Point-of-Care *F2RL3* Mutation Genetic Testing to Inform Antiplatelet Pharmacotherapy

SPECIFIC AIMS

Aim 1: *rs773902* point-of-care assay development *in vitro*. In this proposal, we aim to develop an *in vitro* assay that utilizes novel, isothermal SNP-LAMP technology to fluorescently detect the *rs7733902* SNP, implicated in pathological thrombosis, in F2RL3 gene encoding PAR4.

Aim 2: DNA purification methodology development. The limitations of DNA extraction in POCT settings warrants the testing of various single-step lysis and extraction protocols compatible with our assay from aim 1.

Aim 3: *ex vivo* assay validation. Following successful testing of an *in vitro* assay and a DNA purification protocol, an *ex vivo* assay will be optimized and tested to detect the *rs773902* in human cell lines, calculating an optimal fluorescence threshold between alleles.

INTRODUCTION

Antithrombotic therapy is vital to modern medicine. These drugs' mechanisms of action are largely based around platelets, which are disc-shaped derivatives of megakaryocytes[1]. When platelets are activated, they aggregate with a mesh-like protein known as fibrin in a cascading coagulation known as thromboses[1]. Thromboses can form pathologically in various contexts and may lead to myocardial or cerebral infarction[9].

Disparities in thrombosis are a significant contributor to racial healthcare inequalities in the United States, with rates of venous thromboembolism incidence 30-60% higher in African Americans than persons of European descent[2]. Differential activation of the PAR4 platelet receptor across races likely contributes to this disparity[1]. PAR4, a G-Protein Coupled Receptor, is part of a larger class of protease-activated receptors (PARs) found in humans, which are typically activated by thrombin cleavage and stimulate signaling pathways that activate hemostatic activities[1]. PAR4 functions through $G\alpha_q$, activating phospholipase C-β which then leads to signaling pathways releasing clotting factors such as thromboxane A2[10].

The variant of the gene that encodes the PAR4 protein (*F2RL3*) has a single nucleotide polymorphism SNP, *rs773902*, changing residue 120 from an Ala to a Thr[1]. This SNP is hypothesized to be responsible for about 50% of racial differences in PAR4 activation, with 81% of alleles in persons of European ancestry coding for PAR4-Ala120 and 57% of alleles in persons of African ancestry coding for PAR4-Thr120[1]. The wild type PAR4 receptor has a low affinity for thrombin; however, the variant *F2RL3* allele hypersensitizes platelets to thrombin, suggesting

a quickened and sustained hemostatic response[1]. Thus, patients with this variation have an increased risk of stroke, with an odds ratio of 1.166 for each additional copy of the gene, with a 95% confidence interval of 1.006–1.356[3]. *Ex vivo* clotting data suggests that individuals with at least one allele coding for PAR4-Thr120 are resistant to common thrombosis treatments[1]. In this study, standard of care drug combination of aspirin and clopidogrel[11] were less effective at preventing clotting in PAR4-Thr120 platelets relative to wild type PAR4-Ala120 platelets, as these drugs target the inhibition of the release of thromboxane A2 and other similar factors whose receptors' sensitivities have increased as a result of the SNP. However, it has also been found that this difference in anti-clotting efficacy does not appear following treatment with alternative therapies, such as heparin and ticagrelor, which function in other ways[1,3]. This finding suggests that physicians can improve patient responsiveness to antiplatelet medications by making informed, genome-directed prescription decisions.

Recent advances in sequencing technology make genome-directed therapy a viable strategy in high-resource settings. However, many healthcare settings lack access to traditional sequencing services and the necessary expensive equipment. Additionally, traditional testing creates a need for a second, follow-up patient contact to discuss sequencing results and implement treatment adjustments[4]. Inexpensive, fast, and accessible point-of-care testing (POCT) may address these challenges. Recent improvements to loop-mediated isothermal DNA amplification (LAMP) with competitive fluorescent probes for SNP detection is a promising strategy toward this end[5]. Studies have demonstrated this SNP-LAMP system's ability to detect a SNP rapidly and accurately by producing a fluorescent signal in its presence[5].

We propose developing a SNP-LAMP based point-of-care test for the *rs773902* variant which may help correct disparities in antiplatelet therapy efficacy. We envision that such a test could reduce the prevalence of ineffective prescriptions and likelihood of thrombosis in affected individuals.

EXPLANATION OF AIMS

Aim 1: *in vitro* assay development. First, we will develop the *rs773902* detection assay with known, highly purified DNA sequences. Hyman et al. describes fluorescent detection of SNPs following LAMP of genetic material from human cells[5]. This assay proceeds by two sequential reactions. In the first, sample DNA is amplified in a loop-mediated isothermal amplification reaction, and in the second, association with a fluorescent probe creates a differential signal between alleles[5].

The differential fluorescence is created by two sets of dsDNA probes. In the first set, one strand is attached to a fluorophore and the second to a quencher[5]. In the presence of the sequence-specific SNP containing DNA, the strand with the fluorophore will preferentially

associate with the SNP amplicon, dissociating from the quencher, which has bound to a non-fluorescent sink strand; this produces a signal[5]. In the second set, a sink strand is paired with a blocker sequence that will bind preferentially to the wildtype amplicon while the fluorophore remains bound to the quencher, increasing the difference between the fluorescence of these alternatives[5].

LAMP reaction design is facilitated by online design tools. Use of New England BioLabs' NEB LAMP Primer Design Tool[8]. These primers will be tested with NEB WarmStart LAMP kit[8] following the associated protocol and will be evaluated for the presence of our large desired product (>20 kb) via agarose gel electrophoresis[8].

Design of the fluorescent probe and sink sequences is facilitated by an online design tool[5] published in Hyman et al. Inputting the sequence of our LAMP product produces a set of four parts for the assay – the sequence attached to the fluorophore, the sequence attached to the quencher, the sink sequence, and the competitive binding blocker.

In our second experiment, the products of the amplification reaction with both wildtype and variant alleles will be added to the probe and quencher in various concentrations into solution with the assay sequences and read with a standard fluorescence reader to determine an optimal concentration for SNP-differential fluorescence. We expect samples with the variant allele will produce a stronger signal than samples without the variant allele. We will use a student's t-test to determine whether the fluorescent signals are significantly different between the variant and wild type groups. Hyman et al. was able to develop their SNP-LAMP methodology such that the difference in fluorescent signal between wildtype and variant oligos has a p-value of less than 0.0001 using a one-sided t-test[5]. With this degree of signal differentiation being evidently attainable using SNP-LAMP, we will strive to achieve a comparable degree of distinction. In the case that our initial LAMP constructs do not produce a statistically significant difference in fluorescent signal between variant and wild type samples, we plan to iteratively engineer the LAMP constructs until we achieve satisfactory distinction. Namely, we will strive to manipulate the constructs' lengths in order to make our reagents long enough to bind effectively, but short enough to avoid non-specific binding.

Next, we will perform amplification and fluorescence measurements simultaneously to estimate differential fluorescence as a function of starting reagent concentrations and reaction time. This will be helpful in determining initial reaction parameters in a more realistic context. With this established, we will conduct similar testing on samples containing an equal mixture of the two alleles to estimate the efficacy of the assay in heterozygotes.

Aim 2: DNA purification methodology development. Having determined the parameters of a functional assay, we will proceed to its application in context. DNA extraction in POCT settings

represents a challenge to the utility of our assay. We will overcome this challenge by testing various lysis and extraction methods to find one suitable for low resource settings.

DNA extraction methodology will be performed on cultured human cell lines. With the goal of a single pot reaction, we will begin with testing single-step lysis protocols and subsequently adding assay reagents. These will include thermal, acidic, basic, chaotropic, and detergent lysis conditions. Evaluation of each will first include gel electrophoresis to show the presence of our desired product along with conditionally subsequent fluorescence detection.

We anticipate that these lysis buffers may interfere with assay function. In this case, we will proceed to more complex methodologies. A promising option is gentle chemical cell lysis in detergent or chaotropic buffer followed by DNA extraction via anion exchange. A simple anion exchange is described in Byrnes et al. using chitosan, a positively charged polysaccharide, on a nitrocellulose membrane[6]. The membrane for this technique will be prepared by incubation in a chitosan solution, and initially tested for its ability to bind purified DNA, as measured by A260/280 values[6].

Applied to cell lines, following chemical lysis, DNA will be attracted to the positively charged membrane during incubation or lateral flow, and collected off of the membrane in a mildly basic solution and used in subsequent steps without further purification, although perhaps with pH readjustment through dilution of the weak alkaline buffer with a more concentrated neutral buffer[6]. These results will be evaluated as described for more simple methods.

Aim 3: *ex vivo* assay validation. To test our extraction mechanism and PAR4 variant detection assay, we will further evaluate and verify these methods using cell lines both with and without *rs773902*. The prevalence of this variant in the general human population suggests that it is likely present in some commonly used immortalized human cell lines[7]. We plan to sequence these cell lines using conventional methods prior to use in order to understand which contain a variant allele.

Validation of our assay will proceed in two steps—training and optimization, then testing. The first step will entail performing our assay on half of our cell lines. We will suspend the cells in PBS and standard serum components before lysis, extraction and our assay. We are looking to see that our test is able to accurately detect the PAR4 variant in these cell lines, just as it was able to do in our initial assays with DNA fragments. If we observe some inaccuracies, we plan to continually engineer our LAMP components and extraction methods using the same strategies mentioned previously in order to reach a statistically significant difference between the fluorescence readings of variant and wild type cell lines within this training cohort.

Using the data from this training process, we will calculate an optimal fluorescence threshold for the classification between variant and wild type samples. Assuming a task of imbalanced classification based on the population prevalence of the PAR4 variant, we will use a t-test to confirm significant fluorescence difference between sample groups. Then, we will visually select a minimum fluorescence threshold for a positive result, followed by a supervised approach-algorithm to calculate a clinically optimal threshold.

After optimizing our assay in DNA isolated from cells and developing a threshold that enables variant diagnosis based on fluorescent reading, we will begin the testing step of assay validation. We plan to take a second group of cell lines, called the testing cohort, and use our assay to determine whether they contain the PAR4 variant. This phase is intended to mimic how this test will be used in the clinic on many patient samples. We will compare our results with the sequencing data in order to evaluate how accurate our assay is.

We would like to note that in a clinical setting, this methodology presents the challenge of signal detection from a single sample without a carefully matched control. To overcome this challenge, we will have to develop a methodology to model the fluorescent signature of each allele. One potential solution would be to provide a limiting amount of LAMP reagents so that the final concentration of DNA creating the fluorescent signal will always be equal. Then, testing with different proportions of wild type to variant DNA will produce a standardized scale of fluorescence after adjusting for baseline sample absorbance.

HUMAN CENTERED DESIGN

As we begin to pursue this project, we will integrate the principles of human centered design throughout our technology development process. Our team envisions our PAR4 variant test to be used in low-resource settings to diagnose patients with the variant and inform antiplatelet prescription decisions. Because of the high-stakes nature of this application, we are dedicated to making this test easy to use and reliable in order to maximize its utility in healthcare settings.

In order to achieve these goals of implementing human centered design principles into our research process, we have begun to consider who the stakeholders are in our project, plan opportunities to engage with them throughout the execution of our project, and integrate their opinions and needs into our design. The primary stakeholder and user of our PAR4 variant test are clinicians in low-resource settings. To keep these individuals top of mind, we have made the central goal of our project to develop this test such that it is inexpensive and does not require specialized equipment, enabling the assay to be used in our settings of interest. However, we strive to not only make our test able to be done in these environments, but also ensure that it is simple and time-efficient to do so. Without these requirements, physicians would likely halt the use of our assay due to inconvenience. To ensure our project meets these needs, we aim to

develop extremely simple protocols and automate as much of the test as possible in order to reduce the work required by the clinician. We are currently coordinating meetings with doctors, nurses, and other clinical staff that would be running our PAR4 variant test in order to learn of other potential needs and accordingly account for these requirements in our design.

Another key stakeholder in our project is patients, who are invested in obtaining an accurate diagnosis and subsequently an effective antiplatelet therapy prescription. The very existence of our assay and the subsequent development of genome-directed therapy is in the best interest of patients. Re-thinking the standard of care and instead basing prescription decisions on patient-specific genomic data will help all patients gain access to more efficacious medications. This will be particularly beneficial for African American individuals, as they are known to be more likely to have the *rs773902* variant and thus to be given less effective antiplatelet therapies. Further, test accuracy is our team's top priority throughout the design of this test. We have also considered patient needs while designing our fluorescent signal threshold that dictates whether a variant has been detected. Recognizing that a false positive result leading to an alternative, but still effective, drug prescription is preferable relative to a false negative result leading to an ineffective prescription, we have accounted for this consideration in our statistical method for threshold calculation.

BIOSAFETY AND BIOSECURITY

Throughout the ideation of this project, our team has kept biosafety and biosecurity considerations top of mind. The primary risk involved with our PAR4 variant test is the potential for the materials involved in the assay to become ineffective and begin consistently producing inaccurate results. If the reagents for the SNP-LAMP reactions are freeze-thawed excessively, contaminated, or elsehow altered, this could contribute to non-optimal assay results. To combat this, we feel it would be useful to have a short training session with each clinic that implements our test in order to teach the staff how to run the assay, thus avoiding incorrect usage.

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