck via transrectal ultrasonography. Whilst another cause for these clinical changes cannot be ruled out, in the absence of infection and placental separation, it is plausible they were a consequence of WFFS and further research is warranted.

Evaluating 1799 candidate genes for coding variants as potential alternative causes of an EDS identified three genes, in addition to *PLOD*1, for further consideration *(NHS, NPHP3, EPDR1).* An in-frame duplication in the *NHS* gene was identified *(c.3953_3955dupGCA, p.Ser1318dup)* but it occurred in a region that is not highly conserved across species (Figure 4). Additionally, mutations in *NHS* are associated with Nance-Horan Syndrome in humans, an X-linked disorder in which affected male patients develop severe cataracts, dental abnormalities, dysmorphic facial features and developmental delay, but no known skin conditions have been reported [20]. Therefore, given the serine content variability across species and difference in phenotype observed in humans with mutations in this gene, it

is doubtful that *c.3953_3955dupGCA, p.Ser1318dup* contributes to EDS-like symptoms in horses.

Two missense variants were also identified to have a deleterious effect on protein function *NPHP3 (c.1253T>C, p.Leu418Pro) and EPDR1 (c.154G>C, p.Glu52Gln*). No mutations in either gene have been linked to disease in the horse. However, recessive mutations in *NPHP3* have been associated with human nephronophthisis, a group of cystic kidney disorders in infants and juveniles that progresses to renal failure and sometimes involves the liver [21-23]. Variants in *EPDR1* have been linked to Dupuytren's disease in humans, a late onset progressive disease causing irreversible fibroblastic proliferation affecting the palmar fascia leading to flexion of the digits and accumulation of fibroblastic nodules filled with mostly type III collagen fibrils [24,25]. Given that this foal presented with no gross abnormalities of the kidneys and that mutations in *EPDR1* in humans involve a late onset disorder, these genes are unlikely to explain EDS-like skin fragility. If, however, these predicted deleterious mutations were implicated in EDS, based on findings in other species, follow-up studies would have been necessary to first determine if population allele frequencies match predicted disease prevalence to further investigate these as causal variants. Along those lines, given that *EPDR1 c.154G>C, p.Glu52Gln* was found in four of the 34 control horses, while a role in EDS is unlikely, investigating population allele frequencies and performing experiments to test for associations with progressive tendon, ligament, or fascial contracture in horses phenotyped for these conditions is warranted.

This is the first confirmed case of an Ehlers-Danlos like Syndrome affected foal in the Thoroughbred caused by the *PLOD1* variant *(c.2032G>A, p.Gly678Arg)*. Considering the previously identified carrier frequency in the adult Thoroughbred population (2.4%) [5], other cases likely exist in the breed and are either not reported or are lost earlier in embryonic development. Appropriate use of genetic testing of breeding stock for the *PLOD1* variant is recommended in the Thoroughbred, as it is for all other breeds in which the variant has been detected. Genetic testing can inform breeding decisions to avoid mating two carriers, which would result in a 25% chance of producing an affected foal. Previous research has ruled out both the Thoroughbred stallion Dark Ronald (1905-1928) [26] and the Arabian stallion Bairactar Or. Ar. [5] as the founder of the *c.2032G>A, p.Gly678Arg* variant. The origin of the mutation remains unknown. However, considering that this mutation is not exclusively identified in warmblood breeds, having been identified in the Thoroughbred, Haflinger, American Sport Pony, Knabstrupper, Paint Horse and Quarter Horse [5,27], and with a clinical case now confirmed in the Thoroughbred, we propose that the disease be renamed Fragile Foal Syndrome type 1 (FFS).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Data availability statement

The data that support the findings of this study are available at the request from the corresponding author. The WGS data for the case are not publicly available due to privacy and ethical consent restrictions. However, data from the controls can be found at ENA under projects PRJEB28306, PRJEB30871, PRJEB36380, PRJEB36381, and PRJEB36403.

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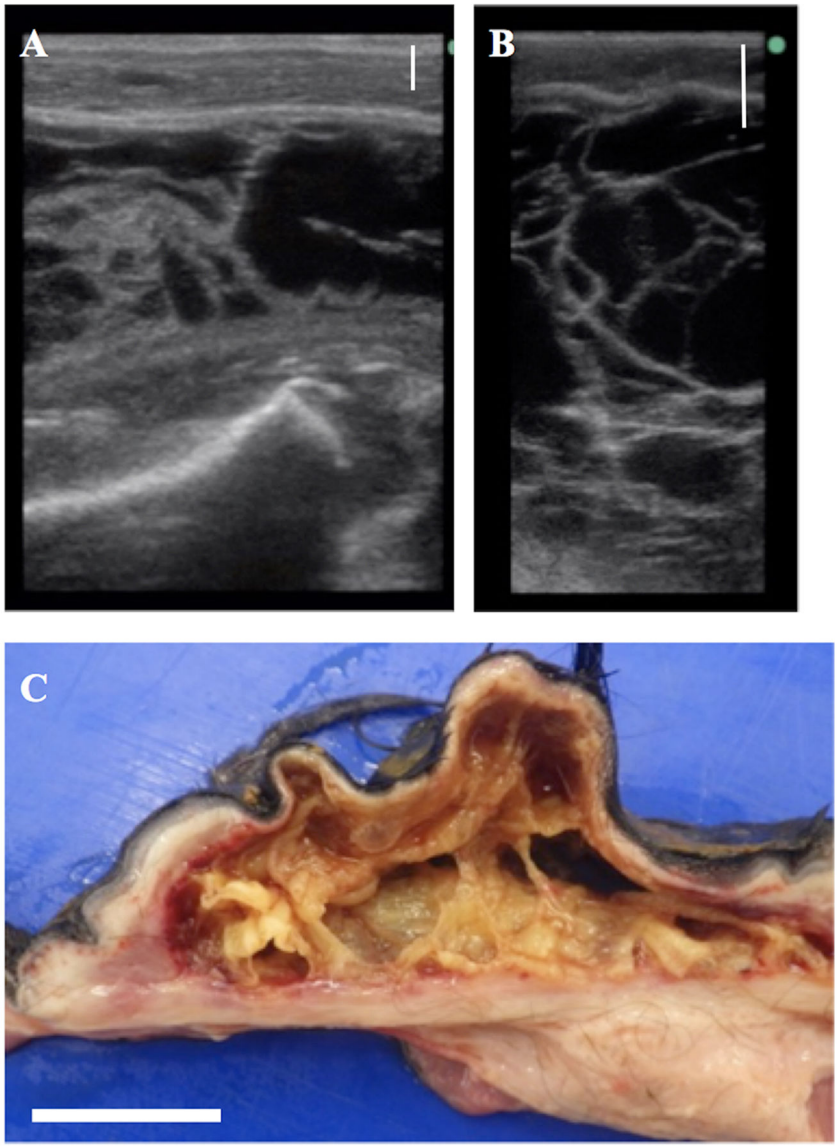

Figure 1:
(A&B) Transrectal ultrasonographic images of a structure located on the dorsal cranial fetal neck of the affected foal. It measured more than 10 cm long, with the images illustrating the compartmentalised cystic appearance. (Scale bar = 1 cm) (C) The structure was confirmed at post-mortem examination to be filled with fibrinous material and an outer wall of granulation tissue (scale bar = 2cm).

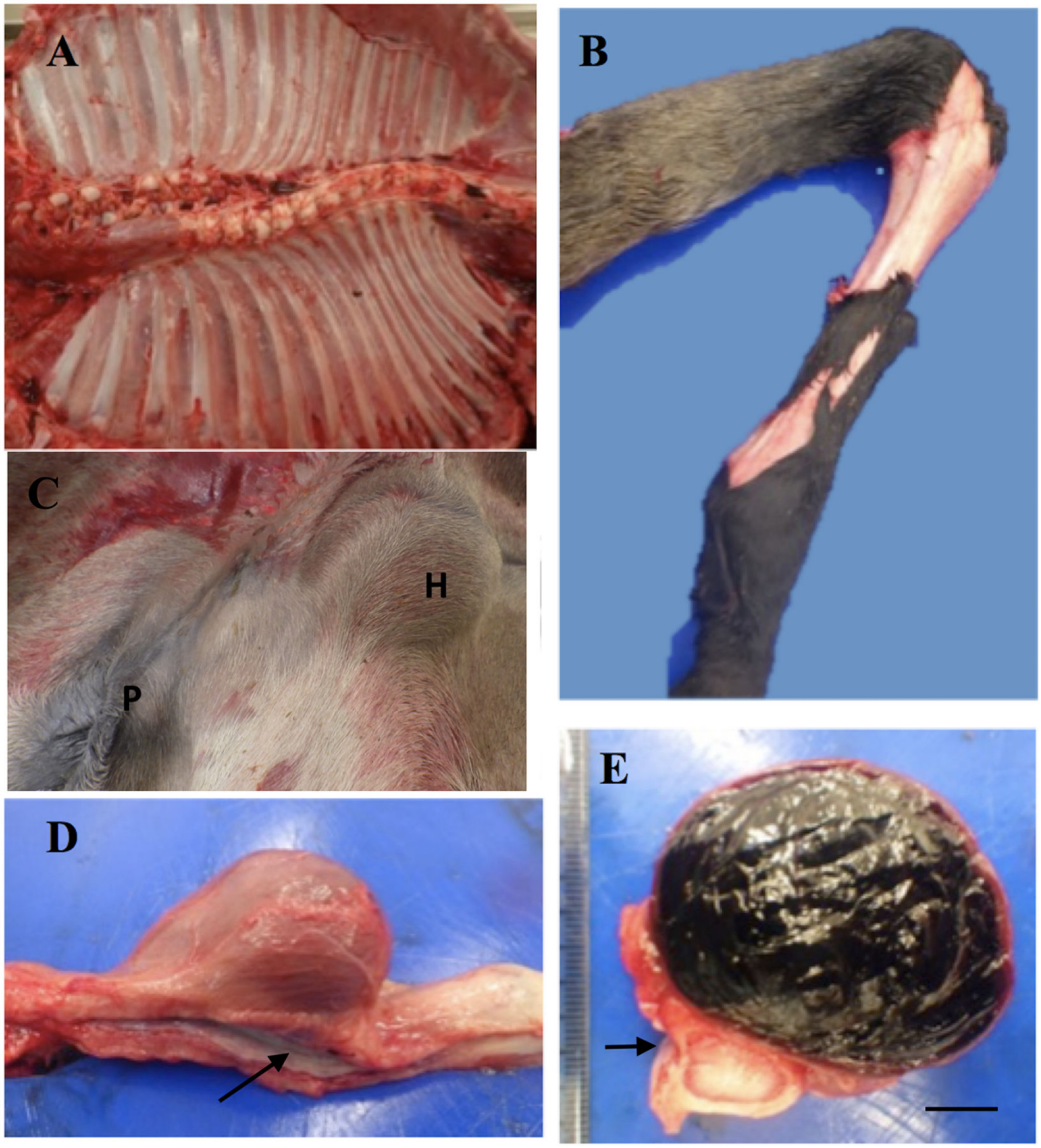

Figure 2:
Gross pathological findings illustrating caudal thoracic scoliosis in the affected foal (A), cutaneous defects with separation of the skin at the level of the deep dermis and superficial fascia (B), a peri-urethral mass in the caudal inguinal region (C-E); prepuce = P, haematoma = H), urethra (incised) is indicated with an arrow (scale bar in E = 1 cm).

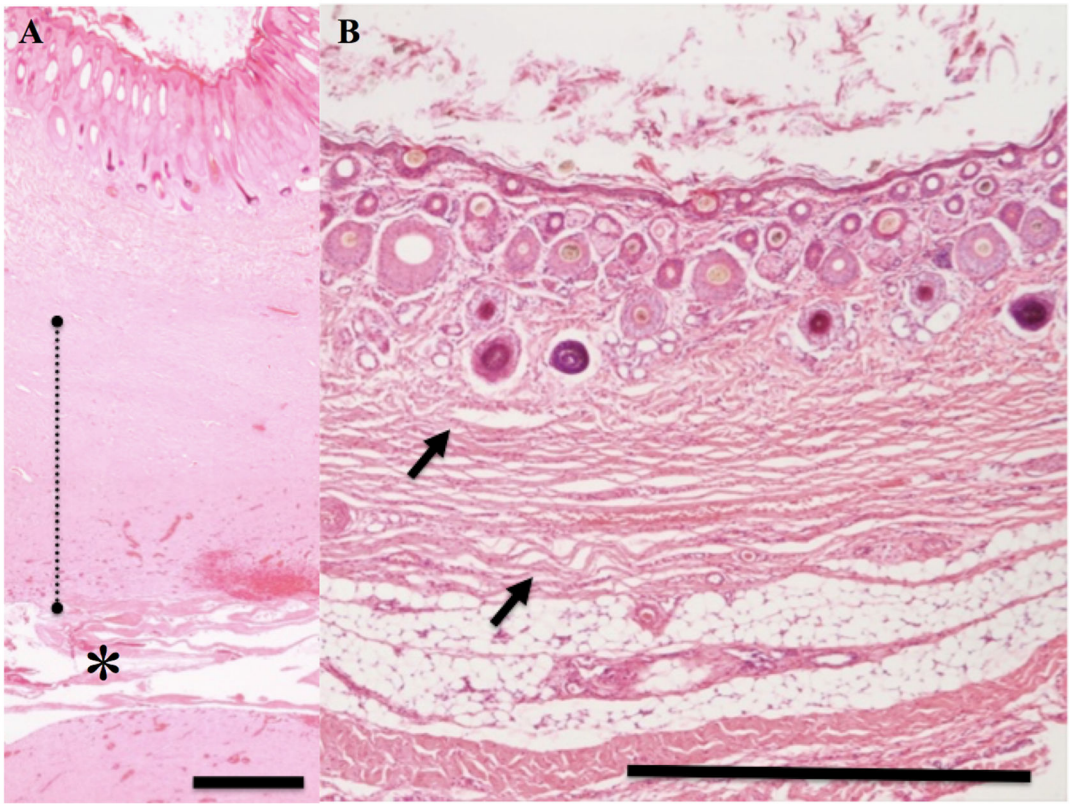

Figure 3:

Histopathology of the skin illustrating the haematoma in the neck of the affected foal (A) and 'non-lesional' skin (B) from an adjacent region of the neck. Central deposits of fibrin (asterisk) within the haematoma expand the deep dermis, surrounded by a zone of organising granulation tissue (dashed line) with increased deposition of collagen, and areas of ongoing acute haemorrhage and mild infiltrates of haemosiderophages (not shown). Sections of skin from adjacent unaffected regions of the neck and other regions revealed relatively normal cutaneous architecture at the light microscopic level, without significantly discernible reduction in density of collagen fibres and only marginal fibre disarray/wavy fibres and very slight clefting in the deep dermis (arrows, Figure 3B) in some areas. H&E staining, scale bars = 1mm.

Figure 4:

Multiple sequence alignment comparing the number of serine residues flanking the prioritised variant in the *NHS* gene (variant) across 16 vertebrate species. Amino Acid numbering for horse is based on Ensemble ID ENSECAG00000020766. The number of serine residues ranged from 2 to 11 across diverse mammalian taxa, suggesting that this duplication likely does not impact NHS gene function.