Veterinary Parasitology 334 (2025) 110379

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Profiling host- and parasite-derived miRNAs associated with *Strongylus vulgaris* infection in horses

Katrine Toft [a,*], Marie Louise Honoré [a,1], Nichol Ripley [b], Martin K. Nielsen [b], Maibritt Mardahl [c], Bastian Fromm [d], Ylva Hedberg-Alm [e], Eva Tydén [e], Lise N. Nielsen [a], Peter Nejsum [f], Stig Milan Thamsborg [g], Susanna Cirera [g], Tina Holberg Pihl [a]

[a] Department of Veterinary Clinical Sciences, University of Copenhagen, Copenhagen, Denmark
[b] M.H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY, USA
[c] Data-set-go, Silkeborg, Denmark
[d] The Arctic University Museum of Norway, UiT, the Arctic University of NorwayTromsø, Norway
[e] Department of Biomedical Science and Veterinary Public Health, Parasitology Unit, Swedish University of Agricultural Sciences, Uppsala, Sweden
[f] Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
[g] Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark

ARTICLE INFO

Keywords:
Nematode
MicroRNA
Host-parasite interaction
Tissue-response
Biomarker

ABSTRACT

The equine bloodworm, *Strongylus vulgaris*, is a common and highly pathogenic parasite in horses due to its migratory life cycle involving the intestinal arteries. Current diagnostic techniques cannot detect the prepatent migrating stages of *S. vulgaris*, highlighting the need for new biomarkers. Parasites release microRNAs (miRNAs) into their environment, which could potentially be detectable in host blood samples. Additionally, host miRNA expression patterns may change in response to infection. This study aimed to identify miRNAs associated with *S. vulgaris* infection by profiling the horse's miRNA response in the larval predilection site, the Cranial Mesenteric Artery (CMA) and examining the circulating parasite and horse-derived miRNAs in plasma of *S. vulgaris*-infected horses. Plasma samples were collected from 27 horses naturally infected with *S. vulgaris* and 28 uninfected horses. Arterial tissue samples from the CMA and Aorta were collected from a subset (n = 12) of the infected horses. Small RNA sequencing (small RNAseq) of a subset of the plasma samples (n = 12) identified miRNAs of interest, followed by quantitative real-time PCR (qPCR) evaluation of selected miRNAs in plasma from a larger cohort of horses. Small RNAseq detected 138 parasite-derived and 533 horse-derived miRNAs in the plasma samples. No difference in parasite-derived miRNA abundance was found between the infected and uninfected horses, but 140 horse-derived miRNAs were significantly differentially abundant between the two groups. When evaluated by qPCR, none of the selected parasite-derived miRNAs were detectable in plasma, but seven horse-derived miRNAs were confirmed differentially abundant in plasma between the two groups. Seven horse-derived miRNAs were differentially expressed in CMA tissue affected by migrating *S. vulgaris* compared with unaffected aortic tissue, with Eca-Mir-223–3p (Log2FC: 4.74) and Eca-Mir-140–3p (Log2FC: −3.64) being most differentially expressed. A receiver operating characteristic curve analysis suggested that Eca-Mir-486–5p and Eca-Mir-140–3p had the best diagnostic performance for distinguishing between infected and uninfected horses, with areas under the curve (AUC) of 0.78 and 0.77, respectively. Notably, Eca-Mir-140–3p was associated with age, and correcting for interaction with age increased the AUC to 0.96. In conclusion, several horse-derived miRNAs were associated with *S. vulgaris* infection and could differentiate between infected and uninfected horses based on their plasma abundance. However, the levels of these miRNAs were influenced by other factors (i.e age, breed), complicating their use as biomarkers. Parasite-derived miRNA abundance did not differ between *S. vulgaris* infected horses and those infected with other parasites using small RNAseq and were below detection limits of qPCR.

* Correspondence to: Department of Veterinary Clinical Science, University of Copenhagen, Agrovej 8, Taastrup 2630, Denmark.
E-mail address: katrine.t.nielsen@sund.ku.dk (K. Toft).
[1] Current affiliation: Novo Nordisk, Vandtårnsvej 108, 2860 Søborg, Denmark

https://doi.org/10.1016/j.vetpar.2024.110379
Received 10 September 2024; Received in revised form 11 December 2024; Accepted 16 December 2024
Available online 17 December 2024

1. Introduction

The gastrointestinal helminth *Strongylus vulgaris* is considered one of the most pathogenic equine parasites. The parasite is characterized by a long prepatent period of six to seven months and a life cycle involving both the horse's intestinal arteries and intestinal tract. After ingestion, the infective third (L3) larval stage penetrates the intestinal mucosa, molts into the fourth (L4) stage larvae and migrates to its predilection site, the Cranial Mesenteric Artery (CMA). Here larvae reside for approximately four months, while they develop into the fifth (L5) larval stage (Duncan and Pirie, 1972). During these four months, the larvae cause endarteritis and thrombus formation in the CMA and nearby branches (Duncan and Pirie, 1972; Morgan et al., 1991). The thrombotic material can subsequently detach and occlude smaller arteries downstream, causing potentially fatal non-strangulating intestinal infarctions (NSII) in the associated part of the intestinal tract (Enigk, 1951; Curtis, 1964; Drudge et al., 1966; Sutoh et al., 1976).

Diagnosis of *S. vulgaris* is challenging, especially due to the long prepatent period, which is also when the parasite causes arterial damage. Currently available methods for *S. vulgaris* detection include coproculture (Henriksen and Korsholm, 1983) with larval identification (Russell, 1948) or fecal qPCR (Nielsen et al., 2008), both methods relying on fecal egg excretion. A serum ELISA test has been developed for the detection of *S. vulgaris* antibodies (Andersen et al., 2013), but this method is currently not commercially available and is more suitable for evaluation of herd exposure to the parasite (Nielsen et al., 2015). Historically, due to *S. vulgaris'* high pathogenicity and high prevalence (Russell, 1948; Slocombe and McCraw, 1973; Lyons et al., 1990), controlling this parasite has long been a main priority in parasite control strategies (Drudge and Lyons, 1966; Drudge, 1979). Presumably, the focus on *S. vulgaris* control and intense treatment regimens with effective anthelmintics led to a drastic decrease in *S. vulgaris* prevalence during the 1980s (Reinemeyer et al., 1984; Lyons et al., 2000). Today, *S. vulgaris* is uncommon in many managed equine operations (Nielsen et al., 2012a; Tzelos et al., 2017; Jürgenschellert et al., 2022). However, studies in Denmark and Sweden have shown increasing prevalences of *S. vulgaris* in recent years (Nielsen et al., 2012b; Tydén et al., 2019), which has been suggested to be related to prescription-only legislations resulting in lower anthelmintic treatment intensities (Nielsen et al., 2012b; Tydén et al., 2019). A more restrictive surveillance-based anthelmintic strategy is currently recommended by parasitologists, due to emerging anthelmintic resistance in a wide range of other parasites (ESCCAP, 2019; Rendle et al., 2019; Nielsen et al., 2024). For optimal outcomes of a surveillance-based strategy, a reliable test for detecting *S. vulgaris* is crucial.

MicroRNAs (miRNAs) are small non-coding RNAs involved in gene regulation at the post-transcriptional level. Since miRNAs were discovered in *Caenorhabditis elegans* (Lee et al., 1993), many studies have explored both their function and possible applications in treatment and diagnostics. Tissue miRNA levels are altered during disease and consequently some miRNAs are secreted into extracellular fluids (circulating miRNAs), where they are remarkably stable (Chen et al., 2008; Mitchell et al., 2008). This phenomenon has been widely explored in cancer research and has further been extended into numerous other research areas. In horses, changes in miRNA expression have been investigated in conditions such as asthma (Issouf et al., 2019), sarcoids (Unger et al., 2019, 2021; Cosandey et al., 2021) and osteoarthritis (Castanheira et al., 2021; Andersen et al., 2024). miRNAs have also shown variable expression patterns in endurance racing (Mach et al., 2016; Cappelli et al., 2018; de Oliveira et al., 2021) and in relation to demographic factors such as breed, age and sex (Pacholewska et al., 2016; Cosandey et al., 2021; Unger et al., 2021).

In parasitology, miRNAs have become an area of interest, especially since it was discovered that parasites excrete/secrete miRNAs, possibly to interact with the host immune system (Buck et al., 2014; Hansen et al., 2019; Liu et al., 2019; Cavallero et al., 2022). Parasite-derived miRNAs are in some cases detectable in blood samples from infected individuals, making them potential candidates as diagnostic biomarkers of infection (Cheng et al., 2013; Hoy et al., 2014; Ghalehnoei et al., 2020; He and Pan, 2022). Furthermore, the host's endogenous miRNA response to parasite infection has been investigated for *A. suum* in pigs, elucidating differences in circulating pig-derived miRNA abundance between infected and uninfected individuals (Hansen et al., 2016; Whitehead et al., 2024).

In a previous study, we annotated the microRNAome of L4 and L5 *S. vulgaris* larvae and described miRNAs excreted from the parasite *in vitro* via excretory/secretory products (Toft et al., 2024) By combining these data with evaluation of horse-derived miRNA expression in response to infection, we aimed to describe horse and parasite-derived miRNAs associated with *S. vulgaris* infection, both locally in arterial tissues and circulating in blood samples. Furthermore, we aimed to evaluate the diagnostic potential of the identified circulating parasite and horse-derived miRNAs as biomarkers of *S. vulgaris* infection.

2. Materials and methods

2.1. Ethical approvals

The Institutional Animal Care and Use Committee at the University of Kentucky (protocol no. 2012– 1046) approved use of horses from the University of Kentucky's parasitology research herd (UKPRH). Inclusion of patients and teaching animals from University of Copenhagen Large Animal Teaching Hospital (LATH) was approved by the Danish animal experimental council (approval no. 2018–15–0201–01480 and 2022–15–0201–01210) and by the ethical board of the LATH. The committee for animal experimentation in Lund, Sweden (approval no. 5.8.18–02993/2022) approved use of samples from patients from the Equine Clinic at the Swedish University of Agricultural Sciences (SLU). Owner consent was obtained for all patients included.

2.2. Study population

Horses included in this study consisted of two overall groups: horses with confirmed *S. vulgaris* infection and uninfected horses. The horses were included from the five subgroups described in Table 1. Overall, 63 horses were initially included, with 27 horses belonging to the *S. vulgaris* infected group and 36 horses to the uninfected group. Methods used for determination of *S. vulgaris* status for each subgroup can be found in Table 1. To exclude horses with significant systemic inflammation and hemolytic samples (factors which might influence circulating horse-derived miRNA levels (Shkurnikov et al., 2016; Unger et al., 2019; Das and Rao, 2022)), we applied the exclusion criteria of serum amyloid A (SAA) levels $>$ 100 mg/L and hemoglobin concentrations $>$ 15 mg/dl, for samples undergoing analysis of horse-derived miRNAs. Each subgroup was further evaluated for other signs of systemic disease as described in Table 1. Eight horses were consequently excluded from evaluation of horse-derived miRNAs, resulting in a final inclusion of 55 horses, with 27 belonging to the *S. vulgaris* infected group and 28 belonging to the uninfected group. All 63 samples were included for analysis of parasite-derived miRNAs. Samples were in some cases used in multiple parts of the study, while others were used in only one part. Table 2 shows the final inclusion of samples in the different parts of the study. All samples for the study were collected between 2018 and 2023.

2.3. Diagnostics and treatment regimen for uninfected control horses

Prior to blood sampling for miRNA evaluation, horses in the CPHunA group were subjected to a diagnostics and treatment regimen.. The regimen was designed specifically to target all stages of *S. vulgaris*. Diagnostics included fecal egg count by modified McMaster (concentration McMaster technique) (Roepstorff et al., 1998), examination for *S. vulgaris* larvae by coproculture (Henriksen and Korsholm, 1983) with

Table 1
Table of the five subgroups included in the study. Subgroup alias: refers to the abbreviation used for each specific subgroup in this paper (complete alias in parentheses). Initially included; indicates the number (n) of horses included prior to the application of exclusion criteria. *Strongylus vulgaris* status indicates if horses in the group were infected with *S. vulgaris* or uninfected.

Subgroup alias	Initially included	*Strongylus vulgaris* status	Subgroup description
KYinfF (Kentucky infected foals)	n = 19	Infected	Foals (<1 year) naturally infected with *S. vulgaris* from the UKPRH (Lyons et al., 1990; Steuer et al., 2022). - Confirmation of pre-patent *S. vulgaris* infection by necropsy - No overt clinical signs or recent history of disease - No macroscopic signs of non-parasite related disease on necropsy
KYinfA (Kentucky infected adults)	n = 8	Infected	Adult horses (>1 year) naturally infected with *S. vulgaris* from the UKPRH (Lyons et al., 1990). - Confirmed *S. vulgaris* egg excretion by fecal qPCR (Steuer et al., 2022) - No overt clinical signs or recent history of disease
KYunA (Kentucky uninfected adults)	n = 8	Uninfected	Adult horses (>1 year) from the UKPRH without *S. vulgaris* infection, but infected with other gastrointestinal parasites (Lyons, 2003) - Free of *S. vulgaris* infection based on herd history of frequent anthelmintic treatment and no findings of *S. vulgaris* on regularly performed necropsies (none of the included horses in this group were necropsied). - No overt clinical signs or recent history of disease
SCANunF (Scandinavian uninfected foals)	n = 16	Uninfected	Foals (<14 days) admitted to the LATH or the SLU. - Negative for migrating *S. vulgaris* based on age - No signs of systemic inflammation based on general demeanor, rectal temperature, WBC, and iron levels.
CPHunA (Copenhagen uninfected adults)	n = 12	Uninfected	Adult horses (>1 year) from the LATH teaching herd. - Negative for *S. vulgaris* based on coproculture prior to anthelmintic treatment - Received anthelmintic treatment on two occasions prior to blood sampling. - Treatment regimen outlined in Section 2.3. - No signs of disease on clinical examination

Abbreviations: LATH: Large Animal Teaching Hospital, University of Copenhagen. UKPRH University of Kentucky's parasitology research herd. SLU: Equine Clinic at the Swedish University of Agricultural Sciences. WBC: White blood cell count

Table 2
Table showing subgroups and numbers (n) of horses included in different parts of the study. Target indicates if the analysis targeted horse or parasite-derived miRNAs. Analysis indicates which method was employed and material indicates if plasma or tissue was analyzed.

Target	Parasite and horse-derived miRNAs	Parasite-derived miRNAs	Horse-derived miRNAs	Horse-derived miRNAs
Analysis	Small RNAseq.	qPCR	qPCR	qPCR
Material	Plasma	Plasma	Plasma	Arterial tissue
Subgroups, number of horses (n)	KYinfF (n = 6) KYunA (n = 6)	KYinfF (n = 19) KYunA (n = 8) KYinfA (n = 8) CPHunA (n = 12) SCANunF (n = 16)	KYinfF (n = 19) KYunA (n = 8) KYinfA (n = 8) CPHunA (n = 11) SCANunF (n = 9)	KYinfF (n = 10)
Total	n = 12	n = 63	n = 55	n = 10

Abbreviations: Small RNAseq: Small RNA sequencing; qPCR: Quantitative real-time PCR; KYinfF: Foals from the University of Kentucky Research herd infected with *Strongylus vulgaris*; KYinfA: Adult horses from the University of Kentucky Research herd infected with *S. vulgaris;* KYunA: Uninfected adult horses from the University of Kentucky Research herd; SCANunF: Uninfected foals from the Large Animal Teaching Hospital of Copenhagen or the Equine Clinic at the Swedish University of Agricultural Sciences; CPHunA: Uninfected adult horses from the Large Animal Teaching Hospital of Copenhagen.

morphological larval identification (Russell, 1948) and identification of tapeworm eggs by Proudman's test (Proudman and Edwards, 1992). Regardless of infection status, all horses were treated with moxidectin at a (400 µg/kg body weight) (Equest, Zoetis, Parsippany, NJ, USA) and an ivermectin/praziquantel combination (200 µg ivermectin and 1.5 mg praziquantel per kg body weight) (Equimax, Virbac, Carros, France) at week 1 and again with Moxidectin (Equest, Zoetis, Parsippany, NJ, USA) at week 8. Blood samples were collected at the end of week 10 after confirmation of successful deworming (full anthelmintic effect) by reiteration of the diagnostic tests. An overview of the diagnostics and treatment regimen can be viewed in Supplementary File 1.

2.4. Blood sampling

For all horses, blood samples were collected in 3.2 % 0.109 M sodium citrate tubes and serum separator tubes (BD, Franklin Lakes, NJ, USA) for plasma and serum analysis respectively. All samples were collected by jugular venipuncture via a vacutainer system or via a venous catheter where the first 10 ml of blood had been discarded. The blood samples were separated by centrifugation at 2000 *g* for 15 min and the plasma or serum fraction were collected, batched and stored at −70 °C before further analysis.

2.5. Necropsy and collection of arterial tissue samples

Included foals from the KYinfF group were euthanized by intravenous injection with pentobarbital and necropsy was performed shortly thereafter. *Strongylus vulgaris* infection was confirmed by visual inspection of the CMA and presence of larvae. Arterial tissue samples were collected from a subset (n = 12) of the necropsied foals. During necropsy, the heart with the most proximal part of the Aorta as well as the Cranial Mesenteric and Celiac Arteries with the connected part of the Aorta, were exteriorized. For each foal, four tissue samples were collected using a 0.4 mm biopsy punch (Integra Lifesciences, Princeton, NJ, USA). Two samples were collected from the CMA and two from the Aorta (serving as a negative control) at the level of the aortic arch. When

an aneurism was present in the CMA, the tissue samples were collected at the site of the aneurism (the largest aneurism was chosen in case of multiple aneurisms encountered). If no aneurism was present, a sample was collected in the area where larvae had been located. The collected tissues were rinsed in cold, sterile PBS before being snap frozen in liquid nitrogen and subsequently stored at −70°C.

2.6. Clinicopathological parameters

Relevant clinicopathological information was collected from the subgroups, according to the inclusion criteria outlined in Table 1**.** Due to their semi-feral nature, clinical parameters from horses in subgroups KYinfF, KYinfA, and KYunA were not obtainable, but health status was based on recent herd history, general demeanor and (subgroup KYinfF only) necropsy results. Relevant clinical and laboratory parameters were obtained from the medical records for horses in the SCANunF and CPHunA subgroups. Serum Amyloid A (SAA) was measured for all groups using an immunoturbidometric assay (VET-SAA, Eiken Chemical Co., Japan) as described by Jacobsen et al. (2019). All plasma samples were assessed for degree of hemolysis using an Atellica CH Analyzer (Siemens Healthcare Diagnostics Inc, Malvern, PA, USA).

2.7. RNA extraction and quality control of RNA integrity

Total RNA was extracted using miRNeasy Serum/Plasma Kit for the plasma samples and the miRNeasy Mini Kit for the arterial tissue samples (both Qiagen, MD, USA). RNA extraction was conducted according to the manufacturer's instructions, with the following minor adjustments: instead of 140 µl, 200 µl chloroform was used and initial centrifugation was 30 min instead of 15 min. A Nanodrop One^{c} spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for assessment of purity and quantification of RNA content in all samples. The tissue samples were furthermore evaluated on a Bioanalyzer (Agilent, Hilden, Germany) using the RNA 6000 Nano Kit (Agielnt, Hilden, Germany). For evaluation of DNA integrity, as an indication of tissue necrosis, a subset of CMA and aortic tissue samples was subjected to DNA purification followed by agarose gel electrophoresis (data not shown). Plasma samples were submitted for both sequencing and qPCR analyses, while the arterial tissue samples were used only for qPCR analysis.

2.8. Small RNA sequencing

Six µl of total RNA per plasma sample (n = 12) were submitted for small RNAseq to an external provider (Omiics, Aarhus, Denmark). Briefly, library preparation was performed using the QIAseq miRNA Library Kit (Qiagen, MD, USA), according to the manufacturer's protocol and unique molecular indices (UMIs) were introduced during the cDNA synthesis. Library quality control using Bioanalyzer High sensitivity DNA Analysis Kit (Agilent, Hilden, Germany) was performed to confirm that the fragments were of the expected size. Size selection was performed using AMPure XP beads (Agencourt, Beckmann-Coulter, USA). The libraries were quantified by the KAPA Library Quantification Kit (Roche, Basel, Switzerland) and pooled equally. The library pool was sequenced on a Novaseq 6000 S4 sequencing machine (Illumina, San Diego, CA, USA) to a depth of 100 million reads per sample.

2.9. Bioinformatics analysis

Overall, a custom bash pipeline was created to obtain read counts mapping to a given reference (horse or *S. vulgaris*), returning a count matrix with a number of miRNA reads in the given sample. A detailed description of the pipeline can be found in Supplementary File 2. The steps of the custom pipeline, which were performed for both horse and parasite-derived miRNAs, were as follows: Reads from each sample were quality control (QC) checked by FastQC (Andrews, 2010) and UMI-tools extract was used to copy the UMI for each read into the fastq identifier line (Smith et al., 2017). Cutadapt (Martin, 2011) was employed for adapter trimming, and to retain only high Phred quality reads of length 18–30nt. Using Bowtie-build, a Bowtie index was built for each reference. Each read was aligned to the resulting bowtie index using Bowtie with the parameters given below for each reference (Langmead et al., 2009). Alignments for parasite-derived miRNAs were performed against our previously annotated *S. vulgaris* precursor miRNAome (Toft et al., 2024). The horse-derived miRNA read counts were mapped to the reference with bowtie -k 1 -v 0 -a –norc –best —strata. The horse reference was the horse precursor miRNAome as annotated by MirGeneDB (Fromm et al., 2022). The resulting sequence alignment (SAM) file was filtered to contain mapped alignments only, with 'samtools view -F 4′ (Li et al., 2009). The SAM file was converted to a binary alignment (BAM) file, sorted and indexed with samtools. Deduplication of PCR duplicated reads was achieved using UMICollapse on the BAM file (Tsagiopoulou et al., 2021). The BAM output file was converted to fastq using samtools fastq (Danecek et al., 2021) and reads in the resulting fastq file were collapsed by Fastx_collapser (Hannon, 2010). Finally, to generate a count matrix, MirDeep-quantify (Friedländer et al., 2012) was used with the following parameters: –weighed –mature5p3p –config –norpm. The alignment counts for parasite and horse-derived miRNAs were quantified using our annotated *S. vulgaris* miRNome and the horse miRNAome as annotated by MirGeneDB (Fromm et al., 2022).

2.10. Differential abundance analysis of sequencing data

All analyses were conducted using the software R studio (Posit team, 2023). For the parasite-derived data, miRNAs with > 10 total raw counts were included for differential abundance analysis, while only miRNAs with > 100 total raw counts were included for the horse-derived miRNA analysis. Raw count data for both the parasite and horse-derived miRNAs were normalized using the geometric mean-based method deployed by the Deseq2 package (Love et al., 2014). A Heat map was generated based on the normalized and rlog transformed counts using the package pheatmap (Kolde, 2022). Paralogues with identical counts and log2 fold changes (Log2FC) were collapsed into a single miRNA, representing all differentially abundant paralogues of the miRNA. Differential expression analyses were likewise conducted using the Deseq2 package, comparing the miRNA abundance in the *S. vulgaris* infected group to the uninfected group, for both parasite and horse-derived miRNAs.

2.11. Quantitative real-time polymerase chain reaction (qPCR)

Two miRNA panels (parasite-derived and horse-derived) were chosen for further qPCR assessment in a larger cohort of horses. The miRNAs in both panels were chosen based on their abundance in the sequencing data and additional horse-derived miRNAs were selected based on studies of miRNAs involved with endothelial pathology in humans (Fu et al., 2015; Stather et al., 2015; Zhang et al., 2015). The miRNAs chosen for evaluation in the CMA and Aorta tissue were likewise based on insights from human studies on endothelial injury (Biros et al., 2014; Borek et al., 2019). Not all the selected miRNAs were included in both panels, since some miRNAs were expected to be of interest in blood but not in arterial tissue and vice versa. In each of the miRNA panels, two miRNAs were included as potential normalizers based on their stable expression in the sequencing data or experience of their stability in plasma or tissue in other studies (Xiang et al., 2014; Balaskas et al., 2018; Castanheira et al., 2021).

Extraction of RNA was performed as described in Section 2.7 of this paper. For all samples qPCR was performed, according to the miRspecific method (Balcells et al., 2011; Cirera and Busk, 2014). cDNA was synthesized in duplicate for each sample according to Balcells et al., (2011). Briefly, a mixture containing 100 ng of total RNA, 10X PAP buffer (1 µL) (NEB, MA, USA) 1 µl RT standard primer CAGGTCCAGTTTTTTTTTTTTTTTVN (10 µM), dNTP mix (1 mM), ATP (1 mM),

M-MuLV Reverse Transcriptase (10 units) (NEB, MA, USA) and Poly(A) polymerase (0,2 μl) (NEB, MA, USA) in a total volume of 10 μL was incubated at 42℃ for 60 min, followed by inactivation at 95℃ for 5 min. The samples were subsequently cooled on ice. Prior to the qPCR procedure, cDNA for plasma samples was diluted 4 times and for tissue samples 8 times, using RNase-free water. Forward and reverse primers were designed using the miRprimerdesign3 software (Busk, 2014) and are listed in Supplementary File 3 and 4. For the qPCR reaction, a mixture was made for each sample, containing 1 μl 10 μM Forward primer, 1 μl 10 μM Reverse primer, 5 μl 2x QuantiFast SyBRGreen (Qiagen, MA, USA), 2 μl Sdw RNase-free water and 1 μl of diluted cDNA. The qPCR reactions were run on a Stratagene MX3005p system (Agilent Technologies, CA, USA), using 96 well white PCR plates (ABgene, Epsom, UK) with the conditions: 95℃ for 5 min followed by 40 cycles of 95℃ for 10 s and 60℃ for 30 s. completed by a melting curve analysis. Samples with Cq-values above 33, unspecific melting curves, or poor PCR efficiency (based on a standard curve made from a 5-fold dilution series from a cDNA pool of all samples included) were excluded from further analysis.

2.12. Search for miRNAs in public equine sequencing data

To explore if the parasite derived miRNAs identified in our study could be found in sequencing data from other equine research, we conducted a search in public datasets for 15 highly abundant parasite derived miRNAs. Furthermore, to validate and compare the abundance of the most abundant horse derived miRNAs, we searched for 19 highly abundant horse derived miRNAs. MicroRNAs of interest were selected based on their overall or differential abundance and the search criteria for both searches were no nucleotide mismatch. Since it would be impossible to identify the origin (parasite or horse) of identical miRNAs found in both *S. vulgaris* and horse, we searched for all miRNAs with identical sequences between the species and excluded them from the search. The search was performed in 13 publicly available datasets from 5 studies exploring miRNAs in horse blood (Kim et al., 2014, 2018; Pacholewska et al., 2016; Cowled et al., 2017; Cappelli et al., 2018). Sequence Read Archive codes for the datasets included in the search were: SRR525254, SRR525255, ERR1462742, ERR1462764, ERR1462745, SRR3034168, SRR6747931, SRR3636185, SRR3636187, SRR3636188, SRR3636192, SRR3636193, and SRR3636194.

2.13. Statistical analyses

2.13.1. Group characteristics

The software R Studio (Posit team, 2023) was employed for all statistical analyses. Continuous variable data were assessed for normality using the Shapiro-Wilk's test and comparisons between the two groups were done by Student's *t*-test for normally distributed data and Mann-Whitney *U* test for non-normally distributed data. Categorical variables were compared between groups with Fisher's Exact test. A P-value < 0.05 was considered significant.

2.13.2. Statistical analysis of qPCR data

Pre-processing of all qPCR data (interplate calibration, PCR efficiency correction, normalization to two stable miRNAs, averaging cDNA replicates, relative quantities calculation based on the lowest expressed sample in each assay and log2 transformation) was performed using GenEx software (MultiD Analyses AB, Västra Frölunda, Sweden). The final selection of normalizers were based on the miRNAs abundance across samples in the qPCR experiment. NormFinder (Andersen et al., 2004) was utilized to rank the miRNAs according to both intra- and intergroup stability and the two most stable miRNAs were chosen as normalizers.

Subsequently, R Studio was employed for statistical comparisons and visualization of the data. To examine differences in miRNA expression in arterial tissue affected by *S. vulgaris* (CMA) and tissue not affected by *S. vulgaris* (Aorta), normality of the data was evaluated by Shapiro-Wilk tests before paired t-tests were performed using the R package limma (Ritchie et al., 2015). Differences in horse-derived miRNA expression between *S. vulgaris* infected and *S. vulgaris* uninfected plasma samples were explored by fitting linear regression models for each miRNA, also using the limma package. The factors age (levels: foal or adult), sex (levels: male or female), and breed (levels: cold-blooded or warm-blooded) were included in the models. For both plasma and tissue samples, corrections for multiple comparisons were done according to the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) and a *q*-value corresponding to a False Discovery Rate (FDR) of 5 % was accepted as significant.

The miRNAs capacity to distinguish between *S. vulgaris* infected and uninfected samples was further assessed for both individual and combined miRNAs, using Receiver Operator Characteristic (ROC) curve analysis with the package pROC (Turck et al., 2011). For this purpose, logistic regression models were fitted for each miRNA and for miRNA pairs, with *S. vulgaris* status as the binary variable of interest and miRNAs as predictor variables. In cases where the miRNAs had shown significant associations with one of the factors age, sex, or breed, the factor was included as an interaction in the model. Diagnostic performance was assessed by determining sensitivity and specificity as well as positive and negative likelihood ratios when establishing the optimal cut-off by the maximum value of Youden's index (Youden, 1950).

2.14. Data accessibility

The data produced in this study have been submitted to NCBI Gene Expression Omnibus (Edgar et al., 2002) and have the GEO Series accession number GSE266592.

3. Results

3.1. Horse characteristics at inclusion

When comparing the demographic characteristics (age, breed, sex) between the infected and uninfected group, no significant differences were found for age and sex. For breed, a significant difference was found between the groups ($P = 0.002$) due to all the horses in the infected group being warmblooded, while 11 of the uninfected horses were coldblooded. Characteristics for the five subgroups and comparisons between the two infection groups are listed in Table 3.

3.2. Necropsy and description of pathological findings

On necropsy, all horses in the KYinfF group were confirmed to be infected with *S. vulgaris*, with *S. vulgaris* larvae present in both the CMA and intestinal walls. Other parasites observed during necropsy were *Parascarids spp.*, *Anoplocephala perfoliata*, *Strongylus Edentatus* and cyathostomins, which were found in all cases. Arterial tissue samples were collected from 12 horses, but samples from two horses were excluded due to technical problems during RNA extraction, resulting in 20 paired tissue samples (CMA and Aorta) from ten horses. All ten horses included for arterial tissue sampling had *S. vulgaris* related pathological changes in the CMA and one had pathology in both the CMA and the celiac artery. No pathologic changes were observed in the Aorta, which was used as control tissue. The parasite status of the uninfected horses in the CPHunA group was evaluated as outlined in Section 2.3. All horses in the group were negative for *S. vulgaris* and tapeworms, both before and after anthelmintic treatment. Prior to treatment, three of the twelve horses had a fecal egg count (FEC) of 0 egg per gram feces (EPG), while the remaining horses had FECs ranging from 20 to 840 EPG. After anthelmintic treatment, all the horses had FECs of 0 EPG. All results of the parasitological testing for the CPHunA group can be found in Supplementary File 5.

Table 3
Group and subgroup characteristics for included horses (n = 63) presented as median and range (age) or as number (n) per category (sex, breed). *Strongylus vulgaris* stats indicates if the subgroups belong to the *S. vulgaris* infected or uninfected group. Foals below 14 days of age are listed as 0.04 years old, while age in years for foals older than one month was calculated individually. *P*-values are based on comparisons between the *S. vulgaris* infected and uninfected group.

Horse characteristics								
S. vulgaris status	Infected		Uninfected			Total		
Subgroup	KYinfF	KYinfA	KYunA	SCANunF	CPHunA	Infected	Uninfected	*P*-value
Total number	19	8	8	16	12	27	36	-
Age in years, median (range)	0.5 (0.3–0.6)	9 (6–15)	3.5 (2–5)	0.04 (0.04–0.04)	13 (6–28)	0.5 (0.3–15)	2 (0.04–28)	0.560
Sex (F/M)	8/11	7/1	8/0	5/11	10/2	15/12	23/13	0.605
Breeds (W/C)	19/0	8/0	0/8	13/3	12/0	27/0	25/11	0.002

Abbreviations: KYinfF: Foals from the University of Kentucky Research herd infected with *Strongylus vulgaris*; KYinfA: Adult horses from the University of Kentucky Research herd infected with *S. vulgaris;* KYunA: Uninfected adult horses from the University of Kentucky Research herd; SCANunF: Uninfected foals from the Large Animal Teaching Hospital of Copenhagen or the Equine Clinic at the Swedish University of Agricultural Sciences; CPHunA: Uninfected adult horses from the Large Animal Teaching Hospital of Copenhagen. SexF/M: Female/Male; Breed C/W: Coldblooded/Warmblooded.

3.3. Quality control of RNA integrity

Spectrophotometric analysis resulted in RNA concentrations with a median of 19.7 ng/μl (range: 11.6–44.6) in the infected plasma samples and 23.7 ng/μl (range: 10.5–48.3) for the uninfected plasma samples. The median RNA concentration for the tissue samples was 1106 ng/μl (range: 335.2–1420) for the CMA samples and 122.1 ng/μl (range: 88.8–222.7) for the aortic samples. Bioanalyzer analysis of the tissue samples resulted in RIN values of 2.6–7.5. Agarose gel electrophoresis on the DNA isolated from the CMA samples showed smearing for 6 of 8 analyzed samples, while none of six analyzed aortic samples showed smearing (data not shown). Total RNA concentrations, 260/280 and 260/230 ratios and Bioanalyzer results for all samples can be reviewed in Supplementary File 6.

3.4. Sequencing results and differential abundance/expression analysis

3.4.1. Parasite-derived miRNAs in plasma

A total of 138 parasite-derived miRNAs were detected with > 10 raw counts across the plasma samples using small RNAseq. Raw parasite-derived miRNA counts can be found in Supplementary File 7. The 10 most abundant parasite-derived miRNAs are listed in Table 4. Differential abundance analysis, comparing *S. vulgaris* infected samples (KYinfF, n = 6) to *S. vulgaris* uninfected samples (KYunA, n = 6), showed no significant difference in parasite-derived miRNA abundance between the two groups.

3.4.2. Horses-derived miRNAs in plasma

Small RNAseq detected 533 horse-derived miRNAs with > 10 raw counts across the plasma samples and 351 were detected with > 100 raw counts. Raw horse-derived miRNA counts can be found in Supplementary File 8. Eca-Mir-486–5p was the most abundant miRNA in plasma, when referring to normalized counts, accounting for 23 % of all normalized counts, followed by Eca-Mir-122–5p with 10 % of the total normalized counts. The top 10 most abundant horse-derived miRNAs in the plasma samples are listed in Table 5**.** When comparing the abundance of horse-derived miRNAs in plasma from *S. vulgaris* infected horses to the abundance in uninfected horses (see Table 2 for subgroups), 140 miRNAs were significantly differentially abundant (see Supplementary File 9). Of these, 74 were more abundant in plasma from *S. vulgaris* infected horses while 66 miRNAs were more abundant in uninfected horses. The heat map in Fig. 1. shows the relative abundance of the differentially abundant horse-derived miRNAs in plasma.

3.5. Parasite-derived miRNAs in plasma detected by qPCR

A panel of six parasite-derived miRNAs were selected for qPCR evaluation in plasma (Novel-4, Svu-Bantam-o6–5p, Svu-Bantam-o4–5p,

Table 5
List of the ten most highly abundant horse-derived microRNAs detected by small RNA sequencing across all plasma from both *S. vulgaris* infected (KYinfF, n = 6) and uninfected horses (KYunA, n = 6). The miRNAs are ranked by overall mean count (Overall) and presented with their mean counts in plasma from *S. vulgaris* infected horses (Infected) and uninfected horses (Uninfected). Counts have been normalized to the geometric mean.

MicroRNAs	Overall	Infected	Uninfected
Eca-Mir−486–5p	118,147	129,836	106,458
Eca-Mir−122–5p	53,244	23,193	83,295
Eca-Mir−15-P2a−5p	31,022	32,023	30,020
Eca-Mir−15-P2b−5p	31,013	32,013	30,013
Eca-Let−7-P2b2–5p	15,362	11,939	18,785
Eca-Mir−191–5p	14,998	14,041	15,955
Eca-Mir−92-P1a−3p	12,823	15,633	10,012
Eca-Mir−92-P1c−3p	12,822	15,633	10,012
Eca-Let−7-P2a1–5p	12,002	11,153	12,852
Eca-Let−7-P2a2–5p	11,988	11,140	12,836

Table 4
List of the ten most abundant parasite-derived microRNAs detected in equine plasma samples (n = 12) by small RNA sequencing. The miRNAs are ranked by overall mean count (Overall) and additionally presented with their mature sequence and mean counts in plasma from *S. vulgaris* infected horses (Infected) and uninfected horses (Uninfected). Counts have been normalized to the geometric mean.

MicroRNAs	Mature sequence	Overall	Infected	Uninfected
Svu-Bantam-o5–5p	AGAGAUCAUGGAUAUGACCAU	33,566	33,223	33,909
Svu-Bantam-o6–5p	UGAGAUCAUCACCAUAAGCACA	18,715	18,565	18,865
Svu-Bantam-o4–5p	UGAGAUCAUGGAUAUGACCAU	15,646	15,594	15,697
Svu-Bantam-o3–3p	UGAGAUCACGCGUAUAUUCGCC	14,836	14,757	14,915
Svu-Let−7-P2–5p	UGAGGUAGUAGGUUGUAUAGUU	14,302	14,520	14,085
Svu-Novel−4–5p	UGGUCAAAUGUCACAAUCUGCU	13,799	13,685	13,912
Svu-Bantam-o1–3p	UGAGAUCACGCGUAUAUUCGCU	13,322	13,248	13,396
Svu-Bantam-o2–3p	UGAGAUCACGCGUAUAUUCGCU	11,975	11,904	12,046
Svu-Mir−71–5p	UGAAAGACAUGGGUAGUGAGACG	9950	9968	9933
Svu-Mir−54-P1–3p	UACCCGUAAUGUUUCCACUGAG	5903	5869	5938

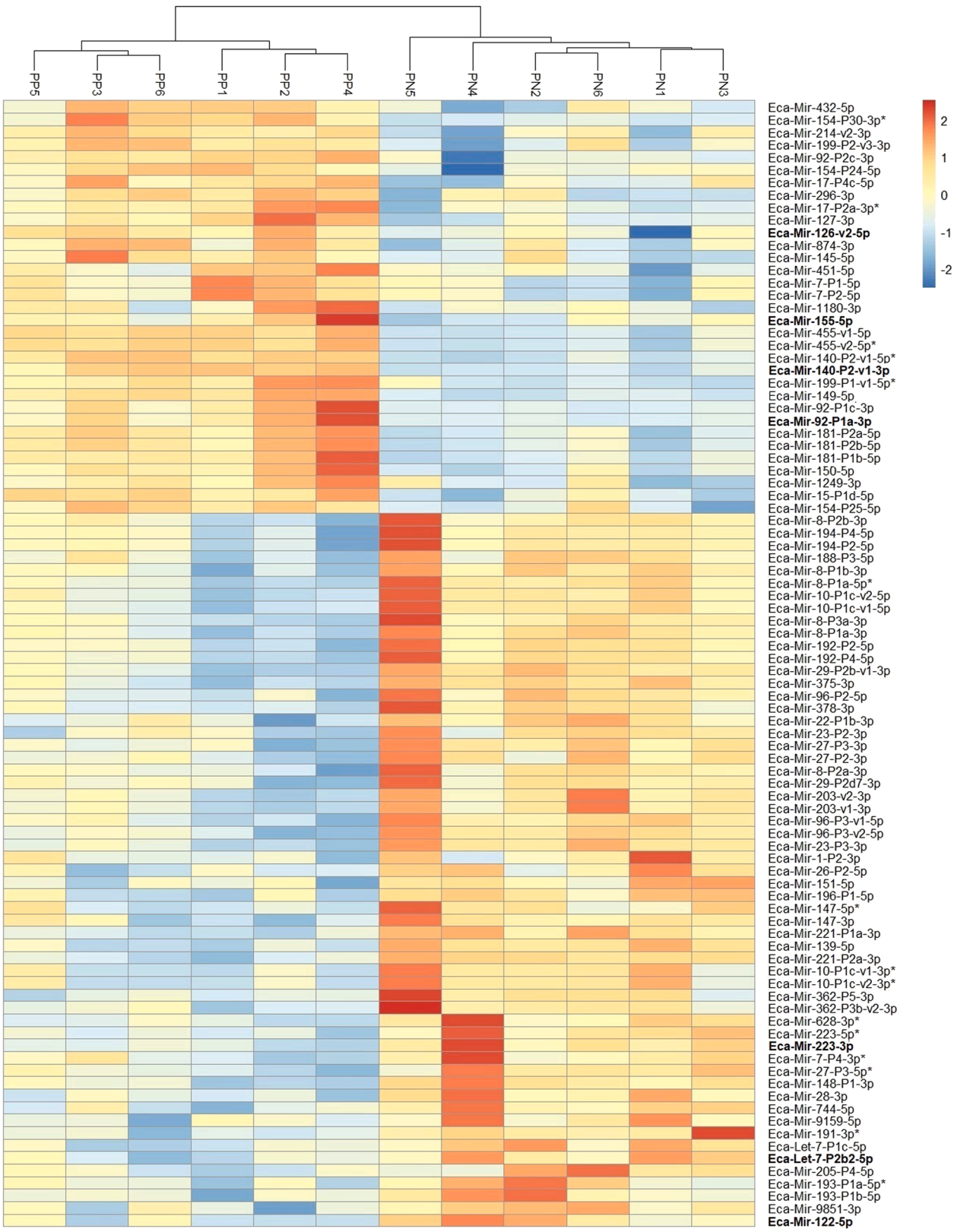

Fig. 1. Heat map for visualization of significantly differentially abundant horse-derived miRNAs, when comparing plasma from six *S. vulgaris* infected horses (PP1–6) to six *S. vulgaris* uninfected horses (PN1–6). The heat map is based on small RNA sequencing results. The color key represents the relative abundance level, red indicating higher abundance and blue lower bundance, when comparing the two infection groups. The dendrogram shows clustering between and within groups. MiRNAs selected for qPCR evaluation are marked in bold. Some miRNAs were selected for qPCR based on their overall abundance or information from literature and are, thus, not represented in the heatmap.

Svu-Bantam-o5–5p, Svu-Mir-54-P1–3p, Svu-Mir-71–5p). The six miRNAs were initially tested in plasma from eight *S. vulgaris* infected horses and two uninfected horses. Amplification was observed in only a few singular replicates, just above the limit of detection and both in the infected and uninfected samples. Overall, none of the parasite-derived miRNAs were deemed quantifiable in plasma samples by qPCR. Consequently, detection of parasite-derived miRNAs in the remaining plasma samples was not attempted.

3.6. Horse-derived miRNAs in arterial tissue detected by qPCR

A panel of 10 horse-derived miRNAs were selected for qPCR evaluation in 20 paired arterial tissue samples (10 CMA and 10 Aorta samples) from infected foals. Selected miRNAs were Eca-Mir-140–3p, Eca-Mir-24–3p, Eca-Mir-126–5p, Eca-Mir-423–5p, Eca-Let-7-P2b2–5p, Eca-Mir-92-P1a-3p, Eca-Mir-223–3p, Eca-Mir-15-P2a-5p, Eca-Mir-155 and Eca-Mir-191. All miRNAs were detectable in all arterial samples. The miRNAs Eca-Mir-155 and Eca-Mir-191 were equally expressed in all samples in the qPCR experiment and were thus selected as normalizers, leaving eight miRNAs for further statistical analysis. When comparing the expression of the miRNAs in the CMA to the control (Aorta), all miRNAs except Eca-Mir-15-P2a-5p ($q = 0.074$) showed significantly differential expression. The miRNAs with the highest Log2FC were Eca-Mir-223–3p and Eca-Mir-140–3p. The miRNAs Eca-Mir-223–3p had a higher expression in the CMA with a log2FCs of 4.74 ($q < 0.001$). In contrast, Eca-MiR-140–3p was downregulated in CMA, with a log2FC of −3.64 ($q < 0.001$). Results for the differentially expressed miRNAs in arterial tissue are visualized as box and whiskers plots in Fig. 2. Log2FC and *q*-values are listed in Table 6.

Table 6
List of differentially expressed horse-derived miRNAs in arterial tissue, when comparing tissue affected by *S. vulgaris* migration from the Cranial Mesenteric Artery (CMA) to unaffected control tissue from the Aorta, using qPCR. The miRNAs are ranked by lowest *q*-value. Higher expression in CMA tissue compared with aortic tissue is indicated by bold font. The remaining miRNAs showed lower expression in CMA compared with Aorta.

MicroRNA	Log2FC	Mean expression	*q*-value
Eca-Mir-223–3p	**4.74**	**3.32**	**< 0.001**
Eca-Mir−140–3p	−3.64	3.07	< 0.001
Eca-Let−7-P2b2–5p	−2.02	2.18	< 0.001
Eca-Mir−24–3p	−2.11	2.49	< 0.001
Eca-Mir−423–5p	−1.61	1.58	< 0.001
Eca-Mir−92-P1a−3p	−1.09	0.92	< 0.001
Eca-Mir−126–5p	**0.90**	**1.63**	**0.014**

Abbreviations: Log2FC = Log2 fold change. Mean expression = relative mean expression across all samples measured by qPCR. *q*-value = *P*-value adjusted for an accepted false discovery rate of 5 %.

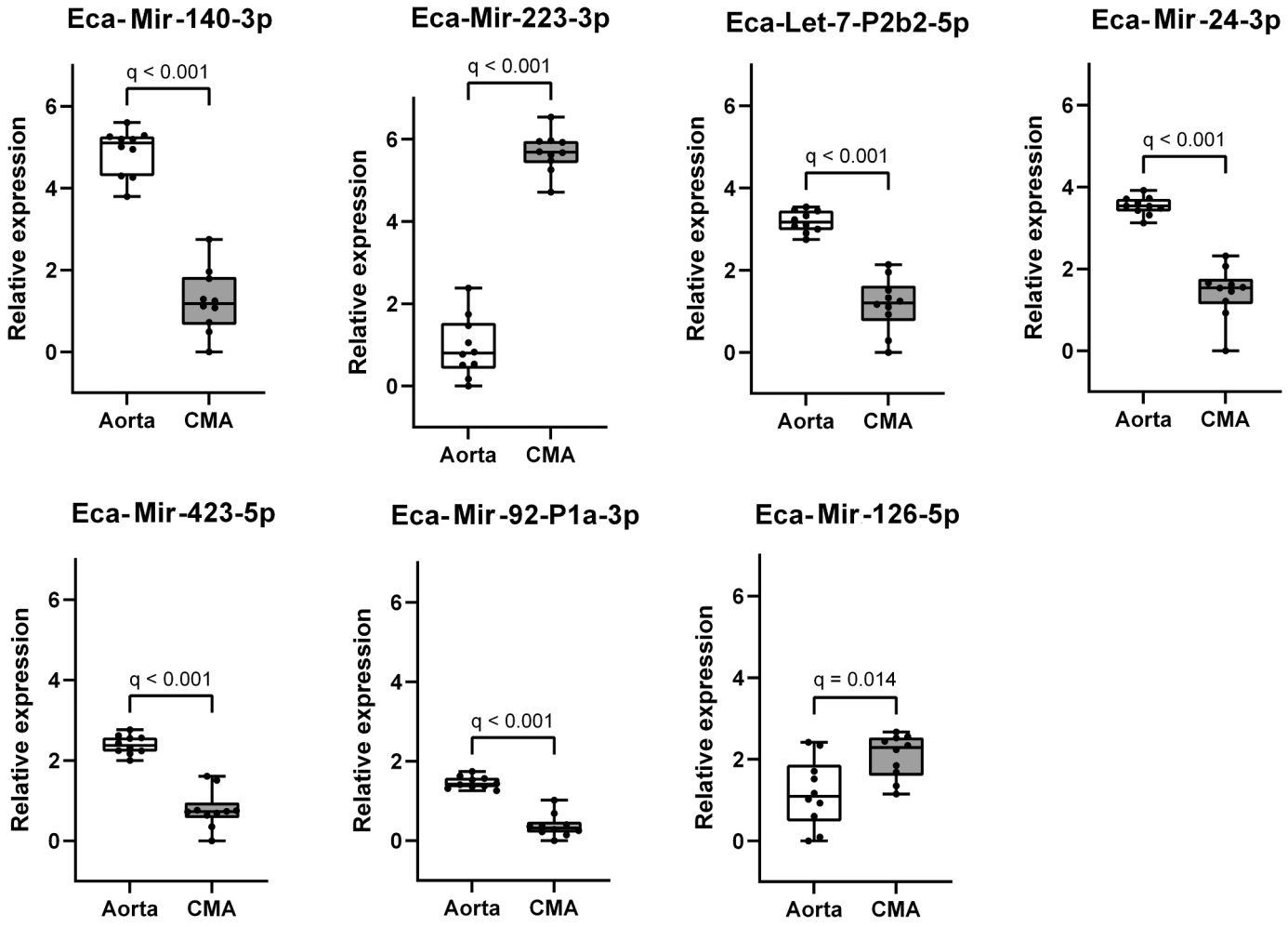

Fig. 2. Differentially expressed horse-derived microRNAs in arterial tissue samples from 10 *S. vulgaris* infected foals, comparing expression in control aortic tissue (Aorta) to tissue from the Cranial Mesenteric Artery (CMA). Boxes show the 25th to 75th percentile and whiskers indicate maximum and minimum values. Dots represent data from the 10 horses. Data are from one qPCR experiment with two technical replicates. Horizontal lines in boxes indicate mean value. *q*-value indicates the FDR corrected significance level of pairwise comparisons (paired t-tests).

3.7. Horse-derived miRNAs detected in plasma by qPCR

A panel of nine horse-derived miRNAs were selected for evaluation in plasma (Eca-Mir-140–3p, Eca-Mir-423–5p, Eca-Let-7-P2b2–5p, Eca-Mir-92-P1a-3p, Eca-Mir-223–3p, Eca-Mir-486–5p, Eca-Mir-191–5p, Eca-Mir-122–5p, Eca-Mir-15-P2a-5p,). All nine miRNAs were successfully amplified and quantified by qPCR.The miRNAs Eca-Mir-191–5p and Eca-Mir-92-P1a-3p were used as normalizers based on their stable abundance across samples in the qPCR experiment, leaving seven miRNAs for further analysis. A significant difference in miRNA abundance between the *S. vulgaris* infected and uninfected group was detected for all miRNAs. The miRNAs Eca-Mir-223-P3–3p, Eca-Mir-486–5p, Eca-Let-7-P2b2–5p, Eca-Mir-423–5p and Eca-Mir-15-P2a-5p had a higher expression in plasma from *S. vulgaris* infected horses, while Eca-Mir-140–3p and Eca-Mir-122–5p showed higher expression in plasma from the *S. vulgaris* uninfected group. Log2FC, sample means, and *q*-values are presented in Table 7. Four miRNAs were significantly affected by age or breed, while sex had no significant effect on the expression of any miRNAs. Effects of these factors on miRNA abundance are presented in Fig. 3.

3.8. Diagnostic performance of horse-derived plasma miRNAs

Based on ROC curve analysis, the best diagnostic performance for individual miRNAs was observed for Eca-Mir-486–5p, Eca-Mir-140–3p and Eca-Mir-122–5p with an area under the curve (AUC) of 0.78, 0.77 and 0.75, respectively. When combining the miRNAs, increases in performance were seen for most combinations. The highest AUC (0.88) was observed for the combination of Eca-MiR-122–5p and Eca-Mir-423–5p. The miRNA Eca-Mir-140–3p had shown significant association with the factor age, which was, thus, included as an interaction in the logistic model for this miRNA, resulting in substantial increase in diagnostic performance (AUC 0.96). ROC curves for miRNAs with significant discriminatory capacity based on AUC and the associated confidence intervals are presented in Fig. 4. All AUCs, sensitivities, specificities, positive and negative likelihood ratios, and associated confidence intervals are listed in Table 8.

3.9. Detected miRNAs in public equine sequencing data

In total, four miRNAs were identical between *S. vulgaris* and horse (Mir-124–3p, Let-7-P5–5p, Mir-1–3p, Mir-10-P2o5–5p) and were excluded from the analysis (Three from the parasite-derived miRNAs and four from the horse-derived miRNAs). This resulted in 12 parasite-derived miRNAs being included in the search (Svu-Mir-71–5p, Svu-Mir-190–5p, Svu-Bantam-o5–5p, Svu-Novel-4–5p, Svu-Mir-54-P1–3p, Svu-Bantam-o4–5p, Svu-Mir-92-P1–3p, Svu-Let-7-P2–5p, Svu-Bantam-o6–5p, Svu-Bantam-o2–3p, Svu-Bantam-o3–3p and Svu-Mir-9-P1–3p.)

Table 7
List of differentially abundant horse-derived miRNAs in plasma, when comparing *Strongylus vulgaris* infected horses to uninfected horses, using qPCR. The miRNAs are ranked by lowest *q*-value. Higher expression in plasma from *S. vulgaris* infected horses is indicated by bold font. The remaining miRNAs showed lower abundance in plasma from *S. vulgaris* infected horses.

MicroRNA	Log2FC	Mean abundance	*q*-value
Eca-Mir−140–3p	−2.56	4.17	< 0.001
Eca-Mir−223–3p	**1.58**	**2.82**	**< 0.001**
Eca-Mir−486–5p	**1.05**	**2.98**	**< 0.001**
Eca-Mir−122–5p	−1.77	4.77	0.006
Eca-Let−7-P2b2–5p	**0.91**	**1.79**	**0.006**
Eca-Mir−423–5p	**0.75**	**1.76**	**0.009**
Eca-Mir−15-P2a−5p	**0.74**	**2.99**	**0.009**

Abbreviations: Log2FC = Log2 fold change. Mean abundance = Relative mean abundance across all samples. *q*-value = *P*-value adjusted for an accepted false discovery rate of 5 %.

and 15 horse-derived miRNAs (Eca-Mir-1-P3–3p, Eca-Mir-1-P2–3p, Eca-Mir-486–5p, Eca-Mir-122–5p, Eca-Mir-574–5p, Eca-Let-7-P2b2–5p, Eca-Mir-15-P2a-5p, Eca-Mir-191–5p, Eca-Mir-423–5p, Eca-Let-7-P2a2–5p, Eca-Mir-223–3p, Eca-miR-140-P2–3p, Eca-Mir-92-P1a-3p, Eca-Mir-146-P4-v1–5p and Eca-Mir-181-P1a-5p). None of the selected parasite-derived miRNAs were found in the publicly available sequencing data, while, on the contrary, all the horse-derived miRNAs were found in at least 4 datasets. The horse-derived miRNA Eca-Mir-486–5p had the highest number of hits in 9 of the 13 datasets. A table listing the number of hits found in the search for each dataset can be found in Supplementary File 10.

4. Discussion

This study investigated miRNAs associated with *S. vulgaris* infection in horses, exploring the horses' local and systemic miRNA patterns in response to infection, as well as parasite-derived miRNAs released to the host. Most notably, several horse-derived miRNAs showed differential abundance in plasma from infected versus uninfected horses and between *S. vulgaris* affected and unaffected arterial tissue from infected horses.

To identify horse-derived miRNAs most likely to be associated with ongoing *S. vulgaris* infection, we investigated the local horse-derived miRNA response to *S. vulgaris* migration in the larvae's predilection site, the CMA. The characteristic pathology occurring in the CMA during a typical *S. vulgaris* infection is probably the most evident reaction to the parasite and, thus, a plausible location of miRNA dysregulation. As anticipated, almost all the horse-derived miRNAs evaluated in the CMA tissue were differentially expressed compared with the Aorta from the same horse, indicating different ongoing processes in the two tissues. Although miRNA expression is probably also affected by the origin of arterial tissue (CMA vs. Aorta), as miRNA have specific tissue patterns (Liang et al., 2007), it is reasonable to argue that some of the difference would stem from the severe inflammatory processes occurring in the CMA during *S. vulgaris* migration (as suggested in a review by (Das and Rao, 2022)). This question could be answered in future studies by comparing the expression in CMA samples from horses without *S. vulgaris* infection.

The miRNAs with the largest differences in expression between CMA and Aorta were Eca-Mir-223–3p and Eca-Mir-140–3p. These miRNAs also showed differential abundance in circulation, when comparing infected and uninfected horses, with a pattern following that detected in the arterial tissue (Eca-Mir-223–3p being upregulated in CMA and plasma from infected horses and Eca-Mir-140–3p being downregulated in CMA and plasma from infected horses). These findings make these two miRNAs of particular interest. Mir-223–3p has previously been linked to parasitic helminth infection with another blood related parasite, *Schistosoma japonicum,* where, similarly to the findings in this study, the miRNA was upregulated in parasite-affected liver tissue and blood from several host organisms (He et al., 2013). Multiple studies have found Mir-223–3p to have immunomodulatory and inflammation regulatory properties (reviewed by Jiao et al., (2021) and Yuan et al., (2021)). As we also observed in the present study, Mir-223–3p expression is often upregulated in inflamed tissue, where it is understood to downregulate production of inflammatory mediators or block inflammatory signaling pathways (Feng et al., 2017; Neudecker et al., 2017; Zhang et al., 2019).

Eca-mir-223–3p was also associated with breed in this study, having a higher abundance in plasma from coldblooded horses. This has not been reported in previous studies evaluating miRNAs in different breeds (Pacholewska et al., 2016; Unger et al., 2021). Conversely, Eca-Mir-122–3p has previously been found to be more abundant in blood from ponies (Pacholewska et al., 2016) compared to warmblooded horses, but in the present study, no significant association with breed was found for this miRNA when evaluated using qPCR. It could, however, be noted that the KYunA (pony) group did overall have a

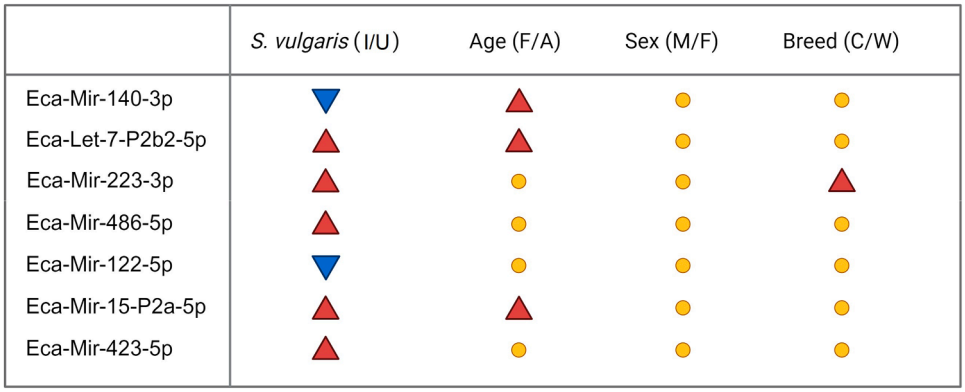

Fig. 3. Chart showing changes in horse-derived miRNA abundance in relation to *Strongylus vulgaris* status, age, sex and breed. Upward triangle ▲ indicates a higher expression of the miRNA in *Strongylus vulgaris* infected horses (I) compared with uninfected horses (U), in foals (F) compared with adults (A), in males (M) compared with females (F) and in cold-blooded (C) compared with warm-blooded (W). Downward triangle ▼ conversely indicates a lower expression in the first factor for the same comparisons. Circle ● indicates no significant association between miRNA expression and factor level.

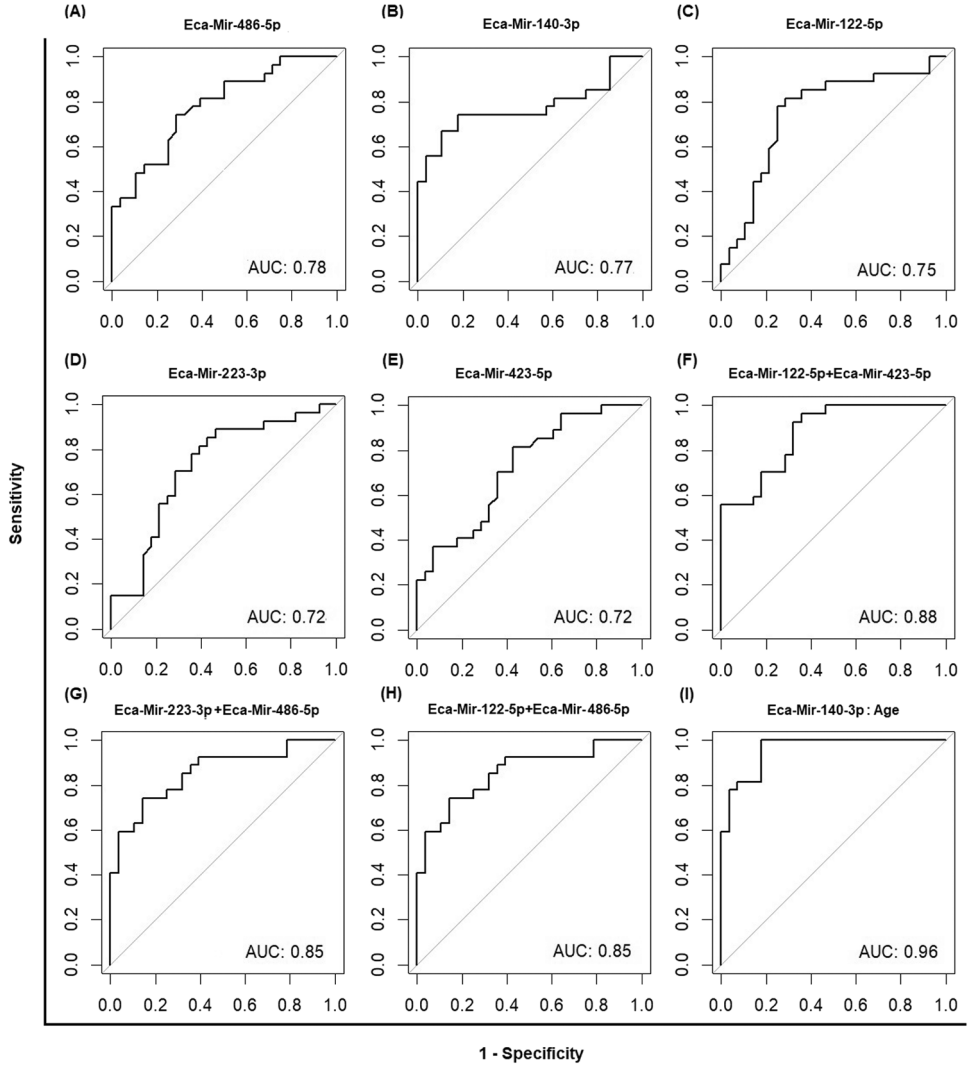

Fig. 4. Receiver operator characteristic (ROC) curves for individual, combined and age corrected horse-derived plasma miRNAs. Plots show performance regarding discriminating between *Strongylus vulgaris* infected and uninfected horses. A-E show performance of individual miRNAs, F-H the top three performing combinations of miRNAs, and I show Eca-Mir-140–3p corrected for interaction with age. Abbreviations: AUC = Area under the curve.

Table 8
Diagnostic performance for individual, combined and interaction corrected horse-derived miRNAs in plasma, with regards to discriminating between *Strongylus vulgaris* infected and uninfected horses.

MicroRNA	AUC	95 % CI (AUC)	Se (%)	95 % CI (Se)	Sp (%)	95 % CI (Sp)	LR+	LR-
Eca-Mir−486–5p	0.78	0.66–0.90	74	59–89	71	54–85	2.59	0.36
Eca-Mir−140–3p	0.77	0.64–0.91	74	68–94	82	68–96	4.15	0.32
Eca-Mir−122–5p	0.75	0.61–0.89	81	67–96	71	54–86	2.85	0.26
Eca-Mir−223–3p	0.72	0.59–0.86	89	78–1	53	36–71	1.91	0.21
Eca-Mir−423–5p	0.72	0.59–0.86	81	67–96	57	39–75	1.90	0.32
Combinations								
Eca-Mir−122–5p +Eca-Mir−423–5p	0.88	0.79–0.96	96	89–100	64	46–82	2.69	0.05
Eca-Mir−223–5p +Eca-Mir−486–5p	0.85	0.77–0.96	74	59–89	86	71–96	5.19	0.30
Eca-Mir−122–5p +Eca-Mir−486–5p	0.85	0.75–0.96	74	56–89	86	71–96	5.19	0.30
Interactions								
Eca-Mir−140–5p:Age	0.96	0.91–1.0	100	100–100	82	68–93	5.69	0.0

Abbreviations: AUC: Area under the curve, CI: Confidence interval, Se: Sensitivity, Sp: Specificity, LR+ : positive likelihood ratio, LR-: negative likelihood ratio.

higher abundance of Eca-Mir-122–3p than the KYinF (warmblooded) group in the sequencing data, but this association cannot be isolated from the effects of age and infection status, which also differ between these groups.

Eca-Mir-140–3p was one of the most highly abundant miRNAs in both arterial tissue and plasma in this study. This miRNA showed a significant downregulation in CMA tissue compared with aortic tissue as well as in plasma from *S. vulgaris* infected horses compared with uninfected horses. Mir-140–3p has also previously been detected with high expression in mouse and human arteries (Wang et al., 2019) and has furthermore been investigated in several arterial injury models. A recurring finding in other species is that Mir-140–3p is downregulated in injured arterial tissue (Zhu et al., 2018; Wang et al., 2019; Li et al., 2021), while upregulation of the miRNA seems to have an angioprotective effect (Wang et al., 2019; Yi et al., 2020). Likewise, (Mo et al., 2024) showed that Mir-140–3p was decreased in plasma from human patients with coronary artery disease compared with healthy patients. The findings in our study align with these previous studies, indicating that Mir-140–3p has an important role in arteries and during arterial injury.

Unrelated to parasite status, a strong association with age was observed for Eca-Mir-140–3p, with foals having a significantly higher abundance in plasma compared with adult horses. Many processes in foals are different to those of adult horses, which, for example, result in different reference intervals for clinicopathological parameters in foals (Faramarzi and Rich, 2019; Barton and Hart, 2020). Differences in plasma miRNA abundance related to age is, thus, not surprising and has also been found in human studies (Lai et al., 2014; Meder et al., 2014) and in a previous equine study, where, however, Eca-Mir-140–3p was not investigated (Unger et al., 2021). Several equine studies have found Eca-Mir-140–3p to be highly related to cartilage development and pathology (Buechli et al., 2013; Andersen et al., 2024; Antunes et al., 2024) and a study exploring the cartilage transcriptome in foals compared with adult horses (Mienaltowski et al., 2008) found that upregulated transcripts in neonatal cartilage were consistent with cartilage growth, while expression patterns in mature cartilage indicate homeostasis. The higher cartilage growth in foals could contribute to a higher release of Mir-140–3p to the circulation and accordingly contribute to the high Eca-Mir-140 abundance detected in the foals in this study.

The overall most abundant plasma miRNA detected by sequencing in this study was Eca-Mir-486–5p, which is consistent with findings in other equine studies (Lee et al., 2016; Pacholewska et al., 2016, 2017; Cowled et al., 2017; Unger et al., 2019; de Oliveira et al., 2021), where this miRNA was also among the most abundant. Eca-Mir-486–5p was also the miRNA with the best diagnostic performance for discriminating between *S. vulgaris* infected and uninfected horses. However, Mir-486–5p is known to be associated with hemolysis (Pritchard et al., 2012; Blondal et al., 2013; Shkurnikov et al., 2016), and great caution should be exercised when using hemolysis-dependent miRNAs as biomarkers (Pritchard et al., 2012). The diagnostic potential of Eca-Mir-486–5p is, thus, questionable.

All thirteen of the most abundant horse-derived miRNAs detected in this study were found when searching publicly available sequencing datasets from studies investigating miRNAs in horse blood. Since the miRNA counts cannot be directly compared between the studies and the parasite statuses of the horses in these studies are not disclosed, no conclusions can be drawn from these findings regarding the usefulness of the miRNAs as *S. vulgaris* biomarkers. It does, however, validate that these miRNAs are commonly found in horse blood.

The second part of this study focused on circulating parasite-derived miRNAs as a potential biomarker of *S. vulgaris* infection. Detection of parasite-derived miRNAs in blood from infected animals has been accomplished for some host-parasite combinations (Cheng et al., 2013; Buck et al., 2014; Hoy et al., 2014; Tritten et al., 2014; Quintana et al., 2015), while found unfeasible for others (Buck et al., 2014; Lagatie et al., 2017; Whitehead et al., 2024). The differences in detectability have been suggested to be due to parasite localization (strictly intestinal or migrating) (Buck et al., 2014) or due to differences in sensitivity of the techniques used (small RNAseq or qPCR) (Cucher et al., 2021). In this study, parasite-derived miRNAs could be detected in plasma samples from horses with *S. vulgaris* infection using small RNAseq, but were detected with similar abundance in plasma samples from horses without *S. vulgaris*. This could relate to the uninfected horses being infected with other parasites than *S. vulgaris*, especially cyathostomins, which are also clade V nematodes, closely related to *S. vulgaris* (International Helminth Genomes Consortium, 2019). Because miRNAs are highly conserved between closely related nematode species (De Wit et al., 2009), the detection of parasite-derived miRNAs in plasma from horses with cyathostomins was not unexpected as such, but since *S. vulgaris* is a hematogeneous parasite and cyathostomins are not, it was surprising that no difference in abundance was detectable. In a previous study (Toft et al., 2024), we detected 136 miRNAs in the excreted/secreted products (ESP) by *S. vulgaris* larvae *in vitro*. Apart from two miRNAs, which were not detected in ESP, there was a 100 % overlap of the parasite-derived miRNAs detected in ESP in the previous study and the miRNAs detected in plasma in the present study. Noticeably, of the 10 miRNAs most abundant in ESP compared to larvae in the previous study, six were represented in the top 10 most abundant miRNAs in plasma in the present study. These findings could support the hypothesis that the parasite derived miRNAs detected in plasma are in fact excreted/secreted by the parasite *in vivo*. When we evaluated the parasite-derived miRNAs using qPCR in a larger cohort of horses, none of the parasite-derived miRNAs were detectable, regardless of infection level. This indicates that the circulating parasite-derived miRNAs were below the limit of detection for qPCR. As noted in a review by Cucher et al., (2021), the same pattern was the case for *Onchocerca volvulus*, with two studies detecting parasite-derived miRNAs in blood from infected animals using small RNAseq (Tritten et al., 2014; Quintana et al., 2015), while two studies using qPCR were unsuccessful (Lagatie et al., 2017; Macfarlane et al., 2020). In future studies, the use of TaqMan probes or

droplet digital PCR could be explored as alternatives to the qPCR methodology utilized in the current study.

When we searched for *S. vulgaris* miRNAs in sequencing data from other equine studies, we were unable to detect them in any of the 13 available datasets. Several factors could contribute to parasite-miRNAs being detectable in our sequencing data, but not in other studies, but since our study had an unusually high sequencing depth (100 mio reads pr. sample), difference in sequencing depth could be an important factor. The parasite status of the horses used in the 13 studies is as noted undisclosed, and it could be speculated that the parasite burden would be lower in these horses than in the KYinF horses used in this study, since most managed horses are routinely treated with anthelmintics. Collectively, however, these findings support the conclusion, that parasite miRNAs are present at very low concentration in host blood and probably do not have potential as biomarkers of *S. vulgaris* infection.

When comparing this study's small RNAseq plasma results for horse-derived miRNAs to the results of qPCR, there are obvious discrepancies. Most noticeable is the abundance of Eca-Mir-223–3p and Eca-Mir-140–3p, which in relation to *S. vulgaris* infection status is reversed for the two techniques. The explanation for this is probably the difference in samples being compared. For the sequenced samples, the uninfected group (KYunA) consisted of adult ponies whereas the infected group (KYinfF) consisted of warm-blooded foals. We showed that Eca-Mir-140–3p was strongly associated with age and the high abundance in plasma from the KYinfF is probably related to their young age, overriding any association with *S. vulgaris* status. Likewise, Eca-Mir-223–3p was associated with a higher expression in ponies compared with warmbloods, which could be the explanation for the high abundance found in the KYunA group on sequencing. We tested if the sequencing and qPCR results were correlated when only including the samples analyzed by both methods, but no significant correlation was found, although no reverse relationship was found either. The groups compared in the sequencing part were not ideal due to the age and breed differences between the groups, and the sequencing experiment was, thus, primarily used as a screening method to identify miRNAs of interest, while the results from the qPCR are more reliable as reflections of *S. vulgaris* infection status. At the time of sequencing, the SCANunF group had not been sampled, but inclusion of this low or possibly parasite free group in the sequencing data, would probably have added valuable information to the study.

Almost all tissue samples in this study had RIN value below 7, which has been suggested as the lower limit for reliable evaluation of RNAs (Ibberson et al., 2009). Low RIN values are a sign of sample degradation, based on ribosomal RNA, but its value concerning estimation of miRNA integrity is debated (Aryani and Denecke, 2015). It has been shown that miRNAs are stable in conditions where other RNAs are not (Chen et al., 2008; Mitchell et al., 2008; Aryani and Denecke, 2015; Glinge et al., 2017) and it has consequently been argued that RIN values do not necessarily reflect miRNA integrity (Aryani and Denecke, 2015). In addition, it is striking that almost all the CMA samples had lower RIN values than the corresponding aortic samples, since the samples were handled nearly identically. The CMA samples were collected from highly inflamed tissue, and histological examinations of *S. vulgaris* induced arteritis in the CMA have shown various degrees of cell necrosis (Morgan et al., 1991; El-Gameel et al., 2022). Necrosis naturally results in DNA fragmentation, which we showed to be present in almost all of the examined CMA samples collected in this study, but in none of the aortic samples. In that sense, the degradation and resulting miRNA expression can also be viewed as a biological/pathological finding, resulting from cell necrosis. Ultimately, however, we encourage interpreting the results from the tissue samples with caution considering the low RIN values.

The design of this study has inherent limitations. All the *S. vulgaris* infected horses were from Kentucky, USA, while most of the uninfected samples were collected from horses in Scandinavia (apart from KYunA). The effect of this geographical difference between the groups is unknown, but it is plausible that differences in management and housing could have an impact on the miRNA profiles. Furthermore, this study was based on naturally infected horses, meaning that the stages of infection could have differed between the horses included. While this is an appropriate model to study the infection in a clinically relevant setting, a more controlled experimentally infected model could also be an option for producing more easily interpretable data. Likewise, a study evaluating miRNA levels before and after treatment of infected horses would be an interesting study design to pursue in the future.

This study uses the miRNA nomenclature system from Fromm et al., (2015) as found in the MirGeneDB database. The nomenclature may, thus, differ from MirBase and for example; Eca-Mir-15-P2a-5p in this study is miR-16–5p in MirBase. The MirGeneDB database and nomenclature was selected, due to its emphasis on manual curation and strict adherence to specific criteria for miRNA annotation, which ensures inclusion of only high confidence miRNAs.

5. Conclusions

Infection with the equine parasite *S. vulgaris* was associated with altered expression of horse-derived miRNAs in their predilection site, the CMA, and several of the same miRNAs showed differential abundance in plasma from *S. vulgaris*-infected horses, when compared with uninfected individuals. These changes could be associated with the horses' parasite status, but several other factors also influenced the plasma miRNA patterns, such as age and breed. Since Eca-Mir-223–3p and Eca-Mir-140–3p were highly abundant and showed substantial differential abundance in both CMA tissue and plasma, they could be of particular interest for further studies. Parasite-derived miRNAs were detectable in plasma from both *S. vulgaris*-infected and uninfected horses, using small RNAseq, but no parasite-derived miRNAs were quantifiable in any samples using qPCR. This appears to preclude their diagnostic value for detection of *S. vulgaris* infection.

Source of funding

The study was funded by the Independent Research Fund Denmark (case number 0136–00101B). The Danish Horse Levy Foundation, University of Copenhagen Graduate School, the Denmark America Foundation, the Hartmann Foundation, the William Demant Foundation, the Sveland Foundation, E-vet, Scanvet and Eickemeyer provided additional support.

CRediT authorship contribution statement

Tina Holberg Pihl: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Nichol Ripley:** Writing – review & editing, Methodology, Investigation. **Martin K. Nielsen:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Maibritt Mardahl:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Bastian Fromm:** Writing – review & editing, Methodology, Formal analysis. **Ylva Hedberg Alm:** Writing – review & editing, Resources. **Eva Tydén:** Writing – review & editing, Resources. **Lise N. Nielsen:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Peter Nejsum:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Stig Milan Thamsborg:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Susanna Cirera:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Katrine Toft:** Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Marie Louise Honoré:** Writing – review & editing, Resources, Methodology, Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tina Holberg Pihl reports financial support was provided by Independent Research Fund Denmark. Tina Holberg Pihl reports financial support was provided by The Danish Horse Levy Foundation. Katrine Toft reports financial support was provided by University of Copenhagen Graduate School. Katrine Toft reports financial support was provided by Denmark America Foundation. Katrine Toft reports financial support was provided by The Hartmann Foundation. Katrine Toft reports financial support was provided by The William Demant Foundation. Katrine Toft reports financial support was provided by The Sveland Foundation. Katrine Toft reports equipment, drugs, or supplies was provided by E-vet. Katrine Toft reports equipment, drugs, or supplies was provided by Eickemeyer Veterinary Equipment Ltd. Katrine Toft reports equipment, drugs, or supplies was provided by Scanvet. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Acknowledgements

The authors would like to thank the laboratory technicians involved with this project: Minna Jakobsen and Tina Bahrt Neergaard Mahler from the Department of Veterinary and Animal Sciences, University of Copenhagen; Maria Rhod and Tina Roust from the University of Copenhagen Large Animal Teaching Hospital; and Holli Sullivan Gravatte from the Gluck Equine Research Center, University of Kentucky. Special thanks are also due to Dr. Bradley Whitehead for his assistance with sample preparations and insightful contributions to parasite miRNA exploration. The authors would also like to thank the staff at the University of Copenhagen Large Animal Teaching Hospital and Equine Clinic at the Swedish University of Agricultural Sciences, who have assisted with sample collection and preparation. Lastly, the authors acknowledge the dedicated farm crew at the Gluck Equine Research Center and the experienced technical staff at the Veterinary Diagnostic Laboratory, both University of Kentucky, whose skilled assistance with horse handling and sample collection was essential for this study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2024.110379.

References

Andersen, U.V., Howe, D.K., Dangoudoubiyam, S., Toft, N., Reinemeyer, C.R., Lyons, E. T., Olsen, S.N., Monrad, J., Nejsum, P., Nielsen, M.K., 2013. SvSXP: a *Strongylus vulgaris* antigen with potential for prepatent diagnosis. Parasites Vectors 6, 1–13. https://doi.org/10.1186/1756-3305-6-84.

Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496.

Andersen, C., Walters, M., Bundgaard, L., Berg, L.C., Vonk, L.A., Lundgren-Åkerlund, E., Henriksen, B.L., Lindegaard, C., Skovgaard, K., Jacobsen, S., 2024. Intraarticular treatment with integrin α10β1-selected mesenchymal stem cells affects microRNA expression in experimental post-traumatic osteoarthritis in horses. Front. Vet. Sci. 11, 1–11. https://doi.org/10.3389/fvets.2024.1374681.

Andrews, S., 2010. FastQC: A Quality Control Tool for High Throughput Sequence Data [WWW Document]. ⟨www.bioinformatics.babraham.ac.uk/projects/fastqc⟩ .

Antunes, J., Salcedo-Jiménez, R., Lively, S., Potla, P., Coté, N., Dubois, M.S., Koenig, J., Kapoor, M., LaMarre, J., Koch, T.G., 2024. microRNAs are differentially expressed in equine plasma of horses with osteoarthritis and osteochondritis dissecans versus control horses. PLoS One 19, 1–19. https://doi.org/10.1371/journal.pone.0297303.

Aryani, A., Denecke, B., 2015. In vitro application of ribonucleases: comparison of the effects on mRNA and miRNA stability. BMC Res. Notes 8, 1–9. https://doi.org/10.1186/s13104-015-1114-z.

Balaskas, P., Anderson, J., Peffers, M., 2018. Identification of microRNAs in equine synovial fluid as potential biomarkers of osteoarthritis. Res. Liverp.

Balcells, I., Cirera, S., Busk, P.K., 2011. Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers. BMC Biotechnol. 11, 70. https://doi.org/10.1186/1472-6750-11-70.

Barton, M.H., Hart, K.A., 2020. Clinical pathology in the foal. Vet. Clin. North Am. Equine Pract. 36, 73–85. https://doi.org/10.1016/j.cveq.2019.11.003.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

Biros, E., Moran, C.S., Wang, Y., Walker, P.J., Cardinal, J., Golledge, J., 2014. microRNA profiling in patients with abdominal aortic aneurysms: the significance of miR-155. Clin. Sci. 126, 795–803. https://doi.org/10.1042/CS20130599.

Blondal, T., Jensby Nielsen, S., Baker, A., Andreasen, D., Mouritzen, P., Wrang Teilum, M., Dahlsveen, I.K., 2013. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods 59, S1–S6. https://doi.org/10.1016/j.ymeth.2012.09.015.

Borek, A., Drzymała, F., Botor, M., Auguściak-Duma, A.M., Sieroń, A.L., 2019. Roles of microRNAs in abdominal aortic aneurysm pathogenesis and the possibility of their use as biomarkers. Kardiochirurgia Torakochirurgia Pol. 16, 124–127. https://doi.org/10.5114/kitp.2019.88601.

Buck, A.H., Coakley, G., Simbari, F., McSorley, H.J., Quintana, J.F., Le Bihan, T., Kumar, S., Abreu-Goodger, C., Lear, M., Harcus, Y., Ceroni, A., Babayan, S.A., Blaxter, M., Ivens, A., Maizels, R.M., 2014. Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. Nat. Commun. 5, 5488. https://doi.org/10.1038/ncomms6488.

Buechli, M.E., Lamarre, J., Koch, T.G., 2013. MicroRNA-140 expression during chondrogenic differentiation of equine cord blood-derived mesenchymal stromal cells. Stem Cells Dev. 22, 1288–1296. https://doi.org/10.1089/scd.2012.0411.

Busk, P.K., 2014. A tool for design of primers for microRNA-specific quantitative RT-qPCR. BMC Bioinforma. 15, 29. https://doi.org/10.1186/1471-2105-15-29.

Cappelli, K., Capomaccio, S., Viglino, A., Silvestrelli, M., Beccati, F., Moscati, L., Chiaradia, E., 2018. Circulating miRNAs as putative biomarkers of exercise adaptation in endurance horses. Front. Physiol. 9, 1–11. https://doi.org/10.3389/fphys.2018.00429.

Castanheira, C., Balaskas, P., Falls, C., Ashraf-Kharaz, Y., Clegg, P., Burke, K., Fang, Y., Dyer, P., Welting, T.J.M., Peffers, M.J., 2021. Equine synovial fluid small non-coding RNA signatures in early osteoarthritis. BMC Vet. Res. 17, 1–12. https://doi.org/10.1186/s12917-020-02707-7.

Cavallero, S., Bellini, I., Pizzarelli, A., Arcà, B., D'Amelio, S., 2022. A miRNAs catalogue from third-stage larvae and extracellular vesicles of *Anisakis pegreffii* provides new clues for host-parasite interplay. Sci. Rep. 12, 9667. https://doi.org/10.1038/s41598-022-13594-3.

Chen, X., Ba, Y., Ma, L., Cai, X., Yin, Y., Wang, K., Guo, J., Zhang, Yujing, Chen, J., Guo, X., Li, Q., Li, X., Wang, W., Zhang, Yan, Wang, Jin, Jiang, X., Xiang, Y., Xu, C., Zheng, P., Zhang, Juanbin, Li, R., Zhang, H., Shang, X., Gong, T., Ning, G., Wang, Jun, Zen, K., Zhang, Junfeng, Zhang, C.Y., 2008. Characterization of microRNAs in serum: A novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 18, 997–1006. https://doi.org/10.1038/cr.2008.282.

Cheng, G., Luo, R., Hu, C., Cao, J., Jin, Y., 2013. Deep sequencing-based identification of pathogen-specific microRNAs in the plasma of rabbits infected with *Schistosoma japonicum*. Parasitology 140, 1751–1761. https://doi.org/10.1017/S0031182013000917.

Cirera, S., Busk, P.K., 2014. Quantification of miRNAs by a Simple and Specific qPCR Method. In: Alvarez, M., Nourbakhsh, M. (Eds.), RNA Mapping. Methods in Molecular Biology, 1182. Humana Press, New York, NY, pp. 73–81. https://doi.org/10.1007/978-1-4939-1062-5_7.

Cosandey, J., Hamza, E., Gerber, V., Ramseyer, A., Leeb, T., Jagannathan, V., Blaszczyk, K., Unger, L., 2021. Diagnostic and prognostic potential of eight whole blood microRNAs for equine sarcoid disease. PLoS One 16, 1–18. https://doi.org/10.1371/journal.pone.0261076.

Cowled, C., Foo, C.H., Deffrasnes, C., Rootes, C.L., Williams, D.T., Middleton, D., Wang, L.F., Bean, A.G.D., Stewart, C.R., 2017. Circulating microRNA profiles of Hendra virus infection in horses. Sci. Rep. 7, 2–11. https://doi.org/10.1038/s41598-017-06939-w.

Cucher, M.A., Ancarola, M.E., Kamenetzky, L., 2021. The challenging world of extracellular RNAs of helminth parasites. Mol. Immunol. 134, 150–160. https://doi.org/10.1016/j.molimm.2021.03.011.

Curtis, R.A., 1964. Mesenteric Aneurism in a Horse. Can. Vet. J. La Rev. Vet. Can. 5, 36–38.

Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., Mccarthy, S.A., Davies, R.M., Li, H., 2021. Twelve years of SAMtools and BCFtools 1–4. https://doi.org/10.1093/gigascience/giab008.

Das, K., Rao, L.V.M., 2022. The Role of microRNAs in Inflammation. Int. J. Mol. Sci. 23, 15479. https://doi.org/10.3390/ijms232415479.

De Wit, E., Linsen, S.E.V., Cuppen, E., Berezikov, E., 2009. Repertoire and evolution of miRNA genes in four divergent nematode species. Genome Res. 19, 2064–2074. https://doi.org/10.1101/gr.093781.109.

Drudge, J.H., 1979. Clinical aspects of *Strongylus vulgaris* infection in the horse. Emphasis on diagnosis, chemotherapy, and prophylaxis. Vet. Clin. North Am. Large Anim. Pract. 1, 251–265. https://doi.org/10.1016/S0196-9846(17)30183-0.

Drudge, J.H., Lyons, E.T., Szanto, J., 1966. Pathogenesis of migrating stages of helminths with special reference to *Strongylus Vulgaris*. In: Soulsby, E.J.L. (Ed.), Biology of Parasites. Emphasis on Veterinary Parasites. Academic Press Inc, New York and London, pp. 199–214.

Drudge, J.H., Lyons, E.T., 1966. Control of internal parasites of the horse. J. Am. Vet. Med. Assoc. 148, 378–383.

Duncan, J.L., Pirie, H.M., 1972. The life cycle of *Strongylus vulgaris* in the horse. Res. Vet. Sci. 13, 374–379.
Edgar, R., Domrachev, M., Lash, A.E., 2002. Gene expression omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30, 207–210. https://doi.org/10.1093/nar/30.1.207.
El-Gameel, S.M., Al-Mokaddem, A.K., Salaeh, N.M.K., Attia, M.M., 2022. Morphomolecular characterization of *Strongylus vulgaris* isolated from donkeys with special references to histopathological study on the affected organs. J. Parasit. Dis. 46, 795–803. https://doi.org/10.1007/s12639-022-01498-y.
Enigk, K., 1951. Weitere Untersuchungen zur Biologie von *Strongylus vulgaris* (Nematodes) in Wirtstiere. Z. Trop. Parasitol. 2, 523–535.
ESCCAP, 2019. A Guide to the Treatment and Control of Equine Gastrointestinal Parasite Infections. ESCCAP Guideline 08 Second Edition.
Faramarzi, B., Rich, L., 2019. Haematological profile in foals during the first year of life. Vet. Rec. 184, 1–5. https://doi.org/10.1136/vr.104461.
Feng, Z., Qi, S., Zhang, Yue, Qi, Z., Yan, L., Zhou, J., He, F., Li, Qianqian, Yang, Y., Chen, Q., Xiao, S., Li, Qiang, Chen, Y., Zhang, Yao, 2017. Ly6G+ neutrophil-derived miR-223 inhibits the NLRP3 inflammasome in mitochondrial DAMP-induced acute lung injury. Cell Death Dis. 8, e3170–14. https://doi.org/10.1038/CDDIS.2017.549.
Friedländer, M.R., MacKowiak, S.D., Li, N., Chen, W., Rajewsky, N., 2012. MiRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic Acids Res 40, 37–52. https://doi.org/10.1093/nar/gkr688.
Fromm, B., Billipp, T., Peck, L.E., Johansen, M., Tarver, J.E., King, B.L., Newcomb, J.M., Sempere, L.F., Flatmark, K., Hovig, E., Peterson, K.J., 2015. A Uniform System for the Annotation of Vertebrate microRNA Genes and the Evolution of the Human microRNAome. Annu. Rev. Genet. 49, 213–242. https://doi.org/10.1146/annurev-genet-120213-092023.
Fromm, B., Høye, E., Domanska, D., Zhong, X., Aparicio-Puerta, E., Ovchinnikov, V., Umu, S.U., Chabot, P.J., Kang, W., Aslanzadeh, M., Tarbier, M., Mármol-Sánchez, E., Urgese, G., Johansen, M., Hovig, E., Hackenberg, M., Friedländer, M.R., Peterson, K. J., 2022. MirGeneDB 2.1: toward a complete sampling of all major animal phyla. Nucleic Acids Res. 50, D204–D210. https://doi.org/10.1093/nar/gkab1101.
Fu, X., Zhou, Y., Cheng, Z., Liao, X., Zhou, X., 2015. MicroRNAs: novel players in aortic aneurysm. Biomed. Res. Int. 2015, 1–9. https://doi.org/10.1155/2015/831641.
Ghalehnoei, H., Bagheri, A., Fakhar, M., Mishan, M.A., 2020. Circulatory microRNAs: promising non-invasive prognostic and diagnostic biomarkers for parasitic infections. Eur. J. Clin. Microbiol. Infect. Dis. 39, 395–402. https://doi.org/10.1007/s10096-019-03715-8.
Glinge, C., Clauss, S., Boddum, K., Jabbari, R., Jabbari, J., Risgaard, B., Tomsits, P., Hildebrand, B., Kaèaèb, S., Wakili, R., Jespersen, T., Tfelt-Hansen, J., 2017. Stability of circulating blood-based microRNAs-Pre-Analytic methodological considerations. PLoS One 12, 1–16. https://doi.org/10.1371/journal.pone.0167969.
Hannon, G.J., 2010. FASTX-Toolkit Version 0.0.13 [WWW Document]. URL ⟨http://hannonlab.cshl.edu/fastx_toolkit/index.html⟩.
Hansen, E.P., Fromm, B., Andersen, S.D., Marcilla, A., Andersen, K.L., Borup, A., Williams, A.R., Jex, A.R., Gasser, R.B., Young, N.D., Hall, R.S., Stensballe, A., Ovchinnikov, V., Yan, Y., Fredholm, M., Thamsborg, S.M., Nejsum, P., 2019. Exploration of extracellular vesicles from *Ascaris suum* provides evidence of parasite–host cross talk. J. Extracell. Vesicles 8, 1–13. https://doi.org/10.1080/20013078.2019.1578116.
Hansen, E.P., Kringel, H., Thamsborg, S.M., Jex, A., Nejsum, P., 2016. Profiling circulating miRNAs in serum from pigs infected with the porcine whipworm, *Trichuris suis*. Vet. Parasitol. 223, 30–33. https://doi.org/10.1016/j.vetpar.2016.03.025.
He, X., Pan, W., 2022. Host–parasite interactions mediated by cross-species microRNAs. Trends Parasitol. 38, 478–488. https://doi.org/10.1016/j.pt.2022.02.011.
He, X., Sai, X., Chen, C., Zhang, Y., Xu, X., Zhang, D., Pan, W., 2013. Host serum miR-223 is a potential new biomarker for *Schistosoma japonicum* infection and the response to chemotherapy. Parasites Vectors 6, 1–8. https://doi.org/10.1186/1756-3305-6-272.
Henriksen, S.A., Korsholm, H., 1983. A method for culture and recovery of gastrointestinal strongyle larvae. Nord. Vet. Med. 35, 429–430.
Hoy, A.M., Lundie, R.J., Ivens, A., Quintana, J.F., Nausch, N., Forster, T., Jones, F., Kabatereine, N.B., Dunne, D.W., Mutapi, F., MacDonald, A.S., Buck, A.H., 2014. Parasite-derived MicroRNAs in host serum as novel biomarkers of helminth infection. PLoS Negl. Trop. Dis. 8, e2701. https://doi.org/10.1371/journal.pntd.0002701.
Ibberson, D., Benes, V., Muckenthaler, M.U., Castoldi, M., 2009. RNA degradation compromises the reliability of microRNA expression profiling. BMC Biotechnol. 9, 1–9. https://doi.org/10.1186/1472-6750-9-102.
International Helminth Genomes Consortium, 2019. Comparative genomics of the major parasitic worms. Nat. Genet. 51, 163–174. https://doi.org/10.1038/s41588-018-0262-1.
Issouf, M., Vargas, A., Boivin, R., Lavoie, J.P., 2019. MicroRNA-221 is overexpressed in the equine asthmatic airway smooth muscle and modulates smooth muscle cell proliferation. Am. J. Physiol. - Lung Cell. Mol. Physiol. 317, L748–L757. https://doi.org/10.1152/ajplung.00221.2018.
Jiao, P., Wang, X., Luoreng, Z., Yang, J., Jia, L., Ma, Y., Wei, D., 2021. miR-223: an effective regulator of immune cell differentiation and inflammation, 17. https://doi.org/10.7150/ijbs.59876.
Jürgenschellert, L., Krücken, J., Bousquet, E., Bartz, J., Heyer, N., Nielsen, M.K., von Samson-Himmelstjerna, G., 2022. Occurrence of strongylid nematode parasites on horse farms in Berlin and Brandenburg, Germany, With High Seroprevalence of *Strongylus vulgaris* Infection. Front. Vet. Sci. 9. https://doi.org/10.3389/fvets.2022.892920.
Kim, H.-A., Kim, M.-C., Kim, N.-Y., Ryu, D.-Y., Lee, H.-S., Kim, Y., 2018. Integrated analysis of microRNA and mRNA expressions in peripheral blood leukocytes of Warmblood horses before and after exercise. J. Vet. Sci. 19, 99. https://doi.org/10.4142/jvs.2018.19.1.99.
Kim, M.-C., Lee, S., Ryu, D., Cui, F., Bhak, J., Kim, Y., 2014. Identification and characterization of MicroRNAs in normal equine tissues by next generation sequencing. PLoS One 9, e93662. https://doi.org/10.1371/journal.pone.0093662.
Kolde, R., 2022. Package "pheatmap": Pretty heatmaps. R. Packag 1–8.
Lagatie, O., Batsa Debrah, L., Debrah, A., Stuyver, L.J., 2017. Plasma-derived parasitic microRNAs have insufficient concentrations to be used as diagnostic biomarker for detection of *Onchocerca volvulus* infection or treatment monitoring using LNA-based RT-qPCR. Parasitol. Res. 116, 1013–1022. https://doi.org/10.1007/s00436-017-5382-5.
Lai, C., Wu, Y., Yu, S., Yu, Y., Lee, S., Liu, C., Hsieh, W., Hwu, H., Chen, P., Jeng, S., Chen, W.J., 2014. Modulated expression of human peripheral blood microRNA from infancy to adulthood and its role in aging. Aging Cell 13, 679–689. https://doi.org/10.1111/acel.12225.
Langmead, B., Trapnell, C., Pop, M., Salzberg, S.L., 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10. https://doi.org/10.1186/gb-2009-10-3-r25.
Lee, R., Feinbaum, R., Ambros, V., 1993. The *C. elegans* Heterochronic Gene lin-4 Encodes Small RNAs with Antisense Complementarity to lin-14. Cell 75, 843–854.
Lee, S., Hwang, S., Yu, H.J., Oh, D., Choi, Y.J., Kim, M.C., Kim, Y., Ryu, D.Y., 2016. Expression of microRNAs in horse plasma and their characteristic nucleotide composition. PLoS One 11, 1–16. https://doi.org/10.1371/journal.pone.0146374.
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352.
Li, H., Liu, X., Sun, N., Wang, T., Zhu, J., Yang, S., Song, X., 2021. Differentially expressed circular non-coding RNAs in atherosclerotic aortic vessels and their potential functions in endothelial injury. Establ. Atheroscler. Mice 8, 1–13. https://doi.org/10.3389/fcvm.2021.657544.
Liang, Y., Ridzon, D., Wong, L., Chen, C., 2007. Characterization of microRNA expression profiles in normal human tissues. BMC Genom. 8, 1–20. https://doi.org/10.1186/1471-2164-8-166.
Liu, J., Zhu, L., Id, J.W., Qiu, L., Chen, Y., Id, E.D., Id, G.C., 2019. Schistosoma japonicum extracellular vesicle miRNA cargo regulates host macrophage functions facilitating parasitism 1–27.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/s13059-014-0550-8.
Lyons, E., 2003. Population-S benzimidazole- and tetrahydropyrimidine-resistant small strongyles in a pony herd in Kentucky (1977-1999): effects of anthelmintic treatment on the parasites as determined in critical tests. Parasitol. Res. 91, 407–411. https://doi.org/10.1007/s00436-003-0983-6.
Lyons, E.T., Drudge, J.H., Tolliver, S.C., 1990. Prevalence of some internal parasites found (1971–1989) in horses born on a farm in central Kentucky. J. Equine Vet. Sci. 10, 99–107. https://doi.org/10.1016/S0737-0806(06)80114-0.
Lyons, E.T., Swerczek, T.W., Tolliver, S.C., Bair, H.D., Drudge, J.H., Ennis, L.E., 2000. Prevalence of selected species of internal parasites in equids at necropsy in central Kentucky (1995-1999). Vet. Parasitol. 92, 51–62. https://doi.org/10.1016/S0304-4017(00)00266-1.
Macfarlane, C.L., Que, S., Pionnier, N., Turner, J.D., Wanji, S., Wagstaff, S.C., Taylor, M. J., 2020. The insufficiency of circulating miRNA and DNA as diagnostic tools or as biomarkers of treatment efficacy for *Onchocerca volvulus* 1–9. https://doi.org/10.1038/s41598-020-63249-4.
Mach, N., Plancade, S., Pacholewska, A., Lecardonnel, J., Rivière, J., Moroldo, M., Vaiman, A., Morgenthaler, C., Beinat, M., Nevot, A., Robert, C., Barrey, E., 2016. Integrated mRNA and miRNA expression profiling in blood reveals candidate biomarkers associated with endurance exercise in the horse. Sci. Rep. 6, 1–15. https://doi.org/10.1038/srep22932.
Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. J. 17, 10–12. https://doi.org/10.14806/ej.17.1.200.
Meder, B., Backes, C., Haas, J., Leidinger, P., Stähler, C., Großmann, T., Vogel, B., Frese, K., Giannitsis, E., Katus, H.A., Meese, E., Keller, A., 2014. Influence of the confounding factors age and sex on microRNA profiles from peripheral blood. Clin. Chem. 60, 1200–1208. https://doi.org/10.1373/clinchem.2014.224238.
Mienaltowski, M.J., Huang, L., Stromberg, A.J., Macleod, J.N., 2008. Differential Gene Expression Associated with Postnatal Equine Articular Cartilage Maturation 14. https://doi.org/10.1186/1471-2474-9-149.
Mitchell, P.S., Parkin, R.K., Kroh, E.M., Fritz, B.R., Wyman, S.K., Pogosova-Agadjanyan, E.L., Peterson, A., Noteboom, J., O'Briant, K.C., Allen, A., Lin, D.W., Urban, N., Drescher, C.W., Knudsen, B.S., Stirewalt, D.L., Gentleman, R., Vessella, R. L., Nelson, P.S., Martin, D.B., Tewari, M., 2008. Circulating microRNAs as stable blood-based markers for cancer detection. Proc. Natl. Acad. Sci. U. S. A. 105, 10513–10518. https://doi.org/10.1073/pnas.0804549105.
Mo, P., Tian, C., Li, Q., Teng, M., Fang, L., Xiong, Y., 2024. Heliyon decreased plasma miR-140-3p is associated with coronary artery disease. Heliyon 10, e26960. https://doi.org/10.1016/j.heliyon.2024.e26960.
Morgan, S.J., Stromberg, P.C., Storts, R.W., Sowa, B.A., Lay, J.C., 1991. Histology and morphometry of *Strongylus vulgaris*-mediated equine mesenteric arteritis. J. Comp. Pathol. 104, 89–99. https://doi.org/10.1016/S0021-9975(08)80091-6.
Neudecker, V., Haneklaus, M., Jensen, O., Khailova, L., Masterson, J.C., Tye, H., Biette, K., Jedlicka, P., Brodsky, K.S., Gerich, M.E., Mack, M., Robertson, A.A.B., Cooper, M.A., Furuta, G.T., Dinarello, C.A., O'Neill, L.A., Eltzschig, H.K., Masters, S. L., McNamee, E.N., 2017. Myeloid-derived miR-223 regulates intestinal inflammation via repression of the NLRP3 inflammasome. J. Exp. Med. 214, 1737–1752. https://doi.org/10.1084/jem.20160462.

Nielsen, M.K., L, M., Grice, A., Erskine, M., Graves, E., Vaala, W., Tully, R.C., French, D. D., Bowman, R., Kaplan, R.M., 2024. AAEP Internal Parasite Control Guidelines 1–25.

Nielsen, M.K., Olsen, S.N., Lyons, E.T., Monrad, J., Thamsborg, S.M., 2012a. Real-time PCR evaluation of *strongylus vulgaris* in horses on farms in Denmark and Central Kentucky. Vet. Parasitol. 190, 461–466. https://doi.org/10.1016/j.vetpar.2012.07.018.

Nielsen, M.K., Peterson, D.S., Monrad, J., Thamsborg, S.M., Olsen, S.N., Kaplan, R.M., 2008. Detection and semi-quantification of *Strongylus vulgaris* DNA in equine faeces by real-time quantitative PCR. Int. J. Parasitol. 38, 443–453. https://doi.org/10.1016/j.ijpara.2007.07.014.

Nielsen, M.K., Scare, J., Gravatte, H.S., Bellaw, J.L., Prado, J.C., Reinemeyer, C.R., 2015. Changes in serum *Strongylus Vulgaris*-specific antibody concentrations in response to anthelmintic treatment of experimentally infected foals. Front. Vet. Sci. 2, 1–6. https://doi.org/10.3389/fvets.2015.00017.

Nielsen, M.K., Vidyashankar, A.N., Olsen, S.N., Monrad, J., Thamsborg, S.M., 2012b. *Strongylus vulgaris* associated with usage of selective therapy on Danish horse farms—is it reemerging? Vet. Parasitol. 189, 260–266. https://doi.org/10.1016/j.vetpar.2012.04.039.

de Oliveira, G.P., Porto, W.F., Palu, C.C., Pereira, L.M., Reis, A.M.M.M., Marçola, T.G., Teixeira-Neto, A.R., Franco, O.L., Pereira, R.W., Teixeira-Neto, A.R., Franco, O.L., Pereira, R.W., 2021. Effects of endurance racing on horse plasma extracellular particle miRNA. Equine Vet. J. 53, 618–627. https://doi.org/10.1111/evj.13300.

Pacholewska, A., Kraft, M., Gerber, V., Jagannathan, V., 2017. Differential expression of serum MicroRNAs supports CD4+ T cell differentiation into Th2/Th17 cells in severe equine asthma. Genes (Basel) 8, 383. https://doi.org/10.3390/genes8120383.

Pacholewska, A., Mach, N., Mata, X., Vaiman, A., Schibler, L., Barrey, E., Gerber, V., 2016. Novel equine tissue miRNAs and breed-related miRNA expressed in serum. BMC Genom. 17, 1–15. https://doi.org/10.1186/s12864-016-3168-2.

Pritchard, C.C., Kroh, E., Wood, B., Arroyo, J.D., Dougherty, K.J., Miyaji, M.M., Tait, J.F., Tewari, M., 2012. Blood cell origin of circulating MicroRNAs: a cautionary note for cancer biomarker studies. Cancer Prev. Res. 5, 492–497. https://doi.org/10.1158/1940-6207.CAPR-11-0370.

Proudman, C.J., Edwards, G.B., 1992. Validation of a centrifugation/flotation technique for the diagnosis of equine cestodiasis. Vet. Rec. 131, 71–72. https://doi.org/10.1136/vr.131.4.71.

Quintana, J.F., Makepeace, B.L., Babayan, S.A., Ivens, A., Pfarr, K.M., Blaxter, M., Debrah, A., Wanji, S., Ngangyung, H.F., Bah, G.S., Tanya, V.N., Taylor, D.W., Hoerauf, A., Buck, A.H., 2015. Extracellular onchocerca-derived small RNAs in host nodules and blood. Parasites Vectors 8, 1–11. https://doi.org/10.1186/s13071-015-0656-1.

Reinemeyer, C.R., Smith, S.A., Gabel, A.A., Herd, R.P., 1984. The prevalence and intensity of internal parasites of horses in the U.S.A. Vet. Parasitol. 15, 75–83. https://doi.org/10.1016/0304-4017(84)90112-2.

Rendle, D., Austin, C., Bowen, M., Cameron, I., Furtado, T., Hodgkinson, J., McGorum, B., Matthews, J., 2019. Equine de-worming: a consensus on current best practice. UK-Vet. Equine 3, 1–14. https://doi.org/10.12968/ukve.2019.3.s.3.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., Smyth, G.K., 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies, 43. https://doi.org/10.1093/nar/gkv007.

Roepstorff, A., Nansen, P., Hansen, J.W., 1998. Epidemiology, diagnosis and control of helminth parasites of swine. Food and Agriculture Organization (FAO), Rome.

Russell, A.F., 1948. The development of helminthiasis in thoroughbred foals. J. Comp. Pathol. Ther. 58, 107–127. https://doi.org/10.1016/S0368-1742(48)80009-3.

Shkurnikov, M.Y., Knyazev, E.N., Fomicheva, K.A., Mikhailenko, D.S., Nyushko, K.M., Saribekyan, E.K., Samatov, T.R., Alekseev, B.Y., 2016. Analysis of plasma microRNA associated with hemolysis. Bull. Exp. Biol. Med. 160, 748–750. https://doi.org/10.1007/s10517-016-3300-y.

Slocombe, J.O., McCraw, B.M., 1973. Gastrointestinal nematodes in horses in Ontario. Can. Vet. J. = La Rev. Vet. Can. 14, 101–105.

Smith, T., Heger, A., Sudbery, I., 2017. UMI-tools: Modeling sequencing errors in Unique Molecular Identifiers to improve quantification accuracy. Genome Res 27, 491–499. https://doi.org/10.1101/gr.209601.116.

Stather, P.W., Sylvius, N., Sidloff, D.A., Dattani, N., Verissimo, A., Wild, J.B., Butt, H.Z., Choke, E., Sayers, R.D., Bown, M.J., 2015. Identification of microRNAs associated with abdominal aortic aneurysms and peripheral arterial disease. Br. J. Surg. 102, 755–766. https://doi.org/10.1002/bjs.9802.

Steuer, A.E., Anderson, H.P., Shepherd, T., Clark, M., Scare, J.A., Gravatte, H.S., Nielsen, M.K., 2022. Parasite dynamics in untreated horses through one calendar year. Parasit. Vectors 15, 1–13. https://doi.org/10.1186/s13071-022-05168-z.

Sutoh, M.M., Saheki, H., Inui, S., 1976. Occurrence and pathology of a disease of foals caused by larval migration of *Strongylus vulgaris*. Exp. Rep. Equine Heal. Lab. 60, 78.

Posit team, 2023. RStudio: Integrated Development Environment for R.

Toft, K., Honoré, M.L., Ripley, N.E., Nielsen, M.K., Fromm, B., Mardahl, M., Nielsen, L.N., Nejsum, P., Milan, S., Cirera, S., Pihl, T.H., 2024. The microRNAome of *Strongylus vulgaris* larvae and their excretory/secretory products with identification of parasite-derived microRNAs in horse arterial tissue. Int. J. Parasitol. https://doi.org/10.1016/j.ijpara.2024.10.005.

Tritten, L., Burkman, E., Moorhead, A., Satti, M., Geary, J., Mackenzie, C., Geary, T., 2014. Detection of circulating parasite-derived MicroRNAs in filarial infections. PLoS Negl. Trop. Dis. 8, e2971. https://doi.org/10.1371/journal.pntd.0002971.

Tsagiopoulou, M., Maniou, M.C., Pechlivanis, N., Togkousidis, A., Kotrová, M., Hutzenlaub, T., Kappas, I., Chatzidimitriou, A., Psomopoulos, F., 2021. UMIc: A Preprocessing Method for UMI Deduplication and Reads Correction. Front. Genet. 12, 1–10. https://doi.org/10.3389/fgene.2021.660366.

Turck, N., Vutskits, L., Sanchez-Pena, P., Robin, X., Hainard, A., Gex-Fabry, M., Fouda, C., Bassem, H., Mueller, M., Lisacek, F., Puybasset, L., Sanchez, J.-C., 2011. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinforma. 8, 12–77.

Tydén, E., Enemark, H.L., Franko, M.A., Höglund, J., Osterman-Lind, E., 2019. Prevalence of *Strongylus vulgaris* in horses after ten years of prescription usage of anthelmintics in Sweden. Vet. Parasitol. X 2, 100013. https://doi.org/10.1016/j.vpoa.2019.100013.

Tzelos, T., Barbeito, J.S.G., Nielsen, M.K., Morgan, E.R., Hodgkinson, J.E., Matthews, J. B., 2017. Strongyle egg reappearance period after moxidectin treatment and its relationship with management factors in UK equine populations. Vet. Parasitol. 237, 70–76. https://doi.org/10.1016/j.vetpar.2017.02.018.

Unger, L., Abril, C., Gerber, V., Jagannathan, V., Koch, C., Hamza, E., 2021. Diagnostic potential of three serum microRNAs as biomarkers for equine sarcoid disease in horses and donkeys. J. Vet. Intern. Med. 35, 610–619. https://doi.org/10.1111/jvim.16027.

Unger, L., Gerber, V., Pacholewska, A., Leeb, T., Jagannathan, V., 2019. MicroRNA fingerprints in serum and whole blood of sarcoid-affected horses as potential non-invasive diagnostic biomarkers. Vet. Comp. Oncol. 17, 107–117. https://doi.org/10.1111/vco.12451.

Wang, D., Wang, H., Liu, C., Mu, X., Cheng, S., 2019. Hyperglycemia inhibition of endothelial miR-140-3p mediates angiogenic dysfunction in diabetes mellitus. J. Diabetes Complicat. 33, 374–382. https://doi.org/10.1016/j.jdiacomp.2019.02.001.

Whitehead, B., Sørensen Rossen, L., Boysen, A.T., Indira Chandran, V., Skallerup, P., Thamsborg, S.M., Nejsum, P., -vch, W., 2024. MiRNA profiles of host extracellular vesicles are modulated by *Ascaris suum* infection but parasite extracellular vesicle miRNAs are undetectable systemically using in-depth miRNA sequencing. Int. J. Parasitol. https://doi.org/10.1016/j.ijpara.2024.08.001.

Xiang, M., Zeng, Y., Yang, R., Xu, H., Chen, Z., Zhong, J., Xie, H., Xu, Y., Zeng, X., 2014. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. Biochem. Biophys. Res. Commun. 454, 210–214. https://doi.org/10.1016/j.bbrc.2014.10.064.

Yi, M., Li, Y., Wang, D., Zhang, Q., Yang, L., Yang, C., 2020. KCNQ1OT1 exacerbates ischemia – reperfusion injury through targeted inhibition of miR-140–3 P, 43, 1832–1845. https://doi.org/10.1007/s10753-020-01257-2.

Youden, W.J., 1950. Index for rating diagnostic tests. Cancer 3, 32–35. https://doi.org/10.1002/1097-0142(1950)3:1< 32::AID-CNCR2820030106> 3.0.CO;2-3.

Yuan, S., Wu, Q., Wang, Z., Che, Y., Zheng, S., Chen, Y., Zhong, X., Shi, F., 2021. miR-223: an immune regulator in infectious disorders. Front. Immunol. 12, 1–16. https://doi.org/10.3389/fimmu.2021.781815.

Zhang, D., Lee, H., Wang, X., Groot, M., Sharma, L., Dela Cruz, C.S., Jin, Y., 2019. A potential role of microvesicle-containing miR-223/142 in lung inflammation. Thorax 74, 865–874. https://doi.org/10.1136/thoraxjnl-2018-212994.

Zhang, W., Shang, T., Huang, C., Yu, T., Liu, Chen, Qiao, T., Huang, D., Liu, Z., Liu, Changjian, 2015. Plasma microRNAs serve as potential biomarkers for abdominal aortic aneurysm. Clin. Biochem. 48, 988–992. https://doi.org/10.1016/j.clinbiochem.2015.04.016.

Zhu, Z.R., He, Q., Wu, W.Bin, Chang, G.Q., Yao, C., Zhao, Y., Wang, M., Wang, S.M., 2018. MIR-140-3p is involved in in-stent restenosis by targeting C-Myb and BCL-2 in peripheral artery disease. J. Atheroscler. Thromb. 25, 1168–1181. https://doi.org/10.5551/jat.44024.