Bachelor Degree Project

UNIVERSITY OF SKÖVDE

DETECTION OF EXOSOMAL MIRNA FROM DIFFERENT VOLUMES OF BIOFLUIDS AS BIOMARKERS FOR THE DIAGNOSIS OF SEPSIS

FUTURE DIAGNOSTICS OF SEPSIS

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ABSTRACT

Sepsis, a life-threatening condition which results from a dysregulation of host response to infection and leads to multiple organ dysfunction, is a cause for great concern. The current gold standard of detection – Blood culturing – is a highly time-consuming process and so, research has proposed the use of biomarkers. Current biomarkers, C-reactive protein and Procalcitonin, though good indicators, individually show certain limitations with respect to the specificity and sensitivity. Hence, as a step forward from singleplex biomarkers, the development of a multi-marker panel was suggested. For this purpose, the use of microRNAs (miRNAs) were employed to serve as potential biomarkers for the detection of sepsis. The aim of this study was to determine whether a higher concentration of miRNA would be obtained from a larger volume of plasma as well as to see if the miRNA present in blood can be used for the diagnosis of sepsis. Extractions were carried out using the QIAGEN exoRNeasy Plasma: Midi & Maxi Kits from plasma and Norgen's Total RNA Purification Kit from blood. The samples were analysed and quantified using the Qubit® microRNA assay kit & Qubit® 3.0 Fluorometer and the NanoDrop™ 2000 Spectrophotometer. Statistical analysis of the results revealed that there was a significant difference between miRNA concentrations in the two volumes of plasma analysed. Based on the accurate Qubit measurements and readings, it was concluded that a larger volume of plasma, does yield a higher concentration of miRNA. In addition, it was also established that the miRNA detected in blood, could be used as probable biomarkers for the diagnosis of sepsis.

LIST OF ABBREVIATIONS

miRNA	Micro Ribonucleic acid
CRP	C – Reactive Protein
PCT	Procalcitonin
IL-6	Interleukin – 6
RPM	Revolutions Per Minute
NBP	New Blood Plasma
NB	New Blood
RT	Room Temperature
OOR	Out Of Range
df	Degrees of Freedom
MM	Master Mix
qRT-PCR	Quantitative Reverse transcription polymerase chain reaction

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INTRODUCTION

Sepsis is a life-threatening organ dysfunction, caused by a dysregulated host response to infection (Singer et al., 2016). It is an imprecise clinical syndrome, with a variety of clinical presentations (Angus & van der Poll., 2013). Diagnosis is typically based on suspicion of infection, combined with primary signs of organ dysfunction (Cohen et al., 2015). Early diagnosis of sepsis and administration of antibiotics is vital as progression to severe sepsis or septic shock has serious consequences (Angus & van der Poll., 2013). Unfortunately, differentiating between sepsis and other inflammatory conditions is usually challenging in seriously ill patients. An international study conducted included 10,069 patients from Europe (54.1%), Asia (19.2%), America (17.1%), and other continents (9.6%) to check for the incidence of sepsis (Sakr et al., 2018). Sepsis-3, defined as a medical condition associated with organ failure, was identified during the intensive care units (ICU) stay study in 2973 (29.5%) patients, including which 1808 (18.0%) were already at ICU admission (Wittebole et al., 2018). Occurrence rates of sepsis varied from 13.6% to 39.3% in the different regions. Sepsis persists as a major health problem in ICU patients worldwide and is associated with high mortality rates. However, there is wide variability in the sepsis rate and outcomes in ICU patients around the globe.. Sepsis is a major cause of morbidity and mortality in modern ICUs (Sakr et al., 2018). Due to its high mortality, its alarmingly high rate of organ dysfunction, and its high costs to hospitals, the importance of early detection of sepsis is crucial (Dolin et al., 2018). The current “gold” standard of testing for detection of sepsis is blood culturing (Benz, Roy, Trautwein,Roderburg & Luedde, 2016). Although effective and most frequently used there are certain drawbacks to this technique. There is low sensitivity and specificity, analysis is tedious, this is a long procedure as it involves multiple aspects and is highly time consuming (Sinha et al., 2018). A standard blood culture takes about 72 hours to produce a definite result (Nunes, Marra, Edmont,da Silva Victor & Pereira,, 2013). Detecting bacterium in the blood is a key step in the diagnosis of sepsis, a prevailing infection, and initiating treatment with antimicrobials (Cohen et al., 2015). However, blood cultures are negative in 60 to 70% of patients with severe sepsis (Cohen et al., 2015), and > 80% were negative in the study by Cartwright et al. (2016). Furthermore, microbiology takes too long a time to influence first line therapy against pathogenic bacteria. This is far too prolonged as the time between when sepsis progresses to septic shock is very short. Septic shock causes death. Therefore, fast, early and precise detection of sepsis by alternate methods, such as biomarkers, may save lives, especially if it can be a

point-of-care test, thereby reducing the cost and time taken from the patient (Pernestig., 2019, personal communication, 1 February).

Biomarkers are a "characteristic" that can be objectively measured and evaluated as an indicator of normal biological processes, pathological processes, or pharmacological responses to a therapeutic intervention (Puntmann., 2009; Biomarkers Definitions Working Group., 2001). Even though the diagnostic criteria of sepsis have changed, the symptoms of sepsis have not, thus highlighting the validity for improved and more accurate diagnostic tools with biomarkers. Current biomarkers, including C-reactive protein (CRP), Procalcitonin (PCT) and Interleukkin-6 (IL-6) though good indicators, individually show certain limitations with respect to the specificity and sensitivity. This concluded that singleplex markers were not enough to make a concrete diagnosis (Benz et al., 2016). Recent studies have suggested the use of new biomarkers as well as the combination of new and pre-existing biomarkers (multiplex, 5-15 markers) for rapid sepsis diagnosis. This includes the analysis of exosomal circulating microRNAs (miRNAs), by the development of a multi-marker panel.

Exosomes are nano-vesicles present in the circulation that are involved in cell-to-cell communication and regulation of different biological processes. miRNAs are part of their cargo and are potential biomarkers (Sanz-Rubio et al., 2018). The exosomal miRNA may be present in vesicles, which makes them less susceptible to damage and being invaded from the external environment and agents. This is one property that makes miRNA highly stable (Pernestig., 2019, personal communication, 5 February). The properties that these species have in addition to the conventional biomarkers are their higher stability, selectivity, and specificity (Browne et al., 2017).

Here, miRNAs have been hypothesized to be used as a biomarker for detection of sepsis. Previous literature has also proposed biomarkers as being useful in diagnostics for different types of cancer as well as other diseases, including viral infections, nervous system disorders, cardiovascular disorders, and diabetes (Wang et al., 2016).

miRNAs are about 18–24 nucleotides long, non-coding RNA, that play a significant role in controlling human gene expression by post-transcriptional gene regulation or silencing (Yoon et al., 2013; Pernestig et al., 2018). Changes in miRNA expression contribute to a wide variety of human disease states and disorders. The miRNA are not only present in human peripheral blood but also in other body tissues and fluids, like saliva, milk, urine, cerebrospinal fluid and it is these characteristics which further indicate that the miRNAs are potential biomarkers for diagnostic purposes (Wang et al., 2018). It is hypothesized that using high-throughput screening with access to biochemical and

physiological data clinomics and intensive data mining, a multi-marker panel can be established for easy, quick detection of sepsis (Wang et al., 2012). This arises simply from the fact that single biomarkers, by themselves are not sensitive or specific enough to detect particular miRNA coding for specific diseases. Continuous research and techniques like RNA extraction, qRT-PCR and microarray analysis have been used to study the role and characteristics of miRNA and their potential to act as biomarkers for various diseases (Zhang et al., 2018). Beyond sepsis, overprescribing antibiotics and the rise of antimicrobial resistance is of considerable concern. The O'Neill report, which provides recommendations for tackling drug-resistant infections globally, advises developing diagnostics for bacterial infections to guide the use of antimicrobials (O'Neill., 2016).

RNA isolation is an essential step in order to obtain miRNA for analysis (Moret et al., 2013). This is due to the fact that the miRNA reside in the exosomes. These exosomes have a sturdy, stable outer layer, hence making it difficult for the miRNA to be released and thus detected by the assays. Once the exosomes are broken down by the extraction process, the miRNA are released and become more accessible and easily detectable by the procedures and techniques used. The QIAGEN exoRNeasy Serum/Plasma Kits are designed for rapid purification of non-coding exosomal miRNA from plasma — Up to 1 ml with the Midi Kit & up to 4 ml with the Maxi Kit. They use a membrane-based affinity binding step to isolate exosomes and other extracellular vesicles (EVs) from plasma. Norgen's Total RNA Purification Kit provides a rapid method for the isolation and purification of total RNA from blood and various other biofluids and substances.

The aim of this project was to measure and analyse the concentration of miRNA and total RNA in different volumes of plasma and whole blood and from this, determine the minimum quantity containing the highest concentration of miRNA for the eventual, timely detection of sepsis with minimum invasiveness. In other words, it was hypothesised that a larger volume of plasma, would give a higher concentration of miRNA. A secondary aim was to detect and quantify circulating miRNA and total RNA in healthy blood to determine if the miRNA in blood instead of plasma can be used to detect sepsis.

MATERIALS AND METHODS

Plasma

miRNA isolation

miRNA isolation was carried out using both the QIAGEN exoRNeasy Plasma Kits: Midi and Maxi, following the manufacturer protocol included in the kit. The entire process has been summarised in Figure 1. The plasma samples, provided from the Skaraborg sepsis study biobank, were removed from the -80 °C freezer and kept to thaw on ice for 15 minutes. The samples were then moved to room temperature for 15 minutes. The Midi Kit allowed an extraction of 500 µl of plasma, while the Maxi Kit allowed 2000 µl of plasma, for miRNA isolation. This kit was selected since it has been tested and proved to be one of the best available methods to attain an enriched miRNA fraction from plasma samples (Berchem, Bernardin, El-Khoury, Kaoma, & Pieron, 2016). The kit uses a membrane-based affinity binding step along with a phenol/guanidine-based combined lysis and elution step. This is followed by silica-membrane– based purification of total RNA. QIAzol Lysis Reagent, included in the kit, is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis. After the lysis and elution step and addition of chloroform, the lysate was separated into aqueous and organic phases by centrifugation. The aqueous layer was removed and further treated with buffers and centrifuged. Next, 22 µl of RNase-free water was then added to elute the required nucleic acid. Final eluted volumes from each aliquot was 20 µL, of which 10 µL was stored at -20 °C for analysis and the remaining 10 µL was stored at -80 °C for future use.

Quantification of miRNA.

Qubit® microRNA assay kit

The Qubit® microRNA Assay Kit for use with the Qubit® 3.0 Fluorometer allows easy and accurate quantification of small RNA, including miRNA. This occurs even in the presence of common contaminants such as salts, free nucleotides, solvents, detergents, and protein. The assay detects all types of small RNA, including microRNA and siRNA, both single stranded and double stranded (Life technologies., 2015). The miRNA samples stored at -20 °C were taken out and placed on ice for 15 minutes to thaw. The samples were pipetted up and down repeatedly to mix them well to prevent any sedimentation of the sample. Next, 1 µl of the sample was added to 199 µl of the Master Mix in the respective Qubit assay tubes to make up a total volume of 200 µl. The mix was vortexed and incubated for 2 minutes in the dark by covering the tubes with aluminium foil. The standards were

measured to calibrate the device. The samples were measured with the Qubit® 3.0 Fluorometer's miRNA detection program. Final concentration values expressed in ng/µL were obtained, given by the fluorometer.

NanoDrop™ 2000 Spectrophotometer

Thermo Scientific™ NanoDrop 2000 is a full-spectrum, absorbance-based spectrophotometer used to quantify DNA and RNA molecules. It is based on 260 nm-absorbance with a range of RNA detection from 2 ng/µL to 15 µg/µL. It was used to quantify 1 µL of the plasma samples. The samples stored at -20 degrees were taken out and placed on ice for 15 minutes to thaw. The samples were pipetted up and down repeatedly to mix them well to prevent any sedimentation of the sample. 1 µl of the sample was taken from the sample tubes and placed on the NanoDrop™ 2000 spectrophotometer. The program was run and the sample was analysed for its purity.

Blood

miRNA isolation

Norgen's Purification Technology: Purification is based on spin column chromatography using Norgen's proprietary resin as the separation matrix. The entire process has been summarised in Figure 1. The blood samples were removed from the -80 °C freezer and kept to thaw on ice for 15 minutes. They were then left at room temperature for 15 minutes. The RNA is preferentially purified from other cellular components such as proteins without the use of phenol or chloroform. The process involves first lysing the cells or tissue of interest with the provided Buffer RL. Ethanol is then added to the lysate, and the solution is loaded onto a spin-column. Norgen's resin binds RNA in a manner that depends on ionic concentrations. Thus only the RNA will bind to the column, while the contaminants will be removed in the flow-through or retained on the top of the resin. The bound RNA is then washed with the provided Wash Solution A in order to remove any remaining impurities, and the purified total RNA is eluted with the Elution Solution A. The purified RNA is of the highest integrity, and can be used in a number of downstream applications (Norgen Biotek Corp., 2018).

miRNA isolation was carried out using Norgen's Total RNA Purification Kit, following the manufacturer protocol included in the kit: Lysate preparation, Binding RNA to Column, On-Column

DNA Removal, Column Wash, RNA Elution with the help of buffers and centrifugation steps as per the protocol along with an additional variant. Since frozen blood samples were used instead of fresh blood, a test was carried out to check if this affected the result and quantity as well as quality of miRNA extracted. After thawing the blood at RT post removal from the -80 °C freezer, half of the blood was centrifuged at 2000 rpm for 2 minutes before processing and the other half was treated directly without centrifugation. This was done to check if there was any difference in the concentration of miRNA obtained. Since miRNA may be used to diagnose sepsis, but is present in such small amounts in plasma, blood and other biological fluids, it is important to obtain miRNA as pure as possible. For RNA elution, 50 µl of elution solution A was added to the column and spun. Two elutions were done for each sample: Elution 1 and Elution 2. Final eluted volumes from each aliquot was 50 µL, of which 20 µL was stored at -20 °C for analysis and the remaining 30 µL was stored at -80 °C for future use.

Quantification of miRNA.

Qubit® microRNA assay kit

The Qubit® microRNA Assay Kit (Thermo Fisher Scientific®) was used along with the Qubit® 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific®) for the quantification of miRNA. The miRNA samples stored at -20 °C were taken out and placed on ice for 15 minutes to thaw. The samples were pipetted up and down repeatedly to mix them well to prevent sedimentation of the sample. Master Mix (MM) was prepared for all the samples. A volume of 199 µl of MM was taken into separate tubes and 1 µl of the sample was taken from the sample tubes and added to the respective tubes containing MM. The standards were measured to calibrate the device. The samples were then measured to determine the concentration of miRNA.

NanoDrop™ 2000 Spectrophotometer

The miRNA samples stored at -20 °C were taken out and placed on ice for 15 minutes to thaw. The samples were pipetted up and down repeatedly to mix them well to prevent sedimentation of the sample. A small volume of 1 µl of the sample was taken from the sample tubes and placed on the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific®). The program was run and the sample was analysed for its concentration and purity.

During extraction and analysis, each of the tubes were given a unique identity to identify them easily and to understand how the study progressed. The following are the tube labels: For plasma extractions using the QIAGEN exoRNeasy Serum/Plasma Midi Kit, the tubes were labelled NBP2019 S1 1-8 which stands for New Blood Plasma 2019 Sample 1 Extraction 1-8. Similarly, for plasma extractions carried out using the QIAGEN exoRNeasy Serum/Plasma Maxi Kit, the tubes were labelled NBP2019 Maxi 1-11 which stands for New Blood Plasma 2019 Maxi Extraction 1-11.

For the extractions carried out on blood, the tubes were labelled as NB2019 1 – 10 E1 meaning New Blood 2019 Extraction 1 – 10, Elution 1. Similarly, the tubes labelled NB2019 1 – 10 E2, corresponded to New Blood 2019 Extraction 1 – 10, Elution 2, the second elution carried out.

Statistical Analysis

Statistical analysis was carried out on the data obtained from the Qubit® microRNA Assay Kit and NanoDrop™ 2000 spectrophotometer using the statistical analysis software, IBM SPSS Statistics. Tests for normality were carried out to check if the data was normally distributed using the Shapiro-Wilks test and by performing a Q-Q Plot. The p value threshold was set at 0.05. Next, depending on whether the data was normally distributed or not, either a parametric statistical test or a nonparametric statistical test respectively was done to compare the results. Since the normality tests carried out showed the data to be normally distributed, an independent t-test was conducted.

Pros and cons of method

The advantages the QIAGEN Kit has brought to the extraction process is that it is found to recover a higher amount and better isolate miRNA (El-Khoury et al., 2016). In addition, different volumes can be tested. At the same time, there are some drawbacks to the technique. The kit involves the use of chloroform and phenol, which are both, a health as well as an environmental hazard. An important kit component, trizol also poses an environmental hazard, causing damage to sea life. A step further, an improvement, could be to use the new phenol free QIAGEN Kit. The kit is said to produce higher yields of miRNA without the need of phenol.

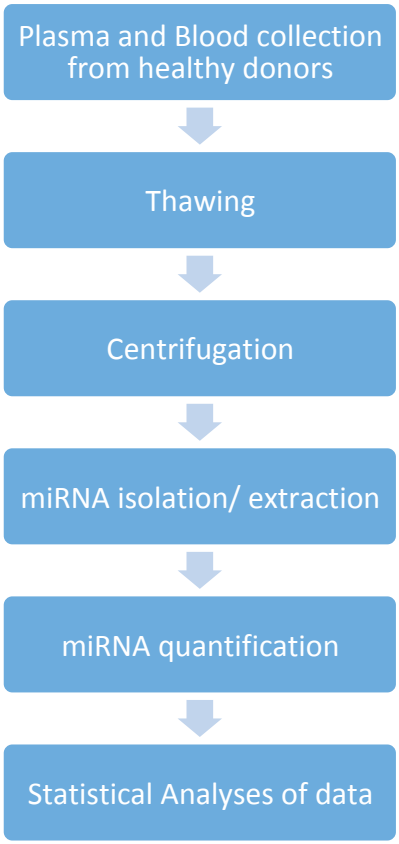

Figure 1. Workflow of the entire extraction procedure

RESULTS

PLASMA

Quantification

Comparison of miRNA yield – Quantity.

In order to be able to compare the concentration of miRNA between the two volumes – 500 µl and 2000 µl – the Qubit® microRNA Assay Kit and Qubit® 3.0 Fluorometer was used. The plasma was processed using the exoEasy Midi and Maxi kits and the extracted miRNA was then analysed using the Qubit® microRNA Assay Kit (table 1). Detailed data is shown in the Appendix - table 8 & 9.

Table 1. Mean miRNA concentration in different plasma samples isolated with the exoEasy Midi and Maxi kits analysed using Qubit® microRNA Assay Kit

	Qubit microRNA Assay
	Mean miRNA concentration ng/µl (±1 SD)
Midi Kit (500 µl) (n=8)	0.53 (± 0.14)
Maxi Kit (2000 µl) (n=11)	3.93 (± 2.18)

Comparison of RNA purity - Quality

In order to be able to compare the concentration and purity of RNA between the two volumes – 500 µl and 2000 µl – the NanoDrop™ 2000 Spectrophotometer was used. The plasma was processed using the exoEasy Midi and Maxi kits and the extracted miRNA was then analysed using the NanoDrop™ 2000 Spectrophotometer (table 2).

Table 2. Mean RNA purity in different plasma samples isolated with the exoEasy Midi and Maxi kits analysed using NanoDrop™ 2000 Spectrophotometer

	Nanodrop	
	Mean miRNA concentration ng/µl (±1 SD)	**260/280**
Midi Kit (500 µl) (n=8)	20.95 (± 10.15)	1.59 (± 0.22)
Maxi Kit (2000 µl) (n=11)	12.6 (± 4.23)	1.55 (± 0.11)

BLOOD

Quantification

Comparison of RNA yield - Quantity.

In order to be able to quantify and determine the concentration of miRNA in 100 µl of blood samples the Qubit® microRNA Assay Kit and Qubit® 3.0 Fluorometer was used (Table 3). The blood was processed using the Norgen's Total RNA Purification Kit and the extracted miRNA was then analysed using the Qubit® microRNA Assay Kit. Detailed data is shown in the Appendix - table 10 & 11.

Table 3. Mean miRNA concentration from different blood samples isolated with the Norgen's Total RNA Purification Kit analysed using Qubit® microRNA Assay Kit

	Qubit microRNA Assay
	Mean miRNA concentration, ng/µl (±1 SD)
Elution 1 (n=10)	9.64 (±3.29)
Elution 2 (n=10)	0.90 (±0.81)

Comparison of RNA purity - Quality

In order to be able to compare the concentration and purity of RNA in 100 µl of blood samples the NanoDrop™ 2000 Spectrophotometer was used. The blood was processed using the Norgen's Total RNA Purification Kit and the extracted miRNA was then analysed using the NanoDrop™ 2000 Spectrophotometer.

Table 4. Mean RNA concentration and purity in different blood samples isolated with the Norgen's Total RNA Purification Kit analysed using NanoDrop™ 2000 Spectrophotometer

	Nanodrop	
	Mean RNA concentration, ng/µl (±1 SD)	**260/280 (±1 SD)**
Elution 1 (n=10)	20.88 (±3.03)	1.89 (±0.07)
Elution 2 (n=10)	5.31 (±0.76)	2.30 (±0.55)

STATISTICAL ANALYSIS

Firstly, the data was analysed to check the normality. In order to determine if the data was normally distributed, two tests were carried out: Shapiro-Wilks test and by performing a Q-Q plot and histogram. The p value threshold was set at 0.05. Next, depending on whether the data was normally distributed or not, either a parametric statistical test or a nonparametric statistical test respectively was done to compare these results. The following results were obtained from these tests.

PLASMA

Qubit Analysis

The Shapiro-Wilk test was used to test the data obtained from the Qubit analysis for normality (Appendix – Table 12). The null hypothesis of these tests states that the data was normally distributed. The p value (Sig.) of this test is greater than 0.05 for both 1 (p=0.070) and 2 (p=0.165). Thus, we do not reject the null hypothesis and conclude that the dependent variable, Concentration of miRNA, is normally distributed for each group (i.e., "Midi - 500 µl" and "Maxi – 2000 µl ").

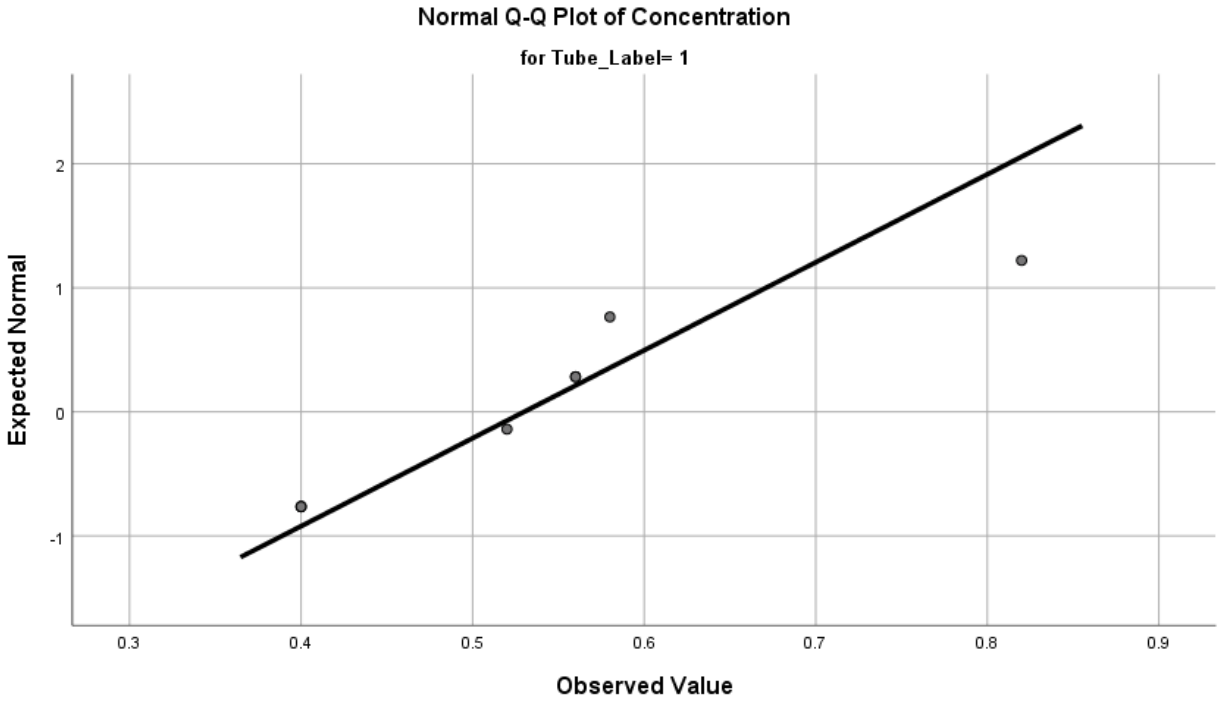

Figure 2: Normal Q-Q Plot of miRNA concentration in 500 µl of plasma samples with a line of best fit depicting the normal distribution of data

The data is said to be normally distributed from the above given p value and the same is seen when applying the Q-Q plot analysis (Figure 2). All the data points lie close to the line of best fit further proving the data is statistically significant. The outlier could be a result of error in sampling.

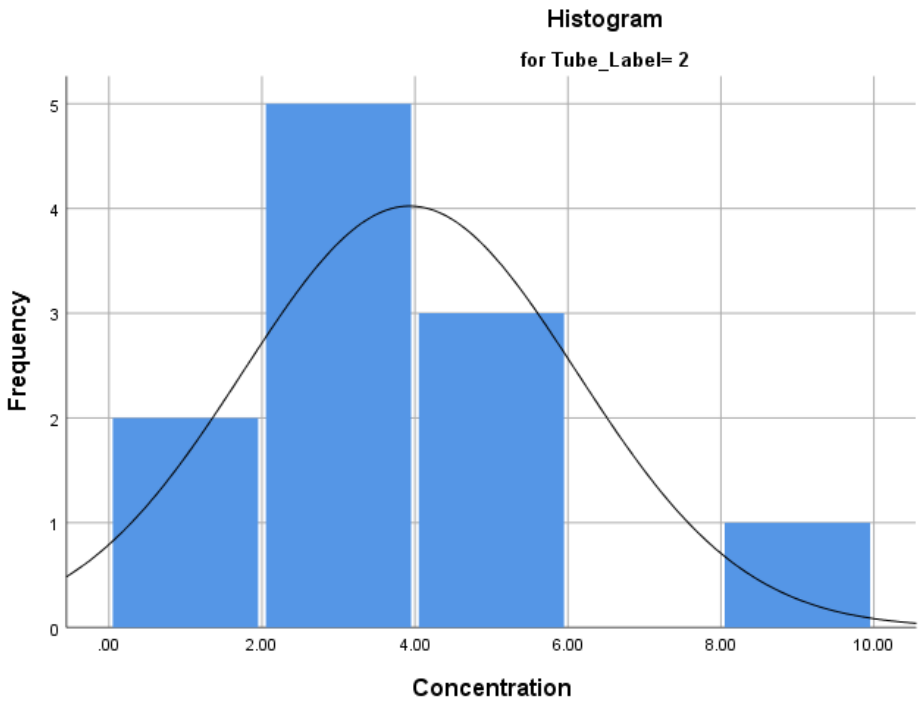

Figure 3: Histogram with the normal distribution curve depicting the normal distribution of data for miRNA concentration in 2000 µl of plasma

The data is said to be normally distributed from the above given p value and the same is seen in the normal distribution curve over the histogram (Figure 3). The normal distribution curve makes a perfect bell shape over the histogram further proving the data is statistically significant.

Next, an independent t-test was carried out to interpret the difference between the means of miRNA concentration among the two volumes in order to determine the significance.

Table 5. Independent t-test carried out on the data from Figure 2 and 3. The p value of 0.000 shows the data was highly significant

Independent Samples Test				
		t-test for Equality of Means		
		T	df	Sig. (2-tailed)
Concentration	**Equal variances assumed**	-4.371	17	.000

The sig. (2-tailed) column represents the p value and the result of 0.000 states that the data was highly significant.

An independent-samples t-test was conducted to compare the concentration of miRNA in two different volumes. There was a significant difference in the scores for 500 µl (M=0.53, SD=0.14) and 2000 µl (M=3.93, SD=2.18) volumes; t = 4.37, p = 0.00. These results suggest that the data was highly significant. Specifically, our results suggest that a larger volume of plasma, contains a higher concentration of miRNA.

Nanodrop Analysis

The Shapiro-Wilk test was used to analyse the data obtained from the Nanodrop analysis results (Appendix – Table 13). This was done in order to check for normality, that is, if the data was normally distributed. The null hypothesis of these tests stated that the data was normally distributed. The p value (Sig.) of this test was highly greater than 0.05 for both 500 (p=0.945) and 2000 (p=0.867).
Thus, we do not reject the null hypothesis and conclude that the dependent variable Concentration of RNA is normally distributed for each group (i.e., "Midi - 500 µl" and "Maxi – 2000 µl ").

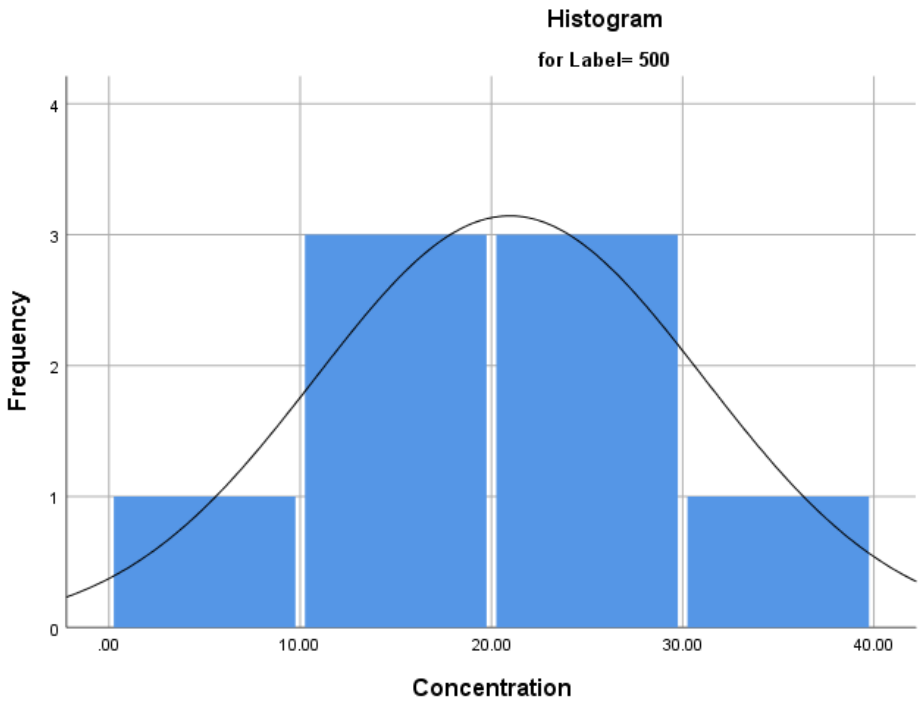

Figure 4: Histogram with the normal distribution curve depicting the normal distribution of data for RNA concentration in 500 µl of plasma.

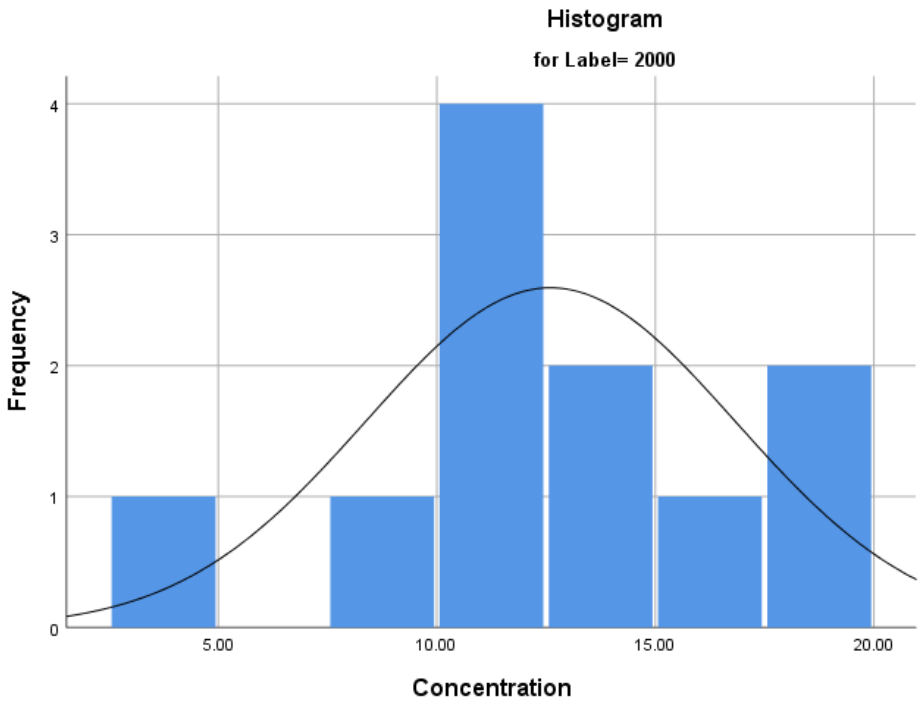

Figure 5: Histogram with the normal distribution curve depicting the normal distribution of data for RNA concentration in 2000 µl of plasma.

The perfectly bell shaped normal distribution curve over the histogram (Figures 4 & 5) state that the data was normally distributed. This proves that the data was statistically significant.

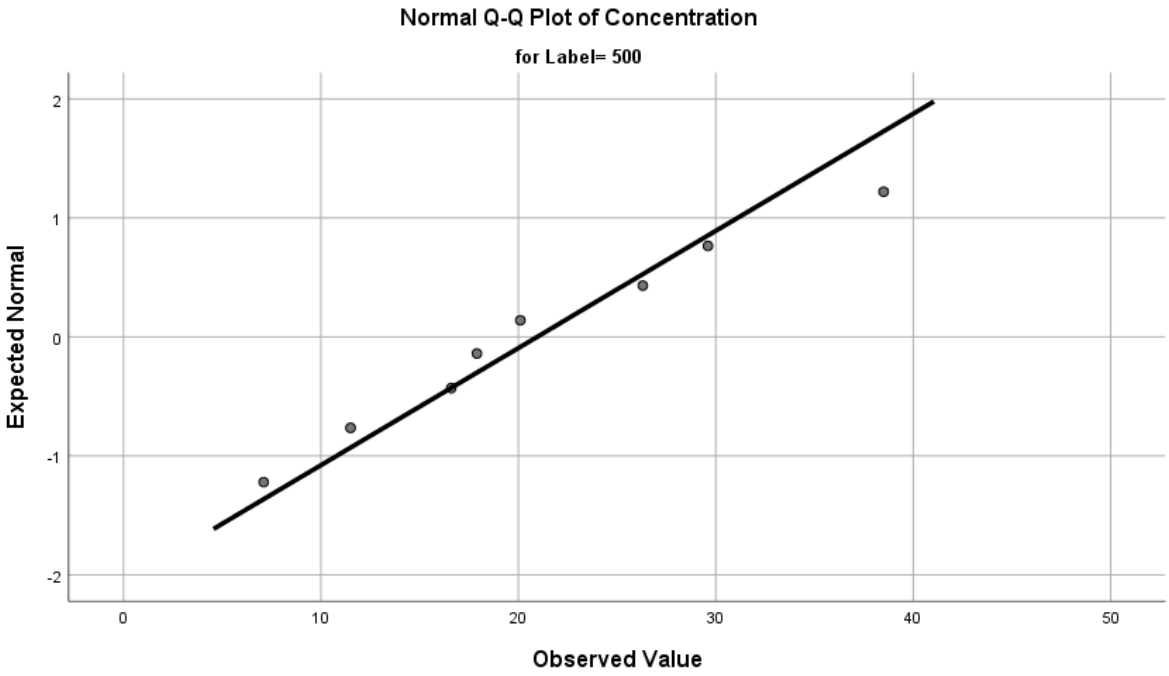

Figure 6: Normal Q-Q Plot of Concentration with a line of best fit depicting the normal distribution of data in 500 µl of plasma sample

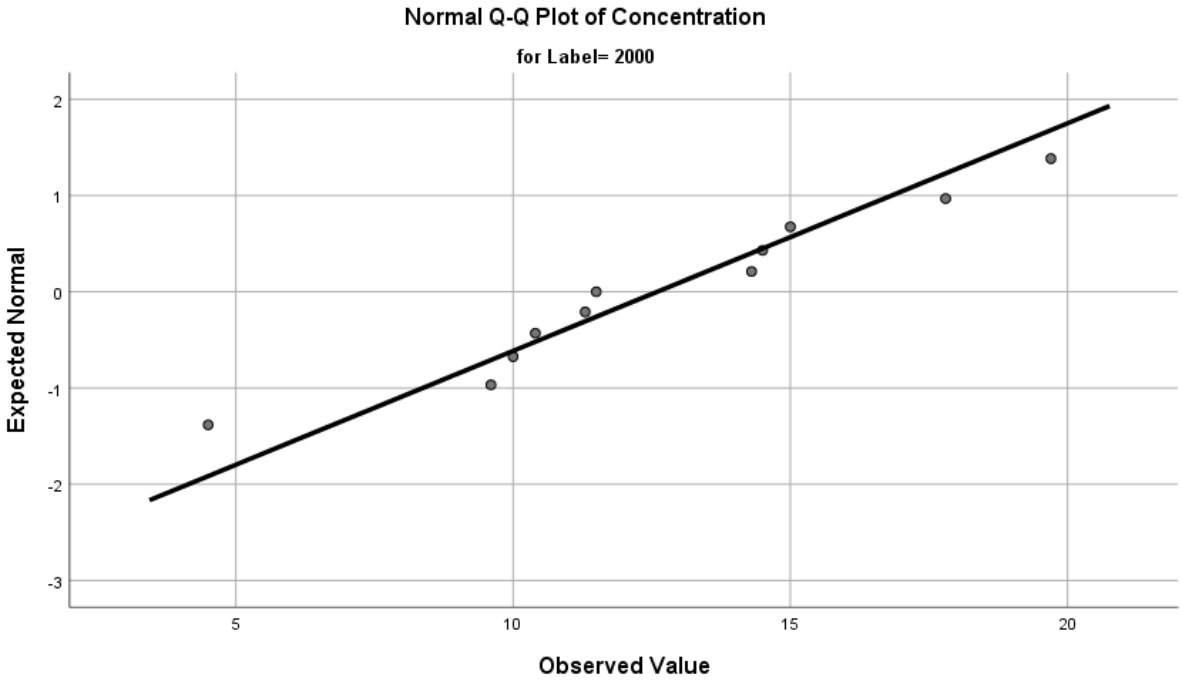

Figure 7: Normal Q-Q Plot of Concentration with a line of best fit depicting the normal distribution of data in 2000 µl of plasma sample

The Q-Q plots shown above (Figures 6 & 7) are an additional plot of data to show that the data was normally distributed. All the data points lie close to the line of best fit further proving the data was statistically significant. The outlier (Figure 6) could be a result of error in sampling.

Table 6: Independent t-test carried out on the data showing it is significant (0.024)

		t-test for Equality of Means		
		t	**df**	**Sig. (2-tailed)**
Concentration	**Equal variances assumed**	2.469	17	.024

The sig. (2-tailed) column represents the p value and the result of 0.024 states that the data was significant.

An independent-samples t-test was conducted to compare the concentration of RNA in two different volumes. There was a significant difference in the scores for 500 µl (M=20.95, SD=10.15) and 2000 µl (M=12.60, SD=4.23) volumes; t = 2.47, p = 0.24. These results suggest that the data was statistically significant.

BLOOD

Table 7: Paired sample t-test showing the difference between Elution 1 and Elution 2

Paired Samples Test			
	t	**df**	**Sig. (2-tailed)**
Concentration - Concentration2	8.543	9	.000

Since the p value is extremely low as seen in table 7, it is said that the data is highly significant.

DISCUSSION

MicroRNAs (miRNAs) are a very promising biomarker used to detect various kinds of disease (Wang et al., 2018). This is because they are present throughout the body, in all biofluids and they are strong, robust, sturdy and sensitive materials. However, they are present in extremely low concentration in plasma. Due to this shortcoming, one may not yield the initial amount of miRNA content required by some assay platforms (Garcia-Elias et al., 2017). This study aimed to try to determine the minimum optimum volume of plasma required to obtain an adequate amount of pure miRNA as well as to determine if the miRNA detected in blood could be used as potential biomarkers for the detection of sepsis. This was done by comparing and carrying out repeats of multiple extractions of different volumes of plasma samples: 500 µl and 2000 µl. RNA extraction is a vital step in order to carry out the study. The miRNeasy® kits used for the extractions from plasma have been said to be efficient, allows better isolation and gives superior performance in comparison to other similar kits used for the same purpose (Berchem et al., 2016). The above mentioned explanations were the reasons this kit was chosen. Extractions on plasma were carried out using the QIAGEN exoEasy Midi and Maxi kits where the Midi Kit (QIAGEN) was used for eight 500 µl extractions and the Maxi Kit (QIAGEN) was used for eleven 2000 µl extractions. One of the components in these kits – QIAzol Lysis Reagent – contained a chemical solution, TRIzol® which is both, a health and environmental hazard. Therefore, all extractions using these kits, were carried out in the safety of a fume hood as per biosafety regulations. Extractions from blood samples were carried out using Norgen's Total RNA Purification Kit. Here, ten extractions of 100 µl were done. The resulting eluate were analysed using quantification methods like Qubit® microRNA Assay Kit (Thermo Fisher Scientific) and NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific). From the statistical analysis carried out using the statistical analysis software SPSS, it was observed that the mean value of miRNA concentration varied greatly in the two different volumes analysed. The study showed that using diverse methods of quantification, produced wide-ranging results. The Qubit has been said to provide the most accurate and precise quantification of miRNA content (Garcia-Elias et al., 2017) and so this was the primary instrument used to measure the miRNA concentration. The results for the Qubit analysis were looked at first to determine whether the hypothesis of if a larger volume of plasma gave a higher concentration of miRNA held true or not. The Qubit® microRNA Assay showed smaller values ranging from 0.53 (± 0.14) ng/µl to 3.93 (± 2.18) ng/µl, which corresponded with the results published in previous literature by (Garcia-Elias et al., 2017). The Qubit assay and fluorometer produced results as expected, true to the hypothesis.

The secondary instrument used, the NanoDrop™ 2000 spectrophotometer detected a greater range of values ranging from 12.6 (± 4.23) ng/µl to 20.95 (± 10.15) ng/µl. This shows that the method of quantification greatly affected the results. There was notable variability between miRNA quantified spectrophotometrically by Nanodrop vs. fluorometrically by Qubit™. While spectroscopic methods provide adequate estimation at concentrations higher than 10 ng/µl, fluorometric quantification will arguably be more specific and more accurate for quantification (Berchem et al., 2016). However, the NanoDrop™ 2000 spectrophotometer was not as highly specific in its detection as it detects total RNA on the whole and not miRNA in particular. Therefore, it cannot be said for sure that all of the concentration obtained was pure miRNA. The following information explains the statement made above. Nucleic acids have absorbance maxima at 260 nm. The ratio of this absorbance maximum to the absorbance at 280 nm has been used as a measure of purity in both DNA and RNA extractions. A ratio of ~2.0 is generally accepted as "pure" for RNA (Thermo Scientific NanoDrop Products., 2012). However, the results obtained showed values differing from this range (Table 2). Abnormal 260/280 ratios usually indicate that a sample is contaminated by residual phenol, guanidine, or other reagent used in the extraction protocol, in which case the ratio is normally low. Inaccurate ratios may also be encountered at very low concentrations (< 10 ng/µl) of nucleic acids (Thermo Scientific NanoDrop Products., 2012).

The fluorometer of the Qubit® microRNA assay analysis showed some of the samples to be Out of Range (OOR) or 'Too Low'. Upon reflection, it was noted that this could be due to a number of reasons. The sample may have not contained the minimum concentration of miRNA detectable by the Qubit™, the Qubit™ reagent and working solutions might not have been well protected from light, the temperature at which the samples were measured might not have been optimal. The assay tubes should not be held in the hand before a measurement, because holding the tubes warms the solution and results in a low reading (Invitrogen by Life technologies., 2010). Another reason could be that extraction was not carried out optimally.

For calculation, tabulation and statistical analytical purposes, throughout the study, all values that appeared on the fluorometer as "Out of Range" were taken as 0.4 ng/ µl. This value was chosen as this value falls just below the lowest amount of concentration of miRNA that can be detected by the Qubit® 3.0 Fluorometer as per previous literature (Garcia-Elias et al., 2017). This was done so the sample size was large enough to produce rich statistical results.

On analysis of the blood samples, it was consistently observed that Elution 1 contained a higher concentration of miRNA than Elution 2 (Table 7). In addition, presence of miRNA in Elution 2 was detected despite eluting from the same extraction filer as Elution 1. Though it was in small amounts, the fact that miRNA was present, indicates that it could be used as a potential biomarker for diagnosis since even such minute quantities were detectable in the blood.

For the Qubit® microRNA Assay, the reading given by Standard #2 should be at least ten times higher than that of Standard #1 (Garcia-Elias et al., 2017). The standards are measured to calibrate the device. From table 1, it is seen that Standard 2 i.e 6672.73 is in fact ten times higher than Standard 1 i.e 167.39. This proves that the instrument has been calibrated optimally.

While carrying out analysis on the blood sample, it was noticed that the column kept clogging post thawing the frozen sample. For this reason, it was proposed to carry out two variants (Pernestig., 2019, personal communication, 26 March)**.** The first was where the sample was centrifuged post thawing, before beginning extraction. In the second variation, the sample was not centrifuged after thawing, but extraction was carried out straight away. This was done to check if there was any difference in the concentration of miRNA obtained due to usage of frozen blood as opposed to recommended fresh blood. No difference was seen in the concentration of miRNA as a result of this trial.

Though the study produced results as somewhat expected and other additional, useful information, some improvements that could be made for further analysis suggested, are as follows. The introduction of a new phenol free kit in the market was learned about towards the end of this study. This QIAGEN phenol-free miRNeasy® Serum/Plasma Advanced Kit was shown to produce higher miRNA yields without the use of phenol. The omission of phenol serves better for the environment without affecting the quality and quantity of miRNA obtained (©QIAGEN., 2019). Another improvement could be to use fresh blood instead of frozen samples provided. This improvisation would probably elevate yield of miRNA (Balzano et al., 2015). Further analysis could also be carried out using qRT-PCR for a more accurate and precise quantification.

Since work was carried out with blood and plasma, some ethical considerations were taken into account. Due to the availability of only small volumes of plasma, the study was not carried out on plasma samples from sepsis patients. Instead, the different kits were tested on healthy people first. Patients in the emergency department with suspected community onset sepsis and which were

above 18 years of age were informed about the study and asked to participate and sign an informed consent. The samples from patients who gave an informed written consent were collected and stored in a biobank. The research area *Future diagnostics of sepsis* use this unique biobank for identification of sepsis patients by measuring biomarkers in the blood of the patients. In this thesis project, healthy adult blood and plasma donor samples were used, for which no ethical approval was needed (Pernestig., 2019, personal communication, 5 February).

This study was a stepping stone toward a larger purpose. It aimed to standardize a control - Determine a minimum, fixed, optimum volume of biofluid containing maximum concentration of miRNA that can be used to detect diseases like sepsis, liver cirrhosis and cancer with minimum invasiveness (Dolin et al., 2018). This would in turn mean lesser usage of antibiotics in the future which would slow down and combat the global issue of the rising rate of resistance the microbes are building up due to advancement in medication (Prestinaci, Pezzotti & Pantosti, 2015). Research pertaining to use of miRNA for rapid diagnosis of various diseases is moving at high momentum and contains great potential (Wang et al., 2016). It is seen to be highly beneficial in the future as quicker detection would mean that more lives could be saved in the future thereby leading to a better patient outcome.

CONCLUSION

From this study of measuring and analysing the concentration of miRNAs in different volumes in order to determine whether they can be used as potential biomarkers, a great deal has been understood. As hypothesized, that a larger volume of biofluid, would mean obtaining a higher concentration of miRNA, this was seen to be true on the basis of the accurate Qubit® microRNA Assay results.

This study was a means to "standardize a control" for future studies in order to determine a minimum, fixed, optimum volume of biofluid with minimum invasiveness.

In line with the aims of this study, from the results obtained, it was concluded that plasma showed to follow the hypothesis. A higher concentration of miRNA was obtained from increasing volumes of plasma. However, this was observed only in the accurate Qubit analysis and so, deemed final. The same was not observed in the Nanodrop results for which the reasons are explained above.

Keeping in mind the latter part of the study, it was also concluded that the miRNA detected in blood could probably be used as potential biomarkers for the diagnosis of sepsis due to its presence in small quantities despite multiple elutions.

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APPENDIX

DATA TABLES

Table 8. miRNA concentration in different volumes of plasma samples isolated with the exoEasy Midi and Maxi kits analysed using Qubit® microRNA Assay Kit and the Qubit® 3.0 Fluorometer.

Qubit microRNA assay				
Readings using Fluorometer				
Standard 1	167.39			
Standard 2	6672.73			
Sample Number	Tube Label	Midi Kit (ng/µl)	Tube Label	Maxi Kit (ng/µl)
1	NBP2019 S1 1	OOR	NBP Maxi 1	3.62
2	NBP2019 S1 2	OOR	NBP Maxi 2	3.88
3	NBP2019 S1 3	0.580	NBP Maxi 3	OOR

4	NBP2019 S1 4	0.820	NBP Maxi 4	1.6
5	NBP2019 S1 5	0.560	NBP Maxi 5	9.04
6	NBP2019 S1 6	0.520	NBP Maxi 6	4.46
7	NBP2019 S1 7	0.560	NBP Maxi 7	3.94
8	NBP2019 S1 8	OOR	NBP Maxi 8	2.82
9			NBP Maxi 9	5.3
10			NBP Maxi 10	4.22
11			NBP Maxi 11	3.98

OOR = Out of Range

Tube Labels are mentioned in the Discussion.

Table 9. RNA concentration and purity in different plasma samples isolated with the exoEasy Midi and Maxi kits analysed using NanoDrop™ 2000 Spectrophotometer

Tube Name	**Concentration**	**A260**	**A280**	**260/280**	**260/230**
	(ng/µl)	(10 mm path)			
NBP2019 S1 1	7.1	0.179	0.09	1.99	-0.05
NBP2019 S1 2	16.6	0.415	0.278	1.5	-0.12
NBP2019 S1 3	29.6	0.741	0.493	1.5	0.3
NBP2019 S1 4	17.9	0.448	0.261	1.72	-0.15
Blank					
NBP2019 S1 5	26.3	0.658	0.46	1.43	0.41
NBP2019 S1 6	38.5	0.963	0.703	1.37	0.64
NBP2019 S1 7	11.5	0.288	0.164	1.76	0.39
NBP2019 S1 8	20.1	0.502	0.355	1.42	0.49
Blank					

NBP2019 Maxi 1	4.5	1.111	0.799	1.39	0.5
NBP2019 Maxi 2	14.3	0.357	0.233	1.53	0.32
NBP2019 Maxi 3	11.5	0.287	0.165	1.74	0.19
NBP2019 Maxi 4	17.8	0.445	0.296	1.5	0.36
Blank					
NBP2019 Maxi 5	11.3	0.282	0.192	1.47	0.3
NBP2019 Maxi 6	15	0.374	0.249	1.5	0.06
NBP2019 Maxi 7	14.5	0.363	0.253	1.44	0.16
NBP2019 Maxi 8	19.7	0.493	0.317	1.56	0.31
NBP2019 Maxi 9	10.4	0.259	0.16	1.62	0.32
NBP2019 Maxi 10	10	0.249	0.151	1.65	0.11
NBP2019 Maxi 11	9.6	0.239	0.141	1.69	0.3

Table 10**.** miRNA concentration from different blood samples isolated with the Norgen's Total RNA Purification Kit analysed using Qubit® microRNA Assay Kit.

Qubit microRNA assay						
Readings using Fluorometer						
Standard 1	167.39					
Standard 2	6672.73					
Extraction No.	**Tube Label**	**Concentration**		**Tube Label**	**Concentration**	
		ng/µl	**ng/ml**		**ng/µl**	**ng/ml**
		Elution 1			**Elution 2**	
1	NB2019 1 E1	6.22	31.1	NB2019 1 E2	1.13	5.65
2	NB2019 2 E1	9.5	47.5	NB2019 2 E2	OOR	
3	NB2019 3 E1	12.6	62.8	NB2019 3 E2	1.07	5.35
4	NB2019 4 E1	9.8	49	NB2019 4 E2	3.04	15.2

5	NB2019 5 E1	7.9	39.5	NB2019 5 E2	OOR	
6	NB2019 6 E1	8.38	41.9	NB2019 6 E2	OOR	
7	NB2019 7 E1	14.4	71.9	NB2019 7 E2	0.88	4.4
8	NB2019 8 E1	4.68	23.4	NB2019 8 E2	OOR	
9	NB2019 9 E1	14.5	72.5	NB2019 9 E2	0.88	4.4
10	NB2019 10 E1	8.46	42.5	NB2019 10 E2	OOR	

Table 11. RNA concentration and purity in different blood samples isolated with the Norgen's Total RNA Purification Kit analysed using NanoDrop™ 2000 Spectrophotometer.

Tube Name	**Concentration**	**A260**	**A280**	**260/280**	**260/230**
	(ng/µl)	(10 mm path)			
NB2019 1 E1	18.9	0.473	0.245	1.93	0.14
NB2019 2 E1	19.3	0.482	0.243	1.98	0.3
NB2019 3 E1	19.6	0.49	0.258	1.9	0.1
NB2019 4 E1	15.3	0.382	0.196	1.95	0.22
NB2019 5 E1	24.2	0.605	0.325	1.86	0.22
Blank					
NB2019 6 E1	20.7	0.518	0.291	1.78	0.31
NB2019 7 E1	23.6	0.589	0.326	1.81	0.16
NB2019 8 E1	19.1	0.479	0.254	1.89	0.09
NB2019 9 E1	25	0.626	0.338	1.85	0.19

NB2019 10 E1	23.1	0.578	0.293	1.97	0.05
Blank					
NB2019 1 E2	4.4	0.109	0.044	2.49	0.1
NB2019 2 E2	5.4	0.136	0.069	1.96	0.23
NB2019 3 E2	6	0.15	0.07	2.13	0.19
NB2019 4 E2	6.2	0.156	0.078	2	0.19
NB2019 5 E2	5.9	0.147	0.11	1.33	0.35
Blank					
NB2019 6 E2	4.2	0.105	0.038	2.8	0.23
NB2019 7 E2	5.3	0.133	0.056	2.39	0.22
NB2019 8 E2	5	0.124	0.051	2.41	0.13
NB2019 9 E2	6.2	0.154	0.072	2.14	0.36
NB2019 10 E2	4.5	0.111	0.033	3.38	0.2

Table 12. Using the Shapiro-Wilk test to test for Normality from the data above (Table 1 & 7). 1 = Midi Kit (500 µl); 2 = Maxi Kit (2000 µl) – Qubit analysis

Tests of Normality				
	Tube_Label	**Shapiro-Wilk**		
		Statistic	**df**	**Sig.(p value)**
Concentration	1	.837	8	.070
	2	.896	11	.165

Table 13. Using the Shapiro-Wilk test to test for Normality from the data above (Table 2 & 8). 500 = Midi Kit (500 µl); 2000 = Maxi Kit (2000 µl) – Nanodrop analysis

Tests of Normality				
	Label	**Shapiro-Wilk**		
		Statistic	**df**	**Sig.**

Concentration	500	.977	8	.945
	2000	.968	11	.867