SPECIFIC AIMS

Defined by low platelet count in the absence of any other causes of thrombocytopenia, immune thrombocytopenic purpura (ITP) affects over 4,000 US children and 8,000 adults each year [1]. While the majority of ITP cases resolve themselves [2–4], ITP is associated with an enhanced risk of bleeding, as approximately 10% of children have major bleeding, and 0.5% of children have life-threatening intracranial hemorrhage [5]. Clinicians typically treat these conditions with corticosteroids or intravenous immunoglobin G (IVIG), which have significant side effects. Moreover, there is no diagnostic test or biomarker available to identify which patients need treatment and to provide clinically actionable data. Studies showing that the platelet count is only loosely tied to bleeding [6–9] led to the development of a bleeding score to grade patient symptoms, which has improved treatment but ultimately remains subjective. Other existing tests of platelet function, such as platelet aggregometry (Plt Agg) and platelet function analyzers (PFA) do not work with low platelet counts and provide no useful data. As such, an ongoing debate in the field of clinical hematology centers around both which specific patients require therapy and what that therapy should entail [2–4].

The **research objective** of this Junior Faculty Focused Award is to investigate a novel hypothesis: that the contractile force of individual platelets correlates with bleeding phenotype independent of traditionally used biological markers or assays of hematological function. We recently published in *Nature Materials* the development and validation of a high-throughput platelet contraction cytometer (PCC) that measures the contraction forces of large numbers of individual platelets [10]. Our latest results of small study of patients with primary ITP (n=22) suggests that 1) the force profile of platelets in patients with ITP, regardless of bleeding phenotype, is significantly different from healthy individuals, and 2) that low platelet forces correlate with symptomatic bleeding at a single time point. However, both how platelet forces change with time in a patient and the possible mechanism for impaired contraction remain unclear. Here, we propose a prospective study (n=30) with two independent aims that seek to gather preliminary data for an R01 application that will propose a meticulous clinical study of using platelet contraction forces as an objective biophysical biomarker in patients with ITP.

Aim 1: Prospective study of platelet contraction force in pediatric ITP patients. Based on our preliminary data *we hypothesize that patients with ITP have substantially different platelet contractile forces than healthy controls that vary with time and symptoms*. We test this hypothesis with a 1-year prospective study on 30 newly diagnosed patients with ITP. In Aim 2a we will test whether the distribution of contractile forces at the first time point in the 30 patients is different from 30 healthy controls. Based on our preliminary data, we expect healthy controls to have normal distribution of contractile force, whereas patients with ITP have a bimodal distribution of contractile forces. In Aim 2b, we will test whether bleeding symptoms and their resolution correlate to a change in the contractile force of platelets. We are also interested in identifying whether platelet forces could help identify patients with acute or chronic ITP.

Aim 2: Mechanistic studies of low platelet contractile force in ITP patients. Noting that the force distribution of ITP patient platelets is markedly different from healthy controls, *we hypothesize that these platelets are morphologically and biochemically distinct from healthy controls*. As our PCC enables immunofluorescent staining on conjunction with contractile force measurements, we will test this hypothesis by examining 2a) actin & myosin and 2b) immature platelets. For Aim 2a, we will test whether actin and myosin formation are correlated to increased contractile force, as in healthy controls. Based on the unique force distributions, it is unclear whether this relationship exists in ITP. For Aim 2b, we will examine the contractile function of immature platelets, which represent a substantial proportion of platelets in patients with ITP. The exact role of this population is whole unclear [11], as some studies suggest immature platelets are correlated with fewer bleeding symptoms [8,9], while others suggest they correlate with increased bleeding [12].

BACKGROUND & SIGNIFICANCE

Defined by low platelet count in the absence of any other causes of thrombocytopenia, Immune Thrombocytopenic Purpura (ITP) affects approximately 2-6 per 100,000 children each year [1]. While the majority of ITP cases resolve themselves [2–4], ITP is associated with an enhanced risk of bleeding, as approximately 10% of children have major bleeding, and 0.5% of children have life-threatening intracranial hemorrhage [5]. Clinicians typically treat these conditions with corticosteroids or intravenous immunoglobin G (IVIG), which have significant side effects. As such, clinicians must balance risk-factors associated with ITP with those of the currently used medications, and an ongoing debate in the field of clinical hematology centers around both which specific patients require therapy and what that therapy should entail [2–4]. Moreover, a minority of patients develop chronic thrombocytopenia that is resistant to most therapeutic modalities, and it is impossible to predict which patients will develop chronic ITP.

The key barrier to managing pediatric ITP is identifying which patients need treatment as there is no diagnostic test or biomarker available to determine which patients are at risk for bleeding or developing chronic ITP. Various studies have shown that platelet count is only loosely associated with bleeding [6–9], leading to vigorous debate on whether patients with low platelet counts (< 20,000/μL) should be treated [2,13–18]. Using Bleeding scores [19] has led to better predicted outcomes than management with platelet count alone [2,20], but remain subjective. Other tests of platelet function, such as Plt Agg and PFA depend on the platelet count and cannot be used. Although recent work has shown higher bleeding scores are linked to impaired platelet function [8,9], the measurements have high patient-to-patient variability and lack diagnostic power. In a substantial departure from current clinical practices and based on my preliminary data, I propose to study the forces of individual platelets as a biomarker for ITP bleeding risk

EXPERIMENTAL DESIGN & METHODS

New device enables high-throughput single platelet force measurements and shows impaired platelet contractile response as expected. Previously in *Nature Materials* [10], we published the development and validation of a "**platelet contraction cytometer (PCC)**" that simultaneously measures the contractile force of hundreds of individual platelets adherent on substrates spanning the physiological range of mechanical clot stiffnesses, while enabling control of the biochemical and shear microenvironment [10]. In our system, an activated platelet adheres to and spans on a fibrinogen microdot pair and contracts the microdot pair together (**Fig 1**). Since contraction force is proportional to only the monomeric fibrin microdot area and microdot displacement, high-throughput measurements can be conducted with single cell resolution. We have used this device to define the platelet contractile response of healthy individuals across a range of stiffnesses and thrombin conditions that are characteristic of the clotting environment [10]. To validate our system, we show that platelets from patients with actin-myosin defects, (Wiskott Aldrich & May Hegglin), have impaired contraction and symptomatic bleeding as expected.

Preliminary data shows impaired contractile force in ITP patients with bleeding symptoms. Importantly, our data shows that platelet contractile force is completely *independent of known markers of platelet activation and platelet count*. With a validated

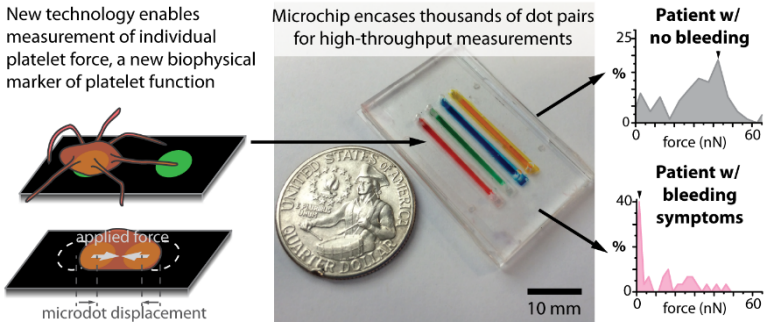

***Fig 1**: Our new technology to measure single platelet contraction force may be a promising new diagnostic for individuals with symptomatic bleeding in ITP. Over 20,000 fibrinogen microdot pairs are microprinted on the surface of each hydrogel encapsulated in each microchannel. Patients with symptomatic bleeding have significantly lower contractile forces and few highly contractile platelets.*

device, we explored platelet contraction in a small cohort of patients (n=22) with primary ITP who were categorized as having or not having bleeding symptoms. We found that highly contractile platelet subpopulations present in healthy controls are conspicuously absent in all the patients with symptomatic bleeding (**Fig 2**), suggesting that we have discovered an objective biophysical biomarker of symptomatic bleeding in ITP.

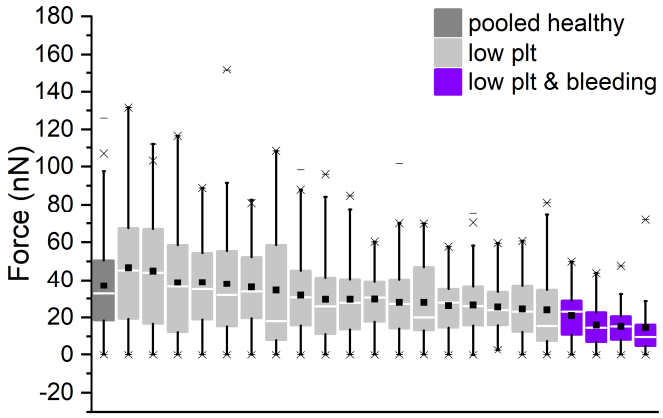

Fig 2: *Impaired platelet contraction in ITP patients with bleeding symptoms suggests a new biophysical diagnostic. Specifically, highly contractile platelets are conspicuously absent from patients with bleeding. No existing objective test or biomarker identifies these patients.*

Preliminary data shows unique force distributions of ITP platelets. When examining histograms of platelet contractile forces, patients with ITP have significantly different contractile forces than healthy controls (**Fig 3**). Healthy individuals have a distribution of contractile forces with a single peak. Patients with ITP have two prominent force peaks, one with a low contractile force and one with higher contractile force. Surprisingly, patients with bleeding symptoms have an absence of the high contractile force platelets, suggesting that highly contractile platelets may be a biophysical biomarker for bleeding.

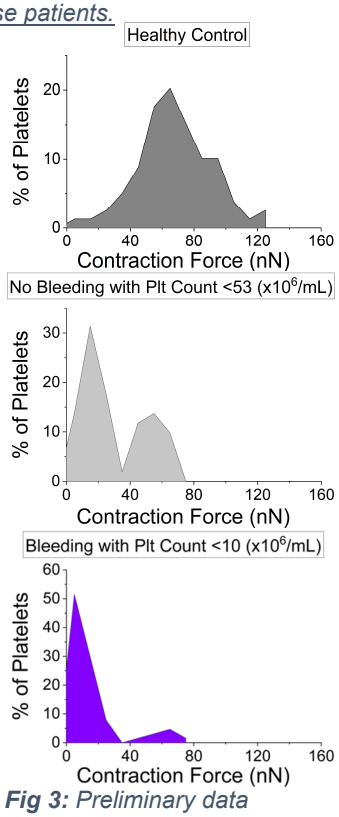

Fig 3: *Preliminary data suggests two distinct contractile (low/high) platelet populations appear in ITP. Patients without the highly contractile population experience bleeding. Here we seek to examine whether this changes in patients over time.*

Limitations with current data: 1) the sample size is small for definitive conclusions. 2) only one timepoint has been measured. 3) no mechanisms have been explored. The current proposal seeks to address these limitations through two independent aims.

Study Population: I will enroll a diverse populations of 30 patients with ITP and 30 healthy volunteers. *The 30 ITP patients will be enrolled in a prospective study and followed during monthly visits over the course of one year*. Patients will have a diagnosis of primary ITP and a platelet count of <50,000/μL, with no other exclusion criteria. Due to the small sample, patients bleeding will be scored by Buchanan-Adix [19] into no bleeding (BA<0.5) or bleeding (BA>1), and there will be no exclusion based on sex, race, etc. Healthy individuals will be excluded if they received pharmacological modifiers of hemostatic function (NSAIDS, proton pump inhibitors, aspirin) within two weeks.

Aim 1: Prospective study of platelet contraction force in pediatric ITP patients

Aim 1a: Test the hypothesis that platelet contraction is different from healthy controls in pediatric patients with ITP. Our preliminary data supports this hypothesis yet additional data is needed for more definitive conclusions. This hypothesis is supported by recent flow cytometry based studies that found links to symptomatic bleeding and thrombin-receptor activated platelet activation as measured by phosphatidylserine exposure, p-selectin exposure, and $\alpha_{IIb}\beta_3$ activation [9]. Whereas the high patient-to-patient variation precludes the use of this approach as a diagnostic, our data appears to have stronger clinical associations and therefore, diagnostic potential.

Aim 1a Experimental Design: Dr. Bennett and I have a well-established protocol of

scheduling, consenting, and enrolling patients for similar platelet studies. We regularly use couriers to ferry blood samples from Scottish Rite to Dr. Lam's GT or Emory labs. All samples will be prepared and tested using established protocols for the PCC [10]. Here, platelet contraction data collected from the 30 ITP patients initial visit and the 30 healthy controls will be analyzed. We will test that these distributions come from a single normal population by the Anderson-Darling test for normality and by visualization of the histograms/ quantile-quantile (QQ) plots, or from a mixture of two normal distributions (i.e. resulting in a bi-modal distribution). Based on the results of histogram, we may utilize Hartigan's dip test to confirm the bi-modal distribution. We will compare the cumulative distributions of healthy controls and ITP patients using the Kolmogorov Smirnov test, quantile-quantile plot, testing shifts in the median (Wilcoxon test) and comparing means with the t-test. With 30 subjects in each group, we have at least 80% statistical power to detect a moderate-large effect size (d=.736) using a two sample t-test. In the event that there is correlation within a subject's platelets with contractile force then we will estimate these main effects (i.e. patient group of healthy controls vs ITP) using a random effects model which will account for any correlation within subject that is present.

Aim 1b: Test the hypothesis ITP resolution over time correlates with presence of highly contractile platelets. ITP is categorized by three disease groups: newly diagnosed (0-3 months), persistent (3-12 months), and chronic (>12 months). These groups help guide management strategies as patients who have ITP for shorter periods of time have higher rates of remission than the chronic cases. Treatment strategies for each of these groups are different. While clinicians focus on monitoring and increasing platelet counts when needed in newly diagnosed patients, persistent patients need steroid sparing agents, and long-term safety aspects must be considered for chronic patients [3]. Based on studies showing links between platelet function and bleeding scores over time [8], as well as our own data, we hypothesize that platelet contractile forces change with patient symptoms over time.

Aim 1b Experimental Design: To test the relationship between platelet contractile force, disease group and bleeding symptoms over time, we will use a linear mixed model. If we enroll the 30 ITP patients uniformly over the year and each patient submits a sample monthly, we would have 200 samples. Assuming some missed clinic visits or a patient enrollment later in the year, we expect 150 samples over the year. We will test whether patients with symptomatic bleeding have different platelet contractile force from those without bleeding. We will adjust for time and test for the interaction with time to estimate how patient's platelet contractile force changes during the year of follow up. We will not present power calculations here because while we expect 20% of patients to be bleeders at the initial visit, it is not clear how many we resolve during follow up. Thus, it is not clear how many bleeders and non-bleeders we will have over the 150 clinic visits during the year. While we will be unable to test chronic samples prospectively, throughout the study, we will still be able to gather data on these patient's contractile forces at the time of initial visit.

Benchmarks for Success / Potential Pitfalls/ Alternative Strategies: Data supporting clinical associations between platelet biophysics and symptomatic bleeding would represent a remarkable breakthrough in diagnostics by bridging the fields of cell mechanics with clinical medicine. The primary challenge associated with this Aim will be securing enough patient samples to perform statistical testing. To mitigate this, we have an established protocol and work schedule that ensures that we are always ready to test patient samples, especially as typical clinic hours are M-F, 7-5pm. In addition, our estimates for patient numbers are conservative estimates based on previous experimental studies [10]. Also, if time allows, we will seek to gather preliminary data on existing patients who already have chronic ITP.

Aim 2: Mechanistic studies of low platelet contractile force in ITP patients

Aim 2a:Test the hypothesis that actin and myosin formation in ITP correlate with single platelet contractile force. Actin and myosin form and drive platelet contraction in response to local biochemical and biomechanical stimuli. Characterizing this formation is a critical first step

to any mechanistic studies of contractile force. Our previous preliminary data indicates that F-actin increases with increasing contractile force in healthy individual [10]. However, noting the dramatically different distributions of platelets in ITP with and without bleeding, it is unclear if this relationship is maintained in ITP or if these platelets experience altered actin and myosin.

Aim 2a Experimental Design: The same platelets isolated from the healthy controls or ITP patients in Aim 1 will be plated into separate PCC's for Aim 2a. After contraction, actin and myosin will be stained with Alexa Fluor 546 Phalloidin and anti-myosin IIa polyclonal antibodies (rabbit), respectively, using immunofluorescent protocols for the PCC [10]. To test that actin and myosin are altered in low contractile force platelets in patients with ITP, we will first establish that actin and myosin correlate with contractile force, as seen in previous results with healthy patients. To test the relationship between actin and myosin with contractile force, at the platelet level, we will first estimate the correlation coefficient among 30 healthy subjects and 30 ITP patients separately based on preliminary data (**Fig 3**) that these groups had different contractile force distributions. For each subject group (healthy controls and ITP), the minimum correlation coefficient to attain statistical significance with 80% power is 0.5. We will also categorize contractile force by patient group and compare the actin and myosin. We will separate contractile force into the following groups<15, 15-30, 31-45, >45. With 4 groups, we will have 80% statistical power to observe at least 30.7% of the variance in actin or myosin will be due to the platelet contractile force (i.e. medium effect size). In the case where normal assumptions do not hold, non-parametric tests will be used.

Aim 2b:Test hypothesis that immature platelets have impaired contractile forces. Immature, or reticulated, platelets have recently been released from the megakaryocyte, and may be identified by the presence of a small amount of RNA [21]. New research suggests a correlation between the immature platelet fraction and bleeding [8,11,12]. However, the role of the immature platelet fraction is hotly debated [11] with some arguing that increased immature platelets correlate to lower bleeding scores whereas other argue a correlation to higher bleeding scores [11].

Aim 2b Experimental Design: This same approach as Aim 2a will be followed except that only ITP patients will be used, and only immature platelets will be stained with thaizole-orange [22] (no actin-myosin staining). Immature platelets will be categorized as immature or not based on staining intensity. We will first test immature platelets and contractile force at the platelet level, and then at subject level. At the platelet level, we will compare the % immature platelets with contractile force split at the median. With 30 ITP patients, we expect a minimum of 600 platelets. Based on preliminary data, it would be more reasonable to obtain 1500 platelets but our statistical power will reflect more conservative estimates. Splitting at the median contractile force, we have 80% statistical power to observe a .229 (i.e. small) effect size or standardized difference in immature platelets between groups. We will also split contractile force at 35nN as a hypothesized cutoff point between low and high force and then test the amount of immature platelets between groups.

Benchmarks for Success / Potential Pitfalls/ Alternative Strategies: Aim 2a may show no difference between ITP and healthy patients. However, as actin-myosin formation is the first upstream pathway to platelet contraction, establishing normal formation is a critical step to identifying other affected pathways. For Aim 2b, any result will help better our understanding of immature platelets in ITP. For both studies, we anticipate using the Zeiss LSM 710 confocal microscope with 20x high resolution lens as done previously [10].However, we have experience with and are able to use labor intensive high-res imaging if needed [10].

Timeline: A key challenge relies on testing a limited patient population. To maximize productivity, Aim 1 & 2 will be completed simultaneously. This is feasible since: 1) the sample schedule is known, 2) only a small number of platelets is needed for each test and 3) samples may be fixed for later staining and imaging. Timeline in Career Dev. Plan.

CONCLUSION & FUTURE OUTLOOK: This research could significantly impact clinical practice given the pressing need for an ITP diagnostic biomarker.

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CAREER DEVELOPMENT PLAN

Candidate Background**:** I have undertaken rigorous training in microsystems design and in experimental hematology. My PhD thesis involved applying advanced microsystem design, fabrication, and circuitry to create force sensors so sensitive that they could detect the forces created by an ant crawling on a diving board. I pushed the field of microsystems forward by translating devices from the bench to extreme temperature and shock environments[1] as well as onto automotive components[2]. Moreover, I published in the *Journal of Micro-electro-mechanical Systems* (JMEMS), the premier journal of my field, with a novel analytical model to reduce temperature-induced sensor errors[3]. Motivated by my desire to see improved biomedical microsystems, I undertook a postdoctoral fellowship under the mentorship of Wilbur A Lam, MD, PhD, an energetic and creative physician-engineer mentor. Due to my extensive background in microsystem design, I was able to create increasingly complex *in vitro* microsystems to study endothelial cell interactions[4,5], neutrophil mechanics[6], and platelet forces[7].

Career Goals and Objectives**:** Almost all current biomedical micro-chip based systems are: 1) conceived by researchers with no clinical training, 2) impractical, and 3) fail to address a significant clinical problem. I seek to become a new breed of biomedical researcher, a translational microengineer-researcher, capable of bridging this divide and moving microchip-based devices from the cleanroom into the clinic. My long-term career goal is to be an independent translational investigator working at the interface of microengineering and medicine at Emory, a Tier 1 research institution. My short-term career goal is to receive new training in clinical research that will enable me to design devices that will be useful in the clinic from the start (Table 1). Designing microsystems for clinical use is incredibly difficult in the absence of real-world hands-on experience in the healthcare setting. I will learn clinical research through a combination of formal coursework and supervised clinical research in pediatric hematology. With protected time I will learn clinical research through a combination of formal coursework and supervised research as well as form *new collaborations with clinical researchers.*

Table 1: Planned activities of the award duration

Category	Activity	Q1	Q2	Q3	Q4
Original Research	Aim 1: Prospective Study of ITP patients	x	x	x	x
	Aim 2: Single platelet mechanistic studies	x	x	x	x
Additional Res. Skills	Performing clinical study (Lam / Bennett)	x	x	x	x
	Pediatric Clinical Observership (Bennett)	x			
Formal Coursework	MSCR 761 – Intro to Clin & Trans. Res	x	x		
Professional Meetings	Amer. Soc. of Hematology Annual Meeting				x
	The Pediatric Alliance Conference		x		
Lab Meetings/Seminars	Weekly Lab Meeting	x	x	x	x
	Hemostasis Journal Club (weekly)	x	x	x	x
	Clinical Research Journal Club (monthly)	x	x	x	x
	Primary Mentor Meeting (weekly)	x	x	x	x
	Secondary Mentor Meeting (monthly)	x	x	x	x
Manuscript Prep	Correlation of bleeding with low platelet force in ITP		x		
	Mechanism of low contraction force in ITP				x
Grant Writing	R01 on platelet contraction for ITP			x	x

Overview of Planned Activities: Working with Dr. Lam and Dr. Bennett, I will learn to plan, conduct, and analyze data from a prospective clinical study. To better understand the clinical environment, I will participate in observership opportunities at Scottish Rite with Dr. Carolyn Bennett. Much of this planning and analysis will be done through meetings with Dr. Lam and Bennett. This hands-on clinical training will be supplemented with the formal course MSCR 761 – Introduction to Clinical and Translational Research, offered by the Georgia Clinical and Translational Science Award (GaCTSA). I will also begin attending the monthly Clinical Research Journal Club (3rd Tues each month, 1 hr), where members are given the opportunity to either discuss a current research paper or to present their research progress and receive clinically relevant feedback. To ensure that I remain current on the latest hemostasis/thrombosis research, I will continue to attend the weekly Hemostasis Journal Club (every Wednesday at 8:30am, 1hr), led by Pete Lollar. I will also attend the annual American Society of Hematology Meeting and the Pediatric Alliance Conference to remain current on the latest research and form new connections with other researchers.

Mentoring: Wilbur A. Lam, M.D., Ph.D. (Primary Mentor & Sponsor): Dr. Lam is an Associate Professor in the Department of Pediatrics at Emory University, as well as an Affiliate Professor in the Wallace H. Coulter Department of Biomedical Engineering at Georgia Institute of Technology. Dr. Lam is a new breed of physician-scientist – a physician-engineer, who works at the interface of microfluidics, cellular biophysics and mechanics, and hematology. Dr. Lam has already make significant contributions into our understanding of endothelial cells biology, sickle cell disease, and platelet mechanics. As both a clinician and engineer, Dr. Lam has the extremely rare, but necessary, experience and resources to provide critical insight on all phases of the project from bench to the bedside. Moreover, his office door is adjacent to the lab and he has an open-door policy that facilitates daily communication between us. In addition to providing key insight and advice on blood-based microfluidics, Dr. Lam will coordinate the efforts of Dr. Bennett to ensure that I receive the appropriate additional training. Dr. Lam will also continue to be my career mentor and help me navigate promotion and advancement in the department.

Clinical Mentoring: Carolyn Bennett, MD, MS (Co-mentor): Dr. Bennett is an Assistant Professor at Emory University and the Aflac Cancer and Blood Disorders Center. Dr. Bennett's clinical research focuses on inherited and acquired bleeding disorders, with an emphasis on the pathophysiology and treatment of pediatric immune thrombocytopenia (ITP). Dr. Bennett's extensive experience in designing and performing clinical studies makes her well suited to guide and advise on the clinical research aspect of this project. Dr. Bennett and I have a well-established and active collaboration that began with the initial studies included in the *Nature Materials* publication. Dr. Bennett already regularly identifies and recruits patients for some of our ongoing studies. She is also available at any time via phone, text, or email.

Planned Grants & Leveraging of Resources: The proposed project is distinct but complementary to data that I propose to collect for my pending R21 Trailblazer Award (impact score = 10), which focuses on basic platelet biology and symptomatic bleeding. This award specifically enables me to focus on my promising ITP data, which is the most compelling that I have taken to date with the platelet contraction cytometer. However, to further explore this area, I need additional training in order to perform a prospective study. This study will better define how the absence of highly contractile platelets correlates with bleeding and also changes over the natural history of ITP. This data in combination with data gathered from the R21 award will form the basis of an R01 to the NHLBI (and specifically the hemostasis and thrombosis study section) that seeks to fully investigate the clinical utility of platelet contraction cytometry in ITP. A key part of this application will be ensuring that I have a strong publication record, as outlined, to demonstrate expertise in platelet contraction cytometry and ITP.