Blood Biomarkers of Pulmonary Hypertension in Sickle Cell Disease: A Cross-Sectional Proof-of-Concept Study

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Abstract

Background. Sickle cell disease (SCD) is an inherited blood disorder that is associated with hemolysis, inflammation, and coagulopathy. Pulmonary hypertension (PH) is a comorbidity of SCD that impacts many adult patients. These patients have a much higher mortality risk when compared to their counterparts without PH. There is little knowledge on the proteomic profile of PH in SCD. Our aim was to conduct a proof-of-concept study to explore targeted serum protein biomarkers that are differentially expressed in SCD patients with elevated tricuspid regurgitation velocities (TRV) to explore their corresponding pathways.

Methods. We used data from the Walk-PHaSST study for this investigation. One group of patients had a TRV $\leq$ 2.6 m/sec (n=35) and another had TRV $\geq$ 2.9 m/sec (n=35). The serum concentrations of 92 protein biomarkers were measured using an OLINK cardiovascular panel. The Hochberg method was used to calculate the false discovery rate. A volcano plot was created using the Bonferroni correction. We tested the correlation of the differentially expressed biomarkers with clinical variables measured in blood samples of the participants.

Results. Six proteins passed a Bonferroni corrected overall critical p-value < 0.00054. This includes T-cell surface glycoprotein (CD4), lymphotactin (XCL1), SLAM family member 7 (SLAMF7), galectin-9 (GAL9), TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2), and tumor necrosis factor receptor superfamily member 11A (TNFRSF11A). CD4, XCL1, GAL9, and TNFRSF11A are involved in T-cell regulation, and immunological and inflammatory

functioning. TRAILR2 is involved in apoptosis activation. All six biomarkers are involved in endothelial dysfunction. Among these biomarkers, CD4, GAL9, TRAILR2, and TNFRSF11A, were slightly positively correlated with white blood cell ($r > 0.20$). A significantly negative correlation was observed between neutrophil count and TNFRSF11A, GAL9, XCL1, SLAMF7, and CD4 ($r < -0.21$). We observed a strong, positive correlation between all proteins and serum NT-proBNP levels ($r > 0.44$).

Conclusion. Circulatory protein markers of immune response, apoptosis, and endothelial function are highly expressed in SCD patients with elevated TRV. This provides evidence of the public health significance of these protein biomarkers, as they have potential to be utilized as diagnostic, predictive, prognostic, or therapeutic markers for PH in patients with SCD.

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Preface

       I would like to thank Dr. Nancy Glynn, Dr. Seyed Nouraie, Dr. Yingze Zhang for their continued mentorship and support. Dr. Seyed Nouraie and Dr. Yingze Zhang have been pillars in my growth as a scientist and a future physician, as they have always pushed me to think critically and strive for excellence. Dr. Nancy Glynn has been the backbone of my public health academic journey. Thank you for always encouraging me to pursue my dreams and goals in public health and beyond. Finally, I would like to thank my family and friends who have provided me with the strength and courage to keep going.

1.0 Introduction

1.1 Sickle Cell Disease

Sickle cell disease (SCD) is a broad overarching term that refers to a group of inherited disorder of the red blood cells. Individuals with sickle cell disease have an abnormal hemoglobin, hemoglobin S in their red blood cells, which is caused by a Glu6Val mutation in *HBB*.[1] Hemoglobin S polymerizes, which causes the red blood cells to form a sickle form, and ultimately can result in hemolysis. The most common form of sickle cell disease is sickle cell anemia, but other forms of sickle cell disease include HbSC, and HbSβ thalassemia.[1]

Sickle cell disease triggers a variety of effects that simultaneously contribute to the pathophysiology of the disease.[2] For example, hemoglobin S has reduced oxygen affinity, which is one factor that promoted hemoglobin polymerization. This ultimately alters the red blood cell membrane, which causes sickling. This hemolysis results in oxidative stress in sickle cell, which further damages the cell membrane of other organs, including the blood vessel. Due to this oxidative stress and tissue damage from ischemia-reperfusion, there is an increase in extracellular hemoglobin in the plasma, which function to activate the innate immune system and lead to adhesion of sickled cells.[2] This clumping of cells and blockage of vessels is dubbed vaso-occlusion. These vaso-occlusive pain crises are commonly associated with sickle cell disease.

A variety of acute and chronic clinical complications are associated with sickle cell disease.[2] Some acute complications include acute pain events, pneumonia, gallstones, sickle hepatopathy, and stroke, among others. Some chronic complications include leukocytosis, anemia,

cardiomegaly, renal insufficiency, diastolic heart failure, complications of pregnancy, and hypertension, among others.[2]

SCD is the most common inherited blood disorder and one of the most common severe monogenic disorders in the world.[3] The Center for Disease Control and Prevention estimate that approximately 100,000 Americans are affected by SCD.[4] Moreover, they find that the prevalence of SCD among underrepresented racial minorities, such as Black and Hispanics is significantly higher.[4] They estimate that approximately 1 in every 365 Black babies and 1 in every 16,300 Hispanic babies have sickle cell disease in the United States.[2] Similarly one study conducted in 2010 using data from 13 US states found that sickle cell trait was found in approximately 73.1 cases per 1000 black births and 6.9 cases per 1000 Hispanic births.[5] However, the incidence of sickle cell trait in white individuals was approximately 3.0 per 1000 births.[5] These racial disparities of sickle cell disease can be explained by the geographical distribution of the β^S allele. There is evidence to indicate that the hemoglobin S trait significantly protects against severe *P. falciparum* malaria, which is most prevalent in tropical regions, such as sub-Saharan Africa, Middle East, India, and the Mediterranean.[2] As a result of the advantageous nature of this sickle cell trait, individuals who have ancestry from these regions are much more likely to have this trait. Due to population migrations, the prevalence of sickle cell disease has also increased in the Americas and Europe.

In terms of global distribution of sickle cell disease, an estimate from 2010 indicated that approximately 230,000 incidence sickle cell anemia births were estimated in sub-Saharan Africa.[2] This estimate would account for approximately 75% of the world's sickle cell anemia births.[2] In Brazil, a study from 2016 approximated an incidence of 1071 newborn babies with SCD, while there was a prevalence of approximately individuals with SCD overall.[2]

SCD is also associated with an increased risk of mortality. A study from California, Illinois, and New York indicated that approximately 1% of children with sickle cell anemia disease died due to SCD complications during their first three years of life.[4] In contrast, an estimate 50-90% of children under 5 years with sickle cell anemia die in sub-Saharan Africa.[4] When considering mortality in adults, a population-based surveillance study was conducted in California and Georgia from 2004 to 2008 found that 615 of a total 12,143 patients with SCD died.[6] They found that this mortality rate among SCD patients was greater than the rate of all-cause mortality among African American and the total population rates between those aged 5-74 years.[6] Another study from the United States analyzed sickle cell related deaths from 1979 to 2005 and found that the mean age of death was approximately 33.4 years for males, while it was approximately 36.9 years for females, which is significantly lower than that of the general population.[7]

1.2 Pulmonary Hypertension

Pulmonary hypertension (PH) is a type of increased blood pressure in the vessels between the heart and lungs. These vessels include the pulmonary artery, veins, and capillaries. The main purpose of the pulmonary artery is to carry deoxygenated blood from the right ventricle in the heart to lungs, while the purpose of the pulmonary vein is to carry oxygenated blood from the lungs to the left atrium of the heart. The purpose of the pulmonary capillaries is to rapidly exchange gases. Pulmonary hypertension is clarified into five categories by the World Health Organization.[8] The first group is pulmonary arterial hypertension, which is due to thickening of the arteries in the lungs. The second group is pulmonary hypertension due to left heart disease. The PH in this category is caused by the inability of the left heart to contract and relax appropriately, which causes

an overflow of blood in the lungs, which increases pressure. The third group is pulmonary hypertension due to lung disease, such as chronic obstructive compulsive disorder or emphysema. In this category, PH is caused due to tightening of arteries in the lungs, which causes increased pressure throughout the lungs. The fourth group is chronic thromboembolic pulmonary hypertension. In this category, PH is caused by non-dissolvable blood clots in the lungs, which increases pressure. The fifth group of pulmonary hypertension is pulmonary hypertension caused by diseases that are not yet well-explained. This category includes PH due to sickle cell disease, sarcoidosis, chronic hemolytic anemia, and splenectomy.

Pulmonary hypertension can be diagnosed with a variety of different testing methods. The first measure that can be used to screen for pulmonary hypertension is an echocardiograph, which measures tricuspid regurgitation velocity.[9] Additionally, other echocardiographic signs can be used estimate the probability of PH. These include a right ventricle/left ventricle basal diameter ration of > 1, a right ventricular outflow Doppler acceleration time <105 milliseconds, mid systolic notching, early diastolic pulmonary regurgitation velocity (>2.2 m/s), inferior vena cava diameter of >21 mm with decreased inspiratory collapse, among others.[10] However, it is important to note that some complicated or early cases of PH may not be detected with an echocardiography, and therefore a normal echocardiography cannot eliminate PH.[9] Other diagnostic tests include pulmonary function tests, cardiopulmonary exercise testing, ventilation/perfusion lung scanning, and chest computed tomography. However, the most important tool necessary to establish a PH diagnosis is right heart catheterization.[9] Right heart catheterization specifically measures resting mean pulmonary artery pressures. PH is defined as an increase in mean pulmonary artery pressures to greater than or equal to 25 mmHg at rest.[11] However, right heart catheterization is an invasive procedure, so it cannot be used to easily assess PH.

Pulmonary hypertension impacts individuals all over the world. Global estimates suggest that approximately 1% of the global population are impacted by PH.[12] PH prevalence is approximately 10% in individuals who are 65 years or older. It is important to note that the majority of PH is accounted for PH World Health Organization categories 2 and 3, and almost 80% of PH are from developing countries. The review also found that independent of the categorization of PH, the development of PH is associated with increased risk of mortality.

Information on the prevalence and incidence of the overarching category of pulmonary hypertension in the United States is lacking. However, in terms of mortality, a surveillance study in the United States found that the PH death rate was 5.5 deaths per 100,000 in 2001, but this increased to 6.5 per 100,000 in 2010.[13]

1.3 Pulmonary Hypertension in Sickle Cell Disease

PH is one of the main chronic clinical complications of sickle cell disease. Pulmonary hypertension has been estimated to affect approximately 6-10% of the adult patients with sickle cell disease, as confirmed by right heart catheterization.[12] The World Health Organization estimates that approximately 20-25 million individuals are impacted by sickle cell disease. Therefore, using these estimates approximately 2.5 million individuals are impacted by PH caused by SCD.[12] Similar estimation for North America, indicate that approximately 8000 people are affected by SCD associated PH.[12] SCD associated PH accounted for approximately 5% of PH in all continents, except Africa, where it accounted for 9% of PH. Additionally, it was found that PH in SCD increases with advancing age. Studies also found PH to be a significant comorbidity of PH as those SCD patients with PH had twice the likelihood of impaired function and mortality.[12] Since

SCD and infectious diseases which cause pulmonary hypertension are primarily prevalent in developing countries, a greater proportion of PH patients are younger in developing countries when compared to more developed countries.[12]

The complication of PH is associated with increased mortality in SCD. A study from France indicated that approximately 12.5% of SCD patients with PH died over a three year period, which was higher than the 0.8% mortality in SCD patients without PH.[1] Similarly in Brazil, a study with a three year follow-up discovered that there was a statistically significant difference in mortality between SCD patients with and without PH (37.5% vs 5.6%).[1] Finally, a study in the United States with a 6-year follow up period found a statistically significant difference in mortality between SCD with and without PH (37% vs 17%).[1] Moreover, previous reviews have found that PH in SCD patients have numerous other comorbidities including cardiovascular function, which contributes to the increased mortality association with PH.[14]

The pathways of SCD leading to PH is not yet fully elucidated. Our current understanding of the pathway is that sickle cell anemia leads to diastolic dysfunction, which causes increased pulmonary vascular resistance, smooth muscle cell and intimal proliferation, and thrombosis, which leads to PH, and may eventually cause right heart failure.[15] However, it is important to note that the underlying cause and link between SCD and diastolic dysfunction is not fully understood.[15] Another pathway between anemia and PH includes the fact that an increase in reactive oxygen species and nitric oxide leads to pulmonary vascular resistance that can then lead to PH.[15]

Multiple methods can be used to screen for pulmonary hypertension. The measurement of tricuspid regurgitation velocity (TRV) during echocardiography gives an appropriate estimate of the systolic pulmonary artery pressure, which would indicate pulmonary hypertension. The tricuspid valve is a health valve between the right ventricle and right atrium. Sometimes the valve

does not close fully, and blood can flow backward (regurgitation) into the right atrium. The TRV measures this flow. A normal TRV is less than 2.5 meters/second, and PH is possible if TRV between 2.5 to 3.0 meters/second.[15] TRV values greater than 3.0 meters/second are very likely to indicate PH.[15] Pulmonary hypertension can be confirmed when measured using right heart catheterization. Previous studies have found statistically significant positive correlations between TRV values measured with echocardiography and systolic pulmonary pressure measured using right heart catheterization in patients with SCD.[1] It is important to note that right heart catheterization is the most sensitive measure of PH, and TRV is not as specific of a measure. However, when TRV values are used in combination with NT-proBNP levels, studies have found increased sensitivity of identifications of patients who are at higher risk of pulmonary hypertension.

1.4 Biomarkers

Biomarkers are a measurable substance that can be collected from a human to test the presence of a disease or complication. Biomarkers can play numerous roles that ultimately allow for better patient outcomes. These roles include diagnosis of diseases, severity assessment, risk assessment, prediction of drug effects, and monitoring.[16] They can be broadly categorized into four main types: diagnostic, prognostic, predictive, and therapeutic. Currently, PH diagnosis methods are invasive (right heart catheterization) or not as accurate (echocardiography). Therefore, to appropriately diagnose PH, we need diagnostic biomarkers. Moreover, in order to risk stratify patients, a prognostic biomarker would be necessary. Predictive biomarkers allow for early detection of PH, so appropriate measures can be taken to prevent or attenuate future effect of PH.

Some biomarkers are a proxy of the disease pathway. These biomarkers can play an important role in helping to further study the development of an under-researched disease, such as sickle cell disease associated PH. Other biomarkers can be targeted using drug therapy to help resolve the disease or associated symptoms.

1.5 Biomarkers of Sickle Cell Disease

There are numerous biomarkers that have been collected from the blood and urine that can be used to understand the various pathways contributing to SCD symptoms. All of the following biomarkers are proxies for various complications and responses in the sickle cell disease pathway.[17] For example, fetal hemoglobin and alpha thalassemia are two biomarkers that alter HbS concentrations and polymerization. Biomarkers identified for red blood cell dehydration include irreversibly sickled cells. Biomarkers for red cell adhesion include the receptors CD36 (Glycoprotein IV) and integrin $\alpha4\beta1$ (very late activation antigen-4). Some biomarkers for white cell adhesion include L-selectin and $\alpha M\beta2$. Some biomarkers for inflammation include total white cell count, C-reactive protein, erythrocyte sedimentation rate, secretory phospholipase A2, interleukins, serum levels of prostaglandin-E2, CA 15-2, tumor necrosis factor-α, angiopoietin 1, among others. Some of the biomarkers of hemolysis include hemoglobin concentration, RBC survival, reticulocyte count, serum lactate, and haptoglobin. When assessing oxidative stress, biomarkers such as reduced glutathione, oxidized glutathione, and glutamine concentrations. Biomarkers for vasculopathy and endothelial dysfunction include triglycerides, apolipoprotein A-1, vascular endothelial growth factor , placental growth factor, and endothelin 1. When measuring

these factors in SCD patients, abnormal levels of any of these biomarkers can indicate a SCD-associated complication.

1.6 Biomarkers of Pulmonary Hypertension

Similar to the biomarkers of SCD, numerous protein biomarkers have been identified that are associated with PH, and also various specific complications of PH. Natriuretic peptides in general are associated with PH including atrial natriuretic peptide and brain natriuretic peptide.[18] Endothelin, uric acid, nitric oxide, asymmetric dimethylarginine, cGMP, D-dimer, serotonin, and plasma von Willebrand factor are all biomarkers associated with PH. These biomarkers are notable as they can be used in screening and even potential treatment of PH.

1.7 Biomarkers of Pulmonary Hypertension in Sickle Cell Disease Patients

Although there have been numerous biomarkers identified which indicate both pulmonary hypertension and sickle cell disease independently, it is important to identify factors that are unique to the combined condition of pulmonary hypertension and sickle cell disease. One study has found that sCD163, a hemoglobin-haptoglobin receptor, was found to be associated with PH in children with SCD.[19] Another study looking at a cohort of SCD patients found that increased levels of lactate dehydrogenase, blood urea nitrogen, creatinine, total bilirubin, N-terminal pro-brain natriuretic peptide, and soluble vascular cell adhesion molecule-1 to be associated with PH in SCD patients.[20] Additionally they found that lower levels of fetal hemoglobin levels were associated

with PH patients.[20] Another study found that serum uric acid was a strong biomarker of PH when studying adult SCD patients.[21] Another biomarker of interest is asymmetric dimethylarginine levels. When studying a cohort of SCD and healthy children, researchers found that higher levels of asymmetric dimethylarginine were correlated with elevated TRV indicative of PH.[22] Finally, N-terminal pro-brain natriuretic peptide is one biomarker that has found to be consistently associated with PH in SCD and higher mortality.[23] However, it is important to note that despite the discovery of these biomarkers of PH in SCD, more information is needed in how these and other biomarkers may fit in the mechanistic explanation of PH in SCD.

1.8 Walk-PHaSST Study

The Treatment of Pulmonary Hypertension and SCD with Sildenafil Therapy (Walk-PHaSST) study was a randomized clinical trial that took place between June 2007 and October 2009. The primary objective of the Walk-PHaSST study was to determine whether sildenafil, a medication to reduce PH symptoms was safe and effective for SCD patients who have PH. This study recruited patients between 12 and 70 years old who were diagnosed with sickle cell disease. Participants were categorized as having mild to severe PH, as defined by a echocardiography measurement of a TRV or greater than or equal to 2.7 meters per second.

Data from this study has been used in numerous other previous studies. For example, one study found that exercise-induced (six-minute walk test) changes of vital signs had prognostic value, as it was associated with anemia and TRV.[24] Another study found that serum albumin may be a biomarker and mediator or SCD, as lower albumin was associated with higher mortality.[25] Another study using the Walk-PHaSST data found a strong association between elevated TRV

levels and pulmonary embolisms in SCD, which has implications in screening patients with high TRV for venous thromboembolic events.[26] A fourth study investigated genetic factors, such as *S100B* that contribute to avascular necrosis and acute pain crisis in SCD.[27] Another study has even established and validated a composite vascular high-risk profile for SCD patients.[28] Researchers have also identified 17 signatures of common biomarkers are associated with many symptoms and comorbidities that ultimately indicate severity of SCD.[29] A sixth study investigated the differential prevalence of albuminuria and mortality in HbSS vs HbSC.[30] Another study found that high TRV has the highest risk for death when compared to any other variable measured in the trial.[31] Additionally, researchers have found associations between hemolytic rate and various clinical measurements and mortality.[32] Finally, a study found that left ventricular diastolic dysfunction and echocardiography-estimated elevated pulmonary artery systolic pressure were associated with worse exercise ability in SCD patients.[33]

1.9 Gaps in Knowledge

There is a drastic contrast between the wealth of information available on biomarkers in PH and SCD separately as compared to biomarkers for PH in SCD. It is clear from the published information that all biomarker research regarding PH in SCD is in its early stages, where correlations between biomarker levels and PH in SCD patients is being established. However, to our knowledge, there is little to no in-depth mechanistic information on how these significant biomarkers uniquely contribute to PH development specifically in SCD patients.

Information regarding a biomarker can be extremely useful in improving the health outcomes of patients. For example, a biomarker can be prognostic meaning that it can help to

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indicate is future occurrence of a disease is likely. In this context, a prognostic biomarker can help to predict whether a SCD patient has a greater likelihood of developing PH. This information is valuable to ensure that the condition is being closely monitored, potential preventative medicine can be administered, and any necessary lifestyle changes can take place. Another example of a useful biomarker is a predictive biomarker, which can provide information about whether a certain therapy or intervention will effective. In this case, a predictive biomarker can provide help to predict whether a certain medication will be effective in a patient with PH. In a similar vein, information about a biomarker that is key in the pathway of disease progression can be targeted in drug discovery and therapy research. The Walk-PHaSST study concluded that the drug sildenafil did not have the same therapeutic effect among PH patients with SCD.

Finally, a biomarker can be diagnostic in nature. Previous research has indicated that TRV measurement using echocardiography is correlated with PH in SCD patients and is often used instead of right heart catheterization to categorize PH even though it may be less accurate that right heart catheterization. The reason for this sacrifice in accuracy of results is because of the ease with which echocardiography can be performed. Similarly, if a biomarker can be discovered that is even more highly correlated than TRV, it may be advantageous to measure this for diagnosis of PH in SCD patients.

More specifically protein biomarkers are one of the most useful types of biomarkers in prognosis and therapy, as proteins are the most sensitive biomarkers to changes in the body. Furthermore, proteins are much easier to measure in patients from a cost and technology perspective when compared to genomics or other biomarkers.

Ultimately, further biomarker research and discovery in the field of PH in SCD is essential in further understanding the pathophysiological pathway of PH in SCD. Furthermore, this is a necessary step in the quest to discover a solution for this issue.

1.10 Public Health Significance

Protein biomarker discovery and research has the potential to eventually serve as a cornerstone in diagnosis, prognosis, and treatment of PH in SCD patients. Given that PH is such a significant comorbidity of SCD that is associated with greater mortality in patients with SCD, research on this topic will help to save more lives.

Additionally, it is important to note that there is a greater need for SCD research. Although SCD is a rare blood disorder in the United States, it is three times more prevalent than another rare disorders like cystic fibrosis (CF).[34] However, one study found a statistically significant expenditure difference for SCD compared to CF.[34] There was on average more than three times greater spending for CF than SCD.[34] Moreover, this study found significantly more Food and Drug Administration approvals and research articles on CF than SCD.[34] It is important to note that there is also a significant racial distribution difference between SCD and CF.[34] While SCD primarily impacts those of color, CF affects mainly white individuals. Given this stark difference and others, we see a clear need to address racial disparities in healthcare and contributing to research and understanding of sickle cell disease will help to address this need in public health.

The various comorbidities with SCD puts a great strain on patients and our healthcare system at large. In 2004, it was estimated that the hospital stays because of complications of SCD were estimated to be around $448 million in the United States.[35]

Overall, research into protein biomarkers of PH in SCD serves as the steppingstone into a world of possibilities of diagnosis and therapy options that will help to reduce the burden faced by public health system and improve the quality of life of patients in marginalized groups.

2.0 Objectives

The primary aim was to conduct a proof-of-concept cross-sectional study to explore targeted serum protein biomarkers that are differentially expressed in SCD patients with elevated TRV (indicating PH). The hypothesis was that protein biomarkers will be associated with PH in SCD patients. Additionally, we hypothesized that positive control protein biomarkers for PH in SCD, such as BNP, will be positively correlated with SCD patients who have PH. Therefore, we are expecting that SCD patients with PH will have higher BNP levels when compared to patients without PH.

3.0 Methods

3.1 Study Population

We used data from a multicenter study Walk-PHaSST. In the screening phase, 720 patients with SCD were recruited from 9 US centers and 1 UK center, which were referral centers.[36] Study participants underwent clinical and lab examinations, as well as an echocardiography. We selected two groups of patients: one group of patients with a TRV≤2.6 m/sec and another with TRV≥2.9 m/sec (N =35 in each).

3.2 Measurements

The serum levels of 92 protein biomarkers, known as potential human markers of cardiovascular disease and inflammation, was measured using an OLINK cardiovascular panel. This panel assessed a range of biological processes including inflammatory response, angiogenesis, cell adhesion, coagulation, and response to hypoxia. The OLINK system uses proximity extension assay (PEA) technology, which only requires a small amount of sample to assess biomarkers.[37] The technology works by matched antibodies with DNA-tags binding to respective protein in sample. Then, correctly matched DNA tags hybridize. These tags are then extended to an amplicon. Then, using qPCR, a digital readout of unique DNA barcodes is created. This produces data indicating the relative concentrations of proteins. This specific cardiovascular panel was selected because it provides a broad range of potential protein biomarkers that would be

most relevant to sickle cell disease due to their important role in cardiovascular disease. Therefore, the disease targeted panel allows for an increased likelihood of high-yield results. The OLINK method was used because it is high throughput. Additionally, the OLINK system has higher sensitivity than ELISA for some of the markers.[38]

3.3 Statistical Analysis

T-tests were performed between the aforementioned groups for each of the serum biomarkers. The Hochberg method was used to calculate the false discovery rate. Through this method the q values were calculated for the proteins. This method helps to correct for false discovery, but it is not as conservative as the Bonferroni correction. A volcano plot was created using the Bonferroni correction. Finally, we tested the correlation of the differentially expressed biomarkers with clinical variables measured in blood samples of the participants. These variables include white blood cell (WBC), neutrophil count, thrombospondin-1 (TSP as measure of coagulation), placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) (growth factors involved in angiogenesis), and NT-proBNP (elevated in pulmonary hypertension).

4.0 Results

With regards to the descriptive characteristics of the study sample, the elevated TRV group has 15 females and 20 males, while the normal TRV group has 20 females and 15 males (Table 1). The age of the SCD patients ranged from 19 to 84 years old. Age varied significantly between the normal and elevated TRV groups (p-value = 0.0025). Measures of kidney function, such as estimated glomerular filtration rate and serum creatinine, varied significantly between the normal and elevated TRV groups (p-value = 0.0015 and 0.0069, respectively). Hemoglobin levels were also significantly different between the groups (p-value = 0.0063). Variables such as sex, smoking status, hydroxyurea treatment use, severe hemoglobin SS genotype, history of acute chest syndrome, and number of severe pain episodes all did not differ between the normal and elevated TRV groups. Similarly, many of the clinical variables measured including WBC, neutrophil, TSP, PIGF, and VEGF did not differ between the groups. However, NT-proBNP levels did differ significantly (p-value = 0.003).

Overall, there were 41 proteins that were statistically significantly different between the elevated and non-elevated TRV group without the Bonferroni correction (p<0.05). Using a false discovery rate of 0.01, we found 14 significantly differentially expressed proteins. Six of them passed a Bonferroni corrected overall critical p value < 0.00054 (Figure 1). These included T-cell surface glycoprotein (CD4), lymphotactin (XCL1), SLAM family member 7 (SLAMF7), galectin-9 (GAL9), TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2), and tumor necrosis factor receptor superfamily member 11A (TNFRSF11A). We observed up to a 1.2-fold increase in these 6 protein biomarker levels in the high TRV groups. All of the significant protein biomarkers and a majority of the analyzed protein biomarkers had a fold-change greater than 1

(positive fold change) when comparing the elevated TRV group to the normal TRV group. It is also important to note that BNP had the largest positive fold change.

The correlogram (Figure 2) indicated that there was a slightly positive correlation between WBC and CD4, GAL9, TRAILR2, and TNFRSF11A ($r > 0.20$). For neutrophil count, we observed significantly negative correlation with selected markers including TNFRSF11A levels ($r = -0.51$), GAL9 ($r = -0.35$), XCL1 ($r = -0.27$), SLAMF7 ($r = -0.21$), and CD4 ($r < -0.24$). A significantly negative correlation between TSP and GAL9 levels ($r = -0.31$) was also observed. Finally, we observed a strong, positive correlation between all proteins and serum NT-proBNP levels ($r > 0.44$).

5.0 Discussion

Our proof-of-concept study yielded results that support our hypothesis that protein biomarkers will be associated with PH in SCD patients. Depending whether a more conservative or liberal approach was taken, we were able to identify between 6 to 41 statistically significant proteins.

Similar to the findings of other studies, we found that BNP had the largest fold change between our two groups. This not only supports the hypothesis we had for the positive control biomarker in our study sample, but it provides further support for the claim that those with elevated TRV in this study had PH.

From the six top biomarkers that we identified, CD4, XCL1, GAL9, and TNFRSF11A are all involved in T-cell regulation, and immunological and inflammatory functioning.[39] SLAMF7 is a surface antigen that helps in isolation of plasma cells. TRAILR2 is involved in apoptosis activation.[39] Additionally, all these biomarkers are involved in endothelial dysfunction.[39] Therefore, there is prior mechanistic research and rationale that these proteins could be involved in the pathway of PH in SCD patients.

Overall, circulatory protein markers of immune response and coagulation are highly expressed in SCD patients with elevated TRV. This provides evidence that these protein biomarkers have potential to be utilized as prognostic markers for pulmonary hypertension in patients with SCD.

One limitation of this study was the limited sample size. Due to the proof-of-concept nature of the study and the broad nature of the OLINK panel used, a total sample size of only 70 patients was used. Future research should target this shortcoming by increasing the sample size of the

analysis. Additionally, it is important to note that the stratifying variable of TRV is not perfect indicator of PH in sickle cell disease patients. However, because a fairly high TRV threshold was used (2.9 m/s), and because the BNP values were also high among an elevated TRV group, we can be fairly confident that most, if not all, of our elevated TRV group patients have PH. A third limitation of our study analysis was the statistical analysis used. The Bonferroni correction is a quite conservative method, which may yield a higher false negative rate. Similarly, the Hochberg method is also conservative, though not as conservative as the Bonferroni correction. However, both these methods greatly reduce false positive results. Thus, although special attention should be given to these proteins, it is important to consider all proteins when moving forward with a specific protein to further analyze. Finally, due to the cross-sectional nature of this study, we cannot establish temporality between the biomarker and the outcome of pulmonary hypertension. Therefore, using the given data we can only be certain about the association between biomarkers and pulmonary hypertension.

In terms of strengths of this study, the OLINK CVD panel is extremely broad and comprehensive in terms of the proteins that it targets. This is useful because this allows for a broad range of proteins to for the future step of pulmonary hypertension analysis in a larger sample size. Another strength of this study was that the biomarkers from the OLINK panel would be correlated and analyzed with the clinical variables measured in the blood sample of participants.

Using the information from the proof-of-concept study, we performed a systematic literature review of all 41 proteins that were statistically significant (p<0.05) to select biomarkers for follow-up in future studies. These proteins of interest were narrowed based on the following four factors 1) the statistical significance of the protein in Walk-PHaSST data, 2) the plausibility

(indicated by the results of the literature search), 3) specificity to sickle cell, and 4) the novelty of the biomarker.

The literature search was completed using PubMed, including many different terms that would relate to sickle cell disease and the pulmonary hypertension pathway, such as endothelial dysfunction, cardiomyocyte dysfunction, heart disease, inflammation, blood coagulation, thrombosis, etc. From this search, we found 15 notable proteins: CD4, PGF, TM, ACE2, BNP, FS, GT, KIM1, PAR1, FGF23, MARCO, IL27, THBS2, RAGE, and IL6.

Next, the following five proteins were excluded because the literature search produced less than four articles for each protein: ACE2, FGF23, MARCO, IL27, and THBS2. Then, when considering the criteria of specificity to sickle cell disease and the relevance of the articles produced by the literature search, we excluded the following five proteins: FS, GT, KIM1, PAR1, and RAGE. Finally, based on the criteria of the novelty of the biomarker, four more proteins were excluded: CD4, IL6, PGF and BNP. Therefore, the only protein that satisfied all these criteria, and the protein we will propose to move forward with is thrombomodulin (TM). The p-value for TM was 0.001.

TM plays an important role in SCD, although its role has not been fully elucidated. TM gene variants associated with thrombosis in African American populations was associated with thrombosis in a sickle cell disease cohort when compared to healthy controls.[40] Another study found that TM was significantly elevated in hemoglobin SC patients when compared to healthy controls.[41] Moreover, it was found that mutations in endothelial function genes (specifically including TM) were associated with sickle cell disease children with cerebrovascular disease.[42] In-vivo studies found that hemoglobin SS mice had higher concentrations of cleaved non-functional TM in comparison to hemoglobin AA mice.[43] In longitudinal follow up in SCD patients, TM

biomarker predicted the risk for multi-organ failure.[43] This previous research shows that TM is a significant biomarker that is associated with sickle cell disease.

From the hypertension perspective, thrombomodulin levels have been found to play a role in hypertension broadly. For example, TM levels have been found to be significantly associated with preeclampsia when compared with chronic hypertension, gestational hypertension, and normotensive controls.[44] Another study found that, in general, TM is significantly higher in individuals with hypertension compared to control subjects.[45] A third study found that TM is elevated in scleroderma associated pulmonary hypertension.[46] In general, TM is a marker of endothelial dysfunction.[43]

Although TM is plausible biomarker for hypertension and for sickle cell disease, it is an under-researched protein biomarker in terms of PH in sickle cell disease patients. Evaluation of the association between TM and PH in sickle cell disease in a larger cohort using immunoassays, such as ELISA, will be an essential next step. Further analysis with knockout animal models of TM will allow us to further elucidate the pathways of PH in SCD patients.

Therefore, this research is an essential step taken towards identifying biomarkers that can eventually be used as prognostic, predictive, and diagnostic markers of PH in SCD to reduce the burden of PH in SCD. Additionally, these biomarkers can be targets of future therapy to attenuate the negative effects of PH in SCD. Thus, this study is contributing to research that will help to reduce racial health disparities, reduce public health expenditure, and ultimately improve the quality of life of SCD patients.

Table 1: Walk-PHaSST Study Participant Characteristics by Tricuspid Regurgitation Velocity (TRV) level,

N=70

Variable	Normal TRV (n=35)	Elevated TRV (n=35)	P-value[a]
Age[b]			**0.003**
Median	33.3	45.9	
25th Percentile	20.7	34.2	
75th Percentile	44.3	53.3	
Sex (n, %)			0.23
Female	20 (57.1)	15 (42.9)	
Male	15 (42.9)	20 (57.1)	
Smoking Status (n, %)			0.45
Never	23 (65.7)	19 (55.9)	
Former	5 (14.3)	9 (26.5)	
Current	7 (20)	6 (17.6)	
Current Hydroxyurea Treatment (n, %)			0.08
Yes	15 (42.9)	8 (22.8)	
No	20 (57.1)	27 (77.1)	
Severe Hemoglobin SS Genotype (n, %)			0.45
Yes	26 (76.5)	27 (81.8)	
No	8 (23.5)	6 (18.2)	
History of Acute Chest Syndrome (n, %)			0.45
Yes	21 (60)	24 (68.6)	
No	14 (40)	11 (31.4)	
Estimated Glomerular Filtration Rate[c] (mL/min)			**0.002**
Mean	135.53	103.39	
Standard Deviation	29.15	49.64	
Hemoglobin[c] (g/L)			**0.006**
Mean	9.72	8.44	
Standard Deviation	1.90	1.90	
Number of severe pain episodes[b]			0.69
Median	2	2	
25th Percentile	1	0	
75th Percentile	4	5	
Serum Creatinine[b] (mg/dL)			**0.007**
Median	0.68	0.80	
25th Percentile	0.55	0.66	
75th Percentile	0.77	1.35	
White Blood Cell Count[b]			0.50
Median	9.5	10.7	
25th Percentile	7.5	7.49	
75th Percentile	11.6	13	
Neutrophil[c] (%)			0.29
Mean	55.7	52.3	
Standard Deviation	10.4	12.3	
Thrombospondin[b] (ng/mL)			0.78
Median	806.4	1010.7	

25th Percentile	405.4	548.7	
75th Percentile	2126.5	1302.4	
Placental Growth Factor[b] (pg/mL)			0.31
Median	26.1	29.1	
25th Percentile	22.9	22.5	
75th Percentile	32.1	42.4	
Vascular Endothelial Growth Factor[b] (pg/mL)			0.95
Median	80.5	82.5	
25th Percentile	70.1	62.4	
75th Percentile	126.4	135	
NT-proBNP[b] (pg/mL)			**0.003**
Median	43.8	162	
25th Percentile	17.7	39.4	
75th Percentile	86.9	455	

[a] Chi-square test for categorical variables, t-test for normally distributed continuous variables, Wilcoxon for non-normally distributed continuous variables

[b] non-normally distributed variable
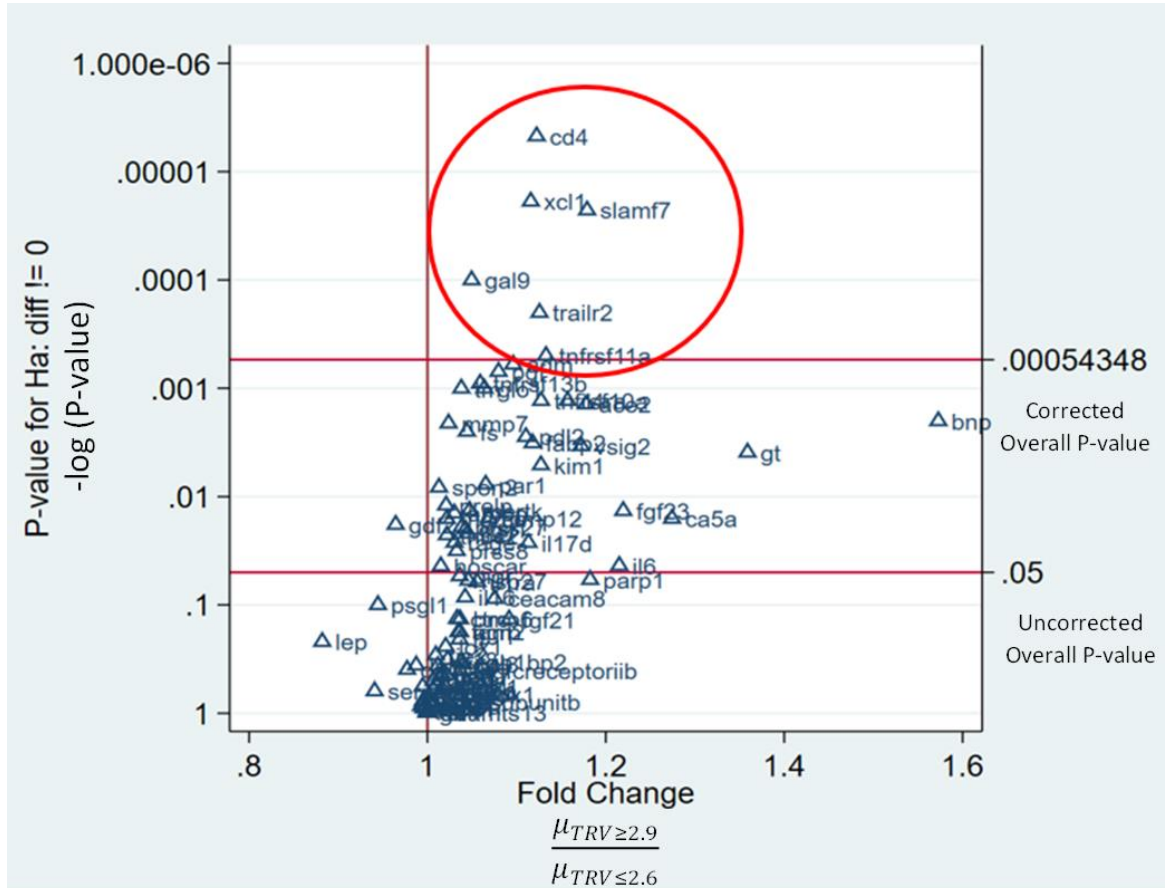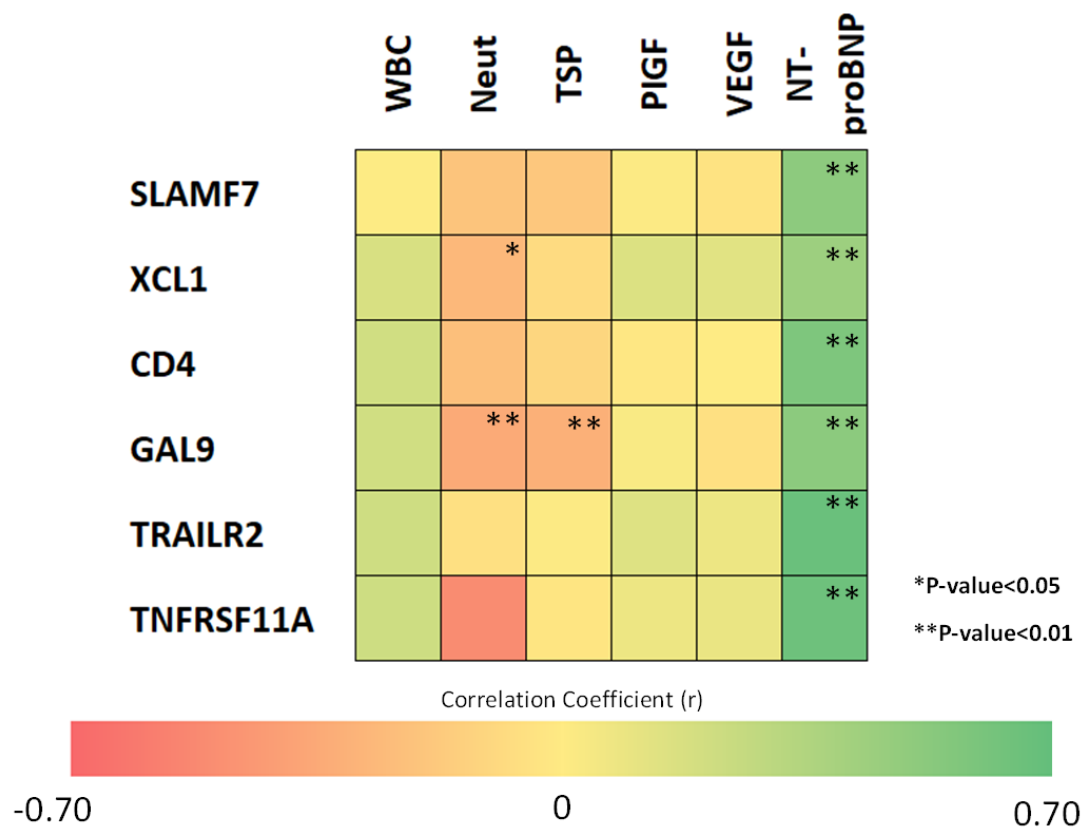
[c] normally distributed variable

Figure 1: Volcano plot of 92 protein biomarkers

The six significant proteins are CD4 (T-cell surface glycoprotein CD4), XCL1 (Lymphotactin), SLAMF7 (SLAM family member 7), GAL9 (Galectin-9), TRAILR2 (TNF-related apoptosis-inducing ligand receptor 2), TNFRSF11 (Tumor necrosis factor receptor superfamily member 11A).

Figure 2: Correlogram of selected biomarkers and clinical measures

Correlogram depicts the association between the six selected protein biomarker levels and seven clinical measures relevant to pulmonary hypertension pathways. Both high and low TRV groups are included in this analysis

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