An equine Endothelin 3 cis-regulatory variant links blood pressure modulation to elite racing performance

Endothelin 3 affects blood pressure regulation and performance in horses

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Abstract

A previous selective sweep analysis of horse racing performance revealed a 19.6 kb candidate region approximately 50 kb downstream of the Endothelin 3 (*EDN3*) gene. EDN3 and other endothelin family members are associated with blood pressure regulation in humans and other species, but similar association studies in horses are lacking. We hypothesized that the sweep region includes a regulatory element acting on *EDN3* transcription, ultimately affecting blood pressure regulation and athletic performance in horses. Selective sweep fine-mapping identified a 5.5 kb haplotype of 14 SNPs shared within Coldblooded trotters (CBT) and Standardbreds (SB). Most SNPs overlapped potential transcription factor binding sites, and haplotype analysis showed significant association with all tested performance traits in CBTs and earnings in SBs. From those, two haplotypes were defined: an elite performing haplotype (EPH) and a sub-elite performing haplotype (SPH). While the majority of SNPs in the haplotype were part of the standing variation already found in pre-domestication horses, there has been an increase in the frequencies of the alternative alleles during the whole history of horse domestication. Horses homozygous for EPH had significantly higher plasma levels of EDN3, lower levels of EDN1, and lower exercise-related blood pressure compared to SPH homozygous horses. Additionally, a global proteomic analysis of plasma from EPH or SPH homozygous horses revealed higher levels of proteins involved in pathways related to immune response and complement activation in the SPH horses. This is the first study to demonstrate an association between the *EDN3* gene, blood pressure regulation, and athletic performance in horses. The results advance our understanding of the molecular genetics of athletic performance, exercise-related blood pressure regulation, and biological processes activated by intense exercise.

Keywords: Athletic performance, Blood pressure, Endothelin, *Equus caballus*, Exercise, Genetics, Harness racing, Horse, Trotting

Author summary

The horse is one of the most common species used for studying athletic performance. For centuries, horses have been used by humans for transportation, agriculture and entertainment and this has resulted in selection for various traits related to athletic performance. A previous study discovered that a genetic region close to the *Endothelin3* gene was associated with harness racing performance. Endothelin3 is known to be involved in blood pressure regulation and therefore we hypothesized that this region influences blood pressure and racing performance in horses. In this study we have used additional horses and fine-mapped the candidate region and we also measured blood pressure in Coldblooded trotters during exercise. Horses with two copies of the elite-performing haplotype had higher levels of Endothelin3 in plasma, lower blood pressure and better racing performance results, compared to horses with two copies of the sub-elite performing haplotype. We also discovered that horses with the sub-elite performing haplotype had higher levels of proteins related to the immune system in plasma. This study is the first to link Endothelin3 to blood pressure regulation and performance in horses. It broadens the understanding of the biological mechanisms behind blood pressure regulation as well as inflammation and coagulation system in relation to racing performance.

Background

The use of domestic animals as models for genomic research has provided not only basic knowledge concerning gene function and biological mechanisms but also a complementary view on genotype–phenotype relationships (1). The horse is one of the most popular species used to study athletic performance. They have been intensively selected for centuries, to engender the optimal physical capacity for strength, speed, and endurance (2,3). Additionally,

their recent population history, involving closed populations with similar phenotypic traits within breeds and large variations across breeds, has created a favorable genome structure for genetic mapping. These factors combined make the horse an optimal model for studying the molecular genetics underlying athletic performance and the complex biological processes activated by exercise (1,2,4,5). Previous work in horses has started to reveal the effect of genes linked to complex traits such as muscle mass and locomotion patterns on athletic performance, and many potential candidate genetic variants have been identified (6–12). However, understanding the mechanisms by which the identified variants exert their functions is much more challenging.

A selective sweep study on harness racing breeds revealed a 19.6 kb genomic region on chromosome 22, located about 50 kb downstream of the *Endothelin 3* (*EDN3*) gene, under selection in Coldblooded trotters (CBTs) (13). Five SNPs in high linkage disequilibrium (LD r^2 = 0.92–0.94) were significantly associated with racing performance, including the number of victories, earnings, and racing times. SNP NC_009165.3:g.46717860C>T (EquCab3.0) showed the strongest association within the sweep and was further genotyped in 18 additional horse breeds. The favorable T allele was found in high frequency in breeds used for racing, while it generally remained at low frequency in ponies and draught horses. It was hypothesized that the identified region might contain a regulatory element influencing either the expression of *EDN3* or other genes nearby (13).

The endothelin system encompasses the EDN ligands (e.g., EDN-1 to -3) and their G protein-coupled receptors (endothelin receptor type A and B). Together, they are involved in a broad range of biological functions, including cell proliferation and vasoconstriction (14). EDN2 and -3 can be considered as natural antagonists for EDN1 (15). The endothelin receptor type

A (EDNRA) has the highest affinity for EDN1 and EDN2 but a low affinity for EDN3 (16). The endothelin receptor type B (EDNRB) has equal affinity for all three isoforms of endothelin (17). During exercise, endothelins are involved in the dynamic redistribution of blood flow to the lung and active muscles, limiting the flow to non-exercise-essential tissues, such as kidneys and intestines (15). Generally, EDNRA activation causes vasoconstriction and increased blood pressure, while EDNRB activation results in nitric oxide release, vasodilatation, and decreased blood pressure (18).

To date, most studies of the EDN system have focused on the role of EDN1. In human health studies, *EDN1* genetic variants were found to be implicated in hypertension (19). In performance trials, increased EDN1 plasma levels were observed in relation to exercise duration (20), with elevated blood pressure leading to impaired exercise capacity in elite athletes (20,21). There have also been performance studies in horses, where EDN1 plasma levels largely mirrored the results found in humans, showing that the EDN1 response to exercise challenge is conserved across both species (22). There is a clear role for vasoconstriction in exercise performance, but the action of the EDN3 ligand is unclear. In this study, we aimed to fill this gap of knowledge. First through the fine-mapping of the *EDN3* region flanking the selective sweep, then the characterization of the spatial and temporal distribution of key genetic variants within horse populations, and finally by evaluating the effect of opposite haplotypes on blood pressure, as well as EDN1 and EDN3 plasma levels, before and during exercise. We also performed a global proteomic study on plasma to identify differentially expressed proteins amongst the various *EDN3* haplotypes. While the key breed of the study was the CBT, a popular harness racing breed in Sweden and Norway, the results are placed in the context of additional racing breeds. The results presented here on

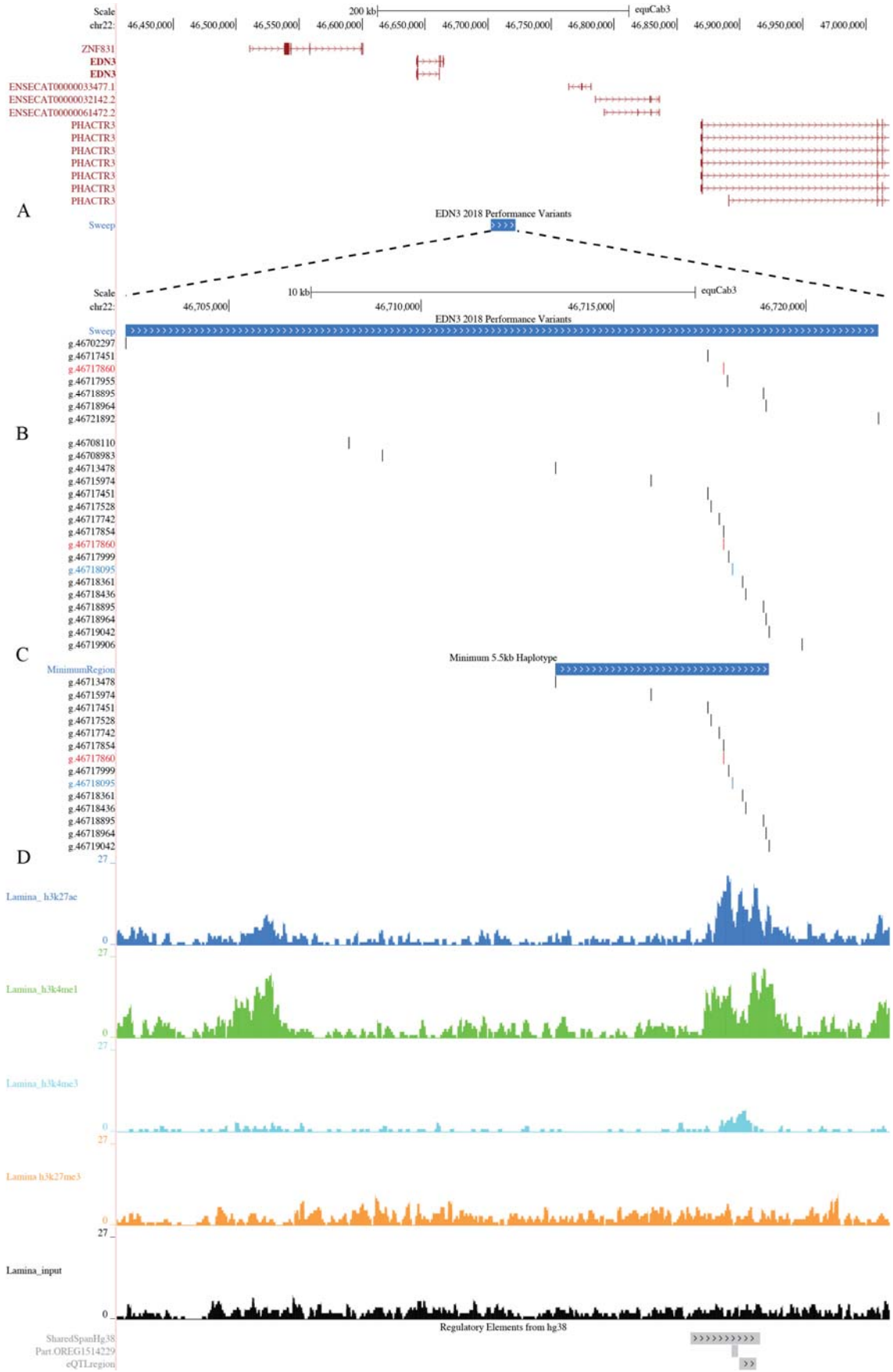
the endothelin pathway not only give new insights into genetic regulation of its established vasomodulatory properties, but also its emerging role in inflammation.

Results

Fine-mapping and comparative analyses suggest a regulatory role of the selected region

A 2018 study using the sparse Illumina SNP50 Genotyping BeadChip, identified a 19.6 kb selective sweep shared between SBs and CBTs (ECA22:46,702,297-46,721,892; Figure 1A). The sweep was located between *EDN3* and ENSECAG00000039543 (lncRNA), and five SNPs within this region were found to be associated with harness racing performance (13). From those five SNPs, g.46717860 C>T was the most significant. With a four-step process, we reduced this 19.6 kb sweep region to a minimal 5.5 kb region (Figure 1).

In step one, we performed a sweep-performance association analysis using additional horse material (n=661) and SNP genotypes extracted from the 670K Axiom Equine Genotyping Array (Figure 1A). However, supplementing the 400 CBTs used originally in the 2018 study(13) with 221 additional CBTs did not provide increased resolution into the 19.6 kb sweep. Seven SNPs within the original sweep span were extracted from the array, including g.46717860. However, pairwise LD ($r^2 > 0.6$) to g.46717860 resulted in the same five SNP set as found in (13), with all SNPs remaining significantly associated with harness racing performance traits in linear models (i.e., number of victories, earnings, and race times, $P \leq 0.05$) (Supplementary Table S1).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g.46721892
g.46708110
g.46708983
g.46713478
g.46715974
g.46717528
g.46717742
g.46717854
g.46717999
g.46718095
g.46718361
g.46718436
g.46719042
g.46719906
MinimumRegion
Minimum 5.5kb Haplotype
Lamina_h3k27ac
Lamina_h3k4me1
Lamina_h3k4me3
Lamina_h3k27me3
Lamina_input
Regulatory Elements from hg38
SharedSpanHg38
Part.OREG1514229
eQTLregion

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Figure 1. A. Overview of the 19.6 kb 2018 selective sweep region including all annotated genes in the region (13). B. Zoomed in view of the 2018 selective sweep region including the 17 SNPs used in the haplotype analysis. C. Overview of the 5.5 kb minimum shared haplotype within the Coldblooded trotters and Standardbreds, including all SNPs from the haplotype analysis. D. Horse functional data as generated by chip-seq of histone modifications from hoof lamina (from Equine Functional Annotation of Animal Genomes, FAANG) indicating an active enhancer in our region of interest. In addition, ~ 2 kb of the associated haplotype region aligned with the human reference, including segments of an ORegAnno regulatory element, pinpointing a curated regulatory annotation of this region, (OREG1514229), GTEx cis-eQTL variants regulating *EDN3* in esophagus mucosa tissue.

In step two, we performed variant discovery to increase variant density across the 19.6 kb sweep region. Here, Illumina short-read whole genome sequences (WGSs) were generated from two CBTs and two SBs (the most common breed used for harness racing). In each breed, the horses were selected to be homozygous for different alleles at SNP g.46717860 (CC and TT, respectively) and to have either high (TT) or low (CC) earnings per start (Supplementary Table S2). From the 19.6 kb sweep region, 78 SNPs and six indels (one 400 bp deletion, one 23 bp deletion, three single bp deletions, and one single bp insertion) were identified. To possibly detect new structural variants, targeted long-read sequencing using Oxford Nanopore technology (ONT) was performed across the sweep region for eight horses: four CBTs with g.46717860-TT genotype and high performance, and four CBTs with the opposite features, including the two CBTs used for WGS (Supplementary Table S2). However, no new structural genetic variants were identified.

In step three, we prioritized variants for further analysis. While the whole genome sequenced elite CBT (high earnings per start) was homozygous for the 400 bp deletion (ECA22:46,714,602-46,715,003), the elite SB was heterozygous, and the deletion was not

detected in the low-performing (low earnings per start) horses. The deletion was, thus, tested for association with racing performance traits in 479 CBTs using linear models, but the results were not significant (Supplementary Table S3). From the remaining variants identified, a total of 24 SNPs and one single base pair deletion, evenly distributed within the region, were selected (see methods) and genotyped in four donkeys and 412 horses sampled from 13 different breeds. SNP g.46717860 was not included in this set but was genotyped separately in 170 horses and imputed in the remainder. At this stage, 24 SNPs and 407 horses passed quality control measures. Pairwise LD estimation with SNP g.46717860 revealed 15 SNPs in strong LD ($r^2 \geq 0.53$, 14 SNPs with an r^2 score > 0.98) (ECA22: 46,708,983-46,719,042). LD decayed outside the region ($r^2 < 0.07$), leaving 17 SNPs (15 SNPs in LD and two flanking SNPs) for haplotype analysis (Figure 1B). Phasing revealed 15 different haplotypes, five showing a frequency > 2 %. All haplotypes with a frequency > 5 % in the total sample set or within each breed are presented in Table 1 (disregarding breeds represented by less than five horses).

Table 1. Haplotype frequencies in different breeds

Haplotype (ECA22: 46,708,110-46,719,906)	All breeds (n=411)	CBT (n=222)	Arabians (n=29)	TB (n=30)	SB (n=45)	Ardennes (n=20)	NSH (n=18)	Exmoor (n=17)	Icelandic (n=7)
1) GCAAGCGTTTCTCAAAA	0.30	0.27	0.74	0.68	0.36	0.00	0.03	0.00	0.00
2) GTGGTTACCCTCTGGGA	0.28	0.25	0.00	0.07	0.00	0.60	0.86	1.00	0.00
3) ACAAGCGTTTCTCAAAG	0.27	0.32	0.26	0.25	0.41	0.10	<0.01	0.00	0.00
4) ACGGTTACCCTCTGGGG	0.11	0.14	0.00	0.00	0.20	0.00	0.01	0.00	0.43
5) ACGGTTACCCTCTGGGA	0.03	0.00	0.00	0.00	0.04	0.18	0.03	0.00	0.36
6) ACAAGCGTTTCTCGAAA	<0.01	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00
7) ACGGGCGTTCCTCAAAG	<0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07
8) GCAAGCGTTTCTCAAGG	<0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07
9) GCAAGTGTTTCTCAAGG	<0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07

[1] Order of SNPs. Chr 22: 46708110, 46708983, 46713478, 46715974, 46717451, 46717528, 46717742, 46717854, 46717860, 46717999, 46718095, 46718361, 46718436, 46718895, 46718964, 46719042, 46719906

CBT: Coldblooded trotter, TB: Thoroughbred, SB: Standardbred, NSH: North-Swedish draught horse

The association between haplotype and harness racing performance traits was performed in CBTs (n=180) and SBs (n=38) using generalized linear model regression (GLM) analysis in the statistical software R (23) (Table 2 and 3). Only haplotypes with a frequency > 2 % in the population were included in the analysis. Of the four haplotypes in CBTs, haplotype 3 (H3), carrying the g.46717860-T high performance associated allele, was the most common (0.32) and was defined as the base haplotype. Two haplotypes, H2 and H4, respectively, demonstrated a significant negative effect on all performance traits tested, and each carried the g.46717860-C low performance associated allele (Table 2). Four haplotypes were also available for testing within SBs with performance data. Three haplotypes were shared with CBTs (H1, H3 and H4) and one (H5) was shared with other breeds (Table 3). As for the CBTs, H3 was the most common haplotype (0.43) and was defined as the base haplotype in this analysis. Two haplotypes demonstrated significant negative effects on performance traits: H4 (earnings and earnings per start) and H5 (all traits tested). The above analysis revealed a minimum 5,564 bp, 14 SNP shared haplotype, significantly associated with racing performance within CBTs (ECA22: 46,713,478-46,719,042). The same variants were also significantly associated with racing performance in SBs. This allowed for the definition of an elite-performing haplotype (EPH: AAGCGTTTCTCAAA), and a sub-elite-performing haplotype (SPH: GGTTACCCTCTGGG) (Supplementary Table S4) (Figure 1C). Of note, the reference EquCab3.0 genome represents the SPH variant.

Table 2. Haplotype frequencies, haplotype coefficient and *P*-values for the performance analysis in Coldblooded trotters (n=180)

	Haplotype[2] (H3)		**Haplotype (H1)**		**Haplotype (H2)**		**Haplotype (H4)**	
Haplotype[1]	ACAAGCGTTTCTCAAAG		GCAAGCGTTTCTCAAAA		GTGGTTACCCTCTGGGA		ACGGTTACCCTCTGGGG	
Frequency	0.32		0.27		0.27		0.14	
	Haplotype coefficient	P-value[3]	Haplotype coefficient	P-value[3]	Haplotype coefficient	P-value[3]	Haplotype coefficient	P-value[3]
No. of starts[5]	-	-	-0.30	0.43	-1.00	**0.004**	-1.05	**0.02**
No. of wins[5]	-	-	-0.04	0.31	-0.14	**<0.001**	-0.17	**<0.001**
No. of placings[5]	-	-	-0.06	0.13	-0.12	**<0.001**	-0.14	**0.002**
Earnings (SEK)[4,5]	-	-	-0.31	0.05	-0.53	**<0.001**	-0.56	**0.002**
Earnings/start[5]	-	-	-0.19	0.07	-0.45	**<0.001**	-0.42	**<0.001**
Best time (sec/km)[5]	-	-	0.03	0.16	0.07	**<0.001**	0.08	**0.002**

[1]Order of SNPs. Chr 22: 46708110, 46708983, 46713478, 46715974, 46717451, 46717528, 46717742, 46717854, 46717860, 46717999, 46718095, 46718361, 46718436, 46718895, 46718964, 46719042, 46719906

[2]The most common haplotype was used as the base haplotype

[3]A GLM regression analysis was performed in R. Sex, age, birth country, number of starts (when applicable) and *DMRT3* genotype were included as fixed effects. Significant results ($P \leq 0.05$) in bold

[4] SEK, Swedish kronor

[5] Log transformed values (log10 +1) were used for wins and placings. Number of starts were square root transformed. Earnings and earnings per start were transformed applying ln(Earnings + 1000).

CBT: Coldblooded trotter, TB: Thoroughbred, SB: Standardbred, NSH: North-Swedish draught horse

Table 3. Haplotype frequencies, haplotype coefficient and *P*-values for the performance analysis in Standardbreds (n=38)

	Haplotype[2] (H3)		**Haplotype (H1)**		**Haplotype (H4)**		**Haplotype (H5)**	
Haplotype[1]	ACAAGCGTTTCTCAAAG		GCAAGCGTTTCTCAAAA		ACGGTTACCCTCTGGGG		ACGGTTACCCTCTGGGA	
Frequency	0.43		0.34		0.18		0.04	
	Haplotype coefficient	P-value[3]	Haplotype coefficient	P-value[3]	Haplotype coefficient	P-value[3]	Haplotype coefficient	P-value[3]
No. of starts[5]	-	-	0.12	0.82	0.48	0.41	-0.61	**0.003**
No. of wins[5]	-	-	-0.02	0.74	-0.08	0.35	-0.47	**<0.001**
No. of placings[5]	-	-	-0.03	0.63	-0.11	0.06	-0.25	**<0.001**
Earnings (SEK)[4, 5]	-	-	-0.17	0.53	-1.42	**<0.001**	-1.32	**<0.001**
Earnings/start[5]	-	-	-0.10	0.66	-1.14	**<0.001**	-1.12	**<0.001**
Best time (sec/km)[5]	-	-	-0.10	0.37	0.15	0.24	0.65	**0.01**

[1]Order of SNPs. Chr 22: 46708110, 46708983, 46713478, 46715974, 46717451, 46717528, 46717742, 46717854, 46717860, 46717999, 46718095, 46718361, 46718436, 46718895, 46718964, 46719042, 46719906

[2]The most common haplotype was used as the base haplotype

[3]A GLM regression analysis was performed in R. Sex, age, birth country and number of starts (when applicable) were included as fixed effects. Significant results ($P \leq 0.05$) in bold

[4] SEK, Swedish kronor

[5] Log transformed values (log10 +1) were used for wins and placings. Number of starts were square root transformed. Earnings and earnings per start were transformed applying ln(Earnings + 1000).

CBT: Coldblooded trotter, TB: Thoroughbred, SB: Standardbred, NSH: North-Swedish draught horse

In step four, we assessed the potential functional impact of the minimal 5.5 kb region and each of the 14 SNP haplotype variants. Using horse functional data from two Thoroughbreds (from the Equine Functional Annotation of Animal Genomes, FAANG), we found three SNPs (g.46717860, g.46718095 and g.46718113) that overlapped potential active enhancers, i.e., H3K4me1 and H3K27ac marks (24,25) (Figure 1D). Both Thoroughbreds were

homozygous for EPH, with the histone marks indicating the presence of an active enhancer in the hoof lamina, present but less active in lung and ovarian tissue, and repressed in the skin(24,25). In comparative analyses, a genome liftover to hg38 indicated that, although not well conserved, ~ 2 kb of the associated haplotype region aligned with the human reference, including segments of an ORegAnno regulatory element (OREG1514229), GTEx cis-eQTL variants regulating *EDN3* in esophagus mucosa tissue (Figure 1D). These combined regulatory signals prompted us to test the potential for transcription factor binding affinity changes in relation to the EPH and SPH alleles. Most SNP allele changes (12 out of 14 SNPs) altered transcription factor binding (Supplementary Table S4). For EPH, this meant the perturbation of matrices for stress responders, e.g., IRF2 and NFKB1, while creating sites for developmental transcription factors such as NR4A2 and HOXA5.

The elite performing haplotype has an additive effect on performance traits.

Given that all 14 associated variants within the minimum shared 5.5 kb haplotype were in perfect LD, we used the genotypes at g.46718095 as a proxy for EPH (C allele) and SPH (T allele) haplotypic pairs and re-assessed the association between haplotypes and racing performance. For CBTs (n=539), EPH homo- or heterozygotes outperformed the SPH homozygotes (Table 4). In SBs, there were no statistically significant differences between the three genotypes (Table 5). However, when analyzing horses carrying at least one T allele versus CC horses, the results were significant for several performance traits (Table 5), including the number of wins, placings and earnings. However, the number of wins and placings was higher in horses with at least one T allele, while the CC horses earned more money than the TT/TC horses (Table 5).

Table 4. Performance results in 539 Coldblooded trotters for SNP g.46718095 T>C

Genotype	**TT (n=65)**		**TC (n=256)**		**CC (n=218)**		**P[2]**		
Performance trait[1]	**Mean**	**Median**	**Mean**	**Median**	**Mean**	**Median**	**TT/TC**	**TT/CC**	**TC/CC**
No. of starts	31.9	22.0	40.2	28.0	41.3	31.0	**0.01**	**0.02**	1.00
No. of wins	2.8	1.0	5.7	3.0	5.9	3.0	**0.003**	**0.02**	0.65
No. of placings (1-3)	9.3	4.0	14.2	9.0	14.5	9.0	**0.005**	0.07	0.38
Earnings (SEK)	207,295	51,772	438,700	140,161	453,911	115,312	**0.006**	**0.05**	0.58
Earnings per start (SEK)	4,752	2,976	9,666	4,362	8,469	3,771	**0.003**	0.08	0.20
Best km time (sec)[3]	90.9	91.4	88.5	88.3	88.9	88.9	**0.001**	**0.02**	0.44

[1]Log transformed values (log10 +1) were used for wins and placings. Earnings and earnings per start were transformed applying ln(earnings + 1000). Square root transformations were used for number of starts

[2]A linear model was performed in R. Sex, age, birth country, number of starts and *DMRT3* genotype were included as fixed effects (when significant). Least square means and Tukey were used as post hoc tests. Significant results ($P \leq 0.05$) in bold

[3]Only races where the horse did not gallop were included

Table 5. Performance results in 271 Standardbreds for SNP g.46718095 T>C

Genotype	**TT (n=7)**		**TC (n=62)**		**CC (n=202)**		**P[2]**			
Perf. trait[1]	**Mean**	**Median**	**Mean**	**Median**	**Mean**	**Median**	**TT/TC**	**TT/CC**	**TC/CC**	**CC/ TT+TC**
No. of starts	43.0	48.0	42.9	38.0	35.6	31.0	0.85	0.60	0.52	0.20
No. of wins	6.7	5.0	6.3	4.0	5.7	5.0	0.82	0.99	0.10	**0.04**
No. of placings (1-3)	15.6	12.0	16.1	16.0	13.7	11.0	0.86	0.96	0.08	**0.03**
Earnings (SEK)	700,008	322,300	730,124	332,300	1,075,529	369,675	0.92	0.43	0.06	**0.01**
Earnings per start (SEK)	11,981	5,463	16,673	9,765	35,346	9,897	0.86	0.44	0.16	**0.04**
Best km time (sec)[3]	73.1	73.9	73.3	72.8	73.1	72.9	0.85	1.00	0.19	0.10

[1]Log transformed values (log10 +1) were used for wins and placings. Earnings and earnings per start were transformed applying ln(earnings + 1000). Square root transformations were used for number of starts

[2]A linear model was performed in R. Sex, age, birth country and number of starts were included as fixed effects (when significant). Least square means and Tukey were used as post hoc tests. Significant results ($P \leq 0.05$) in bold

[3]Only races where the horse did not gallop were included

Our racing performance analyses revealed that the g.46718095 genotypes did not distribute according to HWE. This prompted us to ask if there had been selection on this position or

perhaps the minimal haplotype in other breeds. While each individual's performance status is unknown, a trend for increased frequency of the C allele in traditional performance breeds was observed, e.g., Arabian horses, Thoroughbreds and Warmbloods. In contrast, this allele was low or absent in draft horses and ponies (Table 6).

Table 6. Allele frequency of SNP g.46718095 T>C

Breed	**n**	**T**	**C**
Arabian horses	30	0.00	1.00
Thoroughbred	30	0.07	0.93
Standardbred	306	0.14	0.86
Warmblood	3	0.17	0.83
Coldblooded trotters	539	0.36	0.64
Finnhorses	4	0.50	0.50
Gotland pony	2	0.50	0.50
Shetland pony	18	0.64	0.36
Ardennes	20	0.78	0.22
Icelandic horses	11	0.86	0.14
North-Swedish draught	25	0.88	0.12
Exmoor pony	23	1.00	0.00
Fjord horse	1	1.00	0.00
Przewalski	4	1.00	0.00

Increased frequency of the favorable haplotype coincides with the time of horse domestication

For all 14 SNPs within the minimum shared haplotype in the trotters, we investigated the allele frequency over time using the mapDATAge package (26). For all SNPs but two (SNP 6 and 7 in Figure 2) there was an increase of the alternate allele from 7,500-5,500 years ago (Figure 2). For all SNPs considered, the two alleles segregated in specimens pre-dating the rise and spread of the DOM2 genetic lineage of modern domestic horses (data not shown). The dataset considered included 431 ancient genomes previously characterized (2,27–31).

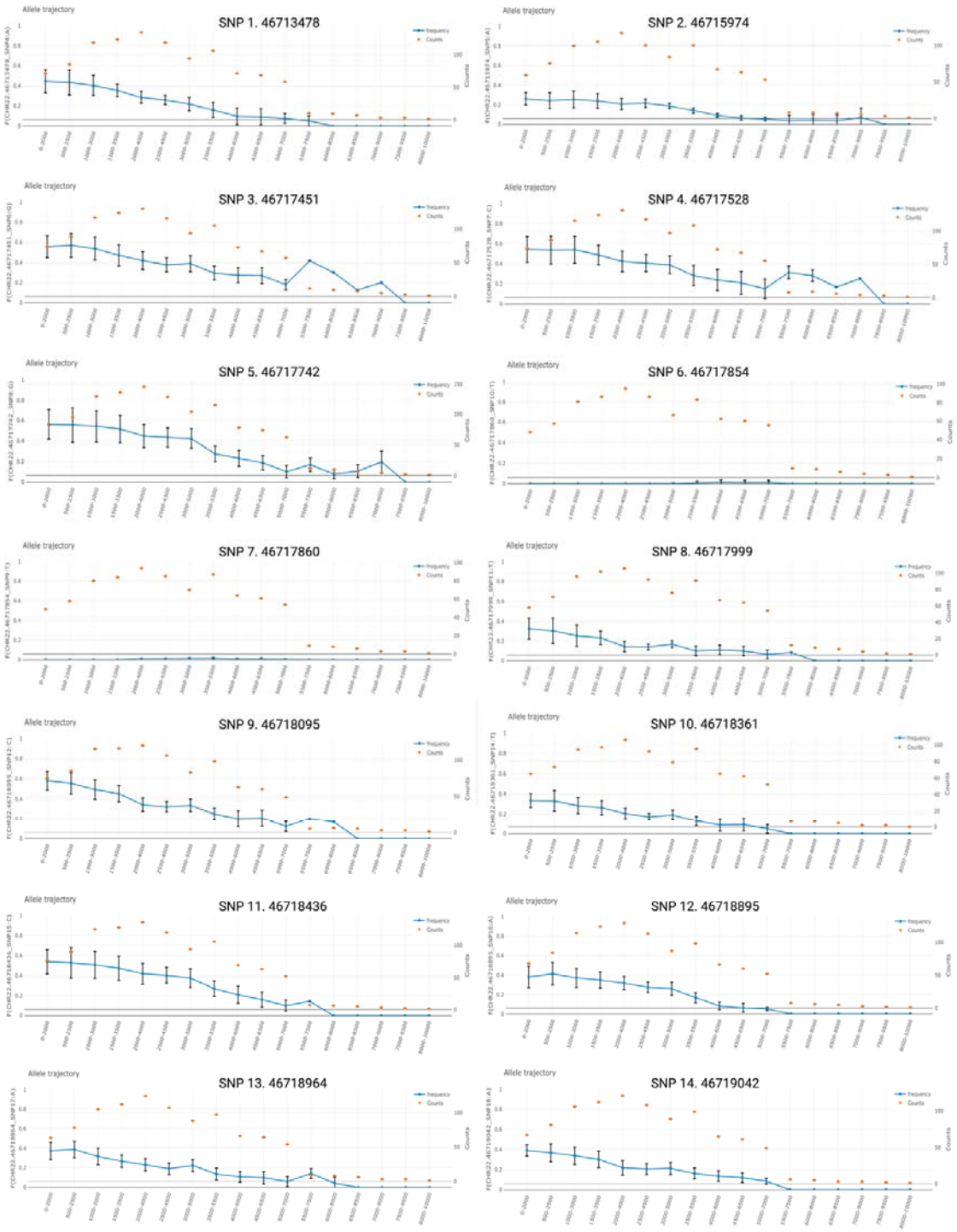

Figure 2. Frequency of the alternative allele over time from the mapDATAge package (26) for all 14 SNPs within the minimum shared haplotype in the trotters. The orange dots represent the number of horse samples considered in a given time bin of 2,000 years, and the blue line represents the frequency for the alternative allele estimated from allele counts. Figure created with Biorender.com.

Elite performance haplotype linked to lower blood pressure

To link genotype to phenotype, we assessed the physiological difference between CBTs homozygous for SPH (n=5) and EPH (n=13) before, during and post-exercise (see methods). Blood pressure measurements were also taken from eight heterozygous horses (HET) at rest. Horses homozygous for SPH had, on average, significantly higher systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP) during exercise, measured directly after the last uphill interval (Figure 3A-C). In addition, five mins after the uphill interval, the SPH group showed significantly higher SBP, MAP, and pulse pressure (PP) relative to the EPH group (Figure 3D-F). Horses homozygous for SPH also had significantly higher SBP and PP 20 mins after completing the exercise, compared to EPH horses (Figure 3G-H). Twenty mins after the exercise EPH horses were back to resting values, while it took ten additional mins for SPH horses to recover to resting values. There were no statistically significant differences in blood pressure measurements at rest, before the exercise. Furthermore, SPH horses had a higher heart rate than EPH horses during exercise (97.5 vs. 86.4) and five mins after exercise (85.7 vs. 77.0), although the difference was not significant. All blood pressure values are presented in Supplementary Table S5.

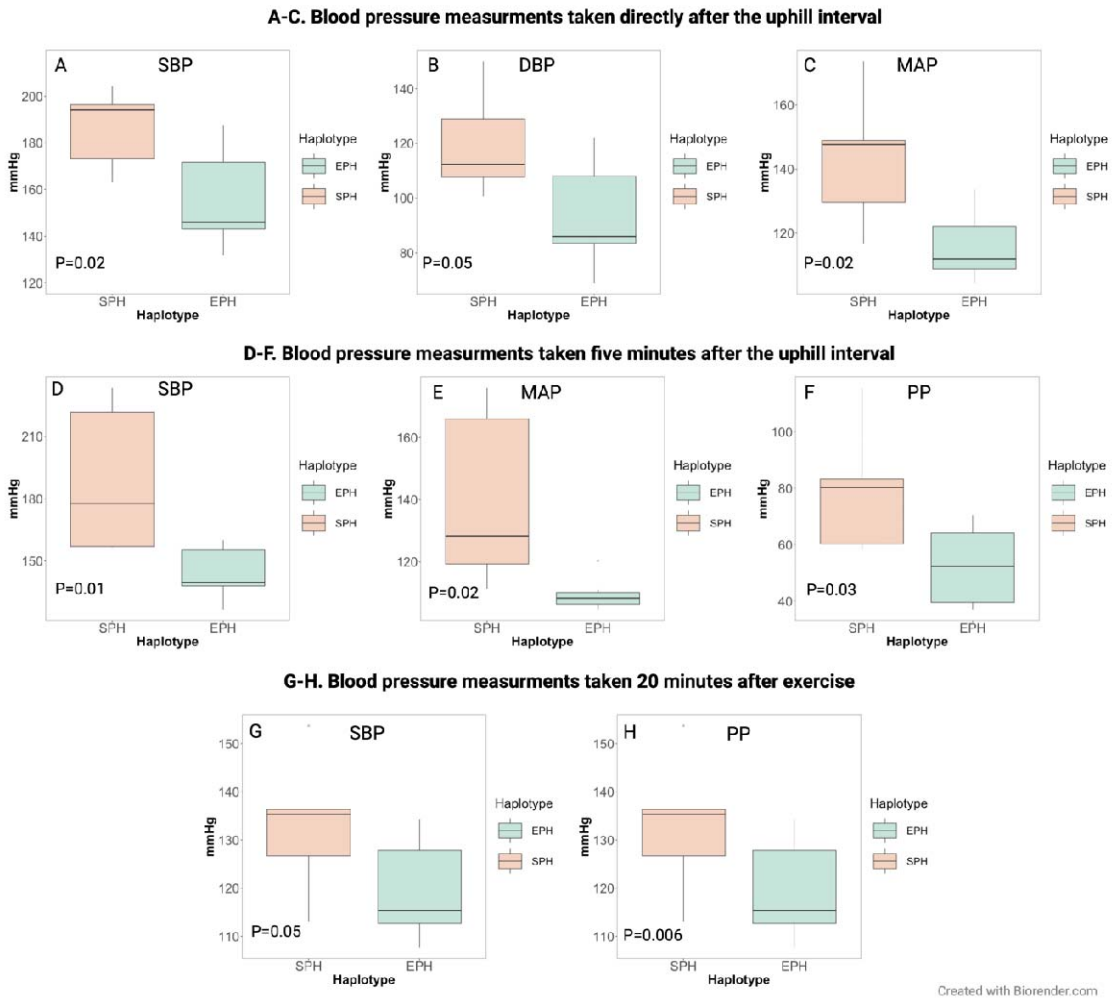

Figure 3. Blood pressure measurements during and after exercise in horses homozygous for the sub-elite performing haplotype (SPH) and horses homozygous for the elite-performing haplotype (EPH). A-C: Blood pressure during exercise measured directly after the uphill interval (SPH n = 5, EPH n=7). D-F: Blood pressure during exercise measured five mins after the uphill interval (SPH n = 5, EPH n=7). G-H. Blood pressure measured 20 mins after completing the exercise (SPH n = 5, EPH n=11). SBP: Systolic blood pressure, DBP: Diastolic blood pressure, MAP: Mean arterial blood pressure, PP: Pulse pressure. PP is defined as the systolic blood pressure minus the diastolic blood pressure.

Plasma concentrations of EDN1 and EDN3 were significantly different between the haplotype groups

ELISA tests were used to measure the plasma concentration of EDN1 and EDN3 at rest and during exercise for each haplotype group (Figure 4). Horses homozygous for SPH (n=8) had a significantly higher plasma concentration of EDN1 both at rest and during exercise compared to the other groups (Tukey´s HSD test). In addition, SPH horses had a significantly lower plasma concentration of EDN3 at rest and during exercise (borderline significant) compared to horses homozygous for the EPH (Figure 4). Individual plasma values are presented in Supplementary Tables S6 and S7.

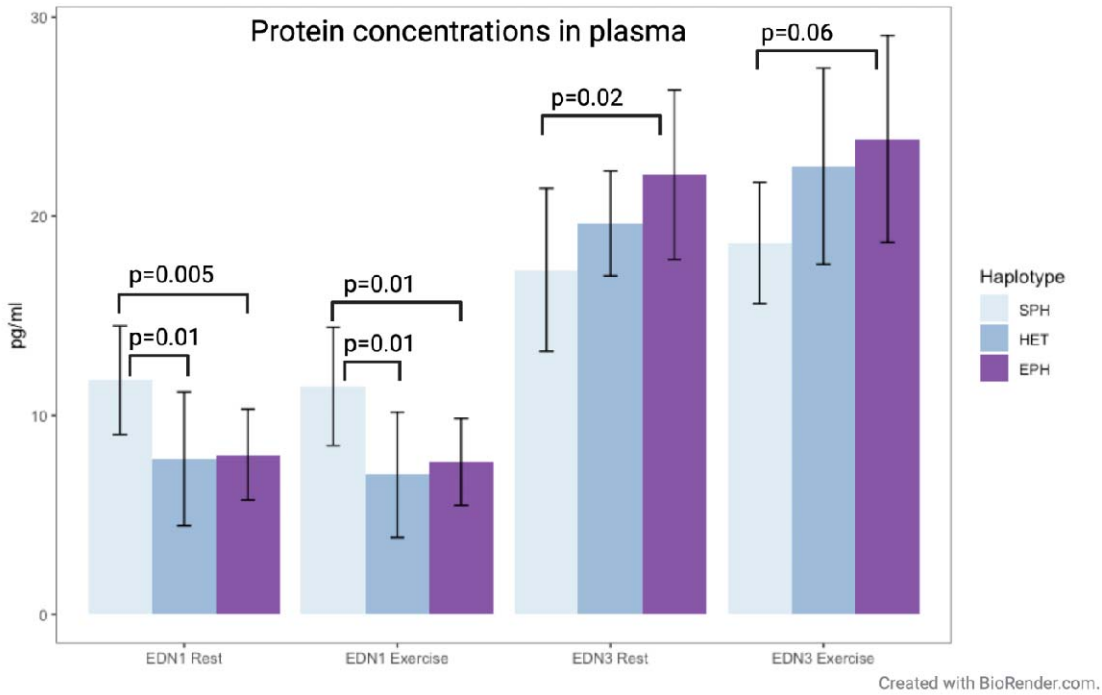

Figure 4. Plasma concentrations (pg/ml) of EDN1 and EDN3 at rest and during exercise in horses homozygous for the sub-elite performing haplotype (SPH) (n=8), horses heterozygous for the EPH and SPH (HET) (n=11) and horses homozygous for the elite-performing haplotype (EPH) (n=21). ANOVA and Tukey´s HSD test were performed. Standard deviations are presented as error bars.

Significant differences in protein expression between haplotype groups

To identify potential biological pathways including EDN1 and EDN3, and to compare the expression of different proteins between EPH (n=6) and SPH (n=6) homozygous carriers, we performed a global relative quantitative proteomic analysis using Tandem Mass Tag (TMT) and mass spectrometry (MS), using plasma samples taken at rest or during exercise. In total, 582 distinct proteins were identified, but neither EDN1 nor EDN3 (Supplementary Table S8). Proteins quantified from at least three horses from each haplotype group were included in the statistical calculations. This resulted in 383 proteins that were analyzed in the five following comparisons; 1) exercise vs. rest in SPH, 2) exercise vs. rest in EPH, 3) EPH vs. SPH at rest, 4) EPH vs. SPH during exercise, 5) paired ratio analysis, where the individual difference at rest and exercise was compared between EPH and SPH groups.

Fifty proteins were found to show significantly different expression levels across the five comparisons ($P < 0.05$; Supplementary Table S8). Table 7 presents the top proteins with the highest fold change in each comparison, including all proteins with different levels that were significant in more than one comparison.

Place for Table 7, see end of document

When comparing protein levels during exercise vs. at rest (comparisons 1 and 2), there were 21 proteins in the SPH group and three proteins in the EPH group with significantly different levels (Supplementary Table S8). A gene ontology (GO) analysis for the 21 proteins showing changing expression profiles at rest vs. during exercise in the SPH group revealed pathways related to blood coagulation, hemostasis, coagulation and regulation of body fluid levels (Figure 5A) (32). When comparing EPH and SPH at rest (comparison 3), there were 23

proteins upregulated in the SPH group (Supplementary Table S8), implicated in pathways related to immune response and complement activation (Figure 5B). There were no statistically significant differences in protein levels when comparing EPH and SPH during exercise (comparison 4). The paired ratio comparison (comparison 5) revealed 19 proteins showing significant difference in the individual difference at rest and exercise in SPH and EPH groups (Supplementary Table S8). These proteins are involved in pathways related to immune response, complement activation and catalytic and hydrolase activity (Figure 5C).

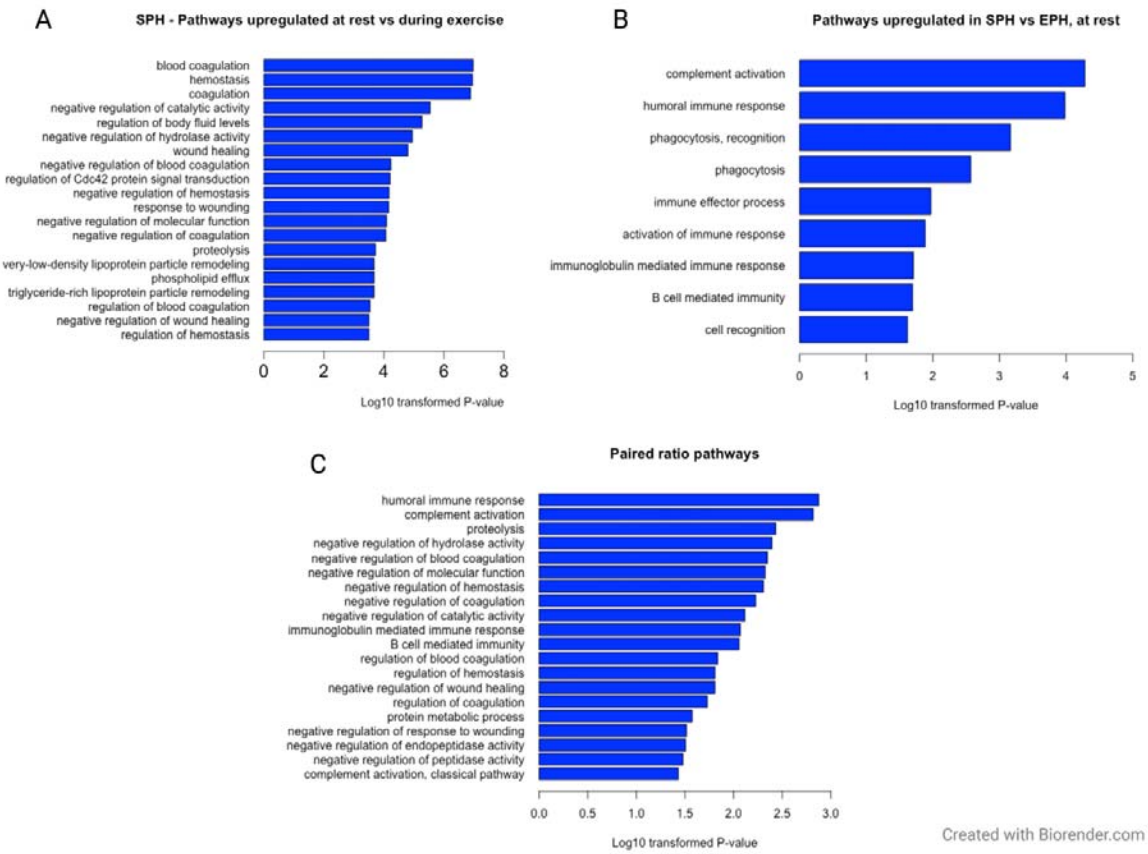

Figure 5. A. The 20 most significant pathways from the GO analysis based on the significant proteins in the comparison of exercise vs. rest in the SPH (sub-elite performing haplotype) group. B. All significant pathways from the GO analysis based on the significant proteins in the comparison of SPH (sub-elite performing haplotype) and EPH (elite performing haplotype) at rest. C. The 20 most significant pathways from the GO analysis based on the significant proteins in the paired ratio analysis of SPH vs. EPH.

Discussion

The EDN3 protein has an established functional role in blood pressure regulation in humans and other species. However, to the best of our knowledge, there are no published reports establishing associations between the *EDN3* gene and equine athletic performance, except for our previous selective sweep study (13). Given the role of EDN3 in blood pressure regulation, we hypothesized that 1) an underlying phenotype for elite athletic performance is blood pressure regulation, and 2) the identified non-coding region harbors a regulatory element that acts on the *EDN3* gene.

All the SNPs defining the haplotypes were found to segregate in the genome of horses that lived prior to the rise and spread of the modern domestic lineage around ~4,200 years ago (DOM2) (31). In fact, temporal allelic trajectories portray an increase in the frequency of the alternative allele, from 7,500-5,500 years ago, prior to the earliest archaeological evidence of horse husbandry (30,33). The concerted rise in frequency of all alternate alleles following the spread of the DOM2 lineage is compatible with a haplotype undergoing positive selection, possibly following artificial selection for animals showing improved athletic performance. Interestingly, the allele trajectories at *EDN3* are in striking contrast with those previously described at the *MSTN* locus, driving performance in short-distance racing (6), which indicated selection starting within approximately the last 1,000 years only. This may indicate performance traits representing recurrent selection targets in the history of horse domestication.

We next demonstrated significant differences in blood pressure values, as well as protein levels of EDN1 and EDN3, when contrasting homozygous EPH to SPH CBT carriers. The 5.5 kb region contained multiple marks pointing at a potential *cis*-regulatory module. First, the region spanned an equine enhancer (H3K4me1) active in tissues relevant to racing

performance. Second, comparative evidence from human gTEX v8 implicated this region in the regulation of *EDN3* expression across multiple tissues. Third, of the 14 SNPs forming the haplotypes, alleles from 12 showed differential transcription factor binding affinities, suggesting differential regulatory activity between the EPH and SPH carriers. Intriguingly, the SPH haplotype was predicted to have binding affinities for both immune system actors, NFATC2 and IRF2, where IRF2 has been shown to be a negative regulator of NFAT target genes (34) (Supplementary Table S4). On the other hand, the EPH haplotype harbored motifs for factors involved during embryogenesis and cell differentiation, e.g., SRF, HOXA5 and CREB1 (35–37) (Supplementary Table S4). These differing regulatory roles may point to the multifaceted role of EDN3, from early neural crest development and proper function of enteric neurons and melanoblasts (38). The EDN3 ligand has a broad expression range across tissues and has been implicated in diseases such as Hirschsprung's disease, Multiple Sclerosis and Waardenburg syndrome in humans, as well as melanocyte development in mice (39–43). However, a key role for this ligand is in blood flow homeostasis.

Endothelins are involved in one of the most potent vasoregulatory systems, where EDN1 and EDN3 have opposite effects and act synergistically to regulate blood pressure (18). EDN1 increases blood pressure by vasoconstriction, while EDN3 is involved in nitric oxide release, which results in vasodilation and decreased blood pressure (18). Additionally, EDN3 stimulates the secretion of vasopressin, which increases the blood volume by retaining water in the kidneys, while EDN1 has the opposite effect in the kidneys (44–47). In the present study, horses homozygous for EPH showed significantly higher plasma concentrations of EDN3 and lower blood pressure during exercise. In contrast, horses homozygous for SPH had a significantly higher plasma concentration of EDN1 and higher blood pressure during exercise. Blood pressure depends on cardiac output and resistance in peripheral blood vessels,

while total peripheral resistance is affected by the diameter of the arteries and the viscosity of the blood. An increase of EDN3 likely leads to a decrease in blood pressure and an increase in vasodilation of the blood vessels, primarily in the working muscles and the lungs. This means that blood can flow more easily, allowing improved arterial and venous oxygenation. This would, in turn, be beneficial for athletic performance. In humans, infusion of EDN3 caused a decrease in mean arterial blood pressure and vasoconstriction to the visceral organs (48). On the other hand, infusion of EDN1 leads to an increased mean arterial pressure and decreased cardiac output (49,50). This is comparable to a study on horses during anesthesia, which revealed that horses receiving nitric oxide, a vasodilator, had better arterial and venous oxygenation and lower EDN1 plasma concentration than the control group without nitric oxide administration (51). In our study, we did not see any effect of exercise on the endothelin levels, as there were no statistically significant differences in EDN1 or EDN3 levels when contrasting resting and exercising. This is in concordance with previous studies in which no differences in EDN1 levels before and during exercise were observed in healthy horses (22,52). In terms of exercise performance, the present study showed that SPH homozygous horses had a slower recovery than the horses carrying the EPH. This could be because the SPH group had higher blood pressure during the exercise, so it took longer for these individuals to return to resting blood pressure values.

Although the current study demonstrated statistically significant differences in EDN1 and EDN3 levels between the different haplotype groups, there were no differences in blood pressure at rest. In adult horses, normal blood pressure at rest is approximately 130/95 mmHg (systolic/diastolic), in line with the values observed in the current study (53). In humans, it has been demonstrated that elevated blood pressure at rest is negatively correlated with athletic performance (21). Individuals with elevated blood pressure had significantly lower maximal oxygen consumption, ventilatory anaerobic thresholds and heart rate reserves

(difference between an individual's resting heart rate and maximum heart rate) (21). However, unlike humans, high blood pressure in horses is uncommon and most blood pressure measurements on horses are performed to evaluate and monitor hypotension. An increase in blood pressure at rest is most commonly seen as a result of diseases such as laminitis, chronic renal failure or equine metabolic syndrome (54–56).

The spleen represents one important organ for the distribution of blood, acting as a reservoir for red blood cells. The equine spleen reservoir is larger than in any other domestic animal (57). When there is an increased demand, red blood cells stored in the spleen can be released into the system (57). During exercise, the spleen is contracted, which causes an increase in the volume of circulating blood cells, while the volume of plasma is unchanged or even reduced. This leads to an increase in the packed cell volume/hematocrit (i.e., the proportion of blood made up of red blood cells), hemoglobin concentration, and red blood cell count (57). Previous studies have demonstrated significantly larger spleen size in horses used for racing compared to breeds that are not traditionally used for racing (58). In addition, one study demonstrated a significant correlation between blood pressure and spleen volume (59), with the splenic volume decreasing when hypertension was induced. However, when hypotension was induced, there were no significant changes in the splenic volume (59). The results by (59) demonstrated the importance of the spleen for the cardiovascular system in horses. Further studies examining the relationship between blood pressure, spleen volume and expression of EDN3 represent promising avenues for enhancing understanding of the cardiovascular system in horses in relation to exercising.

Our global proteomic analysis resulted in the identification of a total of 582 proteins. Only one such protein, Cathepsin X, was encoded as part of the 20 protein-coding genes located

within 2 Mb surrounding the genomic region investigated (ECA22: 45,718,095 – 47,718,095). In contrast to the ELISA analysis, none of the endothelins were detected. A major obstacle in MS-based plasma proteomics is the large dynamic range in protein concentrations and that the abundant high molecular weight proteins mask the identification and quantification of lower molecular weight proteins, such as the endothelins. The highly abundant proteins contribute to approximately 90 % of the total protein content in plasma. In this study, plasma depletion of the most abundant proteins was performed to facilitate the quantification of less abundant proteins and to improve the sequence coverage (60). However, a limitation of the present study is the quality of the available proteome database for *Equus caballus*, which only includes a limited number of proteins. Additionally, many proteins were excluded due to a low number of biological replicates, and in total, 383 proteins could be included in the comparisons between groups.

When comparing protein levels at rest and during exercise for the different haplotype groups, we observed a clear difference in the number of regulated proteins (Supplementary Table S8). In the SPH group, 21 proteins were detected at higher levels at rest, than during exercise. The GO analysis of these proteins identified several pathways related to injuries, including blood coagulation, hemostasis, and wound healing (Figure 5A). Several of these proteins also have functions related to the immune system. When comparing the protein levels between the haplotype groups at rest, 23 proteins were found regulated. The GO analysis revealed significant pathways mainly related to the immune system. This is consistent with previous studies in which an increase of plasma proteins involved in pathways related to inflammation, coagulation and immune modulation was observed (61,62). A previous genome-wide association analysis of racing performance in CBTs also found correlations for genes related to immune response (63). Racing horses perform intense exercise, which induces a physical

stress response to obtain energy for the working muscles. The released stress hormones (e.g., cortisol) stimulate gluconeogenesis and lipolysis (64). Additionally, the stress hormones affect inflammatory and coagulation processes (61,62,65,66). In a non-inflammatory state, intact endothelial cells maintain blood flow and control coagulation. However, chronic inflammation, oxidative stress and mechanical stress from turbulent blood flow negatively affect the function of the endothelial cells (67,68). Furthermore, in a human study, multi-omics changes in response to exercise were observed, involving pathways related to energy metabolism, oxidative stress and immune response (69). Potentially, SPH horses have more difficulties recovering from exercise due to elevated blood pressure, resulting in higher levels of immunity-related proteins and proteins related to injury healing in the plasma at rest.

It is interesting to note that the protein showing the highest expression difference between SPH and EPH groups at rest was the proteasome subunit beta (PSMB10). Interestingly, the *psmb10* gene has previously been linked to blood pressure regulation in mice. Knockout of the *psmb10* gene was protective for high blood pressure, cardiac fibrosis and inflammation in DOCA salt-treated mice (70). Although the *PSMB10* gene may be involved in blood pressure regulation in the horse, it is notable that there were no differences in blood pressure between the haplotype groups at rest. In the EPH group comparison of protein ratios at rest versus exercise, there were only three proteins with significant differences. Notably, one of the proteins detected was the secretoglobin family 1D member (LOC102150542). Secretoglobins are known to have anti-inflammatory effects in airway disorders such as asthma in both horses and humans (71,72). The increased levels of this protein may play an anti-inflammatory role during exercise by preserving the function of the vascular endothelial cells, contributing to a higher tolerance for intense exercise.

Several studies have demonstrated the effect of the *EDN1* gene and the endothelin receptor gene, *EDNRB*, in horses and humans (73–81). Studies have suggested EDN1 as a marker for different diseases in horses, for example, hyperinsulinemia, neonatal diseases, and cardiac disease (76–78). Moreover, the EDNRB receptor is known to be associated with lethal white foal syndrome (73–75). However, to the best of our knowledge, there is only one study performed on horses involving the *EDN3* gene (13). The current study demonstrates significant differences in blood pressure and plasma levels of both EDN3 and EDN1 in CBTs with different haplotypes. Although the identified gene variant may also act on other genes in the region or other parts of the genome, we believe that we have identified a regulatory variant influencing the transcription of *EDN3*. Based on the ELISA results and racing performance data, the identified candidate SNP appears to have an additive effect, with homozygous carriers and heterozygous horses performing significantly better than homozygous wild-type horses. The obtained results in horses emphasize the importance of the endothelin system as critical for performance and support the functional role previously shown for EDN3 in human blood pressure regulation.

Conclusion

Here we demonstrate, for the first time, a significant association between *EDN3*, blood pressure regulation and athletic performance in horses. We identified a 5.5 kb candidate genomic interval and showed that horses homozygous for the elite performing haplotype had a higher plasma concentration of EDN3, lower plasma concentration of EDN1, and lower blood pressure during exercise compared to horses with the sub-elite performing haplotype. Horses carrying the elite performing haplotype also recovered from exercise faster than those with the sub-elite haplotype. The results reported here are important for understanding the biological mechanisms behind blood pressure regulation, as well as inflammation and

coagulation systems in relation to racing performance in both horses and other mammals, including humans.

Materials and Methods

Horse material and DNA extraction

A summary of the horses included in the study, and specifying which experiments they were used for, is presented in Supplementary Table S2. Genomic DNA from all horses was extracted from hair roots or blood samples. For hair preparation, 186 µL of 5 % Chelex® 100 Resin (Bio-Rad Laboratories, Hercules, CA, USA) and 14 µL of proteinase K (20 mg/mL; Merck KgaA, Darmstadt, Germany) were added to each sample. The mixture was incubated at 56°C for two hours at 600 rpm and inactivated with proteinase K for 10 mins at 95°C. Blood samples were extracted on the Qiasymphony instrument with the Qiasymphony DSP DNA mini or midi kit (Qiagen, Hilden, Germany).

Selective sweep racing performance association analysis with additional horse material

We used the previously identified 19.6 kb selective sweep region, associated with CBT racing performance, as the starting point for this analysis (ECA22:46,702,297-46,721,892; EquCab3.0) (13). In that study, g.46717860-T was the variant most significantly associated with elite performance, and LD to this variant was used to define a five SNP haplotype (r^2 > 0.9). We extracted the 19.6 kb region from an existing 670K Axiom Equine Array genotyping experiment of 661 CBTs (including the 400 horses used for the haplotype analysis in the 2018 study) (13,63). Using g.46717860 as the lead variant, we calculated pairwise r^2 across the region, and performed racing performance association analyses

between this variant, and any additional variant with $r^2 > 0.6$. Given the high LD, a significance threshold was set at $P \leq 0.05$.

The available phenotypic records included a) pedigree information b) *DMRT3* genotypes and c) performance data as individual race records for each horse (Swedish Trotting Association (Svensk Travsport) and the Norwegian Trotting Association (Norsk Rikstoto). The *DMRT3* variant was genotyped using StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with the custom TaqMan SNP genotyping assay (82), and included as a factor in the model, since previous studies have demonstrated major effects of the variant on harness racing performance (10,12). Only competitive races were included (i.e., premie and qualification races were excluded). The performance data included: number of starts, number of wins, number of placings (the number of times a horse finished a race in first, second or third place), fastest kilometer (km) time in a race where the horse did not gallop (in seconds), earnings and earnings per start. The earnings for most horses were recorded in Swedish currency (SEK). Earned prize money in Norwegian currency was converted to SEK based on the average exchange rate for the year the race took place.

All statistical analyses were performed in the R statistical environment. The performance data were tested for normality by computing the skewness coefficient using the package moments v0.14. Non-normally distributed values were transformed, i.e., log transformed values (log10 +1) were used for wins and placings, number of starts were square root transformed and earnings and earnings per start were transformed according to the previously reported formula (ln(earnings + 1000)) (83). Genetic variants were tested in a linear model using ANOVA as a *post hoc* test. Number of starts, age at first start, sex, birth year, country of birth, and the *DMRT3* genotype were included as fixed effects, when significant. Significant

values ($P \leq 0.05$) were further tested using the function lsmeans (Least-Squares Means) with the package emmeans followed by the multiple comparison test Tukey's HSD-test.

Additional variant discovery

To find and assess variants missing from the Axiom Equine Array, but with the potential to influence racing performance, we performed both Illumina short-read WGS and MinION Oxford Nanopore targeted sequencing of PCR products.

First, two CBTs and two SBs were selected for WGS based on their g.46717860 genotype and their performance values. From each breed, horses homozygous for opposite alleles of SNP g.46717860 (CC respectively TT) with either high (TT) or low (CC) earnings per start were selected for sequencing (Supplementary Table S4). Paired-end 150 bp Illumina HiSeqX data was generated to a depth of 15X. Read data were processed according to the GATK 3.8-0 best practices (https://software.broadinstitute.org). Briefly, raw data were trimmed with TrimGalore 0.4.4 and quality checked with QualiMap 2.2. The reads were aligned to the EquCab3.0 reference genome (84) (GCF_002863925.1) using BWA-MEM 0.7.17. SAM files were converted to BAM files using SAMtools, and Picard 2.10.3 was used to sort the BAM files and remove potential PCR duplications. The BAM files were recalibrated in two steps using BaseRecalibrator and PrintReads. Variant calling was performed using HaplotypeCaller with a list of known variants in horses downloaded from the NCBI web page annotation release 103 (https://www.ncbi.nlm.nih.gov). Structural variants were called using default parameters in FindSV comprising the software programs Manta, TIDDIT and CNVNator (https://github.com/J35P312/FindSV).

Next, as input for Oxford Nanopore sequencing (ONT, Oxford, UK), a 25 kb PCR product was designed to span the 19.6 kb sweep region and adjacent repeats. Primers were designed in Primer3Plus and included unique 10 bp barcodes (Supplementary Table S9). DNA input

was taken from four CBTs with g.46717860-TT genotype and high performance and four CBTs with the opposite features, including the two CBTs from the WGS analysis (horse selection as per WGS above and Supplementary Table S2). The PCR region was amplified on a ProFlex PCR cycler (Thermo Fisher Scientific, Waltham, MA, USA) and the long-range PCR using AccuPrime™ Taq DNA Polymerase, High Fidelity kit following manufacturers specifications. The sizes of PCR products were confirmed on 0.8 % TBE Agarose gels, and product concentration quantified with Agilent 4150 TapeStation using the Agilent Genomic DNA ScreenTape Assay (Agilent Technologies, Santa Clara, CA, USA). Samples of the respective PCR products were pooled in equimolar concentration and libraries were prepared using the Genomic DNA by Ligation (SQK-LSK109) protocol. The samples were loaded and sequenced on the MinION. Demultiplexed sequencing data were mapped to EquCab3.0 ECA 22 (GCF_002863925) using minimap2 v. 2.4 sequence alignment program (https://github.com/lh3/minimap2). The resulting SAM files were converted to BAM format using samtools v. 1.8. Single nucleotide variants were called using the variant calling tool longshot v. 0.4.3 with default parameters (https://github.com/pjedge/longshot). Structural genomic variations were identified with NanoSV v. 1.2.4 software (https://github.com/mroosmalen/nanosv), with default parameters. Only variants with a quality above 20 were retained.

Genotyping

The largest INDEL identified from the WGS data was analyzed for association with racing performance traits in 369 CBTs, using linear models. For MassArray SNP genotyping the number of variant positions available was limited to 25. For this reason, 24 SNPs and one INDEL, evenly distributed across the 19.6 kb region, were selected for genotyping. In total, DNA from 412 horses of 13 different breeds and four donkeys were genotyped

(Supplementary Table S10). The horses were carefully selected to include both traditional performance horse breeds, as well as non-performing breeds. Genotyping was performed at the Mutation Analysis Facility at Karolinska University Hospital (Huddinge, Sweden), using iPLEX® Gold chemistry and the MassARRAY® mass spectrometry system (85) (Agena Bioscience, San Diego, CA, USA). Multiplexed assays were designed using MassARRAY® Assay Design Suite v2.2 software (Agena Bioscience, San Diego, CA, USA), genotyping the 25 markers in one reaction per sample. The protocol for allele-specific base extension was performed according to Agena Bioscience's recommendation. Analytes were spotted onto a 384-element SpectroCHIP II array (Agena Bioscience, San Diego, CA, USA), using Nanodispenser RS1000 and subsequently analyzed by MALDI-TOF on a MassARRAY® Analyzer 4 mass spectrometer (Agena Bioscience, San Diego, CA, USA). Genotype calls were manually checked by two persons individually using MassARRAY® TYPER v4.0 Software (Agena Bioscience, San Diego, CA, USA).

The variant g.46717860 was not included as part of the MassArray, but was genotyped for 170 horses (9 Ardennes, 96 CBTs, 4 donkeys, 4 Finnhorses, 2 Fjord horses, 2 Gotlandsruss, 10 North-Swedish draught horses, 3 Pzrewalski and 40 SBs) using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) and the custom TaqMan SNP genotyping assay (82). The reaction volume of 15 µl comprised 1.5 µl DNA, 0.38 µl Genotyping Assay 40X, 7.5 µl Genotyping Master Mix 2X, and 5.62 µl deionized water.

Haplotype analysis

Pairwise r^2 calculations with all 24 SNPs and g.46717860 were performed using the LD-function in R. Based on the high LD between all genotyped SNPs, it was possible to impute g.46717860 alleles for ungenotyped samples. All SNPs with a pairwise r^2 value >0.6, plus

two flanking SNPs were selected for haplotype analysis. Haplotype racing performance association tests used a GLM regression analysis, which was performed in 180 CBTs and 38 SBs using the haplo.glm function from the haplo.stats package in R (86). The model included the effects of sex, age, country of registration, *DMRT3* genotype (only for the CBTs) and number of starts, when significant. All CBTs had been genotyped for the DMRT3 mutation, using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with the custom TaqMan SNP genotyping assay (82). Haplotypes with a frequency < 2 % were considered rare, and were not analyzed for association with performance. A minimum shared haplotype of 14 SNPs between CBTs and SBs was used to define the elite-performing haplotype (EPH) and the sub-elite performing haplotype (SPH).

Association analysis with athletic performance

Allele calls at SNP g.46718095 were a proxy for elite-performing haplotype (EPH) and the sub-elite performing haplotype (SPH). Samples not genotyped on the MassArray were genotyped using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with the custom TaqMan SNP genotyping assay (82). Association with racing performance was performed as per "Selective sweep racing performance association analysis with additional horse material" using 539 CBTs born between 1994 and 2017 and 271 SBs born between 2005 and 2014. Horses of 13 different breeds and three other types of equids (wild ass, onager and donkey) were also genotyped to investigate the distribution of allele frequencies.

Functional bioinformatic analyses

ChIP-seq data were provided by the Equine Functional Annotation of Animal Genomes (FAANG) team (www.faang.org) (24,25) including four histone marks (H3K4me1, H3K4me3, H3K27ac and H3K27me) across nine tissues (adipose, brain, heart, lamina, liver,

lung, muscle, ovary and skin) from two Thoroughbreds. FAANG bam and bed files from the different available tissues, the four histone marks and the input DNA control (project: PRJEB35307) were downloaded to a local server. First, the integrity of the data was checked using checksums. Bam files were sorted with samtools and bedgraphs were subsequently created using bedtools (87,88). The Integrative Genomics Viewer (IGV) browser was used to visualize the output data along with the EquCab3.0 fasta file and its gff3 annotation file (84,89). The presence of H3K27ac and H3K4me1 was considered to mark active enhancers, whereas H3K4me1 and H3K27me3 marked poised enhancers. H3K27ac and H3K4me3 marked active promoters and H3K27me3 was considered to mark repressed regions. The region defined by the minimum shared haplotype (see above) was investigated using IGV for the different histone marks in all available tissues.

A comparative analysis to hg38 was conducted for the minimum shared haplotype region. Co-ordinates were lifted in UCSC (liftOver) and potential overlaps between ENCODE Candidate Cis-Regulatory Elements (cCREs), ENCODE Integrated Regulation tracks and ORegAnno Regulatory elements were annotated (90,91). For each of the SNPs in the minimum shared haplotype region, the potential of alleles to alter transcription factor binding potential was tested with sTRAP (92). Here, 31 bp sequences centered on the allele of interest were tested using JASPAR vertebrates, on a background model of human promoters. In the cases where two SNPs were within 10 bp of each other, they were tested as a single phased fragment.

Ancient DNA screening

We leveraged the availability of an extensive ancient genome time-series in the horse to assess the temporal trajectory and spatial distribution of the SNPs in the minimum shared

haplotype within the CBTs, in the past. More specifically, we re-aligned against the EquCab3.0 reference genome (84) supplemented with the Y-chromosomal contigs from (93) and a sub-selection of the sequencing data from (2,27,29,30,94,95), representing a total of 431 ancient genomes. Alignment files were generated using the Paleomix pipeline (96), and further rescaled and trimmed, following the procedures described in (31). This procedure ensured minimal sequencing error rates, especially at sites potentially affected by post-mortem DNA damage. The number of occurrences of each individual allele was counted for each individual using ANGSD (v0.933), with the -baq 0, -remove_bads -minMapQ 25 -minQ 30 -rmTriallelic 1e-4 -SNP_pval 1 -C 50 parameters (97). The resulting occurrences were tabulated together with metadata available for each individual, consisting of their corresponding archaeological site (name and GPS coordinates) and their average calibrated radiocarbon date (or age as assessed from the archaeological context otherwise). The tabulated file was visualized using the mapDATAge package (26), which provides spatial distributions within user-defined time periods and/or spatial ranges and estimates allelic trajectories (i.e., allelic frequencies through time) based on the resampling of individual allelic counts. The final allelic trajectories were assessed considering time bins of 2,000 years (step-size = 500). To explore the genetic variation for the SNPs within the haplotype, we considered the 14 polymorphic sites i.e., ECA22:46703380, 46708110, 46708983, 46713478, 46715974, 46717451, 46717528, 46717742, 46717854, 46717999, 46718095, 46718113, 46718361, 46718436, 46718895, 46718964, 46719042, 46719906, 46720030, 46720999, and 46721822.

Exercise test, sample collection and blood pressure measurements

Horse material

Thirty horses were enrolled in the exercise test (Supplementary Table S2). All horses were genotyped for SNP g.46717860 and g.46718095 using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with the custom TaqMan SNP genotyping assay as above (82). The two SNPs were used as a proxy for the haplotype variants homozygote EPH (TT respectively CC) or homozygote SPH (CC respectively TT). Horses were then divided into three groups based on haplotype variant, i.e., homozygote EPH or SPH and HET (heterozygote). The horses were carefully selected to include an even sex and age distribution across the different groups. The age of the horses ranged from two to 13 years (mean age = 5.5 years). All horses, except for the two-year old, were in competing condition. However, due to various reasons including practical problems, blood pressure measurements were not taken from all horses at all time points and some horses only had measurements at rest (Supplementary Table S2). For the ELISA and proteomics analysis, plasma samples were collected from 40 horses, before and/or during exercise. As for blood pressure measurement, some horses only had samples collected at rest.

Exercise

All horses, except for the two two-year old horses, were challenged on the same standardized exercise session (n=28). The test was performed on several days during autumn and winter of the years 2020 and 2021. Each horse, with a trainer, completed a 1.5-hour exercise route that included both flat and uphill interval training. This route was commonly used in their daily training program. A Polar M460 sensor with GPS (Polar Electro, Kempele, Finland) was used to record heart rate (HR), speed, distance and amplitude during exercise. The horses performed the exercise on a track with hilly terrain in the forest. The uphill interval section

consisted of the horses trotting four times up a hill with four degrees inclination. Each uphill interval lasted for five mins with a distance of 1.7 km. The HR for each horse reached above 200 beats/min. After the uphill intervals, the horses walked down to the flat part of the track, where they completed 10 mins of intervals, at a speed of 35 km/h and HR of about 180-200 beats/min. Finally, the horses jogged one km back to the stable at a speed of 15-20 km/h. During the exercise, the average HR reached above 190 beats/min for approximately eight mins. Due to their young age the two-year old horses (n=2) performed a standardized exercise session at the racetrack. Following warm-up, they performed a number of intervals with HR reaching over 200 beats/min. The two-year old horses performed the exercise test at the same time.

Blood pressure measurements

The following measurements were collected with a Cardell Veterinary Monitor 9402 cuff (Cardell 10), at the middle coccygeal artery: systolic, diastolic, mean arterial pressure, pulse pressure, and heart rate. The same cuff size was used for all the horses. The monitor has previously been successfully evaluated for use in horses (98). Blood pressure was measured at rest in the stable before the exercise and during exercise (directly and five mins after the last uphill interval) (Figure 6). Additionally, blood pressure was measured every ten mins after exercise, up to 40 mins after the exercise was completed. In order for the horse to acclimate to the equipment, the Polar M460 sensor and the cuff were put on at rest, before the exercise route, and the horse was left in the box for 10 mins before measurements were taken. Similarly, blood pressure was measured five times at rest for the horse to get used to the measurement procedure. The measurements with the highest and lowest systolic blood pressure values were removed. During and after exercise the blood pressure was measured three times and an average calculated. For the association analyses with blood pressure, the mean values of the triplicates were analyzed using ANOVA and Tukey's HSD test.

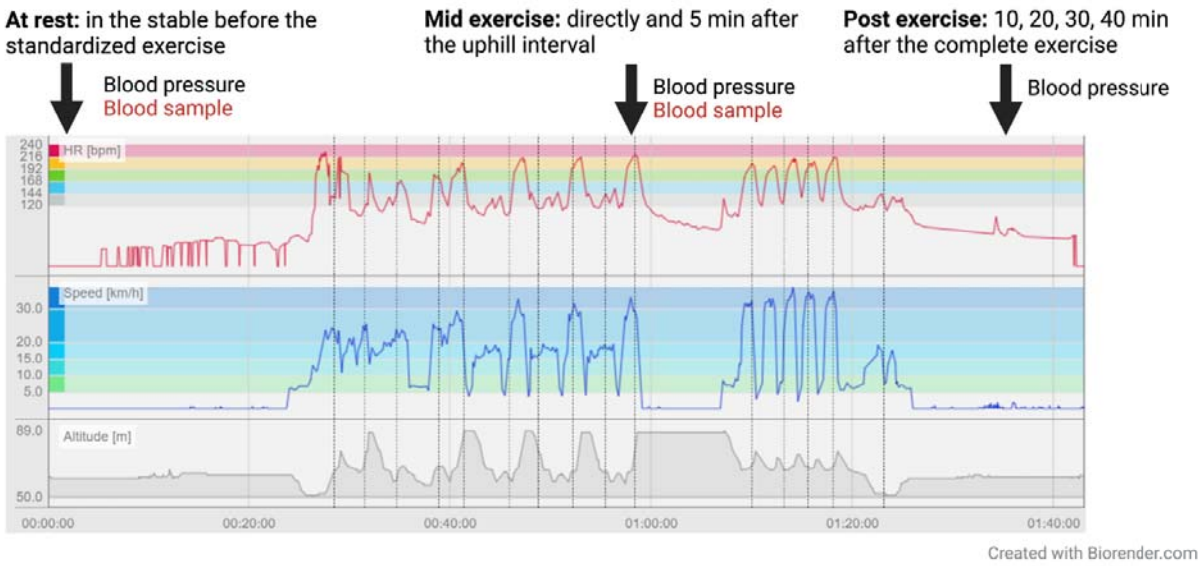

Figure 6. A schematic overview of the parameters recorded during the exercise for each horse, including heart rate, speed and altitude. The arrows indicate when blood samples and blood pressure measurements were taken.

Biological sample collection and analysis

Blood samples were collected at rest before the exercise and during exercise directly after the last uphill interval. Blood was also collected for plasma extraction. Here, a sample was collected in an EDTA tube and mixed gently four times after sampling, to allow the EDTA to mix with the blood, then centrifuged at 1000 x g (3000 rpm) for 20 mins as soon as possible and within two hours after sampling. The tubes were kept cold until centrifugation. The EDTA plasma was aliquoted in Eppendorf tubes and immediately frozen at -20°C and within five days put at -80°C until analysis.

ELISA analysis of EDN1 and EDN3

Plasma EDN1 concentrations were measured using the Horse Endothelin ELISA Kit PicoKine™ EK0945-EQ (Boster Bio, Pleasanton, CA, USA). Plasma EDN3 was quantified with the horse EDN3 ELISA Kit MBS9901200 (MyBioSource, San Diego, CA, USA). The analyses were performed according to the manufacturer's manual. A standard curve was created, and the samples were run in duplicates. Mean values of the concentration (pg/ml) were used in the statistical analysis, and the standard deviation (SD) and coefficient of variation (CV) were calculated. Differences in concentrations between the three groups (EPH, HET and SPH) before and during exercise were analyzed using ANOVA and Tukey´s HSD test. Significance was set at P-values ≤ 0.05.

Relative quantitative proteomic analysis

A global relative quantitative proteomic analysis was performed to identify differentially expressed proteins in plasma before and during exercise. Six horses of each haplotype group (EPH or SPH homozygote) were included in the analyses (Supplementary Table S2). The horses were selected based on earnings per start, age and sex. The two-year-old horses were matched, one from each haplotype group, as they did not perform the same standardized exercise as the other horses.

Sample preparation

The plasma samples and references (10 µl) were depleted from albumin and IgG, using the Pierce™ Albumin/IgG Removal Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The reference was a representative pool that included four groups: EPH at rest, EPH during exercise, SPH at rest and SPH during exercise. The samples were processed using the modified filter-aided sample preparation (FASP) method (99). Half the volume, corresponding to approximately 30 µg, was concentrated on

Microcon-30kDa Centrifugal Filter Units (Merck & Co, Kenilworth, NJ, USA), reduced with 100 mM dithiothreitol at room temperature for two hours, washed several times with 8 M urea and once with digestion buffer (DB) (25 mM TEAB, 0.5 % sodium deoxycholate) before alkylation with 10 mM methyl methanethiosulfonate in digestion buffer for 30 mins. Digestions were performed by adding 1 µg Pierce MS grade Trypsin (Thermo Fisher Scientific, Waltham, MA, USA) in DB and incubating overnight at 37°C. An additional portion of 1 µg trypsin was added, and the samples were incubated for another three hours. Peptides were collected by centrifugation and labeled using Tandem Mass Tag (TMTpro 16plex) reagents (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The labeled samples were combined into two sets, where both sets included all four groups and a reference. The paired samples were analyzed in the same set. Sodium deoxycholate was removed by acidification with 10 % TFA and the sets were desalted using Pierce peptide desalting spin columns (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The two sets were pre-fractionated into 40 fractions with basic reversed-phase chromatography, using a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA), an XBridge BEH C18 column (3.5 µm, 3.0x150 mm, Waters Corp, Milford, MA, USA) and a gradient from 3 % to 90 % acetonitrile in 10 mM ammonium formate with pH 10.0 for 25 mins. The fractions were concatenated into 20 fractions, dried, and reconstituted in 3 % acetonitrile and 0.2 % formic acid.

Liquid chromatography–Mass Spectrometry/ Mass Spectrometry analysis

The fractions were analyzed on an Orbitrap Fusion Lumos Tribrid mass spectrometer interfaced with an Easy-nLC 1200 liquid chromatography system (Thermo Fisher Scientific, Waltham, MA, USA). Peptides were trapped on an Acclaim Pepmap 100 C18 trap column (100 µm x 2 cm, particle size 5 µm, Thermo Fisher Scientific, Waltham, MA, USA) and

separated on an analytical column (75 μm x 35 cm) packed in-house with Reprosil-Pur C18 material (particle size 3 μm, Dr. Maisch, Ammerbuch, Germany) at a flow of 300 nL/min using a gradient from 4 % to 80 % acetonitrile in 0.2 % Formic Acid over 90 mins. The Orbitrap was equipped with the FAIMS Pro ion mobility system alternating between the compensation voltages of -50 and -75. MS scans were performed at 120,000 resolutions, and the 12 most abundant doubly or multiply charged precursors were isolated using 0.7 m/z isolation window and dynamic exclusion within 10 ppm for 60 seconds. The precursors were fragmented by collision induced dissociation (35 %) and detected in the ion trap, followed by multinotch (simultaneous) isolation of the top 10 MS2 fragment ions within the m/z range 400-1400, MS3 fragmentation by higher-energy collision dissociation (55 %) and detection of the reporter ions in the Orbitrap at 50 000 resolution, m/z range 100-500.

Proteomic data analysis

The data files in each set were merged for identification and relative quantification using Proteome Discoverer version 2.4 (Thermo Fisher Scientific, Waltham, MA, USA). The Uniprot *Equus caballus* proteome database (July 2021, combined SwissProt and TrEMBL 44,488 entries) and Mascot search engine v. 2.5.1 (Matrix Science, London, UK) were used in the database matching with peptide and fragment ion tolerance of 5 ppm and 0.6 Da. Tryptic peptides with zero missed cleavage, variable modifications of methionine oxidation and fixed cysteine alkylation were accepted. ProTMT modifications of N-terminal and lysine were selected. Percolator was used for the peptide-spectrum match validation with a strict false discovery rate (FDR) threshold of 1 %. The TMT reporter ions were identified with 3 mmu mass tolerance and no normalization was applied to the samples. For the protein quantification, only unique peptide sequences at a strict FDR threshold of 1 % with a minimum synchronous precursor selection match of 65 % and an average S/N above 10, were

taken into account. The reference in each set was used to calculate protein ratios and quantified proteins were filtered at 5 % FDR.

Processing of quantitative proteome data

The protein ratios of quantified and normalized proteins were processed in Microsoft Excel to calculate the group means and fold changes. All plasma samples were grouped into two sets; before and after exercise. Only proteins quantified in at least three horses from each haplotype group (EPH and SPH), i.e., proteins with at least three biological replicates in each haplotype group, were included in the analyses. Perseus version 1.6.15.20 was used to analyze differences between the groups, by applying t-test on log2 protein ratios. Five different comparisons were made; SPH and EPH at rest and during exercise as well as rest versus exercise within each haplotype. Additionally, paired ratio analysis was performed, to compare differences in protein levels during exercise vs. at rest for each haplotype i.e., (SPH-rest/SPH-exercise)/(EPH-rest/EPH-exercise). For each comparison, fold change values were calculated by dividing the average values for the compared groups and P-values ≤ 0.05 were considered to be significant. GO-annotations downloaded from the Uniprot database into Perseus were used to support the identification of changing biological processes. Furthermore, the GO enrichment analysis and visualization tool g:GOst from g:Profiler was used to identify the biological processes affected in each of the different contrasts (32).

Declarations

Ethics approval and consent to participate

Blood sample collection was approved by the ethics committee for animal experiments in Uppsala, Sweden (number: 5.8.18-15453/2017 and 5.8.18-01654/2020).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study will be deposited to the SRA.

Competing interests

The authors declare that they have no competing interests.

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Authors´ contributions

G.L. designed the project and led the genetic mapping together with J.R.S.M. M.K.R. was supervised with main input from G.L. and R.N. to perform the sampling, genetic mapping and blood pressure measurements. M.Å. designed genotyping assays. A.J. performed genotyping. M.K.R. and K.F. performed haplotype analysis. L.O. performed the mapDATAge analysis. R.N. designed the long-read sequencing experiment, performed ChIP-sequencing and proteomics data analysis. A.T. performed proteomic experiments and analysis. B.D.V. contributed with supervision of M.K.R. and A.R. contributed with mentoring of K.F. B.E .advised the blood pressure measurements. C.M.M. assisted with human comparisons and in the interpretation of the blood pressure results. G.A. performed transcription factor binding site analyses. J.R.S.M. assisted with the comparative genomic

analysis and performed the transcription factor binding site analysis. K.F. lead the writing of the manuscript and created figures, with input from all co-authors.

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References

1. Andersson L. Domestic animals as models for biomedical research. Ups J Med Sci. 2016;121(1):1–11.

2. Fages A, Hanghøj K, Khan N, Gaunitz C, Seguin-Orlando A, Leonardi M, et al. Tracking Five Millennia of Horse Management with Extensive Ancient Genome Time Series. Cell. 2019 May;177(6):1419–35.

3. McGivney BA, Han H, Corduff LR, Katz LM, Tozaki T, MacHugh DE, et al. Genomic inbreeding trends, influential sire lines and selection in the global Thoroughbred horse population. Sci Rep. 2020 Jan 16;10(1):466.

4. Petersen JL, Mickelson JR, Rendahl AK, Valberg SJ, Andersson LS, Axelsson J, et al. Genome-wide analysis reveals selection for important traits in domestic horse breeds. PLoS Genet. 2013;9(1):1–17.

5. Meadows JRS, Lindblad-Toh K. Dissecting evolution and disease using comparative vertebrate genomics. Nat Rev Genet. 2017 Oct;18(10):624–36.

6. Hill EW, Gu J, Eivers SS, Fonseca RG, McGivney BA, Govindarajan P, et al. A sequence polymorphism in MSTN predicts sprinting ability and racing stamina in thoroughbred horses. PloS One. 2010 Jan 20;5(1):1–6.

7. Tozaki T, Sato F, Hill EW, Miyake T, Endo Y, Kakoi H, et al. Sequence variants at the myostatin gene locus influence the body composition of Thoroughbred horses. J Vet Med Sci. 2011 Dec;73(12):1617–24.

8. McGivney BA, Browne JA, Fonseca RG, Katz LM, Machugh DE, Whiston R, et al. MSTN genotypes in Thoroughbred horses influence skeletal muscle gene expression and racetrack performance. Anim Genet. 2012 Dec;43(6):810–2.

9. François L, Jäderkvist Fegraeus K, Eriksson S, Andersson LS, Tesfayonas YG, Viluma A, et al. Conformation Traits and Gaits in the Icelandic Horse are Associated with Genetic Variants in Myostatin (MSTN). J Hered. 2016 Sep;107(5):431–7.

10. Andersson LS, Larhammar M, Memic F, Wootz H, Schwochow D, Rubin CJ, et al. Mutations in DMRT3 affect locomotion in horses and spinal circuit function in mice. Nature. 2012 Aug;488(7413):642–6.

11. Jäderkvist Fegraeus K, Johansson L, Mäenpää M, Mykkänen A, Andersson LS, Velie BD, et al. Different DMRT3 Genotypes Are Best Adapted for Harness Racing and Riding in Finnhorses. J Hered. 2015 Dec;106(6):734–40.

12. Jäderkvist K, Andersson LS, Johansson AM, Árnason T, Mikko S, Eriksson S, et al. The DMRT3 “Gait keeper” mutation affects performance of Nordic and Standardbred trotters. J Anim Sci. 2014 Oct;92(10):4279–86.

13. Jäderkvist Fegraeus K, Velie BD, Axelsson J, Ang R, Hamilton NA, Andersson L, et al. A potential regulatory region near the EDN3 gene may control both harness racing performance and coat color variation in horses. Physiol Rep. 2018 May;6(10):1–12.

14. Horinouchi T, Terada K, Higashi T, Miwa S. Endothelin Receptor Signaling: New Insight Into Its Regulatory Mechanisms. J Pharmacol Sci. 2013;123(2):85–101.

15. Rapoport RM, Merkus D. Endothelin-1 Regulation of Exercise-Induced Changes in Flow: Dynamic Regulation of Vascular Tone. Front Pharmacol. 2017;8:1–9.

16. Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. Nature. 1990 Dec 20;348(6303):730–2.

17. Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, et al. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature. 1990 Dec 20;348(6303):732–5.

18. Rossi GP, Sacchetto A, Cesari M, Pessina AC. Interactions between endothelin-1 and the renin-angiotensin-aldosterone system. Cardiovasc Res. 1999 Aug 1;43(2):300–7.

19. Ostrander EA, Huson HJ, Ostrander GK. Genetics of Athletic Performance. Annu Rev Genomics Hum Genet. 2009;10(1):407–29.

20. Davis PG, Ferguson MA, Alderson NL, Pate RR, Bodary PF, Durstine JL. Effect of exercise duration on plasma endothelin-1 concentration. J Sports Med Phys Fitness. 2005 Sep;45(3):419–23.

21. Mazic S, Suzic Lazic J, Dekleva M, Antic M, Soldatovic I, Djelic M, et al. The impact of elevated blood pressure on exercise capacity in elite athletes. Int J Cardiol. 2015 Feb;180:171–7.

22. McKeever KH, Antas LA, Kearns CF. Endothelin response during and after exercise in horses. Vet J Lond Engl 1997. 2002 Jul;164(1):38–46.

23. R. Foundation for Statistical Computing, Vienna, Austria. R: The R Project for Statistical Computing [Internet]. [cited 2022 Jan 17]. Available from: https://www.r-project.org/

24. Kingsley NB, Kern C, Creppe C, Hales EN, Zhou H, Kalbfleisch TS, et al. Functionally Annotating Regulatory Elements in the Equine Genome Using Histone Mark ChIP-Seq. Genes. 2019 Dec 18;11(1):3.

25. Kingsley NB, Hamilton NA, Lindgren G, Orlando L, Bailey E, Brooks S, et al. “Adopt-a-Tissue” Initiative Advances Efforts to Identify Tissue-Specific Histone Marks in the Mare. Front Genet. 2021 Mar 26;12:1–9.

26. Liu X, Orlando L. mapDATAge: a ShinyR package to chart ancient DNA data through space and time. Wren J, editor. Bioinformatics. 2022 Aug 10;38(16):3992–4.

27. Schubert M, Jónsson H, Chang D, Der Sarkissian C, Ermini L, Ginolhac A, et al. Prehistoric genomes reveal the genetic foundation and cost of horse domestication. Proc Natl Acad Sci [Internet]. 2014 Dec 30 [cited 2022 Aug 17];111(52). Available from: https://pnas.org/doi/full/10.1073/pnas.1416991111

28. Librado P, Der Sarkissian C, Ermini L, Schubert M, Jónsson H, Albrechtsen A, et al. Tracking the origins of Yakutian horses and the genetic basis for their fast adaptation to subarctic environments. Proc Natl Acad Sci. 2015 Dec 15;112(50):6889–97.

29. Librado P, Gamba C, Gaunitz C, Der Sarkissian C, Pruvost M, Albrechtsen A, et al. Ancient genomic changes associated with domestication of the horse. Science. 2017 Apr 28;356(6336):442–5.

30. Gaunitz C, Fages A, Hanghøj K, Albrechtsen A, Khan N, Schubert M, et al. Ancient genomes revisit the ancestry of domestic and Przewalski's horses. Science. 2018 Apr 6;360(6384):111–4.

31. Librado P, Khan N, Fages A, Kusliy MA, Suchan T, Tonasso-Calvière L, et al. The origins and spread of domestic horses from the Western Eurasian steppes. Nature. 2021 Oct;598(7882):634–40.

32. Raudvere U, Kolberg L, Kuzmin I, Arak T, Adler P, Peterson H, et al. g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Res. 2019 Jul 2;47(1):191–8.

33. Outram AK, Stear NA, Bendrey R, Olsen S, Kasparov A, Zaibert V, et al. The Earliest Horse Harnessing and Milking. Science. 2009 Mar 6;323(5919):1332–5.

34. Carneiro FRG, Ramalho-Oliveira R, Mognol GP, Viola JPB. Interferon Regulatory Factor 2 Binding Protein 2 Is a New NFAT1 Partner and Represses Its Transcriptional Activity. Mol Cell Biol. 2011 Jul 15;31(14):2889–901.

35. Miano JM. Role of serum response factor in the pathogenesis of disease. Lab Invest. 2010 Sep;90(9):1274–84.

36. Jeannotte L, Gotti F, Landry-Truchon K. Hoxa5: A Key Player in Development and Disease. J Dev Biol. 2016 Mar 25;4(2):13.

37. Zheng T, Huang J, Xiang X, Li S, Yu J, Qu K, et al. Systematical analysis reveals a strong cancer relevance of CREB1-regulated genes. Cancer Cell Int. 2021 Dec;21(1):530.

38. Bondurand N, Dufour S, Pingault V. News from the endothelin-3/EDNRB signaling pathway: Role during enteric nervous system development and involvement in neural crest-associated disorders. Dev Biol. 2018 Dec;444:S156–69.

39. Baynash AG, Hosoda K, Giaid A, Richardson JA, Emoto N, Hammer RE, et al. Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. Cell. 1994 Dec;79(7):1277–85.

40. Kusafuka T, Wang Y, Puri P. Mutation analysis of the RET, the endothelin-B receptor, and the endothelin-3 genes in sporadic cases of Hirschsprung's disease. J Pediatr Surg. 1997 Mar;32(3):501–4.

41. Kusafuka T, Puri P. Mutations of the endothelin-B receptor and endothelin-3 genes in Hirschsprung's disease. Pediatr Surg Int. 1997 Jan;12(1):19–23.

42. Monti L, Arrigucci U, Rossi A. Insights into Endothelin-3 and Multiple Sclerosis. Biomol Concepts. 2020 Jun 25;11(1):137–41.

43. Read AP, Newton VE. Waardenburg syndrome. J Med Genet. 1997 Aug 1;34(8):656–65.

44. Emori T, Hirata Y, Ohta K, Kanno K, Eguchi S, Imai T, et al. Cellular mechanism of endothelin-1 release by angiotensin and vasopressin. Hypertension. 1991 Aug;18(2):165–70.

45. Imai T, Hirata Y, Emori T, Yanagisawa M, Masaki T, Marumo F. Induction of endothelin-1 gene by angiotensin and vasopressin in endothelial cells. Hypertension. 1992 Jun;19(6_pt_2):753–7.

46. Rossi NF. Effect of endothelin-3 on vasopressin release in vitro and water excretion in vivo in Long-Evans rats. J Physiol. 1993 Feb;461:501–11.

47. Rossi NF. Cation channel mechanisms in ET-3-induced vasopressin secretion by rat hypothalamo-neurohypophysial explants. Am J Physiol-Endocrinol Metab. 1995 Mar;268(3):E467–75.

48. Weitzberg E, Hemsen A, Lundberg JM, Ahlborg G. ET-3 is extracted by and induces potent vasoconstriction in human splanchnic and renal vasculatures. J Appl Physiol. 1995 Oct;79(4):1255–9.

49. Tycho Vuurmans JL, Boer P, Koomans HA. Effects of endothelin-1 and endothelin-1-receptor blockade on renal function in humans. Nephrol Dial Transplant. 2004 Nov 1;19(11):2742–6.

50. Weitzberg E, Ahlborg G, Lundberg JM. Long-lasting vasoconstriction and efficient regional extraction of endothelin-1 in human splanchnic and renal tissues. Biochem Biophys Res Commun. 1991 Nov 14;180(3):1298–303.

51. Grubb T, Edner A, Frendin JH, Funkquist P, Rydén A, Nyman G. Oxygenation and plasma endothelin-1 concentrations in healthy horses recovering from isoflurane anaesthesia administered with or without pulse-delivered inhaled nitric oxide. Vet Anaesth Analg. 2013 Nov;40(6):e9–18.

52. Benamou AE, Art T, Marlin DJ, Roberts CA, Lekeux P. Effect of exercise on concentrations of immunoreactive endothelin in bronchoalveolar lavage fluid of normal horses and horses with chronic obstructive pulmonary disease. Equine Vet J Suppl. 1999 Jul;(30):92–5.

53. Heesch CM, Kline DD, Hasser EM. Control Mechanisms of the Circulatory System. In: Duke´s Physiology of Domestic Animals. 13th ed. John Wiley & Sons, Inc.; 2015. p. 353.

54. Navas de Solis C, Slack J, Boston RC, Reef VB. Hypertensive cardiomyopathy in horses: 5 cases (1995-2011). J Am Vet Med Assoc. 2013 Jul 1;243(1):126–30.

55. Moore JN, Garner HE, Coffman JR. Haematological changes during development of acute laminitis hypertension. Equine Vet J. 1981 Oct;13(4):240–2.

56. Bailey SR, Habershon-Butcher JL, Ransom KJ, Elliott J, Menzies-Gow NJ. Hypertension and insulin resistance in a mixed-breed population of ponies predisposed to laminitis. Am J Vet Res. 2008 Jan;69(1):122–9.

57. Poole DC, Erickson HH. Exercise Physiology of Terrestrial Animals. In: Duke´s Physiology of Domestic Animals. 13th ed. John Wiley & Sons, Inc.; 2015. p. 444.

58. Kline H, Foreman J. Heart and spleen weights as a function of breed and somatotype. Equine Exerc Physiol. 1991;(3):17–21.

59. Lorello O, Heliczer N, Casoni D, Schüpbach G, Navas de Solis C. Correlation of Blood Pressure With Splenic Volume in Horses, Daily Variation in Blood Pressure, and “White Coat Hypertension.” J Equine Vet Sci. 2018 Apr 1;63:41–5.

60. Tu C, Rudnick PA, Martinez MY, Cheek KL, Stein SE, Slebos RJC, et al. Depletion of Abundant Plasma Proteins and Limitations of Plasma Proteomics. J Proteome Res. 2010 Oct 1;9(10):4982–91.

61. Scoppetta F, Tartaglia M, Renzone G, Avellini L, Gaiti A, Scaloni A, et al. Plasma protein changes in horse after prolonged physical exercise: a proteomic study. J Proteomics. 2012 Jul 19;75(14):4494–504.

62. Donovan DC, Jackson CA, Colahan PT, Norton N, Hurley DJ. Exercise-induced alterations in pro-inflammatory cytokines and prostaglandin F2alpha in horses. Vet Immunol Immunopathol. 2007 Aug 15;118(3–4):263–9.

63. Velie BD, Fegraeus KJ, Solé M, Rosengren MK, Røed KH, Ihler CF, et al. A genome-wide association study for harness racing success in the Norwegian-Swedish coldblooded trotter reveals genes for learning and energy metabolism. BMC Genet. 2018 Dec;19(1):80.

64. Goff JP. The endocrine system. In: Duke´s Physiology of Domestic Animals. 13th ed. John Wiley & Sons, Inc.; 2015. p. 635–6.

65. Kostrzewa-Nowak D, Kubaszewska J, Nowakowska A, Nowak R. Effect of Aerobic and Anaerobic Exercise on the Complement System of Proteins in Healthy Young Males. J Clin Med. 2020 Jul 23;9(8):E2357.

66. Barton MH, Williamson L, Jacks S, Norton N. Effects on plasma endotoxin and eicosanoid concentrations and serum cytokine activities in horses competing in a 48-, 83-, or 159-km endurance ride under similar terrain and weather conditions. Am J Vet Res. 2003 Jun;64(6):754–61.

67. Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat Rev Immunol. 2007 Oct;7(10):803–15.

68. Dinh QN, Drummond GR, Sobey CG, Chrissobolis S. Roles of inflammation, oxidative stress, and vascular dysfunction in hypertension. BioMed Res Int. 2014;2014:1–11.

69. Contrepois K, Wu S, Moneghetti KJ, Hornburg D, Ahadi S, Tsai MS, et al. Molecular Choreography of Acute Exercise. Cell. 2020 May;181(5):1112-1130.e16.

70. Yan W, Bi HL, Liu LX, Li NN, Liu Y, Du J, et al. Knockout of immunoproteasome subunit β2i ameliorates cardiac fibrosis and inflammation in DOCA/Salt hypertensive mice. Biochem Biophys Res Commun. 2017 Aug;490(2):84–90.

71. Mootz M, Jakwerth CA, Schmidt-Weber CB, Zissler UM. Secretoglobins in the big picture of immunoregulation in airway diseases. Allergy. 2022;77(3):767–77.

72. Côté O, Clark ME, Viel L, Labbé G, Seah SYK, Khan MA, et al. Secretoglobin 1A1 and 1A1A differentially regulate neutrophil reactive oxygen species production, phagocytosis and extracellular trap formation. PloS One. 2014;9(4):e96217.

73. Metallinos, A.T. Bowling DL, Rine J. A missense mutation in the endothelin-B receptor gene is associated with Lethal White Foal Syndrome: an equine version of Hirschsprung Disease. Mamm Genome. 1998 Jun 1;9(6):426–31.

74. Santschi EM, Purdy AK, Valberg SJ, Vrotsos PD, Kaese H, Mickelson JR. Endothelin receptor B polymorphism associated with lethal white foal syndrome in horses. Mamm Genome. 1998 Apr;9(4):306–9.

75. Yang G. A dinucleotide mutation in the endothelin-B receptor gene is associated with lethal white foal syndrome (LWFS); a horse variant of Hirschsprung disease. Hum Mol Genet. 1998 Jun 1;7(6):1047–52.

76. Gauff F, Patan-Zugaj B, Licka TF. Hyperinsulinaemia increases vascular resistance and endothelin-1 expression in the equine digit: Endothelin-1 and vasoconstriction after hyperinsulinaemia. Equine Vet J. 2013 Sep;45(5):613–8.

77. Gehlen H, Shety T, El-Zahar H, Hofheinz I. Measurement of plasma endothelin-1 concentration in healthy horses and horses with cardiac disease during rest and after exercise. J Vet Med Sci. 2019;81(2):263–8.

78. Giordano A, Castagnetti C, Panzani S, Paltrinieri S, Freccero F, Veronesi MC. Endothelin 1 in healthy foals and in foals affected by neonatal diseases. Theriogenology. 2015 Sep;84(5):667–73.

79. Bye A, Klevjer M, Ryeng E, Silva GJJ da, Moreira JBN, Stensvold D, et al. Identification of novel genetic variants associated with cardiorespiratory fitness. Prog Cardiovasc Dis. 2020 May;63(3):341–9.

80. Kim DS, Wheeler MT, Ashley EA. The genetics of human performance. Nat Rev Genet. 2022 Jan;23(1):40–54.

81. Rautureau Y, Schiffrin EL. Endothelin in hypertension: an update. Curr Opin Nephrol Hypertens. 2012 Mar;21(2):128–36.

82. Hui L, DelMonte T, Ranade K. Genotyping Using the TaqMan Assay. Curr Protoc Hum Genet [Internet]. 2008 Jan [cited 2022 Sep 12];56(1). Available from: https://onlinelibrary.wiley.com/doi/10.1002/0471142905.hg0210s56

83. Árnason T. The Importance of Different Traits in Genetic Improvement of Trotters. In Proceedings of World Congress on Genetics Applied to Livestock Production. University of Guelph, Guelph, Canada; 1994.

84. Kalbfleisch TS, Rice ES, DePriest MS, Walenz BP, Hestand MS, Vermeesch JR, et al. Improved reference genome for the domestic horse increases assembly contiguity and composition. Commun Biol. 2018 Dec;1(1):197.

85. Jurinke C, van den Boom D, Cantor CR, Köster H. Automated genotyping using the DNA MassArray technology. Methods Mol Biol Clifton NJ. 2001;170:103–16.

86. Lake SL, Lyon H, Tantisira K, Silverman EK, Weiss ST, Laird NM, et al. Estimation and Tests of Haplotype-Environment Interaction when Linkage Phase Is Ambiguous. Hum Hered. 2003;55(1):56–65.

87. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009 Aug 15;25(16):2078–9.

88. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinforma Oxf Engl. 2010 Mar 15;26(6):841–2.

89. Thorvaldsdóttir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform. 2013 Mar;14(2):178–92.

90. The ENCODE Project Consortium, Abascal F, Acosta R, Addleman NJ, Adrian J, Afzal V, et al. Expanded encyclopaedias of DNA elements in the human and mouse genomes. Nature. 2020 Jul 30;583(7818):699–710.

91. Lesurf R, Cotto KC, Wang G, Griffith M, Kasaian K, Jones SJM, et al. ORegAnno 3.0: a community-driven resource for curated regulatory annotation. Nucleic Acids Res. 2016 Jan 4;44(D1):D126-132.

92. Thomas-Chollier M, Hufton A, Heinig M, O'Keeffe S, Masri NE, Roider HG, et al. Transcription factor binding predictions using TRAP for the analysis of ChIP-seq data and regulatory SNPs. Nat Protoc. 2011 Dec;6(12):1860–9.

93. Felkel S, Vogl C, Rigler D, Dobretsberger V, Chowdhary BP, Distl O, et al. The horse Y chromosome as an informative marker for tracing sire lines. Sci Rep. 2019 Dec;9(1):6095.

94. Der Sarkissian C, Ermini L, Schubert M, Yang MA, Librado P, Fumagalli M, et al. Evolutionary Genomics and Conservation of the Endangered Przewalski's Horse. Curr Biol. 2015 Oct;25(19):2577–83.

95. Orlando L, Librado P. Origin and Evolution of Deleterious Mutations in Horses. Genes. 2019 Aug 28;10(9):649.

96. Schubert M, Ermini L, Sarkissian CD, Jónsson H, Ginolhac A, Schaefer R, et al. Characterization of ancient and modern genomes by SNP detection and phylogenomic and metagenomic analysis using PALEOMIX. Nat Protoc. 2014 May;9(5):1056–82.

97. Korneliussen TS, Albrechtsen A, Nielsen R. ANGSD: Analysis of Next Generation Sequencing Data. BMC Bioinformatics. 2014 Dec;15(1):356.

98. Söder J, Bröjer JT, Nostell KE. Interday variation and effect of transportation on indirect blood pressure measurements, plasma endothelin-1 and serum cortisol in Standardbred and Icelandic horses. Acta Vet Scand. 2012 Jun 10;54:37.

99. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009 May;6(5):359–62.

Table 7. Fold-change and P-value for the top most upregulated proteins in each comparison in the quantitative proteomic analysis, including all proteins significant for more than one comparison

					Comparison							
					1. SPH exercise vs. rest		**2. EPH exercise vs. rest**		**3. EPH vs. SPH rest**		**5. EPH vs. SPH Paired ratio**	
Protein function		**Protein**	**Gene**	**Accession**	**FC**	**P-value**	**FC**	**P-value**	**FC**	**P-value**	**FC**	**P-value**
Immune System	*Adaptive immune system	Proteasome subunit beta	*PSMB10*	F6SZ35					6.03	0.03	8.2	0.03
	*Adaptive immune system, angiogenesis	CD34 molecule	*CD34*	F6S1S0	4.33	0.03			3.42	0.03	17.97	0.04
	*Antigen binding	Ig-like domain-containing protein	*Unknown*	A0A3Q2HY39					5.58	0.02		
	*Antigen binding	Ig-like domain-containing protein	*Unknown*	A0A3Q2GXU9					5.44	0.03		
	*Complement	Complement C1r	*C1R*	F6Z5L1	4.69	0.02					4.09	0.03

activation, complement and coagulation cascades									
*Complement activation, inflammatory response	C4a, anaphylatoxin	*LOC100059239*	F6XSF7			2.08	0.04	5.6	0.04
*Complement activation, inflammatory response	C3-beta-c	*LOC100060505*	A0A3Q2HWQ6	2.54	0.05			5.83	0.05
*Complement and coagulation cascades, metabolism of proteins	Carboxypeptidase B2	*CPB2*	F6PSH3	4.31	0.01			7.94	0.03
*Innate immune system	Cystatin C	*CST3*	A0A3Q2I5T2	2.32	0.02	4.23	0.007	5.91	0.02

	*Innate immune system	Canopy FGF signaling regulator 3	*CNPY3*	F7CNK7	5.05	0.04						
	*Innate immune system, neutrophil degranulation, angiogenesis	Serine peptidase inhibitor, Kunitz type 1	*SPINK5*	A0A5F5Q0T5	3.22	0.04			4.65	0.03	18.49	0.04
	*Small molecule binding, allergenic capacity	Equ c 2 allergen	*LOC106781327*	A0A3G1Z1K7			0.28	0.001				
	*Small molecule binding, allergenic capacity	Major allergen Equ c 1	*LOC100056556*	A0A5F5PQP7			0.24	0.04				
Metabolism	*Metabolism gluconeogenesis	Malate dehydrogenase 1	*MDH1*	A0A5F5PWG8	4.11	0.05						
	*Metabolism of proteins	Tripeptidyl aminopeptidase	*TPP1*	F6TJX5					2.88	0.04	10.13	0.05

Neuronal	*Neuronal system	Neurexin 2	*NRXN2*	F6YED9					5.85	0.005		
Others	*Serine-type endopeptidase inhibitor activity	Serine peptidase inhibitor Kazal type 5	*SPINT1*	A0A3Q2IKF1							6.35	0.04
	*Amino acid metabolism	T1-TrpRS	*WARS1*	A0A3Q2KTL8	3.51	0.02			3.48	0.01	13.11	0.005
	*Binding of androgens and other steroids	Secretoglobin family 1D member	*LOC102150542*	A0A3Q2HLK8			0.17	0.0009				
	*Hemostasis and regulation of insulin-like growth factor (IGF) transport and uptake	Fibrinogen gamma chain	*FGG*	A0A5F5PPB8	2.8	0.05					4.28	0.05