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Selected microRNAs as biomarkers in sarcoid-affected horses under immunotherapy with a mistletoe extract

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Abstract. We investigated microRNAs (miRNAs) as potential prognostic biomarkers for equine sarcoid (ES) disease. In a breed-, age-, and sex-matched case-controlled study involving 45 ES-affected and 15 control horses, we assessed the diagnostic, prognostic, and theragnostic value of 3 miRNAs (eca-miR-127, eca-miR-379, eca-miR-432) in horses treated with European mistletoe (*Viscum album*) extract versus placebo. Whole-blood miRNA concentrations were measured using reverse-transcription quantitative real-time PCR (RT-qPCR) at 3 different times. We found that eca-miR-432 expression was lower in ES-affected (median = −1.93; 95% CI: −2.03 to −.86) compared to control (median = −1.71; 95% CI: −1.92 to −1.6) horses ($p = 0.03$, $r = 0.3$; 95% CI: 0.024–0.57) with a median difference of −1.93 versus −1.71, respectively. The ROC curve analysis indicated an area under the curve of 0.71 (95% CI: 0.51–0.84; $p = 0.005$) with a sensitivity of 74% (95% CI: 61–88%) and a specificity of 73% (95% CI: 39–94%) to diagnose ES. However, none of the miRNAs evaluated had prognostic potential or significant changes in expression following treatment. Additionally, miRNA expression was not influenced by breed, sex, or season. Although whole-blood eca-miR-432 had moderate diagnostic potential for ES, identifying prognostic miRNA biomarkers for ES remains a challenge.

Keywords: biomarkers; equine sarcoid; horses; miRNA; mistletoe; *Viscum album* extract.

Equine sarcoid (ES) is the most prevalent skin tumor in horses and donkeys globally.[40,48] Its clinical course is difficult to predict; dramatic disease exacerbation with aggressive widespread or locally invasive growth is possible, as is long-standing quiescence or spontaneous regression.[3,24] The multifactorial etiology of ES primarily involves bovine papillomaviruses 1 and 2 (BPV1, BPV2; *Papillomaviridae*, *Deltapapillomavirus 4*), and other external factors, as well as a genetic predisposition.[9,11,29,41] MicroRNAs (miRNAs) are emerging as potential players in the intricate molecular landscape of ES pathogenesis.[4,37,43]

MiRNAs are small non-coding RNA molecules that have gained attention as epigenetic biomarkers in various diseases, including cancer.[35] MiRNAs induce mRNA degradation and translational repression and therefore lead to the downregulation of gene expression.[35] In cancer research, great efforts are made to identify miRNAs in tissue and fluids to predict histologic subtypes, metastasis, responsiveness to therapy, and overall survival.[10,34,56]

In ES, the role of miRNAs extends to tissue-specific expression and quantifiability in circulation, making them potential candidates for noninvasive diagnostic and prognostic tools.[13,20,36,45–47] Histopathology remains the gold standard for diagnosing ES, but a biopsy is required, which can sometimes trigger lesion growth.[19] Furthermore, no reliable prognostic test is available to predict the growth behavior of sarcoids.

Investigations have identified specific whole-blood miRNAs (eca-miR-127, eca-miR-379, eca-miR-432), all encoded by a miRNA cluster on equine chromosome 24, as potential prognostic biomarkers for ES.[47] MiRNAs in this cluster correspond to the 14q32 miRNA cluster, the largest miRNA tumor suppressor cluster in humans.[4,47,54] Whole-blood eca-miR-432 has shown some prognostic potential in 2 RT-qPCR validation studies for predicting the emergence of ES lesions in young, sarcoid-free male horses.[13,20] Furthermore, its expression levels were decreased following effective treatment.[13,20]

Despite the potential prognostic value of circulating miRNAs, challenges related to biological variables, including breed,

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sex, and age, have been identified and prevent their use in daily equine practice.[1,13,20,36,45] The influence of these factors on miRNA expression levels must be thoroughly assessed to ensure the reliability of these biomarkers for clinical applications.[13,47]

We evaluated the diagnostic, prognostic, and theragnostic potential of whole-blood eca-miR-127, eca-miR-379, and eca-miR-432 in ES-bearing horses treated either with mistletoe extracts or placebo. Then we compared them with the miRNA signatures of horses without ES. European mistletoe (*Viscum album* subsp. *Austriacus*) extract (Iscador VAE; Iscador) has long been advocated for its antitumoral properties attributed to mistletoe lectins and viscotoxins, glycoproteins, and polypeptides.[16] Subcutaneous injections of VAE have shown efficacy as a systemic treatment directed against ES.[12] However, in a 2024 study, neither SC nor PO administration of VAE was significantly more effective than placebo for the treatment of ES.[2]

Materials and methods

Study cohort

As part of a randomized, placebo-controlled, double-blinded trial, blood samples were collected from 45 ES-affected horses (3–12-y-old) enrolled in a study investigating the efficacy of PO- and SC-administered VAE for the treatment of ES.[2] The clinical diagnosis of ES was established using a validated scoring protocol and corroborated by a BPV DNA–positive swab or, in one instance, histologic confirmation.[21] Our study was approved by the Ethical Committee of the Kanton of Bern, Switzerland (BE 125/2020).

Among the 45 ES-affected horses, 29 received VAE either SC (14; SC VAE) or PO (15; PO VAE); 16 horses constituted the placebo group (Suppl. Table 1). Horses were treated with either VAE or a placebo (0.9% sodium chloride solution) for 7 mo, 3 times/wk. Depending on the study group, horses received treatment in 1 of 3 ways: PO VAE and SC placebo, PO placebo and SC VAE, or placebo via both SC and PO. The VAE treatment followed a structured dosing regimen over 28 wk, consisting of alternating cycles of 2 series: series 1 (wk 1–5 and 15–19) with escalating doses of 0.1–10 mg, and series 2 (wk 5–10 and 19–24) with doses increasing from 1.0–20 mg. Additionally, during wk 10–14 and 24–28, horses received 2 cycles of seven 20-mg ampoules.[2] All dosages correspond to the amount of fresh plant material in 1 mL of Iscador VAE. Blood collection occurred at 3 times: time 0 (T0) = the first sampling time directly before the initiation of VAE or placebo treatment; time 1 (T1) = the second sampling time at 7 mo ± 8 d later, immediately after the end of the VAE or placebo treatment; and time 2 (T2) = the third sampling time at 14 mo ± 8 d (7 mo after the end of VAE or placebo treatment).

Additionally, blood samples were collected from 15 ES-free control horses without clinical signs of ES or any other skin tumors. The control horses were stabled in the same locations as the ES-affected horses and selected by group matching. Particular attention was paid to achieving an even breed and sex distribution in the ES and control groups. Control horses were 3–12-y-old. The control horses underwent no therapies or interventions directed against ES during the study period. Blood samples were collected from the control horses during the winter (T0; November 2021–January 2022), summer (T1; June and July 2022), and winter (T2; December 2022–February 2023).

All animals were client-owned and stayed with their owners during the trial. The blood samples were taken at the horses' respective stables. Venous blood was drawn from the jugular vein into PAXgene RNA blood tubes (Qiagen/BD). The samples were transported in a refrigerator (5–7°C) to the laboratory in ≤24 h and subsequently stored in the same tubes at −80°C for up to 24 mo.

The sample size calculation was based on a study comparing the PO and SC administration of VAE for treating ES,[2] in the course of which blood samples were taken from ES-affected horses for our present study. The target number of cases per group (SC VAE, PO VAE, placebo) was set at a minimum of 14 horses. To account for early dropouts, the number of cases per group was increased to 15 horses. We added 15 control horses, resulting in a total of 60 horses. The group sizes matched the smallest acceptable group size of 15–20 individuals established in previous miRNA studies in comparable study cohorts.[13]

Pre-analytics

Frozen blood samples were thawed on ice at room temperature for 12 h before extraction. MiRNA was extracted (PAXgene blood miRNA kit; Qiagen/BD) according to the manufacturer's protocol. In step 7, a synthetic spike-in control—cel-miR-39-3p (3.5 µL, 33 nM; Integrated DNA Technologies [IDT])—was added to each sample to monitor miRNA-extraction efficiency. The miRNA was eluted in 40 µL of BR5 buffer (Qiagen/BD). Immediately after extraction, the RNA concentration was measured (Qubit 4 fluorometer; ThermoFisher), in combination with an RNA quantification assay (Qubit RNA BR assay kit; ThermoFisher). The extracted RNA was stored at −80°C for 7 mo until used in the RT reaction within 10 d.

RT-qPCR

The candidate miRNAs (eca-miR-127, eca-miR-379, eca-miR-432) were selected based on their demonstrated potential as diagnostic and/or prognostic markers in 3 comprehensive studies.[13,20,47] For robust normalization and control purposes, eca-miR-30d was chosen as the endogenous control; cel-miR-39-3p was the exogenous control, aligning with established recommendations.[13,47] Efficiencies of the TaqMan miRNA assays were determined with synthetic RNA (IDT) in

the range of 0.1–0.00001 nmol/L (6×10^7–6×10^3 copies/µL RNA; Suppl. Table 2).

For the RT reaction, miRNA was diluted 1:2 with RNase-free water. The RT reactions were performed (TaqMan microRNA reverse transcription kit; ThermoFisher), and sequence-specific stem-loop RT primers were used for the candidate miRNAs as well as endogenous and exogenous controls (TaqMan miRNA assays; ThermoFisher). Each 10-µL RT reaction contained 2 µL of miRNA, 0.34 µL of 5× miRNA-specific stem-loop primer, 1.0 µL of 10× RT buffer solution, 0.1 µL of 100 mM dNTP solution, 0.13 µL of RNAse inhibitor, 0.67 µL of reverse transcriptase (MultiScribe; ThermoFisher), and 5.76 µL of nuclease-free water. The eca-miR-432 reactions contained 0.085 µL of 20× miRNA-specific stem-loop primer and 6.015 µL of nuclease-free water; the other ingredients were as stated above. The eca-miR-432 no-RT reactions contained 0.67 µL of nuclease-free water instead of reverse transcriptase. The RT reaction conditions were as follows: primer annealing at 16°C for 30 min, extension phase at 42°C for 30 min, and 85°C for 5 min to stop the reaction with subsequent cooling at 4°C.

The subsequent qPCR reactions, using specific primers and the RT product, were conducted in triplicate using a 384-real-time PCR system (CFX Opus 384; Bio-Rad). The 15-µL reaction consisted of 7.50 µL of enzyme mix (2× qPCRBIO probe blue mix separate-ROX; PCRBiosystems), 0.38 µL of 20× specific primer, 5 µL of RT product diluted 1:3.5 with nuclease-free water, and 2.13 µL of nuclease-free water. The qPCR reactions were started with a denaturing step at 95°C for 3 min, followed by 40 cycles of denaturing at 95°C for 10 s and annealing and elongation at 60°C for 30 s. A RT-negative control of the eca-miR-432 assay was run simultaneously in triplicate as this assay had occasional signals in RT-negative reactions. We excluded 12 measurements in the eca-miR-432 assay that had <5 Cq cycle differences between RT-positive and RT-negative reactions.[26]

To assess the quality of the assays, we scrutinized the raw Cq values for the tested miRNAs among the 3 technical replicates for each candidate miRNA within each sample. A maximum Cq difference of 0.5 was deemed acceptable; we excluded technical replicates above this threshold.[20] Moreover, we excluded the 3 triplicate measurements if none of the pairs within the triplicates had a difference <0.5. Furthermore, 4 measurements gave no signal.

Statistical analysis

Statistical analysis was performed using statistical software (v.23.0.2.2023; NCSS). The following breed classification was used: warmblood horses/warmblood-related breeds, Franches-Montagnes horses, pony breeds, and Thoroughbreds. Horses were either categorized as male (gelding) or female (mare). Age categories were not considered confounding factors for miRNA expression, as all horses were young or middle-aged (3–12-y-old). Thus, no major effect of age on miRNA expression was expected. According to the classification applied in a previous study using the same study cohort,[2] horses were allocated to different groups based on treatment response and ES progression: regression (complete or partial) versus progression or stable (no change in tumor burden) disease (regardless of whether horses received PO or SC VAE therapy). Horses that received either PO or SC VAE were classified further as successful therapy (complete or partial regression in response to therapy) or failure of therapy (progression or stable disease in response to therapy), respectively.

The miRNA expression data were not normally distributed according to normality tests (Shapiro–Wilk W, Kolmogorov–Smirnov, D'Agostino skewness, D'Agostino kurtosis, D'Agostino Omnibus) and therefore were log-transformed. The Grubb test was utilized to identify outliers for each candidate miRNA within ES-affected and ES-free horses for each time separately.[30] The miRNA data identified as outliers were excluded from further analysis. The miRNA data from 1 ES-free control horse were excluded because of outlier data points for all 3 candidate miRNAs. In 2 other control horses, outlier data points were identified at 1 sampling time each for eca-mir-379; those miRNA data were excluded from further analysis.

The Pearson chi-square test was used to compare breed distribution and the Fisher exact test to compare sex distribution in ES-affected versus ES-free horses, in horses with ES regression versus ES progression or stable disease, and in horses treated with VAE versus placebo. In ES-free horses, the effect of sampling seasonality (winter T0, summer T1, winter T2) on the expression of the candidate miRNAs was investigated using repeated measures of one-way ANOVA. In ES-affected horses, the effect of seasonality was not evaluated because the sampling times were distributed unevenly over the year study subjects were recruited.

We used a general linear model to assess the 1) diagnostic and 2) prognostic potential of the candidate miRNAs and whether this was influenced by sex or breed. MiRNA expression was compared between ES-affected and ES-free horses, horses with regression versus horses with progression or stable disease, and horses with successful therapy versus failure of therapy at T0. Expression levels of the tested miRNAs were compared between groups using the Wilcoxon rank sum test.

For miRNAs with significant variations in expression between groups, a receiver operating characteristic (ROC) curve was created to identify the area under the curve (AUC) and to calculate their specificity (Sp) and sensitivity (Se) as diagnostic and/or prognostic biomarkers. The positive likelihood ratio (LR+), negative likelihood ratio (LR–), diagnostic odds ratio (DOR), pre-test probability (PRP), and post-test probability (POP) were reported.

To determine whether VAE therapy affected the expression of candidate miRNAs, miRNA expression was compared between horses treated with VAE (PO or SC) versus

Table 1. Breed and sex distribution in equine sarcoid (ES)-affected versus ES-free horses.

Biological variable	ES-affected (%)	ES-free (%)	Total population (%)
Breed			
Warmblood, warmblood-related breeds	32/45 (71)	10/14 (71)	42/59 (71)
Franches-Montagnes	6/45 (13)	4/14 (29)	10/59 (17)
Pony breeds	3/45 (7)	0/14 (0)	3/59 (5)
Thoroughbreds	4/45 (9)	0/14 (0)	4/59 (7)
Sex			
F	25/45 (56)	8/14 (57)	33/59 (56)
M	20/45 (44)	6/14 (43)	26/59 (44)

F = female (mare); M = male (gelding).

placebo using the Wilcoxon rank sum test. To determine whether miRNA profiles changed over the study period depending on the clinical and therapeutic outcome, the expression levels of the candidate miRNAs were compared separately 1) in the regression versus progression or stable disease groups and 2) in the groups of horses with successful therapy versus failure of therapy at sampling times T0, T1, T2 using repeated-measures ANOVA. Effect size and its 95% CI were calculated and interpreted as follows: effect size 0.1–0.2 was considered a small effect, >0.2–0.3 was considered a medium effect, and >0.3–0.5 was considered a large effect.[27] *P*-values ≤0.05 were considered significant.

Results

One ES-affected horse in the SC VAE group was euthanized 7 d after being enrolled in the study for reasons unrelated to the study and was therefore replaced by another participant. Due to the blinded nature of the study, the newly enrolled horse was placed in the placebo group. This led to 15 horses in the PO VAE group, 14 in the SC VAE group, and 16 in the placebo group. One horse in the control group developed ES, leading to the exclusion of all samples (T0, T1, T2) from this individual. Another control horse was in foal at the time of the T2 sampling. Due to the possible influence of gestation on the expression of miRNAs of the equine chromosome 24 miRNA cluster, we also excluded this T2 sample from further analysis.[15]

The breed distribution was not significantly different in ES-affected versus ES-free horses ($p=0.30$; Table 1), in horses with ES regression versus ES progression or stable disease ($p=0.75$), and in horses treated with VAE versus placebo ($p=0.52$). Sex distribution was not significantly different in ES-affected versus ES-free horses ($p=1$; Table 1), in horses with ES regression versus ES progression or stable disease ($p=0.75$), and in horses treated with VAE versus placebo ($p=1$). The concentration of the RNA extracted from whole-blood samples was 9.8–182 ng/µL ($\bar{x}=73.5$ ng/µL; SD = 24.0 ng/µL).

In ES-free horses, there was no influence of season (winter T0, summer T1, winter T2) on the expression of eca-miR-127, eca-miR-379, or eca-miR-432 (Suppl. Fig. 1).

Diagnosis (ES vs. ES-free) had a significant effect on the expression of eca-miR-432 ($p=0.04$), with ES-affected horses (median = −1.93; 95% CI: −2.03 to −1.86) having significantly ($p=0.03$) lower expression of eca-miR-432 than ES-free horses (median = −1.71; 95% CI: −1.92 to −1.6), with a medium effect size ($r=0.3$; 95% CI: 0.024–0.56). In contrast, diagnosis (ES vs. ES-free) did not have a significant effect on the expression of eca-miR-127 ($p=0.48$) and eca-miR-379 ($p=0.66$; Fig. 1). Expression levels of eca-miR-127 were not significantly different between ES-affected (median = −1.9; 95% CI: −2.0 to −1.8) and ES-free (median = −1.8; 95% CI: −2.2 to −1.42) horses, with negligible effect size ($r=0.091$; 95% CI: −0.16 to 0.35). The same applied for expression levels of eca-miR-379 in ES-affected (median = −2.4; 95% CI: −2.5 to −2.2) versus ES-free (median = −2.3; 95% CI: −2.43 to −2.07) horses, with a small effect size ($r=0.18$; 95% CI: −0.08 to 0.45).

The expression of none of the tested miRNAs was influenced by the sex variable. The interaction between sex and diagnosis had no significant effect on the expression of the candidate miRNAs (Table 2). The expression of none of the tested candidate miRNAs was influenced by the breed category (eca-miR-127: $p=0.38$; eca-miR-379: $p=0.82$; eca-miR-432: $p=0.65$). The interaction between breed and diagnosis had no significant effect on the expression of all tested miRNAs (eca-miR-127: $p=0.32$; eca-miR-379: $p=1$; eca-miR-432: $p=0.99$). Hence, the use of eca-miR-432 as a diagnostic biomarker is not affected by sex or breed.

The ROC curve for whole-blood eca-miR-432 as a diagnostic biomarker for ES disease had an AUC of 0.71 (95% CI: 0.51–0.84; $p=0.005$), with a Se of 74% (95% CI: 61–88%) and a Sp of 73% (95% CI: 39–94%; Fig. 2). The LR+ and LR− for eca-miR-432 in diagnosing ES were 2.8 and 0.4, respectively, with a DOR of 7.5, a PRP of 79%, and a POP of 91%.

None of the 3 candidate miRNAs had significantly different expression between horses with regression versus progression or stable disease (Suppl. Table 3A; Suppl. Fig. 2A–C) or between horses with successful therapy versus failure of therapy (Suppl. Table 3B; Suppl. Fig. 2D–F) at T0. The expression of none of the miRNAs tested was influenced by the sex variable (Suppl. Table 3). The breed category did not influence the

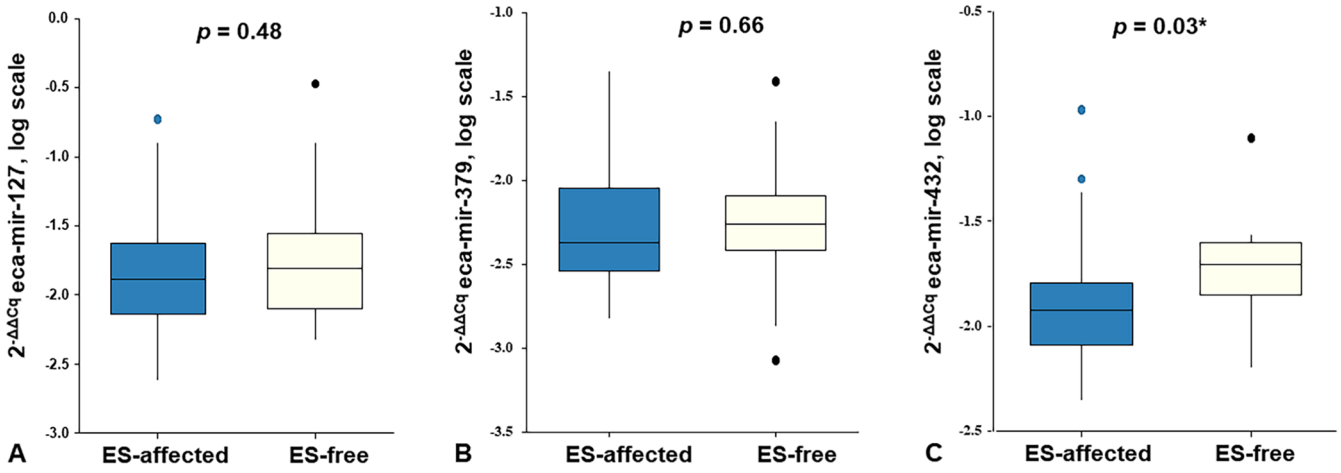

Figure 1. Box and whisker plots of expression of **A**) eca-miR-127, **B**) eca-miR-379, and **C**) eca-miR-432 in equine sarcoid (ES)-affected versus ES-free horses at the first sampling time. The y-axis gives the $2^{-\Delta\Delta Cq}$ values of the candidate miRNA on a log scale. The central horizontal line in the box is the median. The bottom and top of the box indicate the first and third quartiles. The whiskers extend to the upper and lower adjacent values: the upper adjacent value is the largest observation that is less than or equal to the third quartile plus 1.5 times the interquartile range (IQR), and the lower adjacent value is the smallest observation that is greater than or equal to the first quartile minus 1.5 times the IQR. Statistically significant results/comparisons are marked with an asterisk.

Table 2. Influence of diagnosis, sex, and their interaction on the expression of the candidate miRNAs at T0.

Variable	eca-miR-127	eca-miR-379	eca-miR-432
Diagnosis	0.40	0.81	**0.04**
Sex	0.77	0.76	0.32
Interaction	0.66	0.99	0.29

Statistically significant *p*-values are shown in bold.

capability of the candidate miRNAs either to predict regression versus progression or stable disease (eca-miR-127: $p=0.52$; eca-miR-379: $p=0.76$; eca-miR-432: $p=0.72$) or to predict the therapeutic outcome (successful therapy vs. failure of therapy: eca-miR-127, $p=0.66$; eca-miR-379, $p=0.93$; eca-miR-432, $p=1$).

MiRNA profiles did not change significantly in the course of the study period either 1) in regression versus progression or stable disease (Suppl. Table 4; Suppl. Fig. 3) or 2) in the groups of horses with successful therapy or failure of therapy (Suppl. Table 4; Suppl. Fig. 4) at the 3 different sampling times (T0, T1, T2).

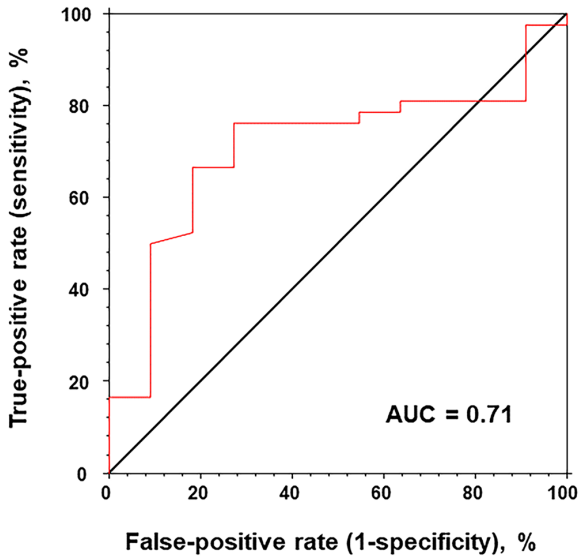

Figure 2. Receiver operating characteristic curve analysis assessing the power of eca-miR-432 for discrimination of equine sarcoid (ES)-affected and ES-free horses at the first sampling time. AUC = area under the curve.

Discussion

Our study is the most recent of our 3 RT-qPCR validation studies of whole-blood miRNAs as prognostic biomarkers for ES, based on the promising results of an initial pilot study using next-generation sequencing (NGS).[13,20,47] The 3 tested candidate miRNAs encoded by the miRNA cluster on equine chromosome 24 have been shown to be differentially expressed in sarcoid tissue and the blood of ES-affected equids.[4,47] We found that only whole-blood eca-mir-432 had diagnostic potential. None of the examined miRNAs had the ability to predict therapeutic responses based on their expression levels.

In human medical research, studies have highlighted the importance of sex-specific differences in the expression of miRNAs, especially in the expression of clustered miRNAs.[17] This divergence in expression patterns of specific miRNAs between male and female individuals is vital for accurately interpreting miRNA data and identifying potential

biomarkers.[14,17,55] Sexual dimorphism of miRNA expression has been further documented in other mammalian species, birds, and insects.[31,50] In contrast to our 2 previous RT-qPCR studies, in both of which sex influenced the expression of eca-miR-127, and in 1 of which sex influenced the expression of eca-miR-379 and eca-miR-432, we could not confirm sex-biased miRNA profiles in our present study.[13,20] The smaller sample size used in our present study compared to the previous 2 studies may have limited our ability to detect subtle sex-based differences. Most circulating equine miRNA biomarker studies have so far not accounted for sex as a potential source of bias.[7] However, sex-biased miRNA expression, both under normal conditions and in disease, warrants further investigation in equids.

In humans, miRNA expression can vary between ethnic groups.[5,22,38] Likewise, there is evidence for breed-specific miRNA fingerprints in horses.[1,36] This should be considered when identifying diagnostic and prognostic biomarkers. The study population in our study was dominated by warmblood and warmblood-related horse breeds, making it difficult to draw clear conclusions about the influence of breed on miRNA expression despite our attempt to categorize the horse breeds. As in our 2 previous validation studies, we could not confirm an influence of breed on any of the tested miRNAs.[13,20] However, it has been shown that breed influences the suitability of eca-miR-127 as a diagnostic biomarker for ES. This whole-blood miRNA had an excellent Se of 91% and Sp of 83% as a diagnostic biomarker in Franches-Montagnes horses. However, in a mixed population of different horse breeds and donkeys or in warmblood horses, the same whole-blood miRNA was not a diagnostic biomarker.[13,20] In accord with these findings. eca-miR-127 was not suitable as a diagnostic biomarker for ES in our study population, which consisted mainly of warmblood horses. The number of Franches-Montagnes horses was too small to test the diagnostic power of eca-miR-127 in this subpopulation. Either way, a breed-specific biomarker does not meet our needs. Our goal is to find a biomarker that can be applied to the general horse population.

We found that whole-blood eca-miR-432 had satisfactory diagnostic Se and Sp. However, this Se/Sp is still inferior to the purely clinical diagnosis of ES and thus is not a real advantage in the diagnostic process in a clinical setting.[25] Among possible diagnostic serum miRNAs, no miRNA has yet been identified as superior in its diagnostic significance to purely clinical diagnosis, nor can a serum miRNA reliably distinguish sarcoids from other skin tumors.[45,46] Histopathology remains the gold standard for diagnosing ES, although the growth behavior of ESs after biopsy is unpredictable, and both spontaneous regression in tumor size and growth exacerbation can occur.[19,23] Research has focused on less-invasive approaches, such as collecting BPV DNA swabs from ES lesions. According to 2 studies, a positive BPV DNA swab had positive predictive values of 98% and 100%, Se of 70% and 88%, and Sp of 92% and 100%, respectively.[18,32]

Whole-blood eca-miR-432 did not show any potential as a prognostic indicator in our study, nor did the other 2 candidate miRNAs tested. The combined results of our 3 validation studies regarding the prognostic significance of candidate miRNAs in the course of ES disease are rather sobering. The use of a blood sample for miRNA profiling to predict the course of ES, for example, in the clinical setting of pre-purchase examinations or to help decide for or against aggressive or expensive targeted therapy, is still out of reach.

Mistletoe therapy had no measurable effect on the equine miRNA profile tested in our study. In another study, the administration of herbal preparations led to alterations in the miRNA profiles of horses.[8] After corn syrup supplementation, horses had serum miRNA fingerprints that differed significantly from those they had before supplementation. Furthermore, corn-specific plant miRNAs were detected in the serum and muscle of those horses.[8] Most medicinal plant miRNAs appear to exhibit adequate stability during the herb preparation process.[53] Almost 700 conserved and >1,000 novel *Viscum album* plant miRNAs have been identified, some detectable in the blood after administration of the herbal preparation to humans.[52] In human medicine, the cross-kingdom effect of plant miRNAs on health is debated, and hopes have been raised that plant miRNAs have a certain therapeutic potential.[39] We did not specifically search for plant miRNAs in the whole blood of horses treated with VAE.

A 2019 study explored the potential impact of seasonal variations on miRNA profiles.[28] Seasonal variations of miRNA expression may be explained by physiologic seasonal adaptation and response to environmental changes or disease susceptibility for disorders with a seasonal nature, such as allergies and asthma.[33,44] In our group of ES-free control horses, we did not observe any seasonal variations in the miRNAs examined, which is a positive aspect for their potential use as biomarkers for ES. The possible influence of seasonality on equine miRNA expression should be examined in larger cohorts.

Our study has limitations. The selection of suitable reference genes is important in preventing bias in miRNA quantification.[6] There is no universally accepted endogenous control, and consensus on the most appropriate reference miRNAs in biofluids remains elusive.[51] Our selection was based on studies in which eca-miR-30d emerged as the sole miRNA meeting the criteria for an endogenous control.[13,20] Methods that allow absolute quantification and therefore do not require a reference gene for normalization would be an alternative. For example, droplet digital PCR generates more consistent results and better reproducibility than RT-qPCR.[42,49] Furthermore, the size of our groups was in the lower range of or—due to the necessary exclusion of some individuals—even below the minimum sample size calculated based on the results of the initial NGS pilot study, particularly in the control group.[47] This limitation could be overcome through cooperation with multicenter studies to include more individuals. On the other hand, our study design was unique. The therapy and follow-up were carried

out in a standardized manner as part of a clinical study on the efficacy of VAE. It is therefore much more standardized compared to our previous studies, in which we included ES-affected horses treated with various approaches from our clinic's patient population.[20]

Not all results of our study align with the findings of previous studies, which could be attributed to a combination of population-related, methodologic, clinical, and environmental factors. Thus, the development of a practical miRNA test for assessing the long-term prognosis and response to therapy in horses with ES remains challenging. Studies with larger cohorts and spanning several years are needed to identify suitable miRNA candidates for this purpose. Our study is another step in exploring the complex functioning of miRNAs in horses and their potential suitability as biomarkers. Our results may also provide a basis for miRNA biomarker research in other animal species.

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Declaration of conflicting interests

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Supplemental material

Supplemental material for this article is available online.

References

1. Barrey E, et al. Muscular microRNA expressions in healthy and myopathic horses suffering from polysaccharide storage myopathy or recurrent exertional rhabdomyolysis. Equine Vet J Suppl 2010;38:303–310.
2. Beermann A, et al. A randomized placebo-controlled double-blinded study comparing oral and subcutaneous administration of mistletoe extract for the treatment of equine sarcoid disease. J Vet Intern Med 2024;38:1815–1824.
3. Berruex F, et al. Clinical course of sarcoids in 61 Franches-Montagnes horses over a 5–7 year period. Vet Q 2016;36:189–196.
4. Bogedale K, et al. Differentially expressed microRNAs, including a large microRNA cluster on chromosome 24, are associated with equine sarcoid and squamous cell carcinoma. Vet Comp Oncol 2019;17:155–164.
5. Bovell LC, et al. The prognostic value of microRNAs varies with patient race/ethnicity and stage of colorectal cancer. Clin Cancer Res 2013;19:3955–3965.
6. Bustin SA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611–622.
7. Calewaert A, et al. Comparison of serum microRNA in healthy horses and horses with moderate to severe mitral valve regurgitation using a commercially available canine cardiac panel. Equine Vet J 2025;57:1001–1008.
8. Carver C, et al. Effects of corn supplementation on serum and muscle microRNA profiles in horses. Food Sci Nutr 2023;11:2811–2822.
9. Chambers G, et al. Association of bovine papillomavirus with the equine sarcoid. J Gen Virol 2003;84:1055–1062.
10. Chen S, et al. Identification of prognostic miRNA signature and lymph node metastasis-related key genes in cervical cancer. Front Pharmacol 2020;11:544.
11. Christen G, et al. Inheritance of equine sarcoid disease in Franches-Montagnes horses. Vet J 2014;199:68–71.
12. Christen-Clottu O, et al. Treatment of clinically diagnosed equine sarcoid with a mistletoe extract (*Viscum album austriacus*). J Vet Intern Med 2010;24:1483–1489.
13. Cosandey J, et al. Diagnostic and prognostic potential of eight whole blood microRNAs for equine sarcoid disease. PLoS One 2021;16:e0261076.
14. Cui C, et al. Identification and analysis of human sex-biased microRNAs. Genom Proteom Bioinform 2018;16:200–211.
15. Dini P, et al. Expression profile of the chromosome 14 microRNA cluster (C14MC) ortholog in equine maternal circulation throughout pregnancy and its potential implications. Int J Mol Sci 2019;20:6285.
16. Eggenschwiler J, et al. Mistletoe lectin is not the only cytotoxic component in fermented preparations of *Viscum album* from white fir (*Abies pectinata*). BMC Complement Altern Med 2007;7:14.
17. Guo L, et al. MiRNA and mRNA expression analysis reveals potential sex-biased miRNA expression. Sci Rep 2017;7:39812.
18. Gysens L, et al. Cross-sectional comparison of superficial swab and fine-needle aspiration: improving the diagnostic workup of horses with sarcoids. Vet J 2022;289:105916.
19. Gysens L, et al. Longitudinal pilot study examining the effect of punch biopsy on equine sarcoid growth dynamics. Vet Dermatol 2024;35:148–155.
20. Hamza E, et al. The potential of three whole blood microRNAs to predict outcome and monitor treatment response in sarcoid-bearing equids. Vet Res Commun 2023;47:87–98.

21. Haspeslagh M, et al. The clinical diagnosis of equine sarcoids—Part 2: assessment of case features typical of equine sarcoids and validation of a diagnostic protocol to guide equine clinicians in the diagnosis of equine sarcoids. Vet J 2018;240:14–18.
22. Huang RS, et al. Population differences in microRNA expression and biological implications. RNA Biol 2011; 8:692–701.
23. Knottenbelt DC. A suggested clinical classification for the equine sarcoid. Clin Tech Equine Pract 2005;4:278–295.
24. Knottenbelt DC. Pascoe's principles and practice of equine dermatology. Elsevier Health Sci, 2009.
25. Koch C, et al. The clinical diagnosis of equine sarcoids. Part 1: assessment of sensitivity and specificity using a multicentre case-based online examination. Vet J 2018;242:77–82.
26. Laurell H, et al. Correction of RT-qPCR data for genomic DNA-derived signals with ValidPrime. Nucleic Acids Res 2012;40:e51.
27. Lee DK. Alternatives to *p* value: confidence interval and effect size. Korean J Anesthesiol 2016;69:555–562.
28. Ludwig N, et al. Spring is in the air: seasonal profiles indicate vernal change of miRNA activity. RNA Biol 2019;16:1034–1043.
29. Lunardi M, et al. Bovine papillomavirus type 13 DNA in equine sarcoids. J Clin Microbiol 2013;51:2167–2171.
30. Mangla P. Anomaly detection: how to find outliers using the Grubbs test. PyImageSearch. 2025 Jan 6. https://pyimagesearch.com/2025/01/06/anomaly-detection-how-to-find-outliers-using-the-grubbs-test/ 2025.
31. Marco A. Sex-biased expression of microRNAs in *Drosophila melanogaster*. Open Biol 2014;4:140024.
32. Martens A, et al. PCR detection of bovine papilloma virus DNA in superficial swabs and scrapings from equine sarcoids. Vet J 2001;161:280–286.
33. Mukherjee K, et al. Seasonal phenotype-specific expression of microRNAs during metamorphosis in the European map butterfly *Araschnia levana*. Arch Insect Biochem Physiol 2020;104:e21657.
34. Nagy Á, et al. Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. Sci Rep 2018;8:11515.
35. O'Brien J, et al. Overview of microRNA biogenesis, mechanisms of actions, and circulation. Front Endocrinol (Lausanne) 2018;9:402.
36. Pacholewska A, et al. Novel equine tissue miRNAs and breed-related miRNA expressed in serum. BMC Genomics 2016;17:831.
37. Pawlina K, et al. Comprehensive characteristics of microRNA expression profile of equine sarcoids. Biochimie 2017;137:20–28.
38. Pollard J, et al. Differential expression of microRNAs in breast cancers from four different ethnicities. Pathobiology 2018;85:220–226.
39. Sanchita, et al. Dietary plant miRNAs as an augmented therapy: cross-kingdom gene regulation. RNA Biol 2018;15:1433–1439.
40. Schaffer PA, et al. Cutaneous neoplastic lesions of equids in the central United States and Canada: 3,351 biopsy specimens from 3,272 equids (2000–2010). J Am Vet Med Assoc 2013;242:99–104.
41. Staiger EA, et al. Host genetic influence on papillomavirus-induced tumors in the horse. Int J Cancer 2016;139:784–792.
42. Stein EV, et al. Steps to achieve quantitative measurements of microRNA using two step droplet digital PCR. PLoS One 2017;12:e0188085.
43. Terron-Canedo N, et al. Differential expression of microRNAs in bovine papillomavirus type 1 transformed equine cells. Vet Comp Oncol 2017;15:764–774.
44. Tiwari A, et al. Seasonal variation in miR-328-3p and let-7d-3p are associated with seasonal allergies and asthma symptoms in children. Allergy Asthma Immunol Res 2021;13:576–588.
45. Unger L, et al. Diagnostic potential of three serum microRNAs as biomarkers for equine sarcoid disease in horses and donkeys. J Vet Intern Med 2021;35:610–619.
46. Unger L, et al. MicroRNA fingerprints in serum and whole blood of sarcoid-affected horses as potential non-invasive diagnostic biomarkers. Vet Comp Oncol 2019;17:107–117.
47. Unger L, et al. Differences in miRNA differential expression in whole blood between horses with sarcoid regression and progression. J Vet Intern Med 2019;33:241–250.
48. Valentine BA. Survey of equine cutaneous neoplasia in the Pacific Northwest. J Vet Diagn Invest 2006;18:123–126.
49. Wang C, et al. Droplet digital PCR improves urinary exosomal miRNA detection compared to real-time PCR. Clin Biochem 2019;67:54–59.
50. Warnefors M, et al. Sex-biased microRNA expression in mammals and birds reveals underlying regulatory mechanisms and a role in dosage compensation. Genome Res 2017;27:1961–1973.
51. Xiang M, et al. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. Biochem Biophys Res Commun 2014;454:210–214.
52. Xie W, et al. Identification of *Viscum album* L. MiRNAs and prediction of their medicinal values. PLoS One 2017;12:e0187776.
53. Xie W, Melzig MF. The stability of medicinal plant microRNAs in the herb preparation process. Molecules 2018;23:919.
54. Zehavi L, et al. Silencing of a large microRNA cluster on human chromosome 14q32 in melanoma: biological effects of mir-376a and mir-376c on insulin growth factor 1 receptor. Mol Cancer 2012;11:44.
55. Zhong B, et al. Identification and analysis of sex-biased microRNAs in human diseases. Genes (Basel) 2023;14:1688.
56. Zhong S, et al. MiRNAs in lung cancer. A systematic review identifies predictive and prognostic miRNA candidates for precision medicine in lung cancer. Transl Res 2021;230:164–196.