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High-Field NMR for Metabolic Profiling and Metabolomics

Gilles J P Rautureau

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Université de Lyon - École Normale Supérieure de Lyon

Mémoire en vue de l'obtention
de l'Habilitation à Diriger des Recherches

High-Field NMR for Metabolic Profiling and Metabolomics

Gilles Rautureau

Centre de Résonance Magnétique Nucléaire à Très Hauts Champs
UMR 5082 CNRS, UCBL, ENS Lyon



8 octobre 2021

Examiné par un Jury composé de :

- Pr. Christian Damblon (CREMAN, Liège)
- Pr. Elise Dumont (Laboratoire de Chimie, ENS de Lyon)
- Dr. Bénédicte Elena-Herrmann (IAB, Grenoble)
- Pr. Patrick Giraudeau (CEISAM, Nantes)
- Pr. Sami Jannin (CRMN, Lyon)
- Dr. Hubert Vidal (CarMeN, Lyon)
- Dr. Emmanuelle Vulliet (ISA, Lyon)

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Enfin Baptiste, je me demande comment seraient mes projets scientifiques si nous n'avions pas encadré ensemble ces TP à l'ENS ? radicalement différents et beaucoup plus calmes, c'est certain. Je râle souvent, je n'arrive pas à canaliser ton énergie scientifique, mais au final nous nous complétons !

Je ne cesserai pas de remercier Françoise Vovelle, Laurence Jouvensal, Christian Damblon, Mark Hinds et Neil Ferguson pour m'avoir intégré dans le monde de la recherche. Je me rends compte de l'investissement que cela représente et de la confiance que vous m'avez faite.

Enfin, je voudrais dédier cette thèse à mes amis fidèles et ma famille qui m'ont offert toutes les conditions pour « découvrir le monde » et trouver ma voie. ... Ainsi qu'à tous et à toutes. En espérant que ce travail participe, même modestement, à l'idéal scientifique... mieux connaître le monde pour contribuer à son épanouissement !

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ASC	Adipocyte Stem Cells
BCAA	Branched-Chain Amino Acids
BMI	Body Mass Index
BMRB	BioMagResBank
CFPS	Cell-free protein synthesis
CK	Creatine Kinase
COPD	Chronic Obstructive Pulmonary Disease
CPMG	Carr-Purcell-Meiboom-Gill
CV	Cross Validatory
dDNP	dissolution Dynamic Nuclear Polarization
DMG	Dimethylglycine
DNP	Dynamic Nuclear Polarization
FDR	False Discovery Rate
FFA	Free Fatty Acid`
FID	Free Induction Decay
GC	Gas Chromatography
HBV	hepatitis-B virus
HCA	Hierarchical Clustering Analysis
HFHSD	High-Fat High-Sucrose Diet
HMDB	Human Metabolome Database
HR-MAS	High-Resolution Magic Angle
HSQC	Heteronuclear Single Quantum Coherence
LC	Liquid Chromatography
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation/Time Of Flight
MS	Mass Spectrometry
MVDA	Multivariate Data Analysis
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser effect spectroscopy
O-PLS	Orthogonal Partial Least Squares
PC	Principal Component
PCA	Principal Component Analysis
PLS	Partial Least Squares
RRL	Rabbit ReticulocyteLysate
RT-NMR	Real-Time NMR
SAT	Subcutaneous Adipose Tissue
SD	Standard diet
SOM	Self-Organizing Map
SRV	Statistical Recoupling of Variables
SVM	Support Vector Machines

TCA	Tricarboxylic Acid Cycle
TG	Triglyceride
TOCSY	TOTAL Correlation Spectroscopy
UTI	Urinary Tract Infection
VAT	Visceral Adipose Tissue
WHO	World Health Organization

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1. Curriculum vitae

1.1. Déroulé de carrière

1.1.1. Formation et diplômes

2021 Préparation pour l'habilitation à diriger les recherches. CRMN. ENS de Lyon

2006 Doctorat en Biologie et Biophysique Moléculaires, Bourse MRT. Moniteur à l'Université d'Orléans. Thèse sous la direction de Françoise Vovelle. (11 décembre 2006) "Expression, purification et étude structurale par RMN de PEBP de drosophile". Centre de Biophysique Moléculaire, CNRS d'Orléans.

2003 DEA / Master de Biologie et Biophysique Moléculaires. Université d'Orléans. Mention TB.

2002 Maîtrise de Biochimie Structurale. Université de La Rochelle. Mention TB.

1.1.2. Parcours professionnel

Depuis Septembre 2012 : Maître de Conférences à l'École Normale Supérieure de Lyon

- Partie enseignement : Département de Chimie, ENS de Lyon.
- Partie recherche : Centre de RMN à très hauts champs, 69100 Villeurbanne.

2010-2012: Post-Doctorant.

Conway Institute of Biomolecular & Biomedical Research, Dublin, Irlande – Equipe du Dr Neil Ferguson : Production et étude des protéines du virus de l'hépatite B pour le développement d'agents thérapeutiques nouveaux. Biochimie, Biophysique et Biologie Structurale

2007-2010: Post-Doctorant.

Walter and Eliza Hall Institute for Medical Research, Melbourne, Australie – Equipe du Dr Mark Hinds, Peter Colman : Étude de la structure et des interactions protéine/protéine et protéine/ligand de différentes protéines de la famille Bcl-2 impliquées dans l'apoptose. Biologie Structurale. RMN et cristallographie

2006-2007 : ATER à l'Université d'Orléans

1.2.Situation actuelle

1.2.1. Activités de recherche

Avec une **formation initiale en biochimie et spécialisé dans l'utilisation de la résonance magnétique nucléaire (RMN)** pour l'étude des biomolécules, j'ai démarré ma carrière avec une thèse puis 2 postdocs en biochimie et biologie structurale des protéines, essentiellement par RMN. J'ai ensuite changé de domaine de recherche lors de mon recrutement à l'ENS de Lyon pour m'orienter vers la métabolomique, je travaille actuellement au Centre de RMN à très hauts champs de Lyon (CRMN).

Depuis mon recrutement à l'ENS de Lyon en 2012, et mon intégration à l'équipe de Bénédicte Elena-Herrmann, j'ai participé à un grand nombre de projets de recherche collaboratifs avec des partenaires français et internationaux, privés et publiques en métabolomique par RMN. Biochimiste, je me positionne à l'interface entre la chimie analytique, l'enzymologie des systèmes complexes, la chimiométrie et la biologie pour offrir des nouveaux outils pour des applications biomédicales. Je m'intéresse particulièrement au **développement de méthodes en métabolomique pour des applications dans les thématiques obésité, diabète et syndrome métabolique par exemple**. L'essentiel de mes recherches est réalisé en partenariat avec Baptiste Panthu (MCF UCBL, Faculté de Médecine Lyon Sud) et le laboratoire CarMeN (Cardiovasculaire, Métabolisme, Diabétologie et Nutrition).

En 2018, j'ai intégré le groupe de Sami Jannin, leader dans le développement la polarisation dynamique par dissolution dDNP. Cela m'a permis d'intégrer la dDNP dans mes projets de recherche, notamment pour les approches de métabolomique par **RMN en temps réel**, par exemple pour étudier les réactions métaboliques du sein de mitochondries fraîches et isolées (projet soumis à l'ANR 2021). La dDNP permet un gain substantiel en intensité du signal RMN qui permet l'observation en temps réel de réactions métaboliques fines au sein des mitochondries intacts.

1.2.2. Activités d'enseignement

Je suis Maître de conférences à l'ENS de Lyon depuis septembre 2012. Parallèlement à mes activités de recherche, j'ai assuré un service complet (>220 h eq TD/an), tous les ans depuis mon recrutement, en prenant en compte la décharge des deux premières années). L'intégralité de mon service est réalisée à l'ENS de Lyon et je n'ai pas bénéficié de période CRCT.

J'interviens de la L3 au M2 pour **enseigner la biochimie, notamment des protéines, et la biologie structurale**. J'enseigne à l'interface entre le département de biologie (pour les approches plus chimie/biochimie pour les élèves biologistes) et le département de chimie (pour des aspects plus biologie/biochimie pour des étudiants chimistes).

Depuis mon recrutement en 2012, je me suis impliqué dans l'organisation des maquettes de cours et **je suis maintenant responsable de 4 UE d'enseignement** :

- *UE protéines et protéomique (L3, dpt de Biologie, 34h eq TD)*
- *UE de TP: chimie et biochimie pour les biologistes (L3, dpt de Biologie, 80h de TP)*
- *UE Européenne : Machines Supramoléculaires et cibles thérapeutiques, (co-encadrée avec P. Gouet) (M2, dpt de Biologie, 15h eq TD)*
- *UE Caractérisation structurale et dynamique par RMN du solide (co-encadrée avec depuis 2019 avec S. Jannin), (M2, dpt de Chimie, 15h eq TD)*

J'interviens aussi dans d'autres UE dont je ne suis pas responsable (cours, TD, et TP). Enfin, je participe à la **préparation à l'agrégation** en tant que correcteur et tuteur de leçons et de montages. Ces dernières années je me suis intéressé et formé à la **pédagogie inversée et au e-learning**.

Au niveau des responsabilités au niveau de l'ENS, j'ai été élu au **conseil d'administration de l'ENS de Lyon** comme représentant des personnels enseignants et assimilés, et siégé de 2014 à 2019, et assumé les fonctions liées : conseils restreints, conseils de discipline, commission des finances. Je participe régulièrement aux commissions de recrutement : 2 postes MCF, 7 ATER, 6 AGPR/PRAG. J'ai été **coordinateur et examinateur** pour le concours d'entrée BCPST à l'ENS (2014 à 2017) et je suis actuellement **évaluateur pour le concours d'entrée médecine-sciences**.

Être enseignant à l'ENS et faire partie du CRMN est un privilège que j'apprécie chaque jour mais qui est lié à des attentes importantes, tant du côté enseignement, que du côté recherche. La fonction d'enseignant chercheur est parfois complexe, mais je pense que c'est général pour tous les collègues qui sont tiraillés entre l'enseignement et la recherche ! et c'est dans le fond un bon problème.

1.3. Activité pédagogique

1.3.1. Formation pédagogique.

J'ai suivi une **formation à la pédagogie** durant mon contrat de moniteur à l'Université d'Orléans (2003-2006, CIES d'Orléans-Tours). Cette formation a eu lieu sur 3 ans, avec un ensemble de présentations, séminaires et ateliers, pour un total de 250 heures et l'obtention d'un certificat d'aptitude. J'ai de plus été suivi par un tuteur pédagogique à l'Université d'Orléans. Cette formation, réelle et efficace, m'a aidé à appréhender ma carrière d'enseignant chercheur avec sérénité.

J'ai suivi un **MOOC de formation à l'enseignement supérieur** (« Se former pour enseigner dans le supérieur », 2017), dont l'objectif est de soutenir la formation et l'accompagnement des enseignants-chercheurs du supérieur dans leurs connaissances des processus d'apprentissage et dans leurs pratiques d'enseignement et d'évaluation. Les parties sur la gestion de la **pédagogie inversée** en amphithéâtre et l'utilisation des nouveaux moyens électroniques pour soutenir l'apprentissage actif, notamment grâce aux **solutions de e-learning**, m'ont particulièrement

intéressées. Ces stratégies et outils sont largement développées à l'ENS ce qui permet des échanges pédagogiques fréquents entre collègues.

Ces formations ont eu un impact sur ma manière d'enseigner, tant par rapport à la théorie de l'apprentissage que pour l'utilisation d'outils pédagogiques. Tout cela a été utile lors des 2 périodes de **confinement de l'année 2020**, durant lesquelles j'ai été contraint de basculer mon enseignement vers un enseignement 100% numérique. J'ai réussi à maintenir l'ensemble de mes cours et TD, mais aussi et surtout TP. Les retours et évaluation des étudiants ont été excellents. Je suis convaincu que leur formation n'a pas souffert de cette adaptation des méthodes pédagogiques. Les ressources que j'ai développées seront aussi des outils pour les années qui viennent.

1.3.2. Présentation de l'activité d'enseignement

Les modules les plus importants dans lesquels j'interviens et contribue à l'évolution sont détaillés dans la suite.

1.3.2.1. UE protéines et protéomique (L3, dpt de Biologie, cours et TD)

Il s'agit d'une UE importante pour les étudiants en biologie à l'ENS de Lyon car c'est la seule UE dédiée à biochimie fondamentale dans leur cursus à l'ENS. L'objectif est d'aborder les notions en biochimie des protéines qui sont indispensables pour tous les biologistes, tant au niveau conceptuel que pratique au laboratoire.

Cette UE était initialement pilotée par le Prof. Sylvie Ricard-Blum. J'en suis intégralement responsable depuis 2014. J'assure les 4/5 des cours et je suis aidé par un autre enseignant UCBL (Lionel Ballut, MCF).

Nous couvrons toutes les bases de la biologie structurale, depuis les niveaux d'organisation des protéines, jusqu'aux aspects thermodynamique du repliement. Les relations entre structure, fonction, activité, régulation, mécanisme d'action et dynamique sont explorés pour des conditions normales et pathologiques ainsi que pour les protéines intrinsèquement non repliées. Les techniques d'étude et de protéomique sont mises en avant pour assurer la compétence scientifique des étudiants face aux enjeux actuels de la biologie.

1.3.2.2. UE de TP : chimie et biochimie pour les biologistes (L3, dpt de Biologie, 2 semaines)

Ce TP est à l'origine de mon recrutement à l'ENS. J'en ai pris la responsabilité dès mon recrutement en 2012. Depuis, le TP a été intégralement repensé, pour passer d'un TP d'enzymologie fondamentale, à un **TP d'exploration des techniques de biophysique et d'initiation à l'approche scientifique expérimentale**. Mon objectif est de démontrer aux étudiants qu'un ensemble de techniques autrefois réservées à des biochimistes experts sont maintenant non seulement

accessibles aux biologistes mais aussi indispensables pour répondre aux questions scientifiques modernes de la biologie. Un autre objectif, absolument assumé, est de casser l'image négative et ennuyeuse de ces techniques de biophysique auprès des étudiants biologistes.

En prenant l'exemple concret de la créatine kinase, on analyse les propriétés fonctionnelles et structurales des différentes isoformes, les propriétés d'attachement du substrat au site actif, la cinétique de la catalyse enzymatique à deux substrats, la formation de complexes protéiques, les interactions protéine-membrane... Ainsi, les techniques de biophysique sont introduites, présentées et utilisées directement par les étudiants, parfois au cours de visites au sein de laboratoires de recherche comme l'IBCP ou le CRMN : électrophorèse native, photométrie, fluorimétrie, dispersion de la lumière, microcalorimétrie (DSC), résonance plasmonique de surface (Biacore) et résonance magnétique nucléaire (RMN). L'équipe pédagogique regroupe des chimiste, physicien, biochimiste et biologiste moléculaire pour développer une approche interdisciplinaire des problématiques biologiques présentées abordées.

Ce module remporte un franc succès et il est généralement complet (la jauge est limitée à 12 étudiants, afin de pouvoir réaliser des expériences dans les laboratoires de recherche). **L'approche pédagogique est de laisser les étudiants déterminer eux même les problématiques scientifiques qu'ils souhaitent étudier en utilisant les méthodes disponibles. Ils proposent ensuite une approche expérimentale qui est discutée et validée avant d'être appliquée.** Ils décident complètement des expériences et protocoles à réaliser. Je suis généralement assisté durant le TP d'un moniteur ou d'un ATER, et c'est à cette occasion que j'ai rencontré Baptiste Panthu en 2013.

1.3.2.3. UE Européenne Machines Supramoléculaires et cibles thérapeutiques, (M2, dpt de Biologie).

Les UE européennes du département de biologie se déroulent sur 2 semaines. Chaque jours, 2 présentateurs extérieurs / étrangers présentent sur une ½ journée chacun leur thématique de recherche, en anglais, sous la forme d'un séminaire, légèrement adapté pour améliorer la compréhension à nos étudiants. Les séances sont animées par les étudiants eux-mêmes. Je suis co-responsable de l'UE avec le Prof. Patrice Gouet. Nous avons intégralement construit cette UE et l'organisons depuis 2016.

Les approches génomiques et post-génomiques à haut débit apportent une masse formidable de données sur les génomes de nombreux organismes. Un défi majeur proposé désormais aux biologistes consiste à comprendre ces données au niveau moléculaire, c'est-à-dire à déterminer la structure et les mécanismes biochimiques d'action des différents acteurs : protéines, acides nucléiques, cofacteurs, et les complexes supramoléculaires dynamiques que tous ensemble ils composent.

A ces fins, les outils et approches de la biologie structurale sont essentiels et incontournables. Ils acquièrent continuellement plus de puissance et de facilité d'utilisation, et les résultats qu'ils fournissent sont présents dans une proportion sans cesse croissante des publications. Cette UE

s'appuie sur l'excellence internationale des ressources scientifiques régionales avec le Centre de Résonance Magnétique Nucléaire à très haut champs (CRMN) à Lyon et le synchrotron européen (ESRF) à Grenoble, et présente les techniques les plus modernes de la biologie structurale (RMN, cristallographie, cryo-microscopie électronique, microscopie super-résolution), dont la connaissance des pouvoirs et des limites est désormais indispensable à tout biologiste soucieux de comprendre en profondeur les mécanismes du vivant.

1.3.2.4. UE Caractérisation structurale et dynamique par RMN du solide (co-encadrée avec le S. Jannin), (M2, dpt de Chimie, Cours et TP)

Je suis responsable de ce module depuis 2012, et co-responsable depuis 2019 avec Sami Jannin. Ce cours est destiné aux étudiants de M2 en Chimie de l'ENS et qui ont déjà suivis des cours avancés de RMN. Il se présente sous la forme de cours/TP qui ont lieu au CRMN. Il est en constante adaptation pour suivre l'évolution des techniques en RMN.

L'idée est de présenter aux étudiants **les techniques les plus à la pointe actuellement en RMN**, en se basant sur les défis scientifiques actuels et les solutions mises en œuvre pour y répondre. Après un brainstorming sur les problématiques scientifiques, les techniques sont présentées, discutées et accompagnées de démonstrations directement sur les spectromètres du centre. Le CRMN est une plateforme européenne qui possède un parc d'instruments unique au monde (spectromètre 1 GHz), instrumentation DNP, sondes à rotation ultra rapide pour la RMN du solide etc.), il s'agit d'une belle opportunité pour les étudiants pour voir des équipements de pointe et d'un argument solide pour leur recherche de stages en RMN.

Les domaines concernés vont de la catalyse à l'étude des matériaux en passant par des applications biologiques. Le cours se présente actuellement sous la forme de 5 séances de 3 heures, au CRMN :

- *L'étude des solutions complexes. Application à la métabolomique par RMN.*
- *RMN biologique, solide et liquide pour l'étude des protéines (structure et dynamique).*
- *RMN solide non-biologique, rotation à l'angle magique (catalyse et matériaux).*
- *DNP, polarisation dynamique nucléaire et rotation à l'angle magique (RMN du solide).*
- *DNP par dissolution (RMN liquide).*

1.3.2.5. Répartition et organisation des enseignements.

La répartition de mes activités d'enseignement de cette année universitaire **2020-2021** est détaillée dans le tableau ci-dessous, avec un nombre total d'heures d'enseignement de **218 h** eq TD. L'ensemble de ces heures est réalisé à l'ENS de Lyon :

Département	Formation	Heures (eq TD)	Détails

Chimie/SDM	M2 - Chimie - Caract. struct. par RMN du solide	15	CM-TP
Biologie	M2 Biologie - Molecular and supramolecular machines	15	CM-TD
Biologie	L3 Biologie - S5 TP de biochimie	24	TP
Biologie	L3 Biologie - S6 / Protéines et protéomique	34	CM-TD
Biologie	L3 Biologie S6 - TPs chimie, biochimie pour les biologistes	80	TP
Chimie & Biologie	Préparation à l'agrégation	12	
Chimie/SDM	Tutorat d'étudiants	38	

1.3.2.6. Responsabilités pédagogiques et montage de formations.

Comme mentionné précédemment, je suis **responsable de 4 UE d'enseignement**, et globalement coordinateur de **l'axe biochimie-biophysique-biologie structurale** à l'ENS qui fait l'interface entre les départements de Biologie et Sciences de la Matière. Au-delà de l'enseignement des aspect moléculaires de la biologie, je suis particulièrement impliqué dans **l'élaboration des maquettes d'offre de formation** (je participe actuellement à la 3^e refonte des enseignement et programmes de l'ENS), tant au niveau des département Sciences de la Matière et Biologie de l'ENS de Lyon.

J'assure par ailleurs, comme tous les enseignants permanents de l'ENS, le **tutorat individuel de 6 à 8 étudiants par an**. J'apprécie le contact individuel avec les étudiants, et la possibilité d'interagir avec eux sur des sujets autres que des aspects purement scientifiques. Nous parlons d'orientation, de stages, de stratégie professionnelle qui deviennent exceptionnellement compétitives et difficiles.

Au niveau recherche, outre le fait que j'organise une **UE de RMN pour étudiants en M2** (UE ouverte aux étudiants en chimie de l'UCBL, mais aussi des autres structures et écoles lyonnaises, comme la faculté de Pharmacie), j'ai participé à la mise en place, avec Bénédicte Elena-Herrmann, d'une formation professionnelle dédiée à la métabolomique par RMN, qui a été ouverte de 2015 à 2018 dans le catalogue de **formation CNRS : formation 17292 : RMN HR-MAS pour l'analyse des échantillons biologiques**. Cette formation d'une journée, avait pour objectif pédagogique de permettre d'acquérir les connaissances de base, théoriques et pratiques, spécifiques à l'analyse haute résolution à l'angle magique (HR-MAS) pour l'étude des échantillons semi-solides : tissus biologiques, cellules ou petits organismes intacts, en vue de leur analyse métabolomique.

1.3.3. Diffusion, rayonnement, sociétés savantes.

Je suis impliqué dans les réseaux spécialisés **MARS (Métabolomique Auvergne Rhône-Alpes Suisse Francophone)** et **RFMF (Réseau Francophone de Métabolomique et Fluxomique)** qui mènent de très nombreuses actions (journées scientifiques, écoles chercheurs, ateliers, formations, annuaire des sites & compétences, etc.) couvrant l'ensemble des champs d'applications de la métabolomique. J'interviens régulièrement autant pour l'organisation des conférences et journées scientifiques, que pour la mise en place et l'animation d'ateliers dédiés à la métabolomique par

RMN et au traitement statistique des données obtenues. Enfin je suis membre de la **Metabolomics Society**.

1.3.4. Encadrement d'étudiants

J'ai encadré de nombreux étudiants, depuis le niveau BTS jusqu'en M2 ou École d'ingénieur, de plus en plus indépendamment avec les années. Voici une liste des étudiants que j'ai encadré à 100% depuis mon recrutement à l'ENS.

Nom	Prenom	Début	Fin	Niveau	Duree (semaine)	Titre
Perrin	Marion	01/05/2014	31/07/2014	L3	13	Signature métabolique de mutant d'un recepteur aux hormones thyroide chez la souris
Furster	Eline	05/01/2015	30/05/2015	M1	21	Étude par RMN de l'impact des hormones thyroïdiennes sur le métabolisme hépatique dans des souris porteuses de mutations des récepteurs TR α et/ou TR β des hormones thyroïdiennes spécifiquement dans le cerveau.
Busi	Baptiste	21/09/2015	18/12/2015	M1	13	Etude métabolomique par RMN des conséquences de mutations sur les récepteurs aux hormones thyroïdes.
Perrier	Johan	01/03/2017	01/06/2017	M1	13	Etude de l'effet Warburg et de la lactagénèse par RMN et temps réels
Perrier	Johan	01/10/2017	01/06/2018	M2	35	Etude du role des hexokinase HK2 et HK4 dans les cellules cancéreuses

J'ai été très activement impliqué dans la formation doctorale de plusieurs étudiants :

Johan Perrier, actuellement en 3^e année de thèse (dir: Charles Thivolet/Baptiste Panthu ;). Titre: Metabolic modifications of β cells during glucose and/or palmitate excess). Article: Cell-Free Protein Synthesis Enhancement from Real-Time NMR Metabolite Kinetics: Redirecting Energy Fluxes in Hybrid RRL Systems. Panthu B, Ohlmann T, **Perrier J**, Schlattner U, Jalinot P, Elena-Herrmann B, **Rautureau GJP**. ACS Synthetic Biology. 2017 Oct 2. doi: [10.1021/acssynbio.7b00280](https://doi.org/10.1021/acssynbio.7b00280)

J'ai activement participé à la formation de plusieurs étudiants en thèse dans le groupe de Bénédicte Elena-Herrmann. Notamment pour les thèses de :

Houda Boumaza (soutenance mars 2019), (encadrement 30%) : détermination par RMN d'une signature métabolique liée à une maladie découverte récemment impliquant un récepteur aux hormones thyroïdiennes. J'ai suivi Houda durant toute sa thèse et participé à sa formation tant au niveau RMN que pour l'analyse des données. Article : *Metabolomic Profiling of Body Fluids in Mouse Models Demonstrates that Nuclear Magnetic Resonance Is a Putative Diagnostic Tool for the Presence of Thyroid Hormone Receptor α 1 Mutations*. **Boumaza H**, Markossian S, Busi B, **Rautureau GJP**, Gauthier K, Elena-Herrmann B, Flamant F. *Thyroid*. 2019 Sep;29(9):1327-1335. doi: [10.1089/thy.2018.0730](https://doi.org/10.1089/thy.2018.0730).

Manhal Mili (soutenance décembre 2019) (encadrement 40%). Elle a beaucoup travaillé sur un protocole d'extraction de métabolites et sur un projet d'étude métabolomique de co-cultures d'adipocytes et de cellules stromales. Article : **Mili, M.**, Panthu, B., Madec, A. M., Berger, M. A., **Rautureau, GJP**, Elena-Herrmann, B. (2020). Fast and ergonomic extraction of adherent mammalian cells for NMR-based metabolomics studies. *Analytical and bioanalytical chemistry*, 412(22), 5453–5463. doi : [10.1007/s00216-020-02764-9](https://doi.org/10.1007/s00216-020-02764-9).

Enfin, et de manière significative, j'ai été très impliqué dans la **formation des étudiants en thèse du groupe de Neil Ferguson à Dublin**, lors de mon second postdoc. En effet, le groupe était constitué de 5 étudiants en thèse (**Crispin Alexander, Maike Jurgens, Maggie O'Connor, Annika Urbanek, Judith Voros**), Neil Ferguson et moi. J'ai été particulièrement sollicité pour encadrer l'équipe. Durant 2 années, j'ai assuré le **suivi quotidien et la formation pratique en laboratoire des 5 thésards simultanément (encadrement 50%)**.

- *Articles 1: Large-scale production and structural and biophysical characterizations of the human hepatitis B virus polymerase. **Vörös J*, Urbanek A*, Rautureau GJ***, O'Connor M, Fisher HC, Ashcroft AE, Ferguson N. (* Contributed equally) *Journal of Virology*. 2014 Mar; 88(5): 2584–2599. doi: [10.1128/JVI.02575-13](https://doi.org/10.1128/JVI.02575-13). Spotlight: Articles of Significant Interest Selected by the Editors.*
- *Article 2: The hepatitis B virus preS1 domain hijacks host trafficking proteins by motif mimicry. **Jürgens MC, Vörös J, Rautureau GJ**, Shepherd DA, Pye VE, Muldoon J, Johnson CM, Ashcroft AE, Freund SM, Ferguson N. *Nature Chemical Biology*, 2013 Sep;9(9):540-7. doi: [10.1038/nchembio.1294](https://doi.org/10.1038/nchembio.1294)*

1.4. Contrats de recherche

Je suis intervenu dans un grand nombre de projets de métabolomique appliqués, dont voici une liste des dernières années (R : coordinateur, P : partenaire, budget total/budget dédié)

R : Émergence ENS. 2020. Coupler dDNP et analyses par RMN en temps réel. 20/20 k€.

R : Convergence ISA. 2017 – soutien pour les analyses par RMN en temps réel 2017. 10/10 k€.

P : Olga Triballat (Baptiste Panthu, CarMeN). 2019. Études d'exosomes de jus de fruits, 70/5 k€.

P : INSERM (CHU de Rouen). 2018-2019. Caractérisation d'un nouveau modèle animal. 370/5 k€

P : SINFONI INSERM (Cyrielle Caussy, CarMeN). 2018-2022. Microbiote et obésité. 320/40 k€.

P : SFD (Baptiste Panthu, CarMeN). 2018-2021. Glycine, Metformine et obésité. 30/15 k€.

P : Glofood INRA (Anne Mey, CarMeN). 2017. Rôle des cellules souches dans l'obésité. 35/5 k€

P : ANR Thyromut (F. Flamant, IGFL). 2015-2019. Étude d'une maladie due à la mutation d'un récepteur aux hormones thyroïde, TRa. 482/115 k€.

P : Biomérieux – 2014 (B. Elena-Herrmann). Identification de bactéries responsables d'infections urinaires. 85/85 k€

1.5. Liste des publications

Mon profil de recherche ORCID est disponible : <https://orcid.org/0000-0002-1064-0293>

1. *Dietary obesity in mice is associated with lipid deposition and metabolic shifts in the lungs sharing features with the liver.*

Rautureau GJP, Morio B, Guibert S, Lefevre, Perrier J, Alves A, Chauvin MA, Pinteaur C, Monet MA, Godet M, Madec AM, Rieusset J, Mey A, Panthu B.

Scientific Reports volume 11, Article number: 8712 (2021) doi : [10.1038/s41598-021-88097-8](https://doi.org/10.1038/s41598-021-88097-8)

This article demonstrates how the determination of individual organ metabolite profiles allows to evidence metabolic shifts in organs and lipid deposition in the lungs of mice fed an obesogenic diet. This unprecedented observation may be important for the observed prevalence of lung diseases among obese patients. From a technical point of view, this study presents results from a physiological compartment that is rarely examined at the metabolomics level. Those results nourish a discussion of the interconnection between compartments and the integrated observations that can be done on central biofluids such as serum or urine.

2. *A hexokinase isoenzyme switch in human liver cancer cells promotes lipogenesis and enhances innate immunity.*

Perrin-Cocon L, Vidalain PO, Jacquemin C, Aublin-Gex A, Olmstead K, Panthu B, **Rautureau GJP**, André P, Nyczka P, Hütt MT, Amoedo N, Rossignol R, Filipp FV, Lotteau V, Diaz O.

Commun Biol. 2021 Feb 16;4(1):217. doi: [10.1038/s42003-021-01749-3](https://doi.org/10.1038/s42003-021-01749-3).

3. *Metabolomic Approaches to Study Chemical Exposure-Related Metabolism Alterations in Mammalian Cell Cultures.*

Balcerzyk A, Damblon C, Elena-Herrmann B, Panthu B, **Rautureau GJP**.

International Journal of Molecular Sciences. 2020;21(18):E6843. doi:[10.3390/ijms21186843](https://doi.org/10.3390/ijms21186843)

4. *Use of Nanovesicles from Orange Juice to Reverse Diet-Induced Gut Modifications in Diet-Induced Obese Mice.*

Berger E, Colosetti P, Jalabert A, Meugnier E, Wiklander OPB, Jouhet J, Errazurig-Cerda E, Chanon S, Gupta D, **Rautureau GJP**, Geloën A, El-Andaloussi S, Panthu B, Rieusset J, Rome S.

Molecular therapy. Methods & clinical development, 18, 880–892. doi : [10.1016/j.omtm.2020.08.009](https://doi.org/10.1016/j.omtm.2020.08.009)

5. *Fast and ergonomic extraction of adherent mammalian cells for NMR-based metabolomics studies.*

Mili M, Panthu B, Madec AM, Berger MA, **Rautureau GJP**, Elena-Herrmann B (2020).

Analytical and bioanalytical chemistry, 412(22), 5453–5463. doi : [10.1007/s00216-020-02764-9](https://doi.org/10.1007/s00216-020-02764-9)

6. *Discrimination of Escherichia coli and Shigella spp. by Nuclear Magnetic Resonance Based Metabolomic Characterization of Culture Media.*

Rautureau GJP, Palama T, Canard I, Mirande C, Chatellier S, van Belkum A, Elena-Herrmann B. *ACS Infectious Diseases*. 2019 Nov 8;5(11):1879-1886. doi: [10.1021/acsinfecdis.9b00199](https://doi.org/10.1021/acsinfecdis.9b00199).

This metabolomics study presents an NMR-based strategy for bacterial identification. We demonstrate, using hard to discriminate bacteria species, that the media bacteria metabolites consumption or liberation profiles during culture constitutes an excellent signature that can be used to discriminate bacteria species. This article emphasizes the potential of NMR to deliver methods that are relevant not only for fundamental studies but also for medical and technological applications, in this case, microbial identification.

7. *Metabolomic Profiling of Body Fluids in Mouse Models Demonstrates that Nuclear Magnetic Resonance Is a Putative Diagnostic Tool for the Presence of Thyroid Hormone Receptor $\alpha 1$ Mutations.*

Boumaza H, Markossian S, Busi B, **Rautureau GJP**, Gauthier K, Elena-Herrmann B, Flamant F. *Thyroid*. 2019 Sep;29(9):1327-1335. doi: [10.1089/thy.2018.0730](https://doi.org/10.1089/thy.2018.0730).

8. *Metabolic phenotyping of adipose derived stem cells reveals a unique signature and intrinsic differences between fat pads.*

Lefevre C, Panthu B, Naville D, Guibert S, Pinteur C, Elena-Herrmann B, Vidal H, **Rautureau GJP**, Mey A. *Stem Cells International*, vol. 2019. doi: [10.1155/2019/9323864](https://doi.org/10.1155/2019/9323864).

We characterized the metabolic differences between stem cells derived from 2 types of adipose tissues (subcutaneous adipose tissues (SAT) and visceral adipose tissues (VAT)). This discovery is important in the field of stem cells and regenerative medicine as adipose tissues are an abundant source of stem cells with important medical applications. Stem cells from the 2 types of adipose tissues present significant metabolic differences which may be related to their different physiological and differentiation properties. This study emphasizes the potential of NMR for the discrimination and characterization of very similar biological objects.

9. *Cell-Free Protein Synthesis Enhancement from Real-Time NMR Metabolite Kinetics: Redirecting Energy Fluxes in Hybrid RRL Systems.*

Panthu B, Ohlmann T, Perrier J, Schlattner U, Jalinot P, Elena-Herrmann B, **Rautureau GJP**. *ACS Synthetic Biology*. 2017 Oct 2. doi: [10.1021/acssynbio.7b00280](https://doi.org/10.1021/acssynbio.7b00280)

This article describes real-time NMR development for metabolic reaction measurements. This was my first scientific study being the principal investigator, from the project establishment to running experiments and writing the final article. The NMR methodology was completely novel in the institute. This article delivers important results for the field of mammalian cell-free protein expression technologies. This article also presents an overlooked NMR method that can deliver crucial results for metabolic profiling in complex media.

10. *Identification of Bacterial Species by Untargeted NMR Spectroscopy of the Exo-metabolome.*
Palama TL, Canard I, **Rautureau GJP**, Mirande C, Chatellier S, Elena-Herrmann B.
Analyst, 2016 Aug 7;141(15):4558-61. doi: [10.1039/c6an00393a](https://doi.org/10.1039/c6an00393a)
11. *Large-scale production and structural and biophysical characterizations of the human hepatitis B virus polymerase.*
Vörös J*, Urbanek A*, **Rautureau GJ***, O'Connor M, Fisher HC, Ashcroft AE, Ferguson N. (* Contributed equally)
Journal of Virology. 2014 Mar; 88(5): 2584–2599. doi: [10.1128/JVI.02575-13](https://doi.org/10.1128/JVI.02575-13)
Spotlight: Articles of Significant Interest Selected from This Issue by the Editors.
- This article presents the production method and characterization of HBV polymerase, the key therapeutic target for hepatitis B. Despite 20 years of research, both by academic and private groups, the protein was believed to be impossible to produce recombinantly, blocking the development of targeted viral inhibitors and structural biology studies. Using state-of-the-art automated approaches, we managed to produce for the first time a form of the HBV polymerase that is both soluble and active. This constitutes a very important step forward in the field of hepatitis B research. This paper finalized a 2-years postdoctoral research project in Dublin (I was in the field of structural biology until my recruitment at the ENS).
12. *The hepatitis B virus preS1 domain hijacks host trafficking proteins by motif mimicry.*
Jürgens MC, Vörös J, **Rautureau GJ**, Shepherd DA, Pye VE, Muldoon J, Johnson CM, Ashcroft AE, Freund SM, Ferguson N.
Nature Chemical Biology, 2013 Sep;9(9):540-7. doi: [10.1038/nchembio.1294](https://doi.org/10.1038/nchembio.1294)
13. *The restricted binding repertoire of Bcl-B leaves Bim as the universal BH3-only prosurvival Bcl-2 protein antagonist.*
Rautureau GJ, Yabal M, Yang H, Huang DC, Kvensakul M, Hinds MG.
Cell Death & Disease, Nature publishing. 2012 Dec 13;3:e443. doi: [10.1038/cddis.2012.178](https://doi.org/10.1038/cddis.2012.178).
14. *Detection of Bcl-2 family member Bcl-G in mouse tissues using new monoclonal antibodies.*
Giam M, Mintern JD, **Rautureau GJ**, Hinds MG, Strasser A, Bouillet P.
Cell Death & Disease, Nature publishing. 2012 Aug 23;3:e378. doi: [10.1038/cddis.2012.117](https://doi.org/10.1038/cddis.2012.117).
15. *CARD-mediated autoinhibition of cIAP1's E3 ligase activity suppresses cell proliferation and migration.*
Lopez J, John SW, Tenev T, **Rautureau GJ**, Hinds MG, Francalanci F, Wilson R, Broemer M, Santoro MM, Day CL, Meier P.
Molecular Cell. 2011 Jun 10;42(5):569-83. doi: [10.1016/j.molcel.2011.04.008](https://doi.org/10.1016/j.molcel.2011.04.008).
16. *Intrinsically Disordered Proteins in Bcl-2 Regulated Apoptosis.*
Rautureau GJP, Day CL and Hinds MG.

International Journal of Molecular Sciences. 2010 Apr 16;11(4):1808-24. doi: [10.3390/ijms11041808](https://doi.org/10.3390/ijms11041808).

17. *The Structure of Boo/Diva Reveals a Divergent Bcl-2 Protein.*

Rautureau GJP, Day CL, Hinds MG.

Proteins: Structure, Function, and Bioinformatics. 2010 Jul;78(9):2181-6. doi: [10.1002/prot.22728](https://doi.org/10.1002/prot.22728).

18. *NMR structure of a Phosphatidyl-ethanolamine Binding Protein from drosophila.*

Rautureau GJP, Vovelle F, Schoentgen F, Decoville M, Locker D, Damblon C and Jouvensal L.

Proteins: Structure, Function, and Bioinformatics. 2010 May 1;78(6):1606-10. doi: [10.1002/prot.22682](https://doi.org/10.1002/prot.22682).

19. *Solution structure of Psb27 from cyanobacterial photosystem II.*

Mabbitt PD, **Rautureau GJP**, Day CL, Wilbanks SM, Eaton-Rye JJ, Hinds MG.

Biochemistry. 2009 Sep 22;48(37):8771-3. doi: [10.1021/bi901309c](https://doi.org/10.1021/bi901309c).

20. *Expression and characterization of the PEBP homolog genes from Drosophila.*

Rautureau GJP, Jouvensal L, Vovelle F, Schoentgen F, Locker D, Decoville M.

Archives of Insect Biochemistry and Physiology. 2009 Jun;71(2):55-69. doi: [10.1002/arch.20300](https://doi.org/10.1002/arch.20300).

21. *¹H, ¹⁵N and ¹³C Resonance Assignments of CG7054, a new PEBP from Drosophila melanogaster.*

Rautureau GJP, Jouvensal L, Schoentgen F, Vovelle F.

Journal of Biomolecular NMR. 2007 Jun;38(2):187. DOI: [10.1007/s10858-006-9112-z](https://doi.org/10.1007/s10858-006-9112-z)

22. *Cloning, high yield over-expression, purification, and characterization of CG18594, a new PEBP/RKIP family member from Drosophila melanogaster.*

Rautureau GJP, Jouvensal L, Decoville M, Locker D, Vovelle F, Schoentgen F

Protein expression and purification. 2006 Jul;48(1):90-7. DOI: [10.1016/j.pep.2006.01.020](https://doi.org/10.1016/j.pep.2006.01.020)

2. Research activities

Biology and biomedical research, from fundamental to applied research, has developed profound and timely needs for bio-molecular phenotyping methods able to dissect the extraordinary complex processes of Life. In the meantime, analytical chemistry methods have made spectacular progress and now offer great solutions and possibilities of development and customization toward the quantitative and qualitative analysis of all types of bio-molecules.

Unfortunately, both communities suffer from a far-from optimal scientific permeability: analytical needs and offers, and technological platforms availability are barely aligned: biologists are scarcely aware of the analytical solutions available and the possibility to develop custom methods to their research needs, and analytical chemists on the other sides have difficulties understanding the needs from the bio-medical communities.

Since my beginnings in the research community, my scientific engagement has consisted in bridging the gap between both communities that depend on each other to thrive, but have rough times interacting. While I had been doing protein structural biology during my PhD and postdocs, I changed of field of research in 2012. Since my recruitment at the ENS de Lyon, I have focussed on metabolism profiling using NMR metabolomics approaches and developed a series of applications for metabolomics in the team of Bénédicte Elena-Herrmann and then Sami Jannin.

I am intimately convinced of the value of NMR metabolomics for its exceptional discrimination potential, which offers unprecedented applications for example for human health diagnosis, prognostic, personalized medicine etc. I believe that we are about to live a bio-medical revolution when all -omics technologies will be integrated into routine healthcare. Unfortunately, while definite proofs of concept are available and preliminary applications are already integrated into research hospitals, we still have much development and validation to work on before metabolomics, and other -omics technologies, are rendered accessible to public health. Outside health applications, -omics technologies, complemented by many other approaches, are also excitingly allowing to reach new layers of comprehension to Biology, and Life in general.

This document begins with a general discussion of metabolomics approaches for metabolic profiling. I then present several applications of NMR for metabolomics projects where I explored a variety of biological specimens, from bacteria to full organs studies, and subcellular fractions. An important aspect of my work is the profiling of the dynamic processes of metabolism, which I assessed either from the measurement of matter fluxes between cells and their culture media or by measuring complex enzymatic pathways activities in cell lysates.

2.1. An overview of metabolomics and metabolic phenotyping in life sciences

2.1.1. Metabolic phenotypes and metabolic plasticity

Biological organisms are constantly adapting to their environment that constantly triggers a continuous flow of stimuli-dependent adaptations. The diversity of the stimuli contributes to the multiplicity of induced biological effects, including activation, stimulation or inhibition of physiological processes and toxicity. Metabolism, as the foremost phenotype and manifestation of life, has proven to be acutely sensitive and highly adaptive to stimuli. Studying metabolism not only permit us to better apprehend the mechanisms of Life, but also offers an excellent diagnostic of the physiological state of an organism.

The metabolome is defined as the complete ensemble of small molecules (<1.5 kDa), or metabolites, present in a biological sample. The biological sample can be a cell, a cellular compartment, an organ, a tissue, a tissue extract, a biofluid or an entire organism [1]. The metabolome includes both endogenous metabolites that are naturally produced by an organism (such as amino acids, organic acids, nucleic acids, fatty acids, sugars, co-factor, etc.) as well as exogenous chemicals (such as vitamins, drugs, environmental contaminants, food additives, and other xenobiotics) that are not naturally produced by an organism [2,3].

Metabolism, as the foremost phenotype and manifestation of life, has proven to be sensitive, adaptive and superbly reflective of biological physiology [4]. As metabolites are the final products of many levels of interaction et regulation between the environment and genetic material, metabolites are considered the most sensitive indicators to probe the phenotype of an organism. Indeed, literature reports multiple examples where alterations in genes or proteins expression do not correlate to changes in the organism's phenotype. For example, changes in the cellular level of an enzyme are not automatically linked to a proportional change in metabolic flux [5]. So while genomics and proteomics identify what might happen, metabolomics characterizes what is happening in the biological system as a result of internal or external factors [6].

Metabolites are the substrates and products of metabolism and sustain, directly or indirectly, every cellular function such as the production of building blocks for biosynthesis, energy production or storage. Their concentration and rates of production/consumption reflect the physiological state of organisms. Importantly, while some metabolites are produced directly by the host organism, others originate from the microbiota, dietary and other exogenous sources. As metabolomic approaches aim to identify the ensemble of metabolites present in a biological sample, the presence of exogenous metabolites (exometabolites) complexifies not only highly the chemical analysis of the living matter, but also the comprehension and reconstitution of the molecular networks of the ecosystem of Life.

Metabolomics defines the scientific approach that aims to the identification and quantification of the metabolites present in a biological system and determines their modification in response to a perturbation. Metabolomics relies on high-resolution analytical methods such as Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) to deliver metabolic fingerprints of biological samples.

2.1.2. Metabolomics and Systems biology

At every level, from single cells to full multicellular complex organisms, biological structures are immersed in a chemical and physical environment. Life depends on the ability of biological structures to adapt to this environment. Cells and organisms do so by controlling every level of biological regulation from gene expression to protein regulations, to modulate their physiological processes to maintain Life homeostasis. A defy for 21st-century biology is to understand the associations and interactions between the multitude of stimuli and the extraordinary ability to adapt using a variety of biological responses. Such understanding is expected to shed light on the full machinery of Life and deliver concrete applications for human health diagnosis, therapy and precision medicine for example [7].

The phenotype of a biological structure, and its adaptation to conditions, is dictated by the multiple layers of regulation, such as the epigenetic, transcriptional, translational and post-translational status of its genetic information. To determine functional associations between genotype and phenotype, it is hence critical to characterize the intermediate levels of genetic expressions, such as transcript, protein or metabolite abundance [8]. Under this objective, numerous initiatives in -omics sciences (such as genomics, transcriptomics, proteomics or metabolomics approaches) helped by bioinformatic computing, have been developed and are gathering unprecedented volumes of data that are being integrated by systems biology analyses. These biological molecular characterising methods are expected to deliver critical knowledge over Life adaptability and regulation and stand at the forefront of future medicine while supporting the development of systemic molecular diagnosis, preventive medicine and personalized treatments [9,10].

Metabolomics and metabolic phenotyping play a key role in the translational -omics cascade [11,12], and thrive in every area of biology, from bio-medical to environmental studies. Metabolomic studies are integral for a vast range of fundamental to pre-clinical studies and proved particularly efficient for example to detect the effect of drugs or chemicals on biological systems [13,14]. Of interest, the analysis of metabolomes has also developed as a cornerstone approach for the development of drug discovery and precision medicine [15].

Numerous projects associate multiple -omics studies to profile biological systems. Exposing cells to modifications of their environment triggers all biological regulatory systems and impact all types of biomolecules. This explains why -omics approaches have become central to all facets of life science. The opportunity to operate multi-omics studies to capture the full dynamic range of a biological response is an unprecedented opening of research, recently enabled by the development of adequate analytical, computational and visualization solutions and strategies [16–18]. Multiple

investigations demonstrate that the integration of multiple -omics leads to a considerable increase of sensitivity, for example, to detect xenobiotics effects on biological systems. They also open doors to complete identification of molecular mechanisms of biological adaptations, providing a systemic understanding of stimuli-induced perturbations [19–22].

2.1.3. Metabolic phenotyping of biological samples

Metabolomics methods, mostly based on NMR and mass spectrometry, have greatly progressed to accommodate almost every type of biological sample, including tissues, cells and biofluids. Solid samples analyses are most often studied after metabolites extraction from the complete tissue material [23] but integral biopsies or small model organisms such as *C. elegans* can also be studied without extraction, for example using NMR HR-MAS methods [24,25]. Tissue subregional, cellular and even subcellular metabolite profiles can also be studied and provide insight over metabolic compartments; this ultrastructural deconstruction can be particularly relevant in complex heterogeneous biological structures such as brain and heterogeneous cancer tumours [26] and appear very attractive when studying localized and specific responses to stimuli and pathogenesis. On the other side, metabolomic analyses of body fluids, such as serum or urine, appear as a solution of choice to investigate animal physiology or for medical purposes. Body fluids metabolic profiling deliver systemic overviews of an organism's state but at the cost of obtaining an averaged picture about the behaviour of cells that underlie changes in the homeostasis of the organism [13,27].

Metabolomics analyses are well adapted to study model systems such as cell cultures. The benefits of using cell models include low experimental costs, easy condition testing and facilitated biological data reproducibility. Cell cultures allow the determination of metabolite biomarkers that characterize physiological and pathological states of the cell and importantly provide data easier to interpret than those obtained in animal models or human biofluids [27]. Cell culture offers a large panel of options regarding the choice of tissues-specific derived cells, allowing to target cell-type-specific responses [28]. Recent developments in metabolomics technologies take advantage of cell culture profiling to extrapolate the results to whole systems [10,29]. So far, metabolomic analyses have been used to identify the biochemical response of cells to drugs or xenobiotics in the context of pharmaco-toxicological studies [28,30–32], to characterize bioproduction [33,34], cell plasticity and carcinogenesis [35], environmental [36,37] or nutritional interventions [38,39].

2.1.4. Analytical strategies for metabolic phenotyping

Comprehensive metabolic profiling requires high-resolution analytical techniques that should ideally be able to profile thousands of small molecules of highly variable physical and chemical properties [40], with dynamic concentration ranges distributed over 6 to 9 orders of magnitude [25]. While stunning technological progress has been achieved over the last decades [41], modern analytical platforms still cannot identify and quantify the entire metabolome of a biological system/sample [42].

Two broad options can be considered for metabolomic investigations of biological samples: targeted or untargeted approaches [43]. They differ conceptually and analytically, but the two of them aim to the acquisition of quantitative data, determination of the relative abundance, or simple detection of metabolites [44]. Targeted approaches focus on a set of predefined metabolites to be measured in biological samples, such as metabolites from a given pathway, or belonging to a certain class of molecules (e.g. amino acids). Targeted metabolomics relies on hypotheses or preliminary data about the mode of action of a perturbator, and have proven useful for assessing the response of biological organisms to environmental xenobiotic exposure [45,46]. On the other hand, untargeted metabolomics studies rely on comprehensive pictures of biological systems obtained by collecting all possible data available from a given technique, without any a priori knowledge on the compounds detected. Untargeted analytical approaches provide access to hundreds or thousands of metabolite signals [41], and sophisticated statistical methods are required to extract and identify relevant metabolite alterations and biomarkers [47,48].

To date, NMR spectroscopy and mass spectrometry largely dominate the field of metabolomics [41,49], both for targeted or untargeted studies. The two platforms rely on very different physical and chemical strategies to generate metabolic profiles from biological samples. Interestingly, their outputs only partially overlap in terms of detected metabolites. Both technologies have proven to deliver robust results for metabolomics studies, with complementary advantages and disadvantages [50]. Recent advances tend to associate MS and NMR through integrated studies to maximize the sensitivity and precision of metabolomics studies [41,51,52].

NMR and MS generate information-dense datasets that necessitate advanced computational and statistical analyses to identify event-related metabolite changes and potential biomarkers [53]. The signal processing, assignment, and data mining processes are key components of metabolomics studies that have seen tremendous progress over the last decade [41].

2.1.4.1. NMR and MS technologies for metabolomics

Though metabolomics is conceptually independent of any particular analytical method, two analytical techniques dominate the field: Nuclear Magnetic Resonance [54–56] and Mass Spectrometry [41,57]. Both have been extensively developed, applied, and validated in the context of metabolomics studies [58].

NMR spectroscopy

NMR is a fast and highly reproducible analytical technique that is based on the interaction between a nuclei spin and an external magnetic field. High magnetic fields generate the strongest nuclei polarization levels, which translates into optimal NMR sensitivity and resolution. NMR spectroscopy at 600 MHz (^1H resonance frequency) is currently considered the gold standard for biomedical metabolomics research, offering a good compromise between adequate and cost-effective analytical performance and broad access to the scientific community [54,55]. NMR is a non-destructive technique, where samples are fully recovered after analysis. Its conventional setting in

solution requires minimal pre-analytical sample processing [59], with no chemical derivatization, and is a robust quantitative technique that offers excellent repeatability and reproducibility across large numbers of samples, NMR spectrometers and NMR facilities [60]. Typical NMR analyses for metabolic profiling are fast (5 to 30 minutes per sample) and cost-effective. The main limitation of NMR is its low intrinsic sensitivity, with a detection limit in the micromolar range for metabolomics applications. Typically, NMR can detect between in the order of 100 metabolites in cell extracts derived from a few hundred thousand to a few million of eukaryotic cells (typical amount for 5 mm NMR tube is a 10 cm culture dish at 80% of confluency), and identify and quantify 50 to 70 of them in cell extracts and culture media [61,62].

NMR is also a flexible technique that has long been developed to study cell metabolism under multiple settings, such as flow-probe perfusion systems or miniaturized bioreactors inside the NMR spectrometers that allow profiling cell metabolism in real-time [63]. Whole-cell pellets or intact biological tissues can also be investigated using the HR-MAS (high-resolution magic-angle spinning) NMR technique, avoiding bias induced by extraction protocols [25].

The nuclei observed by NMR and compatible with metabolomics NMR studies are spin 1/2 isotopes present at the various natural isotopic abundance in biological material: ^1H (proton), ^{13}C , ^{15}N , and ^{31}P [55]. Protons are the most common NMR observables, due to their relatively high sensitivity, 99.98% natural abundance and omnipresence in organic compounds. As a consequence, one-dimensional (1D) ^1H NMR spectra sustain most NMR metabolomics studies. ^{13}C NMR is impractical for high-throughput metabolomics NMR analyses due to the low natural abundance (1.1%) of carbon-13 isotopes in biological material [64]. ^{31}P NMR can be exploited to study intracellular energy states, yet ^{31}P signals from phosphorylated compounds shift heavily upon sample conditions, making signal assignment tedious [65]. Due to an intrinsic lower sensitivity and scarce natural abundance, nitrogen-15 studies rely on isotopic labelling schemes for targeted pathways analysis.

NMR signals, through their spectral position (chemical shift) and multiplicity, carry a vast amount of structural information that is exploited for metabolite annotation, and structure determination of unknown compounds in complex matrices [66]. NMR acquisition relies on a variety of radio-frequency pulse schemes to provide high-throughput detection of 1D metabolic profiles [56,67], which either allow absolute quantification of metabolites (^1H NOESY scheme), or relative quantification when using spectral editing schemes (e.g. ^1H CPMG experiment) [55,68,69]. Two-dimensional (2D) NMR acquisition schemes that require long experimental times (a few hours to days) are typically recorded on a representative sample for metabolite annotation purposes. The traditional assignment strategy relies on a set of 1D and 2D correlation spectra to determine metabolites' ^1H (and ^{13}C) shifts and compare them with spectral reference databases (see section: Metabolite identification and quantification, below).

NMR is quantitative, which means metabolites concentrations can be determined from signal integration using a single reference (e.g. lactate) of known concentrations [70]. Metabolites concentrations are routinely determined from ^1H 1D NOESY spectra, either automatically or interactively, by the integration of well-resolved peaks belonging to a metabolite, or using more advanced curve-fitting dedicated software or algorithms [48,54,71]. Using more advanced spectral

editing NMR techniques, metabolite peaks intensities depend on the specific properties for each metabolite, such as relaxation times, and relative metabolite concentrations are obtained following equivalent procedures [69].

Mass Spectrometry

Mass spectrometry is a highly sensitive and very high-resolution analytical technique that measures mass-to-charge ratios (m/z) of ionized molecules. Mass spectrometry has been a very active research field since the 1980s and a wide range of instruments and approaches have been developed and adapted for metabolomics [72]. MS now offers diverse solutions for metabolomics profiling as various separation techniques, ionization, and mass analyser methods are combined to characterize complex mixtures of metabolites and improve analysis and identification [57,73,74].

MS is generally preceded by a separation step that aims to simplify the sample complexity and aid the mass spectra analysis. Significant advances have occurred in separation-based MS techniques, and liquid and gas chromatography (LC and GC, respectively), or capillary electrophoresis (CE) are the most frequently used separation techniques. GC has been widely used for metabolomics [75] and is most suited for the targeted analysis of volatile compounds, from original or derivatized samples [76]. Liquid chromatography and more recently ultra-high-performance liquid chromatography (UHPLC) techniques have developed to provide high-resolution and high-throughput capacities, with the advantage that metabolites are separated in the liquid phase, which makes derivatization unnecessary and less prone to introduce bias and artefacts [57]. LC-MS coupled to electrospray as an ionization source can now routinely identify and estimate levels of a few hundred metabolites within a single extract [77,78]. On the other side, multiple separation-free MS techniques have been successfully applied to metabolomics with direct infusion-mass spectrometry and matrix-assisted laser desorption ionization (MALDI) mass spectrometry being applicable to cell culture studies. While these techniques benefit from fewer analytical steps, they suffer from an enhanced complexity in the ions annotation and identification [57].

Identification of metabolites from dense and information-rich mass spectra is an ongoing challenge in MS, but remarkable progress has been made over the past decade with the development of hardware strategies, mass databases, and semi-automated or automated computational tools integrated into specialized software [57]. MS signals annotation is mostly based either on comparisons with libraries of existing experimental or theoretical data (see section 0 below, Metabolite identification and quantification) or requires additional experimental data acquisition, such as tandem MS-MS or MS-MS-MS spectra, where further fragmentation of a parent ion provides additional structural information for metabolite identification [57,79,80]. Yet, a large proportion of MS signals remain unidentified. Recent strategies combining NMR and MS show a strong potential for the identification of the unknowns [51,81].

MS can deliver qualitative or quantitative data in the form of tables of metabolites detected in the complex samples [73]. However, the accuracy and reproducibility of the quantitative results are usually recognized to be a weakness as mass spectrometers tend to drift over time, and MS analyses require extensive quality control monitoring. All steps are known to be susceptible to time variations

from the pre-separation steps to the ionization and ion separation steps. Metabolite standards or spiking experiments for each metabolite can be used to estimate concentrations, while relative metabolite concentrations are usually determined from peak intensities comparison.

Generally, the pros of MS include are a very high sensitivity for very dilute metabolites and very high resolution of sub-unit atomic mass differences, which allows MS to identify much more metabolites than NMR while requiring lower amounts of sample [50]. This claim is mitigated by the actual diversity of MS instruments and experimental setups, each having dedicated applications and properties. MS is a destructive technique, i.e., samples cannot be recovered after analysis and requires extensive sample matrix optimization or derivatization.

What is the best analytical technique for metabolomics?

Whether the strategy is to carry out targeted or untargeted analysis, both NMR and MS are adequate to detect and identify metabolite perturbations both qualitatively and quantitatively. Both techniques have their strengths and weaknesses and can deliver largely non-overlapping information from the same metabolomic samples [41,50]. There seemingly exists no universal answer to the question of the choice of an optimum analytical setup in the context of chemical testing of biological samples. Primarily, this choice should depend on the specific focus of the research study, but will importantly consider the accessibility of analytical platforms and availability of technological and methodological expertise for metabolomics studies. Integrating both techniques is an option that may yield the most comprehensive results but requires major resources and specific expertise to integrate datasets [52]. NMR is particularly stable and reproducible, and is well adapted to evaluate the broad cellular metabolism by identifying small variations of mostly common metabolites. NMR is also an excellent tool to discriminate and pre-evaluate metabolic profiles before further detailed MS analyses. NMR results are quantitative can be integrated with other analytical methods. MS strategies offer wider coverage of the metabolome and the ability to assess very low concentration metabolites and are perfectly adapted to identify low concentrated biomarkers [57], although they require more extensive setup optimization and lead to higher complexity of data analysis.

2.1.4.2. The metabolomics workflow

From an analytical point of view, the typical metabolomics workflow for most projects can be divided into three consecutive steps: metabolite extraction and sample preparation, NMR or MS analysis to collect experimental metabolic profiles, and data analysis including statistics to identify the main features associated with a biological perturbator (Figure 2-1) [82]. Unlike other -omics approaches, the metabolome is highly chemically diverse, dynamic, and sensitive to both biological and analytical conditions. As a consequence, all steps along the full procedure are prone to introduce bias and artefacts that will impact the biological conclusions. Experimental design must be thought carefully and suitable standardized protocols for sample preparation should be adopted with proper training of the experimentalist to ensure smooth and reproducible execution. Same

batches of chemicals and consumables are to be used for all samples, and sample handling should be carefully randomized across different sample classes (for the date of sample preparation, sample collection, chemical analysis, etc.) [83,84].

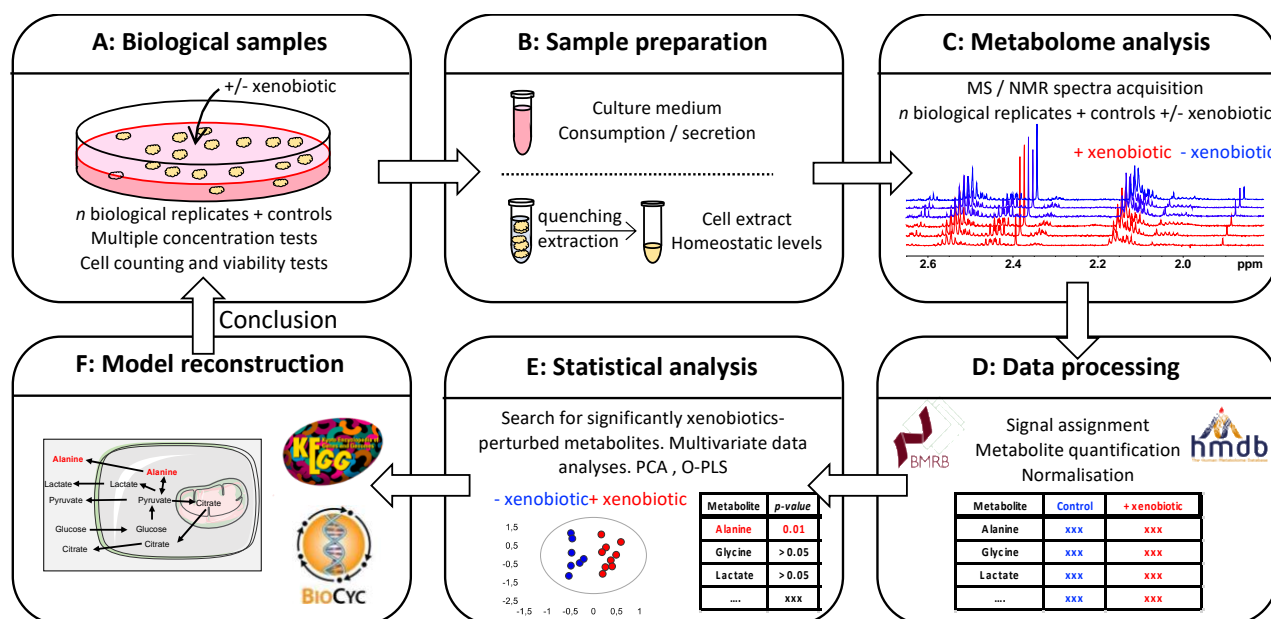


Figure 2-1. Detection of xenobiotics- or drug-induced metabolism alterations in mammalian cell cultures: a general metabolomics workflow. (A) Cell cultures are performed in the presence of a concentration range of xenobiotic or drug. Adequate controls are used: dishes without cells to determine consumption/secretion rates, dishes for cell counting, as well as biological parameters determination such as viability tests. (B) Metabolomics sample preparation: the culture medium that contains extracellular metabolites is isolated by centrifugation; the cell pellet is subjected to metabolism quenching and metabolite extraction protocols to extract the intracellular metabolites. (C) The metabolome analysis by MS and/or NMR data acquisition allows for the obtaining of rich experimental data. (D) Data processing, metabolite identification, quantification, and normalization of the results. (E) Statistical analyses to identify metabolites levels that are altered as a consequence of xenobiotic testing (this step aims to identify individual biomarkers as well as complex metabolites signatures). (F) Model reconstruction and integration of the results over metabolic pathways

Metabolite extraction

While some biofluids and cell culture supernatants can be studied directly, extracting metabolites from biological samples is the first step to most metabolomics studies. Numerous protocols aiming at the extraction of metabolites from biological matrices have been developed for NMR and MS analyses, some of which have been specifically tuned for particular types of samples, such as culture cells [59,61,62,85]. These protocols are quite similar in principle for both analytical platforms but suit individual requirements such as avoiding high concentrations of protonated solvent molecules as concerns NMR profiling. NMR tolerates a certain number of macromolecules in the sample tube, though a lesser number of proteins, lipids, and nucleic acid generate sharper peaks and easier spectral interpretation. A complete arrest of metabolism and enzymatic activity as quickly as possible after culture medium removal is essential for meaningful profiling of the intracellular metabolome [61].

The existence of numerous metabolite extraction protocols stems from the fact that metabolites possess a broad diversity of physical and chemical properties that influence their extraction yields.

Hence, fit-for-all protocols result from experimental compromises, and protocols can be fine-tuned to optimize specific extraction conditions for different classes of metabolites [62]. Beyond differences in metabolites extraction yields, the ergonomics and likely associated reproducibility of protocols, as well as the use of toxic reagents, are also key points to consider, especially when a large number of cell cultures are involved [61]. Most metabolomic extraction protocols eventually target subsets of metabolites, such as water-soluble metabolites or lipids. Various strategies have been optimized using a one-phase liquid extraction, which is usually sufficient to remove most macromolecules such as proteins or nucleic acids [61,62]. Polar metabolites are mainly extracted using methanol or acetonitrile-based solutions followed by centrifugation to precipitate macromolecules and hydrophobic metabolites such as lipids. Biphasic extraction protocols, generally based on a mixture of chloroform, water and methanol, are also employed for the simultaneous extraction of hydrophilic and lipophilic metabolites into two separate phases [59].

Metabolic profiles may also be impacted by metabolites stability in various conditions during processing, storage and analysis, such as drying state, storage temperature (freezing), experimental temperature, pH and buffer conditions, but also by potential chemical cross-reactions between metabolites or oxidation processes, etc. [86,87]. Thus, it appears critical to standardize every step of the protocol and randomize sample handling to minimize biologically irrelevant bias related to sample processing [88]. Under standardized conditions, reliable and reproducible results can be obtained [83,89]. Biofluids, cell supernatants and metabolite extracts are typically stable for a few hours on ice or at 4°C, weeks at -20°C and months at -80 °C [90].

Metabolite identification and quantification

Once NMR or MS spectra have been acquired, metabolite identification is a crucial step and the current limitation to many metabolomic studies. Detailed metabolite annotation strategies are specific to the analytical techniques and instrumental setup. Metabolite assignment is generally obtained by the comparison of peak features (such as multiplicity, coupling constants and chemical shifts for NMR; and m/z ratios, isotope pattern, element composition, fragmentation and/or retention time for (LC-) MS) against the public, commercial or in-house spectral libraries of known metabolites [48,91–93].

The main difficulty lies in the immense repertoire of metabolites in the order of tens to hundreds of thousands for mammalian culture cells, which largely exceeds the size of current databases and the numerous ambiguities for chemical identification [88,94]. Moreover, the use of databases to identify metabolites is specifically limited in the case of unknown molecules, such as most xenobiotics and xenobiotics-derived metabolites. A rigorous approach to both identification and quantification of metabolites in a complex sample remains the use of standards, for example using spiking in NMR [66] or MS experiments [95]. In the latter case, spiking experiments often rely on isotopically labelled compounds [73]. Globally, due to its higher sensitivity and resolution, MS can identify many more metabolites than NMR [96].

Data normalisation and scaling

Metabolite quantitative evaluation is essential to biological interpretations and has been a focus of many studies [25,68,69,73,97]. Normalization aims to allow the comparison of samples of different concentrations. Numerous approaches of normalization for different types of samples have been proposed but normalization remains a very delicate task that can introduce intrinsic bias in the analysis and lead to errors in data interpretation [98,99]. While some biological samples are under strict homeostatic control (e.g. serum and plasma), others are subjected to significant dilution effects (such as urine) [100]. Cell and tissue extract concentrations depend on the quantity of biological from which they were extracted: metabolite concentration is assumed to have a linear correlation to total cell number. As concerns cellular profiles, normalization of the data is essential. Intracellular metabolite quantities are usually normalized to cells number, yet the protein or DNA content can also be used as an alternative [85]. As the exometabolomes are often interpreted in the form of consumption/production rates, normalization should take into account the growth and division of the cells over the experiment time. To count cells, at least one extra seeded culture dish should be added to the experiment for each group [101]. If a condition significantly perturbs the growth capacity of cell culture, collecting supernatants over shorter periods and using smaller volumes of culture media to accentuate metabolites variations, can overcome the difficulty of dealing with different growth rates, moderate normalization issues and facilitate comparison with controls [102].

Since metabolites concentrations vary over multiple orders of magnitude in biological samples, the statistical analysis suffers from variables weighting issues. Without scaling variables to comparable ranges, the most concentrated metabolites would dominate the results of multivariate statistical analysis and outshine low concentrated metabolites. Pareto and Unit Variance scaling are the most widely used scaling approaches. The Pareto scaling offers a good compromise between reducing the influence of highly concentrated metabolites, maintaining the influence of low concentrated metabolites and downgrading the importance of noise [103].

Multivariate data analyses and model construction

Both NMR- and MS-based metabolomics studies generate large amounts of data, from which important features that are associated with a perturbator must be identified among hundreds or thousands of spectral variables. This process of data mining is traditionally achieved using multivariate statistical analyses that include unsupervised and supervised approaches [104,105]. Metabolomics studies aim to identify altered metabolites and metabolic pathways implicated in the response to a perturbation. Metabolites are then considered as individual potential biomarkers alone or in combination, by composing a metabolic signature. These complexes metabolic signatures offer unprecedented classification potential with disruptive innovations and applications, for example in medical diagnosis, prognosis, treatment efficiency follow up or personalized medicine[106]. Furthermore, as identified metabolites are related to pathways that are modified these data not only allow to classify the state of a biological system but also often deliver knowledge about the biological adaptation mechanisms [40].

Unsupervised multivariate statistical analyses approaches, which mainly consist of principal component analysis (PCA) and hierarchical clustering analysis (HCA), examine the global variance over a dataset without knowledge of any class membership. This type of approach is often conducted in the early stages of data analysis and used to check the homogeneity of the dataset, identify the main sources of variation, and pinpoint outlier samples. When the biological effect of the perturbator is strong, it may be readily identified at this early step. Further supervised techniques, such as orthogonal projection onto latent structures (O-PLS) discriminant analysis, exploit information of samples class membership to optimize their discrimination. Such enhanced features detection implies higher risks of overfitting the statistical models and requires careful use of validation methods [104,105,107]. Multivariate data analyses (MVDA) can be employed to examine either unassigned spectral raw datasets or tables of relative or absolute metabolite concentrations. The use of unassigned spectral data tends to offer the highest discrimination potential as the full spectral fingerprint contributes to the statistical model. Yet, discriminatory spectral variables can remain challenging to annotate [49]. On the other hand, MVDA from metabolite concentration tables tend to generate more meaningful results, but miss the discrimination potential of potentially important unassigned signals [104]. Although MVDA are validated statistical tools that have delivered solid results in the field of metabolomics, new algorithms and statistical approaches emerge intending to further improve group separation and reconstruct metabolic models. These exciting methods include machine learning developments such as random forest, support vector machines (SVM) and self-organizing map (SOM) algorithms [108].

Despite the availability of free and commercial computational solutions with user-friendly software, the statistical analyses of metabolomics datasets remain a tedious step in the analytic workflow. No statistical method can yet pretend to offer a universal solution to the metabolomics investigations and work for every dataset [109]. Indeed, over the intrinsic difficulty of identifying weak correlations between metabolites and a perturbator, all computation methods rely on different intrinsic hypotheses. Each method displays unique pros and cons, require method-specific fine data manipulations and handlings, such as normalization or weighting. These manipulations, yet essential, are prone to produce highly variable and possibly false results, artefacts or model overfit, and require dedicated validation. For example, multivariate data analyses are built on linear regression models that hypothesize linearity between stimuli intensity and the biological response, which may not be verified in all cases [6].

Once significant metabolites features are identified, most studies aim at understanding the underlying biological mechanisms and conceptualize a mode of action of the stimuli upon metabolic pathways [40]. This is a challenge as the problem is intrinsically mathematically under-determined (too little data are available to reconstruct a comprehensive or even simplified metabolic model). A single modification of an impacted metabolite at homeostasis in the cell can be explained by multiple metabolic hypotheses, such as upstream or downstream modification of a metabolic enzymatic reaction [110,111]. Also, many complex sensing systems are constantly regulating cellular metabolism at every level, from gene expression to protein activity to compensate for dysregulation [13,112]. However, novel exciting computational tools progress toward this goal to identity

mechanistic models of perturbation, for example by mapping graphically the impacted metabolites onto metabolic pathways or by using elaborate strategies that integrate various levels of experimental and theoretical data [53,113–117]. These rapidly developing approaches can integrate metabolite enrichment estimation on metabolic pathways maps (integrated into databases such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG)) but also data fusion with other -omics datasets and reconstruction of complete metabolic networks based on genome-scale data from transcriptomic analysis [118–120].

2.1.5. Conclusion

As metabolites represent the final expression of all levels of genomic, transcriptomic and proteomic regulations, their measurement offers a fantastic snapshot of cellular physiology. Multiple well-validated methods in metabolomics, from sample preparation, NMR and MS analytical solutions, data processing, statistical analysis and model reconstruction techniques allow us to efficiently identify the perturbation of metabolic and cellular functions and their associated biomarkers. Ongoing developments concerning all these steps constantly open new possibilities, with more metabolites profiled, better precision and accuracy and integration into more precise biological models.

Metabolomics has immensely progressed over the last decade, with developments of analytical methods and computational solutions, to offer disruptive innovations in systems biology for medical research, disease diagnostic, or personalized medicine. However, many challenges remain in experimental methodology to characterize the full chemical complexity of biological samples while diminishing sample-to-sample variability. Much research is active in the field of mass spectrometry and concern all aspects of the analysis, from the pre-separation to ionization, ion separation, and detection steps and too many approaches are being explored to be discussed here. As concerns NMR, dissolution dynamic nuclear polarization (dDNP) opens exciting future perspectives for high-sensitivity NMR metabolomic detection [121,122].

Integration of analytical methods such as MS and NMR should be promoted to potentiate their strengths. This strategy is rendered accessible not only by the compatibility of sample preparation protocols for both methods, but also by the development of integrated analytical platforms. Integrating both NMR and MS results allows for covering a wider range of the metabolome. Cell cultures are ideally appropriate for multi-omics investigations (genomics, transcriptomics, proteomics and metabolomics), and the development of high-throughput automation solutions adapted to most approaches, from cell culture to final integration of results, enables such multi-omics studies. However, beyond the analytical pipeline that relies on well-trained experts to run analyses, handle datasets and computational methods, and integrate results from multiple sources, the achievement of fine metabolomics characterization relies on a deep understanding of metabolism and broad biological processes, their intricate regulations and adaptations. Such complementary expertise is key to the success of metabolomics investigations.

2.2.Applications: metabolic phenotyping at different scales, from bacteria to mice organs and biofluids.

Since my recruitment at the ENS de Lyon, I have focussed on metabolism profiling using NMR metabolomics approaches and developed a series of applications for metabolomics in the team of Bénédicte Elena-Herrmann and then Sami Jannin. This has corresponded to a complete shift of my research field, transitioning from structural biology of drug target proteins to metabolomics studies. My background in biochemistry and NMR has allowed this enriching scientific transition. Since 2012, I have participated in a wild variety of projects and collaborated with researchers from very diverse horizons, from industry, academic laboratories and medical practitioners.

Since my beginnings in metabolomics in the research group of Bénédicte Elena-Herrmann, I have explored many types of scientific questions related to metabolomics and NMR-based analytical chemistry, profiled the metabolites in a wide range of biological specimens, and developed numerous procedures and tools to obtain valuable insights for bio-medical questions. My participation to this variety of projects has opened up my scientific expertise and network. I have met Baptiste Panthu at the ENS in 2012. Baptiste is a biologist with a great interest in cellular metabolism, he now works as a Maitre de Conférences at the CarMeN (Cardiovascular, Metabolism, Diabetes and Nutrition) laboratory. He is now implicated in most, if not all, my research projects. Our work together has seeded interest and collaborations with other researchers from the CarMen (Anne Mey, Béatrice Morio, Jennifer Rieusset, Hubert Vidal, an others)

In this chapter, I have chosen to present 4 projects for which we used NMR-metabolomics approaches to answer very different and specific aims and questions. This chapter emphasizes the relevance of metabolomics to assess a variety of sample types from bacteria to eucaryote cells and organs, and complex reconstituted biological media:

- *The identification of bacterial species using NMR metabolomics approaches.*
- *The metabolic phenotyping and discrimination of adipose-derived stem cells in cell cultures.*
- *The study of tissues and biofluids to determine the impact of a high-fat high-sucrose diet on mice organs.*
- *The use of Real-Time NMR to characterize the energy fluxes in eucaryotic Cell-Free Protein Synthesis systems.*

2.2.1. Identification of Bacterial Species using NMR metabolomics approaches

2.2.1.1. Introduction

In this project, we aimed to use NMR to discriminate bacteria species of medical relevance. We were more interested to achieve a discrimination and classification of bacteria strains responsible for urinal infections, rather than determining the molecular metabolism details at the origin of this classification.

This work was realized with the cooperation of Biomérieux, the world leader industry for microbial identification. Biomérieux has developed and commercialized numerous methods for bacterial identification, ranging from biochemical tests, to genomic and mass spectrometry-based solutions. However, the complexity of microbial species is such that some species remain challenging to identify using state-of-the-art solutions and new approaches are needed.

Biomérieux financed the study and the 16-months postdoctoral fellowship of Tony Palama. The work was led by Bénédicte Elena-Herrmann and I was strongly implicated all along with the realization of the project, from the preliminary data acquisition to the final redaction.

Two articles and one patent were derived from this study:

- Rautureau, G. J. P., Palama, T. L., Canard, I., Mirande, C., Chatellier, S., van Belkum, A., & Elena-Herrmann, B. (2019). *Discrimination of Escherichia coli and Shigella spp. by Nuclear Magnetic Resonance Based Metabolomic Characterization of Culture Media*. *ACS Infectious Diseases*, 5(11), 1879–1886. doi: 10.1021/acsinfecdis.9b00199
- Palama, T. L., Canard, I., Rautureau, G. J. P., Mirande, C., Chatellier, S., & Elena-Herrmann, B. (2016). *Identification of bacterial species by untargeted NMR spectroscopy of the: Exo - metabolome*. *Analyst*, 141(15). doi: 10.1039/c6an00393a
- Patent WO2016198789A1: *Method for the differentiation of Escherichia coli and Shigella bacteria by means of NMR spectrometry*. <https://patents.google.com/patent/WO2016198789A1/en>. Canard, I., Elena-Herrmann, B., Mirande, C., Palama, T., Rautureau, G., & Sprugnoli, C. (2016).

2.2.1.2. Scientific context

Despite intense developments in microbiology, the identification of bacterial species remains a major challenge in many areas such as clinical diagnosis or industrial microbiological control, motivating a constant search for rapid, precise, accurate and low-cost techniques [123]. Improving the characterization of bacterial isolates is of key interest to public health to be able to adapt

suitable antibiotherapies to infections and thus prevent the development of multiple-drug resistance.

Bacterial identification by phenotypic approaches is usually limited by time-consuming preliminary bacterial growth and biochemical reactions. Nevertheless, technological developments have given birth to automated systems (Vitek2, bioMérieux; BD Phoenix, Becton Dickinson) [124,125]. MALDI-TOF mass spectrometry has evolved into a rapid and highly reliable analytical tool for the characterization of micro-organisms in clinical laboratories [126], and other emerging diagnostic approaches have been applied to bacterial studies like genomic analysis [127,128], biochemical sensors [129–132], optical scattering technology [125], or infrared [133] and Raman spectroscopy [134–136].

NMR spectroscopy has been used multiple times to study bacteria, for example, to investigate intra- and extracellular bacterial composition [137–141] or metabolic pathways [137,142,143]. Metabolic footprinting approaches, i.e. extracellular metabolomics analyses that depict the detailed uptake and excretion of nutrients was proposed for microbial clinical diagnosis [144] and several NMR-based strategies have been developed. Tailored analysis for the detection of given species in culture media or bio-fluids from urinary tract infection (UTI) patients have been described, which relies on the targeted detection of one or several metabolite markers [145–147]. Gupta and coworkers have also proposed a targeted strategy based on the NMR profiling of four metabolites in culture media to discriminate Gram-positive vs. negative bacteria [148] without species identification. Intracellular bacterial content has also been described for strain classification, either exploiting low-resolution NMR profiles of bacterial isolate suspensions [149], or high-resolution fingerprints recorded on cell extracts [150].

2.2.1.3. Experimental approach

The collaboration aimed to focus on the discrimination potential of NMR profiles to identify bacteria implicated in urinal infections, an area of interest for Biomérieux. We proposed a generic strategy by untargeted investigation of their exo-metabolome, i.e. the metabolite footprints detected in culture media. Culture media are straightforward to obtain in large quantity using a simple centrifugation at low speed, easy to standardize using appropriate culture media and the intensity of the biological signal can be examined as a function of the culture length.

A schematic workflow of our proposed NMR-based metabolomics approach is depicted in Figure 2-2.

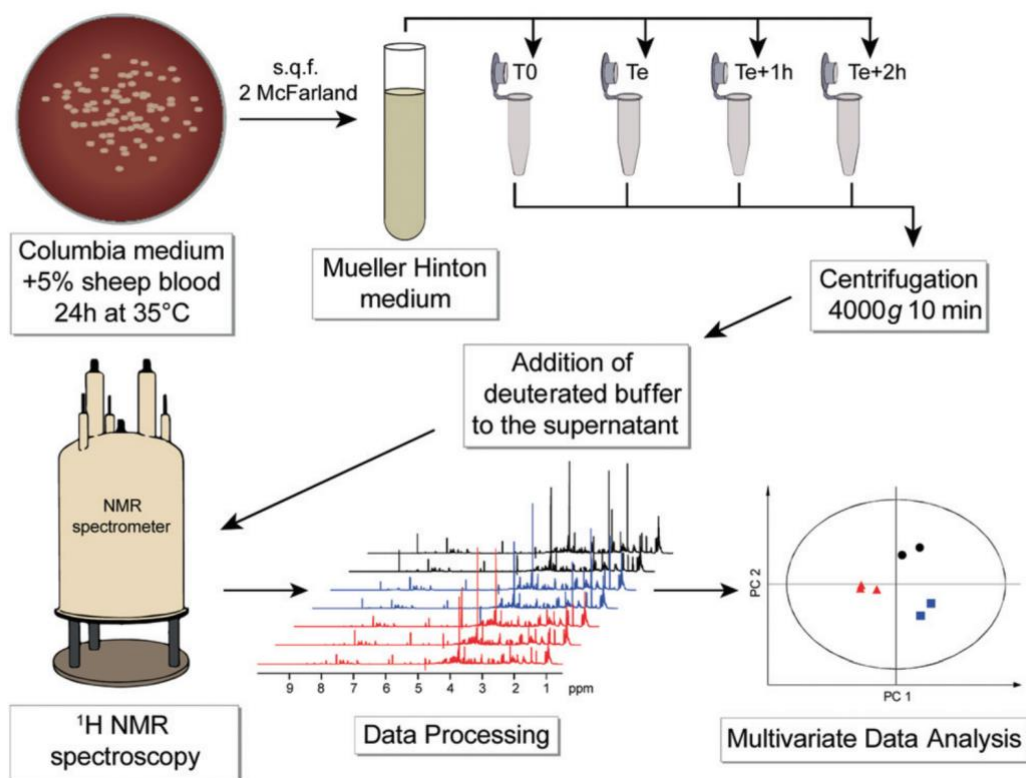


Figure 2-2. Schematic workflow for metabolomics investigation of the bacterial exo-metabolome. Frozen bacteria are first re-isolated on solid media before setting up cultures in Mueller Hinton medium. After a period of growth, bacteria are centrifuged and the culture media samples are profiled using NMR. The metabolite footprints are then processed and discrimination is evaluated using untargeted multivariate data analyses

Studying supernatants allows to evaluate the consumption/production patterns of the different bacteria species, which we demonstrated to allow a strong differentiation of bacteria types. We also examined the length of culture to determine the shortest culture leading to potential discrimination. The biological samples were prepared at Biomérieux' microbiology facilities at La Balme.

2.2.1.4. Identification of Bacterial Species by Untargeted NMR Spectroscopy of the Exo-metabolome.

This study was the first part of our collaboration with Biomérieux and consisted of evaluating the potential of NMR to discriminate the various bacterial species responsible for urinary infections. Six bacterial species were chosen. Gram-negative: Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis and Gram-positive: Enterococcus faecalis, Staphylococcus aureus, Staphylococcus saprophyticus

All strains were cultured in a Mueller Hinton medium until their specific exponential phase times (1.5 to 6 hours). The ^1H NMR profiles were acquired on the culture supernatants at 600 MHz and 576 samples were profiled in this study. Metabolite identification was achieved by comparing spectra with international reference databases [151], and confirmed using two-dimensional NMR

spectroscopy. 43 metabolites were identified. A representative ^1H NMR spectrum of an *E. coli* supernatant culture media sample is detailed in Figure 2-3.

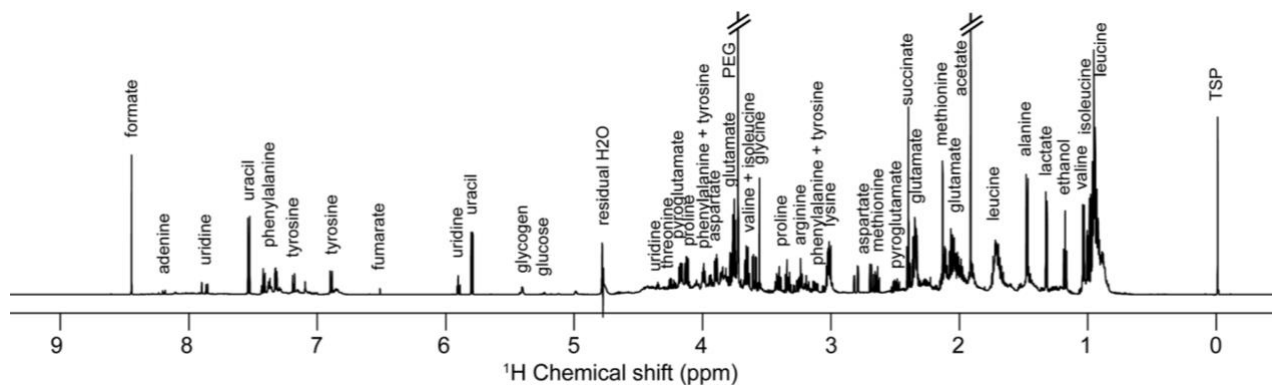


Figure 2-3. Representative one-dimensional ^1H NMR spectrum of an *Escherichia coli* sample (culture supernatant) at exponential growth, i.e. after 1.5 hours of culture in a Mueller Hinton medium.

Unsupervised multivariate data analyses of the NMR profiles, including principal component analysis (PCA) and hierarchical clustering analysis (HCA), were applied to evaluate the datasets and discriminate sample classes.

Strong species-based discrimination was observed at different time points after the bacterial mid-exponential phase was reached (T_e , $T_e + 1$ h and $T_e + 2$ h). The results for the earliest time-point (T_e), more relevant for efficient bacterial identification are presented in Figure 2-4. Strain samples of the same species cluster together into six well-defined branches of the dendrogram. Interestingly, additional levels of similarities are detected between species such as the *P. aeruginosa* and *S. saprophyticus* groups that merge to form a new branch. Similar proximities are observed between *S. aureus* and *E. faecalis*, or *E. coli* and *P. mirabilis*, the latter two consistently belonging to the same Enterobacteriaceae family. To further analyse the discrimination between bacterial species, a PCA model ($N = 144$) is presented in panels B and C of Figure 2-4. The first two principal components of this model explain 62.6% of the variance within the dataset. A clear discrimination of the species groups can be observed on the score plot (Figure 2-4 panel B) except for *E. faecalis* and *S. aureus* samples that are both overlapping but are still distinct from the rest of the species. However, the separation between *E. faecalis* and *S. aureus* samples occurs along with the third principal component.

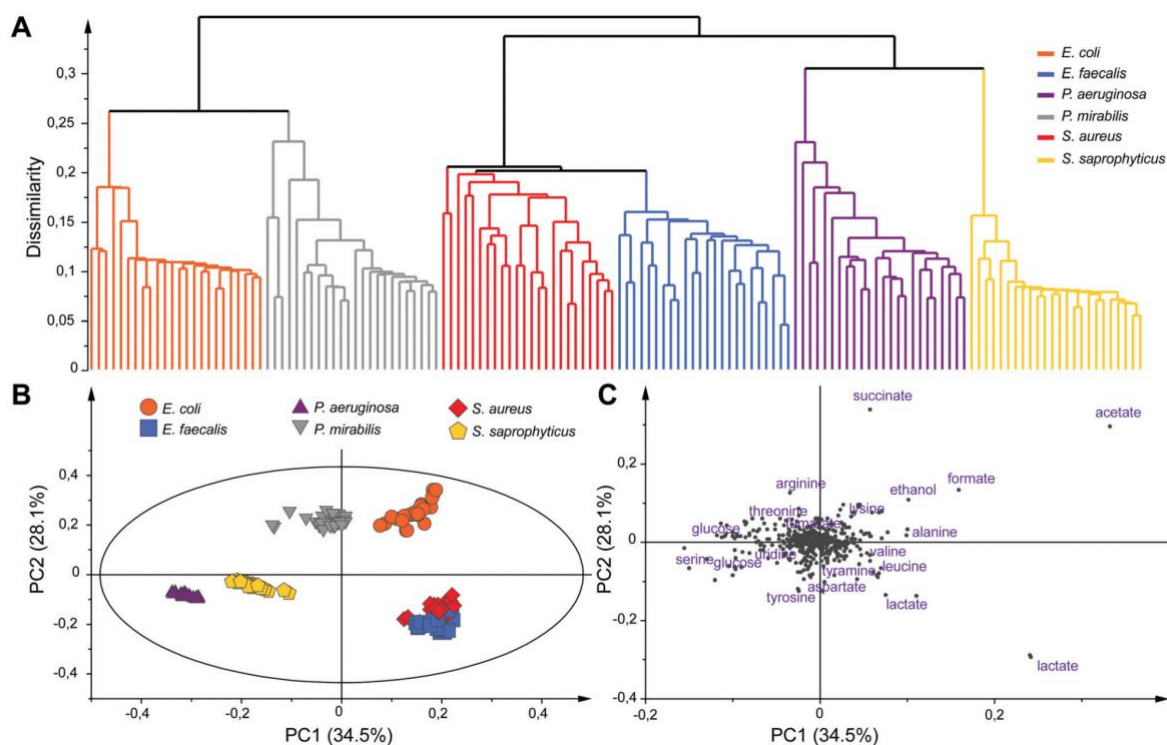


Figure 2-4. Unsupervised multivariate data analysis based on the 144 culture media samples collected at Te and the 947 variables of the NMR 1D profiles. (A) Dendrogram from the hierarchical clustering analysis (HCA), (B) score plot and (C) loading plot of the PCA model (PC1 and PC2) showing the discrimination between the samples and the variables involved in this discrimination, respectively.

A consistent differentiation of bacterial species was obtained at Te + 1 h and Te + 2 h, while samples at all time points were also well separated from those collected at T0. Interestingly, equivalent discrimination of bacterial species is obtained when considering a fixed collection time point after the start of bacterial cultures (e.g., T0 + 3h) for all bacterial species, independently of their respective growth status (data not shown).

Our results show that the concentration of end-products of fermentation in the culture media (acetate, lactate, formate, succinate and ethanol) under anaerobic conditions significantly contribute to the discrimination of bacterial species, notably discriminating groups of strains sharing common sugar fermentation mechanisms, e.g., *S. aureus*/*E. faecalis* vs. *E. coli*/*P. mirabilis*, producing respectively either lactate or succinate among other end-products. These five metabolites have already been identified as broad markers of UTI in the urine of patients against controls [148,152,153], while lactate, succinate and formate were shown to discriminate Gram-positive and negative infections [148]. Additional microbial-mammalian co-metabolites were also shown to characterize *E. coli* infection in urines [147]. Here, robust individual discrimination of the six studied species is based on the full metabolic profiles considered in our multivariate models, notably involving a range of other metabolites such as tyramine, aspartate, leucine, alanine, arginine, threonine, lysine and serine. Further analyses of the data will be carried out in the future to provide a comprehensive picture of the metabolic pathways involved in this bacterial discrimination.

The approach introduced here demonstrates the potential of NMR footprinting for rapid bacterial species discrimination. We have exploited a large-spectrum culture medium that does not restrict

the proposed strategy to a limited set of bacterial species. This rapid and functional approach could be straightforwardly extended to broader selections of bacterial species and/or strains, and provide direct methods for bacterial identification from a single culture footprint. The proposed approach opened up new prospects for accurate and cost-effective microbial clinical diagnosis.

2.2.1.5. Discrimination of *Escherichia coli* and *Shigella* spp. by Nuclear Magnetic Resonance Based Metabolomic Characterization of Culture Media

This study was achieved in collaboration with Biomérieux and corresponds to an extension of the previous work that demonstrated that NMR metabolomics methods of culture media were able to discriminate multiple types of bacteria strains. The next step was to evaluate whether this observation holds true for species that are a struggle to identify, even with modern techniques. We chose to work on the discrimination of lactose negative *E. coli* and *Shigella* spp. that have traditionally been difficult to discriminate using classical (biochemical) techniques as well as more modern ones such as mass spectrometry and nucleic acid amplification and sequencing.

Various pathogenic agents cause dysentery, such as *Shigella* spp. and *Escherichia coli*. Dysentery is a major health threat that dramatically impacts childhood morbidity and mortality in developing countries. Sensitive and precise detection of the infectious agent is important to target the best therapeutic strategy, but the differential diagnosis of these two groups remains a challenge using conventional methods.

Shigella spp. and *Escherichia coli* belong to the family Enterobacteriaceae. They represent species with very high genetic relatedness [154,155]. They could even be classified as one distinctive species in the genus *Escherichia* [156–158]. *Shigella* and *E. coli* remains a challenge for clinical laboratories [156,159]. In particular, a subgroup of *E. coli* isolates that does not ferment lactose, usually termed lactose negative, is the most difficult to separate from *Shigella* [159]. Only recent advances in next-generation sequencing open opportunities for identification [160–162]. However, such analyses still need to be translated into easy-to-use diagnostic tests available in routine settings.

Experimental approach

We studied a total of 144 samples of bacterial growth media collected after 1.5 h (T_e) of culture initiation, corresponding to 48 cultivated strains from the bioMérieux collection, with three independent cultures (biological replicates) each. The aim was to classify three main groups: *Shigella* spp., *E. coli* lactose (+), and *E. coli* lactose (–) strains, with 16 strains per group. The group of *Shigella* species included sets of *Shigella boydii*, *Shigella flexneri*, and *Shigella sonnei* strains. A blind investigation was conducted on an additional cohort of 60 *E. coli* lactose (–) and *Shigella* growth media samples, as independent validation of this work.

Based on our previous results, bacteria were cultured in classical, rich Mueller–Hinton medium for 1.5 h, a delay sufficient to reach exponential growth for both *Shigella* spp. and *E. coli*. As metabolites

were consumed or produced, a complex metabolic footprint was detected by untargeted NMR analysis. Well-resolved ^1H NMR metabolic profiles were obtained for each sample of bacterial culture medium (Figure 2-5a).

Results and discussion

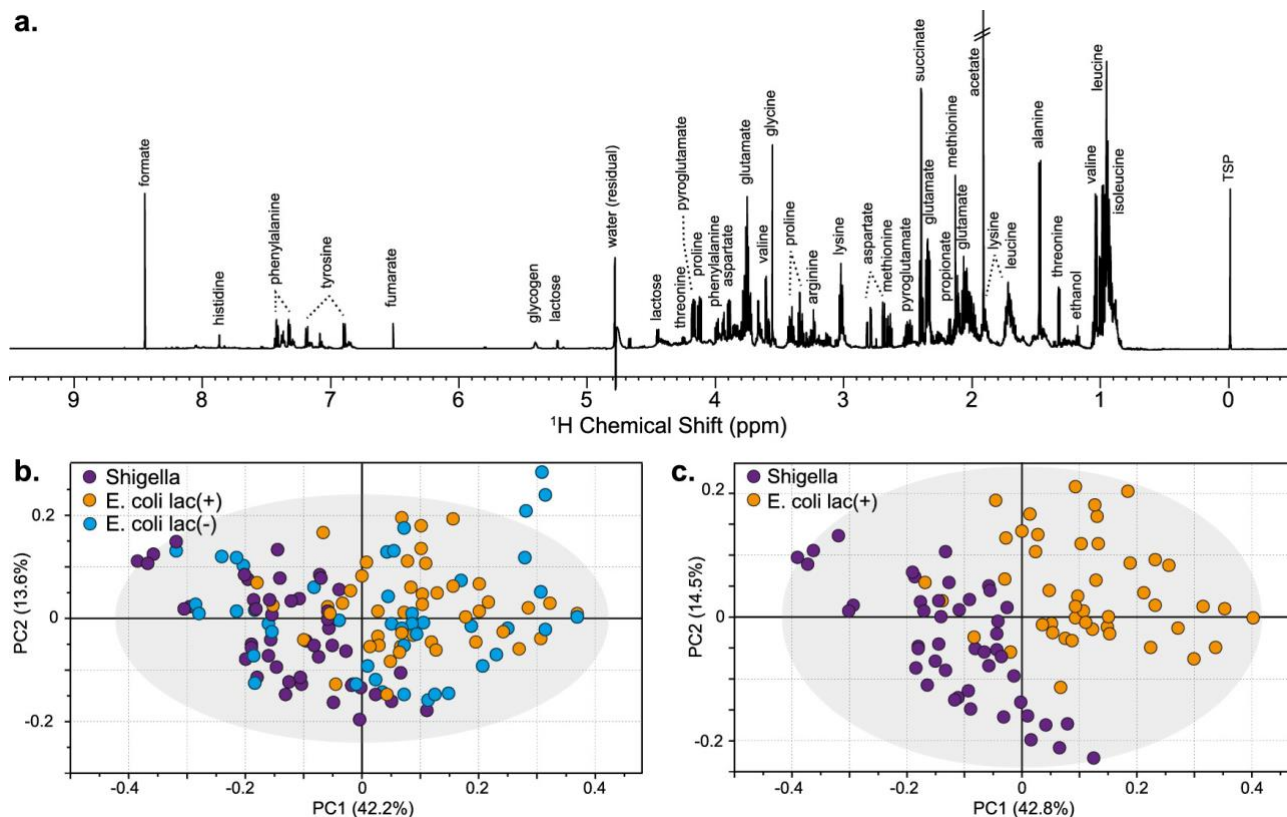


Figure 2-5. NMR analyses of the exometabolomes from *Escherichia coli* and *Shigella* spp. **(a)** Typical ^1H NMR spectrum of a *S. boydii* sample (culture supernatant) at exponential growth (i.e., after 1.5 h of culture in Mueller–Hinton medium). The 950 variables derived from the NMR 1D spectra of the culture media samples (centred variables, with no scaling) were used for unsupervised multivariate data analysis. **(b)** Scores plot of the PCA model (PC1 and PC2) including *E. coli* lactose (+) and (-) and *Shigella* samples ($N = 138$, $R^2 = 0.986$, and $Q^2 = 0.92$ on 32 principal components). *E. coli* lactose (-) and *Shigella* cannot be discriminated. **(c)** Scores plot of the PCA model (PC1 and PC2) of *E. coli* lactose (+) and *Shigella* samples ($N = 91$, $R^2 = 0.95$, and $Q^2 = 0.854$ on 15 principal components). The discrimination between those samples is straightforward.

PCA of the bucketed NMR profiles was first used to evaluate the data set homogeneity and the potential of unsupervised sample class discrimination concerning *Shigella* spp. and *E. coli* footprints. No strong outliers were identified on the PCA scores plot (Figure 2-5b), confirming the reproducibility of our experimental approach. Straightforward species-based discrimination could be observed on this unsupervised model between *E. coli* lactose (+) and *Shigella* spp. but not between *E. coli* lactose (-) and *Shigella*. An independent PCA model of *E. coli* lactose (+) and *Shigella* samples is presented in Figure 2-5c to highlight the corresponding discrimination. The first two principal components of this model explain 57.1% of the variance within the data set, and this model soundly represents the data structure as attested by high values of the goodness-of-fit parameters R^2 (0.985) and Q^2 (0.915), related respectively to the variance explained and predicted by the model. In contrast, *E. coli* lactose (-) and *Shigella* could not be discriminated from the unsupervised

analysis. Altogether, these results confirm our previous observations that NMR analysis of culture supernatants can be adapted to distinguish bacterial species [163], but they also illustrate, at the exometabolome level, the detailed phenotype resemblances between *E. coli* lactose (-) and *Shigella* spp. that lead to identification issues using regular analytical techniques.

To address the discrimination challenge between *E. coli* lactose (-) and *Shigella* spp., a supervised analysis of the NMR metabolic profiles was conducted by O-PLS-DA [107,164]. We obtained a robust predictive O-PLS-DA model ($R^2(X) = 0.861$, $R^2(Y) = 0.962$, and $Q^2 = 0.893$) based on the full NMR fingerprint, as shown in Figure 2-6. (The model validation from permutations of the Y values is provided in the original article [165]).

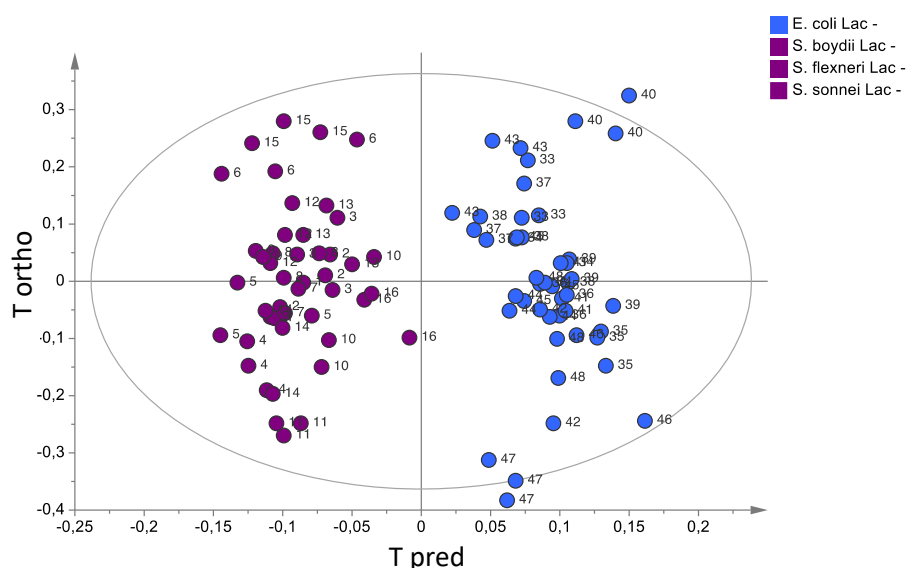


Figure 2-6. Supervised multivariate discriminant analysis (O-PLS-DA) of *S. boydii*, *S. flexneri*, *S. sonnei*, and *E. coli* lactose (-) culture supernatants. The two-class model is built from the full NMR data matrix (950 variables, no scaling) ($R^2(X) = 0.861$, $R^2(Y) = 0.962$, and $Q^2 = 0.893$).

This analysis, carried out using the 950 NMR variables to exploit the full dynamic range of spectral information, was then repeated on a set of 26 quantified metabolites to broaden the applicability of our study as metabolite concentrations can be determined without NMR using a wide range of biochemical methods. The metabolites concentrations were determined using ChemomX software.

Significant discrimination was obtained from the O-PLS-DA scores plot between the *E. coli* lactose (-) and *Shigella* samples (Figure 2-7a), associated with high values of the goodness-of-fit model parameters ($R^2(X) = 0.99$, $R^2(Y) = 0.75$, and $Q^2 = 0.639$). The robustness of the model was validated by permutation testing (1000 permutations) under the null hypothesis, showing clear decreases of R^2 and Q^2 with correlations between the original and permuted class information in the Y matrix (Figure 2-7b). The reliability of the multivariate model was also assessed by analysis of variance (CV-ANOVA), resulting in a p -value of 7.53×10^{-11} . Out of the 26 quantified metabolites, 7 metabolites (succinate, acetate, aspartate, formate, lysine, propionate, and threonine) appeared to contribute significantly to the statistical model, as shown by the variable importance in projection for independent variables (VIP) values being >1 . Most of these metabolites were secreted differently in

the medium by the two species (succinate, acetate, formate, and propionate), lysine and threonine were only secreted by *Shigella* spp., and one (aspartate) was consumed by both at different levels.

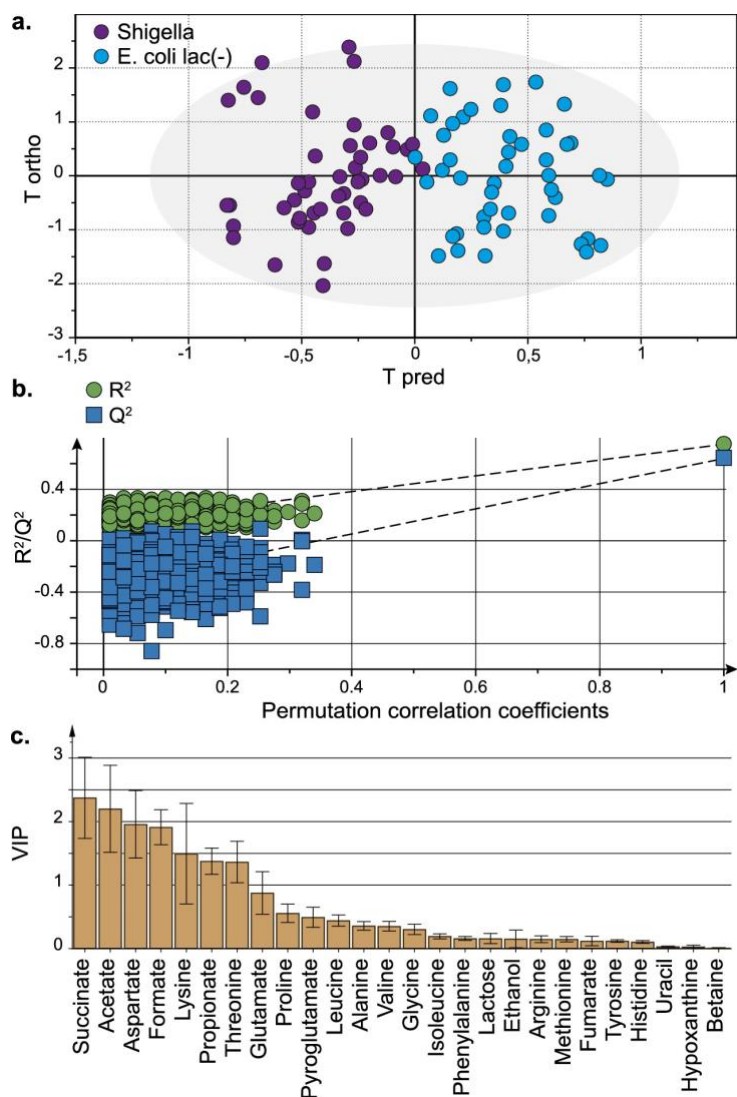


Figure 2-7. O-PLS-DA model based on 26 exometabolite concentrations derived from ¹H NMR profiles of *Shigella* and *E. coli* lactose (-) culture supernatants. (a) Scores plot of the (1 + 7) O-PLS-DA model discriminating 46 *Shigella* samples (in purple) and 45 *E. coli* lactose (-) samples (in blue) ($R^2(X) = 0.99$, $R^2(Y) = 0.75$, and $Q^2 = 0.639$; CV-ANOVA $p = 7.53 \times 10^{-11}$). (b) Validation of the O-PLS model by resampling under the null hypothesis. (c) VIP value of each metabolite.

This proof-of-concept work demonstrates that untargeted proton NMR metabolomics of bacterial culture supernatants can robustly classify *E. coli* lactose (+) and *Shigella* spp. by unsupervised data analysis, and *E. coli* lactose (-) and *Shigella* spp. via supervised approaches. Those are not trivial nor expected results. Our results demonstrate the relevance of NMR footprinting in the field of bacterial identification and discrimination, even in the case of traditionally challenging bacterial species discrimination. This approach, which was patented by Biomérieux, opens up new prospects for accurate microbial clinical diagnosis and demonstrate the potential of NMR metabolomics methods for the discrimination of biological groups.

2.2.2. Studying cell cultures: Metabolic phenotyping of adipose-derived stem cells reveals a unique signature and intrinsic differences between fat pads.

2.2.2.1. Introduction

We have shown in the previous chapter that NMR metabolomics methods were particularly effective to discriminate biological groups, even of significant resemblance. We also demonstrated the relevance and power of untargeted investigation of their exo-metabolome, i.e., the metabolite footprints detected in culture media. Studying supernatants permit to evaluation the consumption/production patterns of fluxes of exo-metabolites. Culture media are straightforward to obtain in large quantity using a simple centrifugation at low speed, easy to standardize using appropriate culture media and the intensity of the biological signal can be examined as a function of the culture length.

In this chapter, we applied a similar approach to discriminate eucaryote cells: adipose stem originating from two different tissues (subcutaneous adipose tissues (SAT) and visceral adipose tissues (VAT)). The results demonstrated that we could discriminate both groups. The interestingness of this project lies in the fact that we then used modified culture media to induce metabolic stresses to the cells and evidence differences in metabolic adaptations. These differences allowed us to get deeper insights over both cell types intrinsic metabolic physiology concerning their functional differences.

This work was performed in cooperation with Anne Mey, Baptiste Panthu and others from the CarMeN laboratory and resulted in the publication of an article:

- *Lefevre, C., Panthu, B., Naville, D., Guibert, S., Pinteur, C., Elena-Herrmann, B., ... Mey, A. (2019). Metabolic Phenotyping of Adipose-Derived Stem Cells Reveals a Unique Signature and Intrinsic Differences between Fat Pads. Stem Cells International, 2019, 1–16. doi: 10.1155/2019/9323864*

2.2.2.2. Scientific context

White adipose tissues are functionally heterogeneous and differently manage the excess of energy supply. While the expansion of subcutaneous adipose tissues (SAT) is protective in obesity, that of visceral adipose tissues (VAT) correlates with the emergence of metabolic diseases. Adipose stem cells (ASC) are stem cells that are maintained in fat pads throughout life. ASC from distinct fat pads have long been reported to present distinct proliferation and differentiation potentials that are maintained in culture, yet the origins of these intrinsic differences are still unknown.

For a long time, ASC properties have been used in regenerative medicine and for cell therapy [166] with an increasing interest in the use of their secretome [167]. Adipose tissue represents a 500 folds

larger pool of stem cells than bone marrow, which makes ASCs an attractive source of stem cells. ASCs present numerous therapeutic properties, for example on angiogenesis, wound healing, and the immune regulatory system [168]. In 2019, 187 clinical trials using adipose stem cells were registered for the treatment of skeletal diseases, gastrointestinal diseases, skin diseases, nervous disorders, autoimmune diseases, diabetes mellitus, lung and heart diseases [169]. ASCs are usually isolated from the patient's adipose tissues and directly re-injected into the wounds or bloodstream. ASCs can directly differentiate into specific cell lineages such as keratinocytes, fibroblast-like cells, and endothelial cells, together with the release of growth factors and cytokines, all that promote angiogenesis, development, migration of fibroblasts, and production of fibronectin and collagen [168]. Many studies have demonstrated that ASC can increase the healing rate and decrease healing time with dramatic clinical applications [169].

White adipose tissue is split into different body regions with two main areas, the subcutaneous (SAT) and the visceral (VAT) white adipose depots that play distinct roles in the control of energy metabolism. SAT expansion is protective in obesity while VAT expansion promotes the metabolic complications of obesity such as resistance to insulin and type 2 diabetes [170]. SAT and VAT have distinct functional properties regarding their capacity of fatty acid storage and the control of inflammation [171]. The metabolic protection by SAT is attributed to its ability to trap free fatty acids through triglyceride esterification (lipogenesis) protecting other organs from lipotoxicity [172] while the deleterious effect of expanding VAT is attributed to its higher lipolytic activity favouring the release of free fatty acids [170] and the delivery of proinflammatory cytokines such as IL6 [173]. It has been shown that VAT expansion occurs when the storage capacity of SAT is saturated [174].

Functional differences exist between ASC from distinct depots, with ASC from SAT (S-ASC) showing higher abilities to proliferate [171], to survive [171,175], to accumulate lipids [171], and to differentiate into adipocytes [171,175,176] than ASC from VAT (V-ASC). This study aimed to evaluate and compare the metabolic phenotype of both cell lines to better apprehend their functional differences.

2.2.2.3. Experimental approach

While not developed in this document, an important part of this study was to develop an efficient isolation and culture conditions of ASC allowing the amplification of stem cells from SAT and VAT collected from mice. This delicate biological work and the obtention of metabolomics samples was accomplished at the CarMeN by Anne Mey and collaborators.

We performed NMR metabolomic profiling of ASC culture supernatants at 600 MHz to characterize their metabolic phenotype in culture. By quantifying the metabolites in the extracellular culture medium, we were able to determine the consumption and secretion rates of a variety of metabolites. We quantified 29 ASC exometabolites over 72 h of cell culture. Our results indicate that V- and S-ASC presented significant metabolic differences among which the role of glutaminolysis and glycolysis were noticeable. To get more details on the metabolic differences between ASC, we examined the role of pyruvate, glucose, and glutamine supplies. Cells were seeded as previously and

cultivated for 72 h. The culture medium was then replaced by a culture medium without pyruvate nor HEPES and supplemented with the indicated concentrations of glucose and glutamine for the last 24h.

2.2.2.4. Results and discussion

Culture supernatants of ASC cultivated during 72 h were analysed by NMR spectrometry and delivered well-resolved ^1H -NMR metabolic profiles of the exometabolomes (Figure 2-8). At 72 h, the steady-state in metabolite changes was not reached even for the most proliferative population.

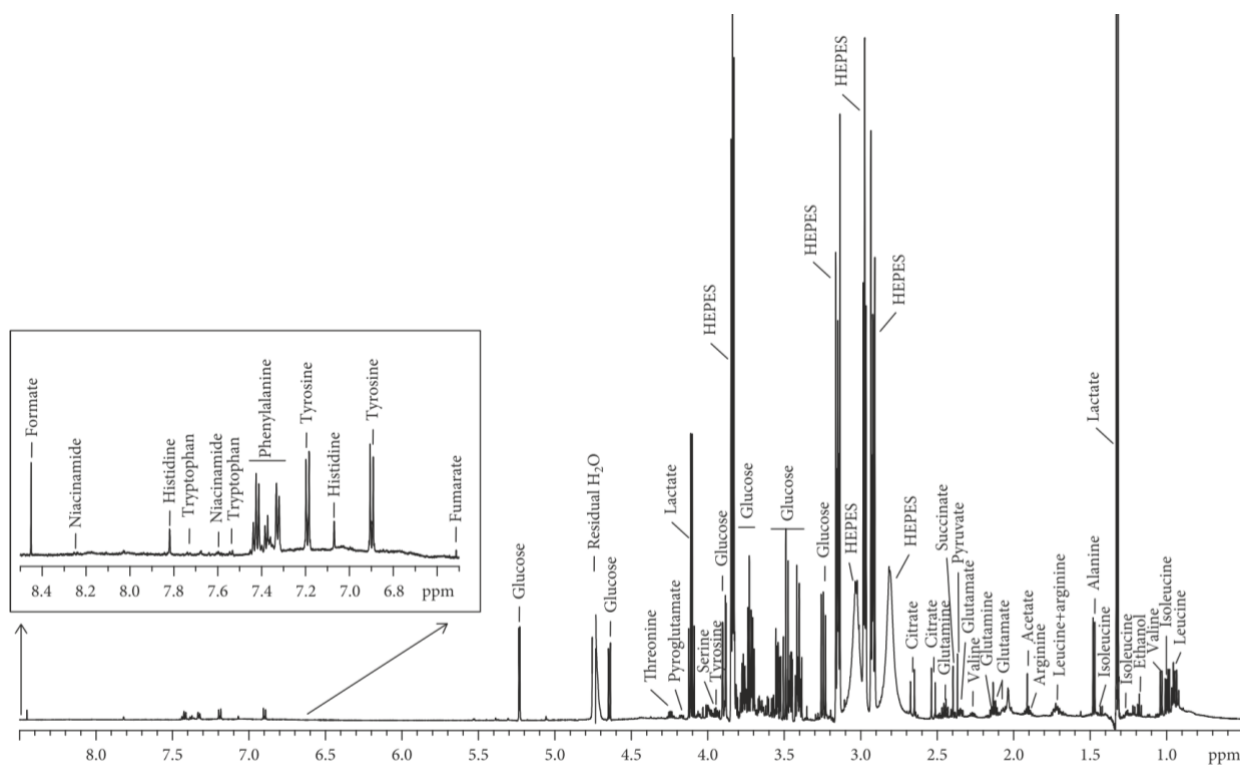


Figure 2-8. Typical ^1H -NMR NOESY spectrum of ASC supernatant (600 MHz, 30.0°C). Spectrum from 72 h culture of S-ASC supernatant is represented. Major metabolite peak assignments are indicated.

We conducted multivariate data analyses of their exo-metabolome to identify the NMR spectra features that correlate with cell types followed by peak identification. Multivariate data analyses were conducted on the absolute value of the NMR spectra bins after subtraction of the original culture medium (0.001 ppm; 9700 NMR spectral variables). PCA unsupervised multivariate data analyses were first used to evaluate the dataset homogeneity and potential sample class discrimination. The dataset showed good homogeneity and straightforward discrimination between S-ASC and V-ASC was observed on the PCA unsupervised model (Figure 2-9a). Remarkably, the first principal component of this model could explain alone 68.4% of the variance within the dataset, indicating significant differences in the metabolic profile between both cell types.

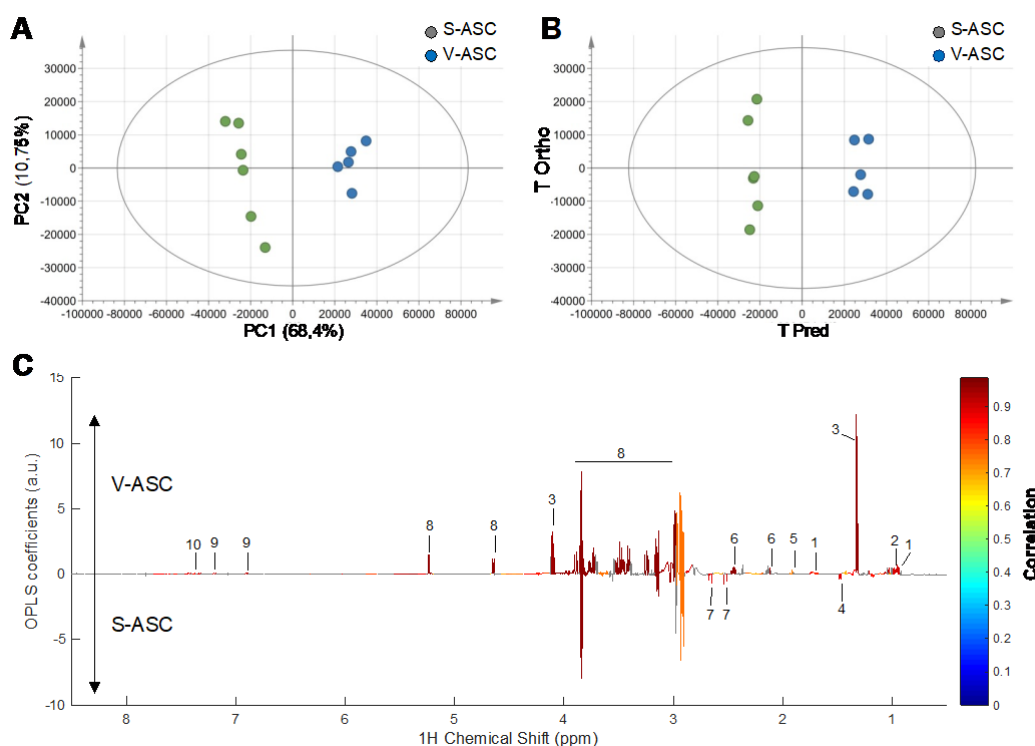


Figure 2-9. Multivariate statistical analyses of the differences between S- and V-ASC. **(A)** Untargeted principal component analysis (PCA) readily evidences the cell type as a major origin of the dataset variance. Score plot of the PCA model (PC1 and PC2) ($n=11$, $R^2=0.962$, and $Q^2=0.843$ on 5 principal components). **(B, C)** Supervised multivariate data analysis (O-PLS-DA). The strong discrimination of the multivariate model is shown by the high values of goodness-of-fit model parameters R^2 and Q^2 ($R^2(X) = 0.796$, $R^2(Y)=0.991$, and $Q^2=0.969$). The discrimination robustness was validated by resampling 1000 times the model under the null hypothesis (data not shown), and the analysis of variance (CV-ANOVA) of the model led to a p -value of 1.20×10^{-4} . **(B)** Score plot of the (1 + 1) O-PLS-DA model. **(C)** O-PLS-DA loading plot after SRV analysis and Benjamini–Hochberg multiple testing correction. Highlighted candidate biomarkers are (1) leucine, (2) valine, (3) lactate, (4) alanine, (5) acetate, (6) glutamine, (7) citrate, (8) glucose, (9) tyrosine, and (10) phenylalanine.

To specifically target the NMR regions discriminating S-ASC and V-ASC, a supervised analysis by O-PLS-DA [107,164] was conducted. We obtained a strongly discriminating O-PLS-DA model (Figure 2-9b). Glucose, leucine, valine, glutamine, tyrosine, phenylalanine, lactate, and acetate appeared to vary more in V-ASC than in S-ASC culture supernatants, while citrate and alanine varied more in S-ASC than in V-ASC culture supernatants (Figure 2-9c). These results define distinct metabolic footprints of V- and S-ASC on their microenvironment and suggest metabolic differences between both cell types.

We did not detect cell type-specific metabolites, but concentrations determined using the Chenomx software revealed quantitative differences. Glucose and glutamine were the most consumed substrates, and their transformation products, lactate and glutamate, were among the most secreted metabolites, along with citrate and alanine (Figure 2-10). When considering glycolysis, the consumption of glucose mirrored the production of lactate indicating active glycolysis in ASC. The high level of lactate secretion demonstrates a high aerobic glycolytic activity in both S- and V-ASC. This singularity was previously described as the Warburg-like effect, a type of noncancer cell metabolism associated with self-renewing stem cells [177].

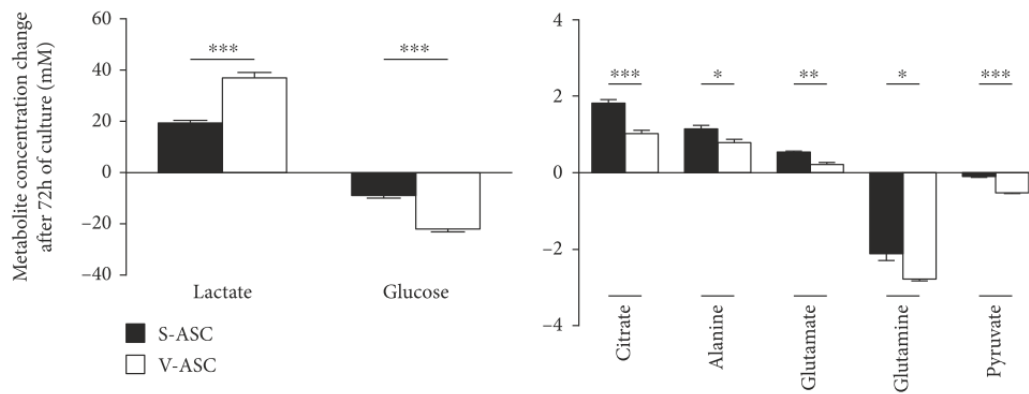


Figure 2-10. Quantitative comparison of S- and V-ASC secretomes. S- and V-ASC at 75% confluency were cultivated for 72 h. Metabolites representing glycolysis (left panel) and other metabolites (right panel) showing significant differences between S- and V-ASC (n=6).

On the opposite, the consumption of glutamine generated few amounts of glutamate, indicating that the carbons generated from glutaminolysis were directed elsewhere, most probably towards the production of additional metabolites and cell growth, as suggested by the strong amino acid consumptions supporting the anabolism associated with protein synthesis.

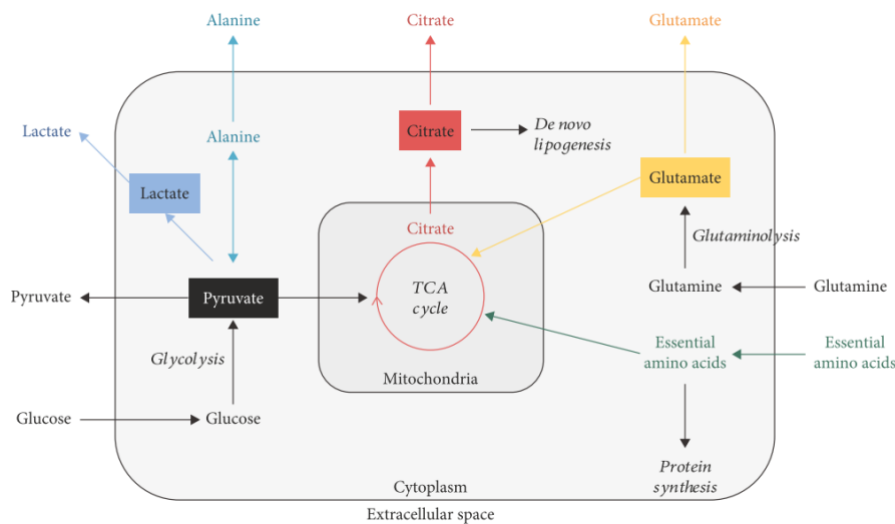


Figure 2-11. Representation of the main carbon metabolic pathways in actively dividing cells. Anabolic and catabolic pathways are represented in italics in relation to the metabolite secretome. Colours identify the metabolic pathways analysed in this study. TCA: tricarboxylic acid cycle; EAA: essential amino acids.

Lactate and citrate are features of highly proliferating stem cells and are, respectively, representative of the use of pyruvate in glycolysis and the mitochondrial TCA cycle. A high level of glycolysis is the signature of undifferentiated cells [178] while citrate can be used for de novo lipogenesis to support the synthesis of new cell membranes [179]. Altogether, our results show that ASC presents the characteristics of proliferating stem cells, with a Warburg-like effect, associated with active amino acid consumption and a specific feature concerning both an elevated citrate secretion and a high glutamine consumption/low glutamate secretion.

To decipher the respective contributions of glucose and glutamine as sources of carbon in ASC, we performed end-point experiments with cells cultivated for 96 h in culture media completely or partially depleted in glucose and/or glutamine during the last 24 h. The replacement of the medium and the measurement on a short period and on cells at confluency was chosen to avoid the depletion of other metabolites than glutamine or glucose. Because pyruvate is the end product of glycolysis and a substrate for the TCA cycle, these experiments were performed in a pyruvate-free medium.

For both ASC, no lactate was secreted in the absence of glucose (Figure 2-12a) and cells even consumed the little amount of lactate initially present in the medium. More importantly, glutamine privation completely switched the metabolism of S-ASC, but not V-ASC, towards lactate synthesis. Indeed, in glutamine-free medium, lactate secretion by S-ASC increased from 6.0 ± 0.7 mM to 11.0 ± 1 mM while glucose uptake decreased from 12.1 ± 1.1 mM to 8.0 ± 0.15 mM (Figure 2-12-a). This indicated that glycolysis S-ASC, but not in V-ASC, was highly sensitive to glutamine availability.

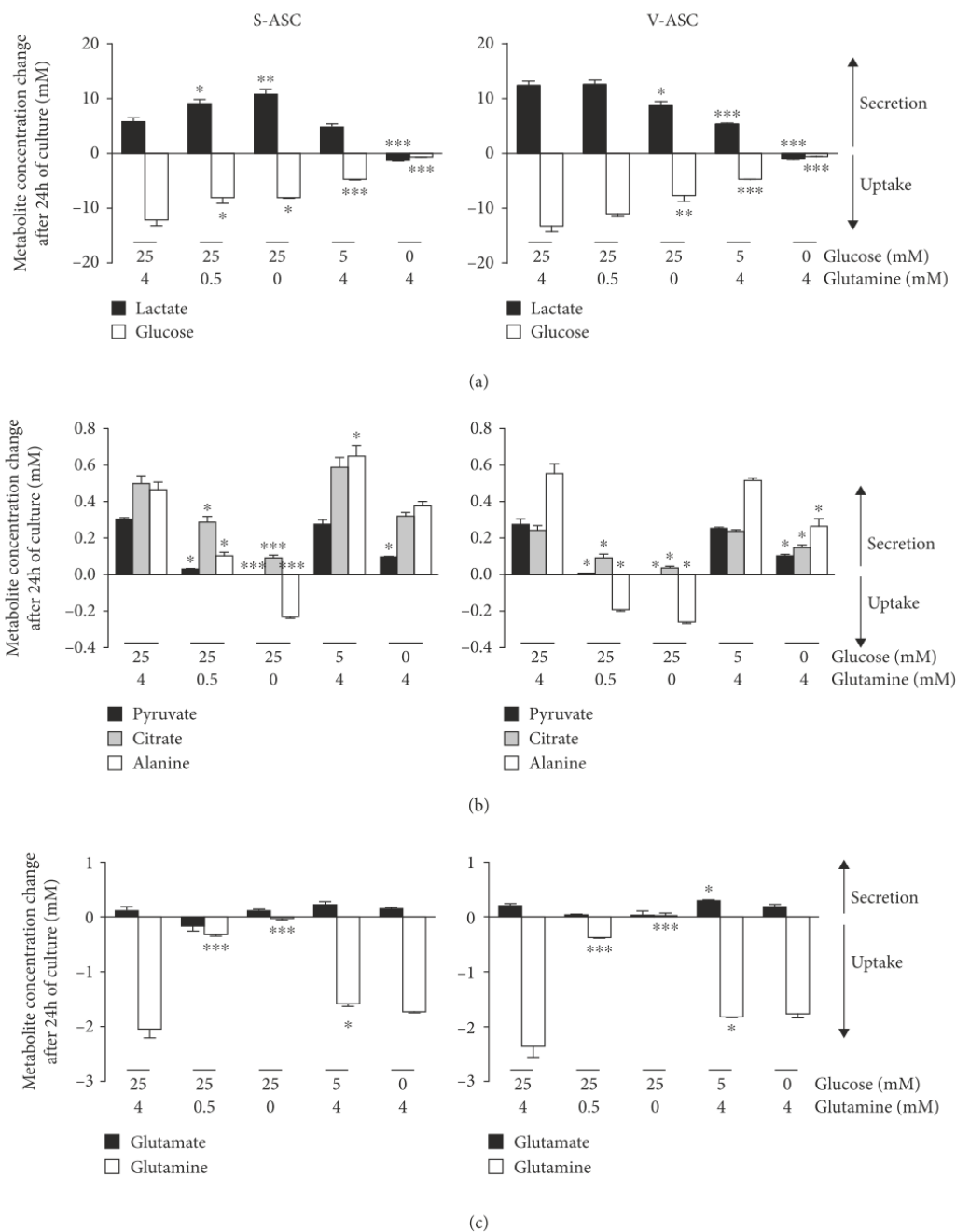


Figure 2-12. Role of glutamine and glucose in the secretome of ASC. ASC was cultivated for 72 h in fresh culture medium containing pyruvate. For an additional 24 h of culture, the medium was replaced by pyruvate-free medium and supplemented with 0, 5, or 25mM glucose in the presence of 0, 0.5, or 4mM glutamine as indicated in the figure. Concentrations in culture supernatants were measured by ¹H-NMR. Results (mean ± SEM; n =3) are the difference between concentrations in cell culture supernatants and the concentration in the initial medium placed in the same conditions (control). Values were not normalized to the cell number. Negative values represent metabolite consumptions and positive values the secretions at concentrations above the control. **(a)** The glycolysis pathway is evidenced by monitoring glucose uptake and lactate secretion. **(b)** Pyruvate, citrate, and alanine secretions and uptakes. **(c)** The glutaminolysis pathway is evidenced by monitoring glutamine uptake and glutamate secretion. Statistics are from the one-way ANOVA test followed by Tukey's multiple comparison test: *p <0 05, **p <0 01, and ***p <0 001 and comparison of the partially depleted medium conditions with the complete medium condition (25mM glucose and 4mM glutamine).

Modulating glutamine and/or glucose supply revealed that the secretion of pyruvate, citrate and alanine was correlated with glutamine concentration but not with glucose concentration. Only complete glucose deprivation decreased pyruvate secretion but to a lesser extent than in the

absence of glutamine (Figure 2-12b). The partial (V-ASC) or total (S-ASC) independence of citrate and alanine secretions regarding glycolysis underlines anaplerosis and the contribution of glutaminolysis for glucose consumption. We did not detect a clear dose-response relationship between glutamate secretion and glutamine uptake, indicating that glutamate secretion in ASC is not exclusively linked to glutamine metabolism. Glucose depletion in the medium did not affect glutamate secretion nor glutamine consumption. This indicated that glycolysis was not involved in the control of glutaminolysis (Figure 2-12c). These results also indicate that glutaminolysis is central to the use of pyruvate in the TCA cycle and the synthesis of alanine in ASC. In S-ASC only, glutamine privation led to a drastic switch of pyruvate consumption towards lactate production, exacerbating the Warburg-like effect.

Our results reveal that sensitivity to glutamine availability is a key feature discriminating both ASC populations. Further analyses involving fluxomics experiments and isotope labelling will be required to finely dissect the underlying mechanisms involved. Our results also demonstrate that ASC have a mixed metabolism based on both glycolysis and mitochondrial activity, similar to what was already reported for bone marrow mesenchymal stem cells [180]. Interestingly, we show that glutaminolysis controls pyruvate consumption for use in TCA cycle and alanine synthesis. Importantly, glutaminolysis prevents the use of pyruvate for lactate production in S-ASC, but not in V-ASC. As a consequence, S- and V-ASC can be discriminated by their relative secretion of lactate and citrate, lactate being more secreted by V-ASC and citrate by S-ASC.

This discrimination demonstrates the potential of NMR metabolomics approaches not only for the identification of biologically very similar entities, but also to deliver insights over the molecular mechanisms at the origin of fine physiological differences. Using metabolic stresses induced by culture media depleted in key nutrients, we reinforce metabolic phenotypes. To our knowledge, this study is the first one to describe at the metabolic level the differences between adipose-derived stem cells isolated from distinct adipose depots. Our results raise the possibility that mechanisms controlling the flux of pyruvate towards the synthesis of either lactate in the cytoplasm or citrate in the mitochondria could be related to the reported differences between S- and V-ASC in their proliferation and differentiation potentials.

2.2.3. Tissues and biofluids study: Dietary obesity in mice is associated with lipid deposition and metabolic shifts in the lungs sharing features with the liver

2.2.3.1. Introduction

In the previous chapters, we have presented examples where NMR metabolomics approaches were applied to bacterial and cellular models. By studying the cell footprint on their culture media, we obtained strong group-discrimination models, even of significant biological resemblance. We demonstrated the importance of profiling the exo-metabolome that allow us to determine the consumption/production patterns of fluxes of exo-metabolites. These data not only allow discriminate groups but also shed light on molecular metabolic mechanisms at the origin of this discrimination.

In this chapter, we jump a step higher, going from cell cultures to the study of entire organs and biofluids. We describe a project where we evaluated the impact of a high-fat high-sucrose diet over the lungs and other individual organs of mice to apprehend the metabolic modifications and disorders associated with the development of obesity, at the organ level. The examination of multiple organs adaptation to the diet coupled with serum profiles permitted to compare the metabolic evolution of multiple biological compartments. As expected, we measured important diet-induced modifications differences between organs, for example, the liver is more impacted by the diet than the brain, but importantly, we evidenced unexpected effects, for example on the lung physiology. This work is also noteworthy as it presents reference quantification tables of metabolites in organs.

This work implicated Béatrice Morio, Sylvie Guibert, Baptiste Panthu and many people from the CarMeN laboratory. The obtention and preparation of NMR samples from mice organs is a multiple months-long and difficult process, from mice breeding and caring, to sacrifice, organ harvesting and metabolite extraction. The ability of metabolomics methods to obtain insights over fine metabolic details directly depends on the quality of the biological samples that require the precise execution along the entire process and perfect coordination between many expert operators. NMR spectra were acquired through the TGIR infrastructure at 800 MHz.

A first article has been published about this project but a large amount of data is still under examination.

- *Rautureau, G. J. P., Morio, B., Guibert, S., Lefevre, C., Perrier, J., Alves, A., ... Panthu, B. (2021). Dietary obesity in mice is associated with lipid deposition and metabolic shifts in the lungs sharing features with the liver. Scientific Reports, 11(1), 8712. doi: 10.1038/s41598-021-88097-8*

2.2.3.2. Scientific context

Obesity has reached epidemic proportions worldwide and affects the metabolism of all organs, impacting therefore whole-body homeostasis. As a consequence, obesity increases the risk of important chronic pathologies such as type 2 diabetes and cardiovascular diseases [181]. Epidemiological studies have long shown that obesity is associated with both chronic and acute respiratory complications such as asthma, infectious diseases or chronic obstructive pulmonary disease (COPD) [182–186]. This topic has gained outstanding relevance with the Covid-19 (SARS-CoV-2) pandemic and its severe pulmonary complications that particularly affect obese patients [187].

Recent studies evidenced lung-localized metabolic alterations that may contribute to the pathophysiology of lung diseases in obesity, such as alterations of the lipid metabolism and the secretion of cytokines, or changes of the arginine metabolism leading to nitric oxide synthesis [182–184,188–190]. At the cellular level, impaired mitochondrial metabolism has also been observed [191,192], and stressed mitochondria have been reported in the airway epithelial cells of high-fat and high-fructose fed mice [193].

2.2.3.3. Experimental approach

Male C57Bl/6J mice were fed either with a standard diet (SD) or a high-fat and high sucrose diet (HFHSD) for 14 weeks, a classical nutritional model of obesity [194]. HFHSD mice gained 65% more weight than SD animals ($p < 0.0001$, $n = 12$ per group) with a 57% increase in the weight of the liver ($p < 0.0001$). The 14-weeks HFHSD diet induced significant alterations in body composition and systemic glucose homeostasis in mice, in favour of a progression towards type 2 diabetes [195].

Mice were sacrificed, serum and five other organs (liver, heart, skeletal muscle, kidneys and brain) were harvested and snap-frozen in liquid nitrogen for both SD and HFHSD mice ($n = 8$ per group). Metabolites were extracted from organs using methanol [196]. The ^1H 1D NMR profiles were acquired at 800 MHz at the CRMN. The careful analysis of the ^1H 1D NOESY spectra and the two-dimensional ^1H - ^1H and ^1H - ^{13}C NMR led to the identification and quantification of 44 metabolites in organs and 32 metabolites in serum. The relative lipid content could be also evaluated [197] and results were confirmed by triglyceride (TG) biochemical quantification.

2.2.3.4. Results and discussion

We analysed the metabolome of the lung by NMR spectroscopy and compared it with the metabolome of serum and five other organs (liver, heart, skeletal muscle, kidneys and brain) in both SD and HFHSD mice ($n = 8$ per group). We determined the absolute quantification of multiple metabolites which let us perform a detailed inter-organ comparison of HFHSD-induced metabolic alterations.

NMR analysis of the lung showed that HFHSD induced significant changes in 9 out of 32 metabolites compared to SD mice (Figure 2-13). The metabolites that varied significantly ($p < 0.05$) with the diet were serine, sn-glycero-3-phosphocholine, glutamine, valine and taurine that increased, while Betaine, myo-inositol, phenylalanine and o-phosphocholine decreased in HFHSD lung compared to SD lung (Figure 2-13-b). Interestingly, broad peaks were modified on lung NMR spectra, that could be assigned to the CH₃, CH₂ and CH₂-CO moieties of heterogeneous lipid species [197]. Their peak area was quantified in Figure 2-14 (panels a and c). To confirm these results, we measured biochemically TG levels in the lung of SD and HFHSD mice (Figure 2-14-b). TG levels were 94% higher in the lungs of HFHSD-fed animals compared to SD controls ($p < 0.001$), confirming lipid accumulation in the lung of HFHSD mice.

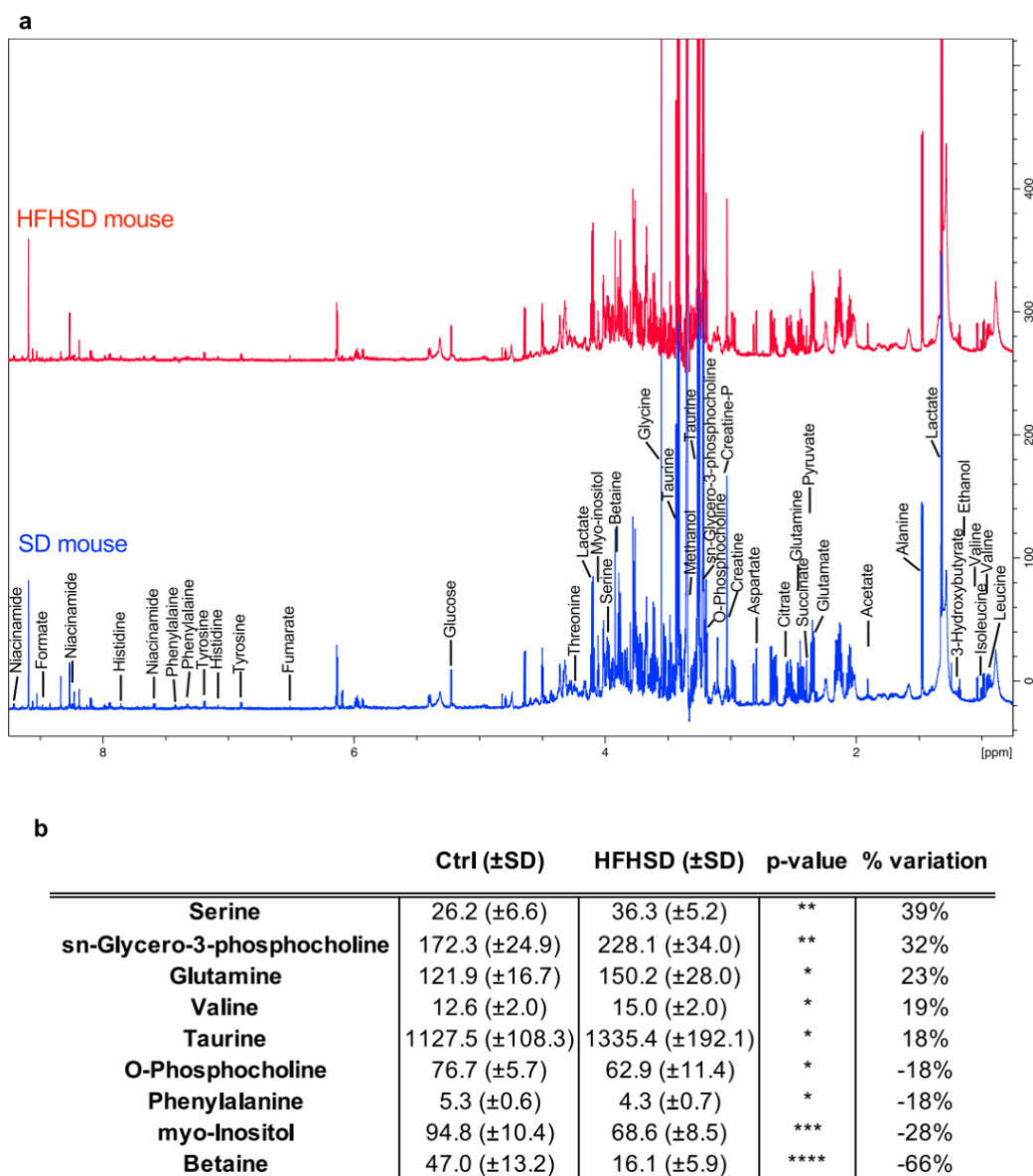


Figure 2-13. Metabolite modifications identified by NMR in the lung of HFHSD mice. (a) ¹H-NMR representative spectra from lung from SD (blue) and HFHSD (red) fed mice. (b) List of metabolites differentially found in the lung of HFHSD mice compared to SD mice. Results are expressed as mean \pm standard deviation (SD) and are in nmol/100 mg of the lung. Data are mean of $n = 8$ mice per group; Results of unpaired two-tailed t-test are indicated $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). Percentages of variation between HFHSD and SD fed mice are indicated.

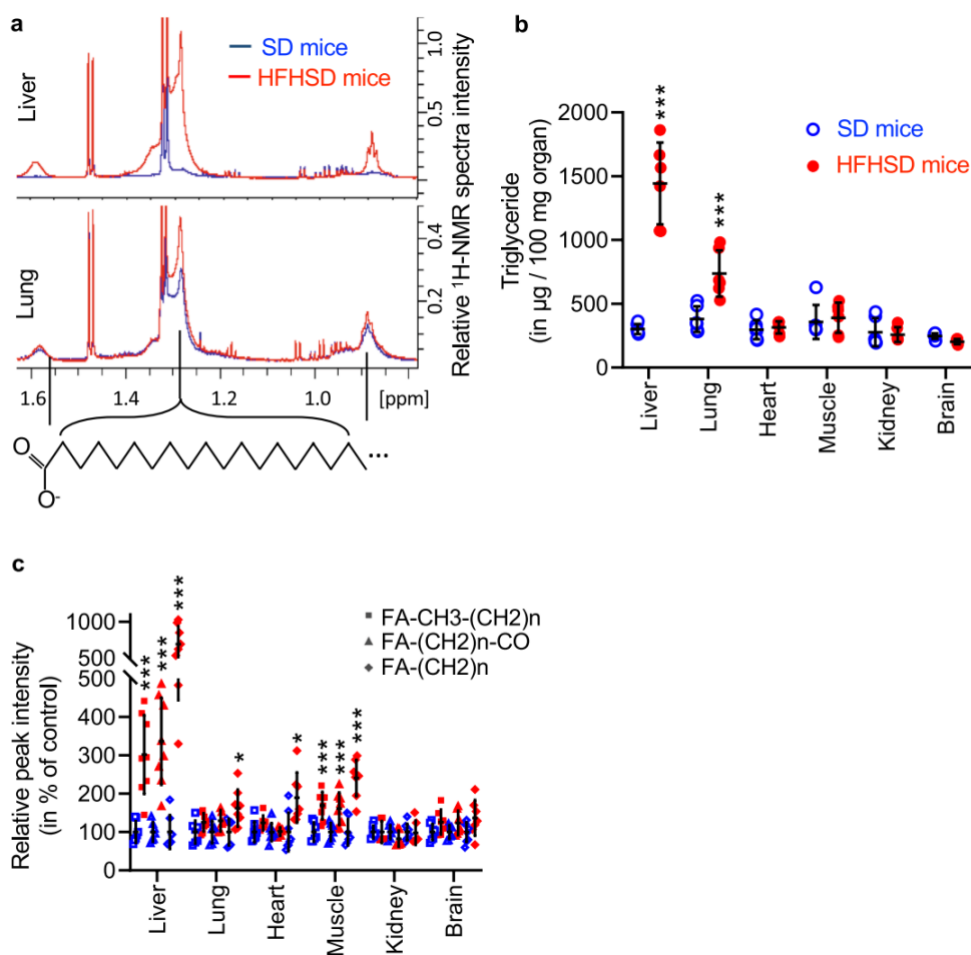


Figure 2-14. Lipids and specifically triglycerides accumulation in the lung in comparison with the liver or all other organs of SD and HFHSD mice. (a) ¹H-NMR representative spectra from the lung (at bottom) and the liver (at top) from SD (blue) and HFHSD (red) fed mice. Spectra zoom highlights fatty acid (FA) areas of -CH₂-CO (1.6 ppm), (CH₂)_n (1.3 ppm) and CH₂-CH₃ moieties (0.9 ppm). (b) Triglyceride contents in liver, lungs, heart, gastrocnemius muscles, kidneys and brain. (c) Relative abundance of each typical lipid moieties -CH₂-CO (triangle), (CH₂)_n (diamond) and CH₂-CH₃ (square) of SD (blue empty) or HFHSD (red full) fed mice estimated by integration of the ¹H-NMR spectra. Data are mean of n = 8 mice per group; Results of unpaired two-tailed t-test are indicated as non-significant (NS) p < 0.05 (*), p < 0.01 (**), p < 0.001 (***) and p < 0.0001 (****).

The serum had glucose concentration increased by 31% in HFHSD mice compared to SD mice (p < 0.02, n = 8 per group). NMR also evidenced lower serum concentrations for allantoin, creatine and phenylalanine, and higher concentrations for alanine and 3-hydroxybutyrate in HFHSD-fed mice compared to controls (p < 0.05). Citrate and succinate concentrations were 28% and 32% higher in the serum of HFHSD-fed mice compared to controls, respectively (p < 0.02). Inversely, glycine, dimethylglycine (DMG) and betaine that derive from the 1C cycle metabolism were 34%, 68% and 54% lower in the serum of HFHSD-fed mice compared to controls, respectively (p < 0.01). No significant alteration of branched-chain amino acid concentrations was observed in serum.

The HFHSD displayed tissue-specific perturbations. Among the 44 quantified metabolites, 16, 8, 8 and 7 were significantly altered in the liver, heart, gastrocnemius muscle and kidneys, respectively. The less impacted organ was the brain which displayed only 2 altered metabolites following HFHSD. All the organs studied harboured one or several unique metabolite alterations which could constitute potential biomarker candidates of the organ physiology adaptation to diet. For example,

pantothenate was 34% lower ($p < 0.05$) in the brain; citrate and acetone were 66% ($p < 0.01$) and 28% ($p < 0.05$) respectively lower in the kidney. Liver showed the most unique metabolite alterations with a notable concentration increase by 3,499% of 2-hydroxybutyrate from 4.8 ± 1.5 nmol/100 mg of tissue to 172.4 ± 61.4 nmol/100 mg ($p < 0.0001$). Important variations in the 1C pathway were observed in several tissues of HFHSD-fed mice. For example, the liver evidenced the strongest decrease in the methionine cycle with 38% lower concentrations in glycine, 60% in sarcosine, 84% in DMG and 93% in betaine in HFHSD-fed mice compared to controls ($p < 0.003$). Similarly, in gastrocnemius muscle, both glycine and DMG concentrations were 30% and 50% lower in HFHSD-fed mice compared to controls, respectively ($p < 0.002$). In kidneys, betaine was 50% lower in HFHSD-fed mice compared to controls ($p < 0.0003$), as observed above in the lung. As observed for the lung, broad peaks on NMR spectra of some tissues (Fig. 2a and 2b), could be assigned to heterogeneous lipid species and evaluated. TG levels were also quantified biochemically in all organs. As showed in Figure 2-2-b, TG levels were 378% higher in the liver of HFHSD mice compared to SD mice but unaltered in other organs than lungs. [198].

2.2.3.5. Conclusion

The lung displayed both specific and shared metabolic changes following HFHSD, compared to serum and/or other organs and was the second most affected organ after the liver. Lung and liver shared reduced one-carbon (1C) metabolism and increased lipid accumulation. Altered 1C metabolism was found in all organs and in the serum. Lastly, tricarboxylic acid (TCA)-derived metabolites were specifically and oppositely regulated in the serum and kidneys but not in other organs. Collectively, our data highlighted that HFHSD induced specific metabolic changes in all organs, the lung being the second most affected organ, the main alterations affecting metabolite concentrations of the 1C pathway and, to a minor extend, TCA.

Lipid content is increased both in the liver and lung of the HFHSD mice, but not in the other organs considered in this study. Lipid accumulation in the liver is well documented, contrary to the lung where a single lipid-targeted metabolomics study showed significant quantitative and qualitative differences in neutral lipids, fatty acids, phospholipids and sphingolipids content in lung between HFHSD and very high-fat diets [184]. As lipotoxicity contributes to the development of non-alcoholic fatty liver diseases, an involvement of the lung lipid storage is possibly involved in obesity-associated complications such as asthma and COPD, or increased susceptibility to infectious diseases such as seasonal flu or SARS-CoV-2.

One important observation of our study is that HFHSD-fed mice presented serum- and organ-specific metabolomics adaptations. Caution should be taken as some metabolic adaptations in organs may not only be a consequence of the diet. Indeed, obesity *per se*, rather than overnutrition, can induce organ-specific metabolic adaptations. This question was raised by Mora-Ortiz *et al.* who examined *db/db* mice organs [199], a natural genetic model of obesity and also reported a decrease of 1C related metabolites in many organs. Unfortunately, they did not include lung in their study.

2.2.4. Subcellular metabolic machinery characterization using Real-Time NMR: Cell-Free Protein Synthesis Enhancement from Real-Time NMR Metabolite Kinetics: Redirecting Energy Fluxes in Hybrid RRL Systems.

2.2.4.1. Introduction

Metabolomics aims to the identification and quantification of the metabolites present in a biological system and determines their modification in response to a perturbation. In most studies, metabolomics profiles are obtained as a snapshot of the steady-state, the metabolites are evaluated either in stable biological samples, such as some biofluids or are extracted from a fixated biological sample. Metabolite concentrations are determined and conclusions are drawn from the variations of concentration of metabolites between two or more conditions. The knowledge obtained from metabolomics studies most often stands as the possibility to distinguish groups (using either individual biomarkers or complex signatures of metabolites), and eventually, mechanistic models explaining the origin of the discrimination. Another approach to defining models of metabolism depends on the obtention of dynamic information such as measures of matter fluxes along metabolic pathways.

Real-time NMR (RT-NMR) is a strategy developed to study composition-evolving solutions. RT-NMR consist in acquiring a series of spectra over time, from which the sample composition, concentration and evolution can be characterized (Figure 2-15) [200]. Real-Time NMR is gaining popularity in the field of metabolism and complex media enzymology due to its unique capacity to measure simultaneously multiple metabolic reactions in one experiment, which allows characterizing whole enchained reactions and metabolic pathways.

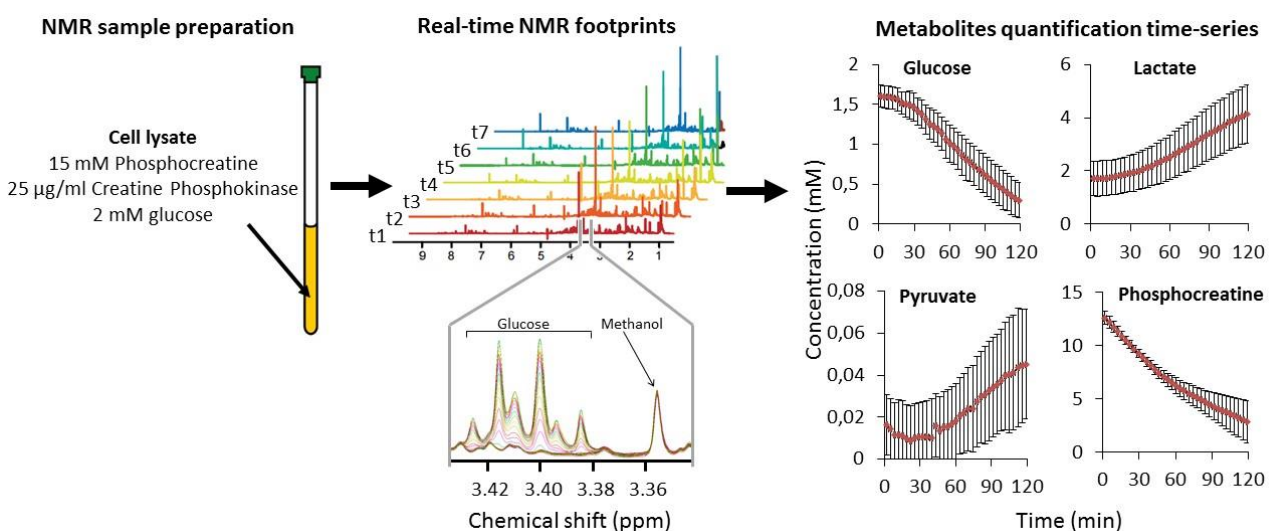


Figure 2-15. RT-NMR allows following the evolution of metabolites in complex enzymatic systems such as cell lysates in which activity is reactivated by adding an energy source (such as phosphocreatine and creatine phosphokinase), and metabolic substrates. NMR spectra are acquired on the active mix. The sample composition, concentration and evolution can then be characterized

In this chapter, we describe how we used RT-NMR to investigate a particular cell-free protein synthesis system (CFPS). CFPSs are cell lysates in which particular cellular functions, such as the translation of recombinant proteins or RNA transcription, are reactivated in vitro for technological applications or scientific research. CFPS require a very delicate formulation to run and even with highly optimised conditions, for example, protein translating, CFPS remain productive for at most a few hours before stopping, as substrates are consumed and waste products accumulate. Cell lysates require complementation with many cofactors (energy sources (in the form of an ATP regenerating system), cellular substrates, metabolites and salts) to be productive.

In this study, we were interested in studying a counter-intuitive cell-free protein synthesis (CFPS) strategy, based on reducing the ribosomal fraction in rabbit reticulocyte lysate (RRL) [201]: reducing the amount of ribosomes allowed to produce higher protein yields. This strategy is at the origin of the development of hybrid systems composed of RRL ribosome-free supernatant complemented with ribosomes from different mammalian cell types. These Hybrid RRL systems present the important advantage of maintaining translational properties of the original ribosome cell types, while delivering protein expression levels similar to RRL.

We followed the CFPS translation reaction using RT-NMR, and managed to determine why a lower amount of ribosome approach was beneficial: persistent ATP energy-consuming metabolic activities associated with the ribosome fraction expends most of the energy supply, independently of protein synthesis. We evidenced that energy metabolism is the most crucial factor for efficient CFPS, and demonstrated that optimising energy supply is essential to sustain long-lasting translation. Using concentrations of ribosomal fractions lower than their native ratios significantly optimises this issue.

This work was performed with Baptiste Panthu, who was studying eucaryote CFPS systems, and with the support of Bénédicte Elena-Herrmann and others. This work led to my first last author article:

- Panthu, B., Ohlmann, T., Perrier, J., Schlattner, U., Jalinot, P., Elena-Herrmann, B., & Rautureau, G. J. P. (2018). *Cell-Free Protein Synthesis Enhancement from Real-Time NMR Metabolite Kinetics: Redirecting Energy Fluxes in Hybrid RRL Systems*. *ACS Synthetic Biology*, 7(1), 218–226. doi: 10.1021/acssynbio.7b00280

2.2.4.2. Scientific context

Cell-free protein synthesis (CFPS) systems have become central tools for protein research or biotechnology [202,203]. For example, they can be used to produce therapeutic proteins such as antibodies, vaccine components or cytokines [204–207], but also key pharmaceutical targets for drug design assays and structural biology [208], or viral particles [209]. CFPS also constitutes excellent opportunities for direct sampling and screening of molecules in a close-to-physiological context, e.g. for numerous in vitro translational assays such as ribosome display techniques [210].

Yet, the development of CFPS systems to their full potential has been hampered by the complexity of their molecular machinery, which involves many cellular processes and regulatory circuits [211]. Only a few active CFPS systems have been successfully developed so far, with lysates derived from *E. coli*, wheat germ, insect cells or rabbit reticulocytes being the most commonly used [202]. *E. coli* and wheat germ can produce large amounts of a given protein but sometimes suffer from problems related to expression, folding and post-translational modifications of eukaryotic proteins [202].

Mammalian CFPS systems are still believed to provide the most adequate machinery for bona fide eukaryotic protein production and downstream processing. Disappointingly, rabbit reticulocyte lysate (RRL), the main commercialized mammalian CFPS system (along with Chinese hamster ovary (CHO) and Hela systems), is so far not optimal to deliver high protein production yield [202]. B. Panthu et al. and others have proposed a CFPS system [212–215] based on rabbit reticulocyte ribosome-free lysate supernatant (ribFreeRRL) complemented with purified ribosomal fractions, either from rabbit reticulocytes (reconstituted RRL or recRRL) or from diverse other cell types (hybrid RRL) (Figure 2-16a). This hybrid RRL approach allows a wide range of biological observations as it maintains cellular properties such as specific mRNA translation regulation.

Interestingly, hybridRRL and recRRL produced higher yields of protein expression when a lower amount of ribosome was used. They initiate translation with the same rate as native RRL but are capable to maintain it for several hours, while translation rapidly levels off after 30mn in native RRL, an effect that is not due to mRNA stability [213]. We aimed to investigate why smaller amounts of ribosomes were beneficial for protein expression.

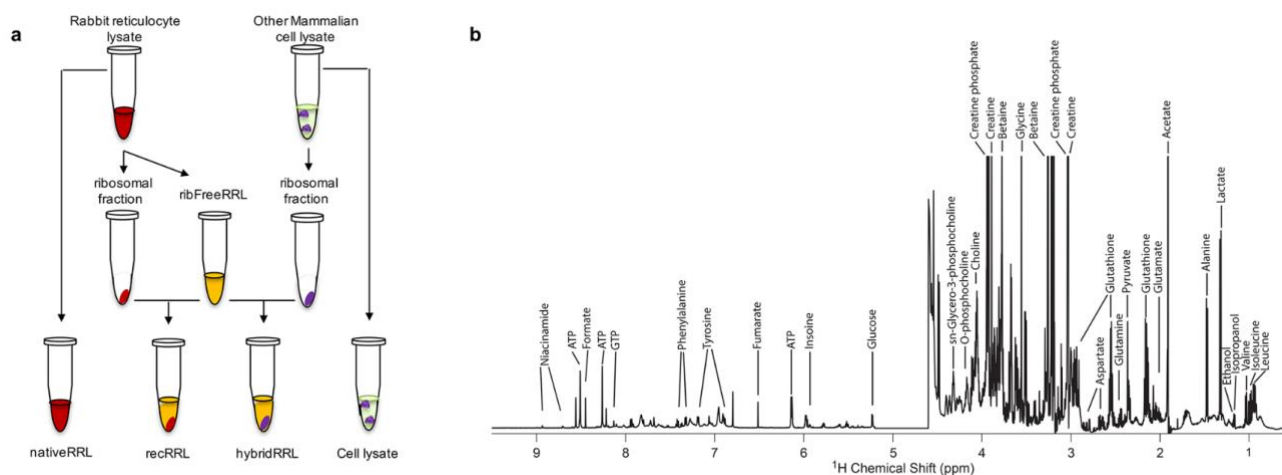


Figure 2-16. CFPS energy consumption monitoring by real-time NMR. (a) RRL-derived CFPS reconstitution approach and nomenclature used in this article. Ribosomal fractions are easily isolated through ultracentrifugation under sucrose cushion. (b) Typical ¹H NMR spectrum of a translating nativeRRL mix. Active CFPS reactions (200 μL) were monitored in real-time inside the NMR spectrometer at 30 °C.

2.2.4.3. Experimental approach

The standard CFPS mix is constituted by commercial untreated RRL (RRL) (Promega) supplemented with 25 μ M hemin, 25 μ g creatine kinase, 5 mg/mL phosphocreatine, 50 μ g/mL of bovine liver tRNAs and 2 mM of D-glucose. The 200 μ l *in vitro* CFPS translation assay was contained 50 % v/v RRL, 27nM of *in vitro* transcribed mRNAs, 75 mM KCl, 0.75 mM MgCl₂ and 20 μ M amino acids mix. The CFPS reaction was realised in the NMR spectrometer at 30°C. The *gloRenilla* construct (a standard reporter of protein translation) was used for every protein translation assays.

For reconstituted CFPS mixes, the strategy and nomenclature are illustrated in Figure 2-16a. Ribosomes were isolated by centrifugation of 1 ml of RRL for 2h15 min at 75000 rpm, 900 μ l of ribosome free RRL was collected, frozen and stored at -80 °C. The ribosomal pellet was then rinsed three times and resuspended in 100 μ L of buffer to get a 10 X final concentration. The reconstituted lysate is then assembled by mixing 100 μ l of ribosome free RRL with a fraction of scale from 0.02 to 1 X ribosomal pellet. Typically, the standard reaction for NMR contains 100 μ l of ribosome free RRL with 20 μ g ribosomal pellet (annotated 0.1 X) in a final volume of 200 μ l.

RT-NMR was performed on a Bruker 600 MHz NMR spectrometer equipped with a 5 mm TCI cryoprobe, at 30.0°C, with 200 μ L CFPS sample in 3 mm NMR tubes. All the solutions were strictly kept on ice until NMR acquisition. Time series of standard ¹H 1D NMR pulse sequence Carr-Purcell-Meiboom-Gill (CPMG) with water presaturation (Bruker pulse program cpmgpr1d) were acquired. 32 free induction decays (FIDs) were collected with 48074 data points and an acquisition time of 1.99 s. The relaxation delay was set to 4s. The total acquisition time for each spectrum was 3min 27sec. The acquisition was repeated for 2 to several hours. Metabolite concentrations were determined using ChenomX.

2.2.4.4. Results and discussion

To study RRL CFPS reactions, we used a quantitative real-time NMR strategy to monitor intact, translating CFPS samples over two hours. As a primary energy source, an ATP regenerating system was used (the CFPS mix is supplemented with 10 mM phosphocreatine (PCr) in the presence of creatine kinase (CK: ADP + PCr \rightarrow ATP + Cr)). From the complex CFPS ¹H-NMR fingerprint (Figure 2-16b), numerous peaks could be unambiguously assigned to specific metabolites and the evolution of their relative concentration followed over time, characterizing production, consumption, or steady-state behaviour. This included PCr consumption and creatine production, but also the time course of other energy intermediates (ATP, GTP, glucose) and other metabolites such as amino-acids.

Classical RRL translation (native RRL) was run as a control experiment. It revealed that the given PCr pool could maintain maximal energy supply for about 60 min, keeping ATP and also GTP concentrations globally stable during this time period (Figure 2-17a). At that time point, glucose was also entirely exhausted, and a drop in ATP and GTP occurred. Protein yield indicated very active

translation during the first 30 min, which then slowed down and ceased after 45 min (Figure 2-17b). These data evidenced a considerable energy flux occurring during CFPS.

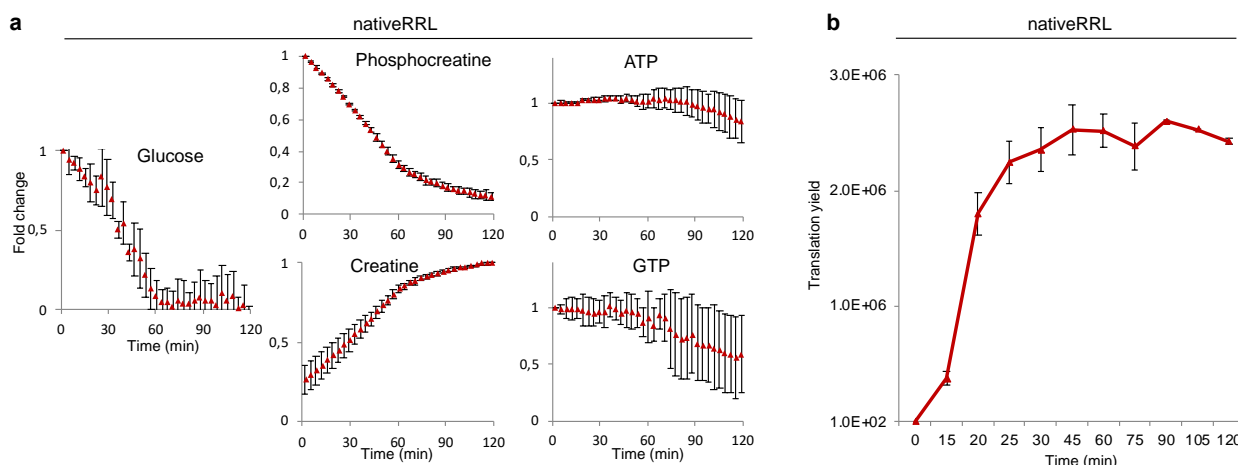


Figure 2-17. Native RRL monitoring by real-time NMR. **(a)** Evolution of metabolite concentrations quantified by real-time NMR during 2 h and normalized to their initial or final concentration. **(b)** Protein production yield based on luciferase activity measurement through a Renilla reporter gene expression. Each batch is supplemented by 27 nM of *in vitro* transcribed mRNA. Error bars represent the standard deviation of three experiments.

We investigated the respective importance of PCr and glucose for protein yield. Removing PCr (or CK) precluded glucose utilization (Figure 2-18a) and protein synthesis (Figure 2-18c). In the absence of an efficient ATP regenerating system, the low net ATP production of glycolysis is insufficient to fuel both glycolysis initial steps and competitive RRL metabolism. Consistently, glucose removal had no apparent effect on steady-state ATP and GTP levels (data not shown), confirming that the PCr/CK system is the primary and essential energy source. Interestingly glucose reduction lowered protein synthesis by about 30% (Figure 2-18c). Glycolysis thus appears to contribute to protein translation, either through NADH regeneration or via production of intermediate metabolites that may indirectly benefit translation. Notably, this is contrary to bacterial lysates, where glucose can be used as the major source of energy [216].

To better understand energy expenditure related to protein synthesis, we conducted experiments in which protein synthesis was blocked by cycloheximide (CHX), which inhibits the elongation process, and RNase A, to induce mRNA degradation. Astonishingly, PCr consumption was only slightly reduced, by less than 20% at 60 minutes of reaction time compared to control (Figure 2-18a, bottom panel). This showed that protein translation was not the main consumer of ATP in the CFPS system. A slight reduction of glucose consumption was also detected and pyruvate and lactate production were reduced (data not shown), indicating that inhibition of translation reduces glycolytic flux.

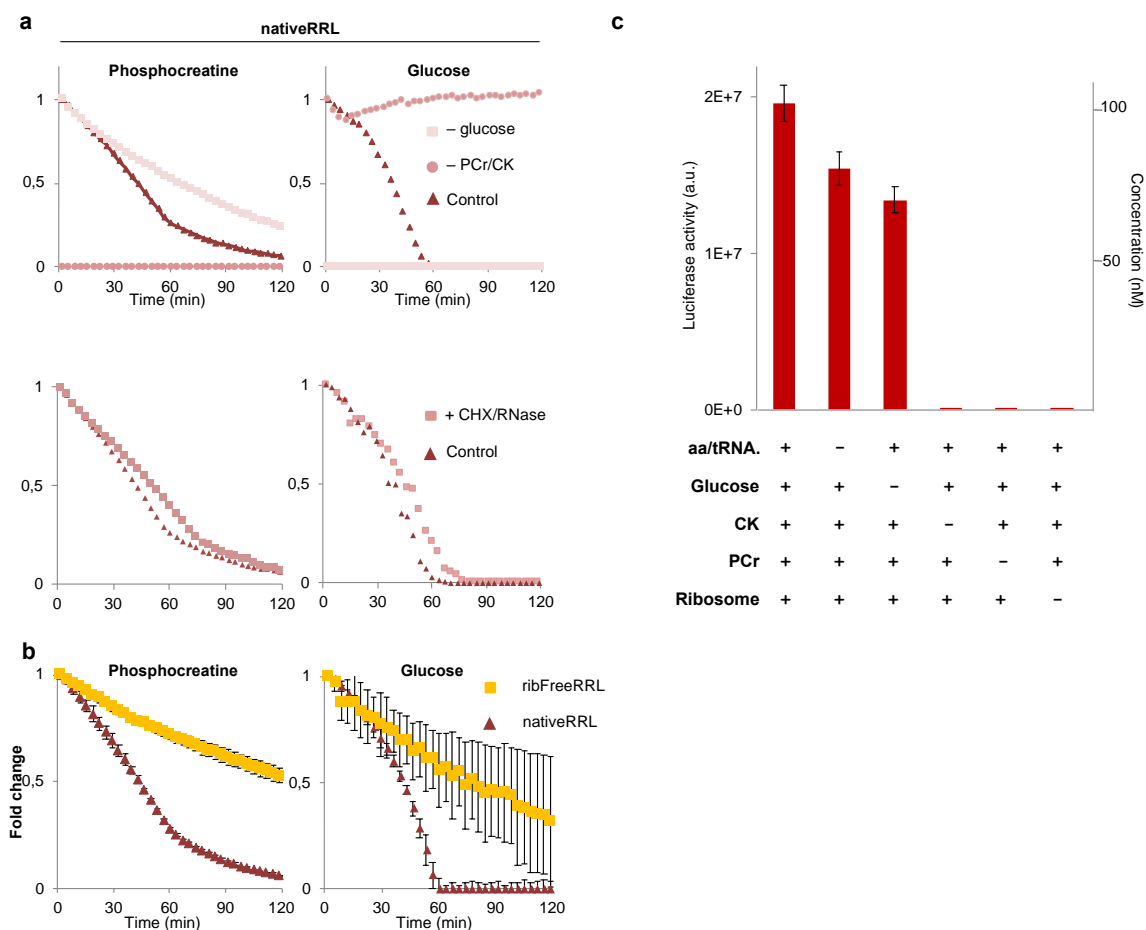


Figure 2-18. Energy consumption in CFPS is maintained even when translation is blocked. **(a)** Phosphocreatine and glucose evolution during 2 h of in vitro translation: nativeRRL control (Δ) versus nativeRRL without glucose (\square) or without CK and phosphocreatine (\circ) supplementation (bottom panel) (top panel) ; comparison of a nativeRRL control (Δ) versus an elongation-blocked nativeRRL (\square) by addition of cycloheximide (CHX) and RNase A; (bottom panel) . **(b)** Comparison of a nativeRRL translating (Δ) versus a ribFreeRRL (\square) system: phosphocreatine and glucose evolution during 2 h of in vitro translation. Error bars represent the standard deviation of three experiments. **(c)** Protein production yield based on luciferase activity measurement through a Renilla reporter gene expression in nativeRRL (left) or without addition of amino acids and tRNA, glucose, CK, phosphocreatine or ribosomal fraction (ribosome). Absolute luminescence intensity is plotted on left y-axis. Concentration was determined using methionine 35S labeling and is plotted on right y-axis. Error bars represent the standard deviation of three experiments.

Finally, we conducted an experiment that omitted the ribosomal fraction, thus avoiding protein synthesis. Under these conditions, PCr and glucose were still consumed, but at a much lower rate compared to the native RRL (Figure 2-18b) or to native RRL after CHX inhibition of protein synthesis (Figure 2-18a). Therefore, translation-independent free energy consumption seems to be largely driven by components co-purified with the ribosomal fraction, or by the ribosome itself.

Our data indicate that while a significant amount of free energy is directed toward protein synthesis (at most 40%), a majority supports alternative pathways that are associated with the ribosomal fraction. The ribosomal fraction has recently been shown to be strikingly complex, with more than 1000 proteins associated to the ribosome machinery, constituting the riboproteome [217] . Those proteins are believed to create in vivo a favourable microenvironment necessary for driving, regulating, and securing specificity of the translation process. Interestingly, the riboproteome

contains also many enzymes involved in common metabolic pathways [217–219], consistent with the free energy-consuming processes that we found associated with the ribosomal fraction.

We then explored the effect of riboproteome concentration on both translation-independent metabolism and protein synthesis in our CFPS system. Reducing the amount of the ribosomal fraction in ribosome free RRL (recRRL; Figure 2-16a) directly correlated with reduced free energy consumption of the system (Figure 2-19a), while protein synthesis was reduced in a more complex manner: reducing the ribosomal fraction of to only 10% (0.1X) the original lysate increased protein yield after 90 min of synthesis by about 4-fold (Figure 2-19b). Further reduction of the ribosomal fraction to 2% (0.02X) reduced protein yield. Under this condition, the yield was nonetheless higher than the one of the 1X controls. At native concentrations, the translation apparatus seems therefore saturated. These differences in protein yield were not apparent at shorter reaction times (30 min), suggesting similar initial translation rates (Figure 2-19b). In fact, the different mixtures differed in the time course, with nativeRRL and 1X recRRL ceasing protein synthesis after 30-45 minutes, while recRRL, depending on the ribosome dilution, remained productive for longer reaction times.

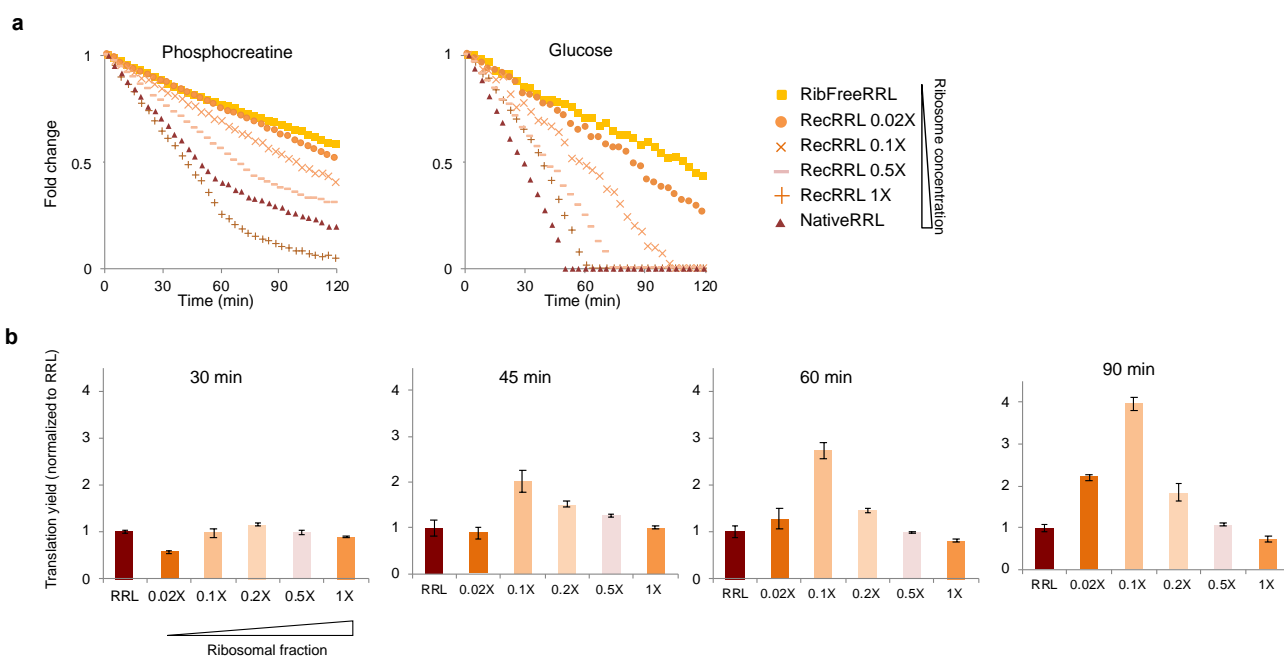


Figure 2-19. Lowering the ribosome ratios increases the protein production yield. (a) Phosphocreatine and glucose evolution during 2 hours of in vitro translation in nativeRRL (Δ), ribFreeRRL (\square), recRRL with 0.02 \times (\circ), 0.1 \times (\times), 0.5 \times ($-$) and 1 \times ($+$) ribosomal fraction addition. (b) Protein production yield evaluated as luciferase activity by luminometry at different time of incubation (30, 45, 60, and 90 min). Protein production was normalized to nativeRRL (RRL). RecRRL was supplemented with an increasing amount of ribosomal fraction from 0.02 \times to 1 \times .

These results demonstrate that the metabolic activities associated to the ribosomal fraction contribute to the reduced translation yield in CFPS reactions by competing for energy supply. Using lower ribosomal fraction ratios than native systems appeared to be very beneficial: diluting the competing free energy-consuming processes liberated energy reserves, which were then efficiently

used by the ribosome translational machinery because of its saturated translation capacity down to concentration ratios of 0.1X.

2.2.4.5. Conclusion

Altogether, we demonstrated that dilution of the ribosomal fraction slows the waste of energy by translation-independent ATP-consuming pathways, promotes significantly longer lasting CFPS reactions, and thus maximises ribosome activity and protein yield. While competing energy-consuming activities of the ribosomal fraction do not appear to contribute to translation, they may be necessary for translation *in vivo*, but appear non-limiting under the conditions tested. Manipulating the ribosome ratio was already used to enhance protein production in yeast CFPS [220], but the underlying energetic aspects were not investigated. The hybrid system approach investigated in this study appears therefore as an accessible, solid and pragmatic tool to improve protein yield and could present a breakthrough for the elaboration of cell-free protein synthesis systems.

This study also illustrates the potential of RT-NMR to follow complex active bio-molecular systems for technological applications. While we concentrated on energy management during the CFPS reaction, a large amount of data was collected about the time evolution of many components of the reaction. Most data were not used in this study but offered a detailed portrayal of the reaction. Such potential is significant and probably overlooked, both from the NMR methodological point of view, but also from the research, development and industrial applications.

Many metabolites were modified during the CFPS reaction, showing that numerous metabolic pathways are still active in the lysate. This led us to postulate that such CFPS and lysates are incredible systems adequate to explore the functional state of the cellular metabolic machinery. Such information would be exceptionally useful to complement standard metabolomics data as it would allow generating more accurate metabolic models by alleviating ambiguities.

3. Research Projects

After being consecutively a member of Françoise Vovelle's, Mark Hinds's, Neil Ferguson's, Bénédicte Elena-Herrmann's, and finally Sami Jannin's groups, I now aim to develop and organize my own scientific exploration along my objective to promote the use of NMR in life sciences and develop NMR analytical solutions to the needs of biologist and biomedical researchers, to push back exploration barriers in life sciences. The CRMN constitutes a perfect environment for that goal, and offers research conditions for which I have been grateful since my recruitment. Not only the CRMN hosts world-class state-of-the-art NMR equipment and unique prototypes, but it also gathers a wide variety of world-recognised researchers and projects that offer complementary expertise in the field of NMR.

I plan to organize my future projects around developments and applications of metabolomics for life-sciences, exploiting the vast possibilities offered by high-field and high-throughput NMR data acquisition:

- *On one side I seek to develop and promote my novel approaches and methods for metabolic phenotyping, for example based on RT-NMR. Among the various projects in which I have been involved over the last years, we have developed with Baptiste Panthu Real-Time NMR approaches to measure the biochemical reactions in cell-free systems and established a proof of concept for the use of RT-NMR to determine metabolic fluxes through metabolic pathways. I believe RT-NMR studies of biologically active samples constitute an overlooked area in metabolomics research, which can deliver otherwise inaccessible dynamic metabolic fluxes data. Developing such an approach will constitute a very unique metabolism phenotyping tool. This project has been proposed at the ANR call 2021 (vide infra.).*
- *On the other side, I seek to collaborate with biomedical researchers and tailor NMR applications to their needs, to deliver at full potential the results that metabolomics promise. Such collaborations are critical to maintain the CRMN in Lyon bio-medical community, and the demand for collaborations in metabolomics is high. Lyon is a European capital for medical research and hosts numerous prestigious research centres. Some collaborations are already active, for example with the CarMeN laboratory of the Faculté de Médecine Lyon-Sud, with which we have active projects, such as the follow up of the study of the organs. Thanks to former collaborations implicating Bénédicte Elena-Herrmann, we have direct contacts with the Hospices Civils de Lyon (HCL), the Centre de recherche en Cancérologie de Lyon (CRCL), Centre Léon Bérard, Centre International de Recherche en Infectiologie (CIRI), Biomérieux, Merial, etc.).*

In the next part, I will present a project proposed to the ANR call 2021, as a collaborative project (PRC) between the CRMN and the CarMeN laboratories. This project aims to profile isolated mitochondria metabolism using RT-NMR. This project integrates a new NMR technology to enhance

NMR sensitivity, that is being developed at the CRMN by Sami Jannin: dissolution dynamic nuclear polarization (dDNP). dDNP constitutes a revolution in NMR as it allows to augment NMR nuclei polarization, leading to a massive signal enhancement. The gigantic gain in signal intensity permits to reduce the acquisition time to single pulsed experiments on a single second timescale, in perfect alignment with RT-NMR approaches. dDNP will allow the characterization of both fast metabolic reactions and low populated metabolic intermediates [122]. The combination RT-NMR and dDNP for metabolic profiling is expected to provide a disruptive technology in the field of metabolism studies and the accessibility at the CRMN of the world-unique equipment's of Sami Jannin's team will allow us to be at the forefront of this field of research.

This project includes 2 partners and 4 participants: at the CRMN, myself and Sami Jannin and at the CarMen: Baptiste Panthu and Hubert Vidal. This project represents the strong collaboration between the CRMN and the CarMen, not only to collaborate along a scientific project but more importantly to develop and integrate new technologies in the biomedical research. Many researchers at the CarMeN, convinced by the active promotion of Baptiste Panthu, have understood the potential of NMR for Life-Sciences and fully support our initiative.

3.1.Mitochondrial metabolism profiling in real-time and application to mitochondrial diversity and drug screening by NMR

The Mitoprofile project concerns the development of an analytical strategy to investigate mitochondria metabolism in real-time: our approach aims to deliver rapid, high-resolution and cost-effective results, with a biomedical community-accessible technology.

We propose to use real-time nuclear magnetic resonance (RT-NMR) to assess the metabolism of freshly isolated mitochondria placed in test media. The RT-NMR approach will deliver a quantitative description of the active metabolic reactions in mitochondria placed in standard conditions. We will use the dissolution dynamic nuclear polarization (dDNP) technic to hyperpolarize substrates and significantly augment their NMR-signal intensity. This gain in sensitivity will let us assess fine and rapid metabolic reactions but also low-populated reactional intermediates. The MitoProfile strategy will offer a versatile and potent tool, that will permit to determine not only the activation level of various metabolic pathways in mitochondria but also the impact of altering conditions or adding chemicals or drugs on mitochondrial metabolism.

To demonstrate the potential of our analytical strategy, we propose to (1) explore the metabolic diversity of mitochondria from various origins and (2) evaluate the activity of well-known drugs targeting mitochondria metabolism.

3.1.1. Objectives and research hypothesis

3.1.1.1. Introduction: Why is studying mitochondria metabolism timely and important?

Long considered the cell's energy powerhouse, mitochondria are nowadays recognized as active organelles that accomplish critical cellular functions and regulate the entire cell physiology [221,222]. Mitochondrial dysfunction has been associated with numerous human diseases, for example, metabolic disorders such as type 2 diabetes and cancer [7]. Deepening our understanding of the role of mitochondria in cell death, oncogenic transformation, cancer metabolism but also to metabolic diseases and response to therapies for example, will open opportunities for diagnosis, therapy and precision medicine [7].

3.1.1.2. The scientific and technical barriers to studying mitochondria metabolism

Mitochondria are symbiotic entities nested in eukaryotic cells. They contain their own DNA, encoding a small number of vital genes, and partially house their own metabolic reactions, regulation and homeostasis. Characterizing mitochondria metabolism inside their host cell, in a comprehensive manner and at the molecular level, is then challenging as their subtle metabolism is hindered in the cytosolic environment. Furthermore, some mitochondrial metabolic reactions are localized inside the mitochondria while others can occur both inside and/or outside the mitochondrial compartment, with complex metabolite transporters and shuttles regulating the chemical fluxes between the cytoplasm and mitochondria. Finally, mitochondria metabolism is complexified by its intermembranous, cristae and matrix sub-compartments that host localized metabolic reactions.

Altogether, despite fantastic progress in analytical technologies, assessing mitochondria metabolism is still challenging, and most biomedical research projects remain limited to the measurement of few parameters, such as O₂ and pH variations, and are constrained to a limited number of samples measured by day (medium-throughput capacity). High-resolution methods, that are based on complex isotope tracing experiments using NMR or mass spectrometry, are restricted to a limited number of samples and require advanced equipment, expertise and resources while lacking real-time and short kinetic potential.

3.1.1.3. The objectives and the research hypothesis

Our objective is to develop a physiologist-accessible analytical approach to obtain high-resolution metabolic profiles of mitochondria, with a medium-throughput capacity. Besides studying metabolite content and concentrations, we seek to portray the dynamic and functional state of

isolated mitochondria metabolism in real-time. Specifically, we aim to provide quantitative data about the main metabolic pathways activity in mitochondria (Krebs cycle, fatty acid oxidation, ketogenesis, transamination, folate cycle, and cardiolipin, quinone and steroid biosyntheses) [221].

To reach this objective, we propose to develop a full new NMR-based methodology, scientific workflow and protocols combining biological sample preparation, real-time Nuclear Magnetic Resonance (RT-NMR) measurements and optional hyperpolarization-based NMR-signal sensitivity enhancement (up to 10,000 times), to enable monitoring real-time metabolic processes in isolated mitochondria, with medium throughput potential, high resolution and high sensitivity. Importantly, our setup will offer facilitated access to conditions and molecules testing.

To demonstrate the ability of our methodology to deliver state-of-the-art data for biological and biomedical sciences, we will (i) perform a complete metabolic profiling of well-characterized mitochondria from Hela-cells, as a model system, and then expand to (ii) explore the metabolic diversity of mitochondria from various cell origins and (iii) evaluate the activity of well-known drugs targeting mitochondria metabolism.

Living cell-lines-derived mitochondria will be placed inside the NMR spectrometer, in the presence of adequate metabolic substrates and in suitable conditions to sustain mitochondrial metabolism [223]. Working on isolated mitochondria will allow to:

- *focus on mitochondria metabolism while eluding other compartments metabolic activities.*
- *reduce the limitations related to a selective plasma membrane.*
- *design a versatile setup offering unique opportunities for chemicals and conditions testing.*

We will use **RT-NMR** to follow the metabolic flux and matter fluxes of unlabelled or ^{13}C -isotopically-labelled substrates. RT-NMR delivers a series of time-resolved spectra, from which about 60 metabolite concentrations and time-evolutions can be determined simultaneously [34,200]. RT-NMR is well-suited to obtain **a broad view of metabolic reactions and a quantitative measure of both the metabolome dynamics and the functional state of the mitochondria.** ^{13}C NMR spectroscopy provides larger signal dispersion than ^1H NMR but at the cost of a loss of sensitivity, hindering the detection of low-populated metabolic intermediates in real-time.

We propose to **use hyperpolarized ^{13}C -isotopically labelled substrates** to enhance sensitivity, selectivity and time resolution and overcome ^{13}C -isotope related limitations. **Dissolution dynamic nuclear polarization (dDNP)**, a revolution in NMR, augments NMR nuclei polarization, allowing a massive signal enhancement (up to 10,000 folds) [224]. The enormous gain in signal intensity permits to reduce the acquisition time to single pulsed experiments on a single second timescale and allows the detection of both fast metabolic reactions and low populated metabolic intermediates [122]. DNP polarization also augments the specificity, as only the polarized substrate(s) and derived product(s) will benefit from signal enhancement [225], producing a very significant intensity gap between polarized and non-polarized metabolites signals. The acquisition time is reduced from 30 minutes using classical ^1H RT-NMR to minutes using ^{13}C dDNP. The recent

installation at the CRMN of a dDNP polarizer will allow us to be at the forefront of this field of research.

3.1.1.4. The expected results and the final products developed

The concrete results and deliveries of this project will be:

1. *A complete series of protocols for metabolic profiling of freshly isolated mitochondria, from biological sample preparation, real-time Nuclear Magnetic Resonance (RT-NMR) acquisition and optional dDNP hyperpolarization-based signal enhancement, and data exploitation guidelines.*
2. *A comprehensive dataset constituted of the metabolic profiles acquired for mitochondria from Hela-cells and a series of the most widely used cell lines (CHO, JURKAT, Huh7, CaCo2, A549) but also from non-immortalized primary cells.*
3. *A comprehensive dataset evidencing the action of commercialized drugs on mitochondria metabolism.*
4. *The proposal of our methodology to academics and industries at CRMN, through the IRICE research infrastructures.*

3.1.1.5. Position of the project as it relates to the state of the art

Analytical methods able to portray mitochondrial metabolism are frustratingly few and present important limitations, creating bottlenecks for biomedical research. **State-of-the-art high-resolution studies**, essentially based on isotope tracing strategies using NMR or mass spectrometry, offer molecular and sometimes atomic resolution but require state-of-the-art non-routine spectrometers, expertise, human resources and important funding. They are most often based on cellular metabolite extracts and cannot portray dynamic processes through real-time measurements, high-temporal kinetic resolution or high-throughput capacities. Those high-resolution methods are mainly restricted to low-throughput, highly detailed research studies.

On the opposite, **analytical methods able to characterize the dynamic aspects** of living mitochondrial metabolism, such as oximetry assays, or that offer medium-throughput capacities, such as the widely used Seahorse technology, are few, relatively expensive to run and offer a deceiving resolution.

Our methodology will allow obtaining high-resolution profiles of mitochondria dynamic metabolic processes. These results will be obtained from a simplified and versatile setup where the isolated mitochondria test medium can be easily altered, constituting a unique analytical platform for conditions and drug testing on mitochondria. Of interest, as the measurement time requirements for a sample is around 30 minutes using widely accessible ^1H -NMR spectrometers, our method is amenable to medium-throughput for drug screening, toxicity or pharmaceutical projects. NMR running costs per sample (estimated in our case to 25-50€/sample for widely accessible ^1H -NMR at 600 MHz) are cost-competitive with both high- and even low-resolution methods such as mass

spectrometry and the Seahorse technologies [226]. The coupling of dDNP-hyperpolarized ^{13}C labelled substrates with RT-NMR will constitute a technological step forward that will allow reaching high-resolution, enlightening selected mitochondrial metabolic pathways, even low-populated.

Our methodology, which is based on an unexplored analytical strategy to study mitochondria, is expected to impact biology and biotechnology innovation and have a strong potential for valorisation. Applications extend to cellular and molecular biology, metabolism research, precision medicine, *in vitro* diagnostics where novel analytical strategies are essential and awaited.

3.1.2. Methodology and risk management

3.1.2.1. Organization of the project

The project is divided in 4 workpackages (WP) to reach our objectives:

- *WP1: obtention of high-quality biological samples, setup of RT-NMR and dDNP hyperpolarization.*
- *WP2: acquisition of a dataset on a well-characterized mitochondria model (HeLa); testing the effect of technological drugs on mitochondria metabolism.*
- *WP3: obtention of medium-throughput results for biological and pharmaceutical research.*
- *WP4: promotion to the scientific community, seeding of the methodology to biomedical projects.*

WP1/ The mitochondria and substrates samples setup

Task 1.1 The isolation of mitochondria (CarMeN). The quality of the biological material, and procedures, is crucial for our project and particular care will be taken to ensure the toughness and reproducibility of the protocols, from mitochondria isolation, purity assessment and storage conditions. The experimental approach to isolate mitochondria is well-validated [223,227] and is routinely employed at the CarMeN: the crude mitochondrial fraction from cell cultures or tissues is purified by differential centrifugation and loaded on a discontinuous Percoll density gradient to obtain pure mitochondria [228]. Cytochrome c (0.05 mg/mL) will be added to test the outer mitochondrial membrane integrity. The inner membrane integrity will be assessed by measuring the respiratory control ratios (state 3/state 4) as previously described [229].

Task 1.2 Determination of the sample conditions and RT-NMR parameters (CarMeN/CRMN). The sample conditions (buffers, salts, additives, substrates) in which the mitochondria metabolism can be maintained and evaluated have long been determined and optimized by Seahorse or Clark electrodes oximetry studies. We have validated the composition of a NMR-compatible mitochondria medium for NMR that allowed both mitochondria maintenance and metabolic measurements in a preliminary RT-NMR setup. We will use HeLa-cells mitochondria and ^1H RT-NMR at 600 MHz to measure the influence of the main substrate concentrations (pyruvate/lactate, NADH, glutamate,

malate, ADP) to delineate their impact on mitochondria metabolism. Even though the isolation protocol requires further optimizations (task 1.1), isolated HeLa-cells mitochondria are already available at the CarMeN laboratory. The metabolic substrates will be injected using an in-house [230] or commercial injector (Bruker Insight Express) directly into the NMR spectrometer where the isolated mitochondria sample will be temperature equilibrated. Substrate concentrations that allow reaching maximal rates will be chosen to obtain comparable data. We will also determine the ideal number of mitochondria in the NMR sample to acquire sensible data. Task 1.2 will also allow the optimization of RT-NMR parameters such as the optimal number of scans and the number of kinetic data points.

Task 1.3 Preparing hyperpolarized metabolites; optimizing the injection of the hyperpolarized substrates into the isolated mitochondria samples (CarMeN/CRMN). Our methodology will be developed on one side using easily accessible ^1H -NMR with unlabelled and unpolarized substrates, and on the other side with dDNP-boosted signal enhancement. To obtain hyperpolarized ^{13}C -labeled substrates and to avoid the effect of contaminants on sensitive enzymatic reactions, we will use hyperpolarising matrices (HYPOP) made up of microporous polymers containing stable radicals [231]. They deliver high levels of ^{13}C polarisation and a highly repeatable hyperpolarisation without any need for extra radicals or glass forming agents. Based on our preliminary results by RT-NMR and literature [232], we aim to produce hyperpolarized ^{13}C -pyruvate, lactate, malate and oxaloacetate in the first approaches. The relaxation parameters of the hyperpolarized molecules will be determined.

The injections of hyperpolarized substrates and external molecules into fragile NMR biological samples may need optimization to maintain mitochondria delicate metabolism and ensure the best NMR signal linewidth. Many injection parameters are available for optimization, such as the injection velocity, volume, substrate concentrations, temperature, or mixing parameters to avoid bubble formation and ensure homogeneity and reproducibility inside the measurement NMR tube. Mitochondria integrity and biochemical properties will be carefully assessed during this optimisation.

The scientific risk associated with task 1.3 is minimal as the substrates have already been hyperpolarized and used for other *in vitro* or even *in vivo* applications [233,234]. A complete injection system is already in use at the CRMN to transfer and inject liquid hyperpolarized molecules into NMR samples placed inside the spectrometers and has already been tested to delicate experiments such as measurement of the interactions of natively folded proteins with ligands. We have already optimized the most critical parameters, such as the temperature of the injected solution, to rule out any major issue and ensure the do-ability of the injection into fragile biological samples.

WP2/ Real-time NMR acquisition on standard mitochondria model isolated from HeLa cells.

Task 2.1 Acquisition of RT-NMR data (CRMN). Our methodology will be developed both using widely accessible ^1H -NMR, and using ^{13}C dDNP techniques where ^{13}C -labeled substrates will be hyperpolarized externally and injected into the cell lysate before real-time NMR analysis [231,235].

We will use easy-to-isolate, robust and well-characterized mitochondria from HeLa cells [232]. Mitochondria will be placed into the adequate buffers, inside the NMR spectrometer, at a controlled temperature and substrates will be injected into the NMR sample before starting the RT-NMR measurements. RT-NMR will deliver a series of time-resolved spectra, from which a variety of metabolite concentrations and time-evolutions can be determined simultaneously [34,200]. The reproducibility of the results will be evaluated and optimized, both from the biological and technical points of view.

For unlabelled tests, the experiments will be straightforward: we will inject the mix of substrates necessary to sustain mitochondrial metabolism [227]. ^1H -RT-NMR acquisitions will allow quantifying substrates consumption (and metabolic pathways downside products) evolution.

For dDNP-enhanced experiments, the hyperpolarization will augment both the substrate(s) and product(s) signal intensities, improving the sensitivity and simplifying the NMR spectra, as only the polarized compounds will benefit from signal enhancement, producing a very significant intensity gap between polarised and non-polarised signals. Moreover, low-populated reaction products and metabolic downside intermediates may become detectable (contrary to classical ^1H NMR spectra where peak crowding, lower sensitivity and low intermediates concentration often hinder intermediates detection). We will test central ^{13}C -labelled substrates (such as pyruvate and lactate, which can fuel almost the entirety of mitochondrial carbon metabolism), but also pathway-specific substrates such as ^{13}C -labelled malate and oxaloacetate, that target the TCA cycle. The other non-hyperpolarized substrates, that are still essential to allow the mitochondria metabolism, will be added as for unlabelled tests. Hyperpolarized substrates will be tested one by one. We will also attempt to enlighten multiple pathways simultaneously by adding mixes of hyperpolarized substrates.

Scientific task 2.1 is probably the most central to this proposal. Yet, given our preliminary results, experience and the optimizations obtained from previous tasks, the scientific risks are managed. The biological feasibility (the maintenance of isolated mitochondria in a metabolically active and measurable state) has long been validated and is routinely performed [223,227]. We have the expertise for RT-NMR measurement of complex biological systems [34] and dDNP-enhanced substrate polarization for biological RT-NMR measurements has been described in numerous studies for *in vitro* and even *in vivo* applications [233,234,236]. Preliminary data have demonstrated that we can detect many metabolites in purified mitochondria extracts, all of them constituting chemical probes of individual metabolic reactions. The main interrogation resides in how many active reactions we will be able to witness. This is essentially a question of threshold detection of metabolite concentration variations over time, which can be modulated either by (i) varying the concentration of mitochondria (more mitochondria will consume substrates faster) or (ii) use signal enhancement (dDNP). Both aspects will be explored.

Task 2.2 Exploitation and metabolomic mapping of the RT-NMR results (CARMEN/CRMN). Metabolites NMR peak will be assigned using both chemical shifts databases and NMR spectra of metabolite standards. Multiple metabolic rates will constitute together a fingerprint of mitochondria metabolism. The results will be submitted to metabolomics webservers such as

MetaboAnalyst and MetExplore, to benefit from computation analyses and obtain quantitative metabolic models.

Task 2.3 Evaluation of integrating drugs to deepen our results (CarMeN/CRMN). Oximetry approaches use sequential technological compounds (activators and inhibitors; oligomycin, FCCP, rotenone, antimycin) to challenge the respiration properties of mitochondria [237]. This allows determining in real-time various physiological and metabolic parameters. We plan to evaluate this strategy in our setup to deliver a comparison of RT-NMR and oximetry measurements realized on identical samples. The successive addition of the drugs on a single RT-NMR experiment may not be doable, but sequential measurements on different samples are realistic. This task will also allow us to evaluate the potential of our methodology for chemical and drug testing on mitochondria metabolism.

WP3/ Methods and model transposition, biological applications.

Multiple applications will demonstrate the phenotyping potential of our setup for biomedical research, its medium-throughput capacity and value for chemicals, drugs and conditions testing.

Task 3.1 Comparison of metabolic profiles of mitochondria from multiple cellular origins (CarMeN/CRMN). We aim to profile mitochondria from the most widely used cell lines (CHO, HeLa, JURKAT, Huh7, A549, CaCO2) but also from non-immortalized primary cells, all of which are available at the CarMeN laboratory. This will allow us to discuss the diversity and adaptability of mitochondria metabolic profiles upon various cellular contexts and provide high-resolution data to confront and complement the available literature. These data will address key scientific questions, such as the comparison of mitochondria from healthy and cancer cells, using healthy liver-derived hepatocytes and hepatocarcinoma derived cells (Huh7).

Exploring the metabolic diversity of mitochondria originating from various cells will deliver an important dataset and demonstrate that our work brings an operational approach for life scientists, rather than a proof-of-concept. This latter point is central to our proposal.

Task 3.2 Evaluation of the effect of mitochondrial-targeting drugs (CarMeN/CRMN). We aim to demonstrate the relevance of our approach to pharmaceutical research. We will evaluate the effect of well-characterized mitochondrial-targeting drugs [7] on HeLa mitochondria, in their active concentration range. We aim to study metformin, nicotinamide mononucleotide, MitoQ, Bendavia, coenzyme Q10 and cyclosporin A to produce a pharmaceutical-relevant portrayal of a panel of drugs over mitochondrial metabolism. Some of these drugs are studied at the physiological level in the CarMeN laboratory [228,238].

WP4/ Promotion and seeding of our methodology.

The concrete translation of our analytical approaches to real biomedical research projects is integral to our proposal. We are not seeking to deliver a proof of concept but rather a usable and tangible method to investigate mitochondria metabolism. Our approach will be integrated into a

series of biological projects at the CarMeN, seeding its use in the scientific community. The rapid and high impact dissemination of results will be ensured by publications in high impact factor journals and by oral communications at appropriate meetings (the consortium is regularly invited to international conferences). The Postdoc fellow will be strongly encouraged to present their work to the international community and will receive financial support to attend conferences. Additionally, we will publicize our work and its objectives through the non-specialist scientific press and newspaper articles.

Task 4.1 Promotion of our methodology to the biomedical community (CRMN/CarMeN).

Website. A project website with an open access area will offer a lively platform for public dissemination for specialists and non-specialists (including reports on past, current and future activities, blogs for discussion and sharing raw data). We will frequently communicate on the progress of the *Mitoprofile* project, which will enhance its visibility and will boost and help research more globally.

Publications. To maximize impact, our scientific results (developments and applications) will be disseminated via articles in international high-impact peer-reviewed journals such as Cell, Cell Metabolism and Nature methods. Special attention will be given to open publication and sharing of raw data among the scientific community.

Conferences. Results will also be presented at international conferences where both partners are regularly invited to deliver plenary lectures and presentations (Euromar, Ismar, ICMBRS, World mitochondria society)

Task 4.2 Seeding and integration of our methodology to real biomedical projects (CarMeN).

Integration to biomedical projects. Hubert Vidal (Director of the CarMeN) will be in charge to integrate our methodology into the mitochondria-related projects available at the CarMeN laboratory to seed its use in the biomedical community.

TGIR access. We expect to offer access to our methodology through the IRICE (for commercial service) and TGIR (for academic researchers) infrastructures that allow external users to access the CRMN unique NMR platform.

3.1.3. Preliminary data and project feasibility and risk assessment.

We have the expertise for both RT-NMR but also cell compartments fractionation [34]. The mitochondria isolation protocols have been validated as well as the conditions allowing their metabolic functions. Fresh isolated mitochondria samples have already been successfully produced. Preliminary RT-NMR and oximetry experiments have confirmed their metabolic fitness and the complete do-ability of this proposal, as we were able to monitor both respiration and key mitochondrial metabolic reactions in real-time using NMR (lactate to pyruvate conversion, the

NAD/NADH coenzymes metabolism and the TCA cycle (succinate, fumarate, malate, citrate) (Figure 3-1, *infra.*)

For the dDNP aspects of the project, we can produce hyperpolarized substrates and mix of substrates using a new generation of hyperpolarizing polymers (HYPOP) to avoid the contamination of the samples with hyperpolarizing radicals, while obtaining excellent hyperpolarization levels [239]. The CRMN dDNP polarizer is coupled to an injection system adapted to inject hyperpolarized samples into biological samples located inside the NMR spectrometers (benchtop, 600 and 700 MHz). The project will benefit from the CRMN's unique NMR resources and worldwide recognized expertise.

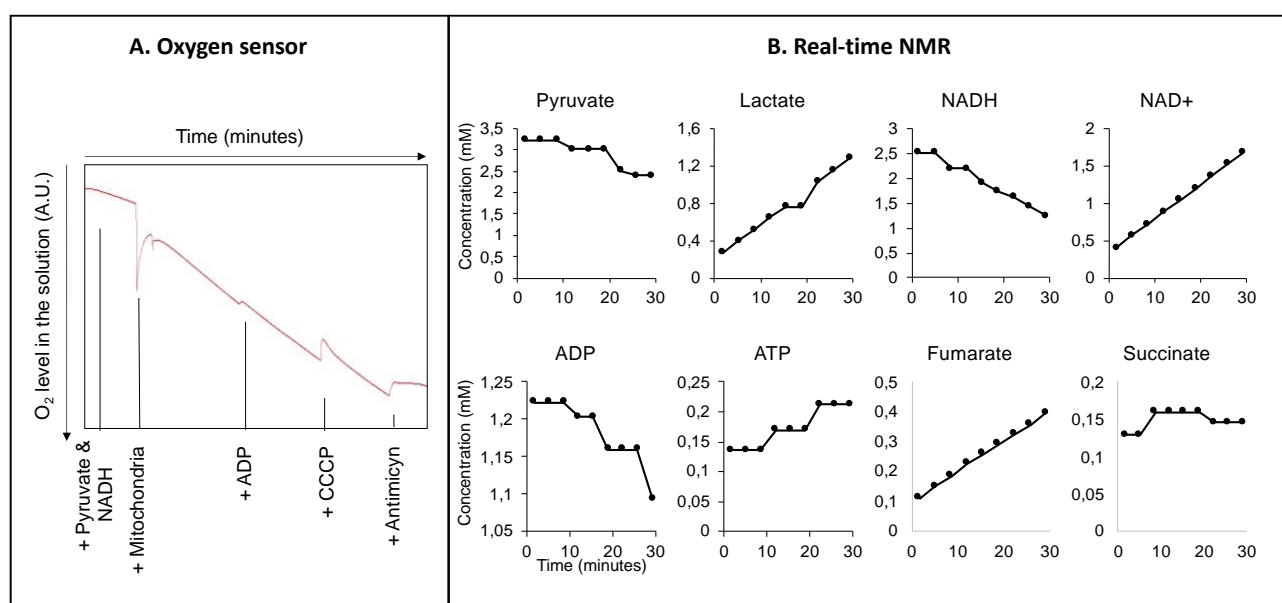


Figure 3-1. Preliminary results obtained for the Mitoprofile project. Hepatocyte mitochondria were isolated and metabolic fitness assessed. A. Real-time O₂ consumption measurement using a Clark electrode confirms the metabolic fitness of the mitochondria. B. RT-NMR results at 600 MHz showing the evolution of key metabolites during the measurement period, demonstrating the consumption and production of metabolites by isolated mitochondria. These results confirm our ability to isolate functional mitochondria and detect mitochondrial metabolism using RT-NMR.

3.1.4. Project follow up and perspectives.

A limitation associated with working with isolated mitochondria is that they lose their critical interaction with the host cell and potential regulations. As a consequence, the rates we aim to measure in this project will not reflect endogenous fluxes, as neither the substrate concentrations will match physiologically identical conditions, nor the regulations by the host-cell will be active. Our project rather aims at assessing the mitochondria metabolic machinery functional status *per se*, in standard conditions, as an objective phenotyping method.

The determination of physiologically relevant metabolic rates using our approach could be obtained by placing mitochondria in cytoplasmic-like environments after cell fractionation [34], or using permeabilised cells (*in situ* mitochondria) to allow substrates to reach the mitochondria while avoiding the limitations of the plasmic membrane barrier. Cell permeabilization can be produced

using digitonin (100 µg/mL) for example. Alternative methods using electroporation have been proposed [240]. All these approaches constitute fascinating projects and offer valuable options to extend further our methodology. They may be explored in WP4/, depending on their need and relevance for real biomedical projects, or as perspectives to this ANR project.

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