

**2017 APS CONFERENCE: PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL  
CONSEQUENCES OF SICKLE CELL DISEASE**

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<p style="text-align: center;"><b>Monday November 6, 2017</b></p>	<p style="text-align: center;"><b>Tuesday November 7, 2017</b></p>	<p style="text-align: center;"><b>Wednesday November 8, 2017</b></p>
<p>2:00 PM – 8:00 PM Registration</p> <p>4:00 PM – 5:30 PM <b>1.0 Educational Workshop</b> Bryan Becker</p> <p>6:00 PM – 6:15 PM <b>2.0 Welcome</b> Harvey Luksenburg</p> <p>6:15 PM – 6:45 PM <b>3.0 Data Blitz I</b></p> <p>6:45 PM – 9:00 PM <b>Poster Sessions and Social</b></p> <p>4.0 NeuralCircuits/ Neurovascular Physiology</p> <p>5.0 Sickle Cell Disease Gene Therapy, Gene Editing and Pharmacological Treatment</p> <p>6.0 Small Molecules to Treat Sickle Cell Disease</p> <p>7.0 Coagulation/Thrombosis</p>	<p>8:30 AM – 8:35 AM <b>8.0 Opening Remarks and Announcements</b> Dexter Lee</p> <p>8:35 AM – 10:00 AM <b>9.0 Neural Circuits and Neurovascular Physiology</b> Thomas Coates</p> <p>10:00 AM – 11:00 AM <b>Poster Sessions</b></p> <p>10.0 Renal and Vascular Physiology</p> <p>11.0 Lung Physiology/ Pathophysiology</p> <p>12.0 Red Cell Physiology</p> <p>11:00 AM – 12:30 PM <b>13.0 SCD Gene Therapy, Gene Editing and Pharmacological Treatment</b> David A. Williams</p> <p><b>12:30 PM – 2:00 PM Buffet Lunch</b></p> <p>2:00 PM – 3:30 PM <b>14.0 Cell Therapy</b> Betty Pace</p> <p>4:00 PM – 5:00 PM <b>15.0 Small Molecules to Treat SCD</b> James Engel</p> <p>5:30 PM – 6:00 PM <b>16.0 Data Blitz II</b></p> <p>6:00 PM – 6:30 PM <b>17.0 Howard University President’s Address</b> Wayne A.I. Frederick</p>	<p>8:30 AM – 10:00 AM <b>18.0 Renal and Vascular Physiology</b> Jennifer Pollock</p> <p>10:30 AM – 12:00 PM <b>19.0 Lung Physiology and Pathophysiology</b> Steffen Meiler</p> <p><b>12:00 PM – 1:30 PM Buffet Lunch</b></p> <p><i>Microsoft Surface Pro 4 drawing will be held during today’s lunch!</i></p> <p>1:30 PM – 3:30 PM <b>20.0 Red Cell Physiology</b> Sergei Nekhai</p> <p>4:00 PM – 5:30 PM <b>21.0 Coagulation and Thrombosis</b> Rafal Pawlinski Felicity Gavins</p> <p>5:30 PM – 5:45 PM <b>Awards Presentations</b></p>

2017 APS Conference  
**Physiological and Pathophysiological Consequences of Sickle Cell Disease**  
November 6–8, 2017, Washington, DC

**APS Council**

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**Acknowledgements**

The Conference Organizers and The American Physiological Society gratefully recognize the generous financial support from the following:

**National Heart Lung and Blood Institute, National Institutes of Health**

**Howard University, Center for Sickle Cell Disease**

**Imara**

**Prolong Pharmaceuticals**

**Global Blood Therapeutics**

**Location:**

The 2017 APS Conference: Physiological and Pathophysiological Consequences of Sickle Cell Disease is held at the Embassy Suites DC Convention Center Hotel, 900 10<sup>th</sup> Street, NW, Washington, DC 20001. Telephone: 1-202-739-2001.

**Onsite Registration Hours:**

Mon., November 6..... 2:00 PM – 8:00 PM  
 Tues. November 7..... 7:00 AM – 5:00 PM  
 Wed., November 8..... 7:30 AM – 5:00 PM

**Student Registration:**

Any student member or regularly matriculated student working toward a degree in one of the biomedical sciences is eligible to register at the student fee. Nonmember postdoctoral fellows, hospital residents and interns, and laboratory technicians do not qualify as students. Non-member students who register onsite must provide a valid university student ID card. APS student members should present their current APS membership card indicating their student category status.

**Postdoctoral Registration:**

Any person who has received a Ph.D. degree in physiology or related field, within five years of the conference start date, as attested to by the department head is eligible to register at the postdoctoral fee. **A statement signed by the department head must accompany the registration form and remittance when registering.**

**Included in your Registration:**

Your registration to this conference includes entry into all oral and poster scientific sessions, buffet lunches on Tuesday and Wednesday and a program book. **Registration is nontransferable.** You must pay the entire fee regardless of the number of sessions/events you attend. Guests of attendees are not permitted in the scientific sessions, opening reception or conference breaks and social events.

**Press Registration:**

Press badges will be issued at the Conference Registration desk to members of the working press and freelance writers bearing a letter of assignment from an editor. Representatives of allied fields (public relations, public affairs, etc.) must register as nonmembers.

**Photograph/Video Recording:**

Photo or video capture of any scientific presentation in whole or part is expressly prohibited. Recording or taking photography of another person without their explicit permission is prohibited.

***Individuals observed photographing or videotaping any presentation, in whole or part, will be asked to leave the conference immediately, forfeiting the registration fee.***

**Code of Conduct:**

APS is committed to providing a safe, productive and welcoming environment for all conference participants and staff. All participants including, but not limited to, attendees, speakers, volunteers, APS staff, hotel staff, service providers and others are expected to abide by the APS Conference Code of Conduct which maintains that all individuals should: be treated with respect and consideration, valuing a diversity of views and opinions; be considerate, respectful and collaborative; communicate openly and with respect, critiquing ideas rather than individuals; avoid personal attacks; be mindful of your surroundings and fellow participants; and, be respectful of the rules APS and the conference venue. Contact the APS staff at the Conference Registration Desk if you notice a dangerous situation, someone in distress, or violations of this Code of Conduct.

**Program Objective:**

The SCD conference seeks to provide a premier forum for physiologists and clinicians to present and discuss their research findings about the effects of SCD on physiology, with the intent that these discoveries will help researchers and clinicians to better understand the disease and its consequences and to devise strategies that prevent and better treat SCD-related pathophysiology. The meeting will support the momentum created by the NHLBI Excellence in Hemoglobinopathies Research Award (EHRA) program to aid in developing studies that will accelerate high-impact multi-disciplinary basic and translational research in the hemoglobinopathies and facilitate maximal collaborations among basic and translational scientists and clinical hematologists located throughout the US.

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**MONDAY, NOVEMBER 6, 2017**
**1.0 EDUCATIONAL WORKSHOP**

Mon., 4:00–5:30 PM, Capital Ballroom A, Lower Level

Chair: **Bryan Becker**, *Univ. of Alabama at Birmingham*

**2.0 WELCOME**

Mon., 6:00–6:15 PM, Capital Ballroom A, Lower Level

**2.1 Harvey Luksenburg**, *NHLBI/NIH*

**3.0 DATA BLITZ I**

Mon., 6:15–6:45 PM, Capital Ballroom A, Lower Level

Chair: **Felicity Gavins**, *LSU Hlth. Sci. Ctr.*

**Crystal Taylor**, *Univ. of Alabama at Birmingham*

*Oral presentations selected from volunteered abstracts.*

**4.0 POSTER SESSION: NEURAL CIRCUITS/NEUROVASCULAR PHYSIOLOGY**

Mon., 6:45–9:00 PM, Capital Ballroom C/D, Lower Level

Board #

- |    |            |   |
|----|------------|---|
| 1  | <b>4.1</b> | Neural Network Analysis of Sickle Cell Disease Patients using Graph Theory. <b>Michelle Case, Huishi Zhang, Yvonne Datta, Stephen Nelson, Kalpna Gupta, Bin He.</b> <i>Univ. of Minnesota, Children's Hospitals and Clinics of Minnesota.</i>   |
| 2  | <b>4.2</b> | EEG Classification of Sickle Cell Patients and Controls using EEG Power during Resting State. <b>Michelle Case, Vishal Vijayakumar, Huishi Zhang, Yvonne Datta, Stephen Nelson, Karim Sadak, Kalpna Gupta, Bin He.</b> <i>Univ. of Minnesota, Children's Hospitals and Clinics of Minnesota.</i>  |
| 3  | <b>4.3</b> | Deriving Phenotypic Markers of the Peripheral Vasoconstriction Response to Heat-induced Pain in Sickle Cell Disease <b>Patjanaporn Chalacheva, Thomas Coates, Michael Khoo.</b> <i>Univ. of Southern California, Children's Hosp. Los Angeles.</i>  |
| 4  | <b>4.4</b> | Microvascular Perfusion is a Physiologic Biomarker of Mental Stress and Fear of Pain in Sickle Cell Subjects and Normal Controls <b>Payal Shah, M. Khaleel, W. Thuptimjang, S. Veluswamy, J. Sunwoo, P. Chalacheva, R. Kato, J. Detterich, J. Wood, J. Tsao, L. Zeltzer, R. Sposto, M. Khoo, T. Coates.</b> <i>Children's Hosp. Los Angeles, Univ. of Southern California.</i>  |
| 4A | <b>4.5</b> | Increased Expression of Cellular Stress Protein, $\alpha$ -synuclein within Normoxic and Hypoxic Brains of SCD Mice: Implications for Neurocognitive Dysfunction in Sickle Cell Disease Patients. <b>Fitz Tavenier, Mariam Hamid, Gratiana Fu, Rebekah Urbonya, Shuaiying Cui, Amrita Pawar, Aaran Varatharajan, Andrew Campbell.</b> <i>Univ. of Michigan, Boston Univ. Sch. of Med., Childrens National Med. Ctr.</i> |

## DAILY SCHEDULE

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### 5.0 POSTER SESSION: SICKLE CELL DISEASE GENE THERAPY, GENE EDITING AND PHARMACOLOGICAL TREATMENT

Mon., 6:45–9:00 PM, Capital Ballroom C/D, Lower Level

Board #

- 5      **5.1**      A Study of the Geographic Distribution and associated Risk factors of Leg Ulcers within an International Cohort of Sickle Cell Disease Patients: The CASIRE Group Analysis. **Charles Antwi-Boasiako, W. Zempsky, B. Inusa, C. J. Strunk, E. V. Asare, D. A. Antwi, D. B. Gifty, S. Perrotta, O. Rodrigues, C. M. Piccone, A. Rivers, D. Manwani, I. Tartaglione, R. Colombatti, F. Sey, B. Andemariam, A. D. Campbell.** *Univ. of Ghana, Connecticut Children's Med. Ctr., Guys St Thomas Hosp., ProMedica Toledo Children's Hosp., Ghana Inst. of Clinical Genetics, Università degli Studi della Campania "Luigi Vanvitelli," Napoli, Italy., Case Western Reserve Univ., Univ. of Illinois at Chicago.*
- 6      **5.2**      Erythrocyte Hypomagnesemia in Patients with Sickle Cell Anemia is Associated with Increased Frequency of Vaso-Occlusive Crises. **Samir Ballas** *Thomas Jefferson Univ.*
- 7      **5.3**      Haptoglobin and Hemopexin Inhibit Inflammation and Vaso-Occlusion in Murine Sickle Cell Disease through Rapid Induction of Heme Oxygenase-1 and CO Production. **John D Belcher, Chunsheng Chen, Julia Nguyen, Fuad Abdulla, Ping Zhang, Hao Nguyen, Phong Nguyen, Trevor Killeen, Tran Vo, Sylvia M Miescher, Nathan Brinkman, Gregory M Vercellotti.** *Univ. of Minnesota, CSL Behring AG*
- 8      **5.4**      A Macrophage-Stimulating Protein Receptor Inhibitor Causes a Greater Reduction in Interferon-Gamma Expression in the Heart of Female Mice with Sickle Cell Disease. **Franklin Duruobasa, Alfia Khaibullina, Zenaide Quezado, Marina Jerebtsova, Dexter L. Lee** *Howard Univ., NIH Clinical Ctr.*
- 9      **5.5**      Effect of the LSD1 Inhibitor RN-1 on HbF, Gene Expression, and Erythroid Differentiation. **Donald Lavelle, Kestis Vaitkus, Vinzon Ibanez, Maria Armila Ruiz, Ramasamy Jagadeeswaran, Angela Rivers.** *Univ. of Illinois at Chicago, Jesse Brown VA Med. Ctr.*
- 10     **5.6**      RNA Trans-splicing Repair of Endogenous  $\beta$ -Globin pre-mRNA in Human Erythroid Cells. **Lloyd Mitchell, Kareem Washington, Naoya Uchida, Brian Mozer, John Tisdale.** *Howard Univ., NHLBI/NIH, RetroTherapy, Rockville, MD.*
- 11     **5.7**      The Case of Eradication of Sickle Cell Anemia Deaths in Africa. **Cornelius Nwora.** *Texas Southern Univ.*
- 12     **5.8**      GBT440 improves rheological properties of sickle cell blood by increasing hemoglobin oxygen affinity. **Mira Patel, Kobe Dufu, Donna Oksenberg, Pedro Cabrales.** *Global Blood Therapeutics, UCSD.*
- 13     **5.9**      Enucleation and Beta-Globin Expression in Induced Red Blood Cells: a Platform to Model Sickle Cell Anemia. **Tolulope Rosanwo, Melissa A Kinney, Linda T. Vo, Vanessa Lundin, George Q Daley.** *Howard Hughes Med. Inst., Boston Children's Hosp., Harvard Med. Sch.*
- 14     **5.10**     Humanized Sickle Mice Are Sensitive to Hypoxia/Ischemia-Induced Stroke, but Respond to Tissue Plasminogen Activator Treatment. **Yu-Yo Sun, Jolly Lee, Henry Huang, Mary B. Wagner, Clinton H. Joiner, David R. Archer, Chia-Yi Kuan.** *Emory Univ. Sch. of Med.*
- 15     **5.11**     Genetic Testing for Alpha Thalassemia Using Droplet Digital PCR **Yu Yang, Mary Jackson, Parker Ruhl, Hans Ackerman** *NHLB/NIH.*

- 16      **5.12**      Development of Novel LSD1 Inhibitors as a Strategy to Treat SCD. **Lei Yu, Jearawiriyapaisarn Natee, Wongkumool Wasinee, Andrew White, James Douglas Engel.** *Univ. of Michigan Med. Sch., Univ. of Michigan College of Pharmacy*

**6.0      POSTER SESSION: SMALL MOLECULES TO TREAT SICKLE CELL DISEASE**

Mon., 6:45–9:00 PM, Capital Ballroom C/D, Lower Level

Board #

- 17      **6.1**      Treatment of the First Sickle Cell Disease Patients with Antagonist of N-Methyl D-Aspartate Receptor Memantine: Biological Outcome of the MemSID Trial. **Anna Bogdanova, Asya Makhro, Elena Seiler, Nikolay Bogdanov, Inga Hegemann, Jeroen Goede, Max Gassmann.** *Univ. of Zurich, Univ. Hospital Zurich, Winterthur Cantonal Hospital, Switzerland.*

**7.0      POSTER SESSION: COAGULATION/THROMBOSIS**

Mon., 6:45–9:00 PM, Capital Ballroom C/D, Lower Level

Board #

- 18      **7.1**      Assay-dependent Results of ADAMTS13 Activity in Sickle Cell Disease. **Ryan Hunt, Ayla Yalamanoglu, Kathryn Hassell, Katie Redinius, David Irwin, Paul Buehler, Chava Kimchi-Sarfaty.** *FDA, Silver Spring, MD, Univ. of Colorado Anschutz Med. Ctr., Aurora.*
- 19      **7.2**      Accelerated Venous Thrombosis with Enhanced Fibrin Deposition and Platelet Accumulation in Sickle Cell Mice. **Erica Sparkenbaugh, Brian Cooley, Camille Faes, Nigel Key, Rafal Pawlinski.** *Univ. of North Carolina at Chapel Hill.*

**TUESDAY, NOVEMBER 7, 2017**

**8.0      OPENING REMARKS AND ANNOUNCEMENTS**

Tues., 8:30–8:35 AM, Capital Ballroom A, Lower Level

Chair: **Dexter Lee,** *Howard Univ.*

**9.0      NEURAL CIRCUITS AND NEUROVASCULAR PHYSIOLOGY**

Tues., 8:35–10:00 AM, Capital Ballroom A, Lower Level

Chair: **Thomas Coates,** *Univ. of Southern California*

- 8:35 AM      **9.1**      Targeting Pain at its Source in Sickle Cell Disease. **Kalpna Gupta.** *Univ. of Minnesota.*
- 9:15 AM      **9.2**      Endothelin Type A Receptors Mediate Sickle Cell Disease-Associated Pain by Up-Regulating Nav1.8 in Primary Sensory Neurons. **Brianna Lutz.** *Rutgers New Jersey Med. Sch.*
- 9:30 AM      **9.3**      Prostaglandin Glycerol Esters Contribute to Hyperalgesia in a Humanized Mouse Model of Sickle Cell Disease. **Donald Simone.** *Univ. of Minnesota.*

## DAILY SCHEDULE

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9:45 AM **9.4** Diminished Cerebral Oxygen Extraction in Anemic Subjects Using Venous MRI Oximetry: Is TRUST Oximetry Calibration Reliable in Sickle Cell Disease? **Adam Bush, Stanford Univ.**

### 10.0 POSTER SESSION: RENAL AND VASCULAR PHYSIOLOGY

Tues., 10:00–11:00 AM, Capital Ballroom C/D, Lower Level

Board #

- 1 **10.1** Activation of Renal Heparin Expression in Sickle Cell Disease Mouse Model. **Nowah Afangbedji, Guelaguetza Vazquez-Meves, Namita Kumari, Charlee Mclean, Naveen Ghuman, Alfia Khaibullina, Zenaide M. N. Quezado, Sergei Nekhai, Marina Jerebtsova** *Howard Univ., NIH Clinical Ctr.*
- 2 **10.2** Hemoglobin Inhibits Uptake of Filtered Proteins by Proximal Tubule Cells: Implications For Sickle Cell Disease. **Megan L. Eshbach, Amandeep Kaur, Youssef Rbaibi, Yash Agarwal, Qiangmin Zhang, Thomas D. Nolin, Jesús Tejero, Ora A. Weisz.** *Univ. of Pittsburgh Sch. of Med.*
- 3 **10.3** Regulation of Renal Heparin in Sickle Cell Disease Mice. **Nowah Afangbedji, Guelaguetza Vazquez-Meves, Zenaide Quezado, Namita Kumari, Alfia Khaibullina, Sergei Nekhai, Marina Jerebtsova.** *Howard Univ. Ctr.for Sickle Cell Disease, Howard Univ., Children's National Health System.*
- 4 **10.4** Which Alpha Globin Gene Is Primarily Expressed in the Vascular Endothelium? **Steven Brooks, Dongying Ma, Yu Yang, Hans Ackerman.** *NHLBI/NIH.*
- 5 **10.5** Endurance Training Does Not Correct Metabolic Abnormalities Related to Ischemia - Reperfusion in Sickle Cell Disease Mice. **Benjamin Chatel, Laurent Messonnier, Christophe Vilmen, Monique Bernard, David Bendahan.** *Aix-Marseille Univ., Univ. Savoie Mont Blanc, France.*
- 6 **10.6** Transient Desaturation Challenge Reveals White Matter Microvascular Disease in Patients with Sickle Cell Disease. **Julie Coloigner, Chau Vu, Adam Bush, SoYoung Choi, Thomas Coates, John Wood.** *IRISA, Rennes France, Univ. of Southern California.*
- 7 **10.7** Transfusion Therapy Improves Multilevel Vascular Dysfunction in Sickle Cell Disease: Are Multiple Levels of the Vasculature Pathophysiologically Linked? **Jon Detterich, Roberta Kato, Michael Khoo, Adam Bush, Thomas Coates, John Wood.** *Children's Hosp. Los Angeles, Univ. of Southern California, Stanford Univ.*
- 8 **10.8** Exercise (In)Tolerance in Sickle Cell Disease: Potential Disruptive Role of Free Hemoglobin on Skeletal Muscle Oxygen Delivery/Utilization Matching and Functional Capacity. **Scott Ferguson, Paul Buehler, Kurt Stenmark, David Irwin.** *Univ. of Colorado, Denver, FDA, Silver Spring, MD.*
- 9 **10.9** Associations Between Cardiorespiratory Fitness and Arterial Function in Adolescents with Sickle Cell Anemia. **Garett Griffith, Nicholas Evanoff, Aaron Kelly, Donald Dengel, Bo Fernhall, Robert Liem.** *Univ. of Illinois at Chicago, Univ. of Minnesota, Ann & Robert H. Lurie Children's Hospital of Chicago.*
- 10 **10.10** Estimation of GFR in Adult Patients with Sickle Cell Disease: Serum Creatinine and Cystatin-C Based Estimation Equations Over-Estimate and Are Poorly Predictive of True GFR. **Antonio Guasch, Sasikala Selvaraj, Mitzi Near, James Eckman.** *Emory Univ.*



- 11      **10.11**    Circulating Exosomes from Patients with Sickle Cell Regulate Pathways of Inflammation and Endothelial Integrity. **Gabrielle Lapping-Carr, Abdelnaby Khalyfa, Joanna Gemel, Chunling Zhang, Radhika Peddinti, Eric C. Beyer, David Gozal.** *Univ. of Chicago, Comer Children's Hospital.*
- 12      **10.12**    Identification of Urinary HGFL Protein as a Potential Marker for the Development of Chronic Kidney Disease in Sickle Cell Disease Patients. **Xionghao Lin, Santosh L. Saraf, Simran Soni, Nowah Afangbedji, Victor R. Gordeuk, Marina Jerebtsova, Sergei Nekhai.** *Howard Univ., Univ. of Illinois at Chicago.*
- 13      **10.13**    The Plasma Proteome of Sickle Cell Pain Crisis. **Dongying Ma, Sajni Patel, Angel Aponte, Allison Ikeda, Marjan Gucek, Hans Ackerman.** *NHLBI/NIH.*
- 14      **10.14**    Urine Proteomic Analysis Identifies Ceruloplasmin as a Biomarker of Chronic Kidney Disease in Sickle Cell Disease Patients. **Sergei Nekhai, Santosh Saraf, Xionghao Lin, Gillian Lee, Elena Adjei, Namita Kumari, Nowah Afangbedji, Charlee Mclean, Victor Gordeuk, Marina Jerebtsova.** *Howard Univ., Univ. of Illinois at Chicago.*
- 15      **10.15**    Effects of Sickle Cell Disease on the Right Ventricle and Pulmonary Vasculature. **David Schreier, Diana Tabima, Tim Hacker, Naomi Chesler.** *Univ. of Wisconsin-Madison.*
- 16      **10.16**    Urinary Oroscomucoid Concentration Correlates with Chronic Kidney Disease in Sickle Cell Disease Patients. **Simran Soni, Santosh Saraf, Nowah Afangbedji, Xionghao Lin, Victor Gordeuk, Marina Jerebtsova, Sergei Nekhai.** *Howard Univ., Univ. of Illinois at Chicago.*
- 17      **10.17**    Heme and Free Iron-Mediated Oxidation of Plasma Lipids in Sickle Cell Disease Patients Undergoing Regular Exchange Blood Transfusion. **Ayla Yalamanoglu, Jeremy Deuel, Jin Hyen Baek, Ryan Hunt, Kathryn Hassell, Katie Redinius, David Irwin, Dominik Schaer, Paul Buehler.** *FDA, Silver Spring, MD, Univ. Hosp. of Zurich, Switzerland, Univ. of Colorado.*

## 11.0      POSTER SESSION: LUNG PHYSIOLOGY/PATHOPHYSIOLOGY

Tues., 8:00–5:30 PM, Capital Ballroom C/D, Lower Level

Board #

- 18      **11.1**      Exposure to Moderate Altitude Enhances Pulmonary Vascular Disease in Berkley Sickle Cell Mice. **David Irwin, Scott Ferguson, Ayla Yalamanoglu, Jin Baek, Katherine Redinius, Kathryn Hassell, Rachell Nuss.** *Univ. of Colorado, Denver, FDA, Silver Spring, MD.*
- 19      **11.2**      Scoping Review of the Literature on Sickle Cell Lung Disease Across the Lifespan. **A. Parker Ruhl, S. Christy Sadreameli, Nancy Terry, Robyn Cohen, Elizabeth Klings.** *NIH Library, Johns Hopkins Univ. Sch. of Med., Boston Univ. Sch. of Med.*

## 12.0      POSTER SESSION: RED CELL PHYSIOLOGY

Tues., 10:00–11:00 AM, Capital Ballroom C/D, Lower Level

- 20      **12.1**      How Does Iron Deficiency Reduce Intracellular Hemoglobin in Mice? **Majed Almashjary, Steven Brooks, Hans Ackerman.** *NHLBI/NIH.*
- 21      **12.2**      Tr4 Haploinsufficiency Results in Decreased Proliferation and Maturation During Erythropoiesis. **Mary Lee, Osamu Tanabe, Lihong Shi, Natee Jearawiriyapaisarn, Daniel Lucas-Alcaraz, James Douglas Engel** *Univ. of Michigan Med. Sch., Tohoku Med. Megabank, Japan, Tohoku Univ., Japan, Inst. of Hematology and Blood Diseases Hosp., Beijing, China,*

## DAILY SCHEDULE

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*Chinese Academy of Med. Sci. and Peking Union Med. Col., Beijing, China, Mahidol Univ., Salaya, Thailand.*

- 22      **12.3**      Haptoglobin Genotype is Associated with Increased Morbidity in Adults with Sickle Cell Disease. **Shaina M. Willen, Joel B. McNeil, Mark Rodeghier, Julie A. Bastarache, Ciara M. Shaver, Shruti Chaturvedi, Michael R. DeBaun, Lorraine B. Ware.** *Vanderbilt Univ. Med. Ctr., Rodeghier Consultants, Chicago, Johns Hopkins Hosp.*
- 23      **12.4**      Annexins are Targeted by Heme During Hemolysis and Fail to Antagonize Phosphatidylserine in Sickle Cell Disease. **Sihem Sadoudi, Sylvai Le Jeune, Dominique Charue, Lubka Roumenina, Laurent Kiger, Jean-Christophe Lambry, Jean-Benoit Arlet, Francois Lionnet, Chantal M. Boulanger, Olivier Blanc-Brude.** *INSERM, Univ. of Paris-Descartes, Hosp. Avicenne, Paris, INSERM, Univ. Pierre and Marie Curie, Hosp. Européen Gorges Pompidou, Hosp. de Paris, France.*

### **13.0      SCD GENE THERAPY, GENE EDITING AND PHARMACOLOGICAL TREATMENT**

Tues., 11:00–12:30 PM, Capital Ballroom A, Lower Level

Chair: **David A. Williams, Harvard Stem Cell Inst.**

- 11:00 AM      **13.1**      Gene Therapy for Hemoglobinopathies: The Challenge to Find a Cure. **Giulianna Ferrari.** *San Raffaele Telethon Inst. for Gene Therapy, Milan, Italy.*
- 11:30 AM      **13.2**      Combined Hydroxyurea and ETA Receptor Blockade Reduces Renal Injury in the Humanized Sickle Cell Mouse. **Crystal Taylor.** *Univ. of Alabama at Birmingham.*
- 11:45 AM      **13.3**      NRF2 Gene Knockout Exacerbates Tissue Pathophysiology in the Sickle Cell Disease Transgenic Mouse Model. **Xingguo Zhu.** *Augusta Univ.*
- 12:00 PM      **13.4**      Discovery of Pharmacologic Fetal Hemoglobin Inducing Agents for Sickle Cell Disease. **Betty Pace.** *Augusta Univ.*

### **14.0      CELL THERAPY**

Tues., 2:00–3:30 PM, Capital Ballroom A, Lower Level

Chair: **Betty Pace, Augusta Univ.**

- 2:00 PM      **14.1**      Control of HbF Silencing: Implications for Genetic and Pharmacologic Induction of HbF for Therapy. **Stuart Orkin.** *Harvard Univ.*
- 2:30 PM      **14.2**      Gut Microbiome Analysis Reveals Major Dysbiosis in Sickle Cell Disease Patients with a Prevalence of Veillonella Strains. **Hassan Brim.** *Howard Univ.*

- 2:45 PM **14.3** The Thermodynamic Hypothesis of Sickle Cell Disease Pathophysiology.  
**Constance Tom Noguchi.** *NIDDK/NIH.*
- 3:00 PM **14.4** CRISPR/Cas9 Enhanced Sickle Gene Correction in Human and Mouse Hematopoietic Stem Cells.  
**Tim Townes.** *Univ. of Alabama at Birmingham*
- 15.0 SMALL MOLECULES TO TREAT SCD**  
Tues., 4:00–5:30 PM, Capital Ballroom A, Lower Level  
Chair: **James Engel,** *Univ. of Michigan*
- 4:00 PM **15.1** KEAP1-NRF2 Antioxidant Response System and Sickle Cell Anemia.  
**Masayuki Yamamoto.** *Tohoku Univ., Japan.*
- 4:30 PM **15.2** Oral Tetrahydrouridine and Decitabine for Non-Cytotoxic Epigenetic Modification of Sickle Cell Disease: A Randomized Phase 1/2 Study.  
**Yogen Sauntharajah.** *Cleveland Clinic.*
- 5:00 PM **15.3** RN-1, an LSD-1 Inhibitor, Induces Hb F in the Baboon (*P. anubis*) and Reduces Mitochondria-containing RBC in a SCD Mouse Model.  
**Angela Rivers.** *Univ. of Illinois.*
- 16.0 DATA BLITZ II**  
Tues., 5:30–6:00 PM, Capital Ballroom A, Lower Level  
Chair: **Malgorzata Kaszta,** *Univ. of Alabama at Birmingham*  
**Brandon Fox,** *Univ. of Alabama at Birmingham*
- Oral presentations selected from volunteered abstracts.*
- 17.0 HOWARD UNIVERSITY PRESIDENT’S ADDRESS**  
Tues., 6:00–6:30 PM, Capital Ballroom A, Lower Level  
**17.1 Wayne A.I. Frederick,** *Howard Univ.*

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**WEDNESDAY, NOVEMBER 8, 2017**


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- 18.0 RENAL AND VASCULAR PHYSIOLOGY**  
Wed., 8:30–10:00 AM, Capital Ballroom A, Lower Level  
Chair: **Jennifer Pollock.** *Univ. of Alabama at Birmingham*
- 8:30 AM **18.1** Sickle Cell Disease: When Endothelin Becomes a Nephrotoxic and Proinflammatory Cytokine  
**Pierre-Louis Tharaux** *INSERM-U970*

## DAILY SCHEDULE

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- 9:00 AM **18.2** Resistance Arteries of Humanized Sickle Cell Disease Mice Display Similar Sensitivity to  $\alpha$ 1-adrenergic and Endothelin-1 Vasoconstriction.  
**Brandon M. Fox.** *Univ. of Alabama at Birmingham*
- 9:15 AM **18.3** Impaired Post-Ischemic Neovascularization in Sickle Cell Disease.  
**Derick Okwan,** *Emory Univ.*
- 9:30 AM **18.4** Role of Macrophage Stimulating Protein 1 (MSP1) in the Development of Endothelial Injury in Sickle Cell Disease.  
**Marina Jerebtsova.** *Howard Univ.*
- 9:45 AM **18.5** Iron accumulation in the Kidney: Potential New Role For Endothelin System.  
**Malgorzata Kasztan.** *Univ. of Alabama at Birmingham*

### 19.0 LUNG PHYSIOLOGY AND PATHOPHYSIOLOGY

Wed., 10:30–12:00 PM, Capital Ballroom A, Lower Level

Chair: **Steffen Meiler,** *Med. Coll. of Georgia*

- 10:30 AM **19.1** Molecular Pathogenesis of Acute Chest Syndrome.  
**Solomon Ofori-Acquah.** *Univ. of Pittsburgh*
- 11:00 AM **19.2** Differentially Expressed lncRNAs in Lungs and Plasma Exosomes of Sickle Cell Mice.  
**Bum-Yong Kang,** *Atlanta Veterans Affairs and Emory Univ. Med. Ctrs.*
- 11:15 AM **19.3** Longitudinal Changes in Diffuse Myocardial Fibrosis in Sickle Cell Anemia.  
**Charles Quinn.** *Cincinnati Children's Hosp. Med. Ctr.*
- 11:30 AM **19.4** Mechanisms of Hypoxia and Its Impact in Sickle Cell Disease  
**Elizabeth S. Klings.** *Boston Univ. Sch. of Med.*

### 20.0 RED CELL PHYSIOLOGY

Wed., 1:30–3:30 PM, Capital Ballroom A, Lower Level

Chair: **Sergei Nekhai,** *Howard Univ. Hosp.*

- 1:30 PM **20.1** Developmental Regulation of Erythroid Self-renewal  
**James Palis.** *Univ. of Rochester Med. Ctr.*
- 2:00 PM **20.2** Pathobiology of Sickle Red Cells: Implications for Pathophysiology of Sickle Cell Disease.  
**Mohandas Narla.** *New York Blood Ctr.*
- 2:30 PM **20.3** Red Blood Cell Rheology and Vascular Dysfunction in Sickle Cell Disease  
**Philippe Connes.** *Univ. of Lyon, France.*
- 2:45 PM **20.4** The Effect of Hemin on Human Red Blood Cell Transformation  
**Elena Senechenkova.** *LSU Hlth. Sci. Ctr.*
- 3:00 PM **20.5** Molecular Resolution of an Active Vaso-Occlusive Crisis in VOC Patients Treated with SANGUINATE™.  
**Ronald Jubin.** *Prolong Pharmaceuticals, South Plainfield, NJ.*
- 3:15 PM **20.6** Intrinsic Cellular Factors Modulate HIV-1 Replication In Sickle Cell Disease.  
**Namita Kumari.** *Howard Univ.*

**21.0 COAGULATION AND THROMBOSIS**

Wed., 4:00–5:30 PM, Capital Ballroom A, Lower Level

Chair: **Rafal Pawlinski**, *Univ. of North Carolina*  
**Felicity Gavins**, *Louisiana State Univ. Hlth. Sci. Ctr.*

- 4:00 PM **21.1** De-clotting Sickle Cell Disease  
**Rafal Pawlinski**. *Univ. of North Carolina*
- 4:30 PM **21.2** Sickle Red Blood Cells Alter Clot Retraction in Mice and Humans.  
**Erica Sparkenbaugh**. *Univ. of North Carolina at Chapel Hill.*
- 4:50 PM **21.3** Sickle Cell Anemia: Hyper- or Hypofibrinolysis state?  
**Anton Ilich**. *Univ. of North Carolina at Chapel Hill.*
- 5:10 PM **21.4** Annexin A1 affords protection in Sickle Cell Disease.  
**Junaid Ansari**. *LSU Hlth. Sci. Ctr.*



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#### 4.0 Poster Session: Neural Circuits/Neurovascular Physiology

##### 4.1

#### NEURAL NETWORK ANALYSIS OF SICKLE CELL DISEASE PATIENTS USING GRAPH THEORY

Michelle Case<sup>1</sup>, Huishi Zhang<sup>1</sup>, Yvonne Datta<sup>2</sup>, Stephen Nelson<sup>3</sup>, Kalpna Gupta<sup>2</sup>, Bin He<sup>1</sup>

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**Background:** Sickle cell disease (SCD) is a lifelong disease that negatively impacts patients' lives. It is not well understood how the long term effects of SCD impacts the brain and the natural dynamics of neural connectivity. In order to gain a better understanding of alterations in neural behavior of SCD patients, we recorded resting state activity of both patients and healthy controls using functional magnetic resonance imaging (fMRI), an imaging modality that measure brain activity. Assessing the natural dynamic of patients and controls was done on a network level using graph theory.

**Methods:** A total of 15 SCD patients and 15 healthy controls were recruited for the study. Resting state fMRI data was recorded on a 3T scanner where sessions lasted for 8 minutes. The CONN toolbox was used to create a connectivity matrix for each subject. Each matrix contains the correlation coefficient between all 136 brain regions used in this study. The connectivity matrices were used to make undirected graphs, which consist of nodes and edges. The graphs were assessed across different sparsity values ranging between 5% and 60%. The characteristic path length, node density, clustering coefficient, global efficiency, and small worldness were calculated to assess the network of each subject.

**Results:** Based on medical history, 2 groups of patients [i] with more severe and [ii] less severe symptoms were examined. The patients with a more severe case of SCD exhibited lower small world values than controls ( $p < 0.01$ ). The more severe patients also had significantly different clustering coefficient values ( $p < 0.03$ ) and global efficiency ( $p < 0.01$ ). The small world value of patients and clustering coefficient of patients also showed a trend associated with the number of hospitalizations in the past 2 years.

**Conclusions:** These results suggest that graph theory can be used as a tool to assess global network connectivity between patients and controls. It was found that patients with more severe SCD tend to have lower small world values and have higher global efficiency. The small world value represents short path lengths and a numerous amount of clusters in the graph. A decrease in small

worldness indicates that patients with more severe symptoms lose the natural organization of neural networks. Graph theory could be utilized as a tool to measure the progression of SCD on neural activity through long term follow up. This work was supported by NIH U01-HL117664 and NSF IGERT DGE-1069104.

##### 4.2

#### EEG CLASSIFICATION OF SICKLE CELL PATIENTS AND CONTROLS USING EEG POWER DURING RESTING STATE

Michelle Case<sup>1</sup>, Vishal Vijayakumar<sup>1</sup>, Huishi Zhang<sup>1</sup>, Yvonne Datta<sup>2</sup>, Stephen Nelson<sup>3</sup>, Karim Sadak<sup>4</sup>, Kalpna Gupta<sup>2</sup>, Bin He<sup>1</sup>

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**Background:** A genetic mutation causes sickle cell disease (SCD) which causes red blood cells to mutate and form a sickle shape. This mutation causes many symptoms including ischemia, pain, inflammation and other comorbidities. Recently, neuroimaging techniques have been applied to patients to gain a better understanding of how SCD affects the natural neural dynamics of patients. The goals of this study were 1) to use electroencephalography (EEG) to differentiate between SCD patients and healthy controls using EEG power and 2) to differentiate two levels of severity in patients. Classifying between patients and controls and classifying severity in patients are important steps to objectively finding neural biomarkers of SCD.

**Method:** Resting state EEG was recorded in 20 patients and 14 controls. EEG sessions lasted 10 minutes and subjects had their eyes open during recordings. The EEG spectral power was found for common frequency bands. The theta and beta bands were found to be significantly different between patients and controls and were used for classification. Two classifiers were implemented, one to discriminate between patients and controls, and the other to discriminate between more severe patients and less severe patients. Patients were grouped as more severe if the number of hospital visits and emergency department visits over the past two years were greater than eight. Several models of each classifier were implemented utilizing independent component analysis to ensure all electrode data was incorporated.

**Results:** The receiver operating characteristic curve was found to determine the performance of the classifiers. The average area under the curve (AUC) for determining

patients from controls was 0.84, where a value of 1 is perfect classification. The best performing model had an AUC of 0.97. The average AUC for determining patient severity was 0.76, and the best performing model had an AUC of 0.86.

**Conclusions:** The classification results show that patients can be differentiated from controls using EEG power. This shows that theta and beta band power are relevant and applicable to SCD patients. Furthermore, these bands were able to distinguish between disease severity in patients indicating that these EEG bands could be used as a potential biomarker of SCD severity. An objective way to measure cognitive health could help improve treatment in patients. This work was supported by NIH U01-HL117664 and NSF IGERT DGE-1069104.

#### 4.3

##### **MICROVASCULAR PERFUSION IS A PHYSIOLOGIC BIOMARKER OF MENTAL STRESS AND FEAR OF PAIN IN SICKLE CELL SUBJECTS AND NORMAL CONTROLS**

P Shah<sup>1</sup>, M Khaleel<sup>1</sup>, W Thuptimdang<sup>2</sup>, S Veluswamy<sup>1</sup>, J Sunwoo<sup>2</sup>, P Chalacheva<sup>2</sup>, R Kato<sup>1</sup>, J Detterich<sup>1</sup>, J Wood<sup>1</sup>, J Tsao<sup>3</sup>, L Zeltzer<sup>3</sup>, R Sposto<sup>1</sup>, M Khoo<sup>2</sup>, T Coates<sup>1</sup>

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**INTRODUCTION:** Sickle cell disease (SCD) is a genetic disorder in which abnormal hemoglobin-S polymerizes upon deoxygenation and forms rigid sickle shaped red blood cells that can occlude the microvasculature leading to the sudden onset of painful vaso-occlusive episodes (VOC). Thus any trigger that decreases microvascular blood flow (MBF) can promote vaso-occlusion and progression to VOC. We previously showed that SCD subjects have an augmented autonomic mediated vasoconstriction response to sigh and pain. Our subjects also showed a decrease in MBF when instructed of upcoming pain, suggesting that neural induced vasoconstriction might be the physiologic link between mental stress triggers and vaso-occlusion.

**OBJECTIVES:** To study the effect of mental stress on autonomic parameters – MBF and heart rate variability (R to R interval; RRI) in SCD.

**METHODS:** 19 SCD and 16 controls performed two standard mental stress tasks with graded levels of difficulty (N-back and Stroop). We also exposed them to a pain anticipation task where they were instructed about upcoming pain, but no pain was applied. We measured MBF using photo-plethysmography (PPG) on the left thumb and calculated the average drop in PPG during the tasks compared to baseline. From the electrocardiogram

we extracted RRI and its spectral index: high frequency power (HFP)  $\approx$  parasympathetic activity.

**RESULTS:** There was a significant decrease in mean MBF, RRI and HFP during N-back and Stroop compared to the baseline ( $p < 0.01$ ), indicating vasoconstriction. While MBF decreased during all the sublevels of N-back and Stroop compared to baseline, there was no difference in MBF between the task sublevels. During pain anticipation task there was a significant decrease in MBF compared to baseline ( $p < 0.001$ ) and N-back ( $p < 0.01$ ). The parasympathetic withdrawal in response to mental tasks and pain anticipation followed a similar pattern.

**CONCLUSIONS:** Mental stress and fear of pain causes significant decrease in regional blood flow and parasympathetic withdrawal in SCD and normal controls. The pattern of responses were not significantly different between the two groups however the consequences of decreased blood flow can be quite different because of the resultant entrapment of sickle cells in the microvasculature in SCD. This could explain how mental stress precipitates VOC in SCD by causing neural mediated vasoconstriction and thus increasing the likelihood of vaso-occlusion. Supported by NIH Grant (U56 HL117718)

#### 4.4

##### **DERIVING PHENOTYPIC MARKERS OF THE PERIPHERAL VASOCONSTRICTION RESPONSE TO HEAT-INDUCED PAIN IN SICKLE CELL DISEASE**

Patjanaporn Chalacheva<sup>1</sup>, Thomas Coates<sup>2</sup>, Michael Khoo<sup>1</sup>  
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In sickle cell disease (SCD), pain is generally thought to be a consequence of vaso-occlusive crises (VOC), but pain itself could trigger a cascade of events that leads to full-blown VOC by promoting regional peripheral vasoconstriction. Thus, knowledge of the magnitude and time-course of the reduction in peripheral blood flow (PBF) in response to pain may be useful for predicting risk for VOC. In this study, we employed our recent model (Chalacheva et al., PLoS One 12(5), 2017) to derive and further investigate the neurogenic response to a standardized pain stimulus in individual SCD and control subjects.

The standardized neurogenic response from each subject was decomposed into the weighted sum of several “wavelets”, each representing different time-frequency characteristics of the response. The largest contributions were derived from 2 wavelets: 1) one with a rapid initial drop in PBF followed by relatively slower recovery towards baseline; and 2) one showing a small rapid initial drop in

PBF followed by a small rebound and subsequently a more prolonged drop. As such, the weighted sum of both wavelets took the form of a relatively more sustained (“tonic”) response, while the weighted difference of these 2 wavelets yielded a “phasic response” to each pain pulse. The sum of the 2 wavelet weight coefficients (Cs) was correlated with the mean drop in PBF during pain from baseline ( $r = -0.47$ ,  $p = 0.015$ ), while the difference between the wavelet coefficients was correlated with the slope between PBF change and temperature ramp accompanying each pain pulse ( $r = 0.48$ ,  $p = 0.014$ ). Cs was also correlated with the normalized high frequency power of heart rate variability (HFPn) during baseline (before pain was applied) in only SCD ( $r = 0.68$ ,  $p = 0.014$ ), but not controls. This result implies that SCD subjects with low baseline parasympathetic activity, as reflected by HFPn, have stronger tonic vasoconstriction patterns; but this association was absent in controls.

In summary, we have introduced a model-based approach for phenotyping the peripheral vasoconstriction response to acute pain in SCD using only noninvasive measurements. Based on our preliminary findings, we speculate that SCD subjects who are autonomically imbalanced are more likely to display prolonged peripheral vasoconstriction, potentially exposing them to higher risk for VOC.

**Funding:** National Institutes of Health National Heart, Lung, and Blood Institute grant U01 HL117718

#### 4.5

#### INCREASED EXPRESSION OF CELLULAR STRESS PROTEIN, $\alpha$ -SYNUCLEIN (SNCA) WITHIN NORMOXIC AND HYPOXIC BRAINS OF SCD MICE: IMPLICATIONS FOR NEUROCOGNITIVE DYSFUNCTION IN SICKLE CELL DISEASE PATIENTS

Fitz Tavenier<sup>1</sup>, Mariam Hamid<sup>1</sup>, Gratiana Fu<sup>1</sup>, Rebekah Urbonya<sup>1</sup>, Shuaiying Cui<sup>2</sup>, Amrita Pawar<sup>1</sup>, Aaran Varatharajan<sup>1</sup>, ANDREW CAMPBELL<sup>1,3</sup> <sup>1</sup> Pediatric Hematology/Oncology, UNIVERSITY OF MICHIGAN, 1500 E. MEDICAL CENTER DRIVE, MPB D4202, ANN ARBOR, MI, 48109, <sup>2</sup> Medicine, Hematology/Oncology, Boston University School of Medicine, 650 Albany St Evans Biomed Research Ctr, Boston MA 02118, Boston, MA, 02118, <sup>3</sup> Pediatric Hematology, Center for Cancer and Blood Disorders, Childrens National Medical Center, 111 Michigan Avenue, Washington, DC, 20010

**Background:** The aggregation of the protein  $\alpha$ -synuclein (SNCA) is linked to neurocognitive dysfunction in Parkinson’s disease and Alzheimers Disease. Neurocognitive dysfunction is also prevalent within sickle-cell disease (SCD) patients including those with strokes, silent and overt. however it is not found within all SCD patients with neurocognitive dysfunctions. SNCA has been

found to be elevated in reticulocytes and pro-inflammatory states and peripheral blood mononuclear cells of hypoxic SCD patients (Zhang et al 2014). Since SCD patients have high retic counts and are in a chronic pro-inflammatory state, we investigated SNCA expression within the Brains of our Wild type and SCD mice under normoxic conditions and hypoxic conditions that would represent an acute pro-inflammatory state. **Materials & Methods:** SNCA qRT-PCR mRNA expression within Brains of Normoxic Wild type (WT) Control Mice ( $n = 5$ , Mean Age=5.5m/o) was compared to expression within the Brains of Normoxic SCD mice ( $n=5$  Mean Age 5.7 m/o). Additionally, in a separate experiment, Brain SNCA expression of Normoxic SCD Mice ( $N=4$ , Mean 5.1m/o) controls was compared to sickle-cell mice, under hypoxic conditions ( $n = 4$ , Mean 5.5 m/o). To simulate hypoxic conditions mice were placed in a chamber for 8 hours, that was infused with nitrogen to lower the oxygen levels to FIO<sub>2</sub> of 7%. The cDNA from normoxic and hypoxic mice brains were synthesized and then subject to qRT PCR to determine the expression level of SNCA in Normoxic WT mice, Normoxic SCD mice, and Hypoxic SCD mice. The fold expression of SNCA was normalized in triplicates was compared to expression of GAPDH. The cDNA samples for each mouse were done in triplicates and the relative expressions of the three samples were averaged. **Results:** SNCA expression were significantly higher in the Brains of Normoxic SCD mice (mean 9.95, sd 3.06) compared to Normoxic WT Control mice (mean 5.04, sd 1.09)  $p = 0.01$ . Further, SNCA Brain expression was significantly higher within the SCD Normoxic Brain (8.75) vs the SCD Hypoxic Brain (15.25). **Conclusion:** There are significant differences in SNCA expression between the brains of wildtype mice and sickle-cell mice. Sickle-cell mice have a higher expression of SNCA in the brain, even more so when subject to hypoxic conditions. SCD complications and associated comorbidities that result in acute and chronic hypoxia could lead to an increased expression of SNCA in the brain, possibly contributing to neurocognitive dysfunction seen in non-stroke patients. **Funding:** NHLBI: K01 HL-03-011

#### 5.0 Poster Session: Sickle Cell Disease Gene Therapy, Gene Editing and Pharmacological Treatment

##### 5.1

#### A STUDY OF THE GEOGRAPHIC DISTRIBUTION AND ASSOCIATED RISK FACTORS OF LEG ULCERS WITHIN AN INTERNATIONAL COHORT OF SICKLE CELL DISEASE PATIENTS: THE CASIRE GROUP ANALYSIS

Charles Antwi-Boasiako<sup>1</sup>, W. Zempsky<sup>2</sup>, B. Inusa<sup>3</sup>, C. J. Strunk<sup>4</sup>, E. V. Asare<sup>5</sup>, D. A. Antwi<sup>6</sup>, D. B. Gifty<sup>6</sup>, S. Perrotta<sup>7</sup>, O. Rodrigues<sup>8</sup>, C. M. Piccone<sup>9</sup>, A. Rivers<sup>10</sup>, D. Manwani<sup>11</sup>, I. Tartaglione<sup>12</sup>, R. Colombatti<sup>13</sup>, F. Sey<sup>5</sup>, B. Andemariam<sup>14</sup>, A. D. Campbell<sup>15</sup>

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The **aim** was to determine the Geographic Distribution and associated clinical and demographic risk factors associated with leg ulcers (LU) in an international cohort of SCD patients. The CASIRE group is an international multi-institutional collaborative group evaluating the clinical severity of adults and children with SCD Sites included U.S., Italy, and Ghana. **Results:** 585 subjects enrolled: 261, 236 and 88 recruited from US, Ghana and Italy respectively. 57(9.6%) had LU. The majority of LUs occurred in adult (>18y/o), (98.2%, p<0.001) and within the Severe Genotype (96.5% vs. 3.5% in Mild Phenotype, p<0.001). Demographically, LU patients were more of Males (62.7%, p=0.010) and from Ghana ( 82.5% N=47 vs. 17.5% N=10 from US/Italy, p<0.001). Clinically, LU patients were hypoxic ( O2 Saturation Room Air: 95.5% vs. 97.4%, p=0.025), underweight ( BMI <5th%tile : 33.3% vs. Non-underweight 8.2%, p<0.001), more anemic (Hemoglobin 7.6 vs 9.3g/dL, p<0.001), more hemolytic ( TBili 3.6 vs. 2.4, p= 0.036, AST 54 vs. 42 p=0.035) with more leukocytosis(12.9 vs.10.4 1000/ul), more thrombocytosis (438 vs. 367 1000/ul, p=0.004). Reported higher Creatinine levels (0.79 vs. 0.52, p=0.003) and more urine acidosis (Urine pH=5.7 vs. 6.0, p=0.002) suggests some associated related renal dysfunction. There was no significant relationship between LUs and microalbuminuria, age, stroke, pain crises patterns, or priapism. **Conclusions:** This is the 1st comprehensive analysis of LUs prevalence and SCD demographic and clinical risk factors within a Cohort in International SCD Patients. West African background, male gender, leukocytosis, thrombocytosis, severe anemia, lower oxygen saturation, and hemolysis, Renal Acidosis and higher creatinine and being an Adult are risk factors for LUs

## 5.2

### ERYTHROCYTE HYPOMAGNESEMIA IN PATIENTS WITH SICKLE CELL ANEMIA IS ASSOCIATED WITH INCREASED FREQUENCY OF VASO-OCCLUSIVE CRISES.

Samir Ballas<sup>1</sup>

<sup>1</sup>Medicine/hematology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA, 19107

The role of magnesium (Mg), if any, in the management of patients with sickle cell anemia (SCA) remains unsettled. Previous studies using oral Mg supplementation showed improvement in the hematological and biochemical markers of RBC in patients with sickle cell disease (SCD) but with no definite conclusion on therapeutic efficacy. Previous single arm trial with historical controls showed that intravenous Mg decreased the length of hospital stay of children with vaso-occlusive crises (VOCs) but a subsequent multicenter randomized controlled trial of intravenous Mg for VOCs in children showed no beneficial effects. However, serum and red blood cell (RBC) Mg levels in these trials were not determined. In this retrospective study, RBC and serum Mg levels were measured in 50 patients with SCA when in the steady state as outpatients between 2002 and 2010. Twelve of the 50 patients had normal RBC Mg level of  $4.7 \pm 0.59$  mg/dL (normal range: 4.0 – 6.4 mg/dL). The remaining 38 patients had significantly lower RBC Mg level of  $2.7 \pm 0.81$  mg/dL (p < 0.001). The serum Mg level was not significantly different in the two groups of patients:  $1.79 \pm 0.12$  mEq/L versus  $1.81 \pm 0.19$  mEq/L (p > 0.5). Normal range of serum Mg level is: 1.3 – 2.1 mEq/L. Nine of the patients with normal RBC Mg levels had  $2.3 \pm 1.00$  VOCs per year that required treatment in the Emergency Department and/or the hospital. Likewise, 28 of the patients with low RBC Mg level had  $4.9 \pm 4.70$  VOCs per year that required treatment in the ED and/or hospital (p < 0.05). Recently, patients with fibromyalgia were reported to have deficiencies in RBC Mg levels.

Together, the data suggest that patients with SCA and RBC hypomagnesemia may be at risk to have frequent VOCs. Further studies including larger number of patients are needed.

Supported in part by the Sickle Cell Program of the Commonwealth of Pennsylvania for the Philadelphia Region.

## 5.3

### HAPTOGLOBIN AND HEMOPEXIN INHIBIT INFLAMMATION AND VASO-OCCLUSION IN MURINE SICKLE CELL DISEASE THROUGH RAPID INDUCTION OF HEME OXYGENASE-1 AND CO PRODUCTION

John D Belcher<sup>1</sup>, Chunsheng Chen<sup>1</sup>, Julia Nguyen<sup>1</sup>, Fuad Abdulla<sup>1</sup>, Ping Zhang<sup>1</sup>, Hao Nguyen<sup>1</sup>, Phong Nguyen<sup>1</sup>,

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Cell-free hemoglobin and heme promote inflammation and vaso-occlusion in murine models of sickle cell disease (SCD). Plasma haptoglobin and hemopexin scavenge plasma hemoglobin and heme, respectively, thwarting these clinical sequelae. Chronic hemolysis depletes plasma haptoglobin and hemopexin in SCD; studies in SCD mice suggest beneficial effects of supplementation. To explore mechanisms mediating this protection and provide a basis for supplementation in patients, dorsal skin-fold chambers were implanted onto Townes-SS mice and stasis (% non-flowing venules) was measured after hemoglobin challenge. Human haptoglobin, hemopexin, or albumin was co-infused with hemoglobin or 1 hour (h) after hemoglobin at equimolar concentrations. SS-mice co-infused with hemoglobin + haptoglobin or hemoglobin + hemopexin had less stasis 1-4h after infusion, compared to hemoglobin or hemoglobin + albumin. Haptoglobin or hemopexin given to SS-mice 1h after hemoglobin, decreased stasis 2-3h after infusion, while venules of SS-mice given albumin remained static. The combination of haptoglobin + hemopexin was similar to either scavenger alone. Plasma hemoglobin and heme levels were unchanged 3-4h after supplementation. Haptoglobin or hemopexin increased hepatic Nrf2 and decreased pro-inflammatory NF- $\kappa$ B phospho-p65 relative to hemoglobin or hemoglobin + albumin. Notably, haptoglobin or hemopexin increased heme oxygenase-1 (HO-1) within 1h in liver and dorsal-skin. Inhibition of HO-1 activity with tin protoporphyrin reversed haptoglobin/hemopexin-mediated inhibition of stasis and NF- $\kappa$ B. Protection was restored by administering the HO-1 reaction product CO, which blocked hemin-mediated Weibel-Palade body P-selectin expression on cultured endothelial cells and hepatic NF- $\kappa$ B activation. Haptoglobin or hemopexin in unchallenged SS-mice induced HO-1 and inhibited stasis for 48h. These data suggest a link between haptoglobin/hemopexin, HO-1, and CO in alleviating SCD vaso-occlusion.

This research was funded by a research grant from CSL Behring and NIH grant R01 HL114567-05.

#### 5.4

##### A MACROPHAGE-STIMULATING PROTEIN RECEPTOR INHIBITOR CAUSES A GREATER REDUCTION IN INTERFERON-GAMMA EXPRESSION IN THE HEART OF FEMALE MICE WITH SICKLE CELL DISEASE

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Interferon-gamma (INF- $\gamma$ ) has been shown to have adverse and beneficial effects during various pathological conditions. Very little information is known about the role of INF- $\gamma$  during human sickle cell disease (SCD). Interleukin-17 (IL-17) has been shown to be increased during vaso-occlusive crisis of sickle cell patients; however little information is known about IL-17 expression in the hearts during SCD. We utilized the Townes mouse model of homozygous sickle cell disease to determine the role of INF- $\gamma$  and IL-17 in the hearts of male and female groups. We hypothesized that the Macrophage-stimulating protein receptor inhibitor (RON-inh) would decrease INF- $\gamma$  expression in male and female homozygous mice. Male and female Townes mice (8 – 10 weeks of age) were divided into T-homozygous and T-controls and injected with the Ron-inh inhibitor (5 mg/kg in 2% DMSO) or DMSO for 14 days. Western-blots were performed on whole heart homogenates to determine INF- $\gamma$  and IL-17 expressions. When comparing male and female INF- $\gamma$  expression in control mice, female controls had a 64% increased expression during RON-inh treatments. The Thomo females during baseline conditions had a  $4.6 \pm 2\%$  increase in INF- $\gamma$  when compared to Thomo males. The RON-inh decreased INF- $\gamma$  expression by  $13.7 \pm 7\%$  and  $13.1 \pm 5\%$  in the Thomo male and Thomo female groups, respectively. IL-17 expression in the hearts were similar between all groups. There were no differences in body weights of Thomo males and females treated with the RON-inh for 14 days. In summary, our results suggest that RON-inh treatment caused a greater reduction in INF- $\gamma$  in female Thomo mice. There were no significant changes in IL-17 expression in male or female groups treated with or without RON-inh. Future studies are needed to determine if macrophage stimulating protein receptor inhibition is more effective in reducing INF- $\gamma$  expression in female hearts with SCD.

#### 5.5

##### EFFECT OF THE LSD1 INHIBITOR RN-1 ON HBF, GENE EXPRESSION, AND ERYTHROID DIFFERENTIATION

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Sickle cell disease (SCD) is caused by a mutation of the  $\beta$ -globin gene that results in polymerization of deoxygenated sickle hemoglobin (HbS). Elevated levels of Fetal Hemoglobin (HbF) inhibit polymerization of HbS and are associated with less severe illness and longer survival. Hydroxyurea, the only approved drug for SCD, increases HbF but is effective in only 50% of patients. Therefore additional strategies to increase HbF are needed. Repression of the  $\beta$ -globin gene is mediated by epigenetic modifications catalyzed by DNMT1, LSD1, and HDACs present within corepressor complexes recruited to the gamma-globin gene by the site-specific DNA binding proteins TR2/TR4 and BCL11A. Our laboratory showed that DNMT inhibitors increased HbF in baboons, long considered as the best animal model to test HbF-inducing drugs because the structure and developmental regulation of genes within the  $\beta$ -globin gene complex is conserved among all simian primates. The effectiveness of DNMT inhibitors has been demonstrated in multiple clinical trials. Following studies that identified LSD1 as an additional therapeutic target (Shi et al, Nat Med 19:291, 2013), we showed that the LSD1 inhibitor RN-1 dramatically increased HbF, F cells and F retics in baboons (Rivers et al, Haematol 101:698, 2015) and these effects were sustained upon long-term treatment (>265d; Ibanez et al, Blood 129:260, 2017). ChIP analysis showed increased levels of Histone H3 di and tri-methyl K4 at the  $\beta$ -globin gene, consistent with LSD1 inhibition. RN-1 increased  $\beta$ -globin mRNA in BM subpopulations enriched in CFUe, but not BFUe, suggesting the CFUe is the earliest cell in the erythroid differentiation pathway "targeted" by the drug. Flow cytometry analysis showed increased proerythroblasts and decreased orthochromatic and polychromatic precursors in BM of RN-1 treated compared to untreated baboons. Increased expression of 120 genes and decreased expression of 18 genes was observed in FACS-purified BM proerythroblasts from RN-1 treated baboons. Among genes with increased expression were GATA-2 and GFI-1b that regulate erythroid differentiation, and BNIP3L (NIX), a key gene in mitochondrial clearance. Preliminary experiments have shown that RN-1 is active when administered orally. The ability of LSD1 inhibitors to increase HbF in non-human primates strongly suggests that further studies be performed to evaluate these drugs for therapy of SCD.

## 5.6

### **RNA TRANS-SPICING REPAIR OF ENDOGENOUS B-GLOBIN PRE-MRNA IN HUMAN ERYTHROID CELLS**

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Sickle cell disease is caused by a single nucleotide mutation in exon 1 of the beta-globin gene. We are developing an RNA trans-splicing approach that corrects all mutations within the coding region of human beta-globin messenger RNA. In our initial proof of principle experiments, we converted ~0.1% of endogenous beta-globin pre-mRNA by trans-splicing, reprogramming the product mRNA to encode for gamma-globin in human erythroid cells. This was accomplished by transducing human CD34+ cells with a lentivirus expressing an RNA trans-splicing molecule (RTM), which is an RNA that consists of the coding sequence of gamma-globin, a 5' splice site, and an anti-sense binding domain to beta-globin intron 2. Upon binding to a beta-globin pre-mRNA, the RTM is able to induce an alternative splicing reaction in *trans*, between the 5' splice site of the RTM and the 3' splice site of beta-globin. Exons 1 and 2 of beta-globin are replaced by the entire coding sequence of gamma-globin and a stop codon provided by the RTM, followed by beta-globin exon 3. Since it follows a stop codon, beta-globin exon 3 is not translated. Alternatively, the RTM may be designed to deliver beta-globin exons 1+2 to produce corrected beta-globin. Further development of beta-globin targeted RTMs is ongoing. Initial results suggest that higher and more clinically relevant levels of trans-splicing are achievable. There are now nearly sixty publications that have reported RTMs targeting other disease genes with efficiencies reaching 40%. High through-put screens have been developed and should lead to the identification of more efficient RTMs.

RNA trans-splicing offers several advantages over gene editing techniques such as CRISPR-Cas. 1) Trans-splicing is mediated by spliceosomes which are abundant in the nucleus of human cells, no enzymes or other components need to be delivered. 2) Cell division is not necessary for RNA trans-splicing, but is required for gene editing repair 3) Since mRNA turns over, off target trans-splicing is transient whereas off-target gene editing leads to permanent mutations. Because gene editing repair requires co-delivery of additional components, requires cell division and the consequences of off-target edits are unknown, it is likely to remain an ex-vivo approach. Generally RTMs are just a few hundred nucleotides longer than the coding sequence they deliver, thus enabling delivery by clinically relevant vectors such as AAV. RTMs can also be delivered as plasmids or as RNA.

## 5.7

**'THE CASE OF ERADICATION OF SICKLE CELL ANEMIA DEATHS IN AFRICA'**Cornelius Nwora, MD.<sup>1</sup><sup>1</sup>Center for Cardiovascular Diseases, Texas Southern University, 15419 West Willowwind Circle, Houston, TX, 77071

Abstract Title: 'The Case of Eradication of Sickle Cell Anemia Deaths in Africa'

Cornelius C. Nwora, MD., RDMS, RRT(S), RCIS, MLS(ASCP).

Sickle Cell Disease (SCD) causes the greatest burden to both survival of children under five years (U5Y) and to the Public Health Management (PHM) in the endemic areas of Africa, India and other developing areas of the world. It is the most dangerous – in terms of rate of morbidity and mortality, of all known hemoglobinopathies.

Several other confounding factors (both natural and inflicted), in the milieu of 'hypoxia' contribute to the expression of symptoms in SCD patients.

"In the late 1960s and early 1970s, as sickle cell anemia was caught up in the torrent of U.S. congressional and presidential politics, the malady became widely characterized as a "neglected disease," a disease of a people whose "pain and suffering" had been ignored for too long, and a disease finally achieving its moment of national recognition". [1], [3].

A neglectful healthcare policy enabled the disease to reach present epidemic proportions to the tune of 150,000 babies with SCD recorded annually in Nigeria – the highest recorded incidence of SCD in the world. [5].

Sickle Cell Disease patients have compromised immunity. There is an increased incidence of meningitis and septicemia and a high mortality. [6]. Serious infections are common in SCD than in other hemoglobinopathies and occur more frequently in patients younger than 5 years of age. [7].

WHO indices for Nigeria showed she suffers a 10 to 40% carrier state, with a prevalence of 2% [9]. A further 75% infant cases, and 80% share of the mortality in the whole of Africa. [11].

It is nearly impossible to eradicate SCD because of the pathophysiology of the disease, but we can ameliorate the scourge or the rate of death due to complications of stroke during the crisis moments.

Transcranial Doppler (TCD) scanning, is the technique of choice to evaluate and diagnose the probable onset of stroke in SCD patients. TCD is a unique technology with many positive attributes - it is non-invasive, portable, relatively cheap, and easy to apply in the hands of experts. It is just one part of a comprehensive health management for TCD cannot be used in isolation. Early intervention

based on the TCD evidence of continuing embolism can prevent stroke from occurring. [16]

A major aim of this project therefore, is to emphasize implementation of TCD scanning in the management and care of SCD patients in an endemic region with comorbidities that trigger sickle cell disease crisis and stroke. This exercise, in the milieu of a comprehensive PHM, I hope, will help ameliorate the morbidity and mortality. Diabetes is a major and dangerous comorbidity leading to adverse cardiovascular complications and blindness in this geopolitical setting (enclave).

Unlike Ebola - a deadly disease with a quick swift decimating rocket style, Malaria and SCD are silent killers with a 'chameleon' tactics and seeking the most vulnerable of all – our children, especially the U5Y age group.

Peter Piot, the Belgian microbiologist who discovered Ebola in 1976 said: "we shouldn't forget that this is a disease of poverty, of dysfunctional health systems – and of distrust." [17].

## 5.8

**GBT440 IMPROVES RHEOLOGICAL PROPERTIES OF SICKLE CELL BLOOD BY INCREASING HEMOGLOBIN OXYGEN AFFINITY**Mira Patel<sup>1</sup>, Kobe Dufu<sup>1</sup>, Donna Oksenberg<sup>1</sup>, Pedro Cabrales<sup>2</sup><sup>1</sup>Biology, Global Blood Therapeutics, 400 East Jamie Court, Suite 101, South San Francisco, CA, 94080,<sup>2</sup>Bioengineering, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA, 92093

In sickle cell disease (SCD), polymerization of deoxygenated hemoglobin S (HbS) leads to the formation of rigid, non-deformable sickled red blood cells (SS RBCs). Loss of RBC deformability causes abnormal blood rheology which contributes to vaso-occlusion of capillaries and reduced blood flow. HbS polymerization increases blood viscosity, sickling and irreversible membrane damage; all these are major contributors to the pathophysiology of SCD. GBT440 is an allosteric modulator of Hb-oxygen (O<sub>2</sub>) affinity. When bound to HbS, GBT440 maintains HbS in an oxygenated state, prevents HbS polymerization and inhibits RBC sickling.

We monitored the effect of GBT440 on SCD patient blood rheology, *ex vivo*, to determine effects on the viscosity and deformability of SS blood and SS RBCs under hypoxic conditions (2-3% O<sub>2</sub> for 2 h for viscosity experiments and 30 min for deformability experiments). Blood viscosity was measured in a cone-plate viscometer at shear rates ranging from 60 s<sup>-1</sup> to 415 s<sup>-1</sup>. SS RBCs deformability was measured in three independent systems which tested: 1) the ability of SS RBC to migrate through a gel filtration

column 2) the pressure required to enable SS RBCs to pass through a 5  $\mu\text{m}$  polycarbonate pore filter and 3) the SS RBCs membrane elasticity module by micropipette aspiration.

We report that GBT440 maintains the deformability of SS RBCs under hypoxic conditions, enabling the unobstructed migration of SS RBCs through a gel filtration column. In addition, GBT440 reduces the SS RBCs elastic modulus during aspiration and the pressure required to pass SS RBCs through the filter under deoxygenated conditions. Moreover, GBT440 dose-dependently reduces the viscosity of SS blood under deoxygenated conditions. Together, these data suggest that inhibition of HbS polymerization by GBT440 helps to maintain SS RBC deformability and improves blood rheological properties. Thus, GBT440 has the potential to reduce the likelihood of vaso-occlusion and preserve microvascular flow in SCD patients.

### 5.9

#### ENUCLEATION AND BETA-GLOBIN EXPRESSION IN INDUCED RED BLOOD CELLS: A PLATFORM TO MODEL SICKLE CELL ANEMIA

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Human induced pluripotent stem cells (hiPSCs) hold promise for both disease modeling and the development of novel therapeutic treatments for sickle cell anemia (SCA). Such models are practical systems to screen new drug therapies and to examine the effects of gene editing. hiPSCs can theoretically produce all cell types including erythroid cells. However, in vitro modeling of SCA with reprogrammed cells has been limited by their inability to differentiate into beta globin-expressing, enucleated erythroid cells. Here, we propose strategies to produce improved in vitro and in vivo models of SCA using these cell types. We derived hiPSCs from sickle cell patients with hemoglobin SS disease seen at our hematology clinic at Boston Children's Hospital. Using a cocktail of transcription factors promoting self-renewal and multipotentiality expressed under the control of a doxycycline-regulated promoter (Erg, HoxA9, RORa, Sox, Myb) we generated conditionally immortalized hematopoietic cell lines that serve as a renewable source of robust erythroid progenitors in vitro. Erythroid progenitors differentiated from these lines underwent globin-switching once engrafted into immune-radiated mice with a 27% induction of beta globin expression. Concurrently, we further improved the in vitro differentiation protocols described to generate 30-40% beta-globin-expressing,

erythroid cells with an enucleation rate of 20-50%. In future studies, we hope to employ hiPSCs to test the therapeutic hypothesis that genetic manipulation of BCL11A, a master regulator of fetal hemoglobin (HbF) expression, will ameliorate sickling. The generation of hiPSC-SCA models will be critical in broadening the current understanding of the molecular mechanisms of this disease, the development of improved pharmacological treatments and a future of autologous cell therapy for the cure of SCA. **Funding:** Howard Hughes Medical Institute, Doris Duke Charitable Foundation.

### 5.10

#### HUMANIZED SICKLE MICE ARE SENSITIVE TO HYPOXIA/ISCHEMIA-INDUCED STROKE, BUT RESPOND TO TISSUE PLASMINOGEN ACTIVATOR TREATMENT.

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Stroke, a devastating complication of Sickle Cell Anemia (SCA), consists of silent cerebral infarct (SCI) and large overt stroke. As high as 37% of this population showed SCI by age 14, and 11% develop large overt stroke by age 20 without prophylactic therapy. The current management relies on blood transfusion without the use of thrombolytic agents. However, a recent study showed that co-existent SCA does not impact the safety of tissue plasminogen activator (tPA) treatment. This finding calls for systemic analysis of the effects of thrombolysis in experimental stroke and there is also a need for predictive markers of SCA-associated SCI for preventive interventions. Here we test the hypothesis that Townes humanized sickle mice (knock-in/out mice that express the human  $\alpha$ ,  $\gamma$ , and sickle- $\beta$  hemoglobin genes) are sensitive to hypoxia-ischemia (HI)-induced stroke, but respond to tPA-thrombolytic therapy.

We report three sets of results. First, three-month-old sickle mice of the SS genotype ( $\beta\text{S}/\beta\text{S}$ ) have a higher resistive index (RI), but normal flow velocity in the common carotid artery, than with AA ( $\beta\text{A}/\beta\text{A}$ ) or AS ( $\beta\text{A}/\beta\text{S}$ ) mice. SS mice were also prone to repetitive-mild HI (rmHI)-induced cerebral infarct and mortality, whereas AA mice were resistant to rmHI. Second, 6-month-old SS mice developed elevated flow velocity and greater RI without stenosis of the carotid artery akin to those previously implicated in large overt stroke in SCA. Further, SS mice showed ectopic P-Selection and plasminogen activator inhibitor (PAI-1) expression in cerebral blood vessels, suggesting a hyper-coagulation state. Finally, six-month-old SS mice endured 20-min transient hypoxia-ischemia (tHI), but showed enhanced leukocyte and platelet adherence to the cerebral blood vessel, as well as,



extensive vascular perfusion deficits and fibrin deposition at 4 h post-injury, followed by greatly increased mortality than AA and AS mice at 24 h recovery ( $p < 0.0001$ ). Importantly, intravenous tPA administration at 0.5 h post-tHI markedly improved vascular reperfusion, mitigated fibrin deposition, and cut the mortality of SS mice by nearly 60%.

These results indicated that humanized sickle mice develop hyper-coagulation and hypersensitivity to HI-induced stroke without large-vessel obstructive vasculopathy at up to 6 months of age. Elevated resistive index may be an early ultrasonic marker for sickle cell vasculopathy and the risk of SCI in SCA. Future studies are warranted to confirm the therapeutic benefits of thrombolytic stroke therapy in SCA.

### 5.11 GENETIC TESTING FOR ALPHA THALASSEMIA USING DROPLET DIGITAL PCR

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In America, 1 in 500 African-American children are born with sickle cell disease. Many develop acute or chronic vascular problems such as stroke, pulmonary hypertension, kidney damage and leg ulcers. These vascular complications of sickle cell disease are prevented or delayed by co-inheritance of one or more alpha globin gene deletions. In order to begin studying how alpha globin gene deletions confer this protective effect, we needed to develop a robust quantitative genotyping method.

Genotyping the alpha globin locus is challenging because each chromosome has two alpha globin genes (*HBA1* and *HBA2*) with nearly identical sequences. The most common alpha globin mutation is a -3.7 kb deletion that reduces gene copy number by one. Therefore, we approached this deletion as a copy number variant, and designed droplet digital polymerase chain reaction (ddPCR) assays to quantify alpha globin gene copy number.

We designed two ddPCR assays, one which targeted exon 2 of the *HBA1* and *HBA2*, and a second assay that targeted a unique (single copy) sequence in the intergenic region between *HBA1* and *HBA2*. We obtained saliva or blood samples from volunteers (NHLBI protocol 03-H-0015) and extracted genomic DNA. For validation, we sent 14 samples representing different alpha globin gene copy numbers to the Mayo Clinic Lab for analysis by multiplex ligation-dependent probe amplification (MLPA). The presence or absence of the -3.7 kb deletion determined by our assays was 100% in agreement with the MLPA gold standard. Statistical comparison our two assays across

hundreds of DNA samples revealed that targeting the intergenic sequence which is present in 0, 1, or 2 copies provided greater precision than targeting exon 2 which is present in 2, 3, or 4 copies. This comparison highlights a real-world performance limitation of ddPCR and provides a technical solution for accurate alpha globin genotyping.

### 5.12 DEVELOPMENT OF NOVEL LSD1 INHIBITORS AS A STRATEGY TO TREAT SCD

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Development of Novel LSD1 Inhibitors as a Strategy to Treat SCD

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β-globinopathies, including α-thalassemia and Sickle Cell Disease (SCD), are the most common hereditary monogenic diseases. Both clinical and experimental evidence indicates that increasing the levels of fetal hemoglobin (HbF; the α<sub>2</sub>β<sub>2</sub> tetramer) alleviates the symptoms of β-globinopathies. Specifically, Hereditary Persistence of Fetal Hemoglobin (HPFH) is a natural human genetic variant in which high levels of fetal β-globin synthesis aberrantly persist into adulthood. It has been observed clinically that when an HPFH mutation is co-inherited with α-thalassemia or SCD, the elevated production of β-globin significantly mitigates the symptoms of the disease. To date, hydroxyurea (HU) is the only current FDA-approved drug for HbF induction and SCD treatment, but only about half of all SCD patients are responsive. Therefore, new therapeutics are required. Lysine-Specific Demethylase 1 (LSD1, KDM1a), an enzyme that removes activating histone H3 (H3K4) methylation marks from chromatin, has been identified as one component of a large multi-protein complex, DRED, that represses the human β-globin genes. Importantly, pharmacologic inhibition of LSD1 leads to dose-dependent increases in β-globin synthesis. We have also reported that *in vivo* inhibition of LSD1 by a second chemical inhibitor (RN-1) in SCD model mice induced HbF synthesis and led to dramatic improvement in many pathological features normally associated with SCD; we collaboratively

demonstrated that RN-1 is also able to significantly stimulate HbF synthesis in baboons, animals that, like humans, exhibit a fetal to adult switch in  $\alpha$ -type globin synthesis. These findings all strongly underscore the possibility that LSD1 might constitute an outstanding molecular target for therapeutic intervention in treating SCD. We report the synthesis and tests for novel HbF-inducing LSD1 inhibitors as potentially safer and more effective  $\beta$ -globin inducers in CD34 cells. One novel compound induces  $\beta$ -globin synthesis up to 24% of total  $\alpha$ -type globin and in a dose-dependent manner with mild side effects at 10-fold lower concentrations than RN-1. These data suggest that the novel LSD1 inhibitors might serve as lead candidates for further in vivo studies.

**6.0 Poster Session: Small Molecules to Treat Sickle Cell Disease**

**6.1**

**TREATMENT OF THE FIRST SICKLE CELL DISEASE PATIENTS WITH ANTAGONIST OF N-METHYL D-ASPARTATE RECEPTOR MEMANTINE: BIOLOGICAL OUTCOME OF THE MEMSID TRIAL**

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We have reported pathologically high abundance of NMDA receptors in red cell (RBC) membranes of sickle cell disease (SCD) patients several years ago. Hyperactivated state of these receptors results in  $Ca^{2+}$  overload, which in turn causes dehydration, and increased proteolytic activity and adhesiveness of RBCs to each other. A pilot clinical trial was initiated in 2015 in which memantine, the antagonist of NMDA receptors, was applied to a limited number of adult patients to estimate safety and tolerability of the compound and the potential benefits of a new treatment (NCT02615847). Four patients have successfully completed the trial that was closed in March 2017. While statistical analysis of the outcome is in preparation, we may share the biological findings related to the effects of memantine of RBCs of the patients. We have observed the changes in membrane stability and an increase in RBC longevity.

These effects were associated in rehydration and substantial reduction in  $K^+$  loss from the cells. Improvement of morphological appearance indicated decrease in proteolytic destruction of cytoskeletal proteins.  $Ca^{2+}$  levels in RBCs were decreasing, as were the numbers of active receptors at the RBC membrane of patients. These changes in RBC properties observed during the treatment were reverted upon the suspension of therapy (challenge). These findings were underlying the substantial improvement of life quality of patients and low number of consultations and hospitalizations.

**7.0 Poster Session: Coagulation/Thrombosis**

**7.1**

**ASSAY-DEPENDENT RESULTS OF ADAMTS13 ACTIVITY IN SICKLE CELL DISEASE**

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**Background:** von Willebrand Factor (VWF) is an adhesive multimeric plasma protein that is acutely elevated in VOC and may play a mechanistic role in the pathogenesis of vaso-occlusion. However, discrepant findings concerning the functionality of ADAMTS13, the VWF-cleaving plasma protease, have been reported in sickle cell disease.

**Objectives:** To characterize ADAMTS13 activity in adult sickle cell patients using multiple *in vitro* assays.

**Methods:** Plasma samples were obtained from adult sickle cell patients undergoing regular exchange transfusion (n=20) and healthy control patients (n=15). Plasmatic ADAMTS13 activity was determined by two VWF A2 domain peptidyl-based assays (FRETs VWF73 and VWF73 GST) and a shear-based assay employing the full length VWF molecule. The level of thrombospondin-1 in plasma was determined by ELISA.

**Results:** In peptidyl-based assays, sickle cell disease plasma displayed significantly lower ADAMTS13 activity relative to healthy controls (mean activity 69.5 vs. 1.11 IU/mL, respectively, for VWF73 GST ELISA,  $P < 0.0001$ ). By contrast, the cleavage potential against the full length VWF molecule was normal or enhanced in sickle cell disease patient plasma. The level of plasma thrombospondin-1, an inhibitor of VWF cleavage which

correlates with disease activity in SCD, was not significantly elevated in this study population.

**Conclusions:** Our findings demonstrate assay-dependent results of ADAMTS13 activity measurements in sickle cell disease, and imply the possible existence of alternative blood proteases capable of VWF cleavage. These findings may have implications for the interpretation of ADAMTS13 activity in sickle cell disease and for the monitoring of ADAMTS13 activity in clinical trials.

## 7.2

### ACCELERATED VENOUS THROMBOSIS WITH ENHANCED FIBRIN DEPOSITION AND PLATELET ACCUMULATION IN SICKLE CELL MICE

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Patients with sickle cell disease (SCD) are at increased risk for thrombotic complications, such as venous thromboembolism and stroke. Previous studies in mouse models have revealed accelerated thrombus formation in the cerebral microvessels of sickle (SS) mice compared to wild type (AA) controls. The objective of this study was to evaluate if SCD alters the dynamics of venous thrombosis in large vessels using a mouse model of electrolytic injury-induced thrombosis in the femoral vein. Anesthetized male AA and SS mice (12 weeks old) were infused with fluorescent antibodies to fibrin (59D8) and platelets (rhodamine 6G) 5 min before positive current was applied to the femoral vein. In the standard injury model, 1.5 V was applied for 30 s. In the severe injury model, 3.0 V was applied for 90 s. Fluorescence was monitored for 60 min after injury using intravital microscopy.

In the standard injury model, AA mice developed thrombi with a fibrin core and platelet cap that peaked at 20 minutes then began to resolve. In SS mice, there was no distinct border between platelets and fibrin in the thrombus, and both continued to accumulate throughout the monitoring period, leading to significantly more fibrin and platelet deposition within the clot compared to AA controls. There was no occlusion of flow in the vein around the developing thrombi in either AA or SS mice. In the severe injury model in AA mice, platelet accumulation began within 10 minutes and was sustained, whereas fibrin accumulation steadily increased over 60 min. In SS mice, there was a rapid increase in both platelet and fibrin accumulation by 15 and 25 min, respectively, which

plateaued for the remainder of the experiment. Moreover, the fluorescence intensities of both platelets and fibrin deposition were nearly 2-fold higher in SS mice compared to AA controls starting at the times mentioned above, and remained significantly elevated throughout the monitoring period. In summary, we have established a model of venous thrombosis that can be used to study the dynamics of venous thrombosis in sickle mice. Future studies will use this model to elucidate the mechanisms of increased risk of venous thromboembolism in SCD, with a focus on the clot stability and rate of fibrinolysis.

This research was funded by NIH U01 HL117659.

## 9.0: Neural Circuits and Neurovascular Physiology

### 9.1

#### TARGETING PAIN AT ITS SOURCE IN SICKLE CELL DISEASE

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Pain, a hallmark of sickle cell disease (SCD), can start in infancy and continue throughout life, leading to increased morbidity and mortality. Yet, how sickle pathobiology evokes pain is not understood. Hemin released upon hemolysis contributes to vascular sickle pathobiology via toll-like receptor 4 (TLR4)-mediated mechanisms. We examined whether hemin contributes to hyperalgesia via TLR4 mediated peripheral and central mechanisms of pain in SCD, using a humanized model of transgenic HbSS-BERK sickle mice. We found that hemin induced hyperalgesia in sickle and control mice. However, genetic deletion of TLR4 reduced hemin-induced chronic hyperalgesia in sickle and control mice, and attenuated hypoxia/reoxygenation (H/R)-evoked acute hyperalgesia. Pharmacological blockade of TLR4 decreased mast cell activation, neurogenic inflammation, IL-6, and substance P in the periphery and spinal cord, as well as p38/MAPK phosphorylation in the spinal cord. TLR4 inhibition attenuated hemin-evoked spinal microglial cell activation by reducing endoplasmic reticulum (ER) stress in vitro. Either inhibiting TLR4 with TAK242 or reducing ER stress with salubrinal ameliorated chronic hyperalgesia in sickle mice in a time-dependent manner. TAK242 pretreatment also reduced the severity of acute pain evoked by H/R and accelerated recovery from H/R-induced hyperalgesia in sickle mice. Collectively, our data demonstrate the pivotal role of TLR4 in evoking chronic and acute pain in SCD. It suggests potential therapeutic benefit of limiting hemin, TLR4 inhibition and ER stress reduction in ameliorating pain and inflammation in SCD. NIH R01HL103773 and U01 HL117664. Reference: Aich A, Beitz AJ, and Gupta K. Mechanisms of pain in sickle cell disease. In, *Sickle Cell Disease*, Inusa B (Ed). November 2016. InTech Publishers, Croatia, EU.

**9.2**

**ENDOTHELIN TYPE A RECEPTORS MEDIATE SICKLE CELL DISEASE-ASSOCIATED PAIN BY UP-REGULATING NAV1.8 IN PRIMARY SENSORY NEURONS**

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Sickle Cell Disease (SCD), a hemoglobinopathy resulting in a mutated  $\beta$  globin gene, is associated with acute painful episodes and persistent intractable pain. Endothelin-1, a known pain inducer, is elevated in the blood plasma of both SCD patients and SCD mouse models. Endothelin-1 binds to Endothelin-type A (ETA) receptors and Endothelin-type B receptors. In dorsal root ganglion (DRG), ETA receptors are found in the neurons, while ETB receptors are expressed in the surrounding satellite cells. We hypothesize that ET-1 binding to ETA receptors in DRG contributes to SCD-associated pain through increased Nav1.8 channel expression. Mechanical, thermal, and cold sensitivity were assessed in 6 months old Townes HbSS and HbAA control mice before and after exposure to hypoxia. The effect of ABT-627, an ETA receptor-specific antagonist, on evoked pain hypersensitivity was also analyzed. Additionally, DRG-specific ETA receptor knockout mice (ETA<sup>flox/cre</sup>) underwent total body irradiation and bone marrow transplantation (BMT) using HbSS and HbAA bone marrow, resulting in ETA<sup>flox/cre</sup> mice expressing human sickle  $\beta$  globin (HbSS marrow recipients) or normal human  $\beta$  globin (HbAA marrow recipients). Pain behavior was assessed in these mice before BMT, after BMT, and after hypoxia. Our results show that HbSS mice possess basal evoked mechanical and thermal pain hypersensitivity and basal spontaneous pain. Subcutaneous injection of ABT-627 attenuated basal and post-hypoxia evoked mechanical and thermal pain hypersensitivity in HbSS mice. Additionally, ETA<sup>flox/cre</sup> mice transplanted with HbSS bone marrow displayed less basal and post-hypoxia evoked mechanical and thermal pain hypersensitivity compared to ETA<sup>flox/flox</sup> mice transplanted with HbSS bone marrow. Electrophysiology recording of HbSS DRG neurons showed an increase in Nav1.8 current and nociceptor spontaneous activity. ABT-627 blocked the increases in Nav1.8 channel current, protein levels, and mRNA levels in the DRG of HbSS mice. Our findings indicate that ABT-627 may be beneficial for the treatment of SCD-associated pain. This work was supported by NIH grants (R01NS072206, R01DA033390, F31NS092310, and U01HL117684).

**9.3**

**PROSTAGLANDIN GLYCEROL ESTERS CONTRIBUTE TO HYPERALGESIA IN A HUMANIZED MOUSE MODEL OF SICKLE CELL DISEASE**

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Sickle Cell Disease (SCD) is a hereditary chronic hemolytic anemia with numerous clinical consequences including ongoing and episodic pain. HbSS-BERK mice which express human sickle hemoglobin (HbSS) and mirror symptoms of human SCD were used to explore the contribution of the prostaglandin glycerol ester, PGE<sub>2</sub>-G, the cyclooxygenase (COX)-2 oxidative metabolite of 2-arachidonylglycerol (2-AG), to mechanical and cold hyperalgesia. Mechanical hyperalgesia was defined as an increase in the frequency of withdrawal evoked by a von Frey monofilament with a force of 3.9 mN applied to the plantar surface of the hind paws. A cold preference test was used to determine cold hyperalgesia. The level of the endocannabinoid 2-AG in dorsal root ganglia (DRG) was determined by HPLS-MS. Hyperalgesia in HbSS mice was accompanied by increased activity of COX-2 in DRGs and decreased levels of 2-AG, the natural substrate for COX-2. Considering that COX-2 oxygenates 2-AG to form PGE<sub>2</sub>-G, we investigated the contribution of PGE<sub>2</sub>-G to the development of mechanical and cold hyperalgesia. Intraperitoneal administration of PGE<sub>2</sub>-G to control HbAA mice produced acute bilateral mechanical hyperalgesia that was dose-dependent. Intraplantar injection of PGE<sub>2</sub>-G produced local hyperalgesia, suggesting a peripheral mechanism of action. *R*-Flurbiprofen is a slow reversible inhibitor of COX-2 that preferentially inhibits the production of PGE<sub>2</sub>-G. Systemic administration of *R*-Flurbiprofen reduced cold and mechanical hyperalgesia in HbSS mice, and this was also dose-dependent. Collectively, our results suggest that COX-2-mediated oxidation of 2-AG results in production of PGE<sub>2</sub>-G in DRGs that may contribute to nociceptor sensitization and to pain in SCD.

This work was supported by: NIH grants R01 HL135895 and U01 HL117664.

**9.4**

**DIMINISHED CEREBRAL OXYGEN EXTRACTION IN ANEMIC SUBJECTS USING VENOUS MRI OXIMETRY: IS TRUST OXIMETRY CALIBRATION RELIABLE IN SICKLE CELL DISEASE?**

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MRI oximetry techniques have opened the door for noninvasive, non-irradiating investigations of cerebral oxygen supply and demand. The most widely used MRI oximetry technique is T<sub>2</sub> Relaxation Under Spin Tagging (TRUST). TRUST works by magnetically isolating blood water in order to determine the blood transverse relaxation (T<sub>2b</sub>). T<sub>2b</sub> is proportional to deoxyhemoglobin and can be used to derive venous oxygen saturation (S<sub>v</sub>O<sub>2</sub>) using predetermined T<sub>2b</sub> calibrations.

To date, studies of TRUST in SCD have reported elevated cerebral oxygen extraction. Some have even concluded that the increased oxygen extraction in SCD exposes patients to increased stroke risk due to diminished oxygen reserve. Unfortunately, these conclusions were made based on T<sub>2b</sub> calibrations developed in bovine blood and derived over a non-anemic hemoglobin range. In order to assess the accuracy of recent conclusions, we derived a SCD specific T<sub>2b</sub> calibration ex vivo and performed TRUST in healthy subjects (CTL), SCD patients and chronic, non-SCD related anemia subjects (ACTL).

**Methods:** Ex vivo, 83 T<sub>2b</sub> measurements from 11 SCD subjects were performed to derive an ex-vivo SCD specific T<sub>2b</sub> calibration. TRUST of the sagittal sinus was performed on 84 subjects (37 CTL, 33 SCD, 14 ACTL) to measure S<sub>v</sub>O<sub>2</sub> and OEF.

**Results:** Ex-vivo, SCD blood demonstrated a distinct T<sub>2b</sub> behavior compared to previously reported bovine and HbA T<sub>2b</sub> calibrations. This difference resulted in diametrically opposed in-vivo oximetry predictions between T<sub>2b</sub> calibrations. For instance, the HbS model predicted higher Y<sub>v</sub> (73±5%) in SCD subjects compared to CTL (61±6%), the HbA model predicted equitable Y<sub>v</sub> (64±4%) values and the bovine calibration predicted lower Y<sub>v</sub> lower values in SCD subjects (60±8% vs 65±5%). In both the SCD and ACTL subjects, use of the appropriate human T<sub>2b</sub> calibration produced extraction estimates that were lower than CTL subjects (table 1).

	CTL	SCD	ACTL	Dunnett (p value)
Bovine Model	0.34±0.5	0.38±0.08	0.32±0.05	SCD(p=0.06)
HbA model	0.38 ±0.06	0.34 ±0.06	0.30±0.04	SCD (p<0.0085), ACTL (p<0.0001)
HbA and HbS model	0.38 ±0.06	0.24 ±0.04	0.30±0.04	SCD (p<0.0001), ACTL (p<0.0001)

**Conclusion:** These finding demonstrate that specific T<sub>2b</sub> models are critical in SCD subjects, suggesting TRUST conclusions in SCD using bovine blood are spurious. Additionally, we report here that cerebral oxygen is actually decreased in SCD and ACTL subjects, suggesting

hyperemic shunting and/or cerebral oxygen supply demand uncoupling.

**10.0 Poster Session: Renal and Vascular Physiology**

**10.1 ACTIVATION OF RENAL HEPICIDIN EXPRESSION IN SICKLE CELL DISEASE MOUSE MODEL**

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Chronic hemolysis and blood transfusions can lead to iron overload and organ iron accumulation in patients with red blood cells disorders. The pattern of iron accumulation within different organs is disease specific. Renal cortical iron deposition is characteristic for sickle cell disease (SCD) but not for β-thalassemia patients; and it does not correlate with the levels of iron overload and number of blood transfusions. SCD mice (Townes) accumulate iron in the epithelial cells of proximal tubules, and can be used to study renal iron metabolism.

The project objective was to measure expression levels of renal iron-regulating proteins in SCD mice. The animal protocol was approved by the Institutional Animal Care and Use Committee at the Children's National Health System. Both SCD and control mice do not express mouse hemoglobin (Hgb). Townes sickling mice express human Hgb S and Hgb F, whereas control mice express human Hgb A1. Kidneys were collected from 4 months old mice. Renal cortexes were used for RNA and protein isolation. Real time RT-PCR, ELISA and Western Blot and immunostaining were performed for characterization of iron-regulating proteins expression.

We detected a significant accumulation of iron in the epithelial cells of proximal tubules in SCD mice. Renal expression of hepcidin was not detected in controls. In contrast renal hepcidin expression was detected in the epithelial cells of proximal tubules of SCD mice. The mRNA levels of FPN, TFR1, DMT1, ferritin and hephaestin were decreased in SCD mice kidney compared to controls. In contrast, protein levels of TFR1, ferritin, and CP were increased. Protein levels of FPN were similar in SCD and control animals. We also observed significant renal macrophages infiltration in SCD mice.

In conclusion, activation of hepcidin expression in renal proximal tubular epithelial cells may induce partial degradation of FPN in SCD mice. Increased levels of iron importers (TFR1 and DMT1) without significant change in FPN levels can saturate iron storage in ferritin and lead to the accumulation of intracellular iron. Activation of renal hepcidin expression in SCD mice may be associated with renal inflammation.

**ACKNOWLEDGMENTS:** This work was supported by NIH Research Grants 1P50HL118006, 1R01HL125005 and 5G12MD007597. The content is solely the responsibility of the authors and does not necessarily represent the official view of NHLBI, NIMHD or NIH.

## 10.2

### HEMOGLOBIN INHIBITS UPTAKE OF FILTERED PROTEINS BY PROXIMAL TUBULE CELLS: IMPLICATIONS FOR SICKLE CELL DISEASE

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Proximal tubule (PT) dysfunction, including tubular proteinuria, is a significant complication in sickle cell disease (SCD) that can eventually lead to chronic kidney disease. The PT is especially susceptible to cytotoxic damage, and tubular dysfunction in SCD is thought to result from prolonged exposure to hemoglobin (Hb) released from damaged red blood cells. Filtered Hb dimers are internalized into PT cells upon binding to the multiligand receptors megalin and cubilin. These receptors bind to numerous filtered proteins, including albumin and vitamin D binding protein, and are important for maintaining vitamin D homeostasis and protein-free urine. We found that concentrations of Hb predicted to enter the tubule lumen during hemolytic crisis profoundly inhibit the uptake of other megalin/cubilin ligands (albumin and vitamin D binding protein) by PT cells. These effects were independent of heme reduction state, occurred in the absence of a cytotoxic response, and appear to be due to direct competition for megalin/cubilin binding. The Glu7Val Hb mutant that causes SCD was equally effective at inhibiting albumin uptake compared with wild type Hb. Haptoglobin restored albumin uptake in the presence of

Hb, suggesting that haptoglobin binding to the Hb  $\alpha\beta$  dimer interferes with Hb binding to megalin/cubilin. BLAST searches and structural modeling analyses revealed regions of similarity between Hb and albumin that map to this region and may represent sites of Hb interaction with megalin/cubilin. Using these data, we established a robust, scalable assay that enables us to screen for selective inhibitors of Hb uptake that preserve PT function. Our studies suggest that the primary cause of tubular proteinuria in SCD is impaired endocytosis of megalin/cubilin ligands due to competition from filtered Hb. Our results have therapeutic implications for SCD, as preventing Hb uptake is predicted to slow the progression of kidney disease. Additionally, our data suggest a potential explanation for the vitamin D deficiency commonly observed in sickle cell patients. Ongoing studies include quantitation of vitamin D metabolites in PT cells to assess the impact of Hb inhibition of vitamin D binding protein uptake, and experiments to refine our screen for inhibitors of Hb uptake. Sources of support: National Institutes of Health R01 DK101484, R01 DK100357, P30 DK079307T32; Pittsburgh Heart Lung and Blood Vascular Medicine Institute P3HVB pilot grant.

## 10.3

### REGULATION OF RENAL HEPCIDIN IN SICKLE CELL DISEASE MICE

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Hemolysis and frequent blood transfusions lead to the iron overload and organ iron accumulation in patients with red blood cells disorders. The pattern of iron accumulation within different organs is disease specific. Sickle cell disease (SCD), unlike  $\beta$ -thalassemia, is characterized by abnormalities of renal iron metabolism. Renal iron deposition does not correlate with iron overload and blood transfusion. Transgenic SCD mice accumulate iron in the epithelial cells of proximal tubules and represent a suitable model to study iron metabolism in SCD patients. To characterize proteins of the renal iron metabolism in SCD mouse model, RNA and proteins were isolated from the kidney of 5 months old transgenic SCD (Townes) and control mice. Western blot, ELISA, and quantitative RT-PCR

were used to measure levels of renal hepcidin, ferroportin, transferrin receptor (TFR1), divalent cation receptor (DMT1), ferritin, and hephaestin in the renal cortex. Immunostaining was used for detection of renal iron accumulation on paraffin-embedded sections. Significant accumulation of iron was found in the epithelial cells of proximal tubules in SCD mice. Also, we found an increased expression of renal hepcidin in SCD mice compared to controls and, surprisingly, decreased mRNA levels of all other proteins involved in renal iron metabolism (ferroportin, TFR1, DMT1, ferritin, and hephaestin). In contrast, increased levels of transferrin receptor, ferritin, and ferroportin were observed alongside with a significant renal macrophages infiltration in SCD mice. These findings suggest that increased levels of renal hepcidin expression in SCD mice may be associated with renal inflammation. Also high levels of locally expressed hepcidin may lead to the partial degradation of ferroportin and significantly impair iron export from renal epithelial cells thus leading to the intracellular iron accumulation.

**ACKNOWLEDGMENTS:** This work was supported by NIH Research Grants 1P50HL118006, 1R01HL125005 and 5G12MD007597. The content is solely the responsibility of the authors and does not necessarily represent the official view of NHLBI, NIMHD or NIH.

#### 10.4

##### WHICH ALPHA GLOBIN GENE IS PRIMARILY EXPRESSED IN THE VASCULAR ENDOTHELIUM?

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People with Sickle Cell Disease (SCD) who co-inherit one or two deletions of the alpha globin gene (a condition called alpha thalassemia) are protected against cerebrovascular and renal complications of SCD. Alpha globin was recently discovered in the endothelium of resistance arteries, where it interacts with endothelial nitric oxide synthase (NOS3) to regulate the diffusion of nitric oxide, raising the question of how endothelial alpha globin may modulate vascular pathophysiology in SCD.

While it is known that alpha globin locus *HBA2* contributes 65% of total alpha globin in red blood cells in humans, the expression profile of each locus in the vasculature is currently unknown. Determining total and relative locus-specific expression of alpha globin in resistance arteries is critical to understand the consequences of *HBA1* or *HBA2* locus deletion. To measure the expression of alpha globin across major organ systems, perfused vessels and whole blood were collected from C57Bl/6J mice. Conduit arteries (thoracic aorta and carotid arteries) and resistance arteries (middle cerebral arteries, skeletal muscle arterioles, mesenteric arteries, and renal arterioles) were dissected

and placed in RNAlater. Total mRNA was isolated from homogenized vessels (RNeasy, Qiagen) and whole blood (RiboPure Blood Kit, Ambion), and converted to cDNA (SuperScript IV VIL0, Invitrogen).

Absolute gene expression of *Hba-a1* (mouse homolog of *HBA2*), *Hba-a2* (mouse homolog of *HBA1*), *Nos3*, and *Ae1* (erythrocyte anion exchanger) was quantified by digital droplet PCR (BioRad). *Ae1* was highly expressed in whole blood, but not in vascular tissue, confirming that vessels were clear of red cells. Conversely, *Nos3* was expressed in all vessels, but not in whole blood. Abundant expression of *Hba-a1* and *Hba-a2* was observed in whole blood, as well as in all vascular tissues. In whole blood, the *Hba-a1/Hba-a2* ratio was (2.55:1), consistent with expression in human blood. However, in all arteries and arterioles, the *Hba-a1/Hba-a2* ratio was inverted (0.60:1).

We report robust, locus-specific expression of alpha globin in six anatomically distinct arteries. The expression ratio of *Hba-a1* and *Hba-a2* is inverted between vascular tissue and whole blood in mice, suggesting differential regulation of alpha globin transcription. Further investigation into the physiologic role of endothelial alpha globin may provide new insights into how alpha thalassemia modulates vascular pathologies associated with SCD.

#### 10.5

##### ENDURANCE TRAINING DOES NOT CORRECT METABOLIC ABNORMALITIES RELATED TO ISCHEMIA - REPERFUSION IN SICKLE CELL DISEASE MICE

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Sickle cell disease (SCD) is a genetic hemoglobinopathy characterized by the occurrence of painful vaso-occlusive crises (VOC) in many tissues including skeletal muscle. Yet, the muscle metabolic abnormalities related to VOC are still unknown in SCD. Our first aim was to evaluate the impacts of ischemia/reperfusion cycle on muscle energetic metabolism in SCD. We aimed secondly at determining whether endurance training could alleviate some of these possible metabolic defects.

Ten control (HbAA), 13 heterozygous (HbAS), 10 sedentary SCD (HbSS-SED) and 9 endurance-trained SCD (HbSS-END) mice were submitted to a standardized rest – ischemia (30 min) – reperfusion (25 min) protocol during which ATP and phosphocreatine (PCr) concentrations, as well as intramuscular pH were measured using <sup>31</sup>-phosphorus magnetic resonance spectroscopy. Forty-eight hours later, skeletal muscles were sampled. While the time-courses of

ATP (that was fairly stable throughout the protocol) and PCr (that decreased linearly) concentrations were similar among groups during the ischemic period, both HbSS-SED and HbSS-END displayed a larger acidosis as compared to the HbAA and HbAS groups ( $p < 0.01$ ) during the same period, with no difference between HbSS-END and HbSS-SED mice. During the reperfusion period, the initial rate of phosphocreatine resynthesis was slower in HbSS-SED and HbSS-END compared to HbAA ( $p < 0.05$ ) and HbAS ( $p < 0.01$ ) animals. The total hindlimb muscles weight was lower in the hindlimb submitted to the ischemia/reperfusion protocol as compared to the control hindlimb ( $p < 0.001$ ). In conclusion, SCD mice displayed an exacerbated intramuscular acidosis in response to ischemia, while the subsequent reperfusion disclosed an impaired skeletal muscle oxidative capacity. Interestingly, these metabolic defects were not improved as a result of endurance training.

#### 10.6

##### **TRANSIENT DESATURATION CHALLENGE REVEALS WHITE MATTER MICROVASCULAR DISEASE IN PATIENTS WITH SICKLE CELL DISEASE.**

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**Introduction:** Patients with sickle cell disease (SCD) have chronic, progressive, white matter disease that impairs cognitive function, quality of life, and employment. Patients with sleep apnea also suffer from similar neurologic sequelae. Since many sickle cell disease patients also exhibit sleep apnea, we devised a MRI imaging protocol to study the impact of transient hypoxia on cerebral blood flow and saturation.

**Methods:** We performed Blood Oxygen Level Dependence(BOLD) MRI and cerebral near infrared spectrometry(NIRS) in 28 SCD patients, 12 patients with non sickle anemia syndromes and 31 age and race matched control subjects. Hypoxia was induced by changing the inhaled gas mixture from room air to 100%

nitrogen for five breaths (20-25 seconds), resulting in transient desaturations of 10% - 30%.

**Results:** Changes in peripheral oxygen saturation were mirrored by increased NIRS deoxyhemoglobin, decreased NIRS oxyhemoglobin, and decreased BOLD signal. NIRS total hemoglobin concentration increased post hypoxia, suggesting a small compensatory hyperemic response. BOLD and NIRS changes were temporally concordant with one another but began prior to detectable changes in pulse oximetry, reflecting shorter transit times to the brain compared with the finger. Peak pulse oximetry, BOLD, and NIRS changes were all inversely related to hemoglobin concentration, consistent with higher cardiac output and cerebral blood flow in anemic subjects. In the BOLD signal, the induced desaturation behaved like a contrast bolus. Anemic subjects had more rapid desaturation wash in, shorter time to peak and more rapid recovery over most of the brain. However, time-to-peak and recovery half-time were pseudonormal (not decreased relative to controls) in white matter regions at greatest risk for stroke.

**Conclusions:** Taken together, patients with chronic anemia had more rapid and severe cerebral desaturation in response to transient hypoxia because of their increased pulmonary and cerebral blood flow. The induced desaturation pulse exposed subtle differences in time-to-peak and recovery half-life consistent with microvascular damage in watershed areas. BOLD changes were mirrored by changes in cerebral NIRS, supporting BOLD as a metric of tissue oxygenation. Subsequent studies will explore the relative importance of hemoglobin S levels compared with changes in total hemoglobin concentration.

#### 10.7

##### **TRANSFUSION THERAPY IMPROVES MULTILEVEL VASCULAR DYSFUNCTION IN SICKLE CELL DISEASE: ARE MULTIPLE LEVELS OF THE VASCULATURE PATHOPHYSIOLOGICALLY LINKED?**

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Objective: To determine whether chronic transfusion therapy improves multiple levels of vascular dysfunction in patients with sickle cell anemia.

Sickle cell disease (SCD), the most common inherited disease in the world, is a model of diffuse vascular disease. The etiology of vascular dysfunction is multifactorial, including decreased nitric oxide bioavailability, chronic inflammation, increased erythrocyte adhesion to the endothelium, and decreased erythrocyte deformability. Together, these affect multiple levels of the vasculature; however, multi-level vascular assessment has not been systematically evaluated. Using a forearm ischemia model, we simultaneously assessed microcirculatory post occlusive hyperemia (PORH) using laser Doppler flowmetry of the nailbed, tissue oxygenation using near infrared spectroscopy (NIRS) over the dorsum of the hand and flow mediated dilation (FMD) of the brachial artery. We evaluated blood viscosity, erythrocyte deformability and erythrocyte aggregation in addition to blood cell counts, markers of hemolysis and inflammation. The Children's Hospital Los Angeles review board approved this protocol. We enrolled 18 healthy, 75 non-transfused SCD and 26 chronically transfused SCD patients. All three levels of the vasculature were diseased in non-transfused SCD patients compared to healthy, with lower FMD ( $P=0.04$ ), lower PORH ( $P=0.003$ ) and lower NIRS ( $P<0.0001$ ). Chronic transfusion improved FMD and NIRS, by 23% ( $P=0.08$ ) and 32% ( $P<0.0001$ ) respectively, but not microcirculatory PORH. Consistent with our previously published data, plasma free hemoglobin was an independent predictor of FMD ( $P<0.0001$ ) and we also found it was an independent predictor of NIRS ( $P=0.003$ ) in non-transfused SCD patients. Further, there is a strong positive association between tissue oxygenation and FMD after controlling for transfusion status ( $P=0.0009$ ). There is no association between microcirculatory PORH and FMD or NIRS, nor did markers of rheology, inflammation or hemolysis predict PORH. Baseline resting laser Doppler flow was the strongest predictor of PORH.

Chronic transfusion therapy improves tissue oxygenation and FMD but not microcirculatory post occlusive hyperemia. FMD and tissue oxygenation have similar pathophysiologic effectors, such as plasma free hemoglobin, while microcirculatory PORH is primarily determined by baseline resting flow.

Funding support: HL099412-01 (JW), NIH# 1 K23 HL 119627-01A1 (JD), NIH# 1U01 HL 117718-01 (TC and JW)

**10.8**  
**EXERCISE (IN)TOLERANCE IN SICKLE CELL DISEASE: POTENTIAL DISRUPTIVE ROLE OF FREE HEMOGLOBIN ON**

**SKELETAL MUSCLE OXYGEN DELIVERY/UTILIZATION MATCHING AND FUNCTIONAL CAPACITY**

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Sickle cell disease (SCD) reduces exercise tolerance likely due to vascular and skeletal muscle abnormalities stemming from increased free hemoglobin (Hb, a known scavenger of nitric oxide). However, there has been little advancement in our understanding of the precise mechanisms responsible for the reduction in physical functionality. Therefore, the purpose of this investigation was 1) determine the degree by which exercise tolerance is impaired in mice expressing human HbSS (BERK) and 2) examine the impact of free Hb on the skeletal muscle microvascular  $PO_2$  ( $PO_2mv$ , the principal driving force that facilitates blood-muscle  $O_2$  flux) at rest and during contractions in rats. We hypothesized that exercise capacity would be lower in BERK relative to wild-type mice (WT) with a lower  $PO_2mv$  observed following Hb infusion. Twenty female mice (WT,  $n=10$  and BERK,  $n=10$ ) performed 4 constant-speed treadmill tests that resulted in fatigue within 1.5 to 20 min. Time to fatigue vs. treadmill speed were fit to a hyperbolic model to determine critical speed (CS).  $PO_2mv$  was measured during 180 s of electrically induced muscle contractions during control, following free Hb infusion (Hb, 50mg), and L-nitro arginine methyl ester superfusion (L-NAME, 1.5 mM) conditions in 9 rats. Speed and time to exhaustion for WT and BERK conformed to a hyperbolic relationship (WT:  $r^2 = 0.93 \pm 0.02$ , BERK:  $r^2 = 0.97 \pm 0.01$ ,  $p>0.05$ ). CS was significantly lower in BERK when compared to WT (WT:  $33.1 \pm 1.5$ , BERK:  $25.2 \pm 0.7$  m/min,  $p<0.05$ ). Following the onset of contractions, Hb and L-NAME significantly increased the amplitude of the fall in  $PO_2mv$  when compared to control, with no significant differences between Hb and L-NAME conditions ( $\Delta 1PO_2mv$ : control:  $9.5 \pm 0.7$ , Hb:  $11.7 \pm 1$ , L-NAME  $10.4 \pm 0.8$  mmHg,  $p<0.05$ ). The increased  $\Delta 1PO_2mv$  resulted in a significantly lower  $PO_2mv$  during the steady-state of muscle contractions in both Hb and L-NAME conditions, with no differences between Hb and L-NAME ( $PO_2mv(\text{steady-state})$ : Control:  $24.1 \pm 0.9$ , Hb:  $21.3 \pm 0.7$ , L-NAME:  $19.6 \pm 1$  mmHg,  $p<0.05$ ). To summarize, exercise tolerance, as measured via CS, was significantly lower in BERK mice relative to WT. Furthermore, the lower  $PO_2mv(\text{steady-state})$  in Hb and L-NAME represents a compromised blood-myocyte  $O_2$  driving force during muscle contractions. Collectively, these data suggest that SCD impacts physical capacity via a disruption in the tight

matching between oxygen delivery and utilization within the skeletal muscle.

**10.9**

**ASSOCIATIONS BETWEEN CARDIORESPIRATORY FITNESS AND ARTERIAL FUNCTION IN ADOLESCENTS WITH SICKLE CELL ANEMIA**

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**Introduction:** Sickle cell anemia (SCA) results in consequences to the cardiovascular system, due to chronic inflammation and endothelial dysfunction. Individuals with SCA have been shown to have a decreased peak aerobic capacity ( $VO_{2peak}$ ); however, the physiological basis for this limitation has yet to be fully understood. Proposed factors include chronic anemia and poor cardiopulmonary function. Endothelial dysfunction, assessed via flow mediated dilation (FMD), may also contribute to reduced  $VO_{2peak}$ . **Purpose:** To explore relationships between  $VO_{2peak}$  and markers of endothelial function in adolescents with SCA. Also, to compare indices of arterial health in adolescents with and without SCA. **Methods:** Eleven adolescents with SCA ( $13\pm 1$  yrs,  $19\pm 4$  kg/m<sup>2</sup>) and 11 ethnicity-, age-, and sex-matched controls ( $13\pm 1$  yrs,  $27\pm 6$  kg/m<sup>2</sup>) underwent a standardized FMD protocol.  $VO_{2peak}$  was measured in SCA using indirect calorimetry on a cycle ergometer. Associations between fitness and arterial function were determined with bivariate correlations, and the Mann Whitney Wilcoxon rank sum test was used to explore potential differences in endothelial function between adolescents with and without SCA. **Results:** Adolescents with SCA tolerated FMD without adverse events.  $VO_{2peak}$  was not associated with FMD % or FMD area under the curve ( $R^2$  of 0.011 and 0.058, respectively). Comparisons of endothelial function between those with and without SCA are shown in Table 1. Baseline as well as peak velocity and shear during FMD testing were significantly higher in the brachial arteries of adolescents with SCA. **Conclusion:** Despite having a greater artery wall stimulus both at baseline as well as during FMD, adolescents with SCA exhibited a similar % change in brachial artery diameter compared to controls. Also,  $VO_{2peak}$  was not associated with endothelial function. Further research with a larger sample size is warranted in adolescents and adults with SCA. **Funding:** NHLBIK23HL094376 (Liem)

**Table 1**

Variable	Group		P-Value
	Sickle Cell	Control	
n	11	11	
SBP (mmHg)	113±9	110±7	0.62
DBP (mmHg)	59±9	56 ± 5	0.26
Baseline Diameter (mm)	3.3±0.004	3.3±0.003	0.76
Baseline Velocity (m/sec)	1.08±0.25	0.58±0.11	<0.001
Baseline Shear (sec <sup>-1</sup> )	331±84	181±35	<0.001
FMD (%)	7±3	9±5	0.44
FMD AUC	681±379	913±635	0.72
Peak Shear (sec <sup>-1</sup> )	561±72	326±70	<0.001
Peak Velocity (m/sec)	1.77±0.23	0.97±0.14	<0.001

Data are mean ± SD. P-value < 0.05 demonstrates significant differences between means.

**10.10**

**ESTIMATION OF GFR IN ADULT PATIENTS WITH SICKLE CELL DISEASE: SERUM CREATININE AND CYSTATIN-C BASED ESTIMATION EQUATIONS OVER-ESTIMATE AND ARE POORLY PREDICTIVE OF TRUE GFR.**

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Epidemiological studies of chronic kidney disease (CKD) in adults with sickle cell disease (SCD) show a high prevalence of abnormal albuminuria but relatively low rates of low GFR, based on serum creatinine (Scr) or creatinine-based eGFR equations. This gap could result from the lower Scr values observed in SCD vs non-SCD patients, possibly related to lower muscle mass and/or differences in tubular creatinine handling. Recently, Cystatin C and cystatin-C based eGFR equations have been suggested to better estimate CKD in non-SCD populations, but they have not been validated in SCD.

Our goal was to compare the ability of Scr and Cystatin-C based eGFR to estimate true GFR in SCD and to analyze the implications of the performance of the different eGFR equations in sickle cell patients.

Eighty-five adult SCD patients (18-65 years, male n=41, female n=44, Hb SS n=69, other sickle Hb n=16) had the GFR measured by the gold-standard urinary clearance of inulin or iothexol on 159 occasions, and were compared with eGFR derived from Scr or Cyst-C based formulas: 1) Cockcroft-Gault, 2) MDRD, 3) CKD-Epi-creat, 4) CKD-Epi-Cysc, and 5) combined CKD-EPI Cr/Cysc equations. Scr ranged from 0.30 to 7.65 mg/dL, serum Cystatin C from 0.45 to 3.4 mg/L, and GFR between 5 and 165 ml/min/1.73m<sup>2</sup>.

All equations overestimated true GFR, ranging between 35% and 60% for different equations. The lowest overestimation was with the CKD-Epi-Cys equation, but still was 35%, on average. We determined the accuracy of

the equations as the percentage of estimated values within 10% (P10), 30% (P30) and 50% (P50) of the true values. All equations lacked accuracy: only about 10-15%, 30-45% and 48-65% of the estimated values were within P10, P30 and P50%, respectively. When restricting the GFR to  $> 90$  ml/min/1.73, the equations performed numerically slightly better, but P10 was only 20-30% of values and P30 ranged between 35-75%.

We conclude that none of the current estimation GFR equations based on Scr or cystatin C accurately estimate true GFR in adults with SCD. Moreover, all of them significantly overestimate the true GFR (35-60% on average). Only patients with advanced renal insufficiency are identified with current equations. We conclude that measurement of GFR is needed when accurate determination of GFR is required, and that new clinical markers or biomarkers of CKD are needed to assess CKD in adults with SCD.

#### 10.11

##### **CIRCULATING EXOSOMES FROM PATIENTS WITH SICKLE CELL REGULATE PATHWAYS OF INFLAMMATION AND ENDOTHELIAL INTEGRITY**

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Abstract; Sickle Cell Disease (SCD) causes intermittent, cumulative endothelial damage, which results in significant end organ damage.(1) We have recently demonstrated that exosomes isolated from patients with SCD cause disruption in endothelial monolayers *in vitro* as measured via electric cell-substrate impedance sensing and as visualized with immunofluorescence.(2,3) The exact pathways regulated by exosomes from patients with SCD remain unknown. Given the critical role of the endothelium in SCD, we hypothesized that exosomes from patients with SCD change behavior of endothelial cells via gene expression. We collected platelet-free plasma when patients were clinically well from patients with SCD and control patients, HgbAA. Patients with SCD were excluded if they had asthma, obesity or a history of Acute Chest Syndrome, as we know that those exosomes also have unique characteristics. Exosomes were isolated from plasma using the Total Exosome Isolation Kit. We then treated HMVEC-d cells for 24 hours with exosomes followed by isolation of RNA for microarray analysis to examine gene expression. Bioinformatic analysis of microarrays was performed including gene ontology and

differential pathway analysis, leading to the identification of 364 genes with a q-value  $< 0.05$  and fold-change  $> 1.2$  that are differentially regulated by SCD-derived exosomes compared to control-derived exosomes. The identified genes are in pathways of innate immune response, cell structure, nitric oxide signaling, and inter-cellular junctions. In conclusion, we provide evidence that exosomes from patients with SCD regulate a unique set of pathways important in maintaining endothelial integrity. Further analysis will be done to validate these genes (including qRT-PCR). Funded by NIH CTSA, Grant numbers: 5K12HL119995, UL1TR000430.

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#### 10.12

##### **IDENTIFICATION OF URINARY HGFL PROTEIN AS A POTENTIAL MARKER FOR THE DEVELOPMENT OF CHRONIC KIDNEY DISEASE IN SICKLE CELL DISEASE PATIENTS**

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Chronic kidney disease (CKD) is common in patients with sickle cell disease (SCD). However, the progression of CKD in SCD and factors associated with such progression remain poorly defined. The purpose of this study was to identify the potential markers associated with CKD progression in patients with SCD. Since glomerular hyperfiltration is an early stage of renal dysfunction, we performed label-free quantitative proteomic analysis for urine samples collected from SCD patients with hyperfiltration (N=3) and normal (N=3). Hepatocyte growth factor-like (HGFL) protein was found to be significantly downregulated (5.52-fold,  $p=8.05 \times 10^{-5}$ ) in samples with glomerular hyperfiltration compared to

normal group. Next, we developed a high resolution/selected ion monitoring (HR/SIM) method by measuring the HGFL peptide ( $m/z$  585.79) with isotope labeled-HGFL peptide ( $m/z$  590.80) as internal standard (IS). HR/SIM quantification was performed for 19 urine samples from SCD patients and 12 urine samples from healthy controls. HGFL levels were found to be significantly downregulated ( $p=0.0084$ ) in the SCD urine samples compared to samples from healthy controls. To further assess the correlation between HGFL level and CDK stage, we expanded the analysis to SCD patients with different CKD stages ranging from 0 to 5 and 19 healthy individuals by ELISA. The result confirmed the finding of HR/SIM quantification, moreover, showed that urinary HGFL level correlated with CKD stage ( $R=0.17$ ) and showed high sensitivity and specificity by ROC analysis ( $AUC=0.78$ ). HGFL protein has been identified as a negative regulator of phosphatidylinositol 3-kinase (PI3K), and PI3K/Akt pathway was found to be activated in the progress of CKD. Therefore, the decrease of HGFL level in urines from SCD patients may indicate the development of CKD. A limitation of our study is the small number of samples from high stage (4 or 5) of CDK patients used for the correlation analysis.

**ACKNOWLEDGMENTS:** This work was supported by NIH Research Grants 1P50HL118006, 1R01HL125005, 5G12MD007597 and K23HL125984. The content is solely the responsibility of the authors and does not necessarily represent the official view of NHLBI, NIMHD or NIH.

### 10.13

#### THE PLASMA PROTEOME OF SICKLE CELL PAIN CRISIS

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People living with sickle cell disease experience severe episodic pain crises that require hospitalization for pain management and supportive care. Hydroxyurea, blood transfusion, and emerging anti-adhesive therapies have been used to reduce the frequency or severity of pain crises; however, questions remain regarding the etiology, diagnosis and optimal treatment of sickle cell pain crisis. We used proteomics to quantify changes in the abundance of plasma proteins that occur during sickle cell pain crisis.

We obtained EDTA plasma from 7 adults with sickle cell disease within 36 hours of admission to the NIH Clinical Center for treatment of pain crisis and at a follow up visit after resolution of pain crisis (clinicaltrials.gov #NCT01568710). We also studied plasma from two independent reference groups: 7 adults with sickle cell

disease in steady state, and 6 healthy African American adults. Samples were TMT-labeled and analyzed by high performance liquid chromatography followed by surface-enhanced laser desorption/ionization time of flight mass spectrometry. Proteins were identified by mass of peptide fragments using Proteome Discovery 2.0beta. We calculated fold change for each protein comparing pain crisis against recovery in paired analyses. In addition, we analyzed quantitative differences between crisis, steady state and healthy groups.

1334±128 proteins were quantified. Cell regulation proteins, metabolic proteins and cell organization proteins accounted for 19.6%,17.5% and 10.4% respectively of the identified proteins. Haptoglobin was significantly lower in plasma from patients with sickle cell disease. We identified changes in the concentrations of proteins involved in immunity, coagulation, and erythrocyte functions. The changes in these proteins, as well as proteins with incompletely defined functions, provide novel insight into the pathogenesis of sickle cell pain crisis.

### 10.14

#### URINE PROTEOMIC ANALYSIS IDENTIFIES CERULOPLASMIN AS A BIOMARKER OF CHRONIC KIDNEY DISEASE IN SICKLE CELL DISEASE PATIENTS

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Chronic kidney disease (CKD) is a complication of sickle cell disease (SCD) that is associated with early mortality. We used high resolution mass spectrometry to analyze urine samples collected before the onset or in the early stage of kidney disease. Urine from patients with late stages of CKD contains large quantities of plasma proteins that overwhelm and complicate mass-spectrometry analysis. As hemoglobinuria is associated with CKD progression in SCD patients and glomerular hyperfiltration is an early stage of renal dysfunction, we compared urine samples from patients with normal GFR and hemoglobinuria (n=2), with glomerular hyperfiltration but not hemoglobinuria (n=12) and with normal GFR but not hemoglobinuria (n=6) to identify biomarkers of the early stages of CKD. Label-free quantitative proteomic analysis showed greater ceruloplasmin (CP) levels in the samples with hemoglobinuria. To test whether urine ceruloplasmin and other proteins of iron metabolism correlate with CKD stage, we measured urine ceruloplasmin, transferrin (TF),

ferritin (FT) and free hemoglobin (Hb) concentrations by ELISA in these patients plus 34 additional SCD patients with CKD stage ranging from 0 to 5 and in 19 healthy individuals. The urinary levels of CP, TF, FT and Hb were all significantly higher in all tested SCD patients comparing to healthy controls. CP concentrations demonstrated a strong correlation with urinary Hb, and both CP and Hb concentrations correlated with CKD disease stage and showed high sensitivity and specificity by ROC analysis. Abnormal renal iron metabolism including cortical iron deposition is characteristic of SCD nephropathy. CP facilitates cellular iron export by ferroportin and iron binding by TF. While TF-bound iron is reabsorbed in renal tubules in healthy individuals, increased urinary TF is found in type 2 diabetes patients along with increased urinary CP. Increased urinary CP may reflect increased intra-glomerular hydraulic pressure. While we found significantly increased urinary TF levels in SCD patients, TF did not correlate with CKD stage. In conclusion, urinary CP may represent a non-invasive biomarker for CKD in SCD patients.

This work was supported by NIH Research Grants 1P50HL118006, 1R01HL125005, 5G12MD007597 and K23HL125984. The content is solely the responsibility of the authors and does not necessarily represent the official view of NHLBI, NIMHD or NIH.

#### 10.15

##### EFFECTS OF SICKLE CELL DISEASE ON THE RIGHT VENTRICLE AND PULMONARY VASCULATURE

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Sickle cell disease (SCD) red blood cells (RBC) are more rigid than healthy RBCs, are frequently trapped in the microcirculation, and lyse easily, leading to chronic anemia. Recent studies demonstrated that coronary microvascular disease, myocardial fibrosis, decreased right ventricular (RV) ejection fraction, RV dilatation and RV hypertrophy are associated with SCD [1,2]. Patients with SCD-related pulmonary hypertension (PH) have low survival -- 50% within 2 years -- and a substantially increased risk of sudden death compared to those with SCD alone [1]. The goal of this study was to quantify how SCD affects RV function, the interactions between the RV and the pulmonary vasculature, and RV afterload. We hypothesized that SCD mice would have poor RV function, impaired ventricular-vascular coupling (VVC) measured with pressure-volume loops, and increased pulmonary vascular resistance as measured by pulmonary vascular impedance (PVZ) compared to healthy control mice, which

would be exacerbated in SCD mice after exposure to acute hypoxia.

**Methods:** 12 male C57Bl6 mice (CTL) and 12 Berkeley SCD mice (SCD) aged 20-24 weeks were cannulated with a pressure-volume (PV) catheter. After initial RV PV measurements were obtained, the inferior vena cava was briefly occluded to calculate end-systolic and end-diastolic pressure relations. In separate CTL and SCD mice, a pressure catheter was advanced into the main pulmonary artery (PA) while echocardiography simultaneously measured PA flow velocity and PA diameter for PVZ. Measurements were obtained under normoxic ventilation (21% oxygen) and, subsequently, after 5 minutes of acutely hypoxic ventilation (10% oxygen) in both studies. It is worth noting that 10 of 12 SCD mice did not survive hypoxia, while all CTL animals survived.

**Results and Discussion:** Cardiovascular hemodynamics in SCD mice were not significantly different from CTL mice under baseline conditions, however pulmonary vascular resistance and RV volume tended to be elevated in SCD mice and VVC tended to be lower. Despite apparently normal ventricular and vascular function absent of development of PH, SCD mice could not tolerate the insult of acute hypoxia.

This work was supported by NIH grant R21HL129047 (NCC), and NIH F31 HL128088-01 (DAS).

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#### 10.16

##### URINARY OROSCOMUCOID CONCENTRATION CORRELATES WITH CHRONIC KIDNEY DISEASE IN SICKLE CELL DISEASE PATIENTS

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Chronic kidney disease (CKD) is a prevalent complication of sickle cell disease (SCD) and associated with early mortality. Discovery and validation of non-invasive biomarkers for early stage renal disease is needed identify and facilitate CKD treatment in SCD. Here, we performed comparative proteomic analysis of urine samples collected from SCD patients with hemoglobinuria (N=2), hyperfiltration (N=3) and normal (N=3). We observed upregulation of orosomucoid in samples with hemoglobinuria versus control (49.93-fold,  $p=1.9 \times 10^{-10}$ ).

We next validated presence of oroscomuoid in urine by ELISA and also expanded the analysis to additional SCD patients with CKD stage ranging from 0 to 5 and in 19 healthy individuals. The urinary level of oroscomuoid was significantly higher in all tested SCD patients comparing to healthy controls. Oroscomuoid concentrations correlated with CKD disease stage and showed high sensitivity and specificity by ROC analysis. Moreover, oroscomuoid concentrations showed strong correlation with urinary free hemoglobin concentrations ( $R=0.45$ ), an established marker of CKD in SCD. Oroscomuoid concentrations also correlated well with ceruloplasmin ( $R=0.62$ ) that we recently identified as a potential biomarker of CKD in SCD. Oroscomuoid is involved in inflammation, and its increased levels were found in urine of type 2 diabetes patients. Oroscomuoid is also an independent factor for diabetic microvascular complication. Microvascular complications and vaso-occlusive crisis are hallmarks of SCD. Taken together, urinary oroscomuoid may represent a non-invasive biomarker for CKD in SCD patients.

**ACKNOWLEDGMENTS:** This work was supported by NIH Research Grants 1P50HL118006, 1R01HL125005, 5G12MD007597 and K23HL125984. The content is solely the responsibility of the authors and does not necessarily represent the official view of NHLBI, NIMHD or NIH.

#### 10.17

#### HEME AND FREE IRON- MEDIATED OXIDATION OF PLASMA LIPIDS IN SICKLE CELL DISEASE PATIENTS UNDERGOING REGULAR EXCHANGE BLOOD TRANSFUSION

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**Background:** Red blood cells of SCD patients are prone to sickling, hemolysis and heme release. Increased plasma heme is linked to vasculopathy. The intercalation of heme into plasma lipoproteins promotes LDL oxidation in vitro and may define heme-to-lipid transfer in plasma. Transient free iron is also common in regularly transfused patients.

Together heme and intermittent iron exposures may be relevant contributors to vascular dysfunction in transfused SCD patients.

**Objectives:** (1) characterize heme, iron, cholesterol, LDL, HDL and endothelial dysfunction markers in human plasma; (2) quantify levels of lipid peroxidation in purified HDL and LDL; (3) define relationships between lipid peroxidation to heme and iron levels; (4) evaluate histological markers in vascular tissues.

**Methods:** Plasma samples obtained from SCD patients undergoing regular exchange transfusion and healthy controls were evaluated for heme, iron, lipid levels, and endothelial dysfunction markers. Extraction of HDL and LDL from plasma was performed by density gradient centrifugation. In purified HDL and LDL, oxidized lipids were measured by MDA and Western blotting. Vascular tissue was evaluated for histopathological markers of injury.

**Results:** SCD plasma showed low cholesterol, HDL and LDL levels as well as an increase in heme, iron and markers of endothelial dysfunction. MDA was increased in purified LDL and HDL from SCD patients relative to control. Western blotting revealed bands of oxLDL. MDA found in purified LDL and HDL correlated with plasma heme and free iron concentrations. Individual SCD patients demonstrated a similar extent of oxidation in both LDL and HDL. Purified control LDL and HDL spiked with different heme-albumin concentrations resulted in a dose dependent increase in MDA development. Histological markers demonstrated evidence of injury in vascular tissue.

**Conclusions:** Chronic hemolysis and release of heme into the circulation triggers oxidation of lipoproteins. The oxidation of LDL and HDL is thereby dependent on heme exposure. In regular exchange transfused patients increased free iron levels may further enhance lipid oxidation. These findings may have implications in SCD progression.

Abbreviations: Sickle cell disease (SCD), Low density lipoprotein (LDL), High density lipoprotein (HDL), Malondialdehyde (MDA), Oxidized LDL (oxLDL)

#### 11.0 Poster Session: Lung Physiology/Pathophysiology

#### 11.1

#### EXPOSURE TO MODERATE ALTITUDE ENHANCES PULMONARY VASCULAR DISEASE IN BERKLEY SICKLE CELL MICE

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A number of sickle cell disease (SCD) patients live at moderate altitude with a mean elevation of 5,900 ft. However, it is unclear if lower barometric pressure affects the disease process. Herein we investigated the impact of disease progression in wild type and Berkley sickle cell mice (BERK-SS) over 2.5 months of altitude exposure associated with sea level, Denver (5280 ft) and moderate (8,000 ft) elevations to assess cardiopulmonary dysfunction. Study end points for hemolysis, pulmonary hypertension, right heart function and pulmonary vascular remodeling were assessed. The adaptive balance between pulmonary vascular endothelial nitric oxide synthase (eNOS) and endothelin (ET-1) was studied as to assess differences in lung vasculature adaptation. Mortality of animals was assessed throughout the study. We hypothesized that BERK-SS mice would demonstrate significant deterioration in the defined parameters of morbidity and mortality when compared to WT mice and to BERK-SS exposed to differing altitudes. As expected BERK-SS mice were significantly different from WT mice in all parameters tested, supporting our hypothesis differences within the BERK-SS cohort demonstrated changes associated with increasing altitude. The primary changes observed were increased pulmonary hypertension and evidence of right heart failure in BERK-SS mice exposed to 8,000 ft. consistent with this were differences in the adaptive response to eNOS/ET-1 balance observed in WT mice. We conclude that exposure to moderate and physiologically relevant altitude enhances the progression of pulmonary hypertension in BERK-SS mice compared to healthy wild type cohorts.

## 11.2

### SCOPING REVIEW OF THE LITERATURE ON SICKLE CELL LUNG DISEASE ACROSS THE LIFESPAN

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**Background:** Despite a high and growing global burden of sickle cell disease (SCD) with an estimated 400,000 births per year by 2050, evidence-based interventions against its pulmonary complications are limited. Pulmonary complications account for much of the accelerated mortality observed in the SCD population, yet we know little about their natural history. While practically every structure and cell type of the lung is affected in SCD patients, the inter-relationship between the airways and vasculature in this population is not well described. One of the limitations in SCD research has been a focus on either the adult or pediatric patient group without recognition of the early life origins of cardiopulmonary morbidity and mortality in adulthood. We know that patients with SCD are at significantly increased risk of lung function abnormalities, respiratory symptoms with progressive dyspnea, and sleep disordered breathing, yet we do not understand how each of these phenomena is associated with overall disease pathogenesis and the increased risk of early mortality. Furthermore, the roles that chronic hemolytic anemia, left-sided cardiac dysfunction, intermittent hypoxia, and thrombosis play in modulating the underlying SCD process are not well understood. Therefore, we undertook a scoping review of the literature with systematic search criteria in order to assess the published literature.

**Methods:** A systematic literature search was performed in four main areas of sickle cell lung disease: 1) Acute chest syndrome 2) Airways disease 3) Sleep-disordered breathing and hypoxia 4) Pulmonary vascular disease and thromboembolic disease. Common search parameters used for each topic included the following: human subjects research in both children and adults in the PubMed and Cochrane Library databases from 1970 to April 20, 2017. In addition, search terms specific to each of the 4 areas of sickle cell lung disease were used to conduct the search strategy.

**Results:** A total of 448 articles met the initial search parameters (acute chest [230], pulmonary vascular disease and thromboembolic disease [102], airways disease [78], and sleep-disordered breathing and nocturnal hypoxia [38]).

**Conclusions:** In conclusion, the number of published articles on 4 specific topics of pulmonary sickle cell disease remains low. More high-quality, multicenter studies are needed in order to evaluate clinical outcomes and treatment interventions in pulmonary sickle cell disease.

**12.0 Poster Session: Red Cell Physiology**

**12.1**

**HOW DOES IRON DEFICIENCY REDUCE INTRACELLULAR HEMOGLOBIN IN MICE?**

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Sickle cell disease (SCD) involves polymerization of hemoglobin-S within the red blood cell (RBC). Cells with a lower mean cell hemoglobin concentration (MCHC) of hemoglobin-S exhibit longer delays between deoxygenation and polymerization.

Sickling delay time is dependent on [HbS]<sup>30</sup>. Iron-deficient erythropoiesis results in RBC with lower MCHC, which could reduce sickling and improve red cell survival. One strategy for lowering MCHC in patients is the induction of iron deficiency anemia. Several clinical case reports and one study in a SCD mouse model suggest that low iron availability is associated with less hemolysis, longer cell survival, and improved symptoms of SCD. However, the mechanisms governing chronic induction of iron deficiency anemia in SCD are not currently well understood.

Data from a four-week pilot study shows iron deficiency anemia induces a reduction in RBC count, hemoglobin, and hematocrit. For this pilot, half of the mice received a single dose of recombinant erythropoietin (EPO, 1000 IU/KG) to accelerate RBC production. EPO effectively stimulated erythropoiesis but did not increase platelets count. At four weeks, MCH and MCV of mature RBCs haven't had changed but reticulocyte hemoglobin content (CHR) was decreased.

To that extent, the purpose of the present study is to characterize the timing of dynamic hematologic changes, that occur within the bone marrow and blood during the onset of iron deficiency anemia, using an iron-restricted diet in 6-week old male and female C57Bl/6J mice over a period of twelve weeks. Blood samples and bone marrow are collected at two-week intervals; blood is processed for CBC and ELISA for serum iron markers, and bone marrow is evaluated by flow cytometry to study erythroblast maturation.

This study will provide a clear picture of the hematologic and physiological changes that occur in the bone marrow and blood during the onset of iron deficiency anemia. This data will help inform a strategy for using iron deficiency anemia to modify MCHC in RBC as a potential treatment for SCD.

**12.2**

**TR4 HAPLOINSUFFICIENCY RESULTS IN DECREASED PROLIFERATION AND MATURATION DURING ERYTHROPOIESIS.**

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The orphan nuclear receptors TR4 (NR2C2) and TR2 (NR2C1) are the DNA binding components of the repressor complex, *direct repeat erythroid-definitive* (DRED), which represses the transcription of  $\epsilon$ - and  $\gamma$ - globin during adult definitive erythropoiesis. Previously, several studies have implied that TR2 and TR4 can act largely in a redundant manner during erythroid differentiation; however, our laboratory has observed variably penetrant phenotypes in the *Tr4* mutants, suggesting that indirect effects of the genetic deletion might be masked by multiple modifying genes. In order to test this hypothesis, *Tr4* heterozygous mutant mice were bred into a congenic C57BL/6 background and their phenotypes were reexamined. Surprisingly, the homozygous *Tr4* null mutant mice expired early during embryogenesis, at approximately embryonic day (E) 7.0 well before erythropoiesis commences. Further examination found that *Tr4*<sup>+/-</sup> erythroid cells failed to fully differentiate and exhibited diminished proliferative capacity and no changes in apoptosis. Furthermore, reduced TR4 abundance resulted in decreased expression of genes required for heme biosynthesis and erythroid differentiation (*Alad* and *Alas2*), but led to significantly increased expression of the proliferation inhibitory gene, cyclin dependent kinase inhibitor 1c, *Cdkn1c*. The cellular differentiation and abundance defects were only observed within the erythroid cell populations. These studies support a vital role for TR4 in promoting erythroid maturation and proliferation, and demonstrate that TR4 and TR2 execute distinct, individual functions during embryogenesis and erythroid differentiation.

**12.3**



### HAPTOGLOBIN GENOTYPE IS ASSOCIATED WITH INCREASED MORBIDITY IN ADULTS WITH SICKLE CELL DISEASE

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**Objective:** Chronic hemolysis is a defining feature of sickle cell disease (SCD), and intensifies during times of illness. This process leads to the release of cell free hemoglobin, which has been associated with an increased odds of developing acute chest syndrome (ACS) in patients with SCD. Haptoglobin, the major endogenous hemoglobin-binding protein, has two alleles in humans (1 and 2) leading to three possible genotypes: HP1-1, HP1-2, and HP2-2. The affinity of haptoglobin for free hemoglobin is genotype dependent with the HP2-2 protein having the lowest binding affinity and increased propensity for oxidative damage due to cell free hemoglobin. We hypothesize that among adults with SCD, those participants with the HP2-2 genotype will have an increase in occurrence of disease specific complications.

**Methods:** Haptoglobin genotype was assayed on an adult cohort of participants with SCD, ages 19-55 years, from the Vanderbilt DNA repository (BioVU), which links DNA samples to de-identified medical record data. We developed an ICD9/10 code based algorithm to identify patients. This was refined through repeated sampling and manual review to achieve >95% sensitivity and specificity. All records in the final cohort were reviewed to collect basic demographic information, lab data at baseline health, and incidence of SCD related complications. Recorded complications included vaso-occlusive pain, ACS, stroke, retinopathy, nephropathy, pulmonary hypertension, and priapism.

**Results:** A total of 58 adults with SCD were included with a mean age of 33.6 years (SD 9.4). Of these 58 participants, 25.9% (N=15) had the HP1-1 genotype, 55.2% (N=32) had the HP1-2 genotype, and 19.0% (N= 11) had the HP2-2 genotype. A total of 90.9% of participants with the HP2-2 genotype had 2 or more complications as opposed to 46.7% of those with the HP1-1 genotype and 56.3% of the HP1-2 genotype. After adjusting for age, sex, SCD genotype, and baseline hemoglobin, bootstrapped logistic regression demonstrated increased odds of having 2 or

more complications in those with the HP2-2 genotype as compared to the HP1-1 and HP1-2 genotypes (OR= 8.6, 95% CI= 1.4-52.2, p=0.019).

**Conclusion:** These findings suggest adults with the HP2-2 genotype are at increased risk for SCD complications. Further research is needed to confirm this finding in a larger, prospective fashion and to determine the role of the haptoglobin genotype and the oxidative effect of cell free hemoglobin in the pathophysiology of SCD.

Funded by: NIH HL103836 and HL087738-12

### 13.0 SCD Gene Therapy, Gene Editing and Pharmacological Treatment

#### 13.1 GENE THERAPY FOR HEMOGLOBINOPATHIES: THE CHALLENGE TO FIND A CURE

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Sickle cell disease (SCD) and  $\beta$ -thalassemia major are congenital anemias caused by mutations in the  $\beta$ -globin gene, resulting in either abnormal hemoglobin structure or reduced/absent production of  $\beta$ -globin chains. They are the most common monogenic disorders associated to morbidity and mortality. Treatment of these diseases is essentially supportive, requiring transfusions, iron chelation and use of hydroxycarbamide in SCD. At present, the only curative approach is represented by allogeneic hematopoietic stem cell transplantation, with a probability to find a well-matched donor of <25%.

Ex vivo gene therapy, using autologous genetically modified hematopoietic stem cells, potentially represents a cure applicable to all patients regardless of donor availability and free from transplant related immunological complications such as graft rejection and GVHD. The development and large scale production of clinical grade lentiviral vectors expressing human globins, and the optimization of gene transfer protocols in hematopoietic stem/progenitor cells have progressed this field to the pioneering clinical trials in France and in U.S.A., and more recently in Italy. The first results of clinical benefit, including early engraftment, hemoglobin expression and transfusion independence were reported for some patients and are proving the potential efficacy of this therapeutic approach.

Although these encouraging results, early clinical studies showed the safety and potential efficacy of this therapeutic approach, as well as the hurdles still limiting its general application. These are the nature and source of hematopoietic stem cells, the suboptimal transduction efficiency and gene expression levels, the toxicity and

efficacy of bone marrow conditioning, and the overall cost and complexity of vector and cell manufacturing. In addition, for both beta-thalassemia and sickle-cell disease, an altered bone marrow microenvironment might reduce the efficiency of stem cell harvesting as well as engraftment.

Our contribution to this field in the last 10 years was devoted to the clinical development of a safe gene therapy approach for  $\beta$ -thalassemia using the GLOBE lentiviral vector GLOBE. The crucial steps leading to the recent start of TIGET BTHAL clinical trial (NCT02453477), as well as preliminary data on treated patients, will be presented.

### 13.2 COMBINED HYDROXYUREA AND ET<sub>A</sub> RECEPTOR BLOCKADE REDUCES RENAL INJURY IN THE HUMANIZED SICKLE CELL MOUSE

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Sickle cell disease (SCD) is a monogenetic hemoglobinopathy associated with an increasing incidence of kidney disease, for which the underlying etiology has yet to be elucidated. Recent data from our lab and others have shown that the vasoactive peptide endothelin-1 (ET-1) plays a critical role in the pathophysiology of sickle cell nephropathy (SCN) and that blockade of its ET<sub>A</sub> receptor improves renal function and protects against renal injury. Hydroxyurea (HU), the only FDA approved therapy for patients with SCD, is commonly prescribed for the treatment of SCN due to its ability to increase fetal hemoglobin levels. Therefore, we hypothesized that combined ambrisentan (ET<sub>A</sub> selective antagonist) and HU treatment has a synergistic effect on renal injury in SCN when compared to HU treatment alone. Male 12 week old humanized sickle mice (HbSS) and their genetic controls (HbAA) were treated with vehicle, HU (50mg/kg/day), ambrisentan (10mg/kg/day), or HU plus ambrisentan. After 2 weeks of treatment, mice were placed in metabolic cages to assess renal function and then euthanized for blood and tissue collection. Vehicle treated HbSS mice exhibited significant proteinuria compared to vehicle treated HbAA mice ( $3.4 \pm 0.4$  vs  $2.1 \pm 0.2$  mg/day, respectively,  $p=0.03$ ). HbSS mice also displayed elevated plasma ET-1 concentration ( $1.09 \pm 0.08$  vs  $0.49 \pm 0.01$  pg/ml,  $p=0.04$ ) and decreased urine osmolality ( $1225 \pm 67$  vs  $1966 \pm 46$  mOsmol/kg,  $p=0.008$ ) compared to HbAA controls. Proteinuria was significantly attenuated in the HU treated animals compared to vehicle treated HbSS mice ( $2.1 \pm 0.3$  vs  $3.4 \pm 0.4$ ,  $p<0.05$ ); however, there was no additional improvement in HbSS mice treated with combined ambrisentan and HU. Ambrisentan also produced a similar decrease in proteinuria ( $1.9 \pm 0.2$  vs

$3.4 \pm 0.4$  mg/day,  $p=0.02$ ). HU alone also reduced nephrinuria ( $4.3 \pm 0.7$  vs  $12.3 \pm 1.0$  ng/ml,  $p<0.0001$ ) and albuminuria ( $13.4 \pm 1.9$  vs  $25.4 \pm 4.4$   $\mu$ g/day,  $p=0.01$ ) similar to what we have previously reported for ambrisentan. However, KIM-1 excretion, a marker of tubular injury, was not attenuated with HU treatment alone. The absence of further attenuation of renal injury with combined treatment suggests that the mechanism of action for both treatments may converge on the same mechanistic pathway.

### 13.3 NRF2 GENE KNOCKOUT EXACERBATES TISSUE PATHOPHYSIOLOGY IN THE SICKLE CELL DISEASE TRANSGENIC MOUSE MODEL

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#### Abstract

The basic leucine zipper transcription factor, nuclear factor (erythroid-derived 2)-like 2 (NRF2) plays a critical role in the cellular antioxidant response to control oxidative stress levels. We and others previously demonstrated that NRF2 activation enhances  $\gamma$ -globin gene and fetal hemoglobin expression in human primary erythroid progenitors. Herein we show the role of NRF2 function in the pathophysiology of sickle cell disease (SCD) in a novel Townes SCD mouse/NRF2 knockout (SCD/NRF2-KO) transgenic model. Loss of NRF2 function reduced  $\gamma$ -globin gene expression during erythroid differentiation from the E13.5 and E18.5 fetal liver to adult spleen and bone marrow stages of hematopoiesis. In peripheral red blood cells, the level of reactive oxygen species was increased 33% ( $p<0.05$ ) and under in vitro hypoxic conditions, the level of sickling was significantly increased by 38%. We next characterized the effect of NRF2 knockout on organ pathophysiology. For the SCD/NRF2-KO mouse, by 8-10 weeks of age, we observed greater splenomegaly and significant inflammation in spleen, lung and liver tissue when compared to SCD/NRF2 wild-type mice. Protein expression profiling by western blotting using adult spleen whole protein extracts, demonstrated downregulation of the antioxidant proteins heme oxygenase 1 (HMOX1), NADPH: quinone oxidoreductase 1 (NQO1) and catalase by 31%, 60%, and 48% respectively. The expression of cellular adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) were significantly increased by 1.7-fold and 2.3-fold ( $p<0.05$ ) while the expression of vascular endothelial growth factor (VEGF) was not changed obviously. In addition, the expression levels of pro-inflammatory molecules interleukin 1 $\beta$  (IL-1 $\beta$ ), IL-6, tumor necrosis

factor  $\alpha$  (TNF- $\alpha$ ), monocyte chemoattractant protein (MCP-1) and macrophage migration inhibitory factor (MIF-1) were elevated in SCD/NRF2-KO mice. These data corroborate a critical role of NRF2 in protecting against the pathophysiology of SCD including red blood cell sickling/oxidative stress and tissue inflammation. Furthermore, the ability of NRF2 to mediate fetal hemoglobin induction provides a rationale for the development of therapeutic agents that activate NRF2 expression. This work was supported by funding from the National Heart, Lung, and Blood Institute to XZ through the Hemoglobinopathy Translational Research Skills Core component of U01 grant HL117684 and R01 grant HL069234 to BSP.

### 13.4

#### DISCOVERY OF PHARMACOLOGIC FETAL HEMOGLOBIN INDUCING AGENTS FOR TREATMENT OF SICKLE CELL DISEASE

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During adult development, production of fetal hemoglobin (HbF) ameliorates the clinical severity of sickle cell disease (SCD) by inhibiting hemoglobin S polymerization. To develop new drugs that induce HbF in erythroid cells, we tested the heme precursor  $\delta$ -aminolevulinic acid (ALA). We demonstrated the ability of 2 mM ALA to activate HbF synthesis by 23-fold ( $p < 0.05$ ) in KU812 erythroid cells. Subsequent studies in primary erythroid progenitors confirmed  $\gamma$ -globin activation and robust HbF induction whereas  $\beta$ -globin gene transcription was not altered. Our lab previously demonstrated the ability of sodium butyrate (NaB) to induce HbF via the p38 MAPK signaling and CREB binding to the  $\gamma$ -globin cyclic AMP response element. Although butyrate induces HbF in adults with SCD by intravenous administration, oral dosing was ineffective. Therefore to address this barrier, we investigated oral acyloxyalkyl (AN233) or butyrylalkyl (AN908) ester prodrugs of ALA and butyric acid. These prodrugs are activated through intracellular esterase-dependent hydrolysis and oral dosing in anemic Balb-c mice increases total hemoglobin and reticulocyte count. Treatment of K562 cells with AN233 and AN908 (0.25 mM) produced up a 1.5-fold increase in  $\gamma$ -globin mRNA and induced HbF synthesis 10-fold ( $p = 0.029$ ). By flow cytometry analysis, the %HbF positive cells increased from 45% to 71% and mean fluorescence intensity from 15% to 25%. Studies in normal and sickle primary erythroid progenitors are in progress to confirm the ability of both prodrugs to induce HbF and the effects on intracellular heme levels, oxidative stress and red blood cell sickling. Support: NIH RO1 HL069234. References: Sangerman J, Lee MS, Yao X, Oteng E, Hsiao CH, Li W, Zein S, Ofori-Acquah SF, Pace BS (2006). Mechanism for fetal hemoglobin induction by histone deacetylase inhibitors involves gamma-globin activation by

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## 14.0 Cell Therapy

### 14.1

#### CONTROL OF HbF SILENCING: IMPLICATION FOR GENETIC AND PHARMACOLOGIC INDUCTION OF HbF FOR THERAPY

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A premise underlying our work has been that an improved understanding of the mechanisms involved in silencing of HbF will provide new avenues for induction of HbF at the adult stage for therapy of the major hemoglobinopathies, beta-thalassemia and sickle cell disease (SCD). Over the past several years we have shown that BCL11A serves as a major quantitative regulator of HbF and collaborated with T. Maeda to identify LRF as a second potent repressor. Both proteins function in concert with the NuRD complex. At this time, these components (BCL11A, LRF, and NuRD) constitute the central factors for HbF silencing. Our hypothesis is that direct targeting of these components, either genetically or through pharmacological means, offers the best hope of effective HbF reactivation. Through near-saturating CRISPR/Cas9 mutagenesis of the erythroid enhancer of BCL11A, we have identified a remarkably discrete region (of  $< 20$  bp) that provides the major activity of the entire 12kb enhancer. Disruption of this vulnerable region by a single cleavage and non-homologous end-joining markedly impairs BCL11A expression, but only in erythroid lineage cells, and represents an attractive therapeutic gene editing target that is actively being investigated. As a genetic therapy will by nature be "low throughput" due to reliance on bone marrow transplantation, a pharmacological approach is greatly needed to meet the clinical need. To achieve this end, we are focusing on BCL11A as a therapeutic target for development of small molecules.

### 14.2

#### GUT MICROBIOME ANALYSIS REVEALS MAJOR DYSBIOSIS IN SICKLE CELL DISEASE PATIENTS WITH A PREVALENCE OF VEILLONELLA STRAINS

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Gut microbiome analysis reveals major dysbiosis in Sickle Cell Disease patients with a prevalence of *Veillonella* strains

**Background:** Sickle cell disease (SCD) is an inherited blood disorder that occurs primarily in patients of African descent and generally associates with frequent pain crises. It has been suggested that the gut microbiome structure and function may have a major impact on host health. Currently, microbiological studies are typically based on cultivable bacteria. Here we used high throughput sequencing technologies to explore the gut microbiome specifics in SCD patients.

**Aim:** To characterize the gut microbiome in patients with sickle cell disease.

**Materials & Methods:** Stool samples from 14 controls and 14 SCD patients were used for DNA extraction. Among the SCD patients, 7 had mild pain crises (< 3 hospitalizations/year) while 7 had severe pain crises (≥ 3 hospitalizations/year). The 16S rRNA gene V4 variable region was PCR amplified, purified using calibrated Ampure XP beads and used to prepare illumina DNA library. Sequencing was performed on a MiSeq following the manufacturer's guidelines. Sequences were joined, and depleted of barcodes. Sequences less than 150bp or ambiguous base calls were then removed. OTUs clustering was performed after the sequences were denoised, and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). The final OTUs were taxonomically classified using BLASTn against a curated database derived from RDP II and NCBI. A LeFSe analysis was used to determine differential bacteria.

**Results:** A major dysbiosis was noticed in the SCD gut microbiome. Several bacterial groups have been depleted from the SCD patients when compared with controls. The SCD gut microbiome has been defined by the prevalence of *Bifidobacteria*, *Campylobacter*, *Veillonella*, *Actinomyces*, *Scardovia* and *Atopobium*. A major shift towards anaerobic bacteria was noted. The analysis among the two SCD groups of revealed a higher prevalence of *Veillonella* and *Oxalobacter* species among SCD patients with severe pain crises.

**Conclusion:** We report a major dysbiosis in SCD patients' microbiota that seems to be driven by local acidosis and hypercapnia that are prevalent condition in these patients. *Veillonella*, a normal oral and colon inhabitant, is known for its ability to form biofilms and as a facilitator of *Streptococcus* strains pathogenesis. Its high prevalence in SCD patients might exacerbate pain crises primarily due to blood vessels occlusion as a consequence of sickle shaped

blood cells. Indeed, *Veillonella* biofilms might block blood vessels as well and increase *Streptococcus* strains virulence.

### 14.3 THE THERMODYNAMIC HYPOTHESIS OF SICKLE CELL DISEASE PATHOPHYSIOLOGY

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Since 1979 we have studied the biophysics and cell biology of the intracellular

polymerization of sickle hemoglobin to better understand the pathophysiology of this disease. Using then new solid-state NMR methods, with Dennis Torchia, we found to our surprise that polymerization is detectable almost to full oxygen saturation of sickle cells and that these results were explicable due to the non-ideal behavior of hemoglobin at red cell concentrations, as modeled by Allen Minton. On the basis of these and many related studies we developed a model of pathophysiology based on the approximate amounts of polymer in red cells in various tissues, as determined primarily by oxygen saturation and intracellular hemoglobin concentration and composition. Polymer amounts are likely close to equilibrium conditions since growth and melting are both very rapid as oxygen levels change in the body. We expect that most impairment of blood flow occurs in the pre-capillary arterioles where resistance to flow is maximal. Variations in the severity of the sickle syndromes and responses to therapies, such as hydroxyurea to elevate fetal hemoglobin, can be quantitatively accounted for by this model.

"Polymerization tendency" can be calculated based on average properties of sickle red cell populations and corresponding hemoglobin solubility at a any given oxygen saturation. Further refinement of this model can be accomplished by detailed data on heterogeneities of intracellular red cell hemoglobin compositions and concentrations but are unlikely to change the overall results greatly. Studies of sickle red cells in various blood vessels as oxygen levels and other physiological parameters vary are equally important in better understanding the relative roles of intracellular polymerization and other factors, such as hemolysis and adhesive interactions, in overall pathophysiology.

### 15.0 Small Molecules to Treat SCD

#### 15.2 ORAL TETRAHYDROURIDINE AND DECITABINE FOR NON-CYTOTOXIC EPIGENETIC GENE REGULATION IN SICKLE CELL DISEASE: A RANDOMIZED PHASE 1 STUDY

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**Background:** Sickle cell disease (SCD) is driven by polymerization of mutated sickle hemoglobin (HbS) in red blood cells (RBC). Fetal hemoglobin (HbF) interferes with this polymerization, but HbF is epigenetically silenced from infancy onward by DNA methyltransferase (DNMT1).

**Methods and Findings:** To pharmacologically re-induce HbF by DNMT1 inhibition, this first-in-human clinical trial (NCT01685515) combined two small molecules, decitabine to deplete DNMT1, and tetrahydrouridine (THU) to inhibit cytidine

deaminase (CDA), the enzyme that otherwise rapidly deaminates/inactivates decitabine, severely limiting its half-life, tissue distribution, and oral bioavailability. Oral decitabine doses, administered after oral THU 10 mg/kg, were escalated from a very low starting level (0.01, 0.02, 0.04, 0.08 or 0.16 mg/kg), to identify minimal doses active in depleting DNMT1 without cytotoxicity. Patients were SCD adults at risk of early death despite standard-of-care, randomized 3:2 to THU-decitabine versus placebo in 5 cohorts of 5 patients treated 2X/week for 8 weeks, with 4 weeks of follow-up. Adverse events were not significantly different in THU-decitabine- versus placebo-treated patients. At the decitabine 0.16 mg/kg dose, plasma concentrations peaked at ~50 nM (C<sub>max</sub>) and remained elevated for several hours. This dose decreased DNMT1 protein in peripheral blood mononuclear cells by >75% and repetitive element CpG methylation by ~10%, and increased HbF by 4-9% (p<0.001), doubling HbF-enriched RBC (Fcells) up to ~80% of total RBCs. Total hemoglobin increased by 1.2-1.9 g/dL (p=0.01) as reticulocytes simultaneously decreased; that is, better quality and efficiency of HbF-enriched erythropoiesis elevated hemoglobin using fewer reticulocytes. Also indicating better RBC quality, biomarkers of hemolysis, thrombophilia and inflammation (LDH, bilirubin, D-dimer, CRP) improved. As expected with non-cytotoxic DNMT1-

depletion, platelets increased and neutrophils concurrently decreased, but not to an extent requiring treatment holds. As an early phase study, limitations include small patient numbers at each dose level and narrow capacity to evaluate clinical benefits.

**Conclusion:** Administration of oral THU-decitabine to patients with SCD was safe and by targeting DNMT1 upregulated HbF in RBC. Further studies are warranted.

## 18.0 Renal and Vascular Physiology

### 18.2

#### RESISTANCE ARTERIES OF HUMANIZED SICKLE CELL DISEASE MICE DISPLAY SIMILAR SENSITIVITY TO $\alpha_1$ -ADRENERGIC AND ENDOTHELIN-1 VASOCONSTRICTION

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Sickle cell disease (SCD) patients at baseline exhibit lower diastolic blood pressure, decreased systemic vascular resistance, and reduced pulse wave velocity compared to controls. In contrast to these cardiovascular alterations in patients, investigation of the vascular reactivity of isolated aorta from SCD mice has demonstrated markedly enhanced  $\alpha_1$ -adrenergic vasoconstriction and has contributed to the hypothesis that enhanced sensitivity to vasoconstrictors plays a role in vaso-occlusive processes in SCD. The aim of this study was to examine vasoconstrictor sensitivity in resistance arteries of SCD mice, as this is directly related to systemic vascular resistance and diastolic blood pressure and has greater relevance to vaso-occlusion in SCD. Humanized SCD mice (HbSS) and humanized hemoglobin A control mice (HbAA) were utilized for all experiments. Vascular reactivity to phenylephrine (PE), endothelin-1 (ET-1), and potassium chloride (KCl) was examined in resistance mesenteric arteries (100-150 $\mu$ m diameter) as well as aortic reactivity to PE and KCl. Resistance mesenteric arteries from HbSS mice displayed similar EC<sub>50</sub> (-5.81  $\pm$  0.12 vs. -5.80  $\pm$  0.07 log[PE, M], p>0.05) and E<sub>max</sub> (133.9  $\pm$  13.4 vs. 118.3  $\pm$  6.7 %KCl, p>0.05) in response to PE compared to HbAA mice. In contrast and consistent with previous findings, aorta from HbSS mice displayed lower EC<sub>50</sub> (-6.22  $\pm$  0.14 vs. -6.90  $\pm$  0.04 log[PE, M], p=0.002) and elevated E<sub>max</sub> (118.7  $\pm$  3.2 vs. 155.9  $\pm$  5.2 %KCl, p<0.001) in response to PE compared to HbAA mice. ET-1 is also an important vasoconstrictor in resistance arteries and enhanced ET-1 signaling has been implicated in the pathophysiology of SCD. In response to ET-1, resistance mesenteric arteries from HbSS mice displayed similar EC<sub>50</sub> (-7.85  $\pm$  0.07 vs. -7.82  $\pm$  0.05 log[ET-1, M], p>0.05) and E<sub>max</sub> (104.2  $\pm$  10.2 vs. 90.9  $\pm$  16.5 %KCl, p>0.05) compared to HbAA mice. Graded concentration responses to KCl were similar

between genotypes in both resistance and conduit arteries. These data suggest regional differences in arterial sensitivity to vasoconstriction, with enhanced  $\alpha_1$ -adrenergic vasoconstriction isolated to the aorta. Additionally, HbAA and HbSS mice have similar sensitivity to multiple vasoconstrictors in resistance arteries, suggesting that enhanced vasoconstriction may not participate in vaso-occlusion and resultant tissue hypoxia in SCD.

### Acknowledgment

This work was supported by funding from the National Institutes of Health - U01 HL117684 to Dr. Jennifer Pollock and Dr. David Pollock, F30 DK107194 to Brandon Fox, and partially supported by MSTP T32 GM008361.

### 18.3

#### IMPAIRED POST-ISCHEMIC NEOVASCULARIZATION IN SICKLE CELL DISEASE

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**Aims:** The appropriate physiologic response to vascular insult is the development of functional collateral vessels to preserve organ function. To that end, first responder immune cells (neutrophils, macrophages) must be tightly recruited to the site of injury, promote repair and resolve inflammation to maintain vascular integrity. Repeat ischemia is the hallmark of sickle cell disease (SCD), leading to many clinical complications such as stroke, pain crises, proliferative retinopathy and sudden cardiac death. However the fundamental response to vascular injury in SCD is unknown, and the roles of first responder immune cells are poorly understood.

**Methods:** We used the hind limb ischemia (HLI) model to determine collateral vessel formation in the humanized Townes sickle cell mice (SS) in comparison to wildtype (AA). Perfusion recovery was measured weekly using LASER Doppler perfusion imaging (LDPI). The voluntary running wheel test was used to measure motor function recovery. Flow cytometry and immunostaining were used to characterize phenotype of neutrophils and macrophages after HLI. Anti-Ly6G antibody was used to deplete neutrophils *in vivo*. Resolvin D1 was encapsulated in liposome and delivered weekly after HLI.

**Results:** Collateral vessel formation was significantly impaired in SS; by day 28, AA mice showed  $76 \pm 13\%$  perfusion recovery, compared to  $34 \pm 10\%$  in the SS mice,  $p < 0.001$ ,  $n=8$  per group). Spontaneous motor recovery was significantly impaired in SS mice after HLI (98% in AA, vs 36% in SS mice.  $p < 0.001$ ). Whereas all neutrophils were

cleared in AA mice by day 5, SS mice demonstrated persistent neutrophils that remained in the ischemic hind limb for weeks. SS neutrophils were highly inflammatory and produced a 2.45 fold increase in hydrogen peroxide. Importantly, *in vivo* depletion of neutrophils reduced oxidative stress and improved collateral vessel formation in SS mice. Administration of resolvin D1, which promotes neutrophil clearance and resolution of inflammation, also significantly improved collateral vessel formation in SS mice after HLI.

**Conclusions:** Our data identify impaired post-ischemic neovascularization as an underlying etiology of the numerous vascular complications in SCD, driven largely by maladaptive inflammation and oxidative stress. Our results also provide potential therapeutic targets to improve vascular function after ischemic injury in SCD.

This work was supported by NIH RO1 HL131414

### 18.4

#### ROLE OF MACROPHAGE STIMULATING PROTEIN 1 (MSP1) IN THE DEVELOPMENT OF ENDOTHELIAL INJURY IN SICKLE CELL DISEASE

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Patients with Sickle Cell Disease (SCD) have an approximately three-fold higher risk of developing chronic kidney disease (CKD) compared to the general population. However, the current standards of diagnosis and care do not prevent and simply delay progression of this disease. The majority of renal complications in SCD are believed to result from vasculopathy and endothelial injury. Hemolysis inside the renal tissue stimulates macrophages infiltration and endocytosis of the products of hemolysed red blood cells (RBCs) by those infiltrating macrophages leads to MT-SP1 protease activation. Macrophage Stimulating Protein 1 (MSP1) is a circulating non-active substrate of MT-SP1.

The objective of this project was to demonstrate a role of MSP1 in the development of renal endothelial injury.

Human renal glomerular endothelial cells (HGEC) and a mouse model of SCD (Townes) were used in this investigation. The animal protocol was approved by the Institutional Animal Care and Use Committee at the Children's National Health System. Townes sickling mice express human Hgb S and Hgb F and control mice express

human Hgb A1. Renal glomerular permeability was measured by a determination of albumin permeability.

MSP1 treatment induced RON receptor activation and downstream signaling evidenced by increased phosphorylation of ERK and AKT kinases. MSP1 also increased motility of HGEC. RON inhibitor (RONi) significantly reduced RON activation and ERK and AKT phosphorylation. We demonstrated a significant accumulation of MSP1 in the glomeruli of SCD mice. Glomerular capillary enlargement and endothelial injury was characterized by PAS staining and immunostaining of ICAM, vWF and CD34. Endothelial injury was significantly increased in SCD mice compared to controls. MSP1 increased glomerular permeability in the mouse whole glomeruli assay. RONi prevented the induction of renal glomerular permeability by MSP1. Treatment of mice with RONi (s.c. daily injections for 14 days) significantly reduced renal endothelial injury in young SCD mice. In conclusion, renal glomerular accumulation of MSP1 is one of the factors involved in the induction of renal endothelial injury in SCD mice. Inhibition of MSP1 receptor RON kinase activation significantly reduced endothelial injury and development of kidney disease.

**ACKNOWLEDGMENTS:** This work was supported by NIH Research Grants 1P50HL118006, 1R01HL125005 and 5G12MD007597. The content is solely the responsibility of the authors and does not necessarily represent the official view of NHLBI, NIMHD or NIH.

## 18.5

### IRON ACCUMULATION IN THE KIDNEY – POTENTIAL NEW ROLE FOR ENDOTHELIN SYSTEM

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Elevated endothelin-1 (ET-1) levels reported in sickle cell disease are associated with microalbuminuria and renal iron deposition early in sickle nephropathy. In humanized sickle cell mice (HbSS), long-term ET<sub>A</sub> receptor antagonism provides robust protection from diverse renal pathologies, including significant attenuation of renal tubular iron deposition in the proximal tubules. These observations led us to hypothesize that ET-1 regulates renal iron trafficking in iron overload-associated sickle nephropathy. We first determined the effect of ET-1 on the expression of iron trafficking mediators in mouse primary proximal tubular epithelial (PT) cells. Expression of the iron importer transferrin receptor 1 (TfR-1) and the iron storage protein, H-ferritin, were increased in a concentration-dependent manner by ET-1. The ET-1-induced decrease in the iron exporter ferroportin-1, FPN-1 (65% reduction), was associated with a doubling in expression of hepcidin, a key

regulator of FPN-1 and iron removal from the cell. In keeping with these observations, cellular iron uptake in response to ET-1 was significantly increased. Addition of plasma from HbSS mice to PT cells increased cellular iron uptake compared to plasma from control HbAA mice (0.098±0.017 vs. 0.004±0.001 ng/μl). Pre-incubation with the selective ET<sub>A</sub> receptor antagonist, BQ123, completely prevented ET-1 induced alterations in all iron import, storage and export mediators, suggesting a likely involvement of the ET<sub>A</sub> receptor in iron trafficking mechanisms. Interestingly, neither selective ET<sub>B</sub> nor combined (ET<sub>A</sub>+ET<sub>B</sub>) receptor antagonism affected ET-1-induced changes in iron trafficking mediators, supporting the hypothesis that the ET<sub>A</sub> receptor mediates ET-1-dependent changes in renal iron handling. Simultaneously, *in vivo* studies showed increased expression of TfR-1, H-ferritin and decreased expression of FPN-1 in cortical tissues of HbSS mice. These effects were prevented by long-term treatment with a selective ET<sub>A</sub> receptor antagonist. Overall, these results reveal a novel role for ET-1 in proximal tubule iron trafficking and provide rational for the use of selective ET<sub>A</sub> receptor blockade as a potential therapeutic approach in iron overload disorders, specifically sickle cell disease.

Support provided by an NIH-funded EHRA U01 HL117684 to D.M.P, ASN Ben J. Lipps Research Fellowship for M.K., and NIH NIDDK K01DK105038 to K.A.H.

## 19.0 Lung Physiology and Pathophysiology

### 19.2

#### DIFFERENTIALLY EXPRESSED LNCRNAs IN LUNGS AND PLASMA EXOSOMES OF SICKLE CELL MICE

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**Rationale:** Sickle cell disease (SCD)-associated pulmonary hypertension (PH) causes significant morbidity and mortality. We recently demonstrated that Townes humanized sickle cell (SS) mice spontaneously develop PH and vascular remodeling. Emerging evidence indicates that long non-coding RNAs (lncRNAs), transcripts longer than 200 nucleotides, play a pivotal role in cellular proliferation, differentiation, and apoptosis. Exosomes, nano-sized extracellular vesicles contain lncRNAs and play an important role in intercellular communication. However, the role of lncRNAs released from lungs and plasma exosomes in SCD-PH pathogenesis has not been defined. To further examine the pathogenesis of SCD-PH, we

hypothesized that lncRNAs are differentially expressed in SS mouse lungs and plasma exosomes.

**Methods:** lncRNAs were isolated from lungs and plasma exosomes of SS mice and littermate control (AA) mice at age 15-17 weeks. Isolated plasma exosomes were characterized for levels of exosomal markers (CD9, CD45, and CD63), and exosomes and lung samples were subjected to measurements of the expression profiles of 90 lncRNAs, 5 housekeeping genes, and one negative control using the LncProfiler™ lncRNA qPCR array (System Biosciences).

**Results:** We found that the levels of four novel lncRNAs (GAS5, Foxn2-as, AK007836-upstream of PCNA, and Adapt33) were increased in the lungs and plasma exosomes of SS mice compared to AA mice. The fold changes ranged from 1.3 to 4.5. Nine lncRNAs (Rmst, Vax2os1, Zeb2NAT, Neat1 v1/MEN epsilon, Six3os-clone9, Kcnq1ot1, lincENC1, linc1242 LINC-Enah, and linc1368) were decreased in both the lungs and plasma exosomes of SS mice.

**Conclusions:** Collectively, these studies establish that lncRNAs are differentially expressed in SS mice. Further characterization of these lncRNAs may determine new mechanisms for SS pathologies and identify a novel therapeutic approach in SCD pulmonary vascular dysfunction and PH. Functional investigation of SS specific lncRNAs should consider the effects of different etiologies of SCD-PH.

Supported by Atlanta VA Merit Review 1101BX001910 (CMH), NIH HL102167 (RLS and CMH), and NIH HL133053-01A1 (BYK).

### 19.3 LONGITUDINAL CHANGES IN DIFFUSE MYOCARDIAL FIBROSIS IN SICKLE CELL ANEMIA

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**Background:** We recently demonstrated that diffuse myocardial fibrosis is a common and novel mechanism of heart disease in sickle cell anemia (SCA) that is strongly associated with diastolic dysfunction (Niss et al. Blood 2017). Diffuse myocardial fibrosis can be detected noninvasively by cardiac magnetic resonance imaging

(CMR). The temporal evolution of diffuse myocardial fibrosis in SCA has not been studied before.

**Methods:** This is an ongoing, prospective, longitudinal study to characterize the cardiomyopathy of SCA. We used CMR measurements of native T1 and extracellular volume fraction (ECV) to quantify diffuse myocardial fibrosis. ECV was calculated from pre- and post-gadolinium T1 measurements of blood and myocardium. Focal myocardial fibrosis was detected by late gadolinium enhancement (LGE). Diastolic function was assessed by echocardiography. We compared paired CMR studies from study entry and 1-year follow-up.

**Results:** We studied 25 individuals with SCA who were 23±11 years of age (mean±SD). All had markedly increased ECV at baseline compared to controls (0.44±0.08 vs. 0.26±0.02, P<0.0001), indicating the presence of diffuse myocardial fibrosis; 1 also had LGE-defined focal myocardial fibrosis. At baseline, 8 (32%) had normal diastolic function, 17 (71%) had diastolic abnormalities, and 7 (29%) met the definition for diastolic dysfunction. The second (1-year follow-up) CMR was performed 12±0.4 months after the baseline. For all participants, the mean difference (year 1 - baseline) in ECV was -0.03 (95% CI: -0.08, 0.03; P=0.34), indicating general stability of the phenotype over 1 year. Similarly, the mean difference in native T1 values was 19 ms (95% CI: -10, 48; P=0.19). None developed new focal fibrosis. Participants with normal systolic and diastolic function at baseline (N=7) had median increases of 23% and 6% in ECV and native T1, respectively, at 1-year follow-up, compared to those with diastolic dysfunction at baseline (N=7) who had a median -0.46% decrease in ECV (P=0.32) and 2% increase in native T1 (P=0.45).

**Conclusions:** Diffuse myocardial fibrosis is a common feature of SCA. Overall, the CMR measurements of diffuse fibrosis, native T1 and ECV, are stable over 1 year in SCA, indicating their utility to monitor anti-fibrotic or disease-modifying therapy for the cardiomyopathy of SCA. Individuals with normal heart function, however, may be at risk of progressive myocardial fibrosis.

**Funding:** NHLBI U01HL117709

### 20.0 Red Cell Physiology

#### 20.1

#### DEVELOPMENTAL REGULATION OF ERYTHROID SELF-RENEWAL

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More than 80% of all the cells in the adult human are red blood cells (RBCs) and maintenance of their steady state level requires a staggering production of  $2.5 \times 10^6$  new RBCs every second. Over 15 million units of RBCs are transfused yearly in the United States to treat patients following trauma or major surgery, or with hereditary anemias, including sickle cell disease, requiring chronic transfusion therapy. Donations, projected to decrease over the coming decades, are the sole current source for RBCs and are associated with infectious risks, high costs of screening, and sporadic shortages, especially for rare blood types required for alloimmunized patients. Ex vivo-generated human RBCs could offer a potential solution by serving as an alternative RBC source. A major obstacle has been generating enough RBCs to constitute even one unit of blood because erythroid precursors from adult sources are only capable of limited self-renewal when cultured in vitro with erythropoietin, stem cell factor, and dexamethasone. We discovered that erythroblasts derived from the murine embryo have a unique ability to self-renew extensively ( $>1060$ -fold) ex vivo, all the while retaining the ability to terminally mature into reticulocytes. We subsequently identified Bmi-1, a member of the polycomb repressive complex-1, as a critical regulator of ex vivo erythroid self-renewal. Bmi1-induced extensively self-renewing murine erythroblasts terminally mature both in vitro and in vivo. Our recent studies indicate that Bmi-1 may also regulate human adult and fetal erythroblast self-renewal. Expanding the proliferative capacity of human erythroblasts lays the groundwork for the in vitro generation of reagent RBCs for improved blood typing of sickle cell patients and ultimately of cultured RBCs for transfusion therapy. Support: NIH grants HL130670 and HL134696, and NYSTEM C030134.

## 20.2

### **PATHOBIOLOGY OF SICKLE RED CELLS: IMPLICATIONS FOR PATHOPHYSIOLOGY OF SICKLE CELL DISEASE**

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Sickle cell anemia is a common inherited red cell disorder with high morbidity. Chronic severe hemolytic anemia, episodic vasoocclusive crisis and end organ damage are clinical features of the disease. Current treatment options are limited and as such the development of new and effective therapies is urgently needed. Our current understanding of the pathobiology of sickle red cells includes: 1) decreased cellular deformability as a consequence of sickling induced structural changes in membrane organization and cell dehydration and 2)

increased adhesion of sickle red cells to vascular endothelial cells and other blood cells as a consequence of increased surface expression of adhesion receptors and plasma factors. Altered rheological properties and increased cellular adhesion of sickle red cells are key contributors to the pathophysiology of the disease. Currently, several treatment options are being explored to minimize or prevent the cellular alterations responsible for clinical manifestations. These include approaches for preventing in vivo sickling by either reactivating fetal hemoglobin synthesis or preventing sickle red cell dehydration and by decreasing the adhesive interactions of sickle red cells with endothelial cells and other blood cells.

## 20.3

### **RED BLOOD CELL RHEOLOGY AND VASCULAR DYSFUNCTION IN SICKLE CELL DISEASE**

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Sickle cell anemia (SCA) is a genetic disease characterized by the presence of abnormal hemoglobin (HbS) that polymerizes under deoxygenated conditions causing a mechanical distortion of red blood cells (RBC). SCA patients have decreased RBC deformability and increased RBC aggregates strength. We recently investigated the contribution of blood rheology and vascular dysfunction in vaso-occlusive crises (VOC). Our findings demonstrated that SCA patients have blunted microvascular reactivity during local thermal heating tests compared to controls. The lower microvascular reactivity was negatively associated with the levels of plasma advanced oxidation protein products and nitrotyrosine suggesting a key role of oxidative/nitrosative stress in vascular dysfunction in this disease. Moreover, we recently observed that circulating exosomes in SCA, originating mainly from RBCs, were able to promote monocytes adhesion to endothelial cells through an increase in P-selectin expression and alter in vitro endothelial cells barrier permeability and the topographic distribution of the tight junction protein ZO-1 in a SCA severity-dependent manner compared to healthy children. These new data suggest that exosomes originating from RBCs could be one of the sub-cellular elements involved in the endothelial dysfunction associated with SCA. Finally, multivariate analyses recently performed in SCA cohorts showed that increased blood viscosity and decreased microcirculatory oxygenation are independently associated with a higher risk to develop frequent VOC episodes. In conclusion, vascular dysfunction and blood hyperviscosity emerge as key factors involved in the severity of SCA and the occurrence of frequent VOC events.

20.4

**THE EFFECT OF HEMIN ON HUMAN RED BLOOD CELL TRANSFORMATION**

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**Background.** Lysis of red blood cells (hemolysis) is associated with pathological states such as Sickle Cell Disease (SCD), ischemia reperfusion injury, and malaria, resulting in high levels of free hemoglobin (Hb). Hb degrades and formats hemin, a highly toxic molecule that triggers increased oxidative reactions, leading to cell damage and increased membrane permeability. Naturally in plasma, during mild to moderate hemolysis, extracellular Hb and hemin are neutralized by a number of pathways scavenger proteins, receptors, and enzymes. At the same time in case of pathological states such as anemia (SCD), the level of hemin exceeds the neutralization capacity and thus leads to different health complications and even death. Thus, the aim of the current study was to ascertain the effect of hemin on RBC transformation.

**Materials and methods.** Washed human RBCs ( $0.5 \times 10^{12}/L$ ) were exposed to a standard saline solution. Hemin concentrations less than 20  $\mu M$  (described as fatal) were used. RBC membrane transformations and microparticle production (MPs) were assessed using flow cytometry (Navios, BeckmanCoulter, USA) and low angle laser scattering (Lasca-TM, BiomedSystems, Russia). EC50 of hemin was used as the marker for the description of RBCs membrane transformations triggered by hemin.

**Results.** We found that hemin action on RBCs induced a biphasic dose-dependent transformation in: i) membrane transformation from biconcave to spherocyte under low concentrations of hemin (EC50 [hemin]  $\sim 120nM$ ), and ii) MP formation and hemolysis (EC50 [hemin]  $\sim 5\mu M$ ) with MPs sized  $\sim 0.6$  micron.

**Conclusions.** Our results demonstrate the effects of hemin on RBCs, which can be distinguished into two stages, those being: 1) spherisation, and 2) MP formation and hemolysis. The established effects of hemin on RBC transformation may be used in clinical practice to elucidate a current state of RBCs in SCD patients.

20.5

**MOLECULAR RESOLUTION OF AN ACTIVE VASO-OCCLUSIVE CRISIS IN VOC PATIENTS TREATED WITH SANGUINATE<sup>®</sup>.**

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SANGUINATE<sup>®</sup> (PEGylated carboxyhemoglobin bovine) is a gas transfer agent that can deliver oxygen directly to the hypoxic (sickled) RBCs of sickle cell disease (SCD) patients. Importantly, the transfer of oxygen also promotes rapid conversion of the sickled RBCs to a normal morphology. SANGUINATE<sup>®</sup> is currently being investigated as a clinical candidate for the treatment of acute severe Vaso-Occlusive Crisis (VOC) in SCD patients (NCT02411708).

Consenting SCD patients in acute severe VOC were treated with a single IV infusion of SANGUINATE<sup>®</sup> (8ml/Kg) with blood samples collected pre-treatment and at two post-treatment points; VOC resolution (discharge time) and 72hrs later. Image-based multi-parameter flow cytometry was performed on all samples (including placebo controls) and the extent of sickled/unsickled cell populations was determined across all enrollees at these time points.

Patient samples treated with SANGUINATE<sup>®</sup> showed a shift towards a more normal morphology when the pre- and post-VOC resolution samples were compared for each individual. In contrast, the placebo control patient samples showed worsening or no improvement of the sickled/unsickled ratios. Additionally, only patients treated with SANGUINATE<sup>®</sup> exhibited extended unsickling profiles at 3 days following treatment.

SANGUINATE<sup>®</sup> was capable of promoting the rapid depolymerization of sickled hemoglobin within peripheral blood samples of severe VOC patients treated with this novel gas transfer agent. Despite a half-life of 18-24hrs SANGUINATE<sup>®</sup> treated patients continued to exhibit fewer sickled cells at the 72hr sample point. SANGUINATE<sup>®</sup> can quickly oxygenate patient sickled RBCs effectively reducing both the short-term and long-term sickled RBC levels. Collectively these results support the continued clinical evaluation of SANGUINATE<sup>®</sup> as a rescue agent for rapid resolution of SCD patients in severe VOC in the ambulatory setting.

20.6

**INTRINSIC CELLULAR FACTORS MODULATE HIV-1 REPLICATION IN SICKLE CELL DISEASE**

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Intrinsic Cellular Factors Modulate HIV-1 Replication In Sickle Cell Disease

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Human immunodeficiency virus type 1 (HIV-1) is challenged by intrinsic antiviral restriction factors which it has to counteract with its own viral proteins. We recently showed that in Sickle Cell Disease (SCD), anti-viral restriction factor SAMHD1 is activated and inhibits HIV-1 infection. SAMHD1 activation was due to its reduced phosphorylation by CDK2, which activity was inhibited by reduced intracellular iron levels in SCD peripheral blood mononuclear cells (PBMC). The present study was aimed at identifying additional restriction factors that might be induced in SCD PBMC. This was paralleled by the HIV-1 infection analysis in PBMC from healthy donors treated with hemin and iron chelators to mimic SCD conditions. We used customized array that included known anti-HIV-1 restriction factors to measure mRNA expression of anti-viral factors. The identified factors were further validated with shRNA-mediated knockdowns of the identified genes and their effect on HIV-1 replication in cultured and primary cells. We observed upregulation of 19 genes in SCD PBMC including APOBEC3A, APOBEC3C, TRIM5 $\alpha$ , TRIM22, MX2 and RSAD2 which were significantly upregulated and were further validated using real-time q-PCR. In PBMC treated with hemin, ABOBEC3A, RASD2 and MX2 mRNAs were overexpressed, whereas TRIM5 $\alpha$  expression was increased in PBMC treated with either hemin or iron chelators. ShRNA-mediated knockdown of, APOBEC3C and TRIM5 $\alpha$ , but not APOBEC3A or TRIM22 induced HIV-1 replication. In conclusion, in addition to SAMHD1, restriction factors APOBEC3A, APOBEC3C, TRIM5 $\alpha$ , TRIM22, MX2 and RSAD2 may also contribute to HIV-1 restriction in SCD. Similar to SAMHD1, these factors are likely to be modulated by hemolysis and intracellular iron levels. Our findings further point to the importance of hemolysis and deregulation of iron metabolism as an underground cause for HIV-1 restriction in SCD.

This work was supported by NIH Research Grants 1P50HL118006, 1R01HL125005, and 5G12MD007597. The content is solely the responsibility of the authors and does not necessarily represent the official view of NHLBI, NIMHD or NIH.

## 21.0 Coagulation and Thrombosis

### 21.0

#### PROMOTING THE RESOLUTION OF INFLAMMATION IN SICKLE CELL DISEASE

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Patients with Sickle Cell Disease (SCD) are more susceptible to thrombotic events, in which leukocytes, platelets and erythrocytes have all been implicated in the pathogenesis. However, the underlying mechanisms promoting a pro-coagulant and pro-thrombotic phenotype in SCD (especially in the brain) are still unknown. The causes for the SCD phenotype may relate to a well-established link between thrombosis and inflammation i.e. inflammation can beget local thrombosis, and thrombosis can amplify inflammation. Accumulating data linking inflammation and thrombosis supports the hypothesis that anti-inflammatory therapies may limit thrombosis and that anti-thrombotic therapies may reduce vascular inflammation. One such target is the anti-inflammatory and pro-resolving endogenous mediator Annexin A1 (AnxA1) and its interaction with the Formyl Peptide Receptor (FPR) family. In humans three FPRs (FPR1, FPR2, and FPR3) regulate innate inflammatory responses. Their function has been most extensively investigated in the context of leukocyte recruitment and activation, where FPRs promote cell motility (chemotaxis) and microbicidal respiratory burst. Here we have tested the effect of targeting the AnxA1-Fpr2/ALX pathway as a therapeutic strategy for SCD with a special focus on thrombo-inflammation. Data presented during the talk adds to the current literature suggesting a proinflammatory and pro-thrombogenic cerebral microcirculation is found in SCD mice. Furthermore, targeting the AnxA1-Fpr2/ALX pathway is effective in resolving SCD associated thrombo-inflammation.

### 21.1

#### DE-CLOTTING SICKLE CELL DISEASE

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Sickle cell disease (SCD) is a hematological disorder caused by a single nucleotide mutation in the  $\beta$ -globin gene. Hemolytic anemia and vaso-occlusive crises, resulting from the sickling of red blood cells, are the primary pathologies of the disease that ultimately result in irreversible damage of multiple organs. Furthermore, the contribution of vascular inflammation and the hypercoagulable state to the pathology of SCD has been recently recognized. This hypercoagulable state is mediated by activation of both the intrinsic and extrinsic coagulation pathways. Consistent with the increased markers of coagulation activation, the incidence of venous thromboembolism is higher in SCD patients than matched controls and is associated with increased mortality. In mouse models of SCD, tissue factor expressed on leukocytes activates coagulation and contributes to inflammation via microvascular thrombosis, whereas the non-coagulant form of tissue factor expressed by endothelial cells mediates factor X-dependent activation of protease activated receptor-2 and contributes to inflammation via IL-6 expression. Inhibition of coagulation also protects sickle mice from developing multi-organ pathologies and improves survival. Together, these data point to the coagulation system as an important mediator of end-organ damage in a mouse model of SCD, and suggest that long-term anticoagulation might lead to improved clinical outcomes in sickle cell patients. The question is how to reduce the prothrombotic state in SCD without compromising hemostasis. Concerns about hemorrhagic transformation during ischemic stroke or increased risk of hemorrhagic stroke has limited the use of currently available anticoagulants in SCD. There is growing interest in targeting components of the intrinsic coagulation pathway (factor XII and XI) to prevent thrombotic disorders, primarily because this goal may be attainable without incurring any significant bleeding risk. Preliminary data from our group indicate that FXIIa contributes to the prothrombotic state during both steady state and vaso-occlusive crises. Targeting of FXII(a) could eliminate risk of serious hemorrhage yet still provide benefits associated with reduced thrombosis, vascular inflammation and end-organ damage in SCD. This research was funded by NIH U01 HL117659

## 21.2

### SICKLE RED BLOOD CELLS ALTER CLOT RETRACTION IN MICE AND HUMANS

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A chronic hypercoagulable state and increased risk for venous thromboembolism are prominent features of sickle cell disease (SCD). Red blood cells (RBC) were thought to be merely trapped within a fibrin-rich clot without affecting thrombus formation. However, recent evidence suggests that the retention of RBC within clots by factor XIII directly affects thrombus size. It has been proposed that clot contraction-mediated packaging of RBC is significantly altered in SCD and may affect clot stability. We investigated the cellular mechanism of clot retraction in SCD.

An *ex vivo* clot retraction assay was performed with blood from sickle patients and from Townes sickle mice. Clot formation was initiated in citrated blood by CaCl<sub>2</sub> (10 mM) and tissue factor (1 pM), and after 2 hours the number of RBC extruded from the clot were counted in the serum and expressed as a percentage of initial RBC number.

Dramatically fewer sickle (SS) RBC were extruded into the serum during clot contraction than wild type (AA) RBC (0.8±0.8% vs. 19.4±0.8%, p<0.0001) in sickle mice; a similar result was observed for human RBC. Mixing the platelet free plasma, platelets, and RBC of AA and SS mice demonstrated that the entrapment of SS RBC within the clot is entirely mediated by yet unknown properties of SS RBC. Since SCD is associated with a lower hematocrit (Hct), we investigated if the initial number of RBC affects the extrusion of these cells during clot retraction. Indeed, lowering the Hct of AA mouse blood reduced RBC extrusion, yet normalizing the Hct in SS mouse blood had no effect on the number of SS RBC extruded from the clot, suggesting that the entrapment of SS RBC within the clot is not simply caused by lower RBC number.

Clots formed from AA blood had a soft gel-like structure, and electron microscopy revealed that these normal RBC had polyhedral shapes tightly packed in the clot core. In contrast, SS clots were more firm and stiff, while many SS RBC had undergone sickling and were not compacted within the clot. RBC sickling was also observed in clots formed in the inferior vena cava of SS mice after vessel stenosis. Our data indicate that SCD alters the structure and dynamics of clot formation. The effect of these differences on the increased risk of pulmonary embolism and stroke in SCD is currently being investigated.

This research was funded by NIH U01 HL117659.

## 21.3

### SICKLE CELL ANEMIA: HYPER- OR HYPOFIBRINOLYSIS STATE?

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**Background.** Sickle cell disease (SCD) is caused by hemoglobin polymerization in hypoxic erythrocytes, due to Glu<sub>6</sub>→Val mutation in the  $\beta$ -globin gene. Numerous studies have been done to evaluate coagulation in SCD; however, fibrinolysis in SCD remains poorly researched. Cellular components play an important role in fibrinolysis, and therefore we used two separate assays, utilizing either whole blood (WB) or the platelet free plasma (PFP) samples.

**Aims.** To evaluate resistance to t-PA-mediated fibrinolysis in PFP and WB of patients with SCD.

**Methods.** *Turbidity Assay.* Citrated PFP from 13 SCD patients (SS genotype) at baseline and 9 controls (race-, age- and sex-matched) was mixed with tissue factor (TF, 1pM), CaCl<sub>2</sub> and t-PA (2.5, 1.25 or 0.625nM) and optical density changes were read spectrophotometrically. Clot lysis time (CLT—the time from half clotting to half lysis) was used to evaluate fibrinolysis.

*Global Assay in WB.* Citrated WB in transparent tubes from 15 patients with SCD and 11 matched controls was mixed with TF, CaCl<sub>2</sub> and t-PA (0.6, 1.2, 2.5, 5 and 10nM). When the clot was formed, a steel ball (d=2mm, w=0.13g) was placed on its surface. The time from coagulation/fibrinolysis activation to the ball reaching the bottom of the tube was measured by capturing time-lapse video.

Sample collection was approved by IRB. Informed consents were obtained.

**Results.** In the PFP assay, we found that CLT was significantly shorter in SS patient samples compared to the AA group with all three t-PA concentrations (CLT mean±SEM SS vs. AA and 'p' value for 2.5, 1.25, 0.625 nM t-PA, respectively – 60.7±2.7 vs. 136.7±12.3, p<0.00001; 92.9±5.9 vs. 182.7±9.2, p<0.00001; and 138.5±12.9 vs. 226.7±0.7, p<0.0001).

In the WB ball sedimentation assay, no significant difference was noted between AA and SS clots at high t-PA concentrations (>2.5nM). However, SS clots challenged with lower tPA concentrations (0.625-1.25nM) were more resistant to fibrinolysis than AA clots (CLT mean±SEM SS vs. AA and 'p' value for 1.25 and 0.625 nM t-PA, respectively – 401.9±59.3 vs. 157.9±57.9, p=0.0085; 651.5±33.9 vs. 369.9±75.4, p=0.001).

**Conclusion.** Plasma clots from SCD patients are more susceptible to t-PA challenge compared to healthy controls. In contrast, in WB, where cell components likely play a role, SS patient samples showed resistance to fibrinolysis (at low t-PA concentrations). Therefore,

patients with SCD appear to have a hypofibrinolytic state, which is present in WB but cannot be detected with plasma assays.

Funded by NIH UO1HL117659

## 21.4

### ANNEXIN A1 AFFORDS PROTECTION IN SICKLE CELL DISEASE

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**Introduction:** Neutrophils have recently been implicated in the pathogenesis of sickle cell disease (SCD) vaso-occlusion potentially by their ability to capture sickle red blood cells and, by the production of neutrophil extracellular traps (NETs). Endogenous anti-inflammatory mediator Annexin A1 and its N-terminal derived peptide (AnxA1<sub>Ac2-26</sub>) have been shown to mitigate cerebrovascular inflammation by engaging with formyl peptide receptors (FPRs). Here we aimed to-determine whether AnxA1<sub>Ac2-26</sub> is able to attenuate SCD vaso-occlusion via a reduction in NET formation.

**Materials and Methods:** All patients and healthy donors gave written consents and Institutional Review Board of the LSUHSC-S approved the study. Following blood collection, neutrophils were isolated and incubated (1 x 10<sup>5</sup>) with AnxA1<sub>Ac2-26</sub> (30μM), the FPR pan-antagonist Boc2 (N-tert-butoxycarbonyl-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe)(10μM) or AnxA1<sub>Ac2-26</sub>+Boc2 for 30 minutes followed by NET stimuli (Ionomycin, 4μM) for a period of three hours. Cells were then stained with Sytox green or fixed, permeabilized and blocked for immunocytochemistry with NET specific antibodies; mouse anti-H3Cit, rabbit anti-NE, followed by species-specific secondary antibodies. NETs were quantified by measuring the percentage of CitH3<sup>+</sup>/NE<sup>+</sup> stained DNA fibers and percentage of Sytox green stained DNA fibers. Extracellular DNA levels were also quantified by analyzing Sytox green intensity.

**Results:** Neutrophils from SCD patients at baseline exhibited enhanced H3Cit<sup>+</sup> NET production versus healthy controls. These effects were further exacerbated by stimulation with Ionomycin (p<0.0001). AnxA1<sub>Ac2-26</sub> was able to significantly reduce the H3Cit<sup>+</sup> NETs (p<0.001), which was abrogated by the addition of Boc2 (p<0.05). Interestingly, there was no change in extracellular DNA levels in stimulated SCD neutrophils compared to controls, and AnxA1<sub>Ac2-26</sub> had no effect in Ionomycin stimulated extracellular DNA production.

**Conclusion:** Our study demonstrates that AnxA1<sub>Ac2-26</sub> is able to inhibit SCD-associated NET production via an engagement with FPRs. Furthermore, these results support the notion of targeting the AnxA1-FPR pathway as a potential therapeutic strategy to prevent neutrophil driven thrombo-inflammation in SCD.



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