Hanoverian F/W-line contributes to segregation of Warmblood fragile foal syndrome type 1 variant PLOD1:c.2032G>A in Warmblood horses

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Abstract
Background: Warmblood fragile foal syndrome (WFFS) is a lethal condition detected in Warmblood horses. Its origin and association with performance traits and fertility among horse populations is unknown.

Objectives: To validate the previously identified WFFS type 1 (WFFST1)-associated missense variant PLOD1:c.2032G>A and to investigate its distribution among various horses with particular focus on Hanoverian breed, as well as its pathomorphological picture. The study aimed at identifying the origin of the mutant allele and its correlation with performance and fertility traits in Warmblood horses.

Study design: Retrospective case-control and association study.

Methods: WFFST1 variant was validated using whole genome sequencing (WGS) in 78 equids. In an affected foal with a homozygous mutant genotype, necropsy was performed. Skin samples were examined using histology and transmission electron microscopy. Pathway analysis was performed to trace back 81 genetic carriers to the most common recent ancestor. Furthermore, generalised linear model analysis was employed to test estimated breeding values (EBVs) for differences in performance and fertility traits among different genotypes in Hanoverian horses.

Results: WFFST1 variant had the lowest minor allele frequency among all variants detected in WGS data in the region of PLOD1. Further genotyping of this variant revealed allele frequencies of 0.14 in Hanoverian horses. Histological investigations of the WFFST1-affected foal showed loosely arranged collagen fibres in the dermis. Ultrastructurally, multifocal areas with degraded collagen fibrils and fibrillar plaques were detected. Further pathway analysis revealed a stallion from the Hanoverian sire F/W line as the most common recent ancestor of all tested genetic carriers. Furthermore, WFFST1 variant was found to be correlated with EBVs for gait-related traits as well as conformation and dressage.

Main limitations: Study evaluated carriers and cases only from Europe.
1 | INTRODUCTION

Collagen fibrils in connective tissues provide structure and strength to the skin and thus are essential for the organisation of the entire dermis. Disorders disturbing this type of tissue are known as Ehlers-Danlos syndromes (EDS) in human subjects and are designated as EDS-like diseases in many other species like cattle, sheep, rabbits, minks, cats, dogs as well as horses. In horses, several case reports on inherited connective tissue disorders exist. They report on dermatosparaxia on an EDS-like disease in Oldenburg foals, in a Coldblood and Warmblood horse, dermal asthenia in Warmblood horses, Hereditary Equine Regional Dermal Asthenia (HERDA) in Quarter horses and Warmblood fragile foal syndrome (WFFS) in a Westphalian. In two of these EDS-like forms, HERDA and WFFS, genetic investigations supported a monogenic autosomal mode of inheritance. Analyses of HERDA-affected Quarter horses revealed a missense mutation in peptidylprolyl isomerase B gene to be potentially causative for this disorder mostly found in older horses. In contrast, WFFS was found to be a characteristically early onset form of inherited connective tissue disorders. For WFFS type 1 (WFFST1) a potential causative variant was identified in the gene procollagen-lysine-2-oxoglutarate-5-dioxygenase 1 (PLOD1, c.2032G>A, NC_009145.3:g.39927817C>T). Clinical signs of WFFST1 were shown to include fragile skin, skin and mucosal lacerations, hyperextension of the articulations, subcutaneous emphysema, oedema and haematomas. The predominant manifestation of WFFST1 was reported to be lethal during late gestation or live birth of nonviable foals.

Initial screenings in pedigree data suggested PLOD1:c.2032G>A segregating among Hanoverian, Selle Francais, KWPN, Oldenburg and Westphalian. Subsequent genetic testing proposed an allele frequency of 5.5% in Warmblood horses in Brazil and 1.2% in Thoroughbred. Due to its clinical importance, it was postulated that further investigations on the prevalence of the mutation and on the correlation between genotype and phenotype are required. Thus, the objective of this study was to validate WFFST1 variant, to investigate the prevalence among the Hanoverian and other horse breeds and its association with performance and fertility traits. We aimed to trace the WFFST1-associated allele to the most recent common ancestor (MRCA) and thus the potential origin of WFFST1. Furthermore, the histopathological and electron microscopical presentation of this inherited connective disorder was examined in an affected foal.

Conclusions: This study provides a comprehensive evaluation of WFFST1 variant and traces it back to its potential origin.

KEYWORDS
horse, skin, fragile foal syndrome, PLOD1, Hanoverian

2 | MATERIALS AND METHODS

2.1 Validation of WFFST1 variant

For evaluation of variants within the region of PLOD1, whole genome sequencing (WGS) data of 77 horses of 35 breeds/populations and one donkey were available. All data are accessible at Sequence Read Archive (NCBI). Sequence IDs can be found in Table S1. Fastq-files were mapped and underwent variant calling as previously described using EquCab 3.0 as reference genome. Data were filtered for variants found within PLOD1 or 1 Mb upstream or downstream of PLOD1 (EC2: 38 926 928-40 953 350 bp). We investigated minor allele frequencies (MAF) and linkage disequilibrium among all variants using SAS/Genetics, version 9.4 (Statistical Analysis System). In addition, all variant effects were estimated using SNPEff predictions. Missense variants were further investigated using SIFT scores.

2.2 Genotyping of WFFST1

In total, 1166 EDTA-blood or hair samples from 1059 horses, 76 ponies and 4 donkeys were obtained. DNA was isolated using standard saline extraction and genotyped for the WFFST1 variant (NC_009145.3:g.39927817C>T) using a Competitive Allele Specific PCR (KASP) assay (LGc Genomics) run on an ABI7300 real-time system (Applied Biosystems) (Table S2).

2.3 WFFS case

A stillborn filly, registered as Oldenburg but a descendant of two Hanoverian sire lines in the third generation, with severe lacerations of the skin underwent necropsy, histopathology and transmission electron microscopy. Skin samples of a Thoroughbred and Warmblood foal served as age-matched controls. In brief, skin samples of various localisations were fixed in 4% formaldehyde for histopathology and transmission electron microscopy. Skin samples of a Thoroughbred and Warmblood foal served as age-matched controls. In brief, skin samples of various localisations were fixed in 4% formaldehyde for histopathology, dehydrated and paraffin-embedded regarding routine protocols. Paraffin sections (4 µm) were stained by haematoxylin and eosin and examined by light microscopy. Histopathological photographs were recorded with an Olympus BX51 microscope. For transmission electron microscopy, skin samples were fixed in 2.5% glutaraldehyde, embedded in epoxy and ultrathin sections were stained using uranyl acetate. Sections were examined using a transmission electron microscope (EM10C, Zeiss).
2.4 | Differences between genotypes

For generalised linear model (GLM) analysis, estimated breeding values (EBVs) based on studbook inspections (SBI) and mare performance tests (MPT) from progeny of the investigated 195 Hanoverian stallions as well as EBVs for stallion fertility and embryonic survival were used. EBVs for fertility were estimated using an animal model for the trait pregnancy rate per oestrus cycle and the random additive genetic effects of stallion and embryo through relationship matrices. In addition, EBVs were corrected for systematic environmental effects including age of the stallion, breeding season, period within breeding season and insemination centre. A successful artificial insemination in an oestrus cycle was encoded 1, otherwise the trait value was 0. All EBVs were standardised onto a mean of 100 and a standard deviation of 20. EBVs > 100 mean higher conception rates than the population average. All 195 individuals were also genotyped for the WFFST1 variant. The analysis was done to evaluate genotype effects on EBVs of Hanoverian stallions for performance and fertility traits using SAS. GLM for LS (least square) means was computed using the genotype as an independent and performance and fertility traits as dependent variates.

2.5 | Evaluation of the MRCA

Analysis for the MRCA of the identified genetic carriers for WFFST1 variant in this study was performed using pedigree certificates from 76 individuals. To extend these data to 25 generations for each individual, further pedigree information was obtained from online databases (https://www.rimondo.com, access: 31.10.2019; https://www.allbreedpedigree.com, access: 31.10.2019). All data were stored in a graph database ArangoDB (version 3.5) with nodes designated as the individual horses and edges representing the relationships between the nodes. After plausibility check of pedigree data based on the dates of birth of ancestors compared with progeny, a collection table was produced containing all 76 animals and their ancestors up to 25 generations and aggregated using Arango Query Language. In a second step, further data from five horses (KWPN, Rhenish Warmblood), which were designated as WFFS-carriers according to breeders reports (https://de.rimondo.com/horse-list/439/Traeger-des-Gendefekts-WFFS, access 31.10.2019) were included in the analysis. Pathways to the MRCA were computed using the aggregated data and displayed as graph using vis.js (https://visjs.org/) network component (vis-network, version 6.1.1).

3 | RESULTS

3.1 | Evaluation of WGS and genotyping data

In order to validate WFFST1 variant and exclude further potential candidate variants for WFFS in the region of PLOD1, a screening of variant calling results in WGS data for the region of ECA2
at 38 926 928-40 953 350 bp was performed. This region, harbouring PLOD1, was seen in a total of 340 variants in 78 analysed horses (Table S1). Among all these variants, the MAF was lowest (MAF = 0.028) for the potential causative variant for WFFS (WFFST1, PLOD1:c.2032G>A—reverse strand, NC_009145.3:g.39927817C>T) including only six heterozygous Hanoverian. None of the investigated variants was in linkage disequilibrium with PLOD1:c.2032G>A. Further evaluation of protein coding variants in the region of PLOD1 revealed six missense variants of which only WFFST1 was exclusive for the Hanoverian (Table 1).

Genotyping of WFFST1 in 1166 equids revealed this variant exclusively in Hanoverians (Table S3). The frequency of the WFFS-associated allele T (reverse strand A) in 849 tested Hanoverian was 0.14. The earliest horse harbouring a heterozygous genotype in this analysis was a Hanoverian stallion born in 1967 (Table S4). Furthermore, a mating of two genetic carriers registered as Oldenburg horses, descendants of Hanoverian sire lines two generations back, resulted in a stillborn filly with a homozygous mutant genotype A/A.

### 3.2 Macroscopic, histopathological and ultrastructural findings

The stillborn filly, aborted 2 weeks before the expected date of birth, was fully developed (bodyweight: 31.8 kg). It had protruding bony prominences with poor muscular development (Figure 1A). Multiple severe skin ruptures (length: 1-16 cm, width: 1-7 cm) were found particularly at both front limbs and shoulders, at both sides of the neck, beneath the right eye, at the left ear and ventral abdomen (Figure 1B-D). The skin could be torn easily and had a thickness of 0.1-0.2 cm. Furthermore, the abdominal cavity was open with parts of the small intestine protruding due to a rupture of the lateral abdominal muscles (Figure 1D).

In comparison to age-matched controls, the skin of the affected foal had a reduced number of thin, loosely arranged collagen fibres in the region of the ears, neck, left shoulder, pasterns, croup and left side of the thorax in haematoxylin-eosin stained slides (Figure 2A). In contrast, a control animal showed large bundles of three-dimensional cross-linked collagen bundles (Figure 2B). Ultrastructurally, a severely reduced number of collagen fibrils in comparison to the control (Figure 2C,D) was detected in the dermis of all examined locations. The diameter of these fibrils was varying between 47.257 and 100.17 nm (abdomen, control: 44.346-77.803 nm) or 74.411 and 127.332 nm (croup, control: 50.196-105.322) in the WFFST1-affected foal. Additionally, all examined areas showed swollen collagen fibrils with loss of normal architecture (degraded fibrils, Figure 2E,F) and multifocal areas with deposition of an irregular shaped, electron dense material accompanying or intermingled with collagen fibres (fibrillar plaques). Longitudinal sections of collagen fibrils showed a regular cross striation. Sometimes, intrafibrillar deposits of an amorphous material were detected. Multifocally, spirally rotating fibres were present.

### 3.3 Association of WFFS and performance

The genotype effects on EBVs were significant for a range of traits recorded at SBI including outer appearance complying with breed characteristics (breed type) and characteristics of stallion or mare (sex type), quality of body conformation, correctness of gait, elasticity (trot), head, neck, frame, saddle area, front and hindlimbs. Furthermore, associations with MPT parameters including gallop and rideability and breeding values for limb conformation, riding
horse points and dressage were also significant (Table S5). No significant differentiation was observed with the inbreeding coefficient and EBVs for the embryonic and stallion effect.

3.4 | Evaluation of the MRCA

Pedigree analysis for 76 genotyped genetic carriers across 25 generations resulted in about 792,000 individuals in the data collection table including 31,000 nodes (horses) and 54,000 edges (relationships). A comparison of all these data revealed a Hanoverian stallion (Stallion A), born in 1861, as the MRCA of all investigated genetic carriers. This was also confirmed in the second approach using additional five Warmblood horses of different breeds, which were designated as genetic carriers according to the breeders’ reports. Only 872 edges (representing the relationships between individuals) were required to link all analysed 81 horses (Figure 3). Further horses found in all 81 pedigrees (Figure 4) were Stallion B (892 edges), Stallion C (900 edges), Stallion D (905 edges), Stallion E (912 edges), Mare F (915 edges), Stallion G (918 edges), Stallion H (918 edges), Stallion I (920 edges) and Stallion J (925 edges), which were all closely related to Stallion A and all had one Thoroughbred stallion (Stallion K) as a common ancestor. Two Thoroughbreds which have previously been suggested as the MRCA were not identified in the pedigree of four investigated genetic carriers for WFFS including a KWPN stallion. In further 77 analysed genetic carriers, one of these two Thoroughbreds was found to be a common ancestor (641 edges) but with more edges required to link all individuals compared with the MCRA of this subgroup (Stallion L, 606 edges).

4 | DISCUSSION

In the current study, comprehensive analysis for WFFS validated the WFFST1 variant and gave a deeper view into its clinical and pathomorphological picture, genotypic distribution and origin.
First of all, we confirmed PLOD1:c.2032G>A variant to be most likely the causative variant for WFFST1 considering the genomic region harbouring PLOD1. Although the frequency of the mutant allele was shown to be quite high considering estimations of approximately 5.5% in a small Warmblood test population used for patent application, 5.5% in Warmblood horses in Brazil as well as 14% according to our investigations in a large sample size of Hanoverian horses, we did not identify another variant with a lower MAF. Thus, due to the low number of known cases of WFFST1-affected foals, we consider it unlikely that the disease is caused by a different variant in linkage with the already suggested missense mutation PLOD1:c.2032G>A, harbouring a higher frequency of the mutant allele in Warmblood horses and/or other equids.

Furthermore, we also identified a homozygous mutant genotype in an affected foal with EDS-like signs. Histopathological investigations of the skin confirmed previous findings in an affected foal of a markedly thinned dermis and irregular collagen bundles. Ultrastructural findings resembled those that have been reported elsewhere in horses, dogs, rabbits, human subjects and mice. However, besides a more severe degradation of collagen fibrils as observed in the previously investigated WFFST1-case, we encountered new findings using ultrastructural examinations, which highlighted fibrillar plaques and collagen fibres embedded in an amorphous matrix. Similar observations were made in an Oldenburg foal with EDS-like signs but no genetically confirmed disease. The irregular shaped electron dense material was found to represent an increased amount of collagen type I. Thus, the ultrastructural findings indicate that PLOD1 protein
affected by the WFFST1-missense mutation is specifically modifying the architecture of collagen fibrils leading to their degradation.\textsuperscript{38} PLOD1 was shown to be an important enzyme hydroxylating lysyl residues in collagen sequences, which are essential for attachments of carbohydrate units.\textsuperscript{38} This is in turn important for cross-linking of collagen fibrils, supporting their mechanical stability. Investigations of different connective tissues from human Ehlers-Danlos Syndrome Type VI patients revealed different expression levels of \textit{PLOD1} in collagen types I and III.\textsuperscript{39,40} These findings suggest that the signs of the disease can vary among different tissues, localisations at the body or even among different individuals dependent of the expression pattern.

In contrast to the severely affected horses harbouring a homozygous mutant genotype, genetic carriers of the recessively inherited disease can pass the mutant allele unnoticed to their progeny. In respect to WFFST1 mutant allele, the distribution was found to be wide in diverse Warmblood horse populations but also Thoroughbred from various countries like Germany, Brazil and USA.\textsuperscript{23-25} We suppose that this wide distribution can be explained due to the admittance of foreign populations in Warmblood breeding and subsequent reciprocal gene flow, as well as due to the origin of WFFST1 variant a long time back in the past. In fact, we identified a Hanoverian stallion (Stallion A) as the MRCA of 81 tested genetic carriers, who was shown to be an important stallion of the traditional sire line F/W in the Hanoverian.\textsuperscript{41} Also, in the Hanoverian breed, there are traditional sire lines designated by letters signifying the names of influential stallions including A/E, F/W, D and G lines.\textsuperscript{41} We assume that the fact that we found the MRCA as late born as in 1861 with a major contribution to the gene pool, explains the wide distribution of WFFST1 among Warmblood horses. Furthermore, we propose that the original horse in which the variant spontaneously occurred can be traced back further in the past to the Thoroughbred breed. Thoroughbred contributed to about 34.8\% to the genes of the Hanoverian population (reference population 1980-2000).\textsuperscript{41} Furthermore, a genotyping study of 716 Thoroughbred racehorses for WFFST1 variant confirmed that the mutant WFFST1 allele is present in the Thoroughbred population with a relatively low frequency of 1.2\%.\textsuperscript{25} The transmission of WFFST1 mutant allele from a Thoroughbred horse and the wide distribution in Warmblood horses might therefore be explained by the fact that Thoroughbred stallions which influence Warmblood breeding are no longer used for breeding in the Thoroughbred population. However, the previous suggestion that two specific Thoroughbreds represent the common ancestors of all genetic carriers,\textsuperscript{42} was not confirmed in our analysis. Our findings of significant correlations of WFFST1 variant with breeding values for performance traits like gaits and rideability which are essential attributes for dressage\textsuperscript{43} suggest a particularly wide distribution of WFFST1 variant into dressage breeding lines with huge genetic impact on population development. This perfectly fits with the identification of Stallion A as MRCA, as he is the ancestor of very successful dressage horses. We assume that the frequent use offspring achieving great dressage success has contributed to the wide distribution of WFFST1 variant. In contrast, we found no significant correlation with the embryonic effect and stallion fertility, which emphasised a previous suggestion that mutant WFFST1 probably does not result in an embryonic loss, but losses occur in later gestation or with live birth of nonviable foals.\textsuperscript{23}

To conclude, our study confirmed WFFST1 variant in WGS data as well as in an affected foal with EDS-like signs due to severe degradation of collagen fibrils. A Hanoverian stallion, Stallion A, was shown to be the major contributor to the wide distribution of the mutant allele in the Warmblood population.

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CONFLICT OF INTERESTS
No competing interests have been declared.

AUTHOR CONTRIBUTIONS
J. Metzger and O. Distl designed the study. J. Metzger, O. Distl, O. Kreft, G. Martinsson, H. Sieme, W. Reineking and M. Hewicker-Trautwein collected samples and data, performed sample preparation and carried out experiments. J. Metzger, O. Kreft and O. Distl performed bioinformatics analyses and completed the manuscript. All authors read and approved the final manuscript.

ETHICAL ANIMAL RESEARCH
EDTA-blood or hair samples were obtained according to the approval of the state veterinary office Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit in Oldenburg (no. 02A/138).

OWNER INFORMED CONSENT
Written informed approval was acquired from the horse owners and breeders to collect samples for current research.

DATA ACCESSIBILITY STATEMENT
All data are accessible at Sequence Read Archive (NCBI, see summary of all IDs in Supplementary Item 1).

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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