NHLBI Clinical Proteomics Program Holds Meeting with External Advisors

The Clinical Proteomics Program will hold its first meeting with external advisors on January 10-11, 2007. Principal investigators from network sites, their collaborators and the External Advisory Board will review the program and, with the assistance of the advisors, will develop plans for future collaborative studies and other activities.

The NHLBI will be represented by Dr. Susan Shurin, Deputy Director of NHLBI, Pothur Srinivas, PhD, Program Officer and other Program staff. Dr. Srinivas has been instrumental in maintaining open lines of communication between the Institute and the investigative group. He has also provided assistance with the initiation and organization of the Clinical Proteomics Program. The NHLBI has assembled a panel of distinguished experts in the field of biomarker identification, quantification and validation. This group includes Peipei Ping, Ph.D, Department of Physiology, University of California Los Angeles; Daniel Chan, Ph.D., Department of Pathology, Johns Hopkins University; Polly Parsons, M.D., Department of Medicine, University of Vermont; David Ransohoff, M.D., Department of Medicine, University of North Carolina; and Ziding Feng, Ph.D., Fred Hutchinson Cancer Research Center.

The University of Colorado investigators attending will be led by PI Frank Accurso and co-PI’s Ron Harbeck and James Murphy. This group is focused on four pediatric lung conditions associated with high morbidity and mortality: asthma, bronchopulmonary dysplasia (BPD), cystic fibrosis (CF), and pulmonary arterial hypertension (PAH). There are pressing needs for development and validation of biomarkers to aid in the diagnosis and management of these groups of pediatric lung diseases. In particular, combinations of protein biomarkers that can be obtained non-invasively offer great promise. Large, well-characterized pediatric patient populations and extensive expertise in biomarker research will be utilized to accomplish these goals.

The Harvard University and Mass General Hospital delegation will be led by PI Robert Gerszten. This group is focused on the development of functional trend analysis software for proteomics datasets for biomarker triage and the development of high throughput targeted LC-MS techniques to validate novel biomarkers of cardiovascular disease. Co-PI, Marc Sabatine, is working to establish the infrastructure necessary for the validation of novel biomarkers of myocardial injury by: (1) rationally triaging candidate biomarkers using novel bioinformatics and functional pathway analysis, (2) characterizing candidate biomarkers in small, well-phenotyped cohorts, (3) prospectively validating the diagnostic and prognostic utility of novel biomarkers in large, diverse populations, and (4) establishing multimarker risk scores and decision-making algorithms using advanced biostatistical approaches.
The Mayo College of Medicine investigators are led by Iftikhar Kullo, Associate Professor of Medicine and co-PI George Klee, Professor of Laboratory Medicine. The goals of this project are to promote systematic, comprehensive, large-scale validation of existing and new candidate protein markers that are appropriate for routine use in the diagnosis and management of vascular disease, facilitating validation of protein panels that may be used to predict disease susceptibility or to assist in differential diagnosis, disease staging, selection of individualized therapies and monitoring of treatment responses.

Vanderbilt University investigators are led by PI Lorraine Ware, Assistant Professor of Medicine. The Vanderbilt project will utilize a multi-disciplinary clinical proteomics approach that draws on existing expertise in measurement and interpretation of biological markers, high throughput protein assay design, bioinformatics and biostatistics and cutting edge mass spectroscopy proteome discovery efforts. This multidisciplinary team approach, combined with ongoing access to large clinical trial datasets and samples will be used to target major areas of unmet need in both clinical practice and clinical research in ARDS. Vanderbilt also houses the Administrative Core for the Program, Gordon Bernard, MD, Professor of Medicine, core director.

University of Colorado Researchers Demonstrate Added Benefits to Protein Biomarker of Early Disease in Cystic Fibrosis.

University of Colorado researchers of the NHLBI Clinical Proteomics Program have recently demonstrated potential uses of serial determinations of immunoreactive trypsinogen, a pancreatic enzyme precursor, in early Cystic Fibrosis.(1) Specifically, trypsinogen was shown to be a potential measure of treatment outcome in young children with CF as well as the basis of a quantitative phenotype that can be used for studies of modifier genes.

Cystic Fibrosis (CF) is a serious medical problem affecting 30,000 individuals in the United States and more than 100,000 worldwide. CF is a hereditary condition resulting from abnormalities in a gene coding for the protein termed the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). The CFTR is a chloride channel expressed on certain epithelial surfaces. Dysfunction of CFTR results in impaired mucociliary clearance, infection, inflammation and scarring of the airways. This process is accompanied by significant respiratory morbidity and mortality. One of the earliest findings in CF is progressive destruction of the exocrine pancreas. Although the mechanism of exocrine pancreatic destruction is not completely clear, it is believed to involve obstruction of the pancreatic duct system leading to the duodenum.

Immunoreactive trypsinogen is a known biomarker of pancreatic injury in CF in the immediate newborn period. It has been used for more than 20 years as the basis for newborn screening programs for CF since it is elevated in the blood of infants with CF compared to normal infants. (2,3) This elevation is thought to result from early exocrine pancreatic injury and “leakage” into the circulation. There are a number of reasons why studying pancreatic injury in CF may contribute to understanding or evaluation of treatment of the lung disease, the major cause of death. The pancreas is destroyed much more rapidly than the lung in CF, as shown by the University of Colorado Researchers. The destruction of the exocrine pancreas occurs by
a few years of age whereas the lung is destroyed over decades. Thus it is possible to follow the course of organ destruction in the pancreas over a more reasonable time frame than the lung. In addition, the pathophysiology in the pancreas is not believed to result from infection and inflammation as in the lung. Hence, it is in some sense less complex.

University of Colorado researchers found that trypsinogen declined steadily in pancreatic insufficient patients with CF after birth. Furthermore, there were rapid and slow decliners, shown schematically in Figure 1. This rate of decline in trypsinogen levels was also heritable when studied in a group of siblings. The rate of decline of trypsinogen provides a potential outcome measure for clinical trials of CFTR altering agents in early childhood. Effective agents might slow or abolish the decline of trypsinogen. In addition, heritability of trypsinogen decline demonstrates that it is a potential target for studies of modifier genes in CF. A modifier gene of early pancreatic injury might also contribute to pulmonary morbidity and hence could be investigated in the pulmonary setting. Targets for treatment of pancreatic disease alone might also be useful in treatment of lung disease in CF because it is believed that the lung disease is often complicated by undernutrition. Given the nutritional treatment burden in CF, any means of prolonging exocrine pancreatic function would be useful to individuals and families.

![Image](image.png)

**Figure 1.** Schematic representation of trypsinogen decline in early CF. Infants with CF have elevated trypsinogen levels at birth compared to normal infants, forming the basis of newborn screening. In some patients, decline in trypsinogen to below than the normal range occurs rapidly and in some patients more slowly. Decline to below the normal range indicates very limited exocrine pancreatic function. Trypsinogen levels over time could provide an important biomarker for treatment effect of early intervention in CF. In addition, rate of decline in trypsinogen is heritable suggesting that examination of this protein biomarker could be useful in the search for modifier genes.
The statistical group at the University of Colorado, sponsored by the NHLBI Proteomics Program, NIDDK and the Cystic Fibrosis Foundation, led the analyses for this study. Drs. Marci Sontag and Gary Zerbe, Clinical Proteomics Program investigators, developed several new statistical approaches to perform this study. First, they constructed a longitudinal model of a clinically useful test, trypsinogen determination, but with the added complication of routine upper limit truncation. Second, they developed a statistical approach to examining heritability in a statistically modeled quantitative phenotype. They are now pursuing the addition of other protein biomarkers to trypsinogen decline in order to determine whether there is added benefit through consideration of panels of biomarkers in early CF.

(2) Kaye CI; Committee on Genetics; Accurso F, La Franchi S, Lane PA, Northrup H, Pang S, Schaefer GB. Introduction to the newborn screening fact sheets. Pediatrics. 2006 Sep;118(3):1304-12.

**Update from the Mayo Clinic Vascular Proteomics Program**

We have completed measurement of ~30 candidate proteomic markers in several etiologic pathways of vascular disease. Data ‘clean up’ is in progress and analyses will begin shortly. We are continuing to explore assay platforms with multiplexing capability and have discussed collaborative efforts with industry.

From the educational standpoint, we held the Biomarkers of Cardiovascular Risk: State of the Art conference on Nov 16-17, 2006. Conference Directors were George G. Klee and Ifitkhar J. Kullo. The meeting was well attended and generally there was positive feedback from both faculty and participants. The conference opened with a keynote presentation by Dr. Peter Libby entitled “Cardiovascular Biomarkers: Insights from Atherosclerosis Biology.” Dr. Libby highlighted the pivotal role of inflammation and thrombosis in atherosclerosis biology and also discussed the potential clinical utility of markers in these pathways – not only to enhance our ability to refine risk prediction but also to speed the evaluation of novel therapeutics. Dr. Christie Ballantyne followed Dr. Libby with an overview of markers of inflammation. He discussed how inflammatory markers could improve risk assessment for the development of cardiovascular diseases (CVD) and diabetes as well as individualized therapy. He also discussed how these markers might guide selection for intensity of therapy and provide surrogate markers of efficacy of therapy. He highlighted emerging data for LpPLA2 as a novel inflammatory marker for assessing cardiovascular risk. Dr. Gordon Lowe spoke next and discussed markers of thrombogenicity in assessing cardiovascular risk, in particular, fibrinogen. He felt that D-dimer merits evaluation and in assessing CHD risk.

Dr. Paul Holvoet presented an overview of assays for oxidized LDL, its association with cardiovascular disease, and comparison of the various assays for detecting oxidized LDL. He compared two antibodies for oxidized LDL – the MAB-4E6 and the DLH3. Dr. Holvoet also compared the Mercodia competition ELISA assay with the Mercodia sandwich ELISA assay. He presented data about oxidized LDL as a risk marker in 3,000 patients from the Health ABC cohort. Risk fac-
tors that correlated with oxidized LDL after adjustment for LDL-C included: BMI, triglycerides, HDL, glucose, insulin, CRP, and fibrinogen. Dr. Holvoet also presented data about the relationship between oxidized LDL and other cardiovascular risk factors and subclinical CVD in different ethnic group in the MESA study. Of note, levels of oxidized LDL were higher in blacks than Hispanics. Metabolic syndrome was associated with higher levels as well.

Dr. Peter Wilson started out by pointing out that several risk factor algorithms for CHD prediction are used around the world. He felt that the following issues needed further study: the analytic and biological variability of measurements, the fact that effects may occur at extreme values, (e.g. quartiles), and the initial need for high sensitivity and follow-up need for high specificity, and time to event, and the type of event. He discussed ROC curves as well as the concept of reclassification using CRP as an example. Dr. Wilson also discussed the need for assessing lifetime risk for CHD instead of just the 10-year probability. Lastly, he discussed the possibility of developing algorithms for the risk of type II diabetes.

Dr. Vasan Ramachandran’s talk was entitled “Assessing Clinical utility of Biomarkers”. He discussed issues related to clinical and analytical validity of biomarkers and pointed out that there is imprecision in assessing CVD risk that needs to be improved. He described the characteristics of an ideal biomarker and also discussed assessment of novel risk factors by using the C-statistic as well as risk reclassification and model calibration. Dr. Ramachandran discussed the utility of a multimarker approach as well as the likelihood that many new candidate markers will be identified as a result of the ‘OMICS’ revolution.

Dr. Shah Ebrahim described the concepts underlying Mendelian Randomization. He described the potential utility of Mendelian Randomization in assessing new biomarkers as well as the limitations of this approach. Dr. Iftikhar Kullo followed Dr. Ebrahim and described the integration of proteomic, genomic, and imaging modalities in refining cardiovascular risk. Dr. Pothur Srinivasa then gave an overview of the NHLBI’s proteomics programs.

Dr. George Klee presented an overview of informatics and an assessment of assay validity. In particular he highlighted the importance of assay traceability and harmonization to translate research into practice. Next, Dr. Gutman provided the FDA perspective on regulatory issues as related to cardiovascular biomarkers. Following Dr. Gutman, Dr. Mehmood Khan highlighted the need for new biomarkers to facilitate personalized medicine. He felt that there was a need to integrate biomarker validation at every stage of the regulatory review for drug, diagnostic, and biological applications.

In the next session, Dr. Ken Kupfer provided an overview of Biosite’s multiple marker panels for diagnosis and prognosis. He described the MMX as a systematic method for combining inputs from several measurements. Dr. Moon followed and provided an overview of the Illumina express Bead XPRESS technology, a novel platform for multiplex detection of protein and nucleic acids. Following Dr. Moon, Dr. Chang Liu described his research in developing a chip-based multiplex detection platform for protein biomarkers and Dr. Dennis Cook described statistical methodologies for dimension reduction in the context of multiple markers focusing particularly on principal components. Finally, Dr. Hanno Langen described biomarker discovery efforts at ROCHE as well as their new multimarker platform called IMPACT.
The Center is comprised of a consortium of cooperating institutions, including the Massachusetts General Hospital (PI: Robert Gerszten, MD), Brigham and Women’s Hospital/Thrombolysis In Myocardial Infarction (TIMI) Study Group (co-PI: Marc Sabatine, MD), Harvard Medical School (Co-Investigator: Fritz Roth, PhD), and the Broad Institute of Harvard University and the Massachusetts Institute of Technology (Co-Investigator: Steve Carr, PhD). The multidisciplinary group of investigators in our Center contributes expertise in cardiovascular basic science, diagnosis and treatment of acute coronary syndromes, epidemiology and bioinformatics, basic and clinical chemistry, and mass spectrometry. The first goal of our Center is to establish the infrastructure necessary for the validation of novel biomarkers of myocardial injury. Our efforts will focus on markers that participate in functional pathways contributing to or triggered by an ischemic insult, thus providing not only diagnostic and prognostic information, but also mechanistic insight into therapeutic responses and novel targets for intervention. When the infrastructure is complete, the next goal is to build on our initial observations and working hypothesis that simultaneous assessment of multiple biomarkers examining different pathophysiological axes will provide complementary information to improve diagnosis, clarify prognosis, target therapeutic interventions, and monitor treatment responses in patients with coronary disease (Sabatine et al, A multimarker approach to risk stratification in non-ST elevation acute coronary syndromes: Simultaneous assessment of troponin I, C-reactive protein, and B-type natriuretic peptide. Circulation 2002;105:1760-3).

Vanderbilt Investigators Identify Novel Markers of Alveolar Epithelial Injury and Nitric Oxide Production in Acute Lung Injury

In collaboration with co-investigator Michael Matthay’s group at UCSF, Vanderbilt Clinical Proteomics Program investigator Lorraine Ware has recently studied two new biological markers with important implications for diagnosis and prognosis in acute lung injury. The first biomarker, Receptor for Advanced Glycation Endproducts (RAGE) is a membrane protein that is very highly expressed in alveolar epithelial type I cells, the predominant cell lining the airspaces. In both rat studies and human studies, high levels of RAGE are released into the alveolar compartment and plasma in the setting of acute lung injury. In rats, both serum and bronchoalveolar lavage fluid levels were associated with the degree of lung injury. These findings were published in the American Journal of Respiratory and Critical Care Medicine 2006; 173:1008-15 and suggest that RAGE may be a novel marker of alveolar epithelial type I cell injury in acute lung injury. Studies are now ongoing to characterize RAGE levels in large groups of patients at risk for acute lung injury after lung transplantation and with established acute lung injury from a variety of causes.

The second biomarker, urine levels of nitrate and nitrite (NOx), is a measure of endogenous nitric oxide (NO) production. Based on a preliminary single center study of plasma levels of NOx, Dr. Ware and colleagues had originally hypothesized that higher levels of urine NOx would be associated with adverse outcomes in patients with acute lung injury. Contrary to their hypothesis, higher levels of urine NOx were independently associated with better outcomes even with adjustment for other clinical covariates. These exciting findings suggest that higher levels of endogenous NO production could either be protective in and of themselves in patients with acute lung injury, or might be a marker of better preservation of normal endothelial function. Further studies to determine the underlying mechanisms of NOx production and to determine whether plasma NO levels show the same pattern are underway. This work was published in the American Journal of Respiratory and Critical Care Medicine 2006, epub Nov 2.
Proteomics Education at Vanderbilt: Focus on Fellowship Training

As part of the educational mission of the Clinical Proteomics Programs, Vanderbilt is currently training three postdoctoral fellows. Training in Clinical Proteomics at Vanderbilt includes both formal coursework in Proteomics and other clinical research topics through the Vanderbilt Masters in the Science of Clinical Investigation Program and mentored research in biomarker development. A summary of our current trainees is below:

Minerva Covarrubias M.D.

Dr. Covarrubias completed her medical school training at the Robert Wood Johnson Medical School and her medical residency at the University of Rochester. She is currently in her third year of pulmonary and critical care fellowship training. Under the mentorship of Dr. Lorraine Ware she is investigating novel biomarkers for prediction and diagnosis of acute lung injury after lung transplantation, also known as primary graft dysfunction. She has successfully obtained funding for her work through a Minority Supplement Award from the NHLBI. Her work on ICAM-1 and von Willebrand factor antigen as novel markers of primary graft dysfunction was recently presented at the American Thoracic Society International Meeting.

Timothy Girard M.D.

Dr. Girard completed his medical school training at University of Texas Southwestern Medical School and his medical residency at University of Virginia Health System. He is currently in his fourth year of pulmonary and critical care fellowship training. He will complete his Masters in the Science of Clinical Investigation in June. Under the mentorship of Dr. Wes Ely and Dr. Lorraine Ware his research focuses on biomarkers of delirium and long-term cognitive dysfunction in critically ill patients. He recently completed a large clinical trial studying the effect of daily awakening and spontaneous breathing trials on duration of ventilation and long-term cognitive outcomes in critically ill patients.

Richard Fremont M.D.

Dr. Fremont completed his medical school training at State University of New York at Syracuse College of Medicine and his medical residency at Emory University. He is currently in his second year of pulmonary and critical care fellowship training. Working with Dr. Lorraine Ware, he is studying biomarker panels for the diagnosis of acute lung injury in critically ill trauma and surgery patient populations.
Major National Meetings Featuring NHLBI Clinical Proteomics Program Participants

Twentieth Annual North American Cystic Fibrosis Conference

The North American Cystic Fibrosis (CF) Conference was held in Denver in November 2006. Clinical Proteomics was featured in two large sessions and in a number of abstracts, several by NHLBI Clinical Proteomics Program researchers.

Dr. Frank Accurso co-chaired a workshop entitled “Clinical Proteomics and Inflammation.” Inflammation plays a prominent role in the progressive lung disease of CF, hence a number of studies have targeted potential protein biomarkers of inflammation. In summarizing the workshop, Dr. Accurso highlighted potential benefits of biomarker applications in CF and stressed the need for thorough validation of biomarkers.

In the “Tools for Measuring Early CF Lung Disease,” Dr. Accurso gave an invited presentation, “Proteomic Biomarkers of Lower Airways Disease” emphasizing different routes to discovery of protein biomarkers. These routes include unbiased proteomic experiments as well as targeted multiplex analysis. The unbiased proteomic experiments involve analysis of plasma or respiratory samples such as sputum, bronchoalveolar lavage fluid and breath condensate, with the goal of identifying and quantitating as many proteins as possible. Then the protein profiles are compared to those obtained from patients with other conditions, normal individuals and other patients with CF who have differing clinical and laboratory characteristics. Discovery proteomic experiments may isolate hundreds or thousand of proteins. In a targeted multiplex approach, investigators assemble a panel of between 5 and 20 candidate proteins based on what is known about the pathogenesis of the disease. Patient samples are then interrogated with respect to these candidates. Current studies of respiratory samples from the early CF airway have isolated a number of neutrophil related proteins including those in the S-100 group and chemoattractants including IL-8 and IL-17. The clinical utility of these proteins is being explored.

Dr. Scott Sagel, of the University of Colorado, presented a poster examining protein biomarkers in a multicenter trial in young children with Cystic Fibrosis. This trial examined the use of rhDnase in children six to ten years of age in a randomized, placebo controlled study. The children were specifically selected for having mild lung disease by pulmonary function testing. The pulmonary function test used is the Forced Expiratory Volume in one second (FEV1), which is known to correlate with outcome in CF. The children in this study had FEV1 greater than 85% predicted for normal children their age and thus were very close to normal. Dr. Sagel examined a multiplex panel of 13 potential protein biomarkers including pro-inflammatory cytokines, antiinflammatory cytokines, and growth factors. Rantes, CRP and IGF-1 each were significantly correlated with some clinical outcome. Although the analysis of whether some combination of these proteins may provide additional value, this study at least shows that even in young children with mild lung disease, protein biomarkers of disease state can be identified.

HUPO 2006

The international Human Proteome Organization met this past fall in Long Beach, California. The theme of the meeting was “Translating Proteomics from Bench to Bedside.” Robert Gerszten, MD, served as one of the moderators for discussions at the burgeoning Cardiovascular Disease Initiative—one of several new groups within the broader organization working to harness proteomics technologies towards specific disease focuses. The Cardiovascular Disease Initiative is establishing a website as a mechanism for the exchange of protocols and reagents, as well as to help coordinate collaborative studies around available clinical cohorts. Dr. Gerszten and Dr. Pothur Srinivas (NHLBI Clinical Proteomics Center Program Officer) also chaired an NHLBI-sponsored Clinical Proteomics Workshop.

American Heart Association

The recent meeting in Chicago in November, 2006 highlighted a spectrum of proteomics efforts, including a session dedicated entirely to Clinical Proteomics. Studies by multiple representatives of the Clinical Proteomics Centers in particular were highlighted. Marc Sabatine of the Harvard group presented studies of early markers of myocardial injury, for which none of the presently available biomarkers are adequate. His studies focused specifically on “the Road to Translation.” Ifitkar Kullo from the Mayo Clinic, in turn, described his group’s studies in richly phenotyped chronic ateriovascular populations to identify novel markers of large vessel (peripheral artery), small vessel (coronary artery) as well as microvessel (renal) diseases. Two speakers also highlighted work devoted principally to lung disease. Lorraine Ware from the Vanderbilt group described their studies to define diagnostic and prognostic profiles of the acute lung injury, another pathology for which no definitive circulating biomarkers exist. Frank Accurso then described studies dedicated to the diagnosis of cystic fibrosis in children, highlighting the development of immunoreactive trypsinogen.

American Physiological Society Conference

Investigators from the Vanderbilt and Denver Children’s Hospital Clinical Proteomics Programs were among the invited speakers at the recent American Physiological Society “Physiological Genomics and Proteomics of Lung Disease” Conference in Ft. Lauderdale from November 2nd-5th, 2006. The conference, sponsored by the American Physiological Society, the NHLBI, the NIEHS and Sepracor Inc. brought together experts in genomics, genetics and proteomics to discuss recent developments in “omics” approaches to lung disease. Richard Caprioli, director of the Mass Spectrometry Research Facility at Vanderbilt University presented a summary of the groundbreaking work by his laboratory in the development of in situ tissue imaging by mass spectrometric protein profiling. Mark Duncan from the University of Colorado Health Sciences Center and a member of the Denver Children’s Clinical Proteomics Program presented a proteomic analysis of bronchoalveolar lavage fluid and pulmonary edema fluid from patients with acute lung injury. Lorraine Ware, the principal investigator of the Vanderbilt Clinical Proteomics Program presented a summary of her work on both targeted and discovery proteomics in plasma from patients with acute lung injury with a focus on sampling handling issues for proteomic research. A summary of the proteomic presentations from the conference will be published in an upcoming issue of the American Journal of Physiology Lung Cell and Molecular Physiology.
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