SERUM EXOSOMAL MICRORNAS AS CLINICAL BIOMARKERS FOR THE DETECTION OF BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

By

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Submitted to the graduate degree program in Clinical Research and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

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Chairperson: Brooke L. Fridley, Ph.D

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ABSTRACT

Background: Barrett's esophagus (BE), caused by Gastroesophageal Reflux Disease (GERD), is the main premalignant condition for esophageal adenocarcinoma (EAC). The majority of BE and EAC patients are missed or diagnosed late resulting in dismal outcomes. Multiple studies have proposed serum microRNAs (miRNAs) as promising biomarkers for benign and malignant conditions. MicroRNAs contained in serum exosomes have increased stability and are a unique, non-invasive strategy for BE and EAC diagnosis.

Methods: Serum from patients with GERD, BE and EAC (n=8 each) were differentially centrifuged to isolate exosomes. RNA was extracted with Trizol and next-generation sequencing was performed on the Illumina HiSeq2500 Sequencing platform. Analysis of the data was conducted using mirDeep2/EdgeR and prediction classification analysis. A False Discovery Rate (FDR) < 0.05 was considered significant.

Results: All subjects were white males with the following mean ages: GERD: 58±9 years; BE: 63±8 years; EAC: 64±3 years (p=0.3833). Comparison of serum exosomal miRNA profiles among the 3 groups found most significant differences between EAC patients compared to BE and GERD patients (79 and 85 miRNAs were found in EAC vs BE and GERD, respectively). Comparison of BE versus GERD patients found 17 differentially expressed serum exosomal miRNAs. Additionally, for each of the disease comparisons, approximately half of the significant miRNAs were up-regulated and the half were down-regulated. Prediction analysis identified a signature of 34 miRNAs that could be used to distinguish these conditions.

Conclusion: We have identified promising candidate serum miRNAs for distinguishing GERD patients with and without BE and EAC. These findings need to be further validated to develop blood-based assays for clinical use.

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BACKGROUND AND CLINICAL SIGNIFICANCE

The eponym "Barrett's Esophagus (BE)" is named after Norman Rupert Barrett, a distinguished thoracic surgeon. Interestingly, however, the condition that bears his name was first described by a Boston pathologist named Wilder Tileston nearly a half a century before Barrett published a paper describing the disease. In 1950, Barrett characterized BE in an article titled "Chronic peptic ulcer of the oesophagus and oesophagitis" but his suggested mechanism has since been proven to be incorrect [1]. He proposed that a congenitally short esophagus led to extension of the stomach into the mediastinum and thus could explain the tendency of esophageal ulcers to be surrounded by gastric type mucosa [2]. Earlier, in 1906, Tileston, had described how the mucosa surrounding esophageal ulcers bore resemblance to the mucosa of the stomach. In the same paper, he proposed a mechanism that is widely accepted today as the pathogenesis of Barrett's Esophagus; Tileston wrote, "The first requisite for the formation of the peptic ulcer of the oesophagus is an insufficiency of the cardia." In other words, gastric contents were not being contained within the stomach, which is essentially what physicians diagnose as Gastroesophageal Reflux Disease (GERD) today.

The estimated prevalence of GERD in the United States is 20%, which equates to approximately 10 million people [3, 4]. The prevailing hypothesis is that residual cells at the gastroesophageal junction migrate proximal during acid-induced injury leading to abnormal placement of those cells [5, 6]. Subsequently, the normal squamous epithelium is replaced by the columnar epithelium [7-9]. The resulting condition is termed Barrett's esophagus (BE), an epithelial metaplasia from squamous to columnar. BE is important because it is the primary premalignant condition for esophageal adenocarcinoma (EAC). In patients with chronic GERD,

the prevalence of BE is 10–15 % [10]. The progression of BE to EAC involves histological changes that result from sequential accumulation of genetic and epigenetic changes: non-dysplastic Barrett's metaplasia, low-grade dysplasia, high-grade dysplasia, and ultimately, adenocarcinoma.

The US incidence of esophageal adenocarcinoma has risen faster than any other malignancy in recent years, its incidence makes it the most rapidly increasing cancer in the United States[11]. Unfortunately, it is also highly lethal with a dismal 5-year survival rate of less than 15% [12]. Although the absolute annual risk of Barrett's patients developing adenocarcinoma is lower than previously thought (0.12-0.3%), the cumulative lifetime risk in patients with BE has been estimated to be 7-14% [13, 14]. Moreover, the risk of developing esophageal cancer is 50-100 times more likely in those patients with BE compared to the general population (standardized incidence ratio of 11.3) [13].

Smoking, diet, abdominal obesity, age >50 and hiatal hernia have all been implicated as risk factors for GERD. Obesity is an important modifiable risk factor and is associated with increased risks for complications such as BE and EAC [15, 16]. The rising prevalence of EAC as well as its major risk factor obesity is worrisome. Over the past 35 years, obesity rates have more than doubled with more than one-third of U.S. adults currently considered obese [17]. Age is also important risk factor for EAC, leading to another cause for concern is the increase in the aging population. Americans ages 65 and older make up 13% of the US population. By 2030, when all the baby boomers will have passed age 65, the over-65 persons will reach 20% of the population [18]. The increasing prevalence of the aging population and the association of obesity with these conditions contribute to the daunting cumulative effect they have on EAC rates and related public health and healthcare spending [19].

Diagnosis of Barrett's esophagus

According to the recommendations by the American Gastrointestinal Association (AGA), patients with multiple risk factors for BE, such as white race, chronic GERD and age >50 should be screened by esophagogastroduodenoscopy (EGD). The two main criteria proposed by the AGA to define BE are, a) columnar lined esophagus based on the appearance of "salmon" colored distal esophageal mucosa and b) biopsies that demonstrate the presence of intestinal metaplasia by detection of goblet cells, a specialized columnar epithelium that bears resemblance to the intestinal mucosa. This latter requirement is limited to the United States [4]. The European definition simply requires the presence of endoscopically visible columnar lined esophagus that on biopsies could either have gastric-type or intestinal-type epithelium. Once BE is identified, the patients are entered into a program of surveillance endoscopies to detect dysplasia and EAC [16]. Under this program, biopsies are obtained every 1-2 cm along the length of BE for histologic evaluation of dysplasia and EAC. To standardize the description of BE, the Prague Classification system of circumferential (C) and maximal length (M) has been widely accepted [20, 21]. On endoscopy, the gastroesophageal junction is identified and the absolute distance of circumferential and proximal columnar lining from this point is noted. If BE was circumferential for 2 cm above this junction, for example, and the maximal extent of non-circumferential BE (including the tongues) was 5cm above this junction, the BE in this situation would be Prague classification: C2M5 (Figure 1). BE lengths can vary from 1 cm to 20 cm depending on the patient. Currently, patients undergoing surveillance receive an endoscopy every 3–5 years for BE without dysplasia, every 6-12 months for low-grade dysplasia, and every 3 months for high-grade dysplasia.

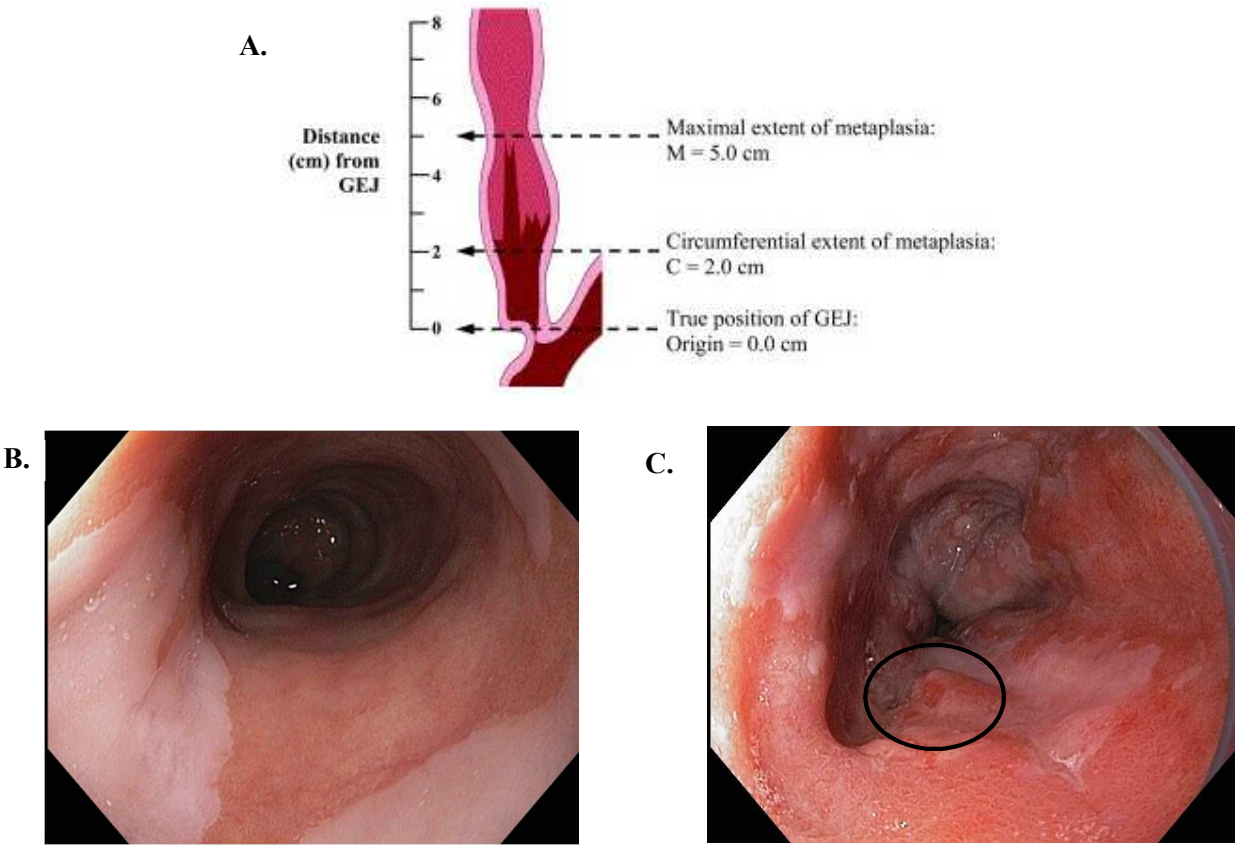

Figure 1. *A) Depiction of Barrett's esophagus (dark red) with a Prague classification C2M5. Gastroenterology. 2006 Nov; 131(5):1392-9. Epub 2006 Aug 16. B.) Barrett's esophagus (salmon colored columnar mucosa) and normal, non-diseased esophagus (whitish squamous mucosa) on endoscopy. C.) Advanced Barrett's esophagus. The open circle marks a nodule, a sign of early cancer.*

It is widely accepted that there are some significant problems with the current practice of BE screening and surveillance. As mentioned above, there are millions of individuals with GERD at risk for BE and, therefore at risk for EAC. However, it is expensive and impractical for the 10 million adults with GERD to go through an invasive upper endoscopy, the current gold standard for BE screening [4]. Also, periodic surveillance endoscopies are not cost-effective. There are other problems such as sampling error of biopsies and substantial intra-observer and inter-observer (κ= 0.30) variability in the diagnosis of dysplasia among pathologists [22-24].

Two major problems that form the basis of the current proposal are that the majority of BE remains undiagnosed and that greater than 80% of EACs are diagnosed late without a prior diagnosis of BE [25]. Although this raises the question of whether EAC arises *de novo* from a pathway alternate to BE, data suggests that this is unlikely to be the case. In greater than 90% of patients with EAC, the BE mucosa becomes visible again once the cancerous tissue sloughs away with chemoradiation [26]. This indicates that the cancer had overgrown the BE epithelium making it invisible. Thirty percent of patients with BE do not experience reflux symptoms and therefore may remain unscreened [7]. These data suggest the screening eligibility criteria are limited in predicting those with BE and therefore leads to a large number of patients with BE undiagnosed in the population [25].

This lack of timely diagnosis of BE is a missed opportunity for early diagnosis of EAC. The majority of patients wait until symptoms such as dysphagia develop and present with an advanced-stage esophageal cancer instead of an earlier stage when treatment options are possible. Early diagnosis could impact survival as effective endoscopic therapy for BE dysplasia and cancer is now available. Intervention in the premalignant stage of EAC may save the lives of patients who develop incurable EAC each year. Thus, there is a need for an alternative, simple, less invasive and more effective diagnostic tool to screen GERD patients for BE and to identify those patients with BE that have cancer.

Due to the above limitations, recently there has been much interest in non-endoscopic detection methods for timely diagnosis of BE with the goal of preventing esophageal adenocarcinoma (EAC) [27-30].

Exosomes and MicroRNA

One emerging strategy is the use of exosomal contents as a diagnostic biomarker.

Exosomes are approximately 40-100 nm membrane-bound vesicles that are secreted from nearly all cell types. Exosomes are ubiquitous in various body fluids such as serum, saliva, breast milk, ovarian follicular fluid and urine [31-35]. Their contents are thought to play a crucial role in cellular communication and reflect properties of the body's benign or disease state. Exosomes are produced when the membrane of parental cells buds inwardly to form an endosome, then subsequent inward budding of the endosome membrane forms intraluminal vesicles (Figure 2).

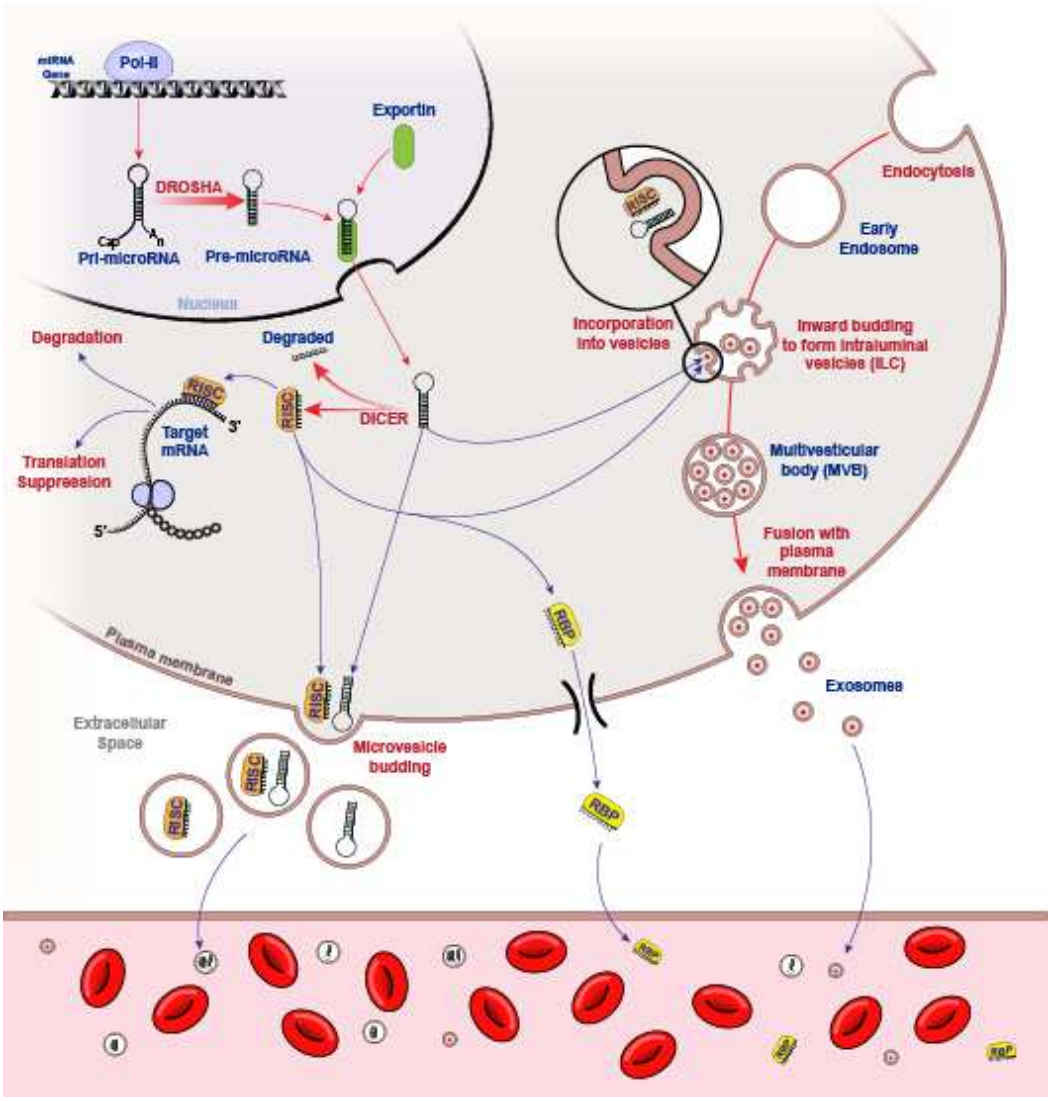

Figure 2. *Schematic overview of MicroRNA and Exosome Biogenesis. RBP: RNA Binding Protein such as Argonaute and Nucleophosmin*

This allows for lipids, cytokines, proteins and RNA to be incorporated in the vesicles [36]. Once this occurs the structure is called a multivesicular body (MVB). Some MVBs become degraded in lysosomes while others fuse to the plasma membrane and release the vesicles into the extracellular space.

Exosomes have proven to be stable in various conditions including the extracellular environment; because of this they are able to protect their cargo from being denatured or degraded [37-40]. Once exosomes are released into the extracellular space recipient cells can uptake the cargo. This results in a “message in a bottle" mechanism allowing distant cells to communicate and regulate the cellular processes of recipient cells [41, 42].

In order to catalog the identified contents of extracellular vesicles, the ExoCarta (http://www.exocarta.org) database was developed [43]. Recent data suggest that miRNAs can be found in exosomes [44]. MicroRNAs (miRNAs) are small (18–25 nucleotides long), endogenous, non-coding RNAs [26]. They have been found in plants, animals, and viruses. Specifically in humans, mature miRNAs function as regulatory molecules in a wide variety of fundamental cellular processes, such as proliferation, death, differentiation, motility, and invasiveness [27]. MicroRNA are being discovered and validated often and they currently they constitute 2-5% of the human transcriptome, equating to approximately 2,000 miRNA. Pri-miRNAs, are the initial transcripts resulting from RNAPol-II activity. Once pri-miRNA fold to form hairpins, they are processed into pre-miRNA, which are 60 to 100 nucleotides in length. Exportin exports pre-miRNA from the nucleus to the cytoplasm where they are typically cleaved by the endonuclease Dicer; this results in a miRNA-miRNA* complex. Sometimes pre-miRNA can bypass Dicer and be released into the extracellular space contained in a microvesicle or exosome. For those that do not go this route, one strand, generally the miRNA strand, is designated to become a mature

miRNA and the other, most often the miRNA* strand, becomes degraded. In the cytoplasm the mature miRNA strand can bind with the RNA-induced silencing complex (RISC); this complex can then bind to specific target mRNA or get released in the extracellular space.

Depending on the degree of complementarity, miRNA binding can either block translation or promote mRNA degradation. MicroRNAs are predicted to regulate as much as 30% of all gene expression and have been intensively studied in cancer research [45]. A single miRNA can influence the expression of numerous genes transcripts and, thereby, may affect the physiology of a cell. Higher levels of a specific miRNA can lead to down regulation of key genes and, similarly, lower levels may lead to up-regulation of other key genes. They can give insight on the state of the body's condition and have shown promise as specific tissue and serum biomarkers in multiple premalignant and malignant conditions [46-49]. Multiple studies have shown them to be good clinical markers for detection of cancerous and benign disease states [50-52]. In a previous study, miRNA profiles were found to mirror the developmental lineage and differentiation state of the tumors more accurately than mRNA[48].

A blood-based diagnostic test for BE and EAC would be a significant advancement, one that is capable of changing patient outcomes. To date, no useful exosomal miRNAs have been used to diagnosis or distinguish GERD, BE or EAC from another. In this study, we sought a novel approach towards addressing the limitations of current diagnostic methods for these conditions in their population based applicability. Here, we isolated serum exosomes and preformed comprehensive miRNA profiling by next generation sequencing to identify a unique miRNA signature that is able to distinguish GERD, BE and EAC from each other.

METHODS

Study Population and Tissue Sample Collection

The patients for the current study were selected from an ongoing repository of controls and patients with GERD, BE and cancer. Frozen patient sera were randomly selected from this biorepository to include 8 patients each with GERD, BE and cancer. The creation of the repository and the use of specimens for this study was approved by the Institutional Review board at the Veterans Administration Medical Center Kansas City, Missouri. Specimens were obtained voluntarily after written informed consent. Adult patients (>18 years old) were enrolled and subjects with the following conditions were excluded: chronic liver disease, diagnosis of cancer in the last 3 years, an inability to discontinue the use of blood thinners, severe uncontrolled coagulopathy, history of unresolved drug or alcohol dependency, and prior history of esophageal or gastric surgery were excluded from the repository.

All participants completed a validated GERD questionnaire. Patients who answered affirmative to the presence of GERD symptoms (heartburn, regurgitation, etc.) were defined to have GERD. After endoscopic examination, patients with a GERD were sub-classified based on evidence of mucosal injury; those with non-erosive reflux disease (NERD) and the others with erosive esophagitis (EE). For this initial study, only those GERD patients who had erosive esophagitis (EE) were included because presence of erosive esophagitis definitively diagnoses GERD. Biopsies were obtained according to a standard protocol. Biopsied sections were reviewed by an experienced gastrointestinal pathologist for intestinal metaplasia, dysplasia and cancer according to the criteria described by Montgomery et al.[53]. Biopsies are graded and if there is concern for high-grade dysplasia and/or cancer, the biopsies are reviewed by a second pathologist. BE was defined as presence of columnar lined esophagus at least 1 cm in length on

endoscopy with demonstration of intestinal metaplasia based on the presence of goblet cells in biopsies. For this study, the BE group included only those BE patients that did not have dysplasia to minimize the impact of dysplasia grade on miRNA expression.

Isolation of exosomes

Blood was collected in red-topped tubes and centrifuged at 3000 rpm for 10 minutes at the Veterans Administration Medical Center Kansas City, Missouri. The serum was then aspirated and stored at -80°C. Randomly selected sera were thawed and differentially ultra-centrifuged to isolate exosomes. Following thawing serum was centrifuged at 2000 g for 20 min at 4°C and the resulting supernatant was centrifuged at 12 000 g for 45min. The supernatant was then transferred to a new tube and was ultra-centrifuged at 100 000 g for 120 min at 4 °C to pellet exosomes and microvesicles. The extracellular vesicle pellet that formed was re-suspended in 4 mL of PBS and centrifuged for a final wash at 100 000 g for 90 min at 4 °C. After this, the supernatant was discarded and the remaining pellet was re-suspended in 100µL of PBS.

Characterization of exosomes: Transmission Electron Microscope

A 1:1 mix of exosomes in PBS to 2% glutaraldehyde was vortexed and 30 µL was placed on a spot dish. Carbon-coated copper grids were glow-discharged in order to make them more hydrophilic. Next, a treated grid was floated on the drop for 20 minutes. The grid was then removed and allowed to float on 6 serial wells filled with deionized distilled water for rinsing. The grid was placed onto a drop of 1% uranyl acetate for ~10 s, excess liquid was drained by touching the edge of the grid against a piece of clean filter paper. The grid was allowed to dry for several minutes and then examined using a JEM-1200 EX microscope (JEOL, Akishima, Japan).

Exosomal RNA extraction

Total RNA was extracted using TRIzol as per the manufacturer's protocol (Ambion, Austin, TX). Briefly, 100μL of chloroform was added to 500μL of TRIzol and the resuspended pellet. After this, it was mixed vigorously and allowed to incubate at room temperature. The mix was centrifuged for 15 minutes at 4 degrees. The upper clear phase was transferred to a fresh tube and glycogen (1μL) and isopropanol (250μL) were added to precipitate the RNA. This was followed by vigorous mixing and centrifugation. The supernatant was carefully decanted and the remaining pellet was washed in 75% ethanol. After removing the ethanol, the RNA pellet was re-suspended in DEPC-treated and nuclease free water. RNA quality was evaluated on Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

Next-Generation Sequencing

Next-generation sequencing was performed to obtain a complete profile of exosomal miRNA from each patient. MicroRNA sequencing was performed using the Illumina HiSeq2500 Sequencing System at the University of Kansas Medical Center – Genomics Core. TruSeq Small RNA library preparation protocol (Illumina, San Diego, CA) was used to generate libraries from EAC (n=8), GERD (n=8), and BE (n=8) patients. The exosomal RNA was ligated with 3' and 5' RNA adapters followed by a duplicated reverse transcription reaction and 15 cycle PCR amplification. The cDNA library was purified and size selected by using 3% marker F gel cassettes on the Pippin Prep Size Fractionation System (Sage Science, Beverly, MA). The Agilent 2100 Bioanalyzer was again used to validate the purified libraries and were quantified with the Roche LightCycler96 Real Time PCR System using FastStart Essential DNA Green Master (Roche Diagnostics, Basel). Following quantification by Cq values, libraries were

adjusted to the same concentration and pooled for multiplexed sequencing. Twelve random samples per lane were multiplexed resulting in two lanes for all of the 24 samples.

Libraries were denatured, diluted and clustered onto the sequencing flow cell using the Illumina TruSeq Rapid Single Read (SR) Cluster Kit-HS. The clustered flow cell was sequenced using the Rapid Read mode with a 1x50 cycle read and index read. Sequence data was converted from .bcl file format to FASTQ files and de-multiplexed into individual sequences for further downstream analysis.

MicroRNA signature for GERD, BE, and EAC

To identify previously reported miRNAs present in our samples, the miRDeep2 software package was used [54]. The following steps were executed sequentially: Sequences with more than one mismatch read or ones that contain characters other than A, C, G, T, U, and N were discarded. Adapter sequences were trimmed from the primary reads and sequences were mapped to the human reference genome, hg19 UCSC. Further, only sequences with at least 18 nucleotides, after adapter removal, are retained and duplicate sequencing alignment reads are removed. miRDeep2 then tabulated the number of reads that aligned to the reference genome. Raw reads counts for each miRNA were normalized to the total number of miRNA reads in each sample.

Differential pairwise exosomal microRNA analysis was performed using the EdgeR package from Bioconductor [55]. EdgeR uses a generalized linear model to assess differential expression between groups assuming the negative binomial distribution. As over-dispersion may differ across genes with the dispersion parameter difficult to estimate per feature / tag, moderated estimates of the dispersion parameter from EdgeR were used. The obtained read counts for the

2,946 identified miRNA were first filtered based on a minimum requirement of >5 counts in at least 8 of the 24 patients; this yielded 353 miRNAs. Next, the counts were normalized by scaling for library size factors in order to deal with variation among samples. The normalized counts of the 353 miRNAs were considered for the pairwise comparisons. The results were adjusted for multiple testing using the Benjamini and Hochberg method. A False discovery rate (FDR) of 5% was considered significant [56].

In order to retrieve insight into diagnosis group variation, development of a classification signature was developed using Prediction Analysis for Microarrays (PAM) [57]. The PAM statistical method identifies the subsets of miRNA that best characterize each class by using the nearest shrunken centroid method. We used PAM to determine which miRNA are the most predictive of GERD, BE, and EAC based on their miRNA expression patterns. The analysis was done by randomly splitting the data set into training (21 samples) and test groups (3 samples). The shrinkage parameter or threshold was chosen by minimizing the prediction error using 7-fold cross-validation within the training set. Choosing a larger threshold value would yield a smaller number of genes to include in the signature; thus making it more practical for clinical settings. The threshold value that returned the lowest classification error with the fewest yet strongest genes was estimated to be 2.0.

Pathway Analysis

Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, CA) was used to determine potential pathways involving the differential miRNAs and their suspected target gene transcripts. IPA provides insight into the interaction of these miRNAs and potential target genes in the disease process of GERD, BE and EAC. Differentially expressed miRNAs (FDR<0.05) from the

pairwise comparisons were imported into the "Core Analysis" module of IPA. Confidence levels were set to "highly predicted" and "experimental observed," based on the Ingenuity Knowledge Base. Enriched networks from identified miRNA targets were reported after analysis. IPA ranks networks according their significance based on Fisher Exact test p-value (p-score = ($-\log_{10}$[P value])).

Statistical Analysis

Statistical analysis was performed using the SAS software package (SAS Institute Inc., 2013). For categorical variables, the data were expressed as N (%) and continuous variables, the data were expressed as means (standard deviation). The relationships among demographic and clinical factors were analyzed using the following tests: Kruskal-Wallis test was used to examine the differences between continuous demographic information in all three groups; Wilcoxon Mann-Whitney was performed to compare the differences between mean BE length for patients with BE and EAC; and Fishers exact was used for categorical data to evaluate the correlation between disease status and patient demographics. Differences were considered statistically significant at the level of $P<0.05$.

RESULTS

Patient Demographics and Clinical Characteristics

All patients for this study were white males reflecting the underlying demographic predisposition of this disease and our attempts to evaluate homogeneous populations. The demographic information and the clinical characteristics of the subjects included in this study are summarized in Table 1. The average BMI of patients with esophageal adenocarcinoma was higher than those of patients with GERD (34.4± 11.92 versus 27.0± 5.34) but the difference was

not statistically significant ($p = 0.3833$). Over 63% of the patients in all three groups had a history of smoking but approximately one-third of participants in each patient group currently smoked. Similarly, greater than 63% of patients had a hiatal hernia present on endoscopic examination. K-Wallis Test did not confirm a significant difference in age or mean hernia length among the three patient groups. The mean lengths of columnar lined esophagus in patients with BE and EAC were C3.5M5.5 and C4.3M5.9 respectively, ($p = 0.7251$, Table 1). EAC patients were less likely to report GERD symptoms compared to the other two groups ($p = 0.021$).

Table 1. *Patient Characteristics and Clinical Parameters*

	GERD Cases n= 8	BE Cases n= 8	EAC Cases n= 8	
Characteristics				*P value*
AGE				
Mean (SD)	64.4 (10.2)	58.8 (12.7)	64.1 (10.2)	0.3833
BMI				
Mean (SD)	27.02 (5.34)	32.03 (5.90)	34.37 (11.92)	0.3826
SMOKING				
Current Smoker	3 (38%)	3 (38%)	2 (29%)	1.00
Past Smoker	5 (63%)	6 (75%)	5 (71%)	1.00
HIATAL HERNIA	5 (63%)	7 (88%)	7 (88%)	0.5573
Mean Length of Hernia in cm (SD)	4.8 (2.2)	3.4 (1.9)	3.3 (1.4)	0.4834
GERD SYMPTOMS	7 (88%)	8 (100%)	3 (38%)	0.0206
FAMILY HISTORY				
GERD	1 (13%)	3(38%)	6 (75%)	0.5573
BE	0	0	1 (13%)	1.00
EAC	0	0	1 (13%)	1.00
MEDICATION USE				
PPI	7 (88%)	8 (100%)	8 (100%)	1.00
Aspirin	5 (63%)	3(38%)	3 (38%)	1.00
PRESENCE of BE		8 (100%)	8 (100%)	0.001
Mean BE Length (SD)	-	5.5 (3.7)	5.9 (3.2)	0.7251
Mean Prague Classification	-	C3.5M5.5	C4.3M5.9	-
Pathological Stage				
I	-	-	4 (50%)	-
II	-	-	1 (13%)	-
III	-	-	1 (13%)	-
IV	-	-	2 (25%)	-

Values reported as N(%) unless otherwise noted. GERD, gastroesophageal reflux disease; BE, Barrett's esophagus; EAC, Esophageal adenocarcinoma

Exosome characterization

After isolation, presence of exosomes was confirmed by transmission electron microscopy (TEM). TEM demonstrated numerous characteristic, cup-shaped, 30-100nm vesicles (Figure 3). Figure 3 is representative of exosome preparations using the differential centrifugation technique described under methods.

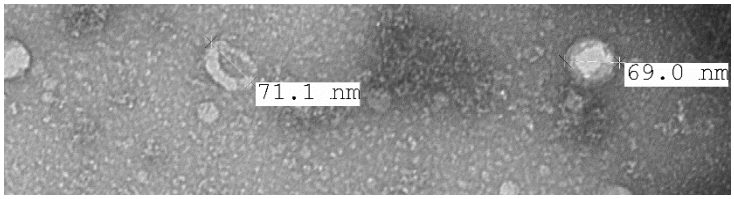

Figure 3. *Transmission Electron Microscopy of negatively stained exosomes*

Next-generation sequencing alignment and analysis

Next-generation sequencing (NGS), generated 11.8 million reads per sample. Low quality scores for sequenced samples can lead to a large number of false positive variant alignments. However, for the samples in this study, 97.8% of bases had a quality score greater than 30. This is equivalent to the probability of an incorrect base call 1 in 1000 times or a base call accuracy of 99.9% [58]. After processing and filtering with MirDeep2, an average of 2.4 million reads per sample mapped to mature and validated miRNA sequences annotated in hg19 UCSC (average: 25%, range: 8%-42%). Additionally, MiRDeep2 predicted more than 1,700 novel microRNA unannotated sequences in serum exosomes.

To get a general idea about the serum exosomal miRNA diversity for each condition, Venn-diagrams were generated with each circle depicting the number of unique miRNA found in serum for each patient group and the overlap indicating the number of common miRNA. Patients with GERD had the greatest miRNA diversity in their serum miRNA with 73 miRNA being unique to its condition (Figure 4a). Notably, there was substantial overlap in unique miRNA when the Venn-diagram inclusion criteria was changed from a read count >1 to a read count >100; neither BE or EAC had unique miRNA under this condition. However, the serum miRNA in patients with GERD still exhibited the highest number of unique miRNA (Figure 5b).

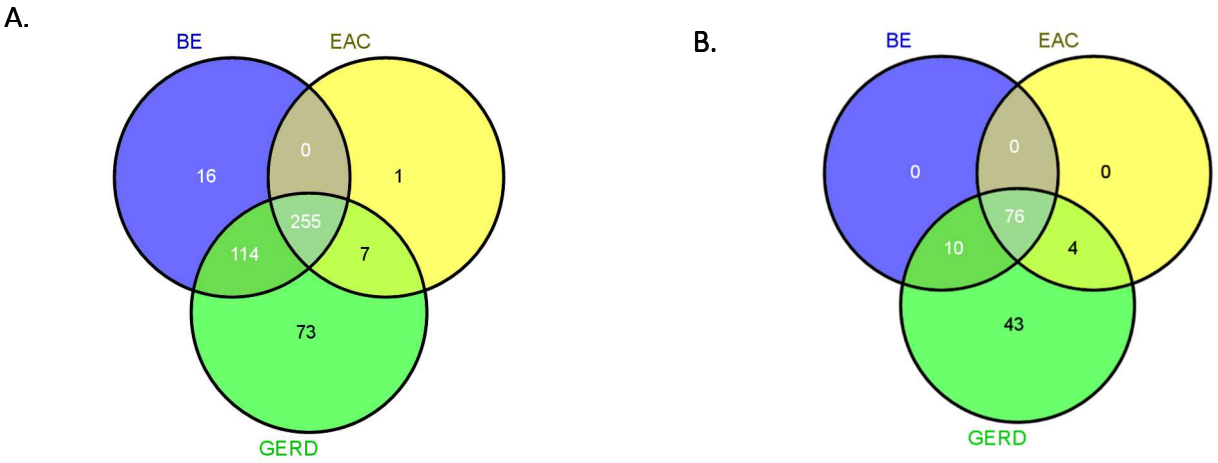

Figure 4. *Venn-diagram of miRNA found in serum exosomes for each patient population. (A) Number of miRNA present in all eight patients for each group (read>1). (B) Number of miRNA present with at least 100 raw counts in all eight patients for each group*

These results are consistent with the clinical observations that GERD patients have two distinct profiles with respect to complications. Some GERD patients are predisposed to BE and EAC whereas others will not develop BE and EAC despite continued GERD.

Next, the serum exosomal miRNA profiles of GERD, BE and EAC were compared using EdgeR. The most significant differences were found when EAC patients were compared to BE

and GERD patients; 79 and 85 differentially abundant miRNAs in EAC versus BE and EAC versus GERD respectively (Table 2). In comparison, 17 serum exosomal miRNAs were different when comparing BE vs. GERD patients. For each comparison, approximately half of the differentially expressed miRNAs were up-regulated and the other half were down-regulated (Table 2). Figure 5a, b and c show volcano plots of statistical significance against fold-change between disease states (the axes are hard to read). A volcano plot of GERD vs EAC and BE vs EAC show seven miRNAs (Figure 5b) and 42 miRNAs (Figure 5a) with a FDR <0.05, respectively (red points). GERD patients, when compared to those with BE, had no miRNA with a FDR <0.05. Tables with a complete list significant genes for each pair wise comparison and fold change can be found in Appendix A, B and C.

Using unsupervised hierarchical clustering a heat map was generated with significantly different miRNA (FDR< 0.05) from the pairwise EdgeR analysis. We found that 4 discrete clusters of serum exosomal miRNAs can differentiate patients with Barrett's esophagus from patients with esophageal adenocarcinoma (Appendix D).

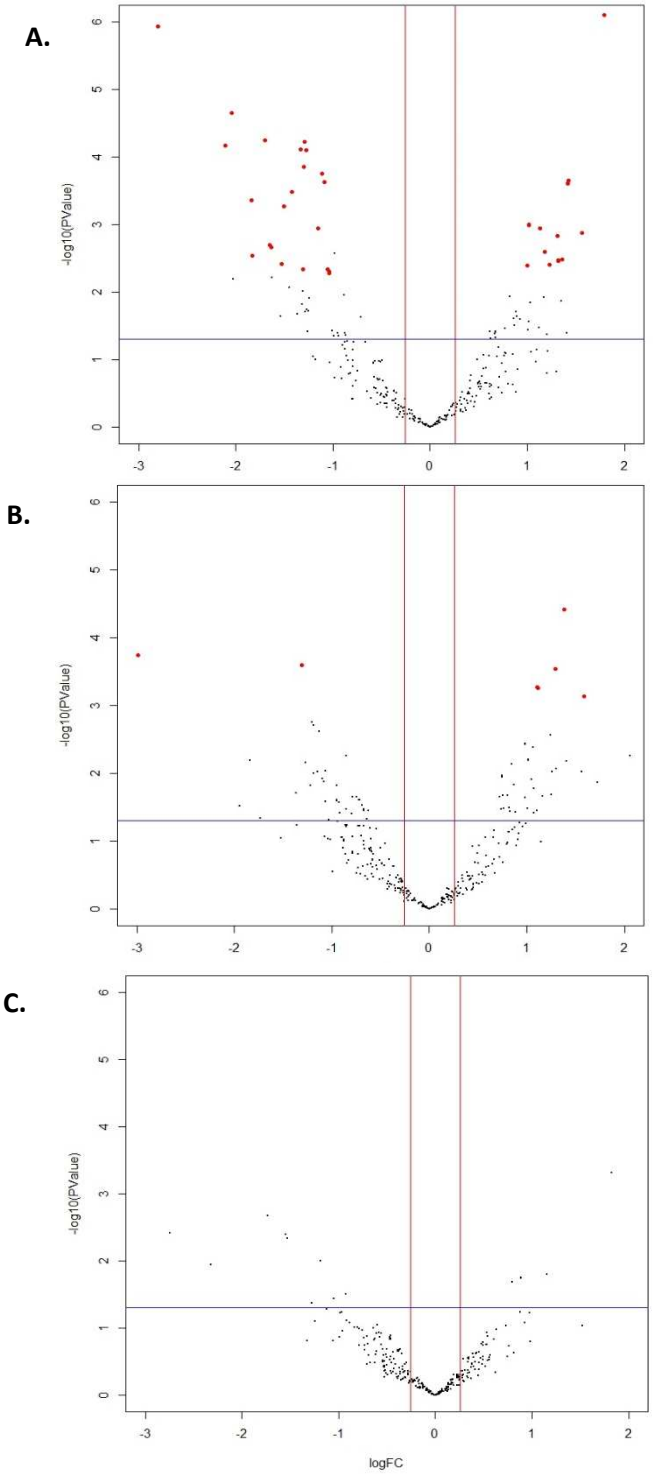

Figure 5. *Volcano Plots of Pairwise comparisons and summary table. The* x*-axis shows the* log_2 *fold-change in circulating miRNAs' expression between (A) BE vs EAC (B) GERD vs EAC and (C)GERD vs BE .The* y*-axis shows the* $-log_{10}$ *of the* P *value for each miRNA. The volcano plots show a visual representing of the strength of the association and magnitude of fold change. The blue line indicates comparisons with* $P < 0.01$*). The two vertical lines (red) are the fold change boundaries. MicroRNA with an FDR <0.05 are seen as red points on the plots.*

Table 2. *Number of differentially abundant miRNA for each comparison.*

	Increased	Decreased	Total
GERD vs BE	9	8	17
GERD vs EAC	40	39	79
BE vs EAC	39	46	85

n= 8 , P value<0.05

Prediction Analysis for Microarrays (PAM)

We used Prediction Analysis for Microarrays to generate a miRNAs list under optimal classification capabilities (threshold of 2.0) to distinguish between the three disease states (Figure 6). Seven-fold cross-validation yielded a 34 miRNA signature showing the greatest potential for distinguishing GERD, BE and EAC (Figure 7) with an overall misclassification rate of 0.36. The PAM model was able to accurately classify 64% of patients to the appropriate group (Figure 7b).

A great deal of the miRNA in the PAM signature correlated with the differentially expressed miRNA from the pairwise comparisons. Interestingly, 21 out of the 42 miRNA considered significant (FDR<0.05) from the EdgeR analysis were found in the PAM miRNA classifiers. This is a 62% concordance from the two analysis techniques. Figure 8 shows the heat map obtained using the miRNA gene signature of 34 genes. There were similar miRNA clusters that noticeably distinguished BE and EAC from those determined by the EdgeR analysis. The

miRNA clusters failed to distinguish the GERD population from others. Following the generated PAM signature, box plots were produced for the 34 miRNAs (Figure 9).

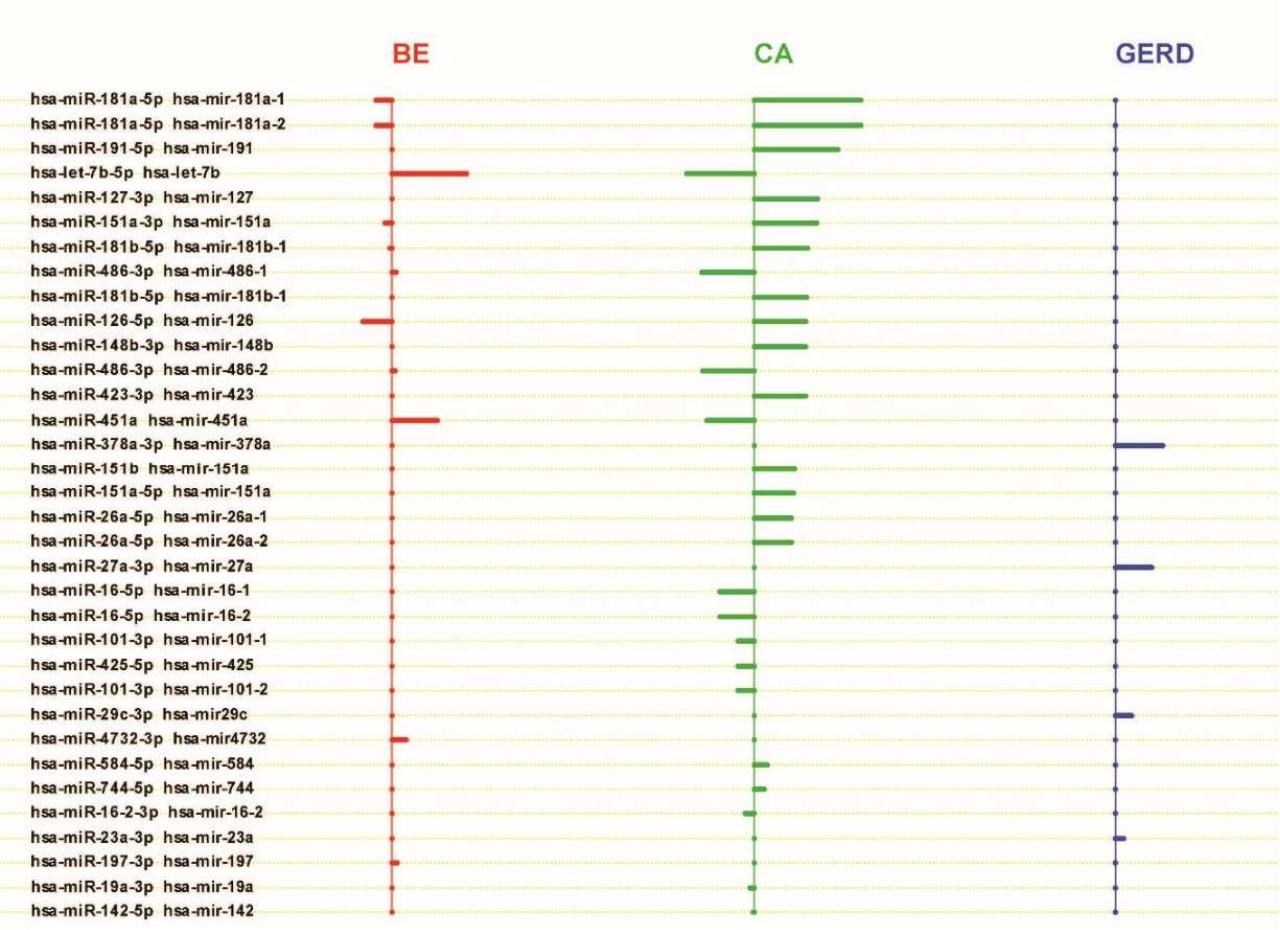

Figure 6. *Prediction Analysis for Microarrays miRNA signature. Plot depicts the shrunken centroids (expression level) for a threshold of 2.0 with the ranked list of significant miRNAs. Bars facing left represent negative shrunken centroid scores indicating decreased expression and the opposite is true for the bars facing right.*

A.

B.

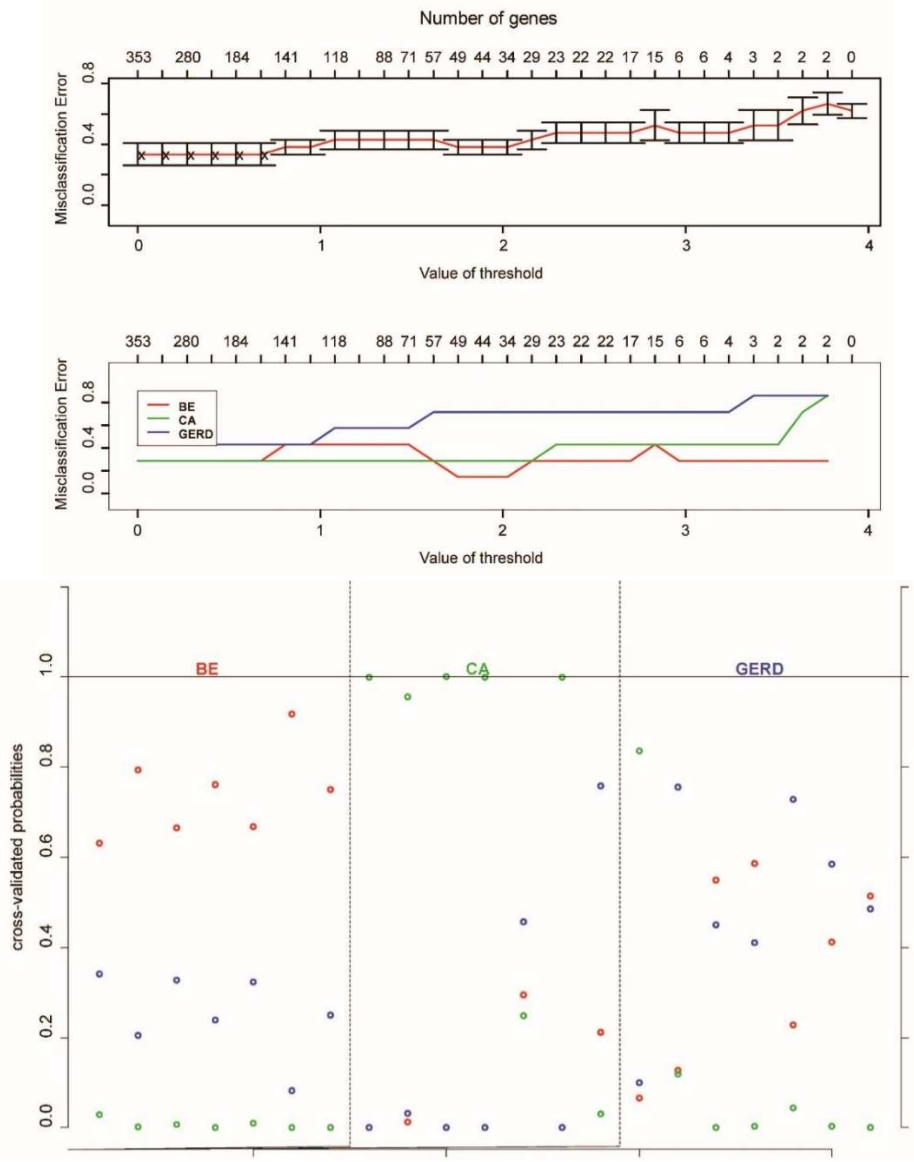

Figure 7. *A) Misclassification error (predictive power) over various thresholds. The upper panel shows misclassification error on the y axis. The labels across the top axis gives the potential number of miRNAs used in the classification; from the total number of miRNA in the entire set to a single one (far left to right). The lower panel shows the misclassification error for each diagnosis, with BE cases in red, EAC in green and GERD in blue. B) Cross-validation errors of the classifying miRNA. The X-axis represents the patient sample number and the Y-axis represents the Test Set probability of diagnosis. The 10X cross-validation probabilities of a given sample belonging to each patient clinical phenotype are indicated by the various color on the graph. BE cases are in red, EAC in green and GERD in blue. The sum of probabilities for a given sample should equal 100%.*

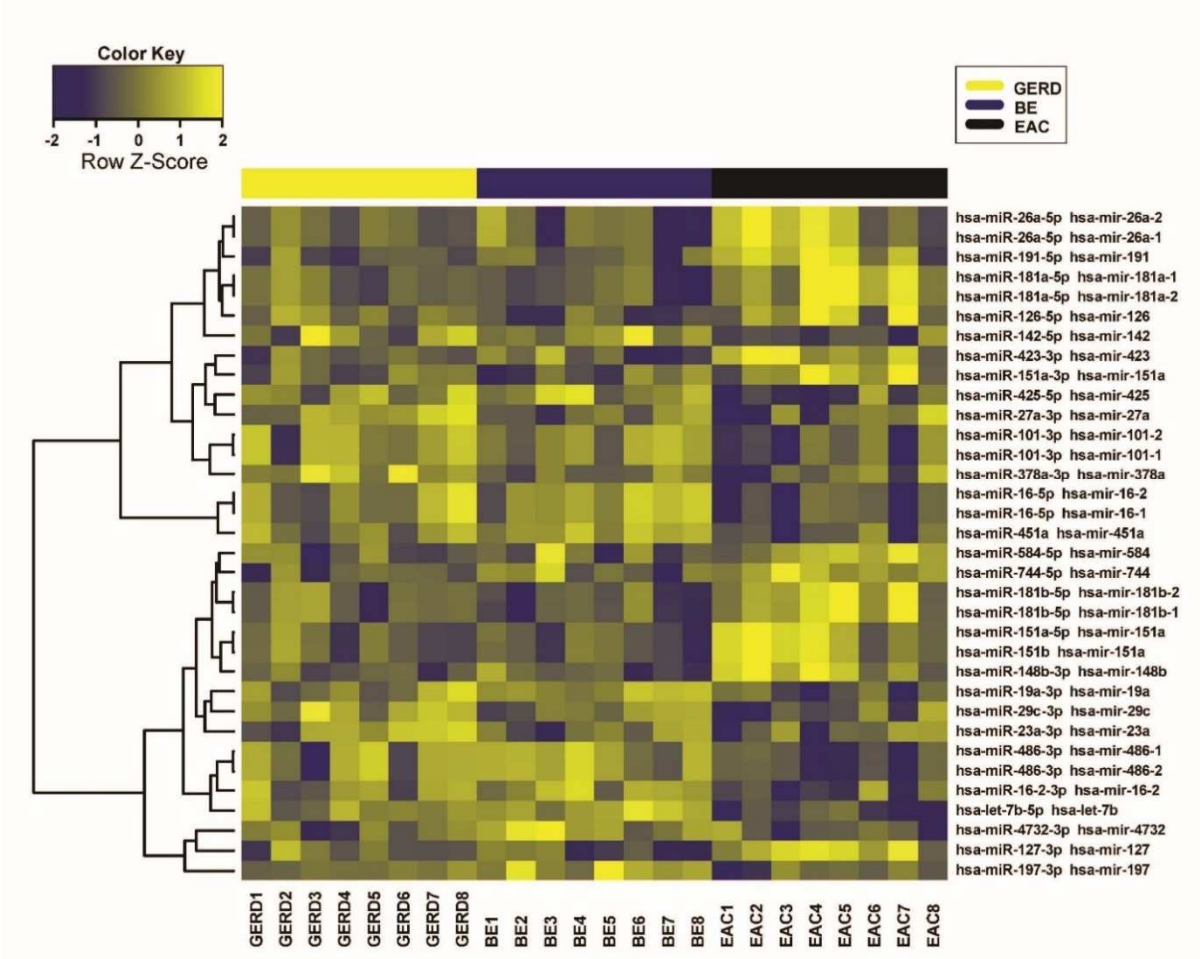

Figure 8. *Heat map. Columns represent patient samples and rows represent the significant genes from PAM analysis. Scale bar indicates expression signal where yellow denotes genes with relative increased expression while blue denotes genes with relative decreased expression.*

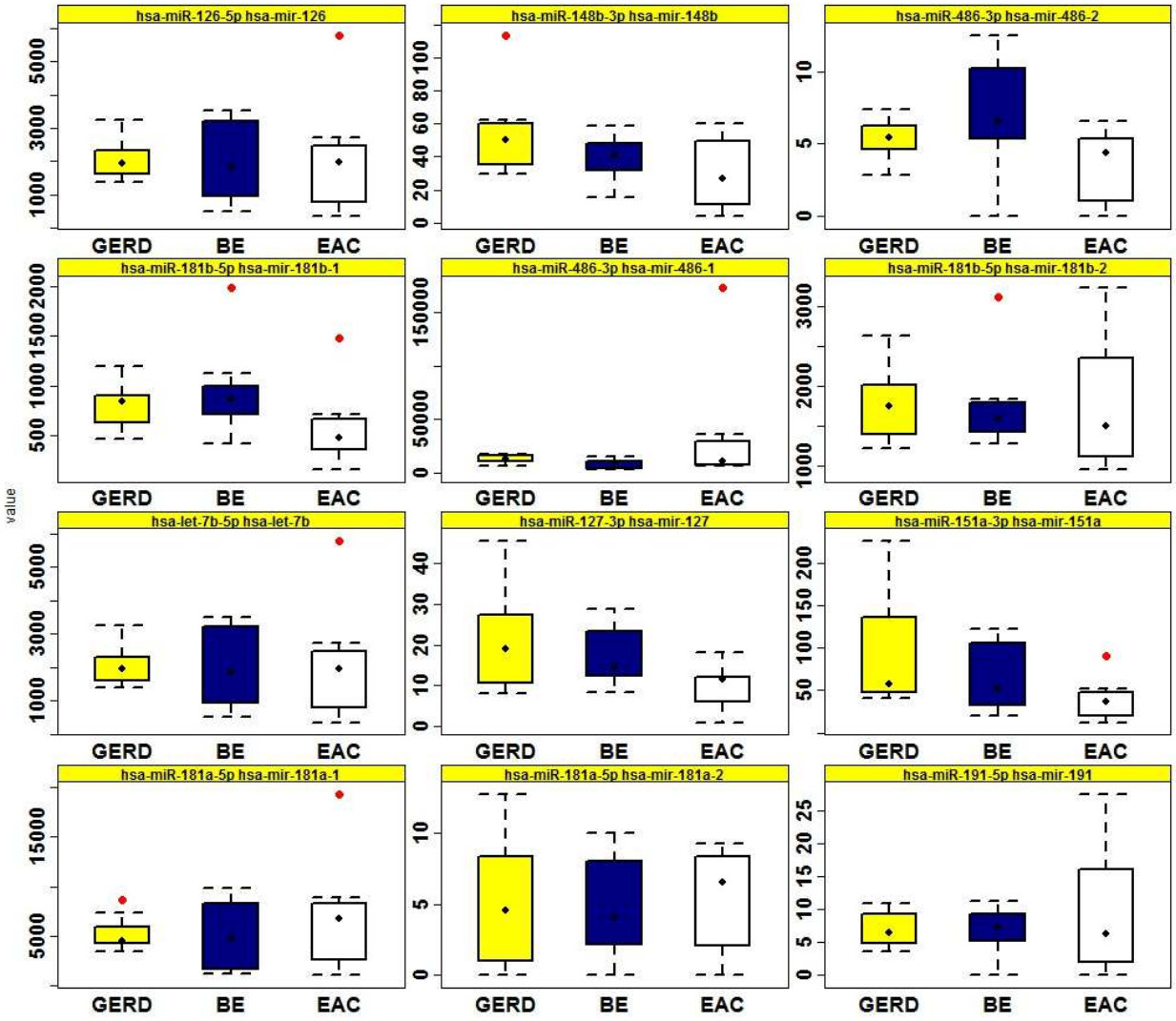

Figure 9. *Distribution of miRNA abundance. Box plot of normalized counts for PAM signature miRNAs. 1 of 3*

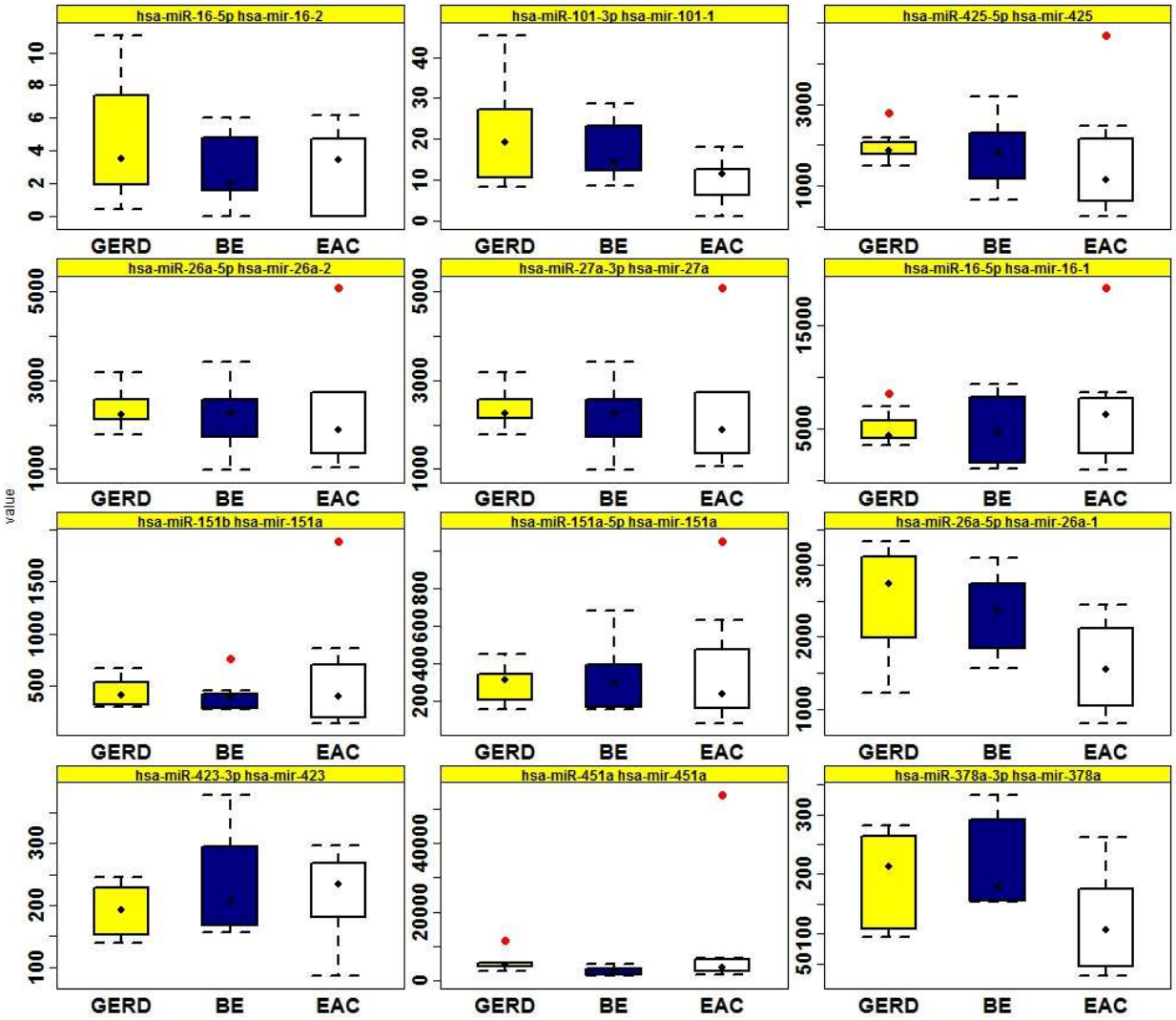

Figure 9. *Distribution of miRNA abundance. Box plot of normalized counts for PAM signature miRNAs. 2 of 3*

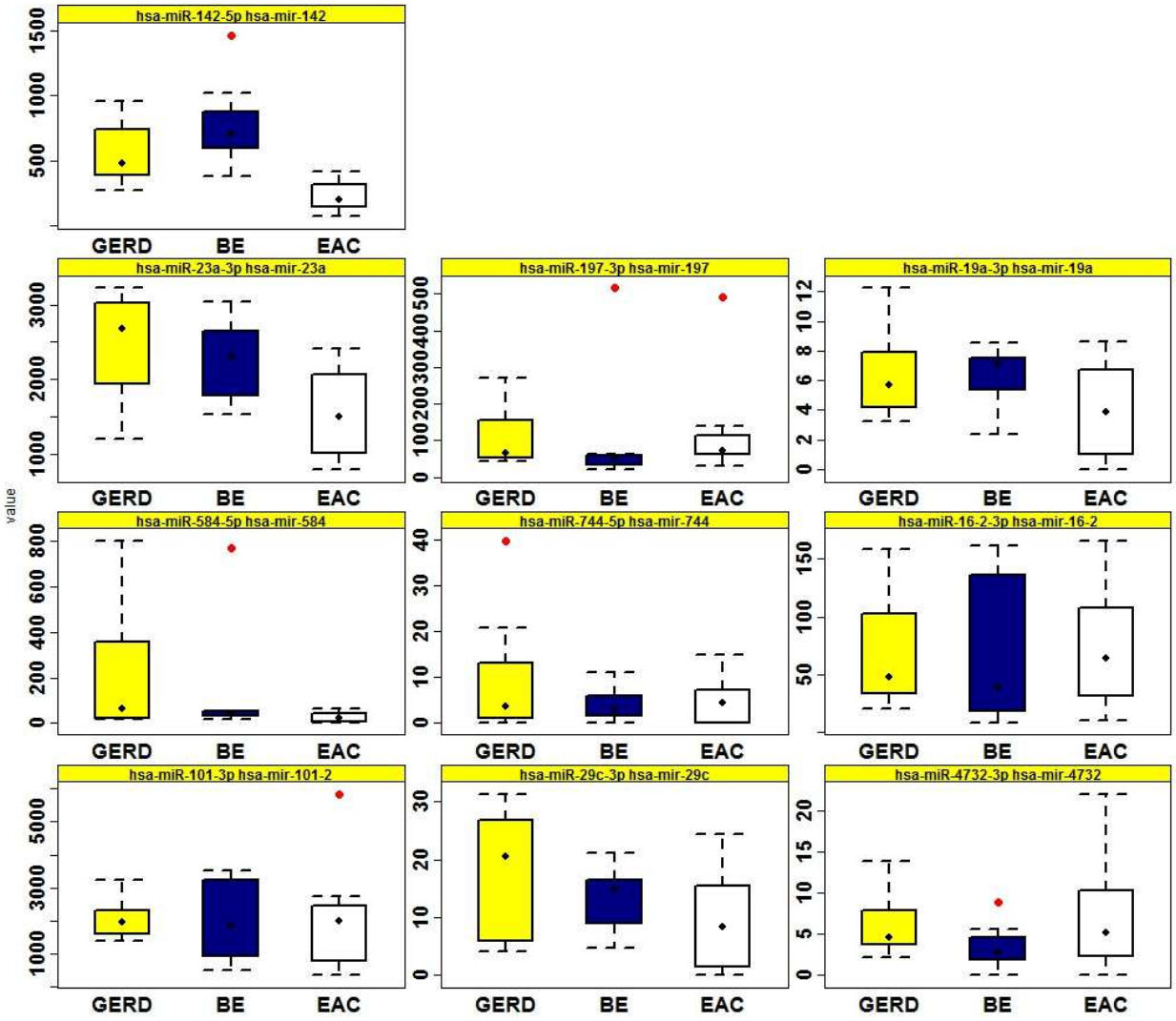

Figure 9. *Distribution of miRNA abundance. Box plot of normalized counts for PAM signature miRNAs. 3 of 3*

Ingenuity Pathway analysis

To assess whether certain regulatory networks were enriched in the three conditions, we performed a series of investigations using IPA. Comprehensive network analysis of the pairwise comparison data showed correlations with top networks relevant to the development of cancer (Table 3). The differentially enriched miRNA gene targets constituted about 40% of the molecules involved in cancer and inflammatory-associated networks. Similarly, the diseases and

function analyses showed analogous biological processes impacted by these miRNA such as inflammatory disease and response, cancer, and organismal injury (Appendix E).

Table 3. *IPA Network summary for each pairwise comparison*

(A) GERD vs BE

ID	Top Diseases and Functions	Focus Molecules	Score
1	Skeletal and Muscular Disorders, Developmental Disorder, Hereditary Disorder	9	24

(B) GERD vs EAC

ID	Top Diseases and Functions	Focus Molecules	Score
1	Cancer, Organismal Injury and Abnormalities, Reproductive System Disease	25	66
2	Connective Tissue Disorders, Inflammatory Disease, Inflammatory Response	23	57
3	Infectious Disease, Organismal Development, Hematological Disease	1	3
4	Cell Cycle, Cellular Movement, Cancer	1	2

(C) BE vs EAC

ID	Top Diseases and Functions	Focus Molecules	Score
1	Developmental Disorder, Hereditary Disorder, Skeletal and Muscular Disorders	20	43
2	Cancer, Organismal Injury and Abnormalities, Reproductive System Disease	19	41
3	Cancer, Organismal Injury and Abnormalities, Reproductive System Disease	10	22

DISCUSSION

MicroRNAs have huge potential as biomarkers for malignant as well as benign conditions. Multiple studies have shown their discriminatory ability between health and disease. We tested the potential role of miRNAs as biomarkers for BE and esophageal adenocarcinoma, a cancer that has increased by 6-fold over the past decade and has a 5 year survival of ~20%. The current diagnostic gold standard, upper endoscopy, is neither cost effective nor widely applicable

to detect the entire population at risk. Our study is unique because we focused on the miRNAs concentrated within serum exosomes. Circulating miRNAs are stable in two forms, one concentrated in membrane bound vesicles called exosomes and the other as protein bound complexes. Majority of work on the diagnostic potential of miRNAs did not discriminate between the two compartments that could have added to the variability of the signature and even lead to the dilution of the signature. We hypothesized that serum exosomal miRNA could be used as disease-specific diagnostic biomarkers because changes in their expression is influenced by the presence or absence of BE and cancer. Here, we compared exosomal miRNA profiles to determine a diagnostic signature for BE and EAC in patients with GERD. Our promising findings suggest that our signature has the potential to be developed into novel clinical assays for the diagnosis of BE and EAC.

The field of exosomes in bodily fluids is rapidly evolving[40]. There are commercially available kits that can isolate serum exosomal miRNAs. The current literature contains evidence for positive[59] and negative results with these simple to use assays [40]. It appears that these commercial isolation kits may not select for exosomes exclusively and precipitate membrane-free macromolecular aggregates and other types of membrane bound vesicles[40]. Ultracentrifugation is the gold standard for isolation of serum exosomes and was used in this proposal for comprehensive identification of novel serum exosomal miRNAs in BE and cancer.

The current study is one of the first studies to examine the role of serum miRNAs for BE and cancer diagnosis. A recent Dutch study presented only in abstract form explored differentially expressed miRNA in plasma and found miRNAs -382, -133, -136, -194, and -451 to be different between GERD, BE and EAC [60]. These miRNAs were absent from our PAM signatures. There were some differences between the Dutch study and our study. We specifically

focused on the miRNAs expressed in exosomes. We used next generation sequencing unlike miRNA microarray used in the Dutch study. Next generation sequencing is the gold standard for high-throughput miRNA analysis [61].When we compare our results of pairwise comparisons from EdgeR, there were some similarities. MicroRNA -451 was significant in BE vs. EAC and GERD vs. EAC comparisons. MicroRNA-194 was significant in GERD vs. BE and BE vs. EAC comparisons and microRNA-133 was significant BE vs. EAC comparisons. Although the purpose of this study was strictly to explore miRNAs as diagnostic biomarkers, it is possible that several of the discovered miRNAs may play a role in BE carcinogenesis. The IPA analysis suggested that several of the genes regulated by the identified miRNAs map to pathways associated with cancer development and inflammatory cascades. These three miRNA have been associated with gastrointestinal tumors with microRNA-133 with a possible tumor suppressive role.

Our study has some limitations. Multiple physiological factors can affect serum exosomal miRNA content. Since blood is a complex mixture of biomolecules and cells, it is likely that some miRNA signals are from the cells other than esophageal. It is possible that serum miRNAs are markers for systemic process that predispose to BE. Irrespective of origin, the purpose of the serum miRNA evaluation in BE is to develop novel biomarkers. If the BE serum miRNA signature successfully fulfills the primary purpose of disease classification, then it will have clinical utility [62]. This study included 24 subjects for the high-throughput analysis. The sample sizes were relatively small because of cost constraints. We applied a sound statistical methodology for data analysis and discovered several potential candidates. Future studies should include larger numbers to validate our results. We evaluated exosomal miRNAs. It is possible that non-exosomal miRNAs may carry diagnostic information. We have stored the non-exosomal supernatant and will be able

to test them if, upon validation, the exosomal signatures are not accurate. The primary control group in this study was participants with GERD instead of healthy controls. This is appropriate because GERD patients are the recommended population to undergo screening and are at the highest risk for BE and EAC, hence, they are logical controls for this type of investigation. Clinically, the accuracy and efficacy of the serum exosomal miRNA signature to diagnose BE will depend on the pre-test probability of disease that would be highest in the GERD population. An ideal disease signature should be able to differentiate among the stages of BE progression to stage 4 cancer. However, we included BE patients with the extreme phenotypes of "no dysplasia" and "cancer" to maintain homogeneity for this preliminary work.

This study has several strengths. Both clinical and laboratory protocols were strictly adhered to ensure the reproducibility and consistency of the results. Many recent studies have conducted similar research with the use of microarrays. However we feel the use of sequencing in our study gives us a more comprehensive picture of the possible differences in serum exosomal miRNA content. Additionally, with the information obtained from NGS we have the opportunity to discover unknown miRNAs. In this study, we focused our efforts on a minimally invasive blood-based diagnosis strategy, a significant improvement over conventional tissue based diagnosis.

Two different methods, EdgeR and PAM were used for analysis of the miRNA profiles between GERD, BE and EAC. EdgeR is a tool that is intended to calculate the statistical probability of differentially expressed miRNAs between groups. It can also control for false discovery rates. However, it does not provide any information on the ability of patterns of gene expression in disease classification. PAM is able to classify samples based on the gene

expression data and also estimates prediction error based on cross validation. The output includes a list of genes that are characteristic of each disease category i.e. GERD, BE and EAC. The overall goal of the PAM algorithm is to find the smallest set of miRNAs that can accurately classify the samples. Its efficiency at identifying the smallest number of miRNAs that contribute the most to the classification makes this method ideal for creating a diagnostic signature. Concordance in miRNA analysis from both EdgeR and PAM suggest our signature is robust and discriminatory for the target conditions. Based on the overall goal and results, we will pursue the classifier signature generated by the PAM algorithm.

Investigations in the immediate future will need to be conducted to validate the miRNA signature with alternative methods such as qRT-PCR. Subsequently, the signature need to be validated in independent cohorts. The development of a novel blood-based assay, independent of endoscopy, will provide a range of medical professionals the ability to accurately suspect BE and cancer with minimal effort with more convenience for the patient. The ability to timely diagnose BE and EAC has the potential to improve patient outcomes and survival.

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APPENDIX

Appendix A: GERD vs BE-Significant miRNAs by ascending p-value

Gene ID	logFC	Fold Change	PValue	FDR	Average Read Count for GERD	Average Read Count for BE
hsa-miR-197-3phsa-mir-197	-1.82	0.28	4.81E-04	1.70E-01	35	124
hsa-miR-411-5phsa-mir-411	1.73	3.32	2.12E-03	3.25E-01	10	3
hsa-miR-208b-3phsa-mir-208b	2.75	6.72	3.81E-03	3.25E-01	17	3
hsa-miR-194-5phsa-mir-194-2	1.55	2.92	4.03E-03	3.25E-01	139	48
hsa-miR-194-5phsa-mir-194-1	1.53	2.89	4.60E-03	3.25E-01	127	44
hsa-miR-378a-3phsa-mir-378a	1.19	2.27	1.01E-02	4.92E-01	2769	1217
hsa-miR-4792hsa-mir-4792	2.32	4.98	1.14E-02	4.92E-01	13	2
hsa-miR-181d-5phsa-mir-181d	-1.15	0.45	1.61E-02	4.92E-01	3	8
hsa-miR-941hsa-mir-941-1	-0.88	0.54	1.78E-02	4.92E-01	54	99
hsa-miR-941hsa-mir-941-5	-0.88	0.54	1.80E-02	4.92E-01	54	99
hsa-miR-941hsa-mir-941-4	-0.88	0.54	1.81E-02	4.92E-01	54	99
hsa-miR-941hsa-mir-941-2	-0.88	0.54	1.81E-02	4.92E-01	54	99
hsa-miR-941hsa-mir-941-3	-0.88	0.54	1.81E-02	4.92E-01	54	99
hsa-miR-4732-3phsa-mir-4732	-0.80	0.58	2.08E-02	5.24E-01	77	133
hsa-miR-410-3phsa-mir-410	0.92	1.90	3.15E-02	7.40E-01	28	15
hsa-miR-30a-3phsa-mir-30a	1.05	2.07	3.67E-02	8.11E-01	26	12
hsa-miR-125b-2-3phsa-mir-125b-2	1.28	2.42	4.23E-02	8.78E-01	62	26

Appendix B: GERD vs EAC-Significant miRNAs by ascending p-value

Gene ID	logFC	Fold Change	PValue	FDR	Average Read Count for GERD	Average Read Count for EAC
hsa-miR-148b-3phsa-mir-148b	-1.39	0.38	3.90E-05	1.38E-02	124	326
hsa-miR-122-5phsa-mir-122	2.99	7.92	1.82E-04	2.54E-02	214	27
hsa-let-7b-5phsa-let-7b	1.31	2.48	2.53E-04	2.54E-02	555	223
hsa-miR-191-5phsa-mir-191	-1.29	0.41	2.88E-04	2.54E-02	2073	5077
hsa-miR-151bhsa-mir-151a	-1.11	0.46	5.43E-04	3.22E-02	188	407
hsa-miR-151a-5phsa-mir-151a	-1.11	0.46	5.48E-04	3.22E-02	679	1132
hsa-miR-127-3phsa-mir-127	-1.59	0.33	7.40E-04	3.73E-02	43	128
hsa-miR-486-3phsa-mir-486-2	1.20	2.30	1.76E-03	7.70E-02	690	300
hsa-miR-486-3phsa-mir-486-1	1.19	2.28	1.96E-03	7.70E-02	700	307
hsa-miR-29c-3phsa-mir-29c	1.13	2.19	2.42E-03	8.56E-02	225	102
hsa-miR-99b-5phsa-mir-99b	-1.24	0.42	2.77E-03	8.89E-02	86	204
hsa-miR-181a-5phsa-mir-181a-2	-0.98	0.51	3.70E-03	1.00E-01	2409	4760
hsa-miR-181a-5phsa-mir-181a-1	-0.98	0.51	3.70E-03	1.00E-01	14	26
hsa-miR-222-3phsa-mir-222	-1.06	0.48	4.12E-03	1.04E-01	245	513
hsa-miR-16-5phsa-mir-16-1	0.85	1.81	5.46E-03	1.10E-01	26873	14867
hsa-miR-150-3phsa-mir-150	-2.06	0.24	5.47E-03	1.10E-01	26	108
hsa-miR-16-5phsa-mir-16-2	0.85	1.81	5.50E-03	1.10E-01	551	353
hsa-miR-26a-5phsa-mir-26a-1	-1.01	0.49	6.34E-03	1.10E-01	2494	5039
hsa-miR-26a-5phsa-mir-26a-2	-1.01	0.50	6.43E-03	1.10E-01	2509	5066
hsa-miR-574-5phsa-mir-574	1.84	3.58	6.46E-03	1.10E-01	8	2
hsa-miR-10b-5phsa-mir-10b	-1.41	0.38	6.60E-03	1.10E-01	12951	34304
hsa-let-7c-5phsa-let-7c	1.27	2.41	6.88E-03	1.10E-01	94	39
hsa-miR-744-5phsa-mir-744	-0.85	0.56	7.31E-03	1.12E-01	130	233
hsa-miR-409-3phsa-mir-409	-1.30	0.41	8.52E-03	1.21E-01	49	120
hsa-miR-19a-3phsa-mir-19a	1.06	2.09	9.14E-03	1.21E-01	203	97
hsa-miR-184hsa-mir-184	-1.56	0.34	9.47E-03	1.21E-01	15	46
hsa-miR-182-5phsa-mir-182	-1.26	0.42	9.53E-03	1.21E-01	1443	3453
hsa-miR-29a-3phsa-mir-29a	1.14	2.21	9.61E-03	1.21E-01	491	222
hsa-miR-378a-3phsa-mir-378a	1.19	2.27	1.01E-02	1.23E-01	2769	1217
hsa-miR-181b-5phsa-mir-181b-2	-0.75	0.60	1.09E-02	1.28E-01	141	237
hsa-miR-181b-5phsa-mir-181b-1	-0.75	0.60	1.13E-02	1.28E-01	140	234
hsa-let-7a-3phsa-let-7a-3	1.10	2.14	1.19E-02	1.31E-01	21	10
hsa-miR-330-3phsa-mir-330	-1.05	0.48	1.24E-02	1.33E-01	6	13
hsa-let-7a-3phsa-let-7a-1	1.08	2.12	1.31E-02	1.36E-01	21	10
hsa-miR-221-5phsa-mir-221	-1.72	0.30	1.37E-02	1.38E-01	3	11
hsa-miR-301a-3phsa-mir-301a	-0.87	0.55	1.46E-02	1.42E-01	77	141
hsa-miR-32-5phsa-mir-32	0.95	1.93	1.50E-02	1.42E-01	724	1556

hsa-miR-378ihsa-mir-378i	1.22	2.33	1.53E-02	1.42E-01	39	16
hsa-miR-671-3phsa-mir-671	-1.07	0.48	1.68E-02	1.52E-01	10	20
hsa-miR-185-5phsa-mir-185	1.37	2.58	1.98E-02	1.61E-01	8	3
hsa-miR-589-5phsa-mir-589	-1.25	0.42	2.06E-02	1.61E-01	50	118
hsa-miR-584-5phsa-mir-584	-0.75	0.59	2.10E-02	1.61E-01	143	241
hsa-miR-125a-5phsa-mir-125a	-0.80	0.57	2.14E-02	1.61E-01	110	192
hsa-miR-374a-3phsa-mir-374a	-1.16	0.45	2.19E-02	1.61E-01	10	23
hsa-miR-423-3phsa-mir-423	-0.72	0.61	2.24E-02	1.61E-01	880	1449
hsa-miR-142-5phsa-mir-142	0.75	1.68	2.25E-02	1.61E-01	76	130
hsa-miR-451ahsa-mir-451a	0.79	1.72	2.25E-02	1.61E-01	17275	10018
hsa-miR-335-3phsa-mir-335	-0.99	0.51	2.30E-02	1.61E-01	9	19
hsa-miR-199a-3phsa-mir-199a-1	0.71	1.64	2.46E-02	1.61E-01	13	24
hsa-miR-199b-3phsa-mir-199a-1	0.71	1.64	2.46E-02	1.61E-01	529	328
hsa-miR-130a-3phsa-mir-130a	0.72	1.65	2.46E-02	1.61E-01	609	369
hsa-miR-199a-3phsa-mir-199b	0.71	1.64	2.47E-02	1.61E-01	527	321
hsa-miR-199b-3phsa-mir-199b	0.71	1.64	2.49E-02	1.61E-01	527	321
hsa-miR-19b-3phsa-mir-19b-1	0.95	1.93	2.49E-02	1.61E-01	592	306
hsa-miR-19b-3phsa-mir-19b-2	0.95	1.93	2.51E-02	1.61E-01	593	307
hsa-miR-4732-5phsa-mir-4732	1.06	2.09	2.61E-02	1.64E-01	29	13
hsa-miR-342-3phsa-mir-342	0.93	1.90	2.65E-02	1.64E-01	162	85
hsa-miR-199b-3phsa-mir-199a-2	0.69	1.61	2.95E-02	1.78E-01	527	321
hsa-miR-199a-3phsa-mir-199a-2	0.69	1.61	2.97E-02	1.78E-01	527	321
hsa-miR-208b-3phsa-mir-208b	1.95	3.85	3.05E-02	1.79E-01	17	4
hsa-miR-181a-3phsa-mir-181a-1	-0.82	0.57	3.20E-02	1.79E-01	2409	4760
hsa-miR-181d-5phsa-mir-181d	-1.01	0.50	3.25E-02	1.79E-01	3	7
hsa-miR-142-3phsa-mir-142	-0.77	0.59	3.30E-02	1.79E-01	5262	3128
hsa-miR-151a-3phsa-mir-151a	-0.74	0.60	3.35E-02	1.79E-01	189	410
hsa-miR-548ar-5phsa-mir-548ar	0.85	1.81	3.37E-02	1.79E-01	22	12
hsa-miR-101-3phsa-mir-101-1	0.67	1.60	3.41E-02	1.79E-01	2465	1544
hsa-miR-101-3phsa-mir-101-2	0.67	1.59	3.51E-02	1.79E-01	2530	1589
hsa-miR-320bhsa-mir-320b-1	-1.11	0.46	3.56E-02	1.79E-01	86	44
hsa-miR-532-5phsa-mir-532	0.62	1.54	3.57E-02	1.79E-01	221	143
hsa-miR-320bhsa-mir-320b-2	-1.10	0.47	3.59E-02	1.79E-01	721	1552
hsa-miR-199a-5phsa-mir-199a-1	-0.83	0.56	3.63E-02	1.79E-01	13	24
hsa-miR-199a-5phsa-mir-199a-2	-0.83	0.56	3.65E-02	1.79E-01	529	328
hsa-miR-30b-5phsa-mir-30b	-0.89	0.54	3.77E-02	1.80E-01	103	191
hsa-miR-210-3phsa-mir-210	0.92	1.89	3.80E-02	1.80E-01	112	59
hsa-miR-411-5phsa-mir-411	-1.07	0.47	3.83E-02	1.80E-01	10	22
hsa-miR-15a-5phsa-mir-15a	0.79	1.73	3.91E-02	1.82E-01	2629	1518
hsa-miR-885-5phsa-mir-885	1.73	3.33	4.55E-02	2.08E-01	53	16

hsa-miR-16-2-3phsa-mir-16-2	0.64	1.56	4.68E-02	2.12E-01	26871	14863
hsa-miR-3605-5phsa-mir-3605	1.03	2.04	4.90E-02	2.19E-01	8	4

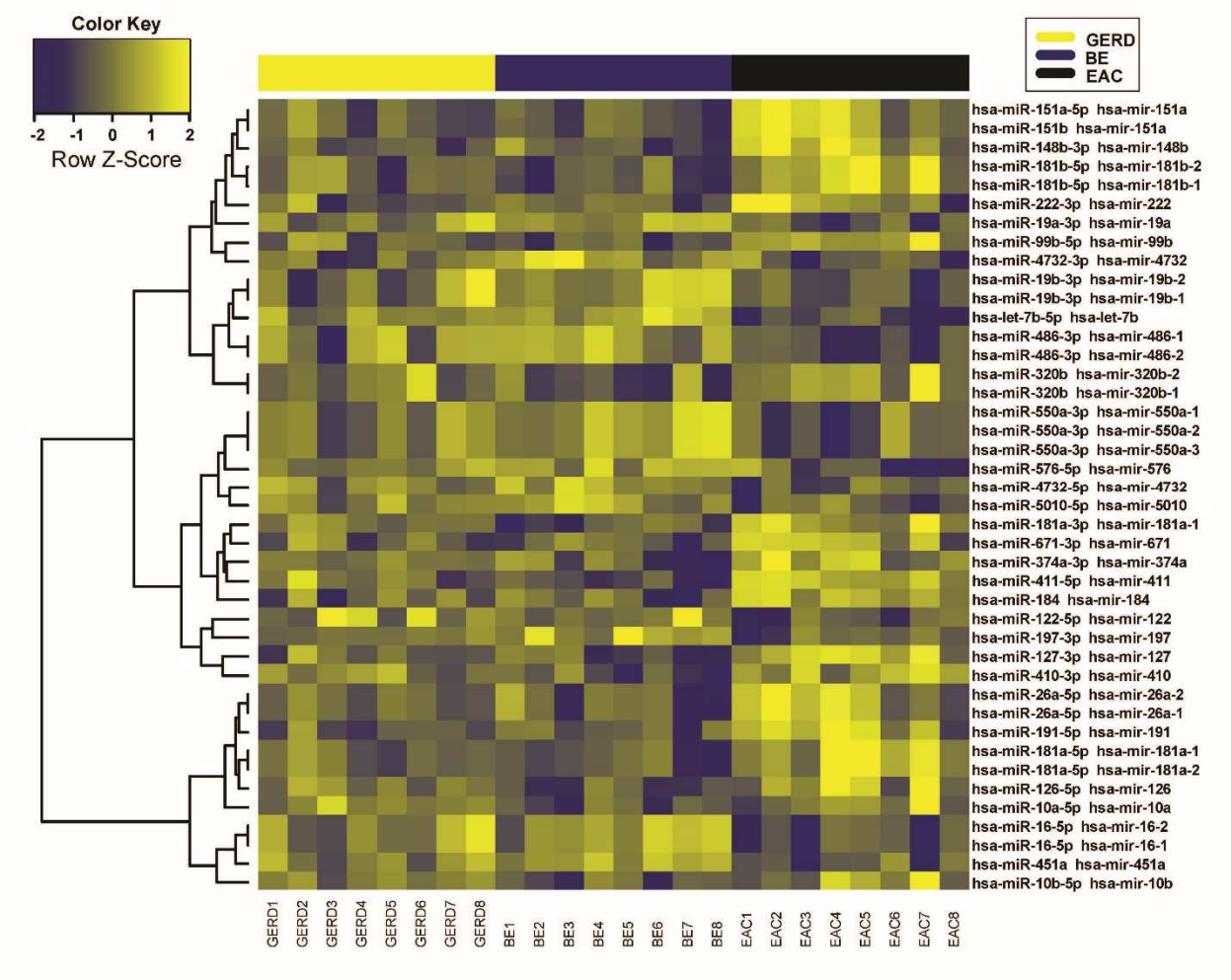
Appendix C : BE vs EAC-Significant miRNAs by ascending p-value

Gene ID	logFC	Fold Change	PValue	FDR	Average Read Count for BE	Average Read Count for EAC
hsa-let-7b-5phsa-let-7b	1.79	3.46	7.97E-07	2.06E-04	773	223
hsa-miR-411-5phsa-mir-411	-2.81	0.14	1.17E-06	2.06E-04	3	22
hsa-miR-127-3phsa-mir-127	-2.04	0.24	2.24E-05	2.34E-03	31	128
hsa-miR-197-3phsa-mir-197	2.23	4.69	2.65E-05	2.34E-03	124	26
hsa-miR-99b-5phsa-mir-99b	-1.70	0.31	5.63E-05	3.09E-03	63	204
hsa-miR-151bhsa-mir-151a	-1.30	0.41	5.96E-05	3.09E-03	166	407
hsa-miR-10b-5phsa-mir-10b	-2.11	0.23	6.82E-05	3.09E-03	7972	34304
hsa-miR-148b-3phsa-mir-148b	-1.34	0.40	7.79E-05	3.09E-03	130	326
hsa-miR-151a-5phsa-mir-151a	-1.28	0.41	7.87E-05	3.09E-03	169	410
hsa-miR-181a-5phsa-mir-181a-2	-1.30	0.41	1.41E-04	4.52E-03	1936	4760
hsa-miR-181a-5phsa-mir-181a-1	-1.30	0.41	1.41E-04	4.52E-03	1936	4760
hsa-miR-181b-5phsa-mir-181b-1	-1.12	0.46	1.78E-04	5.25E-03	108	234
hsa-miR-486-3phsa-mir-486-2	1.43	2.69	2.23E-04	5.83E-03	807	300
hsa-miR-181b-5phsa-mir-181b-2	-1.09	0.47	2.35E-04	5.83E-03	111	237
hsa-miR-486-3phsa-mir-486-1	1.42	2.67	2.48E-04	5.83E-03	819	307
hsa-miR-181a-3phsa-mir-181a-1	-1.42	0.37	3.26E-04	7.20E-03	9	26
hsa-miR-10a-5phsa-mir-10a	-1.84	0.28	4.33E-04	8.99E-03	2879	10305
hsa-miR-410-3phsa-mir-410	-1.50	0.35	5.41E-04	1.06E-02	15	41
hsa-miR-16-5phsa-mir-16-1	1.01	2.02	1.01E-03	1.79E-02	30015	14863
hsa-miR-16-5phsa-mir-16-2	1.01	2.02	1.02E-03	1.79E-02	30019	14867
hsa-miR-451ahsa-mir-451a	1.13	2.19	1.14E-03	1.84E-02	21898	10018
hsa-miR-191-5phsa-mir-191	-1.16	0.45	1.14E-03	1.84E-02	2279	5077
hsa-miR-4732-5phsa-mir-4732	1.56	2.95	1.32E-03	2.03E-02	41	13
hsa-miR-19a-3phsa-mir-19a	1.31	2.48	1.46E-03	2.15E-02	240	97
hsa-miR-320bhsa-mir-320b-1	-1.65	0.32	2.03E-03	2.87E-02	496	1552
hsa-miR-320bhsa-mir-320b-2	-1.63	0.32	2.20E-03	2.99E-02	502	1556
hsa-miR-576-5phsa-mir-576	1.18	2.26	2.52E-03	3.29E-02	31	15
hsa-miR-126-5phsa-mir-126	-0.98	0.51	2.70E-03	3.40E-02	2184	4310
hsa-miR-184hsa-mir-184	-1.83	0.28	2.86E-03	3.48E-02	13	46
hsa-miR-122-5phsa-mir-122	2.28	4.86	3.29E-03	3.62E-02	131	27
hsa-miR-5010-5phsa-mir-5010	1.36	2.56	3.31E-03	3.62E-02	25	10
hsa-miR-550a-3phsa-mir-550a-2	1.32	2.49	3.35E-03	3.62E-02	21	9
hsa-miR-550a-3phsa-mir-550a-1	1.32	2.49	3.38E-03	3.62E-02	21	9
hsa-miR-550a-3phsa-mir-550a-3	1.32	2.49	3.50E-03	3.64E-02	21	9
hsa-miR-374a-3phsa-mir-374a	-1.53	0.35	3.88E-03	3.76E-02	8	23

hsa-miR-19b-3phsa-mir-19b-1	1.23	2.34	3.95E-03	3.76E-02	717	306
hsa-miR-19b-3phsa-mir-19b-2	1.23	2.34	3.96E-03	3.76E-02	719	307
hsa-miR-4732-3phsa-mir-4732	1.00	2.00	4.05E-03	3.76E-02	133	67
hsa-miR-222-3phsa-mir-222	-1.05	0.48	4.60E-03	4.07E-02	247	513
hsa-miR-671-3phsa-mir-671	-1.31	0.40	4.61E-03	4.07E-02	8	20
hsa-miR-26a-5phsa-mir-26a-1	-1.04	0.48	5.01E-03	4.31E-02	2444	5039
hsa-miR-26a-5phsa-mir-26a-2	-1.04	0.49	5.19E-03	4.36E-02	2463	5066
hsa-miR-629-5phsa-mir-629	-1.63	0.32	6.14E-03	5.04E-02	4	13
hsa-miR-150-3phsa-mir-150	-2.03	0.25	6.40E-03	5.14E-02	26	108
hsa-miR-133a-3phsa-mir-133a-1	-1.45	0.37	8.56E-03	6.60E-02	25	69
hsa-miR-133a-3phsa-mir-133a-2	-1.45	0.37	8.61E-03	6.60E-02	25	69
hsa-miR-30a-3phsa-mir-30a	-1.31	0.40	9.85E-03	7.40E-02	12	31
hsa-miR-151a-3phsa-mir-151a	-0.88	0.54	1.12E-02	8.22E-02	613	1132
hsa-miR-16-2-3phsa-mir-16-2	0.82	1.76	1.18E-02	8.46E-02	621	353
hsa-miR-223-3phsa-mir-223	1.17	2.25	1.21E-02	8.46E-02	328	146
hsa-miR-409-3phsa-mir-409	-1.24	0.42	1.22E-02	8.46E-02	51	120
hsa-miR-150-5phsa-mir-150	1.35	2.55	1.35E-02	9.19E-02	2081	818
hsa-miR-342-3phsa-mir-342	1.03	2.04	1.42E-02	9.44E-02	174	85
hsa-miR-589-5phsa-mir-589	-1.32	0.40	1.50E-02	9.78E-02	47	118
hsa-miR-194-5phsa-mir-194-1	-1.27	0.41	1.80E-02	1.16E-01	44	106
hsa-miR-194-5phsa-mir-194-2	-1.25	0.42	1.93E-02	1.20E-01	48	113
hsa-miR-106b-5phsa-mir-106b	0.88	1.84	1.97E-02	1.20E-01	217	118
hsa-miR-21-3phsa-mir-21	-1.28	0.41	1.98E-02	1.20E-01	131	318
hsa-miR-320chsa-mir-320c-1	-1.36	0.39	2.11E-02	1.26E-01	14	36
hsa-miR-320chsa-mir-320c-2	-1.54	0.34	2.29E-02	1.34E-01	7	19
hsa-miR-32-5phsa-mir-32	0.89	1.85	2.31E-02	1.34E-01	82	44
hsa-miR-423-3phsa-mir-423	-0.71	0.61	2.35E-02	1.34E-01	884	1449
hsa-miR-15a-5phsa-mir-15a	0.86	1.82	2.46E-02	1.38E-01	2764	1518
hsa-miR-769-5phsa-mir-769	0.93	1.90	2.50E-02	1.38E-01	63	34
hsa-miR-1180-3phsa-mir-1180	1.02	2.02	2.75E-02	1.49E-01	7	4
hsa-miR-93-3phsa-mir-93	0.87	1.82	3.14E-02	1.68E-01	15	8
hsa-miR-3173-5phsa-mir-3173	1.12	2.17	3.41E-02	1.78E-01	8	3
hsa-miR-361-3phsa-mir-361	0.77	1.70	3.43E-02	1.78E-01	50	29
hsa-miR-143-3phsa-mir-143	1.01	2.02	3.63E-02	1.83E-01	1955	970
hsa-miR-24-3phsa-mir-24-2	-1.01	0.50	3.71E-02	1.83E-01	62	124
hsa-miR-24-3phsa-mir-24-1	-1.00	0.50	3.74E-02	1.83E-01	62	124
hsa-miR-425-5phsa-mir-425	0.61	1.53	3.82E-02	1.83E-01	1042	680
hsa-miR-10a-3phsa-mir-10a	-1.26	0.42	3.82E-02	1.83E-01	3	7
hsa-miR-20a-5phsa-mir-20a	0.67	1.60	3.84E-02	1.83E-01	149	94
hsa-miR-142-5phsa-mir-142	0.68	1.60	3.95E-02	1.83E-01	4999	3128

hsa-miR-330-3phsa-mir-330	-0.88	0.54	3.99E-02	1.83E-01	7	13
hsa-miR-501-3phsa-mir-501	-0.95	0.52	4.00E-02	1.83E-01	23	44
hsa-miR-34a-5phsa-mir-34a	1.40	2.65	4.04E-02	1.83E-01	13	5
hsa-miR-92a-3phsa-mir-92a-2	0.67	1.59	4.12E-02	1.84E-01	92518	58200
hsa-miR-185-5phsa-mir-185	1.20	2.30	4.27E-02	1.88E-01	6	3
hsa-miR-30b-5phsa-mir-30b	-0.87	0.55	4.35E-02	1.89E-01	105	191
hsa-miR-144-5phsa-mir-144	-0.94	0.52	4.45E-02	1.91E-01	915	1752
hsa-miR-381-3phsa-mir-381	-0.99	0.50	4.53E-02	1.93E-01	11	21
hsa-miR-942-5phsa-mir-942	0.67	1.60	4.76E-02	1.99E-01	27	16
hsa-miR-92a-3phsa-mir-92a-1	0.63	1.54	4.78E-02	1.99E-01	95502	61833

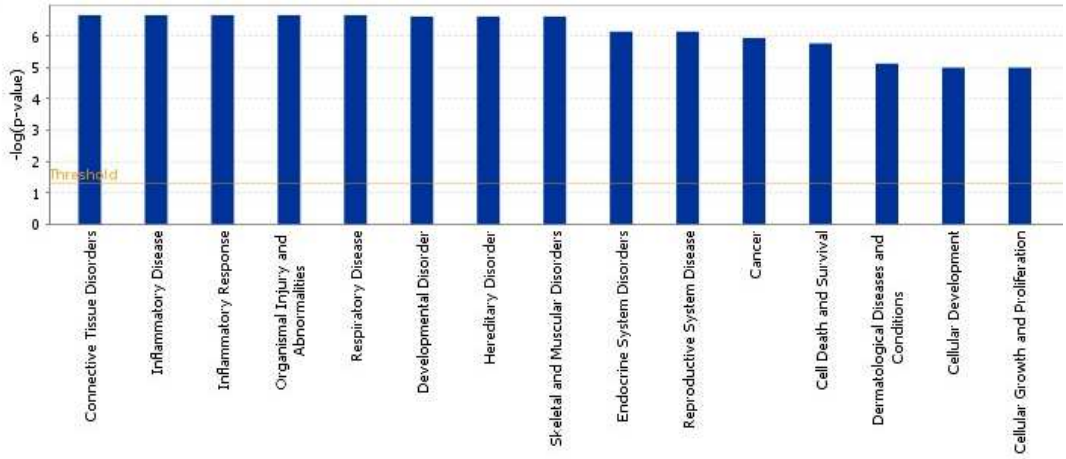
Appendix D: EdgeR Unsupervised clustering and heat map.



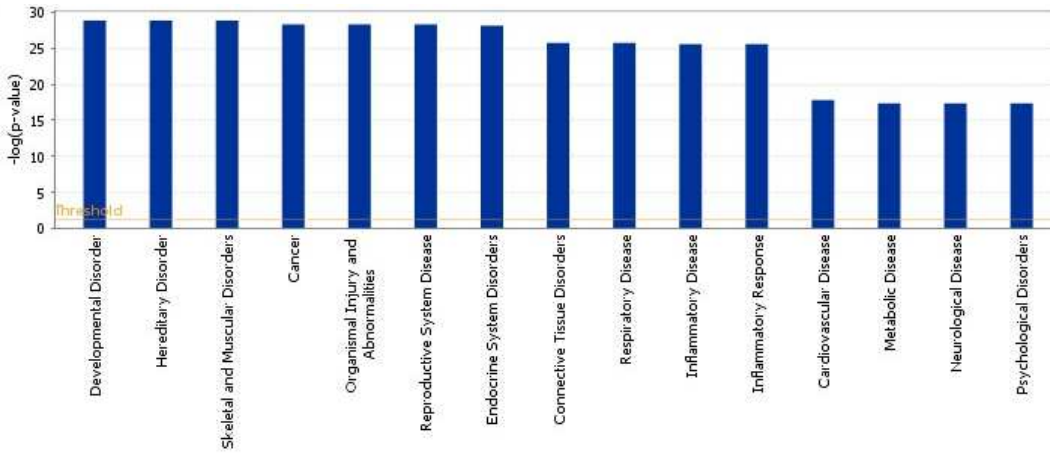
Columns represent patient samples and rows represent the significant genes from EdgeR analysis (FDR<0.05). Scale bar indicates expression signal where yellow denotes genes with relative increased expression while blue denotes genes with relative decreased expression.

Appendix: E: **Top 15 disease and functions from IPA on each pairwise comparison**

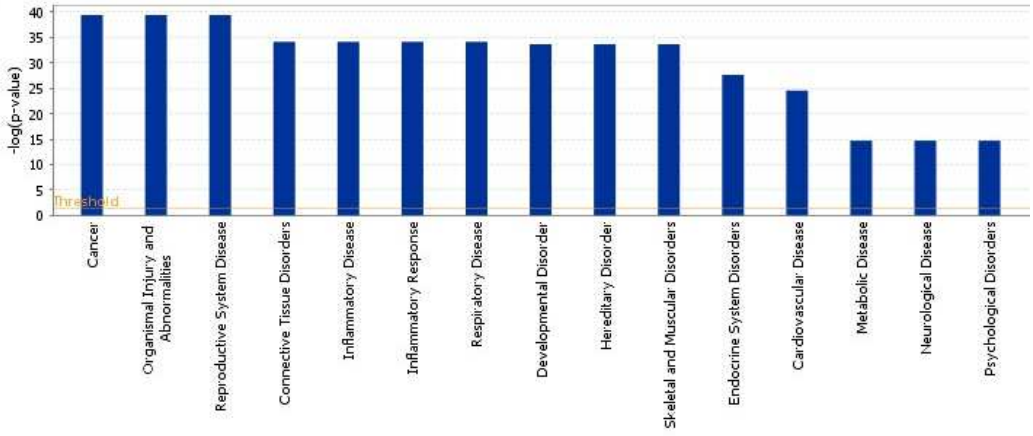
A.



B.



C.



(A) GERD vs BE (B) GERD vs EAC (C) BE vs EAC