

DETECTING ENDOTHELIAL CELLS FROM AVF ANGIOPLASTY BALLOONS:
FROM PROOF OF CONCEPT TO TECHNICAL OPTIMIZATION

By

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ABSTRACT

Over 450,000 patients are currently on hemodialysis in the United States. For each dialysis patient, vascular access is considered to be the “lifeline,” their connection with the dialysis machine that purifies their blood, keeping them alive. Among all the current forms of vascular access, arteriovenous fistulas (AVF) have consistently shown the most efficient results. However, almost 50% of AVFs fail to mature and are in need of intervention. Balloon angioplasty is a type of intervention that is used to restore blood flow through stenotic vessels. Angioplasty balloons are currently discarded and may contain cells that help yield useful information. In order to test the feasibility of endothelial cell adherence to angioplasty balloons, samples were collected and immediately transferred to RNA later®. RNA was extracted to assess quantity and quality. RT-PCR was performed from synthesized cDNA using specific primer probes for vascular endothelial genes. In conclusion, endothelial cells are indeed transferred onto the balloon in the process of intervention. For future directions, cells within the stenosed blood vessel could provide significant information to indicate (1) biomarkers that are predictive of the number of times intervention may be needed and (2) the cause(s) of stenosis in vessels.

INTRODUCTION

1.- The Kidney.

The kidney is responsible for many of the body's functions (**Figure 1**). One of the primary functions of the kidneys is to filter blood in an effort to remove waste products and extra fluid through the production of urine. The kidneys are important because they maintain stability in the blood that is free of toxins and allow the body to function properly. In addition to preventing the buildup of waste products and extra fluid, functional kidneys maintain electrolyte homeostasis and produce hormones. In return, these hormones help regulate blood pressure, make red blood cells and preserve bone strength.¹

Each of the two kidneys is made of roughly one million nephrons, a filtering unit. An individual nephron only filters a small amount of blood, but collectively, they filter roughly 120 to 150 quarts of blood every day. The nephron is composed of both the glomerulus and a tubule. In a simplistic approach, the filtration process done by the nephron can be categorized into two steps. Initially, the glomerulus allows fluids and waste products to permeate through it; however, it excludes blood cells and other large molecules. The tubule portion of the nephron is composed of the proximal convoluted tubule, the descending and ascending Loop of Henle, the distal convoluted tubule and the collecting duct (**Figure 2**). Together they select minerals that should be referred back to the blood and remove waste that will in turn be excreted through the production of urine.¹

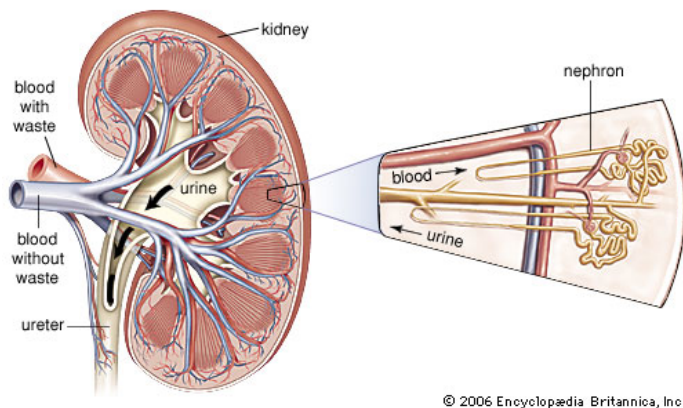


Figure 1: Illustrates a schematic representation of a kidney²⁶

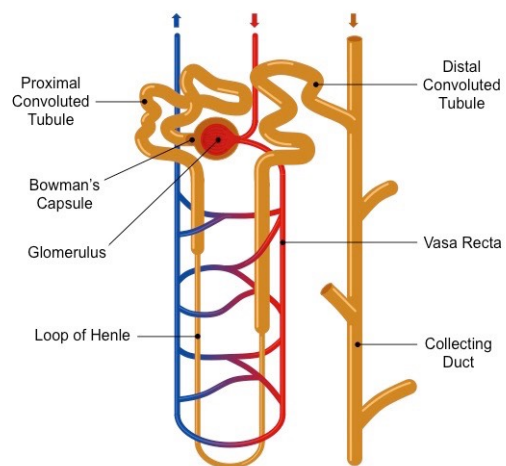


Figure 2: Depicts the anatomical structure of a nephron²⁷

2.- Chronic Kidney Disease.

Chronic Kidney Disease (CKD) is defined as a complex of heterogeneous disorders that progressively reduce the function and structure of the kidney^{2, 3} leading to End-Stage Renal Disease (ESRD) where only transplant or dialysis treatments can keep patients alive. CKD is defined as heterogeneous due to its variability in cause and pathology, severity and rate of progression.⁴ Common causes of CKD in developed countries include diabetes, hypertension, obesity and cardiovascular disease. CKD is a major health concern, presenting a prevalence of nearly 1800 cases per million and an average survival of 3-5 years in the U.S., the disease projected an 8% annual growth rate, with the fastest growth expected in developing nations. Over 661,000 Americans have kidney failure and of those, 468,000 individuals are on dialysis. The incidence rate of ESRD had steadily increased from the 1980's until the early 2000's, however, it leveled off in 2006 and is now at a slight decline.⁵

CKD is generally characterized by an abnormal Glomerular Filtration Rate (GFR, which refers to the rate at which kidneys filter waste and extra fluid from the blood) and the presence of clinical signs of kidney damage. However, it is unclear, which signs of kidney damage, expressed as albuminuria (the presence of albumin in the urine) or the severity of the loss of filtration capacity (expressed as estimated eGFR) best predicts whether a patient will reach ESRD.⁶

However, CKD progression is commonly divided into 5 stages primarily based on the GFR value: Stages 1 and 2 include patients with slight to mild kidney damage presenting close to normal kidney function, while Stage 5 refers to patients that have achieved the kidney failure stage and require either dialysis or a transplant for survival. Stage 5 CKD is also known as End Stage Renal Disease (ESRD).

| | | | | Albuminuria stages, description and range | | |
|---|---------------|----------------------------------|-------|---|----------------------|--------------------|
| | | | | A1 | A2 | A3 |
| | | | | Normal to mildly increased | Moderately increased | Severely increased |
| | | | | <3 mg/mmol | 3-29 mg/mmol | ≥30 mg/mmol |
| GFR stages, descriptions and range (ml/min per 1.73m ²) | Stage 1 (G1) | Normal or high | ≥90 | | | |
| | Stage 2 (G2) | Mildly decreased | 60-90 | | | |
| | Stage 3 (G3a) | Mildly to moderately decreased | 45-59 | | | |
| | Stage 3 (G3b) | Moderately to severely decreased | 30-44 | | | |
| | Stage 4 (G4) | Severely decreased | 15-29 | | | |
| | Stage 5 (G5) | Kidney failure | <15 | | | |

Figure 3: CKD stages chart based on GFR and Albuminuria²⁸

During its early stages, kidney disease is often asymptomatic and can easily go undetected until it is too advanced to completely prevent ESRD. As a result of prolonged kidney failure, the risk of having a stroke or heart attack is augmented. Risk factors for kidney disease include diabetes, high blood pressure and a family history of kidney disease.

3.- Hemodialysis.

When CKD patients progress to ESRD/kidney failure they are in need of renal replacement therapy to stay alive. Only a kidney transplant or dialysis treatment can prevent these patients from dying. Dialysis provides support to 1) remove waste, salt and extra water to prevent build-up, 2) maintain a safe level of chemicals in the blood (i.e. potassium, sodium and bicarbonate) and 3) control blood pressure. In essence, dialysis functions as an artificial kidney, with limitations. These limitations include the inability to produce natural hormones, such as erythropoietin (to regulate red blood cell production) or renin (which helps maintain adequate arterial blood pressure). There are 2 main forms of dialysis treatments: Hemodialysis (in which blood is removed from the body, filtered through an artificial membrane called a dialyzer, and returned to the body) and Peritoneal Dialysis (PD, type of dialysis that uses the peritoneum as the membrane through which fluids and dissolved toxins are exchanged with the blood). Even though PD may present better short-term outcomes, it is less efficient in removing waste and toxins than hemodialysis, adding a high peritoneal infection risk as well. Hemodialysis is the best dialysis modality for many physicians.

About 2 million individuals around the world are in need of hemodialysis. Hemodialysis heavily impacts the quality of life of a patient depending on how often they need the treatment. Factors such as kidney function, fluid weight gain between treatments, human stature (height and weight) and the amount of waste produced determine the frequency and duration of treatment. On average, hemodialysis treatment lasts about 4 hours and is completed three times a week.⁷

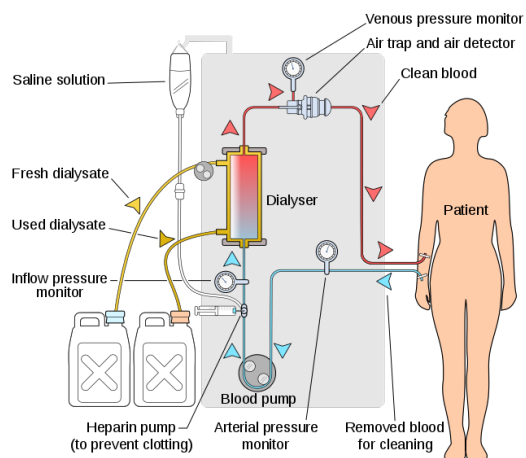


Figure 4: Illustrates the clinical application of hemodialysis²⁹

inside, but the smaller molecules, such as urea and creatinine pass through the small holes of the dialyzer into the dialysate solution and are removed from the blood.⁸

In order to deliver hemodialysis treatment, access to the patient's blood vessels ("vascular access") must be gained in order to remove the blood, filter it through the dialyzer and return it back to the patient. Blood passes through the tubular conducts towards the dialyzer, that acts as filter between the blood and the dialysate (a solution made up of water, an acid solution and bicarbonate/lactate solution). The dialysate solution remains outside the hollow filters and allows for toxins to permeate outwards. During hemodialysis, the larger molecules (blood cells and proteins) remain

4.- Vascular Access.

Vascular access is essential in order to deliver hemodialysis treatment to patients, that is the reason why it is considered the “lifeline” for hemodialysis patients.⁹ Without proper patency and cannulation into blood vessels, dialysis patients are not able to receive proper filtration of their blood. There are currently three main methods of attaining vascular access for hemodialysis and those include the Tunneled Dialysis Catheter (TDC), the ArterioVenous Graft (AVG) and the ArterioVenous Fistula (AVF).

Tunneled dialysis catheters are used to gain immediate and efficient vascular access. However, the use of TDCs often leads to access occlusions, chronic venous stenosis, and more importantly, infection with a high associated mortality.¹⁰ Due to these common complications, The National Kidney Foundation Dialysis Outcomes Quality Initiative (NKF-KDOQI) recommends that less than 10% of chronic maintenance hemodialysis patients should be supported by TDCs as their permanent form of vascular access.¹¹ During AVF maturation, TDCs are commonly used as a bridge until the fistulae has fully formed. Dialysis catheters commonly present a split lumen that bifurcates in the outer portion into the arterial (Red) and venous (Blue) ends, intended to be used to draw blood towards the dialysis machine (Arterial) or back towards the body (Venous) (**Figure 5**). The presence of the catheter creates a direct communication between the interior of the cardiovascular system and the outside of the body that is highly susceptible to infection. Since the inner portion of the catheter lays on the right atrium, catheter related infections commonly spread through the systemic circulation and cause sepsis.

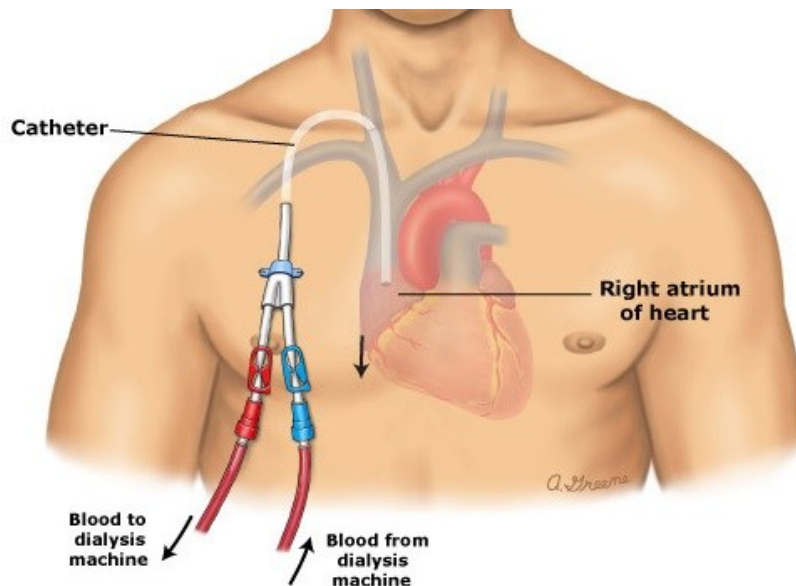


Figure 5: TDC anatomical positioning. Inserted in the right internal jugular vein and advanced until the tip is located in the right atrium³⁰

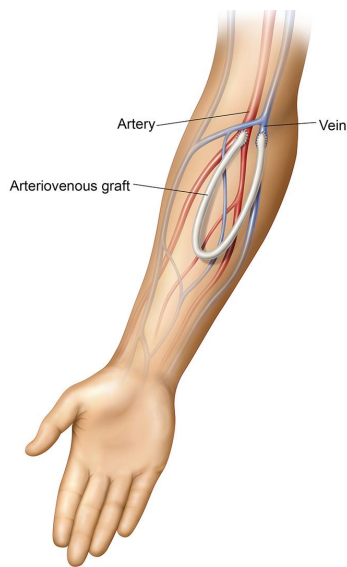


Figure 6: Schematic representation of Arteriovenous Graft (AVG) in the forearm³¹

The grafts in the AVG consist of a flexible synthetic tube commonly made of polytetra-fluoroethylene (PTFE) that directly connects an artery to a vein (**Figure 6**). When the arterial blood transported down the high resistance arterial system encounters an opening towards the low resistance venous system, most of the blood flow is directed towards the graft, allowing enough flow to pass through to enable dialysis treatment.

The down side of AVGs resides on the low primary patency rates that have been reported to be as low as 23% after one year.¹² Additionally, this type of access requires frequent interventions, such as angioplasty and stent or graft placement, in order to maintain patency. As a result, AVGs increase the number of hospital admissions and the cost of access maintenance all while reducing the quality of life of patients.

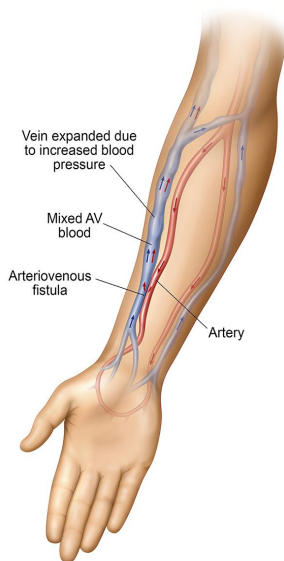


Figure 7: Schematic representation of Arteriovenous Fistula (AVF) in the forearm³²

Last but not least, arteriovenous fistulas (AVFs) are another means of attaining vascular access. AVFs are unanimously the preferred method of hemodialysis vascular access. They are defined as a surgical connection (anastomosis) between an artery and a vein (**Figure 7**) usually in the arm, either at the wrist or the elbow.

The purpose behind connecting a vein to an artery is to promote the movement of blood from a high pressure / high flow arterial system into the low pressure/ low flow / high capacity venous system in order to induce vascular remodeling in the vein, obtaining an overall dilation and thickening of the receptor vein sufficient to support dialysis.

After the surgical creation, the vein destined to become a successful fistula undergoes a remodeling process that is referred to as *maturation*, involving the overall compliancy and responsiveness of the vessel to dilate when exposed to an increase in velocity of blood flow as a result of a newly implemented connection.¹³ Although the AVF is the preferred method of attaining vascular access, roughly 50% of patients with an AVF fail to mature and are in need of intervention.

4.- AVF Maturation.

In order for an AVF to be able to support dialysis, and therefore be considered mature, blood flow must increase to a level that provides an adequate delivery to the dialysis machine. In addition, the vessel must increase in diameter to accommodate patency and the vessel wall must thicken to permit repeated cannulation.¹⁴ Although there is no universal agreement, an average vessel must contain a blood flow of 500ml/min in order to receive dialysis treatment.¹⁵ AVF maturation failure continues to be a topic of concern for clinicians and scientists. Potential contributing factors of AVF failure include, but are not limited to, the surgical procedure, patient comorbidities, blood flow patterns and genetic influences. The complex and heterogeneous clinical scenario needs continuous research effort in order to fully understand, predict and appropriately treat AVF maturation failure.

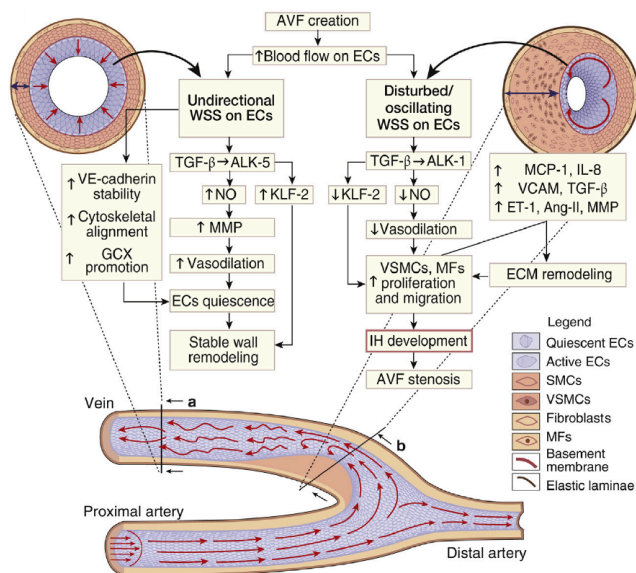


Figure 8: Vascular remodeling pathways that influence AVF maturation³³

Figure 8 illustrates different vascular remodeling pathways involved in the success or failure of the arteriovenous fistula maturation. While a successful maturation path includes outward remodeling in which the vessel wall expands and therefore the lumen of the vessel increases, a non-successful maturation process involves a lack of outward remodeling and/or a reduction in lumen due to an ingrowth of the intimal layer of the vessel, reducing the overall lumen.

Vascular walls are composed of endothelial cells, smooth muscle cells and fibroblasts. Endothelial cells receive sensory information gathered from environmental changes and integrate the information into intercellular communications. These intercellular signals target specific cellular processes, such as cell growth, cell death, cell migration as well as the synthesis/degradation of the extracellular matrix.¹⁶ Complications begin to arise once the lumen begins to narrow. Despite the beneficial outcomes that are associated with outward remodeling, the positive results may be diminished with the presence of intimal hyperplasia. Intimal hyperplasia refers to the growth or hypertrophy of smooth muscle cells in the tunica media. As a result, the vessel appears to be enlarged, but in reality, it is jeopardizing the diameter of the lumen.

When any form of intervention takes place within 3 months after the AVF has been placed, it is considered “assisted maturation.” Clinically, AVF non-maturation results in several endovascular and surgical interventions to aid maturation, which in turn results in prolonged duration of tunneled dialysis catheter use. This is only one of the reasons why there is such a high demand for effective predictors of AVF non-maturation. In doing so, we begin to shift from a “one-size-fits-all” fistula-first approach to a more individualized procedure.⁹

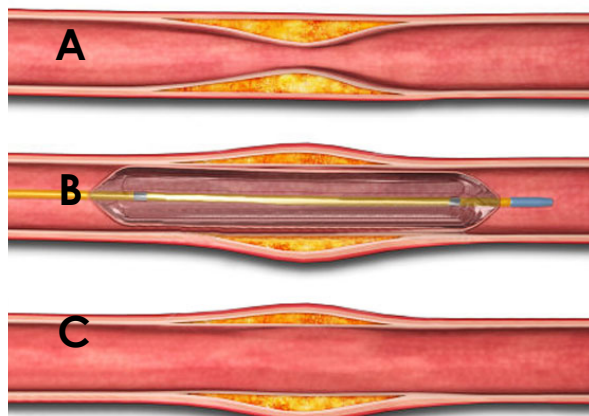


Figure 9: Angioplasty procedure. **A:** Stenotic vascular lesion. **B:** Angioplasty balloon inflated to dilate lumen. **C:** Post angioplasty vascular lumen restored³⁴

AVF non-maturation is often due to venous stenosis (narrowing of the lumen) and the most common treatment for stenosis is balloon angioplasty. Balloon angioplasty is an interventional endovascular technique in which a long catheter is inserted into the vascular system of the patient, advanced towards the location of the stenotic lesion using fluoroscopy assisted visualization (X-ray based), and once there, a collapsed balloon is inflated at a predetermined pressure in order to stretch and open the lumen restoring or improving the blood flow through the area (**Figure 9**).

Since angioplasty balloons are used to treat stenosis, by direct contact at high pressures, in the exact point where the narrowing has developed, our group hypothesized that vascular cells from the narrowed vessel may adhere to the balloon during the expansion, and moreover, these cells may remain there after its extraction from the patient. This is a huge opportunity to take a closer look at the specific gene expression profile of the cell populations responsible for the failure of vascular access. In doing so, it can shed light on the pathophysiology of the clinical problem and possibly provide relevant information for the prognosis and treatment of these patients. The information gathered from this study could lead to further investigation that focuses on potential (1) biomarkers that are predictive of the number of times intervention may be needed and (2) the cause(s) of stenosis in vessels.

Since AVF angioplasties are performed routinely on a daily basis in vascular access centers all around the country, and these balloons are currently discarded directly after use, our group established an agreement with the largest vascular access center in town to test the hypothesis by processing the discarded balloons after use.

OBJECTIVES

Based on the clinical need and the hypothesis previously described, the objectives set for this study are the following:

- To use current standard laboratorial techniques to determine the presence or absence of endothelial cells on angioplasty balloons recovered from AVF patients subjected to therapeutic angioplasties.
- To optimize, in case the hypothesis is confirmed, the collection and processing of the angioplasty balloons to obtain the best RNA quality and quantity from these cells in order to maximize downstream application options.

METHODOLOGY

After obtaining all of the required permissions and approvals from the research regulatory committees at the UA, the study began by establishing a research collaboration with the biggest vascular access center in town. Our group provided a set of sterile Falcon® tubes prefilled with an RNA preservation solution (RNA Later®) along with an anonymous collection form. The form aimed to collect some technical and demographic data that could influence the cell recovery success.

In order to facilitate the description and understanding of the design and methodology, the study was divided into two phases:

-In the first phase of the study, the experiments were designed to test the study's hypothesis: the presence of endothelial cells on the angioplasty balloons recovered from the collaborating vascular access center.

-In the second phase of the study, once the hypothesis was confirmed, the experiments aimed to optimize the collection and processing of the samples in order to maximize the ability to detect endothelial cells.

During both phases of the study the balloons were collected immediately after each procedure, aseptically introduced to the RNA preservation solution inside sterile Falcon® tubes and stored in biohazard collection bags together with the collection form. Samples were picked up by the lab staff and processed all at once for RNA extraction at the research group lab located in BIO5. Finally, after assessing the quantity and quality of the RNA present in the samples, RT-PCR was used to determine the presence of a series of endothelial-specific genes (**Figure 10**).

| | |
|-------------|---|
| FLT1 | - Promotes endothelial cell proliferation, survival and angiogenesis ¹⁷ |
| KDR | - Role in the regulation of angiogenesis, vascular development, vascular permeability - Promotes proliferation, survival, migration and differentiation of endothelial cells ¹⁸ |
| FLT4 | -Produces vascular endothelial growth factor receptor 3 (VEGFR-3) that regulates the growth, movement and survival of lymphatic cells ¹⁹ |
| TIE1 | -Role in angiogenesis and blood vessel stability ²⁰ |
| TEK | -Facilitates communication between endothelial cells and smooth muscle cells -Directs angiogenesis and ensures integrity of blood vessels ²¹ |

Figure 10: Table highlighting the primary roles of endothelial cells: FLT1, KDR, FLT4, TIE1 and TEK

PHASE 1: HYPOTHESIS TEST. PROOF OF CONCEPT

1.- RNA Extraction from Angioplasty Balloon



Figure 11: Two Falcon® tubes filled with RNA Later® (left) and two with balloon and RNA Later® (right)

During the first phase, samples were collected from the vascular center once a week. Angioplasty balloons were directly placed, after the surgical procedure, into the pre-labeled Falcon® tubes provided by our research group. At the time of balloon collection, a survey form was filled out with basic demographics and procedural characteristics. Balloons then remained in preservation solution (RNA later®) between 1 to 5 days until they were processed in the lab (**Figure 11**).

In order to process any type of samples for RNA extraction it was of extreme importance to conduct the experiment in strict sterile conditions (**Figure 12**) in order to minimize contamination of the samples with RNases. RNases are RNA specific degrading enzymes that are extremely ubiquitous and some of them may even resist autoclave processing. In our case, considering that the amount of RNA

expected to be isolated was minimal, all the measures aimed to prevent degradation became even more important. For balloon processing and sample handling, material was autoclaved, and sterile material was used. RNA-zap spray and wipes were constantly used on the handling material (forceps, scissors) all while using nitrile gloves. Adequate PPE (Personal Protective equipment), including lab coat, nitrile gloves and face mask, was worn at all times in order to protect lab staff and also to prevent the contamination of the samples.

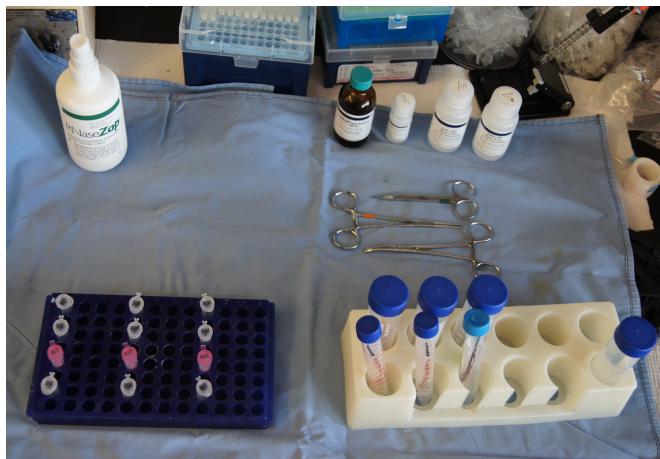


Figure 12: Sterile working area consisting of RNA-Zap spray, reagents, Falcon® tubes with angioplasty balloons, Eppendorf® tubes and handling material (i.e. forceps and scissors).

During processing, angioplasty balloons were cut at the distal end, including the catheter, using sterilized scissors. The same scissors were used to create a slit that ran along the longitudinal side of the balloon (**Figure 13A/B**). The balloon was peeled back in a position that was parallel to the bench and perpendicular to the catheter. The balloon was then placed above a 50mL Falcon® centrifuge tube where it was trimmed into small sections. 1.4 mL of QIAzol Lysis Reagent were added thoroughly to cover all areas of the balloon (**Figure 13C**). The mixture was incubated at room temperature for roughly 5 minutes.

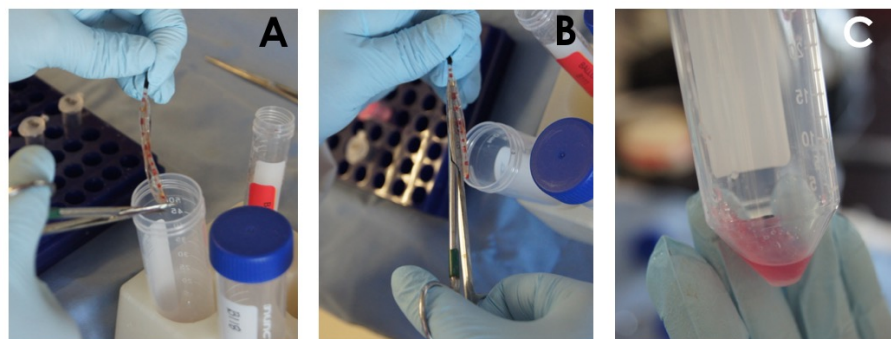


Figure 13: Processing of balloons following sample submergence into RNA Later®; **A:** Snipping of the end of the balloon; **B:** Longitudinal cut along balloon; **C:** Soaking of fragmented balloon inside of lysis buffer.

The liquid solution was then mixed and transferred into a collection tube using a pipette. 240 μL of chloroform were then added into the solution and vortexed to ensure homogenization. This was followed by an incubation period of 3 minutes. The solution was then centrifuged for 15 minutes at 12,000 x g at 4°C. Following centrifugation, the aqueous phase was transferred to a new collection tube (**Figure 14A**). 100% ethanol was added at an equivalence of 1.5 times the volume of the aqueous phase. Using a pipette, the solution was thoroughly mixed and transferred to a RNeasy® Mini column in a 2mL collection tube. Using QIAgen's miRNeasy Mini Kit, 700 μL of Buffer RWT were added to the column and centrifuged for 15 seconds at 8,000 x g. The content in the collection tube (not the column) was discarded. 500 μL of RPE were then added into the column and centrifuged for 15 seconds at 8,000 x g. The content in the collection tube was discarded once again (**Figure 14B**). The previous 2 steps were repeated.

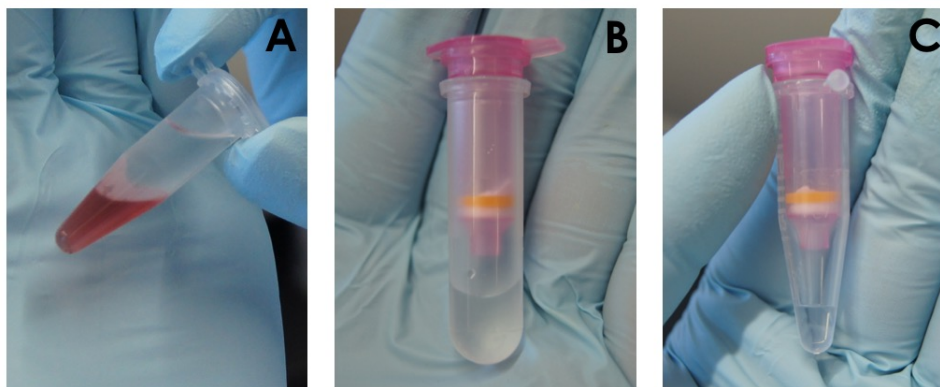


Figure 14: Isolation of pure RNA **A:** Visual representation between organic phase (lower) and aqueous phase (upper); **B:** Removal of debris using RWT and RPE buffers on spin column; **C:** Elution of RNA from spin column after centrifugation using water.

In order to remove any excess buffer that had not been removed, the column was placed in the centrifuge for an additional 3 minutes at 13,000 rpm. The collection tube was replaced with a new one and 20 μL of RNase-free water were added onto the column. In doing so, the tip on the pipette made contact with the column filter to secure absorption of water. The water was allowed to settle for 1 minute. Elution was complete using centrifugation for 1 minute at 8,000 x g (**Figure 14C**). The column was discarded and the eluted RNA and water mixture in the collection tube was preserved at -80°C .



Figure 15: Balanced spin columns embedded into centrifuge tube for RNA isolation following buffer placement.

2.- NanoDrop™. RNA concentration.

In order to narrow down the number of samples to further process for Real Time-PCR a Nanodrop™ spectrophotometer was used, this instrument is able to determine average concentrations of nucleic acids including RNA.

Spectrophotometric analysis is based on the principle that nucleic acids absorb ultraviolet light in a specific pattern. In the case of RNA, samples are exposed to ultraviolet light at a wavelength of 260 nanometers (nm). The measured absorbance is calculated into ng/ μL of nucleic acid (RNA).²²

We divided the samples according to their RNA concentration into two different groups based on the lab's previous experience with extremely reduced concentration samples. Samples with concentration values less than 5 ng/ μL were completely discarded while samples with ≥ 10 ng/ μL were further processed for cDNA synthesis. A reduced number of samples had concentration values between 5-10 ng/ μL , some of these samples (the ones closer to the higher end) were used for cDNA and Real Time-PCR to confirm the validity of the proposed cut-off.

3.- Complementary DNA (cDNA) Synthesis

In order to generate cDNA from mRNA a commercially available cDNA synthesis kit was used (QuantaBio® qScript™ cDNA Synthesis Kit). An aliquot of 15 μL of RNA from each sample was used, each aliquot was transferred to a sterile Eppendorf® tube and the following reagents were added:

- ~5 μL of Nuclease-free water
- 1 μL of concentrated qScript™ reverse transcriptase enzyme
- 4 μL of 5X concentrated master mix

Reverse transcriptase is the enzyme that allows for the creation of a complementary set of DNA from an RNA template. The concentrated master mix contains a titer primer blend (oligo dT(20) and random hexamer), qPCR-optimized dNTP blend and flexible magnesium titration. Oligo dT(20) is a string of 20 deoxythymidylic acid residues that attaches to the tail of mRNA. This allows for an anchor for reverse transcriptase to work on. dNTP stands for deoxynucleoside triphosphate and is a set of four nucleotides that are used by reverse transcriptase to extend an annealed primer.²³

Applied Biosystem's 2720 Thermal Cycler® facilitates the polymerase chain reaction by eliciting changes in temperature that are optimal for cDNA synthesis. The following settings were used:

1. 22°C for 5 minutes; to allow for the annealing of the primer to RNA template
2. 42°C for 42 minutes; DNA polymerization
3. 85°C for 5 minutes; deactivation of reverse transcriptase
4. 4°C for an indefinite amount of time; allows for protection of biological components, i.e. cDNA



Figure 16: Applied Biosystem 2720 Thermal cycler used in the study³⁵

4.- Real Time-PCR

Once cDNA from the samples were synthesized, Real Time-Polymerase Chain Reaction (RT-PCR) was used to quantify the gene expression of the selected endothelial-specific genes. In addition to endothelial cells, the lumen contains red blood cells, white blood cells, platelets, etc., which is why cDNA was used for Real Time-PCR- to detect the following endothelial-specific genes: FLT1, KDR, FLT4, TIE1 and TEK. We analyzed the samples in two steps.

STEP 1: GAPDH

In order to test the viability of the synthesized cDNA, samples obtained for RT-PCR first tested the expression of a housekeeping gene, GAPDH. GAPDH is necessary for basic cell function²⁴, since this gene is stable and constitutively expressed at high levels on most tissues it is commonly used as a housekeeping gene in most of the Real Time-PCR protocols. GAPDH will allow for an easy distinction of desired cDNA samples that may be further analyzed for the presence of endothelial-specific genes. Each PCR sample received 10 μL of 2X TaqMan Mix, 7 μL of PCR water, 1 μL of primer probe (GAPDH) and 2 μL of cDNA (specific to labeled balloon RNA). Default settings on *QuantiStudio*[®] remained constant, however, volume was changed to 20 μL and number of cycles was updated to 60.

STEP 2: Endothelial-specific genes

Based on Step 1, we filtered out the cDNA samples that did not contain GAPDH. Each PCR sample contained 10 μL of 2X TaqMan Mix, 6 μL of PCR water, 1 μL of GAPDH, 1 μL of primer probe (FLT1, KDR, FLT 4, TIE1 or TEK) and 2 μL of cDNA (specific to labeled balloon RNA). Each of the 5 individual primer probes was tested separately on cDNA. Default settings on *QuantiStudio*[®] remained constant, however, volume was changed to 20 μL and number of cycles was updated to 60.

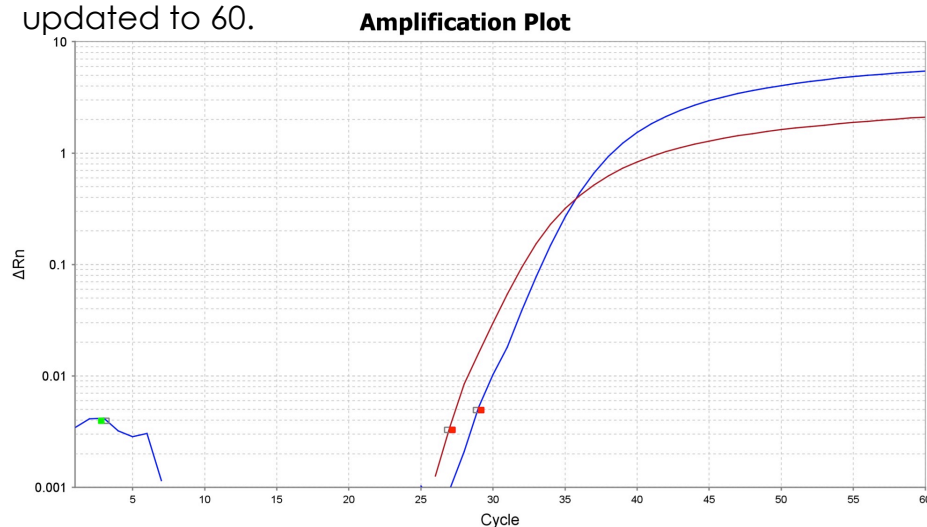


Figure 17: Real Time-PCR graphic output from a balloon sample showing GAPDH (Red) and FLT1 (blue) expression at 26 and 27 cycles, respectively.

PHASE 2: TECHNICAL OPTIMIZATION

For the second phase of the study, the main objective was to optimize the sample processing to improve the quantity and quality of the RNA samples obtained in order to maximize the number of samples suitable for downstream applications. During the first phase, all samples were collected from the same vascular access center, the main difference was that all samples were picked up by the lab staff on a daily basis and were processed within 8 hours post OR collection.

1) RNA Extraction from Angioplasty Balloon

Angioplasty balloons were collected and processed on the same day. The exact same steps were taken as indicated in Phase 1. The only exception is that 60 μL of RNase-free water were used to elute the RNA out of the filter in the column, not 20 μL in order to maximize the RNA elution from the cartridge filter.

2) RNA Concentration

The RNA extracted was concentrated to 20 μL to ensure that all RNA was used at its optimal concentrations for downstream applications. Savant Speed Vac® settings were set to 45°C, while time was manually accounted for until 20 μL were reached. Eppendorf® tubes containing extracted RNA were balanced and remained open to allow for evaporation of water. Since 60 μL of RNase-free water were added, the initial time was set to roughly 10 minutes. Samples containing more than 20 μL after the first speed vac session remained in the speed vac for increments of 2-5 minutes. The concentration of each sample was collected and recorded using the NanoDrop® instrument.

3) Real Time-PCR

Considering the housekeeping gene, GAPDH, may be affected by uremia, ribosomal protein coding gene, RPS18, was used in conjunction to GAPDH to assess the correlation between the two. We did not want to discard samples that may otherwise be helpful.

4) BioAnalyzer®

The BioAnalyzer® measures the quality of RNA by evaluating the size of rRNA fragments. The principle behind sample analysis using bioanalyzer/chip (Pico chip) platform is based on electrophoresis, similar to that used in gel-electrophoresis. The chip format, however, reduces separation time and sample consumption. Moreover, the BioAnalyzer® algorithm calculates the RNA integrity number (RIN) by comparing the amount of 18S and 28S ribosomal RNA. In other words, we can obtain information about RNA quality by calculating ribosomal RNA which constitutes the majority of total RNA. RNA Integrity Number (RIN) values range from 1-10; one being the most degraded.

Similar to the “RNA Extraction” phase, sterility was maintained at all times, using RNA-zap spray and wipes, nitrile gloves and appropriate PPE to protect the delicacy of the RNA composition.

We used the Agilent RNA 6000 Pico kit. The gel-dye mix was prepared by vortexing room temperature RNA dye concentrate for 10 seconds, spinning it down and adding 1 μL of dye into a 65 μL aliquot of filtered gel. The solution was vortexed and centrifuged for 10 minutes at 2,000g. An RNA chip (**Figure 18**) was placed in a chip priming station and 9 μL of gel-dye mix were transferred into the well marked “G” with a black background. The plunger was positioned at 1mL and then the chip priming station was slowly closed. The plunger was slowly pressed down and held for a total of 30 seconds before pressure was released. The plunger remained without pressure for another 5 seconds at which point it was slowly pulled back until it was at the 1 mL position. The chip priming state was opened to make sure that there were no bubbles in the well. If there were bubbles, they were popped using a pipette by airing it out. 9 μL of gel-dye mix were added into the 2 wells also marked with a “G,” but with a white background.

5 μL of RNA marker were placed in all 12 sample wells in addition to the well labeled with a ladder. In the meantime, the PCR machine (**Figure 16**) was set to 70°C in order to cause the RNA samples and the aliquoted ladder to “denature,” meaning the RNA G-complexes straightened out and allowed for interaction. 1 μL of RNA sample was then added into each of the 11 sample wells. Afterwards, 1 μL of the heat denatured ladder was placed into the well labeled with a ladder. 1 μL of RNA marker was placed into each unused sample well. The chip was then vortexed for 1 minute. Following these steps, the chip was assessed using the Agilent 2100 BioAnalyzer® within 5 minutes (**Figure 19**). Information was immediately recorded.



Figure 18: RNA Pico Chip used for BioAnalyzer®³⁶



Figure 19: Agilent 2100 BioAnalyzer®³⁷

RESULTS

In order to set up the context of this study, the first section in the results portion of the paper is used to describe the patient population demographics.

1.- Demographics

The demographics of the patient population from Phase 1 is illustrated in the figures below. With regards to ethnicity and race, the rate of non-reported data was significantly high. While the predominant ethnicity reported was Hispanic/Latino, the second most prevalent group was “non-reported,” which impedes our ability to correlate a reflective demographic representation. A similar trend was observed with regards to race, where the box corresponding to “other” consisted of 35.6% of the total patients.

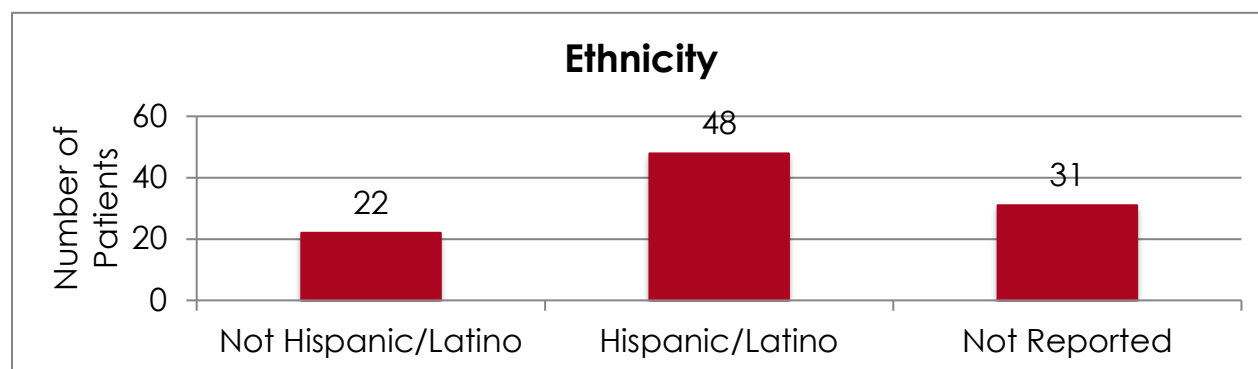


Figure 20: Reported ethnicity distribution from a total of 101 balloons processed during Phase 1.

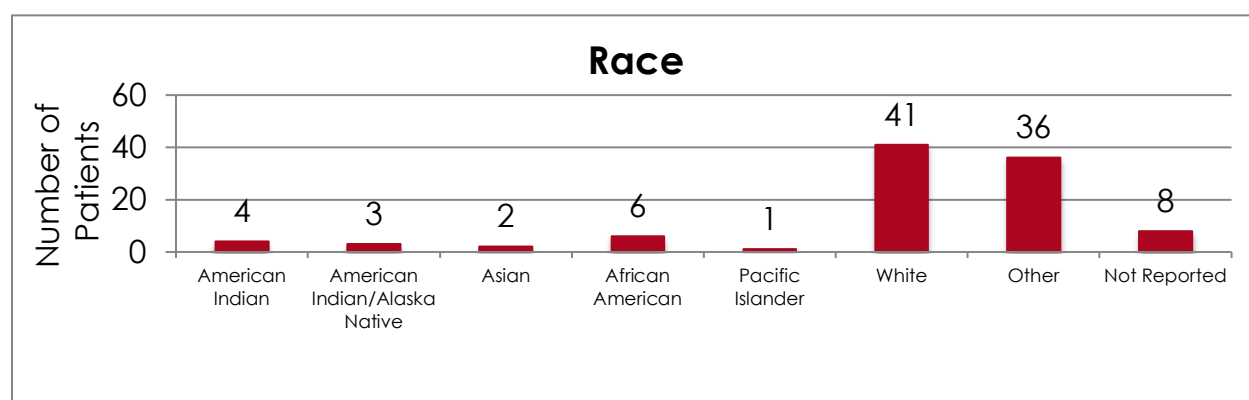


Figure 21: Reported racial distribution from a total of 101 balloons processed during Phase 1.

Age and gender distribution of the study population correlates very closely with the reported hemodialysis patient distribution nationwide. Hemodialysis patient gender distribution in the U.S. is 46.3% in females and 53.6% in males, while in our study 38% of the balloon donor patients were female while 61% were male. The incidence of hemodialysis reaches its peak on the age range between 60-69 nationwide²⁵ exactly the same age range that has the higher incidence in our patient population.

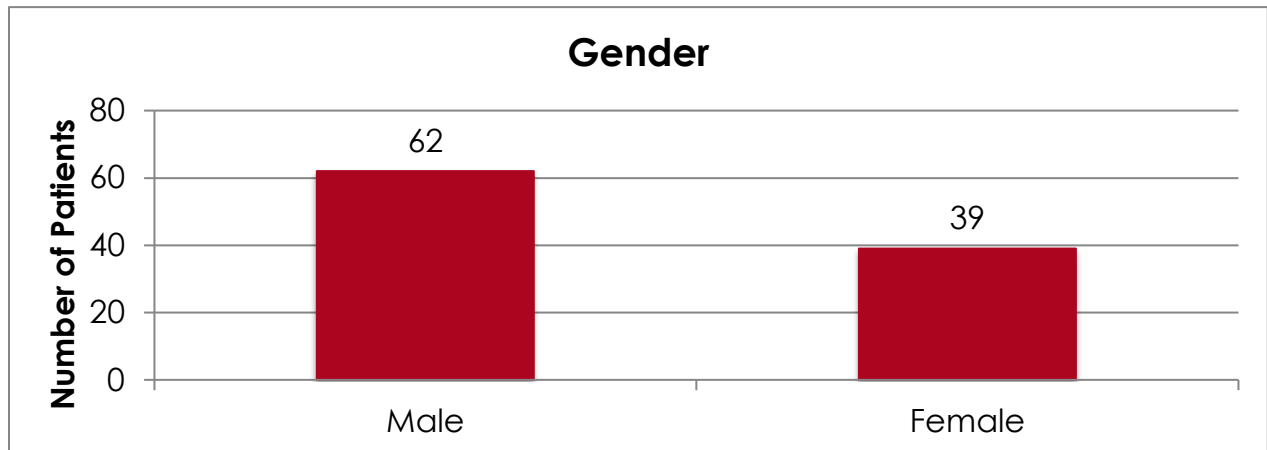


Figure 22: Gender distribution among the hemodialysis patients balloon donor population.

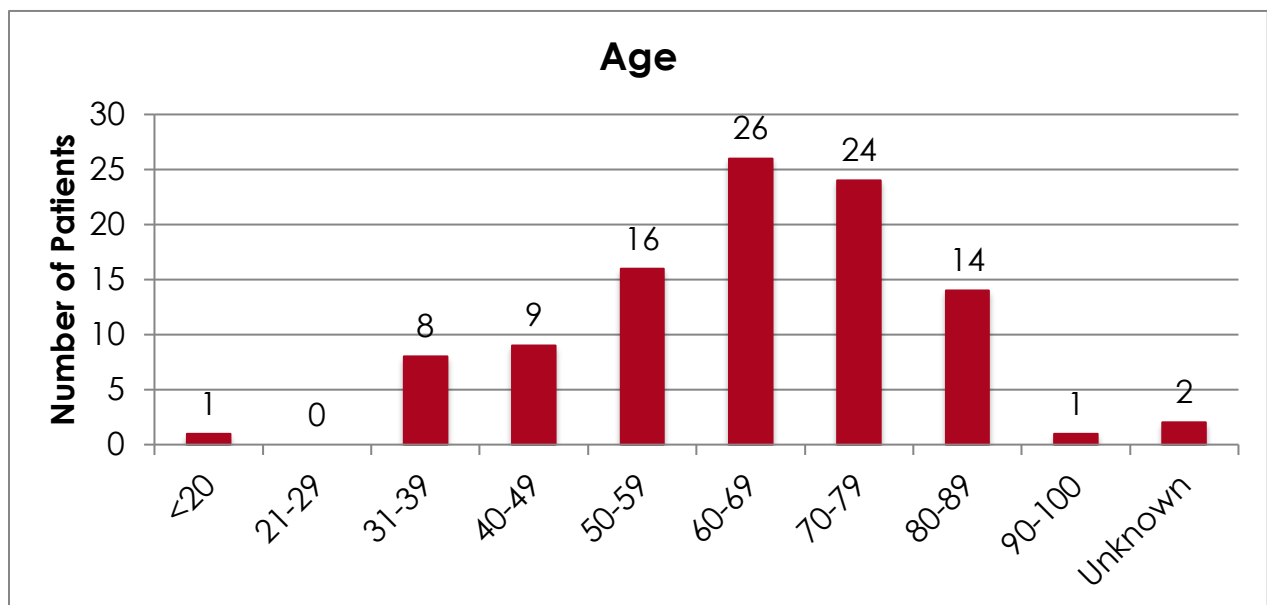


Figure 23: Age range distribution among the hemodialysis patients balloon donor population.

PHASE 1: HYPOTHESIS TEST. PROOF OF CONCEPT

NanoDrop™- RNA Concentration

In order to select the samples to be used for Real Time-PCR, we divided the total number of samples into 3 groups based on the concentration of mRNA as follows:

1. Insufficient amount = $<5 \text{ ng}/\mu\text{L}$
2. Questionable amount = $5\text{-}10 \text{ ng}/\mu\text{L}$
3. Sufficient amount = $>10 \text{ ng}/\mu\text{L}$

From the initial pool of 101 patients, sufficient amount ($>10 \text{ ng}/\mu\text{L}$) of RNA to enable downstream applications of (RT-PCR) was detected in only 44 of the samples (43.6%), a questionable amount was found in 28 samples (27.7%) and an insufficient amount was detected in 29 samples (28.7%) (**Figure 24**)

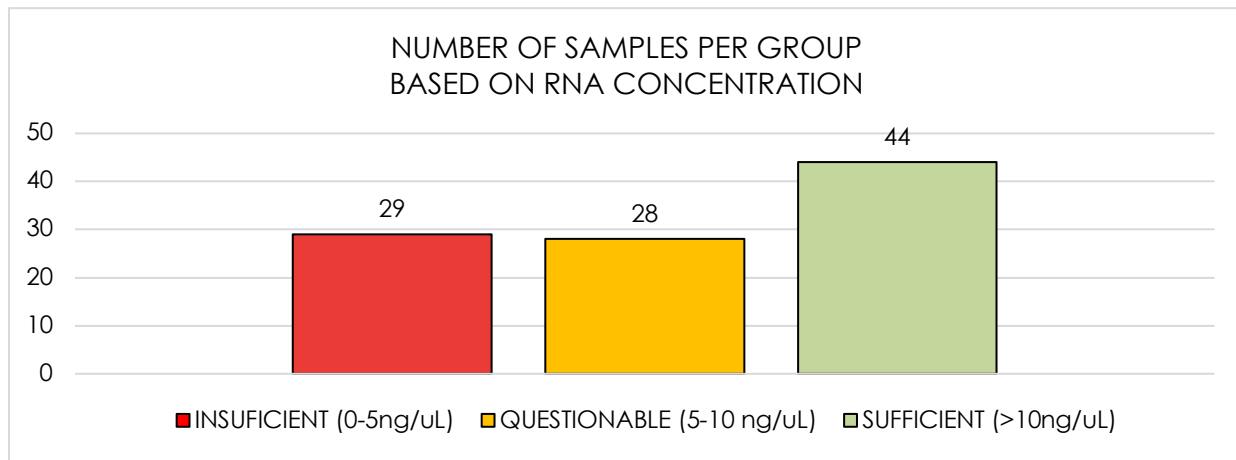


Figure 24: Distribution of samples into groups based on the mRNA concentration detected.

The average concentration in each group is represented in **Figure 25**, the insufficient group had an average concentration of $2.5 \pm 1.2 \text{ ng}/\mu\text{L}$, while the questionable and sufficient group showed concentrations of $7.3 \pm 1.4 \text{ ng}/\mu\text{L}$ and $22.6 \pm 10.3 \text{ ng}/\mu\text{L}$, respectively.

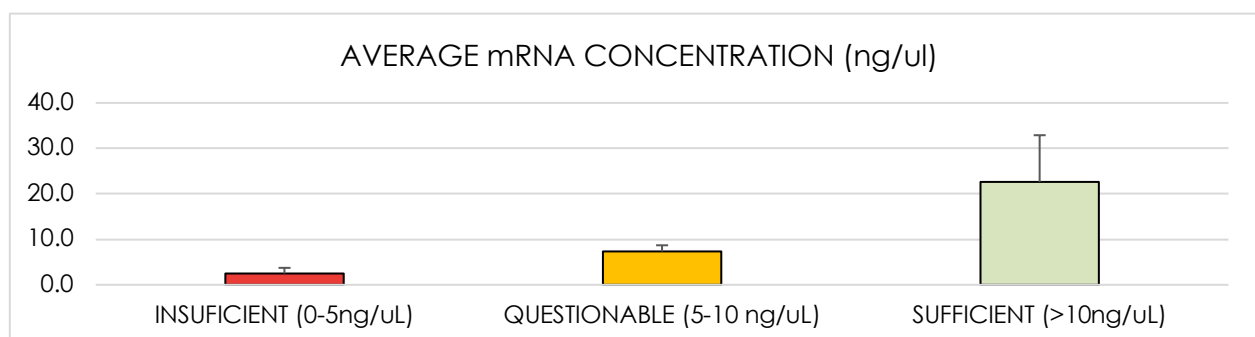


Figure 25: Average concentration of isolated mRNA per group.

RT-PCR- Gene Expression

Initially only the samples from the group presenting sufficient RNA were going to be processed for cDNA synthesis and Real Time-PCR (RT-PCR) for the detection of housekeeping and target genes, but since only 44 samples met the criteria we decided to process both groups, "Sufficient" and "Questionable", which together represented a total of 72 samples (71.3%).

cDNA was successfully obtained from all the samples processed, and subsequently tested on RT-PCR for the expression of GAPDH (the selected housekeeping gene), only the samples expressing GAPDH were tested for the expression of the selected endothelial-specific genes.

Out of the 72 samples, only 18 (25%) expressed GAPDH. Out of the 18 positive samples tested, 14 (77.7%) were from the "Sufficient" mRNA group while only 4 (22.3%) of them were from the "Questionable" group.

Figure 26 illustrates the RT-PCR output after testing for the presence of GAPDH. This specific example is only testing a total of 36 samples, of which 7 tested positive because they exceeded the 0.04 reaction threshold.

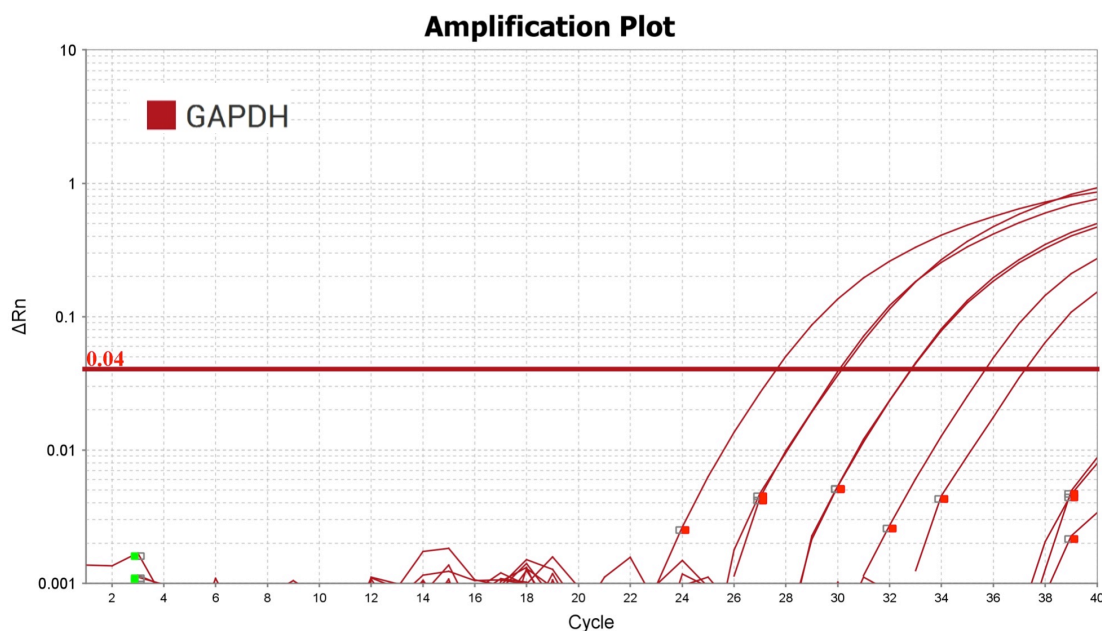


Figure 26: Real Time PCR output showing the expression of GAPDH in 7 samples at different cycles.

The 18 samples that tested positive for the presence of GAPDH were then analyzed once again to search for the presence of endothelial-specific gene structures: FLT1, KDR, FLT4, TIE1 and TEK. The samples were simultaneously evaluated for both the presence of GAPDH and a corresponding endothelial-specific gene. **Figure 27** is the result of a RT-PCR evaluation done on a sample for the presence of GAPDH and all five endothelial-specific genes, which tested positive for all of them.

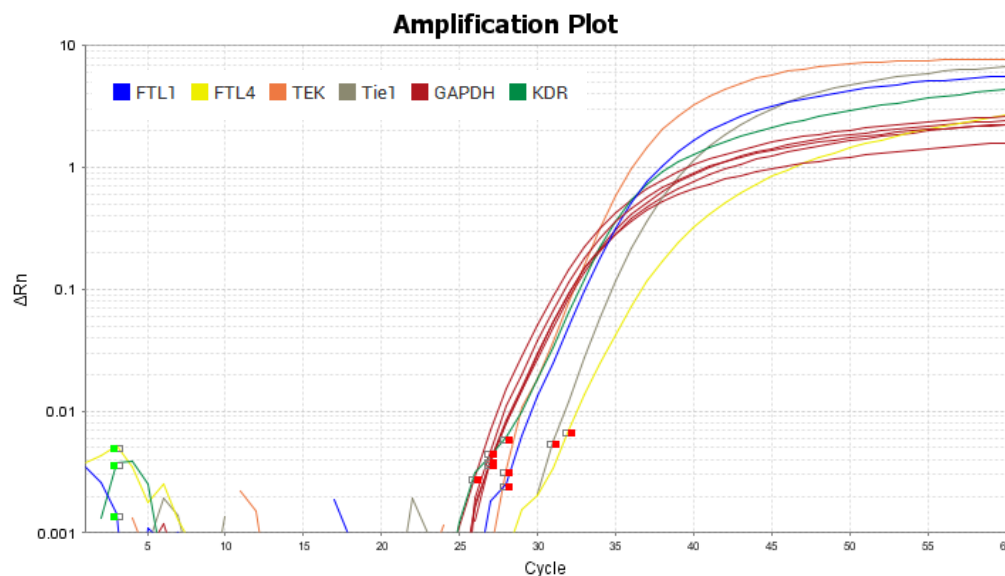


Figure 27: Real Time PCR output showing the combined expression of GAPDH and all the five target genes (FLT1, FLT4, TEK, Tie1, KDR).

9 out of the 18 (50%) samples that tested positive for any of the target endothelial-specific genes are shown below, FLT1, KDR, FLT4, TIE1 and TEK. As shown, the samples did not necessarily express all of the targeted genes. Some samples tested positive for as little as only one targeted gene.

| Balloon ID | FLT1 | KDR | FLT4 | TIE1 | TEK |
|------------|------|-----|------|------|-----|
| B15 | X | | | | |
| B52 | | X | | | |
| B136 | X | | | | X |
| B202 | X | X | X | X | X |
| B105 | X | | | X | |
| B1 | X | X | | X | X |
| B2 | X | | | | |
| B24 | X | | X | X | X |
| B34 | X | | | | |

Figure 28: Selection of all 9 samples that tested positive for any of the target genes using Real Time-PCR during the first phase of the study

BioAnalyzer®- RNA Quality

Since the conversion rate from sufficient mRNA to expression of housekeeping gene was very low, we decided to test the quality of the mRNA present in our samples using the BioAnalyzer®. We began by testing the samples that expressed GAPDH in order to assess if the quality (low degradation) of the samples was appropriate.

Surprisingly, only 2 of the total 27 samples tested with the BioAnalyzer® actually resulted in a calculated RIN (RNA Integrity Number) value, with values of 1.1 and 2.5. The remaining 25 samples were unable to be read using the Agilent RNA 6000 Pico Kit, which either indicates contamination of the samples or a very high degradation of the RNA.

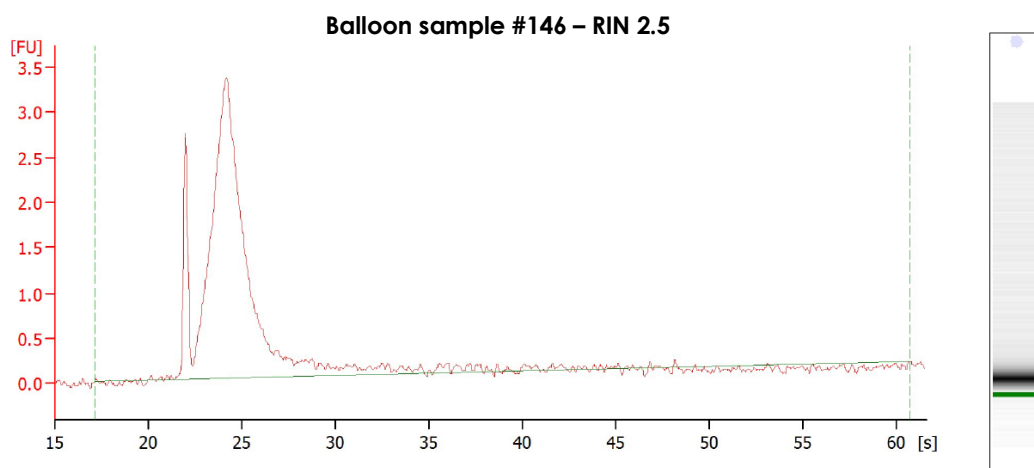


Figure 29: BioAnalyzer® output showing a RIN number of 2.5.

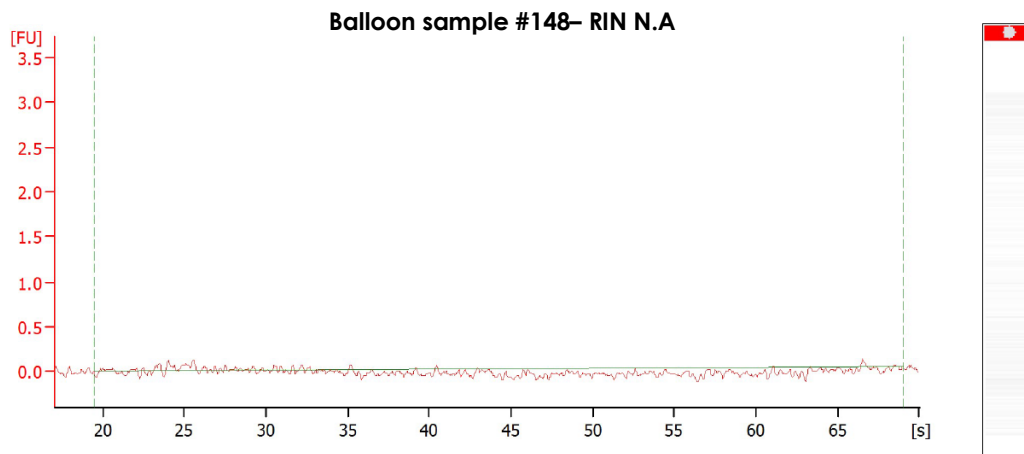


Figure 30: BioAnalyzer® output showing a total absence of ribosomal RNA fragments which results in an absence of RIN value.

PHASE 2: TECHNICAL OPTIMIZATION

After analyzing the data of the first phase of the study, we were able to prove (although on a reduced percentage of samples) the presence of endothelial cells in the samples extracted from the balloons. We confirmed that the quality of the mRNA obtained was extremely low. We wondered if the reason for the low percentage of samples showing housekeeping and target genes was due to the bad quality of the mRNA obtained. For the second phase, we tried to improve the quality of the mRNA (by the approaches described on the methodology section), to test if that would translate into a higher percentage of samples showing both housekeeping and endothelial specific genes.

NanoDrop™- RNA Concentration

Based on the results of the first phase, we decided to consider sufficient mRNA samples that had a concentration above 10 ng/μL. A total of 52 samples were analyzed in the second phase of the study, 37 of them (72%) presented a sufficient quantity with an average concentration of 31.6 ± 31.2 ng/μL, while the insufficient group averaged 7.4 ± 1.6 ng/μL.

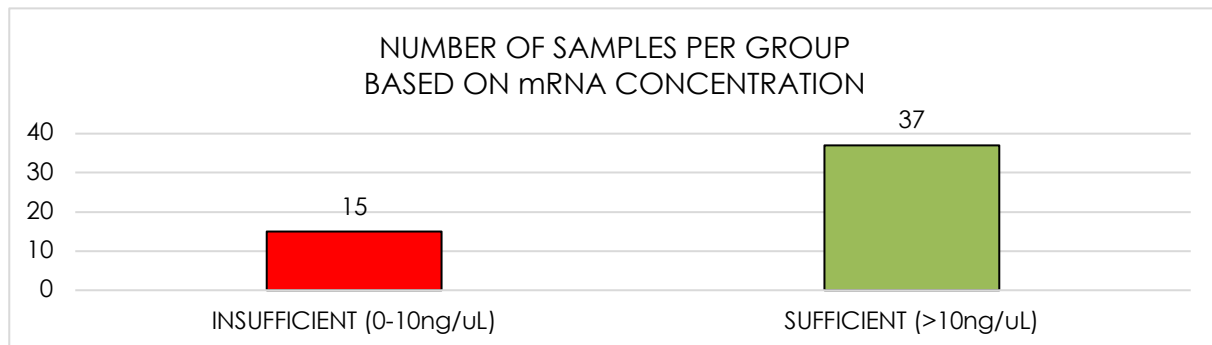


Figure 31: Distribution of samples into groups based on the mRNA concentration detected.

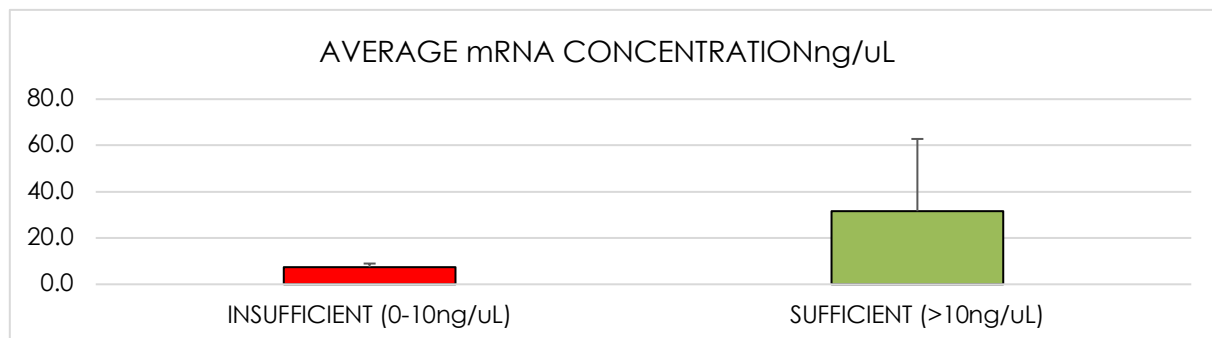


Figure 32: Average concentration of isolated mRNA per group

RT-PCR- Gene Expression

cDNA was successfully obtained from the 37 samples in which sufficient mRNA concentration was detected. Real Time-PCR was subsequently conducted in all these samples for the expression of GAPDH and Ribosomal RNA (housekeeping genes). The samples that tested positive for GAPDH also tested positive for Ribosomal RNA. Once again, the samples that expressed GAPDH were further tested for the expression of the selected endothelial-specific genes.

Out of the 37 samples, only 9 (24.3%) expressed GAPDH and Ribosomal RNA. Out of the 9 positive samples, 6 (66.6%) expressed at least one of the selected endothelial specific genes.

| Balloon ID | FLT1 | KDR | FLT4 | TIE1 | TEK |
|------------|------|-----|------|------|-----|
| B10 | | | | | X |
| B15 | | | | X | |
| B135B | | | | | X |
| B60 | | | | X | X |
| B53 | | | | | X |
| B54 | | | | X | X |

Figure 33: Selection of all 6 samples that tested positive for any of the target genes during the second phase of the study

BioAnalyzer®- RNA Quality

We used the BioAnalyzer® once again to test the quality of the mRNA present on the 37 samples that expressed the housekeeping genes (both GAPDH and Ribosomal RNA). In the second phase, only 7 out of the 37 samples (18.9%) analyzed actually resulted in a RIN (RNA Integrity Number) value, the average RIN number on those samples was 2.5 ± 1.8 .

DISCUSSION

The results gathered from Phase 1 indicated, through Real Time-PCR, that endothelial cells attach to angioplasty balloons during angioplasty procedures on AVFs, and moreover, some of these cells still remain on the balloon surface once it is extracted from the patient, confirming our initial hypothesis. With that being said, the primary goal in Phase 2 was to optimize the technical approach used in Phase 1 to maximize the ability to detect endothelial cells. As a result of the optimization adjustments, we were able to: (1) increase the average mRNA concentration on the "sufficient" ($>10\text{ng}/\mu\text{L}$) group from 22.6 ± 10.3 to 31.6 ± 31.2 , (2) increase the percentage of samples with enough mRNA from an initial 44% to 72%, (3) maintain the percentage of viable samples (expressing housekeeping gene) from 25% to 24.3% (although at a slight decrease), and (4) augment the percentage of viable samples (from the "sufficient group") expressing any of the endothelial-specific genes from 43.7% to 66.6%.

However, the quality of the mRNA remained consistently and extremely low throughout both phases of the study. Samples tested in the BioAnalyzer® resulted in a RIN number in only 7.4% of the samples in Phase 1 and 18.9% in Phase 2. Although the obtained results were slightly better on the second phase, all of the obtained RIN numbers represented a low quality/high degradation of the samples. This is a major technical impact that must be dramatically improved in order for the proposed recovery technique to be useful for additional downstream applications.

Although the high degradation of the samples was evident, many of them still display the presence of housekeeping genes and/or endothelial specific genes after Real Time-PCR analysis. We speculate, that this could be due to the fact that the amplicons (PCR products) synthesized during Real Time-PCR are of very short length (less than 80bp). Thus, in a population of very short degraded pieces of nucleic acid we can still detect the presence of certain gene(s) due to its small size.

Moving forward, different strategies could still be implemented to potentially improve both the quantity and quality of RNA to facilitate further extraction and reliability of information. For example, one major concern regarding the current methodology includes the interaction between the lysis buffer and the plastic component of the balloon, that could accelerate the formation of mRNA fragments. Another approach could include an alternative methodology for the initial processing of the balloon material: Instead of cutting the angioplasty balloon into pieces, and submerging it into a lysis buffer, the whole balloon could be submerged into a Trypsin solution to induce the detachment of

vascular cells from the exterior of the balloon. The solution can then be centrifuged, and the cells could be sorted for individual analysis.

At the time of sample collection, an anonymous collection form was filled out by the vascular center. The form aimed to collect some technical and demographic data that could influence the cell recovery success. Our lab is currently trying to correlate the cell recovery success rate to technical/demographical data such as the number of times the patient had received a form of intervention, the number of inflations used during the procedure, patient comorbidities, the site of procedure, etc.

ACKNOWLEDGEMENTS

I would like to begin by thanking Dr. Prabir Roy-Chaudhury for the opportunity to work in his lab. He has always been supportive and incredibly knowledgeable. Diego Celdran-Bonafonte has been such a great teacher throughout my time in the lab. I appreciate the numerous tutoring lessons that he provided for me to fully understand the material. Jaroslav Janda was also kind enough to share his expertise. He always seemed to be able to put a smile on my face. Aous Jarrouj has been a huge form of support. He always updated me on different ongoing projects, which created a sense of inclusivity on a much larger scope. Dr. Cindy Rankin has been an immense inspiration. She genuinely encouraged me to fulfill my studies in the Honors College.

REFLECTION

My honors thesis has provided me with a more comprehensive perspective of translational research, patience and teamwork. Although the BLAISER summer program served as an initial exposure to research in Dr. Roy Chaudhury's lab, my individualized thesis allowed me to conduct a mini project from beginning to end. I now truly understand the amount of time that is dedicated to the advancement of scientific knowledge with respect to brainstorming, preparation, bench work, the supply of lab materials, lab meetings, documentation, analysis and most importantly, the distribution of ideas. In the larger picture, the purpose behind investigating innovative ideas is for the advancement of society. My admiration for translational research has grown dramatically because I can see how it is directly applied to the medical world. My end goal is to study medicine and my thesis has provided me with the tools to appreciate the direct impact biomedical research has on medicine. In my case, my interest in my thesis project was strengthened with the *potential* benefit that dialysis patients could receive as an end result.

Now that my project is complete, and I reflect on my thesis experience, I realize just how much I have learned. The Physiology Poster Session opened my eyes to it. I enjoyed being able to share my work with others. Students, faculty and staff that attended the poster session were incredibly supportive and genuinely interested in our work. Overall, I am grateful for this opportunity because it allowed me to apply my classroom knowledge to a translational scenario, all while learning about myself in the process.

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FIGURES

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