# The 2nd Meeting of the Italian Metabolomics Network

Firenze
Campus Novoli
15th – 16th December 2025

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Discussione Risonanze Magnetiche

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## MONDAY, DECEMBER 15TH, 2025

9:30 – 10:45 Registration
10:45 – 11:00 Welcome & Introduction (GIDRM, IMaSS, IMN)

***SESSION 1 - Chair: P. Turano, A. Armirotti***

11:00 – 11:40 Plenary I: **G. Theodoridis** "*LC-MS Metabolomics. Constraints, Perspectives and Potential for Biomarker Discovery and Clinical Application*"

11:40 – 12:00 **A. Noto** "*Metabolomic Signatures in Toddlers and Adolescents with Autism Spectrum Disorder: Insights from Ten Years of the UNICA Experience*"

12:00 – 12:20 **G. Solarino** "*Untargeted Fingerprinting in Bipolar Disorder: Chemometric Insights from the BORDER project*"

12:20 – 12:40 **G. Petrella** "*Urinary Metabolomics Reveals Inflamation, Immune Inhibition, and Gut Dysbiosis as Predictors of Bladder Cancer Recurrence*"

12:40 – 13:00 **A. Vignoli** "*Studying Alzheimer's Disease Through an Integrative Serum Metabolomic and Lipoproteomic NMR-Based Approach: the Medea Study*"

13:00 – 13:20 **A. Gallo** "*Exploring the Urinary Metabolomic Fingerprint of Human Cytomegalovirus: a $^{1}$H-NMR Study on Congenitally Infected Newborns*"

13:20 – 14:20 **LUNCH AND NETWORKING**

***SESSION 2 - Chair: P. Franceschi, D.O. Cicero***

14:20 – 14:40 **E. Bossi** "*Mass Spectrometry-Based Workflow Optimization for Combined Metabolomics and Lipidomics Analysis from Blood Microsamples*"

14:40 – 15:00 **A. Ottas** "*Transferable Machine Learning Models for Cardiovascular Disease Prediction through NMR Metabolomics data and Optimal Transport*"

15:00 – 15:20 **L. Brunelli** "*Plasma gut-microbiota-derived metabolites trajectories to uncover the link between gut-microbiota dysbiosis and frailty in older adults*"

15:20 – 15:40 **N. Iaccarino** "*From Transcriptome to Metabolome: Uncovering the Cellular Impact of G-Quadruplex Ligands in Cancer Cells*"

15:40 – 16:00 **L. Tenori** "*Deriving Three One-Dimensional NMR Spectra from a Single Spectrum*"

16:00 – 17:00 **COFFEE BREAK AND POSTER SESSION (ALL POSTERS)**

***SESSION 3 - Chair: A. Randazzo, G. Pieraccini***

17:00 – 17:15 Sponsorship lecture: ***Bruker Mass Spec***
**G. Calza,** "*Beyond Isomer Separation | Trapped Ion Mobility Spectrometry (TIMS) Boosts Annotation Confidence in 4D-Metabolomics & 4D Lipidomics*"

17:15 – 17:35 **M. Spada** "*Metabolomics Reveals Metabolic Adaptations Following Glutamine Metabolism Impairment In Colorectal Cancer Cells*"

17:35 – 17:55 **S. Serrao** "*From Primary Tumor to Circulating Tumor Cells and Metastasis: Tracing Metabolic Reprogramming in Lung Cancer*"

17:55 – 18:15 **G. Picone** "*Influence of IMTA-RAS and Probiotics on the Molecular Profile of Solea Senegalensis: a $^{1}$H-NMR Metabolomics Approach*"

18:15 – 18:35 **G. Meoni** "*Integrating NMR- and MS-Based approaches to characterize coffee micro-lots: elemental, polyphenolic, and metabolomic fingerprints*"

## TUESDAY, DECEMBER 16TH, 2025

***SESSION 4 - Chair: L. Atzori, V. Ghini***

9:00 – 9:20 **S. Zampieri** "*Metabolomics Overcomes Age Bias in Non-Invasive Fibrosis Staging: the GP Index*"

9:20 – 9:40 **V. Balloni** "*Combined Tissue and Liquid Biopsies Reveal the Metabolic Interactions Among Blood, Liver, and Adipose Tissue in Bariatric Surgery of Morbid Obesity*"

9:40 – 10:00 **M. Nobile** "*Exercise-Induced Metabolic Adaptations During Cardiac Rehabilitation: a Longitudinal Metabolomic Investigation on DBS*"

10:00 – 10:20 **V. Righi** "*NMR Based Metabolomics to Investigate Molecular Mechanisms in ADLD Neurodegenerative Disorder*"

10:20 – 10:40 **M. Gallo** "*Saliva Metabolomics as an Emerging Tool for Advanced Diagnostics: the Leukoplakia Paradigm*"

10:40 – 10:55 Sponsorship lecture: ***Sciex***
**M. Armelao** "*Quantitative and Qualitative Analysis of Oxylipins Using Highresolution Mass Spectrometry*"

10:55 – 11:15 **COFFEE BREAK AND POSTER SESSION**

***SESSION 5 - Chair: A. Passoni, M.R. Chierotti***

11:15 – 11:35 G. Boschetti "*Metabolomics and Cystic Fibrosis Drugs: Exposure of Dams to Tezacaftor During Pregnancy and Breastfeeding Induces Molecular Alterations in Mice Brain*"

11:35 – 11:55 C. Marino "*The Metabolomic Dysfunctions of Spinal Muscular Atrophy: a Journey from Animal Models to Human CSF*"

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| 11:55 – 12:15 | C. Guerrini "*Unravelling Urinary Metabolic Alterations Induced by Thirdhand Smoke Exposure: from Untargeted Analysis in Animal Models to Targeted LC-MS Validation in Children*" |
| 12:15 – 12:30 | Sponsorship lecture: ***Bruker Biospin***<br>**M. Zani** "*Building the Ecosystem for Standardized and Automated NMR Metabolomics*" |
| 12:30 – 13:10 | Plenary II: **O. Millet** "*Integrative $^{1}$H-NMR and Machine Learning Framework for Large-Scale Serum Metabolomics and Disease Classification*" |
| 13:10 – 14:20 | **LUNCH AND NETWORKING** |
| 14:20 – 15:20 | Meeting IMN |

# Invited Lectures

# LC-MS METABOLOMICS. CONSTRAINTS, PERSPECTIVES AND POTENTIAL FOR BIOMARKER DISCOVERY AND CLINICAL APPLICATION

**G. Theodoridis**[1,2], H. Gika[1,3]

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Metabolomics show strong growth as the field can offer new insights in disease/health/wellness biochemistry. Liquid Chromatography Mass Spectrometry (LC-MS) takes the largest part of the metabolomics market due to its agility, performance, sensitivity, fit for the analysis of biological samples, direct applicability, large numbers of instruments and practitioners. LC-MS proves superior in the discovery, development of new biomarkers and their application in disease, nutrition, wellness, exposure or safety assessment. Recently LC-MS is also entering clinical chemistry practice, however its application in the clinic is not problem free. Translational aspects and application of research findings to the clinical practice, proceeds slowly. As metabolomics evolve as a technology protocols differ between laboratories. These differences hinder harmonization and may result in problems in replication of findings and the adoption of biomarkers. We discuss the constraints that slow the finalization and uptake of LC-MS metabolomics biomarker development.

To illustrate the strong perspective of metabolomics, examples of biomarker discovery will be presented with focus on larger initiatives where metabolic profiles are associated with genetic, gut-microbiome data, clinical and anthropometric data to identify associations of omics profiles with diet or cardiac health. In Corlipid project, blood samples from 1500 cases of coronary disease were analysed by untargeted UPLC-TOF-MS lipidomics, untargeted metabolomics, and new targeted methods to quantify ceramides, carnitines (UPLC-MS/MS), and fatty acids (GC-MS). A machine learning algorithm selected 17 parameters (8 metabolites) to predict the coronary angiography results (syntax score the golden standard for quantifying the complexity of coronary artery disease). In Codiet blood and urine samples from four European cohorts were analysed by six UPLC-MS methods (combining targeted and untargeted modes) to map the metabolome and link the obtained profiles with nutrition. The presentation will emphasize on the needs and the benefits of the development of new analytical methods to effectively map the metabolome. To better illustrate this, we report the development of a new method for the quantitation of aromatic aminoacids and sulphated metabolites. These molecules are important markers in inflammation, cancer and other disorders, however the lack of commercially available standards hinders their quantitation in biological samples. We synthesized 14 sulphated metabolites; after preparative LC purification, NMR and MS verification, we developed a new UPLCMS/ MS that allowed for the quantitation of 30 metabolites in urine, and the subsequent application in biomarker studies and the analysis of clinical samples.

## Acknowledgements

The authors acknowledge funding from the Horizon Europe Project 101079370 — BiACEM WIDERA Twinning “Biomic_AUTh, Center of Excellence in Metabolomics research”.

# INTEGRATIVE $^{1}$H-NMR AND MACHINE LEARNING FRAMEWORK FOR LARGE-SCALE SERUM METABOLOMICS AND DISEASE CLASSIFICATION

A. Ibañez de Opakua and **O. Millet**

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Nuclear Magnetic Resonance (NMR) spectroscopy provides a powerful and reproducible platform for metabolic profiling, enabling non-invasive insights into human health and disease. In this study, we present an integrated computational framework that combines $^{1}$H-NMR spectral analysis with machine learning models to extract clinically relevant information from large-scale serum metabolomics data. From over 30,000 serum samples, 2D J-resolved spectra were used to quantify 51 metabolites with minimized signal overlap, while 1D NOESY spectra were leveraged to estimate 25 clinical parameters through supervised regression models. These derived biochemical and clinical features were then used to train a multiclass disease classifier based on eXtreme Gradient Boosting (XGBoost), designed to distinguish nine health categories encompassing seven disease conditions and two age-defined healthy groups. The model achieved an overall accuracy of 0.79, with area-under-curve (AUC) values between 0.91 and 1.00 across classes, demonstrating high sensitivity and specificity. Misclassifications were primarily observed between physiologically related groups, such as older adults and individuals with metabolic syndrome or long COVID, reflecting underlying metabolic similarities. Feature importance analyses using SHAP values highlighted key metabolites and clinical markers associated with systemic inflammation, lipid metabolism, and energy balance as major drivers of disease discrimination. This framework underscores the potential of NMR-based machine learning for scalable, interpretable, and non-invasive health assessment, supporting precision diagnostics through individualized metabolic phenotyping.

# Oral Presentations

# METABOLOMIC SIGNATURES IN TODDLERS AND ADOLESCENTS WITH AUTISM SPECTRUM DISORDER: INSIGHTS FROM TEN YEARS OF THE UNICA EXPERIENCE

**A. Noto[1*]**, C. Piras[1], M. Spada[1], K. K. Kopec[2], K. Lilliu[1], L. Atzori[1]

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The clinical concept of autism emerged in the mid-20th century, when Leo Kanner (1943) and Hans Asperger (1944) independently described children with "autistic" features. Today, autism spectrum disorder (ASD) is recognized as a complex condition arising from genetic predisposition, epigenetic modifications, and multiple environmental influences. Diagnosis remains behavior-based, as no validated biological test can yet confirm the presence or grade the severity of ASD. Identifying sensitive and specific biomarkers, therefore, remains a significant challenge. In this context, metabolomics offers an unbiased systems approach to identify disease-associated metabolic fingerprints. We aimed to compare the urinary metabolic signatures of ASD toddlers and adolescents with those of a cohort of healthy controls, in order to define shared specific metabolic features [1-5]. In total, 152 subjects were compared: 91 ASD patients and 61 healthy controls. Parents of all participating children provided written informed consent prior to inclusion. ASD patients were recruited at the Child Psychiatry Unit, University Hospital of Rome Tor Vergata (Italy), and the Pediatric Division, University of Bari (Italy). Exclusion criteria for ASD included genetic syndromes, neurological disorders, ongoing acute illnesses, and known inborn errors of metabolism. Urine samples from 152 subjects were analyzed by 1H NMR spectroscopy and/or GC–MS. Resulting spectra and chromatograms were explored using multivariate and univariate statistical approaches. Among the 91 ASD patients, significant and consistent urinary alterations were observed: uric acid was decreased, whereas 3-methylhistidine, hippuric acid, and p-cresol were increased. Erythronic acid, citric acid, and erythritol reached significance only in multivariate analysis. The ASD cohort showed altered urinary concentrations of microbiota-derived metabolites, sugars, and Krebs-cycle intermediates. Taken together, these findings point toward intestinal dysbiosis, increased intestinal permeability, perturbed energy pathways, and involvement of the antioxidant system. The results are in line with the literature and suggest a plausible pathophysiological substrate for ASD comorbidities, such as sleep disturbances and gastrointestinal symptoms, that may exacerbate core features. Further validation in independent cohorts and longitudinal designs is warranted.

## References

1. Noto A, et al. "The urinary metabolomics profile of an Italian autistic children population and their unaffected siblings", J Matern Fetal Neonatal Med. 2014 Oct;27 Suppl 2:46-52. doi: 10.3109/14767058.2014.954784.
2. Mussap M, et al. "Metabolomics of autism spectrum disorders: early insights regarding mammalian-microbial cometabolites", Expert Rev Mol Diagn. 2016 Aug;16(8):869-81. doi: 10.1080/14737159.2016.1202765.
3. Lussu M, et al. "The urinary 1 H-NMR metabolomics profile of an italian autistic children population and their unaffected siblings", Autism Res. 2017 Jun;10(6):1058-1066. doi: 10.1002/aur.1748.
4. Mussap M, et al. "The Urine Metabolome of Young Autistic Children Correlates with Their Clinical Profile Severity", Metabolites. 2020 Nov 23;10(11):476. doi: 10.3390/metabo10110476.

5. Piras C, et al. ”Alterations of the Intestinal Permeability are Reflected by Changes in the Urine Metabolome of Young Autistic Children: Preliminary Results”, Metabolites. 2022 Jan 23;12(2):104. doi: 10.3390/metabo12020104.

# UNTARGETED FINGERPRINTING IN BIPOLAR DISORDER: CHEMOMETRIC INSIGHTS FROM THE BORDER PROJECT

S. Tanilli[1], **G. Solarino*[2]**, M. Massano[3], C. Bretti[4], A. Olarini[2], R. Santalucia[2], M. Pazzi[2], P. Garofano[3], M. Vincenti[2], G. Lando[4], E. Alladio[2]

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Bipolar disorder (BD) is a complex psychiatric condition characterized by recurrent manic and depressive episodes that profoundly impact everyday life [1]. Currently, diagnosis relies on clinical evaluation of symptomatology and family history, yet symptomatic overlap with other mental health conditions frequently leads to misdiagnosis and suboptimal treatment [2]. The BORDER project (Bipolar disORDER: beyond psychology and towards multi-omics analysis) aims to establish a minimally invasive, objective approach to guide both diagnosis and treatment.

In this study, blood and urine samples from 40 individuals with BD and 40 healthy controls were analyzed using Ultra-High-Performance Liquid Chromatography coupled with High-Resolution Mass Spectrometry (UHPLC-HRMS), generating complex, high-dimensional datasets. Data preprocessing (i.e., peak picking, retention time alignment, features normalization, background removal) and independent statistical evaluations were followed by chemometric modeling approaches, including Principal Component Analysis (PCA) and Partial Least Squares – Discriminant Analysis (PLS-DA). These analyses revealed clear group separations and distinctive metabolic fingerprints associated with BD.

These approaches demonstrates the potential of chemometric tools to uncover latent metabolic signatures of BD, highlighting their value as future diagnostic biomarkers. Their structural identification and validation will be crucial for the advancement of personalized medicine.

## References

1. Vieta, E. et al. (2018). "Bipolar disorders", Nature reviews Disease primers, 4 (1), pp. 1–16.
2. Grande, I. et al. (2016). "Bipolar disorder" Lancet, 387(10027), pp. 1561–1572, doi:10.1016/S0140-6736(15)00241-X.

# URINARY METABOLOMICS REVEALS INFLAMATION, IMMUNE INHIBITION, AND GUT DYSBIOSIS AS PREDICTORS OF BLADDER CANCER RECURRENCE

**G. Petrella**[1*], G. Ciufolini[1], S. Lentini[1], F. Montorsi[2,3], A. Salonia[2,3], M. Pieri[4,5], S. Albisinni[6], R. Vago[2,3], D. O. Cicero[1]

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Non-muscle invasive bladder cancer (NMIBC) is characterized by a high risk of recurrence, yet current prediction tools lack sufficient accuracy [3]. This study investigates the prognostic value of urinary metabolites in stratifying recurrence risk using NMR-based metabolomics. Urine samples from 50 NMIBC patients were collected at diagnosis, prior to transurethral resection (TURBT), and patients were followed for three years [2]. Using multivariate and univariate analyses, we identified a panel of metabolites whose levels significantly differed between relapsing and non-relapsing patients. These included markers of systemic inflammation (lactate, valine, pyruvate), immune inhibition (taurine, alanine), and gut dysbiosis (hippurate, trigonelline, N-phenylacetylglutamine, 3-indoxyl sulfate). Seven metabolites showed strong predictive performance (AUC > 0.7), supporting their potential as a non-invasive biomarker panel for recurrence. Our findings suggest that the degree of dysregulation in inflammation, immunity, and microbiome-derived metabolites reflects the risk of relapse and may enhance current clinical risk models. This metabolomics approach may contribute to more personalized surveillance and therapeutic strategies in NMIBC management [1].

## References

1. Buergel, T. et al. (2022) "Metabolomic profiles predict individual multidisease outcomes", Nature Medicine, 28, pp. 2309–2320. doi: 10.1038/s41591-022-01922-9
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# STUDYING ALZHEIMER'S DISEASE THROUGH AN INTEGRATIVE SERUM METABOLOMIC AND LIPOPROTEOMIC NMR-BASED APPROACH: THE MEDEA STUDY

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Alzheimer's disease (AD) is the most common irreversible neurodegenerative disorder in the elderly population. The high variability in AD progression makes it difficult to predict when a patient will convert to dementia. We hypothesize that metabolic alterations in the brain and cerebrospinal fluid may also be reflected at a systemic level in blood serum and that these alterations could serve as prognostic biomarkers.

This study investigates serum biomarkers using $^1$H-NMR spectroscopy in a population that covers the entire spectrum of cognitive impairment: 57 patients with Alzheimer's disease at the dementia stage (AD-dem), 45 patients with AD at the mild cognitive impairment stage (MCI-AD), and 31 patients with MCI not associated with AD (MCI) [1]. A panel of 26 metabolites and 112 lipoprotein-related parameters was quantified. The logistic LASSO regression algorithm was employed to identify the optimal combination of metabolite-lipoprotein features and their ratios for group discrimination. In the training set, our model distinguished AD-dem from MCI with 81.7% accuracy, which was reproduced in the validation set (82.1% accuracy). The progression of MCI-AD patients was evaluated over time, with those showing a ≥1.5-point MMSE (Mini–Mental State Examination test) decline per year classified as fast progressors. Our model, built on four metabolic features, differentiated fast- and slow-progressing MCI-AD patients with 73.9% accuracy. The identification of potential novel peripheral biomarkers of AD paves the way for an innovative and minimally invasive method to identify AD in its early stages. Moreover, our model appears to be able to sub-stratify MCI-AD patients identifying those associated with a faster rate of clinical progression.

## References

1. Vignoli, A. et al. (2025) "Studying Alzheimer's disease through an integrative serum metabolomic and lipoproteomic approach", Journal of Translational Medicine, 23(1), p. 119. doi:10.1186/s12967-025-06148-4.

# EXPLORING THE URINARY METABOLOMIC FINGERPRINT OF HUMAN CYTOMEGALOVIRUS: A $^{1}$H-NMR STUDY ON CONGENITALLY INFECTED NEWBORNS

A. Spadavecchia[1], M. Zoccarato[2], G. Tedone[1], M. Biolatti[3], V. Dell'Oste[3], A. Leone[1], A. Cossard[2], M. Sozzi[4], I. Bresesti[5], E. Bertino[1], R. Gobetto[2], A. Coscia[1], and **A. Gallo[2*]**

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Human cytomegalovirus (HCMV) is the leading cause of congenital infections resulting in severe morbidity and mortality among newborns worldwide. Currently, the most significant prognostic factor of cCMV is the time of maternal infection, with a more severe clinical phenotype if the mother's first outbreak occurs during the first trimester of pregnancy. Nonetheless, the pathogenesis of cCMV infection has still to be completely characterized. In particular, little is known about the metabolic response triggered by HCMV in congenitally infected newborns. As such, urinary metabolic profiling by $^{1}$H-NMR might represent a promising tool to be exploited in the context of cCMV infection.

This study aims to investigate the impact of HCMV infection on the urine metabolome in a population of newborns by $^{1}$H-NMR spectroscopy combined with multivariate statistical analysis. Thirty-three newborns diagnosed with cCMV infection were recruited at the Neonatal Unit of the University of Turin and for each of them a urine sample was collected during admission medical examination. Seventeen healthy newborns without cCMV infection were also included as healthy controls.

The 1H NMR spectra of patients (n=35) and controls (n=15) allowed the identification of an overall amount of 55 metabolites. Principal Component Analysis (PCA) and clustering correctly assigned 49 out of 50 newborns into the infected and control groups. Partial Least-Squares-Discriminant Analysis (PLS-DA) revealed that newborns with cCMV resulted in having increased betaine, citrate, 3-hydroxybutyrate, 4-hydroxybutyrate, acetoacetate, formate, glycolate, lactate, succinate, andthreonine levels intheurine. On the other hand, healthy controls showed increased 4-aminohippurate, creatine, creatinine, fumarate, mannitol, taurine, and dimethylamine levels. These results showed a clear difference in metabolomic fingerprint between newborns with cCMV infection and healthy controls. Thus, metabolomics can be considered a new, promising diagnostic and prognostic tool in the clinical management of cCMV patients.

# MASS SPECTROMETRY-BASED WORKFLOW OPTIMIZATION FOR COMBINED METABOLOMICS AND LIPIDOMICS ANALYSIS FROM BLOOD MICROSAMPLES

**E. Bossi**[1*], M. Nobile[1], S. Serrao[1], P. Reveglia[2], A. Ferrara[2], V. Ramundi[3], C. Prehn[3], G. Corso[2], M. Witting[3], G. Paglia[1]

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Blood microsampling has emerged as a promising alternative to conventional venipuncture for metabolomics studies, granting advantages such as minimal invasiveness, ease of collection, suitability for multiple sampling, and optimal use in longitudinal designs [1]. This study aimed to optimize a liquid chromatography-mass spectrometry-based workflow enabling both untargeted metabolomics and lipidomics analyses from a single dried blood spot (DBS). In parallel, extraction optimization was performed for targeted metabolomics on DBS (Whatman) using TMIC MEGA kits. For the untargeted protocol optimization three commercially available microsampling devices—Capitainer and Whatman (whole blood) and Telimmune (plasma)—were evaluated. Among five extraction solutions tested, pure methanol provided the best compromise for simultaneous extraction of polar metabolites and lipids. Based on these results, a two-step consecutive extraction protocol was developed, using methanol followed by water to enhance the recovery of more polar metabolite classes and improve metabolome coverage. Short-term stability of polar metabolites and lipids was also evaluated at room temperature (RT) for up to five days. Capitainer showed the best results, preserving the stability of all evaluated classes of compounds for up to five days at RT. Regarding targeted protocol optimization, modifications to the original extraction protocol for panels A and B increased metabolite coverage in DBS with the highest improvement observed for Panel B. Overall, this work suggests that methanol extraction enables integrated metabolomics and lipidomics analysis from a single spot, and that a two-step approach can further enhance polar metabolite coverage. Targeted workflow optimization also improved metabolite coverage in DBS, emphasizing the importance of tailoring device selection and extraction protocols to study aims, matrices and analytical scope.

## References

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# TRANSFERABLE MACHINE LEARNING MODELS FOR CARDIOVASCULAR DISEASE PREDICTION THROUGH NMR METABOLOMICS DATA AND OPTIMAL TRANSPORT

**A. Ottas*[1]**, L. Goldoni[2], A. Armirotti[2], , S. Decherchi[3]

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Machine learning and, more in general artificial intelligence methods, represent powerful tools to address clinical questions related to the discovery of biomarkers and the prediction of disease onset through omics data. Nevertheless, these modeling attempts are often plagued by the non-transferability issue across different patients cohorts. This is due to several factors such as different approaches in metabolite absolute quantification, human and instrumental factors and the biological sample stability, which ultimately lead to out-of-domain data distributions which invalidate the underlying assumptions of the learnt models. Here, we investigate this issue for metabolomics data and cardiovascular diseases and propose a protocol, and a software tool, based on the Optimal Transport theory, which is able to calibrate and normalize data such that the learnt models become more transferable on new data. Results show that the learnt models improve systematically their accuracy after the proposed calibration procedure (Figure 1).

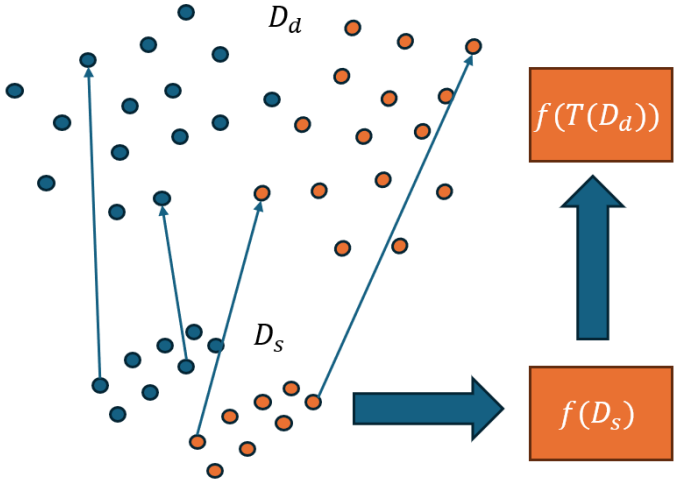


*Figure 1: Adapting the domains of two omics measurements different in time and with different experimental apparatus.*

# PLASMA GUT-MICROBIOTA-DERIVED METABOLITES TRAJECTORIES TO UNCOVER THE LINK BETWEEN GUT-MICROBIOTA DYSBIOSIS AND FRAILTY IN OLDER ADULTS

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Frailty is a geriatric syndrome characterized by a decline in physiological function and increased vulnerability to stressors. Although closely associated with aging, frailty differs between individuals of the same age [1]. Increasing evidence supports a link between frailty development and gut microbiota dysbiosis, wherein altered microbial composition and reduced diversity contribute to frailty progression through enhanced intestinal permeability and systemic low-grade inflammation [2]. This study aimed to build a comprehensive in-house database of microbial-derived metabolites to investigate their association with frailty. Monoisotopic masses of gut microbiota-derived metabolites were retrieved from the Human Metabolome Database (HMDB) and Microbial Metabolites Database (MiMeDB), yielding over 20,000 candidate compounds. After deduplication, the database included monoisotopic mass values, common adducts (+Na, +K), HMDB IDs, and molecular class annotations.

The database was applied to plasma metabolomic profiles of 942 older adults (726 non-frail, 216 frail), defined by the Frailty Index, from the Invecchiamento Cerebrale in Abbiategrasso (InveCe.Ab) cohort. Plasma metabolites were analyzed using flow-injection analysis high-resolution mass spectrometry (FIA-HRMS) and annotated with the EASY-FIA tool, combined with an in-house database [3]. Compound identities were confirmed using MS/MS fragmentation spectra from public databases or in silico predictions.

307 metabolites were annotated to be linked to microbial metabolism. Four of these differed significantly (Wilcoxon Mann-Whitney test) between frail and non-frail older adults. Frail individuals exhibited increased plasma levels of indoxyl sulfate, caproic acid, and 6-deoxy-6-sulfo-D-fructose, alongside decreased levels of rhamnose. The increase in metabolites associated with increased cardiovascular risk, bone disorders, and endothelial dysfunction, along with the decrease of potential beneficial metabolite in frail adults, highlights the critical role of altered microbial metabolism in frailty pathophysiology.

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# FROM TRANSCRIPTOME TO METABOLOME: UNCOVERING THE CELLULAR IMPACT OF G-QUADRUPLEX LIGANDS IN CANCER CELLS

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G-quadruplexes (G4s) are non-canonical DNA structures increasingly recognized as therapeutic targets in cancer. Although numerous ligands have been developed to stabilize G4s, the global cellular and metabolic consequences of this interaction remain largely unknown.

Here, we applied an integrated multi-omics approach, combining transcriptomics, proteomics, and NMR-based metabolomics, to characterize the biological effects of three representative G4 ligands (berberine, pyridostatin (PDS), and RHPS4) in human cervical adenocarcinoma (HeLa) cells.

Our results revealed that PDS profoundly reprograms cellular metabolism, suppressing glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle, leading to reduced ATP, NADPH, and glutathione levels. This energetic and redox collapse was accompanied by accumulation of amino acids such as glutamine and valine, suggesting adaptive anaplerotic responses. In contrast, RHPS4 selectively impaired mitochondrial bioenergetics, consistent with enhanced mitochondrial G4 formation, while berberine exerted minimal effects. Multi-omics data fusion confirmed a coordinated downregulation of key metabolic enzymes (PKM, IDH1, FASN, G6PD) and ribosomal proteins, linking G4 stabilization to both metabolic and translational suppression. Collectively, our multi-omics analysis unveiled the main cellular circuitries that turned out to be perturbed by the investigated G4 binders offering a multi-omics perspective on how G4-binding molecules elicit their anti-tumor activity and potentially guiding the design of more effective G4-directed therapies.

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# DERIVING THREE ONE-DIMENSIONAL NMR SPECTRA FROM A SINGLE SPECTRUM

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Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful tool for analyzing complex mixtures due to its ability to manage matrix complexity, provide detailed molecular insights, and preserve sample integrity. Various NMR experiments, such as NOESY, CPMG, diffusion-edited, and J-resolved spectroscopy (JRES), offer complementary insights into biofluids like serum and plasma. For instance, CPMG selectively detects small molecules, diffusion-edited emphasizes signals from macromolecules, and NOESY captures both, and JRES is particularly useful for signal assignment. However, acquiring multiple NMR spectra can be resource-intensive and time-consuming, especially for high-throughput studies.

Here, we present a simple and efficient strategy to computationally derive CPMG, diffusion-edited, and projected JRES (pJRES) spectra from a single NOESY acquisition using Partial Least Squares (PLS) regression. Serum samples were used as a case study. We used serum NMR data from a total of 1842 individuals enrolled from 18 recruitment centers. The $^{1}$H-NMR spectra for all samples were recorded using a Bruker 600 MHz spectrometer operating at 600.13 MHz. The dataset, comprising serum spectra of samples collected in 17 different recruitment centers, was divided into training (80%) and validation (20%) sets. Furthermore, the spectra of 232 samples from one independent recruitment center were used as the independent test set. Predictive models were created applying PLS regression with 1D NOESY spectra used as independent variables for the prediction of CPMG, diffusion-edited, and pJRES spectra, these latter used as dependent variables. Experimental and predicted spectra were compared in regions with signal intensities at least three times above the noise level. Evaluation metrics included the median relative error (MRE%), root mean square error (RMSE), coefficient of determination ($R^2$), and ratio of performance to deviation (RPD). In the independent test set, MRE% values of 6%, 4%, and 13% were achieved for predicted CPMG, diffusion-edited, and pJRES spectra, respectively.

# METABOLOMICS REVEALS METABOLIC ADAPTATIONS FOLLOWING GLUTAMINE METABOLISM IMPAIRMENT IN COLORECTAL CANCER CELLS

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Cancer cells rewire their metabolism to fulfil the high bio-energetic demand due to their high proliferative rate [1]. Some cancer cells become addicted to alternative bioenergetic sources, as glutamine (Gln) [2], including colorectal cancer (CRC) cells. In this scenario, metabolomics may represent a successful approach to investigate the metabolic alterations presented by cancer cells, to highlight potential therapeutic targets, and to investigate the possible mechanisms of resistance to antitumoral therapies. The present study aims to evaluate the metabolic effects induced by Gln deprivation or by the pharmacological inhibition of glutaminase-1 with CB-839, the first enzyme in Gln metabolism.

Three CRC cell lines (HCT116, HT29, and SW480) were deprived of glutamine or treated with different concentrations of CB-839 (2.5-20 $\mu$M). Cell viability was assessed by MTT assay (48 and 96 h). The metabolomic profile was explored with GC-MS and $^{1}$H-NMR with an untargeted approach, while targeted evaluation of Glutaminolysis and the Krebs'cycle was performed with GC-MS/MS analysis. Data underwent Multivariate and Univariate statistical analysis.

Gln deprivation induced a marked cell viability reduction in all the studied cell lines, while the drug CB-839 induced a cytotoxic effect predominantly in HT29 cells and a less pronounced cell viability decrease in HCT116 cells, while SW480 cells were resistant to the treatment. Metabolomic analysis showed a strong perturbation of the energetic pathways (glycolysis and TCA cycle), the amino acid pool (alanine, leucine, serine), and the antioxidant reserves, in the form of glutathione, following glutamine deprivation in all cell lines. Moreover, metabolomic investigation highlighted that CB-839 treatment induced alterations or adaptations in both the sensitive HT29 cells and the drug-resistant SW480 cells, impacting sugar content, especially glucose, the amino acid pool (alanine, aspartate, phenylalanine), GSH content, TCA cycle intermediates (succinic acid and fumarate), ATP levels, and GABA amount, which is used for TCA anaplerosis. Furthermore, metabolomic analysis also revealed differences between respondent and resistant CRC cells, as fructose, galactose, citric acid, NAD+, lysine, threonine, and leucine levels, suggesting interesting insights for exploring possible resistance mechanisms.

In conclusion, metabolomics offers important support to the research in the oncological field, allowing for the identification of potential therapeutic targets, promising drug combinations, and the investigation of possible mechanisms of resistance to antitumoral compounds.

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# BEYOND ISOMER SEPARATION: TRAPPED ION MOBILITY SPECTROMETRY (TIMS) BOOSTS ANNOTATION CONFIDENCE IN 4D-METABOLOMICS AND 4D-LIPIDOMICS

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Key challenges in high-throughput as well as low-input metabolomics are related to instrument robustness, selective identification and quantitation in complex matrix, and streamlined data processing.

In every single run, many chemically diverse compounds, whose concentrations can cover several orders of magnitude, must be selectively captured. Ideally, they are reliably identified and quantified despite the presence of interferences. In our contribution, we will demonstrate that trapped ion mobility spectrometry (TIMS), which adds a fourth dimension of selectivity to LC-MS/MS workflows, can provide increased profiling depth and annotation confidence beyond traditional (3D) omics approaches. Mobility-based ion sorting minimizes chemical interference in MS and MS/MS.

Cleaner, less chimeric data lends themselves to automated annotation and provide a basis for machine-learning approaches in data exploration. The synchronization of TIMS with downstream QTOF ion handling can be customized to build dedicated 4D methods enabling high coverage profiling of certain classes of biosamples, sensitive quantitation of individual compounds, or hybrid approaches.

With this contribution, we will introduce the recently launched timsMetabo™, a timsTOF platform designed to delivery excellent 4D performance for small molecule bioanalysis. The new system combines speed and robustness, essentials for high-throughput approaches, with the required sensitivity and flexibility for low-input metabolomics and lipidomics. 4D workflows are enabled by dedicated software tools for data processing and annotation. Specifically, the ion mobility-derived information is utilized by the MetaboScape® software package to identify metabolic features, increase annotation confidence and enable easy data review. In addition, we present tools for real-time quality control monitoring as well as system suitability testing for small molecules workflows.

# FROM PRIMARY TUMOR TO CIRCULATING TUMOR CELLS AND METASTASIS: TRACING METABOLIC REPROGRAMMING IN LUNG CANCER

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Lung cancer is the leading cause of cancer-related mortality worldwide, mainly due to its high incidence and late diagnosis. Circulating tumor cells (CTCs), which are released from primary tumors and can be detected in the bloodstream, play a central role in metastatic cascade, representing mobile mediators of metastatic spread from the primary tumor and distant metastases [1]. Despite their prognostic value, CTCs remain poorly characterized, particularly in terms of their metabolic properties. This work aims to investigate metabolic reprogramming, fundamental for tumor progression, survival, and invasion, that occur during the transition from primary tumor (PT) cells to CTCs and finally to metastatic cells (Met) in non-small cell lung cancer (NSCLC). To do this, cell lines PT, CTCs, and Met were isolated from patient-derived xenografts, expanded in vitro, and analyzed with mass spectrometry-based metabolomics/lipidomics.

The results revealed significantly metabolic reprogramming across these disease stages. During the transition from PT to CTC, we observed alterations in purine metabolism, glycolysis, branched-chain amino acid catabolism, and fatty acid β-oxidation, along with an increased recruitment of storage lipids by CTCs. Conversely, the transition from CTC to Met was characterized by a lipid metabolic switch, with increased levels of both storage and membrane lipids in Met, reflecting structural remodeling to boost invasive capacity.

Parallelly, isotope-tracing mass spectrometry experiments with $^{13}$C-glucose and $^{13}$C-glutamine were performed to further investigate the pathways involved in the transition. PT cells exhibited active glycolysis, moderate TCA cycle activity, and fatty acid mobilization. CTCs displayed a reduced TCA cycle and increased purine salvage pathway, increasing ATP levels through regeneration from ribose-5-phosphate and adenine. This indicates a rewiring of energy metabolism to sustain CTCs in bloodstream. In contrast, Met cells showed TCA cycle activity, and a metabolic shift toward alanine metabolism, which reflects in increased α-ketoglutarate levels and acetyl-CoA utilization, and therefore elevated β-oxidation.

Collectively, our results showed a distinctive metabolic profile of CTCs, highlighting dynamic adaptation in energy production, purine recycling and lipid metabolism, that may support CTC survival in circulation and in turn facilitate metastatic colonization, offering potential targets for therapeutic treatment in lung cancer.

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# INFLUENCE OF IMTA-RAS AND PROBIOTICS ON THE MOLECULAR PROFILE OF SOLEA SENEGALENSIS: A $^{1}$H-NMR METABOLOMICS APPROACH

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Aquaculture plays a pivotal role in addressing the global demand for seafood while fostering environmental sustainability and food security. This sector encompasses the controlled farming of finfish, molluscs, crustaceans, and other aquatic organisms, including algae, polychaetes, and cnidarians, contributing to the conservation of marine resources and reducing the pressure on wild stocks. Sustainable approaches such as recirculating aquaculture systems (RAS) and integrated multi-trophic aquaculture (IMTA) have been shown to minimize ecological impact and improve product quality [1,2]. This study investigates the metabolic effects of IMTA-RAS and probiotics on the flatfish Solea senegalensis. High-resolution $^{1}$H NMR-based metabolomics was employed to characterize the molecular profile of sole muscle across three experimental systems: (i) RAS, (ii) IMTA-RAS incorporating Ulva ohnoi for nutrient biofiltration, and (iii) IMTA-RAS enriched with Phaeobacter sp. 4UAC3, a probiotic bacterium known for its antagonistic activity against fish pathogen. Multivariate statistical analyses of NMR spectra revealed significant differences in metabolite concentrations among the three systems (Figure 1).

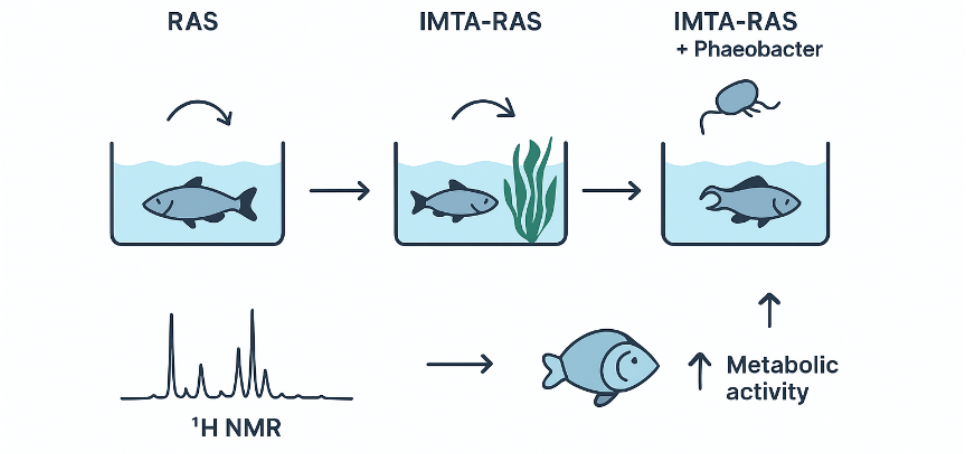


*Figure 1: Graphical abstract illustrating the experimental design and main findings of the study.*

The results indicate that IMTA-RAS, particularly when combined with Phaeobacter and Ulva, enhances energy metabolism, amino acid turnover, and overall metabolic activity in S. senegalensis. These findings suggest that integrating probiotic microflora and macroalgae within recirculating systems creates a more balanced and health-promoting environment, contributing to both fish welfare and the sustainability of aquaculture practices [3].

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# INTEGRATING NMR- AND MS-BASED APPROACHES TO CHARACTERIZE COFFEE MICRO-LOTS: ELEMENTAL, POLYPHENOLIC, AND METABOLOMIC FINGERPRINTS

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Coffee quality and authenticity depend on multiple interacting factors, including genetic background, environmental conditions, and post-harvest processing. Building upon previous NMR-based metabolomic investigations of Coffea arabica microlots from Nicaragua [1], we conducted a comprehensive multi-analytical characterization integrating 1H-Nuclear Magnetic Resonance ($^1$H-NMR), Inductively Coupled Plasma-Triple Quadrupole Mass Spectrometry (ICP-MS-TQ), Inductively Coupled Plasm-Optical Emission Spectroscopy (ICP-OES) and Ultra-High-Performance Liquid Chromatography coupled to High-Resolution Mass Spectrometry (U-HPLC-HRMS).

Eighteen representative green coffee samples from three Nicaraguan farms, each comprising three C. arabica varieties (Catuai Rojo, Bourbon, Maragogype) processed under two fermentation times (12 and 24 h), were analyzed within the framework of the METROFOOD-IT initiative. ICP-MS-TQ and ICP-OES analyses provided a detailed multi-elemental profile, including essential and potentially toxic elements, while UHPLC-HRMS enabled the quantification and identification of key polyphenols. Complementary $^1$H-NMR profiling on aqueous extracts afforded a holistic metabolic fingerprint of each microlot, allowing the integration of inorganic, phenolic, and metabolomic information.

Unsupervise and supervised classification approaches demonstrated that combining NMR and mass spectrometric features enhances discrimination of coffee varieties and farms of origin, even within a narrow geographical area. The joint use of NMR- and MS-based techniques revealed that both varietal and processing variables significantly modulate the chemical signature of coffee, with fermentation time impacting specific elemental and phenolic markers.

This integrative approach provides a robust analytical framework for coffee traceability, authenticity, and quality assessment, demonstrating how the combination of spectroscopic and mass spectrometric fingerprints can generate reproducible and comparable reference data to support coffee characterization and valorization.

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# METABOLOMICS OVERCOMES AGE BIAS IN NON-INVASIVE FIBROSIS STAGING: THE GP INDEX

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Metabolic-associated steatotic liver disease (MASLD) represents a major metabolic disorder in which fibrosis progression critically determines prognosis [1]. However, the current diagnostic standard, liver biopsy, is invasive and unsuitable for routine screening [2], while most non-invasive indices (such as FIB-4) are strongly age-dependent and may misclassify older patients [3]. We harnessed the potential of serum $^{1}$H-NMR metabolomics to uncover metabolic signatures of fibrosis and to design an age-indipendent , non-invasive diagnostic index. Spectra were acquired using the NOESYPR1D pulse sequence to ensure reliable quantification and water suppression, yielding 52 metabolites per sample with high analytical reproducibility [4].

After integration with inflammatory and hematological data (83 total variables), linear regression models were used to identify markers strongly correlated with liver fibrosis that were not influenced by age. Six fibrosis-associated metabolites emerged, primarily involved in amino-acid and short-chain fatty-acid metabolism. Among them, glutamine and propionate displayed the strongest correlation with fibrosis but not with age, forming the basis of a simple diagnostic ratio—the Glutamine–Propionate (GP) Index—able to discriminate mild from advanced fibrosis.

This metabolic signature reflects a shift in nitrogen and microbial co-metabolism pathways, highlighting the interplay between intrinsic hepatic metabolic changes and extrinsic influences from the gut microbiota. The GP Index demonstrates the potential of NMR-based metabolomics to deliver an age-indipendent, noninvasive tool to evaluate fibrosis with a superior predictive power compared to existing scores, like FIB-4 and FIB-3.

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# COMBINED TISSUE AND LIQUID BIOPSIES REVEAL THE METABOLIC INTERACTIONS AMONG BLOOD, LIVER, AND ADIPOSE TISSUE IN BARIATRIC SURGERY OF MORBID OBESITY.

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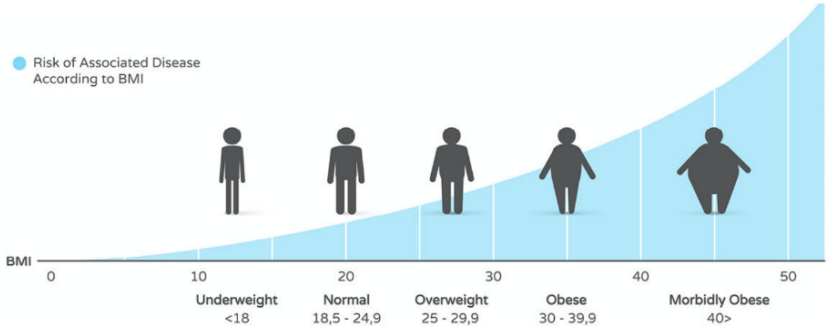
Class III obesity, also known as morbid obesity (MO), is marked by a substantial rise in body mass index (BMI), mainly caused by fat buildup. Individuals with Class III obesity have a BMI of 35 or higher. Recent data show that over 2 billion people globally are affected by obesity or overweight, representing about 30% of the world's population. Epidemiological studies indicate that obesity increases the risk of colorectal cancer (CRC), type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVD), and other chronic non-communicable diseases by 30–70%. Although BMI serves as a helpful tool for diagnosis, it does not account for the metabolic changes or health outcomes often associated with this condition. To address such a gap, we compared the metabolic profiles of serum, liver, and adipose tissue from twenty morbidly obese individuals before and after bariatric surgery by NMR. Although investigating a condition marked by excessive lipid accumulation through profiling polar metabolites may seem counterintuitive, it is important to note that fatty acid metabolism involves fundamental molecules such as acetate, butyrate, and citrate. These and more metabolites were profiled by NMR in an untargeted fashion, combining liquid (serum) and adipose tissue (visceral and subcutaneous). We identified dysregulation of several metabolites related to lipid metabolism, such as ketone bodies, citrate, SCFA, as well as biomarkers associated with the purine salvage pathway and protein methylation. This profile comparison enabled us to classify patients as responders and non-responders. Metabolic profiling of circulating and tissue-specific small molecules in individuals before and after bariatric surgery provided insights into the molecular differences underlying overall and regional metabolic dysregulation.

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# EXERCISE-INDUCED METABOLIC ADAPTATIONS DURING CARDIAC REHABILITATION: A LONGITUDINAL METABOLOMIC INVESTIGATION ON DBS

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Ischemic heart disease remains the leading cause of cardiovascular-related mortality worldwide. Despite the well-established benefits of cardiac rehabilitation (CR) through physical exercise after acute myocardial infarction (AMI), the underlying metabolic adaptations remain only partially understood. This study investigated the metabolic responses to CR in AMI patients using untargeted metabolomics and lipidomics on dried blood spots (DBS). Seventeen male patients recovering from a first AMI completed a three-week supervised rehabilitation program. DBS samples were collected before and after training sessions at three time points: first, mid-protocol, and final training. Untargeted analyses were performed using ultra-high performance liquid chromatography coupled with mass spectrometry to assess exercise-induced metabolic shifts. Throughout the CR protocol, progressive adaptation and increase in energy metabolism was observed, probably correlating with enhanced physical performance at the end of CR protocol. On the other hand, short-term lipid changes were detected at the beginning of the CR. Notably, phosphatidylserine (PS) levels increased significantly after the first training session. Since PS typically decreases following myocardial infarction, previous studies suggested that boosting PS (e.g., oral supplementation) may represent a cardioprotective strategy. Our results suggest that training enhances PS biosynthesis, possibly via activation of phosphatidylserine synthase 1 (PSS1), highlighting a potential role of CR in cardiac repair. Furthermore, N-acetyl-L-tyrosine (NAT) levels increased with rehabilitation time. Since NAT induces mitohormesis-an adaptive mitochondrial stress response-in animals, it may be linked to exercise-induced adaptations in humans. In summary, our findings show that CR induces significant metabolic and lipidomic changes in AMI patients, alongside measurable improvements in physical performance. PS and NAT emerged as promising candidate biomarkers for monitoring rehabilitation progress and may contribute to the benefits of CR on post-AMI recovery.

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# NMR BASED METABOLOMICS TO INVESTIGATE MOLECULAR MECHANISMS IN ADLD NEURODEGENERATIVE DISORDER

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Autosomal Dominant Leukodystrophy (ADLD) is an ultra-rare but underestimated leukodystrophy that occurs in adulthood [1]. This disorder is characterized by progressive degeneration of white matter due to duplication of the LMNB1 gene. Clinically, ADLD progresses slowly and presents with autonomic dysfunction early on, followed by pyramidal and cerebellar signs such as spasticity, tremor, and ataxia. Although affected individuals may survive for over twenty years post-onset, their quality of life is markedly compromised.

As this is an ultra-rare disease, research into reliable biomarkers and the underlying molecular mechanisms of ADLD remains limited. For the first time, we employed NMR-based metabolomics to analyze cerebrospinal fluid (CSF) and blood samples from two patients with confirmed LMNB1 duplication. Using the high-resolution magic angle spinning (HR-MAS) NMR spectroscopy, we present the first metabolic fingerprint of CSF. In this multi-fluid study, non-targeted NMR metabolomics revealed elevated levels of lactate in both CSF and plasma, indicating its potential as a key biomarker for ADLD. We observe, in comparison with CSF no ADLD samples, high level of lactate, together with low alanine levels. This suggests disturbances in energy and nitrogen metabolism within astrocytes. In particular, the absence of glutamate and gamma-aminobutyric acid (GABA), with only glutamine present, indicates possible disturbances in the glutamate/GABA-glutamine cycle, which is essential for the recycling of neurotransmitters by astrocytes. Furthermore, the absence of N-acetyl-L-aspartate (NAA) and low levels of myo-inositol suggest impaired myelin repair by oligodendrocytes. In conclusion, our metabolomic profiling of CSF offers promising non-invasive biomarkers that enhance understanding of the molecular pathology in ADLD. This approach underscores the potential of high-throughput metabolomics to inform diagnostic and therapeutic strategies for this rare disorder, which currently lacks effective treatment options.

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# SALIVA METABOLOMICS AS AN EMERGING TOOL FOR ADVANCED DIAGNOSTICS: THE LEUKOPLAKIA PARADIGM

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Saliva is a complex fluid produced by salivary glands and serves as a valuable source of information for both oral health and systemic conditions. Its non-invasive collection method has led to numerous studies aimed at identifying specific biomarkers linked to both pathological and physiological changes. While genomic and proteomic analyses of saliva have been employed for the early detection of oral diseases, metabolomic analysis is a more recent focus. It provides an overview of an organism's functional state, making it a powerful tool for biomarker identification and the discovery of new therapeutic targets.

Leukoplakia is characterized by white patches on the inner surfaces of the oral cavity and has the potential to progress to oral squamous cell carcinoma (OSCC), underscoring the need for effective screening and early diagnostic procedures. In our pilot study, we employed salivary metabolomics to derive potential biomarkers for leukoplakia, both with and without dysplasia, and compared the resulting profiles with preliminary data from patients diagnosed with OSCC.

Unstimulated saliva was collected from 26 patients with oral leukoplakia (13 with dysplasia and 13 without), 13 patients with OSCC, and 12 healthy subjects. $^{1}$H-NMR spectroscopy enabled the identification and quantification of 72 salivary metabolites. Univariate and multivariate statistical methods were applied to evaluate metabolite concentration profiles. The salivary metabolite profiles of patients with leukoplakia, both with and without dysplasia, showed distinct alterations compared to those of healthy subjects. These metabolic changes were particularly pronounced in cases of dysplastic lesions. Multivariate ROC curve analysis of selected metabolites revealed that the model with the highest diagnostic accuracy effectively distinguished between dysplastic leukoplakia and healthy cases. Additionally, leukoplakia-associated metabolic patterns were evaluated against preliminary data from patients with OSCC.

Our metabolomic approach, based on salivary profiling, revealed promising non-invasive biomarkers for the clinical diagnosis of leukoplakia. With validation in larger cohorts, these tools may enhance clinical monitoring and support precision diagnostics aimed at counteracting disease progression.

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# QUANTITATIVE AND QUALITATIVE ANALYSIS OF OXYLIPINS USING HIGHRESOLUTION MASS SPECTROMETRY

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*Sciex*

Oxylipins, bioactive lipid mediators derived from polyunsaturated fatty acids, play critical roles in inflammation, vascular function, and immune responses. Their low abundance and structural diversity present analytical challenges, particularly in complex biological matrices. This study demonstrates a robust workflow for the comprehensive profiling of oxylipins using high-resolution mass spectrometry (HRMS) on the SCIEX ZenoTOF 8600 system. The method combines high-throughput chromatographic separation with advanced MS/MS acquisition, leveraging Zeno trap-enabled electron-activated dissociation (EAD) to enhance sensitivity and structural elucidation. A targeted panel of hydroxy-, epoxy-, and dihydroxy-fatty acids was analyzed in human plasma, achieving low limits of detection and quantification. The approach enables simultaneous qualitative and quantitative analysis, facilitating the identification of isomeric species and supporting biomarker discovery in lipidomics. This workflow offers a powerful tool for researchers investigating the role of oxylipins in health and disease, with potential applications in clinical and nutritional studies.

# METABOLOMICS AND CYSTIC FIBROSIS DRUGS: EXPOSURE OF DAMS TO TEZACAFTOR DURING PREGNANCY AND BREASTFEEDING INDUCES MOLECULAR ALTERATIONS IN MICE BRAIN

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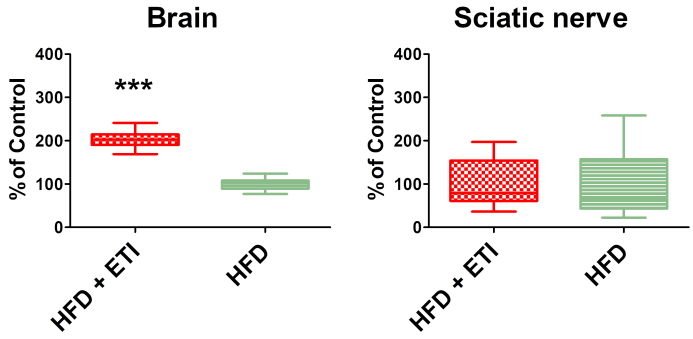
We demonstrated1 that Tezacaftor inhibits the enzyme (DEGS) that converts dihydroceramides (dHCer) into ceramides, thus producing accumulation of dHCer in cells and tissues. We are now conducting an in-vivo safety study, by administering this drug to mice during pregnancy and breastfeeding. The drug was incorporated as powder into mice food in high-fat diet regimen. Drug and dHCer levels in plasma and tissues, as well as changes in the global lipidome and proteome were measured by UPLC-MS. We here present the results observed in pups sacrificed 10 days after birth. We observed a significant accumulation of dHCer in the brains of pups born from drug-fed dams compared to controls. No accumulation was observed in the sciatic nerve, likely due to much lower levels of ETI compared to the brain:




*Dihydroceramides levels the brain (left) and sciatic nerve (right) of pups sacrificed at P10 for the ETI arm compared to the control arm. Data (as sum of all dHCer species) is reported as average ± SEM, N=18 and 15 for ETI and control groups respectively (*** p<0.001 two-tailed t-test).*

We also conducted an untargeted lipidomic survey, which revealed other alterations in lipid metabolism associated with exposure to the drug. We also show that this exposure translates into some physical and behavioral changes, although the animals treated with the drug recover at P28. Our data show that exposure to ETI during pregnancy and breastfeeding is associated with observable molecular changes in the brain lipidome of the pups, which are not likely limited to the inhibition of DEGS. This study is currently ongoing and further data on aging mice are being collected.

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# THE METABOLOMIC DYSFUNCTIONS OF SPINAL MUSCULAR ATROPHY: A JOURNEY FROM ANIMAL MODELS TO HUMAN CSF

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Spinal muscular atrophy (SMA) is an infantile neuromuscular disorder with a high mortality rate. It results from the degeneration of motor neurons caused by a homozygous deletion of the gene coding for the SMN (Survival Motor Neuron) protein. The condition develops when all functional copies of smn1 are lost, leaving the smn2 gene as the only source of functional protein [1]. Although the genetic basis of the pathology has been clarified, the metabolic dysregulations that trigger or worsen the pathophysiology of SMA are not well understood. Based on this evidence, this work aims to outline the neurometabolomic profile of SMA using different animal models: from the simplest, represented by Drosophila melanogaster, to the more complex mouse model SMNΔ7, and then compare these findings with human biofluid—most indicative of dysregulation in the pathology-namely cerebrospinal fluid (CSF) [3].

The exploration of such models was conducted employing an untargeted metabolomic methodology that integrated Nuclear Magnetic Resonance (NMR) with techniques such as high-performance liquid chromatography (HPLC), quantitative RT-PCR (qrt-PCR), Western blotting (WB), and immunohistochemistry (IHC) [2]. The comprehensive and transversal metabolomic approach adopted elucidated specific metabolic dysregulations involving neuromediators and agents of energy metabolism, which were corroborated across multiple systems, thereby providing significant insights into the pathophysiology of SMA, as well as its diagnostic and therapeutic implications.

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# UNRAVELLING URINARY METABOLIC ALTERATIONS INDUCED BY THIRDHAND SMOKE EXPOSURE: FROM UNTARGETED ANALYSIS IN ANIMAL MODELS TO TARGETED LC-MS VALIDATION IN CHILDREN

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Tobacco smoke exposure (TSE), including both second-hand (SHS) and thirdhand (THS) smoke, is responsible for over 7 million deaths annually and has a significant impact on the health of vulnerable populations such as children [1]. Thus, it remains a critical public health concern worldwide. SHS results from direct exposure and inhalation of burning tobacco products, whilst THS consists of persistent tobacco smoke residues on surfaces, clothing, and dust, that can react with environmental compounds to form harmful secondary pollutants [2,3,4]. While the harmful effects of SHS are well established, as well as the ubiquity of tobacco smoke, the metabolic and health effects of THS remain underexplored, particularly in young population [4].

In this work, we present a wide-scope, multiplatform approach aimed at broadening chemical coverage in the study of urinary metabolic alterations associated with THS exposure. Urine samples from mice exposed to THS-contaminated environments, mimicking human exposure conditions, were analysed by untargeted metabolomics using gas and liquid chromatography coupled to high-resolution mass spectrometry (GC-HRMS and LC-HRMS/MS) in positive and negative ionisation modes [5,6,7,8].

LC-HRMS/MS analysis revealed 1805 significantly altered metabolic features compared to controls (filters: intensity > 4000, analytical variability, mass error > 5 ppm, FDR-corrected p-value < 0.05, fold change > 1.5). By GC-HRMS, we detected 290 significantly altered compounds (filters: mass error < 5 ppm, spectral match > 85%, RI error < 1%). Across both techniques, we confidently annotated 77 dysregulated metabolites in exposed-mice (between levels 1 and 2 of confidence [9]), belonging to different chemical classes and associated with 17 metabolic pathways. The tryptophan metabolism pathway was particularly affected by THS, with 15 altered metabolites and increased levels of quinolinic acid/kynurenic acid ratios, suggesting THS-induced neurotoxicity, neuroinflammation and behavioural disorders. To further validate these findings and assess the health risks of TSE exposure in children, we developed a targeted LC-MS method to quantify 21 specific urinary biomarkers, including 16 neurotransmitters, 3 tobacco metabolites, and 2 oxidative stress biomarkers. We analysed the urine of 215 children aged 8 to 12 years, with varying levels of tobacco smoke exposure at home. ANOVA analysis revealed statistically significant differences in the levels of all 21 target metabolites between exposed and non-exposed children (SHS and THS) at home and those not exposed (FDR corrected p-values < 0.05). A one-way hierarchical clustering heatmap displayed 17 up-regulated compounds and 4 down-regulated (kynurenic acid, kynurenine, melatonin and xanthurenic acid) in children exposed to tobacco smole, suggesting TSE-induced metabolic dysregulations, particularly in pathways linked to neurological and hormonal function, and potential carcinogenic activation.

Our findings highlight the need for advanced policies and public health interventions advocating to minimise tobacco smoke exposure and protect children from long-term health effects.

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# 3BUILDING THE ECOSYSTEM FOR STANDARDIZED AND AUTOMATED NMR METABOLOMICS

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This talk will present the development of a comprehensive ecosystem for standardised and automated quantitation of metabolites by NMR, with a focus on delivering robust, reproducible, and fully exchangeable data across a range of biological samples. Leveraging the IVDr NMR platform at 600 MHz, we built the IVDr methods for 800 MHz, enabling standardized acquisition for plasma and urine—the main targets—as well as CSF, saliva, faeces, follicular fluid, and tissue samples using HR-MAS. Furthermore, the efforts to develop fully automated and validated absolute quantitation of metabolites in serum/plasma on a benchtop NMR system will be shared. The approach is complemented by software tools compatible with any field strength, streamlining metabolite quantitation workflows by NMR.

# Posters

# TARGETING THE PHOSPHATIDYLCHOLINE CYCLE IN TRIPLE-NEGATIVE BREAST CANCER: A $^{1}$H-NMR METABOLOMICS APPROACH

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Triple-negative breast cancer (TNBC) is an aggressive subtype characterized by pronounced chemoresistance and profound metabolic reprogramming [1-2]. A distinctive feature of TNBC is the dysregulation of phosphatidylcholine (PC) turnover, partly driven by the activity of PC-specific phospholipase C (PC-PLC) [3]. Through high-resolution $^{1}$H-NMR spectroscopy, we detected markedly elevated phosphocholine (PCho) levels in MDA-MB-231 cells relative to non-tumorigenic breast epithelial cells (HMEC), confirming aberrant activation of the PC cycle. To investigate the pharmacological modulation of this pathway, we assessed the effects of the PC-PLC inhibitor D609 and the antidiabetic agent metformin, which is under active investigation for anticancer applications and acts, in part, by inhibiting mitochondrial complex I (NADH dehydrogenase). NMR-based metabolomic analyses revealed that both compounds significantly impacted cellular energy metabolism, with metformin enhancing glycolytic flux. Notably, the combination with D609 produced a more pronounced antiproliferative effect and deeper alterations in energy-related metabolites, including ATP and ADP. These findings demonstrate that pharmacological targeting of the phosphatidylcholine metabolic pathway profoundly affects TNBC bioenergetics and metabolism and underscore the value of $^{1}$H-NMR spectroscopy as a powerful tool for elucidating drug-induced metabolic reprogramming in cancer cells.

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# METABOLOMIC FINGERPRINTS OF OPIOIDS: A COMMON SIGNATURE BEYOND CHEMICAL STRUCTURE

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New Psychoactive Substances (NPS) represent a complex and diverse group of drugs of abuse, designed as analogs of controlled compounds or synthesized de novo to mimic traditional illicit drugs [1]. Their continuous emergence on illegal markets, often in chemically uncharacterized forms, poses a growing challenge to conventional identification methods that rely heavily on prior structural knowledge [2].

This study adopts a metabolomics-based approach to investigate the systemic effects of four opioids - morphine, brorphine, etonitazene, and fentanyl - in CD-1 mice. Urine samples, collected after administration of equi-effective doses, were analyzed by $^{1}$H-NMR fingerprinting combined with multivariate statistical analysis to identify metabolic signatures reflecting the physiological response to opioid exposure. All compounds induce significant alterations in the urinary metabolic profile, most evident within the first 12 hours post-administration and still detectable at 24 hours. Brorphine exhibits the strongest perturbation, consistent with its pharmacokinetic profile. Importantly, a common metabolic perturbation pattern emerges across these structurally diverse opioids, suggesting potential functional biomarkers of opioid exposure. The alteration involves changes in creatine and 2-oxoglutarate levels, metabolites associated with cellular redox balance and energy metabolism. Despite limitations such as the animal model, limited sample size, and lack of definitive class-specific evidence, this study highlights the potential of metabolomics as a complementary tool in forensic and toxicological research, enabling early detection of novel opioids and reducing reliance on structure-based identification to improve regulatory responsiveness to emerging NPS.

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# URINARY METABOLOMICS OF MUSCLE INVASIVE BLADDER CANCER: A STEP TOWARD SAFER PROGNOSIS

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Muscle-invasive bladder cancer (MIBC) is a clinically aggressive malignancy associated with poor outcomes and high metastatic potential. Diagnosis currently depends on transurethral resection of the bladder tumor (TURBT), a procedure vital for histological confirmation and staging. However, TURBT is invasive and has been associated with complications such as tumor cell dissemination, which may adversely impact prognosis and therapeutic decisions [1,2]. These concerns emphasize the urgent need for reliable, non-invasive diagnostic tools for MIBC.

This study explored the urinary metabolic profiles of 139 bladder cancer patients from two clinical centers using NMR fingerprinting-based metabolomics to distinguish MIBC from non-muscle-invasive bladder cancer (NMIBC). Multiple linear regression on NMR binning data revealed consistent metabolic alterations, including increased lactate and decreased 3-indoxyl sulfate levels in MIBC patients. Moreover, a significant chemical shift variation was observed for creatinine between the two groups, without a corresponding change in concentration, suggesting its interactions with urinary components such as metal ions. This finding underscores the sensitivity of the NMR fingerprinting approach in capturing subtle spectral differences beyond concentration shifts. Finally, a predictive model was developed using NMR data from patients with known tumor staging and applied to 30 urine samples from patients awaiting histological diagnosis. The model's classifications were later compared to actual outcomes, achieving 80% accuracy. This highlights the potential of urinary metabolomics for non-invasive diagnosis while providing insights into the biochemical understanding of tumor progression and systemic responses.

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# $^{1}$H-NMR METABOLOMICS AND LIPIDOMICS PROFILING HIGHLIGHTS SIGNATURES OF CANCER HISTORY IN LYNCH SYNDROME CARRIERS

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The evaluation of metabolomics and lipidomics profiling in liquid biopsies is recently emerging as a powerful and reliable tool for the identification of novel biomarkers to improve early diagnosis and prognosis classification, as well as prediction of treatment benefit in cancer patients. In this context, we evaluated the metabolome and lipidome of a cohort of metastatic CRC (mCRC) patients undergoing liver resection after induction chemotherapy treatment and found two plasma metabolites and three lipid signals associated with favorable disease-free survival and overall survival [1]. More recently, a retrospective single-center study has been conducted involving patients with metastatic stage IV melanoma who received first-line treatment with immunotherapy evidencing a strong correlation between the pretreatment levels of some metabolites and the overall survival of these patients and supporting the potential of these molecules to predict outcomes and to define personalized management and treatment strategies [2].

In this work we decided to evaluate the metabolomics and lipidomics profiling by $^{1}$H-NMR approach in Lynch syndrome (LS) carriers subdivided on the basis of cancer history, because LS is one of the most prevalent hereditary diseases, characterized by a high risk to develop cancer.

We detected twelve metabolites and eight lipid signals able to distinguish LS carriers with cancer history compared to those cancer-free. Through correlation map analysis three clusters involving lipid signals and metabolites were identified as specific for LS carriers with cancer history. Furthermore, we highlighted the metabolites and lipid signals specifically associated to cancer history and comorbidity or lifestyle characteristics, such as smoking and body mass index.

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# DEVELOPMENT OF UNTARGETED METABOLOMICS AND LIPIDOMICS METHODS FOR THE ANALYTICAL CHARACTERIZATION OF EVS AND CONDITIONED MEDIUM DERIVED FROM DRUG SENSITIVE AND MULTIDRUG RESISTANT CELLS

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Extracellular vesicles (EVs) are membrane-bound particles secreted by all living cells, rich in bioactive compounds such as metabolites and lipids. They play an essential role in tumor development and multidrug-resistance (MDR) [1]. MDR leukemic and non-small cell lung cancer (NSCLC) cells differ from drug-sensitive counterparts in metabolism-associated proteins and their EVs can reprogram sensitive cells metabolism, promoting a resistant phenotype [2]. This study aims to provide an exploratory analysis of the metabolomic and lipidomic profile of EVs and conditioned medium (CM) from both drug-sensitive and MDR NSCLC cell lines. EVs were isolated from drug-sensitive and MDR cell lines by ultracentrifugation, then metabolites and lipids were extracted using a Bligh and Dyer method.

Metabolomics and lipidomics analyses based on ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS) were performed to characterize metabolic and lipid alterations. The analysis were conducted in both positive and negative ionization mode with data-dependent acquisition (DDA). Raw data obtained were processed to perform feature detection and annotation through open source softwares. Metabolomics profiling revealed both shared and unique metabolites across conditions, notably acyl-carnitines, vitamin-derived cofactors, tryptophan derivatives and redox-related molecules. Lipidomics analysis identified phosphatidylethanolamines, ceramides, sphingomyelins and triacylglycerols as major lipid classes. These preliminary data reveal distinct extracellular metabolic and lipid signatures associated with drug resistance in NSCLC, pointing to pathways linked to redox homeostasis, fatty acid metabolism and membrane lipid remodeling. Overall, this work represents a preliminar exploration of the extracellular metabolome and lipidome of the two cell lines, providing a basis for future targeted and quantitative analyses to clarify the contribution of EVs to drug resistance in NSCLC.

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# TOOLS FOR THE ASSESSMENT OF THE RESPONSE OF OVARIAN CANCER CELLS TO GOLD CYTOTOXIC COMPOUNDS

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Gold compounds are increasingly studied as potential anticancer agents because of their structural diversity and promising biological activity. This project aims to investigate how different gold compounds affect the metabolism of ovarian cancer cells. Four gold compounds were selected for this study: AF, AuTM, and two gold(I) carbenes (CN1 = Au(NHC)Cl and CN2 = $[Au(NHC)_2]PF_6$).

The human ovarian cancer cell lines A2780 and SKOV-3 were treated at their respective $IC_{50}$ concentrations, and 1H NMR metabolomics was performed on cell lysates and culture media after 24 h and 48 h. Metabolites assignment and quantification were followed by the development of computational approaches aimed to explore possible relationships between chemical structure and biological response, despite the different basal metabolism of the two cell lines [1]. The proposed approach may have broader applicability to the screening of metal-based drug candidate libraries, which is always complicated by their multitarget nature, and could support the comprehensive interpretation of their metabolic actions.

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# NMR-BASED METABOLOMIC INSIGHTS INTO IL-4/IL-13/JAK INHIBITOR RESISTANCE IN ATOPIC DERMATITIS

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Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by epidermal barrier dysfunction, immune dysregulation and Staphylococcus aureus overgrowth. Despite the efficacy of new biological therapies, such as IL-4/IL-13 inhibitors [1] and Janus Kinase (JAK) inhibitors [2], a subset of patients fails to respond or experiences adverse effects, highlighting the need for predictive biomarkers to guide personalized treatment strategies. Metabolomics is a valuable tool to discover molecular alterations underlying this disease by examining small molecules and revealing changes in metabolic pathways and biomarkers. This study aims to deepen molecular insights on AD in mediating resistance to IL-4/IL-13 and JAK inhibitors in patients, analyzing metabolic differences in serum and cell free supernatant samples. We enrolled 25 adults with severe AD (EASI $\geq$ 24) who received either dupilumab (anti–IL-4 receptor $\alpha$ antagonist) or upadacitinib (selective JAK1 inhibitor). Serum samples were collected at baseline, at 8 weeks, and at 16 weeks of treatment. Skin swabs from affected areas (dry, moist, and sebaceous sites) and non-lesional areas were obtained at the three time points. The isolated bacteria were cultured, and their supernatants were analyzed. Nuclear Magnetic Resonance (NMR) spectroscopy combined with statistical analysis was employed to identify metabolomic differences. Univariate analysis identified a panel of key metabolites that significantly varied in samples of patients among the three timepoints. These findings highlight metabolic markers as potential predictors of treatment response in atopic dermatitis, supporting their use to guide personalized therapies and improve clinical outcomes.

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# ANTARCTIC CRYPTOENDOLITHIC COMMUNITIES: ANALYTICAL METHOD OPTIMISATION FOR UNTARGETED METABOLOMICS AND LIPIDOMICS PROFILING

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Antarctic cryptoendolithic communities are microbial assemblages living in the air-spaces of porous rocks that have evolved cryptoendolithism as a survival strategy to persist under different extreme conditions. They are among the most stress-resistant organisms known to date and the best terrestrial analogues for studying the possibility of putative life on Mars. This work constitutes part of the CRYPTOMARS project whose purpose is to understand what set of characteristics would enable a community to resist, adapt and sustain itself on Mars or Mars-like planets. With this aim, we intend to explore both their characteristics and their responses with an untargeted metabolomic and lipidomic study after exposing them to selected stresses that characterize the martian environment in a simulation chamber.

As a starting point, we have focused on adapting the method of sample preparation for untargeted analysis on a pilot experiment of samples similar to those selected for the project. In particular, two different sample extraction methods have been compared: (1) an adaptation of the method previously used for this type of samples [1]; and (2) an optimized double extraction method for the extraction of polar metabolites and lipids. A total of 6 samples from two opposite sun exposure sites (North and South), with an expected different colonization degree, were available for this setup study and they were prepared in duplicate. After metabolic reactivation, the samples were extracted and analyzed by the untargeted metabolomics protocol previously described [2] with slight modifications and by the recently described lipidomics workflow [3].

Among annotated compounds, main metabolites observed in the analyzed samples belong to different chemical classes such as sugars, amino acids, small peptides, organic acids and polyphenols; whereas main lipid classes observed belong to phospholipids, galactolipids and glycerolipids. The optimized double extraction method allowed a better separation of compounds according to polarity. For metabolomics analyses both phases (i.e. aqueous and organic) should be analyzed since polyphenol aglycones tend to dissolve in the organic phase. Samples from the North surface presented higher abundances of identified compounds than samples from South surfaces. Despite the differences observed in the efficiency of compound extraction between the two tested protocols, the overall data from the sun exposure comparison did not show relevant differences based on the sample preparation method used. In other words, both extraction methods revealed the same trend.

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# METABOLOMICS-DRIVEN BIOCHEMOMETRIC PROFILING OF GUIERA SENEGALENSIS LEAVES UNCOVERS ANTIBACTERIAL NAPHTHYL DERIVATIVES

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Plant-derived natural products, owing to their intrinsic chemical diversity, represent a promising avenue to address multidrug resistance (MDR), one of the most pressing emerging threats to global public health [3]. *Staphylococcus epidermidis* is an MDR pathogen and a major contributor to hospital-acquired infections, particularly due to its ability to form biofilms, complicating the treatment of device-associated infections and sepsis, thus requiring advanced diagnostics and therapies to mitigate its impact in healthcare settings [1].

In the omics era, state-of-the-art analytical platforms are essential for revitalizing ethnopharmacological traditions to address urgent challenges by accelerating natural products drug discovery pipeline. In this study, we employed a combined approach integrating untargeted LC-HRMS and Molecular-Networking-based metabolomics and NMR-biochemometrics to investigate *Guiera senegalensis* leaves, traditionally used across African countries to treat infectious and chronic diseases [2]. This methodology enabled both the comprehensive snapshot of *G. senegalensis* leaves metabolome and the identification of key NMR features associated with the anti-bacterial activity of the extract. NMR-fingerprint driven the chromatographic purification allowing the isolation of 7 naphthyl derivatives, including the new naphthyl-butenone guieranone C, and two unprecedented naphtho-$\gamma$-pirones, namely guierapyrone A and B. Isolated metabolites were tested for their anti-bacterial activity against *S. epidermidis* and oxacillin resistant *S. epidermidis* unveiling guieranone A as a good candidate for fighting staphylococcal infections.

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# TACKLING BIOTRANSFORMATIONS IN THE EXTREMELY RARE METABOLIC CONDITION ALKAPTONURIA USING NUCLEAR MAGNETIC RESONANCE

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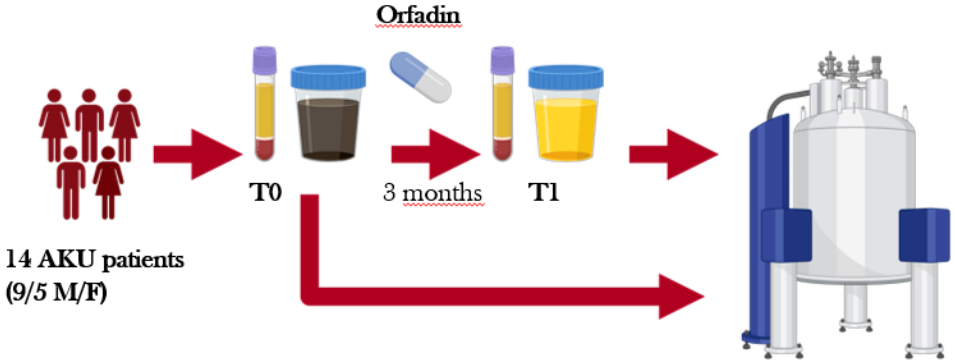
**Introduction:** In the early 19th century, Dr. Archibald Garrod coined the term ”inborn error of metabolism” to describe Alkaptonuria (AKU), an ultra-rare condition with an incidence of 1 in 1’000’000 also known as ”black bone disease.” Garrod rightly linked the discolouration of connective tissue (ochronotic pigment) to a phenolic compound (alkapton) buildup due to a missing enzyme. Nowadays, we know that alkaptonuria (AKU) is an autosomal recessive disorder caused by mutations in the HGD gene, leading to a deficiency of homogentisate 1,2-dioxygenase. This results in the accumulation of homogentisic acid (HGA), which is excreted in the urine and deposited in tissues, causing ochronosis, joint degeneration, valvular heart disease, and the rupture of ligaments, muscles, and tendons. The metabolic defect occurs in the tyrosine catabolic pathway, where HGA accumulates due to a block in the enzyme-mediated breakdown. nitisinone, a drug used to treat hereditary tyrosinemia, has emerged as a treatment for AKU, leading to the approval of Orfadin®for adult AKU patients. By inhibiting 4-hydroxyphenylpyruvate dioxygenase (HPPD), nitisinone reduces the production of HGA, thereby improving symptoms and preventing further metabolic damage. However, nitisinone treatment elevates plasma tyrosine levels, mirroring tyrosinemia type 2 and potentially causing eye damage. This highlights an urgent need for alternative or adjuvant treatments that can reduce HGA without triggering tyrosinemia.

**Aim:** Assess the impact of nitisinone on the tyrosine metabolism before and after treatment, analysing serum and urine samples of alkaptonuria patients via NMR spectroscopy.

**Method:** Serum and urine samples of 14 AKU patients were collected before and after nitisinone treatment. The samples were prepared and analysed through NMR spectroscopy; 15 principal metabolites of the tyrosine pathway were identified and quantified for both serum and urine samples.

**Results:** As predicted, 4-hydroxyphenylpyruvate and tyrosine concentrations increase upon nitisinone administration. Additionally, 4-hydroxyphenillactate, 4-hydroxyphenilacetate and, partially, tyramine also increase in urine samples, suggesting a good metabolic clearance for these compounds. NMR has emerged as a valuable tool for monitoring the metabolic impact of nitisinone administration, with potential applications in other disorders related to tyrosine catabolism.




*Study design for the metabolic analysis of AKU patients treated with Orfadin®*

# METABOLIC PROFILING AND SKIN PENETRATION STUDY OF SERICOSIDE USING HR-MAS NMR AND HPLC-MS TECHNIQUES

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Sericoside is a naturally occurring bioactive compound derived from Terminalia sericea, a medicinal plant known for its rich array of bioactive components such as saponins, flavonoids, tannins, and triterpenic acids. These constituents provide antioxidant, antimicrobial, and skin-rejuvenating effects. Sericoside is important for enhancing skin health and maintaining the integrity of connective tissues, highlighting its potential applications in dermatology and anti-aging treatments [1]. The objective of this thesis is to investigate the potential of Sericoside conducting in vitro experiments focused on assessing the cytocompatibility and regenerative potential of Sericoside in SH-SY5Y neuroblast-like cells. To evaluate the Sericoside metabolic activity ex vivo analysis on cells using HR-MAS NMR spectroscopy, was performed. This methodology enabled us to compare cellular activity and metabolic alterations effectively. Preliminary findings indicated notable changes in choline-containing compounds—crucial for membrane biosynthesis—correlated with cell regeneration outcomes measured via scratch tests. These observations suggest a relationship between membrane metabolism and regenerative responses modulated by sericoside, offering valuable insights into its biological effects. In addition, an ex vivo transdermal delivery study was performed using Franz diffusion cells to evaluate the skin penetration of Sericoside. The amount of compound absorbed into the skin layers and receptor fluid was quantified employing HPLC-MS analysis, offering detailed insights into its percutaneous absorption profile.

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# NMR-BASED METABOLOMIC APPROACH IN SEPSIS-INDUCED ACUTE KIDNEY INJURY

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Sepsis-induced acute kidney injury (SI-AKI) is a frequent complication in critically ill patients, associated with high mortality [1]. The lack of specific biomarkers hampers the early recognition of SI-AKI, which is essential to provide supportive treatment and prevent further organ damage [2]. NMR-based metabolomics is a powerful approach for identifying specific metabolic fingerprints of kidney diseases [3]. Here, we studied 89 urine samples of patients with sepsis to identify urinary metabolomic profiles for the early diagnosis and prevention of SI-AKI. Preliminary statistical analyses showed promising correlations between clinical variability and metabolic alterations. Specifically, multiple factor analysis (MFA) highlighted a distinctive metabolic signature in a group of patients with severe renal and metabolic impairment. These analyses lay the foundation for future investigations aimed at improving diagnostic and therapeutic strategies for patients with sepsis-induced AKI and septic shock.

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# INTEGRATIVE METABOLOMIC AND FLUXOMIC PROFILING REVEALS METABOLIC SHIFTS IN CANCER AND IMMUNE CELLS FOR REFINING PERSONALIZED THERAPEUTIC STRATEGIES AND IDENTIFYING CIRCULATING BIOMARKERS

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Despite the significant progress achieved through chemotherapy and emerging therapeutic approaches, including molecularly targeted drugs and immunotherapy, the prognosis of many cancers, especially in advanced stages, remains unfavorable [1]. These considerations highlight the need for early disease diagnosis and the development of new therapeutic strategies. In this scenario, the identification of circulating biomarkers from biological samples could allow for an early diagnosis and for patient stratification to the therapy. Moreover, the techniques used for the analysis of circulating biomarkers stand out for their lower complexity, invasiveness, and cost [2]. Recently, cellular metabolism has been reported as new relevant hallmark of cancer [3]. Unlike normal cells, which primarily rely on oxidative phosphorylation for energy production, tumor cells exhibit increased glucose consumption and lactate production through aerobic glycolysis. These metabolic features contribute to tumor progression and therapy resistance. Moreover, lactate secretion leads to acidification of the tumor microenvironment, promoting tumor growth, migration, and invasion [4–6]. In this study, we aim to understand the metabolic change underlined to anticancer therapy-resistance, with the ultimate goal of designing new combination therapeutic strategies to treat cancer patients. Then, considering that mitochondrial dysfunction could affect differently several types of cells, we lastly aim to identify metabolic shift in different immune cell populations, in order to propose mitochondrial function as potential biomarker [7–11]. To address our first aim, we took advantage of established cell lines, sensitive or resistant to different anti-cancer therapy, as well as of more complex cancer model as organoids. In detail, the day after seeding the cells or organoids, we performed a dedicated assay to monitor mitochondrial respiration. By using Extracellular Flux Analyzer, we measured oxidative phosphorylation as oxygen consumption rate (OCR) and the glycolysis as extracellular acidification rate (ECAR). Our data showed that progression from an earlier stage of cancer to a more advanced one or from sensitive to resistant cells, induced a basal metabolic shift towards energy-demanding and mitochondria-driven metabolism, as evidenced by differential levels of both OCR and ECAR. Similarly, in 3D cultures, which more accurately recapitulate the tumor microenvironment, both OCR and ECAR values were lower than those observed in 2D cultures. Furthermore, in resistant models, mitochondrial dysfunction was associated to change in endo-metabolites levels, as ATP, lactate and glucose measured by NMR approach. Intriguingly, we observed that, after treatment with appropriate drugs, both sensitive and resistant cells showed lower levels of OCR and ECAR compared to untreated controls. Fluxomics and metabolomic analyses unveil a metabolic shift in drug-resistant tumor cells. Finally, with the aim to identify circulating biomarkers from biological samples and to define metabolic changes in immune cells, we are conducting

a comprehensive metabolic analysis on total PBMCs, T and B cells from healthy donors and patients admitted to intensive care units of Sant' Andrea Hospital. Specifically, PBMCs isolated were sorted, using a gating strategy by Cell Sorter, to obtain T and B cells. Then, all populations were subjected to mitochondrial respiration analysis to assess their metabolic profiles. Preliminary results showed that total PBMCs, T cells, and B cells from patients exhibited reduced ECAR levels compared to healthy donors, indicating decreased glycolytic activity across all populations. Additionally, OCR levels were reduced, particularly in total PBMCs and T cells, suggesting impaired mitochondrial respiration. Interestingly, PBMCs derived from blood sample collected from metastatic colorectal cancer patients, enrolled within a phase 3 study active at IRCCS Fondazione Pascale showed lower OCR and ECAR values compared to healthy donors, corroborating in vitro findings. Overall, our findings suggest that integrating fluxomic approaches enhances the understanding of cancer metabolism, facilitating the identification of predictive and prognostic metabolic biomarkers that may refine personalized therapeutic strategies.

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# UNCOVERING METABOLIC RESPONSES TO FLAVESCENCE DORÉE PHYTOPLASMA INFECTION IN GRAPEVINE LEAVES USING AN UNTARGETED IMAGING APPROACH

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Flavescence dorée (FD) is a serious grapevine disease widespread in Europe, especially in Italy in recent decades [2]. The disease is caused by the so-far-undescribed '*Candidatus* Phytoplasma vitis' and transmitted by the leafhopper insect vector *Scaphoideus titanus* [1]. FD represents a major threat to viticulture worldwide, leading to significant economic losses due to reduced grape yield and quality. For instance, in 2005, Italy allocated €34 million to refund affected growers. Given its economic impact and ongoing spread, there is an unmet need for early detection methods and effective strategies to mitigate phytoplasma infections [3]. The project aims to investigate the metabolic mechanism underlying phytoplasma infection and to identify early candidate diagnostic biomarkers for FD that can differentiate non-infected from infected grapevines through an untargeted mass spectrometry imaging (MSI) approach. Grafted cuttings *Vitis vinifera* plants (cultivar Barbera) were exposed to FD-infected insect vectors, sampled 3 months after, at symptom appearance. Unexposed plants were used as controls. Leaves from control, exposed and symptomatic, as well as exposed and asymptomatic grapevines were analyzed, sampling the upper and lower surfaces, with four biological replicates for each experimental group.

Samples were coated with the $\alpha$-Cyano-4-hydroxycinnamic acid (CHCA) matrix using a SunCollect Sprayer (SunChrom) and analyzed with a high-resolution mass spectrometer (Orbitrap Q-Exactive - Thermo Scientific) coupled with an AP-MALDI (MassTech) source. Data were processed with MSiReader Pro (MassTech) and matched to reference databases for metabolite annotation. Preliminary analyses of non-infected leaves using two databases (HMDB and PMHub 1.0) enabled acquisition of the grapevine leaf's fingerprint and supported the creation of an in-house plant metabolism database. Final leaf data from non-exposed, symptomatic, and asymptomatic exposed plants were analyzed and about 5100 features present in at least three of four replicates were identified. Given the high number of features, it is necessary to prioritize them in order to proceed with annotation [4]. Statistical comparison between the upper and lower leaves for each experimental group revealed that 189, 193, and 5 features were significant for non-exposed, asymptomatic, and symptomatic conditions, respectively. Further statistical analysis will identify significant features across the different experimental groups. The features obtained will subsequently be annotated with MSi Reader Pro software using the previously created in-house database. Future experiments will employ LC-MS/MS analysis to confirm and ensure the accurate identification of annotated metabolites. This strategy could significantly advance and facilitate rapid and early phytoplasma detection. Furthermore, this approach holds potential for broader application to other plant–pathogen interactions and pave the way for improved and sustainable approaches to disease control.

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# UNVEILING THE IMPACT OF EMERGING POLLUTANTS ON LUNG EPITHELIAL CELL LINES VIA METABOLOMICS MASS SPECTROMETRY

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Cell metabolomics represents a powerful approach for exploring cellular responses to environmental or chemical stressors. In this study, we applied an HPLC-HRMS-based metabolomic analysis to investigate metabolic alterations in normal (BEAS-2B) and oncogenic (BEAS-2B KRAS G12C) human lung epithelial cell lines exposed to contaminants of emerging concern (CECs). To assess acute toxicity, cells were incubated with two fluoroquinolone antibiotics, ofloxacin and ciprofloxacin, and their transformation products (TPs) generated by $TiO_2$-mediated heterogeneous photocatalysis. Cell viability assays revealed increased mortality following exposure to TPs (Figure 1).

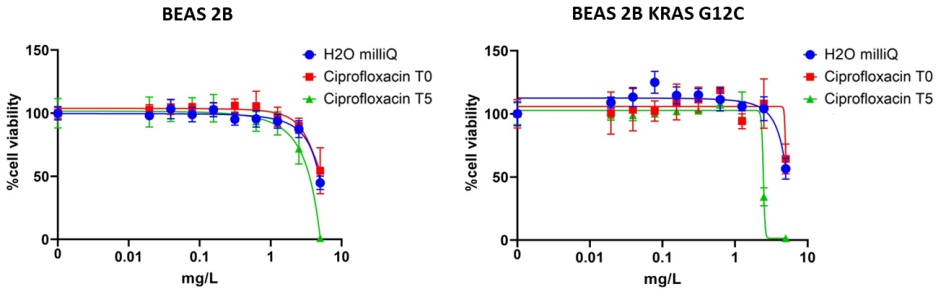


*Figure 1: Cell viability results of ciprofloxacin TPs treatment on BEAS B2 and BEA 2B KRAS G12C*

Metabolomic profiling was performed using an HPLC-HRMS method using ODC and HILIC columns for chromatographic separation and high-resolution mass spectrometric detection operated in data dependent analysis aquisition mode with a resolving power of 60k. A total of 141 and 39 metabolites were annotated [1] in positive and negative ionization modes, respectively. Comparison of metabolite profiles across five exposure conditions (1. ciprofloxacin; 2. photocatalyzed ciprofloxacin; 3. water; 4. TiO2 filtrate; and 5. untreated control; same conditions for ofloxacin) led to the identification of several altered metabolites. Among them, guanidinosuccinic acid (GSA) was significantly overexpressed in TPs-exposed cells. This observation aligns with literature reports [2] describing GSA as a derivative of arginine metabolism capable of inducing oxidative damage, potentially resulting in cellular apoptosis.

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# A HIGH-RESOLUTION MASS SPECTROMETRY APPROACH TO PROMOTE SORGHUM BICOLOR BIOMASS VALORISATION

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*Sorghum (L. Moench)* subsp. *bicolor* (hereafter *S. bicolor*) is an edible grain species native to Africa widely cultivated worldwide due to its adaptability to difficult environment and diverse secondary metabolism, that make it a valuable resource for food, feed and biomass re-use [1]. Despite its potential, a great part of S. bicolor biomass still remains largely undervalued and is often treated as waste. Recent growing interest in its nutritional value and possible health benefits has further highlighted its high potential but the understanding of how environmental conditions influence its metabolic composition is still not complete. Therefore, this study aims to develop a robust mass-spectrometry method for the analysis of the impact of three among the most relevant environmental factors triggering *S. bicolor* develop and growth, the species secondary metabolism, through a comprehensive metabolomic analysis.

Twelve pots were prepared according to a fully cross-cutting experimental design to assess the effects of three considered environmental factors which were water availability, soil enrichment with phosphorous ($Ca(H_2PO_4)_2$) and growth-promotion, obtained using an *ad-hoc* consortium of fungi and bacteria (Micosat F 002). Endogenous metabolites in the leaves of the plants were identified using liquid chromatography (Agilent Technologies, 1200 Series) coupled to high-resolution mass spectrometry (Orbitrap Q-Exactive, Thermo Scientific), operated in both positive and negative heated electrospray ionization (HESI) mode. Analyses were run at four time points to monitor the metabolite production during plant growth phases. Our work starts from a workflow previously applied to study *Lepidium sativum* (cress) metabolism that was adapted and further optimized for the untargeted identification of Sorghum bicolor metabolites.

A linear mixed model was fitted to the longitudinal data to account for repeated measurements and to assess statistical significance across different conditions and time points. Preliminary analysis revealed an extended panel of metabolites belonging from ammino acids, lipids, carbohydrates, phenols and carotenoids classes. These preliminary findings also demonstrated the enhanced efficiency and reliability of this workflow in plant metabolomics. The analysis should highlight key metabolic shifts, providing valuable insights into strategies for enhancing metabolite synthesis. Overall, these findings contribute to the valorisation of underexploited plant resources [2]. Within the framework of the circular economy, valorising plant biomass is essential to minimize waste production, promote environmental sustainability, and obtain high-valuable bioactive compounds. Overall, this study establishes a basis for the metabolic characterization of *S. bicolor* under different environmental conditions and supports its potential for future valorisation.

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# ADVANCING GRAFT EVALUATION: INTEGRATING METABOLOMICS INTO PROGNOSTIC MODELS FOR HEART TRANSPLANTATION

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**Introduction:** Primary Graft Disfunction (PGD) is the most significant cause of early morbidity and mortality after heart transplant, with a reported incidence of 28.2% [4]. Additional complications arise from the use of marginal grafts, obtained by donation after circulatory death (DCD).

**Aim:** This study aimed to integrate tissue metabolomics in the multimodal procedure for graft selection in heart transplantation. The objective was to identify new prognostic markers of PGD.

**Methods:** Endomyocardial biopsies (EMBs) were collected from 21 grafts immediately after organ procurement (T0) and immediately before organ implantation (T1). The myocardial metabolic profile was assessed combining the conventional solution NMR metabolomics workflow [1] with ASICS R-package for automated metabolite identification and quantification [2] Generalized linear models (MetaboAnalyst 6.0 [3]) were applied to assess metabolomic associations with PGD, adjusting for donor covariates.

**Results and conclusions:** Approximately 80 polar metabolites were estimated with ASICS from the 1D-1H NMR spectra of EMBs extracts. T0 up-regulation of malate and pyruvate and T0/T1 down-regulation of aspartate were associated with grafts developing severe PGD. These alterations are consistent with a pre-transplant unbalance of TCA cycle and oxidative phosphorylation in some organs, despite donor selection strictly adhering to international guidelines. Donor cause of death and massive use of inotropic support prior to graft procurement affect the metabolic profile.

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# BETTER TOGETHER: NMR SPECTROSCOPY AND MASS SPECTROMETRY FOR THE METABOLIC PROFILING OF SOLID MATRICES

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Due to highly dynamic concentration ranges, extensive chemical diversity and different physical properties, the metabolic characterization of complex samples may represent a true analytical challenge [1]. In this context, sophisticated analytical techniques, such as Nuclear Magnetic Resonance (NMR) spectroscopy and Liquid Chromatography coupled to High Resolution Mass Spectrometry (LC-HRMS), are considered the "gold standard" for the comprehensive characterization of sample mixtures. In particular, their combination represents a powerful tool to expand metabolic coverage and also to facilitate compound identification and quantification [1,2,3].

When working with complex matrices, such as whole cells, biopsies, or food flours, NMR- and LC-MS-based metabolic profiling is usually carried out at the liquid state after metabolite extraction. Besides requiring time-consuming sample manipulation, also relative and absolute quantification may be influenced by diversified extraction efficiencies. To tackle this problem, the application of NMR analysis under High-Resolution Magic Angle Spinning (HR-MAS) conditions and HRMS employing the Atmospheric Solids Analysis Probe (ASAP) may be considered, since they allow the direct analysis of semi-solid and solid samples with minimal to none sample preparation [4,5]. Here, we report the application of both techniques to the analysis of metabolites contained in five different food flour samples (rice, quinoa, chickpeas, faba beans, and lentils). Qualitative and quantitative data collected by extraction and analysis at the liquid state were compared with those obtained under HR-MAS NMR and ASAP HR-MS conditions. Once validated, this direct approach will be very useful for rapid anti-fraud checks of foodstuffs; also, it will increase the versatility and speed of NMR and MS analysis in resolving complex mixtures of organic compounds in a wide range of applications.

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# METABOLOMIC APPROACH TO EVALUATE THE PROTECTIVE EFFECT OF NGF IN AD CELLULAR MODEL

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Nerve Growth Factor (NGF) is one of the principal neurotrophic factors, regulating neuronal viability, plasticity, and growth. Multiple lines of evidence demonstrate a strong link between NGF and the cholinergic system in the hippocampus, and alterations in the brain's NGF metabolic pathway have been reported in Alzheimer's disease (AD). Based on these premises, the neuroprotective and regenerative effects of NGF on cholinergic neurons have been extensively investigated, yielding positive outcomes in both animal models of AD and clinical studies. Furthermore, some findings support the hypothesis that NGF may act as a suppressor of A$\beta$-induced neurotoxicity [1,2,3].

Although NGF's role in AD is well established, the precise molecular mechanisms underlying its neuroprotective effects remain only partially understood. In this context, elucidating the mechanisms behind NGF's beneficial actions in AD is particularly relevant—not only to better understand its therapeutic potential, but also to clarify why NGF-related alterations occur in the AD brain. To this end, we employed a pharmacometabolomic approach to investigate the effects of NGF on SH-SY5Y cells in the presence of amyloid-$\beta$ peptide, used as a cellular model of AD. Metabolomics, especially when applied in an untargeted manner, offers a powerful tool to uncover unexpected biological responses [4,5]. Based on this premise, we consider this technique highly suitable for comprehensively assessing the global impact of NGF.

In this study, we demonstrated that NGF can protect SH-SY5Y cells from A$\beta(1-42)$-induced toxicity when cells are pretreated for 24 hours. To elucidate the site and nature of NGF's action, we compared the metabolic profiles of SH-SY5Y cells treated with NGF and A$\beta(1-42)$ to those exposed only to A$\beta(1-42)$ and to untreated control cells. This allowed us to evaluate NGF's ability to counteract amyloid-induced metabolic dysregulation. Our analysis demonstrates that NGF's neuroprotective effect is mediated through the modulation of several key biochemical pathways, including phospholipid biosynthesis, bioenergetic processes, and neurotransmitter dynamics.

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# THE METABOLOMIC PROFILE OF PSORIATIC ARTHRITIS PATIENTS UNVEILS THE UNBALANCE OF DISEASE-RELATED MOLECULES AND PATHWAYS

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Psoriatic arthritis (PsA) is a chronic, immune-mediated inflammatory disease characterized by marked clinical heterogeneity, stemming from the variable involvement of multiple disease domains, including cutaneous and nail psoriasis, peripheral arthritis, enthesitis, dactylitis, axial involvement (spondylitis), and sacroiliitis [1]. PsA affects both sexes equally and is frequently associated with progressive structural joint damage, functional impairment, and a significant reduction in health-related quality of life. Inadequate or delayed therapeutic intervention can result in irreversible joint destruction and long-term disability [2]. This study aimed to identify reliable and validated biomarkers needed to enhance diagnostic accuracy and facilitate the longitudinal monitoring of disease activity and progression. Such biomarkers would be pivotal in guiding individualized therapeutic strategies, thereby optimizing clinical outcomes and improving the long-term prognosis for patients with PsA. The plasma metabolomic profile of 38 naive PsA patients, with a disease activity score >14 (no bDMARD treatment in progress), was analyzed and compared to that of 32 healthy controls (HC). Plasma samples were analyzed using a $^{1}$H-NMR spectroscopy-based approach, and the resulting data were processed through multivariate statistical analysis (MVA). The multivariate statistical analysis showed a great separation between PsA and HC, indicating differences in the metabolomics profile between the two groups. Statistically significant metabolites that distinguished PsA from HC were involved in several metabolic pathways such as glucose-alanine cycle and glycine and serine metabolism, glutathione metabolism, as well as selenoaminoacid metabolism, alanine metabolism and tryptophan metabolisms. Despite the limitation of this study due to a small cohort of subjects, our findings suggest that the metabolic differences in PsA patients compared to healthy conditions contribute to elucidating the role of metabolite unbalance on the inflammatory burden, oxidative stress, energy and collagen metabolism but also on peculiar features of PsA disease, as metabolic disorder and diabetes.

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# PERIODIC COOKING: THE PERFECT EGG

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Eggs are biphasic systems composed of albumen and yolk, which require distinct optimal cooking temperatures (≈85 °C and ≈65 °C, respectively) [1]. Conventional methods fail to cook both components evenly, often compromising texture and nutrient quality. In this study, a novel technique termed periodic cooking was developed through computational modeling of heat transfer and protein gelation, which alternates immersion of the egg in hot (100 °C) and cold (30 °C) water. This controlled thermal modulation ensures balanced protein denaturation while preserving nutrients. The periodic cooking was compared with traditional methods, including hard-boiled (100 °C for 12 min), soft-boiled (100 °C for 6 min), and sous-vide (60–70 °C for 1 h) eggs, to evaluate differences in texture, appearance, and metabolomic profile (Figure 1).

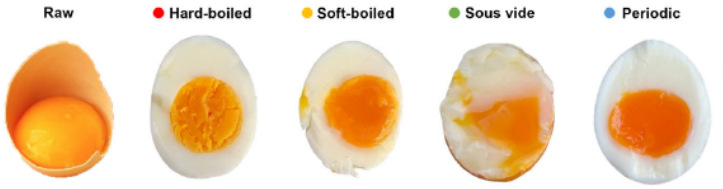


*Figure 1: Comparison of raw and cooked eggs obtained by different methods: raw, hard-boiled (100 °C x 12 min), soft-boiled (100 °C x 6 min), sous-vide (60–70 °C x 1 h), and periodic (100 °C/30 °C cycles).*

The metabolomic impact of this process was explored by untargeted $^{1}$H-NMR spectroscopy on both yolk and albumen, and by UHPLC-HRMS on the yolk, recognized as the main source of polyphenols and bioactive compounds. Principal Component Analysis (PCA) of the NMR spectra revealed a distinct metabolic signature for periodically cooked eggs, clearly separating them from conventionally cooked samples. The HRMS-based PCA further highlighted higher levels of amino acids, branched-chain amino acids, and phenolic compounds such as daidzein and ferulic acid, linked to antioxidant and health-promoting properties [2]. Overall, periodic cooking enhances both eggs' physical texture and metabolomic profile, representing a promising strategy for designing thermally processed foods with improved functional and nutritional properties.

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# AN OPTIMIZED NMR-BASED PROTOCOL FOR PROFILING OF ENDO- AND EXO-METABOLOMES IN A$\beta_{1-42}$ TREATED HUMAN ASTROCYTES FROM HEALTHY AND ALZHEIMER'S DISEASE DONORS

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Astrocytes regulate brain metabolism and respond to neurodegenerative stress. In Alzheimer's disease (AD), their metabolism is dysregulated, but specific alterations remain unclear. We profiled intra- and extracellular metabolites of primary human astrocytes from healthy donors and AD patients exposed to amyloid $\beta_{1-42}$ (A$\beta_{1-42}$) oligomers to identify disease-specific vulnerabilities. Astrocytic metabolic reprogramming contributes to neurodegeneration[1]. Primary astrocytes from six healthy donors and five AD patients were treated with 10 $\mu$M A$\beta_{1-42}$ for five days. Lysates and conditioned media were collected, identifying 41 intracellular and 34 extracellular metabolites.

Samples were analyzed via $^1$H NMR spectroscopy (600 MHz, CPMG), annotated with Chenomx, and quantified using in-house R scripts. Four conditions were compared: untreated and treated cells from both groups[2]. A$\beta_{1-42}$ reduced creatine phosphate in both groups, indicating impaired energy buffering and antioxidant defense. $\beta$-Alanine decreased only in AD astrocytes, suggesting disrupted carnosine biosynthesis. In conditioned media, AD astrocytes showed elevated 2-oxoglutarate, citrate, glycine, and niacinamide, reflecting altered TCA cycle and redox metabolism. Healthy astrocytes showed reduced extracellular leucine and isoleucine, implying increased amino acid uptake. Shared changes indicate general astrocytic stress responses, while AD-specific patterns reveal reduced metabolic adaptability and mitochondrial dysfunction. Altered amino acid and TCA cycle metabolites suggest impaired energy handling and diminished neuronal support[3].

This NMR workflow enables simultaneous profiling of intra- and extracellular metabolites, revealing disease-specific astrocytic responses to A$\beta_{1-42}$ and supporting astrocyte metabolism as a potential target in AD progression.

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# MEASURING ETHANOL CONCENTRATION IN ALCOHOLIC BEVERAGES BY $^{1}$H-NMR: METHODS EVALUATION AND A CASE STUDY ON RED AND WHITE WINES

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Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful analytical technique that is now widely used in the field of food and beverages. Many recent applications are related to quality control, authentication, traceability, fraud detection but also process monitoring such as fermentation and aging effects.

Even if NMR spectroscopy is commonly used for quantification purposes, in the recent literature there are few studies focused on the possibility of using this technique to quantify ethanol in alcoholic beverages [1]. Indeed, some recent works highlighted problems associated with this application, often related to low accuracy [2], occurring also with higher magnetic field intensities [3].

In this context we explore the causes of these results by planning a detailed experimental design using a series of ethanol solutions with increasing concentrations and considering different 1H NMR acquisition parameters, data processing techniques and methods related to ethanol quantification.

To try to avoid the known quantification problems [2,3] related to the use of the proton-proton coupling triplet signal at 1.18 ppm (i.e., one of the two "main" ethanol signals), the carbon-related satellite peak at 0.08 ppm was also tested, building upon the work of Lopez-Rituerto et al. [4]. The main and the satellite signals were modelled separately and compared, and a new correction method to make the satellites quantitative was developed.

In addition, from the data analysis and processing point of view, two different quantification approaches were tested (namely, row sum, and multivariate curve resolution). Also, to evaluate the effects of two deuterated solvents ($D_2O$ and DMSO) and of the suppression of the water signal, ANOVA Simultaneous Component Analysis (ASCA) was applied. Additionally, both internal and external standard quantification approaches were evaluated during this study. To test and further evaluate the model performances, different external wine samples were analyzed following an optimized operating procedure.

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# FROM RAW DATA TO LONGITUDINAL INSIGHT: AN OPEN-SOURCE WORKFLOW FOR UNTARGETED METABOLOMICS OF ULVAN POLYSACCHARIDE FERMENTATION

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Fermentation systems are useful tools to assess the impact of dietary treatments on the gut microbiota in vitro. When combined with untargeted metabolomics, they can reveal temporal and treatment-related metabolic dynamics but require reproducible data processing and robust statistical approaches. Following best practices in nutrimetabolomics [2], this study presents an integrated workflow for data processing, annotation, and longitudinal analysis applied to [3], was subjected to in vitro digestion (INFOGEST 2.0) and colonic fermentation [1], sampling at 0-12-24-48 h. LC-QToF data were acquired in randomized order, with blanks, pooled QC, and external standards ensuring reproducibility. Data processing in MZmine 3 included smoothing, peak detection, alignment, and gap filling. Annotation with SIRIUS 5.8.6 (CSI:FingerID, ZODIAC, CANOPUS, COSMIC) and data filtering retained features with RSD < 20%, and high variance (top 60%). The final dataset (943 ESI and 485 ESI features) included 173 features with MSI 2a confidence. Dynamic responses were modeled using repeated-measures ASCA (RM-ASCA) in RStudio. PC1 (66-72% variance) described the global fermentation trajectory, showing decreased peptides and bile acids and a rise in fatty acid derivatives, while PC2-PC3 captured treatment-specific effects, distinguishing ulvan from control samples. Two cyclic proline dipeptides, cyclo-(Pro-Pro) and cyclo-(Pro-Met), showed opposite time trends and were tentatively linked to Lactobacillus and Bacteroides via MicrobeMASST, suggesting selective stimulation of beneficial microbial groups. Additional ulvan-specific sulfur-containing metabolites, not previously described, may represent markers. This study, building on a previous targeted metabolomic analysis, integrates open-source tools with advanced longitudinal modeling, providing a workflow for untargeted metabolomics data interpretation of ulvan fermentation by human microbiota.

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