A Non-invasive, Biomarker Assay for Detecting Chronic Wasting Disease Pathology in White-tailed Deer

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ABSTRACT

We describe a non-invasive blood test that exploits differences in the abundance of diagnostic miRNA biomarkers associated with prion infections in cervids. Three potentially diagnostic and two control miRNAs were amplified in 93 serum samples from pen-raised white-tailed deer exposed to Chronic Wasting Disease (CWD). Initial analyses revealed sensitivity of the method to interference from hemolysis, requiring exclusion of excessively hemolyzed specimens from further analysis. Results of the miRNA test on the minimally hemolyzed samples from 33 animals were congruent (100% sensitivity, 92.3% specificity, 93.9% accuracy) with results from standard CWD diagnosis by immunohistochemical (IHC) detection of plaques in medial retropharyngeal lymph nodes and obex tissue, performed by a USDA certified laboratory. Overall, the miRNA assay performed as well as, if not superior to, other common CWD testing alternatives to IHC. Thus, this antemortem biomarker-based test offers a new addition to the resources available for monitoring and managing transmission and spread of CWD that is potentially more rapid and sensitive, and amenable to high throughput scaling.

INTRODUCTION

Chronic Wasting Disease (CWD) is a transmissible spongiform encephalopathy (TSE), associated with the deer family Cervidae (Williams and Young 1980; Hannaoui et al. 2017; Slota et al. 2019), that is caused by alternative folding of a prion protein into a pathogenic conformation (*PrP^{CWD}*, Prusiner 1998; Whitechurch et al. 2017; Marín-Moreno et al. 2017; Li et al. 2021). Pathologically folded PrP^{CWD} typically forms amyloid plaques that aggregate predominantly in the central nervous system. Over time, this infection results in a continual decline in the animal's health, invariably leading to death. CWD is related to other transmissible prion diseases (Saá et al. 2006) such as those associated with cattle and bison (*Bos taurus* and *B. bison*; bovine spongiform encephalopathy), sheep and goats (*Ovis aries* and *Capra hircus*; scrapie), and humans (*Homo sapiens*; Creutzfeldt-Jakob disease and kuru). To date, CWD is the only prion disease to be documented in both captive and wild mammals (Sohn et al. 2002; Benestad et al. 2016; Kramm et al. 2017; Vikøren 2019, USGS 2021) and has been shown to be highly contagious within and among most cervid species. As with most TSEs, it is thought that CWD is not easily, if at all, transmissible to humans (Osterholm et al. 2019; Barria et al. 2018), although recent studies provide evidence that CWD transmission could occur across the human (or livestock) barrier (see Waddell et al. 2017, Otero et al. 2021, Pritzkow et al. 2022).

CWD was first identified in captive mule deer (*Odocoileus hemionus*) from Colorado in 1967 (Williams and Young 1980). Recent studies (Osterholm et al. 2019; Slota et al. 2019) indicate that CWD has expanded to include 30 U.S. states, four Canadian provinces, and four foreign countries (Finland, Sweden, Norway, and South Korea). Further, CWD has been detected in captive cervid populations in 18 U.S. states and three Canadian provinces (https://www.usgs.gov/centers/nwhc/science/expanding-distribution-chronic-wasting-disease). Current evidence (Falcão et al. 2017; Mawdsley 2020; Buchholz et al. 2021; Hannaoui et al. 2017; Sohn et al. 2002; Hamir et al. 2011; Nalls et al 2013; Benestad et al. 2016; Vikøren 2019; Cullingham et al. 2020; USGS 2021) indicates that CWD occurs in several native North American cervid species, including white-tailed deer (*O. virginianus*), North American elk

(*Cervus canadensis*), and moose (*Alces alces*); non-North American species such as Norwegian populations of reindeer (*Rangifer tarandus*), sika deer (*C. nippon*), red deer (*C. elaphus*), Reeve's muntjac (*Muntiacus reevesi*), and fallow deer (*Dama dama*); and non-cervid species such as mink (*Vison vison*), ferrets (*Mustela furo*), feral and domestic cats (*Felis catus*), feral and domestic sheep and goats, cows, pigs (*Sus scrofa*), and squirrel monkeys (*Saimiri spp*.). The species promiscuity of CWD is remarkable considering the allelic variation just among individual elk (O'Rourke et al. 1999), reindeer (Güere et al. 2019), or white-tailed deer (Seabury et al. 2020) influences disease susceptibility.

CWD transmission can occur by numerous routes. Several studies indicate that CWD may be transmitted through consumption of carcasses and antlers (bone and velvet), tissues, saliva, feces, urine, blood, placenta, and semen (Mathiason et al. 2006; Haley et al. 2009, 2012; Tamgüney et al. 2009; Maddison et al. 2010; Terry et al. 2011; Mysterud et al. 2020). Infected, asymptomatic individuals can shed large volumes of prions (Mathiason et al. 2009); further, prion shedding can occur several months prior to the visually characteristic "wasting phase" of the disease. Prions also have been shown to remain in soils for several years (Johnson et al. 2006, 2007; Seidel et al. 2007; Dorak et al. 2017; Gemovesi et al. 2007) allowing CWD and other TSEs to persist under multiple environmental conditions. CWD prions can also be taken up by grasses (Pritzkow et al. 2015) and sequestered in stems and leaves, thereby serving as a potential vector for horizontal transfer among herbivore species. Likewise, mineral licks (salt blocks for cattle) in Wisconsin are thought to serve as reservoirs for CWD and potentially represent a conduit for intra- and inter-species infection (Plummer et al. 2018). Even airborne transmission was shown to be possible under controlled laboratory conditions (Denkers et al. 2013), signaling another potential mode for animal-to-animal transmission.

Although little evidence suggests that CWD can be naturally transmitted from cervids to non-cervid species (Belay et al. 2004; Vaske and Lyon 2011; Williams et al. 2018), given the sympatric distribution of cervids and livestock, the introduction of CWD or other potential variant prion disease would be catastrophic to the livestock industry. Free-ranging and captive

populations of deer as well as other cervid species represent a multibillion-dollar industry (Li et al. 2021) that is important from an ecological, socio-cultural, and economical perspective. It is estimated that 5.8 million deer are harvested in the U.S. alone (National Shooting Sports Foundation 2017), not including nearly 100,000 white-tailed deer from captive populations in Texas. Consequently, CWD outbreaks would be financially devastating to landowners participating in the hunting and/or deer breeding industries as some states may require all individual animals in a breeding facility, pen or enclosure, or specific site be destroyed based on a positive test from a single animal. Proactive detection methods that are highly sensitive for CWD would allow hunters, breeders, landowners, and state and federal agencies to enact critical management policies that minimize the impact of this disease at both local and regional scales.

To date, several diagnostic tests have been developed for detecting prions in blood or tissue samples. Protein misfolding cyclic amplification (PMCA, Saborio et al. 2001; Soto et al. 2002; Castilla et al. 2005) was among the first highly sensitive techniques developed for detection of prions in blood samples. Immunochemical techniques, including Immunohistochemistry (IHC) and enzyme linked immunosorbent assay (ELISA) (Hibler et al. 2003; Keane et al. 2008, 2009) led to increased detection rates, simplicity of testing, and the ability to test additional tissue types such as: brainstem (obex), medial retropharyngeal lymph node, muscle, tonsil, and rectal biopsy (Hibler et al. 2003; Daus et al. 2011; Saa et al. 2006; Thomsen et al. 2012).

Atarashi et al. (2007) reported the development of a new method, Real-Time Quaking-Induced Conversion (RT-QuIC), as a highly sensitive assay that amplifies misfolded CWD prions. Haley et al. (2014, 2016) and Holz et al. (2022) further developed this method and extended testing capabilities to include ante-mortem samples such as nasal swabs and rectal biopsies. This method successfully detected prions in skeletal muscles of infected white-tailed deer; leading Li et al. (2021) to promote RT-QuIC as a high-throughput method for monitoring hunter-harvested venison. Further, Christenson et al. (2021) produced a modified version of the RT-QuIC method (MN- QuIC^{TM}) that utilizes gold nanoparticles (AuNPs) to assist in increasing diagnostic sensitivity and to serve as a relatively quick and portable assay.

Slota et al. (2019) used differential expression of miRNAs, small non-coding RNA transcripts approximately 18–22 nucleotides in length, as biomarkers for predicting the presence of CWD prions in elk (*C. canadensis*). These highly specific and sensitive biomarkers circulate in peripheral blood and show tremendous potential in predicting a wide range of diseases such as schizophrenia, diabetes, cancer, and infectious and autoimmune diseases (Richens et al. 2016). Slota et al. (2019) reported that the abundance of 47 microRNAs (miRNAs) differed between 35 CWD positive elk and an equal number of non-infected individuals. Of those 47 variable miRNAs identified, 21 showed potential in producing diagnostic signatures correlated with prion infections in individual animals. Further, Slota et al. (2019) suggested that miRNA surveillance methods held promise at diagnosing sub-clinical prion infections that IHC and PMCA methods would likely miss.

Currently, approved CWD diagnostic assays are restricted to IHC and ELISA methods and must be performed only at USDA-APHIS-approved universities and licensed State and Federal veterinary diagnostic laboratories. Further, many of the biological samples (obex, lymph node, and rectal) required for these "gold standard" tests involve complex, invasive methods that often require anesthesia and/or surgery performed by veterinarians or other highly skilled personnel. Test samples must be obtained post-mortem (e.g., obex) or may be finite with respect to repetitive sampling (i.e., lymph node and rectal samples). Furthermore, criteria for IHC diagnoses vary depending on the tissue examined (obex, retropharangeal lymph nodes, or rectal lymph nodes), and must be assessed by experienced pathologists whose judgements may differ on specimens from, for example, animals that are infected but still in an early stage of disease pathogenesis. Thus, given the sensitivity limitation of IHC and other immunochemical methods that are based on detection of accumulated amyloid plaques that form relatively late in disease pathogenesis, there is a need for a diagnostic method that can identify animals in earlier stages where prion load is increasing but plaque formation is not sufficient to be considered CWD.

The tremendous need for aggressive CWD surveillance worldwide requires new testing methods amenable to high throughput application. The main limitations of current tests include

impossibility (e.g. obex=brain stem) or difficulty (e.g. rectal lymph nodes) in acquiring disease-affected tissues reliably and consistently by antemortem sampling. Here we applied rapidly emerging miRNA technologies to develop a less invasive, non-surgical, antemortem blood test that can be used for early detection of CWD infection in ongoing disease surveillance and management. The goal of this study was to ascertain the potential of using miRNA abundance in blood exosomes as an indicator of a prion load in individual cervids that presumably would lead to symptomatic CWD. Specifically, we identified differences in the abundance of miRNA biomarkers that correlate with CWD diagnosed by the standard IHC method approved for and used by state and federal agencies in their routine CWD surveillance, then devised a three-marker diagnostic protocol that reliably reflects CWD infection status. Finally, we comment on the power of using a consistent set of blood miRNAs for predicting presence of prions and ultimate risk of CWD.

MATERIALS AND METHODS

Sample Collection and Sample Types.—In July 2021, 300 blood and muscle tissue specimens were scheduled for collection from captive white-tailed deer that were euthanized as part of a Texas Parks and Wildlife Department (TPWD)-sponsored CWD research initiative. Samples were obtained by TPWD at a pen-raised deer breeding facility in Uvalde County, Texas, that was CWD-positive and destined for destruction based on TPWD, TAHC, and USDA-APHIS state regulations and guidelines. Individual deer were euthanized by TPWD personnel and biological samples obtained post-mortem. Blood samples, from 168 individuals, were obtained from the jugular vein or collected from blood pooling within the neck near the decapitation area and placed into both lavender K_2 ethylenediaminetetraacetic acid (EDTA) and red/grey serum separator (SST) Vacutainer® collection vials (Becton Dickinson Co., Franklin Lakes, NJ). Skeletal muscle was also collected and either: frozen in liquid nitrogen (later archived at -80° C), placed on ice (stored at either 4° C or -20° C and later archived at -80° C), or preserved in RNA*later* (Ambion, Inc., Austin, Texas) and archived at 37° C for future studies. All samples

were deposited into the Natural Science Research Laboratory (NSRL) where they were assigned a TK number (unique identifying number, placed in a barcoded cryotube, and stored at -80° C, in perpetuity, for future research studies. Acquisition data, including locality, date of collection, sex, tissue type, sampling conditions were incorporated into NSRL's database. Obex and medial retropharyngeal lymph nodes were collected from all 300 individuals and sent to Texas Veterinary Medical Diagnostic Laboratory, Texas A&M University, for independent testing.

Sample Processing.—Whole blood was centrifuged for 3-5 min at 14,000 rpm and serum was drawn off from the SST tubes and placed into 1.5 mL cryogenic vials. Samples were stored on ice until initial centrifugation and sample separation (as above), and then again during transport to Texas Tech University where they were stored at -20° C, and later moved to -80° C. 93 of 168 samples were chosen based on their visual hemolysis scores per CDC guidelines for estimating hemoglobin concentration.

Immunohistochemistry.—Medial retropharyngeal lymph nodes (MRLNs) and obex tissue from deer with a corresponding serum sample were independently tested using the IHC method by the Texas Veterinary Medical Diagnostic Laboratory, Texas A&M University. Those samples served as an independent and blind comparison to validate the diagnostic potential of the miRNA biomarker assays.

RNA Extraction and cDNA Synthesis.—Total RNA was extracted from 200 uL of serum using the miRNeasy Serum/Plasma Kit (Qiagen Inc., Valencia, California, USA) following the manufacture's protocol. First-strand cDNA synthesis was achieved using the miRCURY LNA RT kit (Qiagen Inc., Valencia, California, USA) following the manufacture's protocol and an Eppendorf 5331 MasterCycler thermal cycler.

Diagnostic miRNA Selection.—Five miRNA primer sets (18–22 nucleotides in length; A-E) were evaluated following the protocols established by Convergent Animal Health (St. Louis, MO; patent application 22/63388262) to: 1) establish an endogenous control, independent of hemolysis assay and any known CWD association, for normalizing values of miRNA amplification (miRNA A), 2) determine whether samples had undergone hemolysis (miRNA B),

and 3) determine diagnostic potential of miRNAs as biomarkers to utilize in detection of CWD prion production (miRNAs C, D, E).

miRNA Amplification and Quantification.—cDNA was amplified following the miRCURY LNA miRNA SYBR Green Polymerase Chain Reaction (PCR) Kit for Exosomes, Serum/Plasma, and other Biofluid Samples (Qiagen Inc., Valencia, California, USA). PCR reactions were loaded onto 96-well plates with optical adhesive covers and amplified using a 12K Flex Real-Time PCR thermal cycler (Life Technologies Inc., Carlsbad, CA) following the manufacture's protocol. Crossing threshold values (Ct) of the reference miRNA, the five diagnostic miRNAs, and the hemolysis control miRNA were recorded using the 12K Flex Real-Time PCR software. The relative abundance (ΔCt) of miRNAs B, C, D, and E were obtained by subtracting the mean Ct value (of experimental duplicates) for reference miRNA A from the mean Ct values obtained for the diagnostic and hemolysis control miRNAs (B, C, D, E).

Hemolysis Analysis.—Given the large number of individual deer sampled, the post-mortem timeframe for sample collection ranged from 12-24 hours and could potentially impact sample integrity represented by hemolysis. For sample prioritization, serum quality was scored using three different hemolysis detection methods. Hemolysis was identified based on a read count ratio > 5 between miRNA B known to be abundant in red blood cells (Slota et al. 2019) and miRNA A known to be unaffected by hemolysis (Shah et al. 2016; Slota et al. 2019). We also assessed hemolysis visually (with two independent raters) using the CDC color palette (https://www.cdc.gov/ncezid/dvbd/stories/research-lab-diagnostics/hemolysis-palette.html) and spectrophotometrically (NanoDrop, ThermoFisher Scientific, Waltham MA) by measuring hemoglobin-specific absorbance at 414 nm (see Results).

Exclusion Criteria.—Following the suggestions of Richens et al. (2016) to best evaluate a diverse set of questions (i.e., effects of variation in sample acquisition on miRNA quantification, identification of the optimally diagnostic markers, etc.), three increasingly strict exclusion criteria were established. The first criterion, applied to all samples, was no prior thawing, as freeze-thaw is known to perturb miRNA quantification (Glinge et al 2017). The second and third

criteria were applied sequentially to assess anticipated variation in quality of samples owing to differences in their acquisition. The second criterion excluded samples with amplification of reference miRNA A with Ct > 28, which served as a downstream reference for overall miRNA quantities in the samples. The third criterion excluded samples with a hemoglobin concentration <1.2 mg/ml, assessed either by visual hemolysis score using the CDC color palette (Kosecki et al. 2021) and/or by NanoDrop spectrophotometry (ThermoFisher Scientific).

Based on the criteria described above, samples were divided into three subsets for analyses: the first subset contained 33 samples with minimal serum degradation based on hemoglobin concentration and successful amplification of miRNA A, the second subset contained 39 samples resulting from the addition of 6 samples that were indicative of unexplained RNA degradation where miRNA A Ct > 28, and the third subset contained 71 samples resulting from the addition of 32 samples that were indicative of high serum degradation based on hemoglobin concentration (>1.2 mg/ml). Henceforth, these three groups will be referred to as Group I, II, and III, respectively.

Statistical Analysis.— Discriminant Function Analysis was performed on Groups I-III using JMP Pro software (SAS Institute Inc., Cary, NC, 1989–2021). Subsequent quadratic discrimination function analyses (QDFA) were plotted as canonical correlates to assess the influence of each putative diagnostic miRNA (C-E), depicted as vectors reflecting the contribution of each marker overlaid onto a canonical plot, and to compare their relative association with the single canonical variable. Classification analyses (Group I only) associated with the QDFA were visualized as scatterplot matrices depicting the spatial distribution of positive and negative categories, with 95% confidence interval ellipses estimated and superimposed onto each matrix. These analyses revealed the extent to which individual specimens could be congruently assigned ($P<0.05$) to their respective IHC test categories.

Data Visualization.—To determine the distribution of the calculated ΔCt values, values were grouped (separately for each miRNA) into single cycle ΔCt range bins and plotted against the number of individuals that fell within each range (Fig. 1). Furthermore, an independent

approach to the DFA to assign threshold parameters for miRNA designation was conducted using pair-wise regression analyses of miRNAs coupled with a "visual" partitioning of the *a priori* scoring of individual samples by the TVMDL. Under this approach, a scatterplot of the ΔCt values for each pair-wise combination for the three miRNAs (C, D, AND E was generated. Subsequently, miRNA relative abundance scores were plotted for all non-hemolyzed samples and logistic regression was used to determine correlations and any diagnostic capabilities of miRNAs. Upper and lower thresholds were designated for the miRNAs in each pair-wise comparison. To determine the thresholds, a "box" was superimposed over a scatterplot of the pair-wise ΔCt scores and adjusted to include 100%, 75%, and 50% of the samples identified by IHC as being CWD positive. This partitioning of the data allowed for a projection the minimum and maximum threshold boundaries for assigning miRNAs in the absence of *a priori* IHC data.

RESULTS

Based on results of a previous study (Slota et al. 2019), we initially selected five miRNAs that might serve as serum biomarkers for pathology-associated changes in animals with CWD. Preliminary quantification experiments identified three of the five miRNA targets (designated C, D, and E) as putative diagnostic markers for prion infection. Among the full set of 93 samples, only 71 returned Ct values for the three putative diagnostic and for putative reference and hemolysis miRNAs. For the 71 samples, raw Ct values for the reference miRNA (designated "A") and ΔCt for a putative hemolysis miRNA ("B") and the three diagnostic miRNAs ("C—E") distributed over relatively large ranges of cycles (Fig. 1), with a small number of specimens requiring an excessive number of cycles for amplification. The distribution of miRNA A (Fig. 1, panel A) appeared generally normal, suggesting its abundance is independent of prion infection. We therefore selected this miRNA to serve as an endogenous reference to normalize diagnostic miRNAs' amplification values. The small number of specimens that returned Ct's for the reference miRNA >28 cycles indicated poor (and therefore unreliable, albeit unexplained) amplification, so this result became a possible exclusion criterion for future analyses (see below).

Furthermore, analysis of qPCR data for the candidate biomarkers on the 71 samples raised concerns that poor sample quality, evident as moderate to severe hemolysis, might be masking the diagnostic potential of the markers. In addition, the miRNA previously used as a hemolysis marker (Slota et al.; here designated "B") did not correlate well with hemoglobin concentrations measured either visually (Visual Hemolysis Score, VHS) using the CDC color palette or spectrophotometrically at 414 nm ($R^2 = 0.49$), whereas the latter two methods correlated well (NanoDrop vs VHS regression line slope 0.87, $R^2 = 0.92$) over the 0-1.2 mg/ml hemoglobin concentration range. We therefore set an upper limit of 1.2 mg/ml hemoglobin concentration as an additional exclusion criterion for degraded samples, which is relaxed in comparison to CDC requirements for human blood tests (less than 1 mg/ml). On applying both exclusion criteria, 28 of the 71 samples examined were too degraded for reliable miRNA quantification, and thus were provisionally excluded from some downstream analyses (22 for excessive hemolysis, six for miRNA A Ct >28).

We next applied quantitative discriminant function analysis (QDFA) to the qPCR results for the three putative diagnostic miRNAs to determine the degree of separation in non-linear space among groups of positive and negative individuals (Fig. 2) and to assess congruence between the miRNA biomarker test and diagnoses by IHC (Fig. 3). For all three datasets (Groups I--III), canonical QDFA plots revealed spatial associations among the three diagnostic miRNAs C, D, and E. For Group III (n = 71, Fig. 2. Panel A), miRNA C showed moderate negative association with the Canonical variate 1 (40.1%), whereas miRNA D showed strong positive association (42.3%), and miRNA E showed weak positive association (2.4%). In addition, the length of the vector for miRNA C indicated weaker contribution to variation, whereas length of the vector for miRNA D and E indicated strong contribution to variation. For Group II (n = 39, Fig. 2. Panel B), miRNAs C (9.3%) and E (15.6%) showed weak positive association with the Canonical variate 1, whereas miRNA D showed moderate positive association (25.0%). In addition, the length of the vector for miRNA C indicated weaker contribution to variation, whereas miRNA D and E indicated moderate contribution to variation. For Group I (all

exclusion criteria applied; n = 33, Fig. 2. Panel C), miRNA C showed strong positive association with Canonical variate 1 (47.8%), whereas miRNA D showed weak negative association (16.7%) and miRNA E showed weak positive association (24.6%). In addition, the length of the vector for miRNA C and D indicated moderate contribution to variation, whereas length of the vector for miRNA E indicated strong contribution to variation.

QDFA scatterplot matrices of the ΔCt values for each pair-wise combination of the putative diagnostic miRNAs (C—E, Fig. 3) illustrated the predictive values of all three markers that collectively might constitute a valid miRNA assay for overall classification of specimens from CWD positive and negative animals. For Group I (strictest exclusion criteria applied; n = 33), the miRNA assay classified CWD positive and negative animals with 93.9% accuracy overall (Table 1). All seven individuals in Group I identified as positive by IHC also classified positive by miRNA assay (100% sensitivity), including two animals diagnosed as IHC positive only in retropharangeal lymph node, and 24 of 26 samples identified as negative by IHC also classified as negative by miRNA assay (92.3% specificity). For Group II (no exclusion of specimens with poor reference miRNA amplification; n = 39), the miRNA assay classified CWD positive and negative animals with 84.6% accuracy overall. All nine individuals in Group II identified as positive by IHC also classified positive by miRNA assay (100% sensitivity), and 24 of 30 negative samples identified as negative by IHC also classified negative by miRNA (80% specificity). Finally, for Group III (no exclusion other than specimens previously thawed; n = 71), the overall accuracy of the miRNA assay dropped to 59.2%, with only 14 of 17 samples identified as positive by IHC also classified positive by miRNA assay (82.4% sensitivity), and only 28 of 54 samples identified as negative by IHC also classified negative by mRNA assay (51.9% specificity).

The miRNA assay's high overall accuracy as assessed by QDFA reflected its excellent sensitivity (100% for the Groups I and II datasets), diminished only by lower specificity ("false positives;" animals that tested positive by miRNA but negative by IHC). For an independent assessment of the putative diagnostic miRNAs' utility for detection of CWD infection,

individually and in all combinations, and to parse the effects of false positives on the assay's accuracy, we examined the effects of progressively shrinking the window for identifying positive specimens in pairwise ΔCt scatterplots. Linear regression analyses of each pairwise combination (Fig. 4) revealed positive correlations between each of the three diagnostic miRNAs, with clustering of IHC positive samples at the lower ends of the ΔCt ranges for each miRNA. Despite the positive correlations overall between the putative diagnostic miRNAs' abundance, however, each of the three appeared to be independently informative, especially for identifying IHC negative specimens. Consequently, to test whether altering thresholds for identifying negative specimens would increase specificity (necessarily at the expense of sensitivity), we adjusted ΔCt range quadrants to capture 100%, 75%, and 50% of the IHC positive samples (Figs. 4A-C), and recalculated specificity and accuracy ("False positives" and "Accuracy" columns, Table 2). The analysis revealed that accuracy increased only marginally upon decreasing ΔCt partitions so as to capture progressively fewer IHC positive specimens. Given the importance of avoiding false negatives, and the relative tolerance of CWD tests to false positives (which could, in fact, reflect identification of an infected animal that is presymptomatic), partitioning the data at the higher ΔCt values is most appropriate.

The analyses presented in Figs. 4A-C and summarized in Table 2 also revealed that the three putative diagnostic miRNAs were independently informative. Consequently, to maximize the miRNA test's accuracy, we combined the three partitions into an aggregate method for identifying positive and negative specimens. Specifically, we identified negatives as those returning ΔCt values greater than any one of the three partitions set to capture positives with 100% sensitivity (Table 2). Collectively applying these cutoffs for all three miRNAs raised specificity to 92.3%, and accuracy to 93.9%, which is comparable to the accuracy of the other CWD tests currently in use (Table 3).

DISCUSSION

Herein, we described a non-invasive blood test for prion infection in cervids based on pathology-associated changes in the abundance of select miRNA biomarkers in serum exosomes. This newly emerging technology does not depend on prion detection nor observation of plaques, but instead utilizes the association of miRNAs with presence of prions to preemptively identify potential CWD-positive individuals. Our sample size was limited due to poor sample quality, which was a primary result of the historic collection methods that depend on post-mortem tissues (obex and retropharyngeal lymph node) because of current federal and state agency requirements in regulating CWD. Despite obstacles in sample acquisition, we were able to generate a preliminary dataset demonstrating the potential of a highly sensitive, ante-mortem miRNA CWD test.

Initial hemolysis and reference miRNA amplification classified serum samples into three subsets (Groups I, II, and III) based on serum degradation (minimal, moderate, and high, respectively). Preliminary comparative histogram analyses (Fig. 1) indicated that reference miRNA A was unaffected by prion association across samples confirming its utility as an endogenous control for normalization. By performing regression analyses between all proposed diagnostic miRNAs, we were able to resolve their individual contribution for detecting prions. Setting a threshold ΔCt value for each miRNA increased specificity.

A QDFA with corresponding scatterplots (Fig. 2-3) of Groups I, II, and III generated varying levels of success. Based on the QDFA, miRNA quantification was sufficient for discriminating between positive and negative samples classified by IHC. For Group I, the QDFA classified positive and negative samples with 92.3% accuracy compared to the IHC designation performed by TVMDL. Discrepancies between testing methods occurred primarily as false positive results where samples were positive by miRNA assay and negative by IHC. The disagreement between testing methods could possibly indicate that the miRNA assay was more sensitive or that it was able to detect an earlier-stage infection. Indeed, this interpretation is supported by the observation that the miRNA assay successfully identified two positive animals

(out of seven in Group I) that tested positive by IHC only in retropharangeal lymph nodes. As the sample quality declined (Groups II and III), the DFA classified samples with 84.6% and 59.15% accuracy, respectively. Specifically in Group III, the miRNA assay began to detect false negatives where samples were negative by miRNA and positive by IHC, which would be considered unacceptable for a CWD detection test.

As a companion system to the QDFA, threshold value maxima for each miRNA, assigned by visual partitioning, also reliably identified of positive and negative samples (as determined by IHC). Among the three threshold parameters examined (100%, 75%, and 50%), both 100% and 75% established spatial boundaries with a maximal inclusion of positive samples, while minimizing the inclusion of test discrepancies of both false positives and false negatives by miRNA assay. The modestly improved accuracy achieved by downward adjustment of the ΔCt partitions for capturing positives in each pairwise comparison necessarily came at dramatic expense of sensitivity, which for a CWD test is an unacceptable tradeoff. We therefore propose that setting individual partitions so as to capture all positive specimens, applying a hierarchical diagnostic protocol with miRNA C as the most strongly discriminating component, and collectively applying data from all three diagnostic markers would produce an optimal, miRNA-based, high throughput assay for CWD infection.

Sample quality influenced amplification of miRNAs, demonstrating that the timing of postmortem sampling was critical for the reliability of this assay, and it stressed the need for antemortem sample collection. Hemolysis experiments indicated that degradation of samples may have influenced the quality of RNA and consequently resulted in inaccurate amplification of miRNAs, thus, failing to detect a positive or negative association for CWD. Among the three subsets of data, Group I had discrepancies for two samples where the IHC result was negative and miRNA result was positive. Group II had discrepancies for six samples where the IHC result was negative and miRNA result was positive. Lastly, Group III had both types of discrepancy where 26 samples were determined negative by IHC and positive by miRNA and 3 samples were determined positive by IHC and negative by miRNA. For samples not eliminated by the

exclusion criteria, few disparities will occur, and they occur only in the direction of a false-positive result. As samples became more degraded (Group III), the number of disparities (n = 29) between IHC-negative: miRNA-positive (n = 26) and IHC-positive: miRNA-negative (n = 3) increased, indicating that degradation decreases accuracy of this assay. Both hemolysis and miRNA credibility demonstrated that only a minimum subset of samples met criteria to accurately generate data. Samples acquired during routine animal husbandry (captive deer) or immediately after death (wild deer) are necessary to detect CWD with this technique.

For the minimal serum degradation group, miRNA C appeared to have the strongest degree of association with the canonical variant 1 (Fig. 2) and contributed the greatest weight in determining classification between positive and negative groups in the QDFA analysis. Comparatively, miRNA D and E were less informative. Although it appears that miRNA C was diagnostic, it is premature to ascertain if it is solely responsible for the association with CWD or if the additive combination of miRNAs were synergistic in their classification among positive and negative individuals. miRNA C is less influential on subsets (n = 39 and n = 71). With those two subsets, miRNA D appears to have a moderate degree of association with canonical variant 1. In estimating the minimum and maximum threshold boundaries for each of the three miRNAs, miRNA C had a narrower threshold range than miRNA D and E. This observation indicates that miRNA C possesses a narrow range of variation when classifying samples as either positive or negative. For the remaining two miRNAs, miRNA D is slightly more discriminant than miRNA E when classifying samples as either positive or negative. The above observations are consistent regardless of sample quality in the dataset.

The results by this miRNA assay compared favorably to the IHC test provided by TVMDL with a range of 86-93% accuracy when using samples with minimal to moderate serum degradation. These results (Table 3) are similar to, or in some cases, exceed comparisons of PMCA, Rt-QuiC, and Immuno-QuiC to IHC and ELISA scores as reported in previous studies (80-82%; Saborio et al. 2001; Soto et al. 2002; Castilla et al. 2005; Hibler et al. 2003; Keane et al. 2008, 2009; Daus et al. 2011; Saa et al 2006; Thomsen et al. 2012).

Given the complementary results, ease, and speed at which miRNAs assays can be completed, it appears miRNA techniques may be more favorable than previous tests for high throughput CWD testing. Generally, a sample can pass through the entire process (sample process and RNA isolation to data processing) in under 12 hours. In Groups I and II, the disparities that occurred between miRNA assay and IHC results could be interpreted as 1) a superior assay thereby serving as an early detection assay that would provide hunters, owners, and agencies with information allowing surveillance and identification of asymptomatic animals or 2) capable of detecting prion-related biomarkers at an earlier stage relative to IHC detection method. However, given that the miRNA assay does not directly detect prions or plaques, it is not intended to serve as a replacement for IHC test but instead as a complementary tool.

Although this study was conducted on post-mortem blood samples, the long-term goal is to develop an antemortem blood assay that could prove useful to the scientific community and private sector as an early-detect/rapid response framework for CWD surveillance. Extension of this miRNA test includes ante-mortem sampling that would allow: 1) a high throughput capacity and rapid return of results (same day for a single sample assay, in most cases), 2) repeated testing using the same individual with minimal handling and stress to animals, 3) selective removal of known positive animals rather than removal of all individuals with exposures to the "diseased animal" or prion-contaminated geographic location, 4) continual monitoring or periodic testing in geographic locations or facilities that report positive-individuals; 5) landowners, deer breeders, state and federal agencies to develop methods for management and surveillance for CWD in deer herds to relocate "clean animals", 6) state and federal agencies to monitor wild populations, 7) early detection method (relative to IHC) for asymptomatic individuals, and 8) establish a scientific method to determine an elapsed time-frame from exposure to manifestation of CWD symptoms and death.

Difficulty in acquiring high quality samples significantly hampered our efforts on this study. Indeed, considering the generally poor sample quality (an inevitable consequence of post-mortem acquisition under the difficult field conditions of a July depopulation event in Texas) and

resultant need to limit our analysis to only those samples that met defined exclusion criteria, our findings provide some optimism for future development and implementation of miRNA-based test for CWD infection. Our data provide little insight into how the 12-24 hours post-euthanasia sampling impacted degradation of miRNAs, though other studies identify hemolysis as a strong indicator of specimen degradation. Regardless, acquiring samples under better field conditions so as to increase numbers of samples that meet our exclusion criteria will greatly aid in the ongoing development of miRNA-based tests for high throughput screening of wild and captive animals. Such tests will provide the tools necessary to track disease progression in captive herds and thereby define miRNA changes indicative of different stages of infection (early, onset, late), and in turn define and potentially avoid circumstances that might lead to depopulation of entire herds. Finally, this method may be extended to different sample types (e.g skeletal muscle) that applies postmortem sampling to provide more convenient testing for the hunting industry.

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CONFLICT OF INTEREST STATEMENT

Ned Saleh is a named inventor of a US patent application: "Apparatus and Method for High Throughput Compact Multiplexed micro-RNA Based Rapid Diagnostics with Monetizable Database", N. Saleh; R. Vierling Number: 22/63388262). All other authors declare no conflicts.

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AUTHOR CONTRIBUTIONS

Robert D. Bradley - PI, project organization, communication with many, laboratory supervision (work done in his laboratory), troubleshooting, drafting and editing the manuscript

Emma K. Roberts - organization, laboratory work, troubleshooting, drafting and editing the manuscript

Asha Worsham - laboratory work, data analysis and interpretation, drafting and editing the manuscript

Megan Ashton - laboratory work, data analysis and interpretation, drafting and editing the manuscript

Emily A. Wright - laboratory work, troubleshooting, data analysis and interpretation, editing the manuscript

Ned Saleh – data analysis and resources (experimental protocols, data analysis tools)

Daniel M. Hardy - PI, organization, supervision (work done in his laboratory), troubleshooting, data analysis and interpretation, drafting and editing the manuscript

Table 1. **Classification matrices developed from a discriminant function analysis of the three miRNA markers (C-E) and the three data subsets (Groups I-III).** Data exclusion criteria for the Groups are described in the text. Numbers reflect where individual samples were assigned based on ΔCt values. Assignment to "positive" or "negative" categories (a *priori* assumption) was based on an immunohistochemistry (IHC) assay done by the Texas Veterinary, Medical, and Diagnostic Laboratory (TVMDL). Note that among the seven IHC positive animals in Group I, two were positive only in the retropharangeal lymph node (i.e. were negative in obex), indicating that buildup of pathological prion amplicons had not yet reached the nervous system.

Dataset	**Classification by IHC (*a priori*)**	**DFA Reclassification by miRNA assay**	
		Negative	**Positive**
Group I, n=33	Negative (n=26)	24	2
	Positive (n=7)	0	7
Group II, n=39	Negative (n=30)	24	6
	Positive (n=9)	0	9
Group III, n=71	Negative (n=54)	28	26
	Positive (n=17)	3	14

Table 2. Effect of upper and lower threshold adjustment on miRNA assay accuracy. Constraining the minimal ΔCt diagnostic window in pairwise comparisons of the three miRNA markers (C--E) to 100, 75, or 50% of IHC positive samples (=sensitivity), reflecting 0, 25, and 50% false negatives samples respectively, progressively decreased the percentage of false positives as expected, but at the expense of overall accuracy (two rightmost columns). Number of mismatches was determined by subtracting the number of positive samples from the total number of samples occurring on either side of the threshold boundaries. Note that the ΔCt adjustments variably affected accuracy, with modestly increased accuracy coming at the dramatic expense of sensitivity. Note also that adjusting the capture window required alteration of different miRNAs' ΔCt partitions, indicating that the three markers were not redundantly informative.

Constrained sensitivity (%)	miRNA	ΔCt partition (cycles)	Comparison	False positives	Accuracy (%)
100 %	C	4.00	C vs E	8/15= 53.3%	75.8%
	D	8.75	C vs D	6/13= 46.2%	81.8%
	E	10.00	D vs E	14/21= 66.7%	57.6%
75%	C	3.75	C vs E	2/6= 33.3%	84.8%
	D	7.5	C vs D	2/6= 33.3%	84.8%
	E	8.75	D vs E	5/9= 50.0%	75.8%
50%	C	3.75	C vs E	1/4= 25.0%	84.8%
	D	7.5	C vs D	1/4= 25.0%	84.8%
	E	8.25	D vs E	2/5= 40.0%	81.8%

Table 3. Congruence of current methods for detection of CWD infection. Shown are inter-assay accuracy calculations for Abbreviations are: IHC= Immunohistochemistry, ELISA= Enzyme-Linked Immunosorbent Assay, PMCA= Protein Misfolding Cyclic Amplification, RT-QuiC= Real-time Quaking-Induced Conversion, MN-QuiC= Minnesota Quaking-Induced Conversion, miRNA= this study.

	ELISA	**PMCA**	**RT-QuIC**	**MN-QuIC**	**miRNA**
IHC	78%	89%	83%	100%	94%
ELISA		90%	92%	100%	N/A
PMCA			80%	N/A	N/A
RT-QuIC				100%	N/A
MN-QuIC					N/A

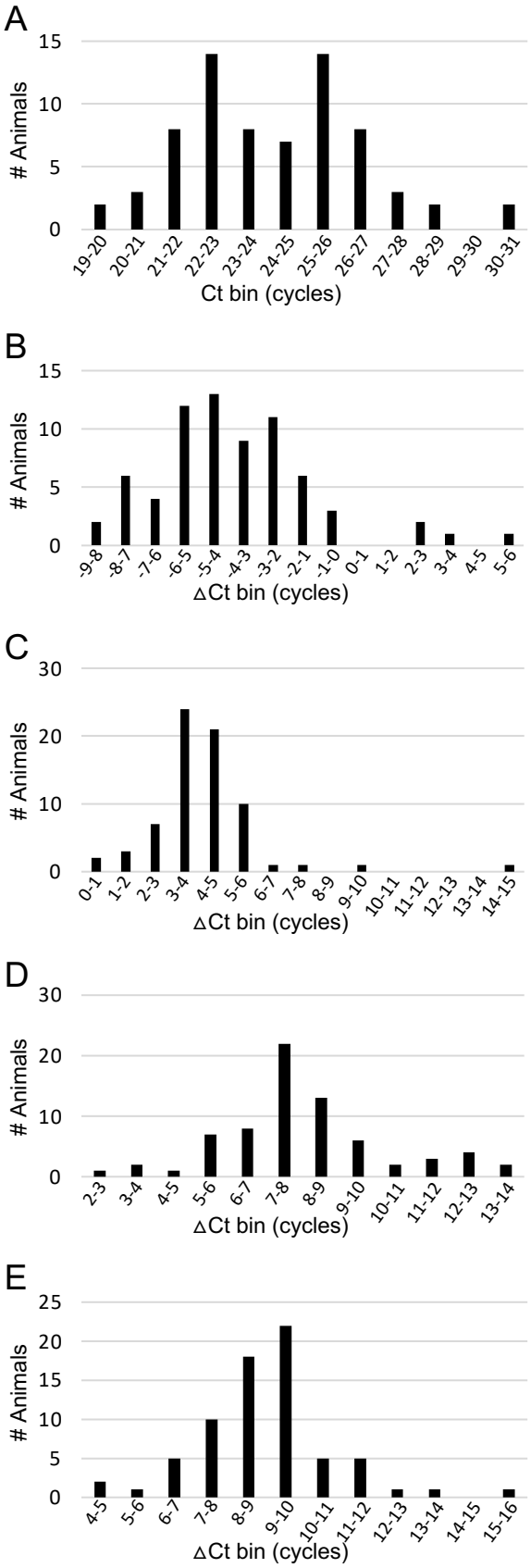

Fig. 1. Distribution of Ct and ΔCt values from amplification of reference and putative diagnostic miRNAs. Y-axis indicates number of animals (out of 71 total) in each Ct or ΔCt amplification range bin (# cycles). Panel A: Raw Ct for reference miRNA. Panels B-E: ΔCt calculated by subtracting the reference miRNA Ct from the indicator miRNA Ct to normalize indicator signals for each animal. Panel B: hemolysis indicator miRNA. Panels C-E: putative CWD indicator miRNAs, hereafter designated "diagnostic C," "diagnostic D," and "diagnostic E," respectively. Note the high reference miRNA Ct values returned for a small number of animals, indicative of poor amplification (thus specimens excluded from some subsequent analyses). Note also the mostly negative ΔCt values returned for the hemolysis indicator miRNA, reflecting robust amplification of that marker (raw Ct less than that for the reference miRNA).

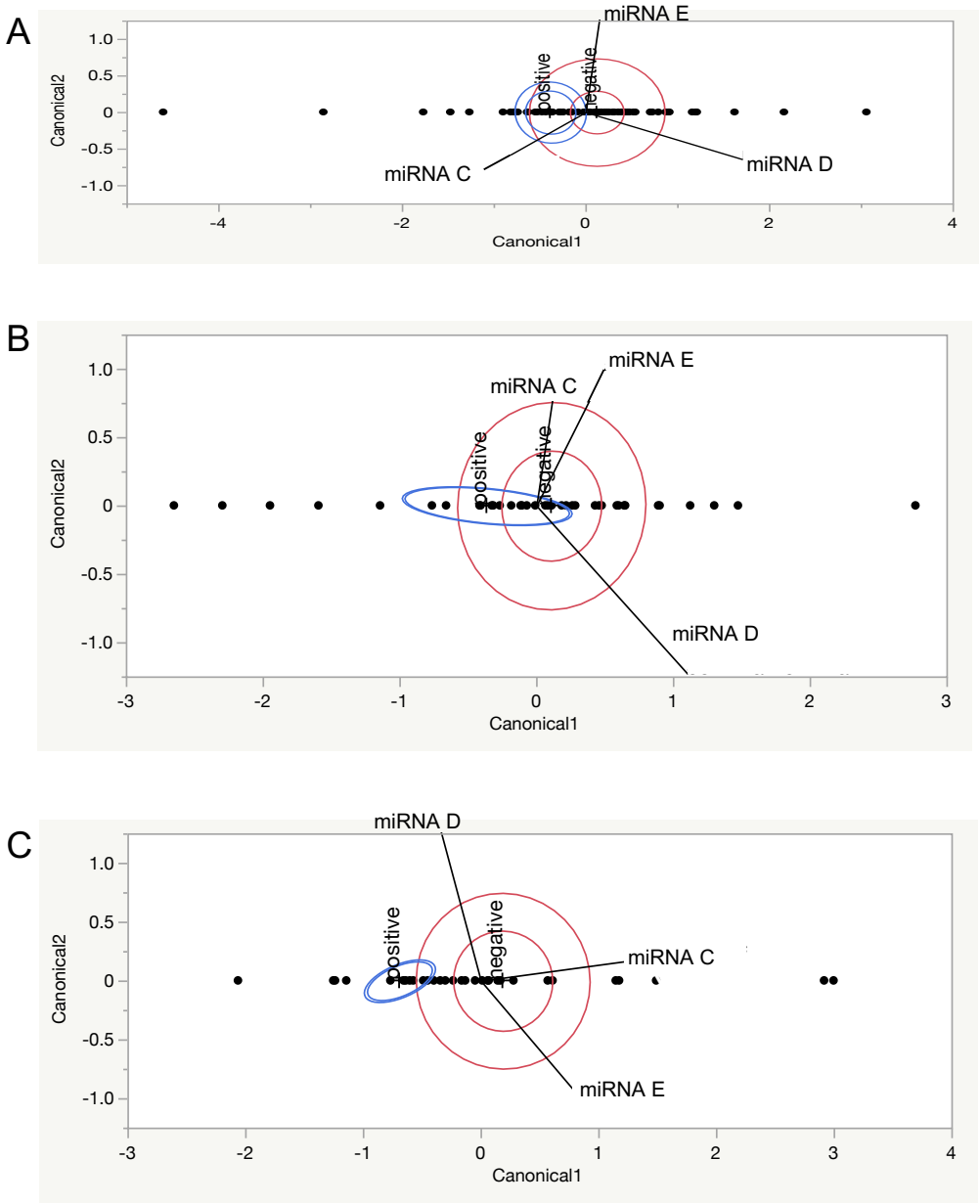

Fig. 2. Canonical Plot for QDFA. Panel A: analysis with no data excluded (n=71). Panel B: analysis with data from excessively hemolyzed specimens excluded (n=39). Panel C: analysis with data from hemolyzed and poor reference miRNA amplification excluded (n=33). Note the progressive improvement in resolution of CWD positive specimens with successive application of exclusion criteria.

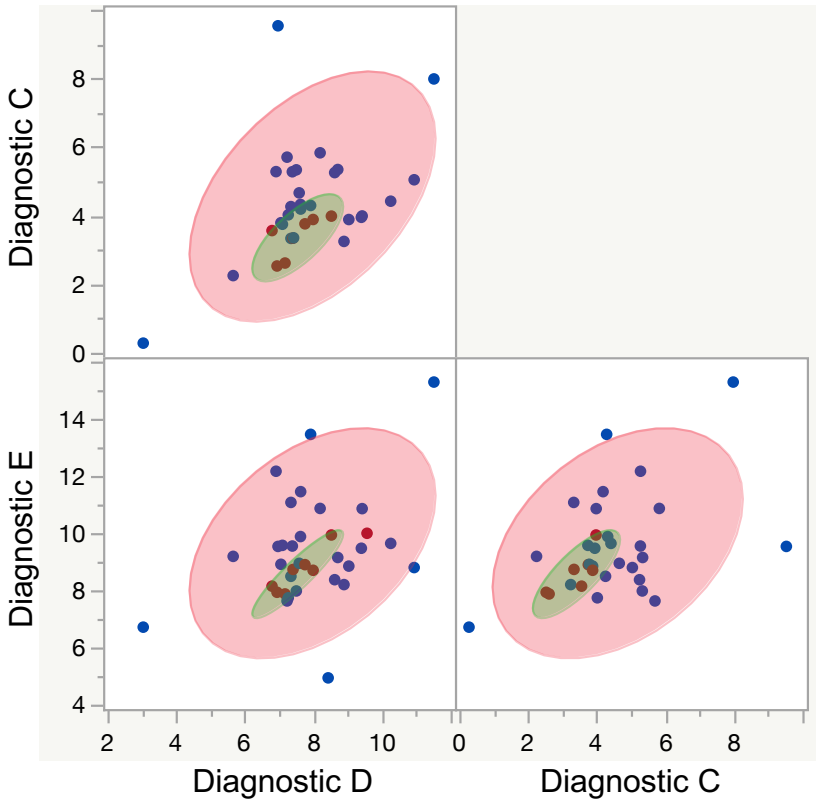

Fig. 3. DFA scatterplot matrix. Pairwise comparisons of qPCR data for putative diagnostic miRNAs reveal combined diagnostic potential. Green ellipse depicts 95% confidence interval for specimens that tested CWD positive by IHC (red dots), and the pink ellipse the corresponding interval for negatives (blue dots).

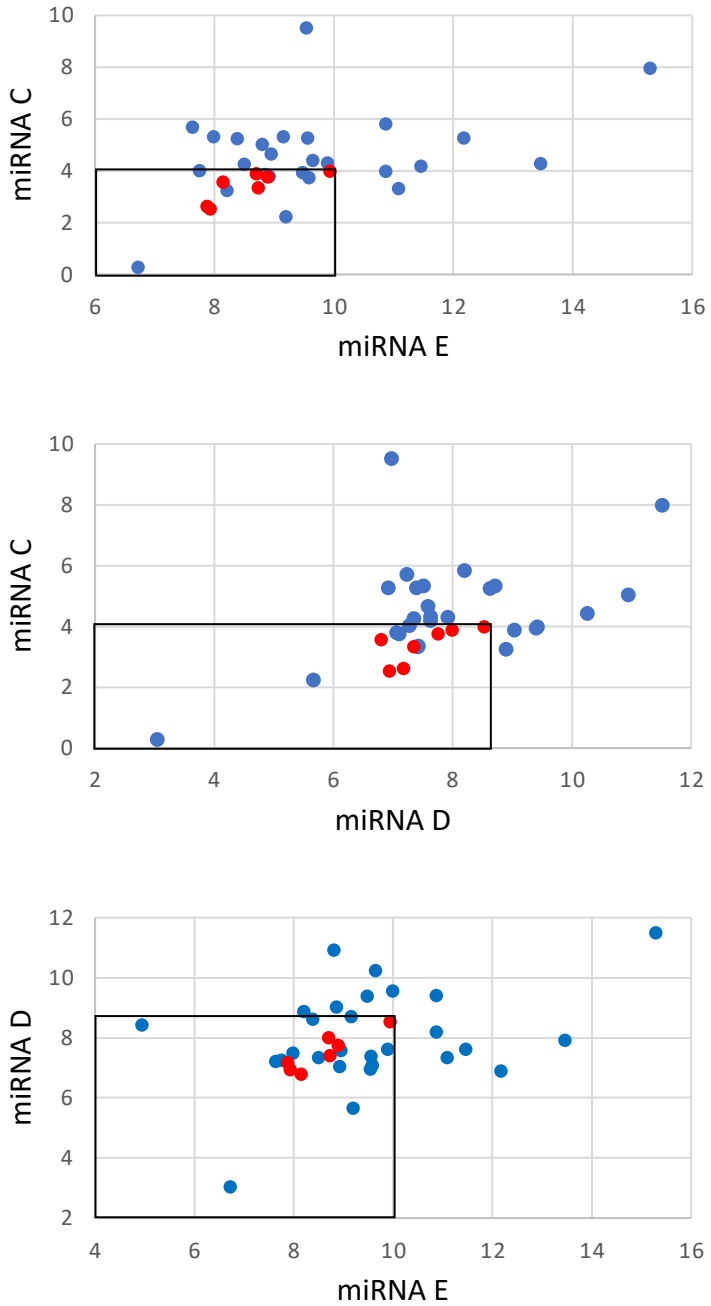

Fig. 4A. Pairwise regression analyses of three diagnostic miRNA values for Group I with Ct partitions drawn to capture 100% (7/7) of positive samples in the lower left quadrant (boxed). Blue indicates IHC negative and red indicates IHC positive samples. See Table 2 for quantitative summary of these analyses.

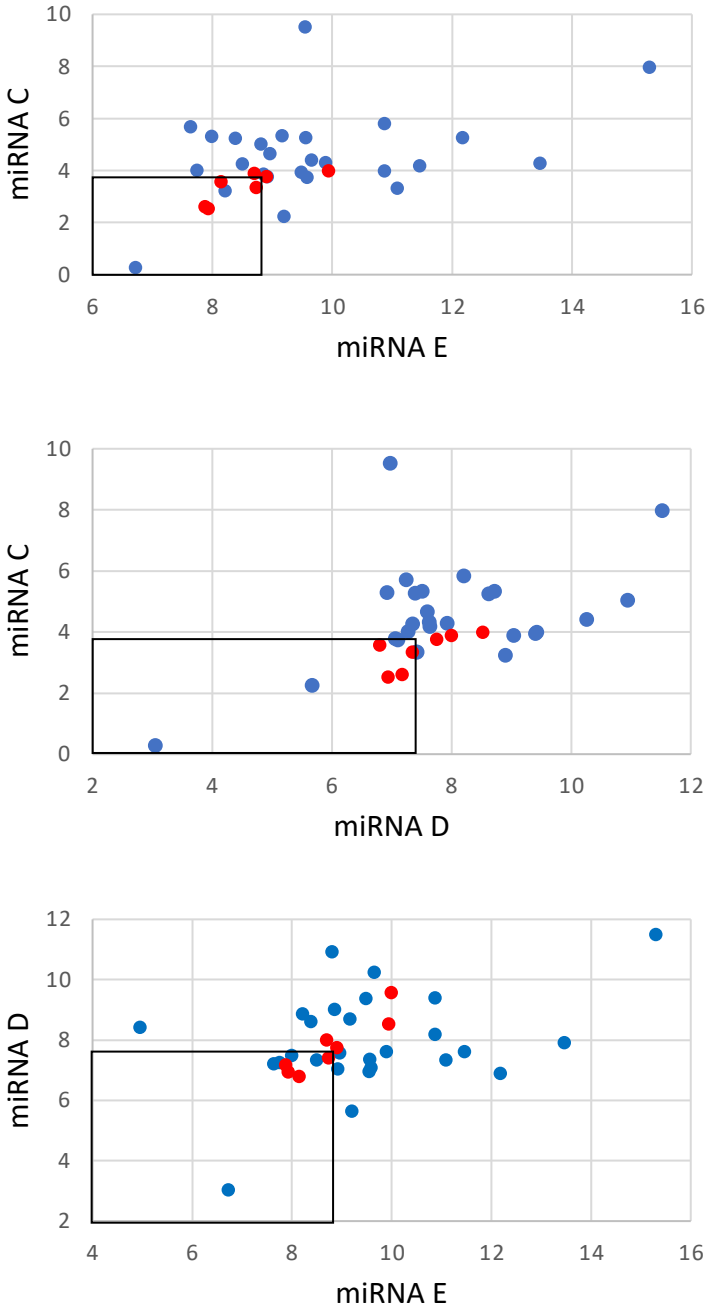

Fig. 4B. Pairwise regression analyses of three diagnostic miRNA values for Group I with Ct partitions drawn to capture 75% (4/7) of positive samples in the lower left quadrant (boxed). Blue indicates IHC negative and red indicates IHC positive samples. See Table 2 for quantitative summary of these analyses.

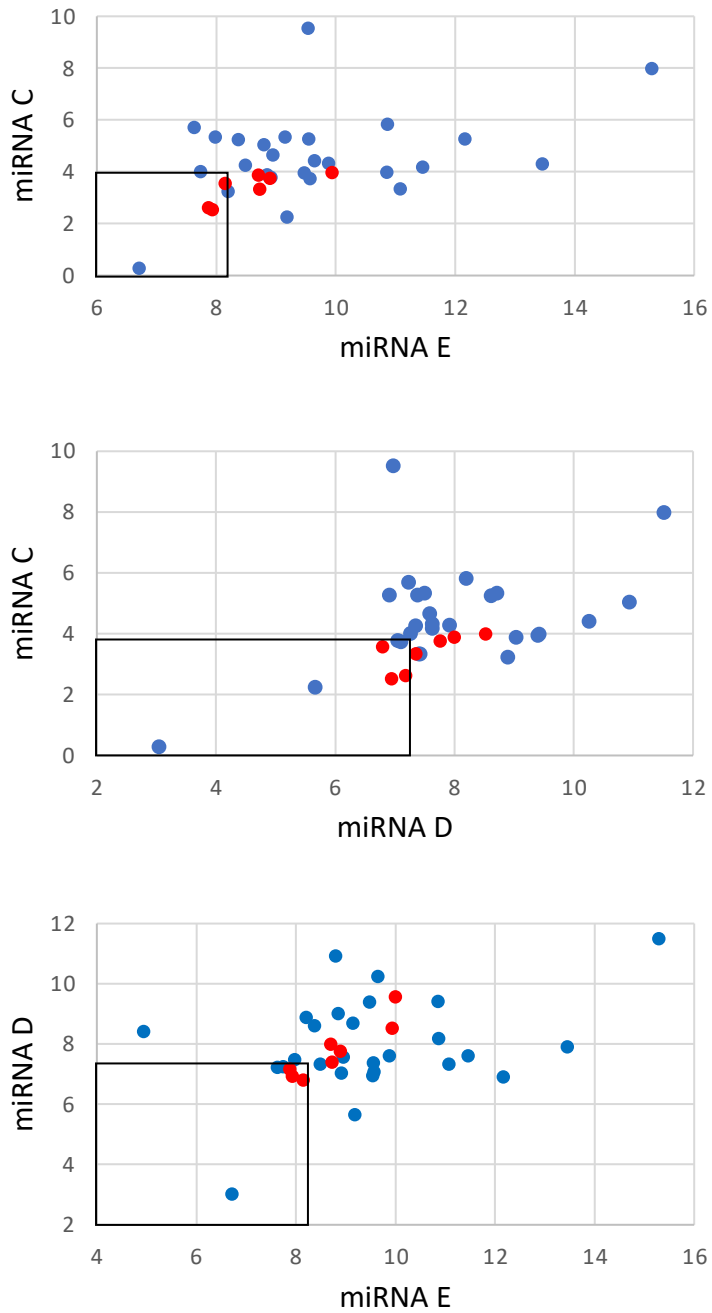

Fig. 4C. Pairwise regression analyses of three diagnostic miRNA values for Group I with Ct partitions drawn to capture 50% (3/7) of positive samples in the lower left quadrant (boxed). Blue indicates IHC negative and red indicates IHC positive samples. See Table 2 for quantitative summary of these analyses.