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Evaluation of circulating microRNAs in plasma from horses with non-strangulating intestinal infarction and idiopathic peritonitis

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ABSTRACT

Non-strangulating intestinal infarctions (NSII) associated with *Strongylus vulgaris* infection and idiopathic peritonitis (IP) share similar clinical presentation but require different treatment approaches. Horses with NSII need surgical intervention, while idiopathic peritonitis cases can be successfully treated with antimicrobials. A correct diagnosis is thus crucial, but because the two diseases overlap in clinicopathological features, differentiation is difficult in clinical practice. MicroRNAs (miRNAs) are non-coding RNAs that exhibit measurable changes in abundance in tissues and circulation during disease. This study aimed to explore differences in plasma miRNA abundance between patients with NSII and IP. Plasma samples were collected from 43 horses, consisting of 21 with NSII and 22 with IP. A subset (n = 12) was submitted for deep small RNA sequencing to identify miRNAs differing between the groups. Next, a panel of nine miRNAs (two were potential normalizers) were selected for evaluation and confirmation by reverse transcription quantitative real-time PCR (RT-qPCR). Small RNA sequencing detected 628 miRNAs in the blood samples, but no miRNAs were differentially abundant between the disease groups. This finding was confirmed by qPCR. In agreement with previous studies, the top abundant miRNAs in both groups included Eca-Mir-122–5p and Eca-Mir-486–5p, as well as Eca-Mir-223–3p, which has previously been associated with inflammation. Target prediction for the most abundant miRNAs additionally predicted targets in inflammatory pathways. Evaluation of clinicopathological parameters revealed differences between the groups in two measures (white blood cell count and blood neutrophil count), which aligns with findings from previous studies. The results demonstrate that NSII and IP elicit similar miRNA profiles in plasma and are characterized by systemic inflammation.

1. Introduction

Non-strangulating intestinal infarctions (NSII) resulting from *Strongylus vulgaris* infection in horses are well described, especially in Denmark (Nielsen et al., 2016; Pihl et al., 2018; Honoré et al., 2019; Poulsen et al., 2023) and Sweden (Hedberg-Alm et al., 2022). The migrating stages of *S. vulgaris* cause thrombotic endarteritis in their predilection site, the Cranial Mesenteric Artery (CMA) and thrombotic material from the area can occlude smaller arteries, compromising intestinal blood supply. This can result in ischemia and necrosis in the associated intestinal sections, which can be fatal. Primary or idiopathic peritonitis (IP), defined as peritonitis of unknown etiology, is a common

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disease in horses (Matthews et al., 2001; Odelros et al., 2019; Arndt et al., 2021; Hedberg-Alm et al., 2022). This condition is characterized by a high survival rate (94–100 %) when treated conservatively with antibiotics (Odelros et al., 2019; Hedberg-Alm et al., 2022). This is in stark contrast to NSII cases, which do not respond to antibiotics and require immediate surgical intervention (Pihl et al., 2018; Hedberg-Alm et al., 2022). The clinicopathological presentations of NSII and IP are, however, very similar. Both groups develop peritonitis and present with colic of varying degrees, and many cases develop pyrexia, hematological alterations, and can have secondary intestinal impactions. Studies have evaluated clinical and clinicopathological features of NSII and IP patients (Hedberg-Alm et al., 2022; Poulsen et al., 2023). These studies identified a few parameters, which could potentially aid in the clinical differentiation, such as fibrinogen concentrations, white blood cell counts, and specific rectal findings, however, none of these parameters allowed for a complete differentiation of the two conditions.

MicroRNAs are involved in gene regulation at the post-transcriptional level and are involved in both normal physiological processes as well as disease processes (reviewed in Bartel, (2004); Cai et al., (2009); Soifer et al., (2007)). MicroRNAs are present in tissues and biological fluids (i.e. blood) and their abundance levels can change in association with disease. This phenomenon has advanced research towards identifying miRNAs as non-invasive biomarkers of many diseases (reviewed by Pogribny, (2018). In horses, few studies have explored miRNAs as biomarkers in conditions such as asthma (Issouf et al., (2019), sarcoids (Unger et al., 2019, 2021; Cosandey et al., 2021), hendra virus (Cowled et al., 2017), and osteoarthritis (Castanheira et al., 2021; Andersen et al., 2024). Our group previously explored miRNAs expressed and released by *S. vulgaris* (Toft et al., 2025b) and investigated both parasite and horse-derived miRNAs as biomarkers of *S. vulgaris* infection (Toft et al., 2025a). Additionally, changes in miRNA abundance have been shown in horses during endurance racing (Mach et al., 2016; Cappelli et al., 2018; de Oliveira et al., 2021) and associated with demographic factors such as breed, age and sex (Pacholewska et al., 2016; Cosandey et al., 2021; Unger et al., 2021).

The aim of this study was to explore circulating miRNA levels in plasma from horses with NSII and IP to investigate whether miRNAs could be used to differentiating between these diseases. Furthermore, the study evaluated clinicopathological parameters and explored possible correlations with miRNA levels.

2. Materials and methods

2.1. Ethical approvals

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. Use of samples from patients at the equine clinic at the Swedish University of Agriculture (SLU) and Evidensia Equine Hospital Helsingborg, Sweden (EEHH) was approved by the committee for animal experimentation in Uppsala (approval no: Dnr. 68/16) and Lund (Dnr. 5.8.18–02993/2022), Sweden. Owner consent was obtained for all included patients.

2.2. Study population

Horses included in this study (n = 43) consisted of two groups: 1. Horses diagnosed with NSII (n = 21); 2. Horses diagnosed with IP (n = 22); Inclusion criteria for each of the groups are listed in Table 1. Samples were collected from horses admitted to one of the three collaborating Scandinavian Equine Hospitals: LATH, SLU, EEHH. All samples were collected between 2018 and 2023. It should be noted that 13 of the horses in this study were also included in a study by Hedberg-Alm et al., (2022), and six horses were included in (Poulsen et al., 2023). In total, 23 horses (8 NSII and 15 IP) were exclusively included in this study.

Table 1
Inclusion criteria for the two groups of horses: Non-strangulating intestinal infarction (NSII) and idiopathic peritonitis (IP).

Group alias	Group description
NSII (n = 21)	Adult horses (>1 year) • Admitted to the LATH, SLU or EEHH. • Diagnosed with NSII by laparotomy or necropsy • NSII was defined as a well demarcated localized ischemic area of the intestine without signs of strangulation or enterocolitis, as previously described (Pihl et al., 2018; Hedberg-Alm et al., 2022).
IP (n = 22)	Adult horses (>1 year) • Admitted to the LATH, SLU or EEHH. • Diagnosed with peritonitis (peritoneal leukocyte count >20 $\times 10^9$ cells/L) • Successfully treated with antibiotics (survived to discharge without surgery)

Abbreviations: LATH: University of Copenhagen Large Animal Teaching Hospital. SLU: Swedish University of Agriculture. EEHH: Evidensia Equine Hospital, Helsingborg.

2.3. Blood sampling

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. Blood samples were collected from all included horses in 3.2 % 0.109 M sodium citrate tubes and serum separator tubes (BD, Franklin Lakes, NJ, USA) for plasma and serum analysis, respectively. The blood samples were separated by centrifugation at 2000*g* for 15 min within a maximum of four hours after sampling and the plasma or serum fraction collected, batched and stored at −70 °C before further analysis.

2.4. Demographic and clinicopathological parameters

Information was collected from medical records of all included horses. Demographical data included age (years), breed (cold-blooded or warm-blooded), and sex (male or female). The time of year for referral to the clinic (month) was likewise recorded. Clinicopathological parameters were collected from records of the first clinical examination performed after admittance. Few blood or peritoneal samples were collected after the initial examinations, and these were evaluated for inclusion on an individual basis. Obtained parameters included heart rate (HR), respiratory rate (RR), rectal temperature, packed cell volume (PCV), total serum protein concentration, white blood cell count (WBC), blood neutrophil count, blood leukocyte count, blood neutrophil/lymphocyte ratio, serum amyloid A (SAA) concentration, fibrinogen concentration, gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), total bilirubin, peritoneal leukocyte count, peritoneal protein concentration, and peritoneal neutrophil percentage. The concentrations for fibrinogen, GGT and GLDH could not be compared directly between the different study centers (different instruments were utilized, resulting in non-comparable results with different reference ranges). These values were consequently recorded as being within or outside the reference range reported by the instrument used.

2.5. RNA extraction and RNA quality control

Total RNA was extracted from 200 µL serum per sample, using miRNeasy Serum/Plasma Kit (Qiagen, MD, USA) according to the manufacturer's instructions, with the following minor adjustments: instead of 140 µL, 200 µL chloroform was used and the initial centrifugation was 30 min instead of 15 min for a better phase separation. A Nanodrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for quantification of RNA content in all samples. All

plasma samples were assessed for degree of hemolysis using spectrophotometry (Nanodrop One) by measuring the absorption at wavelength 414 nm (Shah et al., 2016; ThermoFisher, 2018). In cases where the absorption was > 1, the samples were further assessed for hemoglobin concentration on an Atellica CH Analyzer (Siemens Healthcare Diagnostics Inc, Malvern, PA, USA). Samples were to be excluded if hemoglobin concentration exceeded 15 mg/dl, as suggested by Unger et al., (2019). No samples exceeded this threshold, and no samples were, thus, excluded due to hemoglobin concentration.

2.6. Small RNA sequencing

A subset of 12 plasma samples, comprising of six samples from each disease group, were selected for small RNA sequencing, to identify miRNAs differing between the two disease groups. The selected horses were included based on their adherence to the following criteria to minimize the introduction of confounding factors: each group consisted of three females and three males (geldings) and only warm-blooded horses aged between 5 and 15 years were included. Six μL of total RNA (concentration range 28.7–80.3 ng/μL) per plasma sample (n = 12) were submitted for small RNA sequencing to an external provider (Omiics, Aarhus, Denmark). Total RNA concentration of the samples are given in Supplementary File 1. Briefly, library preparation was performed using the QIAseq miRNA Library Kit (Qiagen, MD, USA), according to the manufacturer's protocol. 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 approximately 50 million reads per sample.

2.7. MicroRNA panel selection

Five microRNAs, (Eca-Mir-140–3p, Eca-Mir-486–5p, Eca-Mir-122–5p, Eca-Mir-223–3p, Eca-Mir-423–5p) were chosen for qPCR evaluation based on our group's previous studies on *S. vulgaris* infection in horses (Toft et al., 2025a), and a confirmed high abundance in the sequencing data in this study. Furthermore, two miRNAs (Eca-Mir-21–5p, Eca-Mir-34-P1–5p) were selected based on a literature review of miRNAs related to intestinal ischemia in other mammalian species (Akbari, 2020). Two miRNAs (Eca-Mir-191–5p and Eca-Mir-92-P1a-3p) were selected for normalization based on their performance as normalizers in our previous study (Toft et al., 2025a).

2.8. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

For all samples, RT-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 50 ng of total RNA, 10x PAP buffer (1 μL) (NEB, MA, USA), 1 μL RT standard primer caggtccagttttttttttttttttvn (10 μM), dNTP mix (1 mM), ATP (1 mM), M-MuLV Reverse Transcriptase (10 units) (NEB, MA, USA), and Poly(A) Polymerase (1 unit) (NEB, MA, USA) in a total volume of 10 μL was incubated at 42ºC for 60 min, followed by inactivation at 95℃ for 5 min. The samples were subsequently cooled on ice. Prior to the qPCR procedure, cDNA samples were diluted 4 times, using RNase-free water. Forward and reverse primers were designed using the miRprimerdesign3 software (Busk, 2014) and are listed in Supplementary File 2. For the RT-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. A non-template control (NTC) and a non-Poly(A) Polymerase control (-PAP) were included as negative controls. The RT-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 and a melting curve analysis was done at the end of the run. Samples with Cq (quantification cycle)-values above 33, unspecific melting curves, or poor PCR efficiency (<80 % or >110 %, based on a standard curve made from a dilution series from a pooled cDNA from all included samples), were excluded from further analysis.

2.9. Bioinformatic analysis of sequencing data

2.9.1. NGS library QC and read count matrix generation

Adapter removal, trimming and quality filtering of fastq files were carried out using miRTrace in "qc" mode (Kang et al., 2018). Reads passing quality control were collapsed using Fastx_collapser. The frequency of reads in the samples were counted against the horse miRNome (available online at https://mirgenedb.org/) using MirDeep-quantify (Friedländer et al., 2012) with the following parameters: –weighed –mature5p3p –config —norpm.

A detailed description of the bioinformatic pipeline can be found in Supplementary file 3.

2.9.2. mRNA target prediction and enrichment analysis

Target prediction was carried out for miRNAs with more than 100,000 read counts across all samples. All miRNA names were converted to mirBase naming, and the corresponding miRNA family was found on the TargetScan 8.0 website (Agarwal et al., 2015). MicroRNAs belonging to the same miRNA family (same seed sequence) were collapsed into one query. Targets were predicted from the human genome, due to a significantly better annotation quality compared to the *Equus caballus* genome. From the TargetScan analysis, the top 100 predicted targets for each miRNA with an aggregate probability of conserved targeting (aP_{CT}) equal to or above 0.95, were selected. This measure represents the conservation status of the target mRNA site and 0.95 represents a 5 % probability that the target's site is conserved by chance. Enrichment analysis was performed using Enrichr (Chen et al., 2013; Kuleshov et al., 2016), and the the Reactome 2022 pathway database (Jassal et al., 2019).

2.10. Statistical analyses

The software R Studio (Posit team, 2023) was employed for all statistical analyses. Continuous variable data were assessed for normality using the Shapiro-Wilks test.

2.10.1. Group statistics for demographical data, clinicopathological parameters and seasonality

Comparisons between the two disease 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 disease groups with Fisher's Exact test. A *P*-value < 0.05 (or adjusted *P*-value where appropriate) was considered significant. Additionally, odds ratios (ORs) with 95 % confidence intervals (CIs) were calculated. ORs for categorical variables were derived from 2×2 contingency tables, while ORs for continuous variables were obtained from univariable logistic regression models. An odds ratio was likewise calculated for the likelihood of IP versus NSII occurring in winter versus summer, with winter defined as October to March and summer as April to September. To further assess seasonal patterns in NSII and IP, separate second-order polynomial regression models were fitted for each disease, using month (January-December) as the predictor variable. A plot including both models was generated using ggplot2 (Wickham, 2016). Seasonality was estimated based on the model fit (coefficient of determination (R^2) and akaike information criterion (AIC)), and confidence

intervals for the fitted values were extracted and compared to determine if significant differences in seasonal trends existed between the two diseases.

2.10.2. Differential abundance analysis of small RNA sequencing data

Only miRNAs with more than 100 total raw counts were included for analysis. Raw count data were normalized using the geometric mean-based method deployed by the Deseq2 package (Love et al., 2014). Heat maps were generated based on the normalized and rlog transformed counts using the package pheatmap (Kolde, 2022). Differential expression analyses comparing the miRNA abundance in the NSII group to the IP group were likewise conducted using the Deseq2 package. A principal component analysis (PCA) plot was generated in R using the package ggplot2, to visualize clustering of samples.

2.10.3. Processing and differential abundance analysis for RT-qPCR data

Pre-processing of all RT-qPCR data was performed using GenEx software (MultiD Analyses AB, Västra Frölunda, Sweden), briefly the steps included: interplate calibration, PCR efficiency correction, normalization to two stable miRNAs selected using NormFinder (Andersen et al., 2004), averaging cDNA replicates, relative quantities calculation based on the lowest expressed sample in each assay, and log2 transformation. Subsequently, R Studio was employed for statistical comparisons and visualization of the data. Differences in plasma miRNAs abundance between NSII and IP cases were explored by fitting linear regression models for each miRNA, using the limma package (Ritchie et al., 2015). Age (numerical), sex (levels: male or female) and breed (levels: cold-blooded or warm-blooded) were included as co-variables in the models. Corrections for multiple comparisons were done according to the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) and a *q*-value (corrected *P*-value) corresponding to a false discovery rate (FDR) of 5 % was accepted as significant.

2.10.4. Correlation between small RNA sequencing and RT-qPCR results

The relationship between small RNA sequencing counts and Cq-values from RT-qPCR was evaluated using Pearson correlation analysis. Prior to analysis, both small RNA sequencing counts and RT-qPCR Cq-values were log transformed, and normality confirmed using Shapiro-Wilk normality test. Scatter plots were generated for each miRNA using the ggplot2 package (Wickham, 2016), including regression lines and 95 % confidence intervals. Statistical significance of the correlations was accepted for a *P*-value < 0.05. A correlation was accepted as weak when the correlation coefficient was below 0.3, fair when it was between 0.3 and 0.5, moderately strong when it was between 0.6 and 0.8, and very strong when it exceeded 0.8, with the same thresholds applied for negative correlations (Chan, 2003).

2.10.5. Correlations between miRNAs and selected clinicopathological parameters

Combinations of clinicopathological parameters and miRNA RT-qPCR profiles were selected for correlation analysis. The combinations were selected based on the miRNAs expected biological functions, as described in the following: Eca-Mir-223–3p was tested for correlation with WBC (numerical), SAA concentration (numerical) and fibrinogen being increased or not (binary categorical), as this miRNA is known to be associated with inflammation and myeloid cells (Johnnidis et al., 2008; Neudecker et al., 2017). Eca-Mir-486–5p was tested for correlation with PCV (numerical) and spectrophotometric absorbance at 414 nm wavelength (numerical) (as a measure of hemolysis), due to known associations with hemolysis and red blood cells (Pritchard et al., 2012; Shkurnikov et al., 2016; de Oliveira et al., 2021). Eca-Mir-140–3p was tested for correlation with WBC (numerical), SAA concentration (numerical), and fibrinogen being increased or not (binary categorical). Eca-Mir-122–5p was tested for correlation with GGT (binary categorical), GLDH (binary categorical), total bilirubin (numerical), as this miRNA is known to be associated with liver and liver disease (reviewed by Jopling, (2012). Pearson correlation was used for normally distributed numeric data and Spearman correlation for non-normal numeric data. Scatter plots with regression lines and 95 % confidence intervals were generated using the ggplot2 package to visualize significant correlations. Logistic regression was employed for relationships involving binary categorical data. The correlations were calculated for all samples as well as separately for the two disease groups. A *P*-value of 0.05 was accepted as significant. Correlation strength for the linear regression was evaluated as described in Section 2.10.4.

2.11. Data accessibility

The data produced in this study has been submitted to NCBI Sequence Read Archive (Leinonen et al., 2011) and the SRA accession number is PRJNA1190892.

3. Results

3.1. Horse characteristics

Descriptive characteristics for the included horses are listed in Table 2. Only age differed significantly between the two disease groups ($P = 0.032$), while no difference was found for the distribution of sex, breed and season of presentation to the clinic, between the disease groups.

3.2. Case seasonality

A significant seasonal pattern was found for both NSII and IP cases, with both diseases presenting more often in the winter than in the summer. The quadratic term, which captures the curvature of the seasonal trend, was significant ($P = 0.0261$) for IP cases with a coefficient of 0.0849, explaining 48.2 % of the variance ($R^2 = 0.482$). This collectively indicates a moderate seasonal pattern for the IP group. Similarly, NSII cases showed a significant quadratic term ($P = 0.004$) with a coefficient of 0.146, explaining 62.5 % of the variance ($R^2 = 0.625$). This collectively suggests a more pronounced seasonal effect compared to IP cases. The models had Akaike information criteria (AICs) of 47.0 and 42.3 for NSII and IP, respectively, indicating a slightly better model fit for IP cases. A visual comparison of confidence intervals for the fitted values

Table 2
Demographic characteristics for non-strangulating intestinal infarctions (NSII) and idiopathic peritonitis (IP) cases, including the total number of cases, sex distribution, breed distribution, age in years (mean and standard deviation (SD), season of referral to clinic (Winter = October-March, Summer = April-September). Odds ratios (OR) for being in the NSII group, with 95 % confidence intervals (CI), relative to the reference category (ref). For the variable age, the OR represents the change in odds per one-year increase. *P*-values are provided for comparisons of sex, breed, age and season of referral between the disease groups. Significant *P*-values are highlighted in bold.

Disease group	NSII	IP	OR (95 % CI)	*P*-value
Total number	21	22	-	-
Sex				0.759
Female	8 (38 %)	10 (45 %)	ref	
Male	13 (62 %)	12 (55 %)	0.74 (0.22–2.49)	
Breed				0.525
Warm-blooded	14 (67 %)	13 (59 %)	ref	
Cold-blooded	6 (28 %)	9 (41 %)	1.62 (0.45–5.81)	
Unknown	1 (5 %)	0 (0 %)	-	
Age in years, mean (SD)	10.0 (4.83)	13.4 (5.22)	0.87 (0.75–0.99)	**0.032**
Season of referral to clinic				0.698
Summer	3	5	ref	
Winter	18	17	0.574 (0.10–2.87)	

across months revealed a consistent overlap between NSII and IP cases, suggesting no statistically significant difference in seasonal trends between the two diseases in this dataset. A plot of the polynomial model with confidence intervals can be viewed in Fig. 1.

3.3. Differences in clinicopathological parameters

The evaluated clinicopathological parameters are listed in Table 3, with *P* and *q*-values indicating if significant differences were found between the disease groups. Significant differences were found for WBC and blood neutrophil count. Boxplots for the two parameters differing between the groups are presented in Fig. 2. The GGT and GLDH data were missing for most horses and few horses had values above the reference value. Comparisons between the groups for these parameters were, thus, not performed.

3.4. MicroRNAs detected by small RNA sequencing

A total of 628 miRNA were detected with > 10 raw counts across all samples and 445 were detected with > 100 raw counts. The most highly abundant miRNAs, after normalization, were Eca-Mir-122–5p, accounting for 23 % of all counts, followed by Eca-Mir-486–5p and Eca-Mir-191–5p accounting for 21 % and 8 %, respectively. The ten most abundant miRNAs are listed in Table 4.

3.5. Clustering by principal component analysis (PCA)

For initial exploration of the sequencing data a PCA plot was generated (Fig. 3). No clear clustering of samples was observed related to the two disease groups.

3.6. Differences in plasma miRNA abundance between disease groups

Differential expression analysis of the sequencing data (subset of 12 horses) identified no significant differences in miRNA abundance between the two disease groups. A heatmap of the miRNAs with the lowest *P*-values are shown in Supplementary file 4. To verify the sequencing results and expand the analysis to all plasma samples from all included horses, we selected nine miRNAs for RT-qPCR analysis (based on the criteria outlined in Section 2.9). The selected miRNAs included Eca-Mir-140–3p, Eca-Mir-486–5p, Eca-Mir-122–5p, Eca-Mir-223–3p, Eca-Mir-423–5p, Eca-Mir-21–5p, Eca-Mir-34-P1–5p, Eca-Mir-191–5p, Eca-Mir-92-P1a-3p, which were evaluated by RT-qPCR in the 42 plasma samples. Eca-Mir-191–5p and Eca-Mir-92-P1a-3p were used as normalizers based on their intra- and intergroup stability. The miRNA Eca-Mir-21–5p, Eca-Mir-423–5p, and Eca-Mir-34-P1–5p were excluded from further analysis due to insufficient amplification or poor melting curves. None of the remaining four miRNAs showed differential expression between the two disease groups or any significant association with horse age, breed or sex. The only Log2FC above one was found for Eca-Mir-122–5p when comparing cold-blooded and warm-blooded horses (data not shown). This miRNA had a Log2FC of −1.77, when comparing the miRNAs abundance in plasma from the two breed groups, indicating a greater abundance in coldblooded horses. With a *P*-value of 0.057 and a *q*-value of 0.183 this finding was, however, not statistically significant. Results of the linear regression models for each miRNA, regarding difference in miRNA abundance between horses with NSII and IP, are shown in Table 5.

3.7. Correlation between small RNA sequencing and RT-qPCR results

The correlation analysis between sequencing counts and RT-qPCR Cq values showed varying levels of agreement across the four analyzed miRNAs. Eca-Mir-122–5p demonstrated a very strong positive correlation ($r = 0.980$, $P < 0.0001$), while moderately strong positive correlations were observed for Eca-Mir-486–5p ($r = 0.649$, $P = 0.022$) and Eca-Mir-223–3p ($r = 0.633$, $P = 0.027$). Eca-Mir-140–3p exhibited a fair, but non-significant correlation ($r = 0.428$, $P = 0.165$). Scatterplots for the correlations are presented in Fig. 4

3.8. Correlations between clinicopathological parameters and miRNA abundance

Correlations between selected miRNAs and clinicopathological parameters were evaluated to explore if miRNA abundance would correspond to expected alterations in clinicopathological parameters. For instance, Eca-Mir-223–3p would be expected to be higher in horses with increased inflammatory parameters, such as WBC and SAA (O'Connell et al., 2011; Fourdinier et al., 2019). Due to the low number of observations for GGT and GLDH, their correlation with Eca-Mir-122–5p was not calculated. Among the selected combinations, a significant correlation was found for two combinations: A fair positive correlation was

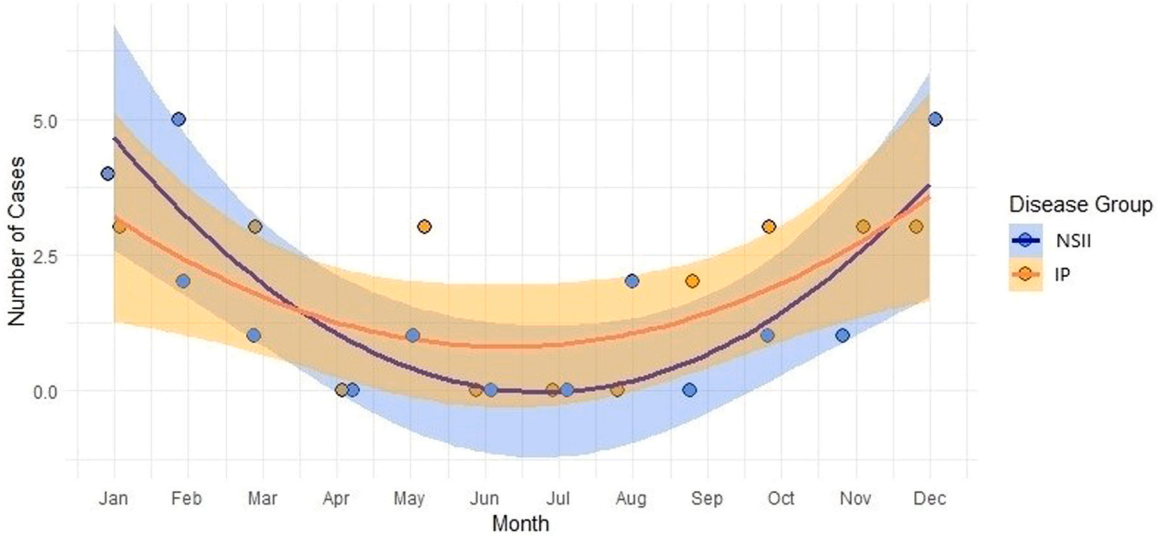

Fig. 1. Seasonal patterns in non-strangulating intestinal infarctions (NSII) and idiopathic peritonitis (IP) cases across months. Second-order polynomial regression models were fitted separately for NSII (blue) and IP (orange) cases, with month (January- December) as the predictor variable. The shaded regions (blue for NSII and orange for IP) represent the 95 % confidence intervals for each fitted curve. Points indicate the observed number of cases per month for each disease group.

Table 3
Comparison of clinicopathological parameters between the horses with non-strangulating infarctions (NSII) and idiopathic peritonitis (IP). Data are presented as mean and standard deviation (SD) for normally distributed data, and as median and range for non-normal data. Odds ratios (OR) for being in the NSII group, with 95 % confidence intervals (CI). For continuous variables, the OR represents the change in odds per unit increase in the given parameter. 'Data missing' indicate the number of missing data for each characteristic in each group. Bold font indicates a significant difference between groups. *q*-value is the *P*-value adjusted for multiple comparisons.

Disease Group	NSII (n = 21)	IP (n = 22)	OR (95 % CI)	*P*-value	*q*-value
Heart Rate, beats/min., mean (SD) Data missing	57 (13.4) 0	51 (12.4) 0	0.96 (0.91–1.01)	0.105	0.255
Respiratory Rate, breaths/min, median (range) Data missing	16 (10−40) 0	12 (10−40) 0	0.98 (0.91–1.04)	0.251	0.411
Rectal Temperature (admission), C°, mean (SD) Data missing	38.1 (0.51) 0	38.3 (0.55) 0	2.21 (0.69–8.03)	0.199	0.3755
Pyrexia prior to referral (>38.5 C°) No Yes Data missing	1 14 6	1 20 1	ref 0.31 (0.08–1.15)	0.070	0.232
Packed Cell Volume, %, median (range) Data missing	36 (25−64) 0	32 (25−42) 1	0.89 (0.78–0.98)	0.055	0.184
Serum protein, g/L, mean (SD) Data missing	65 (11.9) 0	63 (6.2) 0	0.97 (0.91–1.0)	0.466	0.528
WBC, 10^9 cells /L, median (range) Data missing	3.45 (1.6–10.2) 1	7.14 (2.6–14.1) 1	1.47 (1.14–2.00)	**0.004**	**0.043**
Blood neutrophils, 10^9 cells /L, median (range) Data missing	2.50 (0.2–9.3) 1	5.50 (2.2–13.4) 1	1.47 (1.11–2.09)	**0.005**	**0.043**
Blood lymphocyte, 10^9 cells/L, median (range) Data missing	0.90 (0.36–2.6) 2	0.97 (0.3–5.9) 1	1.53 (0.79–4.18)	0.694	0.694
Neutrophil/lymphocyte ratio, median (range) Data missing	3.71 (0.15–17.6) 2	5.53 (0.9–44.6) 1	1.06 (0.97–1.23)	0.065	0.184
Fibrinogen, Within reference Above reference Data missing	7 12 2	14 8 0	ref 2.89 (0.81 – 11.10)	0.594	0.175
Serum Amyloid A, mg/L, median (range) Data missing	1237 (0−11,552) 1	1138 (4–9350) 0	1 (1−1)	0.290	0.411
Total bilirubin, μmol/ml, median, (range) Data missing	51 (22−164) 8	66 (23−101) 6	0.99 (0.96–1.02)	0.140	0.280
GGT, above/within reference Data Missing	0/11 10	2/16 4	-	-	-
GLDH, above/within reference Data missing	1/2 18	0/12 10	-	-	-
Peritoneal leukocytes, 10^9 cells/L, median (range) Data missing	126 (7.9–454.8) 3	152 (7.1–498) 0	1 (1–1.01)	0.286	0.411
Peritoneal protein g/L, mean (SD) Data missing	45 (17) 2	38 (11) 1	0.96 (0.91–1.01)	0.122	0.260
Peritoneal neutrophil percentage %, mean (SD) Data missing	84 (11) 3	86 (6.2) 4	1.03 (0.95–1.11)	0.523	0.556

Abbreviations: GGT: Gamma-glutamyl transferase, GLDH: Glutamate dehydrogenase, SD: standard deviation, WBC: White blood cell count, g/L: grams per liter, 10^9 cells/L: billion cells per liter, mg/L: milligrams per liter.

found between Eca-Mir-223–3p and WBC (r = 0.336, $P = 0.034$), and a fair negative correlation was found between Eca-Mir-140–3p and SAA (r = -0.355, $P = 0.023$). When calculating the correlations for the disease groups separately, the correlation for Eca-Mir-223–3p and WBC became stronger (NSII: r = 0.530, $P = 0.021$, IP: r = 0.587, $P = 0.005$), while the correlation between Eca-Mir-140.3p and SAA became non-significant (NSII: r = -0.42, P = 0.08, IP: r = -0.23 P = 0.30). Scatter plots with linear regression lines for the correlation between Eca-Mir-223–3p and WBC and between Eca-Mir-140–3p and SAA, for all samples, are shown in Fig. 5.

3.9. mRNA target prediction of top abundant miRNAs

Although NSII and IP resulted in similar circulating miRNA patterns, we sought to explore the biological function of the most abundant miRNAs in these diseases, by performing miRNA-mRNA target prediction. MicroRNAs with more than 100,000 read counts across all samples were selected for analysis, resulting in 21 selected miRNAs. These mature miRNAs were all identical to their human orthologues and searching for targets in the better-annotated human genome was thus deemed acceptable. After collapsing miRNAs from the same miRNA families, the final number of miRNAs included in the search was 18. This resulted in prediction of 1764 target genes. When an aP_{CT} cut-off of > 0.94 was applied, several of the miRNAs (Eca-Mir-21–5p, Eca-Mir-23–3p, Eca-Mir-122–5p, Eca-Mir-191–5p, Eca-Mir-192–5p, Eca-Mir-223–3p, Eca-Mir-486–5p, Eca-Mir-342–3p, Eca-Mir-423–5p) did not have any predicted targets above the threshold. These miRNAs and targets were consequently not included in the collected target prediction and enrichment analysis. Inclusion of the eight remaining miRNAs (Eca-Mir-17–5p, Eca-Mir-26–5p, Eca-Mir-30–5p, Eca-Let-7–5p, Eca-Mir-92–3p, Eca-Mir-15–5p, Eca-Mir-1–3p, Eca-Mir-148–3p and Eca-Mir-125–5p) resulted in a total of 472 high confidence targets for further enrichment analysis. Supplementary file 5 shows the miRNA-target gene network, as well as the predicted gene targets for each miRNA.

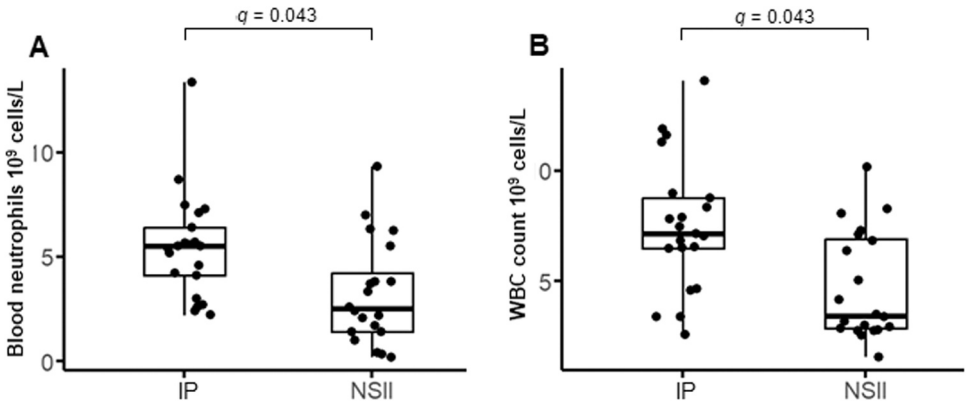

Fig. 2. Box and whiskers plots comparing (A) blood neutrophil count (10^9 cells /L), and (B) white blood cell count (WBC) (10^9 cells /L) between horses with non-strangulating intestinal infarction (NSII) and idiopathic peritonitis (IP). The *q*-value indicates the *P*-value adjusted for multiple comparisons. Dots represent individual horse samples.

Table 4
The ten most abundant miRNAs found by small RNA sequencing in plasma from horses with non-strangulating intestinal infarctions (NSII) and idiopathic peritonitis (IP), ranked by overall mean count across groups.

MicroRNA name	% of all counts	Mean count	NSII	IP
Eca-Mir−122–5p	23	372449	324794	420104
Eca-Mir−486–5p	21	343722	298751	388693
Eca-Mir−191–5p	8	135010	148407	121612
Eca-Mir−15-P2a−5p	6	92856	89348	96364
Eca-Mir−15-P2b−5p	6	92762	89257	96266
Eca-Mir−223–3p	4	74669	77206	72132
Eca-Let−7-P2b2–5p	4	67301	61372	73230
Eca-Mir−423–5p	1	22372	24659	20085
Eca-Mir−92-P2d−3p	1	22050	21531	22568
Eca-Mir−342–3p	1	18676	21347	16005

Table 5
Differences in horse-derived miRNA abundance in plasma, between horses with non-strangulating intestinal infarctions and horses diagnosed with idiopathic peritonitis, using RT-qPCR. The miRNAs are ranked by lowest *q*-value. None of the analyzed miRNAs showed significant differential abundance between the two disease groups.

MicroRNA	Log2FC	Mean abundance	*q*-value
Eca-Mir−486–5p	−0.40	1.73	0.749
Eca-Mir−223–3p	−0.16	1.60	0.87
Eca-Mir−122–5p	0.22	4.93	0.87
Eca-Mir−140–3p	0.05	2.78	0.87

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 %.

3.10. Pathway enrichment analysis of top abundant miRNAs

The enrichment analysis resulted in 37 significantly enriched pathways (top 20 shown in Fig. 6). The most significantly enriched pathway was regulation of RUNX1 expression and activity, while the TGF-beta signaling pathway was represented with four different terms in the top 10 (Signaling by TGFB family members, signaling by TGF-beta receptor complex, TGF-beta receptor signaling activates SMADs and downregulation of TGF-beta receptor signaling).

The full list of significantly enriched pathways is listed in Supplementary File 6.

4. Discussion

This study evaluates, for the first time, the presence and abundance of circulating miRNAs in plasma from horses with NSII and IP.

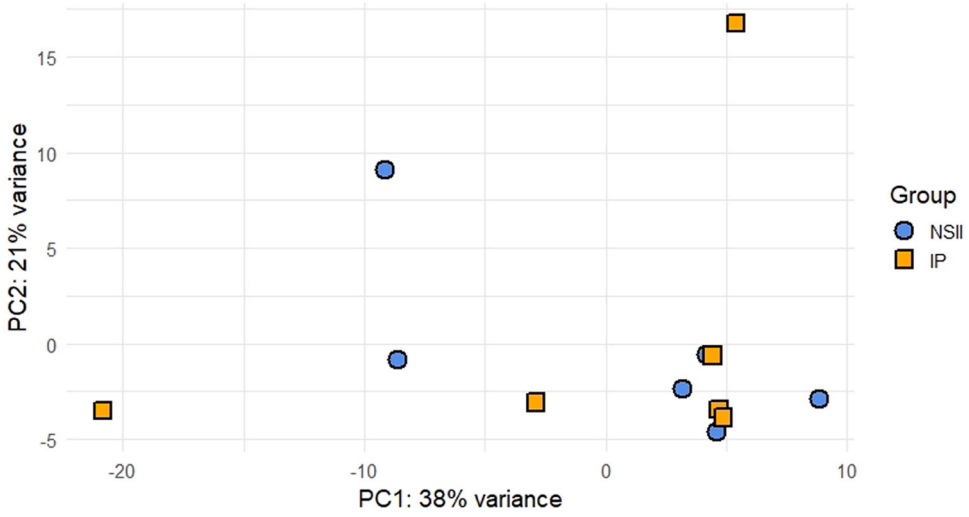

Fig. 3. Principal component analysis plot of miRNA abundance in the twelve sequenced plasma samples. Each point corresponds to one biological replicate (one horse). Blue dots: Plasma samples from horses with non-strangulating intestinal infarctions (NSII). Orange squares: Plasma samples from horses with idiopathic peritonitis (IP). PC1: Principal component 1. PC2: Principal component 2.

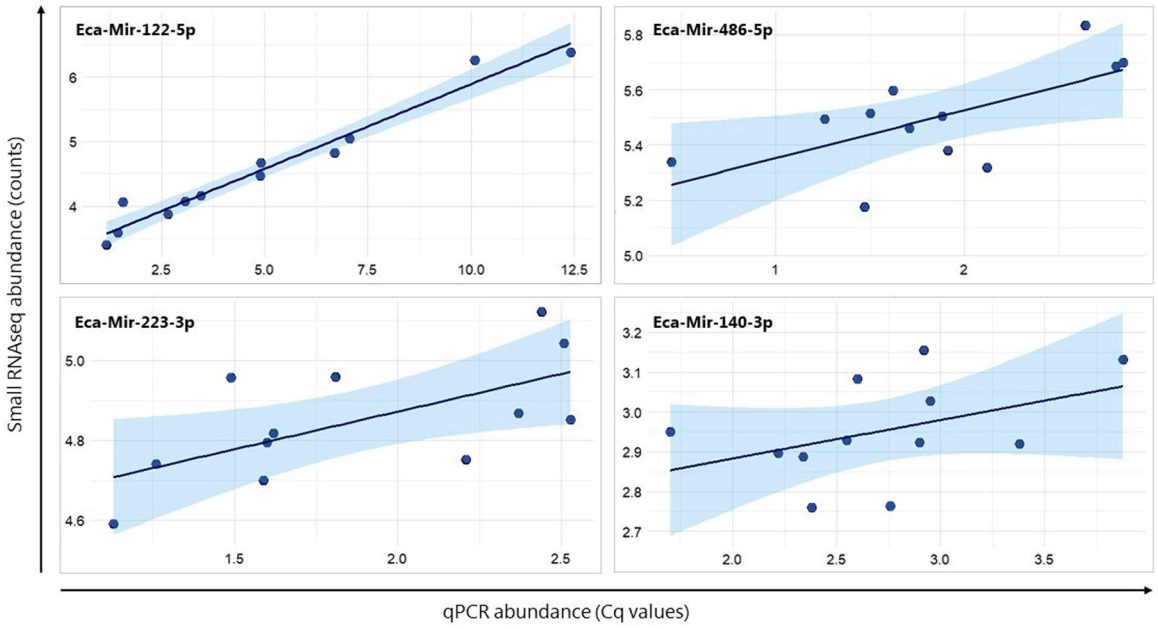

Fig. 4. Scatter plots showing the correlation between qPCR and small RNA sequencing results for Eca-Mir-122–5p, Eca-Mir-486–5p, Eca-Mir-223–3p, and Eca-Mir-140–3p. Each dot represents an individual data point corresponding to the qPCR quantification cycle (Cq) value and count from small RNA sequencing for a specific miRNA. The blue line represents the linear regression trend, showing a positive correlation between sequencing and qPCR results for all miRNAs (Eca-Mir-122–5p: r = 0.980, $P < 0.0001$; Eca-Mir-486–5p: r = 0.649, $P = 0.022$; Eca-Mir-223–3p: r = 0.633, $P = 0.027$; Eca-Mir-140–3p: r = 0.428, $P = 0.165$). The shaded blue areas indicate the 95 % confidence intervals around the regression line.

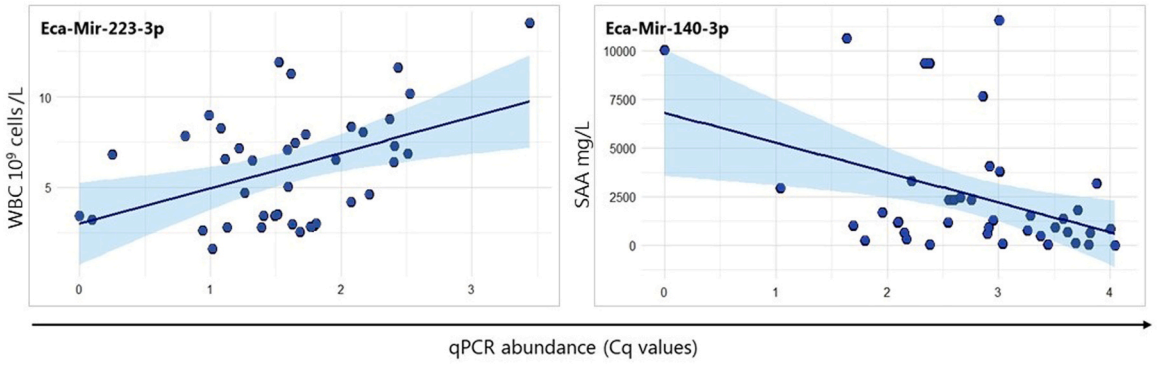

Fig. 5. Scatter plots showing the correlation between qPCR abundance for Eca-Mir-223–3p and Eca-Mir-140–3p with white blood cell count (WBC) and Serum Amyloid A (SAA), respectively. Each black dot represents individual data points corresponding to either Eca-Mir-223–3p and WBC levels or Eca-Mir-140–3p and SAA levels. The blue line represents the linear regression trend, indicating a positive correlation between Eca-Mir-223–3p and WBC (r = 0.336, P = 0.034) and a negative correlation between Eca-Mir-140–3p and SAA levels (r = -0.355, P = 0.023). The shaded blue areas represent the 95 % confidence intervals.

Additionally, we evaluated whether clinicopathological parameters differed between the diseases or correlated with the plasma abundance of specific miRNAs. Finally, we performed target prediction of the top abundant miRNAs to understand their potential biological function within the context of these diseases. The results of this study underpin future studies on miRNAs in horses in general and studies on horses with NSII and IP in particular.

The most abundant miRNAs detected in this study mirror findings from previous equine studies (Lee et al., 2016; Pacholewska et al., 2016; Unger et al., 2019; de Oliveira et al., 2021), with Eca-Mir-486–5p, Eca-Mir-122–5p, Eca-Let-7-P2b2–5p, Eca-Mir-15-P2a-5p, Eca-Mir-191–5p, Eca-Mir-423–5p, Eca-Mir-223–3p being found among the most abundant miRNAs in plasma. The top 10 most abundant miRNAs collectively accounted for 75 % of all miRNA counts, which is consistent with human studies (Tonge and Gant, 2016), which found the top 10 miRNAs in blood samples to account for between 68 % and 90 % of all counts. In our previous study on miRNAs in equine plasma samples (Toft et al., 2025a), the top 10 accounted for 60 % of counts, but this difference probably relates to the much higher abundance of Eca-Mir-122–5p in the present study, with this miRNA being the most abundant miRNA across all samples in both sequencing and RT-qPCR. Eca-Mir-122–5p accounted for 23 % of all normalized sequencing counts, while it only accounted for 12 % in our previous study (Toft et al., 2025a). When further analyzing the normalized data, it became apparent that the greater abundance of this miRNA was primarily driven by the greater abundance in two samples, one horse from the NSII group and one horse from the IP group, with 1791,090 and 2373,058 counts, respectively, accounting for 93 % of the total Eca-Mir-122–5p counts. This finding was also apparent in the RT-qPCR data where the two same samples had the absolute highest abundance of Eca-Mir-122–5p, compared to the other samples where sequencing data was available for comparison. Mir-122 is a well described miRNA in other species, recognized as primarily liver-specific and involved in regulation of gluconeogenesis (Wei et al., 2016), as well as cholesterol and triglyceride metabolism

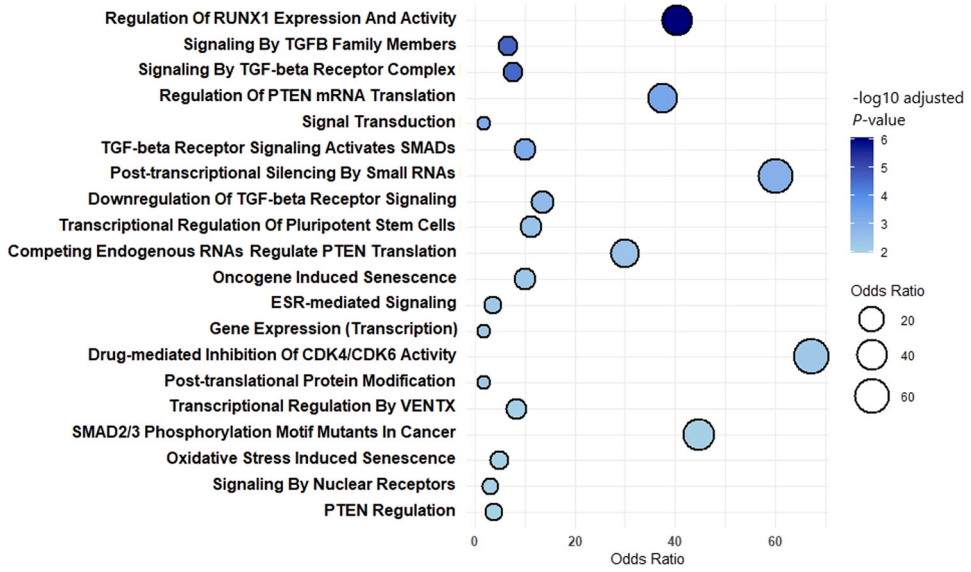

Fig. 6. Pathway enrichment analysis of gene targets associated with eight of the most abundant miRNAs in plasma from horses with non-strangulating intestinal infarctions and idiopathic peritonitis. The plot displays the top 20 enriched Reactome 2022 pathways ranked by their enrichment significance. Each circle represents one pathway, with their position on the x-axis and node size indicating the odds ratio (measure of enrichment strength) and the pathways ranked top to bottom on the y-axis according to the -log10 of their adjusted *P*-value, which is also indicated by their color (light to dark blue).

(reviewed by Jopling (2012) and Wen and Friedman (2012)). Unfortunately, as this very high abundance of Eca-Mir-122–5p was only detected in a few samples, it was not possible to explore associations with for example liver-related parameters.

All horses in this study exhibited signs of systemic inflammation. This finding aligns with the target prediction analysis of the top miRNA families, which revealed components involved in the TGF-ß signaling pathway, including interconnected regulators such as RUNX1, SMAD2/3, CDK4/6, and PTEN. The TGF-ß signaling pathway plays key roles in cell proliferation and differentiation, fibrosis, wound healing, and maintaining homeostasis in the gastrointestinal tract, as well as in modulating inflammatory and immune responses, either amplifying or suppressing them depending on the context (reviewed by Deng et al., 2024). This makes regulation of this pathway in horses with systemic inflammation biologically credible. However, the cellular origin of the circulating miRNAs remains unknown, introducing uncertainty to the predicted regulatory interactions. Further studies are needed to experimentally validate these predictions and confirm the functional relevance of the identified miRNA-mRNA interactions.

An individual miRNA worth noticing in relation to inflammation is Eca-Mir-223–3p. This miRNA, which was found among the top ten most abundant miRNAs in this study, is known to be related to inflammatory processes and myeloid cells in humans and mice (Pritchard et al., 2012; Yang et al., 2015; Neudecker et al., 2017; Yuan et al., 2021). Similarly, Ibrahim et al., (2021) and Lange-Consiglio et al., (2018), found Eca-Mir-223–3p to be involved with regulation of inflammation, and increased levels were found in serum from horse with endometritis compared to healthy controls. This could explain the high abundance of this miRNA in the horses with systemic inflammation in this study. Supporting this, we found a positive correlation between WBC and Eca-Mir-223–3p, although it was not significant when adjusted for multiple comparisons. The association between specific miRNAs and inflammation could have been better explored if a group of horses with no or low grade of inflammation had been included for comparison, but since the difference between NSII and IP was the focus of this study, no such control group was included. In our previous study (Toft et al., 2025a) we did explore miRNAs in plasma from healthy horses with and without *S. vulgaris* infection but direct comparison between different RT-qPCR or sequencing experiments should generally be avoided. It could, however, be noted that this previous study found a higher abundance of Eca-Mir-223–3p in inflamed arterial tissue compared to normal arterial tissue, again supporting a positive association with inflammation for this miRNA.

Eca-Mir-140–3p was similarly a miRNA of interest in our previous study, detected at lower abundance in inflamed arterial tissue and blood from *S. vulgaris* infected horses (Toft et al., 2025a). As the *S. vulgaris* infection status was unknown for most of the horses in the present study, the association with *S. vulgaris* infection could not be investigated. However, it is likely that any changes in miRNA abundance resulting from *S. vulgaris* migration, would be overshadowed by the changes related to general inflammation. It was, nevertheless, interesting to note that Eca-Mir-140–3p was negatively associated with increasing SAA concentrations, echoing the downregulation found in inflamed arterial tissue in our previous study. This miRNA was additionally strongly associated with age in our previous study, with a higher abundance observed in foals (age <1 year) compared to adults. No association with age was found for Eca-Mir-140–3p in this study, but since no foals were included, it could be hypothesized that the increased abundance was related to foals and not age in general.

We additionally compared demographic and clinicopathological parameters between the disease groups. For the demographic parameters, age differed significantly between the groups, being lower in the NSII group. Decreasing age was also a risk factor for NSII in the study by Poulsen et al. (2023), while age did not significantly differ between the disease groups in the study by Hedberg-Alm et al. (2022), although it was slightly lower in the NSII group compared to the IP group. Whether younger horses are at greater risk of NSII (or older horses are at greater risk of IP) is beyond the scope of this study, but since we found an almost total overlap in age between the two disease groups, age does not seem like a valuable parameter for differentiating between NSII and IP. In the study by Hedberg-Alm et al., (2022), significantly more horses with IP presented with pyrexia compared to those with NSII. In this study, there were no significant differences in rectal temperature at admission between the groups, and, overall, only 37 % of horses presented with pyrexia (>38.5°C). It was, however, noted that 20 of the 21 idiopathic peritonitis cases (data missing from one horse in this group) and 14 of 15 NSII cases (data missing from six horses in this group) had pyrexia registered by the referring veterinarian prior to referral. An explanation

for this pattern is probably that a horse presenting with fever and/or colic at home will be treated with nonsteroidal anti-inflammatory drugs (NSAIDs) by the referring veterinarian, often eliminating pyrexia at admission. Overall, pyrexia did not appear to be a relevant parameter for discriminating between NSII and IP in referred patients, especially since NSAIDs will often be administered and influences this parameter. The laboratory parameters WBC and blood neutrophil count were additionally found to be significantly lower in the NSII group than the IP group, which is also in concordance with the two previous studies (Hedberg-Alm et al., 2022; Poulsen et al., 2023) (although not significantly different when corrected for multiple comparisons in Poulsen et al., (2023)). The leukopenia (WBC median 3.45 10^9 cells /L, reference 5.45–12.65 10^9 cells /L) recorded in the NSII group is probably a reflection neutropenia in the same cases. Leuko- and neutropenia in horses can occur in the acute stages of systemic inflammatory responses and endotoxemia, as a consequence of depletion of neutrophils due to increased peripheral leukocyte demands (Vinther et al., 2015, 2016; Lilliehöök et al., 2016). This could suggest that NSII cases progress more acutely compared to IP cases, which have a more sub-acute/chronic leukocyte pattern at admission. This hypothesis is however in contrast to Hedberg-Alm et al. (2022) reporting that more horses had increased fibrinogen concentration in the NSII group than the IP group (which was also the case in this study, although not significant). Since fibrinogen increases more slowly than for example SAA, increased fibrinogen concentrations in the NSII group could indicate that NSII cases present at a less acute stage than the IP cases. It could, however, also reflect a less pronounced inflammatory response in the IP cases, which would align with the leukocyte patterns. The combination of increased fibrinogen and leukopenia was also recorded in NSII cases by Pihl et al., (2018).

We were interested in assessing seasonality of the two diseases, as both NSII and IP have previously been reported to be more common during the winter season (Pihl et al., 2018; Hedberg-Alm et al., 2022; Poulsen et al., 2023). We confirmed this observation in this study, and as also concluded by (Poulsen et al., 2023) there was no significant difference in seasonality between the diseases, although NSII had a slightly stronger seasonality than IP. This seasonal pattern is consistent with *S. vulgaris*' lifecycle, and although several factors could contribute to this shared seasonality, it could also support the theory that some IP cases are caused by *S. vulgaris* infection. This hypothesis is based on the suggestion that less severe NSII lesions might be able to heal without surgery and antimicrobial therapy is consequently effective for treatment of the concurrent peritonitis (Odelros et al., 2019; Hedberg-Alm et al., 2022). This hypothesis is difficult to investigate, especially since IP cases have a very high survival rate.

Ultimately, clinicians still face the question of when to opt for surgery versus when to choose a more conservative approach with antibiotics. Even if IP cases are, in fact, a result of less severe intestinal injuries, it is reasonable to assume that this difference should be measurable. This study sought to find differences in the circulating miRNAs level but concluded that, as other blood parameters evaluated before, there was little to no difference between the disease groups. An alternative approach could involve evaluating the local miRNA expression in the affected intestinal tissues, followed by analysis of the same miRNAs in peritoneal fluid. This would explore a more localized miRNA response but could only be performed in surgical cases or upon necropsy.

A limitation to this study is the relatively small sample size, which likely reduced the statistical power to detect differences in miRNA abundance between the disease groups. At the time of study design, a prospective power calculation was not feasible due to the lack of prior data on variability in equine miRNA abundance. However, post hoc power analyses based on observed standard deviations suggest that approximately 33 samples per group (for RT-qPCR) and 47 per group (for sequencing) would be needed to detect a 1.5-fold change with 80 % power at a significance level of 0.05. While increasing sample size could thus potentially reveal group-level differences, the considerable biological variability observed across individual miRNA profiles suggests that these markers would have limited utility for distinguishing individual cases, even if statistically significant differences were to emerge with a larger cohort.

In conclusion, none of the investigated circulating miRNAs showed potential as biomarkers for differentiation between NSII and IP in referred equine patients. This study did, however, contributed to the still limited knowledge of circulating miRNAs in horses, particularly in cases with systemic inflammation.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2025.106378.

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