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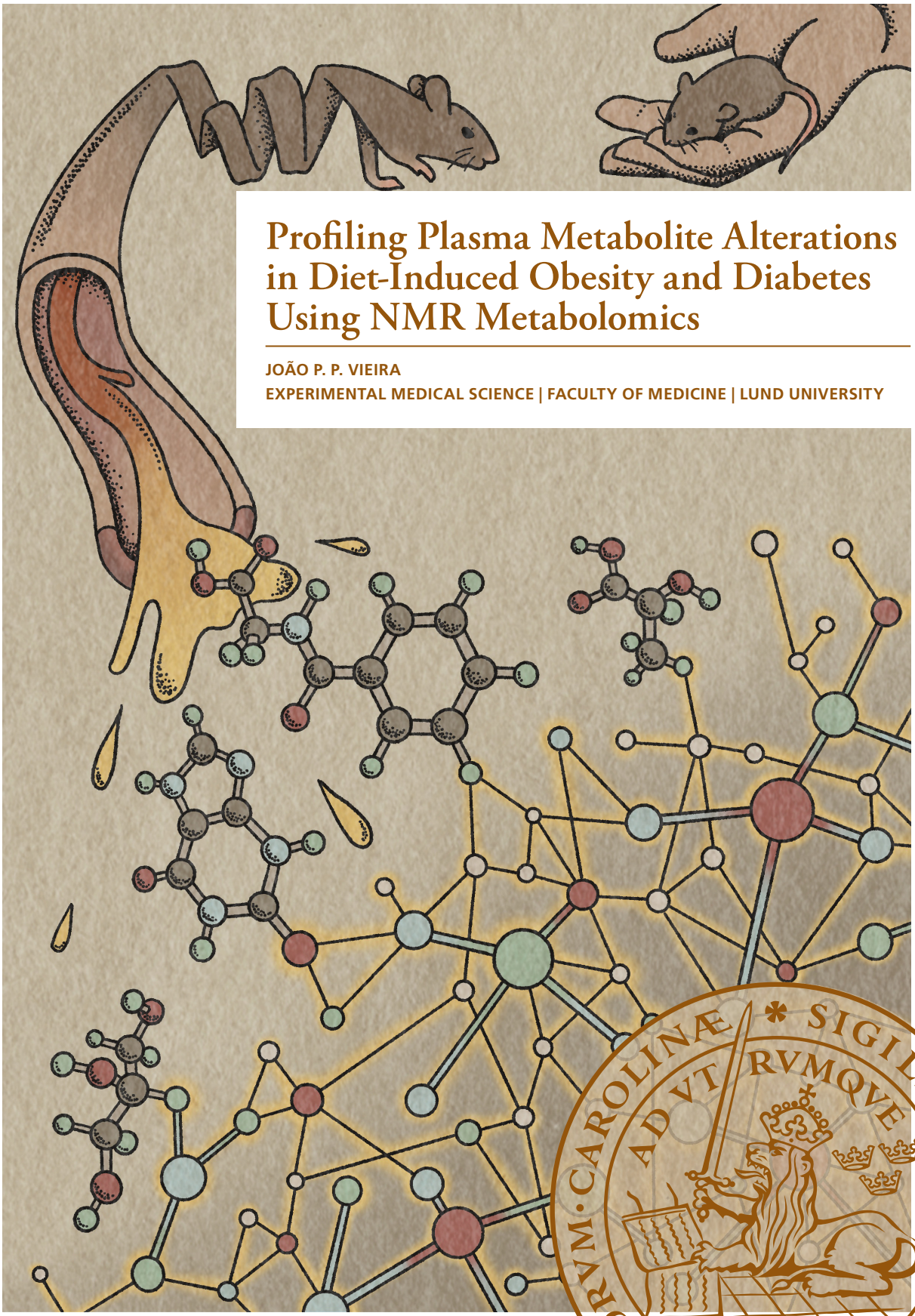

Profiling Plasma Metabolite Alterations in Diet-Induced Obesity and Diabetes Using NMR Metabolomics

JOÃO P. P. VIEIRA
EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY

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About the author

JOÃO P. P. VIEIRA is an accomplished researcher with a passion for unravelling the intricate mechanisms of metabolic imbalances. Equipped with bachelor's and master's degrees in Biochemistry from the University of Coimbra (Portugal), he embarked on a PhD journey under the supervision of João Duarte in the Diabetes and Brain Function Laboratory in November 2019.

His expertise spans a wide spectrum of scientific techniques, including molecular biology, NMR spectroscopy, immunoblotting, confocal microscopy, laboratory animal science, and programming languages. Proficient in various scientific disciplines such as microbiology, biotechnology, and quality control methodologies, João's research focuses on the innovative application of metabolomics tools. He investigates metabolic imbalances induced by obesogenic diets, using plasma and serum samples linked to Type 2 Diabetes and metabolic syndrome. Central to his work is also the exploration of the insulin-dependent regulation of brain energy metabolism.

João is dedicated to applying cutting-edge methodologies to unravel the complex interplay of factors contributing to these conditions, contributing valuable insights to the scientific community's understanding of metabolic disorders.

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Profiling Plasma Metabolite Alterations in Diet-Induced Obesity and Diabetes Using NMR Metabolomics

João P. P. Vieira

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DOCTORAL DISSERTATION

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Abstract:

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Key words: metabolite profiling, diet-induced obesity, type 2 diabetes mellitus, ^{1}H-NMR spectroscopy, metabolism alterations, post-stroke recovery

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from

DIABETES AND BRAIN FUNCTION

Department of Experimental Medical Science, Faculty of Medicine

Lund University, Lund, Sweden

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MADE IN SWEDEN

I dedicate this thesis to those who have helped me throughout this journey and held my hand when I needed it the most.

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Abstract

Diets rich in saturated fat and sedentary lifestyles markedly contribute to obesity and metabolic syndrome-related diseases, including type 2 diabetes mellitus (T2D). Metabolite profiling plays a pivotal role in understanding these metabolic diseases. This thesis comprises insights from four distinct studies to illuminate metabolic imbalances induced by diet-induced obesity (DIO), covering topics related to the duration of dietary regimens, potential benefits of dietary interventions on the brain and metabolism, and the impact of underlying T2D on post-stroke recovery. Using a combination of proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy and mass spectrometry (MS), study I initially demonstrated the superiority of the combined approach in characterizing the effects of DIO on plasma metabolites. The robustness of this method was further validated in a human cohort, underscoring its translational potential in unravelling metabolic imbalances. In study II, female mice exposed to high-fat diet (HFD) exhibited brain metabolism alterations and memory deficits, which were mitigated by taurine and *N*-acetylcysteine (NAC) supplementation. These supplements not only ameliorated HFD-induced memory impairment but also elicited distinct effects on metabolic alterations within the hippocampus. Systemically, ^{1}H-NMR metabolomics data in study III revealed that NAC and taurine treatments impacted plasma metabolites. Ultimately, as explored in study IV using ^{1}H-NMR metabolomics, unique metabolite changes in male mice with T2D following transient middle cerebral artery occlusion were reported. Specifically, metabolite changes that link T2D to poor neurological outcomes after stroke were observed. In summary, this thesis underscores the significance of metabolite profiling in elucidating the complexities of metabolic diseases, memory impairment, and post-stroke recovery in DIO mouse models. It also emphasizes the translational character of such findings to human pathophysiology.

Sumário

Dietas ricas em gordura saturada e estilos de vida sedentários contribuem significativamente para a obesidade e doenças associadas com a síndrome metabólica, incluindo diabetes mellitus tipo 2 (DT2). O perfil metabólico desempenha um papel fundamental na compreensão dessas doenças metabólicas. Nesta tese estão exibidas as principais descobertas relacionadas com desiquilíbrios metabólicos relacionados com obesidade induzida pela dieta (OID) abordadas ao longo de de quatro estudos distintos, focados na avaliação dos efeitos da duração do regime alimentar, em explorar potenciais benefícios de intervenções dietéticas no cérebro e no metabolismo, e o impacto da DT2 subjacente na recuperação após a ocorrência de acidente vascular cerebral (AVC). Usando uma combinação de espectroscopia de ressonância magnética nuclear de protões (^{1}H-RMN) e espectrometria de massa (MS), o estudo I demonstrou inicialmente a superioridade da abordagem combinada em discernir os efeitos da OID nas concentrações plasmáticas de metabolitos e o impacto temporal da dieta nos mesmos em camundongos machos. A robustez deste método foi ainda validada numa coorte humana, sublinhando o seu potencial translacional na resolução de desequilíbrios metabólicos. No estudo II, camundongos fêmeas expostos a uma dieta hiperlipídica (DH) apresentaram alterações no metabolismo cerebral e *déficits* de memória, que foram atenuados pela suplementação de taurina e *N*-acetilcisteína (NAC). Esses suplementos não apenas melhoraram o comprometimento da memória induzido pela DH, mas também provocaram efeitos distintos nas alterações metabólicas no hipocampo. Sistemicamente, os dados metabolómicos de ^{1}H-RMN no estudo III revelaram que os tratamentos com NAC e taurina exerceram impacto nos metabolitos plasmáticos. Finalmente, conforme explorado no estudo IV usando metabolómica baseada em ^{1}H-RMN, foram relatadas alterações metabólicas únicas em camundongos machos com DT2 após oclusão transitória da artéria cerebral média. Especificamente, foram observadas alterações metabólicas que ligam a DT2 a resultados neurológicos negativos após o acidente vascular cerebral. Portanto, esta tese ressalta a importância do perfil metabólico na elucidação das complexidades das doenças metabólicas, comprometimento da memória e recuperação pós-AVC na OID em modelos de camundongos. O carácter translacional de tais descobertas para a fisiopatologia humana é também evidenciado.

List of Publications

I. **Vieira, J. P. P.**, Ottosson, F., Jujic, A., Denisov, V., Magnusson, M., Melander, O., & Duarte, J. M. N. (2023). Metabolite Profiling in a Diet-Induced Obesity Mouse Model and Individuals with Diabetes: A Combined Mass Spectrometry and Proton Nuclear Magnetic Resonance Spectroscopy Study. Metabolites, 13(7), 874. MDPI AG. http://dx.doi.org/10.3390/metabo13070874.

II. Garcia-Serrano, A. M., **Vieira, J. P. P.**, Fleischhart, V., & Duarte, J. M. N. (2022). Taurine and *N*-acetylcysteine treatments prevent memory impairment and metabolite profile alterations in the hippocampus of high-fat diet-fed female mice. Nutritional Neuroscience, 1–13. https://doi.org/10.1080/1028415X.2022.2131062.

III. **Vieira, J. P. P.**, Garcia-Serrano, A. M., & Duarte, J. M. N. (2023). ^{1}H-NMR Spectroscopy Provides Metabolic Predictors Evaluating Taurine and *N*-Acetylcysteine Supplementation in HFD-fed Female Mice. [*manuscript*]

IV. **Vieira, J. P. P.**, Karampatsi, D., Vercalsteren, E., Darsalia, V., Patrone, C., & Duarte, J. M. N. (2023). Nuclear magnetic resonance spectroscopy reveals biomarkers of stroke recovery in a mouse model of obesity-associated type 2 diabetes. [*manuscript*]

Publications not included in the thesis

I. Mohr, A. A., Garcia-Serrano, A. M., **Vieira, J. P.**, Skoug, C., Davidsson, H., & Duarte, J. M. (2021). A glucose-stimulated BOLD fMRI study of hypothalamic dysfunction in mice fed a high-fat and high-sucrose diet. Journal of cerebral blood flow and metabolism: official journal of the International Society of Cerebral Blood Flow and Metabolism, 41(7), 1734–1743. https://doi.org/10.1177/0271678X20942397.

II. Skoug, C., Erdogan, H., Vanherle, L., **Vieira, J. P. P.**, Matthes, F., Eliasson, L., Meissner, A., & Duarte, J. M. N. (2023). Density of Sphingosine-1-Phosphate Receptors Is Altered in Cortical Nerve-Terminals of Insulin-Resistant Goto-Kakizaki Rats and Diet-Induced Obese Mice. Neurochemical Research. https://doi.org/10.1007/s11064-023-04033-4.

III. Jujic, A., **Vieira, J. P. P.**, Matuskova, H., Nilsson, P. M., Lindblad, U., Olsen, M. H., Duarte, J. M. N., et al. (2023). Plasma Galectin-4 Levels Are Increased after Stroke in Mice and Humans. International Journal of Molecular Sciences, 24(12), 10064. MDPI AG. http://dx.doi.org/10.3390/ijms241210064.

Abbreviations

^{1}H-^{1}H TOCSY	Total Correlation Spectroscopy
^{1}H-NMR	Proton-Nuclear Magnetic Resonance
2-HIV	2-Hydroxyisovalerate
3-HIB	3-Hydroxyisobutyric Acid
AD	Alzheimer's Disease
BCAAs	Branched-Chain Amino Acids
BMI	Body Mass Index
CD	Control Diet
DIO	Diet-Induced Obesity
DMGV	Dimethylguanidino Valeric Acid
FDR	False-Discovery Rate
FID	Free Induction Decay
GABA	Gamma-Aminobutyric Acid
GNAT	General NMR Analysis Toolbox
GTT	Glucose Tolerance Test
HbA1c	Glycosylated Haemoglobin
HDL	High-Density Lipoprotein
HFD	High-Fat Diet
HMDB	The Human Metabolome Database
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
IGF-1	Insulin-Like Growth Factor 1
IR	Insulin Resistance
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDL	Low-Density Lipoprotein
LSD	Least significant difference
MDCS	The Malmo Diet and Cancer Study

MRS	Magnetic Resonance Spectroscopy
MS	Mass Spectrometry
NAC	*N*-acetylcysteine
$NADP^+$	Oxidized Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
ND	Non-Diabetic
NLR	Novel Location Recognition
NOR	Novel Object Recognition
NUTS	NMR Utility Transform Software
OPLS	Orthogonal Projection to Latent Structures
PFG-TRP	Pulsed Field Gradient-Total Relaxation Pulse
PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PLS	Partial Least-Squares
ppm	Parts Per Million
Q^2	A measure of the predictive ability of a model
R^2	Coefficient of determination
SAH	*S*-adenosylhomocysteine
SD	Standard Diet
SMPDB	Small Molecule Pathway Database
T2D	Type 2 Diabetes *Mellitus*
TAG	Triacylglycerols
tMCAO	Transient Middle Cerebral Artery Occlusion
UHPLC-QTOF-MS	Ultra-High-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry
UMP	Uridine Monophosphate
VIP	Variable Importance in Projection

Introduction

Obesity, diabetes, and brain function

Obesity prevalence has reached pandemic dimensions over the past 50 years. Obesity can be briefly defined as a visceral and subcutaneous lipid accumulation and body weight gain. Moreover, obesity is frequently accompanied by the deposition of lipids in non-adipose tissues (de Moura e Dias *et al.*, 2021).

The global diabetes burden has been rising quickly and future perspectives are not very encouraging. Sedentary lifestyles and the consumption of large amounts of highly processed food rich in saturated fats and refined sugars are key factors for the development of obesity (Blüher, 2019). Ageing, economic development and increasing urbanisation are other factors that should also be considered for the prevalence of Type 2 Diabetes *Mellitus* (T2D). Although cues about how obesity escalates the pathogenesis of T2D by inducing insulin resistance (IR) development and phenotypical IR-related consequences are already described, there is no consensus for cross-linked mechanisms of IR in T2D (Wondmkun, 2020).

Nevertheless, IR is a common link between T2D and obesity. Despite the insulin resistance often found in obese individuals, many do not experience high blood sugar levels. This is due to the pancreatic β-cells in the islet of Langerhans, which release ample insulin to compensate for reduced insulin levels, thus preserving normal glucose tolerance under usual conditions (Røder *et al.*, 1998). The onset of IR and obesity, pivotal factors in the development of T2D, involves a situation where β-cells cannot entirely offset reduced insulin sensitivity. The release of non-esterified fatty acids from adipose tissue in individuals with obesity raises the plausible hypothesis that a connection exists between insulin resistance and β-cell dysfunction (Kahn, Hull and Utzschneider, 2006).

Obesity also substantially increases the risk of T2D and other metabolic diseases cardiovascular diseases (hypertension, myocardial infarction, and stroke), musculoskeletal disease (osteoarthritis), Alzheimer's Disease (AD) and depression (Blüher, 2019).

A wide range of scientific evidence identifies a connection between T2D and cognitive impairment (Jayaraman and Pike, 2014). In T2D, a desensitization process towards insulin action occurs and also deregulates brain bioenergetics. That also affects synaptic plasticity, learning, memory, and protein metabolism, ultimately promoting the onset of AD, the most common cause of dementia. Brain mechanisms are also disturbed by metabolic and vascular risk factors within the metabolic syndrome, such as dyslipidaemia, hypercholesterolemia and hypertension (Duarte, 2015). Also, it is widely hypothesized that the pro-inflammatory profile linked to T2D, obesity, and metabolic syndrome plays a crucial role in both initiating and progressing AD, fostering significant interconnections between these conditions (Jayaraman and Pike, 2014).

Dietary interventions on the brain and metabolism

Highly caloric and sugar-rich diets have raised concerns about their potential impact on cognitive function and metabolic profiles. Recent research by (Garcia-Serrano, Mohr, *et al.*, 2022) has shed light on how such diets may lead to cognitive impairment and metabolite profile alterations in both the hippocampus and cortex of both male and female mice. Intriguingly, these adverse effects appear to be reversible through dietary modifications.

The brain, an organ highly sensitive to proper fuel availability, responds acutely to changes that compromise this delicate balance (Ashrafi *et al.*, 2017). For instance, diet-induced insulin resistance (IR) can lead to spatial memory deficits and hinder the cognitive-enhancing effects of insulin (Pearson-Leary and McNay, 2012). However, it has been observed that the delivery of exogenous insulin to the hippocampus can enhance memory and metabolism, suggesting a critical role for insulin/ insulin-like growth factor 1 (IGF-1) pathways (Pearson-Leary and McNay, 2012). Dysfunction in these pathways may contribute to the progressive neuronal loss seen in neurodegenerative diseases like AD and Parkinson's (Muhič *et al.*, 2015).

Notably, the development of IR in rodents appears to be intricately linked to feeding time and lipid overload in tissues (Król, Okulicz and Kupsz, 2020). Over time, this process becomes associated with proinflammatory response induced by macrophages, potentially leading to inflammation in both the central nervous system and peripheral tissues (Król, Okulicz and Kupsz, 2020). This pro-inflammatory profile is also implicated in the initiation and progression of AD, obesity, and metabolic syndrome (Jayaraman and Pike, 2014).

Taurine, a β-sulfonated essential amino acid, is naturally synthesized from methionine and cysteine precursors (Król, Okulicz and Kupsz, 2020; Rafiee, García-Serrano and Duarte, 2022). While its accumulation in the brain might not be directly related to memory impairment in obesity and diabetes models, it is associated with neurodegeneration and astrogliosis in mouse models of AD (Garcia-Serrano, Vieira, *et al.*, 2022). Nonetheless, human data on plasma taurine levels and dementia associations remain contentious (Rafiee, García-Serrano and Duarte, 2022). Given the limited rate of taurine biosynthesis in mammals, taurine administration has been suggested not only to prevent neurodegeneration but also to offer systemic benefits for various health conditions, including obesity-induced inflammation (Rafiee, García-Serrano and Duarte, 2022).

Similarly, *N*-acetylcysteine (NAC), a cysteine donor for taurine and glutathione synthesis, has exhibited potential benefits for neurological and psychiatric disorders (Deepmala *et al.*, 2015; Romero-miguel *et al.*, 2023). The primary effects of NAC involve increasing cysteine availability and acting as an antioxidant, offering a multifaceted approach to neuroprotection (Aldini *et al.*, 2018).

Reducing dietary fat content has shown promise in improving health outcomes. Additionally, supplements like NAC and taurine may protect against muscle oxidative stress and lipid peroxidation, respectively, suggesting a link between these stressors and IR in high blood sugar conditions (Haber *et al.*, 2003).

Indeed, non-diabetic overweight or obese men might experience β cell decline due to free fatty acids in the bloodstream. Taurine treatments help counteract this decline, while NAC does not have the same effect (Xiao, Giacca and Lewis, 2008). On the other hand, NAC might be detrimental in regulating methionine metabolism, by promoting the homeostasis of stearoyl-coenzyme A desaturase-1 (Elshorbagy *et al.*, 2013).

Interestingly, the combined use of NAC and taurine effectively alleviates cisplatin-induced nephrotoxicity, likely due to their unique protective actions and restoration of enzymatic antioxidants in renal tissue (Abdel-Wahab, Moussa and Saad, 2017).

Investigating the effects of high-fat diet (HFD) feeding and taurine or NAC supplementation on hippocampal and systemic metabolism holds promise in bridging the gap between systemic and brain metabolisms. This holistic approach may provide valuable insights into potential interventions to counteract the negative consequences of modern dietary patterns on cognitive and metabolic health.

Stroke and diabetes: risk factors and preventive insights

Stroke stands as a leading global cause of mortality, with survivors often grappling with a loss in disability-adjusted life years. This pervasive health issue affects both developed and developing nations, with the latter experiencing a notably higher risk of stroke-related fatalities. Key risk factors for stroke encompass hypertension, diabetes, high cholesterol levels, cigarette smoking, atrial fibrillation, ischemic cardiomyopathy, and carotid stenosis. (Chumachenko, Waseem and Fedorovich, 2022).

Ischemic stroke is the prevailing form of stroke in the European Union (Chumachenko, Waseem and Fedorovich, 2022) and the United States of America (Au, 2018; Yang *et al.*, 2022). This type of stroke results from the occlusion of internal carotid arteries, vertebral/basilar arteries, and most frequently, the middle cerebral artery, leading to the limited blood supply to specific regions of the brain (Au, 2018).

Hence, a decline in cerebral blood flow triggers an energy crisis by disrupting the equilibrium between energy demand and supply (Suissa *et al.*, 2021). Consequently, major changes affecting the release of glycolate, sphingolipids, formate, homocysteine, folate cycle metabolites, Tetrahydrofolate, *S*-adenosylhomocysteine (SAH), cysteine and oxidized, hypoxanthine, lactate and pyruvate, from nerve cells occur during the ischemic episode. These alterations in metabolite levels may be associated with phenomena like excitotoxicity, oxidative stress, and inflammation, among other categories (Sidorov, Sanghera and Vanamala, 2019).

Cardiovascular disease risk is markedly elevated in individuals with T2D when compared to the general population. While factors like dyslipidaemia, inflammation, and oxidative stress offer partial explanations for this increased risk, their precise relationship with T2D and the comprehensive understanding of metabolic status remain somewhat elusive (Huang *et al.*, 2022).

Moreover, T2D appears to hinder the post-stroke reparative neovascularization process, which is typically associated with improved functional outcomes after a stroke. Normalizing glucose through dietary changes may potentially reduce stroke-related complications in the diabetic population (Karampatsi *et al.*, 2021).

Finally, treating diabetes with the goal of preventing strokes could foster cross-disciplinary collaborations in the medical field, leading to a reduction in the overall stroke burden (Kernan and Inzucchi, 2021).

Metabolomics

Since obesity is a risk factor for a plethora of diseases, a profound perturbation of the plasma metabolome should be expected. The plasma metabolome is influenced by metabolites directly linked to endogenous enzymatic activities encoded by the genome or derived from food, medications, the microbiota and the environment (Clish, 2015). Hence, applying metabolomics tools to profile metabolic shifts in obesity could provide a more complete characterization of metabolic signatures characteristic of obesity (Cirulli *et al.*, 2019).

Metabolomics, the most recent field of research within the "omics" approaches (Au, 2018), is a technology-driven discipline that can be broadly described as the comprehensive measurement of all metabolites and low-molecular-weight molecules in a biological specimen. Detecting and measuring the abundance of metabolites in biological specimens is relevant because those molecules have been described as proximal reporters of disease and pathogenic mechanisms (Clish, 2015).

This field of research is constantly evolving and taking advantage of new developments in analytical chemistry, including analytical techniques, instrumentation, analytical software, statistical methods, or computational techniques capable of accelerating or improving data collection, analysis and interpretation (Clish, 2015; Emwas *et al.*, 2019). Ultimately, the metabolomics field can lead to the discovery of biomarkers that may be used to either diagnose a disease or monitor the activity of therapeutics (Clish, 2015; Jové *et al.*, 2015; Casadei-Gardini *et al.*, 2020).

Analytical techniques in metabolomics

In contrast to standard clinical laboratory techniques, mass spectroscopy (MS) and ^{1}H-NMR metabolomics tools enable the detection of a significantly larger number of metabolites (Clish, 2015), which constitute the primary analytical technologies used in metabolomics-based studies (Nagana Gowda and Djukovic, 2014; Emwas *et al.*, 2019).

The relevance of MS and NMR techniques within this field is bolstered by their mutual complementarity and synergy (Nagana Gowda and Djukovic, 2014). While MS is indeed highly sensitive, capable of detecting and elucidating structures at concentrations as low as 10-100 nmol/L, this sensitivity can be influenced by the specific experimental conditions and instrumental settings (Nagana Gowda and Djukovic, 2014; Emwas *et al.*, 2019). Furthermore, it is important to note that MS samples, once analysed in the spectrometer, cannot be repurposed for other uses (Deidda *et al.*, 2015).

Nevertheless, the utilization of ^{1}H-NMR spectroscopy in metabolomics studies has significantly grown, even though it offers lower sensitivity (detectable at concentrations above 1 μmol/L) and faces challenges related to selectivity due to frequency overlaps. This increase can be attributed to its technical advantages, which encompass straightforward sample processing, a non-destructive nature, and high reproducibility, enabling it to effectively address some of the primary limitations associated with MS (Deidda *et al.*, 2015; Emwas *et al.*, 2019).

Currently, ^{1}H-NMR is one of the most reliably employed spectroscopy tools in NMR-based metabolomics studies (Nagana Gowda and Raftery, 2017b, 2017a) because of the ubiquitous character of protons in almost every organic compound and known metabolites.

One-dimensional ^{1}H-NMR spectra are useful for metabolomics studies and theoretically allow the identification of 50-100 metabolites at a time. ^{1}H-NMR spectra are very useful for metabolite quantification because they provide a real representation of the distribution of the proton nuclei within the molecules and the different concentration levels of the corresponding metabolites in a complex mixture (Nagana Gowda and Raftery, 2017b; Emwas *et al.*, 2019). The non-destructive nature of NMR spectroscopy favours experimental reproducibility.

A major advantage of ^{1}H-NMR spectroscopy is the fact that it is not restricted to a specific biological fluid or tissue extract analysis. Nevertheless, urine, plasma and blood serum are the most commonly used biological fluids for metabolomics-based studies because they both contain hundreds to thousands of detectable metabolites and can be collected using non- or minimally invasive methods (Zhang *et al.*, 2010).

This metabolomics tool can be applied in research fields specialized in blood, brain, cancer, cardiovascular diseases, kidney, lung, nutrition, obesity, paediatrics or T2D that can lead to the discovery of pathology-associated biomarkers (Zhang *et al.*, 2010, 2018; Luo, Zhu and Gao, 2012; Sanz-Cortés *et al.*, 2013; Elliott *et al.*, 2015; Liu *et al.*, 2016; Oike *et al.*, 2016; Nagana Gowda and Raftery, 2017b; Chen *et al.*, 2018; Casadei-Gardini *et al.*, 2020).

Metabolomics in metabolic diseases

Studies utilizing ^{1}H-NMR and MS-based techniques have provided crucial insights into metabolic phenotypes associated with obesity and related health risks.

In ^{1}H-NMR studies, Bervoets *et al.*, 2018 found that obese children displayed elevated levels of lipids, *N*-acetyl glycoproteins, and lactate, coupled with decreased levels of certain amino acids, α-ketoglutarate, glucose, citrate, and cholinated phospholipids compared to normal-weight children. Furthermore, the study highlighted that metabolically healthy children showcased distinct metabolic differences, including lower lipid and lactate levels and higher amino acids and cholinated phospholipids compared to unhealthy children (Bervoets et al., 2018).

Aguilar-Ramirez *et al.*, 2022 reported associations between higher adiposity and elevated levels of Apolipoprotein-B, very low-density lipoprotein particles, fatty acids, branched-chain amino acids, and glycoprotein acetyls, independent of general and abdominal adiposity measures. Notably, higher gluteo-femoral adiposity was linked to a more favourable cardiometabolic lipid profile (Aguilar-Ramirez et al., 2022).

In MS-based studies, Cirulli *et al.*, 2019 found 49 metabolites associated with BMI, primarily lipids, amino acids, nucleotides, peptides, energy, carbohydrates, xenobiotics, and co-factors/vitamins. Some of these metabolites, such as DMGV, glutamate, and branched-chain amino acids, have documented associations with BMI and the risk of future type 2 diabetes (Cirulli *et al.*, 2019).

Smith *et al.*, (2020) investigated altered acylcarnitine metabolism and its link to an increased risk of atrial fibrillation within the general population, independent of traditional risk factors (Smith *et al.*, 2020). In a subsequent study Smith *et al.*, 2022 within the same cohort, certain metabolites, including C4:OH-acylcarnitine, ergothioneine, homostachydrine, and acetylornithine, were positively associated with a decreased risk of type 2 diabetes and coronary artery disease. Conversely, metabolites like proline, dimethylguanidino valerate, and isoleucine indicated an unhealthy cardiometabolic state and increased risk of these diseases (Smith *et al.*, 2022).

Metabolic alterations extend beyond obesity. Diaz *et* al. 2011 and Graça *et* al. 2012 in the pipeline methodology have demonstrated applications for describing metabolic changes associated with prenatal disorders. Furthermore, poorly controlled glycaemia in T2D is associated with various comorbidities, including kidney failure, nerve damage, blindness, cardiovascular diseases, and an increased risk for certain cancers (Blüher, 2019; Mora-Ortiz *et al.*, 2019).

Stroke and metabolomics

A collection of recent studies addressing various aspects of stroke research is presented below, showcasing the significance of metabolomics in the field.

Ahmed *et al.*, 2021 assessed the feasibility of repeated sampling of exhaled volatile organic compounds through untargeted metabolomic analysis of plasma collected at various time periods after a stroke (Ahmed *et al.*, 2021).

Balasubramanian *et al.*, 2022 examined the connection between metabolomic profiles and ischemic stroke, with a particular focus on potential sex-specific differences in the serum metabolome, particularly in female participants (Balasubramanian *et al.*, 2022).

Chi *et al.*, 2021 used untargeted metabolomics at the acute stage of ischemic stroke to predict functional recovery and identified platelet activating factor as an important outcome-associated metabolite (Chi *et al.*, 2021).

J. Liu *et al.*, 2021 conducted a case study to comprehensively evaluate metabolic changes in young ischemic stroke patients without common risk factors, highlighting alterations in various metabolites and their connection to specific metabolic pathways (Liu *et al.*, 2021).

Jové *et al.*, 2015 conducted a metabolomic analysis of plasma from transient ischemic attack patients to uncover potential biomarkers associated with stroke recurrence and temporal patterns, with a focus on the potential impact of large-artery atherosclerosis (Jové *et al.*, 2015).

Jung *et al.*, 2011 aimed to identify metabolic biomarkers associated with stroke by utilizing a proton-nuclear magnetic resonance spectroscopy (^{1}H-NMR) metabolomics approach to investigate altered metabolic shifts in plasma and urine from patients with cerebral infarctions (Jung *et al.*, 2011).

Lee *et al.*, 2017 conducted a retrospective cohort study in high-risk stroke patients, revealing low levels of serum lysine metabolites as potential biomarkers for the early diagnosis of high-risk thrombotic stroke patients (Lee *et al.*, 2017).

Suissa *et al.*, 2021 investigated the impact of oral ketone ester administration on brain metabolism, with a specific focus on SLC5A8-deficient transgenic mice (Suissa *et al.*, 2021).

X. Wang *et al.*, 2020 employed targeted metabolomics to examine the association between serum levels of amino acids and functional recovery after stroke (Wang *et al.*, 2020).

Xie *et al.*, 2020 aimed to identify potential biomarkers for diagnosing post-stroke depression in middle-aged stroke survivors (Xie *et al.*, 2020).

Yang *et al.*, 2022 focused on exploring the protective effect of folic acid on ischemic stroke in animal models (Yang *et al.*, 2022). Poupore et al., 2021 investigated sex-specific differences in metabolic profiles and related pathways in patients with acute ischemic stroke using an untargeted metabolomics approach (Poupore *et al.*, 2021).

Z. Huang *et al.*, 2022 identified novel individual metabolites with potential as causal and/or predictive cardiovascular biomarkers in individuals with T2D, specifically in the context of subclinical atherosclerosis and symptomatic atherosclerotic cardiovascular diseases (Huang *et al.*, 2022).

The most exciting results regarding stroke recovery revealed marked disparities in leucine-isoleucine, proline, threonine, glutamic acid, and arginine levels between groups showing good and poor recovery. Notably, proline, glutamic acid, and arginine demonstrated the highest sensitivity and specificity in distinguishing between good and poor recovery groups. The findings suggest a potential link between amino acids related to energy metabolism and excitotoxicity in influencing post-stroke functional recovery. Moreover, arginine emerged as having the most favourable predictive value for assessing the recovery rate after a (Wang *et al.*, 2020).

Literature reports evaluating metabolic shifts at the post-stroke recovery phase in serum are scarce. Furthermore, the strategy reported by our collaborators, as presented in the work by (Karampatsi *et al.*, 2021), involving dietary modifications to normalize glucose metabolism and IR in a T2D mouse model, requires further investigation to better understand its impact on serum metabolite levels.

These findings will increase our knowledge of the reported improvements in functional recovery after stroke and elucidate related outcomes.

Aims of the Thesis

Our major goal is to evaluate the impacts of obesogenic diets on systemic metabolism of a DIO mouse model. By using one-dimension ^{1}H-NMR spectroscopy as the major untargeted metabolomics tool throughout the studies showcased in this thesis, potential biomarkers and metabolic pathways of interest may arise from the results here comprised and become translatable to therapeutic options which may benefit society as a whole.

The specific aim(s) of each paper encompassed by this thesis are discriminated below.

Paper I. Identify metabolic shifts characteristic of DIO and IR in plasma samples of a T2D male mouse model and plasma samples of diabetic and non-diabetic patients from the prospective, population-based cohort "The Malmo Diet and Cancer Study" (MDCS).

Paper II. Assess if treatments with taurine or with NAC could prevent HFD-associated hippocampal metabolite alterations and memory impairment against DIO in a female mouse model.

Paper III. Evaluate potential phenotypical benefits of taurine and *N*-acetylcysteine supplementation against DIO consequences in a female mouse model through NMR of plasma.

Paper IV. Identify potential metabolite biomarkers in a T2D mouse model relevant in the post-stroke recovery phase.

Methodology

The following section provides a summary of the key methods used in the papers included in this thesis. Detailed descriptions of these methods can be found within each respective paper. Furthermore, pertinent statistical information is included in the figure captions in the subsequent sections of this thesis.

Animal Experiments

All animal experiments were conducted in strict accordance with the EU Directive 2010/63/EU. Animal experiments performed in **Papers I**, **II** and **III** were approved by the Malmö/Lund Committee for Animal Experiment Ethics (5123/2021). Animal experiments performed in **Paper IV** were approved the regional ethics committee (approval ID1126, Karolinska Institutet). The Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines were followed.

For **Papers I**, **II** and **III**, aged 8 weeks C57BL/6J mice, were obtained from Taconic Biosciences and housed in groups under controlled conditions. Male mice used in **Paper I** were acclimated to the facility for one week, while female mice used in **Papers II and III** were acclimated for two weeks.

The dietary interventions involved a HFD containing 60% kcal of saturated fat, which induced diet-induced obesity (DIO), and control diet (CD) composed by 10% kcal of saturated fat. Food and water were provided *ad libitum*. The experimental duration varied between 1 and 8 weeks in **Paper I**. In **Papers II** and **III**, dietary and NAC/taurine supplementation experiments occurred simultaneously for 8 weeks.

In **Paper IV**, aged 4 weeks male C57BL/6JRj mice were housed in controlled conditions and subjected to a long-term dietary intervention (a standard diet (SD) vs HFD composed by 60% kcal energy from saturated fat for 10 months). Following this dietary regimen, mice were then subjected to transient middle cerebral artery occlusion (tMCAO) or sham surgery for 30, as previously described (Karampatsi *et al.*, 2021; Augestad *et al.*, 2022).

During the two-month recovery period following the tMCAO or sham procedures, all experimental groups were transitioned to a poststroke balanced diet, consisting of the SD. Blood samples were collected at the experimental endpoint via cardiac puncture and serum was stored at -80°C for analysis.

The Malmö Diet and Cancer Study (MDCS)

Samples from diabetic and non-diabetic patients in the MDCS cohort were utilized in **Paper I** to evaluate the translational applicability of the processing and analysis pipeline that had been optimized in animal experiments. In this study, a cross-sectional analysis was also conducted to investigate the connections between metabolic syndrome parameters and the metabolic variables obtained from the mouse study, employing both MS and NMR methodologies.

Study Background and Participant Details

All participants agreed to participate, and Lund University's Ethics Committee approved the study (LU 51-90).

Cohort description

From 1991 to 1996, a population-based study called "The Malmo Diet and Cancer Study" was conducted in Malmö, Sweden, involving 30,447 individuals born between 1926 and 1945 (Berglund *et al.*, 1993). They provided measurements, blood samples, and completed questionnaires during a baseline examination.

Focus on Cardiovascular Health

To study cardiovascular risk factors and early artery health, a subgroup of 6,103 individuals from the main study was selected. They participated in "The Malmö Diet and Cancer Cardiovascular Cohort," which included measurements, blood samples, and ultrasound examinations of the right carotid artery.

Metabolomics Analysis

Metabolomics analysis, using MS, was performed on plasma samples collected during the baseline examination (1991-96) of this subgroup (Smith *et al.*, 2020, 2022).

Participant Selection and Group Size

28 individuals with prevalent diabetes and 28 without diabetes from this subgroup were randomly chosen for a combined NMR-MS exploratory study. Some samples were used only for MS analysis and not NMR spectroscopy. After quality verifications, the final group size consisted of 23 individuals in each category.

Behavioural Tests

Female mice used in **Paper II** underwent novel object recognition (NOR) and novel location recognition (NLR) tests in a cubic arena (50 × 50 × 50 cm) to assess HFD-induced memory impairment as reported (Garcia-Serrano, Mohr, *et al.*, 2022). To ensure proper acclimatization to the environment, mice spent at least 1 hour in the room before participating in the open field test and object recognition tasks.

Initially, the mice were familiarized with the empty arena for 8 minutes. Their exploration within the arena was monitored and analysed using Any-maze 6 software. Following this, the assessment for NLR involved placing the mice in the arena with two identical objects, allowing them to explore these objects for 5 minutes (familiarization phase). After a 1-hour interval (retention phase), the subjects were reintroduced to the arena for 5 minutes, but with one object relocated to a different quadrant (recognition phase).

Regarding NOR, the familiarization phase employed two identical objects, one of which was substituted with a new object during the recognition phase. The time spent exploring each object was measured. The total distance walked, quadrant crossings, immobility duration, and exploration of the centre of the arena were also recorded.

Magnetic Resonance Spectroscopy (MRS)

MRS was conducted on female mice described in Paper II after receiving 8-week treatments of NAC or taurine, under isoflurane anaesthesia following previously documented procedures (Garcia-Serrano, Mohr, et al., 2022). The administration of isoflurane, delivered through a nose cone at a variable rate between 1% and 2%, ensured a consistent respiratory rate of 60 to 100 breaths per minute. To maintain the body temperature of mice within the range of 36 to 37°C, warm water circulation was maintained throughout the procedure.

The MRS scans were carried out using a preclinical 9.4 T Bruker BioSpec AV III, equipped with a 1H quadrature transmit/receive cryoprobe. The scans utilized STimulated Echo Acquisition Mode with specific parameters: a repetition time of 4 seconds, an echo time of 3 milliseconds, a mixing time of 20 milliseconds, and a spectral width of 4401.41 Hz. Spectra were acquired in a water-suppressed mode, obtained in 20 blocks of 16 scans from a designated volume of interest situated in the dorsal hippocampus (measuring 1.8 mm × 1.2 mm × 1.5 mm).

In addition, an unsuppressed water spectrum from the same volume of interest was obtained in a single block of 16 averages. These spectra were aligned and combined using MATLAB, and the concentrations of metabolites were determined using LCModel v.6.3-1A software.

The analysis through LCModel involved various metabolites. The Cramér–Raolower bound was employed to exclude values surpassing 30%, ensuring the reliability of quantification. Specifically, metabolites such as β-hydroxybutyrate, *N*-acetylaspartylglutamate, glucose, and *scyllo*-inositol were omitted from further analysis. Phosphorylcholine and glycerophosphorylcholine were collectively examined as total choline.

^{1}H-NMR Spectroscopy

^{1}H-NMR Sample Preparation

Plasma or serum metabolites were extracted by mixing plasma or serum and methanol, followed by vortexing and sonication on ice. After centrifugation, supernatants were dried in a Savant SpeedVac concentrator. The dried samples were reconstituted in 100 mmol/L sodium phosphate buffer in D_2O, pH 7.4, complemented with 0.01% NaN_3. Sodium fumarate (0.3 μmol) was used as an internal standard. These samples were then transferred into NMR tubes.

^{1}H-NMR

^{1}H-NMR mouse plasma spectra composing **Papers I**, **II** and **III** were acquired on an 11.7 T Varian VNMRS spectrometer equipped with a 5 mm pulsed field gradient-total relaxation pulse (PFG-TRP) probe and using a Carr–Purcell–Meiboom–Gill sequence with water suppression, spectral width of 8 kHz, acquisition time of 2 s, relaxation delay of 10 s and 520 acquisitions.

^{1}H-NMR human spectra and mouse serum described in **Papers I** and **IV**, respectively, were acquired on a Bruker Avance III HD 14.1 T spectrometer equipped with a standard TCI cryoprobe using the ZGPR pre-saturation pulse sequence for water suppression with a spectral width of 9 kHz, 3 s acquisition time, relaxation delay of 22 s, and 256 acquisitions. In **Paper I**, the number of acquisitions (ranging from 194 to 522) varied according to initial plasma volume to accomplish similar signal-to-noise ratios.

^{1}H-NMR Processing

NMR spectra were processed using MATLAB 2021b with the *General NMR Analysis Toolbox* (GNAT) (Castañar *et al.*, 2018). The fumarate peak was aligned to 6.5 ppm, and phase adjustments were made. The real part of the spectra (0-12 ppm) was retained for analysis. Spectra were aligned using the fumarate peak as a reference.

Spectral points corresponding to residual water, methanol, and specific reference peaks were removed based on their chemical shifts. The remaining spectral data points were normalized to the total spectral area and thresholded at a specific level based on noise measurements between 0 and 0.2 ppm.

Local maxima were used to detect peaks in the spectra. Peaks were assigned to metabolites using Chenomx NMR Suite 9.0 and previously published data (Ala-Korpela, 1995; Sanz-Cortés *et al.*, 2013; Nagana Gowda and Raftery, 2014, 2017b, 2021; Nagana Gowda, Gowda and Raftery, 2015; McHugh *et al.*, 2018).

Plasma taurine determination

Plasma taurine concentration depicted in **Paper II** was determined using NMR Utility Transform Software (NUTS) from Acorn NMR, USA. Free induction decay (FID) signals were processed by applying a 0.2 Hz line broadening to enhance signal quality. Then, a zero-filling function was used to enhance resolution, followed by further signal-to-noise improvement with exponential multiplication. The time domain data was transformed into frequency domain spectra through a complex Fourier Transform. Spectra zero-order phase was adjusted for alignment using a reference signal at 6.50 ppm.

After processing, specific spectral regions for fumarate (6.50 ppm), glucose-α (5.22 ppm), and taurine (3.26 ppm) were analysed. Deconvolution was performed using a line fitting command to fit peaks in the spectrum accurately. This process adjusted multiple parameters, such as frequency, height, width, and line shape. The fitting was optimized through multiple iterations until the error reached its minimum. The resulting fitted lines provided peak areas used to calculate metabolite concentrations. Duplicate analyses were performed for each sample to ensure accuracy and eliminate user-related errors.

MS Spectrometry

Metabolite Extraction and Analysis

Mouse plasma metabolites in **Paper I** were obtained by adding methanol and water to the samples, along with stable isotope-labelled internal standards. After a 30-minute incubation, the samples were spun in a centrifuge, and the supernatant was collected in glass vials.

These extracted samples were then separated using a specific column and analysed using an advanced UHPLC-QTOF-MS system. This system used different solvents for separation, with a changing gradient over time.

MS was performed to analyse the metabolites. Quality control samples were regularly injected to ensure accuracy and reliability in the measurements.

Metabolite Annotation

Metabolites were identified by matching their MS data with The Human Metabolome Database (HMDB) (Wishart *et al.*, 2018) and METLIN (Guijas *et al.*, 2018). Some were also identified using an in-house library of metabolites with known standards.

These annotations were categorized into two levels based on their confidence, as defined by the Metabolomics Standards Initiative (Sumner *et al.*, 2007).

Data Processing

To process the data, a specialized algorithm called "Find by Formula" was used. It employed known molecular formulae and retention times to extract metabolite information from the raw data.

Metabolite intensities were normalized using quality control samples, ensuring the accuracy of the measurements (Dunn *et al.*, 2011).

Data Analysis

Orthogonal projection to latent structures (OPLS) regression of metabolomics data

In **Papers I**, **III**, and **IV**, we employed a consistent analytical approach to examine metabolite data. Z-scores of processed NMR spectral points and MS metabolite concentrations were analysed using SIMCA v.17.0.2 (Umetrics, Umeå, Sweden) through automated OPLS regression.

In **Paper I**, our focus was on diet and time-on-diet for mice and diabetes prevalence for humans. Cross-validation was used to assess predictive power, while permutation tests (100 permutations for each Y variable) validated OPLS regression models. **Paper III** specifically dealt with mouse data from NMR, concentrating on diet and supplementation. Ultimately, **Paper IV** followed a similar protocol.

Variable importance in projection (VIP) scores greater than 1 were considered significant and important for model projection. Model quality was evaluated using cumulative R^2 and cumulative Q^2 obtained from SIMCA.

The OPLS models applied throughout the papers comprising this thesis aligned with guidelines from literature. Specifically, models were validated using permutation tests (100 permutations per each response variable). Briefly, the criteria for avoiding model overfitting (adapted from Mahadevan et al., 2008; Triba et al., 2015) are listed below.

1. All Q^2 values on the permuted data set are lower than the Q^2 value on the actual data set.
2. The regression line (line joining the actual Q^2 point to the centroid of the cluster of permuted Q^2 values) has a negative value of intercept on the y-axis.

Pathway Analyses

In **Papers I**, **III**, and **IV**, metabolite data were subjected to pathway analysis using *MetaboAnalyst* 5.0 (Pang *et al.*, 2021). Variables with VIP scores exceeding 1 were utilized for pathway analysis, considering NMR alone, MS alone, and combined NMR-MS data. This analysis incorporated both the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Small Molecule Pathway Database (SMPDB) as pathway libraries (Frolkis *et al.*, 2010; Jewison *et al.*, 2014; Kanehisa *et al.*, 2021), employing a global test and relative-betweenness centrality parameter for topology analysis.

The approach involved assessing the significance of entire metabolic pathways rather than individual metabolites, with pathway impact scores reflecting their global importance. False-discovery rate (FDR) adjustments were applied to computed P-values to minimize false positive results. Specifically, a minimum impact threshold of 0.1 was defined in **Paper III,** while **in Papers I** and **IV**, pathways of interest were identified when adjusted P-values were less than 0.05.

Results

Paper I

Plasma metabolomics in HFD-fed mice

A combined ^{1}H-NMR and MS metabolomics approach has to potential to increase metabolite coverage in untargeted metabolomics studies. By applying this scarcely reported methodology in a DIO mouse model, short- and long-term effects of HFD intervention were unveiled.

The metabolic characteristics of the mice generally aligned with findings from previous studies (Soares, Duarte and Gruetter, 2018; Soares *et al.*, 2019; Garcia-Serrano, Vieira, *et al.*, 2022). Specifically, the mice fed the HFD in this study developed obesity with glucose intolerance but did not exhibit hyperinsulinemia compared to the mice fed the CD.

The application of OPLS regression to combined NMR and MS data significantly improved the discrimination of the four experimental groups (Figure 1 – A). Rigorous model validation through permutation analysis yielded superior results compared to individual dataset analysis.

In total, 160 NMR and MS variables with VIP scores exceeding 1 were identified (Figure 1 – B). Notable high-scoring metabolites (NMR ppm values in parentheses) include xanthine, hippuric acid, 2-hydroxyisovalerate (2-HIV; 0.827 ppm), ergothioneine, caprylate (0.853 ppm), 1-methylnicotinamide, glucose (3.700 ppm), 2-hydroxybutyrate (0.889 ppm), isobutyrylcarnitine, SAH (4.351 ppm), indoxylsulfuric acid, 2-hydroxy-3-methylvalerate and/or caprate (0.849 ppm), ethylmalonate (0.886 ppm), isovalerate (0.900 ppm), and hydroxycaproic acid. Remarkably, the impact of HFD-induced metabolic changes was more pronounced after 8 weeks compared to 1 week of HFD intervention, as evidenced by z-score differences between HFD and CD.

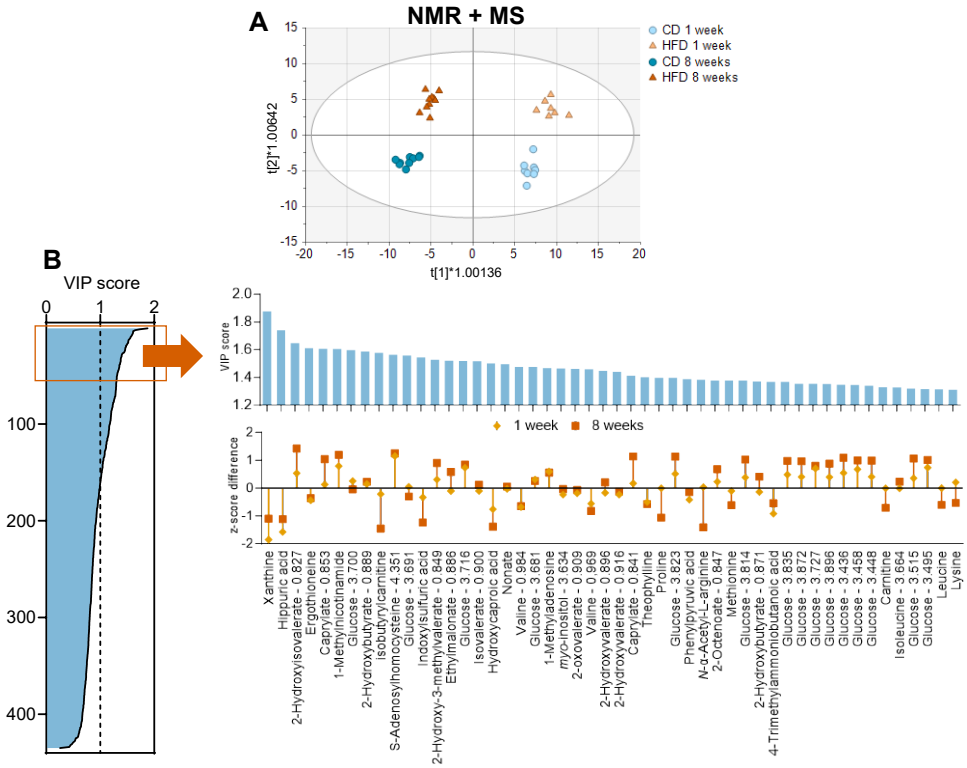


Figure 1 | Multivariate data analysis employing OPLS regression on samples from HFD and CD-fed mice. (A) Plot illustrating group separation with the OPLS score space resulting from the combined analysis of ^{1}H-NMR data and metabolite concentration from MS. (B) Top 50 VIP scores derived from the OPLS model encompassing all MS-identified metabolites and NMR spectral points, with a focus on the top 50 variables exhibiting the highest VIP scores, signifying the most robust discriminators. The calculated z-score differences between HFD and CD at both 1 and 8 weeks are presented alongside their respective VIP scores. Positive and negative values denote HFD-induced concentration increases and decreases, respectively. Variables with z-score differences approaching zero are indicative of potential temporal changes (time-on-diet).

Pathway analysis

Pathway analysis utilized NMR and MS variables with VIP scores exceeding 1. After 1 week of HFD intervention compared to CD, there was a marked influence on purine metabolism. Additionally, pathways related to amino acid metabolism and cellular energy metabolism exhibited discernible impacts induced by the HFD (Figure 2). However, only purine metabolism retained significance following correction for multiple analyses.

Following 8 weeks of HFD feeding, the diet exerted further effects on various pathways, and the prominence of purine metabolism diminished. Specifically, the 8-week HFD intervention significantly perturbed nicotinate and nicotinamide metabolism, pantothenate and coenzyme-A biosynthesis, the degradation of valine,

leucine, and isoleucine, as well as the metabolism of cysteine and methionine. Notably, the integration of MS and NMR data for metabolic pathway analysis revealed a broader spectrum of pathways compared to analysing data from each technique separately.

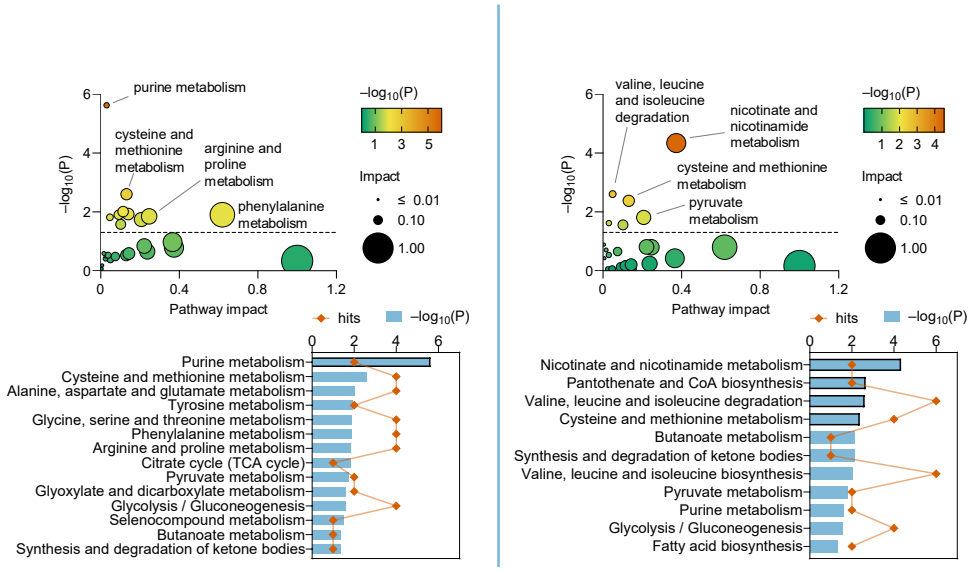


Figure 2 | Metabolic pathway analysis following 1 and 8 weeks of HFD intervention was conducted using MetaboAnalyst with the KEGG database. This analysis exploited z-scores derived from metabolites with VIP > 1 in OPLS regression, utilizing combined MS and NMR spectroscopy data. The pathway impact as determined by MetaboAnalyst, along with their respective significance levels (p-values) is also shown (ball graphs). Dashed lines in the ball graphs signify a p-value of 0.05. Significant findings are highlighted in the bar graphs (blue bars, -log10(P) values), denoting pathways with unadjusted $p < 0.05$. Orange symbols in these graphs represent the number of hits (metabolites analyzed in that pathway). Pathways with a black border around their bars indicate that their significance survives FDR correction. It's worth noting that pathways with an impact score of 0 are not depicted in the ball graphs above but are listed in the bar graphs.

Plasma Metabolomics in Individuals with T2D

The integrated NMR and MS data acquisition, processing, and analysis pipeline, as demonstrated in the animal study, has shown promising potential for translation to human data from the MDCS cohort.

Only partial discrimination of plasma samples of diabetic and non-diabetic subjects was achieved by the used OPLS model. A total of 252 NMR and MS variables with a VIP score exceeding 1 were identified, revealing several prominent peaks that corresponded to glucose signals in NMR spectroscopy (Figure 3).

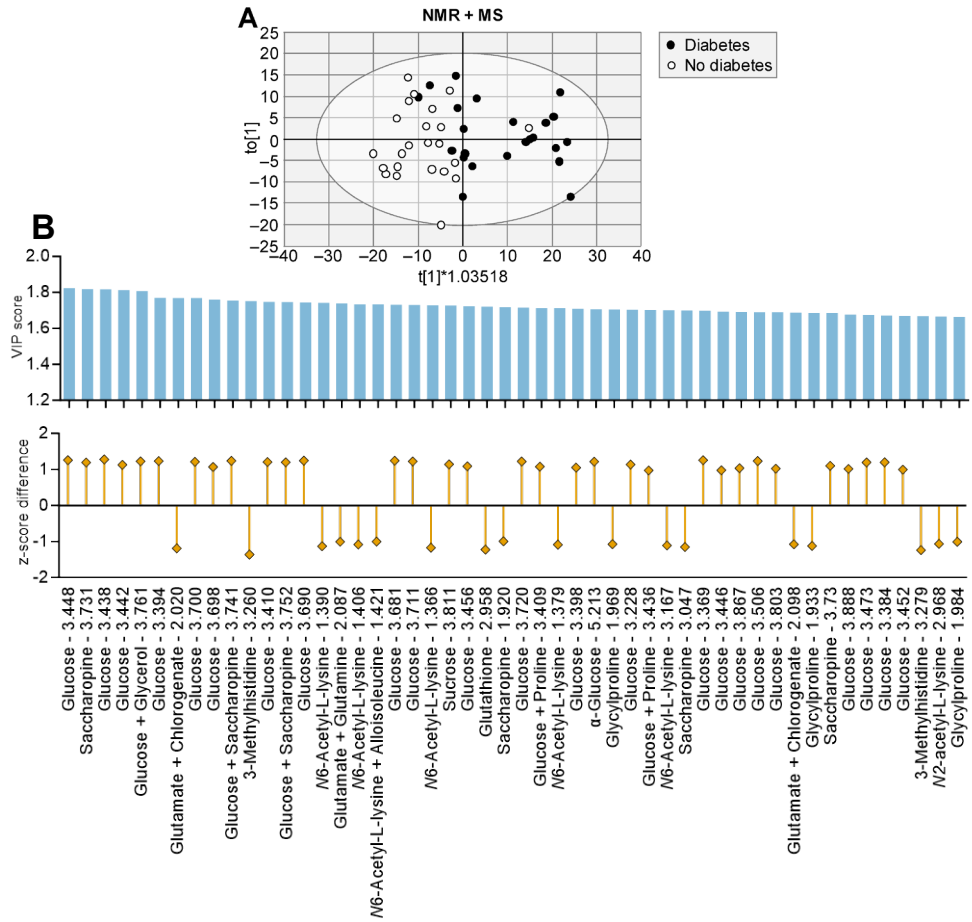


Figure 3 | Multivariate data analysis employing OPLS regression on samples from individuals with and without diabetes. (A) Plot illustrating group separation within the OPLS score space resulting from the combined analysis of ^{1}H-NMR spectroscopy data and metabolite concentrations from MS. (B) Top 50 VIP scores estimated from OPLS model for all MS-determined metabolites and NMR spectral points, highlighting the 50 most discriminative variables, alongside calculated z-score differences between individuals with and without diabetes.

In addition to glucose, the metabolites exhibiting the highest VIP scores, as determined by NMR spectroscopy, were as follows (Table 1).

These metabolites represent significant contributors within the dataset and warrant further investigation for their potential biological relevance.

Table 1 | Non-glucose spectral variables and matching metabolite displaying the highest VIP scores upon OPLS regression of MDCS cohort data. Abbreviations: NADPH, reduced nicotinamide adenine dinucleotide phosphate; UMP, uridine monophosphate.

Metabolite	Chemical shift (ppm)	Metabolite	Chemical shift (ppm)
Saccharopine	3.731	Arginine	1.657
Glutamate + chlorogenate	2.020	3-hydroxyisobutyrate	1.057
Glucose + saccharopine	3.741	4-hydroxybutyrate	3.578
3-methylhistidine	3.260	Butyrate	1.547
N6-acetyl-L-lysine	1.390	Glycocholate	1.064
Glutamate + glutamine	2.087	NADPH	4.049
N6-acetyl-L-lysine + Alloisoleucine	1.421	Methionine	2.153
Sucrose	3.811	3,5-dibromotyrosine	2.872
Glutathione	2.958	2-oxocaproate	1.560
Glycylproline	1.969	5-hydroxylysine	1.550
N-α-acetyl-L-lysine	2.968	Lysine + arginine	1.725
Glutamate	2.041	UMP	3.970
2-oxoisocaproate	2.070	Glutamine	2.141
Lysine	1.883	Alloisoleucine	1.336
N-acetylcysteine	2.049	Dimethylglycine	2.859
Isoleucine	1.497		

Pathway analysis

The pathway analysis unveiled that T2D exerts an influence on a striking 40 out of the 43 metabolic pathways documented in the KEGG database (Figure 4). These encompass all the pathways previously recognized as significantly modified in response to HFD feeding in mice.

Of particular significance are the following pathways, which displayed notable alterations with substantial impact: pyrimidine metabolism, histidine metabolism, alanine, aspartate, and glutamate metabolism, cysteine and methionine metabolism, glutamine and glutamate metabolism, glutathione metabolism, arginine and proline metabolism and arginine biosynthesis.

These identified pathway changes provide valuable insights into the metabolic perturbations associated with T2D, shedding light on potential targets for further research and therapeutic interventions.

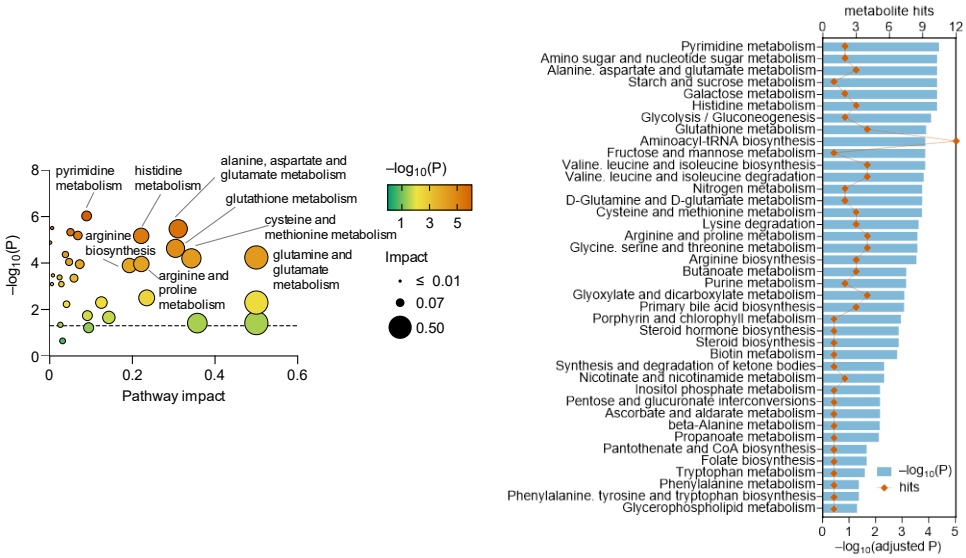


Figure 4 | Metabolic pathway analysis revealing the impact of diabetes on systemic metabolism using MetaboAnalyst and KEGG database: evaluation of z-scores from metabolites with VIP > 1 in OPLS regression combining MS and NMR spectroscopy data. Pathway impact assessed with metaboanalyst, including significance (p-values). Significant outcomes highlighted following FDR adjustment (adjusted $p < 0.05$; bars) and the corresponding number of metabolites analyzed in each pathway (symbols). Pathways with an impact score of 0 are not shown in the ball graph but are listed in the bar graph. A dashed line in the ball graph marks $p = 0.05$.

Cross-sectional analysis between mouse and MDCS data

We conducted a cross-sectional analysis of the MDCS data, examining 34 NMR chemical shifts and MS-measured metabolites to assess their translational potential, as depicted in Figure 5.

Our correlation analysis revealed associations between metabolite levels and parameters related to metabolic syndrome. Notably, impaired glucose homeostasis, as indicated by glycosylated haemoglobin (HbA1c), glucose, insulin, and/or homeostatic model assessment of insulin resistance (HOMA-IR), displayed positive correlations with plasma glucose concentrations and negative correlations with taurine, SAH, oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$), caprylate, arginine, alanine, and 2-HIV.

While somewhat less pronounced, obesity, as indicated by body mass index (BMI) and/or waist circumference, exhibited positive correlations with levels of lactate, kynurenine, homoarginine, glucose, and dimethylguanidino valeric acid (DMGV), and negative correlations with 2-HIV.

Dyslipidemia, characterized by levels of cholesterol, triacylglycerols (TAG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL), also demonstrated correlations with the concentrations of trimethyllysine, taurine, SAH, L-arginine, kynurenine, hippurate, glucose, DMGV, and 2-HIV.

These findings provide valuable insights into the relationships between metabolite profiles and metabolic syndrome parameters, contributing to our understanding of metabolic dysregulation in this context.

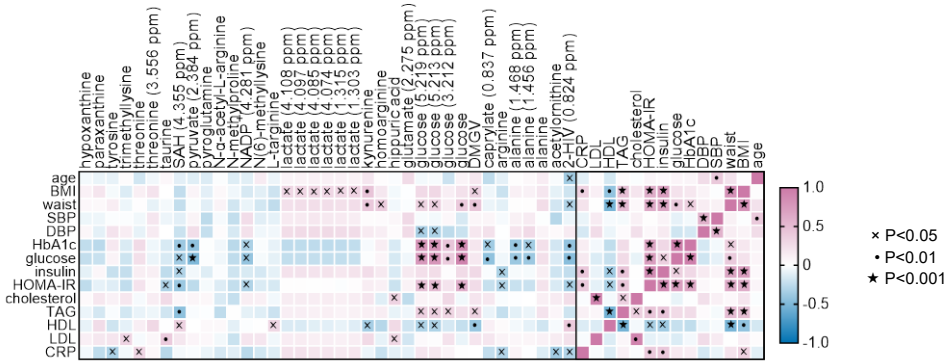


Figure 5 | Analysis of cross-sectional data involving chosen plasma variables among human subjects with and without T2D. Exploratory Pearson correlations examined the relationship between metabolic syndrome parameters and metabolic variables derived from the mouse study utilizing MS and NMR methods. Note that the reported p-values have not undergone correction for multiple comparisons.

Paper II

Taurine and NAC treatments effects in brain function

Obesogenic diets experimentally induce metabolic syndrome, which leads to memory impairment. Prolonged HFD exposure triggers taurine accumulation in the hippocampus when IR is present. We tested the hypothesis that treatment with taurine or NAC could prevent memory impairment and hippocampal changes associated with HFD.

Our findings showed that HFD feeding did indeed lead to memory problems in behavioural tests. Additionally, we observed lower levels of lactate, a reduced phosphocreatine-to-creatine ratio, and a decrease in the neuronal marker *N*-acetylaspartate in the hippocampus. However, treatments with taurine and NAC prevented the memory issues caused by the HFD and also reversed the reduction in *N*-acetylaspartate. Interestingly, NAC, but not taurine, was effective in preventing the decrease in lactate and the phosphocreatine-to-creatine ratio. Magnetic resonance spectroscopy analysis revealed that both NAC and taurine treatments increased glutamate and Gamma-Aminobutyric Acid (GABA) levels in the hippocampus.

Overall, we demonstrated that obesogenic diets lead to memory impairment and specific hippocampal changes. Treatment with taurine and NAC effectively prevented memory deficits and reversed certain neurochemical alterations associated with HFD exposure, highlighting their potential therapeutic value in mitigating the effects of a high-fat diet on cognitive function and hippocampal neurochemistry.

Determinants of memory impairment

A partial least-squares (PLS) regression of a composite variable of memory performance (product of NLR and NOR scores) with hippocampal metabolite concentrations and other independent metabolic parameters was used to identify key predictors of memory performance.

Since hippocampal metabolites and metabolic syndrome parameters were also impacted on taurine and NAC treatments alone, the analysis with the 3-component PLS regression explained less than 50% of the observed variance.

Nevertheless, it allowed good separation of CD and HFD mice, and some level of memory recovery by taurine and NAC treatments can be observed in the distribution of mice in the component space (Figure 6 – A).

VIPs calculated from the PLS model suggest that glucose clearance in GTT, gonadal fat weight, fasting blood glucose and weight gain are important determinants of memory impairment in female mice under HFD (Figure 6 – B).

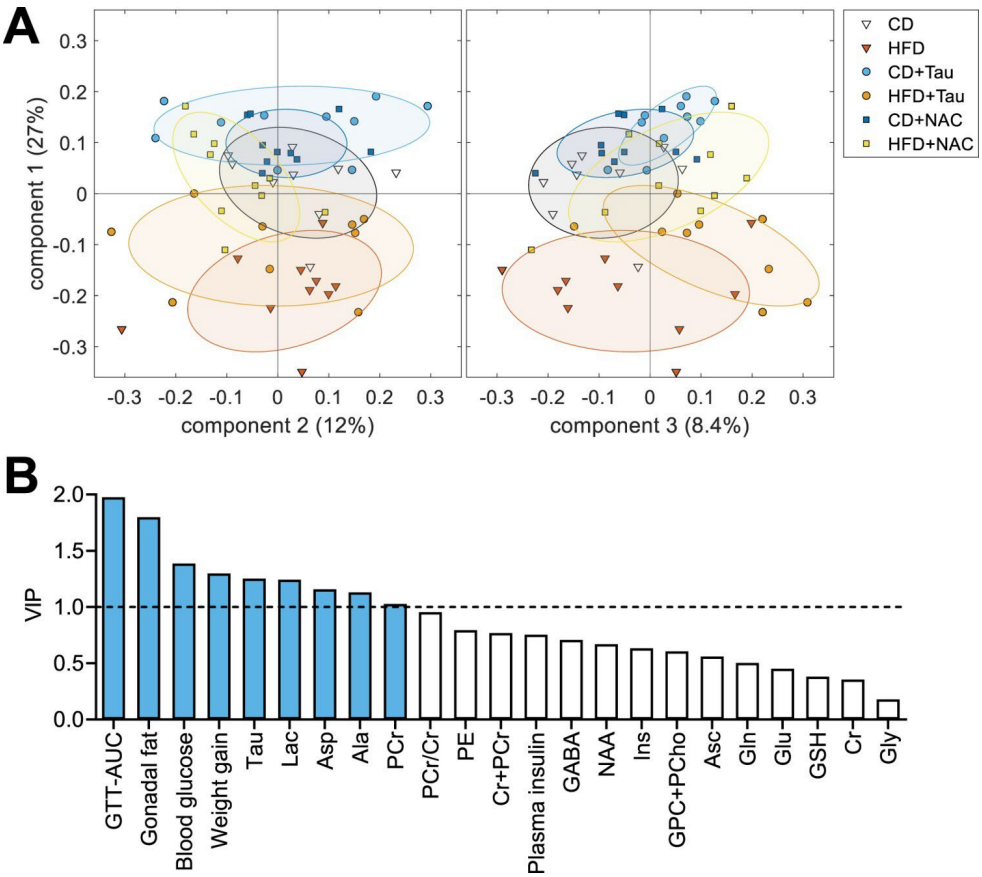


Figure 6 | PLS regression of the metabolite profile and metabolic syndrome parameters to a composite variable of memory performance. (A) component space displaying individual mice (symbols) along with group standard deviation (ellipsoids). (B) VIP scores calculated from the subsequent PLS model.

Effects of HFD and supplementation on phenotype changes

Following the two-month study period, HFD-fed mice developed obesity. NAC treatment effectively prevented it, while taurine treatment did not yield the same results (Figure 7 – A and B).

During glucose tolerance tests, HFD-fed mice across all treatment groups displayed slower glucose clearance compared to their respective control counterparts. However, both taurine and NAC led to improvements in glucose clearance (Figure 7 – C and D).

Interestingly, the HFD did not induce fasting hyperglycaemia or hyperinsulinemia, but it is worth noting that taurine and NAC treatments did cause a slight increase in circulating insulin levels specifically in HFD-fed mice (Figure 7 – E), without a similar effect observed in CD-fed mice.

Notably, taurine accumulation in plasma was only observed in the CD-Taurine treated mice (Figure 7 – F).

Generally, these findings suggested that NAC had a more pronounced and specific effect in counteracting HFD-induced weight gain and fat accumulation compared to taurine in this experimental setting.

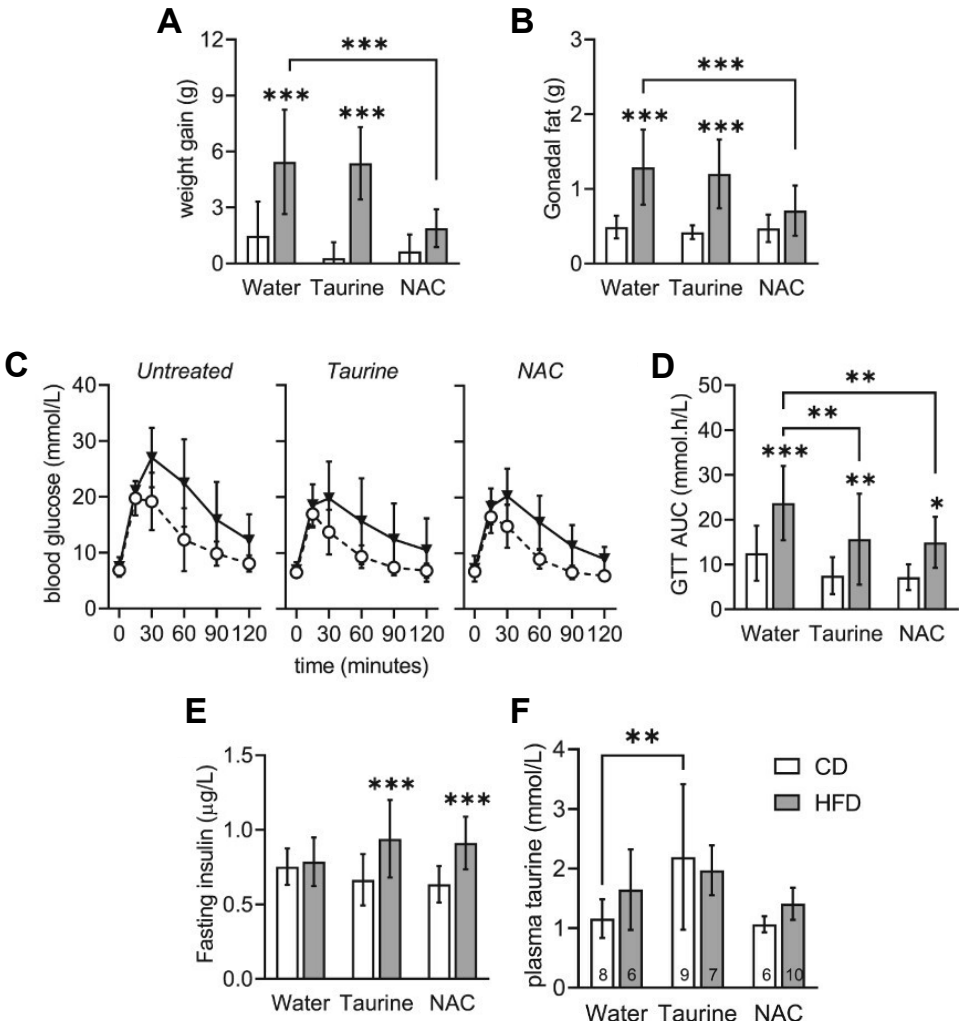


Figure 7 | Effects of HFD and NAC/taurine treatments in phenotypical parameters. Data are presented as mean ± standard deviation. (A) weight gain during treatment. (B) gonadal fat weight. (C) glucose clearance in GTT. (D) area under the curve (AUC) of the GTT. (E) fasting insulin concentrations in plasma. (F) concentration of taurine in plasma. In bar graphs, open bars/symbols correspond to CD-fed mice, while filled bars/symbols represent HFD-fed mice. Statistical significance is denoted as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, determined through Fischer's LSD post-hoc comparison following a significant effect of diet, supplementation, or interaction in ANOVA.

Paper III

Plasma metabolomics in NAC/taurine supplemented female mice

In **Paper II**, we hypothesized that taurine and NAC played a significant role in reversing the changes in female mice caused by HFD. To thoroughly investigate the overall metabolic effects of HFD and the influence of taurine/NAC supplementation in female mice, we conducted an analysis of plasma metabolomics.

In the OPLS scoring space, we observed distinct separation of plasma samples from mice fed a regular CD and those on an HFD. Interestingly, supplementation with either taurine or NAC seemed to induce strong and unique systemic metabolic effects. Specifically, NAC supplementation shifted the clusters of both CD and HFD samples towards the left part of the scoring space, while taurine supplementation shifted sample clusters along t[2] (Figure 8 – A). Moreover, the principal component 1 of the OPLS regression appeared to be indicative of the HFD condition (Figure 8 – B). This dietary stressor indeed had an impact on plasma spectral profiles, and only NAC treatments were able to recover the HFD group to the untreated control levels.

We utilized 227 spectral points for the OPLS regression, with 108 of them having a VIP score greater than 1 (Figure 8 – C). We identified signals from 55 distinct metabolites that played a crucial role in the observed discrimination.

Among these spectral points, those assigned to Glucose + Homoserine (ppm = 3.790) and taurine (ppm = 3.258) had the highest VIP scores (1.513 and 1.487, respectively). For most of these top spectral points, at least one type of supplementation induced noticeable metabolic shifts, either positive or negative, compared to the non-supplemented group.

NAC supplementation led to shifts in uridine and lactate (ppm = 4.124), saccharopine (ppm = 2.371), 4-Carboxyglutamate + *N*-acetylglutamine (ppm = 2.304), choline (ppm = 3.186), and valine + homocystine (ppm = 2.262), which were distinct from the shifts observed in non-supplemented samples. On the other hand, taurine supplementation caused alterations in shifts related to taurine (at ppm = 3.258), glucose + taurine (ppm = 3.414 and 3.402), *cis*-aconitate (ppm = 3.105), *N*-acetyltyrosine (ppm = 3.092), formate (ppm = 8.438), and glucose + trimethylamine *N*-oxide (ppm = 3.246).

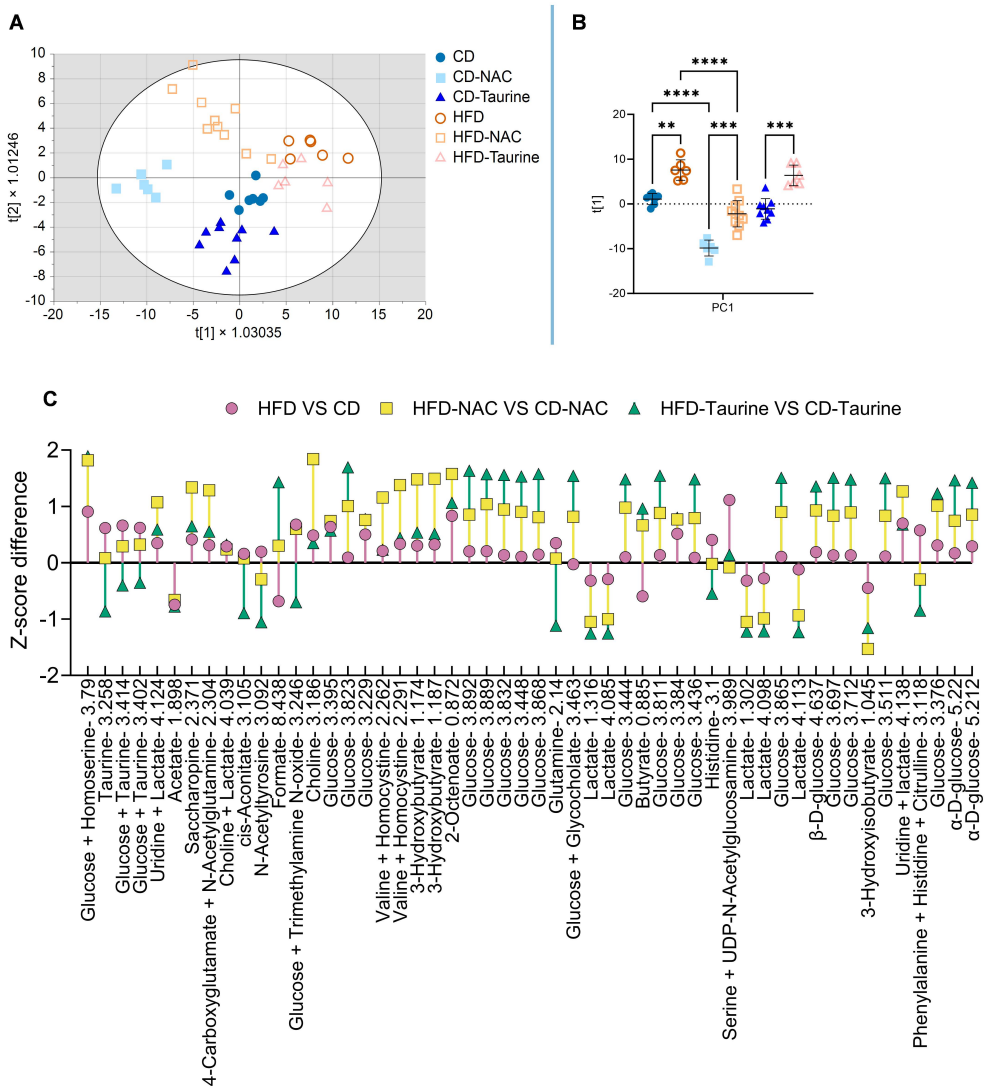


Figure 8 | Multivariate data analysis employing OPLS regression on samples from HFD and CD-fed female mice. (A) Plot illustrating group separation with the OPLS score space resulting from the analysis of ^{1}H-NMR data. (B) Distribution of the experimental groups alongside the first component of the OPLS regression, which seemingly defined HFD effects on the discrimination. (C) Top 52 VIP scores derived from the OPLS model encompassing all NMR spectral points, with a focus on the top 52 variables exhibiting the highest VIP scores, signifying the most robust discriminators. The calculated z-score differences between HFD and CD of untreated and treated groups are presented alongside their respective VIP scores. Positive and negative values denote HFD-induced concentration increases and decreases, respectively.

Furthermore, in the absence of supplementation, two months of HFD led to a significant decrease in phenylalanine levels compared to CD samples. Next, we explored the effects of HFD under the different supplementation strategies. NAC supplementation in HFD- vs CD-fed mice resulted in reduced plasma levels of 3-hydroxyisobutyrate, phenylalanine, and valine, while increasing levels of glucose + homoserine, 2-octenoate, and choline. Taurine supplementation in HFD samples reduced plasma glutamine and creatine content but increased levels of glucose + homoserine, glucose, and caprylate.

Likewise, when comparing CD-NAC or CD-Taurine mice samples to non-supplemented CD samples, both supplementation strategies induced metabolic shifts in the plasma metabolome. CD-NAC samples exhibited decreased levels of glucose and increased plasmatic content of glutamine, 3-hydroxyisobutyrate, and serine + UDP-*N*-acetylglucosamine. CD-Taurine samples showed reduced glucose and acetate levels but increased glutamine, histidine, and taurine plasmatic levels.

Notably, the impact of NAC supplementation on the plasma metabolome of HFD-fed female mice was more pronounced than that of taurine supplementation. While only acetate levels decreased with taurine supplementation, HFD-NAC samples displayed reduced levels of *N*-acetyltyrosine, glucose, glucose + glycocholate, and cystine. Similarly, whereas glucose + ribose shift increased in HFD-Taurine samples, HFD-NAC samples exhibited elevated content of formate, *myo*-inositol, glutamine, glutamate, glucose + ribose, lactate, and ethylmalonate.

Given that these shifts were observed in samples from both CD- and HFD-fed animals, we suggested that NAC supplementation could counteract the dietary effects on plasma spectral profiles. Furthermore, NAC supplementation appeared to have a distinct impact on the plasma metabolome compared to taurine supplementation.

Pathway analysis

Pathway analysis showed no significant metabolic pathway differences between HFD and CD-fed female mice.

However, HFD induced metabolic pathway alterations in NAC and taurine supplementation groups. In the HFD-NAC vs CD-NAC comparison, 15 out of 39 pathways changed, while in CD-Taurine vs HFD-Taurine, 22 out of 39 pathways showed differences (Figure 9).

Remarkably, HFD uniquely influenced pathways related to D-Glutamine and D-glutamate metabolism, alanine, aspartate, and glutamate metabolism, and arginine biosynthesis in taurine-supplementated groups.

On the other hand, the NAC-supplemented groups exhibited notable HFD-induced changes in phenylalanine, tyrosine, and tryptophan biosynthesis, phenylalanine metabolism, tyrosine metabolism, glycine, serine, and threonine metabolism. These pathways were also among the most prominent pathways affected by taurine supplementation.

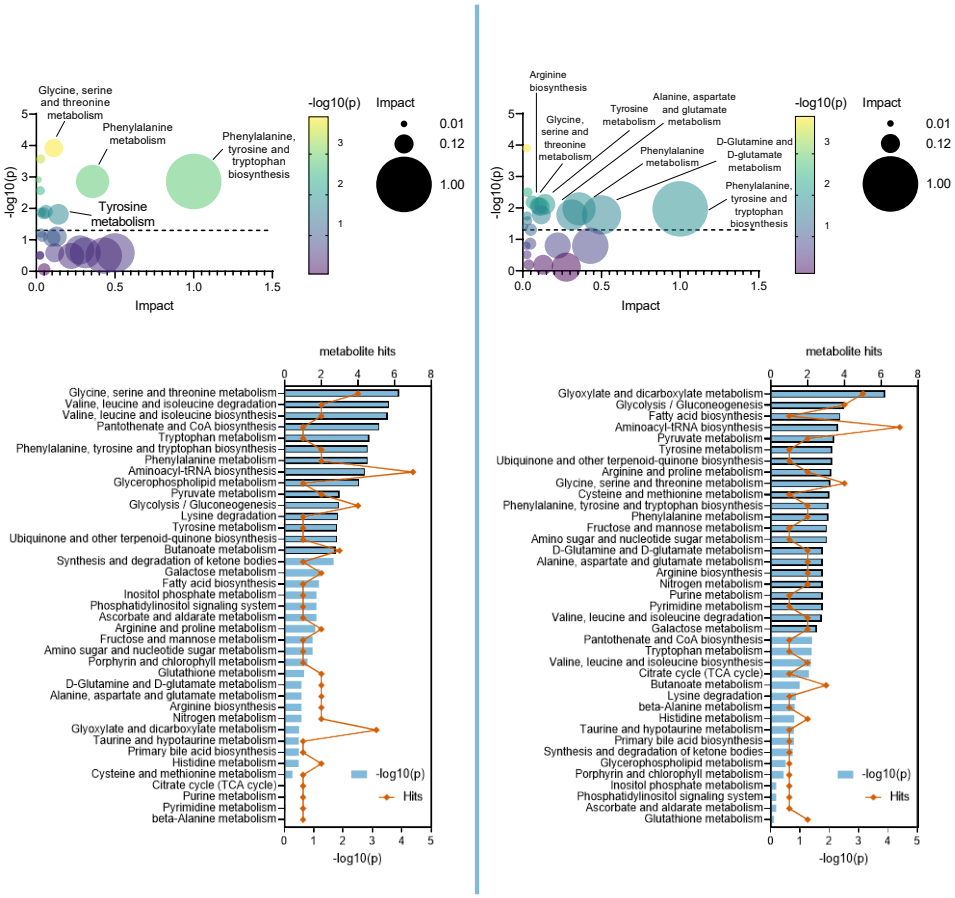


Figure 9 | Metabolic pathway analysis illustrating the effects of NAC or taurine supplementation on systemic metabolism. We utilized MetaboAnalyst with the KEGG database to analyse z-scores derived from metabolites with VIP > 1 in OPLS regression, focusing on NMR spectroscopy data. The ball graphs depict the pathway impact determined by MetaboAnalyst and the associated significance (P-values). Dashed lines in the ball grpahs indicate P = 0.05. The bar graphs specifically showcase significant findings, highlighting pathways with unadjusted P-values < 0.05. Orange symbols and blue colouring represent the number of metabolites analysed within each pathway and -log10(P), respectively. Notably, bars outlined with a black border indicate pathways for which significance withstands false FDR correction.

Both supplements caused substantial pathway changes compared to non-supplemented mice. In CD-NAC and CD-Taurine vs CD groups, 27 and 20 out of 39 pathways were altered, respectively (Figure 10).

Some pathways overlapped, but NAC supplementation significantly affected phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, glutathione metabolism, glycine, serine and threonine metabolism, and arginine and proline metabolism, uniquely.

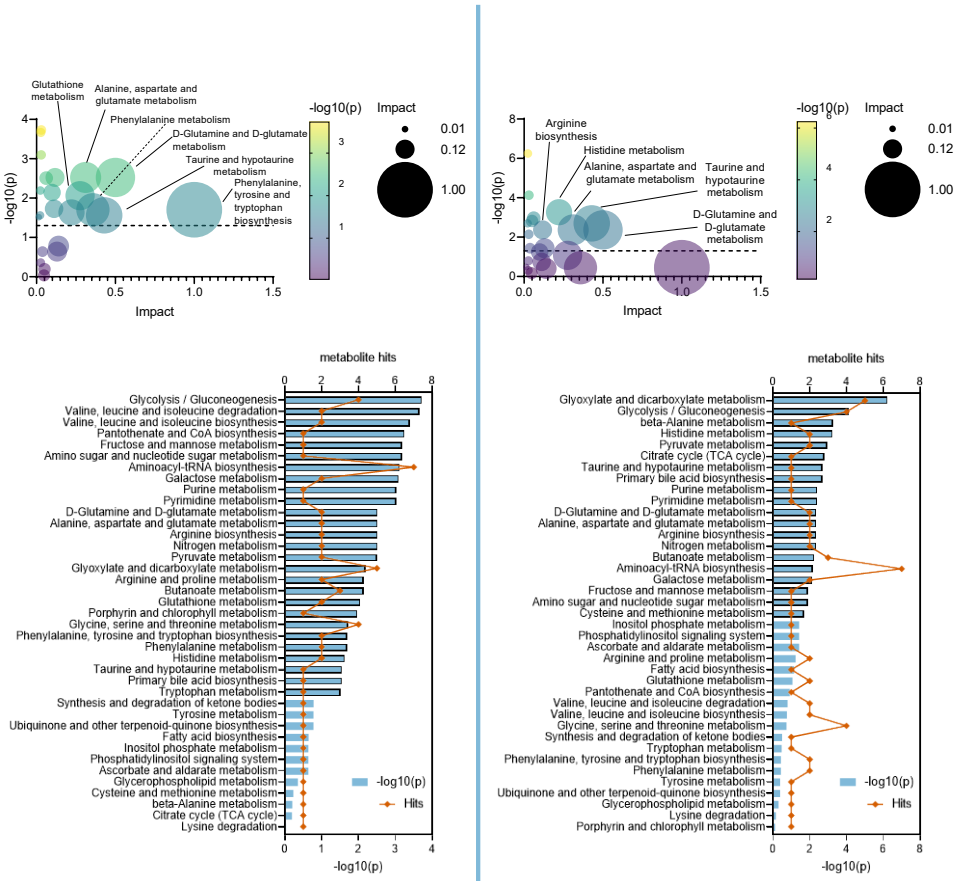


Figure 10 | Metabolic pathway analysis illustrating the impact of NAC or taurine supplementation on control conditions. MetaboAnalyst, utilizing the KEGG database, was employed to analyse z-scores derived from metabolites with VIP > 1 in OPLS regression, focusing on NMR spectroscopy data. The ball graphs present the pathway impact as determined by MetaboAnalyst, along with the respective significance levels (P-values). Dashed lines in the ball graphs indicate a significance level of P = 0.05. The bar graphs specifically highlight significant findings, representing pathways with unadjusted P-values < 0.05. Orange symbols and blue colouring denote the number of metabolites analysed within each pathway and -log10(P), respectively. It's worth noting that bars outlined with a black border indicate pathways for which significance withstands FDR correction.

NAC also induced changes between HFD-NAC and HFD groups, affecting 6 out of 41 pathways, including pterine biosynthesis (Figure 11).

Surprisingly, there were no pathway alterations between HFD-Taurine and HFD groups.

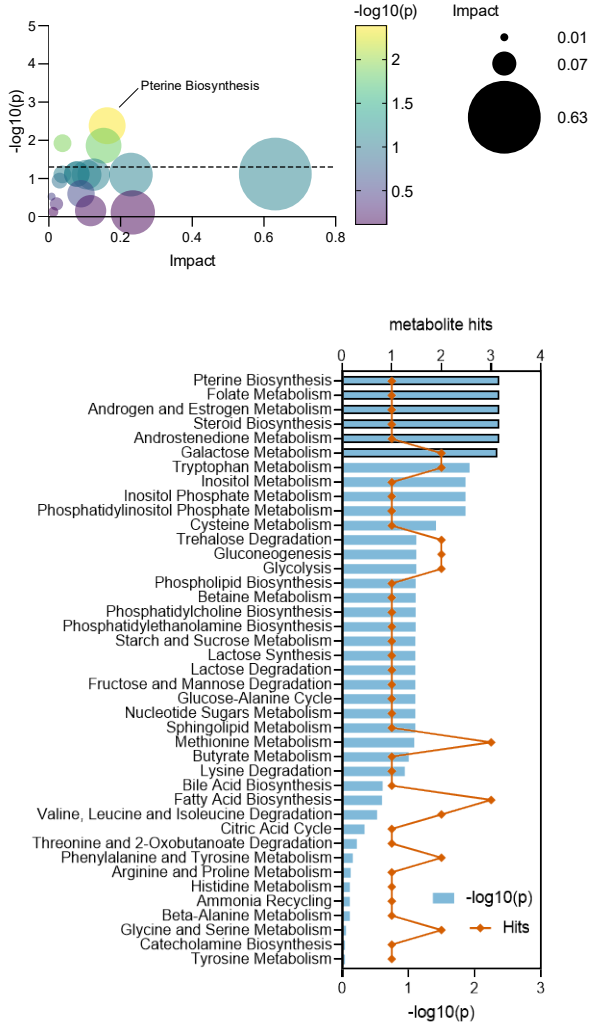


Figure 11 | Metabolic pathway analysis illustrating the impact of NAC supplementation on HFD conditions. MetaboAnalyst, using the SMPDB database, was employed to analyse z-scores derived from metabolites with VIP > 1 in OPLS regression, focusing on NMR spectroscopy data. The ball graph presents the pathway impact determined by MetaboAnalyst, along with the respective significance levels (P-values). Dashed lines in the ball graph indicate a significance level of P = 0.05. In the bar graph, significant findings are specifically highlighted, representing pathways with unadjusted P-values < 0.05. Orange symbols and blue colouring denote the number of metabolites analysed within each pathway and -log10(P), respectively. Notably, bars outlined with a black border indicate pathways for which significance withstands FDR correction.

In summary, HFD effects were reflected by the OPLS model. Metabolic profiling uncovered significantly impacted metabolic pathways. NAC supplementation induced more pathway changes than taurine, suggesting distinct metabolic effects. Taurine supplementation had limited effects in HFD-fed mice.

Paper IV

Underlying T2D condition and stroke recovery

We investigated the impact of obesity and Type 2 Diabetes (T2D) on post-stroke cerebral damage by analysing plasma metabolites. Our aim was to identify biomarkers that shed light on how obesity and T2D influence the recovery process following a stroke.

In our study, we compared the metabolite profiles of T2D mice after stroke recovery with non-diabetic (ND) mice. The OPLS regression analysis applied to NMR data produced a model comprising 1 predictive component and 1 orthogonal component. The OPLS component space demonstrated clear group separation (Figure 12 – A).

A total of 81 chemical shifts exhibited VIP>1 (Figure 12 – B). Notably, the highest VIP scores corresponded to specific metabolites including glycerol, adenine nucleotides, NAD+/NADP+, leucine, isoleucine, valine, butyrate, *O*-acetylcarnitine, homocitrulline, glucuronate, taurine, myo-inositol, or 3-hydroxyisovalerate.

Confirming these metabolite alterations, multiple t-tests comparing average z-scores in HFD-stroke and SD-stroke mice were conducted (Figure 12 – C).

Pathway analysis

Pathway analysis of mice subjected to stroke and affected by Type 2 Diabetes (T2D) revealed various impacted metabolic pathways (Figure 12 D – E).

Notably, the most significantly altered pathways in mice with T2D compared to ND mice after stroke recovery encompassed glutathione metabolism, pentose and glucuronate interconversions, inositol phosphate metabolism and phosphatidylinositol signalling, glycerolipid metabolism, nicotinate and nicotinamide metabolism, as well as ascorbate and aldarate metabolism.

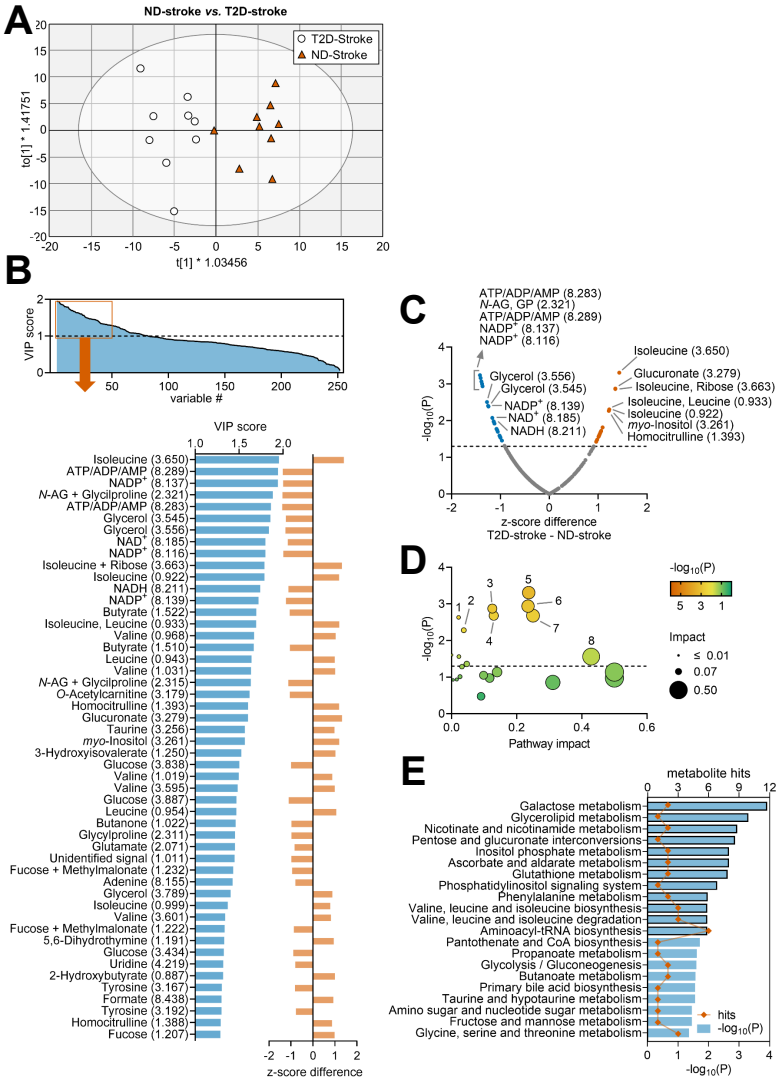


Figure 12 | **Multivariate data analysis using OPLS regression on NMR spectroscopy data obtained from serum samples of ND-stroke mice or T2D-stroke mice.** (A) Group separation in the OPLS score space. (B) VIP scores estimated from the OPLS model, and expansion of the top 50 chemical shifts with highest VIP score presented with the respective z-score difference for T2D-stroke – ND-stroke. (C) Volcano plot showing z-score differences and respective unadjusted P-values resulting from t-tests. (D-E) Metabolic pathway analysis with MetaboAnalyst and the KEGG database on z-scores from metabolites with VIP>1 in OPLS regression. Panel (E) include all pathways with unadjusted P<0.05. Orange symbols and blue bars represent number of hits (metabolites analysed in that pathway) and –log10(P), respectively. Bars highlighted with black border indicate pathways which significance survives FDR correction. Dashed lines in panels D and C indicate P=0.05. Legend in panel (D): 1, Glutathione metabolism; 2, Phosphatidylinositol signaling system; 3, Pentose and glucuronate interconversions; 4, Inositol phosphate metabolism; 5, Glycerolipid metabolism; 6, Nicotinate and nicotinamide metabolism; 7, Ascorbate and aldarate metabolism; 8, Taurine and hypotaurine metabolism. Abbreviations: n-AG, *N*-Acetylglutamine; GP, glycylproline.

Uniquely altered pathways after stroke

From the comprehensive analyses conducted, we were able to discern distinctive metabolic changes induced by Type 2 Diabetes (T2D), stroke, or a combination of both. Interestingly, certain pathways were specifically modified due to the occurrence of a stroke and subsequent recovery, unrelated to T2D. Surprisingly, although stroke led to alterations in several pathways, none of these changes were specifically attributed to T2D-stroke. Comparatively, alterations induced by stroke in ND mice were largely absent when comparing T2D-stroke *versus* T2D-sham (Figure 13 – A). Notably, the KEGG pathways that remained altered in T2D-stroke *versus* T2D-sham mice were the metabolism of arginine and proline, as well as the metabolism of the amino acids alanine, aspartate, glutamate, and glutamine.

Moreover, we delved into identifying metabolite concentrations uniquely affected by stroke, distinct from the influence of T2D and obesity (Figure 13 – B). In ND mice, the unique concentration changes contributing to explaining the effects of stroke were a decrease in isovalerate and an increase in kynurenate, UMP, gluconate, and *N6*-acetyllysine. Conversely, unique concentration changes explaining the effects of stroke in T2D mice were a decrease in *N,N*-dimethylglycine, succinate, and proline, along with an increase in 2-oxocaproate.

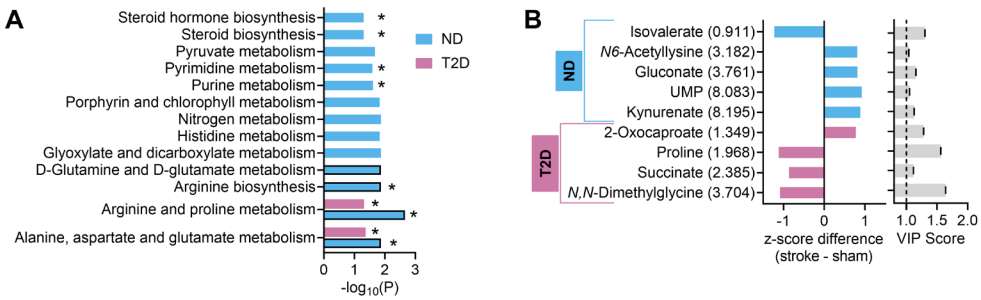


Figure 13 | Identification of distinctively altered pathways (A) and metabolites with VIP>1 (B) in mice with stroke compared to sham controls under conditions of both SD and HFD. (A) Depicts pathways that exhibit significant alterations in stroke vs. sham comparisons, while not displaying significant differences in HFD vs. SD comparisons, are highlighted. Bars outlined with a black border represent pathways for which the significance remains statistically significant after FDR correction. Pathways with an impact score > 0 are denoted by *. (B) Showcases the unique changes in metabolites observed in stroke vs. sham mice, along with their respective VIP scores in the corresponding comparison. Abbreviations: UMP, uridine monophosphate.

Discussion

In **Paper I**, our primary aim was to investigate the temporal effects of HFD feeding and assess the relevance of these findings to diabetic patients. Specifically, we embarked on a journey to compare the early stage/short-term effects *versus* the long-term consequences of HFD feeding using our DIO mouse model.

We had the advantage of conducting both NMR and MS measurements on the same plasma samples. Recognizing that these methods offered valuable insights individually, we chose to adopt a relatively uncommon approach – integrating and analysing data from both tools simultaneously.

This innovative approach resulted in heightened sensitivity and enhanced differentiation between groups in our animal study, which focused on HFD exposure and time-on-diet (or age). Importantly, this approach allowed us to gain a deeper understanding of the metabolic changes induced by HFD.

The three primary metabolites that seemed to contribute to the development of metabolic syndrome during HFD exposure were xanthine, hippurate, and 3-hydroxyisobutyrate (3-HIB).

Notably, our observations revealed that short-term HFD effects primarily perturbed purine metabolism, with noticeable shifts in xanthine levels. The decrease in xanthine levels observed after exposure to a high-fat diet aligns with findings indicating elevated activity of purine catabolism enzymes, namely xanthine oxidoreductase and xanthine oxidase, in the serum of individuals who have obesity, metabolic syndrome, and T2D (Miric *et al.*, 2016; Okuyama *et al.*, 2021; Hernandez-Hernandez *et al.*, 2022).

In contrast, long-term HFD exposure encompassed alterations in branched-chain amino acid metabolism, specifically 3-HIB, as well as changes in cysteine and methionine metabolisms, among others. A connection between 3-HIB and the regulation of glycaemic control has been documented (Yousri *et al.*, 2015; Cobb *et al.*, 2016). An association between plasma 3-HIB levels with T2D has been reported (Mardinoglu *et al.*, 2018). Its role as an adipocyte regulator and involvement in obesity development was also associated with increasements of fatty acid accumulation and reduction of mitochondrial respiration (Nilsen *et al.*, 2020).

Plasma hippurate levels decreased with HFD but did not appear to be linked to diabetes in the MDCS cohort. Indeed, hippurate was connected to higher cholesterol and LDL levels (Figure 5). These differences might be due to variations in the gut microbiome between humans and lab mice. Hippurate is considered a marker of gut microbiome

diversity and a potential risk factor for metabolic syndrome (Pallister *et al.*, 2017; Brial *et al.*, 2021). Interestingly, treating HFD-fed mice 20 nmol/day of hippurate improved glucose tolerance and insulin secretion (Brial *et al.*, 2021).

Moreover, it became evident that HFD-induced metabolic alterations were more pronounced after 8 weeks compared to 1 week of treatment, as indicated by z-score differences between HFD and CD groups (Figure 14).

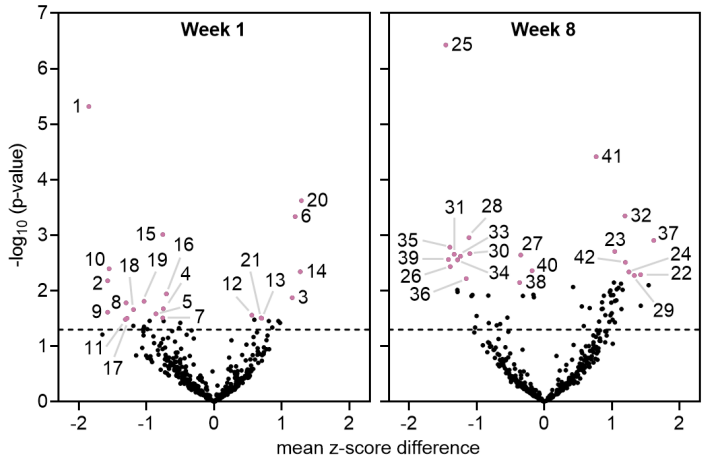
Interestingly, human obesity and overweight are commonly studied using DIO animal models because the dietary challenge promotes the development of a phenotype more similar to human physiopathology (de Moura e Dias *et al.*, 2021).

Therefore, to validate our findings, we focused on the MDCS cohort data, which provided crucial insights. Remarkably, all the metabolic alterations observed in HFD-fed mice were mirrored in individuals with diabetes. In fact, we uncovered a multitude of imbalances in addition to the significant disruptions in glucose metabolism observed in diabetic patients. This was evident from the top 50 VIP metabolite shifts and pathway analyses (Table 1).

We also highlighted the potential role of SAH in modulating metabolic health. SAH is generated during methylation processes and can be broken down into adenosine and homocysteine (Vizán, Di Croce and Aranda, 2021). While adenosine action on the abundant A1 receptors could afford cellular protection, alterations in adenosinergic signalling have been associated with diabetes (Antonioli *et al.*, 2015). On the other hand, elevated homocysteine levels are commonly associated with metabolic disorders and T2D (Huang *et al.*, 2013), and are notably linked to an increased risk of cardiovascular diseases (Herrmann and Herrmann, 2022).

Nonetheless, higher levels of SAH may offer potential health benefits by emulating the positive effects of caloric restriction. This is because increased SAH levels can stimulate the production of *S*-adenosylmethionine, which is believed to have health-promoting properties. This concept is substantiated by research involving *Saccharomyces* cerevisiae (Ogawa *et al.*, 2016).

Furthermore, all the metabolic pathways significantly impacted by HFD feeding in mice were similarly disturbed in individuals with diabetes when compared to healthy controls. These included the cysteine and methionine metabolism pathways, as well as glutathione metabolism, among many others.



Week 1		Week 8	
1	xanthine	22	2-hydroxyisovalerate (0.827 ppm)
2	hippuric acid	23	caprylate(0.853 ppm)
3	S-adenosylhomocysteine (4.351 ppm)	24	S-adenosylhomocysteine (4.351 ppm)
4	acetylornithine	25	indoxylsulfuric acid
5	3-hydroxy-2-methylvalerate	26	N-α-acetyl-L-arginine
6	p-cresol sulfate	27	tyrosine
7	2-hydroxydecanoate	28	phenol sulphate
8	pyruvate (2.389 ppm)	29	glucose (3.214 ppm)
9	2-hydroxybutyric acid	30	N-methylproline
10	cinnamoylglycine	31	lactate (4.089 ppm)
11	alanine (1.458 ppm)	32	pyroglutamine
12	arginine	33	sphingosine 1-phosphate
13	p-cresol glucuronide	34	lactate (4.103 ppm)
14	homoarginine	35	lactate (4.117 ppm)
15	3-phenyllactic acid	36	lactate (1.320 ppm)
16	N(6)-methyllysine	37	L-targinine
17	threonine (3.561 ppm)	38	hyodeoxycholate
18	glutamate (2.278 ppm)	39	hyocholate
19	$NADP^+$ (4.269 ppm)	40	trimethyllysine
20	dimethylguanidino valeric acid	41	kynurenine
21	taurine	42	alanine

Figure 14 | Volcano plots resulting from t-tests comparing z-scores for HFD vs CD. Positive mean differences depict increased concentrations during HFD, while negative mean differences showcase decreased concentrations during HFD (i.e., increased during CD).

Notably, we also observed an increase in circulating plasma taurine levels in mice after just one week of HFD feeding, meaning that changes in taurine concentrations disappear after longer exposure to HFD. This finding aligns with previous research indicating that taurine and its metabolism have potential benefits for metabolic health, including the inhibition of adipogenesis in white adipose tissue (Kim *et al.*, 2019). Therefore, increased taurine concentrations might constitute an early-stage protective response against an HFD insult. Hence, assessing the impact of taurine supplementation might enhance our understanding of these protective mechanisms.

In **Paper II**, we further explored taurine metabolism and its accumulation as a potential compensatory mechanism in female mice. We theorized that augmenting the dietary availability of taurine and enhancing endogenous glutathione synthesis might reduce the risk of cognitive impairment associated with obesity and diabetes.

Overall, our findings demonstrated that both taurine and NAC treatments offer significant benefits for hippocampal metabolism, memory impairment, and metabolic syndrome in mice subjected to an HFD.

As anticipated, taurine treatments resulted in higher taurine levels in both plasma and the hippocampus. Conversely, NAC treatments appeared to be less effective in inducing significant taurine accumulation, especially in plasma. Increased hippocampal taurine concentrations are beneficial, promoting neurogenesis and synaptogenesis (Shivaraj *et al.*, 2012). This observation implies that there is limited taurine synthesis from cysteine derived from NAC.

Taurine treatment exclusively mitigated the HFD-induced reduction in *N*-acetylaspartate levels, a vital marker of neuronal health (Duarte *et al.*, 2012).

NAC supplementation effectively preserved overall hippocampal energy metabolism, preventing the reduction of *N*-acetylaspartate and lactate concentrations, and an unaltered phosphocreatine-to-creatine ratio, despite the influence of HFD.

NAC boosted glutathione levels in the hippocampus of HFD-fed mice, in agreement with a study (Vitvitsky, Garg and Banerjee, 2011) that emphasized the rapid production of glutathione of neurons compared to other metabolites. This heightened synthesis serves as a protective mechanism for the brain against oxidative stress and neuroinflammation (Dwir *et al.*, 2021).

Both supplementation strategies promoted hippocampal glutamate increments. Glutamate is vital for excitatory synapses and memory performance, but proper inhibitory regulation is required for normal neuronal function (Duarte and Xin, 2019).

NAC uniquely promoted the accumulation of GABA, indicating its potential to enhance inhibitory function. Moreover, NAC was the sole treatment that raised glutamine levels in the hippocampus of mice on HFD, a metabolite primarily synthesized by glial cells (Sonnay, Gruetter and Duarte, 2017). This finding suggests that NAC may play a role in modulating the glutamate-glutamine cycle between neurons and astrocytes.

Only NAC treatment prevented obesity development, possibly through the modulation of circulating adipokines, suggesting potential effects on adipose tissue (Shen *et al.*, 2018).

Given this apparent duality of effects, we applied plasma metabolomics as depicted in **Paper III** to gain further insights into the observed systemic observations.

3-HIB is a metabolite derived from branched-chain amino acids (BCAAs) that plays a role in regulating fatty acid transport and IR in animal models (Mardinoglu *et al.*, 2018). This bioactive metabolite has been linked to increased fatty acid uptake, glucose intolerance, and the importance of vascular function in metabolic balance. The secretion of 3-HIB in muscle cells is induced by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a regulator of endothelial fatty acid transport (Jang *et al.*, 2016).

Obesity-related metabolic signatures are often associated with elevated levels of BCAAs (valine, leucine, and isoleucine) and aromatic amino acids (tyrosine, phenylalanine, and tryptophan). Dysregulated BCAAs metabolism can lead to the accumulation of intermediates like branched-chain α-ketoacids and acylcarnitines, which may contribute to lipid peroxidation, oxidative stress, mitochondrial dysfunction, apoptosis, and the activation of stress kinases (Haufe *et al.*, 2016). Valine oxidation and its relationship with acylcarnitine intermediates can offer insights into mitochondrial dynamics (Jensen *et al.*, 2021).

Restricting BCAAs in HFD has been shown to prevent obesity, adipocyte enlargement, adipose tissue inflammation, and IR in mice. This approach maintains body weight, fat and lean mass, subcutaneous and visceral fat mass, as well as serum glucose and insulin levels similar to those in mice on a standard diet, effectively preventing diet-induced obesity (Liu *et al.*, 2022). Given the significance of 3-HIB and BCAAs metabolism in metabolic disorders like IR, it is reasonable to anticipate that HFD-NAC mice may experience favourable outcomes in 3-HIB metabolism, oppositely to male mice after 8 weeks of HFD reported in **Paper I**, which showcased increased concentrations.

NAC treatment in HFD mice caused pathway changes, particularly affecting pterine biosynthesis with formate playing a key role. Formate is crucial for purine synthesis, where increased demand for 1-carbon units may deplete circulating formate levels (Pietzke *et al.*, 2019; Dhayade *et al.*, 2020; Pietzke, Meiser and Vazquez, 2020). This impacts methionine synthesis and methylation reactions through the C1-metabolism pathway (Haag *et al.*, 2013).

Additionally, NAC supplementation induced a beneficial shift in choline metabolism, potentially explaining reduced weight gain in HFD-NAC mice. Choline regulates hepatic lipid secretion, reduces fat accumulation, and alleviates inflammation in adipose tissue (Liu *et al.*, 2023). This aligns with our previous findings in the hippocampus (**Paper II**) and suggests a potential link between NAC, formate, and choline metabolism in combating obesity.

Noteworthily, taurine treatment also impacts systemic metabolism. Between HFD fed mice, taurine supplementation mice revealed decreased acetate concentrations. Acetate production is closely linked to the gut microbiota, which can be influenced by HFD exposure and the subsequent development of IR (Perry *et al.*, 2016). In rats, it has been observed that increased acetate production can potentially activate the parasympathetic nervous system, leading to elevated glucose-stimulated insulin secretion, higher ghrelin secretion, increased appetite, obesity, and related consequences (Perry *et al.*, 2016). Alternatively, dietary acetate supplementation may mitigate HFD-induced obesity in mice by altering the metabolism of taurine-conjugated bile acids. Specifically, in a DIO mouse model, HFD appears to disrupt the balance of bile acids by reducing their production in the liver and increasing their excretion in faeces. Acetate supplementation was found to restore this balance, primarily by promoting the synthesis of taurine-conjugated bile acids in the liver and reducing their excretion in faeces (Wang *et al.*, 2022). Interestingly, it has also been demonstrated in high-cholesterol-fed rats that taurine supplementation can improve cholesterol metabolism by stimulating bile acid production (Murakami *et al.*, 2016).

In the plasma, mice in the CD-NAC group showed a significant increase in glutamate concentrations compared to untreated CD mice, and there was a non-significant trend indicating a glutamate increase in HFD-NAC mice.

In contrast, plasma from HFD-Taurine mice exhibited reduced glutamine concentrations when compared to CD-Taurine mice. Untreated CD mice showed lower glutamine concentrations compared to CD-Taurine and CD-NAC mice. Additionally, HFD-NAC mice showed increased glutamine concentrations compared to untreated HFD mice. This pattern, consistent with our observations in the hippocampus in **Paper II**, suggests that only NAC treatments facilitated this effect.

The changes in glutamate and glutamine concentrations in both plasma and the hippocampus of mice may be interconnected. These amino acids play a role in energy metabolism and are associated with cardiometabolic disorders. High plasma glutamate levels and a low glutamine-to-glutamate ratio have been linked to T2D in a case-cohort study (Liu *et al.*, 2019), while low glutamine levels are expected in the plasma of stroke patients (Jung *et al.*, 2011). However, there appears to be a weaker correlation between metabolite levels in plasma and the brain compared to the correlation between different brain regions in male C57BL/6J mice fed a high-fat diet for 6 weeks. In that study, glutamate and GABA levels were found to decrease due to the high-fat diet (Soto *et al.*, 2018).

Among the treatments, only NAC demonstrated the ability to increase glutamine concentrations in the HFD-NAC group. As a result, we propose that this supplementation may promote a response that enhances metabolic health through systemic metabolism involving glutamate and glutamine in female mice.

Remarkably, both NAC and taurine treatments induced pathway alterations in control conditions, suggesting that the supplementation *per se* being effective. While NAC and taurine induced changes in D-glutamine and D-glutamate metabolism, taurine and hypotaurine metabolism, alanine, aspartate and glutamate metabolism, histidine metabolism and arginine biosynthesis, NAC supplementation uniquely altered phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism and glutathione metabolism.

Our metabolomics approach opened avenues for multi-field applications, and we got the opportunity to apply it in **Paper IV**, where we evaluated changes in the NMR spectral profile of serum metabolites in lean and T2D/obese mice after 2 months of recovery following tMCAO. The experimental design involved prolonged exposure to HFD before tMCAO, leading to obesity, hyperglycaemia, and IR, which are characteristic of T2D (Karampatsi *et al.*, 2021). The goal was to explore the metabolite signatures induced by HFD-induced T2D/obesity during the 2-month recovery phase after a stroke event.

Interestingly, during post-stroke recovery, there were fewer changes in serum metabolites in T2D/obese mice compared to lean mice. Furthermore, the metabolic pathways affected during post-stroke recovery after prolonged HFD feeding did not survive correction for multiple comparisons. Given that T2D/obese mice exhibited worse post-stroke functional recovery (Karampatsi *et al.*, 2021), it suggests that systemic metabolic changes observed in lean mice on a regular diet play a crucial role in enhancing stroke recovery in individuals with T2D.

Among the most significant metabolic pathways uniquely associated with stroke recovery in lean mice were arginine and proline metabolism, as well as the metabolism of the amino acids alanine, aspartate, glutamate, and glutamine. These amino acids are important, yet limited research has explored their role in post-stroke recovery. A recent study found significant differences in proline, arginine, and glutamate levels in the blood of individuals with varying degrees of recovery after stroke (Wang *et al.*, 2020). Additionally, serum arginine levels were predictive of recovery rates after stroke (Wang *et al.*, 2020). Arginine metabolism is involved in nitric oxide production, a pathway that has been proposed to improve cerebral blood flow and functional outcomes after cerebral ischemia (Terpolilli, Moskowitz and Plesnila, 2012).

Increased levels of glutamate, often linked to excitotoxicity and cardiovascular disease risk (Zheng *et al.*, 2016), are reduced in mice recovering from tMCAO, regardless of their pre-stroke diet type. However, these results are based on serum samples taken two months after the stroke event and may not represent the acute phase of stroke, where elevated brain glutamate release could be expected.

Proline metabolism also emerges as a crucial pathway in stroke recovery. Lower proline levels were associated with poorer recovery after stroke in a human study (Wang *et al.*, 2020), and our study found reduced serum proline levels in mice recovering from tMCAO, particularly in T2D mice. This suggests that these pathways identified only in lean mice are likely important for enhancing post-stroke recovery.

Furthermore, specific metabolite alterations were observed after tMCAO in the ND and T2D groups, with no overlap between the two comparisons. For instance, isovalerate levels decreased in ND-stroke mice compared to ND-sham mice, although the relevance of this change to stroke outcomes remains unclear. Notably, levels of N6-acetyllysine were higher in ND-stroke mice, but this change was not observed in HFD-fed mice. Reductions in lysine metabolism metabolites, such as *N6*-acetyllysine, have been reported in individuals at high risk of stroke (Lee *et al.*, 2017).

The kynurenine pathway, which includes neuroprotective kynurenic acid, was more active in ND-stroke mice compared to ND-sham mice. In contrast, metabolites like proline and succinate, associated with poor recovery after stroke, were found at lower levels in T2D-stroke mice compared to T2D-sham mice. Notably, ischemia can lead to the accumulation of succinate, which drives superoxide production upon reperfusion, making it a potential target for reducing brain injury (Mottahedin *et al.*, 2023).

Additionally, *N,N*-dimethylglycine, a product of choline metabolism with neuromodulatory (Lin *et al.*, 2016) and antioxidant properties (Bai *et al.*, 2016), was found at lower levels in T2D-stroke mice. Reduced dimethylglycine levels could contribute to poor recovery from stroke in T2D mice.

Conclusions & Future Perspectives

In summary, this thesis provides valuable insights into the evaluation of how obesogenic diets impact systemic metabolism.

In **Paper I**, specific metabolites were identified, such as xanthine, hippurate, 2-hydroxyisovalerate, SAH, and dimethylguanidino valeric acid, which play a pivotal role in discriminating metabolic disease. Our combined NMR-MS approach has provided valuable insights into metabolic imbalances, offering a more robust analysis compared to individual analytical methods.

In **Paper II**, HFD feeding led to memory impairment, reductions in lactate, phosphocreatine-to-creatine ratio, and *N*-acetylaspartate in the hippocampus. However, taurine and NAC supplementation effectively prevented memory impairment and *N*-acetylaspartate reduction. NAC, in particular, mitigated the reduction of lactate and phosphocreatine-to-creatine ratio. These findings highlight the neuroprotective potential of NAC and taurine in preventing memory impairments and metabolic alterations in HFD-exposed female mice.

In **Paper III**, NAC supplementation induced significant changes in ^{1}H-NMR signals, affecting various metabolites and pathways, distinguishing NAC-supplemented HFD mice from the non-supplemented HFD group. This supplementation had a notable impact on systemic metabolism in female mice, suggesting potential therapeutic implications, particularly related to pterine biosynthesis.

Finally, In **Paper IV**, distinct metabolite changes associated with recovery after tMCAO in mice with and without T2D were identified. These changes may serve as biomarkers for neurophysiological recovery after stroke in T2D individuals. This study provides valuable insights into the unique challenges and recovery mechanisms in T2D-related stroke cases.

Collectively, the research comprising this thesis advances the understanding of metabolic diseases, memory impairments, metabolic alterations, and stroke recovery, opening doors to potential therapeutic applications in various contexts.

Moving forward, conducting targeted metabolomics analyses based on untargeted results presented in this thesis might further validate the reported findings in this thesis.

One promising avenue for future research involves the development of validated and medically certified supplementation therapies to address the consequences of metabolic syndrome.

Additionally, the versatility of the established pipeline holds promise for broader application in the analysis of different biological fluids and the study of various diseases. Particularly, in the realm of neurological complications resulting or aggravated from underlying T2D, such as AD, the application of metabolomics analysis to cerebrospinal fluid offers an exciting opportunity to uncover fresh insights.

Ethical considerations

The pursuit for novel discoveries would have to comply with established ethical guidelines (ALLEA, 2023). Although animal research provides robust pre-clinical data, human sampling might increase the accuracy of the results reduce animal use and suffering.

Patient confidentiality, informed consent, risks, burdens, benefits, and other critical topics outlined in the WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects (Association, 2013) must be rigorously respected and adhered to.

Main limitations

Firstly, in the studies showcased in **Papers I–IV**, we encountered a limitation related to sample size. However, it's crucial to note that each of these investigations aimed at exploring specific aspects of metabolomics, such as metabolic responses to different interventions or the impact of metabolic syndrome in various contexts, which were successfully demonstrated.

Additionally, two of the studies focused on single-sex, either male or female mice, limiting our ability to investigate potential sex-specific differences in metabolic regulation and disease susceptibility.

Notably, addressing this limitation by including both sexes would have reduced the statistical power of these exploratory studies due to observed sex dimorphism in certain metabolic responses (Garcia-Serrano, Mohr, *et al.*, 2022). In males, HFD leads to more severe phenotypical features of metabolic syndrome compared to females. Since females do not develop hyperinsulinemia. The consequences of obesogenic diets in hippocampal metabolism could be assessed without the chronic insulin receptor stimulation.

Moreover, within the shared spectral processing pipeline across **Papers I**, **III** and **IV**, a decrease in the number of variables is accomplished by employing an unbiased strategy using local maxima in the sum of all spectra, after chemical shift alignment, for peak identification. Notably, a key advantage of this approach is its ability to retain signals from metabolites present in low concentrations, thus surpassing the resolution of a binning strategy for processing and condensing ^{1}H-NMR spectral data. (Emwas *et al.*, 2018).

Nevertheless, complex metabolite signals and their substantial overlap in recorded spectra presented challenges, such as signal identification in spectral regions where glucose multiplets were found, among others. Thus, limiting our ability to use overlapping peaks for pathway analysis, as it was challenging to determine which metabolite was responsible for observed signal changes.

As a result, we chose an unbiased analysis of spectral signatures rather than signal quantification to enhance the number of variables for pattern recognition. Future studies could improve peak assignment by incorporating 2D NMR spectra using techniques like total correlation spectroscopy (^{1}H-^{1}H TOCSY). Additionally, considering MS approaches may enhance sensitivity for detecting low-concentration metabolites in serum samples.

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