

Removal of COVID-19 Spike Protein, Whole Virus, Exosomes and Exosomal microRNAs by the Hemopurifier® Lectin-Affinity Cartridge in Critically Ill Patients with COVID-19 Infection

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Case Report

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Abstract

Coronavirus – 19 (COVID-19) has rapidly spread throughout the world resulting in a significant amount of morbidity and mortality. Despite advances in therapy, social distancing, masks, and vaccination many places in the world continue to see an increase in the number of cases and deaths. Viremia is commonly present in severely ill patients with COVID-19 infections and is associated with organ dysfunction and poor outcomes. Exosomes released by activated cells have been implicated in the pathogenesis of COVID-19 infection. We report the experience of two cases of critically ill COVID-19 patients treated with the Hemopurifier; a lectin affinity cartridge designed to remove mannoseylated viruses and exosomes. Both patients tolerated the Hemopurifier sessions without adverse effects. In the first patient removal of exosomes and exosomal microRNAs was associated with improved coagulopathy, oxygenation, and clinical recovery, while in a second patient removal of COVID-19 by the Hemopurifier cartridge was observed. The Hemopurifier is currently under further investigation in up to 40-patients in a safety and feasibility study in ICU patients with COVID-19 infection.

Introduction

On November 17, 2019 the first case of COVID-19, a severe respiratory infection caused by the SARS-CoV-2 virus, was described in Wuhan, China. The virus rapidly spread across the globe with approximately 167,391,920 million cases and 3.47 million deaths as of May 25, 2021 (<https://coronavirus.jhu.edu/map.html>). Fortunately, with mitigation measures, treatments and vaccinations, cases and deaths have decreased in many locales, yet many disease hotspots remain. A need to explore additional treatments, particularly in those most desperately ill, remains.

Early in the pandemic the magnitude of viremia and its clinical importance was underappreciated. A recent meta-analysis has revealed the presence of viremia in 34% of COVID-19 infected patients with higher rates occurring in the critically ill. The presence of viremia was associated with disease severity, the development of multi-organ failure¹. Exosomes, extracellular vesicles ranging in size from 50-150nm, are involved in the cell to cell spread of COVID-19 infection as well as the ensuing inflammation, coagulopathy and complement activation². The Hemopurifier® (HP) cartridge (Aethlon Medical, Inc, San Diego, CA) is a hollow fiber plasma separator filled with an affinity resin containing the lectin Galanthus nivalis agglutinin (GNA) from the Galanthus nivalis plant (the common snowdrop)³. GNA has a high affinity to mannose-rich glycoproteins which are present on enveloped viruses as well as exosomes^{4,5}. A recent in-vitro study demonstrated the removal of the SARSCoV2 spike protein by a mini-Hemopurifier. (Figure 1).

Here, we describe the emergency use of the Hemopurifier in two cases of critically ill patients with COVID-19 infection. The report contains details of the treatments, clinical course, as well as changes in laboratory values, plasma COVID-19 viral load and total exosome and exosomal miRNA concentrations following treatment

Methods

Hemopurifier Lectin-Affinity Treatment

Two patients were treated with the Hemopurifier for 6 hours daily. The cartridge was operated via a standard dual lumen veno-venous hemodialysis catheter. Blood entered the cartridge where plasma was forced through the pores (≈ 200 nm) of the hollow fiber membrane due to the pressure gradient established. Blood cellular elements remained in lumen of the hollow fibers. Plasma entered the extracapillary space where the lectin-affinity resin resides. Glycosylated molecules were bound by the lectin and prevented from re-entering the circulation. The pressure gradient was reversed along the course of the hollow fibers allowing the plasma to flow backward through the hollow fibers to recombine with the blood before re-entering the circulation (Figure 2) ⁶.

Laboratory Measurements

Clinical parameters and laboratories were collected as per standard of care. Blood samples for plasma COVID viral load measurement and exosomal analysis were collected in EDTA blood collection tubes prior to and after each Hemopurifier session. Plasma was isolated, deactivated with viral lysis buffer, and shipped frozen to Aethlon Medical for further analysis. The used Hemopurifier cartridge was sealed and refrigerated and then sent back for viral elution by the following methodology. First, used Hemopurifiers were flushed with a 200 ml solution of 0.5M alpha-methylmannoside (α -MM), a lectin binding competitor⁷, to gently elute a portion of the blood components bound to the resin. Subsequently, Hemopurifier cartridges were flushed with 200 ml of TRIzol reagent (Thermo Fisher) to elute any remaining blood components, proteins, and nucleic acids still bound to the GNA lectin. A spin column capture methodology was used to isolate RNA eluted in the Trizol solution. Briefly, 1ml of the Trizol-eluent (frozen at -80°C) was thawed and mixed with 200 μl of chloroform, vortexed for 15 seconds and incubated at room temperature on the benchtop for 2-3 minutes. The mixture was then spun at 12,000g, 4°C , for 15 minutes and 600 μl of the upper aqueous phase collected and transferred to a new tube. This was mixed with 1.5x volumes (900 μl) of 100% EtOH and then added to the QiaAMP Viral RNA extraction column (Qiagen, cat#52906). The remaining RNA isolation procedure from the column was performed as instructed by the manufacturer, and purified RNA was eluted in 45 μl of AVE Buffer. A one-step RT-qPCR methodology⁸ was used for detection of the SARS-nCoV2 virus with the Taqman 2019 nCov Assay Kit (Thermofisher Scientific, Cat# A47532) targeting three unique SARS-nCoV2 genome sequences (N-protein, S-protein, and ORF1ab) and the Taqman Fast Virus 1-step master mix (Thermofisher, Cat#4444434). An estimate of the viral copy number captured on the hemopurifier was calculated by relative comparison to measurements of the positive control standard (Thermofisher Scientific, #A47533).

Plasma COVID viral load testing was accomplished using the same viral RNA detection techniques. The biological specimens, plasma samples (with EDTA anticoagulant) were collected from each pre- and post-Hemopurifier therapy session, and 140 μl from each plasma specimen was processed in buffer AVL

(Qiagen) to isolate nucleic acids using the QiaAMP Viral RNA extraction kit according to the manufacturer's instructions. Purified RNA samples were collected in 30µl of AVE Buffer and 5µl were used in each RT-qPCR to quantify SARS-nCoV2 viral copy numbers. For comparative analysis of viral loads among distinct plasma samples, calculations were further normalized to the quantity of Ribonuclease P (RNase P) measured in each sample, a recommended methodology to control for differences in SARS-nCoV2 sample collection and processing techniques ⁹.

Exosomes were purified from patient plasma using an established methodology ¹⁰. Briefly, 1ml of patient plasma was precleared through a two-step centrifugation process (2,000g for 10 minutes; 10,000g for 30 minutes) to remove larger plasma particles, then filtered through a 0.2µM PES membrane, and loaded onto a 10ml Sepharose CL-2B column. Exosomes were isolated from the rest of the plasma components through size exclusion chromatography by adding 1ml increments of PBS to the Sepharose column until the Fraction #4 eluent, containing plasma exosomes was collected. Exosome quantification data was collected using nanoparticle tracking analysis (NTA) techniques as previously described ¹¹, using a Nanosight LM10 instrument. In order to obtain reliable quantification measurements, plasma exosome samples had to be diluted in 0.2µM filtered PBS to a concentration of approximately 10⁸-10⁹ exosomes/ml. Approximately 20-100 particles could be observed in the Nanosight field of view once exosome samples had been diluted to the appropriate concentration range. Enhanced detection of smaller exosome populations isolated from the COVID plasma was achieved using a Camera Level of 12 and a Detection Threshold of 3 measurement parameters. Three 30 second capture videos of different segments of the homogenous exosome sample were evaluated with the NTA 3.3 software to obtain particle quantification and sizing measurements.

MicroRNA was isolated from the COVID plasma exosomes using the miRNAeasy Serum/Plasma RNA extraction kit (Qiagen, cat#217184) and incorporating an exogenous miRNA spike-in control (5.6x10⁸ copies cel-miR-39-3p miRNA/sample) to control for variability introduced during the sample preparation process. miRNA was reverse transcribed to a cDNA template using the Taqman Advanced miRNA cDNA Synthesis Kit (Cat#A28007). Specific miRNA targets were amplified on a Quant 3 qPCR machine using specific Taqman Advanced miRNA primer/probe sets (hsa-miR-424-5p, hsa-miR-16-2-3p, Cat#A25576). Quantification of miRNA sequences was done by normalization to the exogenous spike-in cel-miR-39-3p miRNA control, using $\Delta\Delta C_t$ methods ¹². MicroRNAs associated with coagulation, inflammation and acute lung injury were measured.

Case 1

The patient was a 59-year-old female with a past medical history notable for obesity, hypertension, hyperlipidemia, alcohol abuse, and mechanical aortic valve replacement on warfarin. She was admitted in July 2020 with COVID-19 pneumonia and admitted to the general medical ward for oxygen and other therapies. She received a course of dexamethasone and was subsequently given convalescent plasma on hospital day (HD) 8. She developed progressively worsening respiratory failure and acute respiratory distress syndrome (ARDS) despite high flow nasal cannula O₂ followed by BIPAP

therapy. She was transferred to the Intensive Care Unit (ICU) on HD 11 for intubation. After intubation, mechanical ventilation and prone positioning, her oxygenation did not improve. She was not considered a candidate for Remdesivir because of the duration of her disease, but she did receive a course of tocilizumab on HD 12 and was also administered high dose methylprednisolone. Despite all interventions, her PaO₂/FIO₂ ratio continued to decline to the point that she could not maintain adequate oxygenation, while paralyzed on Rocuronium and in the prone position on 1.0 FiO₂ and PEEP of 14 cm H₂O. She was also treated with epoprostenol (EPO) and initially could be supinated while on it but subsequently failed supination even on EPO. She was evaluated for possible ECMO and deemed to not be a candidate.

On hospital day 21 the attending physician and an independent physician determined that the patient had failed maximal medical treatment for COVID-19. A written request was made to Aethlon Medical, Inc. for single patient emergency use of the Aethlon Hemopurifier. In accordance with federal regulations governing emergency use, all regulatory documentation was obtained including signed informed consent as well as IRB approval. On HD 22 the patient received her 1st Hemopurifier treatment. Prior to her 1st HP treatment, she was on maximal ventilatory support (FiO₂ 1.0, PEEP 14 cm H₂O, proned). The venous side of the double lumen internal jugular catheter clotted 20 minutes into the first Hemopurifier treatment. Patency was restored with TPA. The treatment was re-started with a fresh Hemopurifier cartridge, and she successfully completed the prescribed 6-hour treatment. She received a 6-hour HP treatment once daily four days over hospital days 22-25. The patient tolerated the procedure well without evidence of allergic reaction, thrombotic complications, or hemolysis.

The clinical impression of the attending physician following the four treatments was that there was a slight improvement in her clinical status. A review of the patient's laboratories (Table 1) reveals that, prior to treatment, she had evidence of COVID-induced coagulopathy (CAC) with thrombocytopenia and a markedly elevated D-dimer level. The patient also had marked respiratory impairment as indicated by a low paO₂/FIO₂ ratio, systemic inflammation as indicated by hyperferritinemia and tissue damage as indicated by an elevated lactate dehydrogenase (LDH). On HD 27, essentially 2 days after the first 4 HP treatments, the patient had improvements in her markers of coagulation and oxygenation as well as decreases in her ferritin and LDH.

Given the signs of clinical improvement, as well as the thinking that exosomes may still be contributing to the patient's ongoing critical illness, the decision was made to continue the HP treatments for 4 additional days. The patient received 4 additional 6-hour treatments from HD 27 through 30. The patient improved clinically and was able to be maintained supine on an FIO₂ of 50% and a PEEP of 10. She subsequently required a tracheostomy. She was transferred out of the ICU on HD on an FIO₂ of 40% and 5 of PEEP. She was discharged to a long-term care facility where she was able to be successfully decannulated and sent home on nasal O₂ 20 days later.

Throughout the Hemopurifier treatment the patient had plasma samples stored for retrospective analysis of plasma COVID viral load as well as exosomal analysis. Blood samples were collected before and after

the emergency-use Hemopurifier treatment (6hrs/treatment) conducted on 8 different days. The patient's COVID-19 plasma viral load was undetectable at the onset of treatment with the Hemopurifier. Over days 2-7 of the HP treatment the total exosome concentrations decreased from pre to post- HP treatment. Interestingly, the total exosome concentration increased during treatment on Days #1 and #8 (Figures 3&4). A consistent pattern of decreasing exosomal miR-424 concentrations from pre to post- HP treatment was observed over the 8 Hemopurifier treatments coinciding with the improvement in coagulopathy. The concentration of exosomal miR-16 dropped over the first 4 Hemopurifier treatments and then stayed at low levels as the patient's acute lung injury improved (Figures 3&4).

Case 2

The patient was a 67-year-old gentleman with a history of tetralogy of Fallot repair, coronary artery disease, and newly diagnosed diabetes mellitus. He presented to the hospital in January 2021 with a 1-week history of cough and shortness of breath. He was found to be COVID-19 positive by polymerase chain reaction test (PCR) and was admitted to the hospital. The patient was also noted to have acute kidney injury. Despite treatment with Remdesivir, Dexamethasone, Baricitinib, convalescent plasma, and full dose anticoagulation, the patient developed worsening multiple organ system failure. He was on mechanical ventilation with an FIO₂ of 100% and 12 of PEEP, a single vasopressor for hypotension and CRRT for acute renal failure. Given the patient's deterioration despite maximal medical support, the treating physician requested the single patient emergency use of the Aethlon's Hemopurifier on HD 8. In accordance with federal regulations governing emergency use, all regulatory documentation was obtained including signed informed consent as well as IRB approval.

The Hemopurifier treatment was performed on hospital day 9. Prior to the treatment the patient required two vasopressors for hypotension as well as prone position ventilation with an FIO₂ of 0.90 and a PEEP of 8 to maintain oxygenation. The pre-treatment SOFA score was markedly elevated at 13 indicating a predictive mortality of > 80%. The patient received 6 hours and 15 minutes of Aethlon Hemopurifier treatment in series with CRRT. The patient had fluctuations in his oxygenation and blood pressure during the completed HP session. The patient was disconnected from the Hemopurifier without incident. An examination of the Hemopurifier cartridge did not reveal changes suggesting clotting or hemolysis and his haptoglobin level was normal.

An examination of the patient's post-Hemopurifier session labs revealed evidence of clinical worsening with a C-Reactive Protein test (CRP) that had increased from 7.9 to 16.2mg/dl. The d-dimer level and LDH both increased to beyond the upper limit of detection. Following disconnection of the Hemopurifier the attending physicians elected to change out and reinitiate his CRRT circuit. Approximately 15 minutes after the new CRRT circuit was placed the patient's blood pressure began to drop. The patient developed refractory shock and refractory hypoxia and expired due to a pulseless electrical activity (PEA) arrest.

The Hemopurifier cartridge used on the patient was saved for analysis, and plasma samples for viral load testing were collected before and after the Hemopurifier session. PCR testing done on eluent from the

Hemopurifier demonstrated viral capture by the cartridge. Additionally, the plasma COVID viral load normalized for RNase P decreased by 58% in the sample collected at the end of the Hemopurifier treatment compared to the pre-treatment sample. (Table 2)

Discussion

In this publication we describe the first use of a Hemopurifier lectin-affinity plasmapheresis cartridge in two critically ill patients with severe COVID-19 infection. A total of 9 Hemopurifier sessions were tolerated by these two patients despite multi-organ system failure. The cases build on our experience in a critically ill patient with Ebola where viral removal and clinical improvement was observed following a Hemopurifier treatment⁶. Previously the Hemopurifier had been studied in 29 ambulatory patients with > 90 Hemopurifier exposures in clinical studies involving patients with either Hepatitis C¹³ or HIV. The safety profile was benign in these subjects with a side effect profile that is typical for other extra-corporeal therapies.

The first of the two reported cases is notable for the clinical course following Hemopurifier treatments observed in a patient without demonstrable COVID-19 viremia. The absence of viremia was not unexpected given that the patient received her first Hemopurifier treatment on hospital day #22. Following the initial 4 treatments the patient had evidence of improvement in COVID-19 associated coagulopathy (CAC), lung injury, inflammation, and tissue injury. We hypothesize that these improvements were due to the removal of exosomes with noxious microRNA cargo by the Hemopurifier. Exosomes are extracellular vesicles released by activated cells in response to stimuli. Exosomes are involved in cell to cell signaling via cargo including proteins, receptors cytokines, and genetic material such as non-coding microRNAs¹⁴. MicroRNAs are involved in mRNA degradation and inhibition of protein translation¹⁵. Barberis and colleagues compared the exosomes from COVID infected vs healthy controls and found differences in the cargo involving proteins involved in inflammation, coagulation and complement activations². These exosomal proteins correlated with the clinical markers CRP and d-dimer. Gambardella and colleagues found that exosomal miR-424 was upregulated in COVID patients with coagulation activation as indicated by elevated d-dimer levels vs COVID-19 infected patients with normal d-Dimer levels¹⁶. In a rat LPS challenge model of acute lung injury, transfection with an miR-16 overexpression virus was associated with an increased wet/dry ratio of lung tissue and higher NFkappa B levels compared to rats transfected with an empty vector¹⁷.

In our patient we observed decreases in total exosomal concentration from pre to post Hemopurifier treatment over days 2-7. A rise in exosomes was noted on the first and eighth day of treatment. We hypothesize that the rise on the first day may have been related to the clotting in the hemodialysis catheter. The rise on total exosome concentration of the eighth day is unexplained and requires further investigation. We noted a decrease in miR- 424 following the initial 4 days of HP treatment at a time when the patient's coagulopathy improved. We also noted decreases in miR-16 concentrations throughout the treatment period at a time when the patient's oxygenation was improving. These findings

suggests that benefits from the Hemopurifier in COVID-19 may extend beyond viral removal and may be a result of the elimination of mannosylated exosomes.

The second case is novel in that we demonstrate for the first time the *in vivo* removal of COVID-19 from the bloodstream in an infected patient by the Hemopurifier. We were able to elute COVID-19 from the used Hemopurifier cartridge as well as demonstrate a 58% reduction in plasma COVID-19 viral load following the single six-hour treatment. Unfortunately, this patient's disease was quite advanced at the onset of the Hemopurifier therapy, and he succumbed to multi-organ failure. It is possible that viral load reduction earlier in his course of COVID-19 might have provided a clinical benefit in this patient. In a study by Bermejo-Martin and colleagues, the presence of viremia was associated with a dysregulated immune response and development of coagulopathy¹⁸. Post-mortem studies have demonstrated viral dissemination and seeding of organs including the bone marrow, heart, and gastrointestinal tract¹⁹. In a Swedish cohort of critically ill COVID-19 patients, the presence of viremia was associated with the need for renal replacement therapy and poor clinical outcome²⁰.

In summary, we describe the first two cases of critically ill COVID-19 patients treated with the Hemopurifier lectin-affinity plasmapheresis cartridge. The two patients tolerated a total of nine 6-hour Hemopurifier treatments without side effects. For the first time, we demonstrate the removal of COVID-19 from a viremic patient by the Hemopurifier. Additionally, total exosome concentrations and noxious exosomal microRNAs associated with coagulopathy and acute lung injury decreased with Hemopurifier treatments and was associated with clinical improvement in one patient. An IDE study enrolling up to 40 ICU patients with COVID-19 [NCT04595903] is currently underway and should provide more information regarding the safety and feasibility of the Hemopurifier as well as biologic and clinical effects.

Declarations

Financial Support:

The Hemopurifiers were supplied by Aethlon Medical, Inc. Aethlon Medical performed COVID-19 viral load testing and exosomal analysis.

Conflict of Interest: Authors Rosalia de Necochea-Campion, Michael Jacobs, Steven P. LaRosa, and Charles J. Fisher, Jr. are employees of Aethlon Medical, Inc. which manufactures the Hemopurifier device.

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Tables

Date	D-dimer (ng/ml)	Platelet (cells/ml)	PT/INR	Ferritin (ng/ml)	Lactate (mmol/l)	PaO2 /FIO2 ratio	ALC (absolute lymphocyte count) (cells/ml)	LDH (U/L)
HD 14 (8 days prior to therapy)				3599.5 (systemic inflammation)				2370 (tissue injury)
HD16 (6 days prior to therapy)	>7650							
HD 18 (4 days prior to therapy)		115,000			3.6 (tissue hypoxia)			
HD 22 (Day 1 therapy)			1.2 (13.6sec, prolonged)			93	780 (lymphopenia)	
HD 23 (Day 2 therapy)					2.3	98		
HD 24 (Day 3 therapy)						75.5		
HD 25 (Day 4 therapy)						88.57		
HD 27 (Day 5 therapy)	3703	162,000	1.0 (11.3sec, improved)	622.4	0.8 (normal)	136.25	1180	978 (improved)
HD 28-30 (Days 6 - 8 therapy)						>117		
HD 30						175		

Table 1: Laboratory values over time in Emergency Use Case #1

ESTIMATE HEMOPURIFIER CAPTURE					
	1 PCR Rxn	Total RNA	Total Trizol	Total Hemopurifier Eluted Copies	
	Sul/rxn	60/Sul	200/1ml		
HP-P2Eluent	242.1	x12	x200	5.8E+5	
PLASMA VIRAL COPIES NORMALIZED TO RNASEP					
	1 Rxn	Total RNA	Plasma	RNAseP	Plasma
	Sul	45/Sul	1/0.14ml	Normalization	Copies/ml
Pre-plasma	24.3	x9	x7.14	x1	1558.6
Post-plasma	29.5	x9	x7.14	x0.34	648.1

Table 2: Hemopurifier Viral Capture and Change in Plasma Viral Load in Case 2

Figures

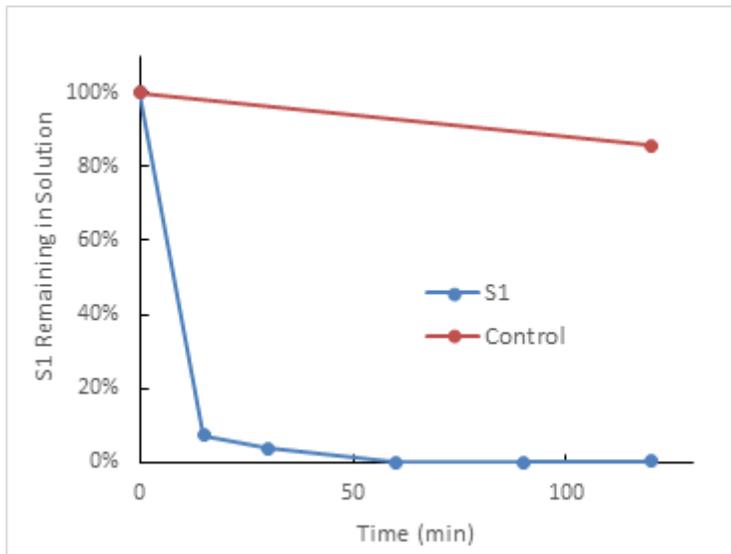


Figure 1

The Hemopurifier captures SARS-CoV-2 spike glycoproteins. This experiment was performed by circulating a solution spiked with the S1 glycoprotein of SARS-COV-2 over the mini-Hemopurifier column in vitro. Briefly, 10 mL of a 1 µg/mL solution of SARS-COV-2 S1 in phosphate buffered saline was circulated over a Hemopurifier column containing 0.7g of affinity resin at a flow rate of 50 mL/min. The rate of viral S1 capture, expressed as a percentage of S1 remaining in solution vs. time, was established by removing fluid samples at defined time intervals. The control consisted of S1 kept on the benchtop for the duration of the experiment (i.e., not run through the device).

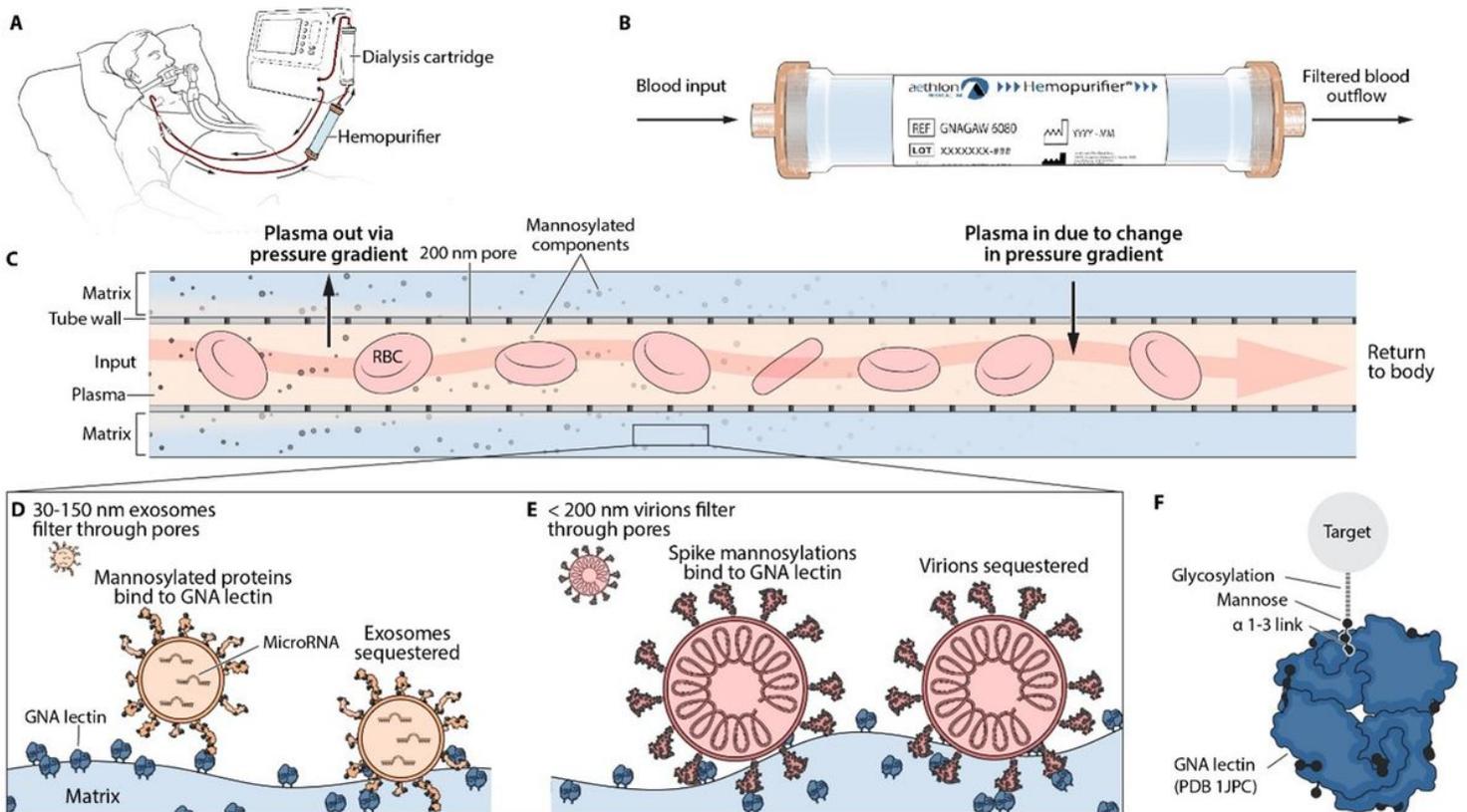


Figure 2

Mechanism of Action of Hemopurifier Lectin-Affinity Cartridge

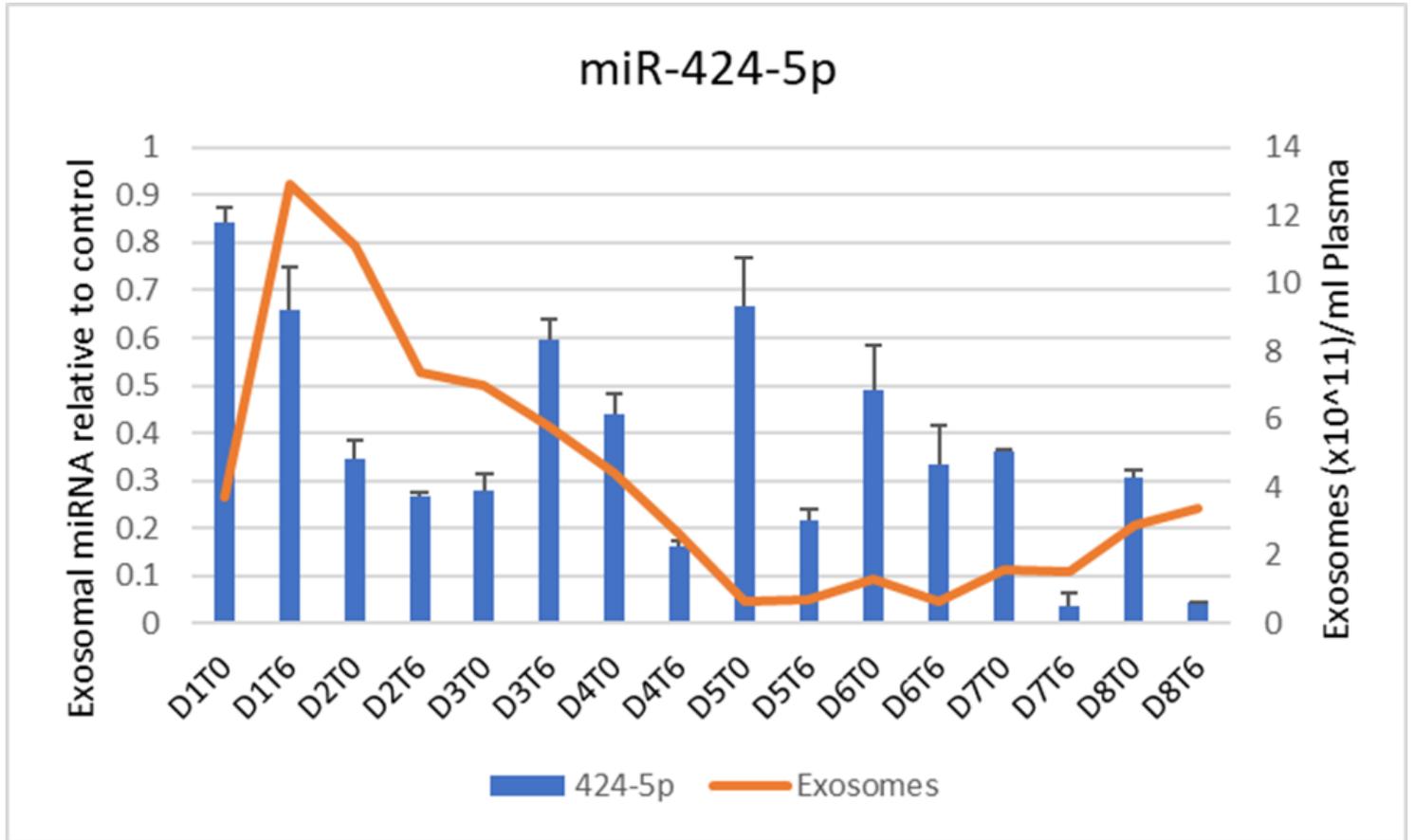


Figure 3

Exosome and Exosomal miR-424 quantification over time in Case 1

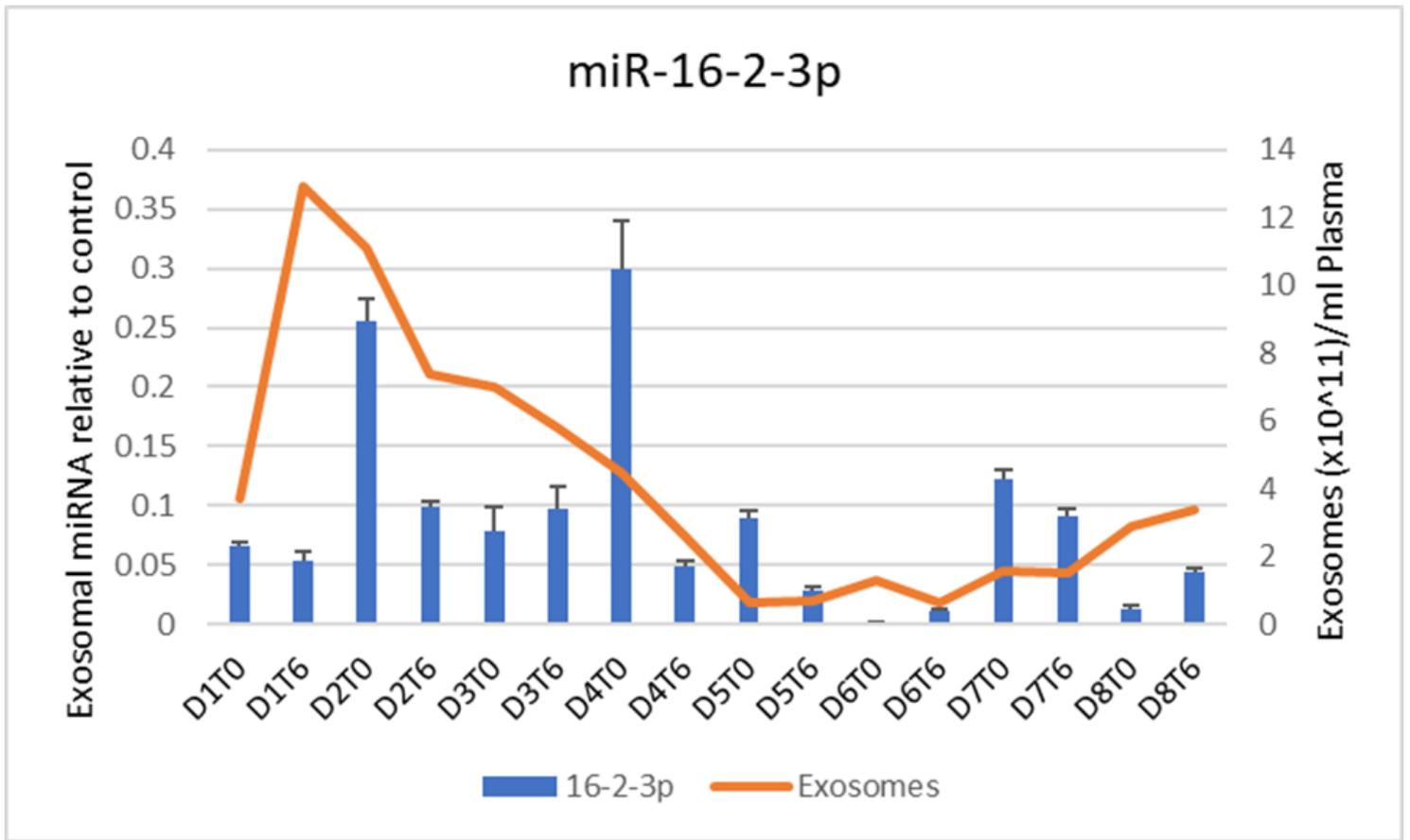


Figure 4

Exosome and Exosomal miR-16 quantification over time in Case 1