EXOSOMAL MICRORNA AS A SEPSIS BIOMARKER

Assessing different volumes of plasma for possible quantification of exosomal microRNA

Bachelor Project in Bioscience G2E BV506G, 30 credits
Spring Semester 2019

Shamika Shenoy

Supervisor: Anna-Karin Pernestig
Examiner: Sanja Jurcevic
Sepsis is a medical emergency and it arises from extreme response of the host to an infection. Current diagnosis in sepsis relies on nonspecific clinical signs and culture-based analysis, which is time-consuming. It is critically important for clinicians to follow a protocol to identify sepsis and administer antibiotic therapy without any delay. Sepsis-specific biomarkers are being assessed for early diagnosis and thus improving the outcome of the sepsis patient. Many cellular molecules have been proposed to be sepsis-specific biomarkers. However, these molecules lack specificity and sensitivity. MicroRNA expression in biological fluids, particularly plasma and other tissues is very specific to disease state and are found to be promising diagnostic biomarkers in sepsis. Therefore, it is essential to extract qualitative and sufficient amount of microRNAs from human plasma for the downstream application of two-tailed RT-qPCR method microRNA needed for detection of sepsis patients. The aim of this study was to find optimal volume of plasma required to measure microRNA as sepsis biomarker. The study also included isolating exosomal microRNA from blood samples to check whether blood can be used for extraction. The study was conducted with healthy donor samples and the extraction is performed using Plasma/Serum Exosome Purification (product 58300, Norgen Biotek Corporation, Canada) and RNA Isolation Mini Kit and Total RNA Purification Kit (product 37500, Norgen Biotek Corporation, Canada). The samples were assessed for its quantity and quality by Qubit® and Nanodrop™ technology. Based on the comparison of amount of exosomal microRNA extracted from different plasma volumes, it can be concluded, that increasing volume of plasma may not give higher quantity of microRNA.
LIST OF ABBREVIATIONS

CRP: C-reactive protein
EDTA: Ethylenediaminetetraacetate
MRNA: Messenger RNA
MiRNA: MicroRNA
PCT: Procalcitonin
qPCR: Quantitative polymerase chain reaction
RNA: Ribonucleic acid
RT-qPCR: Real time quantitative polymerase chain reaction
SIRS: Systemic Inflammatory Response Syndrome
SOFA: Sequential Organ Failure Assessment
WBC: White blood cell
# TABLE OF CONTENTS

**Introduction** 01

- Diagnosis of Sepsis ................................................................. 02
- Biomarkers in Sepsis ............................................................... 03
- Future Diagnosis in Sepsis ...................................................... 04

**Materials and Method** 06

- Ethical Consideration ............................................................... 06
- Sample Collection and Storage .................................................. 06
- Plasma Preparation .................................................................... 06
- Purification of exosomal miRNA from healthy plasma .................. 06
- Purification of total RNA from healthy blood ............................... 07
- Quality control of the extracted exosomal miRNA and RNA ............ 07
- Statistical analysis .................................................................... 08

**Results** 09

- Analysis of miRNA extracted from healthy plasma ....................... 09
- RNA Concentration and from healthy plasma by Nanodrop™ ............ 10
- Analysis of miRNA extracted from healthy blood .......................... 10

**Discussion** ............................................................................. 12

**Conclusion** ............................................................................ 15

**References** ........................................................................... 16

**Appendices** ............................................................................ 24
INTRODUCTION

Sepsis is a complex medical condition caused by dysregulated immune response of the host triggered by an infection and can be life-threatening if left untreated (Sartelli et al., 2018). Sepsis is estimated to affect more than 30 million people worldwide every year, potentially leading to 6 million deaths (World Health Organization [WHO], 2018). An infection if not treated, can progress to septic condition. The populations which are at a higher risk to acquire sepsis include elderly people, pregnant women, neonates, hospitalized patients, and people with HIV/AIDS, liver cirrhosis, cancer, kidney disease, autoimmune diseases (Iskander et al., 2013). The most common sites of infection leading to sepsis are lungs, urinary tract and abdomen. The infection can be caused by either bacterial, viral or fungal attack (Martin, 2012). The immune system releases certain chemicals with a series of biological processes that is localized to the site of an infection. When an infection is particularly severe, it spreads to other parts of the body. This cause the immune system to react extensively; the inflammation damages tissue and interfere with the blood flow giving rise to the septic condition (Poll, Veerdonk, Scicluna, & Netea, 2017). Sepsis is categorized in three stages: sepsis, severe sepsis and septic shock. The first signs and symptoms begin with rapid breathing, confusion, fever and chills, discomfort and high heart rate. If not diagnosed early, sepsis can progress to severe sepsis characterized by organ dysfunction and multiple organ failure (Mahapatra & Heffner, 2019). Severe sepsis is followed by septic shock in which the blood pressure level drops dramatically and may lead to death (Annane, Bellissant, Cavaillon, 2005).

Sepsis is a major cause of concern in critically-ill and hospitalized patients and demand effective management and early diagnosis (Gyawali, Ramakrishna, & Dhamoon, 2019). The manifestations of sepsis are unspecific and attributable to many disease types. It is challenging for the healthcare practitioners to identify sepsis at an early stage (Tavaré, & O’Flynn, 2017). Application of broad – range of antibiotics has increased the resistance of the microorganisms (Pradipta et al., 2015). Despite the development in diagnostics of sepsis, it remains a public health problem because of its high morbidity and mortality (Rudd et al., 2018). In addition to examining clinical signs and symptoms and microbiological analysis, there is an urgent need to develop new diagnostic approaches for early and rapid diagnosis of sepsis (Sinha et al., 2018).
**Diagnosis of Sepsis**

Sepsis is a systemic infection that gives rise to a complex immune response with a wide range of clinical attributes. In 1991 consensus conference, Sepsis-1 definition was introduced which included the use of Systemic Inflammatory Response Syndrome (SIRS) criteria (Singer et al., 2016). The early definition of SIRS was used to describe complex pathophysiologic response to an infection, trauma, or burn; built upon basic clinical and laboratory abnormalities (Balk, 2014). Four SIRS criteria were defined, tachycardia (heart rate >90 beats/ min), tachypnea (respiratory rate >20 breaths/min), fever or hypothermia (temperature >38 or <36 °C) and leukocytosis, leukopenia, or bandemia (white blood cells >1,200/mm³, <4,000/mm³ or bandemia ≥10%) (Marik & Taeb, 2017).

In 2001, limitations in Sepsis – 1 definition were recognized and a new definition of Sepsis-2 was introduced. The Sepsis – 2 definition states that an individual must have at least two SIRS criteria for confirmed or suspected infection (Angus & Poll, 2013). According to the task force convened by Society of Critical Care Medicine and the European Society of Intensive Care Medicine proposed a new definition termed Sepsis – 3 (Bloos, 2018). Sequential Organ Failure Assessment (SOFA)/ qSOFA scores were also evaluated and was shown to be highly specific and preferred method for diagnosis of sepsis. This system evaluates: altered level of consciousness, systolic blood pressure B 100 mmHg and respiratory rate C 22 rpm. However, the poor sensitivity of the qSOFA score excludes it as a screening tool to detect early sepsis. Further validation is required before its use in clinical grounds (Rello, Valenzuela-Sánchez, Ruiz-Rodriguez, & Moyano, 2017).

Currently, in order to diagnose sepsis, microbiological analysis, detecting presence of microorganisms in the blood and other sites of the body is of utmost importance for the clinician (Retz et al., 2017). Gram stains is used to identify the general type of bacteria and provide presumptive results. This is done in conjunction with cultures. The current standard for detection of microorganisms from patients with suspected sepsis is blood culture (Ljungström et al., 2015). An amount of 20 – 30 mL of blood is collected from the patients suspected of infection (Laukemann et al., 2015). Blood cultures allow the microorganisms that may be present in the sample to grow and may also determine the susceptibility to various antibiotics (Marlowe, Gibson, Hogan, Kaplan, & Bruckner, 2003). The limitation to this analytical technique is that the viable microbes require enough time to grow in sufficient numbers to be identified. Further, only 30 to 50% of these patients have positive blood cultures. Some blood cultures may give false-negative results owing to previous use of antibiotics (Vincent, Mira, & Antonelli, 2016). Also, blood culture contamination still remains a challenge, especially in resource-poor settings, due to improper handling of culture bottles. This can lead to difficult in the interpretation of the blood culture results (Patel, 2016). This unavoidable delay of 2-3 days in identification of microorganisms and
lack of relevant results calls for development of novel methods for early identification of sepsis (Wolk & Johnson, 2018).

**Biomarkers in Sepsis**

Sepsis can be fatal, especially if it is not recognized early and treated promptly. Early diagnostics increase the life expectancy of the patients (Daviaud *et al*., 2015). The new era of sepsis diagnostics investigates non-culture based methods (Mancini *et al*., 2010). The usage of biomarkers may aid early diagnosis, and thus early initiation of appropriate therapy in patients with sepsis (Ljungström *et al*., 2017). Biomarker is a defined characteristic that one can measure as an indicator of normal biological processes, diseased states or responses to an intervention (Strimbu and Tavel, 2010). For many years, white blood cell (WBC) count has been used to diagnose any infection in the body. WBC count increases in response to acute infection and the increase is very dramatic in sepsis (Aminzadeh & Parsa, 2011). In a study, a significant percentage of emergency department (ED) patients with blood culture proven sepsis and septic shock have a normal temperature and WBC count upon presentation. Thus, physicians cannot rely solely on WBC to predict this complex disease (Seigel, Shapiro, Howell, & Domino, 2007). C-reactive protein (CRP) is also commonly used to screen early onset of sepsis (occurring during the first 24h of life) because its sensitivity is generally considered to be high (84.3%) in this setting (Faix, 2013). Its primary drawback as a biomarker in sepsis is its low specificity (46.15%) (Pradhan *et al*., 2016). CRP and WBC count ratio are not sufficiently specific for bacterial infections (Hildenwall *et al*., 2017).

In addition to this, procalcitonin (PCT) has been widely evaluated in severe systemic sepsis but is shown to elevate non-specifically in absence of an infection, occur in conditions of massive stress (Lee, 2013). Increased PCT levels are observed in the case of inflammation of non-infectious nature and are also seen in cases of trauma and major surgery. This decreases the specificity of the marker to predict systemic infection (Henriquez-Camacho & Losa, 2014). Though PCT and CRP are currently being used to identify sepsis in patients, it is unlikely that a single biomarker can accurately describe sepsis syndrome. Combination of biomarkers can improve diagnosis of severe bacterial sepsis (Karlsson, Pernestig, & Ljungström, 2015). Advances in the field of biomarkers led to understanding of miRNA-based diagnostic and therapeutic strategies. MiRNAs have emerged as promising biomarkers in various types of cancers (Ha, 2011). It has been studied that alterations in miRNA network can significantly affect the outcome in septic patients (Vasilescu *et al*., 2017).

MiRNAs have been closely associated in various human infectious diseases and thus serve as potential biomarkers in diagnosis of sepsis (Correia *et al*., 2017). MiRNAs are small non-coding
RNA molecules involved in post-transcriptional regulation of gene expression and are highly specific in their expression (Essandoh & Fan, 2014). The expression of miRNA is differentially regulated in pathogenic conditions like cancer, infectious diseases, cardiovascular disorders, neurodegenerative disorders, and many more (Bhardwaj, Arora, Prajapati, Singh, & Singh, 2013). MiRNAs, a class of negative regulators control gene expression by pairing with their target messenger RNAs (mRNAs) (Ha, 2011). Circulating miRNAs are prominently stable analytes and this characteristic makes them potential marker for various infectious diseases (Ojha, Nandani, Pandey, Mishra, & Prajapati, 2018). Exosomes-derived miRNAs has found to contribute significant information in sepsis detection (Reithmair et al., 2017). Exosomes are enriched with molecular cargo and has a functional role in various biological pathways (Simons & Raposo, 2009). Exosomes contain a rich source of miRNA and mRNA, involved in regulating pathogenesis in septic patients (Cheng, Sharples, Scicluna, & Hill, 2014). The stability of miRNA and its certain variability in expression in disease-state makes them good candidate for diagnosis of sepsis (Glinge et al., 2017). Isolation and profiling of miRNAs remains challenging for researchers due to its extremely low concentrations in biological fluids, especially in plasma samples. It is important to standardize a protocol and develop a workflow for isolation and purification of miRNA for its potential use in clinical practice.

Recent discoveries in early recognition and management of septic condition identify miRNA as a promising diagnostic biomarker. Monitoring levels of several biomarkers either singly or in combination has been considered as promising evidence in sepsis diagnostics (Samraj, Zingarelli, & Wong, 2013). Research in sepsis is addressed in developing a novel approach and a standard operating procedure to validate multi-level biomarkers in sepsis diagnostics (Dave et al., 2018). The central point of attention in Future Diagnostics of Sepsis is developing a multi-marker panel by integrating all the vital parameters for an accurate and timely diagnosis of suspected septic patient (Nolan, O’Leary, Bos, & Martin-Loeches, 2017).

**Future Diagnosis of Sepsis**

The project ‘Early Diagnosis of Sepsis’, is an element ‘Future Diagnostics of Sepsis’ within BioMine - data mining for the identification of biomarkers. It involves discovery, selection and validation of multilevel biomarkers along with data obtained from clinical evaluation for early and precise diagnosis of sepsis. The research covers combining large scale data from different sources and measuring multitudes of biomarkers to improve the analysis in interpreting multitudes of data. ‘Early Diagnosis of Sepsis’ involves several standardization and data processing procedures to enable early and correct detection of diagnosis of sepsis patients (Högskolan i Skövde, 2016).
The general aim of the thesis project was to investigate biomarkers, circulating miRNAs, which can distinguish between bacterial sepsis and non-bacterial sepsis, and whether they could be included in the multimarker panel for early sepsis diagnosis. One of the goals of the ongoing sepsis research at the University of Skövde is to develop multimarker panels that enable sepsis patient identification by measuring biomarker levels in the blood (Ljungström et al., 2017), which in the future could help the clinicians in diagnosing bacterial sepsis earlier, than the currently used bacterial culturing method and appropriately implementing anti-microbial treatment. This study aimed to extract qualitative and sufficient amount of miRNA from human plasma and whole blood samples for subsequent miRNA quantification and to see if blood would be feasible to work with in the future sepsis miRNA biomarker research. The future objective is to investigate miR-4488, miR-638 and miR-128-2-5p (unpublished data by thesis supervisor) expression levels in plasma from bacterial sepsis and non-bacterial sepsis patients as well as healthy donors by using the novel two-tailed RT-qPCR method (Androvic, Valihrach, Elling, Sjoback, & Kubista, 2017).

The following study utilized blood and plasma samples from healthy volunteers followed by testing volumes of the biological fluid for optimal extraction of miRNA as a diagnostic biomarker. The results from the quantification of miRNA at different measuring platforms namely Qubit® and Nanodrop™ and appropriate analysis of the ability of the kit used would be essential in further downstream processing of the samples. In this study, a workflow for extracting and purifying sufficient quantity of miRNA from plasma and its subsequent quantification at different platforms was proposed. The thesis also reported whether or not blood can be used for miRNA extraction. The results from this work will contribute to the biomarker knowledge in sepsis diagnosis.
MATERIALS AND METHOD

The prospective study was carried out in Department of Biosciences, University of Skövde, Sweden from February 2019 to June 2019.

Ethical considerations

The Skaraborg sepsis study was approved by the Regional Ethics Committee in Gothenburg (no. 376-11). It took place from September 2011 until June 2012 in Skaraborg Hospital, Sweden to investigate the occurrence of community onset severe sepsis and septic shock in adults. 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 (Biobank Sverige). 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 was used, for which no ethical approval was needed. Both plasma and whole blood samples were stored at -80°C until further needed for analysis.

Sample collection and storage

Whole blood was drawn from healthy volunteers at Unilabs AB in EDTA - treated tubes. In a 2 ml centrifuge tube (Eppendorf tubes®), approximately 0.5 mL of the sample was transferred and stored at -80°C.

Plasma preparation

Cells were removed from the blood samples by centrifugation for 15 minutes at 1790 RPM using the swing - out bucket centrifuge. The samples were maintained at 2-8°C while handling. Plasma supernatant was removed using a plastic transfer pipette, leaving at least 0.5 mL behind to avoid disturbing the buffy coat, and stored at – 80°C (Henry, 1979; Thavasu, Longhurst, Longhurst, Slevin, & Slevin, 1992).

Purification of exosomal miRNA from healthy plasma

The performance of plasma/serum exosome purification and RNA isolation mini kit (product 58300, Norgen Biotek Corporation, Canada) was evaluated using healthy donor plasma samples in order to determine what volume of the biological fluid would be appropriate for extraction of
exosomal miRNA. Because of the limited amount of sample volume, only 100 μL and 500 μL of plasma were tested as the starting volumes with plasma/serum exosome purification and RNA Isolation mini Kit (product 58300, Norgen Biotek Corporation, Canada). The extraction was carried out according to manufacturer’s protocol (product 58300, Norgen Biotek Corporation, Canada), except for the following modifications: before miRNA extraction, the frozen plasma samples were kept on ice for 15 minutes and then transferred to room temperature (21°C) for 15 minutes until completely thawed; after centrifugation, the volume of the cell-free plasma was made up to 1 mL by adding nuclease-free water, as the procedure is outlined for 1 mL of input plasma; to avoid precipitate formation Lysis Buffer A was warmer (60°C) than room temperature when it was added (Section: 2 of the protocol step: 1); for maximum recovery, the centrifugation step was performed (Section: 2 of the protocol step: 10); after extraction, 40 μL of the eluate was stored at -80°C and the remaining 10 μL was aliquoted in a 1.5 mL DNA LoBind Eppendorf tubes® and stored at -20°C for further quantitation. The whole procedure was repeated in the same manner for ten extractions (10 different patients).

**Purification of total RNA from healthy blood**

In order to determine if whole blood (WB) can be used for extracting miRNA and due to limited amount of plasma available in the biobank, healthy donor blood samples were used. Total RNA Purification Kit (product 37500, Norgen Biotek Corporation, Canada) was used to isolate and purify total RNA from healthy blood samples. Only 100 μL of the blood was used as a starting volume. The extraction was performed according to manufacturer’s protocol (product 37500, Norgen Biotek Corporation, Canada) except following modifications: the frozen blood samples were kept on ice for 15 minutes and then at room temperature (21°C) for 10 minutes until completely thawed; five out of ten extractions (10 different patients) were centrifuged before processing- 100 μL of the sample was transferred to RNase-free micro tube and centrifuged at 400 x g (~2000 RPM) for 2 minutes, the remaining were processed without centrifugation; the temperature of the water bath was maintained between 30 - 31°C (Appendix A of the protocol step: 6), for maximum recovery of RNA, the second elution was performed (Section: 2 of the protocol step: 4c), 30 μL of the eluate was aliquoted in RNAse-free tubes and stored at –80°C and the remaining 20 μL was transferred at –20°C.

**Quality control of the extracted exosomal miRNA and RNA**

The quantification of miRNA in the extracted sample was estimated by Qubit® microRNA assay (Thermo Fisher Scientific™) kit with Qubit® 3.0 Fluorometer (Life technologies™) following manufacturer’s protocol except for the following modifications: the sample volumes of 1 μL and 19 μL were tested and the working solutions were prepared accordingly. In order to check the
purity and concentration of RNA in the extracted sample, the samples were also quantified with NanoDrop 2000/2000cc Spectrophotometer™ (Thermo Fisher Scientific™).

**Statistical Analysis**

Shapiro-Wilk’s test was conducted to determine if the sample comes from a population which has a normal distribution or not. Mean, median or mode are measures of central tendency that attempts to describe the set of data by identifying the central position within that set. Arithmetic mean is used when the distribution is assumed normal. However, the median is usually preferred as a measure of central tendency when the data set is skewed (Manikandan, 2011). A p-value below 0.05 was determined to show a significant difference.

A one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test, was used to investigate the differences between groups if the data had a parametric distribution. The Kruskal Wallis H test was used to investigate the significant differences between two or more groups if the data did not have a parametric distribution (Laerd Statistics, 2019). The distribution of the Kruskal-Wallis test statistic approximates a chi-square distribution, with k-1 degrees of freedom, if the number of observations in each group is 5 or more. If the calculated value of the Kruskal-Wallis test ($\chi^2$) is less than the critical chi-square value ($\chi^2_{0.05}$), then the null hypothesis cannot be rejected. If the calculated value of the Kruskal-Wallis test ($\chi^2$) is greater than the critical chi-square value ($\chi^2_{0.05}$), then the null hypothesis is rejected and it can be concluded that one of the samples comes from a different population (Statistics Solutions, 2019). The null hypothesis stated is that the increasing plasma volume may not give higher amount of miRNA ($H_0$).
RESULTS

In order to know which volume of the samples need to be used for optimal recovery of miRNA, quality control of extracted miRNA was conducted using Qubit® and Nanodrop™ as quantification platforms.

Analysis of extracted miRNA from healthy plasma

The purified miRNA from healthy plasma volumes of 100 µL (10 different patients) and 500 µL (10 different patients) were quantified using Qubit®. First, a sample volume of 1 µL in 199 µL of the Qubit® working solution was quantified. All the measurements read ‘out of range’ (OOR), too low. Further, the miRNA concentrations were assessed using a larger sample volume of 19 µL (Appendix 1a).

Prior to conducting the analysis, the assumption of normality was evaluated with the Shapiro-Wilks test. The two data groups did not satisfy the normal distribution at p ≤ 0.05. Thus, for non-parametric distribution, the data set was represented with the median and median absolute deviation (MAD) of miRNA concentration (Table 1a).

<table>
<thead>
<tr>
<th>Sample type (number of donors)</th>
<th>Median miRNA concentration ng/µL (± MAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µL healthy plasma (n = 10)</td>
<td>0.054 (± 0.016)</td>
</tr>
<tr>
<td>500 µL healthy plasma (n = 10)</td>
<td>0.058 (± 0.006)</td>
</tr>
</tbody>
</table>

The hypothesis tested was that the increasing plasma volume may not give higher amount of miRNA (H_0). The alternate hypothesis (H_1) states that the increasing plasma volume may give higher amount of miRNA. A non-parametric Kruskal-Wallis H test was run to determine if there are any differences in miRNA concentrations between plasma input volumes of 100 µL and 500 µL (Appendix 1a).
Here, \( \alpha = 0.05 \), Degree of freedom (df) = 2 - 1 = 1. Therefore, the rejection region for this Chi-Square test is \( R = \chi^2; \chi^2 > 3.841 \). H statistic (\( \chi^2 \)) calculated is 0.321. Since, \( \chi^2 = 0.321 < \chi^2 = 3.841 \). The null hypothesis is not rejected. Also, \( p = 0.5708 > 0.05 \). The null hypothesis is not rejected.

The test showed that there was no significantly different amount of miRNA detected between the two sample groups.

Therefore, the null hypothesis is not rejected. There is not enough evidence to claim that some of the medians of the two sample volumes 100 µL and 500 µL are unequal at \( \alpha = 0.05 \). Thus, it can be concluded that increasing plasma volume may not give higher amount of miRNA.

**RNA concentration and purity from healthy plasma by Nanodrop™**

The mean concentrations of RNA in the sample assessed by Nanodrop™ 2000/2000cc Spectrophotometer™ were 9.12 (± 2.42) ng/µL and 9.26 (± 1.68) ng/µL in initial plasma input volume of 100 µL and 500 µL respectively (Table 1b). The \( A_{260/280} \) ratio were comparatively low (\( A_{260/280} < 2.0 \)) for all measurements. This may be possible due to considerable presence of protein contamination which can be seen by low \( A_{260/280} \) ratio (Appendix 1b).

<table>
<thead>
<tr>
<th>Sample type (number of donors)</th>
<th>Mean RNA concentration ng/µL (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µL healthy plasma (n = 10)</td>
<td>9.12 (± 2.42)</td>
</tr>
<tr>
<td>500 µL healthy plasma (n = 10)</td>
<td>9.26 ± 1.68</td>
</tr>
</tbody>
</table>

**Table 1b. RNA concentrations in samples extracted from healthy donor plasma using Nanodrop™ as a quantification platform.**

**Analysis of miRNA extracted from healthy blood**

In order to check if blood can be used to determine accurate miRNA levels, healthy blood samples were purified to obtain miRNA and quality was checked with Qubit® and Nanodrop™. The miRNA extracted from blood were quantified using Qubit® (Appendix 2a). The assumption of
normal distribution at $p < 0.05$ was satisfied by Shapiro-Wilks test. The data is thus reported as a mean of miRNA concentration with a deviation. The mean value of miRNA concentration showed a greater value in first elution than second (Table 2a).

Table 2a. MiRNA concentrations in samples extracted from healthy donor blood using Qubit® as a quantification platform.

<table>
<thead>
<tr>
<th>Sample type (number of donors)*</th>
<th>Mean value of miRNA concentration ng/µL (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µL healthy blood first elution (n = 10)</td>
<td>19.63 (± 15.7)</td>
</tr>
<tr>
<td>100 µL healthy blood second elution (n = 10)</td>
<td>2.07 (± 1.94)</td>
</tr>
</tbody>
</table>

* Few of the frozen blood samples were subjected to centrifugation prior to extraction with the kit.

On analysis of Nanodrop™ results, a mean concentration of 23.63 (± 7.24) ng/µL and 9.26 (± 1.68) ng/µL of RNA was reported in first and second elutions respectively (Table 2b). The $A_{260/280}$ ratio was less than 2.0 for all the measurements of first elution. In the second elution, the purity at $A_{260/280}$ was improved for most of the samples, $A_{260/280}$ ratio > 2.0 (Appendix 2a).

Table 2b. RNA concentrations in samples extracted from healthy donor blood using Nanodrop™ as a quantification platform.

<table>
<thead>
<tr>
<th>Sample type (number of donors)*</th>
<th>Mean value of RNA concentration ng/µL (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µL healthy blood first elution (n = 10)</td>
<td>23.63 (± 7.24)</td>
</tr>
<tr>
<td>100 µL healthy blood second elution (n = 10)</td>
<td>9.26 (± 1.68s)</td>
</tr>
</tbody>
</table>

* Few of the frozen blood samples were subjected to centrifugation prior to extraction with the kit.
DISCUSSION

In this thesis project, miRNAs were extracted from plasma samples with the kit (Norgen Biotek Corporation, Canada) and subsequently quantified using Qubit® and Nanodrop™. Due to limited amount of plasma, only two volume comparisons (100 µL and 500 µL) were made. The volume comparison was required in order to start on circulating miRNA extraction from the very limited amount of plasma samples obtained from healthy volunteers. MiRNA extraction was also performed on healthy donor blood samples to find out if blood samples could be used instead of plasma, which in future could provide even quicker biomarker detection in the clinical laboratory because plasma preparation would not be required.

The current diagnosis of sepsis is basic microbiological analysis along with examination of clinical signs and symptoms (Evans, 2018). However, the manifestations of this complex condition is very similar to other diseases, thereby giving unreliable results. Culture analysis is very time demanding (Gaieski et al., 2010) and provide preliminary results. Due to incorrect or missed diagnosis of suspected sepsis patients, the administration of antibiotic treatment is hampered (Vincent, Mira, & Antonelli, 2016). At present, several research societies are aiming to introduce a novel mechanism for early detection of sepsis (Otero et al., 2006, Langley & Wong, 2017). There has been a significant interest in using miRNAs as biomarkers in medical field, particularly cancer diagnostics (Sundarbose, Kartha, & Subramanian, 2017). Circulating miRNA influence various cellular processes, including inflammation, proliferation, apoptosis and intracellular communication (Mehta and Baltimore, 2016). Role of miRNAs have also been implicated in pathogenesis and can be considered as a promising evidence as diagnostic biomarker in sepsis Reithmair et al., 2017 also found distinctly compartment-specific regulation of miRNAs between sepsis patients and healthy volunteers.

In this study, exosomal miRNA are investigated for its potential use as biomarkers for sepsis detection mainly due to following reasons: the expression pattern of miRNAs is very specific to the tissue type (Mitchell et al., 2008) and miRNAs are present in human plasma in remarkably stable form as they are protected from endogenous RNA degradation (Shi, 2016). The technique of profiling miRNA expression in serum or plasma as minimally invasive biomarker is encouraged in sepsis diagnostics (Cochetti et al., 2016). However, miRNA signature profiling is challenging because of many reasons: absolute miRNA concentrations in biological fluid, particularly plasma are very low (Williams et al., 2013); miRNAs are very small molecules which exhibit a high degree of homology in its family (Kreth, Hübner, & Hinske, 2018). MiRNA measurement suffers consistency and reproducibility issues; each sample type: blood, plasma,
serum has a varied expression of miRNA; the method of RNA extraction and quantification largely
influence the quality of miRNA; the physical state of the donor affects the amount of circulating
miRNA, thus altering the exact concentration in the biofluid (Kapple and Keller, 2017). The choice
of RNA isolation method and the type and size of input material is considered substantial in the
miRNA measurement (El-Khoury, Pierson, Kaoma, Bernardin, & Berchem, 2016). In order to deal
with low concentrations of miRNA, validation is usually done with specialized qPCR technique
(Goni, García, & Foissac, 2017). Therefore, it is necessary to design a standardized protocol for
reliable miRNA expression profiling to enable quicker diagnosis.

In recent years, several extraction kits have been reviewed for its quality of miRNA
obtained at different measuring platforms (Li, Mauro, & Williams, 2015; El-Khoury, Pierson,
Kaoma, Bernardin, & Berchem, 2016).

In this study, Nanodrop™ was employed to be able to detect the concentration and the
quality of RNA in the samples. This spectrophotometer based methodology is not specific for
miRNA measurement. The concentrations obtained with this technique indicate total RNA in the
samples (Table 1b) absorbed at 260 nm. The A_{260/280} of ~1.8 to 2.1 at pH 7.5 indicates very pure
RNA, and a ratio greater than 1.8 is considered an acceptable indicator of good quality RNA (Fleige
and Pfaffl, 2006). Pure RNA should also give an A_{260/230} Ratio of 2 or slightly above. In this study, it
was observed that the A_{260/230} ratio is on the lower range (Appendix 2b). However, there is no
lower limit of this ratio, as it is not clear which contaminants contribute to a low A_{260/230} Ratio
(Ahlfen and Schlumpberger, 2010). The possible contaminants may include: high salts like
Ethylenediaminetetraacetic (EDTA), used as an anticoagulant and guanidine thiocyanate, a
component of lysis buffer A (product #58300 Norgen Biotek Corporation protocol); proteins that
may have not been completely removed during extraction procedure. These contaminants have
absorbance near 230nm. Regardless of the lower A_{260/230} ratio, Kuang, Yan, Genders, Granata, &
Bishop, 2018 confirmed that there is no significant correlation between A_{260/230} and qPCR
amplification efficiency.

Another important aspect for miRNA assessment is Life Technologies™ Qubit® 2.0
Fluorometer. This assay is most suitable platform for miRNA quantification as it offers a lower
detection range and higher specificity for small RNA molecules compared to other platforms such
as Nanodrop™ (El-Khoury, Pierson, Kaoma, Bernardin, & Berchem, 2016). The results obtained
from Qubit® estimation for both volumes (100 µL and 500 µL) of plasma samples (Table 1a)
suggest that there was no evident difference in the concentration of miRNA when the starting
volume of the plasma sample is increased. The difference in the results obtained from Qubit®
(Table 1a) and Nanodrop™ (Table 1b) for miRNA concentrations is because Qubit® is more
sensitive than Nanodrop™. Mariner et al., 2018 also described a method of miRNA detection
following heat/freeze cycle that allows to detect miRNA from reduced sample volume of plasma and serum, improving miRNA detection and reproducibility.

The quantification of miRNAs in blood is complicated as the measurements often yield high protein content and low RNA concentration (Andreasen et al., 2010). However, the results obtained from estimation of miRNA content in the heathy human blood samples suggests that blood shows elevated levels of miRNA than plasma (Table 2a and Table 2b). The variation in the miRNA may be possibly due to haemolysis during ample collection and handling. Rupturing of red blood cells and the release of contents in the body fluids can give bias results. This technical challenge in extraction of miRNA can alter the true measurement of free circular miRNA (Pegtel et al., 2010). Some studies suggest assessment of free haemoglobin in the plasma or blood samples to determine if the sample is suitable for analysis (Kirschner et al., 2011). Thus, our results suggest that frozen blood that is being thawed, which most certainly will affect haemolysis. To summarize, we suggest that blood can be used for extracting miRNA but requires further validation strategies to provide accurate measurement in qPCR.
CONCLUSION

In conclusion, sufficient and qualitative amount of miRNAs that were extracted from healthy human plasma samples using the kit (*Norgen Biotek Corporation*, Canada) were measured by Qubit®. The research area of biomarkers using unique material from biobanks are limited to technologies, and such assays demand minor volumes of biological fluid. Therefore, one important criteria when considering the design of the standardized protocol for reliable miRNA expression profiling from plasma is the starting volume of the plasma used. The study suggested that the miRNA content in the plasma samples measured by Qubit® assay did not show any major differences in the concentrations for both the volumes (100µL and 500µL). Thus, it can be inferred that increasing starting volume of plasma may not necessarily increase the concentration of miRNA. In case of poor miRNA extraction, quantification by Nanodrop™ platform was performed which allows estimation of RNA in the sample. The results of Qubit® assay for miRNA in blood samples encouraged use of blood for miRNA extraction. Certain steps need to be optimized to assess the degree of haemolysis in the sample for RT-qPCR. These findings also suggests use of fresh blood for qualitative extraction of miRNA and to evaluate the effect of freeze/thaw cycles on miRNA purification.

Our findings will aid in gathering information to develop a standardized workflow for extraction, quality control and analysis of miRNA in early detection of sepsis. This may also help to overcome the drawbacks of current diagnostic techniques of sepsis, thus improving the administration of empirical or standard antibiotic treatment. Early recognition and treatment of suspected sepsis patients will ameliorate health of the sepsis survivors, thereby decreasing its mortality rate.
REFERENCES


Garcia-Elias, A., Alloza, L., Puigdecanet, E., Nonell, L., Tajes, M., Curado, J., ... & Comín-Colet, J. (2017). Defining quantification methods and optimizing protocols for microarray hybridization of circulating microRNAs. *Scientific reports, 7*(1), 7725. [https://dx.doi.org/10.1038%2Fs41598-017-08134-3](https://dx.doi.org/10.1038%2Fs41598-017-08134-3).


APPENDICES

APPENDIX 1

a) Observed original calculated sample concentrations and Qubit® tube concentrations of miRNA extracted from plasma input volumes 100 µL and 500 µL.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Plasma input volume (µL)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Original calculated sample concentration (ng/ µL)</td>
<td>Qubit® tube concentration (ng/ mL)</td>
<td>Original calculated sample concentration (ng/ µL)</td>
</tr>
<tr>
<td>1</td>
<td>0.0669</td>
<td>6.36</td>
<td>0.0505</td>
</tr>
<tr>
<td>2</td>
<td>0.0411</td>
<td>3.9</td>
<td>0.0525</td>
</tr>
<tr>
<td>3</td>
<td>0.0718</td>
<td>6.82</td>
<td>0.0411</td>
</tr>
<tr>
<td>4</td>
<td>OOR*</td>
<td>OOR*</td>
<td>0.0568</td>
</tr>
<tr>
<td>5</td>
<td>0.0263</td>
<td>2.5</td>
<td>0.0615</td>
</tr>
<tr>
<td>6</td>
<td>0.0453</td>
<td>4.3</td>
<td>0.0939</td>
</tr>
<tr>
<td>7</td>
<td>0.0642</td>
<td>6.1</td>
<td>0.0522</td>
</tr>
<tr>
<td>8</td>
<td>0.0379</td>
<td>3.6</td>
<td>0.0592</td>
</tr>
<tr>
<td>9</td>
<td>0.294</td>
<td>27.9</td>
<td>0.235</td>
</tr>
<tr>
<td>10</td>
<td>0.316</td>
<td>30</td>
<td>0.237</td>
</tr>
</tbody>
</table>

*OOR is an abbreviation for 'out of range'. The Qubit® 3.0 fluorometer status displayed 'too low' reading. For statistical analysis, the value for OOR was considered as 0.
b) Observed measurements of extracted RNA from 100 µL of plasma samples by Nanodrop 2000/2000cc Spectrophotometer™ (*Thermo Fisher Scientific™*)

<table>
<thead>
<tr>
<th>Sample number (initial plasma volume 100 µL)</th>
<th>Nucleic Acid concentration (ng/ µL)</th>
<th>A260</th>
<th>A280</th>
<th>260/280</th>
<th>260/230</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.7</td>
<td>0.219</td>
<td>0.126</td>
<td>1.73</td>
<td>0.26</td>
</tr>
<tr>
<td>2</td>
<td>8.2</td>
<td>0.206</td>
<td>0.123</td>
<td>1.68</td>
<td>0.14</td>
</tr>
<tr>
<td>3</td>
<td>7.0</td>
<td>0.175</td>
<td>0.108</td>
<td>1.62</td>
<td>0.75</td>
</tr>
<tr>
<td>4</td>
<td>9.7</td>
<td>0.244</td>
<td>0.143</td>
<td>1.70</td>
<td>0.43</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>0.124</td>
<td>0.070</td>
<td>1.77</td>
<td>0.16</td>
</tr>
<tr>
<td>6</td>
<td>7.1</td>
<td>0.178</td>
<td>0.108</td>
<td>1.65</td>
<td>0.46</td>
</tr>
<tr>
<td>7</td>
<td>11.9</td>
<td>0.299</td>
<td>0.186</td>
<td>1.60</td>
<td>0.30</td>
</tr>
<tr>
<td>8</td>
<td>8.6</td>
<td>0.215</td>
<td>0.126</td>
<td>1.70</td>
<td>0.19</td>
</tr>
<tr>
<td>9</td>
<td>11.8</td>
<td>0.296</td>
<td>0.176</td>
<td>1.68</td>
<td>0.14</td>
</tr>
<tr>
<td>10</td>
<td>13.2</td>
<td>0.331</td>
<td>0.208</td>
<td>1.59</td>
<td>0.41</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>9.12 (± 2.42)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.4</td>
<td>0.180</td>
<td>0.107</td>
<td>1.74</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>9.3</td>
<td>0.234</td>
<td>0.131</td>
<td>1.78</td>
<td>0.26</td>
</tr>
<tr>
<td>3</td>
<td>7.7</td>
<td>0.192</td>
<td>0.118</td>
<td>1.62</td>
<td>0.18</td>
</tr>
<tr>
<td>4</td>
<td>6.4</td>
<td>0.161</td>
<td>0.095</td>
<td>1.69</td>
<td>0.19</td>
</tr>
<tr>
<td>5</td>
<td>11.6</td>
<td>0.289</td>
<td>0.179</td>
<td>1.61</td>
<td>0.89</td>
</tr>
<tr>
<td>6</td>
<td>10.9</td>
<td>0.274</td>
<td>0.172</td>
<td>1.59</td>
<td>0.25</td>
</tr>
<tr>
<td>7</td>
<td>11.0</td>
<td>0.274</td>
<td>0.155</td>
<td>1.77</td>
<td>0.04</td>
</tr>
<tr>
<td>8</td>
<td>10.8</td>
<td>0.269</td>
<td>0.158</td>
<td>1.71</td>
<td>0.05</td>
</tr>
<tr>
<td>9</td>
<td>9.0</td>
<td>0.224</td>
<td>0.128</td>
<td>1.75</td>
<td>0.28</td>
</tr>
<tr>
<td>10</td>
<td>8.5</td>
<td>0.214</td>
<td>0.119</td>
<td>1.79</td>
<td>0.51</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>9.26 (± SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX 2

a) Observed original calculated sample concentrations and Qubit® tube concentrations of miRNA extracted from 100 µL of blood in two elutions.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>100 µL of blood input volume</th>
<th>First elution</th>
<th>Second elution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Original calculated sample concentration (ng/µL)</td>
<td>Qubit® tube concentration (ng/mL)</td>
</tr>
<tr>
<td>1#</td>
<td>8.48</td>
<td>42.4</td>
<td>1.63</td>
</tr>
<tr>
<td>2#</td>
<td>9.08</td>
<td>45.4</td>
<td>0.660</td>
</tr>
<tr>
<td>3</td>
<td>53.2</td>
<td>266</td>
<td>6.08</td>
</tr>
<tr>
<td>4</td>
<td>21.0</td>
<td>105</td>
<td>4.84</td>
</tr>
<tr>
<td>5#</td>
<td>4.42</td>
<td>22.1</td>
<td>OOR*</td>
</tr>
<tr>
<td>6#</td>
<td>10.2</td>
<td>51.2</td>
<td>0.880</td>
</tr>
<tr>
<td>7</td>
<td>15.5</td>
<td>77.4</td>
<td>0.780</td>
</tr>
<tr>
<td>8</td>
<td>18.7</td>
<td>93.5</td>
<td>1.40</td>
</tr>
<tr>
<td>9#</td>
<td>13.8</td>
<td>69.2</td>
<td>1.91</td>
</tr>
<tr>
<td>10</td>
<td>42.0</td>
<td>210</td>
<td>2.58</td>
</tr>
</tbody>
</table>

*OOR is an abbreviation for 'out of range'. The Qubit® 3.0 fluorometer status displayed 'too low' reading. For statistical analysis, the value for OOR was considered as 0.

# The frozen blood samples were centrifuged before extraction with the kit.
b)


<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1#</td>
<td>19.1</td>
<td>0.477</td>
<td>0.256</td>
<td>1.87</td>
<td>0.15</td>
</tr>
<tr>
<td>2#</td>
<td>18.6</td>
<td>0.465</td>
<td>0.249</td>
<td>1.87</td>
<td>0.40</td>
</tr>
<tr>
<td>3</td>
<td>37.8</td>
<td>0.944</td>
<td>0.519</td>
<td>1.82</td>
<td>0.41</td>
</tr>
<tr>
<td>4</td>
<td>26.1</td>
<td>0.652</td>
<td>0.353</td>
<td>1.85</td>
<td>0.29</td>
</tr>
<tr>
<td>5#</td>
<td>16.3</td>
<td>0.407</td>
<td>0.218</td>
<td>1.86</td>
<td>0.27</td>
</tr>
<tr>
<td>6#</td>
<td>26.8</td>
<td>0.670</td>
<td>0.477</td>
<td>1.40</td>
<td>0.78</td>
</tr>
<tr>
<td>7</td>
<td>19.7</td>
<td>0.491</td>
<td>0.273</td>
<td>1.80</td>
<td>0.16</td>
</tr>
<tr>
<td>8</td>
<td>19.7</td>
<td>0.492</td>
<td>0.280</td>
<td>1.75</td>
<td>0.52</td>
</tr>
<tr>
<td>9#</td>
<td>17.0</td>
<td>0.426</td>
<td>0.242</td>
<td>1.76</td>
<td>0.63</td>
</tr>
<tr>
<td>10</td>
<td>35.2</td>
<td>0.881</td>
<td>0.480</td>
<td>1.84</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Mean (± SD) 23.63 (± 7.24)

# The frozen blood samples were centrifuged before extraction with the kit.


<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1#</td>
<td>5.4</td>
<td>0.134</td>
<td>0.076</td>
<td>1.77</td>
<td>0.11</td>
</tr>
<tr>
<td>2#</td>
<td>3.9</td>
<td>0.097</td>
<td>0.046</td>
<td>2.09</td>
<td>0.22</td>
</tr>
<tr>
<td>3</td>
<td>7.2</td>
<td>0.181</td>
<td>0.084</td>
<td>2.16</td>
<td>0.50</td>
</tr>
<tr>
<td>4</td>
<td>6.8</td>
<td>0.169</td>
<td>0.073</td>
<td>2.30</td>
<td>0.18</td>
</tr>
<tr>
<td>5#</td>
<td>3.0</td>
<td>0.074</td>
<td>0.030</td>
<td>2.47</td>
<td>0.38</td>
</tr>
<tr>
<td>6#</td>
<td>4.7</td>
<td>0.119</td>
<td>0.052</td>
<td>2.29</td>
<td>0.22</td>
</tr>
<tr>
<td>7</td>
<td>3.5</td>
<td>0.088</td>
<td>0.040</td>
<td>2.20</td>
<td>0.52</td>
</tr>
<tr>
<td>8</td>
<td>5.0</td>
<td>0.124</td>
<td>0.052</td>
<td>2.38</td>
<td>0.48</td>
</tr>
<tr>
<td>9#</td>
<td>3.5</td>
<td>0.088</td>
<td>0.035</td>
<td>2.51</td>
<td>0.62</td>
</tr>
<tr>
<td>10</td>
<td>6.8</td>
<td>0.169</td>
<td>0.077</td>
<td>2.19</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Mean (± SD) 4.98 (± 1.45)