

EXOSOMAL NON-CODING RNAS FOR MONITORING
BREAST CANCER PATIENTS

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Exosomal non-coding RNAs were explored as a basis for monitoring breast-cancer patients. Specifically, exosomal microRNAs (miRNAs), and especially miR-21, were the focus of this project. Consistent with our hypothesis, we found that miR-21 was highly expressed in exosomes of cancer patients compared to 'healthy' individuals. However, circulating miR-21 levels, when compared in post- versus pre-surgery serum, did not decline for all five experimental patients. This unexpected observation could be the result of small sample size and lack of both endogenous and exogenous miRNA controls. All five patients had early stage breast cancer. We found that our methods were possibly not sensitive enough to detect very small differences between pre- and post-surgery serum. Future experiments might involve samples at a later stage of cancer. High miR-21 levels in post-tumor resection serum raised an issue concerning how long cancerous exosomes can stay in the blood after tumor removal. As well, recovery time from surgery might vary from patient to patient. Consequently, additional serum samples from patients might be obtained at a later time after surgery. This research signals an advance over current modes of cancer detection and involves a more patient-centric approach. Customized-miRNA panels might someday be the standard for each breast-cancer patient. The release of breast-tumor exosomes and their miRNA cargo

into the blood stream allow for disease detection by way of non-invasive methods achieved at lower application costs. This technology, with all its advantages, might result in more frequent screenings of patients and thus an earlier detection of cancer and more efficient patient care.

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ABBREVIATIONS

CCV	Clathrin Coated Vesicle
DLS	Diffusion Light Scattering
dPCR	Digital PCR
ESCRT.....	Endosomal Sorting Complexes Required for Transport
ILV	Intraluminal Vesicles
miR.....	Micro-RNA
MVB.....	Multivesicular Body
NTA.....	Nanoparticle Tracking Analysis
PBS	Phosphate Buffered Saline
RISC	RNA Induced Silencing Complex

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CHAPTER 1

INTRODUCTION

Throughout the world, breast cancer is the second most common type of cancer in women [1]. Although breast-cancer mortality has decreased due to early detection and improved treatments, it still is the main cause of cancer death in females [2]. Approximately one-third of women diagnosed with breast cancer succumb to the disease, mostly through metastasis [3-5]. Exosomes are vesicles that are cell-derived and found in most biological fluids and play a significant role in breast cancer growth and metastasis. Exosomes are secreted in large quantities by cancer cells and have the capacity to merge with and transfer their oncogenic cargo to recipient cells both locally and systemically [6]. Specifically, exosomal microRNAs (miRNAs) are a focus of the emerging field of biomarker research due to their regulatory role in gene expression. Successful applications of exosomal miRNAs as biomarkers could revolutionize early breast cancer detection and further reduce cancer mortality. In the future, early stages of malignancy may be detected even before breast tumors become visible under current screening methods. Circulating miRNAs could soon be used for screening with the benefit of being obtained non-invasively and analyzed rapidly and relatively cheaply

Exosomes

Exosomes are stable membrane-bound nanoparticles (~50-120nm) that are released into the circulation by many cell types, including cancer cells [8, 9]. Originally upon discovery of exosomes in the early 1980s, exosomes were considered cellular waste removers to enable the cell to rid itself of unnecessary proteins [10-12]. Later in the 1990s, their role in the immune system was discovered [13, 14] and they gained more interest due to their intercellular communication capabilities. But it was not until 2007 that transfer of functional mRNA and miRNA to many other cell types was discovered [15]. Especially in cancer research, the discovery of non-coding RNAs being transported in exosomes raised significant enthusiasm in the cancer diagnostics field.

Exosome Formation

The details of exosome formation and their release from cells are not entirely understood. The current hypothesis is that exosomes are developed intracellular via the endosomal pathway [16]. Invagination of the endosomal membrane with the help of Endosomal Sorting Complexes Required for Transport (ESCRTs) create intraluminal vesicles (ILVs), producing structures called Multivesicular Bodies (MVBs)[17]. The MVBs then either fuse with the lysosome, resulting in degradation of their contents, or they merge with the plasma membrane releasing the ILVs, which are now referred to as exosomes, into the extracellular environment [18-20] (Fig. 1).

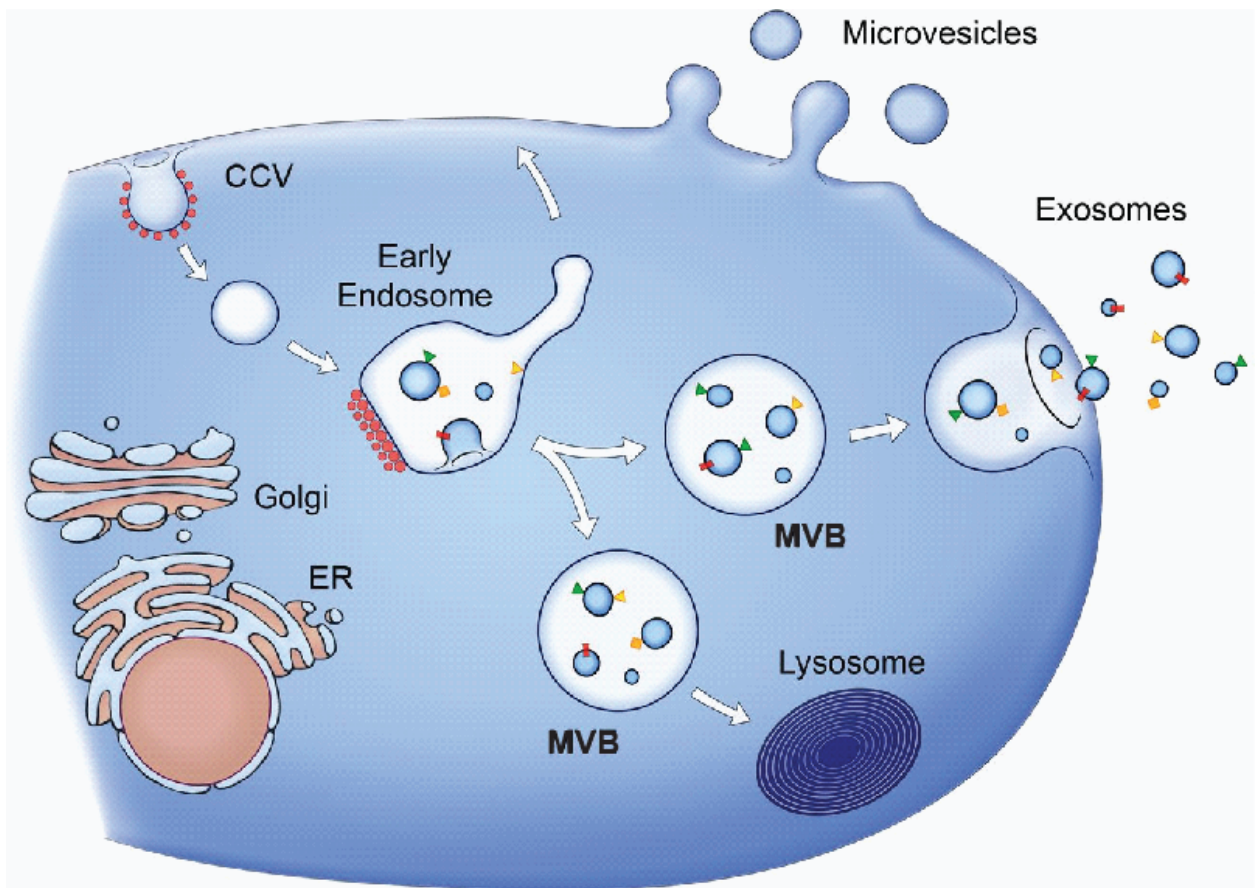


Fig. 1 Biogenesis and release of exosomes. Exosomes are formed by invagination of MVBs and released into the extracellular milieu by the MVB fusing with the cell membrane. In contrast, microvesicles are formed by budding of the cell membrane. MVB, Multivesicular Body; CCV, Clathrin Coated Vesicle. Adapted from Raposo et al. [21]

Exosomal Cargo/structure

Exosomes are composed of a lipid bilayer that encloses a small cytosol with molecular contents from the donor cell including proteins and nucleic acids [22]. The composition of both membrane and cargo of exosomes can greatly differ from the parental cell. This suggests that the biogenesis and packaging of exosomes is a regulated event instead of a random collection of cytoplasmic contents.

Exosomes primarily contain lipids [23, 24], and in particular lipids related to

lipid rafts, including cholesterol, glycerophospholipids, and ceramide [25]. In addition to providing structure to exosomes, it has been suggested that some lipids, such as phosphatidylserine, can be used for long distance cell signaling by interfering with certain processes in recipient cells [26, 27].

Protein content of exosomes depends on the donor cell type. Proteins involved in transport and fusion, MVB formation, and proteins associated with lipid rafts are prominent in exosomes. Exosomes also contain proteins engaged in cell signaling pathways [28, 29]. Various proteins can be used as markers specific to exosomes, for example the tetraspanins CD9, CD63, and CD81, or the cytosolic protein TSG101, since they are not present in other extracellular vesicles [30].

Lastly, exosomes contain nucleic acids such as DNA, mRNA, and small non-coding RNA. Among them, miRNAs are the most abundant RNA species in exosomes [31]. Multiple studies have suggested that the numbers of particular miRNAs found in exosomes differ greatly from the donor cell [15, 32]. This again suggests that miRNAs are carefully sorted by the cell for uptake into exosomes [33].

Exosomal Functions

Exosomes received little attention until the late 1990s when their role in immune responses became known. Since 2007, they have been widely studied after nucleic acids were identified in these vesicles. Although the physiological functions of exosomes are not well understood, they have been shown to be important in immune response [16, 34, 35] and may play a role in short- and long-range intercellular signaling [36-40].

Alteration of immune responses. Exosomes secreted by immune cells (T-cells, B-cells, dendritic cells, etc.) perform significant functions within the immune system [34]. Their role in antigen presentation was derived from the fact that exosomes contain functional MHC-II molecules [13]. In addition, tumor derived exosomes carry immunosuppressive molecules that diminish the proliferation of T-cells and NK-cells [41-44], or have an immunosuppressive effect by increasing the numbers of regulatory T-cells and myeloid cells [45-47].

Intercellular communication. Exosomes serve intercellular communication in three ways. They can bind with membrane proteins such as adhesins and receptors and initiate a signaling cascade in the target cell, they can fuse with the recipient cell, or they can be taken up via endocytosis [48, 49] (Fig. 2). Upon release of their contents into the cytosol, relocated miRNAs can influence translation [50]. The lipid membrane of exosomes protects proteins and RNAs from proteases and ribonucleases, respectively, therefore providing a more stable way of transport compared to naked proteins and RNAs. In addition, exosomes can deliver a higher concentration of messengers to a target cell at one time. It has been hypothesized that exosomes are capable of long-distance communication by targeting signaling pathways in cells far away from the cell of origin, especially in tumor progression and metastasis [36].

Isolation and Characterization Techniques of Exosomes

Exosomes are isolated from most body fluids, including blood [51], urine [52, 53], saliva [54], breast milk [7, 55], amniotic fluid [56], semen [57], cerebrospinal

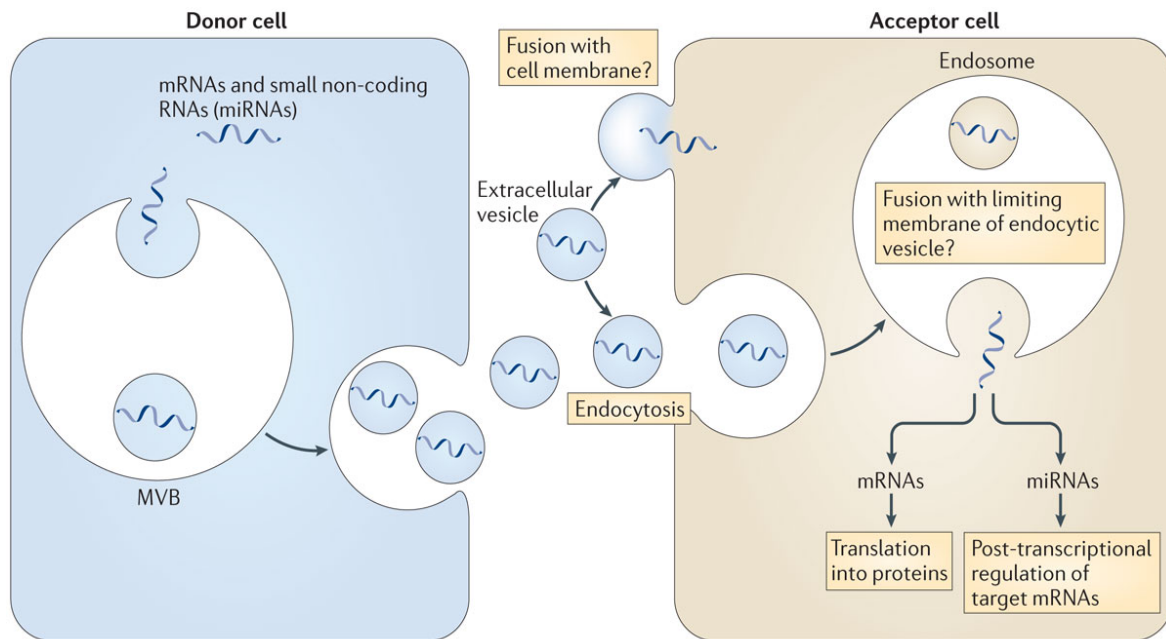


Fig. 2 Intercellular communication via exosomes. mRNAs and non-coding RNAs are packaged into ILVs of MVBs which are then secreted. Exosomes target their cargo to the recipient cell. Adapted from Robbins et al. [83]

fluid [58], nasal secretions [59], malignant ascites [60], and from cell culture medium.

Usually a series of centrifugations are used for pelleting exosomes. In addition, System Biosciences, Inc. developed proprietary reagent called ExoQuick to precipitate exosomes [61]. A comparison of different methods showed that exosomes isolated from urine by ExoQuick-TC produced a better yield and purity than ultracentrifugation [62].

Once exosomes are isolated, many research options are possible. Since exosomes are too small for the resolution of a conventional light microscope, various electron microscopy instruments have been used for the visualization of

exosomes. Transmission electron microscopes (TEM), scanning electron microscopes (SEM) [63, 64], and cryo-electron microscopes [65, 66] have all been used for assessing morphology and size of exosomes. Atomic force microscopy (AFM) provides very high-resolution imaging, up to fractions of a nanometer, and has also been used in numerous studies [67, 68]. Dynamic light scattering (DLS) is useful for characterization of a more homogenous group of vesicles [69, 70].

Quantification of exosomes, however, is more challenging considering their nano-size. Nanoparticle tracking analysis (NTA) is a system developed by NanoSight, to determine the concentration and average size of exosomes in a sample. NTA uses a laser and camera to record the Brownian motion of each particle that is then related to particle size using the Stokes-Einstein equation [71]. It is considered the most precise instrument for phenotyping exosomes [72].

Exosomal contents can be extracted and analyzed through various methods. To extract miRNA, various kits are commercially available, including Total Exosome RNA and Protein Isolation Kit from Invitrogen. The RNA quality, size, and yield can be obtained with the Agilent 2100 Bioanalyzer. Identifying unique non-coding RNAs can be achieved with qPCR or digital PCR (dPCR) analysis [73], microarray assays [74], or next generation sequencing [75] of the RNA sample.

Exosomal Non-Coding RNA

Focus on exosomal biomarkers for detecting disease, especially cancer, has been directed to small non-coding RNAs, including microRNAs (miRNAs)[76, 77]. miRNAs are short (≤ 25 bp) non-coding RNAs, which are transcribed as long primary

transcripts (pri-miRNAs) and then trimmed by the nuclear enzyme Drosha producing a precursor miRNA (pre-miRNA)[78]. Pre-miRNAs are transported via Exportin-5 to the cytoplasm where they are further processed by Dicer, which cleaves the pre-miRNA loop leaving a short-lived double-stranded product [79, 80]. One strand becomes the mature miRNA and assembles with the RNA induced silencing complex (RISC) and targets certain mRNA sequences resulting in silencing [81]. The other strand is usually degraded [82] (Fig. 3).

miRNAs are involved in regulation of translation of many protein-coding genes [81, 85, 86]. By binding to messenger RNA (mRNA), miRNAs can inhibit mRNA translation [50] or encourage mRNA degradation, thereby changing mRNA levels through post-transcriptional regulation. miRNA expression may thus be an additional physiological indicator of mRNA expression [87, 88].

Currently, about 2588 human miRNA sequences are reported in miRBase, a miRNA database [89]. It is anticipated that miRNAs regulate over 60% of protein-coding genes [90] and influence most all cellular processes, including proliferation, differentiation, and apoptosis [81, 91, 92]. Considering that one miRNA can impact many mRNAs, and multiple miRNAs can target a single mRNA [86, 93], the biological impact of miRNAs is vast [94]. miRNAs are considered master-gene regulators that might change between healthy and and diseased cells. Thus the differential expressed miRNAs could be used as biomarkers of various illnesses. miRNAs have been found in their native cells but also in circulation [88]. Naked RNAs are rapidly degraded in blood; therefore, association with protein complexes or packaging within exosomes is required for protection [95]. For example, the bulk of miRNAs

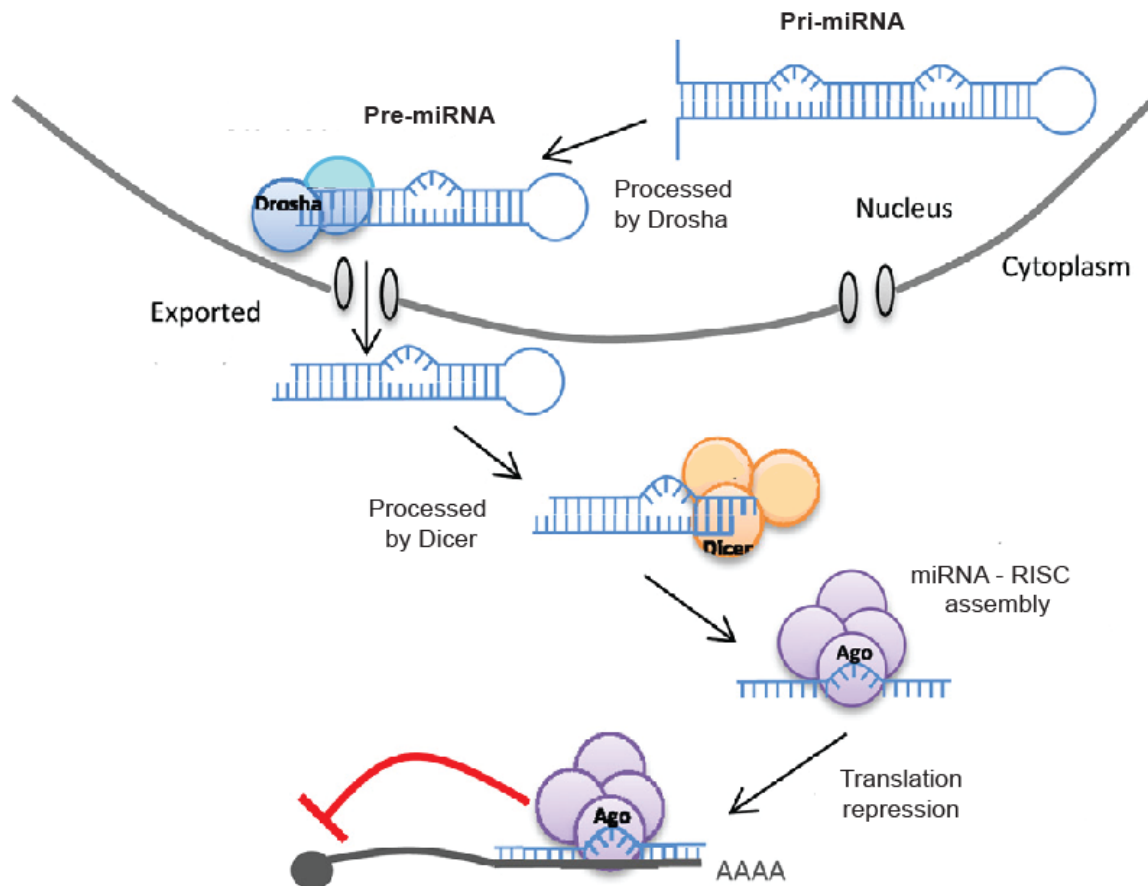


Fig. 3 Biogenesis of miRNAs. Transcribed pri-miRNAs are cleaved in the nucleus by Drosha into pre-miRNAs, which are then transported into the cytoplasm via exportin 5. In the cytoplasm, pre-miRNAs are cleaved again by Dicer creating double stranded RNA segments. One strand combines with the RNA induced silencing complex (RISC) after which it targets mRNA to prevent translation. Adapted from Inns et al. [84]

isolated from saliva was found to be enclosed in exosomes [54]. Another study showed that exosomes protect the miRNAs contained within them, even in severe conditions [96], allowing miRNAs to stay functional when delivered to distant cells. Exosomal transport of miRNAs when combined with the regulatory roles of miRNAs in oncogene expression create the prospect of immense biological consequences and the growing importance of miRNAs in cancer research.

The Role of Exosomal miRNAs in Cancer Biology and Diagnostics

Tumors require interaction with their surroundings in order to develop and progress [35]. Consequently, cancer cells mold their environment, locally and systemically, by secreting molecules to protect tumor cells from immunity [97] and to encourage proliferation, angiogenesis, invasion, and migration [98-100]. A major portion of these molecules, including tumor-derived miRNAs, is secreted via exosomes [54]. A variety of cancer cells secrete more exosomes than normal cells [101]. Some studies found cumulative levels of exosomes in the circulation with increased cancer stages [102]. In addition, malignant transformation in a cell changes the collection of exosome-released miRNAs, resulting in differential gene regulation in recipient cells [103]. These findings suggest that tumors rely heavily on these messengers.

Cancer is caused by deregulation of oncogenes or tumor suppressor gene. Since miRNAs are major regulators, miRNAs can manipulate these genes and, depending on their mRNA target and expression patterns, can support tumor growth or suppression [88, 104]. miRNAs show abnormal expression patterns in

cancerous tissues and have a role in the development and progression of cancer [105, 106]. For example, it was shown that tumor-derived exosomes target healthy pre-metastatic organ cells via miRNAs. These miRNAs mainly modulate metastasis-related processes, such as angiogenesis and invasion [98]. Another model suggests that cancer-associated macrophages enhance the invasiveness of breast cancer cells via exosomal miRNAs [100]. In addition, tumor-secreted exosomes can have adverse effects on the immune system. It was found that, in lung cancer, exosomal miRNA stimulates macrophages to release inflammatory cytokines, which induces metastasis [107]. Collectively these studies show that the association of exosomes and miRNA has powerful implications particularly where cancer-promoting factors are transported to non-cancerous cells and exude their effects systemically.

Exosomal miRNAs are the focus of extensive research since they might be useful as diagnostic biomarkers for cancer. Over the years, various groups have identified exosomal miRNAs that are differentially expressed in healthy versus cancerous individuals. A number of researchers have proposed that exosomal miRNAs can be used for diagnostic, prognostic, and therapeutic purposes in breast [108], colon [109], glioblastoma [110], kidney [111], leukemia [112], lung [113], ovarian [114, 115], prostate [116], and other tissue cancers. In one study, 12 exosomal miRNAs were aberrantly expressed in prostate cancer patients compared to healthy subjects. In addition, 11 miRNAs were found to be upregulated in patients with metastases compared to patients without metastases [117]. Another group found seven exosomal miRNAs extracted from serum that were differentially expressed in colorectal cancer patients versus healthy subjects [109]. This indicates

that changes in exosomal miRNAs may reflect various pathological changes in cancer patients.

In the past few years, multiple companies have been developing exosome-based diagnostic tests. The company Caris Life Sciences developed a proprietary blood-based diagnostic test that focuses on exosomal non-coding RNA, including miRNA, and transcription factors for cancer detection [118]. Exosome Diagnostics is developing a biological fluid-based molecular diagnostic test based on exosomal biomarkers for cancer and other diseases. Recently, this company partnered with QIAGEN to develop a diagnostic test specifically for lung cancer detection [119]. Also Eli Lilly and Company, a global pharmaceutical company, recently partnered with Exosome Diagnostics to identify mutations and exosome expression levels in blood for prediction of drug response and disease recurrence [120]. Exosome Sciences pursues exosome based-diagnostic tools for detection of cancer and other diseases [121]. Lastly, HansaBioMed is an Italian-based company that collaborates globally with numerous academic and commercial research groups and focuses on research and development in exosome-based diagnostic and prognostic applications [122].

Deregulation of Exosomal miRNAs in Breast Cancer

Breast cancer, like many other cancers, has been associated with aberrant miRNA expression. Aberrantly expressed miRNAs can have disease suppressing properties in some cancers as well as disease promoting properties in others. Thus depending on the context, miRNAs that have multiple targets can have varied effects. For example, miR-125b targets multiple transcription factors and is

upregulated in serum of patients with different cancers, such as lung cancer [123] and prostate cancer [124], but downregulated in others, such as breast cancer [125]. Also miR-105, known to be upregulated and distributed via exosomes by metastatic breast cancer cells and shown to induce metastasis in distant organs [108], inhibits tumor cell proliferation in prostate cancer [126].

It is important to recognize distinct miRNA expression profiles that are associated with specific breast-cancer stages and subtypes. During progression of breast cancer, circulating miRNA expression may alter. For example, in breast cancer, miR-10b mediates cell migration and invasion [127]. Increased levels of miR-10b are found in metastatic breast cancer but not early breast cancer [128, 129]. Breast cancer can be categorized into one of five main subtypes (luminal A, luminal B, HER2-enriched, basal-like, and normal-like), indicating molecular characteristics and guides treatment regimes. It has been shown that deregulation of circulating miRNAs tends to be breast cancer-subtype specific [130]. Thus circulating miRNA expression levels are heterogeneous across individual breast cancer patients. Therefore, when focusing on individual-patient profiles, for diagnosis purposes, assessment should focus on differential miRNA expressions found in each patient.

A literature compilation of circulating miRNAs that are differentially expressed in breast-cancer patients is listed in Table 1. Although these miRNAs are not all breast-cancer specific, they are biomarker candidates for breast cancer. As a control for variably expressed biomarkers, 'housekeeping' miRNAs remain relatively stable and are universally expressed in all exosomes whether cancerous

Table 1 Possible biomarkers for breast cancer detection; Underexpressed, Overexpressed, and possible exosomal reference miRNAs. BC, Breast Cancer

Underexpressed in Breast Cancer Patients		
miRNA	Body fluid type	Reference
let-7c	Plasma	[131]
miR-106a	Blood	[130]
miR-1234	Serum	[132]
miR-126	Serum	[133]
miR-127	Plasma	[134]
miR-133a	Serum	[135]
miR-139	Serum	[135]
miR-143	Serum	[135]
miR-145	Serum, Plasma, and Tissue	[135, 136]
miR-148b	Plasma	[134]
miR-17	Serum	[137]
miR-191	Blood	[130]
miR-199a	Serum	[133]
miR-215	Serum	[138]
miR-299	Serum	[138]
miR-335	Serum	[133]
miR-34a	Serum	[137]
miR-365	Serum	[135]
miR-409	Plasma	[134]
miR-411	Serum	[138]
miR-548d	Serum	[132]
miR-605	Serum	[132]
miR-652	Plasma	[134]
miR-760	Serum	[132]

Table 1 Continued

Overexpressed in Breast Cancer Patients		
miRNA	Body fluid type	Reference
let-7a	Blood and Serum	[139, 140]
miR-1	Serum	[141]
miR-103	Serum	[142]
miR-107	Serum and Plasma	[135, 143, 144]
miR-10b	Serum	[141, 145]
miR-122	Serum	146]
miR-1255a	Serum	[132]
miR-125b	Serum and Plasma	[147, 148]
miR-130a	Plasma	[144]
miR-130b	Serum	[132]
miR-132	Plasma	[144]
miR-133a	Serum	[141]
miR-133b	Serum	[141]
miR-138	Blood	[130]
miR-146a	Plasma	[144, 149]
miR-148b	Plasma	[150]
miR-149	Serum	[132]
miR-151	Serum	[132]
miR-155	Serum	[133, 137, 145, 151, 152]
miR-15a	Serum	[135]
miR-16	Plasma & Tissue	[136, 144]
miR-181a	Serum	[132]
miR-182	Serum	[153]
miR-1827	Serum	[132]
miR-18a	Serum	[132, 135]
miR-18b	Serum	[143, 154]
miR-195	Blood and Serum	[139, 140]
miR-21	Serum, Plasma and Tissue	[133, 136, 147, 149, 155, 156]
miR-222	Serum	[132, 142]
miR-23a	Serum	[142]
miR-23b	Serum	[142]
miR-24	Serum	[142]
miR-25	Serum	[142]
miR-27a	Plasma	[144]

Table 1 Continued

miR-29a	Serum	[155]
miR-3136	Serum	[132]
miR-320d	Serum	[132]
miR-34a	Serum	[145]
miR-363	Serum	[132]
miR-373	Serum	[137]
miR-375	Serum	[146]
miR-376c	Plasma	[150]
miR-409	Plasma	[150]
miR-423	Serum	[141]
miR-425	Serum and Plasma	[131, 135]
miR-4306	Serum	[132]
miR-451	Plasma & Tissue	[136]
miR-452	Serum	[138]
miR-486	Serum	[141]
miR-505	Plasma	[147]
miR-589	Plasma	[131]
miR-629	Serum	[132]
miR-652	Serum	[132, 143]
miR-671	Serum	[132]
miR-7	Serum	[141]
miR-744	Serum	[132]
miR-801	Plasma	[134, 150]
miR-92a	Serum	[141]
miR-96	Plasma	[147]

Potential reference micro-RNA's
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miRNA	Body fluid type	Reference
miR-124	BC cell lines	[157]
miR-128	BC cell lines	[157]
miR-1825	Serum	[132]
miR-221	Plasma/ not BC specific	[158]
miR-22	BC cell lines	[157]
miR-30a	Plasma/ not BC specific	[158]
miR-484	Serum/ not BC specific	[159]
miR-99a	Plasma/ not BC specific/BC cell lines	[157, 158]

or normal and are listed as well (Table 1).

miR-21

miR-21 is an extensively studied miRNA, which is dysregulated in breast cancer and thus is the immediate focus of this study. It was one of the first mammalian microRNAs identified. In the human genome, miR-21 is located on chromosome 17q23.2 and codes for the precursor miR-21 (pre-miR-21) which is 72-nucleotides (nts) long. It is subsequently processed into a 22nt mature miR-21 sequence [89] (Fig. 4). miR-21 sequences are strongly conserved throughout evolution across many species, which may suggest that the functions of miR-21 in gene regulation is highly preserved [89].

miR-21 plays an important role in many biological functions as well as diseases, including development [160], immunity [161], epithelial-to-mesenchymal transition [162], cancer [163], cardiovascular diseases [164], pulmonary fibrosis [165], and inflammation [166]. Although miR-21 has important functions in early development and immunity, it is usually present in the blood at low levels. However, in disease and especially cancer, upregulated numbers of miR-21 are detected.

miR-21 is known as a typical onco-miR as its mRNA targets are mostly tumor suppressor genes. Some of the tumor suppressors that are silenced by miR-21 include phosphatase and tensin homolog (PTEN)[167] and programmed cell death 4 (PDCD4)[168], which regulate important cellular processes, such as cell growth, proliferation, and apoptosis [169]. miR-21 is associated with most known cancers. It is consistently upregulated in breast cancer, gastric cancer, lung cancer,

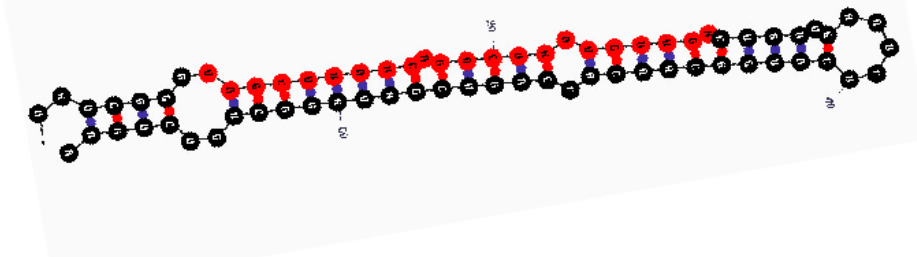
A) Chromosome 17



B) Sequence:

5'-UGUCGGGUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUCUGACA-3'

Length pre-miR-21: 72 nt



C) Mature miR-21:

Predominant Sequence (hsa-miR-21-5p):

5'-UAGCUUAUCAGACUGAUGUUGA-3'

Minor Sequence (hsa-miR-21-3p):

5'-CAACACCAGUCGAUGGGCUGU-3'

Fig. 4 Conserved miR-21 sequence A) Location of miR-21 gene on chromosome 17q23.1. (B) 72-nt-long precursor miR-21 (pre-miR-21) sequence and stem-loop structure, (C) Predominant mature miR-21 and minor strand sequences. Adapted from Selcuklu S.D. et al. [170], and miRNomeMap [171]

esophageal cancer, colorectal cancer, glioma, leukemia, bile duct cancer, nasopharyngeal cancer, osteosarcoma, liver cancer, retinoblastoma, lymphoma, stomach cancer, prostate cancer, ovarian carcinoma, hepatocellular carcinoma, cervical cancer, uterine leiomyomas, head and neck cancer, chronic lymphocytic leukemia, papillary thyroid carcinoma, pancreas cancer, and squamous cell carcinoma of the tongue, as reviewed in the literature [170, 172].

Furthermore, just looking at breast cancer alone, it has been observed in the literature that levels of miR-21 rise in the blood circulation with more advanced

stages of breast cancer [173]. Thus, miR-21 might have potential as a diagnostic biomarker for early breast-cancer detection.

CHAPTER 2

EXOSOMAL NON-CODING RNAS FOR MONITORING BREAST CANCER PATIENTS

Introduction

We hypothesize that pre- and post-tumor resection will result in the differential expression of non-coding RNAs packaged in exosomes from breast cancer cells compared to exosomes released from corresponding “normal” breast-epithelial tissue. A related hypothesis is based upon previous studies that have found that some miRNAs upregulated in breast tumors (e.g. miR21) [174] and known to be involved in cancer progression can also be detected in the blood (serum or plasma) of breast cancer patients. We further hypothesize that a customized panel of microRNAs that change within an individual pre- and post-surgery will provide greater precision in monitoring disease than using a few of the same biomarkers across all patients.

In this study, we first aimed to validate and optimize two methods utilized in succession: nanoparticle tracking analysis and digital PCR. While optimizing those methods, the variation in exosomes and in exosomal miRNAs from healthy women were investigated and negative controls were established. Secondly, for each individual, circulating exosomal miR-21 from the blood was interrogated pre- and

post- breast tumor resection. This allowed us to determine whether changes in circulating miR-21 reflect tumor presence. In addition, circulating miR-21 levels from breast cancer patients were compared with those of healthy women, age matched when possible.

While initially the overall goal of this project was to develop a customized-miRNA panel for each of the five breast-cancer patients, further experiments, including miRNA sequencing, will be scheduled.

Optimization of Exosomal miRNA Detection while Developing a Negative Control Using Serum of Healthy Individuals

Two methods, Nanoparticle Tracking Analysis (NTA) and digital PCR (dPCR), were applied in this study. To create a baseline negative control, control serum samples were investigated with NTA for exosome analysis, and dPCR for the expression of miR-21. Both NTA and dPCR were optimized with the aid of synthetic particles and synthetic miRNA, respectively.

NTA has been adapted for the analysis of microvesicles in many studies [175] and is shown to be efficient for determination of both size distribution and relative concentration of microvesicles in biological fluids [176]. NTA was found to be time consuming and not easily reproducible during initial trials. For this reason, other techniques, including Diffusion Light Scattering (DLS), are commonly used to detect, quantify, and phenotype exosomes. When comparing both NTA and DSL techniques in the literature, it was concluded that for polydispersed samples, NTA was more sensitive[72]. However, NTA has varied software settings, thus optimization was

required to determine the right balance for analysis of exosome preparations.

Digital PCR, a relatively new PCR technique, is a more precise method than qPCR[177]. dPCR is a method used to directly quantify nucleic acids where qPCR involves relative quantification. dPCR incorporates cDNA into droplets where each droplet contains a single cDNA molecule. Each droplet contains also all reagents necessary to amplify, and subsequently count, a target of interest. The high level of quantitative sensitivity of dPCR accommodates measuring very small amounts of RNA extracted from exosomes. Since the partitioning technology of dPCR greatly improves signal to noise separation, the dPCR technique is essential to our study.

MiR-21 is the focus of our study because it is a known onco-miRNA [178] that targets tumor suppressors [179]. In addition, upregulation of miR-21 in primary breast cancer samples is shown to be associated with advanced disease and poor prognosis [173]. In previous experiments, we found that miR-21 was upregulated in cell media exosomes of the breast cancer cell line MCF7, but not in serum exosomes of healthy women (Fig. 2). Thus, we used a synthetic miR-21 sequence to optimize our assays. In conjunction, miR-21 expression levels in exosomes of healthy control samples were assessed.

Nanoparticle Tracking Analysis

Background and Rationale

NTA is a method widely used to estimate concentration and size distribution of exosomes [180, 181]. NTA uses the light-scattering characteristics of laser light on particles undergoing Brownian motion when in solution. A video recording of the

particles is analyzed, particles are tracked, and a hydrodynamic radius is determined and displayed as a particle size distribution [176].

A 60-second video records each sample at 20 frames per second. Particles in a fluid move under Brownian motion and the NTA software uses the hydrodynamic mobility of the exosomes to estimate their size distribution. Exosomes are 30-120nm in diameter and NTA can accurately size particles, which is of use in determining the quality of our exosome preparation [72]. NTA information regarding size distribution and quantification of exosomes further enables normalization of downstream dPCR results.

With the original protocol, we recorded large-concentration fluctuations with repeated NTA measurements from the same exosome preparation. Optimization of the method involved changing software settings such as camera level and detection threshold depending on sample clarity and particle behavior. The overall volume of each sample drawn into the syringe used to inject the sample into the viewing chamber varied from 300uL to 1mL. Injections of samples into the viewing chamber were performed manually applying varying volumes and speeds. Inconsistent results were observed and thus, further optimization of the approach was necessary.

Methods

Samples

Serum samples from 19-healthy women were obtained through Resource for Genetic Epidemiology (IRB# 38201).

Exosome Isolation

Exosomes were isolated using ExoQuick (SBI) following manufacturer's instructions [182, 183]. In brief, 500 μ L serum was centrifuged for 15 minutes at 3000 \times g to pellet any cells. The supernatant was transferred to a new vial and incubated with 126 μ L ExoQuick. After an overnight incubation at 4°C, each tube was centrifuged for 30 minutes at 1,500 \times g to pellet exosomes. The supernatant containing free-circulating miRNAs was discarded. Our standard practice following exosome isolation is to re-suspend in 210 μ L phosphate buffered saline (PBS) and store at -80°C. Most of the exosome preparation is dedicated to miRNA extraction, but 10 μ L aliquots are reserved for exosome quantification with NTA.

The Final NTA Protocol

Prior to analysis, exosomes were diluted in purified PBS (Fisher Scientific) to obtain views of 50 to 100 particles per frame (10^8 to 10^9 particles/mL). The first 100 μ L was not measured but moved through the testing cell and out the other side. The diluted samples were analyzed in duplicate within 5 minutes. The viscosity of the fluid was entered (1.05cP at room temperature for PBS) and adjusted automatically on the basis of temperature measurements. The temperature of the cell was measured manually, and fluctuated between 22°C and 23°C throughout the nanoparticle tracking process. The camera level was set consistently at '14'. The sample was injected slowly via a sterile 1mL syringe into the viewing chamber and was advanced with a syringe pump. The particles were examined twice at a flow rate of 50 and 75, respectively. On average, 75 particles were observed in the field of

view and the typical concentration was approximately 10^9 particles per mL. Five 60-second videos were recorded at 20 frames per second and were analyzed using the NTA software. The detection threshold was set at '5'. The final analysis of the video data assessing the hydrodynamic mobility of particles reported the exosome size distribution and concentration.

Results

Optimization of NTA

NTA was performed using a NanoSight (Salisbury, UK) instrument (model LM10) by illuminating the samples with a 65 mW violet laser (405-nm wavelength). The light scattered by exosomes was captured with a high-sensitivity scientific CMOS camera (OrcaFlash2.8, Hamamatsu C11440). Results were analyzed using the software provided by the manufacturer (NanoSight version 2.3). The samples were diluted and introduced into the testing cell where the concentration was assessed. Subsequent dilutions were adjusted until approximately 50 to 100 particles in the field of view were observed. The standard measurement consisted of five 60-second video captures. The samples were manually advanced after each capture.

1. Adjusted Method

The PBS buffer was examined at various detection threshold settings and a detection threshold of '7' was determined to exclude noise. To rule out any exosome-related effects on the results, size-calibrated standard particles of known concentration (1 to 5×10^8 , $100\text{nm} \pm 5\text{nm}$) (NanoSight) were interrogated. These

particles were measured according to the protocol described above but with 10- video captures instead of five. A consistent concentration of approximately 50 particles per frame was expected with a size around 100nm. Instead concentrations varied depending on injection volume and rate. Sizes measured from 83 to 120nm and did not seem to be affected by injection volume (Table 2, and Fig. 5).

Table 2 NTA results of 10 successive measurements obtained by manually advancing a solution of standard particles. Concentration and particle size were measured in relation to each of the 10 advanced volumes. All but three captures (in red) were advanced with 50 μ L. The captures represented in red were advanced with 150 μ L, 300 μ L, and 150 μ L respectively, for which NTA predicted increasing concentrations. Notably, with advancements of 50 μ L, the concentration steadily decreased. The effect on concentration was due to a static/kinetic issue; time is needed to allow for particles to 'settle' before capture. Size was not affected.

Standard Particles (100nm)

Number of Captures	Volume Advanced (μL)	Concentration (particles/frame)	Particle Size (nm)
1	50	54.2	87
2	50	56.1	94
3	50	41.5	105
4	50	30.5	98
5	150	38.1	120
6	300	52.4	86
7	150	58.4	97
8	50	48.8	105
9	50	38.3	89
10	50	33.9	83

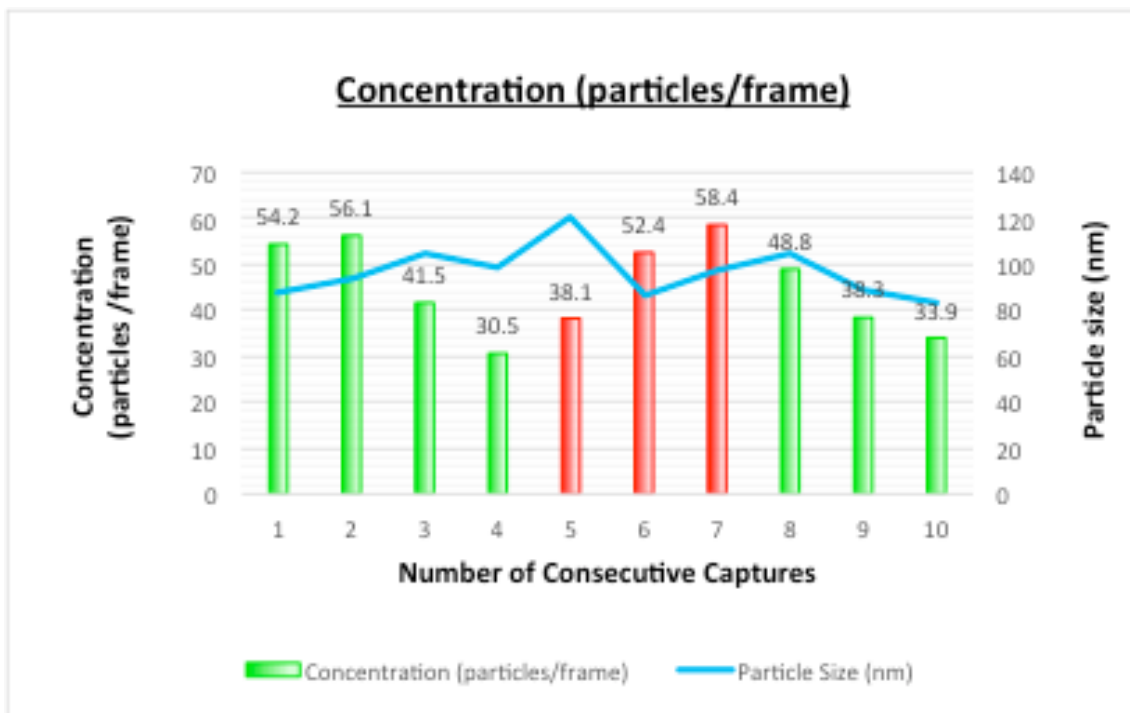


Fig. 5 Graphic representation of NTA concentration and size results shown in **Table 2**. Measurements in green show sample advancements of 50 μ L; red shows advancements of 150 μ L, 300 μ L, and 150 μ L, respectively. Notably, with advancements of 50 μ L, the concentration steadily decreased, where with larger advancements, concentration increased. Size (in blue) was not affected.

2. Adjusted Method

More consistent results were found when the first 100 μ L of liquid was run through the cell without measuring the concentration. Then a 30-second wait period was introduced after each advancement was performed. The wait period allowed the particles to settle before the capture. This adjustment provided more reproducible results. Alternatively, use of a syringe pump, which advances the sample at a set rate, obtained even more reliable results. The flow rate of the syringe pump was set between '50' and '75' to obtain a y-drift of 8 pixels/second. The y-drift refers to the up-and-down movement of particles being tracked as they undergo Brownian motion. The standard particles were evaluated multiple times

with the optimized protocol, which proved to yield reproducible numbers (Fig. 6).

3. Established Negative Control

The negative control was established with the 19-healthy female control samples obtained through Resource for Genetic Epidemiology. Dilutions were made so that the camera level could be kept constant at setting '14'. Subsequently the 19 control samples were run with the optimized settings in duplicate (Fig. 7).

Discussion

Exosome concentration and size distribution data of the 19-control samples were initially not reproducible. This variability was due to inconsistent camera level and detection threshold settings, differences in total volume of samples used for injection, inconsistent rates at which samples were advanced, and differences in settling times between captures.

We adjusted the parameters to be more consistent with the volumes drawn into the syringe, the rate at which the liquid entered into the test cell, the camera levels, and detection threshold settings.

In particular, we used 1mL for each sample, and advanced the solution during readings by use of a syringe pump. The syringe pump was set to obtain a y-drift of approximately 8 pixels per second to best accommodate the narrow bore (1mL) syringe body. We learned that a narrow-bore syringe had the tendency to generate inconsistently high pressures and thus could affect the flow of particles in the test cell. Also, we observed a direct relationship between the volume used for

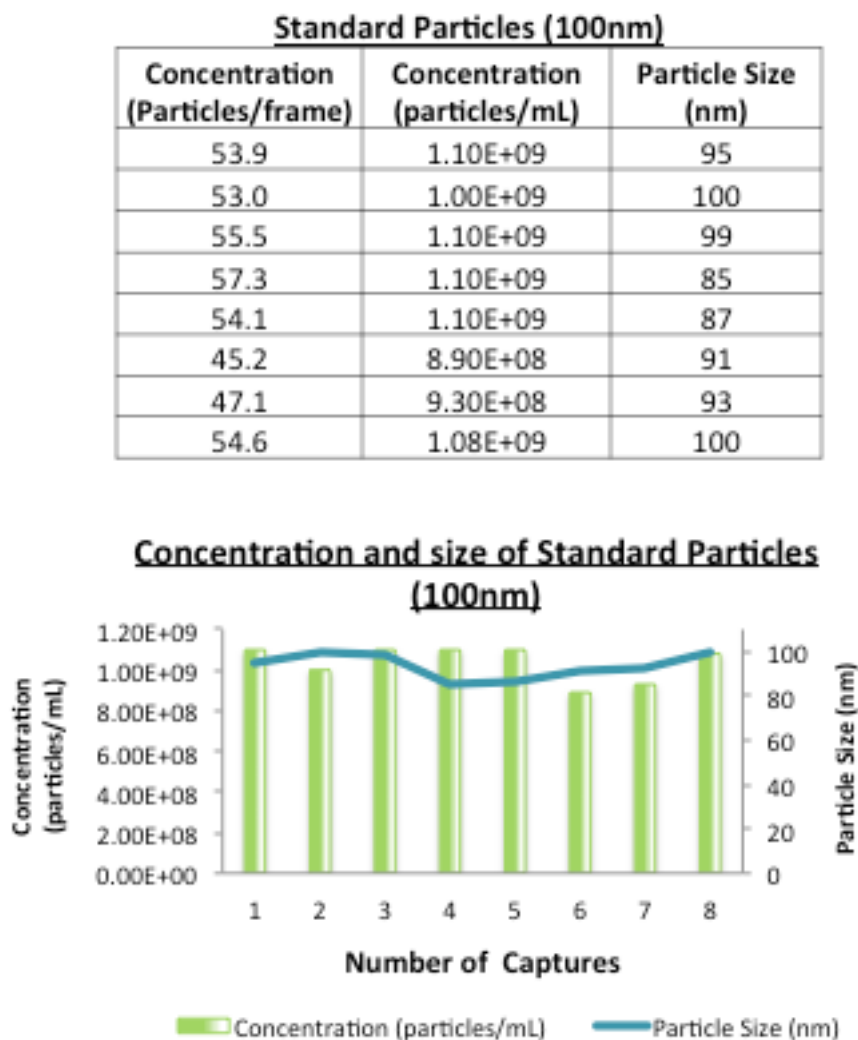


Fig. 6 NTA concentration and size measurements of standard particles (100nm) using syringe pump. The sample was advanced at a syringe-pump speed set at 75. Size is shown in blue, concentration in green.

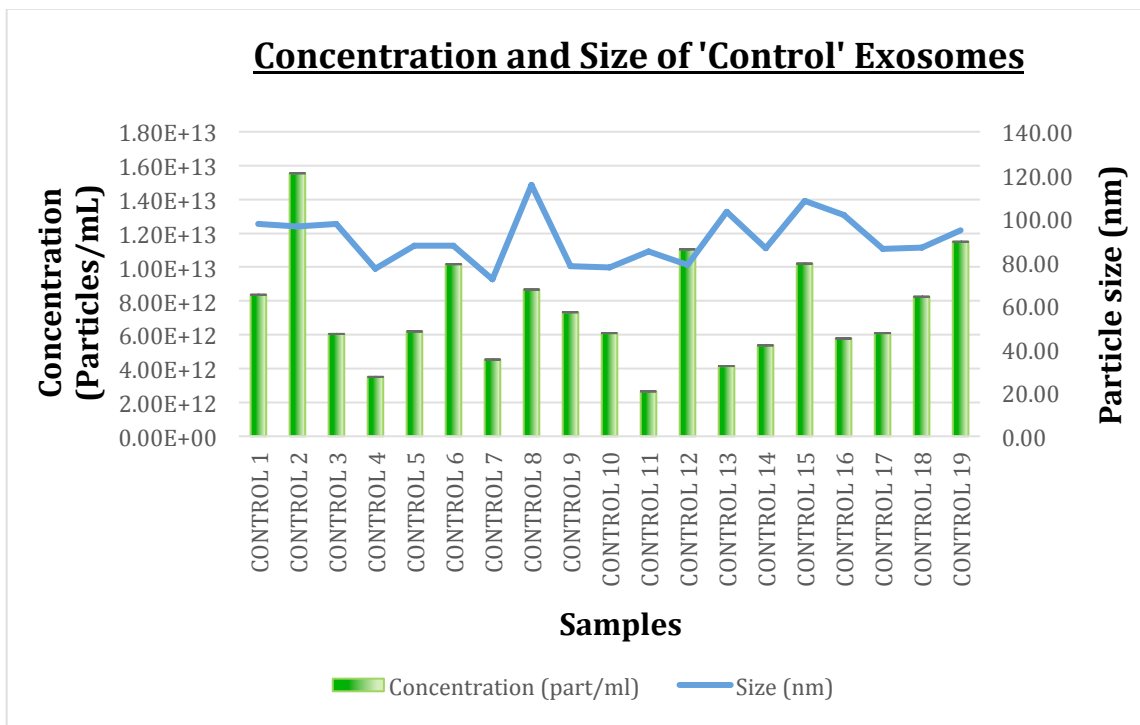


Fig. 7 The concentration and size of 'healthy'-control exosomes. Exosomes of 'healthy' subjects were measured in duplicate and the average of the two measurements is depicted. No trend is detected.

advancement and speed at which the fluid moves into the cell; slower moving particles in smaller volumes have more time to settle compared to the faster moving larger volumes that have less time. In addition, if the first 100uL were pumped through the system prior to taking any measurements, then results were more consistent due to more consistent pressure.

Since exosome preparations are polydispersed samples and can have less reproducible profiles, more reliable data were obtained when more particles were evaluated. Consequently, a continuous-flow rate of particles was maintained during each capture and samples were run in duplicate.

Camera level was kept constant at '14', which allowed for particles to be seen clearly without saturation. The detection threshold was set at '5' to avoid 'noise' and simultaneously to include all particles of interest. See the resulting NTA protocol under the Methods section above.

The negative controls were analyzed at the same time the NTA protocol was optimized. No trend was observed. Varied exosome concentrations and sizes among the 'healthy' control samples were measured (Fig. 7). These results will be compared to exosome samples isolated from breast cancer patients. The varied NTA results of 'healthy' control samples might not be useful for normalization of miRNA numbers obtained with dPCR. Finally, in future experiments, serum samples will be divided into two aliquots before exosome isolation to control for reproducibility.

Digital PCR (dPCR)

Background and Rationale

Digital PCR (dPCR) is a new PCR technology. The main innovation behind dPCR is that the sample is separated into droplets. Each droplet serves as an individual PCR reaction providing exceptional sensitivity and precise quantification of target nucleic acids. In contrast to standard qPCR, dPCR is not dependent on the number of amplification cycles to determine the initial sample amount and hence is a more sensitive and reliable measurement of nucleic acid numbers.

Before the dPCR step, reverse transcription of exosomal miRNA is performed according to an Applied BioSystems TaqMan® assay protocol. After the reverse transcription, the cDNA is then incorporated into droplets using the RainDrop Digital PCR System (RainDance Technologies) such that each droplet contains a single cDNA molecule. Each droplet also holds all reagents necessary to amplify a miRNA target(s) of interest (e.g. miR-21), including a pair of unlabeled PCR primers and a TaqMan probe. An absolute number of miRNA copies per sample input is then calculated from the number of droplets containing a target sequence versus others that do not. Since small amounts of miRNA are extracted from serum exosomes, it is critical to use a highly precise method for quantification.

The new TaqMan® Advanced miRNA Assay was employed because it provided higher sensitivity and more versatility compared to the previously used TaqMan Small RNA Assay for reverse transcription (Fig. 8).

The original TaqMan Small RNA Assay employed a target-specific primer during cDNA synthesis. The protocol required 10uL of miRNA to yield 30uL

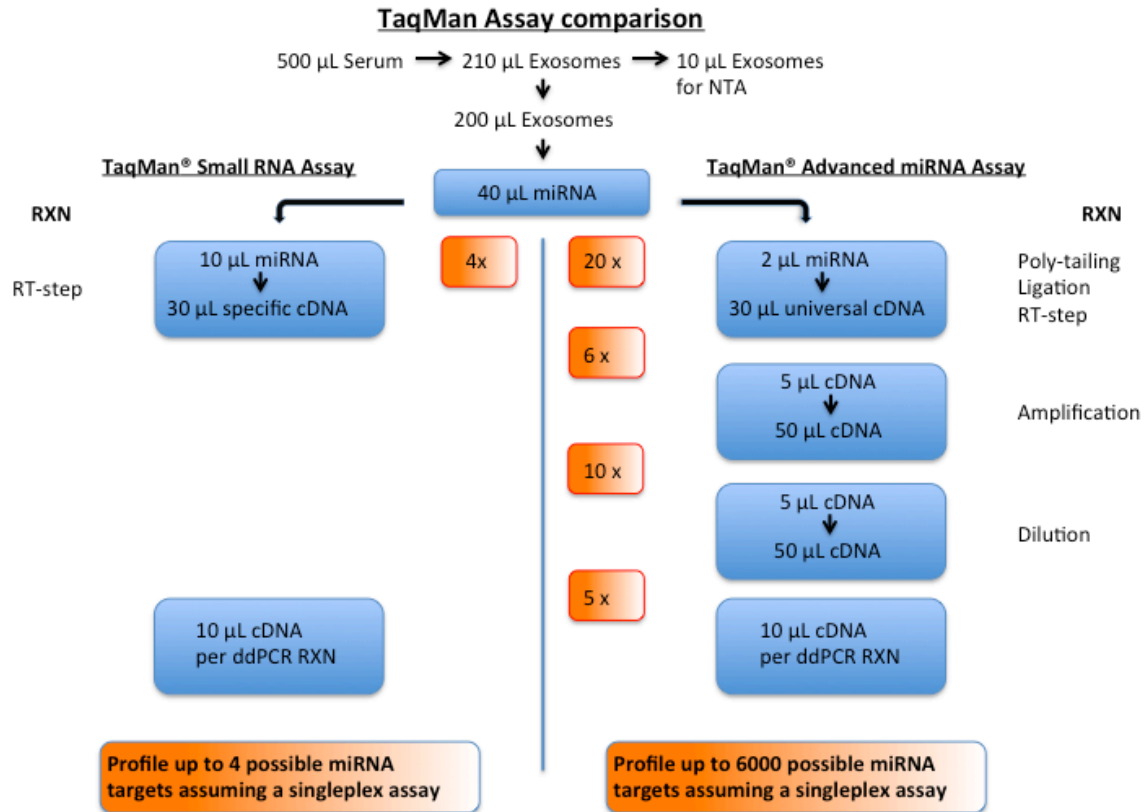


Fig. 8 Comparison of the TaqMan miRNA versus the new TaqMan Advanced miRNA assays. The newer advanced assay allows for exponentially more targets to be profiled (up to 1500 fold). In addition, cDNA amplification allows for expression analysis of very low input miRNA samples.

target-specific cDNA. For each sample, the total miRNA preparation was 40uL, thus only up to four targets could be profiled (assuming a singleplex assay). In 2015, Applied BioSystems came out with the TaqMan® Advanced miRNA Assay. This assay uses a universal RT step and a universal miR-Amp step, which amplifies cDNA to enable more sensitive detection during PCR. Now 2ul of miRNA, not 10uL, is required and could render enough universal cDNA to profile up to 300 targets instead of only one target (assuming singleplexing). That means a 1500-fold increase from the first generation TaqMan assay (Fig. 8). The advanced TaqMan RT

step has a very different approach from the former assay and includes cDNA amplification, thus the subsequent dPCR assay needed optimization.

As described above, miR-21 is the focus of this study. MiR-21 was primarily selected because it is a widely studied onco-miR that is known to be upregulated in the circulation of breast cancer patients. A synthetic miR-21 sequence was used in the process of optimizing the dPCR protocol and was employed as positive control for further experimentation.

Methods

Samples

As previously described, exosomes were isolated and quantified from serum samples from 19-healthy women with the methods described above in section 2.2.1. From the total exosome preparation of 210 μ L per subject, a 5 μ l aliquot of exosome suspension from each control sample was pooled. The pooled samples and the remaining 200 μ L of each sample separately underwent miRNA extraction.

MiRNA Extraction

MiRNA was extracted using the Total Exosome RNA and Protein Isolation Kit (Invitrogen) following manufacturer's procedures[184] with a final elution volume of 40 μ L. Samples were combined with 200 μ L of 2X Denaturing Solution, lysed by vortexing, and then incubated for 5 minutes on ice. 400 μ L of Acid-Phenol: Chloroform was added and vortexed for 60 seconds. Samples were centrifuged (5 minutes at 10000 g) to separate the mixture into aqueous and organic phases and

the upper, aqueous phase was transferred to a fresh tube. 133 μL of 100% EtOH was added, vortexed, and transferred onto a column. After centrifugation (30 seconds at 10000g), the flow-through, which contains the small RNA, was combined with 300 μL of 100% EtOH, transferred onto a new column, and spun down (30 seconds at 10000g). The flow-through was discarded and the column went through two washing steps, after which it was placed into a fresh tube. The RNA was eluted in 40 μL RNase-free water and stored at -80 C for downstream RNA-sequencing and RNA profiling by digital PCR.

Spike-in Synthetic miR-21

Five aliquots of each 2 μL of the pooled control sample were spiked with various concentrations of synthetic miR-21. The duplex sequences for synthetic miR-21-5p used were AmACmAUmCmGUmCUmGAmUAmAGmCUA and 5Phos/UAGCUUAUCAGACUGAUGUmUmGA (Integrated DNA Technologies) for the anti- sense and sense strands, respectively. (m is 2'-O-methyl, a common nucleoside modification of RNA, which acts to prevent degradation.)

RT-step

The miRNA was reverse transcribed with the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied BioSystems) following manufacturer's instructions. The TaqMan Advanced miRNA cDNA Synthesis Kit uses 3' poly (A) tailing and 5' ligation of an adaptor sequence before reverse transcription. Universal RT primers bind to the universal sequences attached to both the 5' and 3' miRNA ends so that all

miRNAs are reverse transcribed to cDNA. The cDNA is then pre-amplified with universal master mix and primers that recognize the 5' and 3' universal sequences. Therefore, the amount of cDNA for each target is uniformly increased while maintaining the relative differential expression levels. cDNA was stored at -20 C until profiling by dPCR.

dPCR

We used TaqMan Advanced miRNA Assays to quantify miRNA targets using dPCR. Applied Biosystems provided the miR-21 TaqMan assay amplification primers and probes. The actual sequences are proprietary (assay ID# 477975). dPCR reactions were prepared to a 25 μ L final volume, using 12.5 μ L TaqMan Fast Advanced Master Mix, 1 μ L TaqMan Advanced miRNA Assay (20X), 0.5 μ L RNase free water, and 1 μ L Drop Stabilizer (RainDance Technologies). Droplets were generated on the Raindrop Source chip (RainDance Technologies) and eluted into PCR tubes. Amplification was done on a thermocycler using the following settings: 95°C for 10 minutes, 50 cycles of 95°C for 15 seconds, 58°C for 15 seconds, 60°C for 45 seconds (slow ramp speed 0.5°C/second), and finally 98°C for 10 minutes. After PCR, samples were transferred into the RainDrop Sense chip (RainDance Technologies) for single fluorescent droplet detection. The data were analyzed using the RainDrop Analyst Software V2 (RainDance Technologies). A count of negative versus positive droplets was recorded.

Results

Optimization of dPCR

Optimization of the dPCR reaction was necessary after changing to the Advanced TaqMan cDNA Synthesis Kit from the previously used TaqMan assay. The reverse transcription method is very different from the former assay. In particular, the cDNA amplification step in the current assay required optimization of the downstream dPCR settings.

The serial dilutions of a synthetic miR-21 were spiked-in with the pooled healthy control miRNA and were optimized. It was initially found that the miRNA concentrations were too high and thus were further diluted to .01nM, .005nM, .002nM, and .001nM, respectively (Fig. 9).

After these modifications, the results of the synthetic miR-21 serial dilutions were consistently linear. The linearity of the serial dilutions of synthetic miR-21 spiked into the pooled control sample reflects the accuracy of the dPCR (Fig. 10). The number of molecules detected was on average 15-fold greater than the number of starting molecules. This indicates that the advanced TaqMan cDNA amplification produces on average 15 times the input molecules (Table 3).

The 19-negative controls were subsequently analyzed with dPCR, including a no-template control, and a synthetic miR-21 positive control. Very low (≤ 50 molecules) miR-21 concentrations were observed in control samples. Controls 5, 6, 8, and 19 reported higher miR-21 numbers constituting total counts of 306, 185, 305, and 434, respectively. In Control 14, 3678 positive droplets were detected; this unexpected result necessitates further study as it may indicate a newly developed

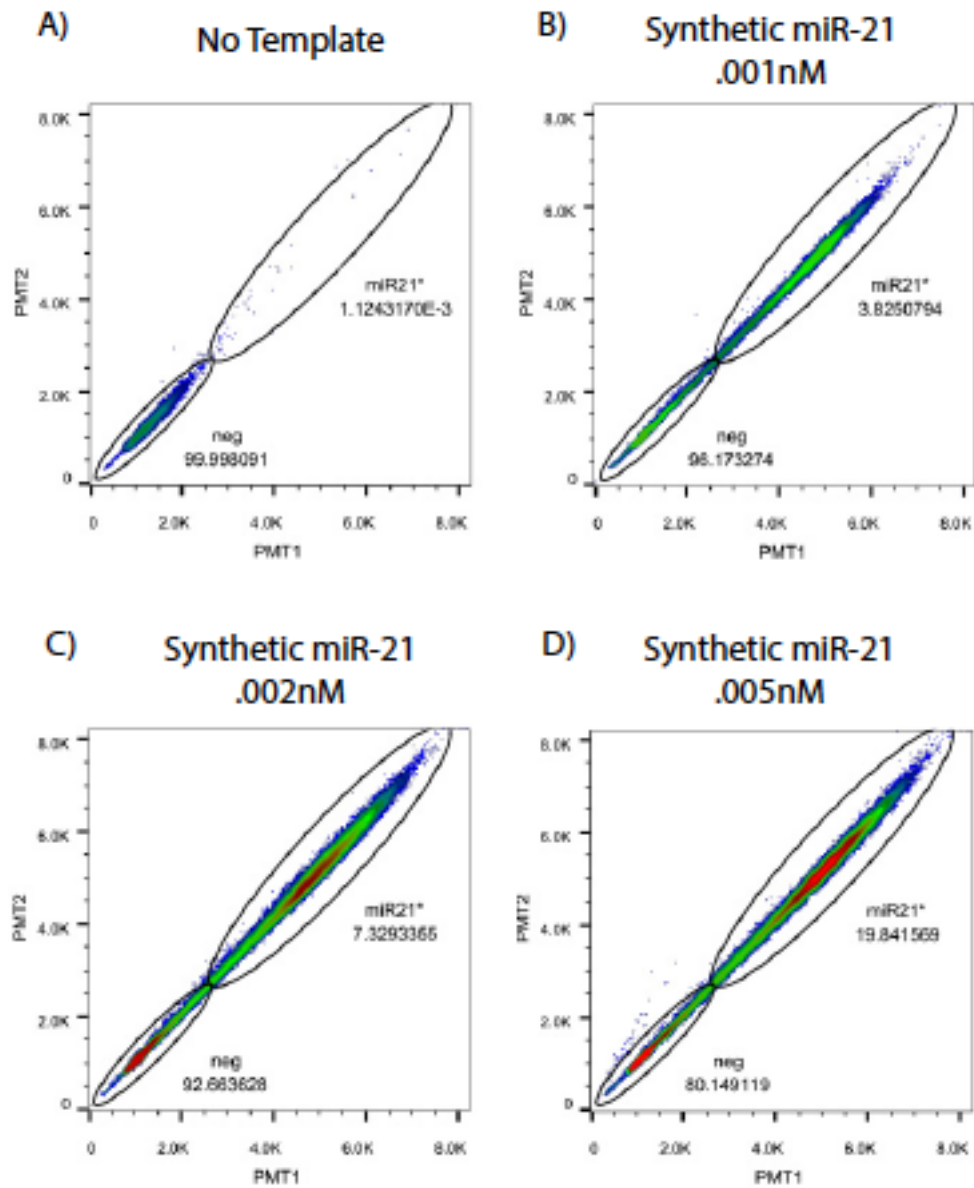


Fig. 9 dPCR dot plots of A) a non-template control, and B)-D) synthetic miR-21 spike-ins of .001nM, .002nM, and .005nM, respectively.

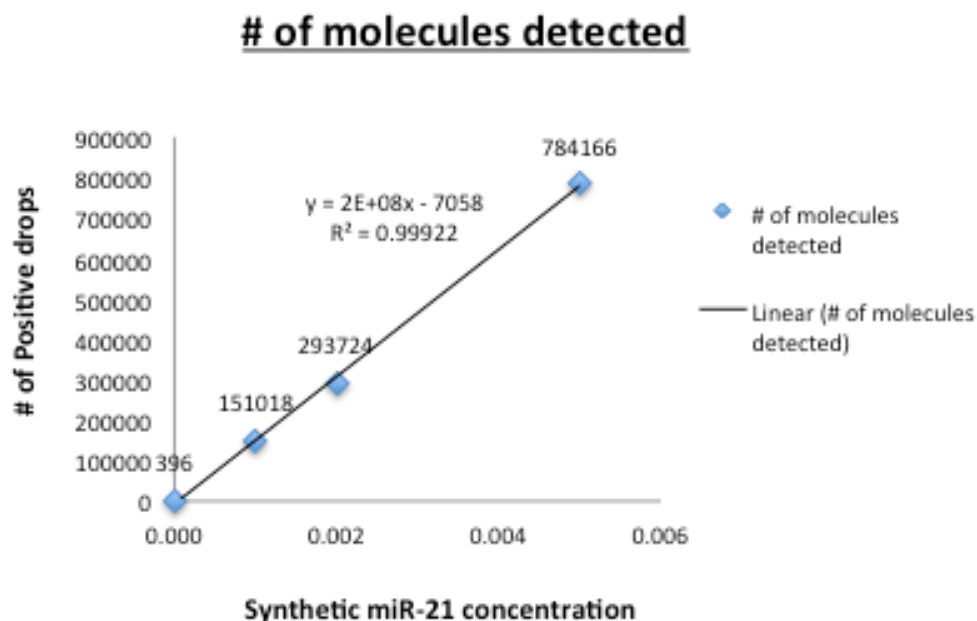


Fig. 10 dPCR results of various concentrations of synthetic-miR-21 spiked into the control pool. Serial dilutions of miR-21, .005nM, .002nM, .001nM, and 0nM, respectively, were spiked into the pooled control sample. The numbers of miR-21 molecules detected were 784,166; 293,724; 151,018; and 396, respectively. The .01nM miR-21 concentration was omitted because saturation of droplets resulted in an inconsistent result.

Table 3 dPCR revealed a 15-fold increase in the number of molecules after cDNA amplification and dPCR with a .6x TaqMan assay. cDNA amplification is part of the protocol of the advanced TaqMan assay.

Average of 15 fold cDNA amplification with advanced TaqMan assay			
miR concentration entered into rt-step (nM)	molecules expected without amplification	molecules detected after amplification	fold increase
0.001	10000	151018	15.1
0.002	20000	293724	14.7
0.005	50000	784166	15.7

cancer in a patient that was considered cancer free, if simply not a false positive result (Fig. 11).

Discussion

The newer TaqMan Advanced miRNA cDNA Synthesis Kit is very different from the first-generation TaqMan assay, which created issues with the dPCR protocol. The universal RT-step and the cDNA amplification of the advanced assay provide the benefits of requiring less sample input and more miRNA targets to be profiled.

Sample	miR-21
PBS	29
miR21_001	59707
Control 1	6
Control 2	6
Control 3	4
Control 4	33
Control 5	306
Control 6	185
Control 7	13
Control 8	305
Control 9	18
control 10	41
control 11	42
control 12	30
control 13	20
control 14	3678
control 15	33
Control 16	42
Control 17	99
Control 18	43
Control 19	434

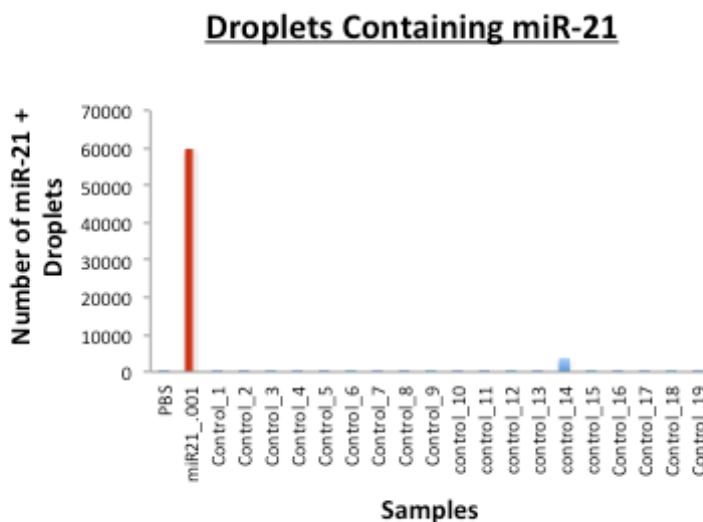


Fig. 11 dPCR data of the negative control samples and .001nM synthetic miR-21 as positive control. The results in the table appear in the graph. In one control sample, control 14, 3678 positive droplets were detected and might reflect the presence of cancer or contamination. Synthetic miR-21 shown in red.

The new assay and the synthetic positive control miR-21 were optimized simultaneously. The spiked-in positive control miR-21 with known miRNA concentrations allows for normalizing dPCR results of target miRNAs with unknown concentrations (Fig. 11).

To test the TaqMan assays' ability to detect the synthetic miRNAs and to determine the right concentration of synthetic miRNA, serial dilutions of synthetic miR-21 were made and spiked into the miRNA of the pooled negative control sample. Initially, poor cluster separation and an unpredictable negative cluster were observed in the dot plot.

Cluster location is assay-specific. Another way to optimize cluster position and separation is by adjusting probe concentrations. The problem is that TaqMan assays come in a premixed tube and the probe concentration alone cannot be adjusted. Instead, decreasing the amount of miRNA-assay mix results in decreasing the amounts of other reagents as well, including primers. We found that lower primer concentration results in decreasing positive droplet numbers (Fig. 12).

In the initial data, the negative cluster moved closer to the positive cluster at higher concentrations (Fig. 13). This was due to saturation. When droplet occupancy becomes satiated, the droplets read by the instrument as 'negative' actually test to be mostly positive. The system under these conditions cannot distinguish negative from positive with clarity. Therefore, the synthetic-miR-21 solution was further diluted to 0.01nM, 0.005nM, 0.002nM, and 0.001nM, respectively. Saturation was still observed in the 0.01nM dilution but the other data points were consistently linear. But when droplet occupancy exceeds 10%, a Poisson-statistical correction

nM	# of molecules after rt	TaqMan assay	nrs after amplification detected with dPCR	fold increase
0.001	4000	1x	70977	17.74
0.001	4000	.75x	65545	16.39
0.001	4000	.50x	53320	13.33
0.001	4000	.25x	37424	9.36

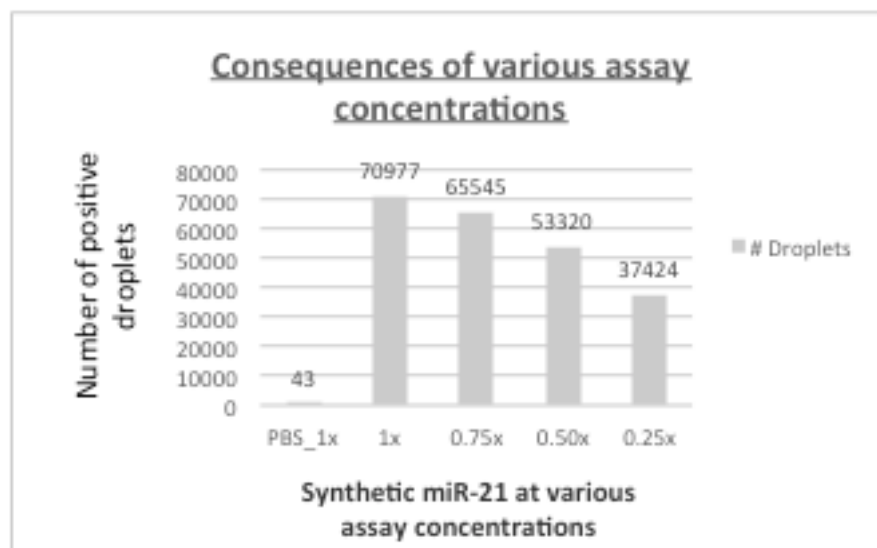


Fig. 12 Consequences of varied TaqMan assay concentrations on detection of the number of positive droplets. While the input of synthetic miR-21 molecules was constant at .001nM, the measured number of positive droplets decreased with decreasing TaqMan assay concentrations; 1x, .75x, .5x, .25x, respectively.

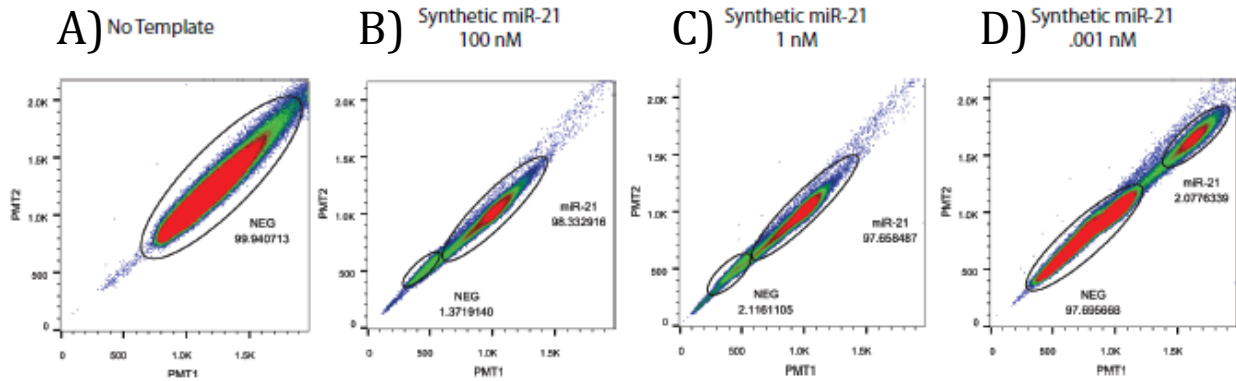


Fig. 13 Cluster movement due to saturation. A) no template control, and synthetic miR-21 at various concentrations from B) 100nM and C) 1nM, both showing saturation, to D) 1nM

has to be applied (Fig. 10).

In addition, poor cluster separation might be due to either weak fluorescent-probe signal or high-probe background noise. As an attempt to increase the fluorescence signal in the positive droplets, the number of PCR cycles was increased by 10, which resulted in 60 cycles total. However, this high number of cycles may have introduced additional errors in the assay. Recently, TaqMan probes and primers became available separately. In future experiments, better cluster separation could be achieved by optimizing probe concentration while keeping a lower number of cycles (≤ 50).

Final dPCR Protocol

Digital PCR reactions were prepared as mentioned above but the PCR amplification was run at 60 cycles instead of 50 to maximize the fluorescent signal. Furthermore, the amount of each TaqMan Advanced miRNA Assay (proprietary

probe and primer mix) added to the reaction requires testing beforehand since the cluster location is assay-specific.

While optimizing the dPCR protocol, a negative control was established. All 19 samples were pooled and profiled for miR-21. The results showed more miR-21 positive is planned in the future. Also, the analysis of 'control14' has to be repeated to exclude a false positive result; small volumes that are amplified are prone to contamination. If this is indeed an individual that has cancer, then this indicates that screening patients for exosomal miR-21 can detect early onset of cancer.

With the current control-sample size of 19 when combined with a lack of information on 'control 14', a noise threshold was preliminarily set at 4000 detected miR-21 molecules. After additional control samples are analyzed, a more accurate noise threshold could be set. Also by following up with these individuals to determine whether they develop cancer, a more precise cut-off line might be established.

Future

In future experiments, the synthetic miRNAs will be added to the exosome preparation instead of the extracted miRNA. For example, cel-miR-39 will be spiked into the exosome solution and will eventually serve as the positive control for all downstream experiments. This would allow us to control for miRNA extraction as well as for downstream cDNA synthesis and dPCR.

Results could be more accurately normalized if endogenous and exogenous control miRNAs could be profiled along with the miRNA target of interest. dPCR

assay multiplexing uses both distinct assay ‘colors’ (e.g. FAM and VIC) and probe ‘intensities’ to quantify each target molecule. But with a premix TaqMan assay, the probe concentration alone cannot be adjusted, as mentioned above, which also proves to be an issue when multiplexing. TaqMan probes and primers became available individually, and will be used in future experiments. Therefore, different assay-probe concentrations will be tested, for example, by adding the probe to something other than 1x in the reaction such as 0.75x or even 0.5x. For comparison, one miRNA-probe could be run at 1x and one at 0.5x. Thus, when multiplexing, finding the right probe concentrations of the assays requires experimenting to obtain proper cluster separation.

Determine Exosomal miRNA Profiles from Blood Samples Taken

Pre- and Post-surgical Resection from Breast-cancer Patients

to Develop Personalized Exosomal Non-coding RNA Panels

Background and Rationale

Presence or absence of breast tumor is expected to be reflected in circulating exosomal miRNA content [103]. miRNAs that are upregulated in the tumor and in the circulation prior to surgical resection are expected to be downregulated in the blood post-surgical resection and vice versa. In addition, it is expected that the exosomal miRNA content differs significantly between breast-cancer patients and healthy individuals. The goal is to develop a personalized miRNA panel, which will allow for closer monitoring of each breast cancer patient prospectively.

For each individual, circulating exosomal miRNAs from the blood will be

interrogated pre- and post-breast tumor resection. This will allow us to determine whether changes in circulating miRNAs reflect tumor presence. In addition, circulating miRNAs from these cancer patients will be compared with those of healthy age-matched women, if possible. This comparison will allow for further development of breast cancer-specific miRNA panels.

Our prospective plan is to assess total RNA content by RNA-sequencing. Genes uniquely expressed in pre-surgery circulating exosomes, but not expressed in post- surgery circulating exosomes, will be identified. DPCR will be used to validate these potential markers and to follow up patients prospectively.

In this study, we focused only on miR-21 as a pilot project. As mentioned above, miR-21 is a known onco-miR that is upregulated in the circulation of breast cancer patients. Furthermore, we have already identified in a previous study (unpublished) miR-21 as uniquely expressed in breast cancer cell line MCF7 and not in healthy women (Fig. 14).

We compared exosome size and concentration as well as miR-21 profiles of pre- and post-tumor resection serum of breast cancer patients. Also exosomes and miR-21 levels in serum of healthy women were analyzed and compared to the breast cancer samples.

Methods

Serum samples from 5 women with breast cancer pre- and post-surgery were obtained from the Bio-repository core at the Huntsman Cancer Institute (IRB # 10924). All five individuals had low-stage breast cancer, ranging from IA to IIB.

Ages ranged from 45 to 79 at the time of surgery (Table 4).

The same array of experiments as in Aim 1 was performed; exosomes were isolated, miRNA was extracted, and cDNA was synthesized. DPCR was used to validate miR-21 numbers.

- Exosomes were isolated from 500 μ L serum using ExoQuick (System Biosciences) and then resuspended in 210 μ L pure PBS.

The exosome concentration and size distribution was then characterized by NTA.

- miRNA was extracted from 200 μ L exosome suspension using the Total Exosome RNA and Protein Isolation Kit and eluted in 40 μ L RNase free water.

miRNA was subsequently reverse transcribed with the TaqMan Advanced miRNA Assay using 2 μ L. The cDNA was then profiled for miR-21 with dPCR. The molecular quantification indicated whether the levels of miR-21 found to be present in exosomes of breast cancer patients differed between pre- and post- resection serum and between healthy individuals.

Results

NTA Results

There was no significant difference detected between exosome size and concentration of breast cancer serum samples and 'healthy' controls or between pre- and post-resection samples. Standard error was determined by NanoSight software version 2.3 (Fig. 15).

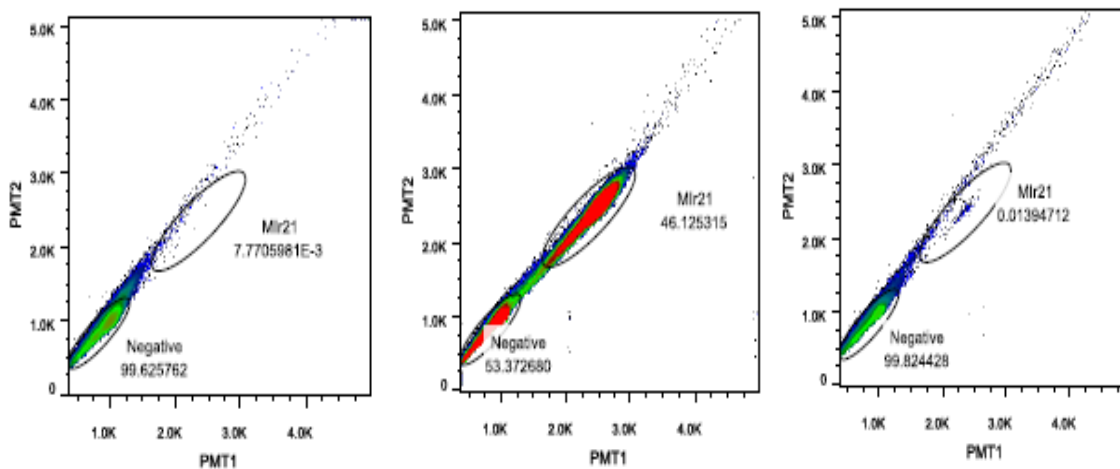


Fig. 14 Quantification of miR-21 expression by ddPCR: (A) empty template control; (B) number of miR-21 molecules in exosomes of MCF7 cells; (C) and normal female serum

Table 4. Patient phenotypes enrolled in this study. All 5 individuals had early stage breast cancer (IA-IIB).

Shadow ID	Pre-treatment (days)	Post-treatment (days)	age at Diagnosis	Grade	Stage		
					T (size)	N (node)	M (metastasis)
2Z2HMP	-11	11	68	1	1A T1a	N0	M0
JQE6NC	-16	11	63	1	1A T1b	N0	M0
PJREBY	-18	9	45	2	1B T1c	N1mi	M0
93WMMV	-24	26	79	3	1A T1c	N0	M0
9K66Q2	-13	28	47	2	11B T2	N1mi	M0

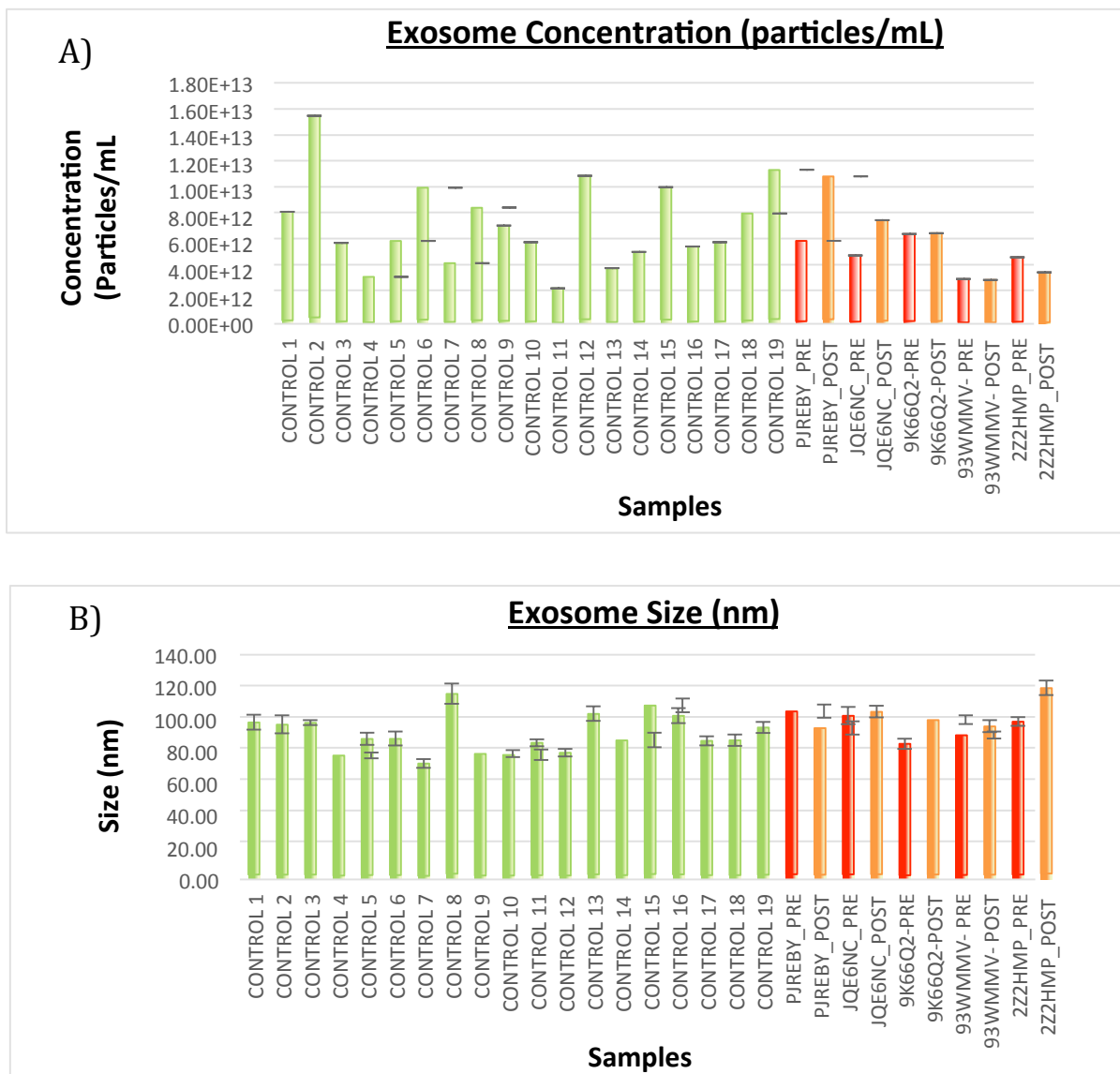


Fig. 15 NTA results of negative control samples (green) and pre- (red) and post- (orange) resection breast cancer samples A) Exosome Concentration, B) Exosome Size

dPCR Results

As expected, breast cancer samples showed a much higher concentration of miR-21 compared to the control samples. Unexpectedly, there was no constant trend in miR-21 expression in pre- versus post-tumor resection serum. Out of the five patients, three show a decrease in miR-21 numbers after tumor resection as expected, but two show an increase in miR-21 after surgery (Fig. 16, see also Fig. 17).

Discussion

NTA

In contrast to what we expected [101, 102], there was no difference observed in exosome concentration between pre- and post-surgery serum, nor was there a distinct trend detected between the breast cancer samples and the healthy control samples. The lack of distinct differences between the cancerous and 'healthy' exosomes might be explained by the small sample size, and the early stage of disease in all five patients, if differences are to be found. Also, exosomes were found to be involved in inflammatory response [185]. Where surgery might cause inflammation, elevated exosome concentrations would be present in post-tumor resection samples.

dPCR

In disagreement with our expectations, exosomal miR-21 profiles did not significantly differ between pre- and post-surgery blood samples. These results may

be explained by the small sample size (5). A larger sample size should be investigated.

While it is important to have endogenous reference controls to normalize expression levels of candidate miRNA targets, such controls have not yet been discovered for exosomal miRNA [186, 187]. This can be explained by previous studies reporting that exosomal miRNAs are selectively packaged by parent cells [33] and thus exosomes have different miRNA compositions compared to their parent cells [48]. Therefore, reference controls for normalization of tissue miRNA are not suitable for exosomal miRNA normalization. Exogenous spike-in controls, such as cel-miR-39, have been reported but have limitations since they cannot correct for miRNA extraction variations [188]. Based on a literature search, we found eight exosomal miRNA reference candidates (Table 1). However, further testing needs to be done to determine whether these eight miRNAs maintain constant levels in healthy controls and breast cancer samples.

The fact that the patients have early stage breast cancer may explain the absence of differentially expressed exosomal miR-21 in pre- and post-resection blood samples. Thus our methods might not be sensitive enough, and we might have to look at patients with more severe stages of breast cancer. It is also possible that the timeline of this experiment may need to be extended a few weeks into chemotherapy to notice a difference in miRNA expression since it is unknown how long cancer exosomes stay in the bloodstream after surgery. In addition, patients might have distinct recovery rates from trauma of surgery that need to be considered.

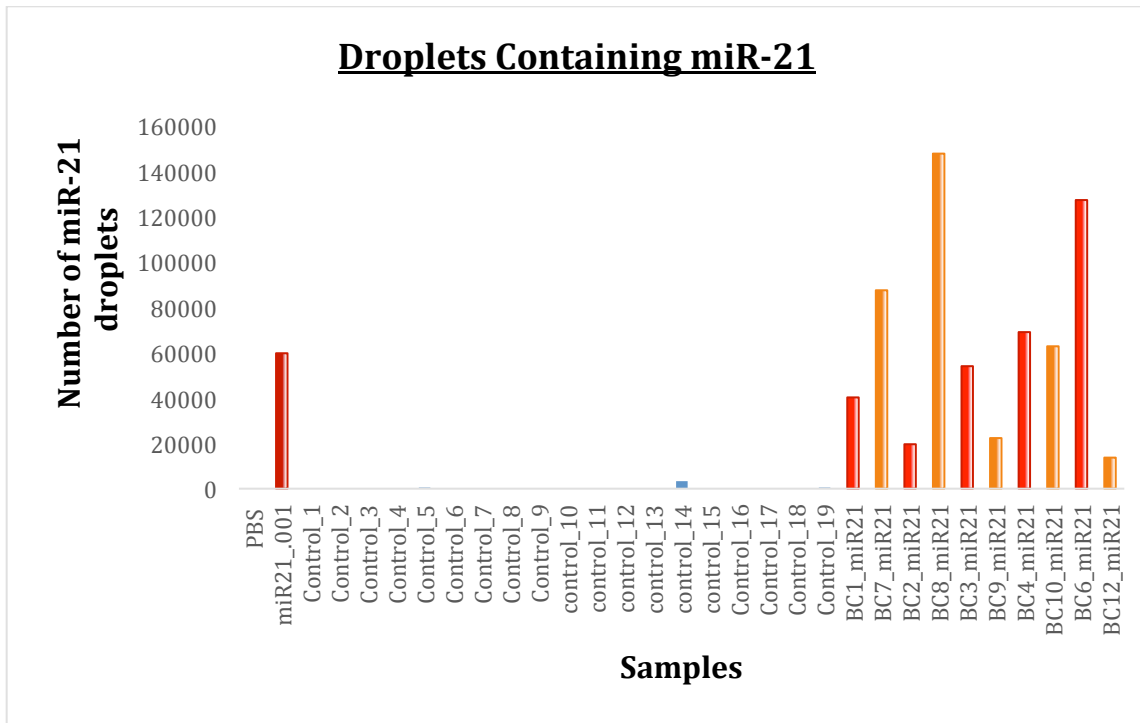


Fig. 16 dPCR results of droplets containing miR-21. Comparing 'healthy' control samples (blue) with the pre- (red) and post- (orange) resection breast-cancer samples. Synthetic miR-21 as control (dark red).

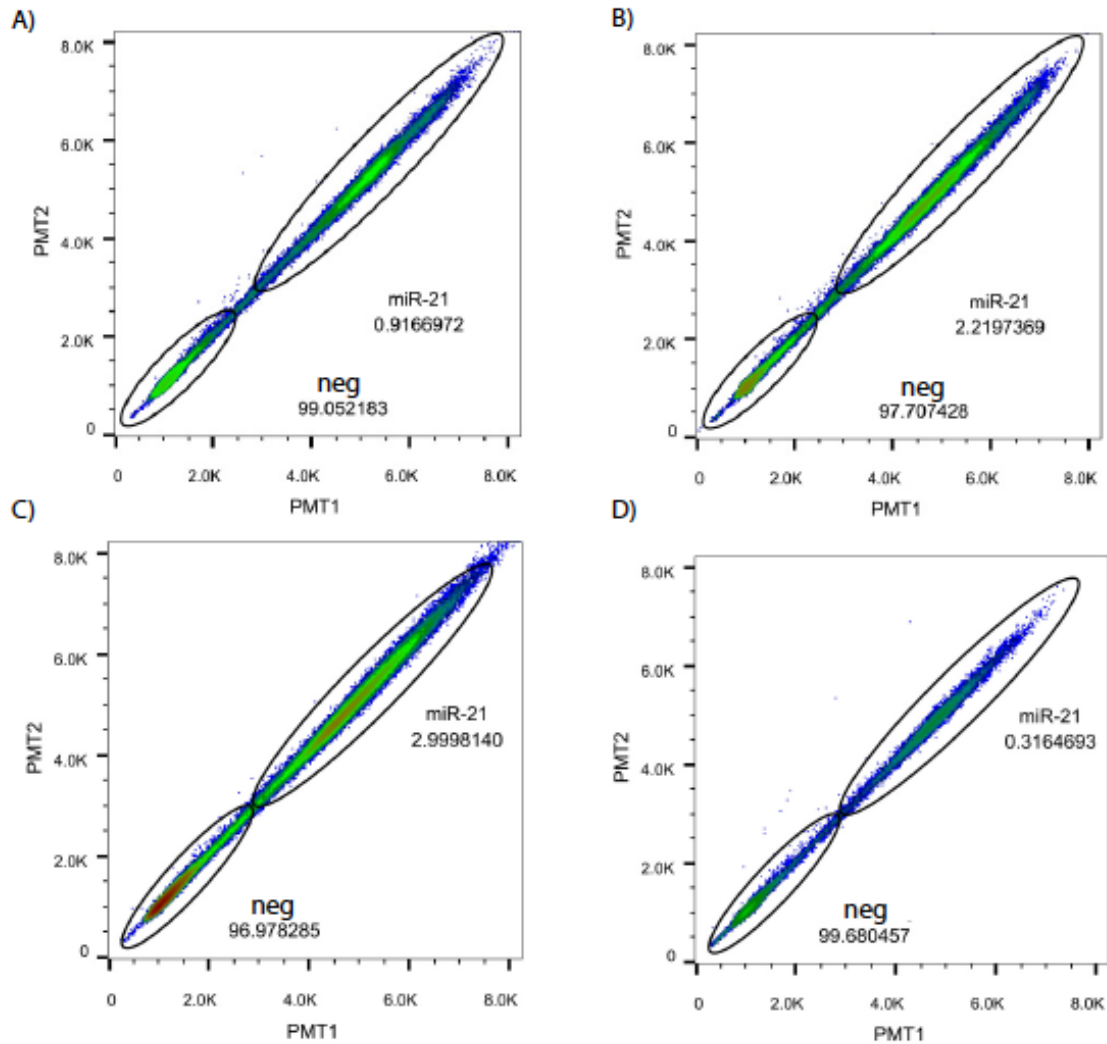


Fig. 17 dPCR dot plots of miR-21 numbers measured in two patients, JQE6NC and 9K66Q2, pre- and post-tumor resection. Dot plots A) and B) show pre- and post-resection miR-21 levels of patient JQE6NC, where miR-21 levels increase after tumor resection. Dot plots C) and D) show pre- and post-resection miR-21 levels of patient 9K66Q2, where miR-21 levels decrease after tumor resection.

Notably, in patients, 2Z2HMP and JQE6NC, the level of miR-21 increased unexpectedly after surgery, in contrast to the other three individuals (Fig. 16). The two subjects had the lowest grade and stage out of the five patients. Also, the blood draws before and after were timed closer together compared to the three patients with more advanced cancer. We might have to assess the serum miR-21 levels of these two individuals again at a later time to determine whether miR-21 levels drop due to the absence of tumor.

As anticipated, we did find differentially expressed miR-21 profiles between cancer patients and healthy individuals. MiR-21 was found in high numbers in breast cancer serum but not in healthy individuals. However, one control sample, 'control 14', showed significantly higher miR-21 levels (Fig. 16) and will have to be reanalyzed as mentioned above. A preliminary data-driven threshold of 4000 is set since any number between 3678 and 13693 separates the two populations. As well, we hope to eliminate false negatives by setting the threshold conservatively. No reason has been found to eliminate 'control 14' as a negative control. However, if this individual has developed cancer, then control 14 can be eliminated as a negative control and the threshold can be adjusted. In addition, this could serve as further evidence that miR-21 profiles could be used for screening and detection of early staged breast cancer.

CHAPTER 3

DISCUSSION AND CONCLUDING REMARKS

At the onset of this project, we hypothesized that we would find differential expression of exosomal miRNAs from breast cancer cells when we compared pre- and post-tumor resection serum. Also, exosomal miRNA released from 'normal' cells were expected to differ from the breast cancer exosomes. It was initially expected that we would establish personalized miRNA panels for 10 women. But, for practical reasons, the research plan was modified to focus just on miR-21 and its role as potential biomarker in breast cancer detection.

As expected, we found that miR-21 was highly expressed in exosomes of cancer patients compared to 'healthy' individuals. However, circulating miR-21 levels, when compared in post- versus pre-surgery serum, did not decline for all five patients. This unexpected observation can be explained on technical grounds, including small sample size and lack of both endogenous and exogenous miRNA controls. And while all five patients had early stage breast cancer, our methods might not be sensitive enough to detect very small differences between pre- and post-surgery serum. Consequently, we might have to analyze samples that involve a later stage of cancer. The results that miR-21 levels did stay high in post-tumor

resection serum compared to healthy serum raise the issue that it is unknown how long cancerous exosomes can stay in the blood after tumor removal. Also, recovery time from surgery might vary from patient to patient. Therefore, additional serum samples from these patients should be obtained at a later time after surgery.

Importantly, it was also unexpected that when observing the exosome concentrations alone, they did not change between the healthy control samples and the breast cancer samples. As such, larger sample size might be analyzed. In addition, exosomes from patients with more advanced cancer might be tested. Also, no difference between pre- and post-surgery exosomes was detected. This might be a consequence of the inflammation resulting from surgery, a known cause of elevated exosomes.

These results relied on optimizing the NTA and dPCR techniques. In the rapidly evolving field of exosomal miRNA research, new techniques are continually developed. We settled on using the new Advanced TaqMan assay, which resulted in redesigning our dPCR protocol. Exosomes and miR-21 levels of negative controls were analyzed at the same time dPCR and NTA were optimized. Notably, one control sample showed elevated miR-21 levels. A possible explanation is that this individual had developed cancer in spite of the fact that she was chosen as a control subject because she had no known history of cancer. While this result might be explained by contamination, she should be further investigated. The overall goal of this work is to develop a customized-miRNA panel for every breast-cancer patient which will require further experiments, including miRNA sequencing.

In the near future, we hope to follow patients with both early as well as more

advanced stages of cancer. We expect this will show a significant relationship between levels of exosomes and exosomal miRNA in the development of breast cancer. Also, patients will be followed over a longer period of time after surgery. Additionally, a large enough cohort of both healthy and cancer patients will be tested to obtain statistically relevant results.

Finally, this research is an advance over current modes of cancer detection and involves a more patient-centric approach. The release of breast-tumor exosomes and their miRNA cargo into the blood stream allow for disease detection via noninvasive methods and at lower costs. This technology combined with its relative low cost allow for more frequent screening and hence earlier detection of cancer and enhances patient care.

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