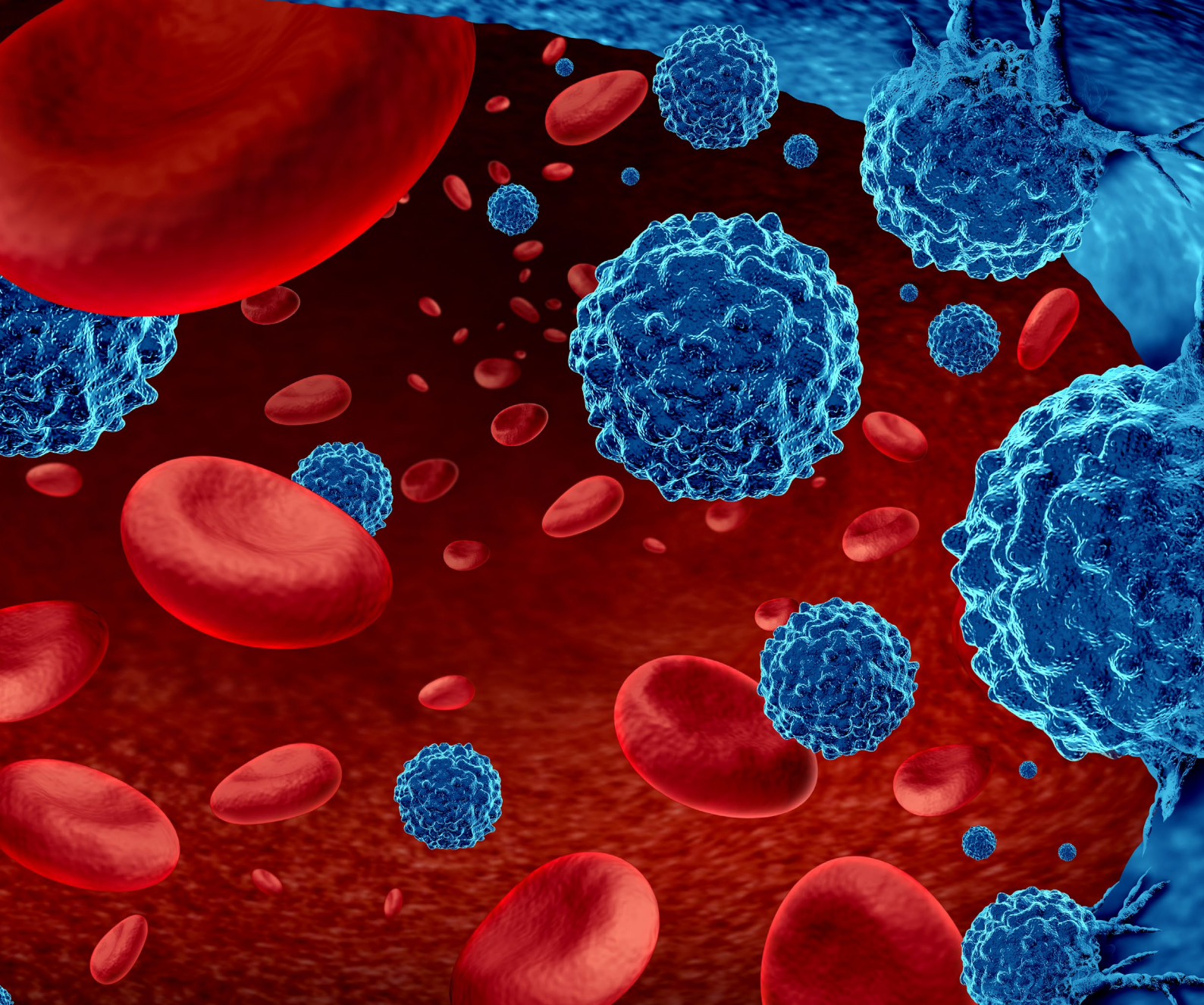




Advancements in Multiomics and Lipidomic Analysis:

Illuminating Therapeutic Targets and
Drug Responses in Various Diseases

Expert Insights



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Introduction

In the realm of modern biomedical research, our understanding of disease processes has evolved immensely, thanks to the remarkable advancements in multiomics and lipidomics. Multiomics combines genomics, proteomics, metabolomics, and more, providing a holistic view of biological systems. These cutting-edge technologies have enabled us to delve deeper into the intricacies of various diseases, offering once unimaginable insights. This eBook serves as a comprehensive exploration of these transformative approaches and their pivotal role in identifying therapeutic targets and understanding drug responses across a spectrum of ailments.

The first article within this compilation by Arif *et al.* [1], focuses on the burgeoning field of multiomics and its application in pulmonary fibrosis research. This complex lung disease has limited treatments available, with the lack of biomarkers being one of the hurdles to its research. This study connects preclinical models with clinical relevance in pulmonary fibrosis treatment, with potential therapeutic targets identified using a multiomics-based framework.

Next, Pergande *et al.* [2] delve into the use of lipidomics for Symptomatic Niemann-Pick, Type C1. It reveals how fatty acid metabolism changes in model mouse livers with the disease. The understanding of lipid metabolism's critical role in disease pathogenesis has opened new avenues for interventions and therapies. Currently, there is no FDA-approved therapy for this disorder, highlighting the necessity of this research. This study, conducted by the University of Illinois and Agilent, investigates the abundance of lipid species in search of potential therapeutic targets.

To fully understand the field of multiomics, we interviewed Daniel Cuthbertson, Ph.D., the Director of the Global Life Science Research Market at Agilent. This interview covers Dr. Cuthbertson's expertise in the field, current trends, and important takeaways from the two Agilent posters highlighted in this eBook. His insights frame the content of this eBook and cover why omics analysis, and powerful instrumentation, are critical in diverse areas of research.

The two Agilent posters "Targeted Lipidomic Analysis of Pediatric Leukemia Cells Using LC/MS/MS Triple Quadrupole" and "Illuminating the Cellular and Molecular Response to Drug Treatment by Combining Bioenergetic Measurements with Untargeted Metabolomics" shed light on the practical applications of multiomics and lipidomics in real-world scenarios, offering valuable insights into the fields of pediatric leukemia research and drug treatment response analysis.

Together, the articles, interview, and posters showcase the powerful synergy between multiomics and lipidomics in unraveling the mysteries of diseases ranging from metabolic disorders to cancer. As we delve into the complexities of multiomics and lipidomics, we encourage you to explore the practical applications of these innovative technologies in the fields of therapeutic discovery and understanding drug responses.

Through the methods and applications presented in this Expert Insights, we hope to share innovative technologies and techniques about multiomics and lipidomics with researchers and to highlight the new Agilent 6495D LC/TQ and Revident LC/Q-TOF systems as the solution for multiomics analysis. For more information, we encourage you to visit [Intelligence that Inspires | Agilent](#) to learn more and explore options to enhance your research.

Christene A. Smith, Ph.D.
Editor at Wiley

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- [1] Arif, M. *et al.* (2023). An Integrative Multiomics Framework for Identification of Therapeutic Targets in Pulmonary Fibrosis. *Advanced Science*. DOI: 10.1002/adv.202207454.
- [2] Pergande, M.R. *et al.* (2019). Lipidomic Analysis Reveals Altered Fatty Acid Metabolism in the Liver of the Symptomatic Niemann-Pick, Type C1 Mouse Model. *Proteomics*. DOI: 10.1002/pmic.201800285.

An Integrative Multiomics Framework for Identification of Therapeutic Targets in Pulmonary Fibrosis

Adapted from Arif, M. *et al.*

Introduction

Pulmonary fibrosis (PF) is a progressive and complex lung disease with limited treatment options and not sufficiently effective medication. The lack of predictive disease biomarkers and challenges in preclinical-to-clinical translation are significant obstacles. The commonly used preclinical model for PF is bleomycin-induced PF in mice, although it has limitations in simulating idiopathic pulmonary fibrosis (IPF) in humans.

This study investigated the changes in lung function, histopathology, transcriptome, and metabolome, in the bleomycin-induced PF mice model, after pharmacological induction by oropharyngeal bleomycin administration. Co-expression network analysis identified key gene subnetworks (G-1 and G-2) relevant not only in diseased rodent phenotype but also in lung biopsy samples from IPF patients, establishing clinical relevance. Transcriptional network analysis identified major regulators (gene tracks T-1 and T-2) of these subnetworks. Combined data suggested a potential therapeutic candidate for PF that could attenuate fibrotic changes. The overactivation of cannabinoid receptor 1 (CB1R) was identified as a contributor to PF pathology across multiple subnetworks and the potential of peripheral CB1R antagonism as a therapeutic strategy for PF was further investigated.

In summary, this research aimed to bridge the gap between preclinical models and clinical relevance in PF treatment. By investigating dynamic changes in the bleomycin-induced PF model and identifying key gene subnetworks and regulators, the study proposed a potential therapeutic target and demonstrated the utility of a multiomics-based framework in guiding drug development and rational combination therapy strategies.

Methods using Agilent Technologies

Hydroxyproline Measurements with liquid chromatography tandem mass spectrometry (LC/MS/MS):

PF was quantified by measuring hydroxyproline, using an Agilent 6470 triple quadrupole mass spectrometer, coupled to an Agilent 1200 LC system.

Untargeted Metabolomics Profiling: Metabolomics analysis was outsourced for capillary electrophoresis mass spectrometry (CE-MS) and liquid chromatography time-of-flight mass spectrometry (LC-TOFMS). Agilent has developed reliable instruments for both procedures.

Agilent's equipment facilitated precise hydroxyproline measurements in the study and provided reliable instruments for untargeted metabolomics profiling, ensuring accurate and comprehensive analysis.

Results

The study investigated the progressive decline in pulmonary function and fibrotic development in a bleomycin-induced PF mouse model. Pulmonary function declined significantly up to 14 days post-bleomycin, with collagen deposition and fibrogenic markers increasing. Transcriptomics analysis revealed significant differential expression of genes (DEGs) occurred at different time points, with the highest number of DEGs at day 14, post-bleomycin. Pathways related to extracellular matrix, cell cycle, cytokine signaling, and focal adhesion were upregulated, while others were downregulated.

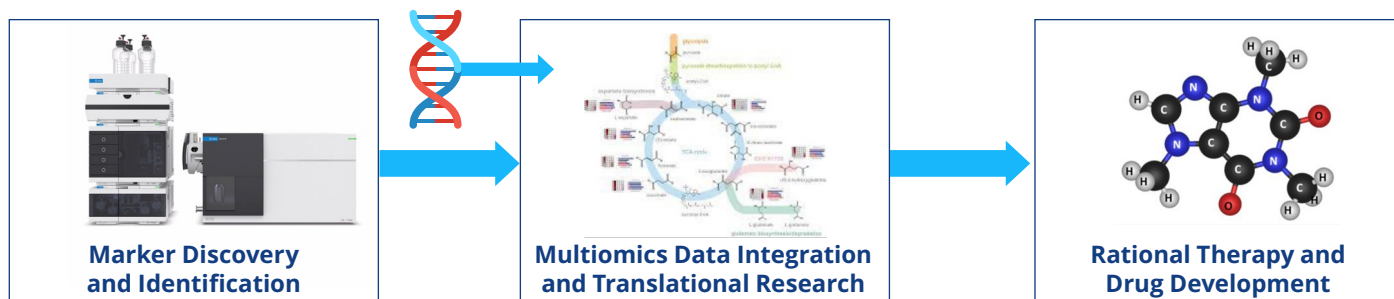
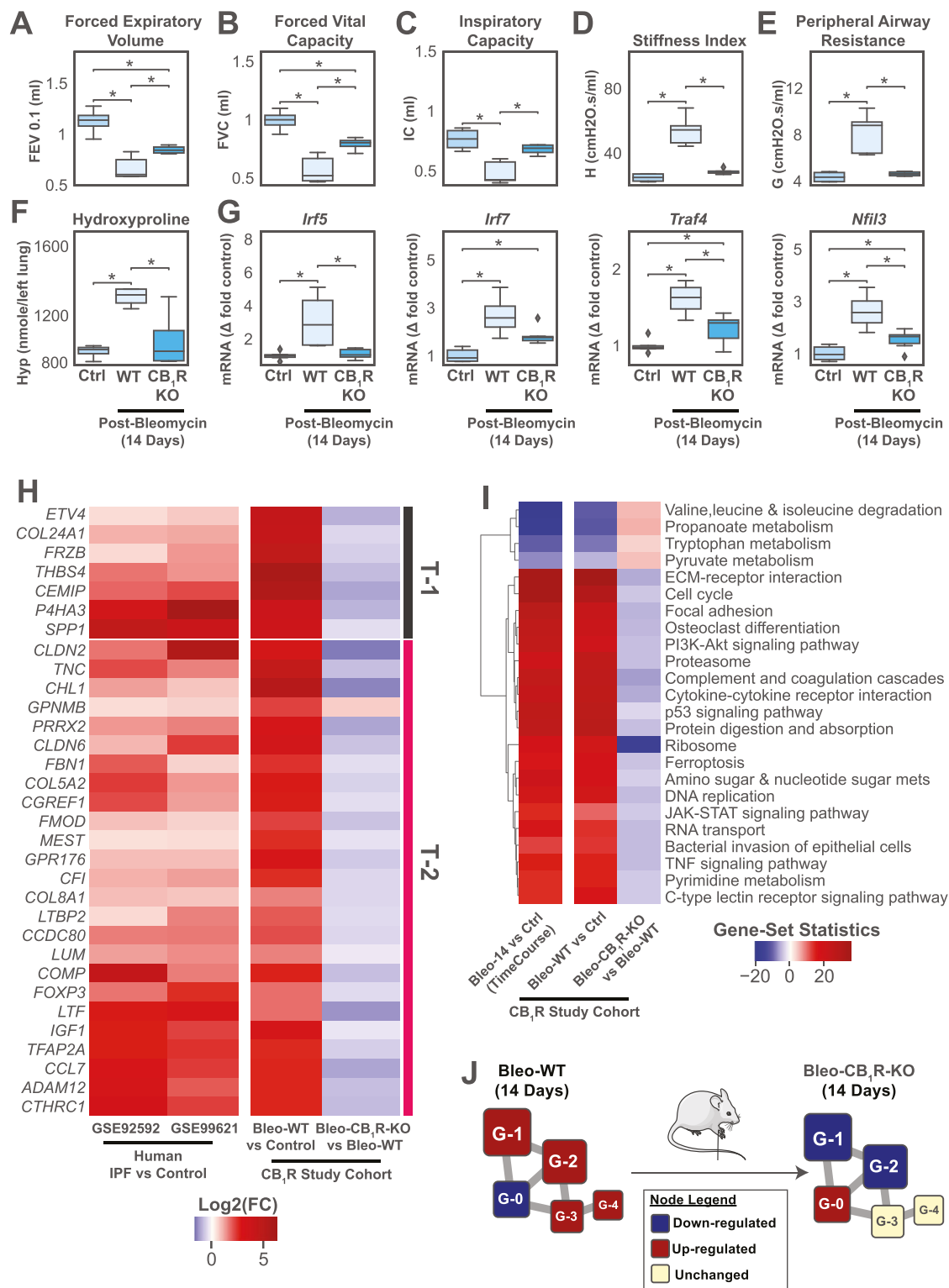


Figure 1



Identifying cannabinoid receptor 1 (CB1R) antagonism as a rational therapeutic target in pulmonary fibrosis. A–E) Deletion of CB1R prevented bleomycin-induced decline in pulmonary function parameters (stiffness index, peripheral airway resistance, inspiratory capacity [IC], forced expiratory volume 0.1 s [FEV_{0.1}], and forced vital capacity [FVC]; n = 4 for Ctrl and CB1R KO, and n = 5 for WT; one-way ANOVA; *: p-value < 0.05). F) Hydroxyproline level decreased significantly in CB1R KO mice compared to wild-type after the administration of bleomycin (n = 4 for Ctrl and CB1R KO, and n = 5 for WT; one-way ANOVA; *: p-value < 0.05). G) Gene expression of *Irf5*, *Irf7*, *Traf4*, and *Nfil3*, transcriptional regulators of T-1 and T-2 gene tracks (n = 6 for Ctrl and CB1R KO, and n = 4 for WT; one-way ANOVA; *: p-value < 0.05). H) PF-induced 31 validated T-1 and T-2 target genes in both humans and mice were significantly attenuated with the deletion of CB1R compared to wild-type mice at 14 days post bleomycin. (p-value < 0.05) I) The majority of important pulmonary fibrosis-related KEGG pathways, such as ECM-receptor interaction, PI3K-Akt signaling, focal adhesion, amino-acid metabolism, and immune and inflammatory pathways, were significantly attenuated with the deletion of CB1R compared to wild-type mice at 14 days post bleomycin. J) The deletion of CB1R in the lung reversed the dysregulation of genes 14 days post-bleomycin in the three biggest GCN subnetworks: G-0 (up-regulated), G-1 (down), and G-2 (down), while G-3 and G-4 remained unchanged, compared to wild-type mouse 14 days post-bleomycin.

The researchers constructed a Gene Co-Expression Network (GCN) to identify subnetworks associated with fibrotic progression. Two central subnetworks (G-1 and G-2) closely interacted with all other subnetworks. Pulmonary function parameters correlated with specific genes in these subnetworks, providing insights into fibrotic changes. Metabolomics analysis demonstrated significant shifts in the lung metabolome post-bleomycin. Differentiated metabolome pathways were upregulated and downregulated, indicating changes in metabolic processes associated with PF progression.

Integration of GCN and Metabolite Correlation Network (MCN) revealed connections between metabolic subsystems and main GCN subnetworks. The most central subnetwork (M-0) was linked to the G-1 and G-2 subnetworks, highlighting potential metabolic regulators of fibrotic pathways. A comparison of transcriptomics data from mice with human IPF patients revealed overlapping gene expression changes in G-1 and G-2 subnetworks. The transcriptomic signature at 14 days post-bleomycin in mice resembled late-onset IPF patients' lung transcriptome.

Using interactive dynamic regulatory events miner (iDREM) analysis, critical gene tracks associated with fibrotic pathways were identified. Transcription factors regulating these tracks were linked to inflammation, fibrosis, and epithelial-mesenchymal transition. IRF5 and IRF7 were potential therapeutic targets due to their association with fibrogenic pathways. Deletion of cannabinoid receptor 1 (CB1R) attenuated fibrosis-related gene expression and improved pulmonary function in mice with bleomycin-induced PF. CB1R deletion also impacted pathways associated with fibrosis and inflammation, supporting its potential as a therapeutic target.

In summary, this comprehensive study provides insights into the molecular and functional changes associated with PF progression, identifies potential therapeutic targets, and highlights the translational relevance of findings from the mouse model to human IPF patients.

Discussion

The study presents a multiomic framework that integrates transcriptomics, metabolomics, and pulmonary function parameters to compare the temporal changes in PF-associated alterations between a commonly used animal model of PF and human IPF and address the translational gaps.

The key findings are as follows:

- **Identification of Major Drivers:** The study identifies major drivers within gene co-expression networks (GCNs), such as G-1 and G-2 subnetworks, that play a central role in PF. These subnetworks are associated with transcriptomic and metabolic alterations.
- **Translational Validation:** The study confirms that transcriptomic signatures observed in fibrotic mouse lungs overlap with those from human IPF, highlighting the translational relevance of the framework.
- **Therapeutic Targets:** The study suggests potential therapeutic targets for PF treatment, including central gene subnetworks G-1 and G-2, as well as upstream regulators like CB1R. Deletion of CB1R is shown to attenuate pathological changes in GCN subnetworks and is proposed as a potential therapeutic strategy.
- **Disease Progression and Treatment Timing:** Temporal analysis indicates that key changes in pulmonary function, transcriptome, and metabolome occur within the first 14 days after bleomycin-induced PF. This suggests that treatment interventions could be targeted at early (days 7-21) or later (days 14-28) stages of disease progression.
- **Biomarker Identification:** The study highlights the importance of identifying early disease-stage biomarkers for effective antifibrotic therapy. Metabolomics studies using lung, bronchoalveolar lavage fluid (BALF), and blood samples are proposed for future biomarker research.
- **Systems Pharmacology Approach:** The study demonstrates how a systems biology and pharmacology approach can be used to identify druggable targets that regulate multiple pathological pathways, thus minimizing potential on-target toxicity and facilitating the translation of preclinical findings into clinical therapies.

Conclusion

In conclusion, the study employs a comprehensive multiomic approach to better understand the molecular mechanisms of PF, identify potential therapeutic targets, and suggest optimal treatment timing strategies. Agilent Technologies played a pivotal role in unraveling the complex interactions in PF and the findings contribute to the development of effective therapeutic interventions for PF patients.

Keywords: bleomycin, CB1R, IPF, Irf5, metabolomics, mouse model, multiomics, network biology, pulmonary fibrosis, transcriptomics, Agilent Technologies

Lipidomic Analysis Reveals Altered Fatty Acid Metabolism in the Liver of the Symptomatic Niemann–Pick, Type C1 Mouse Model

Adapted from Pergande, M.R. *et al.*

Introduction

The article investigates the Niemann–Pick disease, type C1 (NPC1), caused by mutations in the NPC1 gene. It is a lysosomal storage disorder that leads to the abnormal accumulation of unesterified cholesterol and sphingolipids. It can result in pathologies, such as liver disease, hepatomegaly, vertical gaze palsy, and cerebellar degeneration and can become fatal, especially for infantile patients. Currently, no FDA-approved therapy exists, stressing the need for intense research on NPC1.

The present research uses the NPC1 null mouse model (Npc1^{−/−}) that mimics the human condition. Previous microarray analyses, differential proteomics, and lipidomics, by this team and others, have identified altered expression of proteins and lipids in NPC1 mice. Namely, changes in lysosomal enzymes, late endosomal/lysosomal proteins, microRNA-155, GM2 gangliosides, sphingosine, sphinganine, and other lipid species have been reported. As fatty acids are embedded in biological membranes and are involved in signaling pathways,

the researchers hypothesize that fatty acid levels are altered in NPC1 and that untargeted lipidomic analyses will uncover them, along with the pathways they affect, particularly in the NPC1 rodent liver.

This study, conducted with the collaboration of the University of Illinois and Agilent Technologies, investigates the abundance of lipid species in the NPC1 mouse model and a liver cell line, in search of potential therapeutic targets.

Methods using Agilent Technologies

LC/MS: Lipid extracts were detected by an Agilent 6546 LC/Q-TOF system controlled by the Agilent Mass Hunter acquisition software.

GC: Fatty Acid Methyl Esters (FAMES) were analyzed by an Agilent 7820A gas chromatograph (GC).

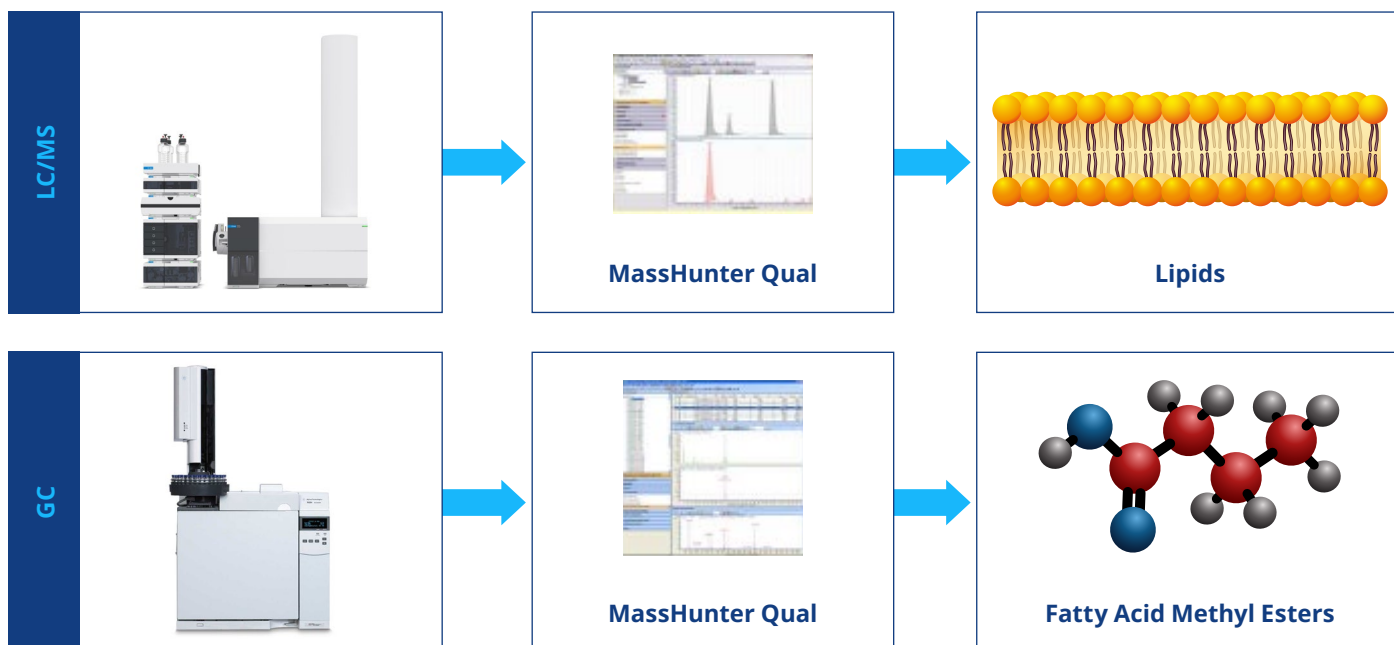
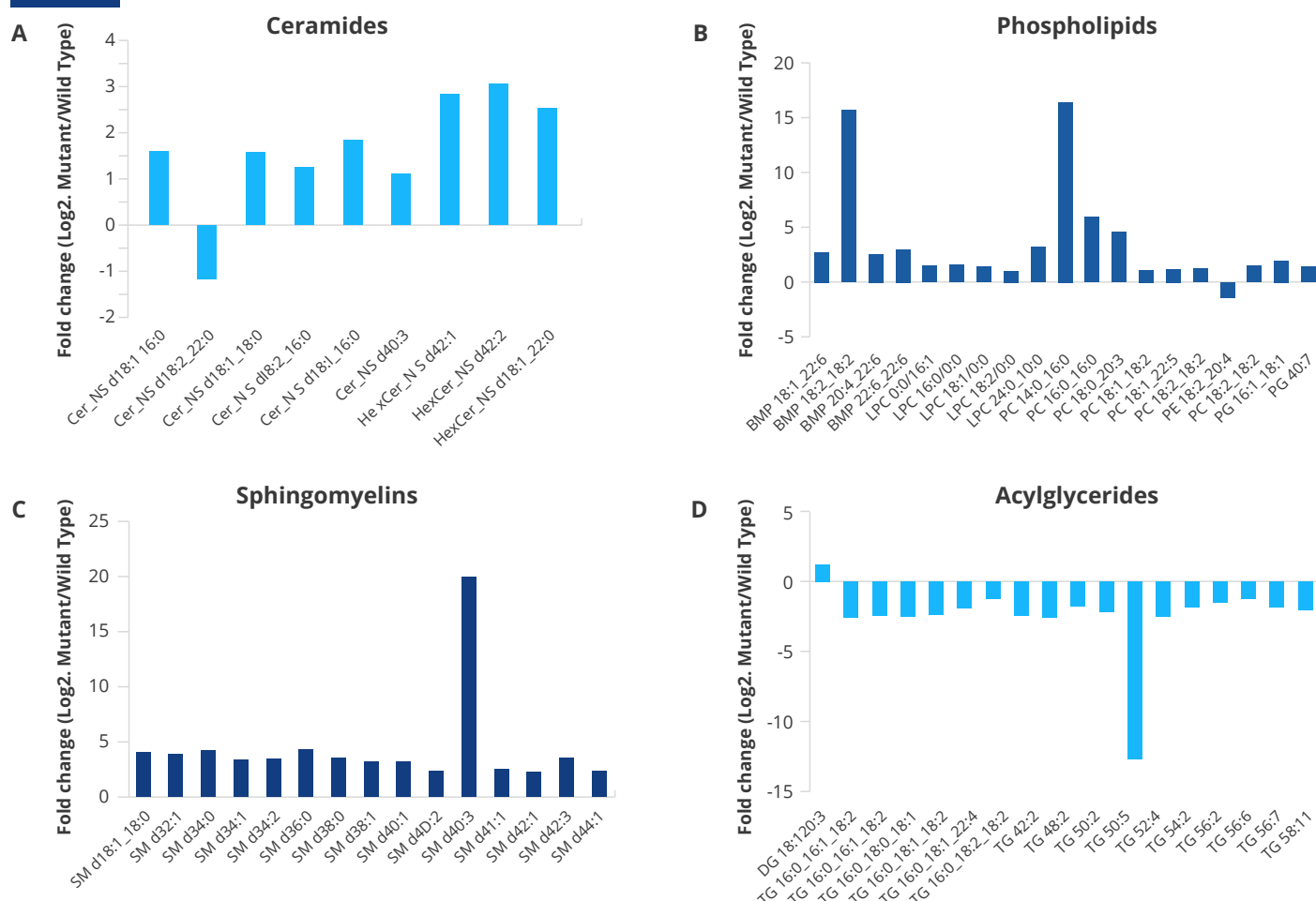


Figure 1



Altered lipid species from the LC/MS analysis of 11-week *Npc1*^{+/+} and *Npc1*^{-/-} mice. Altered lipid species (fold change ≥ 2.0 and $p \geq 0.05$) in *Npc1*^{-/-} mice identified by accurate mass (≤ 10.0 ppm) plus MS2 fragmentation: A) ceramides, B) phospholipids, C) sphingomyelins, and D) acylglycerides. The fold change for lipid species observed in positive and negative ion modes was averaged.

Results

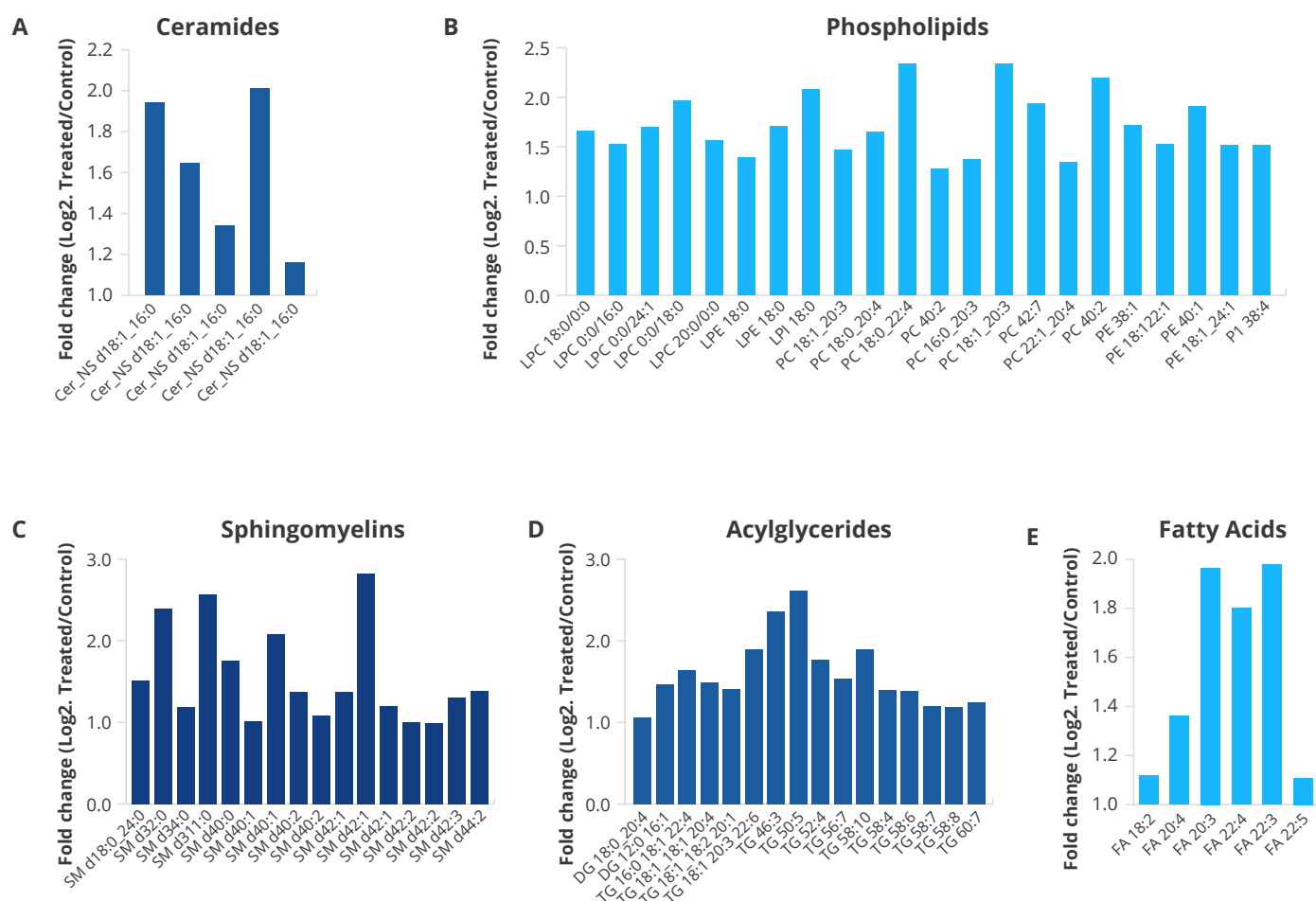
A multifaceted approach was employed, involving targeted measurements of fatty acids, differential lipidomics analysis, and the assessment of enzyme concentration, in liver tissue from both normal and *Npc1* mutant animals. The *Npc1* mutant mice exhibited distinct alterations in their fatty acid metabolism. These changes encompassed reduced levels of various free fatty acids and modifications in the acyl chains of fatty acids.

The researchers also conducted an untargeted lipidomic analysis using Agilent's LC/MS/MS in both positive and negative ion modes, which revealed significant changes in the levels of different lipid classes of the *Npc1* mutant mice liver. Notably, ceramides, phospholipids, sphingomyelins, and triacyl glycerides displayed varying degrees of change (Fig. 1). This finding was complemented by the Western blot analysis of fatty acid regulatory enzymes. The levels of fatty acid desaturase 1 (*Fads1*), elongation of very long chain fatty acids protein

2 (*Elovl2*), elongation of very long chain fatty acids protein 5 (*Elovl5*), fatty acid binding protein 2 (*Fabp2*), and fatty acid binding protein 5 (*Fabp5*) were decreased, while no significant difference was seen in the fatty acid synthase (*Fas*) expression.

The researchers used a cell culture model (HepG2 cells) treated with U18666A, to further investigate the underlying mechanisms. Free docosahexaenoic (DHA) and oleic (OA) acids and tissue arachidonic (AA), stearic (SA), and OA acids increased with U18666A treatment while alpha-linolenic acid (ALA) decreased. Like the *Npc1*^{-/-} mice liver, cells' untargeted lipidomic analysis revealed increased expression of ceramides (Cer), phosphatidylcholines (PC), and sphingomyelins (SM). However, upon U18666A treatment, there was an increase in triglycerides (TG) and phosphatidylethanolamines (PE) contrary to the

Figure 2



Altered lipid species from the LC/MS analysis HepG2 cells treated with the U18666A. Altered lipid species (fold change ≥ 2.0 and $p \geq 0.05$) in *Npc1*^{-/-} mice identified by accurate mass (≤ 10.0 ppm) plusMS2 fragmentation: A) ceramides, B) phospholipids, C) sphingomyelins, D) acylglycerides, and E) fatty acids. The fold change for lipid species observed in both positive and negative ion modes was averaged.

liver tissue. This finding points to a more complex interaction pattern. Western blot analysis showed a significant decrease in the expression levels of fatty acid binding protein 2 (FABP2) and fatty acid binding protein 5 (FABP5), with no statistically significant changes in the enzymes involved in ω -PUFA synthesis.

Discussion

In this study, researchers investigated the lipid profiles within the context of *Npc1* deficiency. They examined both liver tissue from *Npc1*^{-/-} mice and a cell culture model of NPC1. Agilent provided sophisticated mass spectrometry equipment (LC/MS / GC/MS) and software that enabled researchers to perform both targeted and untargeted lipidomic analyses. This technology empowered the precise identification and quantification of diverse lipid species, shedding light on the intricate alterations in lipid profiles and enzyme expressions within the liver tissue and cell line model.

Namely, measurements of free fatty acids and those integrated into different lipid species, such as PG, PE, and PC were performed. A marked decrease in several fatty acids, especially the ω -6 and ω -3, was reported. These changes, along with previously reported alterations in the liver proteome, highlight the significance of disrupted fatty acid metabolism in late-stage *Npc1*^{-/-} mice, contributing to the pathophysiology of NPC1 disease.

An untargeted lipidomic analysis was performed on liver tissue from both control and *Npc1* null mice. The results revealed a pattern of decreased PE and TGs, accompanied by increased levels of PC, PG/BMP, and SMs in the liver tissue of *Npc1*^{-/-} mice at an advanced stage of disease progression. Notably, these observations were further supported by a pharmacological model that mimicked the NPC1 phenotype, providing insight into acute cholesterol storage conditions and the role of the absent NPC1 protein.

The study emphasized the crucial role of endogenous fatty acid synthesis, particularly the downstream synthesis of signaling fatty acids such as AA and DHA. The imbalance in fatty acid metabolism and transport proteins leads to impaired cellular processes not only in the liver but also in other tissues, the brain included. Although further investigation is needed, these findings hold promise as potential markers for disease monitoring and targets for drug therapy design.

Conclusion

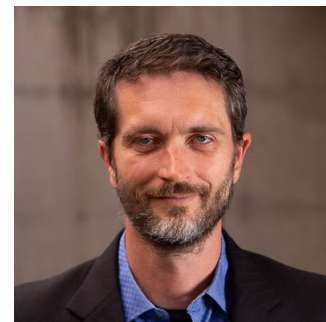
The above-mentioned findings suggest that the imbalance in fatty acid metabolism in the *Npc1*^{-/-} mice liver may not be directly linked to an acute accumulation

of unesterified cholesterol in the lysosome. More likely, the decrease in fatty acids and subsequent triglyceride synthesis correlates with defects in the uptake of essential fatty acids in the gut of these mice, as supported by the observed decrease of *Fabp2*. Agilent Technologies played a pivotal role in unraveling the complex changes in lipid profiles and enzyme expressions, enhancing the understanding of lipid metabolism dysregulation in NPC1 disease.

Keywords: fatty acids, GC, LC/MS, lipidomics, MS, Niemann–Pick, type C, Agilent Technologies

Advancing Metabolomics and Lipidomics with Agilent Technologies

Interview with Daniel Cuthbertson, Ph.D., the Director of the Global Life Science Research Market at Agilent



In this exclusive interview with a prominent omics expert, we delve into the world of cutting-edge science and technology, particularly in the field of metabolomics and lipidomics. Daniel Cuthbertson, a seasoned scientist at Agilent, shares valuable insights into the development of industry credibility, the latest trends in chemometric tools for metabolomics, and the transformative impact of Agilent's equipment in life science research. He also sheds light on significant research involving pediatric leukemia and the dynamic metabolic response of cancer cells to therapeutic compounds, showcasing the pivotal role of Agilent's instruments and workflows in advancing our understanding of these critical areas.

How did you develop your industry credibility, particularly through your scientific consulting, and could you elaborate on the ways this experience has shaped your role at Agilent and your impact within the life science sector?

One of the biggest challenges in modern life sciences especially as they pertain to omics technologies, now commonly used in life science research, has been the appropriate use and understanding of multivariate statistics and other advanced analytics including machine learning. These advanced data analytics have always been a specialty of mine and when I first joined Agilent as a scientific consultant over a decade ago marrying those analytics with omics studies was still in its early stages. As part of Agilent's technical team, these skills were very much in demand and one of the great things about consulting in such a hot area was that I got to see and be involved in a wide variety of studies in labs across the globe, so I truly got to see a bit of everything

and learn how to apply cutting edge omics technologies across the life science spectrum. This experience not only led to developing great relationships and personal friendships with researchers but truly it was an honor to be part of the field as it developed and exchange ideas with top scientists in the field. Looking back to a more mature field now, it's really satisfying to see the ideas that we exchanged for analytical strategies, omics experimental design, quality control, and data analysis are now common techniques and are considered best practices. I'd like to think that during my scientific consulting days taking this knowledge with me from lab to lab really helped facilitate uptake in the community and made a difference.

Can you provide an overview of current trends in the use of innovative chemometric tools and methodologies for metabolomics research?

One of the interesting things to watch in the past decade was the transformation of a metabolomics field that used small data sets to large ones and from one that wasn't particularly statistically savvy with mass spec data to one that's now cutting edge. It's not uncommon now that graduate students have skills in programming and data science. So, as you might imagine this has the field well suited to take advantage of the latest advances in data processing and especially machine learning. This means that you're now seeing a lot of really innovative machine learning algorithms and strategies to understand not only which metabolites are responding to a disease for example but also how to identify unknown metabolites, integrate peaks, and make instruments more intelligent.

But there's another angle to this too, now that omics technologies are becoming relatively common in the life science sector the need to analyze large datasets routinely and confidently is becoming a bottleneck. So, this means that now there's this real need for data analysis solutions like MassHunter Explorer and MassHunter Quantitative Analysis that are not only powerful but are easy for researchers to use routinely

day in and day out. Researchers no longer have the time to spend weeks or months picking through peaks, they need their results as soon as possible and they need them confidently to get ready for the next set of experiments. I'm quite optimistic with the new approaches being pioneered by Agilent we will see this bottleneck becoming less of a challenge moving forward.

What do you consider the most exciting or cutting-edge developments in the field of metabolomics right now?

Presently, one of the things I'm most excited about however is the adoption of instrument intelligence analytics into the newest generation of mass spectrometers by Agilent. We've incorporated AI-based SWARM auto-tune as well as Early Maintenance Feedback that helps you anticipate when maintenance events are upcoming. We've even incorporated Reflexive Injection Logic (intelligent reflex) that has logical controls to ensure samples are within their quality specifications and ensure that you are collecting quality data without manual intervention from the operator. The power of all this is that this is making the use of mass spectrometry equipment for metabolomics and life sciences easier than ever before and researchers that are focused on life sciences and don't have a lot of prior mass spec experience can now more easily bring these cutting-edge metabolomics technologies into their lab and focus on making discoveries.

As an innovator for LC/MS technology development, can you highlight some of the trends you have recognized and adapted to address current scientific needs?

One of the trends that I've really been pleased with in the past couple of years is communities improving their understanding that generating quality LC/MS data is a process that involves more than just a cutting-edge Agilent mass spectrometer. It involves good sample preparation, which in my opinion is one of the most essential components of any LC/MS study. It also involves solid data analysis approaches to extract meaning from data.

This is one of the reasons that I've been leading an initiative at Agilent to develop standardized and highly curated workflows for multiomics, including metabolomics, lipidomics, and proteomics.

We intelligently design these workflows to maximize the biological information from each sample. We integrate these with our advanced data analysis software like [MassHunter Explorer and Mass Profiler Professional](#) to help get to biological meaning as quickly as possible.

In context, this is the new era of metabolomics, more routine, more confident, and more accessible. Our instruments and just as important curated workflows are helping make this happen.

How do you envision the future of metabolomics and chemometrics communities, and what steps do you believe can be taken to make them greater together?

Long-term metabolomics and chemometrics technologies will be routine and easily available in every lab and that's why it's so important that what we are doing in Agilent is helping make this transition possible by helping our customers focus on what's important to them, life sciences. Metabolomics technologies are becoming increasingly routine, enabling larger and larger cohorts of samples to be analyzed. This is something we are seeing a lot of demand for as life scientists. With these large datasets this is where the chemometrics and data science community has a huge role to play and will become ever more important in partnership with our bench scientists. Bench scientists recognizing that machine learning techniques really excel at analyzing ever larger datasets will drive these communities even closer together than they are now. To help that happen it's the responsibility of cutting-edge life science companies like Agilent to help enable this and that's exactly what we are doing with our intelligent instruments and curated workflows. The communities come together when it's about the life science results and what those can do to improve the human condition.

Based on these publications:

Poster 1 - [Targeted Lipidomic Analysis of Pediatric Leukemia Cells Using LC/MS/MS Triple Quadrupole](#)

Poster 2 - [Illuminating the Cellular and Molecular Response to Drug Treatment by Combining Bioenergetic Measurements with Untargeted Metabolomics](#)

Can you highlight the significance of the workflow described in Poster 1 for lipidomic profiles in pediatric leukemia patients?

This study is a really great example of life science and cancer researchers taking advantage of our highly curated lipidomics workflow and adapting it to their needs. In this case, Dr. Mike Snyder's team is already very capable with mass spectrometry systems and method development. So that begs the question what's the advantage of these highly curated workflows for labs that are already at the advanced stage? Because these are workflows that you can trust and are highly vetted, adopting these workflows can save labs months of effort developing new methods and get running with their new equipment even more quickly. In this case we also wanted to highlight that these highly curated workflows are extremely customizable working with our very robust method the Stanford and Agilent teams collaborated to take advantage of the 6495 LC/TQs high number of concurrent MRM measurements to increase the number of lipids detected. Taking advantage of both time savings from our lipidomics workflow and taking that to build on the advanced capabilities of the 6495 LC/TQ Dr. Snyder's team was able to identify predictive lipid biomarkers for pediatric leukemia development and progression. It's studies like these that I really appreciate because they show how Agilent Technologies, and the efforts of our scientific teams, can really make an impact for researchers.

What methodologies were used to investigate the dynamic metabolic response of cancer cells to therapeutic compounds (Poster 2), and how were the drugs SU1498 and AG879 selected for the study?

This is one of my favorite recent studies by the Agilent scientific team because it's a stellar example of how cell analysis techniques like the Seahorse XF Pro and the mass spectrometry tools like the Revident LC/Q-TOF can be integrated to gain holistic biological insights. In this case, the team used the metabolic profiles generated from the Seahorse Agilent Seahorse XF Real-Time ATP Rate Assay to screen these tyrosine kinase inhibitors for metabolic phenotypes of interest. They found an interesting scenario where both AG-879 and SU-1498 exhibited mitochondrial uncoupling in THP-1 cancer cells. However, in the case of SU-1498, the impact of mitochondrial uncoupling in healthy PMBC cells was much reduced from AG-879. With this information in hand from the Seahorse XF screen it led

to additional questions and the desire to dig deeper into the metabolism of the cells treated with these two tyrosine kinase inhibitors. Using a combination of both our targeted HILIC metabolomics workflow on the 6495D LC/TQ and the Revident LC/Q-TOF for untargeted metabolomics the team was able to identify changes in metabolites in the glycolytic and TCA pathways correlating with Seahorse results. Furthermore, untargeted lipidomics studies on the Revident showed an increase of triacylglycerides with SU-1948 treatment which may be related to a buildup of energy precursors for the TCA cycle.

All in all, this is a great example of how multiple technologies were integrated, and the Seahorse informed the mass spectrometry study. We're going to see more integrations like these as researchers see the value of multiple points of confirmation of their studies and how these different technologies produce synergistic data to provide a more complete picture of metabolism.

Revident



What are the most important considerations when selecting an instrument for lipidomics and metabolomics analyses?

One of the most important things of course about selecting instrumentation is what I call performance with productivity. Performance is why the new [Agilent 6495D LC/TQ](#) is one of our most sensitive yet. You need to have the confidence that you will see low abundance metabolites in heavy biological matrices and the advanced electronics of these systems help you monitor as much of the lipidome and metabolomic as accurately as possible.

However, the productivity of your instrument in real terms is just as or in some cases more important than performance alone as you need to be able to make progress for grant renewals and deadlines to turn around data for scientific publications. When you look at productivity, we have enhanced Agilent's already strong reputation for robust instrumentation and building in instrument intelligence which is frankly ground-breaking because it helps you keep your instruments online producing high-quality data even longer and plan for maintenance.

You need to look at the ecosystem around instrument productivity. Agilent has a fantastic scientific community (including consultants!) to help you get up and going as I did for many years. You must also consider workflows that include sample preparation, methods for instrument data acquisition, and data analysis tools. These elements provide tremendous value to the instrumentation and save you loads of time getting started.

What specific Agilent instrument was employed to analyze the samples in these studies, and how did it contribute to the findings?

In the studies highlighted here we saw the Agilent 6495 LC/TQ very prominently using our highly curated targeted metabolomics and lipidomics workflows. Again, the value of these workflows is they've been designed to pack as much biological information into them as possible and they monitor hundreds of metabolites and lipids in each workflow. By casting this intelligently designed but extraordinarily wide net for a targeted assay the teams were able to discover markers of cancer progression and understand metabolic differences in the tyrosine kinase inhibitors.

6495D LC/TQ



In the tyrosine kinase inhibitor study, you also saw the Agilent Revident LC/Q-TOF work in conjunction with the new Agilent 6495D LC/TQ for the untargeted portion of this study. Revident sports the same instrument intelligence as the 6495D LC/TQ. While both platforms concurred on the results of many metabolites there are sometimes advantages to going with an untargeted analysis to cast an even wider net. When doing untargeted analysis on Revident you can take advantage of the same base sample, preparation, and chromatography methods to enhance your productivity. To support this, we have enabled the conversion of MRM methods on the LC/TQ to a PRM method on the Revident QTOF allowing hybrid targeted/untargeted data acquisition for even more flexibility.

I'd also like to highlight one of the unsung heroes enabling both the 6495D LC/TQ and Revident LC/Q-TOF in these studies and that's the [Infinity II Bio LC](#).

Many researchers don't realize that metal surfaces in LCs can interact with metabolites or lipids that have anionic moieties, and this interaction broadens chromatographic peaks, decreasing sensitivity. The Infinity II Bio LC solves this problem with biocompatible alloys in its flow bath that reduce metal interactions while maintaining the same performance as standard systems. Many metabolites that were once considered troublesome, such as organic acids and nucleosides like ATP, are greatly improved by the Infinity II Bio LC. It was precisely the metal interactions that made them problematic in the past!

Can you elaborate on the advantages of the Agilent equipment utilized in this research, and how it compares to other available technologies in the field?

One of the great advantages of going with Agilent instrumentation is performance with productivity. In general, you're going with an LC/TQ system when you want maximum sensitivity and day-in and day-out performance for large cohort studies. In the case of the new 6495D LC/TQ, we've achieved new levels of sensitivity but didn't sacrifice the robustness of the instrument. Competitive high-end instruments have been plagued with robustness issues in heavy matrices where their ion optics easily get fouled quickly lead to reduced sensitivity or worse yet to downtime where you aren't generating data at all. To achieve this breakthrough, we've incorporated our mature 4th generation ion funnel technology that improves ion signal without compromising robustness.

On top of that, you're getting the most advanced electronics allowing you to monitor an unprecedented number of targets simultaneously with the highest precision and that's really one of the pieces of magic that enables our omics workflows.

In the tyrosine kinase inhibitor study, you saw the Revident LC/Q-TOF and when comparing against ion trap-based instruments QTOFs enjoy several advantages. First, ion traps have finite ion capacity and when used in untargeted acquisition mode heavy matrix can cause you to miss important low abundance metabolites and this isn't a concern with beam instruments like QTOFs where you can see those low abundance metabolites without having to worry about trap capacity. Another angle that is important in this context is mass resolving power that is not dependant on acquisition rate like

ion trap-based instruments. In modern large cohort studies, if investigators choose to do an untargeted omics study, they will often select a QTOF instrument because sample throughput becomes a consideration. In these studies, QTOF gives the researchers the freedom to increase the chromatographic speed of the method without compromising mass resolving power. The ability to do this can often halve analysis times, saving days and sometimes weeks in exceptionally large studies. Additionally, it helps reduce costs associated with solvents. The software is designed to process untargeted data files quickly like MassHunter Explorer, in a large study data processing times can often become a bottleneck which is why we've dramatically reduced processing times by streamlining our algorithms to fully take advantage of multithreaded computational systems.

Poster Reprint

ASMS2023
Posternumber WP 564

Targeted Lipidomic Analysis of Pediatric Leukemia Cells Using LC/MS/MS Triple Quadrupole

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Introduction

Various research has shown that lipids play important roles during cancer development, progression, and treatment. Leukemia is the most frequent childhood cancer. A challenge in treating leukemia is eradicating leukemia stem cells (LSCs), which are inherently resistant to chemotherapeutics. Although there is great interest in designing therapeutics to target LSCs, clinical translation has been hampered by limited characterization of the biological properties of these heterogeneous cells. While intensive research efforts have been devoted to characterizing the genetics of these tumors, the comprehensive study of lipids has been relatively unexplored. In this study, an easy and fast lipidomics sample preparation method and a rapid (16-min run), sensitive LC-QQQ based targeted lipidomics workflow was developed to quantitatively study the alternation of lipid profiles in bone marrow leukemia cells acquired at initial diagnosis and at relapse diagnosis and at relapse. The targeted lipidomic approach was applied for identification and quantification of over 1200 lipids from about 50 classes in progenitor cells from leukemia bone marrow acquired at diagnosis. A representative subject cell sample was spiked with the internal standard mix containing 97 isotope labelled compounds at different levels with at least 3 replicates. The method was validated in terms of identification, accuracy, precision, matrix effect and linearity of calibration curves. The quantitation was performed using extracted matrix calibration curve. This validated method allowed the detailed lipid profiling by LC-QQQ to identify predictive lipid biomarkers for pediatric leukemia development and progress.



Agilent 1290 Infinity II LC with 6495 Triple Quadrupole LC/MS System.

Experimental

Chromatographic Conditions-Agilent 1290 Infinity II Bio LC

- ✓ Agilent targeted lipidomics chromatographic method as described previously¹
- ✓ 16-minute RP method designed for comprehensive coverage of major lipid classes
- ✓ Combination of Agilent Deactivator Additive and the Agilent Bio LC improves peak shape and detection for metal-sensitive lipids

¹Huynh, K, et al. A Comprehensive, Curated, High-Throughput Method for the Detailed Analysis of the Plasma Lipidome. Agilent Application Note 5994-3747EN, 2021

MS Conditions-Agilent 6495 Triple-Quadrupole

Parameters	
MS acquisition	Dynamic MRM
Ion source	Agilent Jet Stream electrospray ionization (AJS ESI positive/negative)
Drying gas temperature	150 °C
Drying gas flow	17 L/min
Nebulizer	20 psi
Sheath gas heater	200 °C
Sheath gas flow	10 L/min
Capillary	3500 V ESI+ / 3000 V ESI-
Nozzle voltage	1000 V ESI+ / 1500 V ESI-
High pressure RF voltage	150 V ESI+ / 200 V ESI-
Low pressure RF voltage	60 V ESI+ / 110 V ESI-

Sample Preparation

- ✓ Prepare 1M subject cell pellet
- ✓ Add 75% ice cold methanol spiked with IS
- ✓ Vortex, sonicate
- ✓ Homogenize with ceramic beads
- ✓ Extract with 3 volumes of 1:1:1 acetonitrile : isopropanol : acetone
- ✓ Vortex, centrifuge and collect the supernatant

Experimental

Lipid Classes (52 Sub-Classes)

Lipid Class	Lipid Subclass	Full Name
AC	AC	Acylcarnitine
AC	AC-OH	Hydroxylated acylcarnitine
BA	BA	Bile acid
CE	CE	Cholesteryl ester
CE	dimethyl-CE	Dimethyl-cholesteryl ester
CE	methyl-CE	Methyl-cholesteryl ester
Cer	Cer(d)	Ceramide
Cer	Cer(m)	Deoxyceramide
Cer	Cer1P	Ceramide-1-phosphate
Cer	dhCer	Dihydroceramide
Cer	dhCer1P	Dihydroceramide-1-phosphate
Cer	dhHex2Cer	Dihydrodihexosylceramide
Cer	dhHexCer	Dihydromonohexosylceramide
Cer	dhS1P and dhSph	Dihydrosphingosine-1-phosphate
Cer	Hex2Cer	Dihexosylceramide
Cer	Hex3Cer	Trihexosylceramide
Cer	HexCer	Monohexosylceramide
Cer	S1P	Sphingosine-1-phosphate
Cer	SHexCer	Sulfatide
Cer	SM	Sphingomyelin
Cer	Sph	Sphingosine
CL	CL	Cardiolipin
COH	COH	Free Cholesterol
DE	DE	Dehydrocholesterol ester
DE	methyl-DE	Methyl-dehydrocholesteryl ester
DG	DG	Diacylglycerol
FFA	FFA	Free fatty acid
Glycerophospholipids	LPC	Lysoalkylphosphatidylcholine
Glycerophospholipids	LPC(O)	Lysoalkylphosphatidylcholine (lysoplatelet activating factor)
Glycerophospholipids	LPC(P)	Lysoalkenylphosphatidylcholine (plasmalogen)
Glycerophospholipids	LPE	Lysoalkylphosphatidylethanolamine
Glycerophospholipids	LPE(P)	Lysoalkenylphosphatidylethanolamine (plasmalogen)
Glycerophospholipids	LPG	Lysoalkylphosphatidylglycerol
Glycerophospholipids	LPI	Lysoalkylphosphatidylinositol
Glycerophospholipids	LPS	Lysoalkylphosphatidylserine
Glycerophospholipids	PA	Phosphatidic acid
Glycerophospholipids	PC	Phosphatidylcholine
Glycerophospholipids	PC(O)	Alkylphosphatidylcholine
Glycerophospholipids	PC(P)	Alkenylphosphatidylcholine (plasmalogen)
Glycerophospholipids	PE	Phosphatidylethanolamine
Glycerophospholipids	PE(O)	Alkylphosphatidylethanolamine
Glycerophospholipids	PE(P)	Alkenylphosphatidylethanolamine (plasmalogen)
Glycerophospholipids	PG	Phosphatidylglycerol
Glycerophospholipids	PI	Phosphatidylinositol
Glycerophospholipids	PIP1	Phosphatidylinositol monophosphate
Glycerophospholipids	PS	Phosphatidylserine
GM3	GM3	GM3 ganglioside
MAG	MAG	Monoacylglycerols
OxSpecies	OxSpecies	Oxidised lipids
TG	TG [NL]	Triacylglycerol
TG	TG(O) [NL]	Alkyltriacylglycerol
Ubiquinone	Ubiquinone	Ubiquinone

Subject Cell Collection

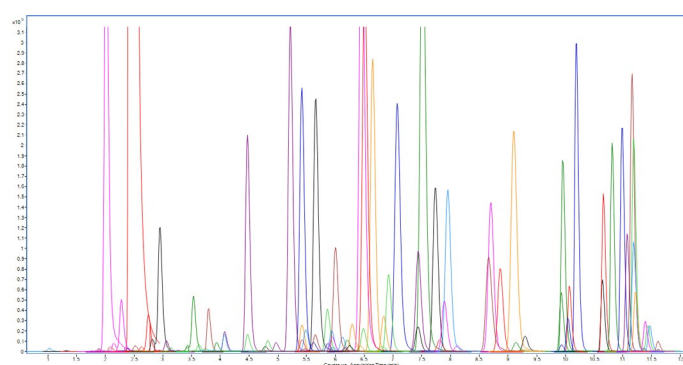
- ✓ Cells were collected from pediatric subjects in accordance with an approved Stanford Review Board protocol
- ✓ The control samples are progenitor cells from healthy young adult bone marrow
- ✓ Leukemia bone marrow cells were acquired at diagnosis and at relapse

Results and Discussion

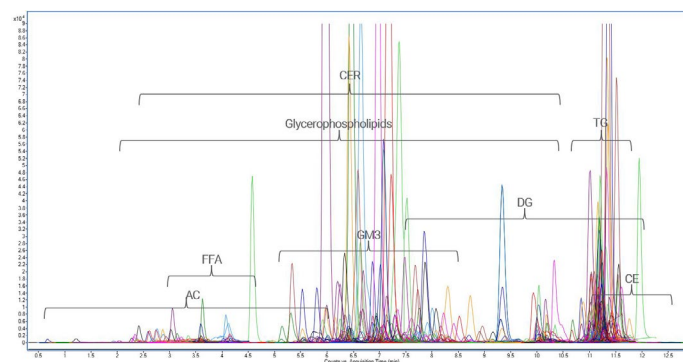
Method Validation Procedure

- ✓ 97 internal standards that represent majority of the included lipid classes were selected to evaluate the method performance
- ✓ Three sets of standards (extracted matrix-matched standards, post-extraction matrix-matched standards and standards in solvent) were prepared at 0.01, 0.02, 0.05, 0.1, and 0.5 $\mu\text{g/mL}$ with 3 or 4 replicates to test the linearity, limit of quantitation (LOQ), accuracy, reproducibility, and matrix effect (ME)

Profile of Internal Standards Spiked in Subject Cell



Profile of Lipids in Subject Cells



Method Validation Results

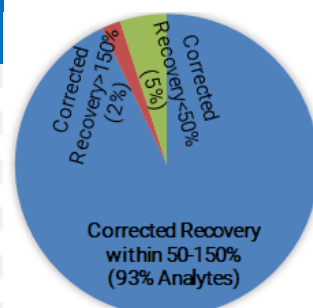
- ✓ Extracted matrix-matched standards provide accurate results by compensating for both matrix effects and potential recovery losses
- ✓ Over 90% of analyte corrected recoveries (CR, within the 50-150% range), CVs ($\leq 30\%$) and matrix effect ($\pm 50\%$) were obtained at and above the LOQs
- ✓ The coefficient of determination (r^2) values of matrix extracted calibration curves were >0.95 for the majority of analytes ranging from 0.01 (or 0.02) to 0.5 $\mu\text{g/mL}$

Results and Discussion

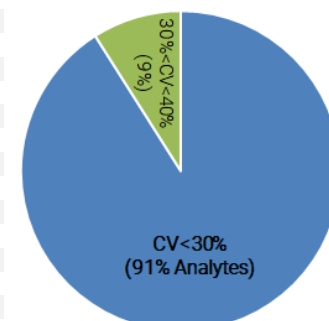
Internal Standard Specific Conditions and LOQ in Cell

Internal Standard	Transition m/z	RT min	LOQ in Cell µg/mL	Internal Standard	Transition m/z	RT min	LOQ in Cell µg/mL
AC(16:0)-d3	403.4 > 85.1	2.45	0.01	PC (17:0_18:1)-d5	779.6 > 184.1	7.51	0.01
CE(14:1)-d7	619.6 > 376.5	11.12	0.02	PC (17:0_20:3)-d5	803.6 > 184.1	7.08	0.01
CE(16:1)-d7	647.6 > 376.5	11.36	0.02	PC (17:0_22:4)-d5	829.6 > 184.1	7.42	0.01
CE(18:0)-d6	676.7 > 376.5	11.60	0.02	PC (P-18:0_18:1)-d9	781.6 > 184.1	8.66	0.01
CE(18:1)-d7	675.7 > 376.5	11.61	0.01	PE (15:0_18:1)-d7	711.6 > 570.5	6.81	0.01
CE(20:3)-d7	699.6 > 376.5	11.42	0.01	PE (17:0_14:1)-d5	681.5 > 540.5	5.92	0.01
CE(22:4)-d7	725.7 > 376.5	11.46	0.01	PE (17:0_16:1)-d5	709.5 > 568.5	6.83	0.01
Cer (d18:1_16:1)-d7	543.5 > 271.4	6.63	0.01	PE (17:0_18:1)-d5	737.5 > 596.5	7.88	0.01
Cer (d18:1_18:0)-d7	573.6 > 271.4	8.69	0.01	PE (17:0_20:3)-d5	761.5 > 620.5	7.43	0.01
Cer (d18:1_18:1)-d7	571.5 > 271.4	7.73	0.01	PE (17:0_22:4)-d5	787.6 > 646.6	7.80	0.01
Cer (d18:1_20:1)-d7	599.6 > 271.4	8.85	0.01	PE (P-18:0_18:1)-d9	739.5 > 348.3	9.13	0.01
Cer (d18:1_22:1)-d7	627.7 > 271.4	9.95	0.01	PG (15:0_18:1)-d7	759.6 > 570.6	5.59	0.01
Cer (d18:1_24:1)-d7	655.6 > 271.4	10.18	0.01	PG (17:0_20:3)-d5	729.5 > 540.5	4.96	0.01
Cer1P (d18:1/12:0)	562.4 > 264.3	4.06	0.01	PG (17:0_16:1)-d5	757.5 > 568.5	5.63	0.01
Cholesterol-d7	411.5 > 411.5	4.46	0.01	PG (17:0_18:1)-d5	785.5 > 596.5	6.47	0.01
Cholic Acid-d4	430.3 > 359.3	1.03	0.01	PG (17:0_20:3)-d5	809.5 > 620.5	6.11	0.01
DG (15:0_18:1)-d7	605.5 > 299.5	9.23	0.01	PG (17:0_22:4)-d5	835.5 > 646.5	6.40	0.01
DG (17:0_14:1)-d5	575.6 > 332.3	8.11	0.01	PI (15:0_18:1)-d7	847.6 > 570.6	5.38	0.01
DG (17:0_16:1)-d5	603.6 > 332.3	9.29	0.01	PI (17:0_14:1)-d5	817.6 > 540.6	4.76	0.01
DG (17:0_18:1)-d5	631.6 > 332.3	10.06	0.01	PI (17:0_16:1)-d5	845.6 > 568.6	5.40	0.01
DG (17:0_20:3)-d5	655.6 > 332.3	9.92	0.01	PI (17:0_18:1)-d5	873.5 > 596.5	6.20	0.01
DG (17:0_22:4)-d5	681.6 > 332.3	10.04	0.01	PI (17:0_20:3)-d5	897.5 > 620.5	5.85	0.01
dhCer (d18:0_8:0)	428.4 > 266.4	4.47	0.01	PI (17:0_22:4)-d5	923.6 > 646.6	6.15	0.01
FFA (18:1)-d9	290.3 > 290.2	3.79	0.01	PS (15:0_18:1)-d7	755.5 > 570.5	5.45	0.01
GlcCer (d18:1_15:0)-d7	693.6 > 271.3	5.84	0.01	PS (17:0_14:1)-d5	725.5 > 540.5	4.82	0.01
Hex3Cer (d18:1_17:0)	1038.7 > 264.3	5.95	0.01	PS (17:0_16:1)-d5	753.5 > 568.5	5.47	0.01
LacCer (d18:1_15:0)-d7	855.6 > 271.3	5.41	0.01	PS (17:0_18:1)-d5	781.5 > 596.5	6.28	0.01
LPC (15:0)-d5	487.3 > 184.1	2.28	0.01	PS (17:0_20:3)-d5	805.5 > 620.5	5.93	0.01
LPC (17:0)-d5	515.4 > 184.1	2.94	0.01	PS (17:0_22:4)-d5	831.5 > 646.5	6.23	0.01
LPC (18:1)-d7	529.4 > 184.1	2.74	0.01	S1P (d18:1) d7	387.2 > 271.3	2.08	0.02
LPC (19:0)-d5	543.4 > 184.1	3.53	0.01	SHexCer (d18:1_12:0)	724.8 > 264.3	3.92	0.01
LPE (15:0)-d5	445.3 > 304.3	2.38	0.01	SM (d18:1_15:0)-d9	698.6 > 193.1	5.41	0.01
LPE (17:0)-d5	473.3 > 332.3	3.06	0.01	SM (d18:1_16:1)-d9	710.6 > 193.1	5.21	0.01
LPE (18:1)-d7	487.3 > 346.3	2.85	0.01	SM (d18:1_18:1)-d9	738.7 > 184.1	5.98	0.01
LPE (19:0)-d5	501.3 > 360.3	3.63	0.01	SM (d18:1_20:1)-d9	766.6 > 193.1	6.91	0.01
LPG (15:0)-d5	493.3 > 304.3	2.02	0.01	SM (d18:1_22:1)-d9	794.7 > 193.1	7.94	0.01
LPG (17:0)-d5	521.3 > 332.3	2.65	0.01	SM (d18:1_24:1)-d9	822.7 > 193.1	9.08	0.01
LPG (19:0)-d5	549.3 > 360.3	3.24	0.01	Sph (d17:1)	286.3 > 268.3	2.01	0.01
LPI (13:0)	548.3 > 271.3	1.30	0.01	TG (41:0) [NL-13:0]-d5	731.6 > 500.4	10.64	0.01
LPI (15:0)-d5	581.3 > 304.3	1.87	0.01	TG (43:1) [NL-15:1]-d5	757.7 > 500.4	10.66	0.01
LPI (17:0)-d5	609.3 > 332.3	2.51	0.01	TG (45:1) [NL-17:1]-d5	785.7 > 500.4	10.81	0.01
LPI (19:0)-d5	637.3 > 360.3	3.11	0.01	TG (47:1) [NL-15:1]-d5	813.7 > 556.4	10.98	0.01
LPS (15:0)-d5	489.3 > 304.3	1.88	0.01	TG (48:1) [NL-18:1] d7	829.8 > 523.5	11.07	0.01
LPS (17:0)-d5	517.3 > 332.3	2.51	0.01	TG (49:1) [NL-17:1]-d5	841.8 > 556.5	11.16	0.01
LPS (19:0)-d5	545.3 > 360.3	3.13	0.01	TG (51:2) [NL-19:2]-d5	867.8 > 556.5	11.18	0.01
PA (15:0_18:1)-d7	685.6 > 570.6	5.97	0.02	TG (53:3) [NL-17:1]-d5	893.8 > 608.5	11.18	0.01
PC (15:0_18:1)-d7	753.6 > 184.1	6.45	0.01	TG (55:4) [NL-19:2]-d5	919.8 > 608.5	11.21	0.01
PC (17:0_14:1)-d5	723.6 > 184.1	5.65	0.01	TG (57:4) [NL-21:2]-d5	947.9 > 608.5	11.39	0.01
PC (17:0_16:1)-d5	751.6 > 184.1	6.50	0.01				

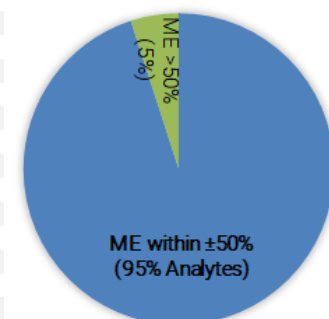
Corrected Accuracy



Reproducibility



Matrix Effect



Statistical results for quantitative analysis based on five spiked levels at 3 or 4 replicates

Conclusions

- ✓ A lipid profiling workflow in cells targeting over 1200 lipids in 54 sub-classes was developed and validated
- ✓ This workflow is ready to be used to accurately quantitate the change of lipidomic profiles in the development and treatment of pediatric leukemia

The Future Plan

- ✓ The lipid profile of 200 samples taken from leukemia bone marrow in relapsed to non-relapsed cases will be compared to identify lipid predictors of relapse, which could guide the development of biomarkers for the prediction of relapse

<https://www.agilent.com/en/promotions/asms>

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RA45051.6948032407

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Published in USA, May 31, 2023

Poster Reprint

ASMS 2023
Poster number ThP 520

Illuminating the Cellular and Molecular Response to Drug Treatment by Combining Bioenergetic Measurements with Untargeted Metabolomics

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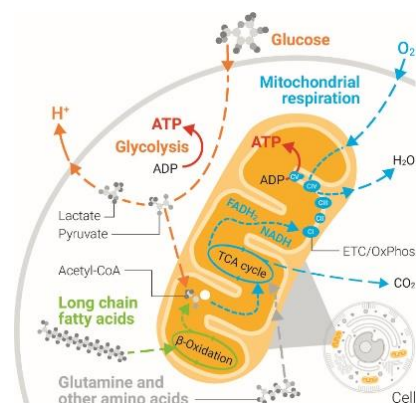
²Agilent Technologies, Inc., Lexington, MA, USA

Introduction

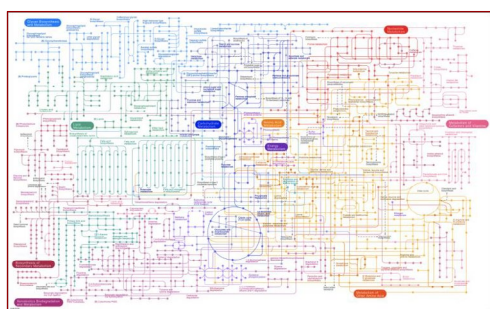
To better understand the biology of cancer cells and their dynamic metabolic response to therapeutic compounds, advanced methodologies are required. We combined results from two technologies that measured metabolic pathway utilization at two scales, cellular and molecular. An acute monocytic leukemia cell line was treated with the drugs SU1498 and AG-879, which were selected from a library of 80 kinase inhibitors based on their modulation of mitoATP production rates. Separately, cells treated with the drugs were lysed, metabolism was quenched, and metabolites and lipids were extracted with an automated sample preparation method. Extracts were directly analyzed with LC/Q-TOF and discovery-based MS software. The combined results provided deeper insight into the cellular and molecular metabolic response to drug treatment.

Seahorse XF analyses provide measurements at a cellular resolution

Cellular oxygen consumption and extracellular acidification rates



LC/MS omics and qualitative flux analyses provide measurements at a molecular resolution



Production and consumption of individual metabolites, lipids and proteins

Experimental

Sample Preparation

THP-1 cells were cultured in supplemented RPMI medium and treated with SU1498, AG-879, or DMSO vehicle for either 1-2 hours (Seahorse XF) or 18 hours (XF and LC/MS). Cells were stained for viability assessment and counted for sample normalization with an Agilent Novocyte Quanteon flow cytometer. Kinetic measurements were made with the Agilent Seahorse XF Real-Time ATP Rate Assay to determine ATP production rates specific to glycolysis and mitochondrial respiration as well as the Mito Stress Test for a deeper investigation of drug impact on mitochondrial respiration rates. For LC/MS, metabolites were extracted with an automated dual metabolite + lipid extraction process with an Agilent Bravo Liquid Handling Platform using a supplementary protocol.¹ Metabolite extracts were separated with HILIC-LC and analyzed with an Agilent Revident LC/Q-TOF. Datasets were analyzed with Agilent MassHunter Explorer software.

Workflow Overview

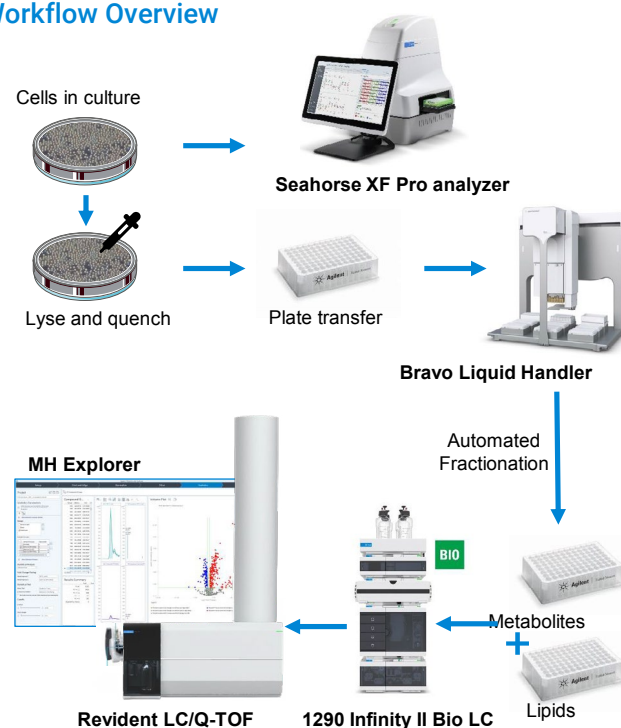


Figure 1. Overview of workflow, from cell culture to Seahorse XF and from cell culture to automated sample preparation to LC/MS analysis.

Results and Discussion

Tyrosine Kinase Inhibitor Screen Identifies Compounds of Interest

A kinase inhibitor library was screened with the Agilent Seahorse XF Real-Time ATP Rate Assay to assess acute effects of the inhibitors on mitochondrial ATP production rates.² Two inhibitors, AG-879 and SU1498, were selected for additional XF and LC/MS analysis, and MitoATP production rate IC₅₀ values were determined.

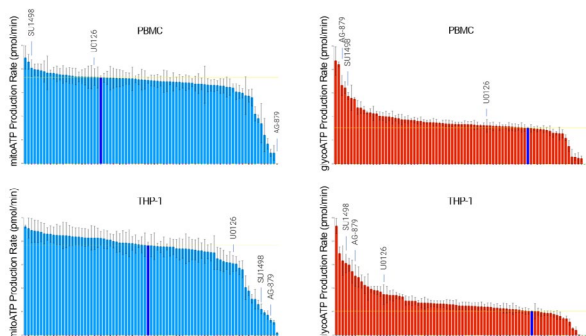


Figure 2. Effect of 80 kinase inhibitors on mitoATP production rate in THP-1 and PBMC cells. AG-879 reduced mitoATP production rates in both THP-1 cancer cells and healthy PBMCs. SU1498 reduced mitoATP production rates in THP-1 cells, but not in healthy PBMCs.

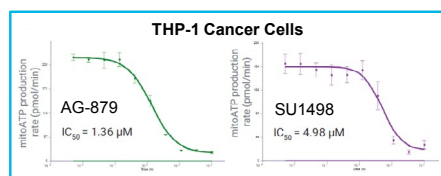


Figure 3. MitoATP production rate dose response curves and IC₅₀ values.²

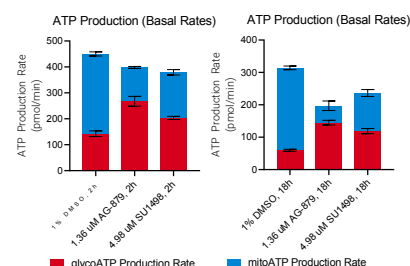


Figure 4. IC₅₀ AG-879 and SU1498 doses increase glycoATP and decrease mitoATP production rates after 2h and 18h. Total ATP production rates decrease relative to vehicle control after 18h with AG-879 or SU1498.

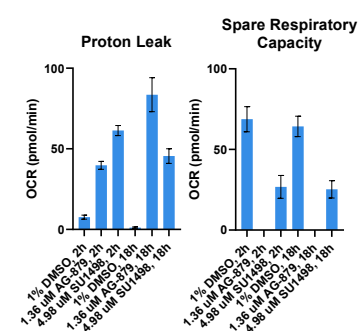


Figure 5. AG-879 and SU1498 increase proton leak by uncoupling oxygen consumption from mitoATP production. AG-879 induced proton leak increases from 2h to 18h of treatment, while SU1498 induced proton leak decreases from 2h to 18h (ANOVA). SU1498 reduces spare respiratory capacity (SRC); AG-879 causes complete loss of SRC.

Untargeted Metabolomics (Mx)

HILIC(-) LC/Q-TOF datafiles were processed in MH Explorer software. Metabolite annotations were made using an AMRT search with a subset of the Agilent METLIN PCDL curated with HILIC-Z retention times.

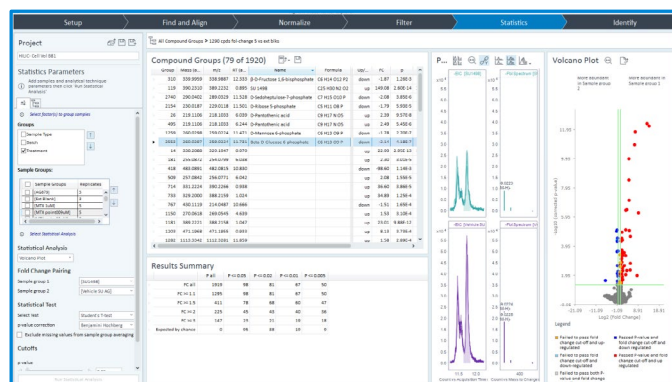


Figure 6. MH Explorer UI showing statistical analysis of HILIC(-) SU1498 treatment versus control samples, with 79/1920 compounds passing significance thresholds. Shown are results for glucose-6-phosphate. Cut-offs: Fold-change >1.5 and p-value <0.05.

Analysis based on the HILIC(-) dataset indicate similar trends for both drug treatments with a smaller magnitude of change for AG-879. Both drugs resulted in a decrease of sugar phosphates, intermediates in glycolysis that may correlate to the increased glycoATP production rate observed in Fig. 4. Conversely, pantothenic acid, a precursor for acyl-CoA required for pyruvate metabolism in the TCA cycle, was increased with SU1498 treatment, which may correlate with the XF measured reduction in mitoATP production.

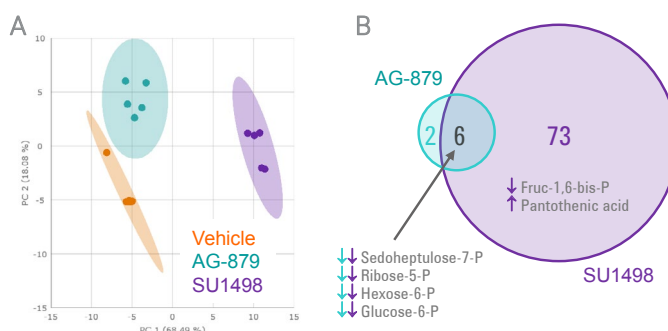


Figure 7. (A) Supervised PCA based on HILIC(-) significant compounds for AG-879 and SU1498 versus vehicle control. (B) Venn Diagram of significant compounds with compound annotations. Direction of arrows indicate significant increase (↑) or decrease (↓) relative to vehicle control.

Results and Discussion

Untargeted Lipidomics (Lx)

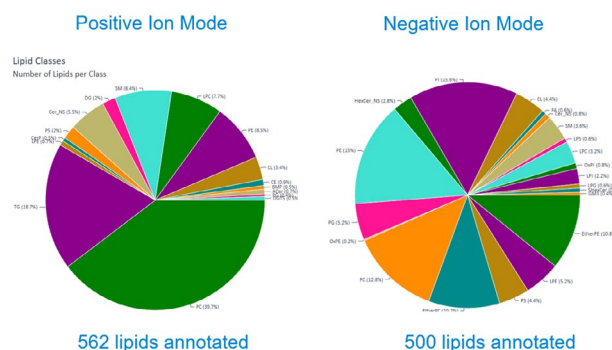


Figure 8. Agilent Lipid Annotator software results from 2 sets of 6 Iterative MS/MS datafiles acquired on pooled THP-1 cellular lipid extracts prepared with the dual automated workflow.

Lipid Annotator results from Fig. 8 were used in MH Explorer for an AMRT database search. A third curated database³ leveraging the same chromatography⁴ was additionally used resulting in 677 lipid annotations out of 4,476 compounds in the positive-ion mode project (right).

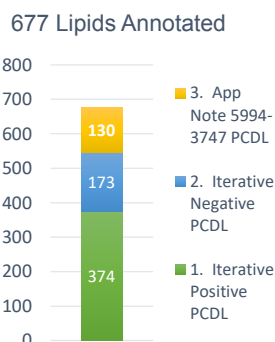


Figure 9. (A) MH Explorer PCA based on 677 annotated lipid compounds. Volcano plots of AG-879 (B) and SU1498 (C) versus vehicle control. 95 significant lipids are shown for the latter, with significant classes noted (Cer_NS, ceramides; TGs, triacylglycerols; CE, cholesterol esters).

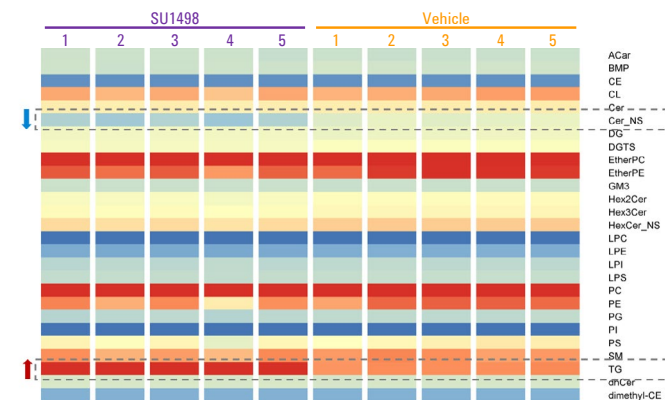
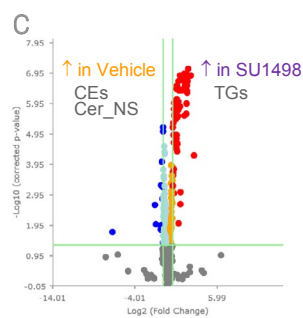
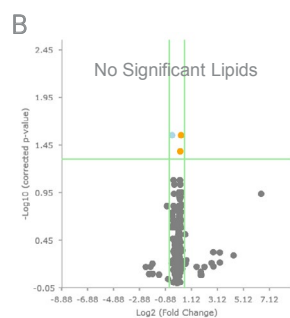
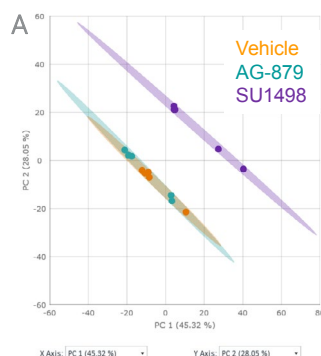


Figure 10. Lipid Class matrix plot in MPP created from exported MH Explorer .pfa results. Notable differences in ceramide (Cer_NS) and triacylglycerol (TG) classes are highlighted.

Conclusions

Combining XF and LC/MS technologies provided deeper insight into the cellular and molecular metabolic response to drug treatment for cancer research.

- XF analysis of AG-879 and SU-1498 corroborated previous results² and newly demonstrated that both drugs cause mitochondrial uncoupling.
- Untargeted Mx identified changes in key metabolites affected in glycolysis and mitochondrial respiration that correlate with XF results.
- Untargeted Lx showed an increase in TG content with SU-1498 treatment which may be related to buildup of energy precursors for the TCA cycle, shown to be reduced with XF.
- Future directions could include 1) further mining of the unidentified significant features, 2) qualitative flux analysis, and/or 3) the Seahorse XF Substrate Oxidation Stress Test to assess fuel dependencies with drug treatment.

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Published in USA, May 31, 2023

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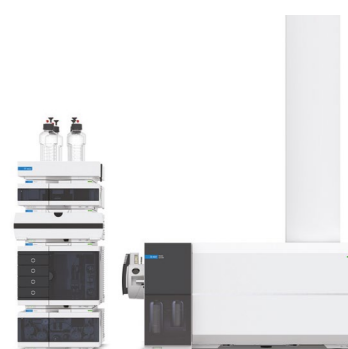
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RA45335.728587963