Metabolomics for NASH R&D

Integrating complexity to enable a clear path forward
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NASH OVERVIEW

A complex interplay of risk factors and molecular pathways challenging therapeutic intervention
NAFLD/NASH: The hepatic manifestation of the metabolic syndrome

Energy surplus results in the deposition of fat in the liver

Obesity and diabetes are metabolic diseases characterized by an imbalance between energy intake and expenditure. The metabolic syndrome, obesity and type 2 diabetes are major risk factors for the development of NAFLD/NASH. It is estimated that 60-80% of the diabetic and obese population is affected by NAFLD. The development of obesity, and by extension NAFLD, is influenced by a combination of factors including genetics, physiology, environment, and daily habits.
NAFLD/NASH

NAFLD/NASH is a liver disease with a spectrum of severity

NAFLD encompasses a spectrum of diseases that range from simple steatosis to NASH, cirrhosis and hepatocellular carcinoma. As the disease progresses, hepatic lipid accumulation is accompanied by excessive inflammation and oxidative stress. Eventually, fibrosis can be detected and used to stage NASH. Fibrosis is the primary clinical predictor of risk for progressing to cirrhosis and eventually liver cancer. Approximately 20-25% of NASH cases will develop into cirrhosis within 10 years.
**Therapeutic development challenges**

Aside from diet and exercise, there are no FDA-approved therapies for NASH and deep mechanistic understanding of the disease is lacking. Consequently, the diversity of targets currently being pursued in NASH is staggering. Fundamentally, lack of a better grasp of disease taxonomy hinders development. As the next section describes, the underlying disease taxonomy involves a constellation of risk factors and molecular pathways.
An unclear constellation of risk factors

The pathology of NAFLD/NASH is not well understood and occurs over a great deal of time. The two-hit hypothesis states that hepatic lipid accumulation weakens the liver and increases susceptibility to the second “hit”, inflammation and fibrosis. The two-hit hypothesis has recently been challenged by research suggesting multiple parallel hits lead to the development of NASH. Additionally, genetics, environmental cues, microbiota metabolism and lifestyle influences come together resulting in an uncertain natural history of disease.
An unclear constellation of molecular pathways

A number of molecular pathways have been implicated in the progression of NAFLD/NASH. Changes in lipid metabolism resulting in the excessive accumulation of hepatic fat content is considered one of the primary drivers of disease. Increases in inflammation and oxidative stress are also observed. More recently dysbiosis of the gut microbiota has been implicated in the onset and progression of NASH. The chronology of these impairments and their precise role during disease progression remain uncertain.
Lipid accumulation and inflammation are hallmarks of NASH

Excessive lipid accumulation and the development of inflammatory markers are established characteristics of NASH. Hepatic liver accumulation results from the combination of increased lipid uptake, synthesis and impaired lipid excretion. NASH is also exemplified by the activation of hepatic stellate cells (HSC) that trigger an immune response causing tissue damage. A subpopulation of NASH patients develop fibrosis which is the primary driver of risk. NASH is predicted to become the leading cause of liver transplants in the near future.
**Redox and NASH**

Oxidative damage is the result of imbalances between reactive oxygen species production and antioxidant capacity

Oxidative stress is one of multiple hits that contribute to hepatic fat accumulation and fibrosis development. Excessive production of reactive oxygen species combined with the decline in anti-oxidant capacity leads to cellular damage (i.e. lipid peroxidation, build up of reactive aldehydes). Metabolon’s platform is capable of detecting lipid peroxidation products and reactive aldehydes. Additionally, we also screen for multiple metabolites with anti-oxidant capacity.
Role of microbiome

It is now appreciated that the microbiome exists in a symbiotic relationship with the host. This association is impacted by external influences such as diet and lifestyle. Disruptions in this relationship lead to pathological states (i.e. NAFLD/NASH). Data suggests that shifts in the gut microbiome can influence a wide range of pathways involved in NAFLD/NASH including energy homeostasis, inflammation and lipid metabolism. Metabolomics offers the unique opportunity to explore the interactions between the host and bacterial metabolism. And when paired with 16S RNA sequencing, offers a comprehensive picture of the connection between the host and the gut microbiome.
Genetic predisposition/approach

**Most traits are complex & individual genetic/biological diversity is high**

Evidence of a genetic component of NAFLD is found in epidemiological, familial and twin studies. However, the penetrance of genetic SNPs is quite variable and often identified genes do not have a known role. For example, PNPLA3 I148M allele is a well known risk factor for NAFLD and NASH; however, the precise function of this gene product and how it influences the disease state is unknown (Dongiovanni P et al, Current Pharmaceutical Design, 2013). Metabolomics potentially serves as a GPS for genomic studies by pointing an arrow at gene function.
Metabolism can be a functional integrator

As you have read on the previous pages, there are multiple inputs and pathways associated with NAFLD/NASH. Metabolism functions as the culmination of disease drivers, pathway interactions and target identification.
Metabolism’s role in NAFLD is well accepted

New technologies allow us to interrogate a broad range of metabolites

Focus is traditionally on lipids and other “one-off” biomarkers; however, this approach has yet to yield a robust biomarker for NAFLD/NASH. We can utilize our global metabolomics platform to survey the entire metabolome across samples to find key changes or differences that are important (i.e. disease vs healthy; drug treated vs vehicle). This approach has the potential to produce “hits” within the metabolome and can provide important insights into associated biomarkers.
WHY METABOLOMICS FOR NAFLD/NASH R&D

A key integrative read-out for many aspects of the disease
Metabolomics primer

Metabolites are a surrogate of the physiological/phenotypic state, making them an ideal way to track changes induced by disease or treatment. The assessment of the metabolic state is particularly important for understanding complex phenotypes where the drivers are numerous (i.e. genetics, environment and microbiota). Metabolomics is the technology for comprehensively measuring and interpreting the metabolic state (i.e. metabolome), as well as an essential technology for defining the key functional attributes of tumors or cancer cells.
Metabolites - a longstanding ally for assessing the phenotype

Since metabolites circulate throughout living systems to maintain all physiological processes, they have had a longstanding history of deciphering how living systems work. This includes mapping how our muscles work, furthering the knowledge of diseases like NAFLD/NASH and diabetes and defining gene function. Furthermore, biochemicals like glucose and cholesterol are used as a staple for clinical-decision making today and have been for over a century.
Current technology opens the aperture

The vast majority of workflows only look at a small proportion of the metabolites and/or molecules, often within the same pathway. Targeted approaches, such as bile acids, provide a limited view under the lamp post. From a NAFLD/NASH research perspective, these are incomplete because the focus is too narrow and limits the capacity to decipher the entire biological story. Improvements in mass spectrometry technology has allowed us to move away from single analyte measurements to large scale metabolic screens. These types of screens provide us the opportunity to interrogate the multiple nodes of NAFLD/NASH in one experiment.
“Pixelated” extremes for insight & biomarkers

Decisions can be abstract based on too little or too much information. Metabolomics, being a proxy to the phenotype, provides a key ingredient to bring things into focus.

Targeted measurements based on what we know often represents a small part of the scenario

Powerful genomic technologies can be a challenge to translate into actionable information

BRINGING IT ALL TOGETHER

METABOLOMICS

A comprehensive, physiologically meaningful, functional readout of the molecular phenotype

- Interpretable
- Integrates other data
USING METABOLOMICS FOR NAFLD/NASH R&D

How metabolomics is being applied to advance NASH R&D
How Groups are Using Metabolomics

Metabolomic profiles are a source of important signals that are often missed by less sensitive (to the phenotype) approaches. Across indication areas it is being applied diversely to derive “signals” that inform on how a target/molecule combination is operating in the context of the model or human subject. These “signals” also often serve as biomarkers.

Signals for:

- Target engagement
  - Efficacy
  - PK/PD biomarkers
- Gauging response and stratifying risk
  - Responders/nonresponders
  - Secondary endpoints
  - Safety
  - Re-purposing potential
  - Compliance
- Unique individual signals
  - Dose
  - Compliance
  - Baseline signatures
Metabolon’s experience in NAFLD/NASH research

Metabolon performs metabolomics studies for most of the world’s leading pharmaceutical and academic research organizations. This includes work across disease indications and from *in vitro* models to clinical studies. Below, our experience in NASH is highlighted across different studies and models.

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Key Applications and Examples

Numerous studies that we have performed for our clients have been published. We share several examples on the following pages. These examples offer insight into the tractability of using metabolomics for your own discovery work within the context of your specific model, cohort and molecule.

1. **TRANSLATIONAL STUDIES**
   - Enriching disease taxonomy
   - Building confidence in biomarkers, molecule and target

2. **DEVELOPMENT**
   - Target engagement
   - Efficacy and safety assessment

3. **DIAGNOSTICS / NEW TARGETS**
Translational studies: Lipidomics revealed the liver lipid signature of patients diagnosed with NASH and NAFLD

Synopsis. Steatosis is defined as the excessive accumulation of fat in the liver and defines just one aspect of the spectrum of liver diseases that extends from NAFLD to NASH. However, the specific types and amounts of lipids that accumulate in NAFLD was unknown. In this study, investigators submitted biopsies from healthy, NAFLD and NASH patients for lipidomic analysis to understand the hepatic lipid composition. This investigation confirmed that diacylglycerol (DAG), triacylglycerol (TAG) and cholesterol were elevated in NAFLD/NASH subjects. Conversely, phosphatidylcholine and many polyunsaturated fatty acids were decreased. Product/precursor ratios suggested upregulation of diacylglycerol acyl transferase (DGAT), the enzyme that converts DAGs to TAGs. In sum, this investigation was among the first to demonstrate alterations in hepatic lipid composition and metabolism in NAFLD/NASH patients.

**Synopsis.** Understanding the liver lipidome is helpful in gaining mechanistic insights into disease taxonomy. However, circulating factors are more practical markers with enhanced clinical application. The circulating lipidomic signature provides a systemic view of global metabolism. Similar to the liver biopsy data, DAG and TAG were elevated in plasma from NAFL and NASH subjects. The quantitative resolution of distinct lipid species (as opposed to class) allowed for more mechanistic insights into disease progression. The increased ratio of 16:1n7 to 16:0 suggested increased activity of stearoyl CoA desaturase (SCD) and *de novo* lipogenesis (DNL) in NAFL patients. The lipidomic analysis also revealed increased levels of oxidized lipids and lipoxygenase (LOX) activation as the disease progressed from NAFL to NASH. Conversely, there were signs of declining peroxisomal function upon the onset of NASH. Opening the aperture with new and improved technologies can provide more insights and reveal targets with therapeutic potential.

**Synopsis.** The metabolic aperture was further opened in a study where plasma from steatotic and NASH patients were subjected to Metabolon’s global metabolic profiling platform. In this study, the entire metabolome (as opposed to the lipidome) was surveyed. These data showed a decrease in glutathione, the major anti-oxidant, and a concomitant increase in circulating bile acids and amino acids as the disease progressed. The global platform is often used to develop targeted assays. After the initial screen, metabolites of interest can be chosen to be a part of a quantitative panel. In this case, Metabolon developed a bile acid panel following the observation that these metabolites were elevated in the plasma of NAFLD and NASH patients. These targeted panels are quantitative and have potential clinical utility.

Summary of translational studies – a translational resource

NASH/Fibrosis – an extensive body of work (~500+ samples) as a translational resource

Finding an appropriate pre-clinical model is a major hurdle in the development of NASH therapeutics. Metabolon has performed lipidomic and metabolomic analysis on multiple human cohorts which has revealed a number of established and less appreciated metabolic signatures. These datasets can be used to evaluate the translatability of your model. Using these tools, many investigators are comparing NASH rodent models with and without treatment to the signatures obtained in these human studies.
Development

Clarifying clinical potential and biomarker strategy
2 Development – Translatable Biomarkers for PK/PD Modeling

Case study example

- Metabolite biomarkers of **efficacy AND target engagement** were identified in translational studies
- Sought to use markers in Phase 1 dose escalation, (PK/PD; efficacy)
  - Across cycles of trial for efficacy
  - More frequent analysis for PK/PD
- Using this “biomarker-rich” data to achieve a precise understanding of dose via a modeling approach to design the phase 2 study with greater clarity

Synopsis. Trial design with the right biomarkers is essential to a successful clinical outcome but they are often limited by a lack of biomarkers that are tightly coupled to target engagement and efficacy – particularly for novel, first-in-class mechanisms. With good markers, precise modeling can be performed on the relationship between PK/PD for both desired and undesired effects, and individual patient characteristics (i.e. “good data” is required to generate models). Robust translational biomarker discovery can deliver a suite of PK/PD & efficacy biomarkers that can provide a deeper line of sight into trial design to maximize the probability of success.

Synopsis Source: Metabolon unpublished example.
Diagnostics/new targets

Building for the future
The potential to develop non-invasive methods to diagnose NAFLD/NASH is consequent to the markers that routinely recur in translational or development studies from metabolomic profiling. Although we believe molecular pathways driving disease may inform on disease taxonomy and new targets will emerge from these types of efforts, we focus the remainder of this section on the diagnostic potential of metabolomics.

### THE GOLD STANDARD: LIVER BIOPSY

- CK-18
- BARD Score
- AST/ALT Ratio
- Ultrasonography
- NALFD Fibrosis Score
- ELF Panel
- FIB-4
- FibroTest
- NASHTest
- NASH FibroSure
- Magnetic Resonance
- NASH CRN Model
- FibroScan

### The Test Landscape.
Tests have not found the desired balance between effectiveness and practicality. In the NASH space there is a major unmet need for non-invasive diagnostic tests. The currently available diagnostics are often limited based on scope and/or practicality. The existing “gold standard” diagnostic tool is a liver biopsy that carries its own set of complications and risks. Additionally, liver biopsies are subject to user bias and have been shown to be inaccurate in up to 20% of cases. The lack of a robust clinical diagnostic increases the difficulty of developing less invasive diagnostic tests.
Why Metabolon in diagnostic development?

As shown previously, metabolites are key to nearly all aspects of NAFLD.

We know metabolites are key to integrating information from other -omics, and environmental influences.

But, most of the efforts have been too narrowly focused for this complex disease (e.g. lipids, liver enzymes, etc.).

This has hampered the development of tools and the clear mapping of the taxonomy of NAFLD.

We believe there is promise, but the aperture needs to be opened further – a case in point – our pilot work in NAFLD utilizing metabolomics approaches.
Synopsis. The quest for non-invasive diagnostic tools requires comparison to liver biopsy, the current “gold standard” NASH diagnostic. Liver biopsies from 200 hundred patients were collected and scored according to NASH CRN Criteria. They ranged from mild (stage 0-1) to advanced (stage 3-4) fibrosis. Information on steatosis, inflammation and hepatocellular ballooning was also collected. The serum from these patients were profiled with metabolomics. When compared to other currently used diagnostic tools, metabolomics analysis out-performed these other methods. It is tempting to speculate that combining these methods could potentially increase accuracy.

Metabolon as a partner

The development of non-invasive diagnostic approaches is critical to the study and treatment of NASH. Metabolomics is a powerful tool that can be used to generate these methods. NASH is a complex disease encompassing a complex amalgam of dysregulated pathways. Metabolon believes that integrating metabolomic data with other –omics tools will provide the best opportunity to develop a non-invasive diagnostic with clinical applications. To this end, Metabolon is prepared to partner with external firms to develop and validate novel diagnostic tools.
Summary
Leveraging the platform to improve our understanding of NASH

As you have just read, mechanistic understanding, diagnosing and treating NALFD/NASH poses unique challenges. We believe that metabolomics offers an excellent tool to explore this disease. Global metabolomics opens the aperture and allows for the interrogation of the many diverse pathways implicated in this disease with one test. Additionally metabolomics is suited for the many stages of drug development from target discovery, to target engagement, safety and biomarker identification.

TRANSLATIONAL STUDIES
- Enriching disease taxonomy
- Building confidence in biomarkers, molecule and target

DEVELOPMENT
- Target engagement
- Efficacy and safety assessment

DIAGNOSTICS / NEW TARGETS

Indication-specific infrastructure through systematic profiling
WORKING WITH METABOLON

What specific technologies and assays can I access?
How do I get more information?
Technology from discovery to the clinic

Metabolites are a surrogate to the physiological/phenotypic state, making them an ideal way to track changes induced by disease or treatment. The assessment of the metabolic state is particularly important in complex diseases where the drivers are numerous and diverse (i.e., genetics, environment and microbiota).

To best survey the metabolome, Metabolon has developed a continuum of capabilities:

- Understanding disease pathology
- Biomarker identification
- Target engagement
- Assessment of multiple pathways in one screen
- Develop diagnostic tools

TARGETED METABOLITE ASSAYS
Narrow your focus to specific pathways, themes or metabolites with custom or pre-developed assays. (~1-160 metabolites/assay)

GLOBAL METABOLOMICS
Cast a wide research net with our global metabolomics technology (Up to 1,000 metabolites/sample)*

LIPIDOMICS
Deploy a uniquely insightful lipidomic approach that comprehensively quantitates lipid species (up to 1,100 lipids/sample)
A translational resource

NASH/Fibrosis – an extensive body of work (~500+ samples) as a translational resource

Finding an appropriate pre-clinical model is a major hurdle in the development of NASH therapeutics. Metabolon has performed lipidomic and metabolomic analysis on multiple human cohorts which has revealed a number of established and less appreciated metabolic signatures. These datasets can be used to evaluate the translatability of your model. Using these tools, many investigators are comparing NASH rodent models with and without treatment to the signatures obtained in these human studies.

- NAFLD, n=154
- NASH (biopsy-proven), n=150
- NASH (biopsy-proven), n=156
- NASH (biopsy-proven), n=200
Facilitating your success – data to knowledge

We are committed to helping you harness metabolomics to achieve your research goals. We’ve designed our process to be a complete solution that produces actionable knowledge and drives research forward. This includes production of the metabolomics data and the provision of interpretation infrastructure (bioinformatics, tools, expertise & institutional knowledge), accessed via the MetaboLync™ client portal. From our initial consultation to the delivery of results, the entire process of working with Metabolon is designed to empower your informed decision-making, so you can move forward with confidence. The entire process is supported by a team of Ph.D.-level biochemists, biologist and bioinformaticists with decades of experience.
Concluding remarks

This eBook provides a sense of the breadth of successful application of metabolomics, but is not limited to what is described. Much of our experience resides confidentially with our clients, and many areas are yet to be explored. If you have a biological question or a need for a biomarker, with the right experimental design, metabolomics can be applied successfully.

Please contact one of our representatives to discuss how our technology can be applied in the area of NASH research or drug development.
Key references

Clinical

Pre-Clinical

In Vitro
Brown M.V. et al. *Obesity*, 000, 1-10 (2013)
FAQS

Do you have a NASH-specific panel?
Due to the diverse set of disease drivers and pathways, we believe that our global metabolic analysis is best-suited for interrogating as many of these pathways as possible.

How much tissue do I need? Can you work with needle biopsies?
Ideally, we would like tens of milligrams – 50 to 100 is ideal. However, biopsies are small and precious. Therefore, we do have a sample preparation method for small biopsies – a soaking method that “leaches” metabolites out of the tissues. When considering a study with small biopsy specimens, it is important to discuss the details with one of our study directors, because powering the study and assessing the sample quantity will be paramount to success.

How easy is it to incorporate gene expression data?
Easy is a relative term. But, what we can say is that most of our clients use the metabolomics data as the “beachhead” from which to find the important signal within a data set. They use the metabolomics signal as “bait” for pulling out the relevant genes.