NORTH CAROLINA DIABETES RESEARCH CENTER METABOLOMICS CORE LAB

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1. Introduction

Comprehensive metabolic analysis, or "metabolomics", is a technology that defines the chemical phenotype of human subjects and animal models. As such, it has unique potential for discovering biomarkers that predict disease incidence, severity, and progression, and for casting new light on underlying mechanistic abnormalities (1, 2). Metabolomics measures chemical phenotypes that respond to genomic, transcriptomic, proteomic and environmental variability, thus providing a highly integrated profile of biological status. The evolution of targeted and non-targeted methods for static profiling of metabolites has been accompanied by advances in metabolic flux analysis, in which heavy atoms from stable isotope-labeled substrates are detected as they label downstream metabolic products. When used together, as they will be in this NIH-funded North Carolina Diabetes Research Center (NCDRC) Core, these assembled tools provide a deep and dynamic view of metabolic functions of cells, tissues/organs, and living animals and humans.

The Sarah W. Stedman Nutrition and Metabolism Center (now part of the Duke Molecular Physiology Institute, DMPI) has operated a research-dedicated metabolomics core laboratory since 2003. In that time, the lab has executed >400 collaborations, resulting in >250 publications co-authored by core lab scientists (see representative publications, 1-38). Today, the laboratory operates a comprehensive suite of 13 mass spectrometry instruments that facilitate both targeted and non-targeted static metabolomics assays as well as metabolic flux measurements that are applied to a wide array of sample types. By deploying these tools, including guidance in data analysis and interpretation, we expect that the core will help the NCDRC consortium in making impactful scientific discoveries that can be translated for improvement of human health.

The NCDRC metabolomics core has several innovative features. First, it has steadily developed one of the most comprehensive platforms for rigorous, quantitative targeted metabolic profiling by MS/MS and GC/MS, backstopped by one of the world's largest libraries of stable isotope-labeled standards. Second, it has developed a retention-time locked spectral library that contains approximately 1300 analytes to support its non-targeted GC/MS platform, allowing rapid provisional identification of metabolites when using GC/MS in a discovery mode. Third, it has recently added capabilities in non-targeted metabolite profiling by LC-QTOF and Q-Exactive/Orbitrap technology, as well as metabolic flux analysis, creating a "one-stop shopping", comprehensive metabolic analysis core for NCDRC consortium members. ***Overall, an important feature of the core over its 17-year history is that it has managed to balance a high level of productive collaborative service with continuous development and deployment of new methods.***

2. Targeted Metabolomics

For targeted analyses, the Duke lab deploys 3 GC/MS and 4 triple quadrupole (MS/MS) instruments to measure approximately 350 metabolites of known identity across multiple analyte modules:

a) A set of approximately 200 acylcarnitines (depending on tissue type and assay method) ranging from 2 to 22 carbons in length and varying degrees of saturation (number of double bonds). These metabolites report on mitochondrial metabolism of glucose, lipids, and amino acids.
b) A set of approximately 60 acyl CoA species ranging from 2 to 20 carbons in length and varying degrees of saturation. Long-chain acyl CoAs are involved in fatty acid oxidation and lipid esterification, while shorter chain species are used for a variety of protein acylation reactions that influence protein functions.
c) A set of 20 organic acids (depending on tissue type) comprising TCA cycle intermediates and related metabolites
d) Amino acids and their metabolic byproducts, including urea cycle intermediates, branched-chain ketoacids, and disease-relevant products of branched-chain amino acid metabolism
e) A set of 31 purine pathway intermediates and nucleotides;
f) A set of 21 ceramides and 30 sphingolipids.

A complete set of analytes measured by the Core Lab's targeted methods is shown in **Figure 1**.

- **Flow Injection MS/MS**
  - **Amino Acids (15 analytes)**
  - **Acylcarnitines (66 analytes)**
  - **Free and Total Carnitine**
  - **Acyl CoAs (57 analytes)**
  - **Ceramides (21 analytes)**
  - **Sphingomyelins (30 analytes)**
  - **Creatinine**
- **LC-MS/MS**
  - **Malonyl CoA**
  - **Tryptophan and Kynurenic Acid**
  - **Glutamate and Glutamine**
  - **Leucine and Isoleucine**
  - **Nucleotides (31 analytes)**
  - **Acylcarnitines (>200 analytes)**
  - **Ketoacids (KIV, KIC, and KMV)**
  - **Disease biomarkers (TMAO, 2-AAA, BAIBA, 3-HIB, etc)**
- **GC/MS**
  - **Organic Acids (20 analytes)**

Figure 1. Analytes measured by targeted MS, NCDRC metabolomics core lab.

Over its years of operation, the Duke lab has accumulated a large library of stable isotope-labeled internal standards that are added to biological samples in known quantities to enable target analyte quantitation. For some modules (e.g. amino acids, organic acids), every targeted analyte has its own cognate stable isotope-labeled standard, whereas for others (e.g. acylcarnitines) representative standards for each size class are included (5, 7, 9, 17). Use of a comprehensive "cocktail" of internal standards for each assay module allows quantitative and reproducible measurement of metabolites, with data expressed in molarities rather than relative units. A complete list of the stable isotope-labeled standards used in our assays is provided in **Figure 2.** Acylcarnitine, acyl CoA, ceramide and amino acid analyses are conducted by flow injection-MS/MS, as previously described (5, 7, 9). GC/MS is used to measure organic acids (7-9). Nucleotides and their precursor metabolites are measured by LC-MS/MS (17). Resolution of isomeric species of amino acids, keto-

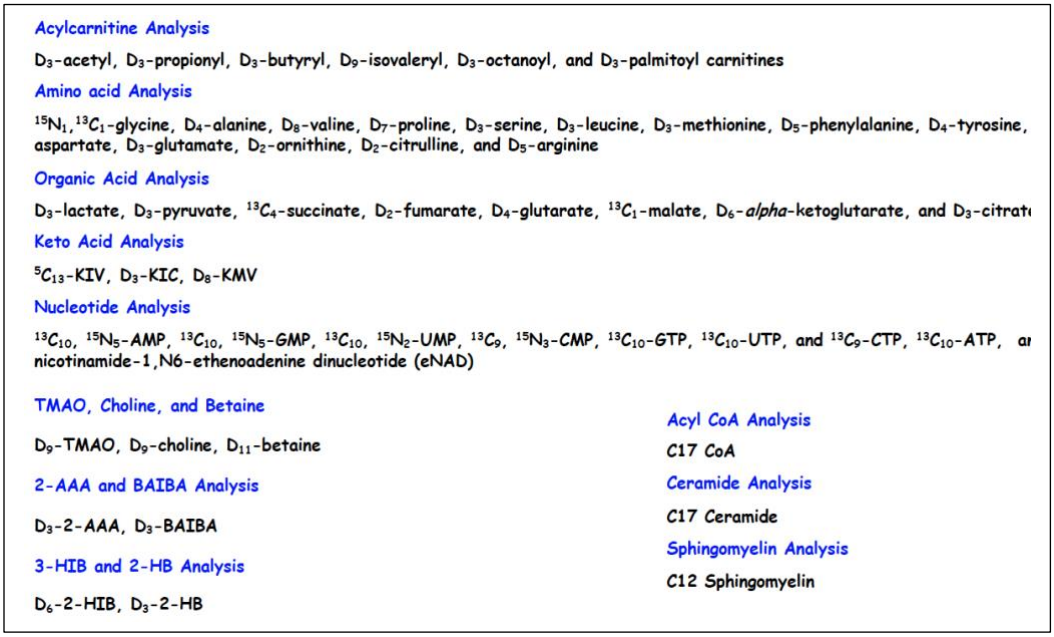


Figure 2. Internal standards used for targeted metabolomics analyses, NCDRC metabolomics core lab.

acids, acyl CoAs and acylcarnitines is achieved by LC-MS/MS, and customized internal standards for such assays are synthesized or acquired as needed (18, 23). Finally, the core operates a Beckman Unicel DxC 600 autoanalyzer for analysis of conventional metabolites such as glucose, lactate, total ketones, β-hydroxybutyrate, free fatty acids, glycerol, uric acid, triglycerides, total cholesterol, LDL, and HDL (9). Although the number of analytes measured by our targeted methods is small relative to the estimated number of metabolites in the human "metabolome", the modules are highly useful because they report on classical pathways of metabolism of the three major classes of macronutrients--lipids, carbohydrates, and protein.

In addition to metabolites found in classical pathways of intermediary metabolism, the Core has an ongoing program for development of targeted assays for emergent disease biomarkers. Recently, this has included development of new targeted assays for disease- and biology-associated branched chain amino acid metabolites

(BAIBA, 3-HIB), other disease-associated amino acid metabolites (2-AAA, 2-HIB), and a suite of metabolites associated with cardiovascular diseases and microbial metabolism--TMAO, choline, and betaine (see Ref 1 for review of disease associations).

For targeted metabolomics, the Core's quality-control (QC) program relies on ladders of stable isotope-labeled internal standards (IS) (5, 7, 9, 17), calibrant series in which biologic matrices are spiked with pure, unlabeled reagents, and longitudinal QC reference materials such as human serum from an aliquoted and frozen preparation. For example, in the assay of amino acids (AA), large lots of IS and calibrants (adult bovine serum spiked with unlabeled AA) are prepared, aliquoted, and frozen at -80 ºC until use. Each 96-well plate contains a calibrant series, a "ghost" (process blank), both high- and low-AA longitudinal QC reference sera, and unknowns. Calibrant series and the two QC sera are analyzed at the beginning and end of each plate. Use of two independent QC samples in each plate enables monitoring of interday and intraday precision of the assay. Similar QC procedures are used for each of the targeted modules, and coefficients of variation (CV) for the targeted assays are generally ≤ 10%.

3. Non-targeted Metabolomics

The laboratory also applies non-targeted MS methods using platforms of intermediate (GC/MS) and higher (LC-QTOF and LC-MS/MS/Orbitrap) resolution. When using GC/MS in a non-targeted mode, metabolite identification is facilitated by a retention-time-locked (RTL) metabolite library originally created by Dr. Oliver Fiehn's group with a set of 700 analytes (39) and marketed by Agilent, and then expanded upon by Dr. James Bain and Dr. Mike Muehlbauer in the Duke lab, such that it currently contains ~1300 analytes. The Duke team, working with Dr. Denise Scholtens at Northwestern University, has developed a mixture model in the R statistical computing environment that accommodates missing metabolite values in non-targeted analyses (40,41). For cross-platform integration, metabolite data gathered using the conventional clinical analyzer, targeted MS methods, and non-targeted GC/MS are subjected to per-metabolite analyses and collective biochemical pathway analyses using UniPathway annotation. We have applied this technology to several biological models, including microbiome studies at Duke and with Dr. Jeff Gordon and associates (19-21) and analyses of effects of maternal metabolism on metabolic health in offspring with Dr. Scholtens and Dr. William Lowe (22, 41, 42).

The laboratory recently received funding from the Duke School of Medicine for purchase of new Thermo Q-exactive (Orbitrap) and Agilent 1290 liquid chromatography (LC)/6546 QTOF mass spectrometer (MS) systems, providing the group with ultra-high resolution non-targeted metabolomics tools that provides complementary coverage when overlaid with the GC/MS methodology. Our recently acquired instruments provide superior resolution and broad dynamic mass range that allow us to expand beyond the metabolites detected by our non-targeted GC/MS methods. In addition, the 6546 QTOF provides the capability to identify trace-level target compounds in the presence of more abundant matrix metabolites with up to five orders of in-spectrum dynamic range. The Agilent MassHunter software and proprietary library of metabolites facilitate compound detection and identification. We employ reverse and normal phase chromatography using Thermo Hypersil Gold and Agilent HILIC-z columns respectively, coupled with positive and negative MS modes to characterize diverse classes of small molecules from a variety of sample types (e.g., cell culture, tissue, plasma, etc.). The extracted *m/z* features are chromatographically aligned across multiple data files using the Agilent Profinder program. This step minimizes the appearance of false positive and false negative features by "binning" in the chromatographic time domain, allowing removal of redundant and non-specific information. The files generated by Profinder are imported into Agilent Mass Profiler Professional (MPP), a chemometrics platform used to determine relationships among two or more sample groups and variables. MPP provides us with comprehensive statistical tools including ANOVA, PCA, volcano plots, hierarchical trees, etc. and visualization options for many types of MS data analysis.

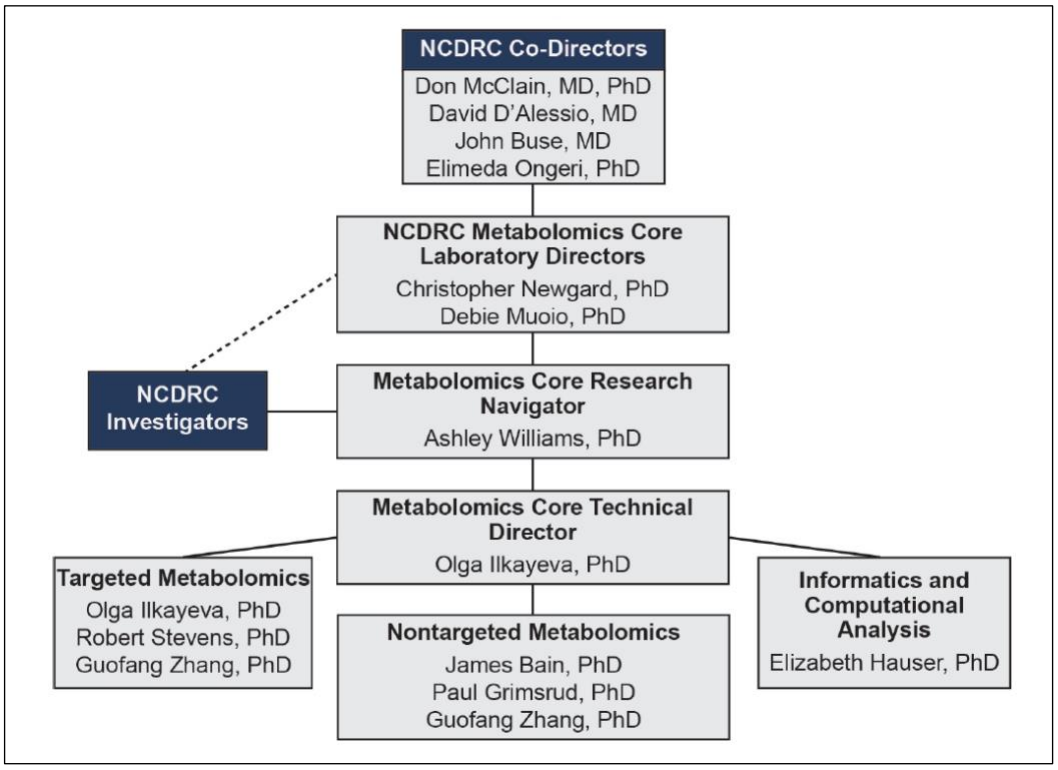
4. Flux analysis

Finally, the Core has installed capabilities for metabolic flux analysis with stable isotope tracers, via recruitment of Drs. Guofang Zhang and Scott Crown to the DMPI. Dr. Zhang joined our faculty after working closely with Dr. Henri Brunengraber at Case Western for several years with MS-based methods for measurement of metabolic fluxes (43, 44). Dr. Crown trained in the laboratory of Dr. Maciek Antoniewicz, a leader in the area of computational analysis of metabolic flux (45, 46). These scientists are working closely with DMPI labs to decipher

mechanisms of glucose-stimulated insulin secretion, mitochondrial metabolism of lipids, changes in branched-chain amino acid fluxes in response to dietary and drug treatments, measurement of lipogenesis in both the in vitro and in vivo settings, and tracking of acyl groups used for post-translational modification of histones and mitochondrial proteins, as illustrated by recent publications (28, 46-48). Their efforts are supported by an AB Sciex UPLC-MS/MS 6500 instrument for sensitive and precise resolution of isotopomer species. Collaborative projects involving flux measurements are already engaged with NCRDC faculty, and more collaborations are anticipated as this program evolves.

5. Core organization, leadership, and operating plan

The figure below summarizes the organizational structure of the proposed NCDRC metabolomics core. The core is co-led by Drs. Christopher Newgard and Debbie Muoio, senior faculty members of the Stedman Center/DMPI who each have decades of experience in diabetes and metabolic disease research. Drs. Newgard and Muoio have overseen the development of the Stedman/DMPI metabolomics core laboratory since its inception in 2003 as an in-house analytical unit, through to its current standing as an internationally-recognized field leader and highly collaborative laboratory, with > 400 executed collaborative studies and > 250 publications. During that time, the laboratory has expanded its technical capabilities to include an array of targeted, non-targeted, and stable isotope flux analysis capabilities. Drs. Newgard and Muoio are committed to work closely with Drs. McClain, D'Alessio, Buse, and Ongeri to coordinate the efforts of the Metabolomics Core with the other NCDRC components.



Dr. Ashley Williams is a senior research scientist in the Muoio lab at DMPI who is deeply trained in metabolic physiology via her PhD studies in the laboratory of Dr. David Wasserman at Vanderbilt. She has continued her training as a postdoctoral fellow and senior scientist in the Muoio lab at the DMPI for the past five years, during which time she has been collaborating with Drs. Muoio, Zhang and Crown to develop new metabolic flux methods, including a novel method for measuring insulin sensitivity via tissue uptake of 2FDG. Her deep experience in metabolic physiology via her training with Drs. Wasserman and Muoio makes her an ideal person to perform a dedicated "navigator" role for NCDRC customers, especially in the area of data interpretation. Dr. Williams will collaborate closely with Dr. Olga Ilkayeva, Technical Director of the DMPI metabolomics core lab, to serve as the Study Coordinators for the NCDRC Metabolomics Core laboratory. Based on our historical experiences, new users will typically approach the Stedman Center/DMPI core laboratory via informal communication with Drs. Newgard or Muoio. When such inquiries come from Duke, Wake Forest, or UNC NCDRC members in the future, they will be referred to Drs. Williams and Ilkayeva, who will interview the affiliate investigator to understand the research project and its fit (or lack thereof) with the suite of metabolomics technologies available at DMPI. Assuming that the project is well-suited for the tools of the Core, investigators will receive information about pricing and sample preparation, and a data storage and analysis plan will be developed. Dr. Ilkayeva will then route samples to the appropriate core scientists for execution of the requested

assays. Following generation of metabolomics data sets and their safe storage in the PEDIGENE® data base housed at DMPI, users will be able to choose between performing their own statistical/computational analysis of the data, or can request assistance from the DMPI computational biology group led by Dr. Scott Crown and James Draper, who will be partially funded by this core grant. ***After data analysis has occurred, a special feature of the NCDRC Metabolomics Core kicks in. Investigators will be able to request assistance in interpretation of their metabolomics data sets from Dr. Williams. When needed, Dr. Williams can seek further consultation with Drs. Newgard or Muoio. Through this approach, we hope to overcome what is clearly a recurrent weakness of "metabolomics for hire" laboratories in both the private and university sectors, which is their failure to properly assist users in interpreting their data in a physiologic or disease context. The proposed structure provides uncommon guidance for users in their use of the metabolomics core lab from start to finish, beginning with assistance in study design and sample collection methods, and ending with assistance in data interpretation.*** A more complete roadmap of a typical investigator interaction with the metabolomics core follows.

6. Road Map for interaction of NCDRC members with the Metabolomics Core

A. The lead contact person for initiating a collaboration with the NCDRC Metabolomics core lab is Dr. Olga Ilkayeva (Olga.Ilkayeva@duke.edu; 919-479-2370). Drs. Williams and Ilkayeva will meet with the prospective user to understand the nature and size of the project, and the projected number of samples. They will introduce the user to the suite of analytical technologies in the NCDRC metabolomics core laboratory and pricing for various kinds of assays. Based on this discussion, Drs. Williams and Ilkayeva will determine if there is a fit between the goals of the user project and the technologies resident in the metabolomics core laboratory, in consultation with Drs. Newgard and Muoio if needed. When a project is deemed in good alignment with the core's capabilities, Dr. Ilkayeva will identify scientists in the core laboratory with the particular expertise for performance of the chosen assays. Over our years of operation, the Duke team has found that some groups of collaborators have indwelling bioinformatics and computational analysis capabilities, and prefer to store and analyze the data themselves. In those instances, the Duke core will simply store the data set on its internal PEDIGENE® system as a backup source of the original data. In other instances, the user seeks assistance with data storage and analysis. In those cases, James Draper and Scott Crown can provide assistance with statistical and computational analyses. Drs. Williams, Ilkayeva, and the chosen analytical and statistical analysis experts will constitute the Project Team.

B. The Project Team will work with the user to develop a study plan. The user will be provided with precise sample collection and processing protocols, and will be instructed to label homogenized/processed tissue or bodily fluid sample containers with bar-coded labels provided by the project team. The user will perform the clinical, animal, or cellular study and will prepare samples as instructed by Dr. Ilkayeva. Once collected, samples will be rapidly frozen, and stored at -80$_{o}$C until they are either hand-delivered or shipped on dry ice to Dr. Ilkayeva at the DMPI metabolomics core laboratory. Dr. Ilkayeva will log in the sample shipment via the bar codes on the sample tubes, and will immediately store samples in -80$_{o}$C freezers available at DMPI.

C. Metabolomics analyses will be performed according to the study plan devised by the Project Team, and data will be stored in the PEDIGENE® data management system resident at DMPI. This system was originally designed by Duke investigators (http://dmpi.duke.edu/research-informatics-shared-resource), and is a flexible, integrated system for managing research informatics needs for hundreds of studies via large databases. It utilizes a multi-layered security system to ensure participant confidentiality, and allows for transfer of data to a wide variety of analysis program formats and storage of analysis results. Storage of data at different phases of analysis ensures data retrievability and provenance. Relevant data will be uploaded to the Common Fund Metabolomics Program Data Repository and Coordinating Center (NCDRCC), no later than the acceptance of the first publication of the findings from the data set. All shared data will be adherent to US Common IRB and HIPAA regulations and will ensure patient confidentiality.

D. Based on our 17 years of experience in the Stedman Center/DMPI metabolomics core lab, we expect to execute numerous NCDRC collaborative projects, many involving large and complex data sets. ***Thoughtful interpretation of the data will be critical for maximizing the impact of research conducted by NCDRC members.*** A highly experienced member of the Duke team, Dr. Ashley Williams, will dedicate a significant

component of her overall effort to work with NCDRC collaborators in interpretation of their data sets. When helpful, Dr. Williams is able to seek assistance from Drs. Newgard or Muoio for additional consultation. Examples of interpretive activities include identifying changes in clusters of metabolites according to the pathways in which they participate, and providing context at a systems biology level for such changes. This includes signatures of metabolic fuel selection, as well as deducing the contribution of different tissues and unique gut microbiome metabolites to global profiles in plasma, urine or other bodily fluids. When warranted by tangible scientific contributions, Dr. Williams will serve as a co-author of manuscripts for publication.

7. Cost recovery: Fees and reimbursement, and explanation of core budget

By NIH policy, NCDRC members will pay the same fees for metabolomics core services provided by the Stedman Center/DMPI metabolomics core laboratory as charged to any and all academic, NIH-funded investigators. The Duke lab has calculated a no-profit cost structure for its targeted metabolomics "modules" that are all-inclusive, including personnel costs, materials and reagents, and instrument time. The costs shown below assume that the NCDRC collaborating laboratory will perform all sample isolation and extraction/homogenization procedures according to detailed protocols provided by Dr. Ilkayeva.

Metabolomics Pricing, FY2020

Service	Price	unit	
Mass Spec assay-Amino Acid Panel (AA)	$52.38	each	
Mass Spec assay- Acylcarnitine Panel (AC)	$52.38	each	
Mass Spec assay- combined panel AA/AC	$77.20	each	
Mass Spec assay-Organic Acid Panel (OA)	$84.30	each	
Mass Spec assay-Acyl Coenzyme A Panel (Acyl CoA)	$100.43	each	
Mass Spec assay-Total and Free Carnitine	$68.33	each	
Mass Spec assay-Nucleotides Panel	$157.86	each	
Mass Spec assay-Ceramides Panel	$102.75	each	
Mass Spec assay-Keto Acids Panel	$116.68	each	
Non-targeted Mass Spec assay	$182.04	each	
MSD Imager assay (1-40 Batch)*	$275.49	**batch**	*kits charged directly as a supply
Plate reader assay (1-40 Batch)*	$217.67	**batch**	*kits charged directly as a supply
Beckman custom assay (undefined)	$5.17	each	
Pyruvate	$5.60	each	
BUN	$4.62	each	
CK	$4.64	each	
HBA1c	$7.29	each	
Microalbumin	$6.55	each	
Glucose assay *	$4.62	each	
Cholesterol assay	$4.64	each	
HDL assay	$5.34	each	
LDL assay	$6.55	each	
TG assay	$4.64	each	
Creatinine assay	$4.62	each	
CRP assay	$7.29	each	
Lactate assay	$5.34	each	
ALT assay	$4.62	each	
AST assay	$4.62	each	
Albumin assay	$4.62	each	
Uric Acid assay	$4.64	each	
NEFA assay	$5.34	each	
Total Ketones assay	$6.91	each	

3-HB assay	$7.40	each
glycerol assay	$5.51	each

The pricing shown for targeted metabolomics modules includes personnel costs for execution of the assay. However, they do not include the core management services provided by Dr. Ilkayeva and Williams, or the interpretive services provided by Dr. Williams; these costs are therefore included in the budget. The pricing also does not include fees for statistical or computational analyses, but partial funding for two members of our team well versed in multi-variate statistics (James Draper) and computational methods associated with metabolic flux studies (Scott Crown) are included in the budget, allowing these core members to engage with collaborative colleagues when requested.

For non-targeted metabolomics we estimate a charge of $182/sample, although this pricing may evolve over the course of the program, as deeper analytic methods are developed. Metabolic flux analysis is also available to NCDRC members, but is not viewed as a "commodity" technology that lends itself readily to fixed pricing. Pricing will be negotiated with collaborators on an individual basis based on the effort commitment of key core personnel and other costs.

8. Pace of service and anticipated user base. As noted above, the DMPI Metabolomics Core already has a large user base, yet the turn-around time for the targeted metabolomics modules averages only 3 weeks. We believe that we will be able to handle the full NCDRC user base with a similar turnaround pace, and with no need for special prioritization of NCDRC users, for the following reasons: i) Many NCDRC members at Duke are already collaborating with the core, and therefore will not contribute to a surge in sample numbers; ii) The core has grown over its 14 years of existence to include 7 PhD-level faculty and staff and 5 technicians, working with 13 different mass spectrometers. Thus, despite its high level of activity, the core is still able to absorb a substantial increase in sample volume with little or no disruption in pace of service. With regard to anticipated users of the proposed core, this is not entirely predictable, but using the Duke user base as a template, 16 of the 35 NIH-funded NCDRC faculty at Duke are current collaborators with the core. In addition, the core is currently engaged in 4 collaborations with Wake Forest investigators, and 4 collaborations with UNC investigators. We believe that with engagement of our Outreach plan, it is reasonable to anticipate engagement in about 25 collaborative projects at Duke and 10 each at UNC and Wake Forest over the 5-year term of the grant.

9. Rigor and Reproducibility. Detailed QC procedures for metabolomics assays were described in Section 2. With regard to data management, data will be stored in the PEDIGENE® computer data management system resident at DMPI, and the features of this system that insure data retrieval, reproducibility and provenance were described earlier. The system allows for the transfer of data to a wide variety of analysis program formats and then stores the analysis results. Importantly, this allows senior core personnel or project PIs to have a traceable record of data handling, ensuring data provenance**.** The Metabolomics core lab has developed a data integrity plan that includes standard operating procedures for maintaining and validating laboratory notebooks, data provenance, and application of appropriate standard operating and Q/C procedures in all work performed in the lab. All laboratory members have read and agreed to the data integrity plan in writing.

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