Development of a Conjugate Vaccine Against SARS-CoV-2

Nathan Wymer, PhD (Department of Chemistry and Biochemistry); Lindsey M. Costantini, PhD and Vijay Sivaraman, PhD (Deparment of Biological and Biomedical Sciences)

Introduction:

The novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first detected in China in late 2019 and has gone on to infect nearly three million people worldwide as of April 2020.[1] The World Health Organization (WHO) declared the SARS-CoV-2 outbreak as a global pandemic in March 2020. Vaccines and other treatments are desperately needed to combat the spread. One area research that has received little attention is the development of conjugate vaccines against SARS-CoV-2.[2,3] Covalently attaching an antigen onto a carrier protein can provide a significantly higher and longer lasting immune response compared to the antigen alone.[4]

The non-toxic mutant of diphtheria toxin, cross-reactive material 197 (CRM197), has a long and successful history as the carrier protein of conjugate vaccines. CRM197-based vaccines have been FDA-approved to combat pneumococcal, *H. influenzae* b, as well as meningitis ACWY infections.[5-8] CRM197 has also been studied to create vaccines against ß-amyloid protein (Alzheimer's disease) and nicotine (smoking cessation).[9,10] The power of the CRM197 carrier protein for antigens comes from CRM197's ability to directly target antigen presenting cells (APCs). After CRM197 binds to the surface of the APC, both the CRM197 and its covalently attached antigen becomes internalized by the APC. The antigen can then be displayed in a class II major histocompatibility complex (MHC) to begin a T cell dependent immune response. The endocytosis of the antigen by the APC is not left to chance since the CRM197 directly transports the antigen directly into the APC. Another advantage of using CRM197 is that the T cell dependent mechanism is active within infants shortly after birth.[6]

The CRM197 binds to the heparin-binding EGF-like growth factor (HB-EGF) on the surface of the APC to initiate endocytosis.[11,12] Initial results from our laboratory have suggested that binding to the HB-EGF rather than a special property of the CRM197 protein is responsible for this endocytosis. As such, an anti-HB-EGF monoclonal antibody (mAb) might be able to be substituted for CRM197 as the vaccine carrier protein. This mAb could provide additional flexibility by increased protein stability as well as using the knowledge for attaching molecules onto a mAb from both the conjugate-vaccine and antibody-drug conjugate fields.

The spike glycoprotein (S-protein) of the coronavirus, particularly the receptor binding domain (RBD) portion, has undergone intense research as a vaccine candidate for both SARS-CoV-1 and SARS-CoV-2 so will be used in this project.[13-20] Performing research directly with SARS-CoV-2 is extremely difficult since this research requires a BSL4 laboratory. As such, this proposal would use two different model systems: the mouse hepatitis virus A59 (MHV-A59) coronavirus[21] and a chemically-activated microbead which allows for protein attachment.[22] This research would attach the MHV-A59 or SARS-CoV-2 S-protein RBD onto CRM197. Inoculated rabbits would have their serum tested with antibody neutralization. Promising vaccine candidates would then be used in mouse survival models. The overall goal of this research is to expand SARS-CoV-2 vaccine research and development in order to help create a safe and efficacious vaccine to protect humanity against this pandemic.

Specific Aim #1: Development and optimization of CRM197-RBD conjugate vaccines for neutralization of MHV-A59, a SARS-CoV-2 coronavirus model system. The MHV-A59 S-protein RBD will be covalently attached to CRM197. The RBD would be attached either through an amino acid (labelled lysine or cysteine) or through its polysaccharide after chemical activation. These vaccine candidates will then be tested with antibody neutralization assays. Promising vaccine candidates will then be tested *in vivo* within a mouse model to protect mice against MHV-A59 infection.

Specific Aim #2: Development and optimization of CRM197-RBD and mAb-RBD conjugates *in vivo* within mice against MHV-A59, a SARS-CoV-2 model system. The SARS-CoV-2 S-protein RBD will be covalently attached to CRM197. The RBD would be attached either through an amino acid (labelled lysine or cysteine) or through its polysaccharide after chemical activation. These vaccine candidates will then be tested with antibody neutralization assays using a modified microbead as a model for the SARS-CoV-2.

Work Plan:

Specific Aim #1: Development and optimization of CRM197-RBD and mAb-RBD conjugates and testing for neutralization in SARS-CoV-2 coronavirus model system

Performing research directly upon SARS-CoV-2 requires a biosafety level 4 (BSL4) laboratory. Few BSL4 laboratories exist so we have selected the mouse hepatitis virus A59 (MHV-A59) coronavirus to serve as a model system. MHV-A59 causes SARS-like symptoms within mice but cannot be transmitted through direct contact.[21] This more difficult mode of transmission will significantly reduce the chances of an outbreak within our animal facility. Like all model systems, using a mouse-infecting coronavirus instead of a human-infecting one makes the system less than ideal but can still provide important proof-of-concept insights. A major difference between MHV-A59 and SARS-CoV-2 is mode of infection. The RBD of the S-protein in MHV-A59 enters the cell by binding to the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) protein while SARS-CoV-2 uses the ACE2 protein for entry.[13,23] While the targets for the SARS-CoV-2 and MHV-A59 coronaviruses is different, both coronaviruses use their S-protein for cellular entry.[24]

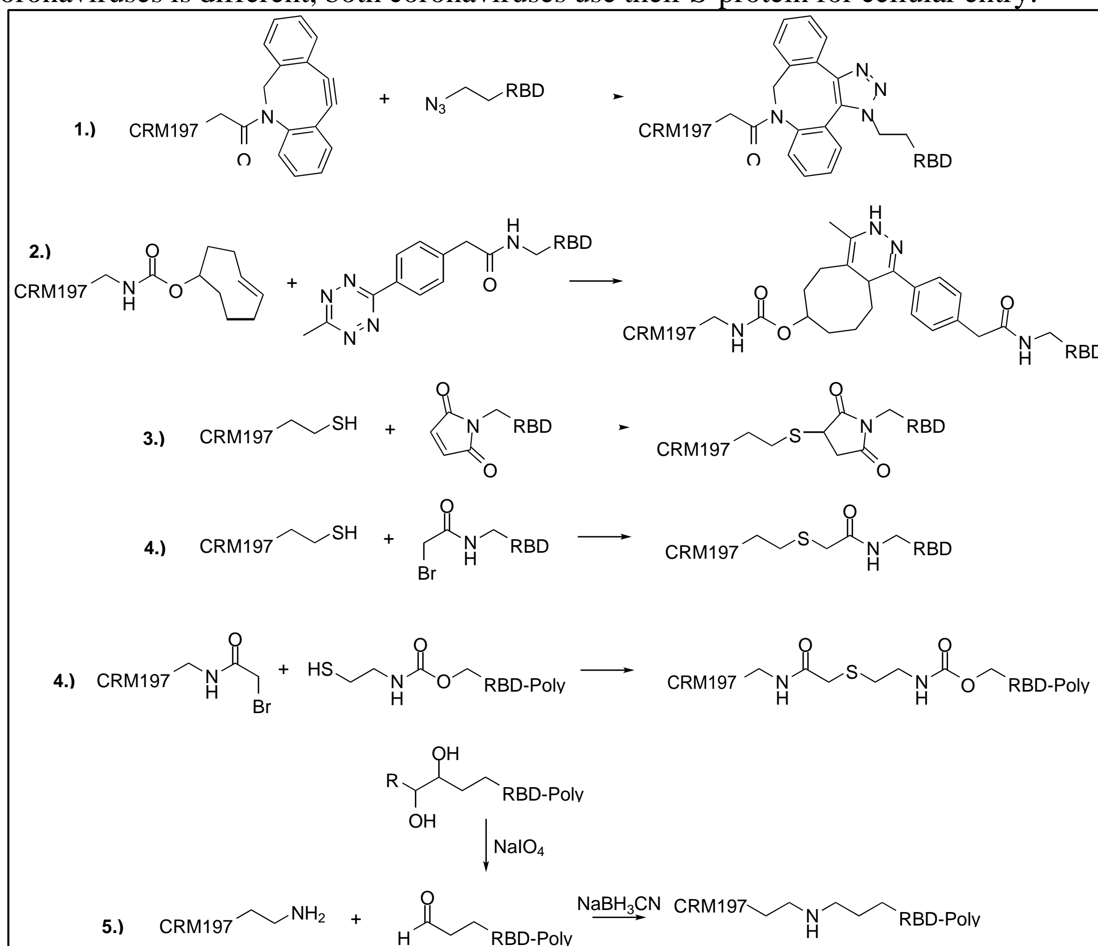

Figure 1: Examples of chemical synthetic strategies for the development of CRM197-RBD protein and CRM197-RBD polysaccharide conjugate vaccines. 1.) Cu-free CLICK chemistry, 2.) *trans*-cyclooctene/tetrazine CLICK chemistry, 3.) thiol/maleimide, 4.) bromoacetamine with a nucleophilic thiol, 5.) Periodate oxidation of the RBD polysaccharide, mixing of the oxidized RBD polysaccharide, and reduction of the resulting imine (Schiff base) to an amine.

The conjugation of CRM197 and RBD can occur in a myriad of ways. Many different chemistries can be attempted, including Cu-free CLICK,[25] cyclooctene/tetrazine,[26] and thiol/malimide.[27] (Figure 1) The reagents to label proteins are readily available and have been formulated for efficient reactions. Our lab has significant experience attaching these molecules onto CRM197. The conjugate could also attach CRM197 onto the glycosylation polysaccharide of the RBD. (Figure 1) This process would allow us to tap the vast experience of CRM197-polysaccharide conjugation already reported within the literature.[28] This flexibility of creating both

protein-protein and protein-polysaccharide conjugates should significantly increase our chances of developing a successful conjugate vaccine candidate.

Once these various conjugates have been synthesized, rabbits would be inoculated with the conjugate candidates. Serum would then be collected and used for *in vitro* antibody neutralization assays to determine if any samples could prevention of MHV-A59 infection of mouse cells. The conjugate vaccine candidates that showed promising results could then be optimized for protective immune response, e.g. modifying CRM197:RBD ratios, vaccine dose size and regiment, and adjuvant.[28]

Vaccine candidates that display the greatest potential will then be tested within *in vivo* mouse models. C57BL/6 mice will be immunized with the vaccine candidates or control compounds, and antibody production will then be monitored. After 14 days, the immunized mice will be challenged by infection with MHV-A59 via internasal inoculation and monitored for mortality and morbidity, e.g. lack of weight gain and proinflammatory cytokine responses. The differences in response in the inoculated mice between those receiving vaccine candidate and controls will then be examined. These results could then inform the further optimization of vaccine candidates.

Specific Aim #2: Development and optimization of CRM197-RBD and mAb-RBD conjugate vaccines against a SARS-CoV-2-like particle *in vitro* model.

The MHV-A59 model system described in specific aim #1 will provide invaluable information against a live coronavirus. However, the overall goal of this research is to develop a vaccine against SARS-CoV-2. Aim #2 will examine the creation a molecule that can mimic SARS-CoV-2 without the infection risk and BSL4 requirements.

Aim #2 would create conjugate vaccines and inoculate rabbits similarly to specific aim #1. However, the SARS-CoV-2 RBD would be attached to the CRM197 or anti-HB-EGF antibody instead of the MHV-A59 RBD. The S-protein RBD and human ACE2 proteins can all be purchased initially to accelerate the research, but the proteins will eventually need to be recombinantly expressed similarly to the proteins discussed in specific aim #1. The rabbit antibodies from the conjugate vaccine candidates could be measured for relative dissociation constant (Kd) for RBD binding using surface plasmon resonance (SPR) and/or a sandwich ELISA.[16] These antibodies would then be used to prevent the attachment of the SARS-CoV-2 mimic onto mammalian cells expressing the human ACE2 on their outer membrane that have been previously demonstrated to be infected by SARS-CoV-2.[14] These cells could then be tracked for "infection" by the SARS-CoV-2 mimic with flow cytometry and/or live-cell imaging.

SARS-CoV-2 has an overall spherical structure of approximately 120 nm in diameter with S-proteins spiking out over the entire structure, similar to orange and clove pomander ball decorations.[24,29] (Figure 2)

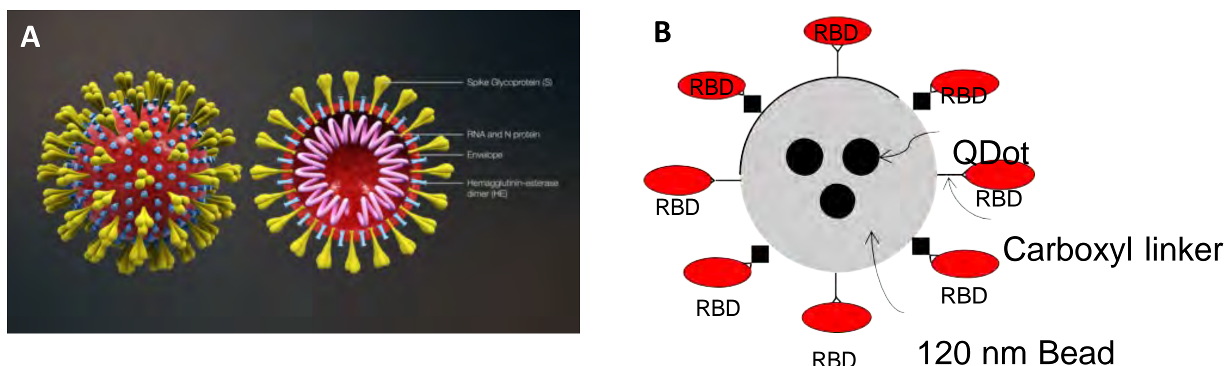

Figure 2: (A) External and internal structure of SARS-CoV-2.[24] (B) SARS-CoV-2 mimic consisting of quantum dots (QDots) affixed inside of an approximately 120 nm polystyrene bead. The bead contains linkers with carboxylate function groups which will facilitate the attachment of the SARS-CoV-2 S-protein receptor binding domain (RBD).[22]

A SARS-CoV-2 mimic should maintain these features while including a direct detection method, such as a fluorophore. Quantum dots (QDots) offer advantages over other fluorophores, e.g. fluorescein, in that their

emission spectrum can be readily tuned by their size, are chemically stable, and do not photo bleach with repeated measurements.[22] This lack of photobleaching would allow for continual monitoring over the course of hours or days. QDots cannot be used directly because they are not available in sizes >20 nm. However, QDots can be encapsulated within polystyrene microbeads in varying sizes, including 120 nm. The beads can also be functionalized to attach biomolecules onto their surface. Therefore, the S-protein RBD protein could be attached to the outside of this QDot microbead in order to create a SARS-CoV-2 mimic for the assays described above. (Figure 2)

Timeline and Milestones:

2020 – Quarter #3

- Procure adequate stocks of reagents and supplies to support the following:
    - >10 mg of SARS-CoV-2 S-protein and/or S-protein RBD
    - Human ACE2-expressing human cell lines
    - Quantum dot-containing activated microbeads
    - MHV-A59 virus and mouse cell lines
- Examine different activation and conjugation strategies to attach SARS-CoV-2 RBD to CRM197 protein
- Ship conjugate vaccine candidates for antibody-containing serum generation (latest: mid-September)
    - Proposed vaccine candidate samples:
        - MHV-A59-RBD attached through protein to CRM197
        - MHV-A59-RBD attached through polysaccharide to CRM197
        - MHV-A59-RBD only
        - SARS-CoV-2-RBD (mammalian-expressed) attached through protein to CRM197
        - SARS-CoV-2-RBD (*E. coli*-expressed) attached through protein to CRM197
        - SARS-CoV-2-RBD (mammalian-expressed) attached through polysaccharide to CRM197
        - SARS-CoV-2-RBD only
        - SARS-CoV-2-Spike protein attached through protein to CRM197
        - SARS-CoV-2-Spike protein attached through polysaccharide to CRM197
        - SARS-CoV-2-Spike protein only
        - CRM197 only (as control)
    - Have company remove and store rabbit spleens to later identify promising B-cells

2020 – Quarter #4

- Optimize antibody neutralization assays for both anti-MHV-A59 and anti-SARS-CoV-2 RBD serum samples
- Once serum samples have been received, evaluate serum samples with appropriate antibody neutralization assays
- Promising anti-MHV-A59 conjugate vaccines can then be used for initial mouse viral-protection assays

Proposed Budget:
Teaching release time: $4000 (Wymer)
Reagents and supplies: $25,000
Rabbit serum generation: $36,000
Mouse viral-protection studies: $10,000
Total: $75,000

Budget Justification:
Teaching release time: $4000 (Wymer)

- This release time will provide funds for the hiring of an adjunct professor to teach CHEM3340 – Organic Chemistry Lab II in order relieve Prof. Wymer of the required laboratory preparation, in-class teaching, and grading time. This time will then be spent on research activities.

Reagents and supplies: $25,000

- Many expensive proteins and reagents are going to be initially purchased instead of produced in-house so as to accelerate the creation and testing of vaccine candidates.

Rabbit serum generation: $36,000

- Initial quotes for rabbit serum production was ~$2000/sample/rabbit. The initial experimental design calls for 11 samples with two rabbits/sample. Additional charges will occur including rabbit spleen remove and storage.

Mouse viral-protection studies: $10,000

- The mice for this study will require extensive isolation in order to prevent MHV-A59 infection throughout the facility. After discussions about the protocols, the experiments will be performed at either the NCCU Animal Research Center or the Animal Studies Core facility at UNC-Chapel Hill with consultation with Prof. Robert Tarran.

Future Funding Target:
NIH-NIAID: Emergency Awards: Rapid Investigation of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and Coronavirus Disease 2019 (COVID-19) (R21 Clinical Trial Not Allowed)
Funding level: $275,000 total over two years.

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OMB No. 0925-0001/0002 (Rev. 08/12 Approved Through 8/31/2015)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Nathan J. Wymer

eRA COMMONS USER NAME (credential, e.g., agency login): nwymer

POSITION TITLE: Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Purdue University, West Lafayette, IN	BS	05/1997	Chemistry
Duke University, Durham, NC	PhD	05/2002	Organic Chemistry and Biocatalysis
Georgia Institute of Technology, Atlanta, GA	Postdoctoral	05/2003	Protein Engineering and Biocatalysis

Please refer to the Biographical Sketch sample in order to complete sections A, B, C, and D of the Biographical Sketch.

A. Personal Statement

I have been researching areas of protein engineering and synthetic organic chemistry since graduate school. These research areas include both pharmaceutical and academic research. While working within the biotechnology and pharmaceutical industries, I researched novel synthetic routes to use enzymes to manufacture pharmaceutical intermediates ranging from milligram to multi-ton scales. I have expanded these interests while in academia to research ways to use engineer proteins in order to deliver medicines and diagnostic probes across the blood brain barrier.

Many different levels of students (high school to PhD-level) have performed research within my research group. To date, one PhD-level, three Masters-level, and twenty-five undergraduate students have worked within my lab. These students have graduate and moved onto various graduate programs and employment. These students have the opportunity to learn a myriad of different skills from molecular biology, analytical chemistry, and synthetic organic chemistry. Exposure to these broad skillsets benefit the students as they begin careers within the multifaceted nature of modern scientific research and biomedical fields.

Examples of former undergraduate research students:

	Current Position
Arielle Harris	Molecular Genetics Technologist at LabCorp
Brittany Carson	Intern at National Tropical Botanical Garden (Hawaii)
Conrad Kovalcik	Medical Student at Edward Via College of Osteopathic Medicine (2018 – present)
Tiffany Nguyen	Associate II at Biogen
Eliran Nunez	Production Technician at Merck and Co.
Hieu Hoang	Graduate Student in Molecular Biology and Biochemistry Doctoral Program at East Carolina University (Entering Fall 2020)
Nnenna Ujah	Lab Manager at McDaniel Laser & Cosmetic Center
Melissa Wooten	Graduate Student in Pharmaceutical Sciences Master's Program at North Carolina Central University

B. Positions and Honors

Positions and Employment

2003-2006 Scientist, zuChem, Inc., Peoria, IL
2006-2011 Principal Scientist, Biocatalysis Center of Emphasis, Pfizer, Inc., Groton, CT
2011-2014 Principal Scientist, Vaccine Manufacturing Science and Technology, Pfizer, Inc., Sanford, NC
2014-Present Assistant Professor of Chemistry and Biochemistry, North Carolina Central University, Durham, NC

Other Experience and Professional Memberships

1994-Present Member, American Chemical Society
2013-2015 Adjunct Assistant Professor of Chemistry, Fayetteville State University, Fayetteville, NC

Honors

2010 Pfizer Global Manufacturing Quarterly Technology Award
2011 Pfizer Green Chemistry Team Award
2011 Pfizer Innovation Excellence Team Award
2016 NCCU Excellence in Teaching Award for the Department of Chemistry and Biochemistry
2020 NCCU Award for Teaching Excellence

Review Committees

2017 NSF - Metabolic Engineering, Bioenergy and related topics – *Ad hoc* member

C. Publications and Patents

Nathan Wymer, Louise Buchanan, Darla Henderson, Nupur Mehta, Catherine H. Botting, Luka Pociavavsek, Eric Toone, James H. Naismith. "Directed Evolution of a New Catalytic Site in 2-Keto-3-deoxy-6-phosphogluconate Aldolase," *Structure*, **2001**, 9, 1-10.

Stephen W.B. Fullerton, Jennifer S. Griffiths, Nathan J. Wymer, Eric J. Toone, James H. Naismith. "Mechanism of the Class I KDPG Aldolase," *Bioorganic and Medicinal Chemistry*, **2006**, 14 (9), 3002-10.

Ryan D. Woodyer, Nathan J. Wymer, F. Michael Racine, Shama N. Khan, Badal C. Saha, "Efficient Production of L-Ribose with a Recombinant *E. coli* Biocatalyst," *Applied and Environmental Microbiology*, **2008**, 74, 2967-75.

Matthew Badland, Michael P. Burns, Robert J. Carroll, Roger M. Howard, Daniel Laity, Nathan J. Wymer, "Application of Biocatalysis Towards Asymmetric Reduction and Hydrolytic Desymmetrisation in the Synthesis of a β-3 Receptor Agonist," *Green Chemistry*, **2011**, 13, 2888-94.

Nathan Wymer, Jeremy Steflik, and Maria Brown, "Investigating the Structure-Activity Relationships Within the *Enterococcus faecalis* 2-Deoxyribose Aldolase," *African Journal of Biochemical Research*, **2011**, 5(6), 172-5.

Rajesh Kumar, Jim Cawley, Michael Karmilowicz, Carlos A Martinez, Nathan Wymer. "6.1 Preparative Method for the Enzymatic Synthesis of 5-Ketogluconic Acid and its Isolation," *Practical Methods for Biocatalysis and Biotransformations 2*, **2012**, 2101, 163-6.

Paul Taylor, Nathan Wymer, F. Michael Racine, "Methods for Production of Xylitol in Microorganisms." United States Patent 8,367,346, Granted **February 5, 2013**

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Nathalie Bravo-Bautista, Hieu Hoang, Anusha Joshi, Jennifer Travis, Melissa Wooten, and Nathan J. Wymer. "Investigating the Deoxyribonuclease Activity of CRM197 with Site- Directed Mutagenesis," *ACS Omega*, **2019,** 4, 11987-92.

Nneka Okehie, Melissa Wooten, Hieu Hoang, Asia Sandridge, Nathan J. Wymer, "Determining the Effects of Naturally-Occurring Mutations within the Heparin-Binding EGF-like Growth Factor (HB-EGF) upon Binding to Cross Reactive Material 197 (CRM197)," *ACS Biological Chemistry*, (**Submitted – May 2020**)

D. Research Support

<u>Current:</u>

2019: NCCU – Innovation Seed Grant – *Developing New Treatments for Metastatic Breast Cancer Tumors Within the Brain* – $25,000

2020: NCCU – RCHDR Research Voucher Program - *Examining the Effects of Naturally-Occurring Mutations Found Within the Serine/Threonine/Tyrosine Kinase 1 (STYK1) in Minority Populations* - $5000

<u>Previously Completed:</u>

Nathan Wymer (PI)
ThermoFisher Cancer Research Grants
Developing New Treatments for Metastatic Breast Cancer Tumors Within the Brain
Accepted March 2019 ($2,833)

Nathan Wymer (PI), Darlene Taylor, and Fei Yan
Department of Defense (DoD) Research and Education Program for Historically Black Colleges and Universities and Minority-Serving Institutions (HBCU/MI) Equipment/Instrumentation
Using NMR to Expand Chemistry Research and Educational Experiences at North Carolina Central University, an Historically Black University
Accepted June 2016 ($348,424)

Nathan Wymer (PI), Paul Taylor, Andreas Bommarius, and David Demirjian
NIH-SBIR Phase II Grant Application
Production of L-Ribose and Other Rare Sugars
Accepted May 2006 ($750,000)

Nathan Wymer (PI), Paul Taylor, and David Demirjian
NIH-SBIR Phase I Grant Application
Production of L-Ribose and Other Rare Sugars
Accepted February 2005 ($100,000)

OMB No. 0925-0001 and 0925-0002 (Rev. 09/17 Approved Through 03/31/2020)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
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NAME: Costantini, Lindsey M.

eRA COMMONS USER NAME (credential, e.g., agency login): LCOSTANTINI

POSITION TITLE: Assistant Professor of Biological and Biomedical Sciences

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Connecticut, Storrs, CT	B.S.	05/2005	Molecular and Cellular Biology
University of Connecticut, Storrs, CT	M.S.	05/2008	Cellular Biology
Albert Einstein College of Medicine, Bronx, NY	Ph.D.	09/2014	Biomedical Sciences
University of North Carolina, Chapel Hill, NC	Postdoc.	07/2018	Virology

A. Personal Statement

Throughout my scientific career I have integrated microscopy techniques into my research. As a graduate student, I developed novel fluorescent protein tools to pair with live cell fluorescence microscopy to visualize cellular processes and measure levels of cellular stress in real time. As a UNC-CH postdoctoral fellow, I expanded my microscopy expertise into electron microscopy and specialized sample preparation techniques, which I have used to visualize cells, organelles, viruses, proteins and nucleic acids. In my future research program, I will continue to conduct research spanning multiple fields including cellular and molecular biology, virology and microscopy (electron microscopy, live cell and fluorescence microscopy). By utilizing highly specialized techniques I am positioned to address questions that can only be answered via direct visualization.

My long-term research focus is on understanding viral replication of human oncogenic viruses. I am investigating Kaposi's sarcoma herpesvirus (KSHV, HHV-8) proteins that control viral DNA replication by: 1) characterizing the *in vitro* activities of viral DNA replication proteins, 2) mapping DNA binding sites of purified viral proteins at the viral origin of replication, and 3) investigating the interactions between viral and host proteins through incorporating relevant host DNA binding proteins. Direct visualization via electron microscopy produces qualitative and quantitative data. The characterization of the core replication machinery of KSHV by biochemical and electron microscopy techniques will provide a greater understanding of the viral proteins involved in this process, the organization of viral origins of replication and the overall mechanisms of KSHV replication.

There is a natural integration of my current research of oncogenic viruses with alcohol induced and cellular stresses. I propose to investigate how alcohol influences the virus lifecycle of herpesviruses. Herpesviruses establish a lifelong infection in the host by oscillating between latent (dormant) and lytic (active) phases. After establishing primary infection, the virus enters the latent stage. Throughout the viral lifecycle, cellular and environmental stresses induce viral reactivation and re-entry into the lytic cycle. I hypothesize that the presence of environmental chemicals and stressors, such as alcohol, will enhance herpesvirus reactivation along the oral-esophageal route. Future studies using methods employed during my graduate studies will test cellular stress and secretory stress pathways that are upregulated by alcohol and viral reactivation. These approaches will be modified to examine the effect of alcohol on additional herpesviruses (HSV-1 and EBV) to understand both the conserved and specialized cellular responses to herpesvirus reactivation in the context of alcohol induced stress. Undergraduate students who train in my lab would thus gain experience in microscopy, cellular, biochemical, and molecular biology techniques in order to make significant contributions to the field of virus biology.

B. Positions and Honors

Positions and Employment

2014 – 2018 Postdoctoral Fellow, laboratories of Drs. Blossom Damania and Jack D. Griffith, University of North Carolina, Chapel Hill, NC
2016 – 2017 Visiting Assistant Professor, Department of Biological and Biomedical Sciences, North Carolina Central University, Durham, NC
2018 – Assistant Professor, Department of Biological and Biomedical Sciences, North Carolina Central University, Durham, NC

Other Experience and Professional Memberships

2007 – Member, American Society of Cell Biology
2010 – 2012 Chair, Graduate Student Government, Albert Einstein College of Medicine
2011 – 2014 Member, Graduate Executive Committee, Albert Einstein College of Medicine
2011 – 2014 Co-founder and Chair, Women's Networking Group, Albert Einstein College of Medicine
2016 – 2018 Chair, Postdoctoral Association, University of North Carolina
2018 Member, Center for Integration of Research, Teaching, and Learning (CIRTL) Advisory Group, University of North Carolina

Honors

2009 – 2012 Cellular and Molecular Biology Training Grant (T32 GM007491)
2012 Student Service Award, Albert Einstein College of Medicine
2014 – 2017 SPIRE Postdoctoral Fellowship (IRACDA K12 GM081259-09)
2017 Invited *Spotlight* session Speaker, International Herpes Workshop Travel Award, Ghent, Belgium

C. Contributions to Science

1. The Optimization of Fluorescent Proteins for Subcellular Environments. At Einstein, I optimized fluorescent tools for use in live cell fluorescence microscopy. I engineered fluorescent proteins for oxidizing cellular environments, such as the secretory pathway lumina, mitochondria intermembrane space and the periplasm of bacteria. Prior to, the use of fluorescent proteins in these subcellular locations produced undesirable consequences. The optimized fluorescent proteins establish a palette of functional fluorescent proteins suitable for the chemically distinct environments of subcellular organelles. My extensive characterization of fluorescent proteins led to the invention of a fluorescent protein derived tool that enhanced protein expression in mammalian cells. Furthermore, I developed biologically relevant assays to assess the performance of fluorescent proteins in cells. One of the assay (OSER assay) has been adopted by experts in the fluorescent protein field and is routinely utilized as a standard measure to determine the suitability of new fluorescent proteins for live cell applications.

a. **Costantini LM**, Baloban M, Markwardt ML, Rizzo M, Guo F, Verkhusha VV, Snapp EL. A palette of fluorescent proteins optimized for diverse cellular environments. Nat Commun. 2015 Jul 9;6:7670. PMID: 26158227; PMCID: PMC4499870.
b. **Costantini LM**, Subach OM, Jaureguiberry-bravo M, Verkhusha VV, Snapp EL. Cysteineless non-glycosylated monomeric blue fluorescent protein, secBFP2, for studies in the eukaryotic secretory pathway. Biochem Biophys Res Commun. 2013 Jan 18;430(3):1114-9. doi: 10.1016/j.bbrc.2012.12.028. Epub 2012 Dec 19. PMID: 23257162; PMCID: PMC3552020.
c. Snapp EL and **Costantini LM**. Fusion Tags for Protein Expression. U.S. Patent 15/152,908
d. **Costantini LM**, Fossati M, Francolini M, Snapp EL. Assessing the tendency of fluorescent proteins to oligomerize under physiologic conditions. Traffic. 2012 May;13(5):643-9. doi: 10.1111/j.1600-0854.2012.01336.x. Epub 2012 Feb 20. PMID: 22289035; PMCID: PMC3324619.

2. Characterization of KSHV DNA Replication Proteins by Electron Microscopy. Herpesviruses encode their own DNA replication machinery. KSHV expresses six DNA replication proteins during viral replication: DNA polymerase (ORF9), polymerase accessory factor (ORF59), helicase (ORF44), primase (ORF56), primase associated factor (ORF40/41), single-stranded DNA binding protein (ORF6), and the regulatory protein, replication and transcriptional activator (ORF50). The activity of ORF50 (RTA), is essential for viral replication

and reactivation. ORF50 binds to the viral lytic origin DNA sequence and ORF59. Together these binding events lead to the recruitment of the additional KSHV core replication proteins and the initiation of viral DNA replication. Previous studies have identified ORF50 binding sites throughout the KSHV genome consistent with replication and transcriptional activities. To investigate ORF50 binding in the context of viral DNA replication, I performed *in vitro* binding assays with purified ORF50 and isolated lytic origin DNA. I mapped ORF50 binding to three distinct regions, two regions represent novel binding locations within the viral origin DNA sequence. In addition, I observed ORF50 binds viral DNA primary as a dimer. ORF50 dimerization may stabilize protein-DNA interaction as well as facilitate the binding of additional KSHV DNA replication proteins. In addition, I visualized purified ORF59 using electron microscopy and observed ORF59 forms two structurally distinct oligomers with a clearly visible center hole. These findings are consistent with the previous observation that a homologous protein from a closely related herpesvirus forms a ring structure *in vitro*. I predict head to tail dimers assemble into higher order oligomeric rings composed of four or six monomers, which may function as a sliding clamp during DNA replication. The use of electron microscopy approaches revealed novel DNA binding sites and the oligomeric state of KSHV DNA replication proteins. The findings would be otherwise undetectable if not for the high-resolution imaging approach. These new insights may result in better approaches to target and inhibit viral replication.

a. **Costantini LM**, Griffith JD, Damania B. Characterization of KSHV DNA Replication Proteins by Electron Microscopy. 42nd International Herpesvirus Workshop, Ghent, Belgium, 2017.
b. **Costantini LM**, Griffith JD, Damania B. Characterization of ORF59 Complexes by Electron Microscopy. 42nd International Herpesvirus Workshop, Ghent, Belgium, 2017.
c. Edwards AB*, **Costantini LM** and Griffith JD. Electron Microscopy Analysis of KSHV Origin of Replication and Viral DNA Replication Proteins. 2017 Annual Biomedical Research Conference for Minority Students (ABRCMS), Phoenix, AZ, 2017.
d. Edwards AB*, **Costantini LM** and Griffith JD. Characterization of Kaposi's Sarcoma Herpesvirus DNA Polymerase and Processivity Factor using Electron Microscopy. 2016 Annual Biomedical Research Conference for Minority Students (ABRCMS), Tampa, FL, 2016.

3. Applications of electron microscopy to visualize molecular interactions and subcellular structures. For nearly a century, electron microscopy has been a standard method to visualize and quantify nanometer to micron sized structures and particles. Due to my expertise with transmission electron microscopy and a variety of electron microscopy preparation methods, I have developed research collaborations and made significant contributions to multiple publications. These studies are across diverse areas of molecular and cellular biology and include: 1) characterizing cellular derived extracellular vesicles, 2) investigating the structural changes induced by cardiomyopathy relevant mutations in actin-filament assembly, and 3) evaluating the consequences of silver nanoparticle exposure to cancer cells. To evaluate the physical characteristics of extracellular vesicles derived from viral infected cells, I acquired electron micrographs of a variety of cell-derived extracellular vesicles. Ultrastructural studies of extracellular vesicles derived from HIV and SIV infected cells align with my primary research interest, KSHV. KSHV infection is highly increased in HIV positive patients, in addition, others describe the functional importance of extracellular vesicles in the infectious cycle and persistence of KSHV infection. More recently, I have developed a collaboration with researchers at NCCU investigating the impact of silver nanoparticle exposure on cancer cells adhesion dynamics. My contributions to these studies including characterization of silver nanoparticle size distributions and particle uniformity. These productive research partnerships are evidence of how currently as well as in the future my microscopy expertise promotes cross disciplinary contributions.

a. Martin ME, Reaves DK, Jeffcoat B, Enders JR, **Costantini LM**, Yeyeodu ST, Botta D, Kavanagh TJ, Fleming JM. Silver nanoparticles alter epithelial basement membrane integrity, cell adhesion molecule expression, and TGF-β1 secretion. Nanomedicine.2019 Jul 24;:102070. doi: 10.1016/j.nano.2019.102070. [Epub ahead of print] PubMed PMID: 31351238.
b. McNamara RP, Chugh PE, Bailey A, **Costantini LM**, Ma Z, Bigi R, Cheves A, Eason AB, Landis JT, Host KM, Xiong J, Griffith JD, Damania B, Dittmer DP. Extracellular vesicles from Kaposi Sarcoma-associated herpesvirus lymphoma induce long-term endothelial cell reprogramming. PLoS Pathog. 2019 Feb;15(2):e1007536. doi: 10.1371/journal.ppat.1007536. eCollection 2019 Feb. PubMed PMID: 30716130; PubMed Central PMCID: PMC6361468.

c. McNamara RP, Caro-Vegas CP, **Costantini LM**, Landis JT, Griffith JD, Damania BA, Dittmer DP. Large-scale, cross-flow based isolation of highly pure and endocytosis-competent extracellular vesicles. J Extracell Vesicles. 2018;7(1):1541396. doi: 10.1080/20013078.2018.1541396. eCollection 2018. PubMed PMID: 30533204; PubMed Central PMCID: PMC6282418.
d. McNamara RP, **Costantini LM**, Myers TA, Schouest B, Maness NJ, Griffith JD, Damania BA, MacLean AG, Dittmer DP. Nef Secretion into Extracellular Vesicles or Exosomes Is Conserved across Human and Simian Immunodeficiency Viruses. MBio. 2018 Feb 6;9(1). doi: 10.1128/mBio.02344-17. PubMed PMID: 29437924; PubMed Central PMCID: PMC5801467.

(*PI mentored undergraduate author)

Complete List of Publications by Lindsey M. Costantini

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing Research Support
1SC2GM136527 (PI: Costantini, Lindsey) 04/15/20-02/28/23 NIH/NIGMS: Support of Competitive Research (SCORE) Pilot Project Award (SC2) Goal: The most recently discovered herpesvirus, Kaposi's sarcoma herpesvirus (KSHV/HHV8) is known to cause three human cancers, Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). No cure or vaccine exists for KSHV infection and its associated malignancies. To identify novel KSHV antiviral targets, we propose to identify virus specific protein functions and protein conformations by employing highly specialized electron microscopy (EM) methods to directly visualize purified KSHV DNA replication proteins and viral DNA sequences during the formation of the DNA replication fork at the viral origins of replication.

Completed Research Support
K12GM000678 (PI: Dykstra, Lysle) 09/15/14-08/31/17 NIH/NIGMS: SPIRE Training Grant Goals: SPIRE (Seeding Postdoctoral Innovators in Research and Education) has established itself as an innovative postdoctoral research and career development training program whose goals are aligned with those of IRACDA: 1) to inspire and motivate undergraduate students from underrepresented groups to engage in science-based course content, research, and to pursue graduate degrees in science while 2) providing training in research, teaching, and career development that promotes success of scholars in their own career goals. The underlying hypothesis of SPIRE is that faculty who combine excellence in research and teaching, and value the importance of mentoring and diversity, will effectively train the next generation of scientists and promote diversity in the scientific workforce.

Role: Trainee, SCHOLAR

T32GM007491 (PI: Kielian) 06/30/2010-06/30/2012 NIH/NIGMS: The goal of this program is to train graduate students for the PhD degree in the broad and interdisciplinary areas of Cell Biology, Molecular Biology and Genetics. The overall objectives of the program are for students to perform significant basic science research projects, to acquire rigorous scientific background and experimental training, and to develop into independent scientists that make long-term contributions.

Role: Trainee, PRE-DOC

OMB No. 0925-0001 and 0925-0002 (Rev. 09/17 Approved Through 03/31/2020)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Vijay Sivaraman

eRA COMMONS USER NAME (credential, e.g., agency login): Vijay_Sivaraman

POSITION TITLE: Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
The New College of Florida	BS	05/2001	Chemistry/Biology
The National Institutes of Health (pre-doc fellow)		07/2003	Immunology
The University of North Carolina, Chapel Hill	PhD	08/2009	Microbiology/Immunology

A. Personal Statement

My long-term research interests involve elucidating immunological interactions between the host-pathogen interfaces that contribute to disease. I am currently an Assistant Professor of Biology at North Carolina Central University. My work focuses primarily on interaction between the microbial pathogens and immune cells within the lung, and I hope to broaden these lines of inquiry with other infectious microbes to generate a more global picture regarding mediators of inflammation for the purposes of generating novel targets for diagnostic, therapeutic and drug development. I am currently interested in the interplay between inhaled “environment determinants” and cells within the pulmonary tract that may lead to health impacts and disparities. I am excited to be part of the “Center for Environmental Health Disparities” at NCCU.

I recently carried a NIAAA-funded U54 Pilot Grant to evaluate pulmonary pathology due to alcohol intoxication. From these studies, my laboratory has recently published a research article in *Alcohol*, and I have published several articles in peer-reviewed journals regarding pathogen-host interaction. I currently teach courses on medical microbiology and immunology and mentor students participating in research in my laboratory. I currently mentor both graduate and undergraduate students to perform active research in my laboratory, and students who had graduated while working in my laboratory have been placed in both the biotech and academic environments.

B. Positions and Honors

Positions and Employment

05/01-07/03	Research Fellow, Department of Immunology; NEI, National Institutes of Health (Supervisor – Dr. J.A. Ragheb)
01/10-02/10	Visiting Teaching Fellow, Department of Microbiology; The New College of Florida; (Supervisor – Dr. K. Walstrom)
02/10-07/13	Post-doctoral Research Associate, Department Microbiology/Immunology; The University of North Carolina-Chapel Hill; Chapel Hill, North Carolina (Supervisor – Dr. W.E. Goldman)
08/13-Present	Assistant Professor, Department of Biological and Biomedical Sciences; North Carolina Central University; Durham, NC

Other Experience and Professional Memberships

	2012-2013 **Chair,** Microbiology and Immunology Postdoctoral Association; UNC-Chapel Hill 2017-Current **Member**, Center for Human Health and the Environment, NCSU

Academic and Professional Honors

2019 **Invited Seminar Speaker**, Dept. of Biology, New College of Florida, Sarasota, FL
2018 **Invited Seminar Speaker,** Pulmonary Health Symposia, CHHE, NCSU, Raleigh NC
2017 **Invited Seminar Speaker**, Dept. of Biology, North Carolina Agriculture and Technology
2015 **Invited Speaker**, North Carolina Academy of Science Meeting, Raleigh NC

2012	**Presentation Award**, Gordon Conference "Biology of Acute Respiratory Infection"; Ventura, CA
2011	**Invited Seminar Speaker**, Dept. of Biology, North Carolina Central University
2011	**Travel Award**. Mid-Altantic Microbial Pathogenesis Meeting, Wintergreen, VA
2010	**Alumni Scholars Teaching Fellowship**, The New College of Florida
2008-2007	**T32 Virology Training Grant**. Dept. of Microbiology/Immunology, University of North Carolina at Chapel Hill
2008	**Infectious Diseases Scholarship Award.** Keystone Symposia: Cell Biology of Virus Entry, Replication and Pathogenesis. Victoria, British Columbia
2007	**Young Investigator Award.** Conference on Retroviruses and Opportunistic Infections, Los Angeles, CA
2001-2003	NIH Pre-Doctoral Intramural Training Award**, National Institutes of Health**

C. Contributions to Science

I. Working with the envelope of a highly pathogenic HIV-1 strain obtained from a Rapid Progressor patient, I determined the role of the HR-2 domain for enhanced fusogenicity and hence pathogenicity of the virus. These data suggested an important role for fusion inhibitors in controlling highly pathogenic strains, but also potentially yielded molecular targets within the HIV envelope for drug design.

    1. Sivaraman, V, Zhang, L, Meissner, EG, and Su L. 2009. The Heptad Repeat (HR) 2 Domain Is the Major Determinant for Enhanced HIV-1 Fusion and Pathogenicity of A Highly Pathogenic HIV-1 Env. Journal of Virology, 83:11715-25 PMCID 2772666

II. At the cusp of the discovery of plasmacytoid dendritic cells, I took particular interest in their roles in viral infections. I aided in the isolation and purification of these cells, for work with Kaposi Sarcoma virus and performed my own work studying HIV-1 infection. This work contributed to knowledge regarding Type 1 Interferon induction by pDC cells and resulting CD4+ T cell depletion, in a thymic organ model of infection.

    1. West, J, Gregory, Sean, Sivaraman, V, Su, L and Damania, B. 2011. Activation of Plasmacytoid Dendritic Cells by Kaposi's Sarcoma-Associated Herpesvirus. Journal of Virology. 85: 895-904 PMCID 3020034.
    2. Sivaraman, V, Zhang, L, and Su, L. 2011. Type 1 interferon contributes to HIV-1 induced CD4+ T cell depletion in human thymus. Journal of Virology, 85: 9243-6 PMCID 3165786

III. My interests in immunological interaction with microbes led me to focus on cellular interactions of Yersinia pestis within the lung, and role these interactions play on inflammation and disease. I worked closely with

my colleague Dr. Pechous to develop a method for characterizing early cellular interaction of Y. pestis with host immune cells within the lung, and their contributions to pneumonia. These studies led to more in depth analysis of Y. pestis interactions with alveolar macrophages, and a novel means of evasion of host immune signaling by this highly virulent pathogen.

1. Pechous, R, Sivaraman, V, Stasulli, N, Price, P and Goldman, W.E. 2013. Early host targets Yersinia pestis during primary pneumonic plague. PLOS Pathogens. 9(10):e1003679 PMCID 3789773
2. Sivaraman, V, Pechous, R, Stasulli, N, Miao, E, and Goldman, W.E.2015. Yersinia pestis activates both IL-1b and IL-1 Receptor Antagonist to modulate lung inflammation during pneumonic plague. PLOS Pathogens. 11(3):e1004688 PMCID 25781467
3. Pechous, R, Sivaraman, V and Goldman, W.E. 2016. Pneumonic Plague: The Darker Side of Yersinia pestis.Trends in Microbiology 3:190-7: PMCID 26698952

IV. My research interests regarding pulmonary inflammation have turned down a novel path of pulmonary disease exacerbation in the context of adolescent alcohol binge exposure. We hope that our work will shed new light on alcohol priming effects on lung inflammation.

Harris B, Mcalister A, Willoughby T, and Sivaraman V, 2018. Alcohol-Dependent Pulmonary Inflammation: A Role for HMGB-1, Alcohol, doi:10.1016/j.alcohol.2018.09.008

V. My research interests in viral immunology and translational sciences have led to novel work regarding HPV detection and cervical cancer.

1. Kienka, T, Varga, MG, Caves, J, Smith, JS, and Sivaraman, V "Epstein-Barr virus, not human cytomegalovirus, is associated with human papillomavirus-associated cervical lesions among women in North Carolina" (Journal of Medical Virology, 2018) PMCID:PMC6331249
2. De Marais, A, Zhao, Y, Hobbs, M, Sivaraman, V, Brewer, NT, Smith, JSS "Home Self-Collection by Mail to Test For Human Papillomavirus and Sexually Transmitted Infections" (Obstetrics and Gynecology, 2018) PMCID:PMC6249061

D. Additional Information: Research and/or Scholastic Performance

Current Research Support

U54MD012392 (Kumar-PI) 7/1/2019-6/30/2021

RCMI: Center for Health Disparities Research: Health Disparity Associated with Exposure to Traffic-Induced Air Pollutants: Pilot Study

Role: project PI

2U54AA019765 (Cole-PI) 9/1/2015- 8/31/2020

NCCU-CAS Mechanisms of alcohol pathology: a collaborative partnership between NCCU and UNC
Project: Pulmonary Inflammation following Binge-Alcohol Intoxication: Pilot Study
Role: Project PI

Previous Research Support

U54 CA156735 (Richarson-PI) 9/1/2015-8/31/2018

NCCU-LCCC Partnership in Cancer Research

Project: Improving Testing, Triage, and Follow up for Cervical Cancer Screening in Medically Underserved Women

Role: Project co-PI