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***Metabolomics by NMR: applications and
challenges from biomedicine to food research***

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*A mia nonna
ai miei genitori.*

Abstract

Metabolomics is an emerging technology that holds promise of powerful insights into many different fields. Nuclear magnetic resonance spectroscopy represents an optimal tool in modern chemical and biochemical research; for this reason, numerous applications can be found in biology, biomedicine and food-stuff studies. Nuclear magnetic resonance spectroscopy offers many advantages to perform metabolomic analysis. Overall, because of its characteristics, NMR is most suited for metabolomics fingerprinting studies. Metabolomics is defined as the analysis of the complete ensemble of low molecular weight molecules, the metabolites, present in a biological specimen. In this methodological thesis, with the aim of demonstrating the potential of untargeted NMR-based metabolomics approach from biomedicine to food research studies, different topics have been addressed and discussed: i) the use of NMR-metabolomics applied on biological fluids to unravel fingerprints/biomarkers, both for drug response phenotypes, providing an effective means to predict variation in response to treatment; and for the diagnosis/differentiation of pathological states; ii) the characterization of a common metabolic drift in a heterogeneous group of different cellular breast cancer subtypes following the development of drug resistance, providing in vitro models which will eventually lead the way to early prediction of treatment inefficacy on patients with breast cancer (personalized medicine); iii) the application of NMR-based metabolomics to demonstrate the potentiality in determining molecular features (biomarkers descriptors of plant stress and/or organoleptic properties) of olive pre-harvest and post-harvest phases to exploit the possibility for the establishment of a new, large-scale applicable protocol (treatments or additional post-harvest manipulations) which will eventually be utilized by olive mills and olive oil producers to improve quantity and quality of the final products; iv) the use of NMR-metabolomics to assess food products provenance, even at the scale of several different farms in a restricted geographical territory, such as small milk farms in Mugello (Tuscany), or coffee plantations of a restricted area of the Huila district in Colombia, and proving that NMR can be an accurate and automated service for determining food quality based on metabolomic descriptors.

Results presented in this thesis, obtained by a combination of biochemistry, analytical chemistry, bioinformatics, and descriptive data, contribute to the demonstration that NMR-based metabolomics can be considered as a whole as a "universal" quantitative analytical technique. Offering an unbiased and untargeted view of samples composition and the ability to simply quantify multiple compounds simultaneously, could be considered a very powerful choice for diagnosis studies as well as for the characterization and quality control of complex biological/natural samples such as biofluids, foods, plants and cellular extracts.

Table of contents

1. INTRODUCTION	1
1.1 WHAT IS METABOLOMICS	1
1.2 OVERVIEW OF MAIN METABOLOMIC APPROACHES	2
1.3 METABOLOMICS BY NMR	5
1.3.1 Type of samples	5
1.3.2 NMR-based metabolomics applications	7
2. AIMS OF THE THESIS	11
3. METHODOLOGICAL ASPECTS	13
3.1 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY: PRINCIPLES AND TECHNIQUES	13
3.2 SAMPLE PREPARATION	15
3.3 NMR DATA PROCESSING	17
3.4 NMR METABOLOMICS APPROACHES	19
3.4.1 Multivariate analysis for classification and modeling	20
3.4.2 Identification, quantification, univariate analysis	23
4. METABOLOMICS IN BIOMEDICINE	25
4.1 NMR-BASED METABOLOMICS IN DISEASE CHARACTERIZATION	25
4.1.1 Metabolic fingerprints between HCV and HBV patients: a possible interference of the two major hepatitis viruses in basal metabolism pathways	31
4.1.2 NMR-based metabolomics in Parkinson's Disease: preliminary results of PROPAG-AGEING project	57
4.1.3 Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics	77
4.1.4 Effect of non-surgical periodontal therapy on salivary metabolic fingerprint of generalized chronic periodontitis using nuclear magnetic resonance spectroscopy	89
4.1.5 NMR-Based Plasma Metabolomics at Set Intervals in Newborn Dairy Calves with Severe Sepsis	107
4.2 TOWARD PERSONALIZED MEDICINE: USE OF NUCLEAR MAGNETIC RESONANCE-BASED METABOLOMICS IN DETECTING DRUG RESISTANCE IN BREAST CANCER	119
4.2.1 Metabolomic analysis of Estrogen Receptor positive breast cancer cell lines by nuclear magnetic resonance (¹ H-NMR) spectroscopy is able to identify cells with acquired resistance to palbociclib	121
5. NMR-BASED METABOLOMICS IN FOOD SCIENCE	141
5.1 PRE- AND POST-HARVEST FACTORS AFFECTING YIELD EFFICIENCY AND OIL QUALITY IN <i>OLEA EUROPAEA</i>	141
5.1.1 Towards the assembly of the olive fruitlet drop puzzle in <i>Olea europaea</i> (cv. Frantoio)	145
5.1.2 Cold water treatment of olive fruit before oil extraction: effect on volatile profile and quality parameters	159
5.2 NMR ON THE SIDE OF SMALL FARMS: METABOLOMICS IN FOOD TRACEABILITY	180
5.2.1 NMR metabolomic fingerprinting distinguishes milk from different farms	183
5.2.2 From beans to brew: NMR based metabolomic approach to assess traceability of coffee producers within a restricted geographical area of Colombia	195
6. CONCLUSIONS	219
BIBLIOGRAPHY	223
7. ACKNOWLEDGMENTS	235

Chapter 1

Introduction

1.1 What is metabolomics

In recent years there has been an increasing interest of life sciences towards a more holistic approach to the study of organisms, considering them as a whole rather than as a sum of individual parts. This approach, commonly known as systems biology, necessarily interdisciplinary, requires the use of physiological, physical, molecular, biochemical and chemical methods to observe and measure the response of an organism to diverse conditions¹. The omics technologies, such as genomics, transcriptomics, proteomics and metabolomics, are particularly important tools for systems biology.

Metabolomics is one of the most recent -omic sciences. It deals with the characterization of the metabolome defined as the whole set of metabolites (small molecules <1500 Da) in a certain biological system (e.g. a cell, a tissue, an organ, an entire organism)².

Metabolomics seats downstream to the flow of -omic sciences, and this has important implications, as illustrated in **figure 1**; the interaction becomes increasingly complex starting from left to right, then metabolome and fluxome (not shown in **figure 1**) represent the most integrative functions among all the other levels of organization.

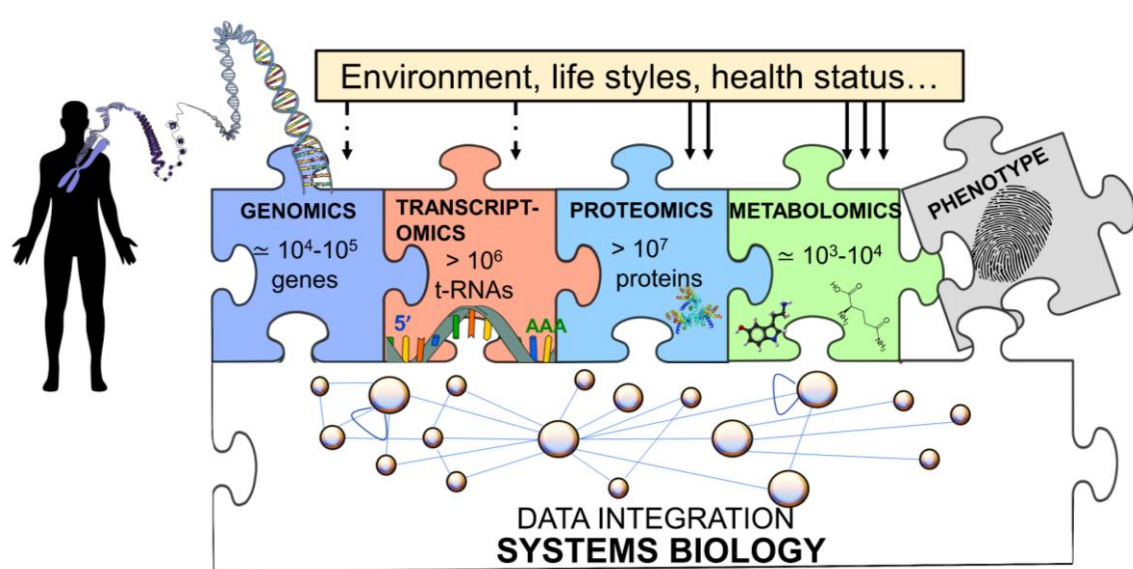


Figure 1. The flow of information in systems biology proceeds from the genome to the transcriptome, the proteome and finally to the metabolome. From left to right they are increasingly variable during an individual lifespan, and all concur to the phenotype. Figure taken from Vignoli et al. ⁶.

However, an important remark is that, unlike mRNA and proteins, it is difficult or impossible to establish a direct link between genes and metabolites. The convoluted nature of cell metabolism, where the same metabolite can participate in many different pathways, complicates

the interpretation of metabolite data³. While the genome is (almost) invariant throughout the lifespan of an individual, the metabolome may change as an effect of lifestyle, stress and, most importantly, onset of pathologies. Moreover, while the number of biological objects increases from 10^4 - 10^5 genes to ca. 10^7 proteins, it decreases down to 10^3 - 10^4 metabolites (**Figure 1**) condensing the information (*e.g.* in yeast cell has been calculated 600-700 metabolites, about an order of magnitude less than the number of protein-encoding genes^{4,5}).

Although microbes may prove to be the richest overall source of metabolites, plants are the source of the most complex individual mixtures. It has been reported that bacterial genomes already sequenced can support the biosynthesis of just a few hundred metabolites (*e.g.* 580 for *Bacillus subtilis* and 800 for *Escherichia coli*), but for individual plants, this value is likely to be in the tens of thousands⁷. This metabolic richness comes not just from the number of genes present (20,000 to 50,000) but also from multiple substrate specificities for many enzymes⁸, subcellular compartmentation, and the occurrence of nonenzymic reactions.

Approximately 50,000 different compounds have been elucidated in plants⁹, and it is predicted that the final figure for the plant kingdom will approach or even exceed 200,000¹⁰⁻¹² (a single species may be producing, depending on genome size, about 5000–25,000 compounds at one point of time in a certain environment)^{13,14}.

Even though in all organisms the total number of these metabolites is relatively small if compared with genes, transcripts or proteins, the metabolite levels strongly depend on several internal stimuli and external perturbations. This makes metabolomics an extremely valuable tool to identify diseases' profiles in the form of endogenous metabolites (gene derived metabolites) and exogenous metabolites (environmentally derived metabolites), which provides precious information on the underlying causes of diseases at a molecular level¹⁵.

1.2 Overview of main Metabolomic approaches

Each metabolomic study requires an evaluation of the sample preparation, of the extraction procedure, and of how they couple to a combination of different analytical techniques in order to achieve as much information as possible.

As there is no single analytical method for the analysis of the metabolome, different terms are used in the field of metabolomics. These terms are summarized in **table 1** and described in more detail in the **methodological section 3.4**.

Metabolomic data can be obtained through targeted and untargeted (non-targeted) approaches. The former ensure a low detection limit and enable the absolute quantification of the sample; therefore, the analytical techniques used, including sample preparation and unambiguous detection methods, should provide maximum sensitivity and selectivity. The latter, including metabolic fingerprinting and profiling, provide both sample classification and identification of metabolites related to specific physiological or pathological conditions¹⁶. However, metabolomics analysis performance also relies on instrument type and on methods implemented. Current applications rely primarily on nuclear magnetic resonance (NMR) spectroscopy and/or gas, liquid chromatography and ultra-high-pressure liquid chromatography

(GC, LC and UPLC) and more recently capillary electrophoresis (CE), usually hyphenated to mass spectrometry (MS).

Table 1. *Metabolomic glossary (adapted from Nielsen and Oliver¹⁷).*

Term	Definition
Metabolites	Low molecular weight organic molecules (~1500 Da) involved in metabolic processes as substrates or products with different biological functions (<i>i.e.</i> fuel, structure, signaling, catalytic and inhibitory effects on enzymes, defense <i>etc.</i>).
Metabolome	The quantitative ensemble of all low molecular weight molecules within an organism, an organ, a biofluid, a tissue or a cell in a particular physiological or developmental state.
Metabolomics	The quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modifications.
Targeted metabolomics	The measurement of defined groups of biochemically and chemically characterized metabolites undoubtedly associated with a specific metabolic condition.
Untargeted metabolomics	The comprehensive analysis of all the measurable analytes in a sample, including chemical unknowns.
Metabolic fingerprinting	Global, high-throughput, rapid analysis of all metabolites presents in a biological sample to provide sample classification (even without metabolite identification).
Metabolic profiling	The identification and quantification of as many as possible metabolites present in a biospecimen.

GC is used in metabolomics and elsewhere in the analytical sciences because it is both sensitive and highly reproducible. Its weakness is that it is limited to compounds that are volatile or can be made volatile via chemical derivatization. Derivatization brings its own problems, examples include glutamine and glutamic acid being converted to pyroglutamic acid¹⁸ and arginine being converted to ornithine¹⁹ and thus producing incorrect readings. LC can detect a large range of metabolites, does not require the samples to be volatile or derivatized and when coupled to MS has high sensitivity.

Nevertheless, liquid chromatography in metabolomics still has issues: polar metabolites, such as sugars and many amino acids are often not retained by conventional reverse phase LC columns²⁰; the increased number of peaks results in correspondingly crowded chromatogram, likely obscuring important biochemical details just as crowded NMR spectra do (see figure 6). Two-dimensional liquid chromatography (2DLC) offers a way to increase peak capacity and separation power. However, there are still problems such as mismatch between the mobile phase used on each dimension and column re-equilibration times^{21,22}.

CE-MS separations are very efficient, require only small amounts of sample, reagents and solvent, and are relatively inexpensive (cheap fused-silica capillaries versus costly LC columns). Reproducibility tends to be one of the major challenges, due to the small size of the samples and analytical conditions.

The applications of NMR spectroscopy are not limited to liquid and solid samples but extend to intact tissue samples with use of high-resolution magic-angle spinning (HRMAS) NMR spectroscopy.

NMR spectroscopy offers many advantages over MS^{23–26}, **table 2**. It allows identification and quantification of the more abundant bulk metabolites present in biofluids, cell extracts, and tissues without complex sample pretreatment or fractionation. Due to its non-selectivity, no prior knowledge of the samples is required. Moreover, it enables the identification of compounds having identical masses, including those with different isotopomer distributions.

Table 2. *Comparison of different metabolic technologies, adapted from table of Wishart²⁵.*

Technology	Advantages	Disadvantages
<i>NMR spectroscopy</i>	Quantitative Non-destructive Fast (2-3 min/sample) Requires no derivatization Requires no separation Detects all organic classes Allows ID of novel chemicals Robust, mature technology Can be used for metabolite imaging (fMRI) Large software and databases for metabolite ID Compatible with liquids and solids	Not very sensitive Expensive instrumentation Large instrument footprint Cannot detect or ID salts and inorganic ions Cannot detect non-protonated compounds Requires larger (0.5 mL x 5mm tube) samples
<i>GC-MS spectrometry</i>	Robust, mature technology Relatively inexpensive Quantitative (with calibration) Modest sample size need Good sensitivity Large software and databases for metabolite ID Detects most organic and some inorganic molecules Excellent separation reproducibility	Sample not recoverable Requires sample derivatization Requires separation Slow (20-30 min/sample) Cannot be used in imaging Novel compound ID is difficult
<i>LC-MS spectrometry</i>	Superb sensitivity Very flexible technology Sample not recoverable Not very quantitative Detects most organic and some inorganic molecules Expensive instrumentation Minimal sample size requirement Can be used in metabolite imaging (MALDI) Can be done without separation (direct injection) Has potential for detecting largest portion of metabolome	Slow (20-30 min/sample) Poor separation resolution and reproducibility (vs. GC) Less robust instrumentation than NMR or GC-MS Limited software and databases for metab. ID Novel compound ID is difficult

Despite these advantages, NMR spectroscopy has its main drawback in sensitivity, with limits of detection on the order of 10 μ M or a few nmol at high fields using new cryoprobes²⁶. In comparison MS is superior in allowing analysis of secondary metabolites where the detection level is of picomole to femtomole. Resonance assignment, which permits to associate each nuclear spin of the sample molecule with a chemical shift, may be not straightforward, in such

case the proposed assignment can be checked by spiking with the candidate compound. Importantly, it is observed that biomarkers indicated by NMR and MS techniques are different, suggesting that these two analytical platforms may have a complementary nature.

Discrepancies in biomarker identification can be also due to different protocols used for sample preparation. Such heterogeneity can mainly result in divergences in the results produced from different places and prevent their generalization.

Despite these discrepancies in biomarker identification Martin JC *et al.*²⁷ have demonstrated a high convergence in the spectral information (untargeted approach) produced from various instruments (5 NMR and 11 LCMS platforms located in three different European countries) in describing the same set of samples, irrespective of the type of samples preparation, standardization, deconvolution method, analyzer brand or configuration (QTOF, TOF, Orbitrap delivered by various LC systems, ionization modes).

Overall, because of its characteristics, NMR is most suited for metabolomics fingerprinting studies (§3.4).

1.3 Metabolomics by NMR

Numerous unsurpassed characteristics of NMR, including its excellent reproducibility, highly quantitative nature, ability to identify unknown metabolites unambiguously, and capacity to detect metabolites using intact biospecimens compensate for its relatively low sensitivity. In the area of methods development, numerous efforts have been focused on enhancing the sensitivity and resolution of NMR, improving unknown identification and quantifying a larger number of metabolites by the improvement of techniques and faster 2D experiments to be routinely used in high-throughput studies^{22,28,29}. Indeed, NMR may show some advantages in the analysis of several samples, especially biofluids.

1.3.1 Type of samples

The types of samples that NMR can analyze are many; some of these are listed in **figure 2**. Samples can be classified in terms of molecular/biochemical complexity, from cells to biofluids and tissues.

Biological samples include plants, plant-based food (*e.g.* fruits and vegetables, wine, oil), animal-based food (*e.g.* milk, cheese, meat), urine, blood plasma or serum, fecal material, organ tissue, culture medium, bacteria, yeasts etc. The NMR detectable part of the metabolome of biological samples corresponds to tens-hundreds of molecules.

They mainly belong to the classes of amino acids, organic acids, alcohols, carbohydrates, other organic acids including amines, nucleotides, osmolytes (such as betaine or choline), phenolic compounds, *etc.* (**Figure 2**), covering several areas of study and addressing major subjects of research such as human health and disease, food safety and security, and industrial biotechnology.

In addition, large and small lipid entities (high-density and low-density lipoproteins; short-chain fatty acids; mono-, di-, and triglycerides; cholesterol esters; cholesterol; glycerophosphocholines; and sphingomyelins) can be detected. Indeed, NMR can contribute also to the definition of the overall lipid composition of a biosystem, the so called lipidomics^{43,44}.

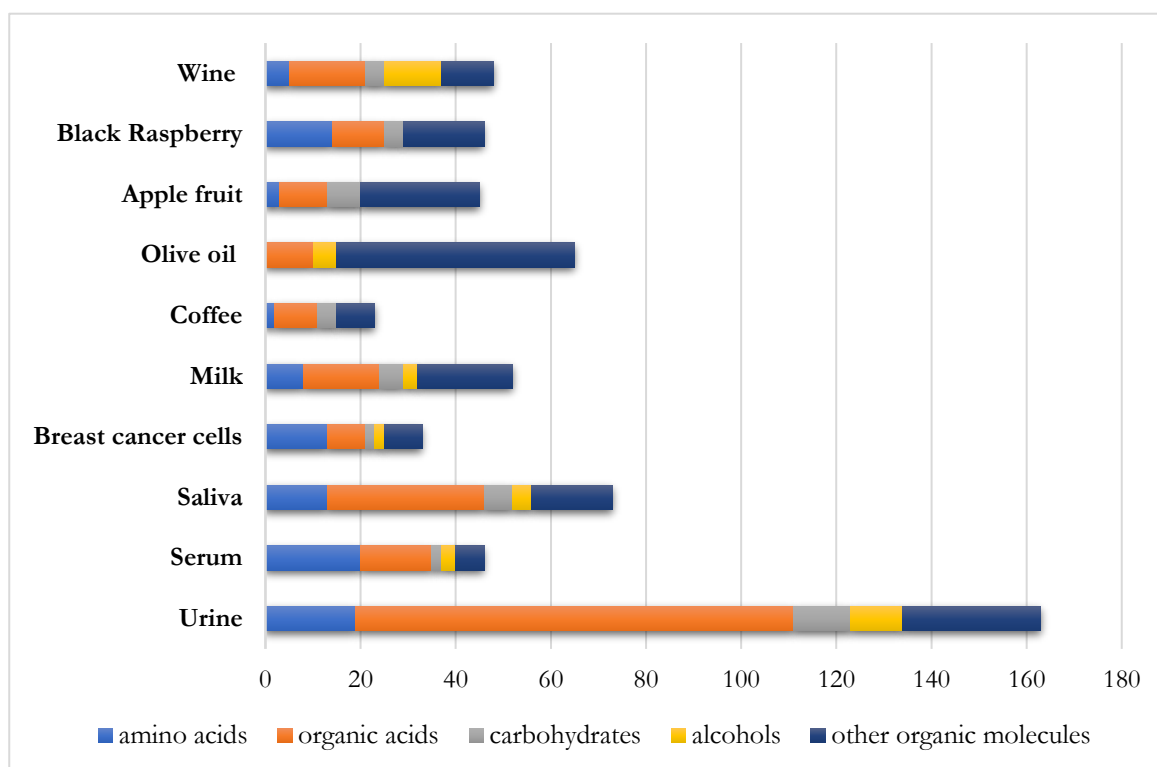


Figure 2. Number of detectable and quantifiable metabolites with $\geq 50\%$ occurrence in the ^1H NMR spectra of different biological fluids (urine,³⁰ serum,¹⁹ saliva,³¹ breast cancer cell lysates^{32,33} black raspberry³⁴, apple fruit³⁵, wine³⁶, olive oil^{37,38}, milk^{39,40} and coffee^{41,42}).

Sample specific considerations need always to be made. Different types of cells are characterized by different metabolomes. Intensity variations in intracellular metabolites (endo-metabolome) induced by treatment with drugs, overexpression of a protein, genetic manipulation, *etc.* can be directly related to the up- or down-regulation of specific pathways. For a comprehensive picture of the biochemistry of cells, the complementary information provided by analyzing culture media (exo-metabolome) is extremely important⁴⁵. The NMR spectra of tissues reflect organ-specific biochemistry (aerobic respiration, lipid metabolism, *etc.*) but also depend on the heterogeneity of the tissue composition and on the contribution from the extracellular matrix and the tissue microenvironment, so that their biochemical interpretation is more difficult than in cultured cells. Nevertheless, samples are extremely valuable as direct reporters of the diseased region, where variations in the metabolome with respect to a healthy status are expected to be most evident: *e.g.* cerebrospinal fluid (CSF) that reflects the biochemistry of the central nervous system or exhaled breath condensate (EBC) that reflects the biochemistry of the respiratory pathways. However, in other compartmentalized samples such as saliva, the metabolome not only changes in correlation with oral disorders^{46,47} but also in the presence of distant pathologies⁴⁸.

Metabolite alterations have been identified in blood samples of Parkinson's disease patients^{49–51} or other neurological diseases⁵².

In general, systemic biofluids, such as urine or blood (serum and plasma), have a fainter biochemical correlation with a diseased organ or apparatus, but present two main advantages: the simple, noninvasive or minimally invasive collection, and the ability to reflect the overall

response of the individual to a disease status. On the other hand, urine and blood are largely different in terms of chemical composition and number of low molecular weight molecules (**Figure 2**), with urine metabolites being heavily influenced by environmental factors such as food and liquid intake, while blood has a better defined and stable metabolome.

Saliva is now increasingly being used as an investigational aid in the diagnosis of oral and systemic diseases⁵³. It is a complex fluid containing a variety of enzymes, hormones, antibodies, antimicrobial constituents and growth factors. Saliva is functionally equivalent to serum, but its low metabolite concentration, compared with serum, may prevent salivary diagnostic from being clinically practical. About 99% of saliva is water and the remaining 1% is a mixture of organic and inorganic molecules⁵⁴. There are two types of saliva: whole and gland-specific saliva. Whole saliva is useful for the evaluation of systemic disorders, whereas gland-specific saliva is collected directly mostly from major salivary glands and the evaluation of the secretions is predominantly useful for the detection of gland specific pathology⁵⁵. Moreover there are two distinct way of collecting whole saliva, at rests (unstimulated saliva) or during stimulation by mastication (stimulated saliva), can affect both its composition and metabolites concentration^{56,57}.

As well as metabolites in milk not only reflect metabolic activity in the mammary gland but they can originate from multiple cell types or metabolisms in the organism, and different origin and sources of metabolites, contribute to the variability of milk metabolite profiles. Milk composition is influenced also by other factors, such as diet⁵⁸, genetics⁵⁹, number and stage of lactation⁶⁰, seasonal variation⁶¹, milk processing⁶² or may also be secreted by microorganisms present in raw milk⁶³.

Generally, fruit metabolomics is extremely complex due to the enormous diversity of metabolite chemical structures present in plants, especially among the secondary metabolites which are specific for every species. All fruits have a specific chemical profile mostly characterized by endogenous components (produced by the plant and by environmental microorganisms) that are strongly connected to their nutritional value, aroma, taste and health-promoting effects.

This great heterogeneity can be ascribed to the fact that plant metabolites are family or species-specific to fulfil important biological functions such as helping plants to survive in specialized ecological niches. These fruit components can be as well ubiquitous metabolites (such as amino acids, sugars, organic acids) involved in the basic functions of the living cells or secondary metabolites that are usually fruit-type specific. These metabolites can be used as potential markers for quality, origin and authenticity of fruit and fruit-derived foods⁶⁴.

From the above considerations preserving the chemical composition of the sample under study (*e.g. in-vivo* metabolome or closest to the one at the time of sampling) and ensuring spectral reproducibility, are key factors for the significance of metabolomic analysis.

1.3.2 NMR-based metabolomics applications

This NMR versatility has permitted numerous studies: the investigations of human diseases, food, microbial, pharmacology, toxicology, plants, systems biology and enzymes have made use of NMR-based metabolomic techniques (**Figure 3**).

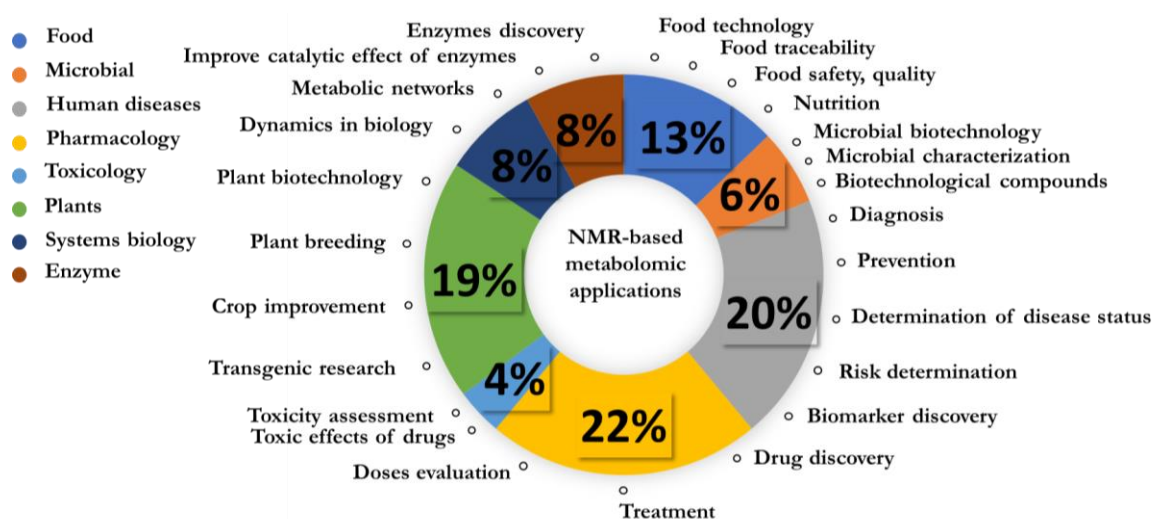


Figure 3. Pie chart of the NMR-based metabolomics in different fields of pure and applied research. The plot is based on the number of peer reviewed publications on metabolomics taken from PubMed, Web of Science and Scopus database (average).

Two big portions of this pie chart relate to human diseases and plant studies. Since metabolomics monitors the global outcome of all exogenous and endogenous factors, without making assumptions about the effect of any single contribution to that outcome, it can be considered a perfect instrument to investigate and understand the molecular mechanisms of mammals health and disease⁶⁵. Several biomedical fields, summarized in three broad areas in **figure 4**, could benefit from metabolomic studies.

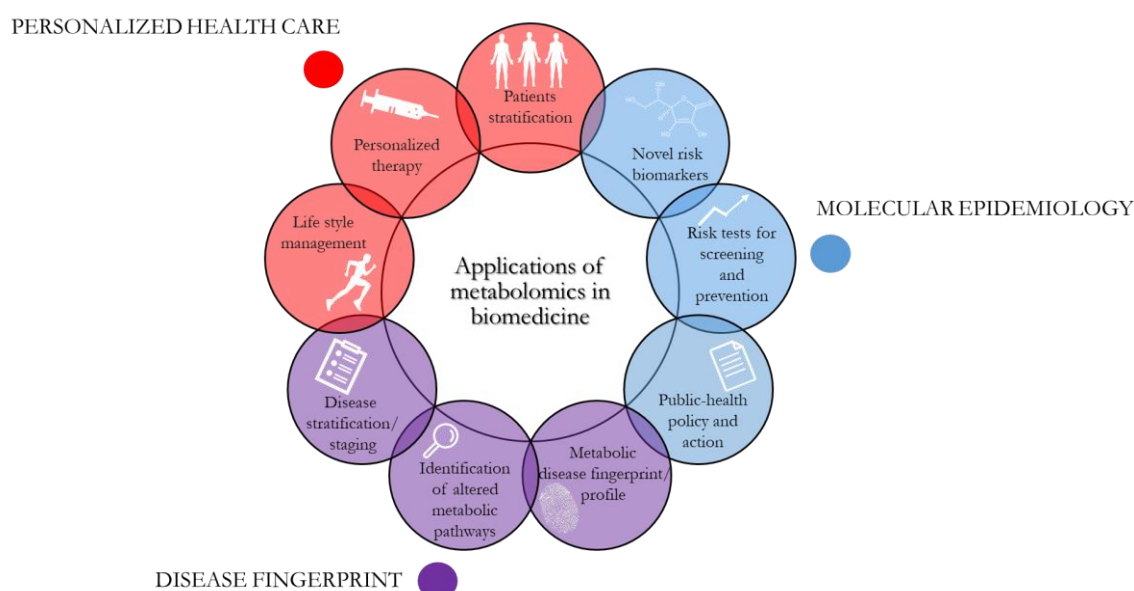


Figure 4. Applications of metabolomics in biomedical research. Adapted from Nicholson et al.⁶⁵ Disease fingerprint area ranging from molecular/profile disease characterization to the identification of altered pathways and disease stratification (purple); personalized healthcare including individual life style management and patient stratification (red); molecular epidemiology studies for public-health policy and action, risk test for screening and for prevention and for novel risk biomarkers (cyan).

As it has been already widely stated, one of the most suited NMR based metabolomics application is represented by *disease fingerprint* (disease diagnosis and characterization).

In particular it has already proved in the past decade to be useful for the characterization of a wide range of pathologies such as cancer⁶⁶, meningitis⁶⁷, infections^{68,69}, neurological disorders⁷⁰, cardiovascular diseases⁷¹, inborn errors of metabolism⁷², and coeliac disease^{73,74}.

However, one of the major challenges nowadays relies in *personalized health care*^{75,76}, aiming at customizing subjects' treatments according to their specific omic-profiles (individual profiling). The so-called "pharmaco-metabolomic" approach that is currently emerging aims to identify the individual metabolomic characteristics able to predict drug effectiveness and/or toxicity⁷⁷. Applications of metabolomics include also monitoring the effects of medical interventions including drugs, surgical and non-surgical interventions, analysis of biochemical pathways and their perturbations resulting from mutations, aging, diet, exercise, or life style.

Above all it offers promises as a clinical tool in *molecular epidemiology* with the most ambitious objective of detection of early metabolic perturbations even before the manifestation of disease symptoms, providing new a challenge for early diagnosis and preventing therapies. This means being able to identify metabolites or fingerprint that can be used as novel risk biomarkers of a certain disease, or of a disease recurrence.

¹H-NMR metabolomics is increasingly applied also *in vitro* (cellular models) studies. "Mammalian cell culture metabolomics" have shown their usefulness in biomedicine^{78–81}; *in vitro* cell metabolomics applications offer several advantages which include easily controlled experimental variables, greater reproducibility, low cost, easily interpreted results and to eliminate compounds likely to cause problems with side effects, but its require well set sample preparation protocols and accurate conditions monitoring.

It is important to underline that the use of metabolomic approach in biomedicine is not confined to human wellness. Several potential applications in veterinary medicine are possible, in particular in the framework of disease diagnosis and investigation, optimization of health and production, drug discovery and animal welfare⁸².

Plant studies is the other hugest area of NMR-based metabolomic investigations. It is widely used to understand biochemical mechanisms involved in plant mechanisms: development, host-plant resistance, stress exposure^{83,84}. NMR based pant metabolomics application fits with biology, botany and agronomy.

The food and beverage industry is constantly growing, and the global expenditure on this sector is twelve times bigger than the global expenditure on health care sector^{85,86}.

It is therefore not surprising that research on food and nutrition has witnessed numerous NMR-based metabolomic studies focused on a variety of topics. These include food processing⁴², quality⁸⁷, taste⁸⁸, composition, adulteration⁸⁹, traceability⁹⁰, contaminants and phytomedicine⁹¹, nutritional values⁹² and effects on cellular models or model organisms^{80,93–95} or directly on human metabolism^{96,97}, significantly contributing as a key tool for the new field of "foodomics"⁹⁸.

The applications of metabolomics in foodomics can be summarized in three main areas (**Figure 5**): i) in the field of human health, which can be divided in food consumption monitoring studies, where, given a particular diet, the consequent metabolome changes are investigated^{99–103}, and in treating/preventing diseases by improving and monitoring patient diet^{73,104}; ii) in food resources area, which can be investigated through the analysis of the composition of food from animal

and plant origin and defined also on the basis of climate, land and cultural practices (*terroir*) that contribute to the traceability and thus to the characterization of a genuine product^{90,105–107}; iii) food processing -which includes the characterization of the most influencing pre- and post-production manipulations on the original product, such as altered growing conditions (e.g. feed, GMOs, chemicals, pesticides etc.), the effect of packaging and storage^{108,109}, and last but not least safety and authenticity control.

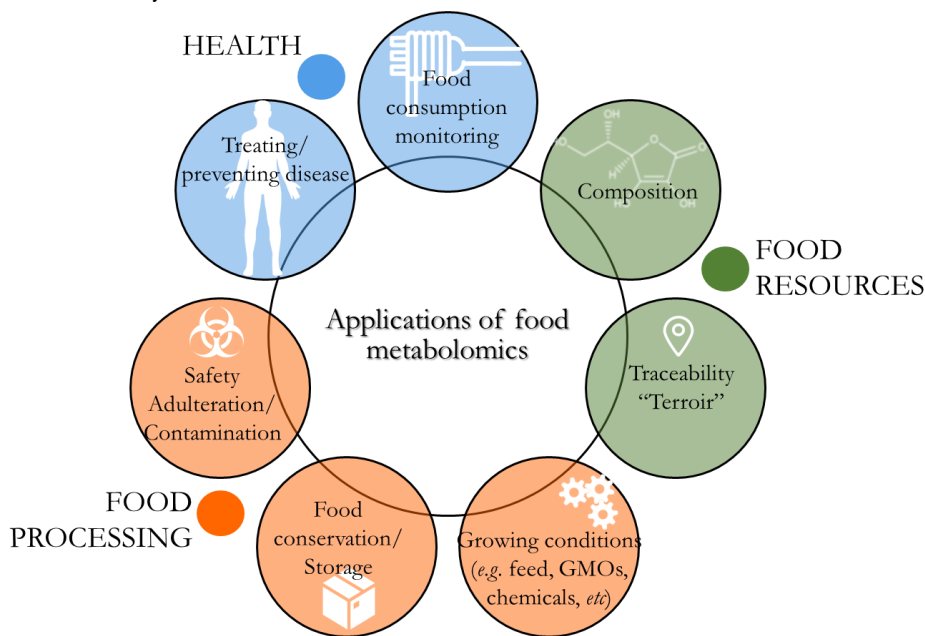


Figure 5. The main applications of food metabolomics. Food consumption monitoring and treating/preventing diseases, for the human health field (blue); chemical and molecular analyses of food composition and its characteristic ecosystem that contributes to the definition of traceability/authenticity of food resource (green); food processing area for the characterization of the effect of pre- and post-harvest manipulations on food (orange). Figure taken from⁶.

Despite the fact that conventional quality, safety and authenticity control in food is based on targeted strategies, high resolution ¹H NMR has found its way into routine food analysis¹¹⁰, offering several advantages if performed under well-defined instrumental specifications and SOPs. NMR provides an extremely reproducible food fingerprint and fully quantitative data by means of a single experiment with minimal or no sample preparation.

Since 1993, NMR has become an AOCS (American Oil Chemists' Society) official method to determine solid fat contents of fats and oils in the food industry, particularly in the bakery, confectionery and margarine industries¹¹¹. Although several analytical methods already exists for the detection of virgin olive oil adulteration, NMR fingerprinting was proven to be a much more effective method in the authentication of virgin olive oils based on their geographical origin^{112–114}. A platform for ¹H NMR based food quality and authenticity control, the FoodScreenerTM platform, has already been introduced by Bruker BioSpin based on an Avance 400 MHz spectrometer, for the analysis and characterization of fruit juices and wines, and with application to honey samples being currently in development¹¹⁰.

Chapter 2

Aims of the thesis

Metabolomics is an emerging technology that holds promise of powerful insights into many different fields. This elaborate is mostly a methodological thesis that covers different topics with the aim of demonstrating the potential of untargeted NMR-based metabolomics approach from biomedicine to food research studies. Particular attention is dedicated to:

- 1) prove the usefulness of metabolomics applied on biological fluids to unravel fingerprints/biomarkers, both for drug/treatment response phenotypes, providing an effective means to predict variation in response to treatment; and for the diagnosis/differentiation of pathological states, such as Hepatitis C infection, Parkinson's disease, periodontitis and sepsis mainly to uncover the underlying molecular mechanisms, and to enable patients' stratification;
- 2) demonstrate the existence of a common metabolic drift in a heterogeneous group of different cellular breast cancer subtypes following the development of drug resistance, providing in vitro models which will eventually lead the way to early prediction of treatment inefficacy on patients with breast cancer (personalized medicine);
- 3) demonstrate the potentiality in determining molecular features (biomarkers descriptors of plant stress and/or organoleptic properties) of olive pre-harvest and post-harvest phases to exploit the possibility for the establishment of a new, large-scale applicable protocol (treatments or additional post-harvest manipulations) which will eventually be utilized by olive mills and olive oil producers to improve quantity and quality of the final products;
- 4) prove that NMR-metabolomics is sensitive enough to accurately assess food products provenance, even at the scale of several different farms in a restricted geographical territory, such as small milk farms in Mugello (Tuscany), or coffee plantations of a restricted area of the Huila district in Colombia. And prove that NMR can be an accurate and automated service for determining food quality based on metabolomic descriptors.

Chapter 3

Methodological aspects

3.1 Nuclear Magnetic Resonance spectroscopy: principles and techniques

Solution-state NMR is an efficient profiling tool that can generate information-rich spectra that have shown success in the study of metabolites and related research areas¹¹⁵. NMR spectroscopy exploits the behavior of molecules when placed in a magnetic field and irradiated with specific radiofrequency waves to excite different nuclei such as ^1H , ^{13}C , ^{14}N and ^{31}P . Because of the high natural abundance of ^1H isotope (99.9%) it is the most used in NMR based metabolomic studies. NMR spectroscopy has been widely applied in plant, food and medical metabolomics.

However, NMR has poor sensitivity if compared to MS approach. Sensitivity and resolution increase with increasing magnetic field strength. Even though an ultrahigh magnetic field NMR spectrometer, such as a 950 MHz instrument, will offer better resolution and higher sensitivity, it is also significantly expensive, and the maintenance is highly costly. Consequently, most NMR-based metabolomics studies are conducted at 500 and 600 MHz NMR spectrometers that are commonly available in research institutes and offer a good compromise between resolution, sensitivity, and cost. 400 MHz instrumentation is most used for food based studies¹¹⁶, while the use of a 600 MHz spectrometer is considered the standard for biofluid and tissue analyses⁶.

The first two issues that are faced in solution-state NMR concern the adequate suppression of solvent resonance and the presence of macromolecules (e.g. proteins and lipoproteins) causing peak broadening in the spectra.

The former is commonly solved using presaturation, where gentle and selective pre-irradiation of the solvent resonances equalizes the spin populations of solvent protons before data acquisition¹¹⁷. Pulse sequences can be also implemented with gradients to improve the solvent suppression quality¹¹⁸. A modulated shaped pulse is often used for the study of food matrices such as alcoholic beverages¹⁰⁷ and edible oils⁹⁰ to suppress, respectively, multiple ethanol resonating signals or the strong lipid signal (e.g. 16-20 frequencies), resulting also in enhancing signals of the minor components.

One of the most used pulse sequences in solution-state metabolomics is 1D ^1H -NOESY-presat (Nuclear Overhauser Effect Spectroscopy); 1D NOESY spectra present signals from both low- and relatively high-molecular weight molecules, providing a highly reproducible, robust and high-quality method to obtain a complete overview of metabolites in the sample.

The latter is commonly solved using CPMG (Carr–Purcell–Meiboom–Gill) spin-echo sequence¹¹⁹, making the acquisition more selective for low-molecular-weight compounds (via T2 filtering). Conversely, the ^1H Diffusion-Edited pulse sequence permits the selective observation of macromolecular components in solutions containing small molecules. These three experiments are commonly used for NMR-based blood (serum and plasma) analyses (**Figure 6**), since blood contains many more macromolecules if compared to other biofluids, such as urine and saliva. However, it should be pointed out that some authors prefer to physically remove macromolecular components via centrifugation with e.g. 3000 MWCO

Amicon Ultra-0.5 filters followed by NOESY acquisition, rather than relying of CPMG filtering⁶.

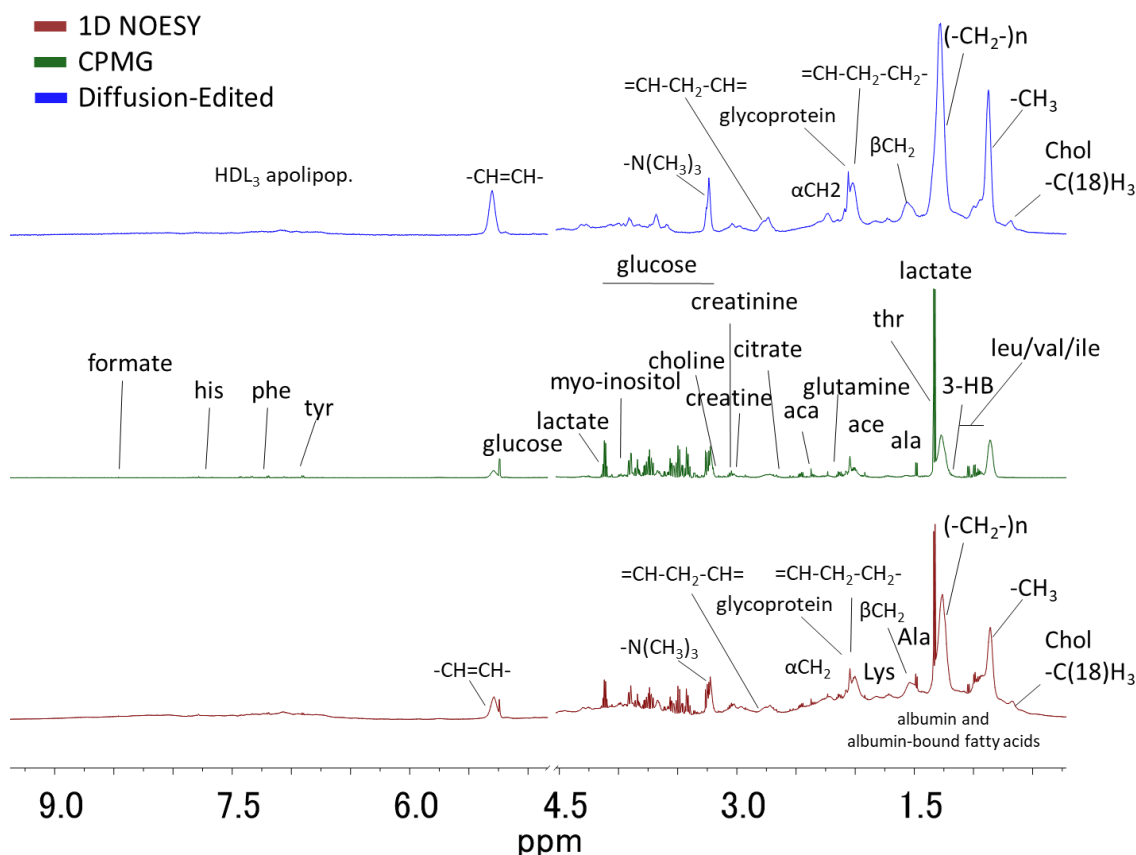


Figure 6. From the bottom to the top: 1D NOESY, CPMG and Diffusion-Edited human serum spectra. Some of the main peaks are assigned. Serum lipoprotein lipids and albumin are the main responsible for the broad signals. The residual water peak region (4.5-5 ppm) is not shown.

2D spectra for metabolomics studies are mainly required to assign new metabolites or for doubtful cases some of the most used 2D experiments are: ¹H-¹H J-resolved (JRES), to provide information about multiplicity and coupling patterns; ii) ¹H-¹H Correlation Spectroscopy (COSY) and ¹H-¹H Total Correlation Spectroscopy (TOCSY), to provide, respectively, short and long range scalar connectivities; iii) natural-abundance heteronuclear experiments that use ¹H in combination with other nuclei. Examples are ¹³C/¹H Heteronuclear Single Quantum Coherence Spectroscopy (HSQC), Heteronuclear Multiple-Quantum Correlation (HMQC) and Heteronuclear Multiple-Bond Correlation Spectroscopy (HMBC), to obtain information on the direct scalar coupling between ¹³C and ¹H nuclei¹²⁰. These 2D experiments generally require much longer acquisition times than the standard 1D pulse sequences, therefore in defining the total acquisition time, one should also consider the stability of the sample under the selected experimental conditions. Common pulse programs for NMR experiments used for metabolomic studies presented in this Ph.D thesis are reported in **table 3**.

Table 3. *Samples analyzed and pulse programs for 1D and 2D NMR experiments selected for the studies reported in this PhD thesis.*

Experiment	Kind of sample	Pulse program
ZG	<i>olive oil</i>	1D ^1H standard single pulse experiment, i.e. RD-P(90°)-acquisition of the free induction decay
NOESY water presaturation	<i>blood, urine, saliva, cell lysate, cell growing medium, milk, olive extract, coffee</i>	1D with spoil gradient using continuous wave presaturation during relaxation delay and mixing time and spoil gradient
NOESY shaped pulse	<i>olive oil</i>	1D with spoil gradient using shaped pulse for multiple solvent presaturation (main solvent peak on resonance) during relaxation delay and continuous wave presaturation during mixing time
CPMG with water presaturation	<i>Blood</i>	1D experiment with T2 filter using Carr-Purcell-Meiboom-Gill sequence with presaturation during relaxation delay
Diffusion Edited	<i>Blood</i>	1D sequence for diffusion measurement using stimulated echo and LED with bipolar gradient pulses for diffusion, 2 spoil gradients and presaturation during relaxation delay ¹²¹
J-resolved	<i>blood, urine, saliva, cell lysate, cell growing medium, milk</i>	homonuclear J-resolved 2D correlation with presaturation during relaxation delay using gradients
^1H-^1H COSY	<i>olive extract, milk</i>	2D homonuclear shift correlation with presaturation during relaxation delay using gradient pulses for selection
^1H-^1H TOCSY	<i>olive extract</i>	2D homonuclear phase sensitive Hartman-Hahn transfer using DIPSI2 sequence for mixing, water suppression using excitation sculpting and gradients ¹²²
^1H-^{13}C HSQC	<i>olive extract, milk</i>	2D H-1/X correlation via double inept transfer using sensitivity improvement phase sensitive using Echo/Antiecho-TPPI gradient selection with decoupling during acquisition using trim pulses in inept transfer and gradients in back-inept ¹²³
^1H-^{13}C HMBC	<i>olive extract</i>	2D H-1/X correlation via heteronuclear zero and double quantum coherence optimized on long range couplings with low-pass J-filter to suppress one-bond correlations no decoupling during acquisition with gradient pulses for selection

3.2 Sample preparation

Molecular analyses of biological samples are necessarily preceded the pre-analytical phase, which includes several steps such as primary sample collection, processing, transport, storage. This phase plays a crucial role in avoiding the generation of artifacts and ensuring the detection of the original metabolome of the sample making it easier to compare metabolomic data collected in multicenter studies. This phase is dependent on sample type, thus proper sampling and proper storage conditions have been designed according to the kind of matrix under study.

For example, during storage (-80°C) and sample preparation reduced temperatures (4°C) are usually employed for samples that tend to denature fast such as urine, blood samples, but also cell lysates. Saliva samples for instance have been demonstrated by Gardner *et al.* to be more stable and less sensitive to multiple thawing steps if compared with the other mentioned biofluids¹²⁴.

The analytical step initiates with NMR sample preparation; for biofluids such as urine, blood and saliva, but also for beverages (*e.g.* wine, fruit juices, *etc.*), minimal processing is needed as samples are already in liquid form. In this case the analytical step consists mainly of buffering the solution to easily reference chemical shift values to existing databases. A measured amount of a reference compound such as tetramethylsilane (TMS) for organic solvents and trimethylsilylpropanoic acid (TMSP) or sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) for aqueous solutions is often added to the sample. This serves both as chemical shift and intensity reference. Deuterated solvents provide a signal for magnetic field stabilization (field-frequency lock) and allow optimization of the resolution of the NMR peaks. Solid samples (*e.g.* fruits, vegetables, green tea) after quenching are freeze-dried and ground or lysate by sonication and then extracted preferably into a deuterated solvent. For solid samples (*e.g.* plants *etc.*) the metabolite extraction process requires a choice of the solvent with a broader polarity range to extract as many possible (in number) classes of metabolites. The most common solvents used in this process are methanol, chloroform, dichloromethane or methanol–chloroform–water, mixed in different proportions.

Chloroform is a non-polar solvent and an excellent choice for non-polar compound extractions, but it may extract a huge amount of lipids that may obscure some other metabolite's signals and make sample pre-concentration more difficult to perform. Furthermore, chloroform can be substituted with other solvents, such as dichloromethane. On the other hand, water has a high polarity and may extract many sugars. Liquid samples with solid debris (*e.g.* urine, saliva, *etc.*) involved ultracentrifugation steps for phases' separation, then the supernatant is collected for analysis. The same measured volume should be placed in the NMR tube for all samples in a given series. Usually, volumes required are 0.5 or 0.6 ml for ^1H -NMR in conventional 5 mm o.d. For each step in the process, several practical considerations (*e.g.* solvent, time, temperature, pH, *etc.*) should be taken into account considering always the kind of sample under study. Standard procedures should be followed to ensure repeatability and comparability when preparing a series of samples.

NMR sample preparation must fulfill the following criteria: i) reproducibility; ii) robustness; iii) ease of use; iv) non-discrimination between samples; v) reduction of unwanted variation in the data due to different pH of samples and so on; vi) maintenance or improvement of spectral quality regarding resolution and sensitivity; vii) freedom of analytically visible artifacts¹²⁵.

3.3 NMR data processing

In the metabolomic work-flow, the data processing step follows the acquisition of the raw spectra and is used to transform the data in a form suitable for subsequent statistical analyses (Figure 7).

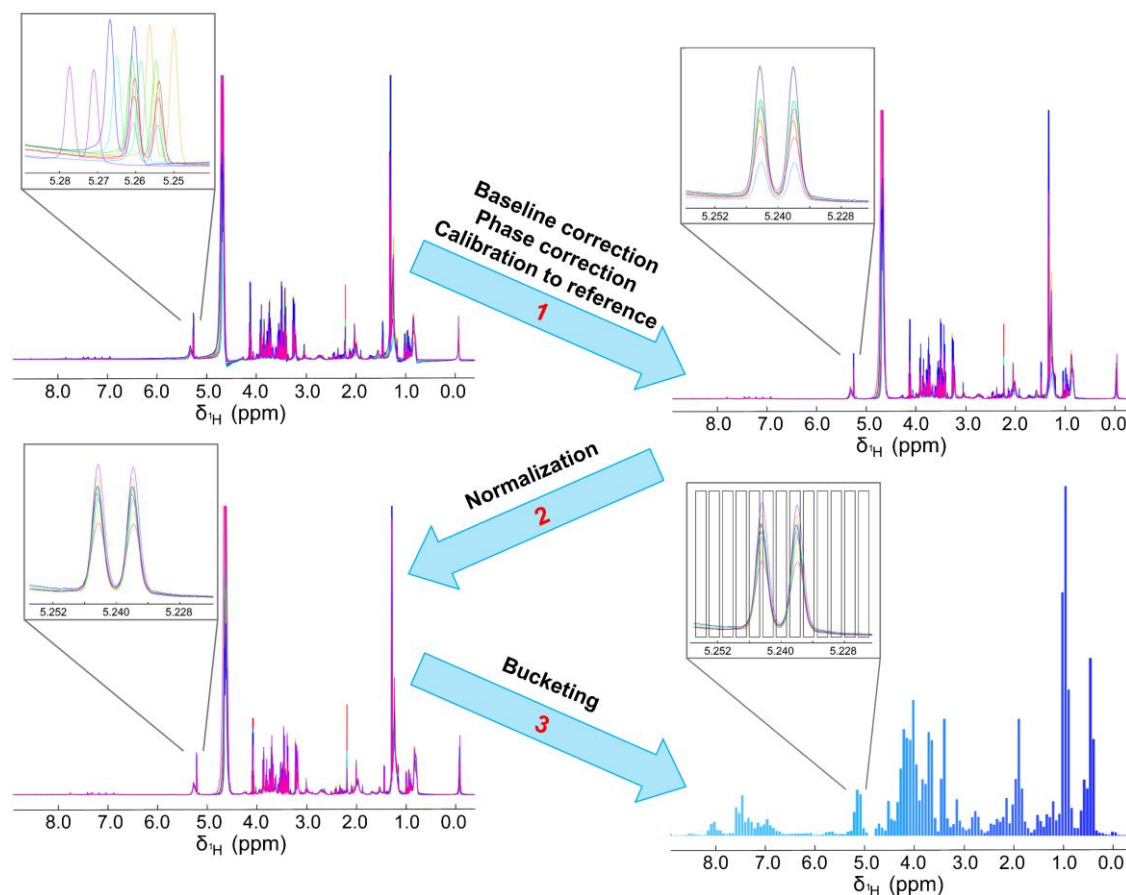


Figure 7. Key stages of NMR spectral processing: 1) baseline correction, phase correction, calibration to an internal reference peak (*e.g.* glucose, TMSP, etc.); 2) normalization; 3) bucketing. Picture taken from Vignoli *et al.* ⁶.

Each NMR spectrum must be properly adjusted for phase and baseline. Both operations are usually performed automatically; manual adjustment is discouraged for metabolomic data, because it can introduce operator-biased artefacts. The spectra need also to be properly aligned. It is necessary to align all spectra acquired so not to have errors in the chemical shifts during the construction of the matrix. Alignment can be done using a known resonance of a reference signal *e.g.* using a chemical shift standard, ideally not interacting with any sample component. For instance, deuterated trimethylsilylpropanoic acid (TMSP) may bind macromolecules present in blood. Thus in samples like serum/plasma, tissues and cell extracts an alternative internal reference is preferred (*e.g.* the anomeric signal of glucose resonating at 5.24 δ_{H} ppm)¹²⁶. For the same reason, use of TMSP as a standard for quantification is discouraged. For the absolute quantification of metabolites, alternative approaches have been proposed, such as the production of an artificial NMR signal based upon PULCON method¹²⁷ or the ERETIC method¹²⁸. There are actually also software systems that can automatically perform both spectral processing and spectral profiling, be able to analyze complex mixtures quickly and accurately

such as FoodScreener™ Bruker platform for fruit juice, wine and honey samples¹¹⁰ and the B.I (Bruker IVDt) platform for analysis and quantification of metabolites in biofluids (urine, cerebrospinal fluid, serum/plasma).

Starting from calibrated full NMR spectra, multidimensional data matrices are then created by means of binning -or bucketing-, a procedure used to reduce the total number of variables and to compensate for small misalignments in the spectra. NMR spectra are simply divided in chemical shift bins (usually with a width of 0.01 or 0.04 ppm) and each bin is integrated to obtain a matrix of variables. Although many more sophisticated algorithms for bucketing exist, this equidistant binning is the most commonly used method¹²⁹ and works quite well despite its simplicity¹³⁰. The main drawback of binning is the drastic loss in resolution that can lead to poor metabolite quantitation.

To compare bucket – or signal - intensities in different samples, one should refer to the same amount of total sample. A preliminary step of correction for dilution effects is needed due to the presence of large physiological variations in concentration. For examples, urines and saliva samples, exhibit significant metabolite concentration fluctuations depending on the hydration state of the individual (in turn related to water/food intake, physical exercise and sweating, etc.), therefore, a global intensity correction by means of normalization is a critical initial step. For biofluids under stricter physiological control (like serum/plasma) normalization is less crucial but could be beneficial to compensate for non-physiological sources of variations emerging from (small) experimental inaccuracies and/or technical artifacts.

Total area normalization and normalization to a reference peak, are two of the most common practices; generally, the normalization algorithm divides each data point (NMR variable) by a constant number that can be represented either by the integral of the reference peak or the sum of binning of all spectra. Although a plethora of strategies/algorithm have been developed the optimal method has not yet been defined. PQN (Probabilistic Quotient Normalization)¹²⁷ is often defined a good choice for biofluids and has been used in this PhD thesis on urine, wine and olive extract NMR data. PQN algorithm works by calculating a reference spectrum (e.g. the median spectrum), then for each spectrum, each bin is divided by the corresponding bin in the reference spectrum, and the median of all these quotients is taken as the normalization factor. PQN assumes that biologically interesting concentration changes influence only few parts of the NMR spectrum, while dilution effects will affect all signals.

Instead, for cells NMR data, it can be assumed that an increase of cell number produces a linear increase of metabolite signal intensities, thus data normalization is usually performed to the number of cells. On the same line, for olive oil and coffee samples of these PhD studies, normalization according to the sample weight has been applied. If weight information is not available, total area normalization represents a good method, as the total spectral area can be considered proportional to the weight of sample. However, prior to this assumption it must to be excluded that the signals that account for a major part of the total area (*e.g.* lactose in milk samples, §5.2.1) vary too much between farms, seasons, diseased groups *etc.*

3.4 NMR metabolomics approaches

Two different methodological approaches can be used in metabolomics, namely targeted and untargeted analysis (**Figure 8**), the most suitable one for the study purpose depends on the experimental question at issue.

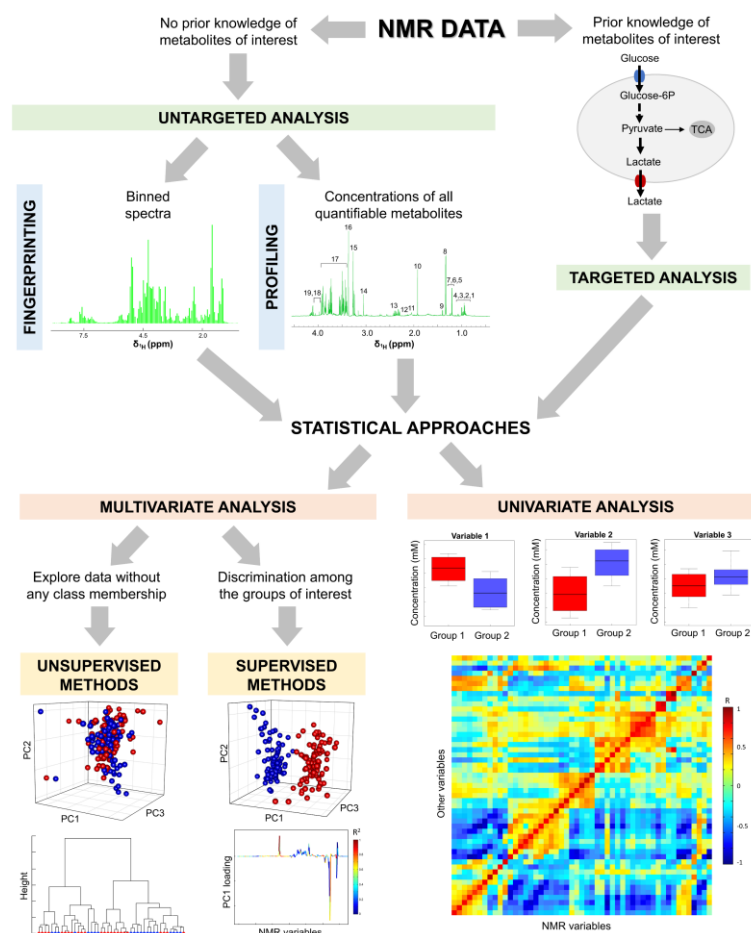


Figure 8. Scheme of metabolomics strategies. Picture taken from published review Vignoli et al.⁶

The targeted one, involves the monitoring of a panel of metabolites selected *a priori* on the basis of known metabolic pathways or pre-identified biomarkers that are undoubtedly associated with the disease or condition of interest. These selected metabolites must be unambiguously assigned and quantified in the samples.

On the other side, the untargeted approach provides a global view of a sample by analyzing all measurable analytes present, including unknown chemicals¹³¹.

The latter purpose can be achieved via two strategies: fingerprinting or profiling. Fingerprinting is a global, rapid evaluation of an NMR spectrum that can be considered a sort of ‘fingerprint’ of all (assigned or unassigned, known and unknown) detectable metabolites present in that sample¹³². The fingerprinting approach is essentially utilized to provide sample classification mainly aiming at discriminating specimens in relation to different biological statuses (e.g. presence/absence of disease, before/after treatment) or at classifying food samples, for example, on the basis of the provenance.

Metabolic profiling deals with the determination of the concentrations of all quantifiable metabolites in a biological sample, it provides considerably more meaningful data from a

biochemical perspective since it also enables the identification of metabolites and metabolic pathways associated with a specific physiological or pathological condition.

3.4.1 Multivariate analysis for classification and modeling

The principal aims of metabolomics can be summarized as: i) visualization of biological data; ii) determination of significant differences possibly present among groups (*e.g.* discrimination between healthy and diseased subjects, classification of fruits according to developmental stage, discrimination among cell samples according to the resistance or the sensitivity to a certain treatment *etc.*); iii) identification of all metabolites/biomarkers that are responsible for the observed differences; iv) construction of a predictive model for classification of new samples¹²⁵. Multivariate statistics is the key to achieve these goals, and it can be performed by either unsupervised or supervised approaches.

Unsupervised methods are utilized to summarize, explore, and discover clusters or trends in the data unlabeled with any class membership; therefore, no prior assumptions or knowledge of the data are needed¹³³. Unsupervised methods represent usually the first step in data analysis, helping to visualize the data and to discover possible outliers. Among the different unsupervised machine learning methods, principal component analysis (PCA)¹³⁴ is the most popular one, it involves the projection of the data in a new space using just few dimensions (data reduction). Each principal component (PC) is a linear combination of the original variables (values of the bucket or metabolites); they are orthogonal and independent from all the others. The first PC (PC1) expresses the maximum percentage of variability inside the data, then the value of variance quickly decreases in such a way that the first PCs are responsible for the great part of variability, while the last PCs are significantly less important and express noise variability. PCA can be interpreted as a linear mixture model, where the data matrix is factorized into two new matrices: score matrix and loading matrix (showed below as plots, **figure 9**). The score matrix contains the new coordinates of the original data in a new rotated coordinate system (defined by the PCs). The loading matrix contains the weights of the original variables to transform them into the score.

If in the score plot of PC1 vs PC2 (**Figure 9b**), a separation between two or more groups of samples is visible, the loadings of the corresponding PCs give us information about which original variables are responsible for that separation.

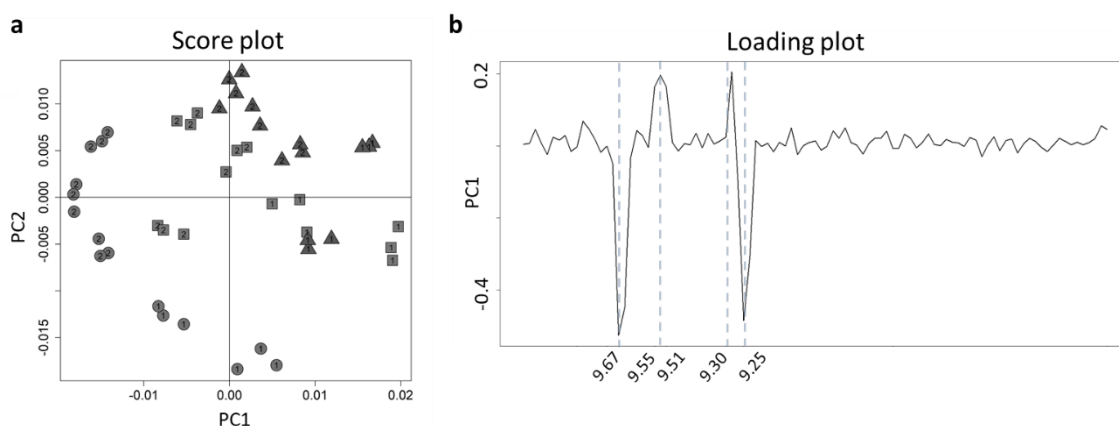


Figure 9. Example of PCA analysis on olive oil samples, §5.1.2. a) Score Plot: PC1 vs PC2, each dot in the plot represents a different sample; circles: group 1 (olives manipulated at room temperature); square: group 2

(olives manipulated at 15°C); triangles: group 3 (olives manipulated at 10°C); 1: olives collected in 2016; 2: olives collected in 2017. It is possible to visualize a separation of samples along the PC1 according to different temperature manipulation. b) Loadings Plot of PC1. Most significant metabolites in the score plot showed the highest loadings in the PC1. δ H 9.55-9.50 ppm: *trans*-2-hexenal; 9.67-9.65 ppm: 4-hydroxy-*trans*-alk-2-enal; 9.26-9.26 ppm: hemiacetal form of secoiridoids; 9.30 ppm: unknown aldehyde.

Instead, supervised approaches use *a priori* knowledge to generate models that are focused on the effects of interest. In other words, they require information about the class identities. These approaches can be divided in two main classes: projection-based methods and machine learning methods.

Among the projection-based approaches, Partial Least Squares (PLS)¹³⁵ and its variant Orthogonal Projections to Latent Structures (OPLS)¹³⁶ are the most widely used. Their robustness in the presence of highly correlated variables and their ability to provide highly predictive models using a small number of latent variables makes them useful to address the study of a large number of systems¹³⁷. PLS can be thought of as the supervised extension of PCA, as it addresses the data reduction problem in a regression-based framework: but instead of describing the maximum variation in the measured data (X), PLS aims at deriving latent variables, analogous to PCs, which maximize the co-variation between the measured data (X) and the response variable (Y) regressed against¹³⁸.

OPLS uses orthogonal signal correction maximizing the explained covariance between X and Y on the first latent variables (n-1 variables, with n the number of groups of interest), and all the orthogonal (or unrelated) variance in the other following latent variables.

The large variation among human subjects can give rise to two problems in the analysis of the data. The first is that small and subtle treatment effects (*e.g.* treatment responses) can be easily overlooked, especially when the effect is smaller than the intrinsic variation among the subjects. The second problem is that the response and the impact of the treatment effect may differ among the subjects. A widely used approach in this case is pairwise methods, like multilevel-PLS (M-PLS), where all subjects act as their own control (cross-over design) eliminating the inter-subject variability^{139,140}. In a multilevel PLS, between-subject variation is separated from the within-subject variation by subtracting the individual specific average, retaining only the within-subject variation.

Both PCA, PLS, OPLS and M-PLS can be used in combination with Discriminant Analysis (DA) to establish the optimal discrimination among samples, and to prediction purpose. Modern clustering/prediction methods comprise machine learning algorithms such as k-Nearest Neighbors (kNN) and Support Vector Machine (SVM) that are extremely used in metabolomic analysis. kNN is probably the simplest and computationally easiest classification technique ever presented: it works in a local neighborhood around a considered test sample to be classified. The neighborhood is usually determined by the Euclidean distance and the closest *k* (*e.g.* $3 \leq k$) objects are used for estimation of the group membership (“majority voting”) of a new sample¹⁴¹ (**Figure 10a**). Of course, the choice of the neighborhood size *k* determines the quality of the results: small *k* values can lead to the construction of a model subject to significant statistical fluctuations, while large *k* values can reduce statistical errors but flatten many details of the distribution.

SVM uses a statistical learning paradigm to construct a “borderline” (a line, a plane, or, for more than three dimensions, a hyper-plane) in the sample space separating the different classes of samples inside the dataset from each other¹⁴². The hyper-plane found by the SVMs in the feature space corresponds to a non-linear decision boundary in the input space using what is called kernel trick (ϕ), **figure 10b**.

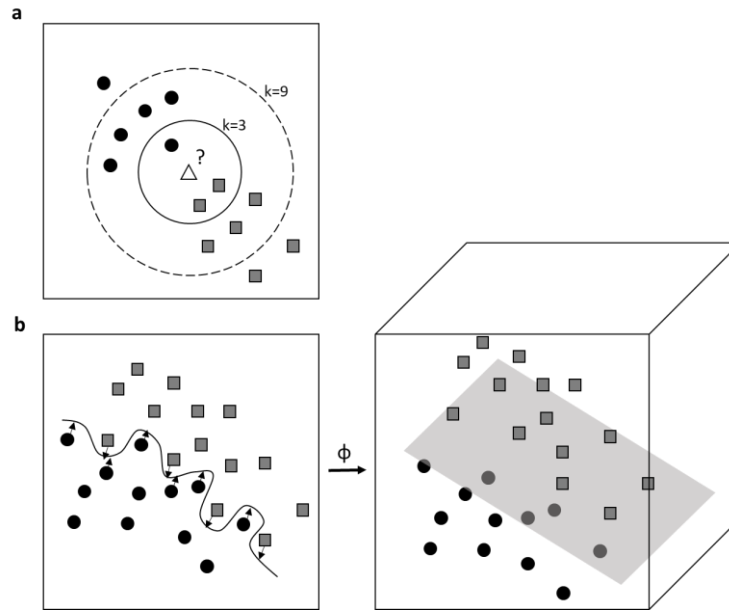


Figure 10. Machine learning algorithms: a) classification of an unknown object (white triangle) among two groups (grey squares and black dots) with k -NN methods; b) the non-linear SVM classifier with the kernel trick.

The risk of supervised approaches is the overfitting, it is usually therefore necessary and advisable to employ an independent validation set (when available) or with more sophisticated approaches like cross-validation schemes⁹², these are useful statistics tool to test and to strength the power of the built models. Cross-validation (CV) techniques create a random internal validation set by removing one (Leave-One-Out, LOO) or many (Leave-Many-Out, LMO) samples from the training set. The model is built on the training set and the removed samples are used to assess the model performances generating a confusion matrix that express sensitivity, specificity, and accuracy. A confusion matrix (**Table 4**) is used with classification models, meaning the response value is categorical. Thus, the model predicts one response value for each observation in the testing set, and each predicted response value is compared to the actual response value for that observation. Sensitivity is defined as the ratio between true positives (TP) and all positives (P), while specificity is the ratio between true negative (TN) and all negatives (N). Accuracy is defined as the ratio between the sum of TP and TN, and the total population analyzed (P+N).

Moreover, permutation test is often advocated to calculate the statistical significance of the results. The use of independent training and validation sets is the preferred approach in the medical community, but requires collecting samples from different hospitals, even from different countries. The selection of the best approach strongly depends on the aim of the research: cross-validation on a unique collection of samples should be recommended for exploratory studies, where the aim is to see whether a fingerprint for a specific condition exists. Independent validation is then recommended to further demonstrate that the fingerprint is still

strong also in the presence of “environmental noise”, useful also in food traceability studies to test the capability of the model to recognized same specimens sampled in different period/condition.

Table 4. *Example of confusion matrix: true positive; TN: true negative; FP: false positive; FN: false negative; N: all negatives; P: all positives.*

Confusion matrix					
		Predicted Class		Specificity	=TN/N
		group 1(yes)	group 2 (no)	Sensitivity	=TP/P
Actual Class	group 1(yes)	TP	FN	Precision	=TP/(FP+TP)
	group 2 (no)	FP	TN	Accuracy	=(TN+TP)/(N+P)

Metabolomics, as well as other omics, generates data to provide biological insight based on statistical inference from datasets that are typically large. As such, the power to detect associations or the flow of information strongly depends on effect size, heterogeneity of the background noise, and sample size, with the latter often being the only parameter controlled by researchers. Unfortunately, human studies are affected by a multitude of confounding factors that are difficult or impossible to control for (*e.g.*, diet and lifestyle choices). Thus, the ability of metabolomic approaches to produce meaningful insight into human disease is very much dependent on available sample sizes, and in many settings an underpowered study may not only be a shot in the dark, missing true signals, but it is also more likely to produce false positive results. An initial power calculation to ensure sufficient sample size and variation in outcomes is increasingly necessary in large-scale studies

3.4.2 Identification, quantification, univariate analysis

The process of metabolite identification in NMR spectra, especially for less common biospecimens, is not straightforward due to the high complexity of their ¹H-NMR spectra. Usually many resonances can be directly assigned in one-dimensional spectra based on chemical shifts and multiplicity. This task is facilitated by databases, often freely available: in particular, for human metabolites, the Human Metabolome Database (HMDB), or food metabolites the FoodDB database. For doubtful cases, the addition of standard molecules (spiking) to the NMR sample could be of help. However, two-dimensional spectra (as already defined in § 3.1) are often required to assign new metabolites. The quest for a completely automatic assignment tool is an active research field, with promising achievements. Indeed, automated tools such as FoodScreenerTM (for fruit juices, wine and honey samples) and B.I (for urine, CSF and serum/plasma samples) have been already developed allowing for automatic identification and quantification of metabolites. Moreover, other tools for semi-automatic quantitation have been developed such as BATMAN tool based on Bayesian¹⁴³, ASICS based linear models¹⁴⁴ or NMR Suite, that is a complete computer-assisted tool for analysis and deconvolution of the spectra, permitting the user-guided fitting, integration, and quantitation of the selected peaks. However, being mostly manual, it is time consuming and it needs a skilled operator. In order to find a metabolite (or a panel of metabolites) that can be considered as a possible biomarker for the specific pathology or condition under investigation, each metabolite needs to be analyzed independently of all the others without considering any possible interactions. Univariate statistical analysis is the key to achieve this goal: statistical tests, correlation analysis are the most

used approaches. On the biological assumption that metabolite concentrations are not normally distributed, non-parametric tests are often utilized. Two of the most used non-parametric approaches are Wilcoxon-Mann-Whitney and Kruskal-Wallis tests, used to assess which metabolites are statistically significant different among classes. These tests are very useful in metabolomics to assess whether the metabolite concentration levels are significantly different in the groups of samples under examination, e.g. between healthy and pathological samples. The null hypothesis (H_0) claims that two randomly selected samples from two populations actually belong to the same population; the alternative hypothesis (H_a) states that H_0 is false and the two population are distinct. Wilcoxon-Mann-Whitney¹⁴⁵ test is the non-parametric analogue of the classical t-test to compare the distribution of a metabolite in two groups it is used to assess whether two independent groups (no more) of samples come to the same population. Unlike the t-test it does not require the assumption of normal distributions. Wilcoxon signed rank test¹⁴⁶ instead can be used for pairwise comparisons where all subjects acts as their own control (same individuals before and after treatment). The Kruskal-Wallis test¹⁴⁷ (the analogue of the parametric Analysis of Variance) is a non-parametric method for testing whether two or more independent groups of samples originate from the same distribution. It does not assume a normal distribution of the residuals, unlike the analogous parametric one-way analysis of variance test. The output of all these tests is a P -value, that expresses the probability of obtaining a result equal to or more extreme than what was observed, assuming the null hypothesis true. Conventionally, when the P -value is less than the significance level of 0.05 or 0.01 the null hypothesis is rejected, and the metabolite is deemed statistically different in the two groups. When several metabolites are tested together, to avoid random false positives, multiple testing corrections need to be adopted: Bonferroni¹⁴⁸ and Benjamini-Hochberg¹⁴⁹ are the most widespread methods. However, P -values need to be taken with care: they are often misused, misunderstood and misinterpreted¹⁵⁰; for this reason statisticians have proposed replacing (or accompanying) P -values with effect size¹⁵¹ (a standardized measure of the magnitude of the observed phenomenon) as an alternative (complementary) measure of evidence. Pearson product-moment correlation coefficient is often used to get information about the correlation (linear dependence) between two variables, giving a value between +1 and -1, where 1 is total positive correlation, 0 is no correlation, and -1 is total negative correlation. In metabolomic, it is commonly used for instance to assess the correlation among different metabolites, among metabolite expression levels and clinical data and/or other biological data.

Chapter 4

Metabolomics in biomedicine

4.1 NMR-based metabolomics in disease characterization

Metabolomics presents a snapshot of the metabolic dynamics that reflect the response of living systems to pathophysiological stimuli and/or genetic modifications and the surrounding environment, giving it an advantage as a diseases diagnostic tool¹⁵². Finding specific biomarkers/fingerprint that can be used for detecting, tracking, and monitoring disease progression or for monitoring the efficacy of a treatment protocol, could significantly influence the treatment of many human diseases.

In this thesis, we assess the ability of an NMR-based untargeted metabolomic approach to characterize different disease signatures using several biological specimens; five studies with distinct focuses are presented in this section.

The first study presented examines the HCV serum profile of 67 infected patients before and after viral clearance by DAAs (Direct Acting Antivirals) and compares naïve HCV to 50 naïve HBV patients and to 43 matched healthy controls (§ 4.1.1).

The originality of this work is mainly due to two factors. One is that it is the first application of NMR metabolomics for the study of the effects induced by this new HCV therapy on serum metabolome conducted at standardized level, the second factor is that actually there are no metabolomics studies based on the characterization of serum samples of HCV infected patients: indeed most of them are focused on patients with liver injury of mixed etiology¹⁵³.

The new direct antiviral therapies, which directly target on the replication of the virus, have granted new opportunities, as in the last two decades interferon (IFN) therapy has been used for the treatment of HCV infection. The recent development of DAAs has not only contributed to an increased SVR (Sustained Virologic Response), from the 50% obtained with interferon to the 95% resulting from DAAs treatment¹⁵⁴, but has provided us the opportunity to obtain a real metabolic picture of patients since DAAs are not biologically active molecules with a systemic action such as IFN. Nevertheless, differences in metabolites levels among the METAVIR subgroups were significant, suggesting that different concentrations of metabolites characterize different fibrosis scores. In agreement with other authors^{155,156}, tyrosine and formate increased going from no/mild fibrosis to severe fibrosis, suggesting that their role as potential fibrosis severity biomarkers should be investigated more deeply.

Differences in the metabolomic profile of HCV patients before and after an effective DAA treatment (three time points for each patient have been considered: before treatment, 12- and 24-weeks after SVR) have been detected. Intriguingly 2-oxoglutarate and 3-hydroxybutyrate levels have been found higher in HCV baseline subjects when compared with HBV, healthy subjects and successfully treated HCV patients (SVR12 and SVR24). Main findings suggest a direct role of HCV in altering biochemical pathways of basal metabolism; this could help explaining the association with some of the most frequent extrahepatic manifestations such as cardiovascular diseases and diabetes mellitus. Longer post-therapy follow-up epidemiological studies evaluating metabolomic fingerprints of HCV SVR patients could confirm that the restoration of altered metabolites levels is related to the decreased mortality rate for extrahepatic causes.

Although it is still an in-progress study, I would like to mention preliminary metabolomic results of PROPAG-AGEING project since they are promising and perfectly fit with the topic of this chapter.

PROPAG-AGEING is an H2020 EU-funded project that aims to identify new molecular signatures for early diagnosis of Parkinson Disease (PD). The main question of this project is trying to answer why the advanced age is the most important risk factor in developing PD. In this project PD is considered as totally embedded within the basic molecular and cellular mechanisms of the ageing process¹⁵⁷. Therefore, the final goal is to identify the combination of these alterations capable of shifting the phenotype of elderly subjects from a physiological decline into PD. This will eventually enable the identification of markers for early PD diagnosis (before motor symptoms occur) using human serum and urine samples. Some of the shared features among ageing and PD are the accumulation of senescent cells, inflammation and a propagation phenomenon. Inflammation seems to have a central role in pathogenesis; senescent cells are indeed capable to secrete cytokines and other inflammatory compounds, which contribute to a chronic and physiological inflammation status called “inflammaging”^{158,159}. This inflammaging status can be transmitted both locally and systematically as it has been demonstrated by using the so called heterochronic parabionts. Rejuvenation (improvement of cognitive function and synaptic plasticity) of an old mouse has been reached thanks to blood molecules of a young one, and vice versa an aging effect on the young mouse when treated with blood components of an old mouse was observed¹⁶⁰.

Our project will examine samples of 700 *de novo* PD patients collected in different European countries. PD samples will be compared to matched healthy controls and centenarian individuals. Besides, to highlight differences between healthy ageing and PD, will be also used a cohort of twin's pairs discordant for PD: this will enable the identification of genetic-independent risk factors. §4.1.2 is part of the discovering phase of PROPAG-AGEING and covers the NMR metabolomics characterization of serum and urine samples from an initial subset of *de novo* PD patients (n= 74) and matched healthy subjects (n=61). Our preliminary results reveal the presence of a subtle metabolomic trace of PD in sera and for the first time in literature also in urine (74% overall discrimination accuracy). Moreover significantly altered metabolites are concordant in describing the presence of a greater oxidative stress (isoleucine and hippurate), energy metabolism disfunction (acetone and pyruvic acid) and phenylalanine levels in sera seem corroborating this metabolite as hallmark of early stage PD⁵¹. The future perspective of this preliminary work is to strengthen the statistical model based on metabolomics data of PD patients thanks to new cohorts of samples. A more reliable statistical model could allow a better validation of candidate biomarkers. Data from centenarians and twins will help respectively in understanding more about the metabolomic traces of healthy ageing and in identifying genetic-independent risk factors.

Recent studies have shown that the coincident loss of normal innate and adaptive immune response capacity with aging, combined with low grade chronic inflammation, coalesce to alter immune-competence and promote the pathogenesis of a diverse number of diseases¹⁵⁸.

Elderly individuals exhibit increased susceptibilities also to a number of autoimmune, infectious, and inflammatory diseases, including atherosclerosis, rheumatoid arthritis and periodontitis^{161–163}. Two studies are reported in this thesis deal with the application of NMR-based metabolomics for the study of periodontitis analyzing the saliva of affected patients¹⁶⁴.

The etiology of periodontal disease is still unknown and it is mainly associated with oral tissue damage, which is primarily initiated by an excessive immune response by the host to subgingival pathogens. It is an inflammation of the periodontal tissues that causes teeth attachment loss

with the consequent formation of pockets, dental mobility, gingival bleeding, suppurations until the loss of teeth. Poor periodontal health, genetics and ageing are thought to be the main causes, approximately 48% of US adults have chronic periodontitis (similar or higher rates reported in other populations) and the 70% of them are elderly people¹⁶⁵. The traditional classification recognizes two major forms of periodontitis: chronic periodontitis (CP) and aggressive periodontitis (AgP), both conditions can be defined as localized or generalized according to the extent of the periodontal destruction. Mainly these two forms diverge in the age of onset (people with aggressive periodontitis are significantly younger than individuals with chronic periodontitis) and rate of progression (chronic periodontitis has traditionally been viewed as a slowly progressing disease)¹⁶⁶. However there are not clinical, histopathological or microbiological parameters providing an unequivocal discrimination between the two conditions.

First study on periodontitis reported here (§4.1.3) was planned primarily, to determine the ability of NMR analysis to discriminate generalized chronic periodontitis (GCP, n=33) from generalized aggressive periodontitis (GAgP, n=28), and secondarily, to assess potential metabolites discriminating periodontitis patients from healthy individuals (HI, n=39) considering whole saliva samples.

Our results, although corroborating substantial differences between periodontitis patients and healthy individuals, have failed in providing a significant discrimination between GCP and GAgP metabolomic fingerprints. This finding is anyway in agreement and confirms the increasing body of evidence that it is almost impossible to use the term “aggressive periodontitis” since there is no proper way to diagnose it¹⁶⁷.

This may suggest that what distinguishes AgP from CP are dissimilarities in the immune-inflammatory host response or differences in the degree of bacterial invasiveness.

Presumably, as far as all new high-throughput technologies have proven the existence of several molecular signatures in distinct periodontal patients, the traditional binomial classification seems not to fit this emerging heterogeneity anymore. New models need to be hypothesized and tested, but because of the disease complexity a simultaneous multi-omics approach should be elected. Conversely, the successful differentiation between healthy and diseased individuals has corroborated the sensibility of metabolomics profiling as a source of potential biomarkers for molecular diagnostic using saliva.

Indeed, while there is almost a spread awareness of a metabolomic trace of periodontitis in human saliva^{46,47,168}, there is still lack of investigations concerning the effect of periodontal treatment on individual metabolic fingerprint.

Therefore, the aim of the second study on periodontitis of this thesis (§ 4.1.4) was planned to determine whether non-surgical periodontal therapy could change salivary metabolomic profile in GCP to one more similar to periodontal health. Non-surgical therapy relies in mechanical removal of bacterial plaques from periodontally involved root surface¹⁶⁹. In the present investigation the conventional quadrant-wise non-surgical therapy induced a compressive shift in the within-subject salivary metabolome of CGP patients as detected by multilevel-PLS (92.5% predictive accuracy), but no individual metabolite resulted *per se* statistically significant following the univariate analysis. The explanation could lie in the small sample size (19 GCP/treated patients) and the extreme heterogeneity and variability within the complex interplay of molecules sustaining the pathogenesis of periodontitis.

Confirming what seen in previous study, metabolites defining the periodontal status could be ascribed to bacterial species (lactate, pyruvate, formate) and to tissue degradation (proline, phenylalanine, tyrosine), but they could also describe host immune response (valine, isoleucine).

These results should be interpreted with caution since actually there is no way to determine unequivocally their true origin since too many factors interact, and this is one of the major problems of oral metabolites. In this case considering individual fingerprint could represent the best choice to describe the global response resulting from the sum of the microbiome presence and individual immune response. Indeed, our results have revealed also that based on individual metabolic fingerprint is not possible to assimilate treated GCP patients to healthy subjects even if they are more projected toward the healthy status with the respect of baseline. One explanation could be the presence of some sites of inflammation in treated patients and/or the fact that non-surgical periodontal treatment could not be resolute in all cases. Further studies should clarify the effect on salivary metabolites of different periodontal therapy (*e.g.* surgical, antimicrobial, daily maintenance, host modulation therapy *etc.*) and longer prospective studies could provide some insight about periodontitis recurrence of treated patients.

The second explanation could be that even if successfully treated, periodontitis patients still hold in their salivary metabolite a signature of their background susceptibility to the disease. Elucidating this aspect could tell us more regarding the individual immune response and could provide more information also regarding other associated chronic inflammatory condition such as diabetes, heart disease and other minor related disease such as osteoporosis, respiratory disease and cancer¹⁷⁰.

In this thesis NMR based approach for disease diagnosis have been applied also to veterinary research. The study reported was planned to identify metabolomic profile of sepsis in plasma samples of newborn calves (1-month-old) by ¹H NMR to assess severity and predict outcomes (§4.1.5). The interest has been driven by the fact that clinical signs of newborn sepsis are often nonspecific, subtle, and inconspicuous and therefore demand a high index of suspicion for early diagnosis. Positive blood cultures are required for a definitive antemortem diagnosis of sepsis, but results take time (not reported within 48–72 hours), and false-negative culture findings are common. The high mortality of sepsis can be seen as an indication of insufficient laboratory diagnostics¹⁷¹.

In this study 113 plasma samples of newborn calves collected at different time points (diseased animals at 0 hour; diseased animals at the 3rd hour; diseased animals at the 6th hour; diseased animals at the 24th hour; diseased animals at the 48th hour; and diseased animals at the 72nd hour) have been analyzed and compared to healthy ones. Collected data indicated that NMR metabolomics is a feasible tool for the identification of septic profile in newborn calves: unsupervised PCA of all plasma spectra is effective in discriminating healthy animals from calves with sepsis, moreover cross-validated OPLS models built on NOESY and CPMG experiments have revealed 90% and 100% predictive accuracy respectively. Metabolites analysis showed alteration in biochemical pathways like aminoacyl-tRNA biosynthesis (histidine, phenylalanine, glycine, valine, alanine, isoleucine, leucine, and proline), valine/ leucine/isoleucine biosynthesis (pyruvate, leucine, valine, and isoleucine), nitrogen metabolism (phenylalanine, histidine, glycine, and formate), glycine/serine and threonine metabolism (choline, glycine, creatine, and pyruvate), and synthesis and degradation of ketone bodies (3-hydroxybutyrate, acetone). Being in accordance with literature evidences most changes occurring during sepsis suggest an energy crisis in non survivors¹⁷². Remarkable increased allantoin of the septic group in this present study can be an indication of microbial overgrowth or oxidative stress. Cell death from cytochrome oxidase inhibition by formic acid believed to result partly from depletion of ATP, reducing energy concentrations so that essential cell functions cannot be maintained. In this regard, gradually increasing of formic acid being decreased at 0 hour can be meaningful for prognosis of septic calves.

This study represents an important trace of this thesis corroborating the use of the fingerprint approach in helping to identify (quickly) a clear disease signature.

Although identified and quantified metabolites may become potential biomarkers for diagnosing newborn sepsis and monitoring therapy of sepsis in calves, the extent of the predictive and prognostic values of this given set of metabolites will be required for clinical trials.

Taken to the extreme, fingerprinting and multivariate analysis would always be the most efficient method, *i.e.* a fingerprint would be, by definition, the best biomarker.

4.1.1 Metabolic fingerprints between HCV and HBV patients: a possible interference of the two major hepatitis viruses in basal metabolism pathways

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Submitted

Candidate's contributions: acquisition of NMR data, statistical analysis and interpretation of data and writing the manuscript.

ABSTRACT

HCV global prevalence is estimated to be 2%, therefore the number of people at risk of developing HCV-related chronic liver disease is substantial. The treatment of HCV-infection has recently experienced a remarkable advancement with the introduction of new direct-acting antivirals (DAA) allowing Sustained Virologic Response (SVR) rate exceeding 90%, 95–97% in compensated cirrhosis and 85–90% in subjects with advanced liver disease.

DAAs provide an ideal model to study the effect of HCV on human metabolism since they are not biologically active molecule such as interferon. Metabolomics is the study of metabolic changes in biological systems and may help identifying specific profiles associated with subtle alterations induced by diseases.

Few studies are available on metabolic changes in liver injuries and this is the first metabolomic study evaluating a group of HCV-positive patients, before and after viral eradication by DAA IFN-free regimens, using ¹H-NMR to characterize and compare their serum fingerprint to naïve HBV-patients and healthy donors. The investigation clearly shows differences in the metabolomic profile of HCV patients before and after an effective DAA treatment. Especially interesting are the high levels of 2-oxoglutarate and 3-hydroxybutrate in HCV samples compared to HBV and progressively decreasing from baseline HCV to SVR24.

Introduction

HCV global prevalence is estimated to be 2% (180 million people), therefore the number of people at risk of developing HCV-related chronic liver disease is substantial.

Anti-HCV therapy has been based on the administration of Pegylated-Interferon (Peg-IFN) plus Ribavirin (RBV) for almost 15 years; the treatment of the HCV-infection has recently experienced a remarkable advancement with the introduction of new direct-acting antivirals (DAA). In large real-life cohorts DAAs allow Sustained Virologic Response (SVR) rates exceeding 90%, 95–97% in compensated cirrhosis and 85–90% in subjects with more advanced liver disease^{173–175}.

In the near future, the DAA regimens will probably reduce the incidence of hepatic decompensation, the development of HCC and the insurgence of extrahepatic diseases¹⁷⁶.

A significant contribution to this new approach has been provided by the so called “-omic” techniques during the last fifteen years. This term refers to innovative technology platforms such as genetics, genomics, proteomics, and metabolomics, which allow us to detect and identify many different molecules that are present in the body. Metabolomics is the latest of the “-omic” techniques and has emerged as an exciting tool for biomedical researchers¹⁷⁷. Metabolomics is the study of the metabolome, i.e. the whole ensemble of small molecules (metabolites) present in body fluids, cells or tissues¹⁷⁸. The chemical nature and the relative abundance of detectable metabolites can be viewed as the fingerprint that characterizes the physio-pathological state of an individual¹⁷⁹. While genomics tells us “what could happen”, metabolomics tells us “what is happening”. Thus, metabolomics plays a significant role in bridging the phenotype-genotype gap and assists us not only in linking gene to function but also in gaining an entirely new insight into how organisms respond to exogenous stimuli and how individuals evolve over time^{180,181}.

Proton nuclear magnetic resonance (¹H-NMR) spectroscopy is one of the main analytical tools that are used in metabolomics. It allows simultaneous identification and quantification of hundreds of small molecules, creating a disease-specific metabolomic profile/fingerprint⁶.

Presently, there are few studies concerning HCV-infection that are based on a metabolomic approach^{182–185}; however, the majority of metabolomic analyses were performed on subjects with chronic liver damage with no stratification according to etiology^{186,187}. The most interesting data regarding infection by HCV and antiviral therapy effects are reported by Saito *et al.* by comparing the metabolic profiles of 10 patients with chronic HCV infection who failed treatment with Peg-IFN and RBV from the profiles of those 10 subjects who obtained an SVR (Sustained Virologic Response, viral eradication) after the same therapy. Despite the limited population and the absence of appropriate controls, the study already mentioned suggests that it could be interesting to evaluate, on a wider and better characterized population, the HCV infection pattern with a metabolomics approach.

The need of a well-designed metabolomics analysis on large cohorts of well-defined patients undergoing standardized therapies, evaluating samples drawn at different time points corresponding to specific clinical situations (before treatment, after treatment at the time of judgement of efficacy, and subsequently at interesting end-points) was also suggested by the most comprehensive review on this topic¹⁵³.

The new direct antiviral therapies, which have a direct target on the replication of the virus, provide an ideal model of study: indeed, the lack of possible confounding effects caused by biologically active molecules with a systemic action such as interferon should allow us to obtain a real metabolic picture of the patient.

The aim of this study was to evaluate a group of HCV-positive patients, before and after viral eradication by DAA IFN-free regimens, through an NMR-based metabolomic approach, comparing their fingerprints to naïve HBV-patients and healthy donors.

Results

NMR spectra of 294 serum samples (67 HCV or baseline, 67 SVR12, 67 SVR24, 50 HBV, 43 HC) were acquired.

As a preliminary unsupervised approach, principal component analysis (PCA) was used to obtain a simplified view of the variation in the data and to check the sample quality excluding the presence of outliers. Some separation in the space of the first five components seemed to have already emerged from this unsupervised approach (**Supplementary Figure 1A and B**).

DAA induced metabolomic changes in HCV patients

With the aim of analyzing within-subject metabolic changes introduced by DAAs therapy, multilevel partial least-squares analysis (MPLS) was performed on 67 x 3 HCV serum samples collected at three different times (baseline-HCV, 12 weeks- and 24 weeks-after SVR). Multilevel PLS models were built on 1D NOESY and CPMG spectra and both proved satisfactory in discriminating baseline from SVR12 and SVR24 subjects (64.5% predictive accuracy from the 1D NOESY model and 70% from the CPMG model).

As it emerges from the confusion matrices (**Table 2**), SVR12 samples mingle with SVR24 samples, indicating that, after the viral eradication patient profiles remain stable for at least the next twelve weeks of treatment.

We tested the influence of therapy duration (12 or 24 weeks), administration of ribavirin (RBV), HCV genotypes and METAVIR score on metabolic fingerprint.

Concerning treatment duration and RBV administration, neither multivariate analysis on spectral buckets (predictive accuracy <50%) nor univariate analysis on metabolite levels highlighted significant differences among HCV subgroups.

Regarding the analysis on HCV genotypes, considering the small number of individuals *per* group as the main limitation, and thus the impossibility to compare accurately all of them, a comparison between the most common HCV viral genotypes (1a/1b and 2a/2c) was attempted without obtaining a reliable discrimination (predictive accuracy = 61 %).

On the other hand, the analysis of HCV subgroups stratified on METAVIR score revealed interesting differences.

The NMR spectra of serum samples from HCV patients with a high score of fibrosis (F3 and F4) and without fibrosis or lower fibrosis (F0/F1 and F2) were analyzed at each time point (Baseline, SVR12 and SVR24) using multivariate and univariate statistical analyses. The OPLS-DA test was not able to discriminate between advanced fibrosis and mild fibrosis, but the concentration levels of several metabolites were significantly altered, as assessed by univariate tests (**Figure 1, Supplementary Tables 3-5**).

In baseline samples, valine, isobutyrate, phenylalanine, 2-oxoglutarate, tyrosine and formate significantly decreased from F4 to F0/F1, and glutamine only increased from F4 to F0/F1.

Comparison among naïve HCV, naïve HBV and healthy subjects

We also performed comparative analyses among the groups and the 67 HCV baseline samples. The 50 naïve HBV patient samples and the 43 serum samples from healthy controls were used to build two OPLS-DA models, one on NOESY spectra and the other on CPMG spectra, with

the aim of defining a statistical model able to accurately predict the healthy or pathological state of a certain subject, simply using a single serum NMR spectrum.

Both OPLS-DA models show a clear-cut discrimination between HCV, HBV and HC, with an overall cross-validated accuracy of 77% on NOESY and 74% CPMG models respectively (**Figure 2 a, b**). For the sake of completeness, confusion matrices and OPLS-DA score plots of each comparisons (HCV vs HBV; HCV vs HC; HBV vs HC) are reported in Supplementary materials (**Supplementary Figure 2 and Supplementary Table 1**), explaining an overall cross-validated accuracy of 86% (using NOESY experiments to build the model) and 84% (using CPMG experiments to build the model) by comparing HCV vs HBV serum profile, 98.7% (NOESY) and 98% (CPMG) by comparing HCV vs HC, and an overall cross-validated accuracy of 80% (NOESY) and 77.6% (CPMG) by comparing HBV subjects with healthy controls (HC).

Detailed analysis of metabolites

With the aim of identifying metabolite level variations related to changes introduced by the therapy, pairwise univariate analysis was applied on thirty-one assigned metabolites.

When the post-hoc Nemenyi test was applied on baseline (HCV), SVR12 and SVR24 serum samples, significant differences were evident (**Table 3**): 2-oxoglutarate levels progressively decrease from baseline (HCV) to SVR12 and SVR24; 3-hydroxybutyrate, formate and acetate levels are significantly higher in serum samples before the therapy; histidine, ornithine, phenylalanine, creatine and tyrosine levels are lower in SVR24 if compared with HCV and SVR12. These alterations are informative both to indicate the presence of HCV/pathological state biomarkers in infected serum samples (e.g. 2-oxoglutarate, 3-hydroxybutyrate, acetate and formate), and for the effect of the therapy on patients' metabolism.

Afterwards, with the aim of characterizing the direct action of HCV in metabolic pathways, serum levels of each metabolite in patients infected by HCV and HBV were compared between themselves (**Figure 3b**) and each of them with healthy controls (HC) as shown in **figure 3a and 3c** (**Supplementary table 2**).

Discussion

To the best of our knowledge, the present NMR metabolomics-based study is the first that examines the HCV serum profile of infected patients before and after viral clearance by DAAs, and the first one comparing a high number of naïve HCV patients to naïve HBV patients and to healthy controls.

DAAs provide an ideal model to study the effect of HCV on human metabolism since they are not biologically active molecules such as IFN.

This investigation clearly shows differences in the metabolomic profile of HCV patients before and after an effective DAA treatment. In this case, differences were detected both by MPLS analysis on the whole ¹H-NMR fingerprint, with an overall predictive accuracy of 70% in the CPMG experiment and by pairwise univariate analysis on metabolite levels.

This means that there is a clearly visible metabolic shift after therapy and viral eradication, which can be recognized in serum samples through ¹H-NMR spectroscopy. Since the CPMG model is the most effective in discriminating between the different groups, this suggests a major contribution of low molecular weight molecules in characterizing the within-subject changes introduced in the individual metabolic profile by the DAAs therapy.

Multivariate analysis on spectral buckets (predictive accuracy <50%) and univariate analysis on metabolite levels, did not highlight post-therapy significant differences among HCV subgroups

stratified on treatment duration and RBV administration. Therefore, DAAs and RBV have no *per se* detectable effects on metabolic profiles and do not represent confounding factors in the main analysis. Unluckily, we could not perform a reliable statistical analysis regarding the different viral genotypes since the genotypes were not equally represented. In fact, from a metabolic point of view, genotype 3 seems to be the most interesting one, being associated with severe steatosis, but also with an accelerated fibrosis progression rate and increased oncogenesis¹⁸⁸. Even if no significantly altered metabolite levels were detected between the two more numerous genotype subgroups (genotype 1b and 2), a further analysis to evaluate the genotype 3 contribution on metabolic fingerprints, with special focus on lipid metabolism, should be useful to clarify the genotype-related differences in liver disease progression.

The OPLS-DA analysis of naïve HCV patient sera stratified according to METAVIR score and grouped in F0-F2, F3 and F4 did not allow to discriminate between advanced and mild fibrosis. Conversely, Cano *et al.* proposed a diagnostic algorithm built using two sphingomyelins and two phosphatidylcholines measured in sera by mass spectrometry (MS), able to accurately classify rapid and slow fibroses after liver transplantation¹⁵⁵.

Nevertheless, in the present study, differences in a few metabolite levels among the three METAVIR subgroups were significant, suggesting that different concentrations of metabolites characterize different fibrosis scores. In agreement with other authors^{155,156}, tyrosine and formate increased going from no/mild fibrosis to severe fibrosis, and their role as potential fibrosis severity biomarkers should be investigated more deeply. Their increase may also be associated with energy supply modulation in the liver^{189,190}.

In addition, the differences in metabolite levels between higher and lower fibrosis scores, detected in patients before the therapy, decreased after SVR, confirming modulation of altered metabolites, most likely due to the liver damage regression induced by viral eradication.

The two OPLS-DA models showed a clear-cut discrimination between baseline HCV, naïve HBV and HC, with an overall cross-validated accuracy of 77% on NOESY and 74% CPMG models respectively. While the main metabolic differences recorded among the three metachronous HCV-samples, collected from the same patient, are due to low weight molecules (CPMG analysis), the comparison between HCV and HBV positive sera showed a good discrimination in the NOESY spectra, which reveals an important contribution of the high molecular weight molecules (i.e. those involved in the lipid metabolism, higher in HCV-related spectra). This information lead to two different deductions; i) In HCV patients, the antiviral therapy is able to positively change the metabolic pattern of small molecules but, in the short post-therapy follow-up it does not seem to affect the serum pattern of molecules involved in lipid metabolism. This is in agreement with steatosis maintenance after SVR in HCV patients, except for those infected by Gt3, which is often observed in the clinical practice¹⁹¹; indeed, HCV Gt 3 is more likely to cause steatosis than the other genotypes¹⁹², but Gt3-related steatosis is significantly reduced by SVR while there was no change in steatosis, after SVR, among patients infected by the other Gts¹⁹¹. As already mentioned, in our analysis, the number of Gt3 infected patients was too low to allow a statistical analysis, but our data, supported by clinical observations, suggest that the other genotypes are able to induce a metabolic perturbation in lipid metabolism that persists after viral eradication; ii) it is well known that while HBV infection is generally free from insulin resistance, steatosis and increased cardiovascular risk, HCV is associated to dysmetabolic syndrome, featuring steatosis, hypocholesterolemia and insulin resistance, determining a substantially increased cardiovascular risk¹⁹³. Our results support these findings, showing a completely different action of the two major hepatitis viruses on basal

metabolism, and the contribution of liver damage severity on the perturbation of metabolic pathways seems to have a lesser impact than the kind of the virus.

In addition, an analysis of specific metabolite levels was performed. 2-oxoglutarate levels progressively decrease from baseline HCV infected patients towards SVR12 and SVR24; 3-hydroxybutyrate, formate and acetate levels were significantly higher before therapy; histidine, ornithine, phenylalanine, creatine, and tyrosine levels are lower in SVR24 if compared with baseline and SVR12.

The OPLS-DA model built on NOESY spectra of HCV, HBV infected patients and healthy controls proved to be effective in predicting the specific infection and the healthy status of studied subjects (predictive accuracies: 85.6 % HCV, 57.7 HBV and 85.9% HC). Moreover, analysis on metabolites levels among these three groups reveals significantly higher levels (adjusted *P*-values < 0.05) of 2-oxoglutarate, fumarate, 3-hydroxybutyrate, acetate, ornithine, formate, lactate, pyruvate, glutamate, acetone, tyrosine, phenylalanine and choline in HCV infected patients and lower levels of glucose, glutamine and isoleucine if compared to HC; higher levels of 2-oxoglutarate, fumarate, 3-hydroxybutyrate, pyruvate, dimethylamine, lactate and phenylalanine and lower levels of isoleucine and leucine if compared to HBV infected patients.

Among these metabolites, especially interesting are the high levels of 2-oxoglutarate and 3-hydroxybutyrate in HCV baseline samples compared to HC and HBV samples. In fact, both metabolites were found to be altered in association to cardiovascular diseases and were therefore previously identified as potential biomarkers of cardiovascular risk. In more detail, high levels of 2-oxoglutarate were present in patients with heart failure^{194,195}, with chronic resistant hypertension¹⁹⁶ and could more generally be considered as biomarkers of cardiovascular risk¹⁹⁷. In addition, high levels of the ketone-body 3-hydroxybutyrate were also found associated to cardiovascular diseases^{11,198} and was identified as part of a panel of cardiovascular risk biomarkers by different analyses^{71,199}. 3-hydroxybutyrate was also identified as one of the first ketone-body to increase in the diabetic ketoacidosis and defined as diabetes biomarkers (even if an early predictive biomarker) in humans and in *in vivo* murine models^{200–203}. Interestingly, cardiovascular diseases and diabetes mellitus are two of the several extrahepatic manifestations associated to chronic HCV – but not HBV - infection^{204–207}. Therefore, it could be speculated that HCV has a direct role in altering biochemical pathways of basal metabolism, and this could help in explaining the association with some of the most frequent extrahepatic manifestations such as cardiovascular diseases and diabetes mellitus. In order to ascertain this hypothesis, further, dedicated studies using *in vitro* models of perturbations of such biochemical pathways should be performed; in addition, longer post-therapy follow-up studies evaluating metabolomic fingerprints of HCV SVR patients could confirm that the restoration of increased metabolites, such as 2-oxoglutarate and 3-hydroxybutyrate, is related to the decreased mortality rate for extrahepatic causes which is reported by large cohort studies^{153,208,209}.

Patients and Methods

Patients and serum collection

Sera were collected before therapy (baseline), at SVR12, and SVR24 time-points, from 67 HCV patients (age 63.4 ± 1.22) successfully treated with IFN-free DAA regimens; as controls, we tested serum samples from 50 naive subjects with chronic HBV infection (pathological controls; HBV, age 56.6 ± 2.3) and 43 healthy donors (healthy controls; HC, age 50.7 ± 18.9 , 58% females).

HCV-positive patients were recruited by the outpatient clinic of the Center for Systemic Manifestations of Hepatitis Viruses (MaSVE), University of Florence, Italy, while HBV subjects and HCs were recruited by the MaSVE Center and by the Infectious and Tropical Diseases Unit of the University of Florence, Florence, Italy.

Blood was drawn, in the absence of an anticoagulant, following routine phlebotomy methods and immediately after centrifugation the serum was placed at -80°C to be subsequently examined by NMR.

The main baseline demographic and virologic characteristics of the HCV and HBV samples were stratified in the three groups, as previously described (REF), and are reported in Table 1. No demographic information was available for HCs who were negative for HCV and HBV and other viral infections as well were not known to suffer of any significant illness relevant to the present study.

NMR sample preparation

Following standard operating procedures, fresh serum samples were stored at -80°C, and then thawed at room temperature at the time of analysis²¹⁰. A total of 350 µL of a phosphate sodium buffer (70 mM Na₂HPO₄; 20% (v/v) ²H₂O; 0.025% (v/v) NaN₃; 0.8% (w/v) sodium trimethylsilyl [2,2,3,3-²H₄]-propionate (TMSP) pH 7.4) was added to 350 µL of each serum sample, and the mixture was homogenized by vortexing for 30 s. A total of 600 µL of this mixture was transferred into a 5 mm NMR tube (Bruker BioSpin srl) for analysis.

NMR analysis

NMR measurements were performed at the CERM/CIRMMP center for Nuclear Magnetic Resonance, University of Florence, Italy.

One-dimensional (1D) ¹H NMR spectra for all samples were acquired using a Bruker 600 MHz spectrometer (Bruker BioSpin) operating at 600.13 MHz proton Larmor frequency and equipped with a 5mm PATXI ¹H-¹³C-¹⁵N and ²H-decoupling probe including a z axis gradient coil, an automatic tuning-matching (ATM) and an automatic and refrigerated sample changer (SampleJet). A BTO 2000 thermocouple served for temperature stabilization at the level of approximately 0.1 K on the sample. Before measurement, to equilibrate temperature at 310 K, samples were kept inside the NMR probe head for at least 5 minutes.

For each sample two one-dimensional proton NMR spectra were acquired with different pulse sequences, allowing the selective detection of different molecular components²¹¹:

(i) a standard nuclear Overhauser effect spectroscopy pulse sequence NOESY 1Dpresat (noesygppr1d.comp; Bruker BioSpin) pulse sequence, using 32 scans, 98304 data points, a spectral width of 30.0459 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s and a mixing time of 0.1 s, was applied to obtain a spectrum in which signals of metabolites and high molecular weight macromolecules are visible (lipids and lipoproteins).

(ii) a standard spin echo Carr-Purcell-Meiboom-Gill (CPMG)²¹²(cpmgpr1d.comp; Bruker BioSpin) pulse sequence applied to a standard 1D sequence, with 32 scans, 73728 data points, a spectral width of 12019 Hz and a relaxation delay of 4 s, was used for the selective observation of low molecular weight metabolites, suppressing signals arising from macromolecules. The acquisition conditions used in this work are perfectly in line with the current SOPs¹²⁵. The same conditions are also recommended by Bruker (NMR manufacturer) for metabolomic analysis²¹³.

Statistical analysis

All data analyses were performed using R, an open source software for the statistical analysis of data ²¹⁴. Multivariate data analysis was conducted on processed NMR spectra.

Principal component analysis (PCA) was used as the first exploratory analysis.

When the unsupervised approach was not effective in obtaining discrimination between groups of interest, Orthogonal Projections to Latent Structures-Discriminant Analysis (OPLS-DA), was chosen as a supervised technique. OPLS-DA is a multivariate projection method which is frequently applied for modelling spectroscopic data. This algorithm is able to separate “response-related” and “response-orthogonal” variations in data, providing benefits in terms of model interpretation compared to PCA or to PLS ²¹⁵. Multilevel partial least square (MPLS, R script developed in-house)²¹⁶ analysis was applied to study the within-subject changes introduced in the individual metabolic profile by DAAs therapy, considering serum samples collected during HCV infection (baseline), 12 weeks (SVR12) and 24 weeks (SVR24) after the sustained virological response. All the accuracies reported and the confusion matrix for different classifications were assessed by means of 100 cycles of a Monte Carlo cross-validation scheme (MCCV, R script developed in-house). In this case, 90% of the data was randomly chosen at each iteration as a training set to build the model. Then the remaining 10% was tested, and sensitivity, specificity and accuracy of the classification were assessed.

The spectral regions related to assigned metabolites were identified in the spectra by the use of matching routines of AMIX 3.8.4 (Bruker BioSpin) in combination with the BBIORFECODE database (Bruker BioSpin), public databases (e.g., HMDB) storing reference and published literature when available.

The concentrations of the various metabolites in the different spectra were calculated by spectral fitting and integration of the signal area using in-house developed Matlab® scripts (Takis et al. in preparation) and the concentrations were analyzed to determine the discriminating metabolites among the groups under study. The Wilcoxon test was chosen to infer differences between two groups of subjects ²¹⁷. The Kruskal-Wallis test followed by Dunn post-hoc analysis was chosen to infer differences among fibrosis groups (F0/F1/F2 vs F3 vs F4). The Friedman test followed by post-hoc Nemenyi analysis was chosen to ascertain pairwise differences among baseline (HCV), SVR12 and SVR24 groups. False discovery rate correction was applied using the Benjamini & Hochberg method (FDR), an adjusted *P*-value <0.05 was considered statistically significant ²¹⁸. Changes in metabolite levels are calculated as the log₂Fold-Change (FC) ratio of the normalized median intensities of the corresponding signals in the spectra of the two groups. The effect size, was calculated for pairwise comparisons using the Wilcoxon signed-rank test with the Rosenthal (1995) qualitative descriptors ²¹⁹ i.e. $r \leq 0.10$ “small”, $r \leq 0.30$ “medium”, $r \leq 0.50$ “large”, $r \leq 0.70$ “very large”, instead Cliff’s delta (Cd) formulation ²²⁰, was also calculated to aid the identification of the meaningful signals giving an estimation of the magnitude of the separation between the different groups. The magnitude is assessed using the thresholds provided in Romano et al. ²²¹, i.e., $|Cd| < 0.147$ “negligible”, $|Cd| < 0.33$ “small”, $|Cd| < 0.474$ “medium”, otherwise “large”.

Ethical guidelines

The study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki; all the anti-HCV treatments were open-label and provided by the National Health Care System, therefore, ethical approval was not required for their administration. All the subjects included

in the study signed an informed consent form. The local institutional review board (Comitato Etico Area Vasta Centro, AOU Careggi, Florence, Italy) approved the study (#10650_bio).

Tables and figures

Table 1. Main demographic, hepatovirological, and clinical baseline characteristics of HCV and HBV infected patients before treatment. Data are expressed as mean $\pm$ standard error; ^Based on liver stiffness assessed by FibroScan; * Alanine Aminotransferase (ALT) normal range: 12-65 U/L; ** Aspartate Aminotransferase normal range: 15-37 U/L; AVT: Antiviral Treatment; ° 3D: ombitasvir, paritaprevir+ritonavir, dasabuvir.

	HCV (n = 67)	HBV (n = 50)	<i>P-value</i>
Age (years)*	63.4 ($\pm$ 1.22)	56.6 ($\pm$	≤ 0.0159
Gender (male/females)	26/41	31/19	<0.015
Metavir score^:			
F0-F1	17	26	
F2	6	6	
F3	20	3	
F4	24	2	
ALT (U/L)*	100.2 (± 10)	47 ($\pm$ 4.7)	<0.0001
AST (U/L)**	76.7 ($\pm$ 15)	25.2 ($\pm$	<0.0001
HCV-RNA (IU/mL $\times 10^6$)	5.8 ($\pm$ 2.5)	/	
HBV-DNA (IU/mL $\times 10^6$)	/	14.4($\pm$ 6.1)	
HCV genotype:			
1°	6 (8.9%)		
1b	39 (58.2%)		
2	16(23.9%)		
3	4 (6%)		
4	2 (3%)		
Previous AVT response:			
Naïve	35	50	
Experienced	32	/	
DAA treatment:			
3D°	8		
3D+RBV	8		
Sofosbuvir+Daclatasvir	4		
Sofosbuvir+Daclatasvir+RBV	5		
Sofosbuvir+ Ledipasvir	7		
Sofosbuvir + Ledipasvir+RBV	7		
Sofosbuvir+Simeprevir	3		
Sofosbuvir+Simeprevir+RBV	5		
Sofosbuvir+RBV	18		

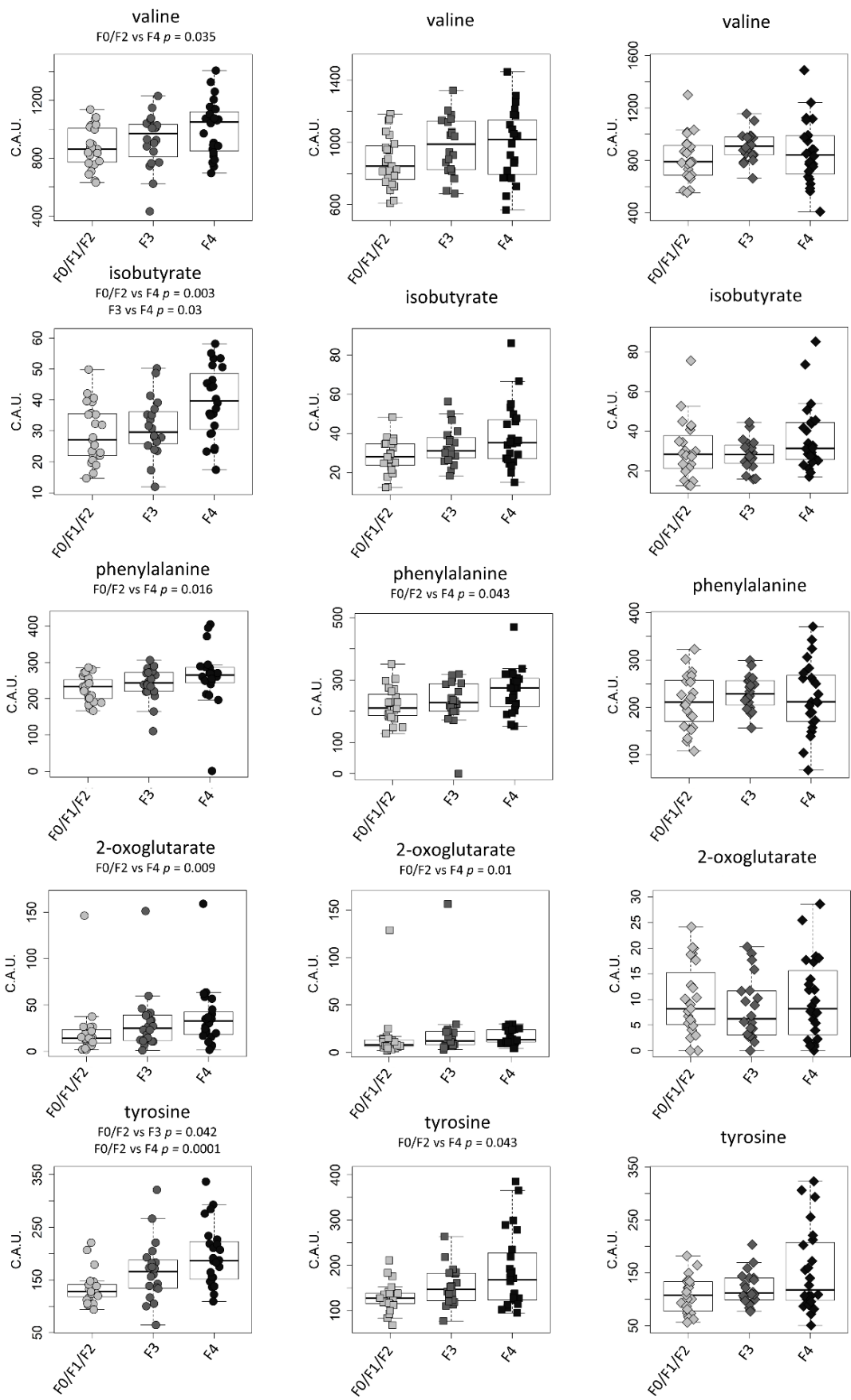
PegIFN+Telaprevir+RBV	1
PegIFN+Sofosbuvir+RBV	1

Table 2. MPLS analysis on 1D NOESY and CPMG spectra. Confusion matrix and prediction accuracy of Monte Carlo cross-validation are reported for both models.

Confusion matrix of NOESY spectra				Confusion matrix of CPMG spectra			
	<i>HCV</i>	<i>SVR1</i>	<i>SVR24</i>		<i>HCV</i>	<i>SVR1</i>	<i>SVR24</i>
<i>HCV</i>	79.7	$\hat{1}2.3$	8	<i>HCV</i>	82.1	$\hat{1}1$	6.9
<i>SVR12</i>	10.1	54.6	35.3	<i>SVR12</i>	11	61.6	27.4
<i>SVR24</i>	8.1	32.6	59.3	<i>SVR24</i>	5.1	27.4	67.4

Table 3. Pairwise metabolites analysis of baseline, SVR12 and SVR24. Adjusted *P*-values (FDR) are reported for each comparison. ↑ in baseline (HCV) vs SVR12 and SVR24, means higher metabolite levels in HCV; ↑ in SVR12 vs SVR24 means higher levels in SVR12. Values in bold are significantly altered (*P*-value < 0.05), effect size is also reported for each comparison (n=negligible, s= small, m= medium, l= large).

Metabolites	<i>P</i> -value (effect size)		
<i>2-oxoglutarate</i>	x	<i>HCV</i>	<i>SVR12</i>
	<i>SVR12</i>	0.0004↑ (m)	-
	<i>SVR24</i>	0.00004↑(m)	0.041↑(s)
<i>3-hydroxybutyrate</i>	x	<i>HCV</i>	<i>SVR12</i>
	<i>SVR12</i>	0.03↑(s)	-
	<i>SVR24</i>	0.03↑(s)	0.608(n)
<i>acetate</i>	x	<i>HCV</i>	<i>SVR12</i>
	<i>SVR12</i>	0.005↑(s)	-
	<i>SVR24</i>	0.02↑(s)	0.095(s) ↑
<i>creatine</i>	x	<i>HCV</i>	<i>SVR12</i>
	<i>SVR12</i>	0.663(n)	-
	<i>SVR24</i>	0.015↑(s)	0.025↑(s)
<i>formate</i>	x	<i>HCV</i>	<i>SVR12</i>
	<i>SVR12</i>	0.03↑(s)	-
	<i>SVR24</i>	0.0071↑(s)	0.996(n)
<i>histidine</i>	x	<i>HCV</i>	<i>SVR12</i>
	<i>SVR12</i>	0.769(n)	-
	<i>SVR24</i>	0.004↑(s)	0.033↑(s)
<i>ornithine</i>	x	<i>HCV</i>	<i>SVR12</i>
	<i>SVR12</i>	0.88(n)	-
	<i>SVR24</i>	0.001↑(s)	0.98(n)
<i>phenylalanine</i>	x	<i>HCV</i>	<i>SVR12</i>
	<i>SVR12</i>	0.306(n)	-
	<i>SVR24</i>	0.0002↑(s)	0.27(n)
<i>tyrosine</i>	x	<i>HCV</i>	<i>SVR12</i>
	<i>SVR12</i>	0.22(s)	-
	<i>SVR24</i>	0.00001↑(s)	0.02↑(s)
<i>lactate</i>	x	<i>HCV</i>	<i>SVR12</i>
	<i>SVR12</i>	0.42(n)	-
	<i>SVR24</i>	0.04↑(s)	0.14(n)



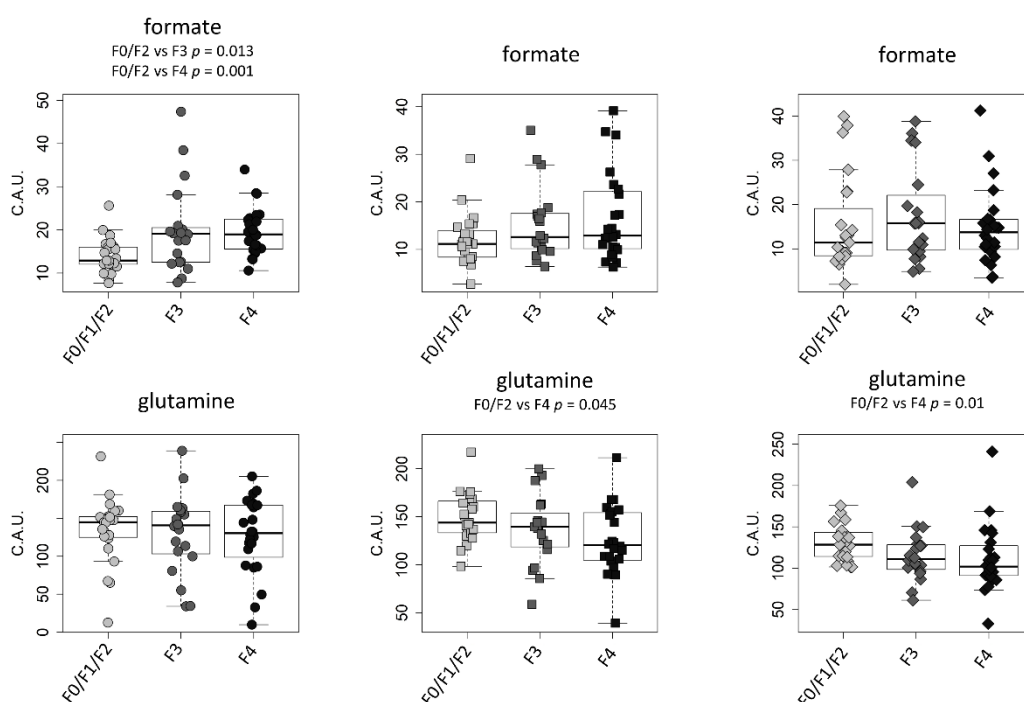


Figure 1. Concentration levels of metabolites in advanced and mild fibrosis. Box plots of the metabolites differentially concentrated in F0/F1/F2 (light grey, $n = 23$), F3 (dark grey, $n=20$) and F4 (black, $n=24$) for each time point: baseline (circles), SVR12 (squares) and SVR24 (rhombus). For each comparison, the adjusted P -value (Kruskal-Wallis test followed by Dunn post-hoc) is also reported.

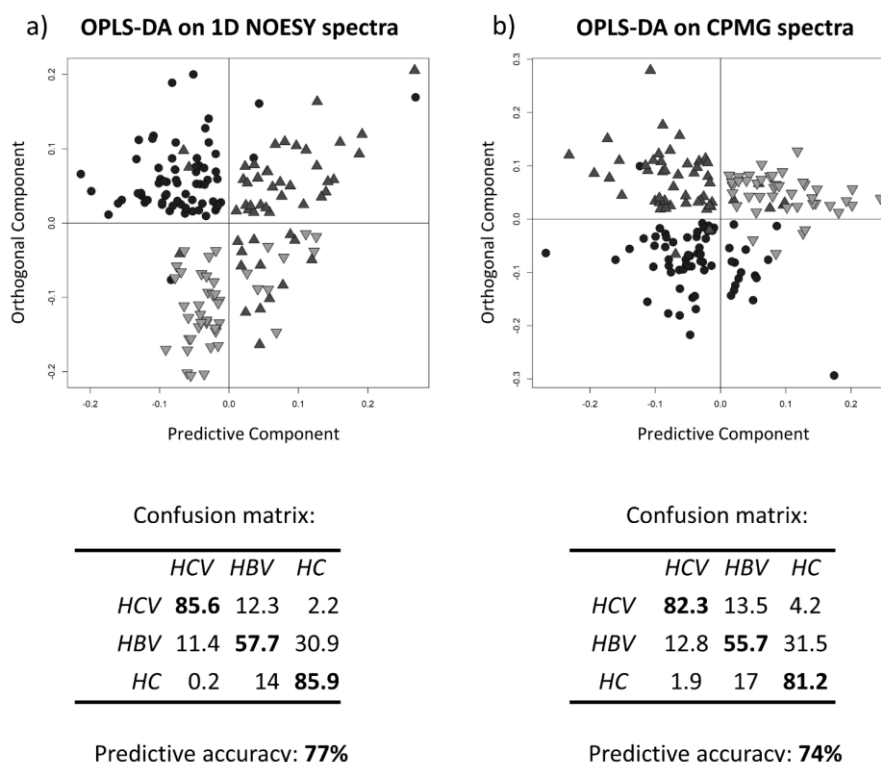


Figure 2. OPLS-DA on HCV, HBV and HC ^1H -NMR serum spectra. Score plots of OPLS-DA, discriminating HCV (dots, $n = 67$), HBV patients (triangles, $n = 50$) and HC (inverted triangles, $n = 43$); a) NOESY experiment; b) CPMG experiment. Confusion matrix and prediction accuracy of Monte Carlo cross-validation are also reported for both models.

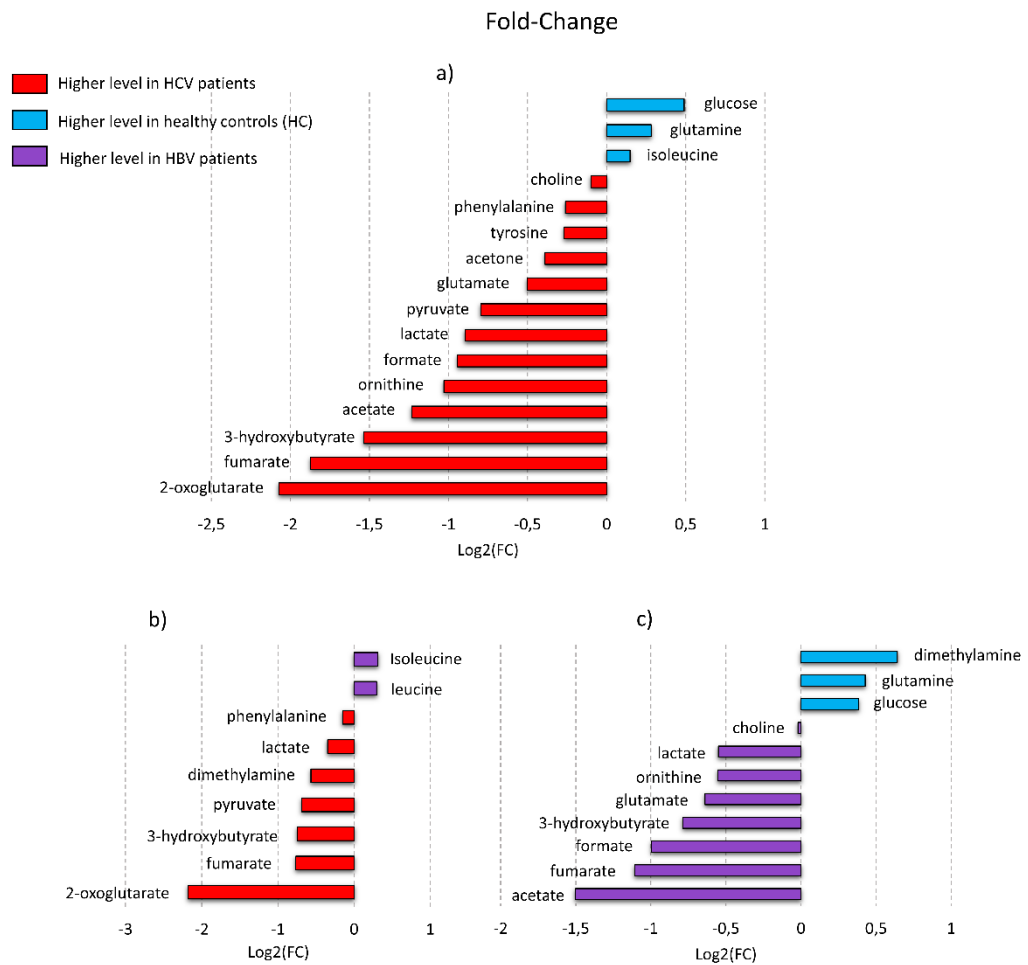
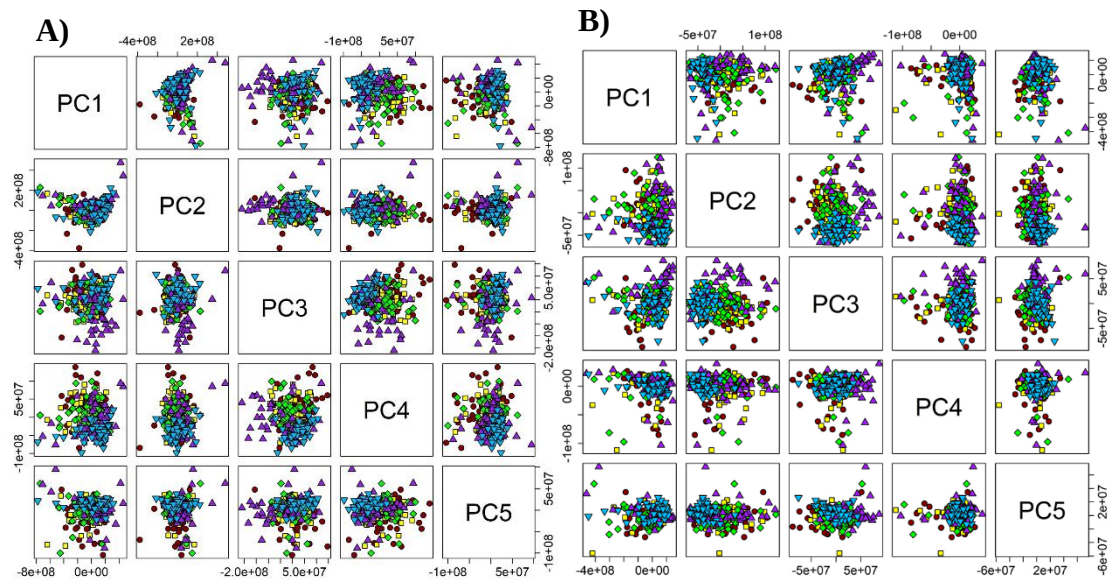
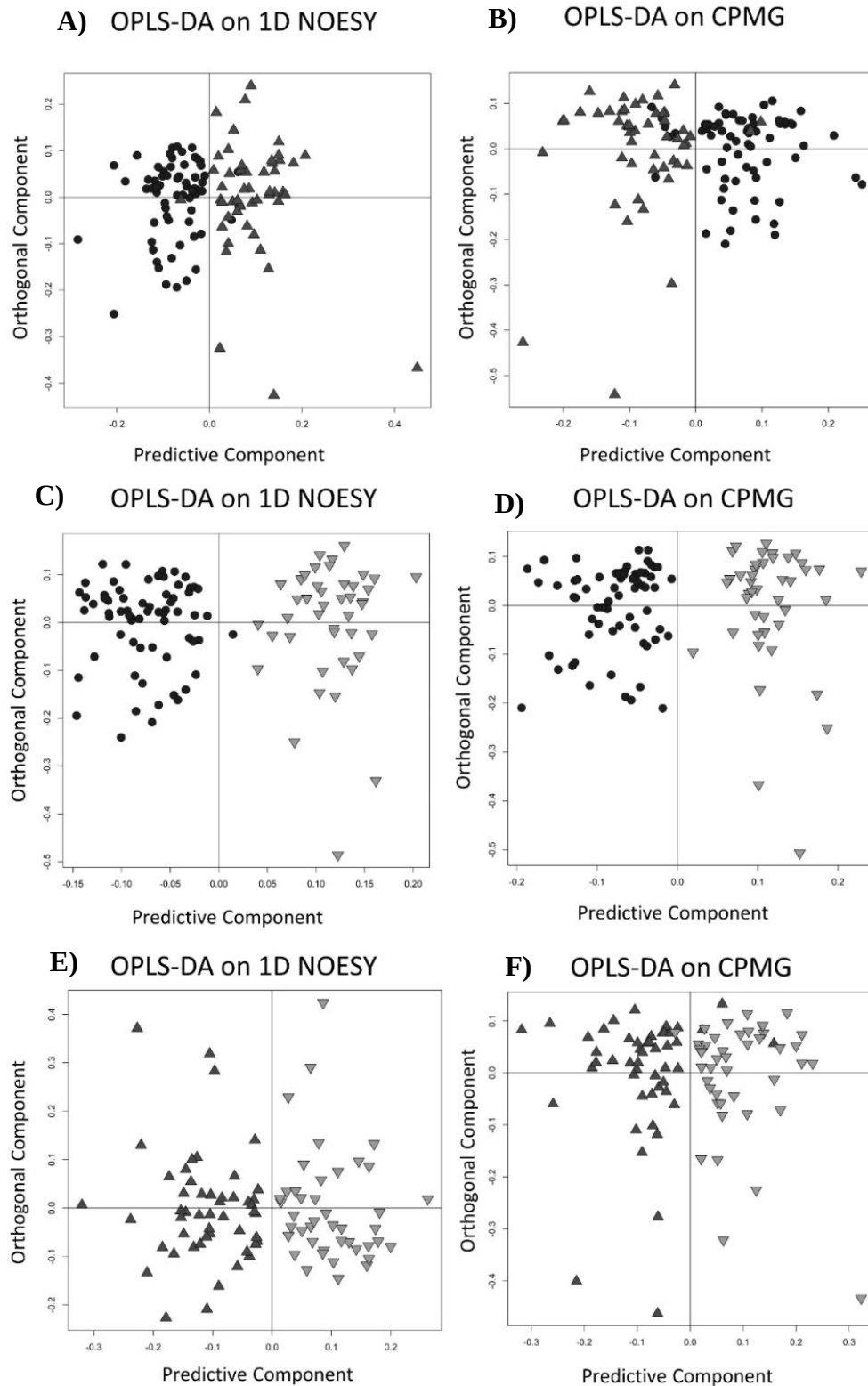


Figure 3. Boxplots of Fold-Change values (FC) for the significantly altered metabolites (P -value < 0.05) in HCV, HBV and HC serum samples. a) comparison between HCV ($n=67$) and healthy subjects (HC, $n = 43$), negative FC values mean higher metabolite levels in HCV serum samples; b) comparison between HCV and HBV patients ($n=50$), positive FC values mean higher metabolite levels in HBV sera; c) comparison between HBV and HC subjects, negative FC values mean higher metabolite levels in HBV serum samples.

Supplementary information



Supplementary Figure 1. PCA analysis on NOESY (A) and CPMG spectra(B): in the score plots are shown the first 5 principal components. Each plot is seen as a transformation in a two-dimensional space where the two components are the new coordinate system. Each dot represents a single NMR spectrum of serum samples. Red circles: baseline (HCV, $n = 67$) patients; yellow squares: SVR12 patients ($n = 67$); green rhombus: SVR24 patients, ($n = 67$); purple triangles: HBV patients ($n = 50$); sky-blue triangles: healthy donors ($n = 43$).



Supplementary Figure 2. Score plots of OPLS-DA, discriminating HCV (red dots, $n = 67$), HBV patients (purple triangles, $n = 50$) and HC (sky-blue triangles, $n = 43$) using both experiments (1D NOESY and GPMG). a,b) HCV vs HBV; c,d) HCV vs HC; e,f) HBV vs HC.

Supplementary Table 1. Confusion matrix and predictive accuracy(%) of Monte Carlo cross-validation are reported for both 1D NOESY and CPMG models.

NOESY			CPMG		
<i>Confusion matrix</i>			<i>Confusion matrix</i>		
	HCV	HBV		HCV	HBV
HCV	85.7	14.3	HCV	81.3	18.7
HBV	13.6	86.3	HBV	12	88
Predictive accuracy:	86%		Predictive accuracy:	84%	

<i>Confusion matrix</i>			<i>Confusion matrix</i>		
	HCV	HC		HCV	HC
HCV	97.9	2.1	HCV	96.8	3.2
HC	0	100	HC	0.2	99.8
Predictive accuracy:	98.7%		Predictive accuracy:	97.9%	

<i>Confusion matrix</i>			<i>Confusion matrix</i>		
	HBV	HC		HBV	HC
HBV	73.1	26.9	HBV	72.4	27.6
HC	11.6	88.4	HC	16.3	83.7
Predictive accuracy:	80.1%		Predictive accuracy:	77.6%	

Supplementary Table 2. Concentrations in arbitrary units [$\log(\text{mean} \pm \text{SD})$] of the metabolites assigned (MSI level 1) in HCV, HBV and HC serum samples. Significantly different *P*-values from each comparison are reported in bold. The magnitude of the effect size is assessed using the thresholds provided in (Romano 2006) and is reported as “n” as “negligible”, “s” as “small”, “m” as “medium” and “l” as “large”.

	HCV (67 SUBJECTS)			HBV (50 SUBJECTS)			HC (43 SUBJECTS)			HCV VS HC	HCV VS HBV	HBV VS HC
<i>METABOLITES</i>	<i>mean</i>	$\pm$	<i>SD</i>	<i>mean</i>	$\pm$	<i>SD</i>	<i>mean</i>	$\pm$	<i>SD</i>	<i>P-value</i>	<i>P-value</i>	<i>P-value</i>
Valine	9.36E+02	$\pm$	1.81E+02	1.00E+03	$\pm$	2.11E+02	9.80E+02	$\pm$	2.14E+02	4.81E-01(n)	1.46E-01(s)	6.21E-01(n)
Isoleucine	1.17E+02	$\pm$	3.35E+01	1.45E+02	$\pm$	4.74E+01	1.32E+02	$\pm$	3.77E+01	4.46E-02(s)	8.13E-03(m)	4.98E-01(n)
Leucine	2.23E+02	$\pm$	6.96E+01	2.70E+02	$\pm$	9.68E+01	2.31E+02	$\pm$	8.14E+01	8.58E-01(n)	1.61E-02(s)	9.07E-02(s)
Isobutyrate	3.34E+01	$\pm$	1.10E+01	3.02E+01	$\pm$	9.99E+00	2.96E+01	$\pm$	7.55E+00	1.55E-01(s)	1.95E-01(s)	9.42E-01(n)
2-Methylsuccinate	1.42E+01	$\pm$	1.22E+01	1.39E+01	$\pm$	1.43E+01	1.21E+01	$\pm$	1.20E+01	3.70E-01(n)	5.86E-01(s)	8.76E-01(n)
3-Hydroxybutyrate	3.45E+01	$\pm$	3.22E+01	2.09E+01	$\pm$	2.15E+01	1.58E+01	$\pm$	2.12E+01	2.39E-05(l)	2.24E-02(s)	3.08E-02(s)
Lactate	4.19E+03	$\pm$	1.20E+03	3.62E+03	$\pm$	1.77E+03	2.42E+03	$\pm$	8.00E+02	3.62E-11(l)	3.06E-02(s)	2.76E-03(m)
Alanine	1.41E+03	$\pm$	3.15E+02	1.47E+03	$\pm$	4.44E+02	1.39E+03	$\pm$	3.49E+02	9.71E-01(n)	8.21E-01(n)	8.74E-01(n)
Acetate	1.88E+02	$\pm$	1.75E+02	3.78E+02	$\pm$	3.89E+02	6.34E+01	$\pm$	3.11E+01	2.41E-10(l)	3.58E-01(n)	2.20E-06(l)
Acetone	2.77E+02	$\pm$	2.13E+02	2.16E+02	$\pm$	1.36E+02	1.92E+02	$\pm$	1.37E+02	1.67E-03(m)	1.19E-01(s)	3.97E-01(s)
Glutamate	1.95E+02	$\pm$	8.42E+01	2.40E+02	$\pm$	1.43E+02	1.36E+02	$\pm$	5.90E+01	6.54E-05(l)	5.20E-01(n)	2.05E-03(m)
Pyruvate	3.24E+02	$\pm$	1.54E+02	2.13E+02	$\pm$	1.50E+02	1.86E+02	$\pm$	8.00E+01	7.39E-07(l)	1.97E-05(l)	8.76E-01(n)
Glutamine	1.30E+02	$\pm$	4.77E+01	9.75E+01	$\pm$	6.90E+01	1.70E+02	$\pm$	3.80E+01	6.22E-05(l)	7.53E-02(s)	4.64E-06(l)
Citrate	9.42E+01	$\pm$	3.50E+01	8.17E+01	$\pm$	3.46E+01	9.26E+01	$\pm$	2.66E+01	6.35E-01(n)	1.51E-01(s)	6.21E-01(n)
Phenylalanine	2.44E+02	$\pm$	5.72E+01	2.21E+02	$\pm$	5.77E+01	2.14E+02	$\pm$	5.14E+01	2.23E-03(m)	1.40E-02(m)	7.33E-01(n)
Dimethylamine	3.29E+01	$\pm$	2.17E+01	2.19E+01	$\pm$	1.57E+01	5.20E+01	$\pm$	7.45E+01	8.00E-01(n)	2.08E-02(s)	1.27E-02(m)
Choline	3.25E+02	$\pm$	2.37E+02	5.49E+02	$\pm$	7.08E+02	4.63E+01	$\pm$	1.47E+02	1.39E-11(l)	8.52E-01(n)	2.52E-07(l)
Sarcosine	4.82E+01	$\pm$	1.25E+02	8.09E+01	$\pm$	9.54E+01	7.41E+01	$\pm$	1.65E+02	9.21E-01(n)	5.07E-02(s)	7.25E-02(s)
2-Oxoglutarate	2.94E+01	$\pm$	3.11E+01	1.88E+01	$\pm$	4.30E+01	6.81E+00	$\pm$	5.10E+00	1.52E-09(l)	1.38E-05(l)	9.42E-01(n)
Ornithine	6.86E+01	$\pm$	3.75E+01	6.33E+01	$\pm$	4.76E+01	3.28E+01	$\pm$	1.70E+01	1.27E-08(l)	1.34E-01(s)	5.44E-03(m)
Proline	5.09E+01	$\pm$	1.45E+01	5.91E+01	$\pm$	2.03E+01	5.31E+01	$\pm$	2.11E+01	9.71E-01(n)	8.28E-02(s)	2.52E-01(s)
Glycine	5.84E+02	$\pm$	1.51E+02	6.28E+02	$\pm$	1.35E+02	6.25E+02	$\pm$	1.19E+02	9.85E-02(s)	9.16E-02(s)	9.33E-01(n)
Glycerol	2.30E+02	$\pm$	8.21E+01	2.42E+02	$\pm$	9.95E+01	2.34E+02	$\pm$	1.44E+02	8.71E-01(n)	5.82E-01(n)	6.21E-01(n)
Creatine	8.23E+01	$\pm$	5.12E+01	1.12E+02	$\pm$	7.44E+01	1.01E+02	$\pm$	6.70E+01	2.32E-01(s)	9.16E-02(s)	7.07E-01(n)

Tyrosine	1.67E+02	±	5.57E+01	1.43E+02	±	4.05E+01	1.30E+02	±	3.72E+01	8.23E-04(m)	8.28E-02(s)	2.72E-01(s)
Creatinine	1.72E+02	±	5.20E+01	2.31E+02	±	2.75E+02	1.90E+02	±	4.88E+01	1.15E-01(s)	8.28E-02(s)	9.42E-01(n)
Glucose	2.43E+03	±	1.14E+03	2.42E+03	±	1.07E+03	2.86E+03	±	4.81E+02	1.22E-05(l)	5.86E-01(n)	2.55E-04(l)
Histidine	1.25E+02	±	2.31E+01	1.51E+02	±	1.43E+02	1.29E+02	±	2.63E+01	4.01E-01(n)	2.93E-01(n)	8.64E-01(n)
Formate	1.78E+01	±	7.20E+00	2.15E+01	±	1.28E+01	8.91E+00	±	2.87E+00	2.11E-12(l)	5.86E-01(n)	2.28E-07(l)
Fumarate	6.34E+00	±	2.87E+00	4.07E+00	±	2.55E+00	1.73E+00	±	1.08E+00	3.64E-13(l)	1.66E-04(m)	2.99E-06(l)
Dimethyl Sulfone	1.07E+02	±	2.51E+02	4.91E+01	±	4.14E+01	5.55E+01	±	2.37E+01	9.71E-01(n)	5.07E-02(s)	7.25E-02(s)

Supplementary Table 3. Concentrations in arbitrary units (mean ± SD) of the metabolites assigned (MSI level 1) in HCV infected patients (Baseline) with different fibrosis levels. Significantly different *P*-values from the comparisons are also reported. The magnitude of the effect size is assessed using the thresholds provided in (Romano 2006) and is reported as “n” as “negligible”, “s” as “small”, “m” as “medium” and “l” as “large”.

	F0/F1/F2 (23 SUBJECTS)			F3 (20 SUBJECTS)			F4 (24 SUBJECTS)			
<i>METABOLITES</i>	<i>mean</i>	±	<i>Sd</i>	<i>mean</i>	±	<i>sd</i>	<i>mean</i>	±	<i>sd</i>	<i>P-values</i>
Valine	872.9914	±	143.0722	923.4722	±	187.1931	1006.024	±	190.0334	F0/F2 vs F4 = 0.035(m) F0/F2 vs F4 = 0.003(l) F3 vs F4 = 0.027(s)
Isoleucine	110.2645	±	19.31842	111.8576	±	22.85543	128.9745	±	47.12133	
Leucine	200.7073	±	44.30786	245.6707	±	99.42027	226.0607	±	53.89349	
Isobutyrate	28.92624	±	9.42736	31.1681	±	9.391529	39.54155	±	11.32954	
2-Methylsuccinate	12.88537	±	11.60661	14.58815	±	12.79503	15.01337	±	12.72507	
3-Hydroxybutyrate	34.59433	±	39.54477	27.37601	±	16.23305	40.35884	±	34.34469	
Lactate	4192.145	±	1330.22	4040.164	±	1028.309	4323.169	±	1244.042	
Alanine	1357.024	±	321.4859	1391.873	±	357.5795	1462.398	±	274.0975	
Acetate	195.8567	±	204.7201	178.3808	±	162.089	188.6576	±	160.5097	
Acetone	284.5237	±	204.513	247.2592	±	113.2241	293.6201	±	281.1121	

Glutamate	190.328	±	80.25048	179.9669	±	85.29092	211.5896	±	87.43645	F0/F2 vs F4 = 0.016(m)
Pyruvate	327.7061	±	126.1987	295.7834	±	118.7823	343.4813	±	200.7553	
Glutamine	134.1233	±	43.94446	129.7077	±	51.69462	126.7755	±	49.41918	
Citrate	89.5828	±	33.52876	92.89142	±	26.01442	99.79614	±	42.63248	
Phenylalanine	227.8844	±	35.16247	241.4996	±	45.19537	261.9931	±	76.80927	
Dimethylamine	30.78331	±	18.88965	33.22431	±	24.29584	34.73956	±	22.79983	F0/F2 vs F4 = 0.009(m)
Choline	310.8827	±	206.8082	356.9217	±	332.3066	312.6665	±	167.7154	
Sarcosine	27.86382	±	53.41223	29.34722	±	28.45348	83.39211	±	198.9539	
2-Oxoglutarate	20.62694	±	28.94839	31.5553	±	31.95039	36.05691	±	31.67711	
Ornithine	61.21947	±	31.49703	58.22912	±	32.29505	84.20022	±	42.73492	
Proline	49.64702	±	15.25496	46.73222	±	12.31658	55.66286	±	14.62757	F0/F2 vs F3 = 0.042(m) F0/F2 vs F4 = 0.0001(l)
Glycine	652.331	±	201.0095	556.2076	±	110.009	542.8855	±	97.90495	
Glycerol	200.0959	±	59.66613	256.951	±	112.5743	235.3082	±	62.6684	
Creatine	95.98974	±	43.17941	70.11794	±	48.1848	79.28722	±	59.09022	
Tyrosine	135.6254	±	30.91254	167.2505	±	58.11155	196.0381	±	57.72867	
Creatinine	174.6617	±	62.66421	180.5294	±	43.83221	161.2994	±	47.20629	F0/F2 vs F3 = 0.013 (m) F0/F2 vs F4 = 0.001(l)
Glucose	2365.527	±	1124.358	2471.909	±	1177.919	2448.565	±	1170.817	
Histidine	125.9417	±	24.67217	119.8494	±	18.24007	127.6986	±	25.24045	
Formate	14.03734	±	4.017313	19.93502	±	9.969093	19.60409	±	5.468339	
Fumarate	5.505981	±	2.52722	6.333263	±	3.267491	7.135679	±	2.701265	

Dimethyl Sulfone	156.0876	±	354.3236	113.2424	±	256.8157	54.24472	±	30.75187
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Supplementary Table 4. Concentrations in arbitrary units (mean $\pm$ SD) of the metabolites assigned (MSI level 1) in SVR12 patients with different fibrosis levels. Significantly different *P*-values from the comparisons are also reported. The magnitude of the effect size is assessed using the thresholds provided in (Romano 2006) and is reported as “n” as “negligible”, “s” as “small”, “m” as “medium” and “l” as “large”.

	F0/F1/F2 (23 SUBJECTS)			F3 (20 SUBJECTS)			F4 (24 SUBJECTS)			
<i>METABOLITES</i>	<i>mean</i>	$\pm$	<i>sd</i>	<i>mean</i>	$\pm$	<i>sd</i>	<i>mean</i>	$\pm$	<i>sd</i>	<i>P-values</i>
Valine	878.5802	$\pm$	164.8128	978.1091	$\pm$	183.3603	982.1636	$\pm$	220.9057	
Isoleucine	104.983	$\pm$	30.7078	119.1752	$\pm$	26.12661	119.0149	$\pm$	35.30863	
Leucine	206.8827	$\pm$	54.06354	229.4831	$\pm$	62.25033	229.3056	$\pm$	84.19148	
Isobutyrate	28.47156	$\pm$	8.655182	33.3635	$\pm$	9.604465	38.32618	$\pm$	15.94306	
2-Methylsuccinate	14.16253	$\pm$	10.15293	15.3847	$\pm$	13.15978	15.65041	$\pm$	16.00054	
3-Hydroxybutyrate	17.18685	$\pm$	14.59583	36.56811	$\pm$	38.34298	19.81219	$\pm$	14.34052	
Lactate	3548.522	$\pm$	1168.12	4358.056	$\pm$	1657.846	4272.276	$\pm$	1124.701	
Alanine	1330.031	$\pm$	266.4937	1401.785	$\pm$	395.6161	1487.159	$\pm$	304.2056	
Acetate	90.60917	$\pm$	39.42667	122.1464	$\pm$	93.28265	118.9622	$\pm$	107.4117	
Acetone	205.4372	$\pm$	91.46179	236.2751	$\pm$	121.9978	266.6749	$\pm$	186.8173	
Glutamate	160.6675	$\pm$	47.72061	179.4132	$\pm$	76.16655	198.8988	$\pm$	79.22456	
Pyruvate	335.9581	$\pm$	109.8447	329.1377	$\pm$	200.3567	352.4094	$\pm$	116.4868	
Glutamine	148.8389	$\pm$	25.55513	136.4283	$\pm$	35.71063	125.9396	$\pm$	35.889	F0/F2 vs F4 = 0.045 (m)
Citrate	102.4793	$\pm$	27.17361	82.59975	$\pm$	39.92617	93.14139	$\pm$	40.03672	
Phenylalanine	221.0171	$\pm$	54.35739	230.2129	$\pm$	71.01162	265.3839	$\pm$	69.20688	F0/F2 vs F4 = 0.043 (m)
Dimethylamine	43.25279	$\pm$	58.58497	31.89837	$\pm$	23.68526	39.50236	$\pm$	27.82123	

Choline	213.7519	±	139.3133	270.4795	±	163.4079	293.3026	±	189.2334	
Sarcosine	18.77254	±	22.27758	58.04898	±	90.9906	104.0718	±	232.2182	
2-Oxoglutarate	14.43368	±	25.49835	21.22785	±	32.74073	16.33092	±	7.656204	F0/F2 vs F4 = 0.01 (m)
Ornithine	50.46928	±	23.95702	61.22086	±	34.84361	71.62092	±	41.2111	
Proline	50.40539	±	16.80852	55.72947	±	16.19476	60.80946	±	29.305	
Glycine	669.1007	±	113.7242	606.4909	±	119.1829	592.9336	±	145.5215	
Glycerol	246.909	±	207.7596	212.0495	±	58.90802	219.0967	±	77.10528	
Creatine	90.11979	±	55.56252	68.48192	±	35.92777	83.00246	±	71.26684	
Tyrosine	129.664	±	31.5404	153.1314	±	42.15222	184.9529	±	83.53053	F0/F2 vs F4 = 0.043 (m)
Creatinine	175.5075	±	67.02307	186.1984	±	34.17521	161.9486	±	62.40044	
Glucose	2501.656	±	1233.932	2364.464	±	767.7128	2509.339	±	956.2962	
Histidine	120.0146	±	25.81705	128.8307	±	22.84923	132.3853	±	28.21402	
Formate	12.04921	±	5.299339	15.41144	±	7.52849	16.50948	±	9.271967	
Fumarate	4.034736	±	2.524734	5.888089	±	3.240363	6.007497	±	2.949352	
Dimethyl Sulfone	170.1642	±	576.6509	62.61559	±	31.3587	59.33534	±	63.91941	

Supplementary Table 5. Concentrations in arbitrary units (mean $\pm$ SD) of the metabolites assigned (MSI level 1) in SVR24 patients with different fibrosis levels. Significantly different P-values from the comparisons are also reported. The magnitude of the effect size is assessed using the thresholds provided in (Romano 2006) and is reported as “n” as “negligible”, “s” as “small”, “m” as “medium” and “l” as “large”.

	F0/F1/F2 (23 SUBJECTS)			F3 (20 SUBJECTS)			F4 (24 SUBJECTS)			
<i>METABOLITES</i>	<i>Mean</i>	$\pm$	<i>sd</i>	<i>mean</i>	$\pm$	<i>sd</i>	<i>mean</i>	$\pm$	<i>sd</i>	<i>P-values</i>
Valine	816.1322	$\pm$	175.2088	910.9055	$\pm$	113.7559	864.3518	$\pm$	241.7014	
Isoleucine	108.3865	$\pm$	38.68138	114.5766	$\pm$	17.27441	110.8553	$\pm$	34.13063	
Leucine	206.6237	$\pm$	76.45991	236.5747	$\pm$	55.28458	228.6679	$\pm$	87.43291	
Isobutyrate	30.6914	$\pm$	14.60333	28.55075	$\pm$	7.728365	36.54976	$\pm$	16.60386	
2-Methylsuccinate	13.50245	$\pm$	14.1309	12.4181	$\pm$	8.682088	12.16944	$\pm$	10.9797	
3-Hydroxybutyrate	21.6248	$\pm$	23.30183	14.29376	$\pm$	10.53473	37.81965	$\pm$	36.98521	
Lactate	3625.53	$\pm$	1503.181	4023.024	$\pm$	1092.116	3518.213	$\pm$	1718.244	
Alanine	1178.56	$\pm$	274.5454	1297.342	$\pm$	175.3452	1284.896	$\pm$	357.863	
Acetate	132.4665	$\pm$	101.229	168.8207	$\pm$	126.7609	123.8931	$\pm$	78.25261	
Acetone	259.8753	$\pm$	149.3608	253.2759	$\pm$	110.8628	318.1729	$\pm$	239.6095	
Glutamate	140.4988	$\pm$	53.49719	192.9817	$\pm$	72.45277	156.2723	$\pm$	104.9528	
Pyruvate	305.4263	$\pm$	101.7993	306.5109	$\pm$	100.7126	299.2185	$\pm$	109.2571	
Glutamine	130.4382	$\pm$	21.46937	115.3625	$\pm$	31.22951	111.3299	$\pm$	39.5484	F0/F2 vs F4 = 0.01(s)
Citrate	86.40278	$\pm$	32.22326	68.43276	$\pm$	40.20644	93.34926	$\pm$	49.00735	
Methionine	41.38683	$\pm$	15.50586	49.07082	$\pm$	16.18497	41.33306	$\pm$	15.8318	
Phenylalanine	213.1676	$\pm$	57.20165	231.2008	$\pm$	35.69953	222.1332	$\pm$	74.65876	
Dimethylamine	49.7225	$\pm$	70.77825	40.8382	$\pm$	57.63194	46.49572	$\pm$	63.99615	
Choline	239.8566	$\pm$	165.2892	270.2267	$\pm$	165.1887	153.1702	$\pm$	184.8209	

Sarcosine	74.05955	±	179.241	85.23142	±	180.6324	171.3496	±	358.8475
2-Oxoglutarate	9.918583	±	6.988234	8.224293	±	6.104633	9.971286	±	7.877852
Ornithine	74.49051	±	46.1415	62.27972	±	35.83145	51.99081	±	31.99896
Proline	44.28869	±	15.5671	49.98553	±	26.20892	51.12165	±	26.02578
Glycine	615.0294	±	132.5945	573.5426	±	125.7025	541.423	±	146.1363
Glycerol	216.0578	±	60.73088	221.7939	±	101.082	240.3614	±	154.2644
Creatine	59.85857	±	37.7463	75.79023	±	66.30669	52.31979	±	44.55576
Tyrosine	108.3306	±	34.32403	120.6797	±	32.32322	153.2354	±	78.51438
Creatinine	156.187	±	42.29091	174.3305	±	38.83627	164.1034	±	73.35583
Glucose	2014.378	±	486.6631	2087.699	±	389.9089	2610.645	±	1368.026
Histidine	107.9622	±	26.79335	115.4834	±	21.79021	106.4334	±	19.4607
Formate	15.38128	±	10.70641	17.47975	±	10.60822	14.85762	±	8.666453
Fumarate	4.576927	±	2.806694	5.766869	±	2.588213	5.637881	±	3.11872
Dimethyl Sulfone	46.17509	±	23.75042	160.1803	±	445.0755	51.60176	±	23.30473

4.1.2 NMR-based metabolomics in Parkinson's Disease: preliminary results of PROPAG-AGEING project

In Preparation

Candidate's contributions: acquisition of NMR data, statistical analysis and interpretation of data and writing the draft.

Introduction

ROPAG-AGEING is an H2020 EU-funded project that aims to identify new molecular signatures for early diagnosis of PD. The main question to answer in this project is why the advanced age is the most important risk factor in developing PD.

Recent discoveries suggest a continuum between healthy aging and neurodegenerative motor disorders, so the project assumes that PD is strictly bound to the basics molecular and cellular mechanisms of aging. The purpose of PROPAG-AGEING is to identify the combination of these cellular and molecular alterations capable of shifting the phenotype of elderly subjects from a physiological decline into PD. This will eventually allow the identification of markers for early PD diagnosis (before motor symptoms occur) using blood and other body fluids.

Various hypothesis suggest that PD is an accelerated/propagated type of ageing which affects more markedly certain types of neurons in brain. Indeed, healthy ageing is characterized by a progressive impairment of motor and cognitive abilities, and elderlies without PD signs, also show neuronal degeneration. Some of the shared features among ageing and PD are the accumulation of senescent cells, inflammation and a propagation phenomenon. Inflammation most of all seems to have a central role in pathogenesis; senescent cells are indeed capable to secrete cytokines and other inflammatory compounds, which contribute to a chronic inflammation status called “inflammaging”. Moreover this inflammaging status can be transmitted both locally and systematically as has been demonstrated by using the so called heterochronic parabionts. Two mice of different ages were conjoined surgically: in this way they were capable to exchange blood and consequently, all contained molecules. The measured effect was a rejuvenating effect on the old mouse thanks to blood molecules of the young one, and vice versa an aging effect on the young mouse¹⁶⁰. Inflammation creates a vicious circle inducing cell senescence which in turn creates a large amount of inflammatory compounds able to propagate locally and systemically. Why inflammation is so crucial for PD development still remains an unresolved question. We currently know that it is able to induce accelerate aging (which can propagate) by ROS-mediated worsening of telomeric dysfunctions, by damage to DNA and by mitochondrial dysfunction: all these factors could lead to cell death. This project will examine samples (blood and urine) of a large cohort (~700) de novo PD patients collected in different European countries; clinical and lifestyle data are also available in order to perform a complete analysis of risk factors. PD patients' samples will be compared to healthy controls represented by elderly and centenarian individuals. Besides, to highlight differences between

healthy ageing and PD, will be used a cohort of twin pairs discordant for PD: this will allow the identification of genetic-independent risk factors.

PROPAG-AGEING will consist in two phases: the discovery phase, in which samples will be analyzed using different omics sciences, and the validation phase, in which all the informative molecules emerged from the first phase will be integrated with environmental and clinical data. PROPAG-AGEING has also the objective to overcome fragmentation of data adopting a system biology approach to the problem: a unified dataset including all the omics data will be created. The present results are part of the discovering phase of PROPAG-AGEING and covers the NMR metabolomics characterization of serum and urine samples from an initial subset of 74 *de novo* PD (not dopaminergic treatment) patients and 61 healthy subjects of a German cohort (DK).

Materials and methods

NMR sample preparation

Serum samples were stored at -80°C and thawed at room temperature. A total of 350 µL of a phosphate sodium buffer (70 mM Na₂HPO₄; 20% (v/v) ²H₂O; 0.025% (v/v) NaN₃; 0.8% (w/v) sodium trimethylsilyl [2,2,3,3-²H₄] propionate (TMSP) pH 7.4) was added to 350 µL of each serum sample, and the mixture was homogenized by vortexing for 30 s.

Frozen urine samples were thawed at room temperature and shaken before use. A 630 µL aliquot of each urine sample was centrifuged at 14 000g for 5 min, and 540 µL of the supernatant was added to 60 µL of buffer solution (1.5 M KH₂PO₄/d₂O, pH 7.4; 2mM NaN₃; 0.1% TSP). 600 µL of each of these urine and serum mixtures were transferred into 5 mm NMR tubes (Bruker BioSpin srl) for the analysis.

NMR analysis

One-dimensional ¹H-NMR spectra for all samples were acquired using a Bruker 600 MHz spectrometer (Bruker BioSpin srl; Rheinstetten, Germany) operating at 600.13 MHz proton Larmor frequency and equipped with a 5 mm PATXI ¹H-¹³C-¹⁵N and ²H-decoupling probe including a $\tilde{z}$ axis gradient coil, an automatic tuning-matching (ATM) and an automatic and refrigerated sample changer (SampleJet, Bruker BioSpin srl; Rheinstetten, Germany).

A BTO 2000 thermocouple served for temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 3 minutes inside the NMR probehead, for temperature equilibration (310 K for serum samples and 300 K for urine samples).

According to common practices^{210,211} for each serum samples three one-dimensional ¹H NMR spectra were acquired with different pulse sequences allowing the selective detection of different molecular components:

- (i) a standard nuclear Overhauser effect spectroscopy pulse sequence NOESY 1D presat (noesygppr1d.comp; Bruker BioSpin) pulse sequence, using 32 scans, 98304 data points, a spectral width of 18028 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s and a mixing time of 0.1 s, was applied to obtain a spectrum in which both signals of metabolites and high molecular weight macromolecules (lipids and lipoproteins) are visible.
- (ii) a standard spin echo Carr-Purcell-Meiboom-Gill (CPMG)²²²(cpmgpr1d.comp; Bruker BioSpin) pulse sequence applied to a standard 1D sequence, with 32 scans, 73728 data points, a spectral width of 12019 Hz and a relaxation delay of 4 s, was used the selective observation of low molecular weight metabolites, suppressing signals arising from macromolecules.

(iii) a standard diffusion-edited²²³ (ledbgppr2s1d.comp; Bruker BioSpin) pulse sequence, using 32 scans, 98304 data points, a spectral width of 18028 Hz and a relaxation delay of 4 s, was applied to suppress metabolites signals.

For urine samples, one-dimensional ¹H NMR spectra with a standard nuclear Overhauser effect spectroscopy pulse sequence (NOESY 1D presat; noesygppr1d.comp; Bruker BioSpin) and with water peak suppression, were acquired²²⁴. The acquisition parameters for urine samples were: 32 scans, 65536 data points, spectral width of 12019 Hz, acquisition time of 2.7s, relaxation delay of 4s and mixing time of 0.01s.

Spectral Processing

Free induction decays were multiplied by an exponential function equivalent to a 1.0 Hz line-broadening factor before applying Fourier transform.

Transformed spectra were automatically corrected for phase and baseline distortions and calibrated (serum spectra are calibrated to anomeric glucose doublet at 5.24 ppm, and urine spectra to TMSP singlet at 0 ppm) using TopSpin 3.2 (Bruker Biospin srl).

Each 1D spectrum in the range between 0.2 and 10.00 ppm was segmented into 0.02 ppm chemical shift bins and the corresponding spectral areas were integrated using AMIX software (version 3.8.4, Bruker BioSpin). Binning is a means to reduce numbers of total variables and to compensate for small shifts in the signals, making the analysis more robust and reproducible²²⁵. Regions containing residual water signal (from 4.5 ppm to 5.20 ppm in serum spectra, and between 4.7 and 4.9 ppm in urine spectra) were removed. Binned urine spectra were normalized to be comparable. Normalization is a preprocessing method, which accounts for different dilutions of samples by scaling the spectra to the same virtual overall concentration. In our case, urine spectra were normalized using Probabilistic Quotient Normalization (PQN)²²⁶ algorithm and total integral intensity (the total spectral area)¹³⁸.

Statistical analysis

All data analyses were performed using R, an open source software for the statistical analysis of data²¹⁴. Multivariate data analysis was conducted on processed NMR.

Principal component analysis (PCA) was used as a first exploratory analysis. Since the unsupervised approach was not effective in obtaining discrimination between DKK (healthy subjects) and DKP (group of Parkinson's disease patients), Orthogonal Projections to Latent Structures (OPLS), was chosen as supervised technique. OPLS is a multivariate projection method which is frequently applied for modelling spectroscopic data. This algorithm is able to separate "response-related" and "response-orthogonal" variation in data, providing benefits in terms of model interpretation compared to PCA or to PLS²²⁷. All the accuracies reported and the confusion matrix for different classifications were assessed by means of 100 cycles of a Monte Carlo cross-validation scheme (MCCV, R script in-house developed). In this case, 90% of the data were randomly chosen at each iteration as a training set to build the model. Then the remaining 10% was tested and sensitivity, specificity and accuracy for the classification were assessed. The procedure was repeated 100 times to derive an average discrimination accuracy for each group of subjects.

The spectral regions related to assigned metabolites were assigned in the spectra by using matching routines of AMIX 3.8.4 (Bruker BioSpin) in combination with the BBIORFECODE database (Bruker BioSpin), public databases (e.g., HMDB) storing reference and published literature when available.

The concentrations of the various metabolites in the different spectra were calculated by spectral fitting and integration of the signal area using in-house developed Matlab® scripts (*Takis et al.*

in preparation) and the concentrations were analyzed to determine the discriminating metabolites among the groups under study. Wilcoxon test was chosen to infer differences among the DKK (controls) and DKP (Parkinson) groups of subjects²¹⁷. Kruskal-Wallis test followed by Dunn post-hoc analysis was chosen to ascertain differences among DKK, DKP1 (mild PD) and DKP2 (more severe PD patients) groups. False discovery rate correction was applied using the Benjamini & Hochberg method (FDR) an adjusted *P*-value < 0.05 was considered statistically significant²¹⁸

Results

Discrimination between healthy subjects (DKK) and Parkinson's disease patients (DKP) in urine samples

NMR spectra of 135 urine samples (61 healthy subjects, DKK and 74 Parkinson's disease patients, DKP) were acquired. As first exploratory analysis PCA analysis (multivariate unsupervised approach) was performed on normalized spectra. PCA allows the identification of 4 outliers: DKK 903, DKK 1161, DKP 1194, and DKP 1373, which were excluded in the following analysis. To differentiate the two diagnosis groups, an OPLS model was built using the PQN normalized spectra of 59 DKK and 72 DKP urine samples. Only the first 15 components were retained in the model. The optimal model correctly identified $78.1 \pm 21.9\%$ of DKK and $70.5 \pm 29.5\%$ of DKP with an overall accuracy of 74%, as evaluated from the results on the outer loop of the double cross-validation procedure. The obtained discrimination between the two categories can be observed in **Figure 1**, where the projection of the samples onto the predictive component and the first orthogonal component of the model is reported.

Discrimination between healthy subjects (DKK) and Parkinson's disease patients (DKP) in serum samples

NMR spectra of 135 serum samples were acquired. Samples corresponding to DKK995, DKK1152, DKP1078 and DKP983 were excluded from data analysis because the spectra were of bad quality.

Processed ¹H spectra were analyzed using the binning procedure described above, and data were thus submitted to OPLS. In the score plot each dot represents the NMR spectrum of a serum sample. Dots are colored according to the diagnosis (green for DKK and blue for DKP). OPLS models were built on NOESY, CPMG and DIFFUSION-EDITED experiments respectively (**Figure 2 a, b, c**) and the first 25 components were retained in the model. Each model was validated using Monte Carlo cross-validation scheme. The NOESY model correctly identified 75.7% of DKK and 74.0% of DKP with an overall accuracy of 74%. The obtained discrimination between the two categories can be observed in **figure 2a**, where the projection of the samples onto the predictive component and the first orthogonal component of the model is reported. The CPMG model correctly identified 75.8% of DKK and 73.9% of DKP with an overall accuracy of 74.7% (**Figure 2b**). Finally, DIFFUSION-EDITED model correctly identified 71.3% of DKK and 71.5% of 72 DKP with an overall accuracy of 71.4% (**Figure 2c**).

Metabolites analysis of urine samples

Thirty-six metabolites, corresponding to well defined and resolved peaks in urine spectra, were assigned. NMR spectra were then analyzed to identify which of the assigned metabolites differ

significantly among DKK ($n = 59$) and DKP ($n = 72$) subjects. All metabolites identified were normalized using both total area and PQN algorithms. The two different normalization techniques shown similar results. In both cases we found seven significantly altered (P -value < 0.05) metabolites: isoleucine, pyruvate, acetone, trigonelline, hippurate, L-aspartate and scyllo-inositol. Parkinson's disease patients are characterized by higher levels of isoleucine, pyruvate and acetone, and lower levels of trigonelline, hippurate, L-aspartate and scyllo-inositol compared to healthy subjects (**Figure 3, Supplementary Table 1**).

Metabolites analysis of serum samples

Thirty-one metabolites, corresponding to well defined and resolved peaks in serum spectra, were assigned.

NMR spectra were firstly analyzed to identify which of the assigned metabolites differ significantly among DKK ($n = 59$) and DKP ($n = 72$) subjects, but only two were significant after tests for multiple correction. Unknown 1 (probably α -oxoisovalerate, to be confirmed) and phenylalanine are both more abundant in PD patients if compared to healthy subjects (**Figure 4, Supplementary Table 2**).

Clinical Analysis

Clinical records were also analyzed. We compared blood analyses results found in clinical records between DKK and DKP. We found five significant items: blood sugar, potassium, Glutamic Oxalacetic Transaminase (GOT), Cholesterol and Creatine kinase (CK). Blood sugar is higher in PD compared to controls while potassium, GOT, cholesterol and CK are higher in healthy subjects (**Table 1, Supplementary Figure 1**). In clinical reports, there is also information about the duration of the disease since diagnosis in months and the United Parkinson's Disease Rating Scale (UPDRS), a scale ranking the disease severity. This rating scale is divided in six different parts which evaluate respectively: mental state of patients, everyday abilities, motor ability, clinical analysis of progression, the Hoehn and Yahr scale and a scale ranking the disability degree. Hoehn and Yahr scale²²⁸ is a simple descriptive staging scale that provided a general estimate of clinical function in PD, combining functional deficits (disability) and objective signs (impairment). Originally designed as a five-point scale (1–5), during the 1990s, 0.5 increments were introduced for some clinical trials. The scale is based on the twofold concept that the severity of overall parkinsonian dysfunction relates to bilateral motor involvement and compromised balance. Increasing parkinsonian motor impairment therefore can be charted as shown in **table 2**.

No correlation between Hoehn and Yahr scale and duration in months of disease was found (correlation index 0.13), meaning that the duration of the disease in months does not affect the severity of the symptoms esteemed by the Hoehn and Yahr scale. We decided to retain only the Hoehn and Yahr information in the following analyses. Thus, all Parkinson's disease patients were divided according to their Hoehn and Yahr scale value. PD patients of our cohort are in a range of 1 to 3 of the Hoehn and Yahr scale and were divided into two groups: one group comprehends patients with values between 1 and 1.5, called DKP1 (mild PD) with 36 PD subjects, the other comprehends patients with H&Y values from 2 to 3, called DKP2 (more severe PD) with 36 subjects. Firstly, we compared relative abundance of urine metabolites within the DKP1, DKP2 and healthy subjects (DKK), to check if variations in metabolite levels occur also with the increase of PD severity (**Figure 5, Supplementary Table 3**). Some metabolites, such as, hippurate, citric acid, propionic acid, dimethylamine, and acetate in urine samples differ significantly (P -value < 0.05) between DKP1 and DKP2. Serum metabolites

levels of DKK, DKP1 and DKP2 subjects were analyzed with the same approach (**Figure 6, Supplementary Table 4**). In particular, phenylalanine and unknown 1 result increasing in the three stages (DKK<DKP1 <DKP2).

Discussion

As preliminary approach PCA has been useful to check the quality of the urine samples and to find the presence of outliers. Although an unsupervised approach was not enough to discriminate urine spectra of PD patients from healthy controls, the model build on the OPLS supervised analysis has been proven to be quite effective in discriminating the two groups (74% discrimination accuracy).

We used OPLS approach also for discrimination of serum samples. OPLS models were built on NOESY, CPMG and DIFFUSION-EDITED experiments respectively. The model built on NOESY spectra demonstrated to be the most effective in discriminating DKK subjects from DKP patients, achieving an accuracy of 75%.

The univariate analysis on urine metabolites revealed higher levels of isoleucine, pyruvic acid and acetone and lower levels of scyllo-inositol, L-aspartate, hippurate and trigonelline in DKP patients respect to DKK subjects. These results are mostly in agreement with literature on PD and other neurodegenerative diseases.

Although, based on model organism, a study on goldfish treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)²²⁹ to induce Parkinson's-like symptoms, revealed a higher level of isoleucine on their brain tissue compared to control goldfish.

Scyllo-inositol, naturally produced by gut microflora, has instead a decreased level in treated goldfish. Isoleucine is one of the essential amino acids that are important for the synthesis of proteins. The increased level of isoleucine could reflect a higher degradation of proteins due to enhanced levels of free radicals during oxidative stress. It is known that free radicals affect both the structure and function of neural cells, and contribute to a wide range of neurodegenerative diseases, including PD. Parkinson disease is also correlated with cerebral accumulation of α -synuclein and recent studies have speculated that scyllo-inositol could be able to inhibit fibrils formation both in mice and humans²³⁰.

Ketone bodies (one of which is acetone) have been examined in studies regarding various neurodegenerative disorders. Glucose hypometabolism is one of the hallmark conditions in Alzheimer Disease (AD): consequently, in addition to the usual alternate energy sources, such as acetoacetate and β -hydroxybutyrate, even acetone could be used by brain. Thus, the reported higher level of ketone bodies could be due to increased beta oxidation²³¹.

Since ketonuria is a common finding in malnutrition, we tried to check if the presence of acetone positively correlates with subjects with lowest BMI. However, no correlation was found between acetone levels and BMI.

Preliminary docking studies on trigonelline regarding Alzheimer Disease, show that this nicotine-related compound could inhibit the conformational change from α -helix to the β -sheet (known as the amyloidogenic conformation). Therefore, trigonelline seems to have anti-neurodegenerative properties²³².

PD is also strictly related to the aging process. Aging has been attributed partly to cellular free radical damage and to exogenous antioxidants. Hippurate is one of the final products of gut microbial metabolism in urine after polyphenols intake. It has been proposed that hippurate could be an indicator of antioxidant molecules synthesis: thus it is not surprising to find a higher level of hippurate in controls, who could have less oxidative stress, in comparison to PD²³³. We

tried to investigate the correlation between hippurate and patients' age to exclude age as confounding factor in the univariate analysis, but hippurate level does not show a positive correlation with increasing age. Pyruvate is the end metabolite of glycolysis. It enters Krebs' cycle as acetyl-coA by the catalysis of enzyme pyruvate dehydrogenase in the presence of the coenzyme NAD⁺. The accumulation of pyruvate or its increased concentration may be due to abnormal activity of pyruvate dehydrogenase complex and its interacting genes in patients. Increased pyruvate CSF has already been reported in Alzheimer's patients²³⁴.

Afterwards, metabolites concentration in serum sample were analyzed, and higher levels of phenylalanine and unknown 1 (tentatively attributed to α -oxoisovalerate) were found in PD patients compared to healthy subjects. Altered phenylalanine level is a hallmark of disturbed phenylalanine metabolism in early-stage PD. Phenylalanine is not only an important amino acid in proteins but is also a precursor for dopamine. In previous studies, the levels of plasma phenylalanine were slightly increased without statistical significance. Molina *et al.* reported differences between levels of phenylalanine in PD patient treated with levodopa and PD patient untreated. Phenylalanine level were higher in treated patients than in those who did not receive this drug as a treatment; other amino acids levels were unchanged^{236,237}. Following this assumption, we repeated the univariate analysis of urine and serum metabolites and on blood tests comparing patients treated with L-Dopa during the four weeks before sampling (n = 24) to untreated patients (n = 49). In all cases no significant differences emerged among the two groups.

The next step was the investigation of clinical records at our disposal. The clinical records analysis showed higher levels of blood sugar in DKP patients and lower concentrations of potassium, cholesterol, GOT and CK with respect to controls.

According to literature, some anti-PD medications have effects on glucose metabolism, since evidence suggests a reciprocal regulation between insulin and brain dopaminergic activity. Patients treated with levodopa have been shown to have decreased glucose tolerance, hyperglycemia and hyperinsulinemia²³⁸.

Concerning levels of potassium, our results seem to be in accordance with literature. Hypokalemia, sometimes severe, was observed in some L-Dopa-treated parkinsonian patients. L-Dopa intake indeed, was found to cause an increased excretion of potassium²³⁹.

A study on serum samples of PD subjects, examining 555 PD patients and 555 age-, gender- and body mass index (BMI)-matched controls, suggests that lower levels of total cholesterol can be related to PD severity²⁴⁰.

GOT transamination is a chemical reaction in which there is an exchange of the α -amino group of one amino acid for the keto group of an α -keto acid, with the resulting synthesis of a second α -amino acid and a new α -keto acid. GOT is an enzyme found primarily in the cells of the liver, heart, kidneys, skeletal muscles, pancreas and to a lesser extent in red blood cells. It is released into serum in large quantities when any one of these tissues is damaged. Currently we are not able to explain the GOT role in this context, thus further studies are needed.

The most significant value among those of the fold change in blood analysis was CK.

CK have shown to be higher in healthy subjects than in DKP patients. High serum levels of CK could be an indication of tissue damage in those tissues where CK is most abundant, such as heart. Therefore, CK can be an indicator of cardiac diseases. To exclude any possible confounding factor, we tried to exclude from our analysis all healthy subjects assuming beta-blockers, but no changes in CK levels are reported. Another indication of subject's heart problems is contained in the SCOPA test. The SCOPA-AUT is a specific 23-item self-completed questionnaire for the assessment of autonomic dysfunction in patients with PD.

The 23 items of the SCOPA-AUT are grouped into six domains: gastrointestinal functioning, urinary functioning, cardiovascular functioning, thermoregulatory functioning, pupillomotor functioning, and sexual function. The score for each item range from 0 (never experiencing the symptom) to 3 (often experiencing the symptom)²⁴¹. We then tried to exclude from our analysis all subjects with value of cardiovascular functioning score different from zero. Even with this adjustment the result does not change and CK is still higher in healthy subjects compared to PD patients.

Takubo *et al.* found a significantly higher serum CK level in patients with PD than in controls. It is well known that serum CK increases after exercise, thus they measured serum CK levels in a small number of parkinsonian patients on the first day of hospital admission and again on the day following admission. Serum CK appears to elevate after even a minor exercise in patients with PD. It has been proven that physical activity mildly increases serum CK levels. The magnitude of CK increase in serum correlates with the strenuousness of the exercise and correlates inversely with the degree of muscle training²⁴².

There are not data regarding patients' physical activity in clinical records, therefore we analyzed data regarding clinical evaluation of motor dysfunctions in parkinsonian patients. We found that 95.94% of patients suffer from bradykinesia, 91.9% suffer from muscle stiffness, 71.62% suffer from tremor at rest and 52.7% suffer of postural instability.

Given these values we can suppose that parkinsonian patients may not have an active style of life and are more sedentary compared to healthy subjects. This could be a valid explanation for higher CK level in controls.

According to Hoehn and Yahr scale we divided all subjects in three groups called DKK, DKP1 and DKP2 as explained above. We compared relative abundance of urine metabolites among the groups to find some variations. Fourteen metabolites showed a significant P-value at least for one comparison: isoleucine, L-alanine, acetate, propionate, pyruvate, citrate, L-aspartate, scyllo-inositol, hippurate, trigonelline, dimethylamine, glycine, acetone, ascorbate. Some of these are the same metabolites we found significantly altered in the first fold change analysis.

Interestingly, as shown in the box plots, some metabolites display different levels between DKP1 and DKP2 patients. Propionate, citrate, hippurate and glycine have lower levels in DKP2 compared to DKP1, while dimethylamine has higher levels in DKP2 compared to DKP1.

Using the same division method, we analyzed differences among serum metabolites concentration in DKK, DKP1 and DKP2 groups. Seven metabolites were found significantly altered: phenylalanine, tyrosine, glycine, glucose, creatinine, lactate, unknown 1. Phenylalanine and unknown 1 were found previously also in the fold change analysis.

As for urine, even for serum samples, some metabolites show differences among DKP1 and DKP2 patients. Lactate, creatinine, glucose, tyrosine and phenylalanine have higher levels in DKP2 group compared to DKP1 group. These alterations need to be further confirmed after the analysis on other cohorts as hallmark of Parkinson's disease severity.

Conclusions

The OPLS supervised analysis has been proven to be the most effective in discriminating PD patients from healthy subjects both in urine and serum samples.

Furthermore, OPLS model built on NOESY experiment achieved the highest accuracy compared to DIFFUSION-EDITED and CPMG models. For serum samples a 75% discrimination accuracy was reached, and similar results are obtained for urine samples. For urine samples this accuracy can be considered a satisfactory result, since evidences about PD signature in urine, through NMR based metabolomics, are not yet present in literature.

The univariate analysis on metabolites of urine samples revealed higher levels of isoleucine, pyruvic acid and acetone and lower levels of scyllo-inositol, L-aspartate, hippurate and trigonelline in DKP patients with respect to DKK subjects.

Isoleucine is often related to an increased level of free radicals and consequently to more oxidative stress. Oxidative stress is a common condition in neurodegenerative disorders like Parkinson's disease and even the lower level of hippurate in DKP subjects could be an effect of this state. Hippurate is indeed considered an indicator of antioxidant molecules synthesis. Our results seem to display an overall description of a metabolic state of oxidative stress. Actually, hippurate shows lower levels among mild PD patients compared to more severe ones, suggesting a hypothetical correlation with PD severity degree.

Neurodegenerative diseases often affect also energy metabolism, and high levels of acetone in DKP subject seem to describe this dysfunction. During glucose hypometabolism, hallmark condition mostly in AD, acetone and other ketone bodies can be used by brain as an alternative energy source, causing higher levels in DKP patients' urine. The accumulation of pyruvate or its increased concentration may be due to abnormal activity of pyruvate dehydrogenase complex and its interacting genes in patients.

Glucose metabolism alteration are also visible in blood analysis results, where DKP subjects show hyperglycemia compared to DKK. Available literature suggests that hyperglycemia could be attributable to side effects of L-Dopa treatment. L-Dopa seems to affect not only glucose but also phenylalanine and potassium levels in DKP patients. However, our analysis on L-Dopa treated patients (patients treated at least 4 weeks before the sampling) versus untreated ones, did not reveal any significant differences both in urine and in serum samples of the two groups. Serum multivariate analysis reveals higher level of phenylalanine in DKP patients respect to DKK subjects. It is known that dysfunction in phenylalanine metabolism is a hallmark of early-stage PD. Our results confirm this last observation since phenylalanine levels increase with the severity of PD ($\text{DKK} < \text{DKP1} < \text{DKP2}$, following the H&Y scale).

One of the leading causes of Parkinson's disease is reported to be the α -synuclein fibrils accumulation. Scyllo-inositol and trigonelline have both been proven to have neurodegenerative properties in this sense. Scyllo-inositol is able to inhibit fibril formation in mice and humans, while preliminary studies on trigonelline show that this molecule can inhibit the change of α -synuclein to the amyloidogenic conformation. These observations are in agreement with our results, where both molecules are less abundant in DKP subjects compared to DKK.

The future perspective for this work is to strengthen the statistical model based on metabolomics data of PD patients thanks to new cohorts of samples including also centenarians and twins. We are really hopeful that at the end by the integration of newly obtained characterization and data with genetics, epigenetics, lipidomics and glycomics, we could provide an important contribution to identify the combination the alterations capable of shifting the phenotype of elderly subjects from a physiological decline into PD. This will eventually enable the identification of markers for early PD diagnosis.

Tables and figures

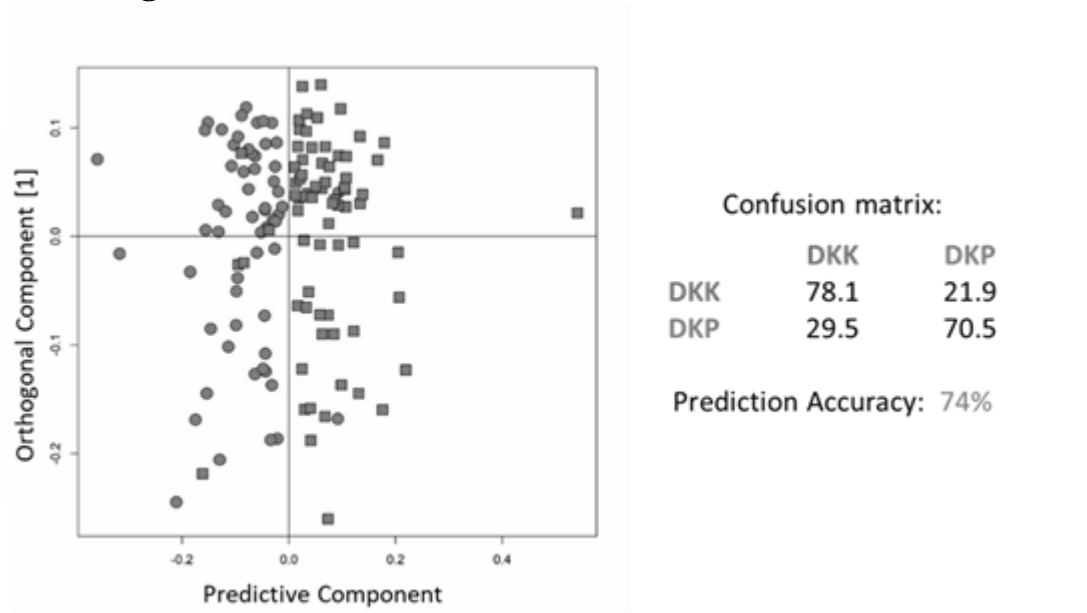


Figure 1. Score plot of OPLS discriminating DKK (dots) from DKP patients (squares) using urine samples. Confusion matrix and prediction accuracy of the Monte Carlo cross-validation also reported.

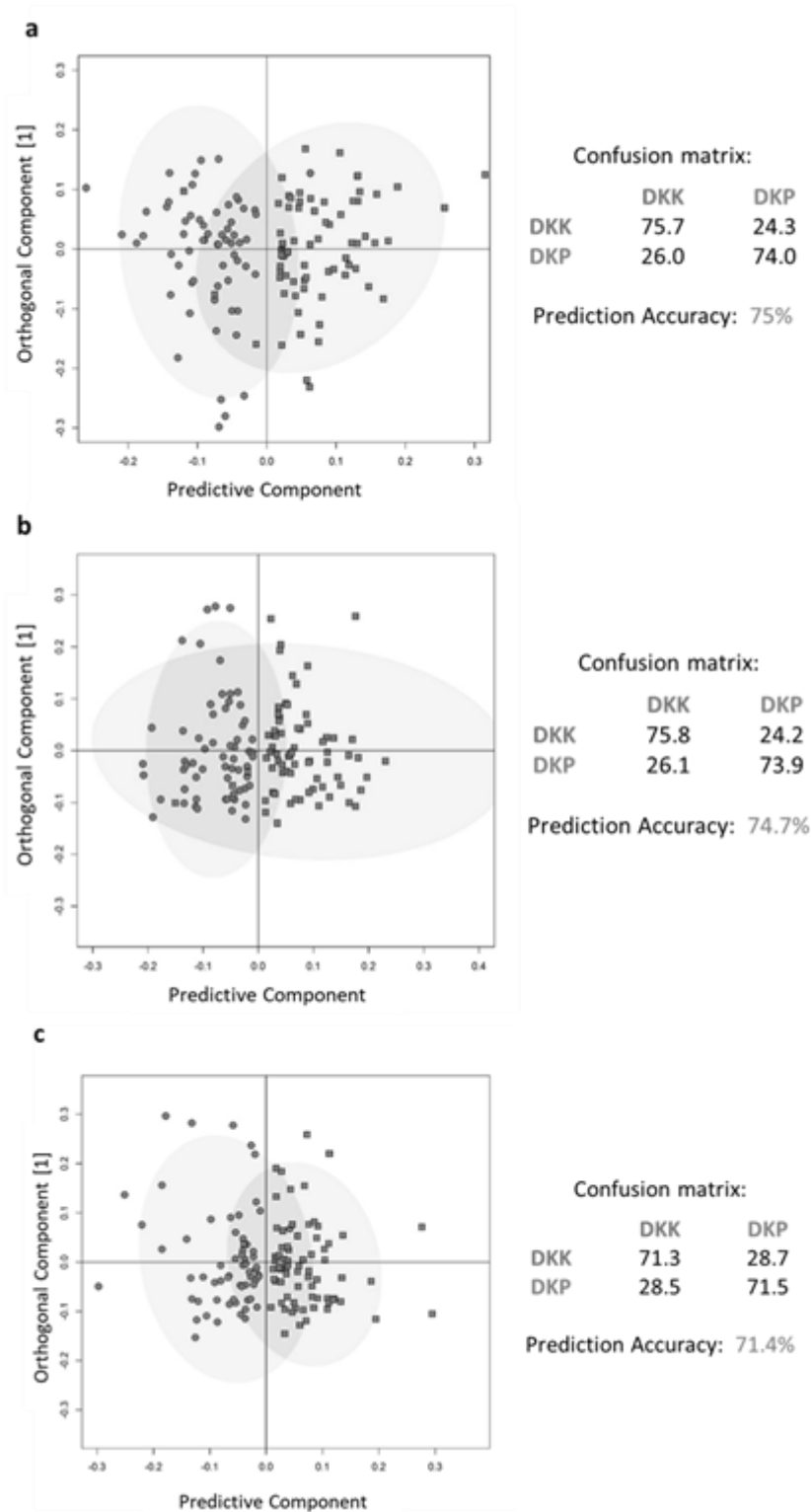


Figure 2. Score plots of OPLS discriminating DKK (dots) from DKP patients (squares) serum samples; a) NOESY experiment; b) CPMG experiment; c) DIFFUSION-EDITED experiment. Confusion matrices and related prediction accuracy are reported for each model.

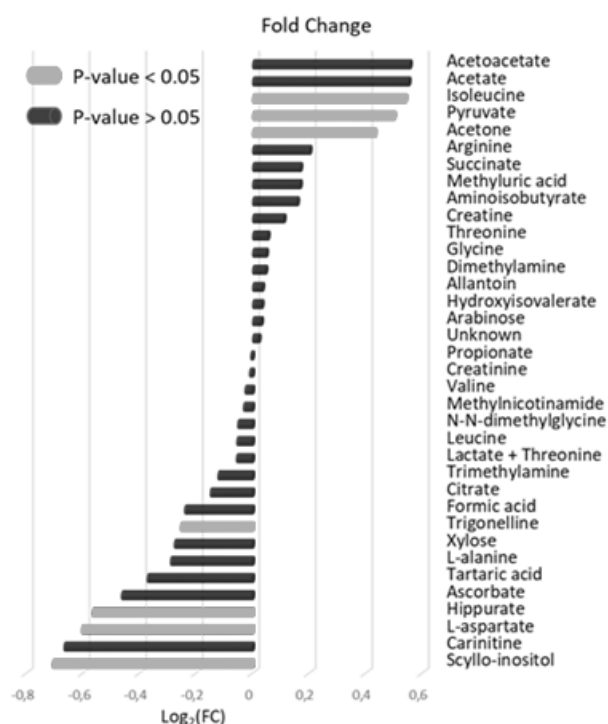


Figure 3. Barplot of fold changes (FC) values for each of the 36 quantified metabolites in urine samples. Negative $\text{Log}_2(\text{FC})$ values means higher metabolite levels in DKK subjects, while positive $\text{Log}_2(\text{FC})$ refers to higher metabolite levels in Parkinson's disease patients. Metabolites that displayed significant concentration changes ($P\text{-value} < 0.05$) are colored in light gray. Shown metabolites are normalized using PQN algorithm. Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics

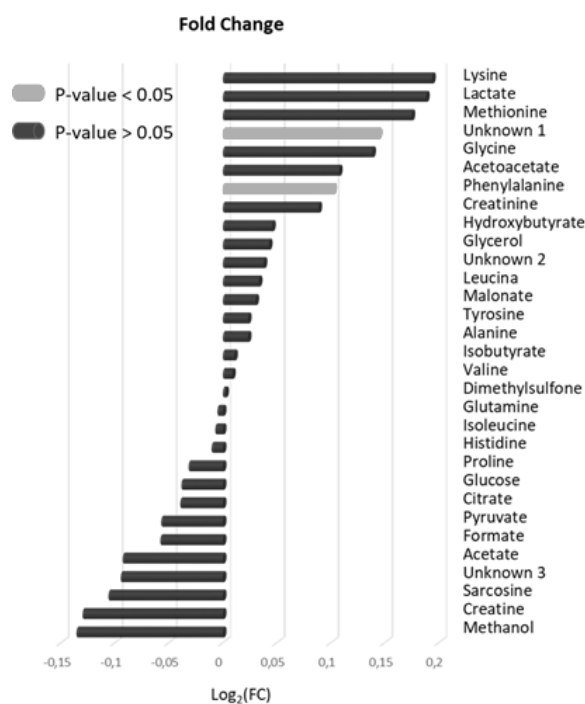


Figure 4. Barplot of the values of the fold changes (FC) for each of the 31 quantified in serum samples. Negative $\text{Log}_2(\text{FC})$ values means higher metabolite levels in DKK subjects, while positive $\text{Log}_2(\text{FC})$

refers to higher metabolite levels in DKK. Metabolites that displayed significant concentration changes (P-value < 0.05) are colored in light gray.

Table 2. Concentrations (mean $\pm$ SD) of significantly altered blood analyses values in the comparison of DKK and DKP subjects. P-values from the comparisons are reported. The signals whose intensities were significantly higher or lower in DKP groups are marked with $\uparrow$ or $\downarrow$.

	DKK			DKP			<i>P-value</i>
	<i>Mean</i>		<i>SD</i>	<i>Mean</i>		<i>SD</i>	
<i>Blood sugar</i>	8.77E+01	$\pm$	1.72E+01	9.37E+01	$\pm$	1.88E+01	7.13E-03 $\uparrow$
<i>Potassium</i>	4.28E+00	$\pm$	3.24E-01	4.09E+00	$\pm$	3.62E-01	1.66E-02 $\downarrow$
<i>GOT</i>	2.81E+01	$\pm$	9.91E+00	2.60E+01	$\pm$	1.05E+01	1.09E-02 $\downarrow$
<i>Cholesterol</i>	2.38E+02	$\pm$	3.73E+01	2.13E+02	$\pm$	4.21E+01	7.13E-03 $\downarrow$
<i>CK</i>	1.41E+02	$\pm$	8.64E+01	8.53E+01	$\pm$	4.13E+01	1.32E-05 $\downarrow$

Table 3. Original and modified Hoehn and Yahr scale. Table adapted from "Movement Disorder Society Task Force report on the Hoehn and Yahr staging scale: status and recommendations", Goetz, C. G. *et al.* (2004).

Hoehn and Yahr scale		Modified Hoehn and Yahr scale	
1	Unilateral involvement only usually with minimal or no functional disability	1.0	Unilateral involvement only
		1.5	Unilateral and axial involvement
2	Bilateral or midline involvement without impairment of balance	2.0	Bilateral involvement without impairment of balance
3	Bilateral disease: mild to moderate disability with impaired postural reflexes; physically independent	2.5	Mild bilateral disease with recovery on pull test
		3.0	Mild to moderate bilateral disease; some postural instability; physically independent
4	Severely disabling disease; still able to walk or stand unassisted	4.0	Severe disability; still able to walk or stand unassisted
5	Confinement to bed or wheelchair unless aided	5.0	Wheelchair bound or bedridden unless aided

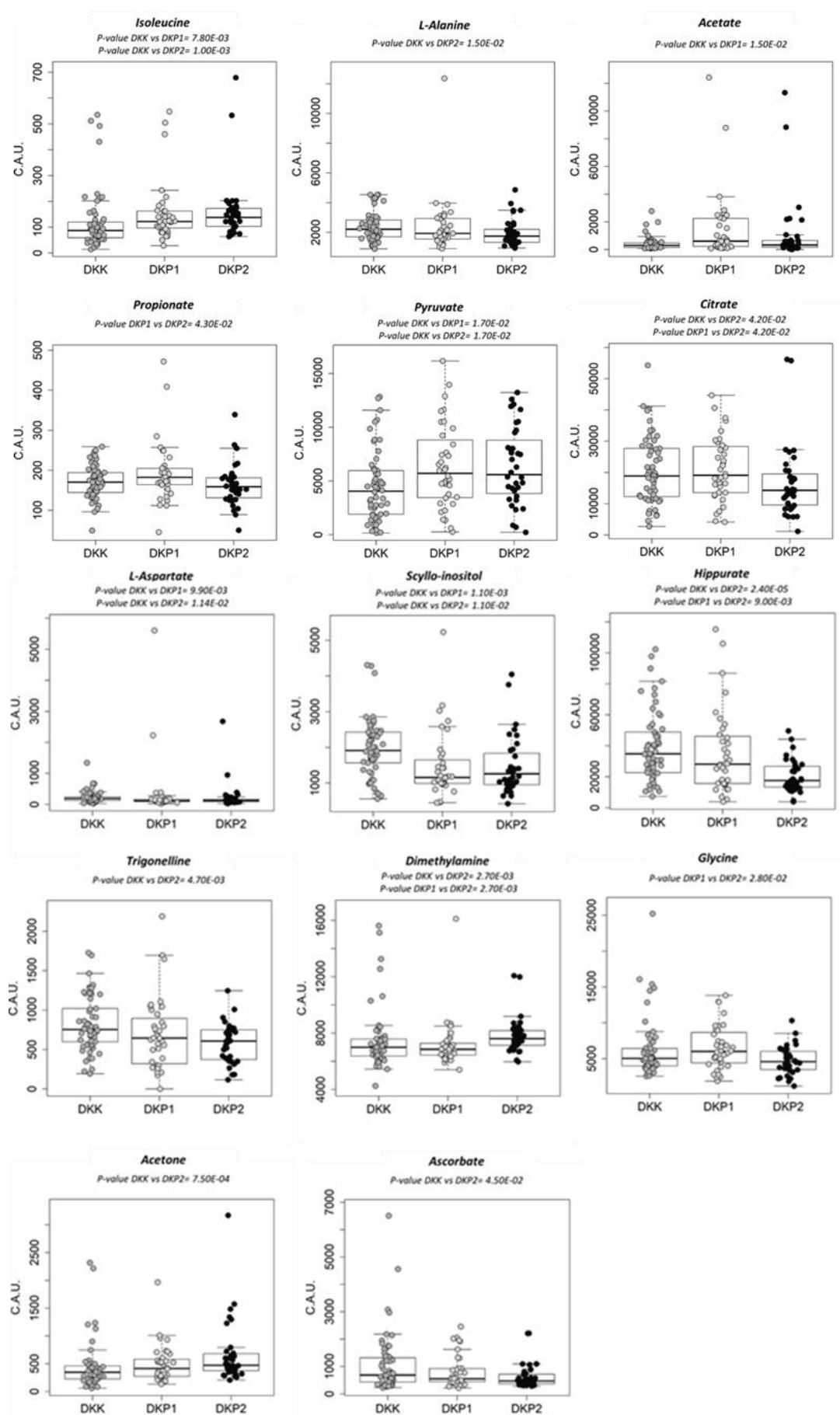


Figure 5. Boxplots of urine metabolites differentially concentrated in DKK (healthy subjects in green),

DKP1 (mild PD individuals in light blue), DKP2 (more severe PD patients in dark blue) according to NMR analysis. For each comparison, the *P*-value (by the Kruskal-Wallis test followed by Dunn post-hoc analysis) is reported. C.A.U: Concentration in Arbitrary Unit. Metabolites are scaled according to PQN algorithm

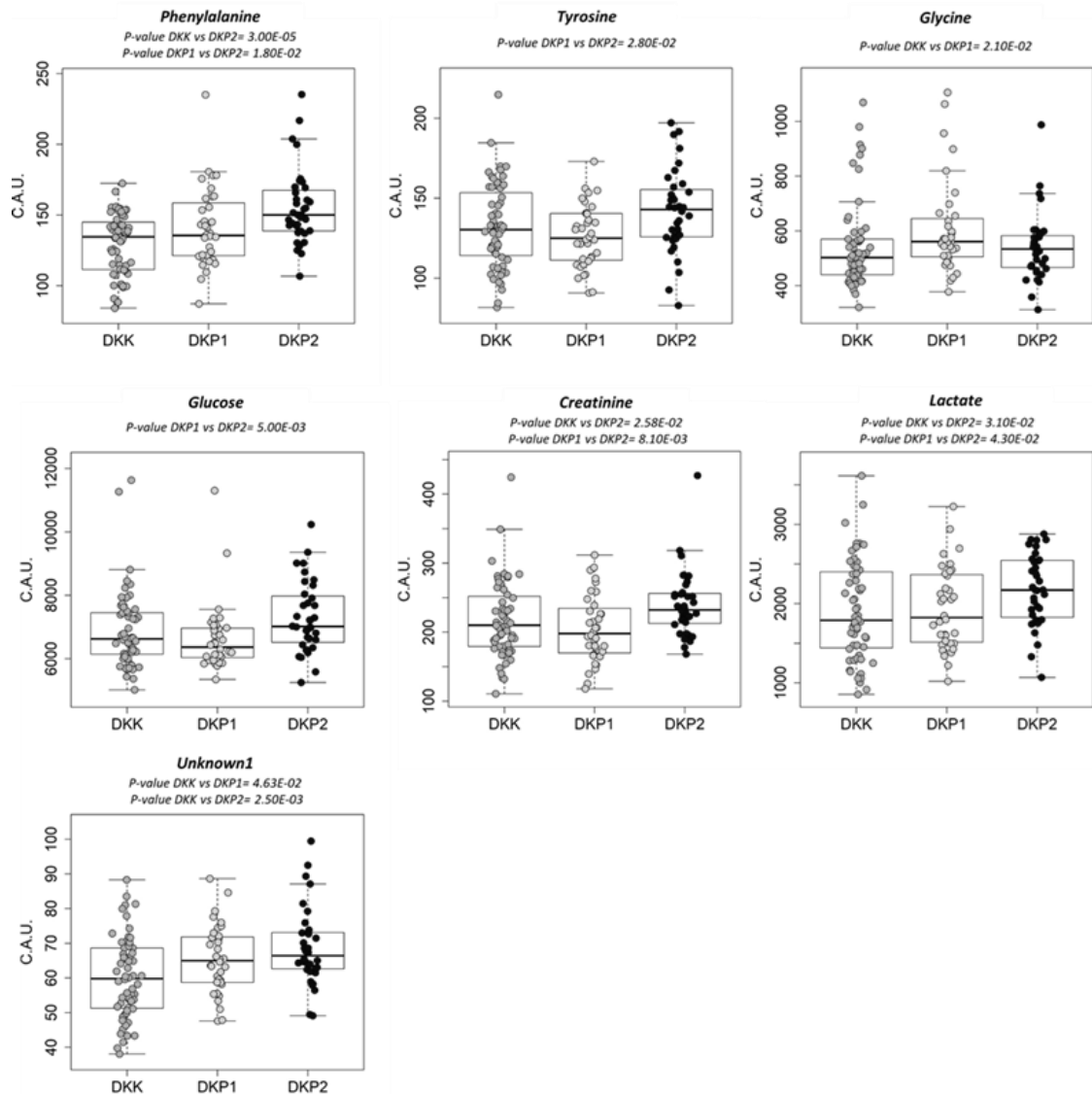


Figure 6. Boxplots of serum metabolites differentially concentrated in DKK (healthy subjects in green), DKP1 (mild PD individuals in light blue), DKP2 (more severe PD patients in dark blue) according to NMR analysis. For each comparison, the *P*-value (by the Kruskal-Wallis test followed by Dunn post-hoc analysis) is reported. C.A.U: Concentration in Arbitrary Unit.

Supplementary information

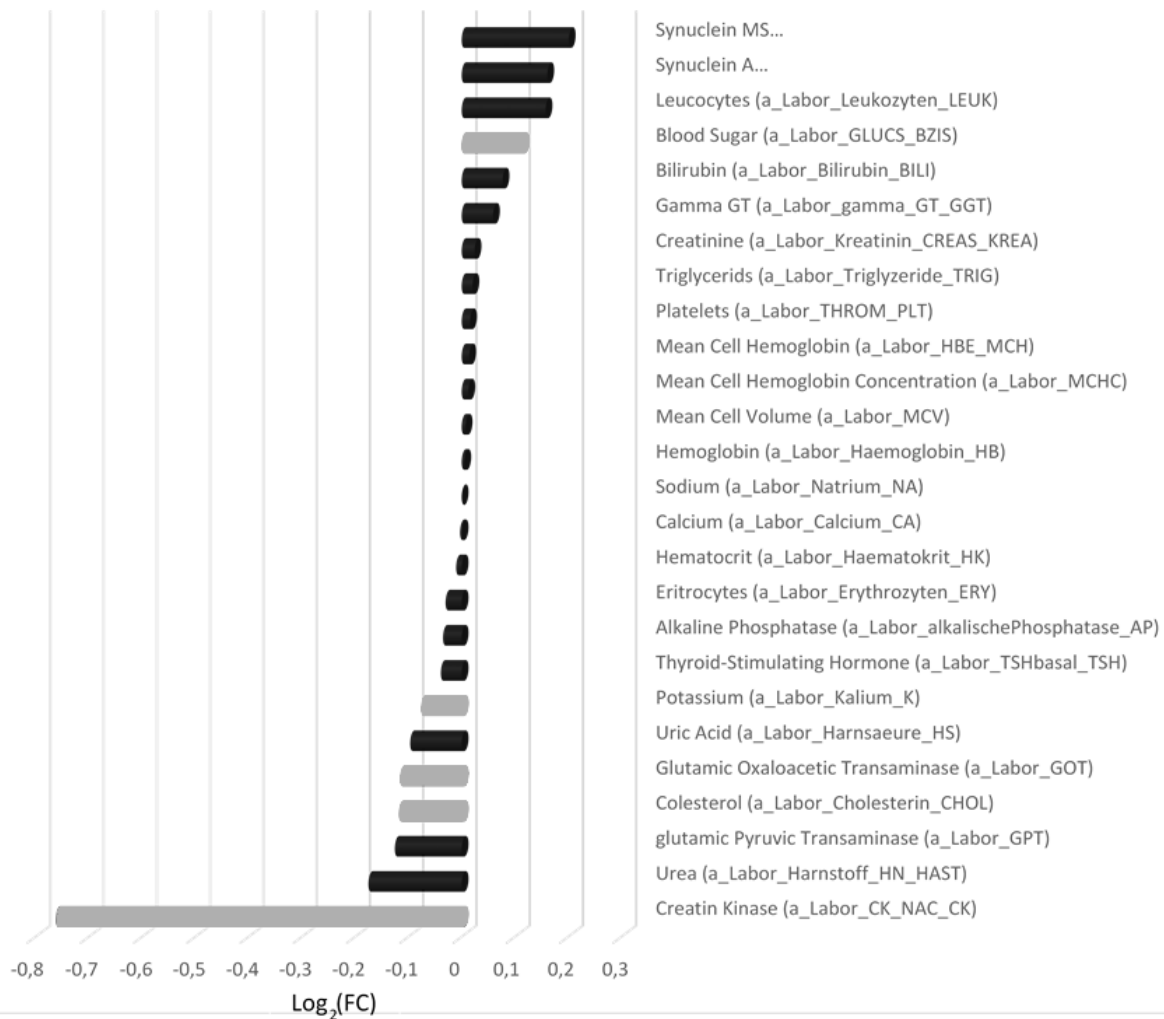
Supplementary table 1. Concentrations (mean $\pm$ SD) of the metabolites assigned in urine samples. P-values from the comparisons are also reported.

Metabolites	DKK		DKP		P-Values
	Mean	SD	Mean	SD	
<i>Isoleucine</i>	1.20E+02 $\pm$ 1.14E+02		1.56E+02 $\pm$ 1.17E+02		1.42E-03
<i>Leucine</i>	2.11E+02 $\pm$ 8.97E+01		2.02E+02 $\pm$ 9.32E+01		6.86E-01
<i>Valine</i>	1.72E+02 $\pm$ 3.72E+01		1.64E+02 $\pm$ 4.83E+01		5.94E-01
<i>Aminoisobutyrate</i>	9.05E+02 $\pm$ 1.25E+03		8.54E+02 $\pm$ 9.49E+02		7.17E-01
<i>Hydroxyisovalerate</i>	1.19E+03 $\pm$ 3.92E+02		1.23E+03 $\pm$ 4.73E+02		8.94E-01
<i>Lactate + Threonine</i>	1.18E+03 $\pm$ 5.16E+02		1.11E+03 $\pm$ 6.47E+02		3.26E-01
<i>L-alanine</i>	2.41E+03 $\pm$ 9.41E+02		2.18E+03 $\pm$ 1.49E+03		6.42E-02
<i>Acetate</i>	4.31E+02 $\pm$ 4.88E+02		1.32E+03 $\pm$ 2.40E+03		6.97E-02
<i>Propionate</i>	1.71E+02 $\pm$ 4.12E+01		1.78E+02 $\pm$ 6.65E+01		9.75E-01
<i>Acetone</i>	4.42E+02 $\pm$ 4.27E+02		5.84E+02 $\pm$ 4.64E+02		4.57E-03
<i>Succinate</i>	3.25E+02 $\pm$ 1.45E+02		3.54E+02 $\pm$ 1.96E+02		6.36E-01
<i>Pyruvate</i>	4.43E+03 $\pm$ 3.26E+03		6.34E+03 $\pm$ 3.71E+03		7.33E-03
<i>Unknown 1</i>	6.95E+02 $\pm$ 1.97E+02		7.16E+02 $\pm$ 2.15E+02		8.27E-01
<i>Citrate</i>	2.05E+04 $\pm$ 1.05E+04		1.89E+04 $\pm$ 1.10E+04		4.93E-01
<i>L-aspartate</i>	2.28E+02 $\pm$ 2.00E+02		2.89E+02 $\pm$ 7.51E+02		4.57E-03
<i>Creatinine</i>	8.99E+04 $\pm$ 1.65E+04		9.09E+04 $\pm$ 1.76E+04		9.57E-01
<i>Creatine</i>	2.14E+03 $\pm$ 2.31E+03		1.95E+03 $\pm$ 1.75E+03		9.57E-01
<i>Scyllo-inositol</i>	1.98E+03 $\pm$ 8.02E+02		1.47E+03 $\pm$ 8.70E+02		1.19E-03
<i>Methyluric acid</i>	2.76E+04 $\pm$ 2.89E+04		2.22E+04 $\pm$ 1.84E+04		8.35E-01
<i>Xylose</i>	5.01E+02 $\pm$ 5.11E+02		3.66E+02 $\pm$ 3.03E+02		2.13E-01
<i>Hippurate</i>	3.94E+04 $\pm$ 2.25E+04		2.79E+04 $\pm$ 2.15E+04		4.57E-03
<i>Threonine</i>	5.86E+02 $\pm$ 1.49E+02		6.11E+02 $\pm$ 2.39E+02		9.66E-01
<i>Tartaric acid</i>	5.90E+02 $\pm$ 8.89E+02		4.82E+02 $\pm$ 1.19E+03		6.42E-02
<i>Trigonelline</i>	8.22E+02 $\pm$ 3.45E+02		6.41E+02 $\pm$ 3.70E+02		6.93E-03
<i>Methylnicotinamide</i>	1.35E+02 $\pm$ 7.68E+01		1.34E+02 $\pm$ 6.95E+01		9.75E-01
<i>Allantoin</i>	3.75E+02 $\pm$ 1.79E+02		3.81E+02 $\pm$ 1.89E+02		8.64E-01
<i>Dimethylamine</i>	7.46E+03 $\pm$ 2.13E+03		7.50E+03 $\pm$ 1.52E+03		2.31E-01
<i>N-N-dimethylglycine</i>	1.37E+03 $\pm$ 5.68E+02		1.41E+03 $\pm$ 9.35E+02		8.64E-01
<i>Trimethylamine</i>	1.01E+03 $\pm$ 5.20E+02		1.03E+03 $\pm$ 6.73E+02		9.69E-01
<i>Arabinose</i>	2.45E+02 $\pm$ 9.16E+01		2.62E+02 $\pm$ 1.05E+02		7.83E-01
<i>Formic acid</i>	4.74E+02 $\pm$ 1.83E+02		4.13E+02 $\pm$ 1.95E+02		1.47E-01
<i>Glycine</i>	6.24E+03 $\pm$ 4.04E+03		5.65E+03 $\pm$ 2.71E+03		9.80E-01
<i>Acetoacetate</i>	1.04E+03 $\pm$ 8.16E+02		1.29E+03 $\pm$ 1.05E+03		2.54E-01
<i>Ascorbate</i>	1.07E+03 $\pm$ 1.09E+03		7.31E+02 $\pm$ 5.34E+02		1.42E-01
<i>Carintine</i>	4.17E+03 $\pm$ 3.77E+03		3.28E+03 $\pm$ 3.49E+03		2.38E-01
<i>Arginine</i>	1.31E+03 $\pm$ 9.39E+02		1.38E+03 $\pm$ 6.92E+02		2.70E-01

Supplementary table 2. Concentrations (mean $\pm$ SD) of the metabolites assigned in serum samples. P-values from the comparisons are also reported.

<i>Metabolites</i>	DKK		DKP		<i>P-Values</i>
	<i>Mean</i>	<i>SD</i>	<i>Mean</i>	<i>SD</i>	
<i>Valine</i>	8.83E+02	$\pm$ 1.43E+02	9.00E+02	$\pm$ 1.23E+02	9.48E-01
<i>Isoleucine</i>	1.16E+02	$\pm$ 2.84E+01	1.14E+02	$\pm$ 2.14E+01	9.95E-01
<i>Leucina</i>	8.83E+02	$\pm$ 1.96E+02	9.10E+02	$\pm$ 1.68E+02	6.40E-01
<i>Isobutyrate</i>	5.66E+01	$\pm$ 1.44E+01	5.95E+01	$\pm$ 1.67E+01	9.48E-01
<i>Alpha-oxo isovalerate</i>	6.00E+01	$\pm$ 1.18E+01	6.71E+01	$\pm$ 1.05E+01	3.29E-03
<i>Hydroxybutyrate</i>	4.49E+01	$\pm$ 3.91E+01	4.90E+01	$\pm$ 3.40E+01	7.20E-01
<i>Lactate</i>	1.91E+03	$\pm$ 6.24E+02	2.06E+03	$\pm$ 4.97E+02	5.74E-01
<i>Alanine</i>	1.20E+03	$\pm$ 2.33E+02	1.23E+03	$\pm$ 2.31E+02	9.48E-01
<i>Acetate</i>	7.88E+01	$\pm$ 2.89E+01	7.93E+01	$\pm$ 3.96E+01	8.54E-01
<i>Glutamine</i>	1.59E+02	$\pm$ 2.00E+01	1.58E+02	$\pm$ 1.78E+01	9.95E-01
<i>Unknown 1</i>	1.08E+02	$\pm$ 1.94E+01	1.08E+02	$\pm$ 1.87E+01	9.95E-01
<i>Acetoacetate</i>	1.14E+02	$\pm$ 8.84E+01	1.40E+02	$\pm$ 1.27E+02	7.20E-01
<i>Pyruvate</i>	1.82E+02	$\pm$ 6.79E+01	1.82E+02	$\pm$ 6.80E+01	9.95E-01
<i>Citrate</i>	2.59E+02	$\pm$ 6.20E+01	2.58E+02	$\pm$ 4.58E+01	9.95E-01
<i>Methionine</i>	4.61E+01	$\pm$ 2.36E+01	5.19E+01	$\pm$ 2.10E+01	4.08E-01
<i>Sarcosine</i>	3.57E+01	$\pm$ 4.64E+01	4.42E+01	$\pm$ 7.21E+01	9.48E-01
<i>Lysine</i>	3.67E+01	$\pm$ 2.47E+01	4.26E+01	$\pm$ 3.53E+01	2.12E-01
<i>Creatine</i>	6.77E+01	$\pm$ 4.15E+01	6.73E+01	$\pm$ 4.25E+01	9.95E-01
<i>Creatinine</i>	2.17E+02	$\pm$ 5.49E+01	2.23E+02	$\pm$ 5.11E+01	7.20E-01
<i>Malonate</i>	2.84E+01	$\pm$ 3.72E+01	1.74E+01	$\pm$ 1.97E+01	7.78E-01
<i>Dimethylsulfone</i>	5.96E+01	$\pm$ 2.40E+01	6.28E+01	$\pm$ 3.38E+01	9.95E-01
<i>Unknown 2</i>	6.41E+01	$\pm$ 3.45E+01	5.88E+01	$\pm$ 3.06E+01	7.78E-01
<i>Methanol</i>	3.35E+02	$\pm$ 7.16E+01	3.05E+02	$\pm$ 6.71E+01	2.12E-01
<i>Glycine</i>	5.42E+02	$\pm$ 1.57E+02	5.74E+02	$\pm$ 1.51E+02	2.12E-01
<i>Glycerol</i>	1.67E+02	$\pm$ 6.98E+01	1.80E+02	$\pm$ 6.40E+01	7.20E-01
<i>Proline</i>	6.53E+01	$\pm$ 2.11E+01	6.37E+01	$\pm$ 1.47E+01	9.95E-01
<i>Glucose</i>	2.90E+03	$\pm$ 4.16E+02	2.90E+03	$\pm$ 4.07E+02	9.95E-01
<i>Tyrosine</i>	1.32E+02	$\pm$ 2.62E+01	1.34E+02	$\pm$ 2.42E+01	9.95E-01
<i>Histidine</i>	1.07E+02	$\pm$ 1.42E+01	1.10E+02	$\pm$ 1.98E+01	9.95E-01
<i>Phenylalanine</i>	1.29E+02	$\pm$ 2.14E+01	1.48E+02	$\pm$ 2.80E+01	3.29E-03
<i>Formate</i>	1.80E+01	$\pm$ 3.38E+00	1.81E+01	$\pm$ 3.59E+00	9.95E-01

Fold Change



Supplementary figure 1. Barplots of the values of the fold changes (FC) for blood test results. Negative Log₂(FC) values means higher values in DKK subjects, while positive Log₂(FC) refers to higher values in DKK. P-values < 0.05 are colored in light gray.

Supplementary table 3. Concentrations (mean $\pm$ SD) of the metabolites assigned in urine samples. P-values from the comparison is also shown.

<i>Metabolites</i>	DKK			DKP1			DKP2			<i>P-values</i>
	<i>Mean</i>	$\pm$	<i>SD</i>	<i>Mean</i>	$\pm$	<i>SD</i>	<i>Mean</i>	$\pm$	<i>SD</i>	
<i>Isoleucine</i>	1.20E+02	$\pm$	1.14E+02	1.54E+02	$\pm$	1.16E+02	1.59E+02	$\pm$	1.19E+02	DKK vs DKP1= 7.80E-03 DKK vs DKP2= 1.00E-03
<i>Leucine</i>	2.11E+02	$\pm$	8.97E+01	1.84E+02	$\pm$	4.51E+01	2.21E+02	$\pm$	1.22E+02	
<i>Valine</i>	1.72E+02	$\pm$	3.72E+01	1.70E+02	$\pm$	5.08E+01	1.58E+02	$\pm$	4.56E+01	
<i>Aminoisobutyrate</i>	9.05E+02	$\pm$	1.25E+03	7.71E+02	$\pm$	6.67E+02	9.37E+02	$\pm$	1.17E+03	
<i>Hydroxyisovalerate</i>	1.19E+03	$\pm$	3.92E+02	1.21E+03	$\pm$	4.68E+02	1.24E+03	$\pm$	4.85E+02	
<i>Lactate + Threonine</i>	1.18E+03	$\pm$	5.16E+02	1.13E+03	$\pm$	5.07E+02	1.09E+03	$\pm$	7.69E+02	
<i>L-alanine</i>	2.41E+03	$\pm$	9.41E+02	2.41E+03	$\pm$	1.89E+03	1.95E+03	$\pm$	9.08E+02	DKK vs DKP2= 1.50E-02
<i>Acetate</i>	4.31E+02	$\pm$	4.88E+02	1.52E+03	$\pm$	2.49E+03	1.11E+03	$\pm$	2.34E+03	DKK vs DKP1= 1.50E-02
<i>Propionate</i>	1.71E+02	$\pm$	4.12E+01	1.93E+02	$\pm$	7.60E+01	1.62E+02	$\pm$	5.20E+01	DKK vs DKP2= 7.50E-04
<i>Acetone</i>	4.42E+02	$\pm$	4.27E+02	4.98E+02	$\pm$	3.37E+02	6.69E+02	$\pm$	5.55E+02	DKK vs DKP2= 7.50E-04
<i>Succinate</i>	3.25E+02	$\pm$	1.45E+02	3.42E+02	$\pm$	1.60E+02	3.67E+02	$\pm$	2.28E+02	
<i>Pyruvate</i>	4.43E+03	$\pm$	3.26E+03	6.32E+03	$\pm$	3.90E+03	6.35E+03	$\pm$	3.58E+03	DKK vs DKP2= 1.70E-02 DKK vs DKP2= 1.70E-02
<i>Unknown 1</i>	6.95E+02	$\pm$	1.97E+02	6.71E+02	$\pm$	2.30E+02	7.60E+02	$\pm$	1.91E+02	
<i>Citrate</i>	2.05E+04	$\pm$	1.05E+04	2.11E+04	$\pm$	1.02E+04	1.67E+04	$\pm$	1.16E+04	DKK vs DKP1= 4.20E-02 DKP1 vs DKP2= 4.20E-02
<i>L-aspartate</i>	2.28E+02	$\pm$	2.00E+02	3.47E+02	$\pm$	9.69E+02	2.32E+02	$\pm$	4.47E+02	DKK vs DKP2= 1.14E-02 DKK vs DKP2= 1.14E-02
<i>Creatinine</i>	8.99E+04	$\pm$	1.65E+04	8.91E+04	$\pm$	1.68E+04	9.27E+04	$\pm$	1.85E+04	
<i>Creatine</i>	2.14E+03	$\pm$	2.31E+03	1.72E+03	$\pm$	1.54E+03	2.18E+03	$\pm$	1.94E+03	
<i>Scyllo-inositol</i>	1.98E+03	$\pm$	8.02E+02	1.48E+03	$\pm$	9.35E+02	1.47E+03	$\pm$	8.13E+02	DKK vs DKP1= 1.10E-03 DKK vs DKP2= 1.10E-02
<i>Methyluric acid</i>	2.76E+04	$\pm$	2.89E+04	2.26E+04	$\pm$	2.35E+04	2.19E+04	$\pm$	1.14E+04	
<i>Xylose</i>	5.01E+02	$\pm$	5.11E+02	3.67E+02	$\pm$	3.58E+02	3.65E+02	$\pm$	2.41E+02	
<i>Hippurate</i>	3.94E+04	$\pm$	2.25E+04	3.54E+04	$\pm$	2.68E+04	2.04E+04	$\pm$	1.04E+04	DKK vs DKP2= 2.40E-05 DKP1 vs DKP2= 9.00E-03
<i>Threonine</i>	5.86E+02	$\pm$	1.49E+02	5.96E+02	$\pm$	1.85E+02	6.27E+02	$\pm$	2.84E+02	
<i>Tartaric acid</i>	5.90E+02	$\pm$	8.89E+02	4.32E+02	$\pm$	6.80E+02	5.32E+02	$\pm$	1.55E+03	
<i>Trigonelline</i>	8.22E+02	$\pm$	3.45E+02	7.00E+02	$\pm$	4.58E+02	5.82E+02	$\pm$	2.47E+02	DKK vs DKP2= 4.70E-03
<i>Methylnicotinamide</i>	1.35E+02	$\pm$	7.68E+01	1.34E+02	$\pm$	7.05E+01	1.33E+02	$\pm$	6.95E+01	
<i>Allantoin</i>	3.75E+02	$\pm$	1.79E+02	3.83E+02	$\pm$	2.00E+02	3.78E+02	$\pm$	1.80E+02	
<i>Dimethylamine</i>	7.46E+03	$\pm$	2.13E+03	7.18E+03	$\pm$	1.70E+03	7.82E+03	$\pm$	1.26E+03	DKK vs DKP2= 2.70E-03 DKP1 vs DKP2= 2.70E-03
<i>N-N-dimethylglycine</i>	1.37E+03	$\pm$	5.68E+02	1.26E+03	$\pm$	5.58E+02	1.56E+03	$\pm$	1.19E+03	
<i>Trimethylamine</i>	1.01E+03	$\pm$	5.20E+02	1.03E+03	$\pm$	6.33E+02	1.04E+03	$\pm$	7.19E+02	
<i>Arabinose</i>	2.45E+02	$\pm$	9.16E+01	2.64E+02	$\pm$	9.18E+01	2.60E+02	$\pm$	1.18E+02	
<i>Formic acid</i>	4.74E+02	$\pm$	1.83E+02	4.23E+02	$\pm$	1.74E+02	4.02E+02	$\pm$	2.16E+02	
<i>Glycine</i>	6.24E+03	$\pm$	4.04E+03	6.54E+03	$\pm$	3.08E+03	4.75E+03	$\pm$	1.94E+03	DKP1 vs DKP2= 2.80E-02
<i>Acetoacetate</i>	1.04E+03	$\pm$	8.16E+02	1.42E+03	$\pm$	1.16E+03	1.16E+03	$\pm$	9.28E+02	
<i>Ascorbate</i>	1.07E+03	$\pm$	1.09E+03	8.30E+02	$\pm$	5.96E+02	6.31E+02	$\pm$	4.51E+02	DKK vs DKP2= 4.50E-02
<i>Carintine</i>	4.17E+03	$\pm$	3.77E+03	3.06E+03	$\pm$	3.80E+03	3.50E+03	$\pm$	3.19E+03	
<i>Arginine</i>	1.31E+03	$\pm$	9.39E+02	1.43E+03	$\pm$	7.52E+02	1.32E+03	$\pm$	6.31E+02	

Supplementary table 4. Concentrations (mean $\pm$ SD) of the metabolites assigned in serum samples. *P*-values from the comparisons are also reported.

	DKK			DKP1			DKP2			
Metabolites	Mean	$\pm$	SD	Mean	$\pm$	SD	Mean	$\pm$	SD	P-values
<i>Valine</i>	8.83E+02	$\pm$	1.43E+02	5.13E+02	$\pm$	1.31E+02	9.30E+02	$\pm$	1.08E+02	
<i>Isoleucine</i>	1.16E+02	$\pm$	2.84E+01	7.24E+01	$\pm$	2.26E+01	1.20E+02	$\pm$	1.87E+01	
<i>Leucina</i>	8.83E+02	$\pm$	1.96E+02	5.40E+02	$\pm$	1.87E+02	9.25E+02	$\pm$	1.49E+02	
<i>Isobutyrate</i>	5.66E+01	$\pm$	1.44E+01	3.55E+01	$\pm$	1.40E+01	6.27E+01	$\pm$	1.87E+01	
<i>Alpha-oxo isovalerate</i>	6.00E+01	$\pm$	1.18E+01	3.59E+01	$\pm$	9.73E+00	6.88E+01	$\pm$	1.10E+01	DKK vs DKP1= 4.63E-02 DKK vs DKP2=2.50E-03
<i>Hydroxybutyrate</i>	4.49E+01	$\pm$	3.91E+01	4.20E+01	$\pm$	3.52E+01	5.19E+01	$\pm$	3.30E+01	
<i>Lactate</i>	1.91E+03	$\pm$	6.24E+02	1.27E+03	$\pm$	5.16E+02	2.18E+03	$\pm$	4.52E+02	DKK vs DKP1= 3.10E-02 DKP1 vs DKP2=4.30E-02
<i>Alanine</i>	1.20E+03	$\pm$	2.33E+02	7.18E+02	$\pm$	1.79E+02	1.27E+03	$\pm$	2.71E+02	
<i>Acetate</i>	7.88E+01	$\pm$	2.89E+01	5.38E+01	$\pm$	3.56E+01	7.56E+01	$\pm$	4.35E+01	
<i>Glutamine</i>	1.59E+02	$\pm$	2.00E+01	8.93E+01	$\pm$	1.75E+01	1.58E+02	$\pm$	1.83E+01	
<i>Unknown 1</i>	1.08E+02	$\pm$	1.94E+01	6.37E+01	$\pm$	1.16E+01	1.11E+02	$\pm$	2.38E+01	
<i>Acetoacetate</i>	1.14E+02	$\pm$	8.84E+01	1.01E+02	$\pm$	1.30E+02	1.54E+02	$\pm$	1.24E+02	
<i>Pyruvate</i>	1.82E+02	$\pm$	6.79E+01	1.25E+02	$\pm$	5.24E+01	2.01E+02	$\pm$	7.68E+01	
<i>Citrate</i>	2.59E+02	$\pm$	6.20E+01	1.60E+02	$\pm$	3.24E+01	2.64E+02	$\pm$	5.61E+01	
<i>Methionine</i>	4.61E+01	$\pm$	2.36E+01	3.48E+01	$\pm$	2.13E+01	5.21E+01	$\pm$	2.10E+01	
<i>Sarcosine</i>	3.57E+01	$\pm$	4.64E+01	4.11E+01	$\pm$	8.95E+01	3.47E+01	$\pm$	4.85E+01	
<i>Lysine</i>	3.67E+01	$\pm$	2.47E+01	3.07E+01	$\pm$	4.23E+01	3.86E+01	$\pm$	2.66E+01	
<i>Creatine</i>	6.77E+01	$\pm$	4.15E+01	5.46E+01	$\pm$	3.93E+01	6.91E+01	$\pm$	4.60E+01	
<i>Creatinine</i>	2.17E+02	$\pm$	5.49E+01	1.36E+02	$\pm$	4.87E+01	2.40E+02	$\pm$	4.79E+01	DKK vs DKP2=2.58E-02 DKP1 vs DKP2= 8.10E-03
<i>Malonate</i>	2.84E+01	$\pm$	3.72E+01	3.28E+01	$\pm$	2.43E+01	1.41E+01	$\pm$	1.31E+01	
<i>Dimethylsulfone</i>	5.96E+01	$\pm$	2.40E+01	4.18E+01	$\pm$	1.89E+01	7.18E+01	$\pm$	4.23E+01	
<i>Unknown 2</i>	6.41E+01	$\pm$	3.45E+01	4.93E+01	$\pm$	2.92E+01	6.54E+01	$\pm$	3.08E+01	
<i>Methanol</i>	3.35E+02	$\pm$	7.16E+01	2.03E+02	$\pm$	7.35E+01	3.07E+02	$\pm$	6.11E+01	
<i>Glycine</i>	5.42E+02	$\pm$	1.57E+02	3.50E+02	$\pm$	1.70E+02	5.42E+02	$\pm$	1.22E+02	DKK vs DKP1= 2.10E-02
<i>Glycerol</i>	1.67E+02	$\pm$	6.98E+01	1.19E+02	$\pm$	5.51E+01	1.88E+02	$\pm$	7.18E+01	
<i>Proline</i>	6.53E+01	$\pm$	2.11E+01	4.32E+01	$\pm$	1.33E+01	6.45E+01	$\pm$	1.61E+01	
<i>Glucose</i>	2.90E+03	$\pm$	4.16E+02	1.66E+03	$\pm$	3.67E+02	2.99E+03	$\pm$	4.26E+02	DKP1 vs DKP2= 5.00E-03
<i>Tyrosine</i>	1.32E+02	$\pm$	2.62E+01	7.93E+01	$\pm$	1.95E+01	1.42E+02	$\pm$	2.60E+01	DKP1 vs DKP2= 2.80E-02
<i>Histidine</i>	1.07E+02	$\pm$	1.42E+01	6.07E+01	$\pm$	1.31E+01	1.11E+02	$\pm$	2.49E+01	
<i>Phenylalanine</i>	1.29E+02	$\pm$	2.14E+01	7.54E+01	$\pm$	2.79E+01	1.55E+02	$\pm$	2.65E+01	DKK vs DKP2=3.00E-05 DKP1 vs DKP2=1.80E-02
<i>Formate</i>	1.80E+01	$\pm$	3.38E+00	1.07E+01	$\pm$	3.21E+00	1.86E+01	$\pm$	3.92E+00	

4.1.3 Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics

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Analysis of salivary phenotypes of generalized aggressive and chronic periodontitis through nuclear magnetic resonance-based metabolomics

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Leonardo Tenori^{2,3} | Stefano Cacciatore⁴ | Mario Aimetti¹¹Department of Surgical Sciences, C.I.R. Dental School, University of Turin, Turin, Italy²Magnetic Resonance Center (CERM), University of Florence, Sesto Fiorentino, Italy³Department of Experimental and Clinical Medicine, University of Florence, Sesto Fiorentino, Italy⁴Department of Surgery & Cancer, Imperial College, London, UK and International Centre for Genetic Engineering and Biotechnology, Cancer Genomics Group, Cape Town, South Africa**Correspondence**Prof. Mario Aimetti, Section of Periodontology, C.I.R. Dental School, Via Nizza 230 10126 Turin (Italy).
Email: mario.aimetti@unito.it**One-sentence summary:** Metabolomic analysis of saliva discriminates healthy individuals from periodontitis patients, irrespectively of the aggressive or chronic periodontitis profile.**Abstract****Background:** Recent findings about the differential gene expression signature of periodontal lesions have raised the hypothesis of distinctive biological phenotypes expressed by generalized chronic periodontitis (GCP) and generalized aggressive periodontitis (GAgP) patients. Therefore, this cross-sectional investigation was planned, primarily, to determine the ability of nuclear magnetic resonance (NMR) spectroscopic analysis of unstimulated whole saliva to discriminate GCP and GAgP disease-specific metabolomic fingerprint and, secondarily, to assess potential metabolites discriminating periodontitis patients from periodontally healthy individuals (HI).**Methods:** NMR-metabolomics spectra were acquired from salivary samples of patients with a clinical diagnosis of GCP ($n = 33$) or GAgP ($n = 28$) and from HI ($n = 39$). The clustering of HI, GCP, and GAgP patients was achieved by using a combination of the Principal Component Analysis and Canonical Correlation Analysis on the NMR profiles.**Results:** These analyses revealed a significant predictive accuracy discriminating HI from GCP, and discriminating HI from GAgP patients (both 81%). In contrast, the GAgP and GCP saliva samples seem to belong to the same metabolic space (60% predictive accuracy). Significantly lower levels ($P < 0.05$) of pyruvate, *N*-acetyl groups and lactate and higher levels ($P < 0.05$) of proline, phenylalanine, and tyrosine were found in GCP and GAgP patients compared with HI.**Conclusions:** Within the limitations of this study, GCP and GAgP metabolomic profiles were not unequivocally discriminated through a NMR-based spectroscopic analysis of saliva.**KEYWORDS**

biomarkers, magnetic resonance spectroscopy, metabolomics, periodontal diseases, saliva

Periodontitis is a multifactorial, chronic, inflammatory disease that leads to loss of periodontal attachment to the root surface and alveolar bone resorption and, if untreated, ultimately results in tooth exfoliation.¹ It is widely accepted that dysbiosis within the human dental plaque biofilm is the

primary initiator of periodontitis,² even though the extent and severity of tissue destruction appear to be host-mediated.^{3,4}

Periodontitis can have heterogeneous clinical presentations. The traditional classification recognizes two major forms of periodontitis, chronic periodontitis (CP) and

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aggressive periodontitis (AgP), differing in rate of progression, prognosis, and need for specific treatment approaches.⁵ At the present, the diagnosis AgP and CP is primarily based on clinical examination and radiographic parameters.⁵ No clinical, histopathologic, or microbiologic assessment provides an unequivocal discrimination between the two conditions.^{6,7} For this reason, there is a strong effort to discover specific molecular arrays as a diagnostic tool to differentiate CP and AgP by oral-health professionals.⁸

Several molecules in the oral fluids, namely gingival crevicular fluid (GCF) and saliva, have been investigated so far in the attempt to provide detailed understanding of the biochemical network of periodontal tissue destruction.⁹ Saliva is particularly promising as it contains locally produced proteins, as well as other molecules from the systemic circulation.⁸ Furthermore, its collection is noninvasive, rapid, and inexpensive.¹⁰ Salivary diagnostics has already proved efficient in identifying alterations in oral and systemic health status.^{11,12} Even so, various challenges persist regarding the use of saliva as a medium for an accurate and cost-effective detection of periodontitis, mainly because of the lack of specific markers of disease.¹³

Metabolomics is a newly emerging field of research dealing with the high-throughput identification and quantification of the whole ensemble of metabolites (small molecules; <1500 Da) in a cell, tissue, body fluids, or ecological systems.¹⁴ The metabolomics profiling reflects the dynamic response of a living system to genetic modification and physiologic, pathologic, and developmental stimuli.¹⁵ Thus, metabolomics offers the potential for a holistic approach to an individualized, patient-centered medicine.

Compared with other high throughput approaches, the main benefit of metabolomics analysis resides in its ability to take a snap at the very end-point of all the complex causal pathways driving periodontal pathogenesis. Small molecules derived from the dysbiotic community and host tissue breakdown, targeted by metabolomics, are potentially able to reflect the real-time molecular phenotype of the disease.⁹ At the same time, it has been proven that saliva is a stable biofluid and that a clear individual metabolic phenotype can be revealed using saliva samples.¹⁶

Untargeted metabolomics by nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) has been previously employed to differentiate healthy and periodontally diseased individuals through the pattern recognition analysis of saliva and GCF, because this approach has the advantage to maximize the number of metabolites detected, including chemical unknowns.^{17–19} Although some studies provided promising preliminary outcomes regarding the detection of some panels of discriminant metabolites, further trials with larger sample sizes are needed to add consistency and external validity to these results.^{17–20}

To the best of our knowledge, the possibility to employ the NMR-based metabolomics analysis to discriminate CP and AgP remains to be explored. This pilot study was designed to test the hypothesis that untargeted metabolomic analysis of saliva could differentiate the biochemical signatures of the generalized forms of chronic periodontitis (GCP) and aggressive periodontitis (GAgP). The secondary aim was related to the detection of a differentially expressed array of metabolites that could be further investigated as potential biomarkers for the development of a rapid diagnostic tool for periodontitis.

1 | METHODS

The protocol of this cross-sectional study was approved by the Institutional Ethical Review Board (protocol number 1503/2016) and the study was conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from all participants. The study was reported according to the STROBE guidelines.²¹

1.1 | Study population

The sample size was set at 100 individuals based on the results of previous studies^{19,22} and the pilot nature of this study. A total of 33 patients with GCP (mean age: 50.5 ± 8.9 years, 63.6% males and 15.2% smokers), 28 patients with GAgP (mean age: 31.1 ± 4.6 years, 64.3% males and 14.3% smokers), and 39 periodontally healthy individuals (mean age: 46.6 ± 8.2 years, 64.1% males and 15.4% smokers) were consecutively recruited from among individual seeking oral health consultation at the C.I.R. Dental School, University of Turin (Italy) from January to September 2017. After being screened, participants were balanced with respect to gender, and smoking habits.

Exclusion criteria included less than 20 teeth; antibiotic intake within the previous 3 months, periodontal treatment during the past 6 months, abnormal salivary function, diagnosis of any disease in oral and hard tissues and other systemic conditions that could influence periodontal status and metabolomic profile (e.g., diabetes mellitus and metabolic syndromes), regular alcohol consumption, pregnancy and lactation.²³

1.2 | Determination of periodontal status and saliva collection

All participants underwent a periodontal examination by two experienced clinicians (V.M, F.R) who were previously trained and calibrated for the periodontal examination and saliva sampling. A set of full-mouth periapical radiographs was taken for each patient. Presence or absence of plaque

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(PI), presence or absence of bleeding on probing (BoP), probing depth (PD), and clinical attachment level (CAL) were measured at six sites around each tooth by manual probing (PCP UNC 15, Hu-Friedy, Chicago, IL.). Interexaminer reliability was determined by having each examiner made dual measurements along with those of the Project Director (M.A.) on 15 nonstudy patients, and intraexaminer reproducibility was assessed by taking replicate measurements on the same patients with an interval of 24 hours between the first and the second recording. The percentage of agreement within 1 mm of PD and CAL ranged between 94% and 97%.

Patients with GCP and GAgP and healthy controls were diagnosed based on the current classification of the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions⁵ and met the following criteria. GCP patients had $\geq 30\%$ of sites with PD and CAL > 5 mm, and presence of BoP.⁵ Patients in the GAgP group were < 35 years of age, and had at least six permanent first molars and incisors with at least one site with PD and CAL > 5 mm as well as a minimum of six teeth other than first molars and incisors also presenting at least one site each with PD and CAL > 5 mm.²⁴ Other factors such as family aggregation, rapid progression, and the relationship between local factors and periodontal destruction were also considered.⁵ The control group comprised healthy individuals (HI) with PD and CAL ≤ 3 mm at all sites on all teeth, no radiographic evidence of alveolar bone loss, and $< 15\%$ of sites presenting BoP.²⁴

At least 24 hours after periodontal measurements to avoid blood contamination, unstimulated whole saliva was obtained by all study subjects between 8:00 and 10:00 am using standard techniques as described by Silwood et al.²⁵ Briefly, all subjects were advised to refrain from using mouthwash and brushing their teeth at least 1 h before sample collection. Each subject was instructed not to force salivation, to allow saliva to be collected in the mouth, and let the saliva drain into a sterile graduated tube for 10 minutes. About 1 ml of saliva was collected from every patient and immediately frozen.

1.3 | NMR sample preparation

Frozen saliva samples were thawed at room temperature and were centrifuged ($5000 \times g$ for a period of 30 minutes at 4°C) to remove debris. A total of $300 \mu\text{l}$ of sodium phosphate buffer (70 mM Na_2HPO_4 ; 20% (v/v) $^2\text{H}_2\text{O}$; 6.15 mM NaN_3 ; 6.64 mM sodium trimethylsilyl [2,2,3,3- $^2\text{H}_4$] propionate (TMSP); pH 7.4) was immediately added to $300 \mu\text{l}$ of each sample, and the mixture was homogenized by vortexing for 30 seconds. NaN_3 was added as a preservative to ensure that metabolites were not generated or consumed because of bacteria present in the saliva during the time of preparation of the samples or of the acquisition of NMR

spectra. A total of $450 \mu\text{l}$ of this mixture was transferred into a 4.25 mm NMR tube (Bruker BioSpin srl, Milan, Italy.) for analysis.

1.4 | NMR spectral acquisition

NMR spectra for all samples were acquired using a spectrometer (Bruker BioSpin srl, Milan, Italy) operating at 600.13 MHz proton Larmor frequency equipped with a 5 mm CPTCI ^1H - ^{13}C - ^{31}P and ^2H -decoupling cryoprobe including a z axis gradient coil, an automatic tuning-matching (ATM) and an automatic sample changer. A BTO 2000 thermocouple served for temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 3 minutes inside the NMR probehead, for temperature equilibration (300 K). For each saliva sample, a ^1H -NMR spectrum was acquired using the pulse sequence NOESY-presat with 64 free induction decay (FID) collected into 65536 data points over a spectral width of 12019 Hz, relaxation delay (RD) of 4 seconds and mixing time of 0.1 seconds.

1.5 | Spectral processing and analysis

Free induction decays were multiplied by an exponential function equivalent to a 1.0 Hz line-broadening factor before applying Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated using a RMN processing software (Tospin version 2.1, Bruker BioSpin srl, Milan, Italy). Spectra were aligned by calibrating the TMSP peak at 0.00 ppm. Each 1D spectrum in the spectral ranges 0.2–4.3 and 6.6–10.0 ppm was segmented into 0.02 ppm chemical shift bins, and the corresponding spectral areas were integrated using a specific software program (Amix software, version 3.8.4, Bruker BioSpin, Milan, Italy). The binning procedure is a mean to reduce the number of total variables, to compensate for subtle signal shifts, and filter noise in the spectra, making the analysis more robust and reproducible.^{26,27} The total spectral area was calculated on the bins and total area normalization was carried out on the data prior to pattern recognition.

1.6 | Statistical analysis

All data analyses were performed blindly using R statistical package. Significance difference among the clinical groups was calculated using analysis of variance for clinical data and post hoc significance of differences between pairs of comparisons was determined using Fisher least significant difference procedure.

Multivariate statistical analysis tools were applied to study the metabolomics profiles of GCP, GAgP and HI groups.²⁸ The supervised statistical procedure applied for data reduction and classification was a combination of Principal Component

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TABLE 1 Metabolites that result discriminant [median $\pm$ mean absolute deviation (MAD), in arbitrary units] between healthy individuals (HI) and patients with GCP and GAgP

Variable	HI Group (<i>n</i> = 39)	GCP Group (<i>n</i> = 33)	FDR ^a HI vs. GCP	GAgP Group (<i>n</i> = 28)	FDR ^a HI vs. GAgP
Isoleucine	0.131 $\pm$ 0.064	0.202 $\pm$ 0.075	0.0029	0.169 $\pm$ 0.071	0.4120
Valine	0.079 $\pm$ 0.04	0.115 $\pm$ 0.075	0.0037	0.114 $\pm$ 0.081	0.166
Propionate	0.598 $\pm$ 0.207	0.592 $\pm$ 0.265	0.9720	0.681 $\pm$ 0.544	0.3395
Isopropanol	0.05 $\pm$ 0.048	0.028 $\pm$ 0.03	0.1308	0.028 $\pm$ 0.041	0.2916
Ethanol	0.055 $\pm$ 0.045	0.037 $\pm$ 0.032	0.1999	0.052 $\pm$ 0.053	0.8935
Lactate	0.249 $\pm$ 0.62	0.139 $\pm$ 0.059	0.0007	0.087 $\pm$ 0.108	0.0044
Alanine	0.21 $\pm$ 0.083	0.245 $\pm$ 0.107	0.1920	0.207 $\pm$ 0.152	0.9922
Butyrate	0.048 $\pm$ 0.032	0.041 $\pm$ 0.031	0.9031	0.058 $\pm$ 0.043	0.5957
Acetate	13.503 $\pm$ 3.046	13.267 $\pm$ 4.108	0.9412	13.462 $\pm$ 5.744	0.8209
N-acetyl-groups	0.747 $\pm$ 0.463	0.375 $\pm$ 0.229	0.0086	0.352 $\pm$ 0.451	0.0481
Proline	0.045 $\pm$ 0.033	0.071 $\pm$ 0.046	0.0221	0.085 $\pm$ 0.059	0.1171
Pyruvate	0.272 $\pm$ 0.343	0.082 $\pm$ 0.05	0.0001	0.088 $\pm$ 0.063	0.0044
Succinate	0.065 $\pm$ 0.126	0.067 $\pm$ 0.082	0.9720	0.171 $\pm$ 0.124	0.33956
Methylamine	0.023 $\pm$ 0.011	0.023 $\pm$ 0.011	0.9031	0.022 $\pm$ 0.011	0.8714
Sarcosine	0.026 $\pm$ 0.02	0.019 $\pm$ 0.012	0.2134	0.013 $\pm$ 0.007	0.0086
GABA	0.128 $\pm$ 0.066	0.125 $\pm$ 0.085	0.9637	0.158 $\pm$ 0.113	0.6568
Choline	0.197 $\pm$ 0.103	0.205 $\pm$ 0.069	0.9412	0.163 $\pm$ 0.146	0.8483
Methanol	0.083 $\pm$ 0.179	0.082 $\pm$ 0.07	0.9720	0.083 $\pm$ 0.177	0.8209
Glycine	0.626 $\pm$ 0.205	0.65 $\pm$ 0.303	0.1920	0.657 $\pm$ 0.549	0.6707
Tyrosine	0.057 $\pm$ 0.0210	0.08 $\pm$ 0.02	0.0297	0.083 $\pm$ 0.037	0.0481
Phenylalanine	0.092 $\pm$ 0.0412	0.143 $\pm$ 0.044	0.0007	0.1430 $\pm$ 0.044	0.0099
Formate	0.0045 $\pm$ 0.021	0.009 $\pm$ 0.0684	0.1197	0.0134 $\pm$ 0.073	0.0086

^aFalse discovery rate correction. Bold face indicates statistically significant inter-group differences.

Analysis (PCA) and canonical correlation analysis (CA) on the PCA scores. K-nearest neighbors (kNN) learning method ($k = 5$) applied on the CA scores was used to predict test samples. The global accuracy for classification was assessed by means of a Monte Carlo cross-validation scheme. Twenty-two metabolites, corresponding to well defined and resolved peaks in the spectra, were assigned. Signal identification was performed using a library of NMR spectra of pure organic compounds, public databases (e.g. HMDB) storing reference. The relative concentrations of the various metabolites in the different spectra were calculated by spectral fitting and integration of the signal area using in-house scripts (Matlab and Statistics Toolbox, Mathworks Inc., Natick, MA).²⁹ The Wilcoxon test was used for the determination of the statistically relevant metabolites. False discovery rate correction (FDR) was applied using the Benjamini and Hochberg method³⁰; an adjusted P -value ≤ 0.05 was considered statistically significant. The changes in metabolites levels between periodontitis and healthy controls spectra were calculated as the $\log_2$ fold change (FC) ratio of the normalized median intensities of the corresponding signals in the spectra of the two groups. A statistical software program was used for pathway analysis (MetaboAnalyst version 3.0, www.metaboanalyst.ca).³¹

2 | RESULTS

2.1 | Characteristics of the study population

Demographic and clinical characteristics of the 100 participants according to the periodontal diagnosis are shown in supplementary Table 1 in the online *Journal of Periodontology*. The mean age in the GAgP group was significantly less than the other two groups, whereas ages were similar for the GCP and HI groups ($P > 0.05$). Smokers in all the three clinical groups smoked less than 10 cigarettes a day (range 5 to 8).

As expected, the mean Full-Mouth Plaque Scores (FMPS), Full-Mouth Bleeding Scores (FMBS), PD, and CAL values were statistically significantly higher in patients with GAgP and GCP compared with the HI; all reached $P < 0.001$. When GCP and GAgP groups were compared, the only statistically significant difference found was in FMPS ($P < 0.001$).

2.2 | Metabolomic profiling of saliva

The clustering of HI, GCP, and GAgP patients was achieved by using PCA/CA on the ¹H-NMR profiles of saliva samples (Figure 1). These analyses revealed 81% predictive accuracy discriminating HI from GCP, and 81% discriminating HI

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FIGURE 1 PCA/CA score plot built on saliva spectra of healthy (HI) and periodontitis patients (GAgP and GCP). Healthy individuals are very well recognized, whereas the saliva samples of chronic and aggressive periodontitis are confounded within the same metabolic space as suggested by cross-validation result

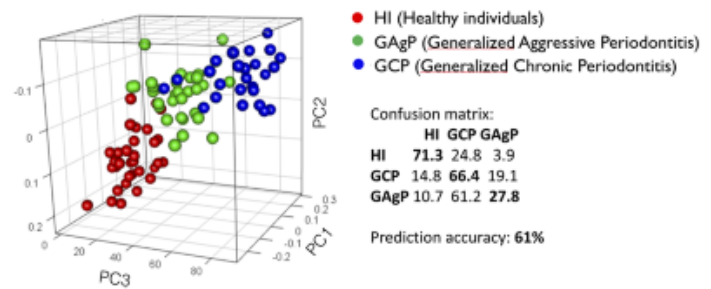


FIGURE 2 PCA/CA score plots. Discrimination between saliva spectra of healthy individuals and chronic periodontitis patients (A) and between healthy and aggressive periodontitis patients (B). In both cases, the discrimination is effective as it arises from the related prediction accuracy

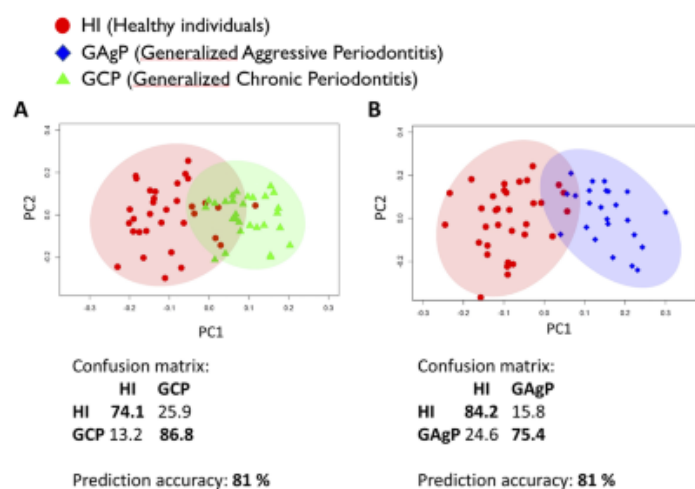
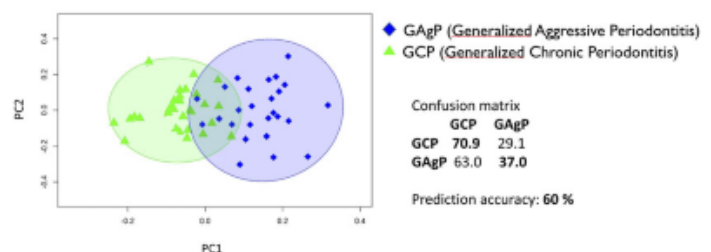


FIGURE 3 PCA/CA score plot. Discrimination between saliva spectra of chronic and aggressive periodontitis subjects. The model is not effective in discriminating the two groups (60% predictive accuracy)



from GAgP affected patients (Figure 2A, B). Permutation test (number of permutations = 1000) results showed statistically significant classification accuracy ($P < 0.001$). The statistical model applied proved to be effective to discriminate HI from GCP and GAgP patients, whereas the same statistical approach was not effective to discriminate GCP from the GAgP counterpart (60% predictive accuracy). Indeed, GAgP and GCP saliva samples seem belonging to the same metabolic space (Figure 3). The predictive accuracy of these models did not change when smokers were excluded from the analysis (81% HI versus GCP, 81% HI versus GAgP, 60% GAgP versus GCP).

2.3 | Metabolites contributing to periodontal disease

The discrimination obtained between saliva samples of GCP and GAgP patients compared with HI, also demonstrated the existence of an altered metabolism in patients affected from periodontitis. Assigned signals in NMR spectra were integrated to obtain the concentration of metabolites in arbitrary units. By comparing the spectra of the saliva samples of periodontitis patients with HI, it results that GCP patients are characterized by lower levels ($P < 0.05$) of pyruvate, *N*-acetyl groups and lactate, and higher levels ($P < 0.05$) of

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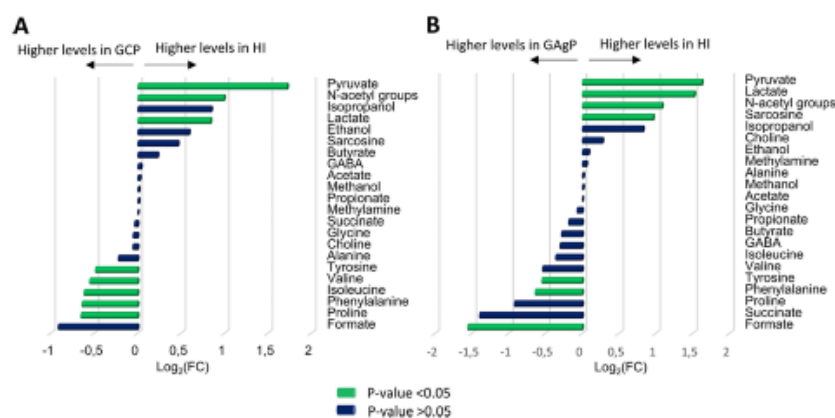


FIGURE 4 Changes in metabolites levels between healthy individuals and chronic periodontitis (A) and between healthy and aggressive periodontitis (B) calculated as the $\log_2$ Fold Change (FC) ratio of the normalized median intensities of the corresponding signals in the spectra of the two groups. Green bars represent significantly altered metabolites ($P < 0.05$)

TABLE 2 An integrated analysis based on MetaboAnalyst software: view of most contributing pathways

Pathway Name	P-value	Holm P-value	FDR ^a	Impact
Phenylalanine metabolism	5.95×10^{-5}	0.005	0.005	0.12
Pyruvate metabolism	0.002	0.127	0.03	0.32

^aFalse discovery rate correction.

proline, phenylalanine, isoleucine, valine and tyrosine, as summarized in Figure 4A and Table 1. Compared with HI, GAgP patients are characterized by lower levels ($P < 0.05$) of pyruvate, N-acetyl groups and lactate and sarcosine, and higher levels ($P < 0.05$) of formate, phenylalanine, and tyrosine (Figure 4B). A simplified list of the most contributing metabolic pathways is reported in Table 2. The analysis showed alteration in biochemical pathways like phenylalanine metabolism (phenylalanine, pyruvic acid, tyrosine, lactic acid) and pyruvate metabolism (pyruvic acid and lactic acid). The analysis was calculated based on adjusted P -value ($P < 0.05$) of the pathway enrichment analysis and an "Impact" (calculated from pathway topology analysis) equal to or greater than 0.1 was considered significant.

3 | DISCUSSION

The present study was designed to test the ability of NMR-based metabolomics to differentiate the biochemical signatures of GCP and GAgP in human saliva. To this purpose, gingivitis and localized manifestations of periodontitis were pointedly excluded because of the risk of flawing the results, and only periodontally HI were selected as controls. However, although corroborating substantial differences

between pathological and healthy periodontal conditions, the multivariate analysis of NMR spectra did not provide a significant discrimination between the GCP and GAgP metabolomics profiles. The latter finding is in agreement with an increasing body of evidence and confirms that it is almost impossible to use the term AgP as long as there is no proper way to diagnose the disease.³² Indeed, the discrimination between CP and AgP is not supported by sufficiently distinct histologic, microbiologic, immunologic, or genetic foundations.^{33–37} Moreover, microbiome exhibits conserved metabolic and virulence gene expression profiles despite the interindividual differences in the disease phenotype.³⁸ This may suggest that what distinguishes AgP from CP are dissimilarities in the immune-inflammatory host response³⁹ or, as advocated by Van der Velden,³² a difference in the degree of bacterial invasiveness. It is unlikely that the sole analysis of the metabolites in oral fluids can detect any pathognomonic benchmarks. Presumably, as far as all new high-throughput technologies have proven the existence of several molecular signatures in distinct periodontal patients,³⁵ the traditional binomial classification seems not to fit this emerging heterogeneity anymore. New models need to be hypothesized and tested, but because of the disease complexity a simultaneous multiomics approach should be elected.

The secondary goal was assessing the potential of oral fluid-based metabolomics to provide robust molecular biomarkers for periodontal diagnosis. Chronic periodontal infections activate the patient's host response to liberate a myriad of metabolic products at the interface between the tooth and the periodontal pocket.¹³ The discrimination between the salivary samples from periodontitis patients, irrespective of the type of disease, and HI strengthens the evidence of a metabolomics trace of periodontitis in human saliva.¹⁷ The Human Metabolome Database reports about 800 metabolites

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detected in saliva. However, according to similar studies, the NMR spectral profiles of this set of subjects are dominated by the signals of 20–30 molecules.^{16–18} The values of the relative concentrations of saliva metabolites were estimated through the integration of the signals in the NMR spectra and were found consistent with the results and the biological interpretation of a previous publication of this same group.¹⁸ The significantly reduced levels of lactate detected in the saliva of patients with GCP are partially explained by its conversion to acetate and propionate by some of the most prevalent periodontal bacterial species.^{40,41} This may reflect on the pyruvate concentrations as the result of the substrate depletion of the L-lactate dehydrogenase. The levels of proline, phenylalanine, isoleucine, valine and tyrosine were higher in the saliva samples of patients with GCP with respect to C, as the amounts (not significant) of fatty acids, dipeptides and monosaccharide. This parallel up-regulation of the lipase, protease and glycosidase activities found in periodontitis is responsible for the overall tissue degradation and offers an ideal environment for bacterial proliferation and immune cells migration.⁴²

The results of the present study should be interpreted with caution, as there are some limitations. One of the major problems with oral metabolites is that there is no way to determine their true origin. They could be essential constituent of the patient unique saliva, they could derive from the breakdown of the host tissues as from the bacterial communities, even from the supragingival plaque. Kuboniwa et al.²² performed supragingival scaling prior to sample collection and found that the discriminating ability of their model was significantly improved. Also, in this study, a scaling session was not performed; being able to find differences in a largely noisy environment could have more impact on the development of a rapid, noninvasive diagnostic tool.

Furthermore, the groups were matched for gender and smoking habits, yet not for age. This was not possible because of the difference in the age of onset of the two clinical forms of periodontitis, therefore GAgP patients were in average younger than GCP patients and the control group was comparable for age only with GCP individuals. Although there are no NMR-based metabolomics studies on the effect of aging on saliva, it is well-known that aging has a drastic effect on the serum metabolome.^{43,44} Concentrations of certain small molecule metabolites in saliva, including some hormones and many pharmaceuticals and drugs of abuse, are known to correlate quantifiably with concentrations in serum. Nonetheless, data from a companion study demonstrated that the salivary NMR fingerprint had low discrimination accuracy between young (14–40 years) and elderly (58–73 years).¹⁸

Finally, because of the limited number of light smokers within this subset, the effect of smoking habits on metabolomics profiling was not specifically analyzed. However, when smokers were excluded from the analysis, the

discrimination accuracy of the predicting models remained unchanged. It was thus plausible to exclude smoking as a confounding factor. This finding corroborates previous data demonstrating that light smoking had a negligible effect on the salivary profile.¹⁸

A major challenge in clinical periodontology is to find a reliable molecular marker of tissue destruction with high sensitivity, specificity and utility. At present, there is still a certain level of noise in metabolites fluctuation occurring in the periodontal microenvironment during the pathogenesis of periodontitis, that is not currently understood.⁴⁵ The hurdle in identifying neat pathological phenotypes is because of the intrinsic heterogeneity of periodontal diseases and the inherent complexity underlying.^{35,46} Presumably, the main concern a clinician should have is not about discriminating between GCP and GAgP, but about the prompt detection of active or inactive phases of supporting tissue breakdown. Future endeavors of salivary biomarkers inquiries should be hence directed towards the real time assessment of disease activity and the molecular characterization of different phenotypes of severe periodontitis. Regarding this issue, metabolomics could be of most interest in future research directions.

4 | CONCLUSIONS

This cross-sectional investigation provided the evidence that NMR-based metabolomics failed to detect an unequivocal biochemical signature discriminating GCP from GAgP. The absence of evidence is not automatically evidence of the absence, but this finding adds consistency to the quest to redefine the current classification of periodontitis. Conversely, the successful differentiation between healthy and diseased individuals corroborated the sensibility of metabolomics profiling as a source of potential panels of biomarkers for molecular diagnostics. Nonetheless, the complex multifactorial etiology of periodontitis will require large clinical trials bringing together a multiomics assessment of saliva to properly address this issue.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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4.1.4 Effect of non-surgical periodontal therapy on salivary metabolic fingerprint of generalized chronic periodontitis using nuclear magnetic resonance spectroscopy

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ABSTRACT

Objective: Metabolomic analysis of saliva proved its accuracy in discriminating patients with generalized chronic periodontitis (GCP) from healthy subjects by identifying specific molecular signatures of the disease. There is lack of investigations concerning the effect of periodontal treatment on individual metabolic fingerprints. Therefore, the aim of this study was to determine whether non-surgical periodontal therapy could change salivary metabolomic profile in GCP to one more similar to periodontal health.

Design: Unstimulated whole saliva of 32 controls and 19 GCP patients were obtained prior to and 3 months after conventional staged non-surgical periodontal therapy. Metabolic profiling was performed using Nuclear Magnetic Resonance (NMR) spectroscopy, followed by univariate and multivariate paired approaches to assess the changes introduced by the therapy.

Results: In GCP group, periodontal treatment led to an improvement in all clinical parameters ($p < 0.001$). The accuracy of the multivariate model in discriminating the metabolomic profile of each GCP patient at two time points was 92.5%. Despite the almost perfect separation of the spectra in the metabolic space, the univariate analysis failed to identify significant variations in single metabolite content. The post-treatment metabolic profile of GCP patients could not be assimilated to that of healthy controls who exhibited different levels of lactate, pyruvate, valine, proline, tyrosine, and formate.

Conclusions: Based on these data, NMR-spectroscopic analysis revealed that, despite significant changes in the overall metabolomic fingerprint after non-surgical therapy, GCP patients maintained a distinctive metabolic profile compared to healthy individuals.

Introduction

Saliva is an easily accessible biofluid and comprises a wide spectrum of both locally synthesized and systemically derived molecules involved in various biological functions (de Almenida Pdel, Grégio, Machado, de Lima, & Azevedo, 2008; Yoshizawa et al., 2013). Detecting changes in the concentration of these compounds allowed identification of individual metabolic fingerprints that can reflect many oral and systemic pathophysiological conditions (Silwood, Lynch, Claxson, & Grootveld, 2002; Dame et al., 2015). Among these conditions, periodontitis is a chronic immune-inflammatory disease with a complex pathophysiology, bringing together microbiological determinant and both host genetic and epigenetic variations (Jiao, Hasegawa, & Inohara, 2014; Kurgan & Kantarci, 2018). Irrespective of the differences among clinical phenotypes of periodontitis, the first phase of conventional periodontal therapy always involves bacterial plaque disruption and thorough cleaning of the root surfaces (Cobb, 2002). Although not resolute in all treated cases, the mechanical removal of the periodontal biofilm has proven effective in controlling inflammation and, generally, in restoring the host-microbial symbiotic state (Mombelli, 2018). However, while advances in technology have introduced high-resolution techniques over the past decades, the determinants affecting the individual response to the treatment remain poorly understood and no biomarkers can accurately estimate the effectiveness of the periodontal therapy (Chambrone, Chambrone, Lima, & Chambrone, 2010; Gul et al., 2017).

Metabolomics is a newly emerging field of research dealing with the high-throughput identification and quantification of small-molecule metabolites in biological fluids and it has the potential to shed new lights into the multilevel complexity related to onset, progression and resolution of periodontitis (Zhang, Sun, Yan, Wang, & Wang, 2015). Nuclear magnetic resonance (NMR) spectroscopy analysis is considered as a robust, reproducible quantitative method (Duchemann et al., 2016) and proved its accuracy in discriminating patients with periodontitis from healthy subjects by identifying specific molecular signatures of the disease in the salivary metabolic phenotype (Aimetti, Cacciatore, Graziano, & Tenori, 2012; Yoshizawa et al., 2013; Kuboniwa et al., 2016; Rzeznik et al., 2017).

This metabolotype remains relatively stable over time and displays traits of individual specificity in periodontally healthy subjects, so there could be a potential way to distinguish which fluctuations from the individual fingerprinting are related to the disease (Wallner-Liebmann et al., 2016). NMR-based metabolomics has been successfully employed in different pathological context providing significant information on oral conditions such as dental caries (Fidalgo et al., 2013), periodontal disease (Aimetti, Cacciatore, Graziano, & Tenori, 2012; Romano et al., 2018), Sjögren's syndrome (Mikkonen et al., 2013) and oral cancer (Mikkonen et al., 2016) as well as on systemic conditions such as dementia (Figueira et al., 2016), neurodegenerative disorders (Yilmaz et al., 2017) and cardiovascular diseases (Ussher, Elmariah, Gerszten, & Dyck, 2016). To date, no attempts have been performed to characterize the changes of human salivary metabolotype after etiological periodontal treatment. Therefore, the present study was conducted to determine whether non-surgical periodontal therapy could change salivary metabolomic profile in patients affected by generalized chronic periodontitis (GCP) to one more similar to periodontal health. This would be important to lay the groundwork for understanding the molecular basis of individual response to periodontal treatment and for identifying molecular targets for diagnostic tests and novel therapies development.

Material and methods

Study design and population

The GCP patients and periodontally healthy controls (H) participating in the study were recruited among the outpatients undergoing periodontal consultation or routine dental examination at the C.I.R. Dental School, Department of Surgical Sciences, University of Turin, Turin (Italy) between January and June 2017. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2002 and was approved by the local Ethical Committee (Protocol n° 1503/2016). Written consent was obtained from all patients prior to the beginning of the study. Due to the lack of previous studies investigating changes in metabolic profile in GCF following periodontal treatment the power analysis was performed retrospectively. According to the Cohen formulation of power analysis²⁴³, and using Wilcoxon as the statistical test (with an alpha of 0.05, and a power of 80%), 32 controls and 19 GCP patients undergoing non-surgical therapy are enough to detect a medium-large effect-size (*Cohen's d* = 0.76). The study appears well powered for this explorative analysis.

The selection and diagnosis of participants was based on the clinical and radiographic criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions (Armitage, 1999). GCP patients were required to have at least 20 natural teeth and to demonstrate radiographic evidence of bone loss, clinical attachment level (CAL) of ≥ 5 mm in $> 30\%$ of intraoral sites and presence of bleeding on probing (BoP) (Lindhe et al., 1999). The severity of periodontal damage was in accordance with the local factors (Armitage, 1999).

The periodontally healthy group had at least 20 natural teeth, no probing depth (PD) and CAL > 3 mm, $\leq 15\%$ of sites with BoP, and no horizontal or vertical bone loss in radiographic examination (Ertugrul, Sahin, Dikilitas, Alpaslan, & Bozoglan, 2013).

All individuals in both groups were in good general health, were non-smokers and none had received periodontal treatment over the previous 6 months or antibiotic medication within the past 3 months. No subjects with a history of systemic conditions such as heart disease, diabetes and other types of disorders that could affect the periodontal tissues or could impact the metabolic profile were included (Guash-Ferré et al., 2016). Enrolled individuals did not use medications that could affect the manifestations of periodontal disease, such as antibiotics, phenytoin, cyclosporine, anti-inflammatory drugs, or calcium channel blockers. None of the women were pregnant or in lactation period.

Clinical Examination and Periodontal Treatment

All participants underwent a comprehensive periodontal examination by an experienced periodontist (GMM). After calibration, a 95.3% concordance within 1 mm for measurements of PD and CAL between the first and the second recording with an interval of 24 h was reached. Clinical measurements were taken at six sites per tooth for all teeth present (excluding third molars), using a North Carolina periodontal probe (Hu-Friedy, IL, USA). Plaque levels (plaque control record) (O'Leary, Drake, & Naylor, 1972), and BoP were recorded dichotomously as present or absent, and PD as well as CAL was measured in millimeter. Full-mouth periapical radiographs were taken with the long cone paralleling using Rinn holders.

After baseline examination, all GCP patients underwent a session of supragingival scaling and received instructions on oral hygiene techniques. One week later, they were subjected to quadrant-wise full-mouth subgingival scaling and root planing (SRP) under local anesthesia in four visits. Mechanical therapy was performed by a single trained periodontist (V.M.) using hand instruments (Grace curettes, Hu-Friedy) and ultrasonic scalers (Cavitron Select, Dentsply,

York, PA, USA) and was completed over a maximum of 28 days. Patients were followed every month for 3 months. Oral hygiene was reinforced and supragingival prophylaxis was carried out at each visit. Clinical monitoring was repeated 3 months post-therapy and the above detailed periodontal parameters were recorded again. All 3-month follow-up visits were completed in December 2017. All data were entered, and statistical analyses were performed at the completion of the 3-month study visits.

Saliva sampling collection

The collection of saliva was done under standardized conditions, between 9:00 and 11:00 a.m., on the day following the clinical examination to prevent blood contamination and was repeated 3 months after non-surgical periodontal therapy for GCP patients (Holmes et al., 1994). Patients and controls were asked to refrain from eating, drinking, brushing and using mouthwash for at least 1 h prior to saliva collection. Each subject was instructed not to force salivation, to allow saliva to be collected in the mouth, and let the saliva drain into a sterile graduated polypropylene tube for 10 min. A minimum of 1.0 ml of unstimulated saliva was collected and stored at -80°C until processing.

Salivary metabolomics profiling

Frozen saliva samples were thawed at room temperature and centrifuged at 5000 g for 30 min to precipitate cells and remove any solid debris. 300 µl of the supernatant were added to 300 µl of sodium phosphate buffer (70 mM Na₂HPO₄; 20 % (v/v) ²H₂O; 6.15 mM NaN₃; 6.64 mM sodium trimethylsilyl [2,2,3,3-²H₄] propionate (TMSP); pH 7.4). An aliquot of 450 µl of this mixture was transferred into a 4.25 mm NMR tube (Bruker Biospin srl, Milan, Italy) for analysis. For each saliva sample, one-dimensional ¹H NMR spectrum was acquired using a standard pulse sequence (noesygpprd.comp, Bruker Biospin) using 128 scans, receiver gain 11.3, 65,536 data points, a spectral width of 12,019 Hz, a relaxation time of 4 s, and a mixing time of 0.1 s. working at 300 K using a Bruker 600 MHz spectrometer. Fourier transformed spectra were automatically corrected for phase and baseline distortions and calibrated using Topspin 2.1 (Bruker Biospin srl). Spectra were aligned to TMSP peak (δ_{H} 0.00 ppm). All spectra were segmented into 0.02 ppm chemical shift bins water resonance region from 4.3 ppm to 6.5 ppm was excluded from the bins), and the corresponding spectral areas were integrated using AMIX software (Bruker BioSpin, version 3.8.4) (Spraul et al., 1994; Holmes et al., 1994). Total area was calculated on the bins and total area normalization was carried out on the data prior to pattern recognition.

Statistical analysis

All calculations were made using the R statistical environment. The Shapiro–Wilk test and Q-Q normality plots were applied to verify the normal distribution of the clinical quantitative variables. The significance of changes in clinical data with time in GCP group was determined using the paired t-test. Differences between GCP and H groups were tested using the unpaired t-test.

Multivariate statistical analysis was applied to study the metabolomics profiles (Madsen, Lundstedt, & Trygg, 2010). The combination of Principal Component Analysis (PCA) and canonical correlation analysis (CA) on the PCA scores was used to discriminate H individuals from GCP patients before and after periodontal treatment. K-nearest neighbors (kNN) learning method (k = 5) was applied on the CA scores to predict test samples. The global accuracy for classification was assessed by means of a Monte Carlo cross-validation scheme.

All metabolites were assigned according to the available literature and reference databases (e.g. HMDB, BIOREFCODE) (Wallner-Liebmann et al., 2016; Gardner, Parkes, Carpenter, & So, 2018). The relative concentration of the various metabolites in the different spectra was calculated by spectral fitting and integration of the signal area (Wishart, 2008). Kruskal-Wallis test followed by Dunn post-hoc analysis test was chosen to infer metabolite differences among three groups (GCP before and after treatment, and H) on the biological assumption that metabolite concentrations are not normally distributed. False discovery rate correction (FDR) was applied using the Benjamini and Hochberg method (2000), an adjusted p-value < 0.05 was considered statistically significant.

The changes in metabolites levels between periodontitis and healthy control spectra were calculated as the log₂ fold-change (FC) ratio of the normalized median intensities of the corresponding signals in the spectra of the two groups. In the GCP group a pairwise analysis was performed to follow the interindividual changes in metabolite levels after non-surgical periodontal treatment. Pairwise Wilcoxon test and multilevel partial least square (MPLS) were used with this aim (van Velzen et al., 2008; Westerhuis, van Velzen, Hoefsloot, Smilde, 2010).

Results

A flowchart of participants in the study is provided (**Supplementary Figure 1**). Saliva samples were collected from nineteen GCP patients (8 males and 11 females, mean age 48.9 ± 7.8 yrs.) and thirty-two controls (14 males and 18 females, mean age of 45.5 ± 7.0 yrs). Participants were balanced with respect to age and gender ($p > 0.05$).

Clinical parameters in GCP and healthy controls are presented in **Table 1**. The number of remaining teeth was similar in both groups. As expected, mean values of all periodontal parameters were significantly higher in GCP than in control group ($p < 0.001$).

The postoperative healing was uneventful in all GCP cases. As reported in **Table 1**, mean FMPS and FMBS decreased significantly in GCP group from baseline to 3 months ($p < 0.001$). At 3 months after therapy, no statistically significant differences were observed for FMPS values between the two groups. Periodontal treatment was also associated with a significant reduction in mean PD and mean CAL values ($p < 0.001$), but they remained significantly higher than in the H group ($p < 0.001$).

In order to evaluate the impact of the non-surgical periodontal therapy on the metabolic signature of GCP, a supervised MPLS pairwise approach was applied. This statistical model seemed to be effective in discriminating the metabolic phenotype of each untreated GCP patient from its treated counterpart with a predictive accuracy of 92.5% (**Fig. 1**). Furthermore, PCA/CA statistical approach was used to assess the separation of H controls profiles from those of GCP patients at baseline (Pre) and 3 months after the completion of the non-surgical treatment (Post), resulting in an overall discrimination accuracy of 82.2% (**Fig. 2A**) and 70% (**Fig. 2B**), respectively. As displayed in Figure 3, GCP treated patients occupied an intermediate metabolic space between their baseline counterpart and H individuals.

Changes in individual metabolites were generally modest when comparing salivary samples collected before and after therapy in GCP, while statistically significant differences in the concentrations of some molecules were identified in periodontitis patients after treatment compared to H controls. The main molecular markers for the observed differences were valine, lactate, proline, pyruvate, tyrosine, and formate (**Table 2, Supplementary Table 1 and Supplementary Figure 2**).

Discussion

To the best of our knowledge, this was the first study conducted in any population regarding the impact of non-surgical periodontal treatment on the salivary metabolic fingerprint of GCP. Our focus on metabolites as biomarkers is based on the fact that metabolites are the final downstream products of the genome and the closest reporters of the functional phenotype of the organism²⁴⁴. Metabolomics looks more workable than the analysis of the proteome or transcriptome because it deals with < 10,000 estimated small molecules in contrast to the millions or tens of thousands of proteins, transcripts and genes. Furthermore, changes in metabolites can be significant even when alterations in the concentrations of proteins and transcripts could not be detectable. Finally, metabolites permit to monitor the pathophysiology of both the host (the individual) and the guest (the microbiome), and their interactions. For this reason, metabolites are susceptible to environmental and external perturbations, even if their levels show a background signature that has proven to be individual and disease-specific (Wallner-Liebmann et al., 2016).

A defined metabolic signature of GCP has been detected in a number of investigations based on the metabolic profile of saliva samples of GCP patients compared to healthy controls, reaching discrimination accuracies up to 84% (Aimetti, Cacciatore, Graziano, & Tenori, 2012; Kuboniwa et al., 2016; Rzeznik et al., 2017). In agreement with previous findings from our group^{47,245}, metabolites defining the periodontal status could mainly reflect bacterial species (lactate, pyruvate, formate)¹⁶⁸ and tissue degradation (proline, phenylalanine, tyrosine)^{246,247}, moreover they can describe host immune response (valine, isoleucine)²⁴⁸.

Selected salivary biomarkers could have utility for monitoring periodontal health and response to therapy (Lee, Chen, Tu, Wu, & Chang, 2018). Previous investigations demonstrated changes in salivary content in terms of pro-inflammatory cytokines and proteinases as a result of non-surgical periodontal treatment (Sexton et al., 2011; Kaushik, Yeltiwar, & Pushpanshu, 2011; Sanchez, Miozza, Delgado, & Busch, 2013). In the present investigation the conventional quadrant-wise non-surgical therapy induced a comprehensive shift in the salivary metabolome of GCP patients as detected in the MPLS score plot. With MPLS approach, the between-subject variations were removed and only the within-subject treatment-related changes were taken into account (van Velzen et al., 2008; Westerhuis, van Velzen, Hoefsloot, & Smilde, 2010). The discrimination accuracy was high, of the order of 92.5%. This result is biologically plausible and can be likely ascribable to a significant quantitative and qualitative change within the oral microbiome and to the overall reduction of periodontal inflammation (Klukowska et al., 2015; Califf et al., 2017). The achieved improvements in clinical inflammatory and disease parameters were in line with those reported in the literature (Hung & Douglass, 2002). Plaque scores were maintained at a low level (<15%) through the study period due to the strict recall program for reinforcement of oral hygiene measures and professional debridement combined with self-performed plaque control.

Interestingly, intra-individual samples collected before and 3 months after treatment were accurately discriminated in the multivariate analysis, but no individual metabolite resulted *per se* statistically significant in the univariate analysis. While the explanation could lie in the sample size, consistent with the explorative nature of the study, it should be taken into account the extreme heterogeneity and variability within the complex interplay of molecules sustaining the pathogenesis of periodontitis. Given the impossibility to reduce this complexity to any single biomarker (Lee et al., 2012) untargeted metabolomics appears to be a promising candidate to study the within- and between-subject differences in the salivary samples through a

multiparametric approach (Barnes et al., 2014). In this case the individual fingerprint could represent the best choice to describe the global response resulting from the sum of microbiome infection and individual immune response¹⁶⁴.

This investigation has also been undertaken to test the hypothesis that periodontitis leads to an imbalance in the individual salivary metabolome that could be partially restored by treatment. Although this could be best corroborated by collecting data before the onset of periodontal disease, due to the inherent difficulties, we compared the salivary metabolome of post-treatment samples with that of a subset of healthy subjects having similar characteristics except for periodontal status.

From the results of the unbiased PCA/CA analysis, it can be inferred that healthy and post-treatment periodontitis samples were projected into distinct metabolomic regions within the 3-dimensional plot. Unlike our expectations, the group of treated patients occupied an intermediate area between the healthy controls and their untreated counterpart. Looking at these data, it did not seem possible to assimilate the treated GCP patients to healthy subjects. Two possible mechanisms can be hypothesized to account for this evidence. First, SRP is not usually resolute against periodontitis and the success of the non-surgical periodontal treatment remains highly unpredictable (Chambrone, Chambrone, Lima, & Chambrone, 2010; Armitage & Xenoudi, 2016). Most of the patients in this sample displayed some sites with persistent inflammation and residual pockets at 3-month reevaluation. Indeed, it could also be speculated that 3 months may represent a too short time to detect any relevant metabolic difference. Furthermore, other strategies, *i.e.* adjunctive delivery of antimicrobials and surgical therapy, may be needed to reach the ultimate goals of periodontal treatment (Lindhe & Nyman, 1975; Becker et al., 2001). The comparison between pre and post-treatment metabolomic profiles focusing only on patients who completely satisfied with the treatment outcomes would presumably allow for ruling out the confounding effect of residual disease after non-surgical therapy.

The second speculation could be that, even though successfully treated, periodontitis patients still hold in their salivary metabolomes a signature of their background susceptibility to the disease. Although a tempting hypothesis, experimental evidences are needed to make it more consistent. Whether this susceptibility lies in the microbiome, in the local immune-inflammatory reactivity or at the systemic metabolic level it is still unknown (Giannobile, 2012; Ebersole et al., 2016).

These findings warrant further investigation. We believe that future research should compare the salivary metabolomes of responders *versus* non-responders to periodontal treatment with the aim of both elucidating the factors responsible for the differential healing phenotype and estimating the specific risk profile of every patient.

Conclusion

NMR-spectroscopic analysis revealed significant changes in the overall metabolomic signature of periodontitis in saliva after the initial non-surgical therapy. However, this effect was not enough to shift the disease-related biochemical profile towards the salivary fingerprint associated with healthy periodontal status. The relatively small sample size, together with the high level of variability in human system biology, likely contributed to the low number of observed differences achieving statistical significance. It remains also not clear which is the exact contribution that microbiome, inflammatory mediators and host specific metabolic signatures (*i.e.* susceptibility) play in determining the intra and interpersonal variability in the salivary metabolome.

Understanding the molecular variations between periodontal health and disease in saliva could lead to the development of more sensitive diagnostic tools and more appropriate interventional strategies for periodontitis. Due to the complex multifactorial etiology of periodontitis, clinical trials with larger sample size are needed to add consistency and external validity to the present results.

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the ethical committee of the “AOU Città della Salute e della Scienza” (Turin, Italy) (Ref: 1503/2016).

Tables and figures

Table 1 Clinical parameters (mean $\pm$ SD) in periodontally healthy individuals and patients with GCP before and after periodontal treatment.

	• Healthy individuals (<i>n</i> =32)	GCP patients (<i>n</i> =19)	
• Parameters		Baseline	3 months
• Sites with BoP (%)	12.7 $\pm$ 2.2	58.4 $\pm$ 23.6 [¶]	13.7 $\pm$ 3.8*
• Sites with Plaque (%)	11.5 $\pm$ 3.2	50.1 $\pm$ 14.7 [¶]	17.6 $\pm$ 4.9 ^{*¶}
• PD (mm)	2.3 $\pm$ 0.4	5.4 $\pm$ 0.8 [¶]	3.7 $\pm$ 0.4 ^{*¶}
• CAL (mm)	2.4 $\pm$ 0.5	6.0 $\pm$ 0.9 [¶]	4.9 $\pm$ 0.7 ^{*¶}
• N° sites with PD $\geq$ 5 mm	0	62.3 $\pm$ 15.3 [¶]	22.2 $\pm$ 8.1 ^{*¶}
• N° teeth	27.0 $\pm$ 1.6	26.3 $\pm$ 2.3	25.9 $\pm$ 2.1

**P* < 0.001 *versus* baseline.

[¶]*P* < 0.001 *versus* healthy controls.

GCP, generalized chronic periodontitis; BoP, bleeding on probing; PD, probing depth; CAL, clinical attachment level.

Table 2 Metabolites that result discriminant (P-values and FDR adjusted P-values) between healthy individuals and GCP patients before and after periodontal treatment.

Metabolites	GCP patients at baseline vs after therapy		GCP patients at baseline vs Healthy individuals		GCP patients after therapy vs Healthy individuals	
	paired Wilcoxon test		Post hoc Dunn test			
	<i>P-value</i>	FDR* adj. <i>P-value</i>	<i>P-value</i>	FDR* adj. <i>P-value</i>	<i>P-value</i>	FDR* adj. <i>P-value</i>
Isoleucine	0.495	0.95	0.0110	0.0330	0.0340	0.0510
Valine	0.832	0.95	0.0050	0.0120	0.0077	0.0120
Propionate	1.000	1.00	0.9100	0.9500	0.8600	0.9500
Isopropanol	1.000	1.00	0.0560	0.1700	0.1770	0.2600
Ethanol	0.304	0.95	0.2400	0.6600	0.4400	0.6600
Lactate	0.671	0.95	0.0027	0.0040	0.0006	0.0018
Alanine	0.393	0.95	0.0960	0.2400	0.9400	0.9400
Butyrate	0.587	0.95	0.1400	0.4200	0.7200	0.7200
Acetate	0.325	0.95	0.4700	0.6400	0.6400	0.6400
N-acetyl-groups	0.043	0.95	0.0947	0.1145	0.0005	0.0016
Proline	0.442	0.95	0.0307	0.0460	0.0074	0.0220
Pyruvate	0.702	0.95	0.0001	0.0003	0.0003	0.0005
Succinate	0.580	0.95	0.5200	0.5200	0.3900	0.5200
Methylamine	0.671	0.95	0.4000	0.6500	0.4300	0.6500
Sarcosine	0.468	0.95	0.0790	0.1180	0.0200	0.0590
GABA	0.776	0.95	0.5300	0.8000	0.3600	0.8000
Choline	0.832	0.95	0.4300	0.7100	0.7100	0.7100
Methanol	0.196	0.95	0.4200	0.8800	0.4400	0.6700
Glycine	0.865	0.95	0.4300	0.8800	0.3800	0.6400
Tyrosine	0.832	0.95	0.0101	0.0150	0.0098	0.0150
Phenylalanine	0.229	0.95	0.0009	0.0028	0.0470	0.0705
Formate	0.325	0.95	0.0732	0.1100	0.0052	0.0160

*False discovery rate correction. Bold face indicates statistically significant ($p < 0.05$) inter-group differences.

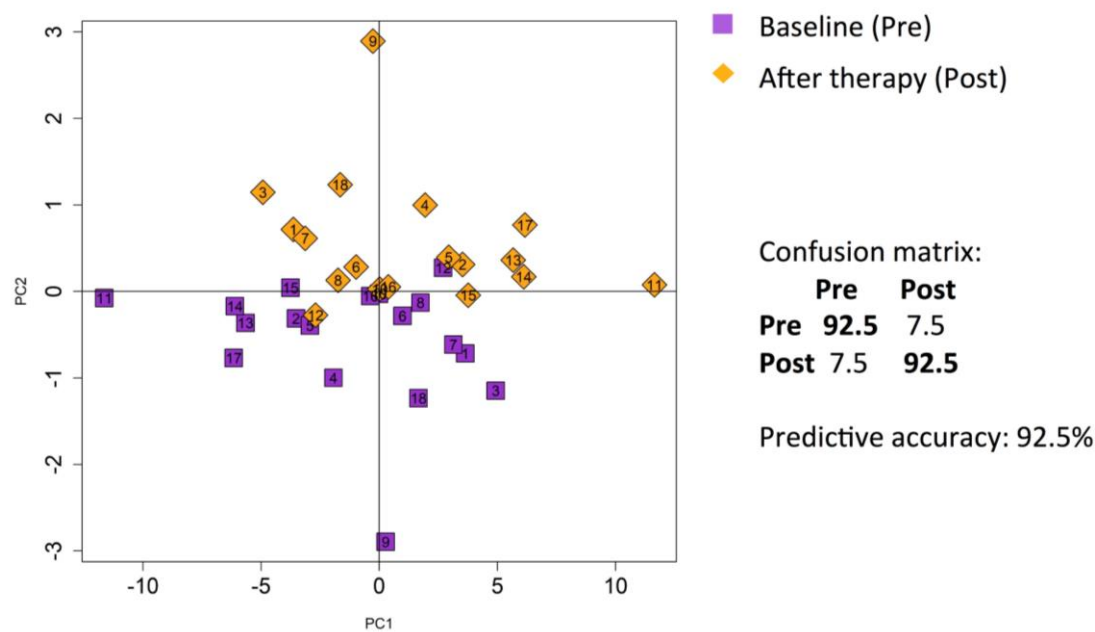


Fig. 1 Score plot of Multilevel PLS (MPLS) pairwise discrimination of pre- (baseline, squares) and post-treatment (after therapy, rhombus) salivary samples in GCP patients. Both sensitivity and specificity are 92.5% in the confusion matrix.

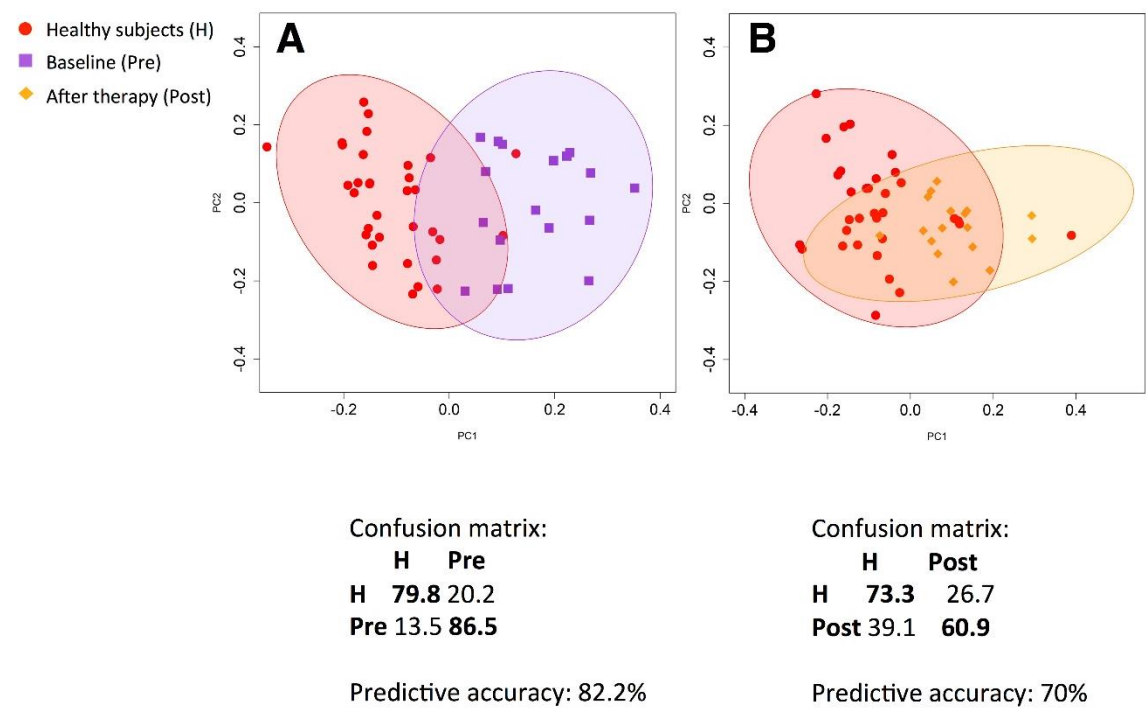


Fig. 2 Score plots of PCA/CA: A) model effective in discriminating healthy subjects (H, dots) and GCP patients at baseline(squares); B) model for the classification of H (dots) and post-treatment GCP patients (rhombus). Confusion matrices and predictive accuracies are reported for both models.

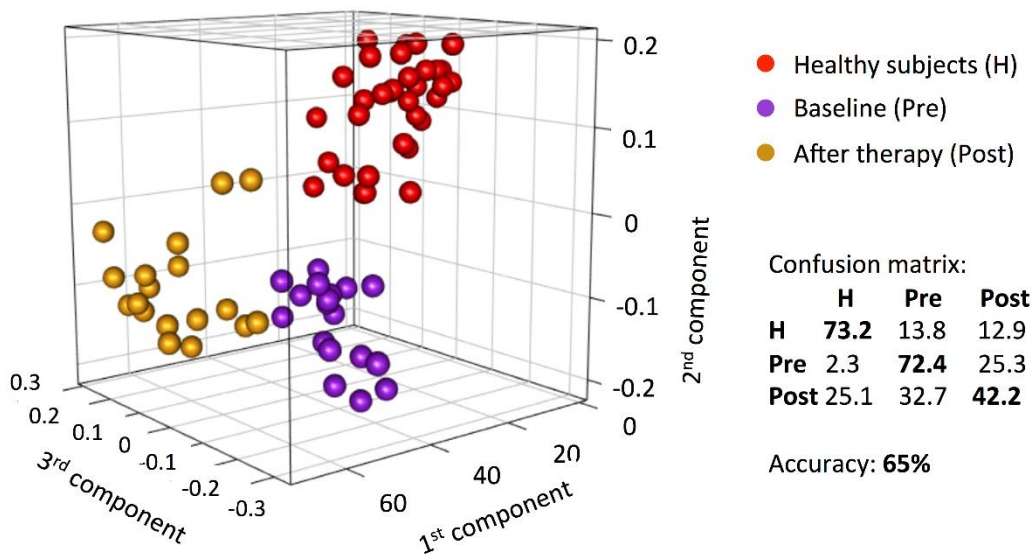


Fig. 3 Projection of the ^1H NMR spectral buckets of the salivary samples into the three most significant dimensions of the PCA/CA considering the subspace for each healthy control (H) and GCP patient before and after periodontal treatment.

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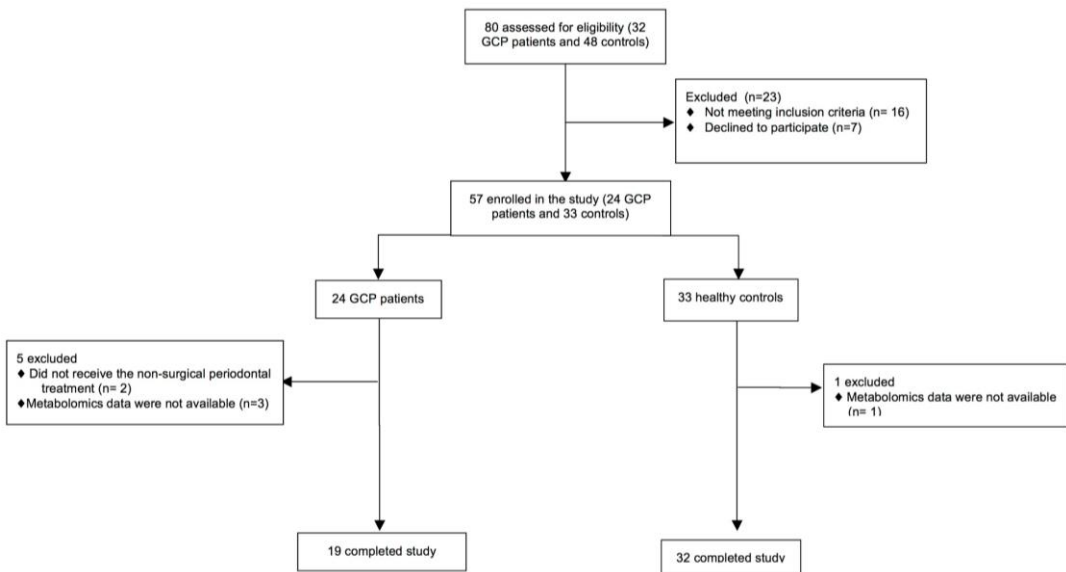
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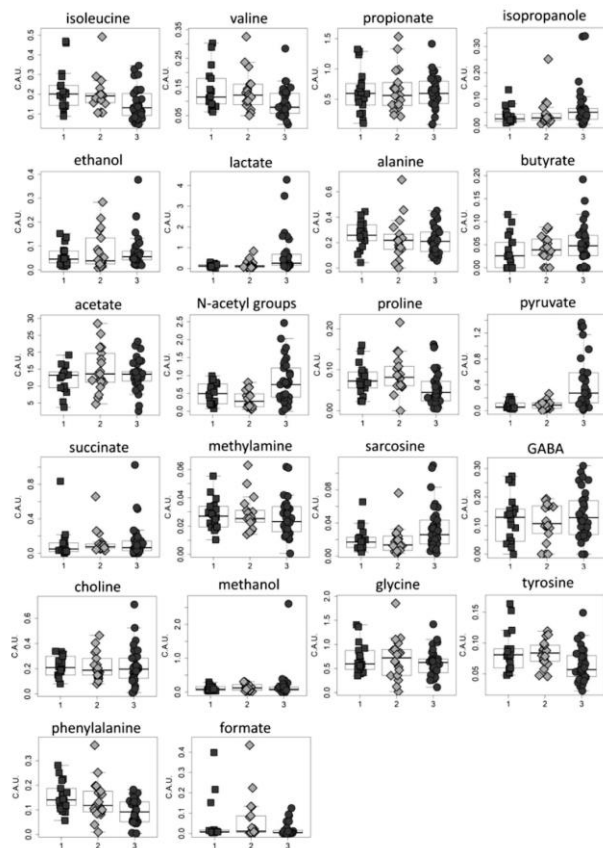
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Supplementary information



Supplementary Fig. 1 Patient flowchart illustrating participant recruitment and disposition.



Supplementary Fig. 2 Box plots of metabolite relative concentrations in GCP patients at baseline (1=squares) and after periodontal therapy (2=rhombus) and in healthy individuals (3= circles).

Supplementary table 1. Relative concentrations (median $\pm$ median absolute deviation [mad]) of the metabolites assigned in saliva samples.

Metabolites	GCP patients at baseline			GCP patients after treatment			Healthy individuals		
	median $\pm$ mad			median $\pm$ mad			median $\pm$ mad		
<i>Isoleucine</i>	0.201	$\pm$	0.077	0.191	$\pm$	0.053	0.131	$\pm$	0.064
<i>Valine</i>	0.114	$\pm$	0.054	0.121	$\pm$	0.049	0.079	$\pm$	0.040
<i>Propionate</i>	0.598	$\pm$	0.274	0.564	$\pm$	0.276	0.598	$\pm$	0.207
<i>Isopropanole</i>	0.026	$\pm$	0.023	0.029	$\pm$	0.034	0.050	$\pm$	0.048
<i>Ethanol</i>	0.045	$\pm$	0.032	0.038	$\pm$	0.064	0.055	$\pm$	0.049
<i>Lactate</i>	0.123	$\pm$	0.060	0.105	$\pm$	0.137	0.249	$\pm$	0.620
<i>Alanine</i>	0.258	$\pm$	0.079	0.218	$\pm$	0.110	0.210	$\pm$	0.083
<i>Butyrate</i>	0.026	$\pm$	0.030	0.039	$\pm$	0.023	0.048	$\pm$	0.033
<i>Acetate</i>	13.239	$\pm$	3.052	13.603	$\pm$	5.251	13.503	$\pm$	3.046
<i>N-acetyl-groups</i>	0.497	$\pm$	0.250	0.280	$\pm$	0.208	0.747	$\pm$	0.463
<i>Proline</i>	0.073	$\pm$	0.028	0.082	$\pm$	0.034	0.045	$\pm$	0.033
<i>Pyruvate</i>	0.058	$\pm$	0.046	0.087	$\pm$	0.049	0.272	$\pm$	0.343
<i>Succinate</i>	0.050	$\pm$	0.103	0.075	$\pm$	0.087	0.065	$\pm$	0.126
<i>Methylamine</i>	0.027	$\pm$	0.008	0.025	$\pm$	0.009	0.023	$\pm$	0.011
<i>Sarcosine</i>	0.017	$\pm$	0.010	0.014	$\pm$	0.011	0.026	$\pm$	0.020
<i>GABA</i>	0.130	$\pm$	0.066	0.107	$\pm$	0.054	0.128	$\pm$	0.067
<i>Choline</i>	0.209	$\pm$	0.065	0.188	$\pm$	0.083	0.197	$\pm$	0.103
<i>Methanol</i>	0.084	$\pm$	0.060	0.123	$\pm$	0.073	0.083	$\pm$	0.179
<i>Glycine</i>	0.598	$\pm$	0.248	0.721	$\pm$	0.328	0.626	$\pm$	0.205
<i>Tyrosine</i>	0.081	$\pm$	0.024	0.084	$\pm$	0.017	0.057	$\pm$	0.021
<i>Phenylalanine</i>	0.141	$\pm$	0.046	0.118	$\pm$	0.061	0.092	$\pm$	0.042
<i>Formate</i>	0.009	$\pm$	0.068	0.012	$\pm$	0.076	0.005	$\pm$	0.021

4.1.5 NMR-Based Plasma Metabolomics at Set Intervals in Newborn Dairy Calves with Severe Sepsis

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Research Article

NMR-Based Plasma Metabolomics at Set Intervals in Newborn Dairy Calves with Severe Sepsis

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The aim of this first study was to reveal the new potential biomarkers by a metabolomics approach in severe septic calves. Sepsis is a common cause of morbidity and mortality in newborn dairy calves. The main challenges with the use of biomarkers of sepsis in domestic animals are their availability, cost, and time required to obtain a result. Metabolomics may offer the potential to identify biomarkers that define calf sepsis in terms of combined clinical, physiological, and pathobiological abnormalities. To our knowledge, this is the first study presenting an NMR- (nuclear magnetic resonance-) based plasma metabolomics at set intervals in neonatal septic calves. Twenty neonatal dairy calves with severe sepsis and ten healthy calves were used. Hematological and biochemical health profiles were gathered in plasma samples at set intervals. Similarly, NMR spectra were acquired. All diseased animals (except one) died after 72 hours. Clinical and laboratory results were in accordance with those of severe septic animals. Multivariate analysis on NMR plasma spectra proved to be an excellent tool for faster identification of calves with severe sepsis from healthy animals. The NMR-based metabolomic profile may contribute to the better understanding of severe sepsis in newborn calves.

1. Introduction

The mortality risk of live-born newborn calves, 1 month of age, has been reported to range from 15 to 30%. The majority of deaths are attributable to infectious diseases; diarrhea, pneumonia, and sepsis are the most common. Sepsis is an acute systemic inflammatory response of the body to microbial infection and a life-threatening condition associated with multiple-organ failure [1]. Newborn sepsis is the third most common cause of calf mortality in the United States (behind diarrhea and respiratory disease) and occurs most commonly in calves associated with failure of passive transfer. The early signs of sepsis in newborn calves are vague and nonspecific and are often indistinguishable from signs of noninfectious diseases or those of focal infections such as diarrhea. Positive blood cultures are required for a definitive antemortem

diagnosis of sepsis, but results are not reported for 48–72 hours, and false-negative culture findings are common. The high mortality of sepsis can be seen as an indication of insufficient laboratory diagnostics [2]. Unfortunately, despite many advances, the majority of laboratory investigations are not sufficiently sepsis specific. The laboratory diagnosis of sepsis is thus a mosaic of different technological and methodological approaches which are vital to the subsequent integration of the clinical picture and outcome of the septic patient [3]. Fortunately, the advent of “omic” technologies may allow for increased diagnostic support. The metabolomics approach offers the possibility to identify variations in fingerprint and metabolite profile that can be used to discriminate disease [4]. Metabolomics may become a promising tool for diagnosing newborn sepsis and monitoring therapy in the near future. Taking into consideration the

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studies carried out so far, it is reasonable to argue that the metabolomics technique can be considered an effective tool for the diagnosis of sepsis [5, 6]. While metabolomic studies are often encountered in human medicine for newborn sepsis [7–14], there is little knowledge related to the same approach for calf sepsis. The aim of the present study was to identify metabolomic biomarkers of sepsis in plasma by ^1H -NMR spectroscopy to assess the severity and to predict outcomes.

2. Materials and Methods

2.1. Animals and Sepsis Criteria. Twenty newborn calves presented for treatment to the teaching hospital, 1–15 days of age with severe sepsis, and 10 clinically healthy age-matched control calves belonging to the faculty farm were used in this study. Inclusion criteria were the following: SIRS (systemic inflammatory response syndrome) criteria: body temperature $>39^\circ\text{C}$ or $<36^\circ\text{C}$, heart rate <100 or >160 beats/min, respiratory rate $>65/\text{min}$ or $\text{pCO}_2 > 50\text{ mmHg}$, total leukocyte count $>12,000/\text{mm}^3$ or $<4,000/\text{mm}^3$, or band neutrophil $>10\%$; sepsis criteria: at least two results above or more than SIRS + infection or infection suspected; and severe sepsis criteria: 4 sepsis results together with at least one organ failure (acute lung damage; coagulation abnormalities; thrombocytopenia; mental state changes; and liver, kidney, or/and cardiac insufficiency or acidosis due to hypoperfusion) [15].

2.2. Blood Sampling. Diseased calves were treated by antibiotics and fluids, and critical care was performed, when needed. Their blood samples were taken at set intervals (0, 3, 6, 12, 48, and 72 h) after admission to the animal hospital, and serum and plasma samples were harvested by centrifuging the blood at $1000 \times g$ for 15 min at 4°C .

2.3. Laboratory Analysis. Laboratory workup including complete blood count analysis (blood cell counts, hematocrit, hemoglobin, MCV, MCH, and MCHC), blood gas analysis (pH, pO_2 , pCO_2 , HCO_3^- , base excess, and O_2 saturation), and biochemical analyses (in serum samples: total protein, albumin, BUN, creatinine, lactate, AST, ALT, ALP, GGT, Na, and K) was performed by use of automated analyzers in the teaching hospital's clinical laboratory.

2.4. ^1H -NMR Sample Preparation. NMR measurements were performed at the CERM/CIRMMP center of the ESFRI Instruct, University of Florence, in Florence, Italy. $350\text{ }\mu\text{L}$ of each plasma sample was added to a total of $350\text{ }\mu\text{L}$ of a phosphate sodium buffer (70 mM Na_2HPO_4 ; 20% (v/v) $^2\text{H}_2\text{O}$; 0.025% (v/v) NaN_3 ; 0.8% (w/v) sodium trimethylsilyl [2,2,3,3- $^2\text{H}_4$] propionate (TMSP), pH 7.4), and the mixture was homogenized by vortexing for 30 s. A total of $600\text{ }\mu\text{L}$ of this mixture was transferred into a 5 mm NMR tube (Bruker BioSpin s.r.l.) for the analysis.

2.5. NMR Analysis. Monodimensional ^1H -NMR spectra for all samples were acquired using a Bruker 600 MHz spectrometer (Bruker BioSpin s.r.l.; Rheinstetten, Germany) operating at 600.13 MHz proton Larmor frequency and equipped with

a 5 mm PATXI 1H-13C-15N and 2H decoupling probe including a z-axis gradient coil, an automatic tuning and matching (ATM), and an automatic and refrigerated sample changer (SampleJet, Bruker BioSpin s.r.l.; Rheinstetten, Germany). A BTO 2000 thermocouple was served for temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 5 minutes inside the NMR probehead, for temperature equilibration (310 K for plasma samples).

For each sample, three one-dimensional proton NMR spectra were acquired with different pulse sequences, allowing the selective detection of different molecular components [16].

- (i) A standard nuclear Overhauser effect spectroscopy pulse sequence NOESY 1Dpresat (noesygprr1d.comp; Bruker BioSpin) pulse sequence, using 32 scans, 98,304 data points, a spectral width of 30,045.9 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s, and a mixing time of 0.1 s, and with water peak suppression [17], was applied to obtain a spectrum which are visible signals of metabolites and high molecular weight macromolecules (lipids and lipoproteins).
- (ii) A standard spin echo Carr-Purcell-Meiboom-Gill (CPMG) [18] (cpmgrp1d.comp; Bruker BioSpin) pulse sequence applied to a standard 1D sequence, with 32 scans, 73,728 data points, a spectral width of 12,019 Hz, and a relaxation delay of 4 s, was used for the selective observation of low molecular weight metabolites, suppressing signals arising from macromolecules.
- (iii) A standard diffusion-edited [19] (ledbgprr2s1d.comp; Bruker BioSpin) pulse sequence, using 32 scans, 98,304 data points, a spectral width of 18,028 Hz, and a relaxation delay of 4 s, was applied to suppress metabolite signals.

The acquisition conditions used in this work are perfectly in line with what is validated and recommended in literature [20]. These conditions are suggested by Bruker (the NMR machine vendor) for metabolomic analysis [21].

2.6. Spectral Processing. Free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line-broadening factor before applying Fourier transform.

Transformed spectra were automatically corrected for phase and baseline distortions and calibrated to anomeric glucose doublet at 5.24 ppm using TopSpin 3.2 (Bruker BioSpin s.r.l.).

Each 1D spectrum in the range between 0.2 and 10.00 ppm was segmented into 0.02 ppm chemical shift bins, and the corresponding spectral areas were integrated using AMIX software (version 3.8.4, Bruker BioSpin). Binning is a means to reduce numbers of total variables and to compensate for small shifts in the signals, making the analysis more robust and reproducible [22]. Regions containing residual water signal were removed.

2.7. Statistical Analysis. For laboratory data, normality test was performed to explain if all data are parametric or non-parametric. For comparing the parametric values, ANOVA and Tukey tests were performed and calculated as mean $\pm$ SD. For comparing the nonparametric ones, Mann-Whitney *U* test was performed and presented as median (SPSS 21.0). Statistical significance was evaluated as *P* value < 0.05 .

Principal component analysis (PCA) was used as a first exploratory analysis. Orthogonal projections to latent structures (OPLS) was chosen as supervised technique. OPLS is a multivariate projection method which is frequently applied for modelling spectroscopic data. This algorithm is able to separate "response-related" and "response-orthogonal" variation in data, providing benefits in terms of model interpretation compared to PCA or to PLS [23]. All the accuracies reported and the confusion matrix for different classifications were assessed by means of 100 cycles of a Monte Carlo cross-validation scheme (MCCV, R script in-house developed). In this case, 90% of the data were randomly chosen at each iteration as a training set to build the model. Then the remaining 10% was tested and sensitivity, specificity, and accuracy for the classification were assessed. The procedure was repeated 100 times to derive an average discrimination accuracy for each group of subjects.

For metabolomic data, twenty-two metabolites, corresponding to well-defined and resolved peaks in the spectra, were assigned. Signal identification was performed using a library of NMR spectra of pure organic compounds, public databases (e.g., HMDB), and literature data.

The molecule 1,4-dioxane was used as reference standard to perform a quantitative NMR (qNMR) analysis on the plasma samples to obtain absolute concentration (μM) values of the metabolites analyzed. The concentrations of the various metabolites in the different spectra were calculated by spectral fitting and integration of the signal area using in-house MATLAB® scripts and the concentrations of peak integrals are compared to the internal standard peak integral to obtain absolute concentrations, using cpmg spectra. The use of CPMG spectra for quantitative analysis is well supported by literature [24]. Kruskal-Wallis test followed by Dunn post hoc analysis was chosen to infer metabolite differences among the groups on the biological assumption that metabolite concentrations are not normally distributed. False discovery rate correction was applied using the Benjamini-Hochberg method (FDR) and adjusted *P* value < 0.05 was considered statistically significant [25]. MetaboAnalyst 3.0 was used for pathway analysis [26].

3. Results

3.1. Clinical and Laboratory Data. The diseased calves were recumbent, weak, dehydrated ($\geq 8\%$), and hypothermic ($T = 36.8^\circ\text{C}$). Poor pulse quality and occasional pulse deficits were revealed by palpation and auscultation, and heart rate (92 beats/min) was within normal limits except two animals in which atrial standstill was observed. The animals had metabolic acidosis, hyperkalemia, lactatemia, and leukocytosis. O₂ saturation remained decreased, and BUN, creatinine, and AST increased at set intervals in spite of therapy

(Supplemental Tables 1, 2 and 3). Three animals died at the 24th hour and at the 48th hour, and two animals at the 72nd hour. The remaining animals (11) died after the 72nd hour.

3.2. NMR-Based Metabolites. NMR spectra of all 113 plasma samples were acquired, sample n°15 (0 h) was removed from our analysis because the spectrum was of bad quality. Processed plasma samples of newborn calves, collected at different time points (Healthy animals (Ha); diseased animals at 0 hour; diseased animals at the 3rd hour; diseased animals at the 6th hour; diseased animals at the 24th hour; diseased animals at the 48th hour; and diseased animals at the 72nd hour), have been analyzed firstly using the unsupervised multivariate method (PCA) to gain an overview on the main changes responsible for sepsis. Figure 1 shows the PCA score plots on 1D NOESY, CPMG, and diffusion-edited spectra of plasma samples. Unsupervised principal component analysis of all plasma spectra is sufficient to discriminate, in all the three experiments, healthy animals (Ha, yellow dots) from calves with sepsis, and particularly 0 h blood samples (brown dots) can be distinguished from the other time points, suggesting that from the 3rd to the 72nd hour, plasma profile/metabolism of newborn calves with sepsis is very similar.

Subsequently, the OPLS-supervised method was employed to extract and visualize latent and hidden variation characteristics of sepsis progression. OPLS models were built on NOESY, CPMG, and diffusion-edited experiments, respectively (Figures 2(a)–2(c)) and the first 10 components were retained in the model. All the three models, as shown by cross-validations (Figure 2), are able to excellently identify healthy animals (Ha) with 90% and 100% accuracy using OPLS models built on NOESY and CPMG experiments, respectively. Interestingly, 0 h plasma samples appear the most discriminated, while from the 3rd hour to the 72nd hour, each group cannot be recognized with good accuracy. Using the same statistical approach, discrimination analysis was also attempted to check whether the metabolic profile at time 0 differed between animals that died early from those that died later to check if the metabolic profile can predict animal outcome before starting the therapeutic treatment. However, the predictive accuracies obtained from the models were very low (predictive accuracy on the NOESY model: 39%, predictive accuracy on the CPMG model: 44%, and predictive accuracy on the diffusion-edited model: 38%); this unsatisfactory result can be ascribed to the fact that the number of samples per group is too low or also because all animals (except one) died after 72 hours leading to the conclusion that all individuals share the same pathological fingerprint.

NMR spectra were also analyzed to identify which metabolites are altered in the seven groups of calves. The twenty-two identified and quantified metabolites are listed in Table 1; *P* values are reported only for metabolites that differ significantly (*P* value < 0.05) (Figure 3). Diseased samples at 0 hour compared with healthy animals appeared to be richer in isoleucine, leucine, valine, alanine, creatine, phosphocreatine, glycine, phenylalanine, and histidine which decreased gradually along hours. Allantoin, 3-hydroxybutyric

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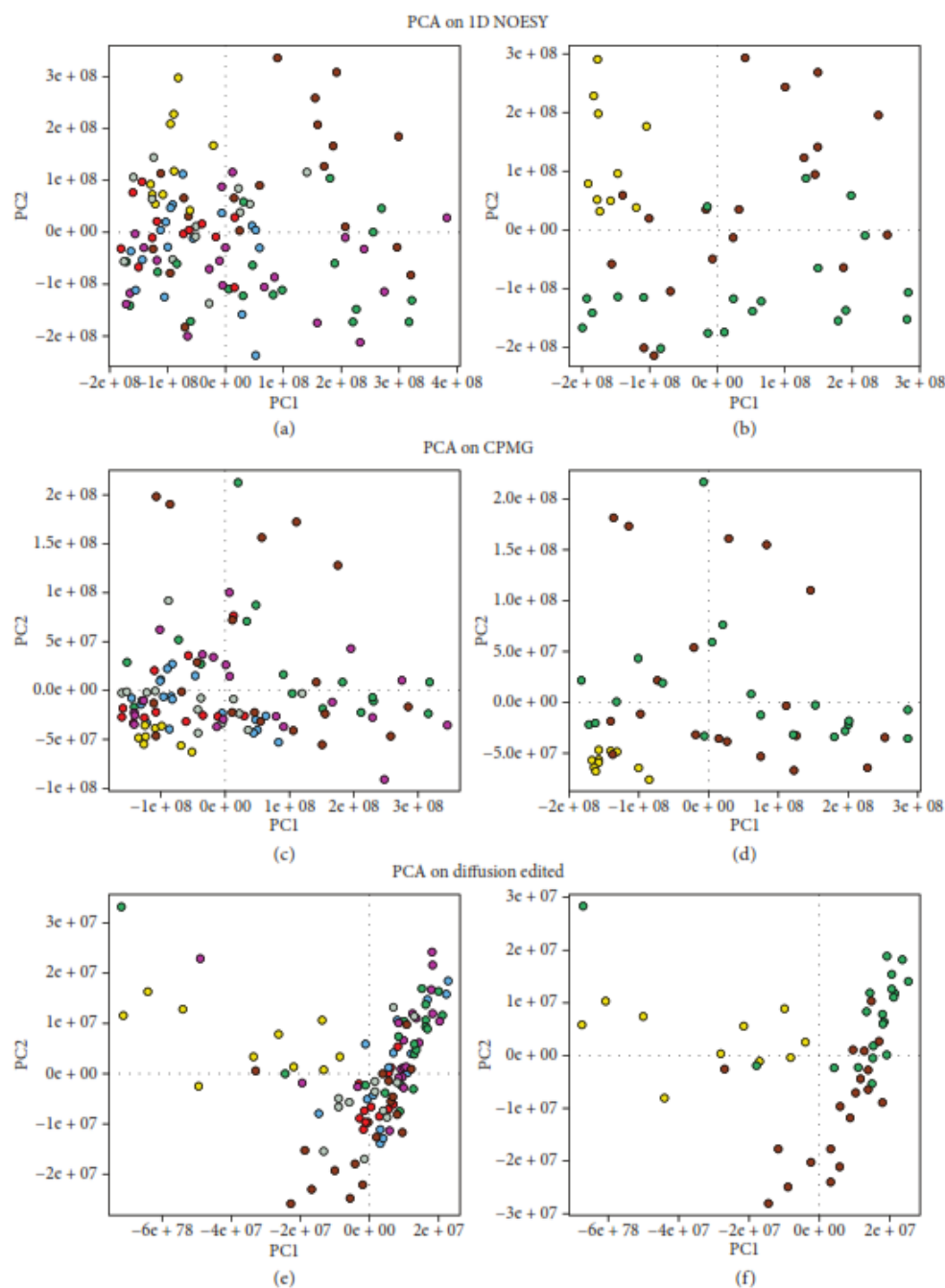


FIGURE 1: Principal component analysis (PCA) score plots. Each dot represents a single NMR spectrum of plasma samples collected at different time points: healthy animals (Ha, yellow dots), 0 hour (brown dots), 3rd hour (green dots), 6th hour (magenta dots), 24th hour (light-blue dots), 48th hour (gray dots), and 72nd hour (red dots). (a) PCA on all 1D NOESY spectra; (b) PCA on Ha, 0 h, and 3rd-hour 1D NOESY spectra; (c) PCA on all CPMG spectra; (d) PCA on Ha, 0 h, and 3rd-hour CPMG spectra; (e) PCA on all diffusion-edited spectra; (f) PCA on Ha, 0 h, and 3rd-hour diffusion-edited spectra.

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TABLE 1: Concentrations in μM (mean $\pm$ SD) of the metabolites assigned in plasma samples. Significantly different P values from the comparisons are also reported.

Metabolites (μM)	Healthy animals ($n = 10$)	0 ($n = 19$)	3rd ($n = 20$)	Diseased animals (hours) 6th ($n = 20$)	24th ($n = 17$)	48th ($n = 14$)	72nd ($n = 12$)	P value
Isoleucine	98.8 $\pm$ 28.3	147.8 $\pm$ 67	122.3 $\pm$ 57.4	98.5 $\pm$ 53	82.3 $\pm$ 36.4	111.5 $\pm$ 27.5	106.1 $\pm$ 36.5	=0.01 (0 h versus 6 h) <0.001 (0 h versus 24 h) <0.05 (3 h versus 24 h)
Valine	227.3 $\pm$ 76.2	434.7 $\pm$ 182.7	380.6 $\pm$ 157	323.6 $\pm$ 144.5	232.9 $\pm$ 80.6	304.6 $\pm$ 56.8	284.9 $\pm$ 100.1	<0.05 (Ha versus 0 h) <0.001 (0 h versus 24 h) =0.005 (0 h versus 24 h) <0.05 (0 h versus 72 h)
Leucine	405.7 $\pm$ 111.2	851.5 $\pm$ 421.8	701 $\pm$ 355	586.4 $\pm$ 303.6	425 $\pm$ 145.3	538.9 $\pm$ 144.2	483.6 $\pm$ 137.9	=0.001 (Ha versus 0 h) <0.05 (Ha versus 3 h) <0.001 (0 h versus 24 h) <0.05 (3 h versus 24 h) <0.05 (0 h versus 72 h)
Alanine	236.9 $\pm$ 142.3	398.1 $\pm$ 154.2	341.8 $\pm$ 333.4	274.6 $\pm$ 191.6	226.2 $\pm$ 51.5	293 $\pm$ 123.2	242.2 $\pm$ 97	<0.05 (Ha versus 0 h) <0.05 (0 h versus 3 h) <0.05 (0 h versus 6 h) <0.05 (0 h versus 24 h) <0.05 (0 h versus 72 h)
3-Hydroxybutyric acid	61.4 $\pm$ 44.6	94.2 $\pm$ 38.9	129.8 $\pm$ 105	134 $\pm$ 146	84.4 $\pm$ 44.2	111.2 $\pm$ 118.2	124.7 $\pm$ 132	<0.05 (Ha versus 3 h) <0.05 (Ha versus 0 h) <0.0001 (Ha versus 3 h) <0.00001 (Ha versus 6 h) <0.05 (Ha versus 24 h) <0.05 (Ha versus 48 h) <0.05 (Ha versus 72 h)
Isobutyric acid	10.9 $\pm$ 3.7	27.1 $\pm$ 16.14	40 $\pm$ 17.8	41.2 $\pm$ 17.9	22.7 $\pm$ 8.5	21.6 $\pm$ 9.8	21.6 $\pm$ 10.6	<0.05 (0 h versus 3 h) <0.05 (0 h versus 6 h) <0.05 (3 h versus 24 h) <0.05 (3 h versus 48 h) <0.05 (3 h versus 72 h) <0.05 (6 h versus 24 h) <0.05 (6 h versus 48 h) <0.05 (6 h versus 72 h)
Acetic acid	54 $\pm$ 17	234.24 $\pm$ 536	110.31 $\pm$ 54.44	86.9 $\pm$ 30.87	116.7 $\pm$ 115.5	77.5 $\pm$ 62.3	67.6 $\pm$ 43.7	
Acetone	6.7 $\pm$ 8.6	10 $\pm$ 9.6	14.6 $\pm$ 11.8	15.4 $\pm$ 14.6	15.4 $\pm$ 11.4	16.6 $\pm$ 25.5	26.3 $\pm$ 33.7	=0.05 (Ha versus 3 h) =0.05 (Ha versus 6 h) =0.05 (Ha versus 24 h) =0.05 (Ha versus 72 h)
Choline	68.5 $\pm$ 29.3	66.8 $\pm$ 29.5	47.5 $\pm$ 18.2	32 $\pm$ 13.5	29.6 $\pm$ 11.7	32.7 $\pm$ 12.3	33 $\pm$ 14.6	<0.001 (Ha versus 6 h) <0.001 (Ha versus 24 h) <0.05 (Ha versus 48 h)

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TABLE 1: Continued.

Metabolites (μM)	Diseased animals (hours)					P value
	0 (n = 19)	3rd (n = 20)	6th (n = 20)	24th (n = 17)	72nd (n = 12)	
Creatine	168.8 $\pm$ 74.5	702.9 $\pm$ 397	628.4 $\pm$ 431.3	533.5 $\pm$ 401.6	309 $\pm$ 144	305 $\pm$ 146.3
Phosphocreatine + creatinine	108 $\pm$ 30.8	399.3 $\pm$ 153.5	378.4 $\pm$ 226.8	333.2 $\pm$ 221	219 $\pm$ 111.2	219 $\pm$ 75.5
Formic acid	51 $\pm$ 17.8	27.3 $\pm$ 10.4	24.6 $\pm$ 9.3	24.7 $\pm$ 9.2	43.4 $\pm$ 16	54.3 $\pm$ 22.3

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TABLE 1: Continued.

Metabolites (μM)	Healthy animals ($n = 10$)	0 ($n = 19$)	3rd ($n = 20$)	6th ($n = 20$)	Diseased animals (hours)	24th ($n = 17$)	48th ($n = 14$)	72nd ($n = 12$)	P value
D-Glucose	1400 $\pm$ 245.5	1811 $\pm$ 1196	1351 $\pm$ 709.5	1816 $\pm$ 1226.4	1697 $\pm$ 652	1783 $\pm$ 439	1714.4 $\pm$ 142.3		
Glycine	171.2 $\pm$ 76.1	223.8 $\pm$ 90.5	160.3 $\pm$ 137	142.2 $\pm$ 86	176.3 $\pm$ 68.2	198 $\pm$ 73.2	170 $\pm$ 77.25		<0.05 (0 h versus 3 h) <0.05 (0 h versus 6 h) <0.05 (3 h versus 48 h) <0.05 (6 h versus 48 h)
Phenylalanine	57.2 $\pm$ 16.7	112.2 $\pm$ 52.7	95.2 $\pm$ 50	77.6 $\pm$ 44.8	83 $\pm$ 66.5	71.3 $\pm$ 17.9	71.6 $\pm$ 21		<0.05 (Ha versus 0 h) <0.05 (Ha versus 3 h) <0.05 (0 h versus 6 h) <0.05 (0 h versus 24 h) <0.05 (3 h versus 6 h)
Proline	108.7 $\pm$ 34.2	84 $\pm$ 38.8	92.8 $\pm$ 14.5	71.2 $\pm$ 21.3	43 $\pm$ 14.5	63.3 $\pm$ 32.2	48 $\pm$ 16.6		<0.05 (Ha versus 3 h) <0.05 (Ha versus 6 h) <0.05 (Ha versus 24 h) <0.05 (Ha versus 48 h) <0.05 (Ha versus 72 h) <0.05 (0 h versus 3 h) <0.05 (0 h versus 6 h) <0.05 (0 h versus 72 h)
Pyruvic acid	21.8 $\pm$ 16.7	49.6 $\pm$ 56	51.2 $\pm$ 21.3	56 $\pm$ 26.8	48.8 $\pm$ 22.6	42.4 $\pm$ 24.4	39.5 $\pm$ 24.3		<0.05 (Ha versus 3 h) <0.05 (Ha versus 6 h) <0.05 (Ha versus 24 h)
Tyrosine	44.64 $\pm$ 23.9	53.39 $\pm$ 16.64	50.74 $\pm$ 27.90	41.22 $\pm$ 17.07	36.54 $\pm$ 14.87	35.89 $\pm$ 12.76	34.62 $\pm$ 14.93		
Dimethyl sulfone	7.91 $\pm$ 1.94	15.06 $\pm$ 8.74	14.11 $\pm$ 9.1	13.19 $\pm$ 9.37	11.91 $\pm$ 6.63	11.15 $\pm$ 4.93	10.54 $\pm$ 4.87		
Allantoin	71.8 $\pm$ 35.05	501.4 $\pm$ 238.9	423.4 $\pm$ 213	376.4 $\pm$ 200.2	256.8 $\pm$ 169.2	268.3 $\pm$ 155.8	238.7 $\pm$ 165.7		<0.00001 (Ha versus 0 h) <0.00001 (Ha versus 3 h) <0.001 (Ha versus 6 h) <0.05 (Ha versus 24 h) <0.05 (Ha versus 48 h) <0.05 (Ha versus 72 h) <0.05 (0 h versus 24 h) <0.05 (0 h versus 48 h) <0.05 (0 h versus 72 h) <0.05 (3 h versus 24 h) <0.05 (3 h versus 72 h)
GPC	32.9 $\pm$ 55.8	44.7 $\pm$ 27	34.9 $\pm$ 27.4	27.6 $\pm$ 23.4	25 $\pm$ 17.8	25.5 $\pm$ 25.4	28.8 $\pm$ 29.9		
Histidine	98.6 $\pm$ 50.6	151.5 $\pm$ 62.2	120.3 $\pm$ 86.9	110.7 $\pm$ 68.9	87.4 $\pm$ 32.9	94.8 $\pm$ 30.3	78.8 $\pm$ 32.5		<0.05 (0 h versus 24 h) <0.05 (0 h versus 72 h)

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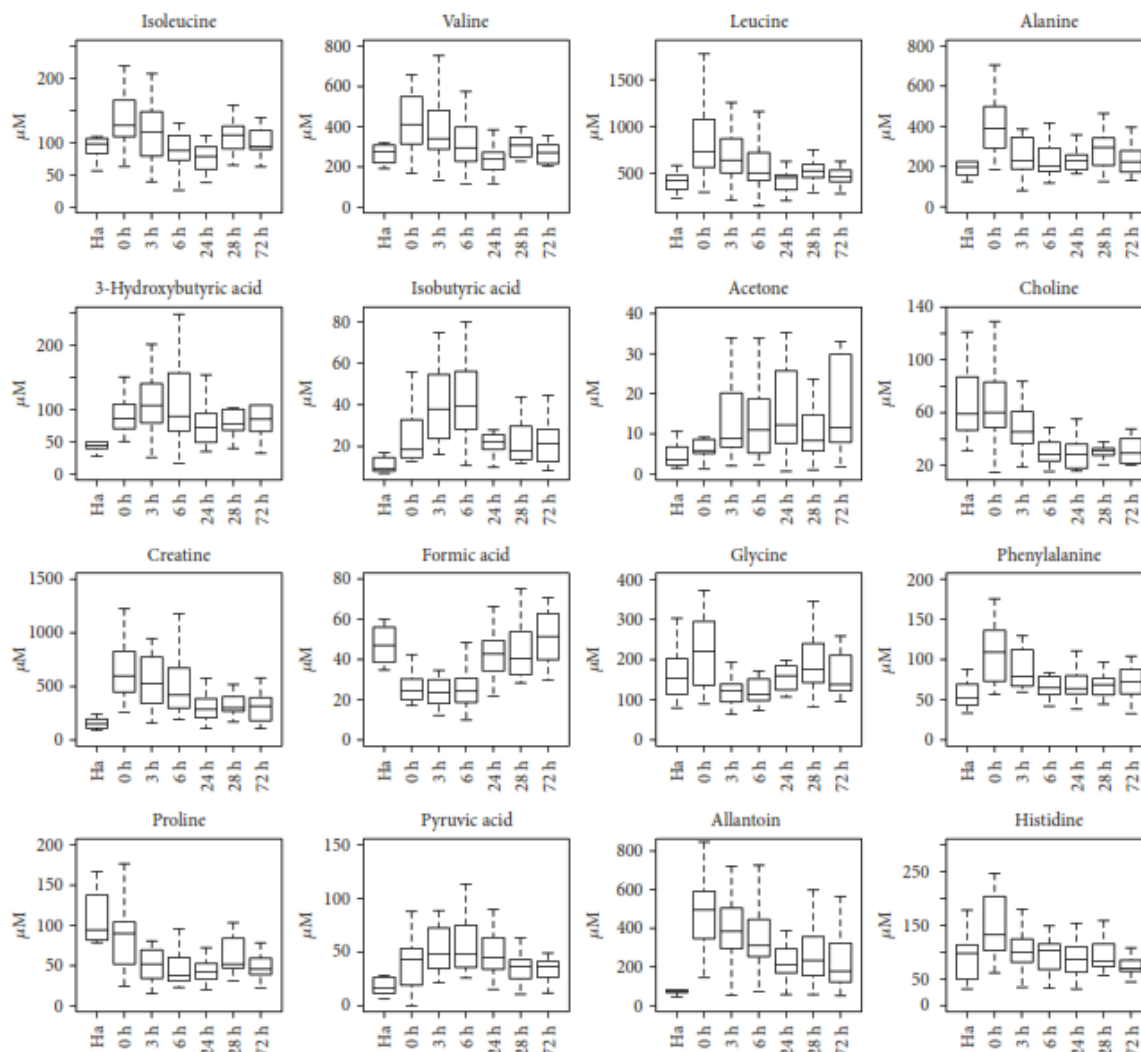


FIGURE 3: Box plots of the metabolites differentially concentrated in the seven calf groups. Ha: healthy animals; 0 h: diseased animals at 0 hour; 3 h: diseased animals at the 3rd hour; 6 h: diseased animals at the 6th hour; 24 h: diseased animals at the 24th hour; 48 h: diseased animals at the 48th hour; 72 h: diseased animals at the 72nd hour.

acid, acetone, and isobutyric acid remained increased at all the hours. While acetone gradually increased, choline and proline gradually reduced along hours. Formic acid is decreased at 0 hour then increased gradually.

4. Discussion

NMR-based metabolomics were evaluated at set intervals, for the first time, in newborn calves with severe sepsis. The data indicated that metabolomics was a feasible tool for the identification of septic plasma profiles and quantification of potential meaningful biomarkers for severe septic newborn calves. Newborn sepsis commonly originates from an abrupt evolution of infections in the first 4 weeks of life.

Clinical signs of newborn sepsis are often nonspecific, subtle, and inconspicuous and therefore demand a high index of suspicion for early diagnosis [2]. In this present study, clinical and laboratory results at admission were in accordance with most references. Clinical findings especially mental status contrary to leukocytosis, metabolic acidosis, lactatemia, and hyperkalemia could not be corrected properly. In the diseased animals, O_2 saturation remained decreased; however BUN, creatinine, and AST were increased along all hours. All the animals (except one) died after 72 h. This may be attributed to multiple-organ failure. These laboratory results on animal serum samples obtained by analytical methods optimized for human matrix may be questionable, even though they may be considered indicative.

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There are a variety of tests that are helpful for screening newborns with sepsis. Traditional biomarkers do not sufficiently discriminate between sepsis and SIRS. Thus, the identification of more sensitive reliable and rapidly measured biomarkers to differentiate sepsis from SIRS and monitor disease progression and treatment efficacy is a matter of intense interest. This study has shown that NMR metabolomics could represent an optimal tool for faster identification of newborn calves with severe sepsis and a complementary tool to classical methods used until now to stratify better the prognosis in septic shock and severe sepsis. The usefulness of metabolomics in exploring underlying biochemical mechanisms of sepsis could—after validation—provide novel candidate mechanisms to confirm or follow-up the progression of newborn early- or late-onset sepsis [27, 28]. Urinary ^1H -NMR and GC-MS metabolomics predict early- and late-onset newborn sepsis [8]. In the study, the variables significantly contributing to the separation of the septic samples from healthy animals in multivariate analysis included several metabolites such as acetate, glycine, glucose, acetone, lactate, and lysine, whereas control samples were characterized by citrate and creatinine. In another ^1H -NMR human study, several metabolites (maltose, glucose, biotine, methylamine, inosine, methylguanidine, creatine, myoinositol, and quinolinic acid) were found significantly changed in septic neonates at the onset of the disease [28]. In this current study, identified and quantified metabolites and their functions are the following: *valine*: synthesis of glutamine and alanine and balance among branched-chain amino acid (BCAA); *proline*: collagen structure and function, neurological function, and osmoprotection; *choline*: synthesis of betaine, acetylcholine, phosphatidylcholine, and sarcosine; *alanine*: inhibition of pyruvate kinase and hepatic autophagy, gluconeogenesis, transamination, and glucose-alanine cycle; *phenylalanine*: activation of BH4 (a cofactor for NOS) synthesis, synthesis of tyrosine, and neurological development and function; *leucine*: regulation of protein turnover through cellular mechanistic target of rapamycin signaling and gene expression, activator of glutamate dehydrogenase, BCAA balance, and flavor enhancer; *isoleucine*: synthesis of glutamine and alanine and balance among BCAA; *histidine*: protein methylation, hemoglobin structure and function, antioxidative dipeptides, and one-carbon unit metabolism; *glycine*: calcium influx through a glycine-gated channel in the cell membrane, purine and serine syntheses, synthesis of porphyrins, inhibitory neurotransmitter in the central nervous system, and coagonist with glutamate for N-methyl-D-aspartate receptors; and *creatine*: antioxidant, antiviral, antitumor, energy metabolism in muscle and brain, and neurological and muscular development and function [29]. Most metabolites increased at 0 hour (Supplemental Table 1) indicated the response to metabolic deficits for early-onset sepsis. These metabolites gradually decreased at other hours following the therapy. Also, the presence of ketone bodies such as acetone, 3-hydroxybutyric acid, and isobutyric acid in the plasma of the septic group at all hours suggests a compensatory reaction to a reduced level of ATP [8]. A simplified list of the most contributing metabolic pathways is reported in Supplemental Table 4 based on MetaboAnalyst software.

The analysis showed alteration in biochemical pathways like aminoacyl-tRNA biosynthesis (histidine, phenylalanine, glycine, valine, alanine, isoleucine, leucine, and proline), valine/leucine/isoleucine biosynthesis (pyruvate, leucine, valine, and isoleucine), nitrogen metabolism (phenylalanine, histidine, glycine, and formate), glycine/serine and threonine metabolism (choline, glycine, creatine, and pyruvate), and synthesis and degradation of ketone bodies (3-hydroxybutyrate, acetone). The analysis was calculated based on the significance value (P value < 0.05) of the pathway enrichment analysis. In our previous study [30] where the measurements were not at set intervals, the whole lipid soluble metabolites such as sphingomyelin and fatty acids including PUFA were reduced and attributed to a great systemic energy deficit during sepsis. Being in accordance with Langley and Wong [31], it is said that metabolomic changes in sepsis suggest an energy crisis in nonsurvivors. Some metabolites such as formic acid in this present study were similar to those in the previous study. Remarkable increased allantoin of the septic group in this present study can be an indication of microbial overgrowth or oxidative stress. Cell death from cytochrome oxidase inhibition by formic acid believed to result partly from depletion of ATP, reducing energy concentrations so that essential cell functions cannot be maintained. In this regard, gradually increasing of formic acid being decreased at 0 hour can be meaningful for prognosis of septic calves.

5. Conclusions

To our knowledge, this is the first study where the measurements were at set intervals showing that ^1H NMR-based metabolomics approach may indeed be a powerful instrument providing knowledge about the factors responsible of the metabolic modifications in newborn septic calves. NMR metabolomics proved to be an optimal tool for faster identification of newborn calves with sepsis using plasma samples. Although identified and quantified metabolites may become potential biomarkers for diagnosing newborn sepsis and monitoring therapy of sepsis in calves, the extent of the predictive and prognostic values of this given set of metabolites will be required for clinical trials.

Ethical Approval

The experimental design was approved by the Committee on Use of Animals in Research of the Selçuk University, Faculty of Veterinary Medicine (Protocol no. 04/2015).

Disclosure

The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Conflicts of Interest

The authors declare no conflict of interest to declare.

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Authors' Contributions

Abdullah Basoglu, Ismail Sen, and Amir Naseri conceived and designed the experiments and performed the laboratory data acquisition, and Gaia Meoni and Leonardo Tenori obtained the NMR data. Abdullah Basoglu wrote the paper. All authors have read and approved the final manuscript.

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Supplementary Materials

Supplemental Table 1: blood gas analysis. Supplemental Table 2: complete blood count analysis. Supplemental Table 3: biochemical profile. Supplemental Table 4: an integrated analysis based on MetaboAnalyst software: view of contributing pathways. (*Supplementary Materials*)

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4.2 Toward personalized medicine: use of nuclear magnetic resonance-based metabolomics in detecting drug resistance in breast cancer

Each year, 1.3 million new cases of breast cancer are diagnosed worldwide, and account for almost 15% of all cancer-related deaths²⁴⁹. Breast cancer is a heterogeneous disease²⁵⁰ with multiple subtypes and cellular/molecular characteristics, and thus, one of the major challenges for successful treatment in the clinic has been lack of reliable molecular predictors. Other essential biochemical information required for therapeutic decisions are hormone receptor status or growth factor receptor status which may also change with cancer progression and treatment necessitating the development of precise biomarkers for subtypes that can be monitored in real-time²⁵¹. Estrogen and estrogen receptors play significant roles in the development of human breast cancer in 70% of breast cancer cases that are ER-positive²⁵². Pharmacological agents that inhibit estrogen signaling are collectively referred to as endocrine therapy and is commonly used as initial treatment option for breast cancer that is ER /PR-positive. Regardless of biochemical subtypes or clinical subgroups, drug resistance in all types of breast cancer remains an unsolved clinical problem. While a vast majority of breast cancers are treated with endocrine therapy, about 40%–50% of these tumors will display *de novo* or acquired resistance^{253,253}. Proteomic studies have recently uncovered ER-associated co-regulators and transcription factors as possible targets in endocrine resistant breast cancers²⁵⁴. The use of metabolomics for assessment of treatment effect, as both a predictive measure of efficacy and as a pharmaco-dynamic marker, has been shown *in vitro* for both traditional chemotherapy and hormonal agents²⁵⁵.

There is an ongoing effort by the National Cancer Institute, researchers, clinicians, and industry to expand the use of metabolomics, for the assessment of therapeutic response²⁵⁶.

Choline phospholipid metabolism intermediates have been deemed a potential biomarker for monitoring treatment efficacy in a variety of human cancers²⁵⁷. In general, a decrease in the tCho signal on ¹H NMR equates to a response to chemotherapy or radiation and may be an early marker of effect as it can be detected before changes moreover on conventional imaging in breast and prostate cancer, brain tumors, and non–Hodgkin's lymphoma²⁵⁸. Studies exploring key metabolic regulators of cellular functions and response to treatment are highly relevant and may help improve the effectiveness of currently available treatment strategies. In general, quantitative metabolomic biomarkers for cancer detection and/or assessment of treatment efficacy are explored preclinically using animal and human cell cultures, followed by validation in biofluid or tumor tissue.

At this point cell metabolomics could offers different advantages respect to animal models or experiments on human subjects, such as easier control of the experiment, greater reproducibility and easier and more direct interpretation of the results.

In the following study, we employed ¹H NMR spectral profiling of breast cancer cell lines to identify the metabolomic alterations associated with resistance/sensitivity to palbociclib, a CDK4/6 inhibitor recently approved for the treatment of HR+ HERneg advanced breast cancer. When we analyzed differentially expressed metabolites between sensitive and resistant cells, Glycerophosphocholine (GCP) was the only metabolite significantly increased in resistant cells after correction for multiple testing, suggesting that choline metabolism might be implicated in resistance to palbociclib; previous evidences already have suggested that alterations in Cho-metabolite levels occur in breast cancer cell lines in response to estrogen signaling²⁵⁹.

Taken together, our data demonstrate for the first time that a metabolomic approach is able to identify changes in metabolic reprogramming accompanying palbociclib resistance in breast cancer cell lines.

The biggest challenge of this work still remains demonstrating that in vitro observations occur also in vivo to reach the final goal of developing a model to predict non-responders before drug administration.

4.2.1 Metabolomic analysis of Estrogen Receptor positive breast cancer cell lines by nuclear magnetic resonance (^1H -NMR) spectroscopy is able to identify cells with acquired resistance to palbociclib

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In preparation

Candidate's contributions: acquisition of NMR data, statistical analysis and interpretation of data, writing and review of the manuscript.

ABSTRACT

Palbociclib, a CDK4/6 inhibitor, in combination with endocrine therapy has recently shown unprecedented activity for the treatment of HR+/HER2neg advanced breast cancer. Identifying patients more likely to benefit from this compound and understanding the mechanisms of resistance is critical. Here, we investigated metabolomic profiles of breast cancer cell lines with acquired resistance to palbociclib (PDR). We have established seven HR+ PDR breast cancer derivatives by chronically exposing cells to escalating doses of palbociclib. PDR cells were not significantly inhibited by palbociclib and showed IC₅₀ values 6 to 30 times higher than their sensitive counterparts (PDS). Whole-cell lysates and conditioned cell culture media from five replicates of each of the PDS and PDR models were analyzed by ^1H NMR fingerprinting. Principal component analysis (PCA) was used as first exploratory analysis and as dimension reduction technique. Canonical Analysis (CA) was used to discriminate different groups. Differentially expressed metabolites between PDR and PDS models and between CAMA-1 and PDS cells were analyzed. Unsupervised PCA analyses of ^1H NMR spectra correctly identified individual cell lines on both whole-cell lysates and conditioned media. However, this unsupervised analysis did not discriminate PDS from PDR within each model. Using a supervised approach, these groups were discriminated with 90% accuracy using whole-cell lysates and of 81% using conditioned media as determined by cross-validation analyses. Metabolites with differential levels between PDS and PDR models in lysates and conditioned media were identified. Glycerophosphocholine levels in lysates were significantly higher in PDR compared to PDS models after correction for multiple testing. In this study, we show that analysis of metabolomic profiles of cells lysates and conditioned media is able to discriminate PDR from PDS cell lines. Future studies investigating metabolomic profiles of patients treated with palbociclib are warranted.

Introduction

Palbociclib is an inhibitor of Cyclin-Dependent Kinases 4 and 6 (CDK 4/6) that has recently received approval in the U.S and in the European Union for the treatment of women with hormone receptor-positive, human epidermal growth factor receptor 2-negative (HR+/HER2-) locally advanced or metastatic breast cancer^{260,261}. The approval is based on the results of three pivotal randomized clinical trials, the PALOMA-1, the PALOMA-2 and the PALOMA-3^{262,263}. PALOMA-1 and -2 are phase II and III randomized trials, respectively, of palbociclib in combination with letrozole versus letrozole alone for previously untreated patients in the metastatic setting; PALOMA-3 is a phase III randomized trial of palbociclib and fulvestrant versus placebo and fulvestrant for the treatment of patients relapsed on or progressed to a previous line of hormonal therapy²⁶⁴. These trials demonstrated the superiority of the combination over hormonal treatment alone in both hormone therapy-untreated and -pre-treated populations. To date, no single biomarker has been validated to select patients for palbociclib treatment. However, data from the PALOMA trials indicate that a proportion of patients do not achieve a clinical benefit (i.e. response or prolonged stabilization) from palbociclib. Biomarkers of sensitivity/resistance to CDK4/6 inhibitors could improve patient selection. In addition, understanding the mechanisms of acquired resistance to palbociclib is vital given the growing number of patients treated with palbociclib in the metastatic setting who will invariably develop resistance.

Metabolomics²⁶⁵ is the study of metabolites (small molecules) in blood, tissue or other biological samples. Metabolomics analyzes the presence and relative concentrations of metabolites, which can be used as evidence of cellular processes and functions or as biomarkers^{80,266,267}. Studies in breast cancer cells have used metabolomics to understand underlying mechanisms of breast cancer progression and response to specific treatments^{268,269}. However, to the best of our knowledge this approach has never been applied to the study of resistance to palbociclib in breast cancer cell lines.

Palbociclib induces a marked senescent phenotype in sensitive cells. Evasion from senescence is one of the phenotypic hallmarks of resistance to Palbociclib. Although senescent cells have reduced proliferative capacity, these cells remain metabolically active. We have developed in our laboratory seven HR+ breast cancer derivatives with acquired resistance to palbociclib (PDR). We hypothesized that the PDR phenotype might be captured by changes in cell metabolism. Therefore, we aimed to investigate whether metabolomic profiles of PDR cells differ from their sensitive counterparts (PDS). In addition, to gain insight into the relationship between sensitivity to Palbociclib and metabolic changes, we analyzed the metabolomic profile of a breast cancer cell line unable to acquire resistance to Palbociclib (CAMA-1).

Materials and methods

Cell lines, cell culture and reagents

Cell lines used in this study are T47D, ZR75-1, MCF7, MCF7 Estrogen Deprivation Resistant (MCF7 EDR), MCF7 Tamoxifen Resistant (MCF7 TamR), CAMA1, MDA MB 361 and BT474. T47D and ZR75-1 cell lines were obtained from Dr Livia Malorni, CNR Avellino, Italy. CAMA1, MCF7, MCF7 EDR and MCF7 TamR from the laboratory of Dr Rachel Schiff at Baylor College of Medicine, Houston, TX, US. MDA MB 361 were purchased from Sigma-Aldrich and BT474 from ICLC (Interlab Cell Line Collection), Genova, Italy.

Cells were cultured at 37°C and 5% CO₂. T47D, ZR75-1, MCF7, CAMA1, MDA MB 361 and BT474 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5g/glucose and L-glutamine (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone) and 1% 200 mM L-glutamine, 10,000 U penicillin and 10 mg streptomycin/mL solution (Sigma-Aldrich). MCF7 EDR and MCF7 TamR were grown in DMEM with 4.5g/glucose and without L-glutamine and phenol red (Lonza) supplemented with 10% charcoal-stripped FBS (CS-FBS) (GIBCO) and 1% 200 mM L-glutamine, 10,000 U penicillin and 10 mg streptomycin/mL solution (Sigma-Aldrich). MCF7 TamR cell medium was supplemented with 100 nM final concentration of (Z)-4-Hydroxytamoxifen (Sigma-Aldrich) dissolved in 100% ethanol.

Palbociclib resistant derivatives (PDR) were generated by long term culturing of cells with increasing concentrations of palbociclib from the starting treatment dose (STD) up to 1 µM. STD was preliminary assessed on each model and defined as the concentration that, after 9 days of treatment, inhibited cells growth rate between 40% and 60%. Cells were considered resistant to palbociclib when they restored the ability to regularly grow in the presence of palbociclib 1 µM.

The palbociclib STD was 50 nM for T47D, ZR75-1, MCF7 EDR and MCF7 TamR cell lines, 100 nM for CAMA 1 and 350 nM for MCF7, MDA MD 361 and BT474 cell lines. PDR cells were then continuously cultured in their original media with the addition of palbociclib 1 µM. Palbociclib was kindly provided by Pfizer and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

All cell lines and their PDR derivatives have been authenticated on January 2016 by short tandem repeat (STR) DNA profiling analysis.

Proliferation and IC₅₀ assays

A total of 3,000 cells/well of PDS or PDR cell lines, cultured in their individual media, were plated in 96-well plates in triplicate, 24 hours before beginning treatments. These consisted of palbociclib 1 µM or 0.01% DMSO (vehicle) for nine days for proliferation assays or doubling dilutions of palbociclib, from 64 µM to 0.97 nM for nine days for the IC₅₀ assays. Media were replaced every 72 h. Cell growth was assessed at zero and nine days. Cells were fixed with 4% glutaraldehyde grade II (Sigma-Aldrich) and stained with 0.05% methylene blu (Sigma-Aldrich). The dye was subsequently extracted with 3% HCl (Carlo Erba) and absorbance measured at 655 nm. Data were normalized to the mean value of absorbance at zero days (O.D. 655 nm at zero or at nine days/mean of O.D. 655 nm at zero days). For proliferation assay, growth fold changes were computed as ratio, dividing growth rate of treated cells by their untreated controls. Mean value of growth fold change of all experiments +/- standard error (SE) was plotted. For IC₅₀, the signals were converted to proportions adjusting on time zero (T₀) and on untreated DMEM control values and fitted using the 4-parameter logistic regression (R package nplr version 0.1-4). The proportion values, y_p , were computed as: $y_p = (y - T_0) / (DMSO - T_0)$, where y are the observed values. The model performance was estimated by a weighted standard error, and a weighted goodness-of-fit. Once the model was fitted, the drug 50% inhibitory concentration (IC₅₀) could be estimated from inhibition rates by simply inverting the function (given a y value = 0.5 survival rate). Mean value of IC₅₀ of all experiments +/- SE was calculated. Student's t tests were performed and *P*-values <0.05 were considered significant. Calculations and plots were performed by R (version 3.3.1). At least three biological replicates for each PDS or PDR cell line were performed.

β -galactosidase senescence assay

PDS and PDR cell lines were plated in 12-well plates in duplicate, in a range of 10,000-20,000 cells/well. Cells were plated in their individual medium which was replaced after 24h with medium containing palbociclib (1 μ M) or 0.01% DMSO (vehicle) as a control. Cells were grown for six days and the media were replaced every 72h. The cellular senescence was assessed by Senescence detection kit (BioVision) according to the manufacturer's instructions. Positive and negative cells were counted in six randomly selected 20X fields and the percentage of positive cells was calculated. Mean value of senescence percentages of all experiments $\pm$ SE was plotted. Student's *t* tests were performed and *P*-values <0.05 were considered significant. Calculations and plots were performed by R (version 3.3.1). At least three biological replicates for each PDS or PDR cell line were performed.

NMR sample preparation

PDR and PDS cells were plated in 100 mm dishes in duplicates in their individual medium. This was changed after 24h with medium containing 0.01% DMSO (vehicle) in PDS cells and replaced every 72h. Cells were grown until they reached 70% confluence. PDS treated cells (D3) were grown in medium supplemented with palbociclib 1 μ M for 3 days. Both conditioned cell culture medium and cell lysates were collected for ^1H NMR analysis. Conditioned cell culture medium was collected from each dish duplicate and an aliquot of 1 ml was snap frozen at -80°C . Dishes were placed onto ice and cells were rinsed twice with ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS) (GIBCO, Life Technologies). Cells from dishes in duplicate were scraped with 500 μL of ice-cold DPBS supplemented with 1% Protease Inhibitor Cocktail (Sigma-Aldrich) and 1% HaltTM Phosphatase Inhibitor Cocktail (Thermo Scientific) and collected in one tube. Cells were lysed by sonication (12 pulses for 15 seconds each) on ice and centrifuged at 14,000 *g* for 30 minutes at 4°C ⁸¹. Supernatants were collected and stored at -80°C . Five biological replicates for both conditioned cell culture medium and cell lysates from each model were collected.

Frozen samples were thawed at room temperature and shaken before use and were prepared for NMR analysis. Briefly, 60 μL of buffer solution (1,5 M $\text{KH}_2\text{PO}_4/\text{d}_2\text{O}$, pH 7.4; 2mM Na_2N_3 ; 0,1% TMSP) were added to 540 μL of cell lysate sample. A total of 300 μL of a phosphate sodium buffer (70 mM Na_2HPO_4 ; 20% (v/v) $^2\text{H}_2\text{O}$; 0.025% (v/v) Na_2N_3 ; 0.8% (w/v) sodium trimethylsilyl [2,2,3,3- $^2\text{H}_4$]propionate (TMSP) pH 7.4) were added to 300 μL of each aspired culture medium sample. In both cases (lysates and media) the mixture was homogenized by vortexing for 30 seconds. 600 μL of this mixture was transferred into a 5 mm NMR tube (Bruker BioSpin srl) for the analysis.

NMR analysis

One-dimensional ^1H NMR spectra were acquired for all samples using a Bruker 600 MHz spectrometer (Bruker BioSpin) operating at 600.13 MHz proton Larmor frequency and equipped with a 5mm PATXI ^1H - ^{13}C - ^{15}N and ^2H -decoupling probe including a *z* axis gradient coil, an automatic tuning-matching (ATM) and an automatic and refrigerate sample changer (SampleJet). A BTO 2000 thermocouple served for temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 5 minutes inside the NMR probe head, for temperature equilibration at 300 K for lysates and 310K for conditioned medium samples.

For each lysate and for sample of conditioned cell culture medium, a one-dimensional ^1H NMR spectra with a standard nuclear Overhauser effect spectroscopy pulse sequence (NOESY

1Dpresat; noesygppr1d.comp; Bruker BioSpin) with water peak suppression was acquired²²⁴. Both signals of metabolites and high molecular weight molecules (lipids and lipoproteins) are visible.

The acquisition parameters for lysate samples was: 32 scans; 65,536 data points, spectral width of 12,019 Hz; acquisition time of 2.7s, relaxation delay of 4s and mixing time of 0.01s. For each sample of conditioned cell culture medium, were applied 32 scans; 98,304 data points, a spectral width of 18,028 Hz; an acquisition time of 2.7 s, a relaxation delay of 4 s and a mixing time of 0.01 s.

Spectral processing

Free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line-broadening factor before applying Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated (lysate samples aligned to alanine doublet at 1.48 ppm, culture media to anomeric glucose doublet at 5.24 ppm) using TopSpin 3.2 (Bruker Biospin srl). Each 1D spectrum in the range between 0.2 and 10.00 ppm was segmented into 0.02 ppm chemical shift bins and the corresponding spectral areas were integrated using AMIX software (version 3.8.4, Bruker BioSpin). Binning is a means to reduce the number of total variables and to compensate for small shifts in the signals, making the analysis more robust and reproducible. The region containing residual water between 5.0 and 4.3 ppm was removed from the spectra. Additionally, for cell lysates, the region between 2.904 and 2.58 ppm of the DMSO peak was also removed. The total spectral area was calculated on the remaining bins and total area normalization was carried out on all the data prior to pattern recognition.

NMR Statistical analysis

All data analyses were performed using R software for the statistical analysis of data. Principal component analysis (PCA) was used as first exploratory analysis and as dimension reduction technique.

Canonical Analysis was used to discriminate different groups. For the purpose of classification K-nearest neighbors (k-NN) method (k=3) was applied on the PCA-CA scores²⁷⁰. All the accuracies reported and the confusion matrix for different classifications were assessed by means of 100 cycles of a Monte Carlo cross-validation scheme (MCCV, R script in-house developed). Briefly, 90% of the data were randomly chosen at each iteration as a training set to build the model. Then the remaining 10% was tested and sensitivity, specificity and accuracy for the classification were assessed. The procedure was repeated 100 times to derive an average discrimination accuracy for each group of subjects.

The spectral regions related to assigned metabolites were assigned in the spectra by using matching routines of AMIX 3.8.4 (Bruker BioSpin) in combination with the BBIREFCODE database (Bruker BioSpin) and published literature^{32,33,80}. These spectral regions were integrated to obtain the concentrations of metabolites in arbitrary units and the concentrations were analyzed to determine the differentially expressed metabolites among PDR and PDS cells.

Wilcoxon test²⁷¹ was chosen to infer differences among the groups on the biological assumption that metabolite concentrations are not normally distributed; false discovery rate correction was applied using the Benjamini–Hochberg method. An adjusted P-value <0.05 was deemed significant. The effect size, using the Cliff's delta (Cd) formulation²²⁰, was also calculated to aid the identification of the meaningful signals giving an estimation of the magnitude of the separation between the different groups. The magnitude is assessed using the thresholds

provided in Romano *et al.*²²¹, i.e., $|\text{Cd}| < 0.147$ “negligible”, $|\text{Cd}| < 0.33$ “small”, $|\text{Cd}| < 0.474$ “medium”, otherwise “large”.

Results

Functional characterization of PDR and PDS cell lines

We were able to develop PDR derivatives for all cell lines with the exception of CAMA1. CAMA1 cells failed to restore growth at 350 nM of palbociclib concentration over two separate attempts. The time to develop resistance for all other models varied from 8 to 22 weeks. Proliferation of all PDS cells was significantly (P -value <0.05) inhibited by palbociclib, while PDR derivatives did not show significant differences in cellular growth when treated with palbociclib 1 μM compared to untreated cells (data not shown). In addition, PDR cells showed IC₅₀ values from 6 to 30 times higher than their PDS counterparts (**Table 1**). With the exception of the MDAMB361 cell line, PDR cells showed significantly (P -value <0.05) lower proportions of senescent cells during palbociclib treatment compared to their PDS counterparts, demonstrating the ability to escape palbociclib-induced senescence (data not shown).

NMR analysis of NOESY spectra from PDR and PDS cells

As first exploratory analysis, we performed an unsupervised principal component analysis (PCA) of ¹H NMR spectra of all cell lines. In this analysis, no information about PDS and PDR phenotype has been included in the statistical model and has been performed on both cell lysates and conditioned media, separately. As shown in **figure 1**, individual cell line lysates and cell line corresponding growing media tend to group together (**Figure 1 a, b**). However is not possible appreciating any clusterization of Palbociclib resistant versus sensitive cells, both using cell lysates and conditioned media. Such unsupervised approach is not too sensitive to reveal metabolic differences among PDS and PDR derivatives, undoubtedly in this case metabolic variations are more subtle than metabolic differences between distinct cell lines, representing a significant confounding factor for our goal.

Therefore, supervised analysis, PCA-CA-kNN have been applied in order to discriminate between PDR and PDS cell lines. The statistical model built using the five replicates of each cell line and validated using Monte Carlo cross-validation analysis shows an accuracy of 90% and 81% on cell lysates and on conditioned media, respectively (**Figure 2**) in recognizing PDS from PDR cells.

An additional supervised approach in which the cell models were randomly divided in two groups: group A, formed by MCF7, BT474 and EDR; group B containing T47D, ZR75-1, MDAMB361 and TamR, has been performed. The statistical model for the identification of PDR and PDS phenotypes has been created and trained using all the replicates from group A, leaving group B for the validation.

The training model used shows 87% accuracy for cell lysates and 74% accuracy for conditioned media (group A); the 70% of cell lysates and the 55% of cultured media have been correctly predicted as test set (group B) as shown in **figure 3 a** and **3b**.

Here we detect an evident drop of the overall accuracy suggesting that the model used as training, is not probably big enough to cover all the (uncommon) variations developed by different cell lines.

Moreover, to rule out the possibility that the differences observed between PDR and PDS cells could be related to the presence of Palbociclib or its metabolites in PDR cells, we assessed ¹H-NMR spectra of MCF7, T47D and ZR75-1 parental cell lines treated with palbociclib 1μM for three days (PDS-D3) and included these spectra in the model. Intriguingly, PDS-D3 cells are almost exclusively predicted as PDR by the model on cell lysates, while using conditioned media cells are randomly assigned as PDS or PDR (**Figure 4**); however, metabolic differences could be identified between the PDS-D3 and PDS or PDR. Indeed, as shown in **figure 5**, PDS-D3 cells are well separated from the other two groups along the first (PC1) and second component (PC2) of the score plot, and they occupy a central position between PDR and PDS on the third component (PC3) both in the analysis of cell lysates and in the analysis of conditioned media. Each group is well recognized by model with very high accuracy, as shown in the diagonal of the confusion matrix (**Figure 5**). These data suggest that the observed differences in the metabolic profile of PDS and PDR models are in part related to the presence of Palbociclib. However, intrinsic metabolic peculiarities of PDS and PDR models are detectable by a supervised way even when the relative contribution of Palbociclib is taken into account, thus suggesting that resistance to Palbociclib induces a peculiar metabolic reprogramming.

NMR-based metabolites in PDR and PDS cells

We next aimed to identify different levels of metabolites between PDR and PDS cells, both on cell lysates and on conditioned cell culture media.

On cell lysates, we identified over 32 different amounts of metabolites (**Table 2**). Among these metabolites, creatine, glycerophosphocholine (GPC), myo-inositol and methionine levels were higher in PDR cells, while taurine, fumarate, 4-aminohippuric acid were higher in PDS cells. However, only GPC levels were significantly higher in PDR cells compared to PDS (adjusted *P*-value: 0,006; **table 2**). When we compared the levels of the differentially expressed metabolites between PDR, PDS and PDS-D3 cells, again GPC was the only metabolite whose levels were significantly higher in PDS-D3 compared to PDS or PDR (adjusted *P*-values: 1,36E-09 and 0,000218, respectively; **supplementary table 1 and 2**).

In conditioned media, ten metabolites were differentially expressed between the two groups. acetate, formate and 2-oxoisocaproate levels were among metabolites higher in PDR, while fumarate levels were higher in PDS. However, none of these differences reached statistical significance (**supplementary table 3**).

NMR analysis of CAMA1 cells

We were not able to induce resistance in CAMA1 cell line. Therefore, a distinct metabolomic analysis of this model was performed to identify characteristics that might explain its peculiar behavior. The model used is effective in predicting CAMA1 as PDS, both on cell lysates and conditioned media (**Figure 6 a,b**). Intriguingly, CAMA1 cell lysates are located at the far right side of the plot, clustering separately from other PDS models, appearing as the “most PDS” cells

The analysis of differentially expressed metabolites between CAMA1 and all other PDS cells, reveals higher levels glutamine and glutamate levels in CAMA1 cell lysate samples compared to all other PDS models (adjusted *P*-value <0.05, **Table 3**).

Discussion

An increasing body of research suggests that the capability to modify, or reprogram, cellular metabolism in order to most effectively support neoplastic proliferation is an emerging hallmark of cancer and might be suitable for possible therapeutic intervention^{272,273}. Studies exploring key metabolic regulators of cellular functions and response to treatment are highly relevant and may help improve the effectiveness of currently available treatment strategies.

In this study, we employed ¹H NMR spectral profiling of breast cancer cell lines to identify metabolomic alterations associated with resistance/sensitivity to palbociclib, a CDK4/6 inhibitor recently approved for the treatment of HR+ HERneg advanced breast cancer^{260,261}.

We included eight ER positive luminal breast cancer cell lines with different degree of sensitivity to palbociclib, including endocrine resistant derivatives, in order to take into account heterogeneity of luminal breast cancers. We developed PDR derivatives by chronically exposing cells to increasing doses of palbociclib. This approach was highly effective as we were able to obtain PDR derivatives for all cell lines with the exception of CAMA1. All PDR derivatives showed significantly reduced sensitivity to palbociclib as revealed by functional analyses investigating proliferation, IC50 and β -galactosidase senescence assay, indicating that these represent reliable models of acquired resistance to palbociclib.

Our analyses were performed both on cell lysates and on conditioned cell culture media in order to have a broader spectrum of metabolic alterations associated with palbociclib resistance. However, the analyses on cell culture media were complicated by the fact that endocrine resistant derivatives, compared to their parental counterparts, grow in a medium deprived of growth factors and cytokines as well as lacking phenol red. This intrinsic difference between the culture media of endocrine resistant vs parental cell lines required an adjustment to allow comparisons between these two groups. Indeed, the regions in the spectra that were specific of the two different types of culture media were deleted. This might explain the general reduced performance of the analyses on conditioned media and the reduced number of metabolites identified.

The initial unsupervised analyses revealed that individual breast cancer cell lines tend to cluster together, suggesting that each cell line shows a distinct metabolic pattern discriminating one cell line from the others. These observations were further confirmed by the supervised analysis which discriminated individual cell lines with very high accuracy. Our results were not unexpected since previous reports already highlighted heterogeneity of breast cancer cell lines, revealing that individual cell lines display unique metabolite patterns^{274,275}. The metabolomic profile of each cell line is probably a prominent feature and masked the subtler differences in metabolic patterns between PDS and PDR phenotypes. However, using a supervised approach, in which the statistical model was trained to discriminate between PDS and PDR, these groups were categorized with high accuracy showing clearly distinct metabolic patterns. Our observation that PDS and PDR models differ from a metabolomic point of view is further strengthened by the fact that a classifier created with only three of the cell lines discriminates cell lysates as well.

Previous studies have shown that metabolic reprogramming might be implicated in transformation of breast cancer cells and cellular response to stimuli and treatments^{259,276–278}; in addition one study in pancreatic cancer demonstrated that CDK4/6 inhibition stimulate glycolytic and oxidative metabolism²⁷⁹. Our results suggest for the first time that adaptation of

breast cancer cell lines to palbociclib treatment may be captured by changes in their metabolome.

In order to exclude that the differences observed between PDS and PDR cells might be related to drug metabolism of palbociclib (that is present experimentally only in the culture medium of PDR cells), we analyzed three cellular models after 3 days of treatment with palbociclib (PDS-D3).

The evidence that the presence of the drug influences our model, is given by the fact that PDS-D3 cell lysates are mostly classified as PDR. However, the model on conditioned media did not clearly categorized PDS-D3 cells as PDR, and when compared PDR, PDS and PDS-D3 show distinct metabolic profiles (both considering lysates and conditioned media analyses). These evidences support the hypothesis that specific metabolic changes occur at the time of acquired resistance to palbociclib.

When we analyzed differentially expressed metabolites between PDS and PDR cells, Glycerophosphocholine (GCP) was the only metabolite significantly increased in PDR cells after correction for multiple testing, suggesting that choline metabolism might be implicated in resistance to palbociclib. The role of choline metabolism in response/resistance to palbociclib needs to be clarified by further molecular studies investigating the determinants of GPC alterations. However an abnormal choline metabolism has been already implicated in oncogenesis, tumor progression and response to treatment^{258,280} and a recent study also showed that alterations in Cho-metabolite levels occur in breast cancer cell lines in response to estrogen signaling.²⁵⁹

CAMA1 was the only cell line unable to develop resistance. We reasoned that some peculiar characteristics of this cell line might explain their inability to develop palbociclib resistance and decided to include this line in the analyses. As expected, CAMA1 model was correctly predicted as PDS, both on cell lysates and on conditioned media. It is interesting to note that in the metabolomic analysis of cell lysates, CAMA1 formed a distinct cluster in the plot, occupying the most distinct position from PDR models compared to all other PDS models. This suggests that the Palbociclib-sensitive behaviour of CAMA1 may indeed be captured by their distinct metabolomic profile.

Palbociclib resistance is going to represent a major clinical challenge in the near future given the growing number of patients treated with this drug. Preclinical studies have suggested molecular determinants of resistance to CDK4/6 inhibitors in breast cancer including genetic loss of RB transcriptional corepressor 1 (*RB1*), amplification of Cyclin Dependent Kinase 6 (*CDK6*), over-expression of cyclin E1 and high levels of a gene expression signature of Retinoblastoma loss-of-function^{281–283}. Molecular analysis of tumour specimens of patients with advanced breast cancer are difficult to perform due to the frequent lack of available material from metastatic tissue. As a consequence, prognostic and predictive biomarkers are often evaluated on the primary tumour with the intrinsic limitation of not taking into account the possible biological divergence occurring during the metastatic process. Previous studies demonstrated that metabolic signatures can be detected in the sera of patients with breast cancer and might help predict recurrence risk, outcome and response to neo-adjuvant chemotherapy,^{267,284}. A serum derived metabolomic signature of response/resistance to palbociclib could be a potentially useful biomarker for patients with metastatic breast cancer both to identify patients more suitable to initiate a treatment with palbociclib and to monitor disease progression over time during therapy. Our data support further investigations in patients with breast cancer treated with palbociclib.

Taken together, our data demonstrate for the first time that a metabolomic approach is able to identify changes in metabolic reprogramming accompanying palbociclib resistance in breast cancer cell lines.

Future work should address some key questions: first, are the metabolic alterations observed in vitro similar to those that occur in vivo? Our experiments were performed on tumour cell lines and under controlled conditions. Translating our findings in patient's samples has obvious challenges. Indeed, the metabolic profile of an individual is influenced by numerous factors, including age, sex, ethnicity or external influences such as diet. Moreover, tumour metabolism itself might vary upon influence from the surrounding stroma or immune infiltrate^{285,286}. Nevertheless, the fact that we observed that different cell lines acquire common metabolic changes during acquisition of resistance supports the hypothesis that metabolomic studies conducted on patients treated with palbociclib might give some valuable information about development of resistance.

Second, what are the molecular bases of the alterations observed? Our results suggest that choline metabolism might be implicated in response/resistance to palbociclib. Understanding how genes and proteins regulate the levels of GPC during treatment and at the time of resistance might further clarify our observations and elucidate the role of GPC in palbociclib resistance. Finally, we observed high heterogeneity in the individual metabolic profile of ER positive breast cancer cell lines. In this study, we focused on common metabolic alterations that occur at the time of resistance to palbociclib which remained significant despite the heterogeneity of individual cell models. It might be interesting to analyse breast cancer cell lines separately to investigate specific metabolic alterations.

Further investigations clarifying the role of metabolism in palbociclib resistance are warranted.

Tables and Figures

Table 1. IC50 values of PDS, PDR and CAMA1 cell lines.

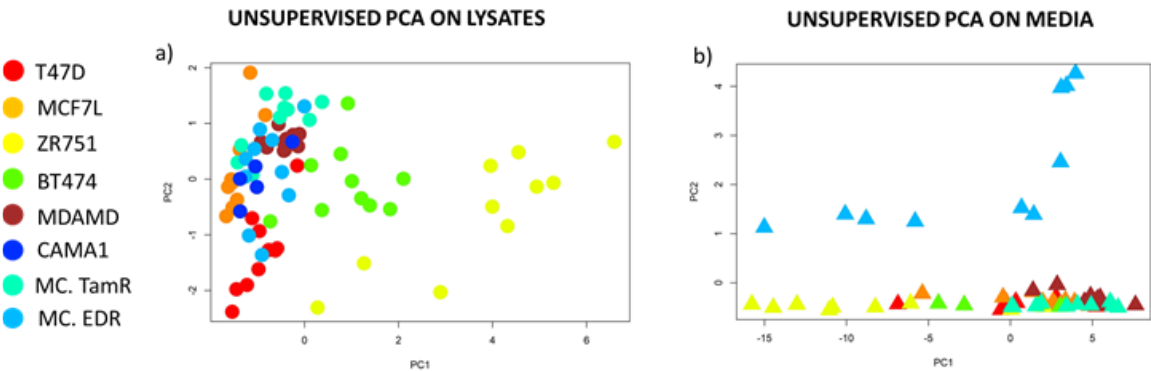
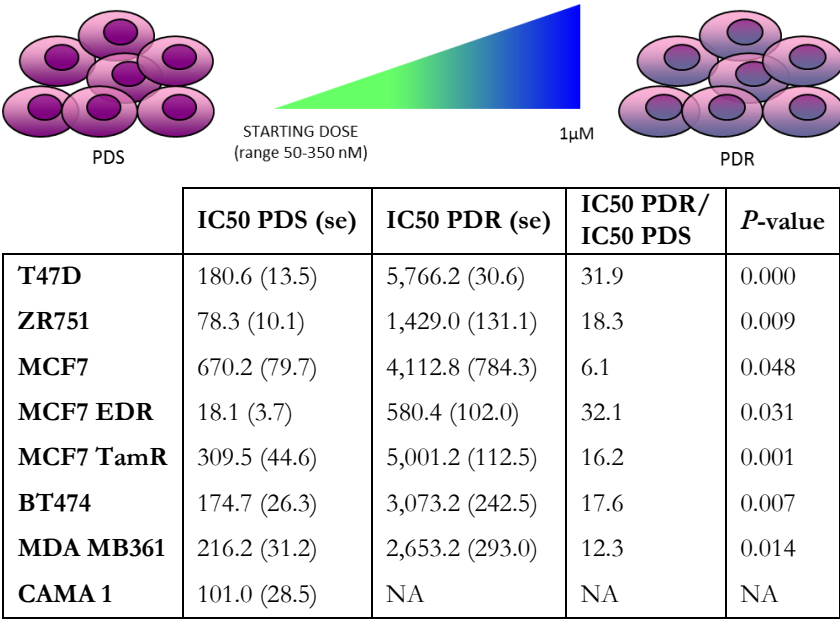


Figure 1. Identification of individual cell lines.

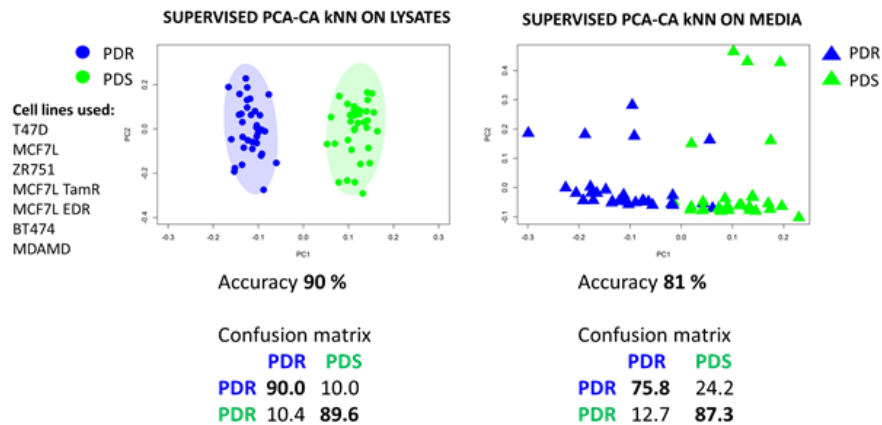
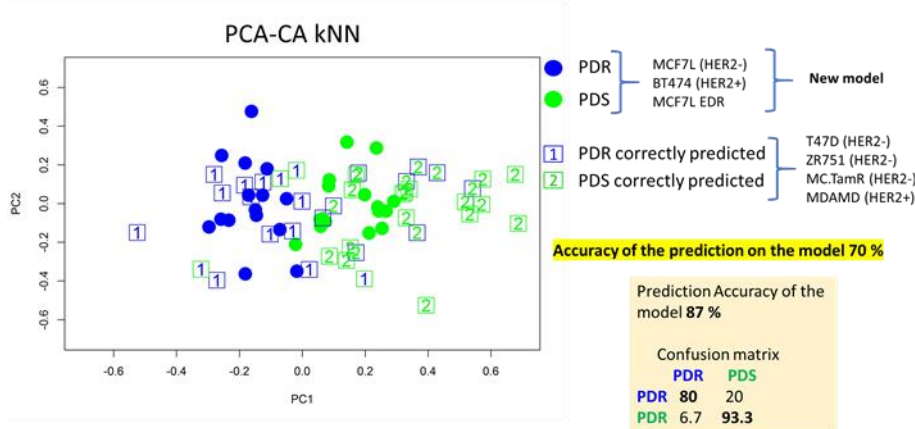


Figure 2. Discrimination of PDS and PDR cell lines based on a supervised PCA-CA-kNN analysis and the related confusion matrix obtained from MCCV.

a) LYSATES SUPERVISED TRYING TO PREDICT PDR AND PDS CELLS WITHIN A NEW MODEL



b) MEDIA SUPERVISED: TRYING TO PREDICT PDR AND PDS CELLS WITHIN A NEW MODEL

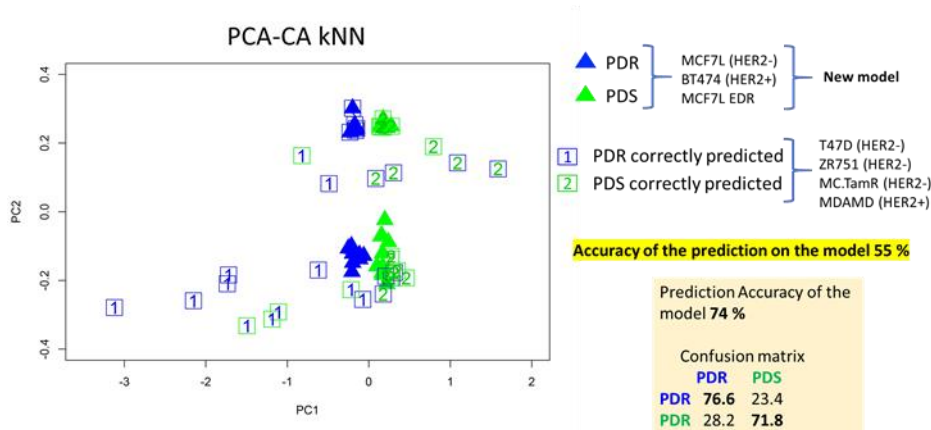


Figure 3. PDS and PDR classification using a random training (group A MCF7, BT474 and EDR) and test set (group B T47D, ZR75-1, MDAMB361 and TamR).

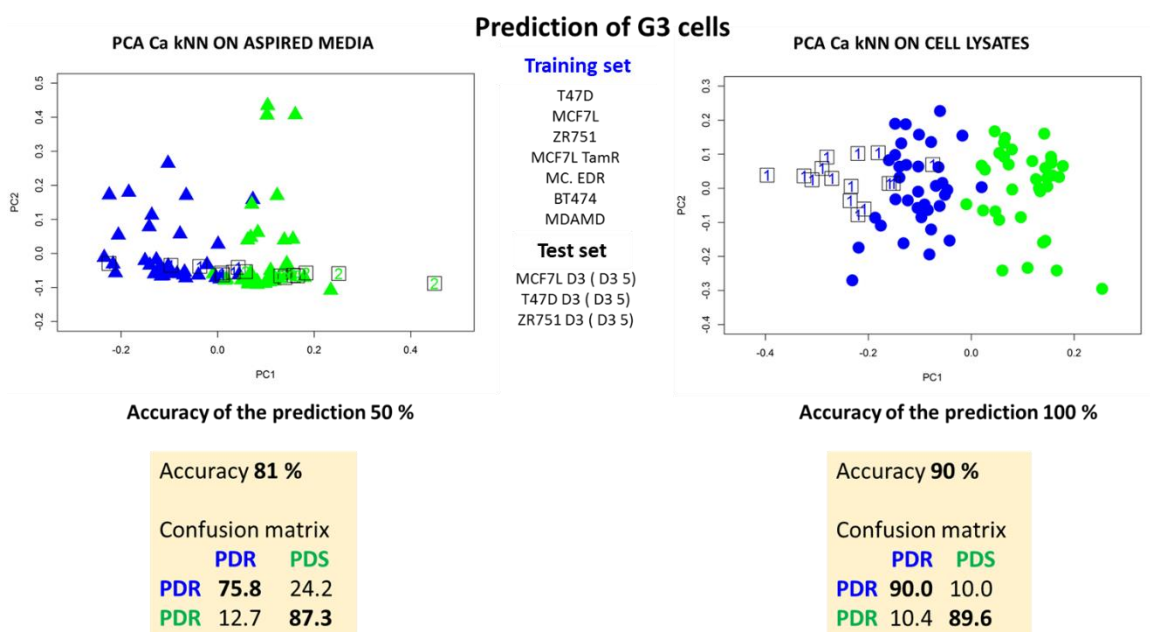


Figure 4. D3 cell lysates and growing media classification on a PDS/PDR model.

Analysis of the metabolic space.

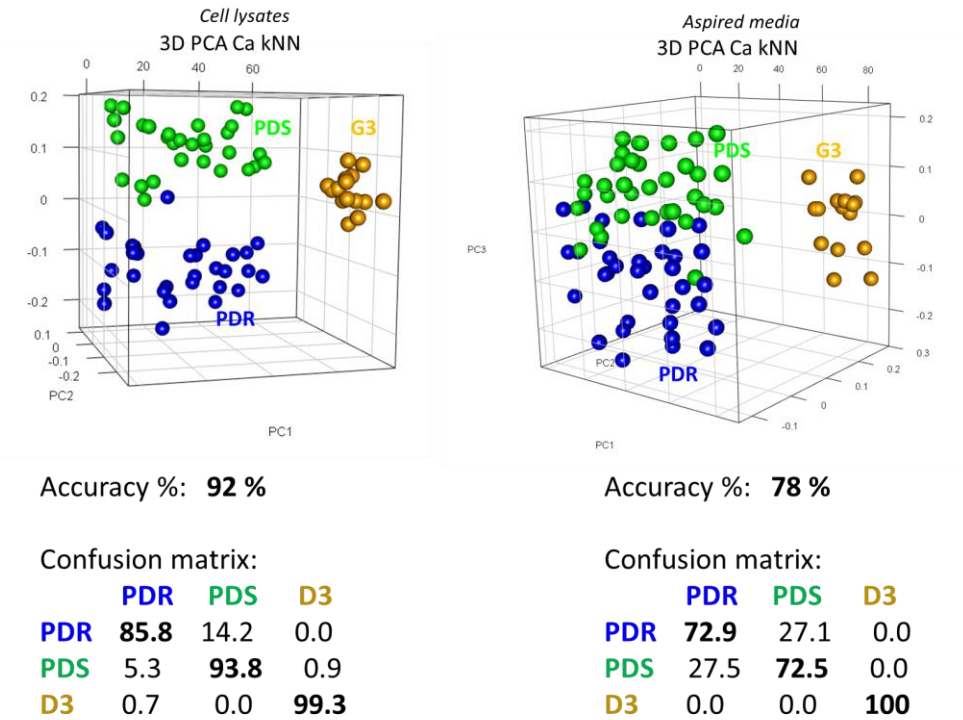


Figure 5. PCA-CA kNN revealing metabolic space of PDS, PDR ad D3.

CAMA 1 PREDICTION

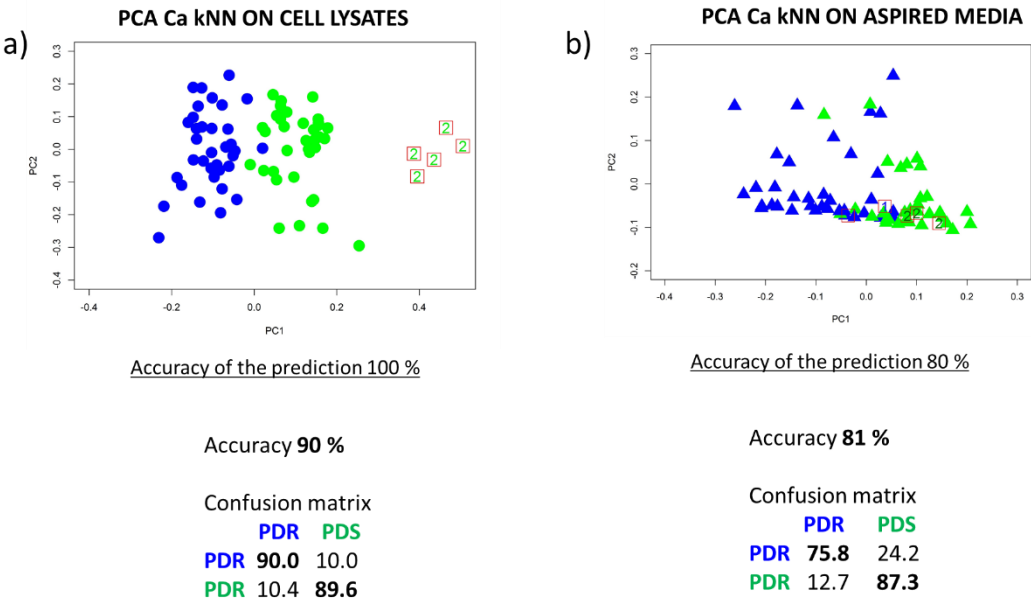


Figure 6. CAMA1 (red squares) line classification on PDS (green) and PDR (blue) cell lines. Nr 2 on square means CAMA1 predicted as PDS.

Table 2. Metabolites analysis of PDR vs PDS. P-value, adjusted P-value(FDR), Cliff's delta (Cd) effect size and Log₂(FC) are reported for PDR vs PDS comparison. Values in bold are significantly different (P-value<0.05, Cd=large, medium) in the PDR vs PDS lysates comparison. Negative Log₂(FC) means higher levels in PDR cells

Metabolites	Chemical shift (ppm)	P-value	Adjusted P-value	Cliff's Delta	Log ₂ (FC)
<i>Glucose 1-phosphate</i>	3.45 (m)	6.91E-01	8.40E-01	negligible	-2.20E-01
<i>Glucose</i>	3.9 (d)	3.26E-01	7.94E-01	negligible	-2.20E-01
<i>GPC</i>	3.23 (s)	1.90E-04	6.46E-03	large	-6.45E-01
<i>Phosphocreatine</i>	3.05 (s)	9.91E-01	9.91E-01	negligible	-1.44E-02
<i>4-Aminobippuric acid</i>	7.62 (d)	2.10E-01	7.94E-01	small	2.55E-01
<i>Acetate</i>	1.91 (s)	4.27E-01	7.94E-01	negligible	-1.69E-02
<i>Alanine</i>	1.5 (d)	2.32E-01	7.94E-01	small	-6.59E-02
<i>Choline</i>	3.2 (s)	9.63E-01	9.91E-01	negligible	-4.10E-02
<i>Creatine</i>	3.03 (s)	1.90E-01	7.94E-01	small	-1.01E+00
<i>Ethanol</i>	1.18 (m)	9.16E-01	9.91E-01	negligible	3.89E-02
<i>Fumarate</i>	6.52 (s)	1.16E-01	7.94E-01	small	3.41E-01
<i>Glutamate</i>	2.33 (d)	2.61E-01	7.94E-01	small	1.13E-01
<i>Glutamine</i>	2.47 (s)	6.66E-01	8.38E-01	negligible	9.76E-02
<i>Glutathione</i>	2.97 (s)	9.63E-01	9.91E-01	negligible	4.58E-02
<i>Glycine</i>	3.56 (s)	1.36E-01	7.94E-01	small	2.00E-01
<i>Histidine</i>	7.1 (s)	4.88E-01	7.94E-01	negligible	6.82E-03
<i>Isoleucine</i>	1.01 (d)	4.98E-01	7.94E-01	negligible	1.07E-01
<i>Lactate</i>	1.3 (s)	9.53E-01	9.91E-01	negligible	1.41E-01
<i>Leucine</i>	0.97 (s)	4.48E-01	7.94E-01	negligible	5.74E-02
<i>Methionine</i>	2.06 (s)	5.14E-01	7.94E-01	negligible	-3.50E-01
<i>Myo-Inositol</i>	4.05 (t)	1.22E-01	7.94E-01	small	-5.56E-01
<i>O-phosphocholine</i>	3.2 (s)	5.91E-01	7.94E-01	negligible	4.63E-02
<i>Phenylalanine</i>	7.2 (l)	6.07E-01	7.94E-01	Negligible	2.28E-01
<i>Succinate</i>	2.4 (s)	5.28E-01	7.94E-01	Negligible	8.35E-02
<i>Taurine</i>	3.43 (s)	5.67E-02	7.94E-01	Small	4.17E-01
<i>Threonine</i>	4.26 (d)	5.91E-01	7.94E-01	Negligible	-1.37E-01
<i>Tyrosine</i>	6.9 (m)	4.55E-01	7.94E-01	Negligible	-2.66E-02
<i>Unknown1</i>	2.3 (s)	3.38E-01	7.94E-01	Negligible	-1.33E-01
<i>Unknown2</i>	5.95 (s)	5.75E-01	7.94E-01	Negligible	1.60E-01
<i>Unknown3</i>	5.97 (s)	2.10E-01	7.94E-01	Small	2.35E-01
<i>Unknown4</i>	6 (s)	8.89E-01	9.91E-01	Negligible	4.77E-02
<i>Valine</i>	1.04 (d)	5.99E-01	7.94E-01	Negligible	-1.28E-01

Table 3. Metabolites analysis of CAMA1 vs PDS; P-value, adjusted P-value(FDR), Cliff's delta (Cd) effect size and Log₂(FC) are reported for CAMA1 vs PDS comparison. Values in bold are significantly different (P-value<0.05, Cd=large, medium) in the CAMA1 vs PDS lysates comparison. Negative Log₂(FC) means higher levels in PDS cells.

Metabolites	P-value	adjusted P-value	Cliff's Delta	Log ₂ (FC)
<i>Glucose 1-phosphate</i>	3.37E-01	6.03E-01	small	3.29E-01
<i>Glucose</i>	8.43E-01	9.55E-01	negligible	-1.62E-01
<i>GPC</i>	3.37E-01	6.03E-01	small	-6.70E-01
<i>Phosphocreatine</i>	1.85E-02	7.85E-02	large	-1.25E+00
<i>4-Aminobippuric acid</i>	7.84E-02	2.22E-01	large	4.05E-01
<i>Acetate</i>	6.34E-01	7.98E-01	negligible	2.12E-02
<i>Alanine</i>	1.23E-02	7.85E-02	large	1.05E+00
<i>Choline</i>	7.75E-01	9.08E-01	negligible	4.10E-01
<i>Creatine</i>	2.98E-01	5.95E-01	small	-5.54E-01
<i>Ethanol</i>	3.80E-01	6.45E-01	small	-1.81E+00
<i>Fumarate</i>	4.02E-01	6.51E-01	small	-5.22E-01
<i>Glutamate</i>	4.87E-03	4.89E-02	large	5.38E-01
<i>Glutamine</i>	2.13E-05	7.23E-04	large	7.75E-01
<i>Glutathione</i>	1.97E-01	5.15E-01	medium	-1.97E-01
<i>Glycine</i>	1.41E-02	7.85E-02	large	4.42E-01
<i>Histidine</i>	2.98E-01	5.95E-01	small	4.29E-01
<i>Isoleucine</i>	7.51E-01	9.08E-01	negligible	8.63E-02
<i>Lactate</i>	5.83E-02	1.80E-01	large	-1.19E+00
<i>Leucine</i>	9.68E-01	9.68E-01	negligible	-9.30E-02
<i>Methionine</i>	4.73E-02	1.79E-01	large	1.37E+00
<i>Myo-Inositol</i>	1.85E-02	7.85E-02	large	-9.71E-01
<i>O-phosphocholine</i>	8.74E-01	9.59E-01	negligible	1.13E-02
<i>Phenylalanine</i>	2.12E-01	5.15E-01	medium	4.21E-01
<i>Succinate</i>	4.73E-01	6.70E-01	small	-6.62E-01
<i>Taurine</i>	9.37E-01	9.65E-01	negligible	-4.57E-02
<i>Threonine</i>	2.28E-01	5.16E-01	medium	2.89E-01
<i>Tyrosine</i>	4.73E-01	6.70E-01	small	2.09E-01
<i>Unknown1</i>	5.24E-01	6.86E-01	small	3.21E-01
<i>Unknown2</i>	9.37E-01	9.65E-01	negligible	-7.38E-02
<i>Unknown3</i>	4.49E-01	6.70E-01	small	1.37E-01
<i>Unknown4</i>	8.81E-05	1.50E-03	large	4.73E-01
<i>Valine</i>	4.98E-01	6.78E-01	small	2.38E-01

Supplementary information

Supplementary table 1. Metabolites' analysis of PDS vs D3. P-value, adjusted P-value (FDR), Cliff's delta (Cd) effect size and Log₂(FC) are reported for PDS vs D3 comparison. Statistically significant values are deemed as P-value<0.05 and Cd=large or medium.

Negative Log₂(FC) means higher levels in PDS cells.

Metabolites	P-value	Adjusted P-value	Cliff's Delta	Log ₂ (FC)
<i>Glucose 1-phosphate</i>	4.50E-01	6.38E-01	negligible	-1.87E-02
<i>Glucose</i>	1.77E-01	4.02E-01	small	3.10E-01
<i>GPC</i>	4.00E-11	1.36E-09	large	1.12E+00
<i>Phosphocreatine</i>	5.92E-03	1.01E-01	large	-2.84E-01
<i>4-Aminohippuric acid</i>	7.38E-01	7.84E-01	negligible	1.09E-01
<i>Acetate</i>	6.91E-01	7.58E-01	negligible	2.00E-01
<i>Alanine</i>	5.02E-01	6.83E-01	negligible	-2.93E-01
<i>Choline</i>	3.08E-02	2.46E-01	medium	4.95E-01
<i>Creatine</i>	4.34E-02	2.46E-01	medium	1.29E+00
<i>Ethanol</i>	3.34E-01	5.41E-01	small	-4.77E-01
<i>Fumarate</i>	7.88E-02	2.68E-01	small	-7.84E-01
<i>Glutamate</i>	1.34E-01	3.50E-01	small	-1.51E-01
<i>Glutamine</i>	6.91E-01	7.58E-01	negligible	5.09E-02
<i>Glutathione</i>	7.89E-02	2.68E-01	small	-5.16E-01
<i>Glycine</i>	5.86E-01	7.11E-01	negligible	5.96E-02
<i>Histidine</i>	2.65E-01	4.51E-01	small	-5.92E-01
<i>Isoleucine</i>	2.65E-01	4.51E-01	small	-3.45E-01
<i>Lactate</i>	3.90E-01	5.76E-01	small	-2.50E-01
<i>Leucine</i>	6.15E-01	7.21E-01	negligible	-1.64E-01
<i>Methionine</i>	1.71E-01	4.02E-01	small	8.45E-01
<i>Myo-Inositol</i>	7.52E-02	2.68E-01	small	8.24E-01
<i>O-phosphocholine</i>	1.37E-02	1.56E-01	medium	-3.98E-01
<i>Phenylalanine</i>	1.18E-01	3.34E-01	small	-4.76E-01
<i>Succinate</i>	8.67E-01	8.67E-01	negligible	-5.68E-03
<i>Taurine</i>	5.43E-01	6.84E-01	negligible	-1.52E-01
<i>Threonine</i>	2.39E-01	4.51E-01	small	-3.15E-01
<i>Tyrosine</i>	1.92E-01	4.07E-01	small	-2.25E-01
<i>Unknown1</i>	5.29E-01	6.84E-01	negligible	6.32E-02
<i>Unknown2</i>	1.08E-01	3.34E-01	small	-3.96E-01
<i>Unknown3</i>	7.52E-02	2.68E-01	small	-7.98E-01
<i>Unknown4</i>	8.18E-01	8.43E-01	negligible	-5.64E-02
<i>Valine</i>	2.06E-01	4.13E-01	small	-2.73E-01

Supplementary table 2. Metabolites analysis of PDR vs D3. P-value, adjusted P-value (FDR), Cliff's delta (Cd) effect size and Log₂(FC) are reported for PDR vs D3 comparison. Values in bold are significantly different (P-value<0.05, Cd=large, medium) in the PDR vs D3 lysates comparison. Negative Log₂(FC) means higher levels in PDR cells.

Metabolites	P-value	adjusted P-value	Cliff's Delta	Log ₂ (FC)
<i>Glucose 1-phosphate</i>	2.84E-01	5.67E-01	small	-2.39E-01
<i>Glucose</i>	4.63E-01	6.47E-01	negligible	9.01E-02
<i>GPC</i>	6.41E-06	2.18E-04	large	4.76E-01
<i>Phosphocreatine</i>	3.98E-03	6.77E-02	large	-2.98E-01
<i>4-Aminohippuric acid</i>	2.14E-01	5.60E-01	small	3.64E-01
<i>Acetate</i>	3.34E-01	5.67E-01	small	1.83E-01
<i>Alanine</i>	7.17E-02	3.48E-01	small	-3.59E-01

<i>Choline</i>	4.21E-02	2.46E-01	medium	4.53E-01
<i>Creatine</i>	4.76E-01	6.47E-01	negligible	2.87E-01
<i>Ethanol</i>	3.23E-01	5.67E-01	small	-4.38E-01
<i>Fumarate</i>	3.56E-01	5.67E-01	small	-4.43E-01
<i>Glutamate</i>	3.67E-01	5.67E-01	small	-3.90E-02
<i>Glutamine</i>	9.00E-01	9.17E-01	negligible	1.48E-01
<i>Glutathione</i>	9.03E-02	3.84E-01	small	-4.70E-01
<i>Glycine</i>	2.06E-01	5.60E-01	small	2.59E-01
<i>Histidine</i>	5.97E-01	6.97E-01	negligible	-5.85E-01
<i>Isoleucine</i>	6.15E-01	6.97E-01	negligible	-2.38E-01
<i>Lactate</i>	3.67E-01	5.67E-01	small	-1.08E-01
<i>Leucine</i>	8.67E-01	9.17E-01	negligible	-1.06E-01
<i>Methionine</i>	5.43E-01	6.84E-01	negligible	4.95E-01
<i>Myo-Inositol</i>	2.74E-01	5.67E-01	small	2.68E-01
<i>O-phosphocholine</i>	4.34E-02	2.46E-01	medium	-3.52E-01
<i>Phenylalanine</i>	2.65E-01	5.67E-01	small	-2.48E-01
<i>Succinate</i>	5.29E-01	6.84E-01	negligible	7.79E-02
<i>Taurine</i>	2.39E-01	5.67E-01	small	2.65E-01
<i>Threonine</i>	1.64E-01	5.07E-01	small	-4.51E-01
<i>Tyrosine</i>	4.01E-01	5.93E-01	small	-2.51E-01
<i>Unknown1</i>	9.17E-01	9.17E-01	negligible	-6.93E-02
<i>Unknown2</i>	1.64E-01	5.07E-01	small	-2.36E-01
<i>Unknown3</i>	3.14E-02	2.46E-01	medium	-5.63E-01
<i>Unknown4</i>	8.51E-01	9.17E-01	negligible	-8.72E-03
<i>Valine</i>	5.72E-01	6.94E-01	negligible	-4.01E-01

Supplementary table 3. Metabolites analysis of PDR vs PDS cells in conditioned media. P-value, adjusted P-value (FDR), Cliff's delta (Cd) effect size and Log₂(FC) are reported for PDR vs PDS comparison. Values in bold are significantly different (P-value<0.05, Cd=large, medium) in the PDR vs PDS conditioned media comparison. Negative Log₂(FC) means higher levels in PDR conditioned media.

Metabolites	Chemical shift (ppm)	P-value	adjusted P-value	Cliff's Delta	Log ₂ (FC)
<i>3-Methyl-2-oxovalerate</i>	0.88 (s)	1.74E-01	3.73E-01	Small	-2.46E-01
<i>2-Oxoisocaproate</i>	0.93 (s)	6.52E-02	2.44E-01	Small	-4.46E-01
<i>Isobutyrylglycine</i>	1.13 (d)	1.19E-01	2.99E-01	Small	-9.53E-02
<i>2-Oxobutyrate</i>	1.08 (s)	4.13E-01	5.17E-01	Negligible	-3.28E-03
<i>Acetate</i>	1.9 (s)	3.25E-02	1.63E-01	Small	-8.60E-01
<i>Glutamate</i>	2.34 (2)	4.07E-01	5.17E-01	Negligible	-4.85E-02
<i>2-Oxoglutarate</i>	3.0 (1)	8.25E-01	8.25E-01	Negligible	-1.66E-01
<i>Fumarate</i>	6.52 (1)	3.56E-01	5.17E-01	Negligible	3.43E-02
<i>Formate</i>	8.46 (1)	8.84E-03	6.63E-02	Medium	-5.84E-01
<i>Lactate</i>	4.10 (q)	7.65E-03	6.63E-02	medium	-4.19E-01

Chapter 5

NMR-based metabolomics in food science

5.1 Pre- and post-harvest factors affecting yield efficiency and oil quality in *Olea europaea*

Current research in plants makes extensive use of metabolomics due to the interest regarding benefits produced on human health by various products of plant origin (*e.g.* food, pharmaceuticals, industrial raw materials, *etc.*). However plant metabolomics is being increasingly used for understanding physiological processes such as responses to stress conditions²⁸⁷. Indeed, unravelling plants' biological and physiological mechanisms contributes to agricultural or industrial production by increasing the crops amount, helping in finding solutions that compensate damages caused by climate change, drought or salinity, or in the prevention of pathogens, with beneficial outcome for the growers and the society.

In this thesis NMR based metabolomics has been used to study some features of pre-harvest and post-harvest phase in order to obtain advantages in yield of olive and in quantity and quality of olive oil. Olive is one of the most extensively cultivated fruit crops in the world, however if compared with other fruit species it suffers, in terms of yield, of low production problems mainly due to very low fruit-set (number of fruits per number of flowers), alternative bearing and high levels of fruitlet drops. Furthermore, the constant consumers' demand regarding olive oil of high quality has driven the olive growers to earlier harvest, leading to reduced yield since less oil is accumulated by the fruit when compared with the late harvest.

In recent literature, the biological model, the molecular and biochemical mechanisms regulating fruitlet drop in *Malus domestica*, has been proposed and described in detail^{288–290}. No specific information is yet available to better understand the mechanisms inducing fruitlet abscission in olive trees. The elucidation of these phenomena represents a key step towards the improvement of olive tree productivity. We therefore aim to unravel the physiological mechanisms characterizing olive fruitlet drop in order to understand the biological basis of this process, an essential step to find possible practical solutions to reduce the incidence of fruitlet shedding and increase the final yield (§5.1.1). Olive (cv. Frantoio) were sampled in at various developmental stages (5, 8, 16, 23, 30 days after full bloom, DAB) and were discriminated as “developing” and “prone to abscission” and on the basis of growth pattern (fast growing, big, and slow growing, small), for a total of fifty olive extracts analyzed. Results pointed out a more immature profile of dropped fruitlets with respect to olives collected at the same time without the same abscission potential, indicating something blocking the development of these small fruits prone to fall. The OPLS model built shown its efficacy in discriminating “not dropped” fruitlets from “dropped” ones, suggesting two distinct fingerprints of two groups. Among the nineteen identified metabolites, gallic and caffeic acid, 3,4-dihydroxyphenylglycol and arabinose were higher in developing fruitlet, while the latter remained almost stable throughout the sampling points for

the abscising fruitlet, suggesting an impairment in stress response mechanisms in premature abscising fruits accordingly to that described by Eccher G. *et al.*²⁹⁰ in *Malus domestica*. Data collected until now will be integrated soon with UPLC-MS/MS hormonal, transcript (RNASeq) profiles and Quantitative Real-time PCR results to gain a complete picture of the molecular mechanism leading olive fruitlet abscission. The understanding of this phenomenon could help in developing effective treatment strategies for limiting premature abscission in olive tree.

Another challenge that characterizes modern Olive culture is the constant demand of enhanced quality of olive oil. The volatile organic compounds (VOCs) and the phenolics are the main elements markedly affecting the sensory profile of the olive oil^{291,292}.

From a physiological standpoint, a key factor affecting the olive oil quality and composition is the ripening stage of the processed fruit. Polyphenols tend to decrease as ripening progresses while VOCs, and particularly C5 and C6 aldehydes are progressively replaced by their alcohol derivatives. These changes have a strong influence on the sensorial characteristics of the olive oil and its stability: the riper the fruit, the higher the oil yield, but the less “green, cut-grass”-like and bitter the oil flavor. Additionally, various off-flavor compounds are formed by oxidation, which may be initiated in the olive fruit during the advanced stages of ripening and are induced/enhanced by the high temperatures²⁹³. Actually, numerous studies have been conducted in respect to application of high temperature on olive during malaxation, which indicated an increase in quantity but at the same time a decrease of its quality^{294–296}. On the contrary, little is known regarding the effects of a short application of relatively low temperatures on post-harvest olive fruit in respect to olive oil quality^{293,297}. Therefore, our aim was that of preliminarily assessing the effect of lowering temperature of fruit, prior to crushing, in respect to the modifications of soluble and of the volatile organic compounds of the resulting oil (§5.1.2).

Olive (*Olea europaea*, cv Leccino) fruit, were harvested in mid-October, early and mid-November. Fruit were processed within 24h after harvest, divided in three groups of equal weights and submerged in washing water kept at three different temperatures (4, 12 and 19°C) until reaching mesocarp temperature of 10, 15 and 19 °C for the three water conditions respectively. Confirming that the effect of temperature on olive oil ¹H-NMR profiles, OPLS-DA discriminates with a very good accuracy oil obtained at room temperature (ctr) from olive oil obtained at 12 °C (medium) and 4°C (low): 98% predictive accuracy. Overall, the results point out that the protocol of pre-processing conditioning of the fruit undoubtedly has an effect on the aroma profiles of the different oils, as well as on the polyphenol content and the metabolites. NMR and MS results on metabolites concordantly suggest that molecules responsible for the green, fresh and fruity sensory impact of the oil (trans-2-hexenal, 2-hexenal and hexanal), as well as polyphenols content, are positively influenced by lowering temperature. Moreover, Ethyl alcohol, acetic acid, 1-penten-3-ol, 2-penten-1-ol (Z)- and 1-penten-3-one were reported significantly lower when the fruit were conditioned in cold temperature for the first two harvests, with ethyl alcohol to display the most profound decrease. The presence of ethyl alcohol, acetic acid, 1-penten-3-ol, 2-penten-1-ol (Z)- and 1-penten-3-one in olive oil has been associated with the perception of winey, vinegary, mold, ripe and metallic perception, respectively. Therefore, the results suggest that by lowering the temperature of the pulp before the fruit undergo crushing, results in the reduction of aroma compounds responsible for specific defects. PLS performed on NMR and GC-MS identified metabolites has shown that lowering

fruit temperature has a more pronounced effects on oils extracted from ripe and ripe+ (overripe) olives. In addition, the oil samples obtained from ripe and ripe+ olives cooled down to 10 and 15°C lose the association with alcohols and 1-penten-3-one, negatively correlated traits with bitter, spicy and green and positively associated with ripe fruit trait.

The results obtained up to now show that by manipulating low temperatures more ripe olives at, it is possible to obtain an oil of a quality really similar to that obtained with the most appreciated less ripe olives but with the possibility to obtain higher amount of oil as confirmed by sensory results of panel test.

5.1.1 Towards the assembly of the olive fruitlet drop puzzle in *Olea europaea* (cv. Frantoio)

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In Preparation

Candidate's contributions: acquisition of NMR data, statistical analysis and interpretation of data and writing the draft.

ABSTRACT

Olea europaea is one of the most important and widespread fruit trees in the Mediterranean basin. About 73% of the global olive oil production comes from European Union countries and 97% comes from Spain, Italy, and Greece.

Olives are a cash crop of great economic importance even for the great adaptability to dry spells and drought. However, olive trees suffer several production problems, especially in challenging conditions. Generally, olives have a very low fruit-set and this is also due to the high levels of fertilized fruitlet drop. Another important problem affecting yield and productivity, is the climate change. Each crop and even each variety has different climatic and environmental requirements for normal growth (e.g., light, nutrients, soil, temperature, and water availability). Climate change mainly influences these variables. Climate warming will affect olive yield and quality across the Mediterranean basin and at local scale and profitability of small olive farms will decrease. Irrigation is not an easy task in several hilly areas and it may negatively affect water supplies, leading to water shortages for other purposes. The low yield (and the high costs of the grove management) makes olive growing a less profitable cultivation if compared with other crops with the risk of land abandonment. New and innovative solutions to improve olive tree productivity must be found. As above stated, olives are characterized by extremely high levels of post-fertilization/early growth fruitlet abscission. Furthermore, no specific information is available to better understand the molecular mechanism and the metabolic processes inducing fruitlet abscission. The elucidation of these phenomena represents a key step towards the improvement of olive tree productivity. This part of the project focuses on the metabolic basis of mechanisms involved in fruitlet shedding using Nuclear Magnetic Resonance (NMR)-based metabolomics (600 and 900MHz Bruker spectrometers). Olive fruitlet samples, collected at different time points and categorized as “fast-“and “slow-growing”, and as “dropped” according to their size and/or their tendency to drop, have been analyzed for the first time. The PCA

analysis performed on one-dimensional spectra, proved to be effective in discriminating olive groups according to the developmental stage. The supervised approach built on the OPLS model is highly effective in discriminating the “not dropped” fruitlets from the “dropped” ones, meaning that the two groups have distinct profiles. Moreover, several water-soluble metabolites differ among groups: higher choline levels and lower levels of gallic acid, caffeic acid and 3,4-dihydroxyphenylglycol in “dropped” fruitlets suggest, according to literature, an impairment of stress response mechanisms. Instead, lower arabinose levels can be ascribed to an incomplete development in “dropped” fruitlets.

Introduction.

Olea europaea is one of the most important and widespread fruit trees in the Mediterranean basin (Loumou & Giourga, 2003). About 73% of the global olive oil production comes from European Union countries and 97% comes from Spain, Italy, and Greece. Olives are a cash crop of great economic importance even for the great adaptability to dry spells and drought (Tanasijevic et al., 2014). However, olive trees suffer several production problems, especially in challenging conditions. Generally, olives have a very low fruit-set and this is also due to the high levels of fertilized ovaries/fruitlet drop, particularly abundant in this fruit tree species. Another important problem affecting yield and productivity is the climate change. Each crop and even each variety has different climatic and environmental requirements for normal growth (e.g., light, nutrients, soil, temperature and water availability). Climate change mainly influences these variables (Galán et al., 2001). Furthermore, due to the global warming new olive groves are established in northern regions, which are characterised by a shorter production season (late blooming) and, consequently, lower yield (Moriondo et al., 2010). Climate warming will affect olive yield and quality across the Mediterranean basin and at local scale, profitability of small olive farms will decrease (Ponti et al., 2014). Irrigation is not an easy task in several hilly areas and it may negatively affect water supplies for the area, leading to water shortages for other purposes. Furthermore, the constant consumers’ demand regarding olive oil of high quality has driven the olive growers to earlier harvest, leading to reduced yield since less oil is accumulated by the fruit when compared with the late harvest. The low yield (and the high costs of the grove management) makes olive growing a less profitable cultivation if compared with other crops with the risk of land abandonment. New and innovative solutions for improving olive tree productivity must be found. As above stated, olives are characterized by extremely high levels of post-fertilization/early growth fruitlet abscission. Olive trees do not display specific trends of physiological fruitlet drop after pollination or at the early stages of development as other fruit crops such as apple. Furthermore, no specific information is available to better understand the molecular mechanism and the metabolic processes inducing fruitlet abscission. The elucidation of these phenomena represents a key step towards the improvement of olive tree productivity. This study focuses on the physiological, metabolic and molecular basis of mechanisms involved in fruitlet shedding using Next Generation Sequencing (NGS) and Nuclear Magnetic Resonance (NMR)-based metabolomics. NGS methods are rapid and relatively cheap technologies to perform high-throughput gene expression profiling. RNA sequencing employs NGS to produce a complete picture of the on-going molecular mechanisms in a biological sample at a given moment in time, leading to the detection of changes in gene expression over time, or between different groups or treatments. Metabolomics plays a significant role in bridging the phenotype-genotype gap and thus assisting us not only to link gene to function but also enabling us to gain wholly new insight into how plant organs respond to environmental stimuli. NMR one of the

most suitable techniques to obtain “high-throughput” analytical methodologies in metabolomics, gives a complete view of the foodstuff (e.g., olive oil, wine, fruit juice, honey, fresh fruit) metabolites together with suitable statistical analysis. Results reported here concern the NMR part of the study, the work is awaiting hormonal UPLC-MS/MS and NGS results to be assembled in its complete form.

Methodologies

Sample preparation and NMR analysis

Olive methanol extracts were dissolved in D₂O (700 uM). 600 µL of this mixture were transferred into a 5 mm NMR tube for the analysis.

One-dimensional ¹H NMR spectra for all samples were acquired using a Bruker 600 MHz spectrometer (Bruker BioSpin). For each olive extract sample, a one-dimensional ¹H NMR spectra with a standard nuclear Overhauser effect spectroscopy pulse sequence (NOESY 1Dpresat; noesygppr1d.comp; Bruker BioSpin) was acquired working at 300 K. The assignment of ¹H spectra was obtained by means of 2D experiments, namely ¹H–¹H COSY (Correlation Spectroscopy), ¹H–¹H TOCSY (Total Correlation Spectroscopy), ¹H–¹³C HSQC (Heteronuclear Single Quantum Coherences) and ¹H–¹³C HMBC (Heteronuclear Multiple Bond Coherences), acquired using a Bruker 950-MHz spectrometer equipped with a 3-mm TCI Cryo Probe working at 300 K.

1D Spectral Processing

Each 1D spectrum in the range between 0.2 and 10.00 ppm was segmented into 0.02 ppm chemical shift bins and the corresponding spectral areas were integrated using AMIX software (version 3.8.4, Bruker BioSpin). Binning is a means to reduce the number of total variables and to compensate for small shifts in the signals, making the analysis more robust and reproducible. The regions containing residual methanol from 3.20 to 3.50 ppm and residual water between 5.0 and 4.0 ppm were removed from the spectra. The binned spectra were normalized to be comparable. Normalization is a preprocessing method, which accounts for different dilutions of samples by scaling the spectra to the same virtual overall concentration. In our case, all the spectra were normalized using both Probabilistic Quotient Normalization (PQN)²²⁶ and total area, carried out separately on all the data prior to pattern recognition, in order to choose the most appropriate method.

Statistical analysis

All data analyses were performed using R, an open source software for the statistical analysis of data. Multivariate data analysis was conducted on processed NMR spectra. Principal component analysis (PCA) was used as a first exploratory analysis. Data reduction was carried out by means of projection into an Orthogonal projections to latent structures (OPLS)²⁹⁸ that was applied to obtain the supervised separation of the analyzed groups.

All the accuracies reported and the confusion matrix for different classifications were assessed by means of 100 cycles of a Monte Carlo cross-validation scheme (MCCV, in-house developed R script). In this case, 90% of the data were randomly chosen at each iteration as a training set to build the model. Then the remaining 10% was tested and sensitivity, specificity and accuracy for the classification were assessed. The procedure was repeated 100 times to derive an average discrimination accuracy for each group of olives.

Nineteen metabolites, corresponding to well defined and resolved peaks in the spectra, were assigned. Signal identification was performed using a library of NMR spectra of pure organic compounds, public databases (e.g., FooDB, PhytoHub, PhenolExplorer) storing reference and literature data. The concentrations of the various metabolites in the different spectra were calculated by spectral fitting and integration of the signal area using in-house Matlab® scripts (Takis *et al.* in preparation) and the concentrations were analyzed to determine the discriminating metabolites among the groups of olives. Kruskal-Wallis test followed by Dunn post-hoc analysis was chosen to infer differences among the ten groups, on the biological assumption that metabolite concentrations are not normally distributed. Wilcoxon signed-rank test was chosen to infer differences among the two groups of “dropped” and “not dropped” olives. False discovery rate correction was applied using the Benjamini & Hochberg method (FDR)²⁹⁹ an adjusted *P*-value <0.05 was considered statistically significant.

Results and discussion

Multivariate analyses on fruitlet spectra

NMR spectra of all 50 olive extracts (ten groups: T0, T1 small, T1 big, T1 dropped, T2 small, T2 big, T2 dropped, T3 small, T3 big, T3 dropped) were acquired, sample n°15 (T1s) was removed from our analysis because the spectrum was of bad quality.

Olive fruitlet samples, collected at different time points and categorized as “fast-”(big) and “slow-growing”(small), and as “dropped” according to their size and/or their tendency to drop (**Figure 1**), have been analyzed firstly using unsupervised multivariate method (PCA) to gain an overview on the main changes responsible for the detachment process. Figure 2 shows the PCA score plots of olive fruitlet spectra normalized with both PQN algorithm (**Fig. 2a**) and total area (**Fig. 2b**).

The two different normalization techniques (PQN and total area) gave similar results (**Figure 2a, 2b**), confirming that with this extraction procedure and sample preparation it is possible to obtain good quality and highly reproducible spectra as it can be seen also from the 1D ¹H-NMR spectra shown in figure 2. The principal component analysis of all olive spectra allowed to identify clusters. Since T0, T1 and T2 dropped tend to group together, this could mean that they have a similar profile/metabolism, and therefore an incomplete development which has led the fruitlets to fall early from the tree. T3 dropped fruitlets tend to cluster with the preceding time point (T2small and T2big). Among all profiles T3 big olives seem to show a peculiar profile, probably because they are metabolically more developed. Different NMR profiles are clearly visible even at first sight (**Figure 3**). Sequentially, OPLS supervised method has been employed to extract and visualize latent and hidden variations characteristic of the abscission phenomenon. Therefore, olive fruitlets were divided according to their tendency to drop. The processed ¹H spectra were analyzed using the bucketing procedure described above, and the PQN-normalized data were submitted to OPLS. At first, the NMR data from 35 ND (not dropped fruitlets) and 15 D (dropped) samples were used to build an OPLS model to differentiate the two types of fruitlets. The optimal model correctly identified 97.8% of 35 ND fruitlet and 100% of 15 D with an overall accuracy of 98.5%, as evaluated from the results on the outer loop of the double cross-validation procedure. The obtained discrimination between the two categories can be observed in **figure 4**, where the projection of the samples onto the first two latent variables of the model is reported.

Univariate analyses on metabolites of methanol fruitlet extracts

NMR spectra were analyzed to identify which of the twenty metabolites identified differ among the ten groups of fruitlets (Supplementary Table 1, Supplementary Figure SF1).

We then aimed to identify different levels of metabolites between ND and D fruitlets. Among the twenty metabolites, gallic acid, caffeic acid, 3,4-dihydroxyphenylglycol and arabinose were higher in ND fruitlet samples, while choline was higher in dropped (D) fruitlets (**Figure 5**.) Concerning choline metabolism, a study on tomato fruit³⁰⁰ suggests that one of the reasons for choline accumulation may be the release of choline from membrane phospholipids by phospholipase D. Choline is the precursor of membrane phospholipids, intracellular messengers diacylglycerol and ceramide, signaling lipids platelet-activating factor and sphingosylphosphorylcholine, and neurotransmitter acetylcholine³⁰¹. Also, another methyl donor betaine and an osmoprotectant Gly betaine are synthesized from choline³⁰². In plants, Gly-betaine has been reported to confer tolerance to environmental stresses such as salinity and drought³⁰³. The impaired conversion of choline in Gly-betaine (with a consequent lower protection against stressful conditions) could be the reason why choline is higher in dropped fruitlets. The cell walls of the inner and outer pericarp of fruits usually contain arabinose as one of the most prominent components. Arabinose plays an important role in regulating the chemical structure of glucuronoarabinoxylan (GAX) and xyloglucan (XG) cell wall matrix polysaccharides³⁰⁴. α -L-Arabinofuranosidase and β -D-xylosidase are responsible for the hydrolysis of XG and GAX. B-D-Xylosidase and α -L-arabinofuranosidase have recently been identified in developing and ripening tomato fruits³⁰⁵. The activity of both enzymes was highest during early fruit growth, before decreasing during later development and ripening. In tomato fruits arabinose content has been found decreased in the early ripening stage and increased in the later ripening stage in the skin, mesocarp/endocarp, and septum. Thus, lower levels of arabinose in “dropped” fruitlets can be ascribed to an incomplete ripening.

The most common phenolic acids in fruits are caffeic and gallic acid, and the total phenol content is usually determined in terms of caffeic or gallic acid equivalents. 3,4-dihydroxyphenylglycol is considered a major C₆-C₂ phenolic compound in *olea europaea* fruits³⁰⁶. Fruits also contain polyphenolic compounds, derived from the polymerization of simple phenolics. The most well-known group of these compounds is tannins. Tannins are segregated into hydrolysable tannins, with a base unit of a gallic acid moiety, and condensed tannins, with a flavone base unit. Tannins are the main responsible of the astringency effect which causes the dry and pucker feeling in the mouth following the consumption of unripened fruit. It is often reported in the literature that astringency decreases during maturation, usually accompanied by a decrease in the concentration of tannins. However, a recent study on transgenic non-isoprene emitting gray poplars³⁰⁷ shows a down-regulation of phenolic and tannin pathways under high temperature and high light conditions. Behnke et al. found in transgenic non-isoprene trees only lower quantities of phenolic end products, total phenolics, anthocyanin and condensed tannins, whereas the concentrations of intermediates of the Shikimate pathway and aromatic amino acid biosynthesis were not affected. Indeed, isoprenoid emissions have often been found to be sustained when carbon supply becomes scarce under stress conditions. The function of isoprene might be considered relevant for the plant under stress, and a role has been proposed in apple (*Malus domestica*), in altering the oxidative status of plants under stress condition²⁹⁰. Considering that we found lower concentration of gallic acid, caffeic acid and 3,4-dihydroxyphenylglycol (major phenolic compounds in fruits and in *olea europaea*), and also vanillic acid (phenol lower in “D”, but not significantly altered) in dropped fruitlets, and considering the available literature,

we can speculate on the presence of alterations in the mechanisms regulating the isoprene production (similar to that one proposed by Eccher et al.2013).

Conclusions

The PCA analysis seems to be effective in the discrimination of olive groups according to the developmental stage. Dropped fruitlets shows a more immature profile/metabolism with respect to olives collected at the same time but without the same abscission potential. The supervised approach built on the OPLS model is highly effective in discriminating the “not dropped” fruitlets from the “dropped” ones, meaning that the two groups have distinct profiles. Several water-soluble metabolites differ among the ten subgroups of fruitlets, but further studies need to be performed in order to elucidate specific pathways involved. Anyway, normalization of metabolite concentrations with different approaches (PQN and total area), gave similar results, except for D-glucose and 3,4-dihydroxyphenylacetic acid in T3small and T3big (Supplementary Figure 1, delimited by the black rectangle). The water-soluble metabolites that are significantly altered in one of the two groups (“not dropped” vs “dropped”) maintain the sign and *P*-values significance using both normalizing approaches.

Higher choline levels and lower levels of gallic acid, caffeic acid and 3,4-dihydroxyphenylglycol in “dropped” fruitlets suggest, according to literature, an impairment of stress response mechanisms. Instead, lower arabinose levels can be ascribed to an incomplete development in “dropped” fruitlets.

Tables and Figures

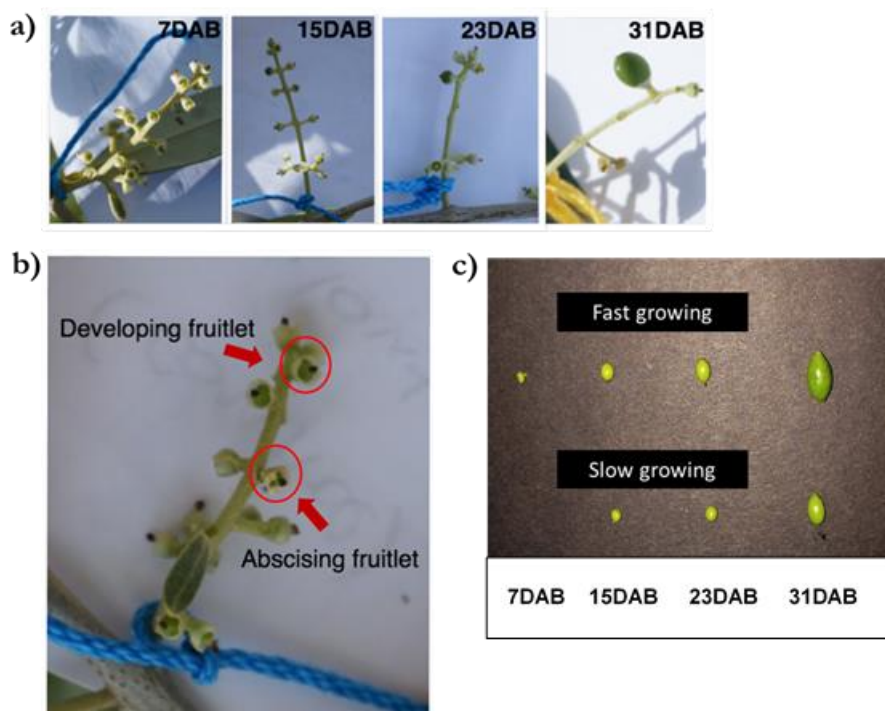


Figure 1. Fruit set. a) olive sampled from DAB (Days After Blooming) for four consecutive weeks: 7 (t0), 15 (t1), 23(t2), 31 (t3) DAB; b) olive discriminated as developing fruitlets and abscising fruitlets; c) developing fruitlet discriminated on the basis of growth pattern: fast growing (big) and slow growing (small) fruits.

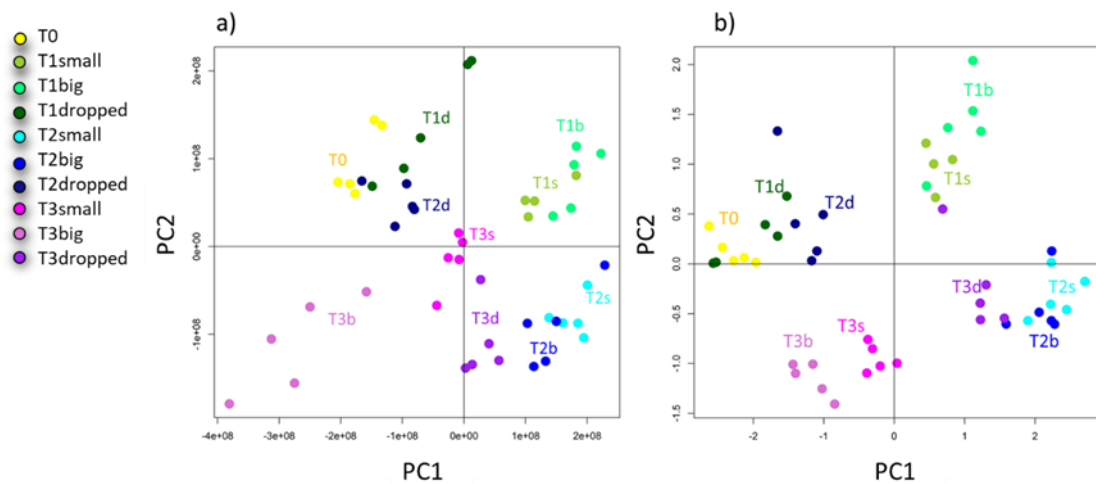


Figure 2. Principal component analysis (PCA) score plots. Each dot represents a single NMR spectrum of olive fruitlet samples collected at different time points and categorized according to size and dropping tendency. A) NMR spectra normalized with PQN algorithm. B) NMR spectra normalized on total area.

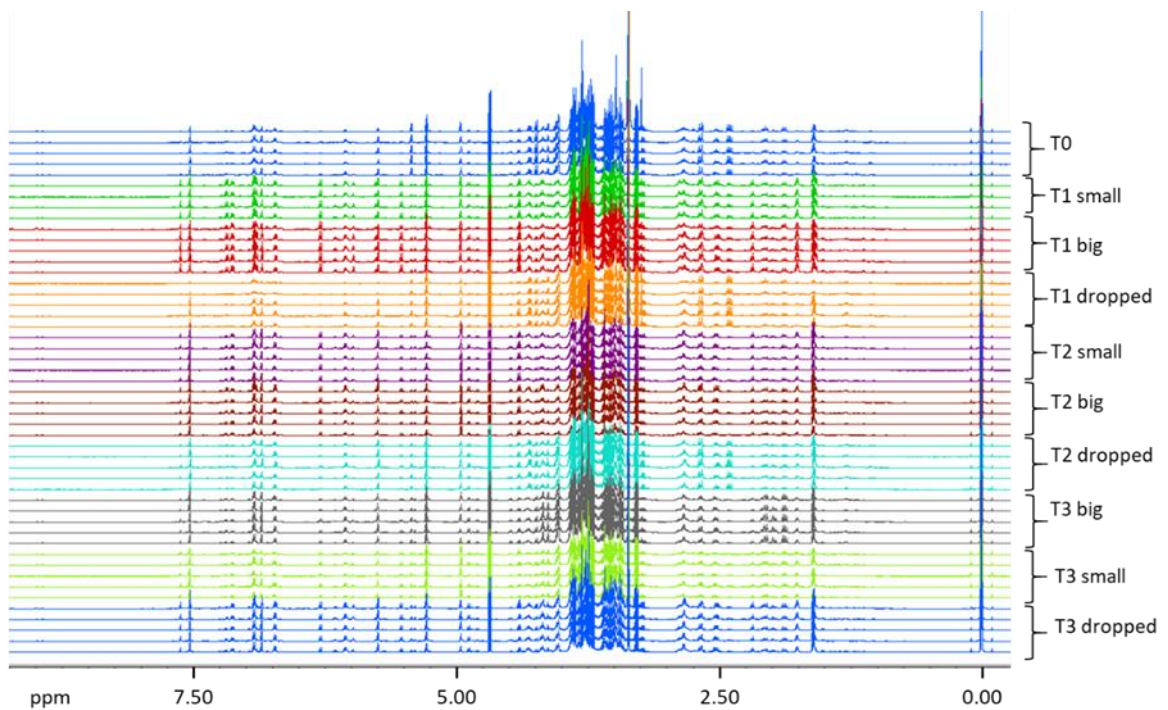


Figure 3. ^1H -NMR spectra of olive methanol extract samples

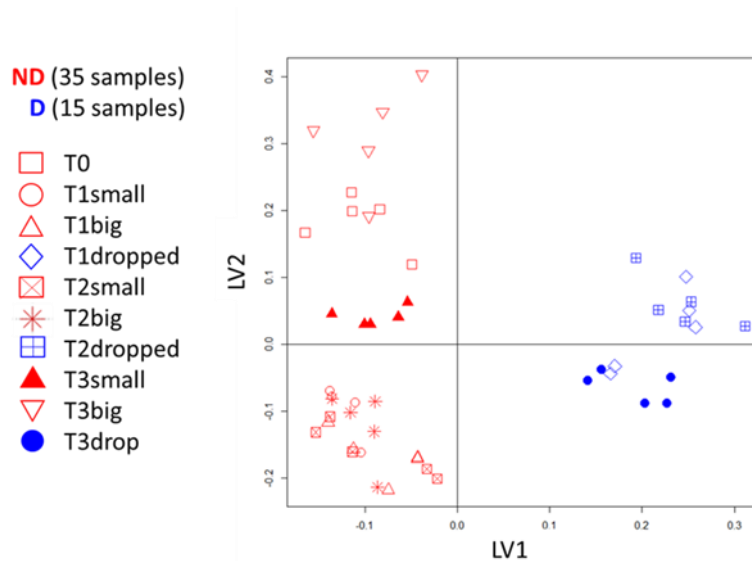


Figure 4. OPLS score plot of not-dropped (ND) and dropped (D) fruitlet methanol samples. Projection of the samples onto the space spanned by the two significant latent variables of the model (red and blue). Each different sign represents a distinct subgroup.

Metabolites	P-value	adj. P-value (FDR)
Choline	1.04E-05	2.19E-04
D-glucose	7.72E-01	8.53E-01
Saccharose	3.34E-02	1.00E-01
D-mannose	4.88E-02	1.28E-01
Mannitol	2.07E-01	2.90E-01
Quinic acid	5.12E-01	6.68E-01
Arabinose	1.38E-04	9.69E-04
Guanine	1.51E-01	2.86E-01
Uracile	1.63E-01	2.86E-01
Xylose	1.07E-01	2.24E-01
Dihydrocaffeic acid	5.41E-01	6.68E-01
4-hydroxybenzoic acid	1.99E-01	2.90E-01
Gallic acid	6.39E-03	3.32E-02
Vanillic acid	8.46E-02	1.97E-01
Caffeic acid	1.35E-02	4.72E-02
3,4-Dihydroxyphenylacetic acid	6.60E-01	7.70E-01
3,4-dihydroxyphenylglycol	7.90E-03	3.32E-02
Acetic acid	9.40E-01	9.57E-01
Pantothenic acid	2.07E-01	2.90E-01

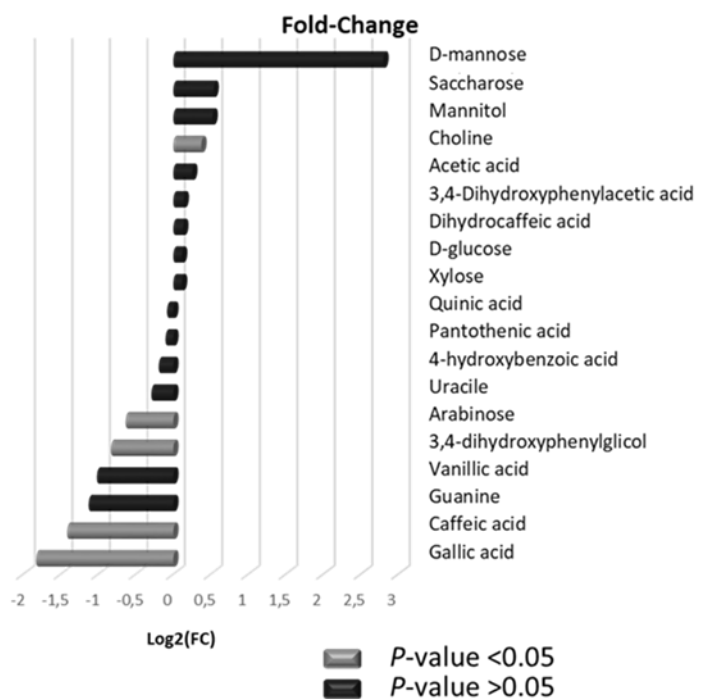


Figure 5. Metabolites whose concentrations are significantly different (P-values <0.05) in ND (not dropped) samples compared to D (dropped) olive fruitlet samples. The values of Log₂ (FC) and the P-values and adjusted P-values (FDR) are provided in the barplot and in the table on the left respectively. Metabolites with Log₂ (FC) negative values have higher concentration in ND samples with respect to D samples. Metabolites with Log₂ (FC) positive values have lower concentration in D olive samples.

Supplementary Material

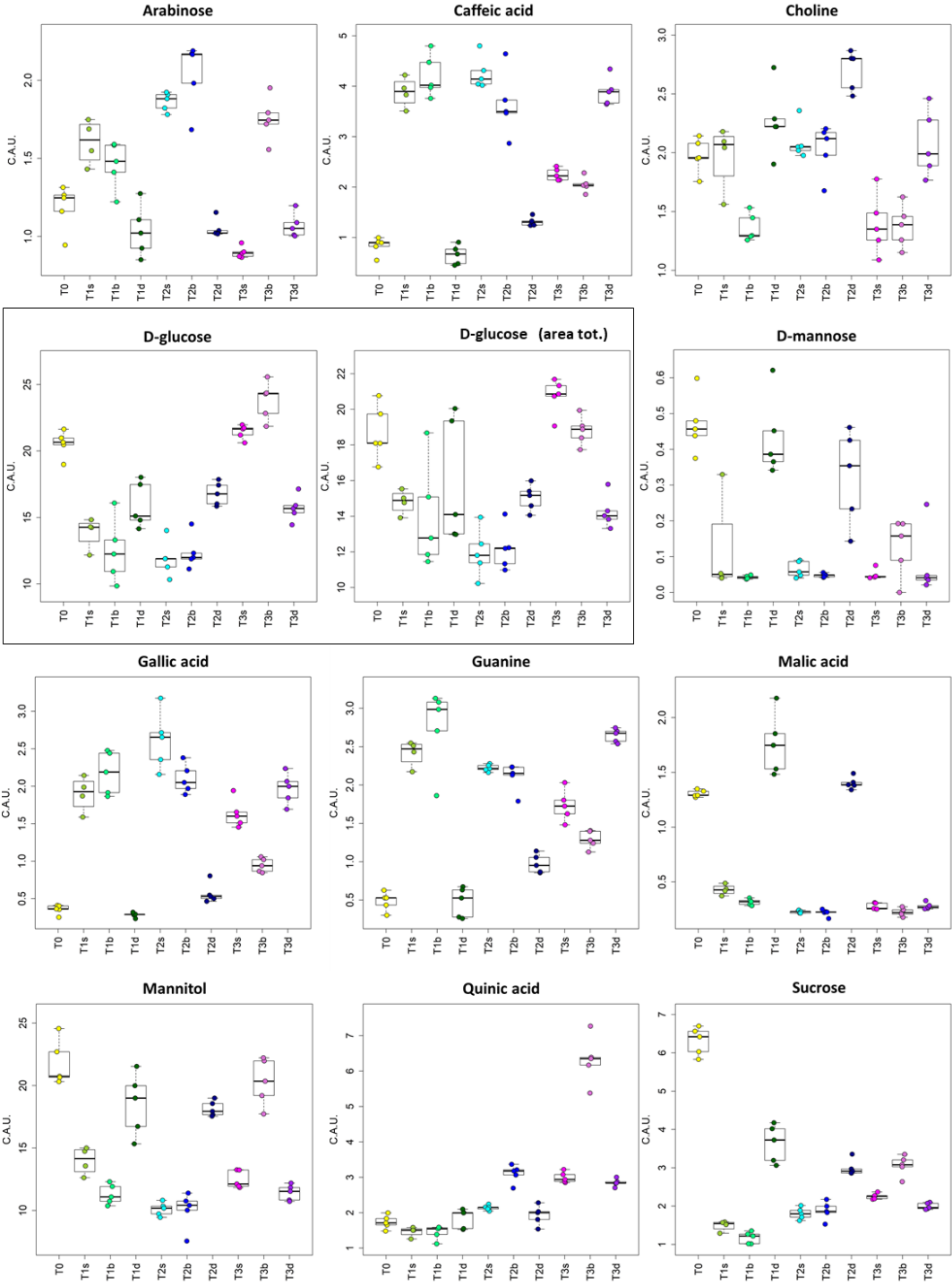
Supplementary Table 1. Concentrations (mean $\pm$ SD) of the metabolites assigned (MSI level 1) in methanol fruitlet extracts. Significantly different P-values from the comparisons are also reported.

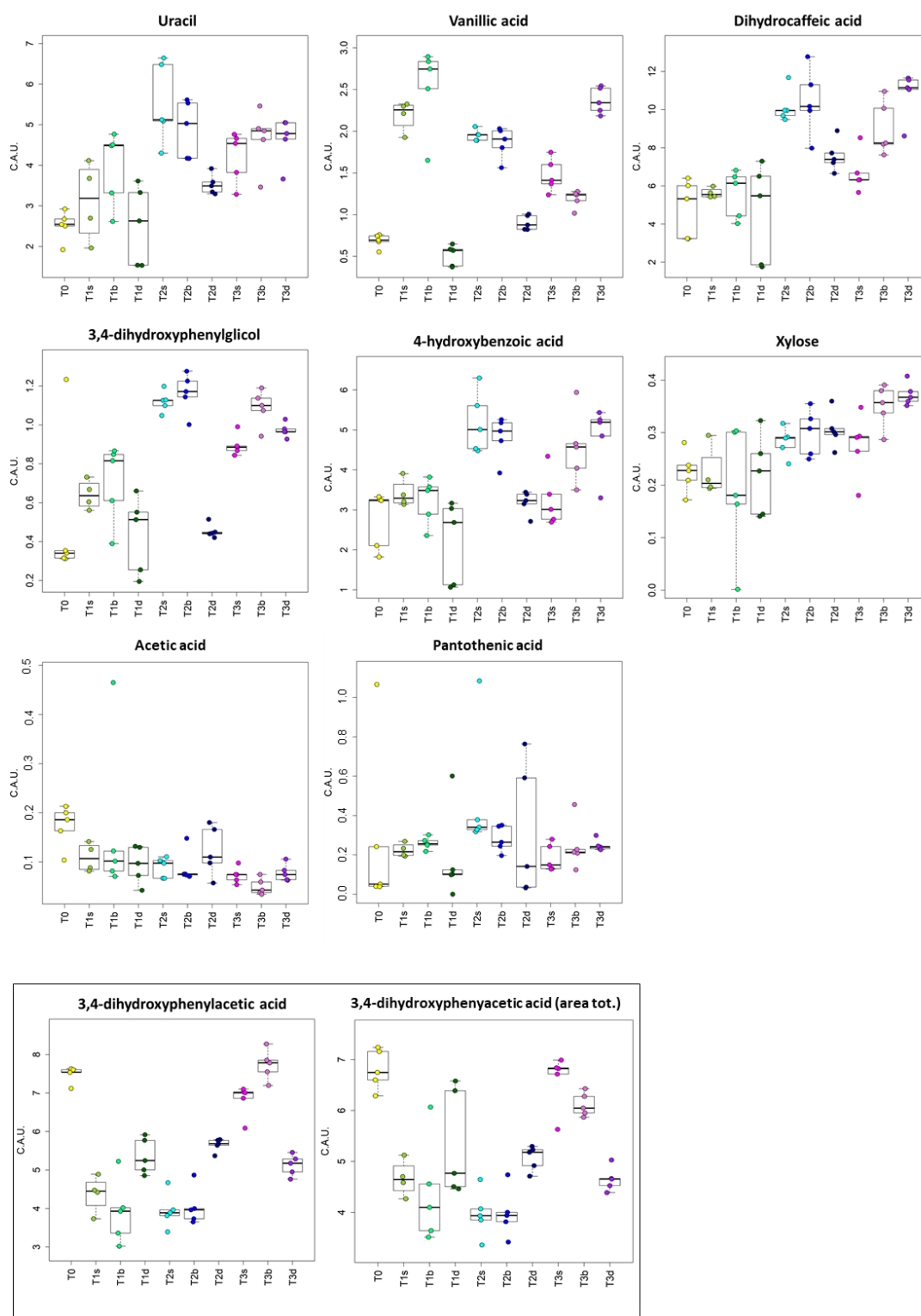
Metabolites	T0	T1small	T1big	T1drop	T2small	T2big	T2drop	T3small	T3big	T3drop	Significant comparisons (P-value <0.05)
Choline	1.98 $\pm$ 0.15	1.97 $\pm$ 0.28	1.36 $\pm$ 0.12	2.27 $\pm$ 0.29	2.09 $\pm$ 0.15	2.03 $\pm$ 0.22	2.70 $\pm$ 0.17	1.39 $\pm$ 0.26	1.38 $\pm$ 0.18	2.08 $\pm$ 0.29	(T1b vs T1d), (T1b vs T2d), (T1d vs T3s), (T1d vs T3b), (T2d vs T3s), (T2d vs T3b)
D-glucose	20.55 $\pm$ 0.98	13.88 $\pm$ 1.17	12.48 $\pm$ 2.40	15.91 $\pm$ 1.72	11.88 $\pm$ 1.36	12.36 $\pm$ 1.28	16.78 $\pm$ 0.87	21.44 $\pm$ 0.54	23.79 $\pm$ 1.45	15.70 $\pm$ 0.98	(T0 vs T1b), (T0 vs T2s), (T0 vs T2b), (T1s vs T3s), (T1s vs T3b), (T1b vs T3s), (T1b vs T3b), (T1d vs T3b), (T2s vs T2d), (T2s vs T3s), (T2s vs T3b), (T2b vs T3s), (T2b vs T3b), (T3b vs T3d)
Sucrose	6.31 $\pm$ 0.37	1.49 $\pm$ 0.14	1.18 $\pm$ 0.15	3.63 $\pm$ 0.49	1.81 $\pm$ 0.15	1.88 $\pm$ 0.24	2.99 $\pm$ 0.21	2.25 $\pm$ 0.08	3.06 $\pm$ 0.27	2.00 $\pm$ 0.09	(T0 vs T1s), (T0 vs T1b), (T0 vs T2s), (T0 vs T2b), (T0 vs T3d), (T1s vs T1d), (T1s vs T2d), (T1s vs T3b), (T1b vs T1d), (T1b vs T2d), (T1b vs T3s), (T1b vs T3b), (T1d vs T2b), (T2s vs T3b)
D-mannose	0.47 $\pm$ 0.08	0.12 $\pm$ 0.14	0.04 $\pm$ 0.00	0.43 $\pm$ 0.11	0.06 $\pm$ 0.02	0.05 $\pm$ 0.01	0.32 $\pm$ 0.13	0.05 $\pm$ 0.01	0.13 $\pm$ 0.08	0.08 $\pm$ 0.09	(To vs T1s), (To vs T1b), (To vs T2s), (To vs T2b), (To vs T3s), (To vs T3d), (T1b vs T1d), (T1b vs T2d), (T1d vs T2b), (T1d vs T3s), (T1d vs T3d), (T2d vs T3s), (T2d vs T3d)
Mannitol	21.79 $\pm$ 1.81	13.98 $\pm$ 1.10	11.28 $\pm$ 0.81	18.51 $\pm$ 2.49	10.10 $\pm$ 0.53	10.02 $\pm$ 1.47	18.14 $\pm$ 0.62	12.48 $\pm$ 0.71	20.29 $\pm$ 1.89	11.42 $\pm$ 0.63	(T0 vs T1b), (T0 vs T2s), (T0 vs T2b), (T0 vs T3s), (T0 vs T3d), (T1b vs T1d), (T1b vs T3b), (T1d vs T2s), (T1d vs T3d), (T2s vs T2d), (T2s vs T3b), (T2b vs T2d),

											(T2b vs T3b), (T3b vs T3d)
Quinic acid	1.73±0.19	1.46±0.14	1.44±0.20	1.83±0.27	2.14±0.07	3.10±0.25	1.93±0.28	2.99±0.15	6.31±0.67	2.85±0.11	(T0 vs T2b), (T0 vs T3s), (T0 vs T3b), (T1s vs T2b), (T1s vs T3s), (T1s vs T3b), (T1b vs T2b), (T1b vs T3d), (T1b vs T3s), (T1b vs T3b), (T1d vs T2b), (T1d vs T3s), (T1d vs T3b), (T2s vs T3b), (T2d vs T3b)
Arabinose	1.19±0.15	1.60±0.14	1.46±0.15	1.04±0.16	1.86±0.06	2.04±0.21	1.05±0.06	0.90±0.04	1.75±0.14	1.07±0.08	(T0 vs T2s), (T0 vs T2b), (T1s vs T3s), (T1b vs T3s), (T1d vs T2s), (T1d vs T2b), (T1d vs T3b), (T2s vs T2d), (T2s vs T3s), (T2b vs T2d), (T2b vs T3s), (T2b vs T3d), (T2d vs T3b), (T3s vs T3b), (T3b vs T3d)
Guanine	0.48±0.12	2.42±0.17	2.75±0.52	0.47±0.19	2.22±0.05	2.11±0.18	0.97±0.12	1.73±0.21	1.29±0.12	2.65±0.09	(T0 vs T1s), (T0 vs T1b), (T0 vs T2s), (T0 vs T2b), (T0 vs T3d), (T1s vs T1d), (T1s vs T2d), (T1b vs T1d), (T1b vs T2d), (T1b vs T3b), (T1d vs T2s), (T1d vs T2b), (T1d vs T3d), (T2d vs T3d), (T3b vs T3d)
Uracile	2.51±0.37	3.11±0.97	3.94±0.93	2.53±0.97	5.53±1.00	4.90±0.71	3.53±0.25	4.21±0.64	4.66±0.78	4.64±0.57	(T0 vs T2s), (T0 vs T3b), (T0 vs T3d), (T1s vs T2s), (T1d vs T2s), (T1d vs T3b), (T1d vs T3d), (T2s vs T2d)
Xylose	0.23±0.04	0.22±0.05	0.19±0.12	0.22±0.08	0.28±0.03	0.30±0.04	0.31±0.04	0.28±0.06	0.35±0.04	0.37±0.02	(T0 vs T3b), (T0 vs T3d), (T1s vs T3b), (T1s vs T3d), (T1b vs T3b), (T1b vs T3d), (T1d vs T3b), (T1d vs T3d)
Dihydro- caffeic acid	4.84±1.52	5.62±0.26	5.58±1.26	4.58±2.61	10.15±0.88	10.44±1.77	7.58±0.83	6.70±1.09	9.02±1.42	10.81±1.25	(T0 vs T2s), (T0 vs T2b), (T0 vs T3b), (T0 vs T3d), (T1s vs T2s), (T1s vs T2b), (T1s vs T3b), (T1s vs T3d),

											(T1b vs T2s), (T1b vs T2b), (T1b vs T3d), (T1d vs T2s), (T1d vs T2b), (T1d vs T3b), (T1d vs T3d), (T2b vs T3s), (T3s vs T3d)
4-hydroxy- benzoic acid	2.75±0.72	3.41±0.35	3.23±0.59	2.22±1.04	5.19±0.77	4.81±0.54	3.18±0.29	3.24±0.67	4.54±0.91	4.81±0.87	(T0 vs T2s), (T0 vs T2b), (T0 vs T3b), (T0 vs T3d), (T1b vs T2s), (T1d vs T2s), (T1d vs T2b), (T1d vs T3b), (T1d vs T3d), (T2s vs T2d), (T2s vs T3s), (T2b vs T2d), (T2b vs T3s), (T3s vs T3d)
Gallic acid	0.36±0.06	1.90±0.23	2.18±0.29	0.28±0.03	2.61±0.39	2.10±0.20	0.57±0.13	1.63±0.19	0.95±0.09	1.97±0.21	(T0 vs T1s), (T0 vs T1b), (T0 vs T2s), (T0 vs T2b), (T0 vs T3d), (T1s vs T1d), (T1b vs T1d), (T1b vs T2d), (T1d vs T2s), (T1d vs T2b), (T1d vs T3b), (T2s vs T2d), (T2s vs T3b), (T2b vs T2d)
Vanillic acid	0.69±0.08	2.19±0.18	2.53±0.51	0.51±0.13	1.95±0.07	1.86±0.19	0.90±0.09	1.47±0.20	1.19±0.10	2.37±0.16	(T0 vs T1s), (T0 vs T1b), (T0 vs T2s), (T0 vs T2b), (T0 vs T3d), (T1s vs T1d), (T1s vs T2d), (T1b vs T1d), (T1b vs T2d), (T1b vs T3b), (T1d vs T2s), (T1d vs T2b), (T1d vs T3d), (T2d vs T3d), (T3b vs T3d)
Caffeic acid	0.84±0.17	3.88±0.30	4.21±0.42	0.66±0.19	4.26±0.32	3.64±0.64	1.32±0.09	2.25±0.12	2.05±0.15	3.89±0.28	(T0 vs T1s), (T0 vs T1b), (T0 vs T2s), (T0 vs T2b), (T0 vs T3d), (T1s vs T1d), (T1s vs T2d), (T1b vs T1d), (T1b vs T2d), (T1b vs T3b), (T1d vs T2s), (T1d vs T3d), (T2s vs T2d), (T2s vs T3s), (T2s vs T3b), (T2d vs T3d)
3,4-Dihydroxy- phenylacetic	7.49±0.21	4.38±0.48	3.91±0.84	5.36±0.47	3.95±0.46	4.04±0.48	5.65±0.17	6.82±0.42	7.73±0.40	5.13±0.27	(T0 vs T1s), (T0 vs T1b), (T0 vs T2s), (T0 vs T2b),

<i>acid</i>											(T1s vs T3s), (T1s vs T3b), (T1b vs T3s), (T1b vs T3b), (T2s vs T3s), (T2s vs T3b), (T2b vs T3s), (T2b vs T3b), (T3b vs T3d)
<i>3,4-dihydroxy-phenylglycol</i>	0.51±0.40	0.64±0.07	0.71±0.20	0.44±0.20	1.12±0.05	1.16±0.10	0.45±0.04	0.90±0.06	1.09±0.09	0.97±0.04	(T0 vs T2s), (T0 vs T2b), (T0 vs T3b), (T1s vs T2s), (T1s vs T2b), (T1b vs T2s), (T1b vs T2b), (T1d vs T2s), (T1d vs T2b), (T1d vs T3b), (T1d vs T3d), (T2s vs T2d), (T2b vs T2d), (T2d vs T3b)
<i>Acetic acid</i>	0.17±0.04	0.11±0.03	0.17±0.11	0.09±0.04	0.09±0.02	0.09±0.03	0.12±0.05	0.07±0.02	0.05±0.02	0.08±0.02	(T0 vs T3b)
<i>Pantothenic acid</i>	0.29±0.44	0.22±0.03	0.26±0.03	0.19±0.24	0.49±0.33	0.28±0.07	0.31±0.34	0.19±0.07	0.25±0.12	0.25±0.03	





Supplementary Figure1 Boxplots of the metabolites differentially concentrated in T0 (yellow), T1small (light green), T1big (green), T2 dropped (dark green), T2 small (light blue), T2 big (cyan), T2 dropped (dark blue), T3 small (magenta), T3 big (pink) and T3 dropped (purple) according to NMR analysis. For each comparison, the P-value (by the Kruskal-Wallis test followed by Dunn post-hoc analysis) is reported in Supplementary Table 1. C.A.U: Concentration in Arbitrary Unit. Metabolites are scaled according to PQN algorithm, in black boxes also results using total area are reported.

5.1.2 Cold water treatment of olive fruit before oil extraction: effect on volatile profile and quality parameters

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Introduction

Olive oil, which is the basis of the Mediterranean diet, has increased its consumption worldwide by 1.8-fold during the last 20 years (International Olive Oil Council, 2016). This higher consumption leads to an increasing demand for olive oil of high quality and thus it becomes imperative to explore and adopt strategies in order to improve both the sensorial and the nutraceutical parameters of the oil while maintaining high yield and profitability.

The volatile organic compounds (VOCs) and the phenolics are the main elements markedly affecting the sensory profile of the olive oil (Angerosa 2002; Aparicio, 2002). The phenolic compounds are responsible for the characteristic bitter and pungent taste of the oil and, together with other molecules, provide the highly-appreciated antioxidant properties, with benefits for the consumer's health but also for the stability of the oil itself against oxidation (Fregapane and Salvador, 2013). Olive oil VOCs have a great influence on the overall flavor, highly discriminating oils in terms of sensorial but also commercial quality (e.g. defects due to the presence of off-flavors). In fruit, VOCs are generated from both primary and secondary metabolites and, in addition to the impact on flavour, a correlation between health and the volatiles that contribute to a positive perception, has been postulated (Goff and Klee, 2006). From a physiological standpoint, a key factor affecting the olive oil quality and composition is the ripening stage of the processed fruit. In relation to the degree of ripening the abundance, accumulation and profile of polyphenols, tocopherols, fatty acids and VOCs in the oil markedly change (Gutierrez et al., 2000). Polyphenols tend to decrease as ripening progresses while VOCs, and particularly C5 and C6 aldehydes are progressively replaced by their alcohol derivatives. These changes have a strong influence on the sensorial characteristics of the olive oil and its stability: the riper the fruit, the higher the oil yield, but the less "green, cut-grass"-like and bitter the oil flavour. Additionally, various off-flavour compounds are formed by oxidation, which may be initiated in the olive fruit during the advanced stages of ripening and are induced/enhanced by the high temperatures (Kritsakis, 1998). In the last decades, the effects of climate changes are resulting in an accelerated fruit development and ripening with a consequent earlier harvest (lower yield) and fruit processed with higher inner temperature. General metabolism and enzyme activities in the olive fruit are highly sensitive to the temperature. Among the different pathways responsible for VOCs biosynthesis, the lipoxygenase (LOX) pathway, which generates the abundantly present C6 compounds, is highly responsive to different temperature regimes, as observed in several fruit crops during storage (El Hadi et al., 2013; Brizzolara et al., 2018) resulting in altered profiles of aldehydes, alcohols and esters.

Based on what reported above, there is a need of setting up unified strategies aimed at reducing the negative impact on EVOO quality of the high temperatures (even 30°C or above) of early harvested olives, and/or processing ripe olives with a reduced risk of developing off-flavours. Ruling out the option of storing olive fruit before processing (all delays in oil extraction lead to a reduction of quality traits), a recent published paper (Veneziani et al., 2017) reports that a rapid cooling treatment of olive paste right after crushing and just before malaxation led to an increase in several phenolic fractions in the oil, and showed quantitative and qualitative modifications of volatile compounds, possibly due to changes in enzyme activities of the LOX pathway in the paste. An alternative approach to modulate the volatile profile of the oil is represented by controlling (reducing) the temperature of the fruit immediately after harvest, when fruit are washed and just before they undergo crushing. The aim of this study was that of assessing the

effect of lowering fruit temperature, on the VOC profiles and the polyphenol content of the resulting oil.

Materials and methods

Olive fruit and treatments

Olive (*Olea europaea*, cv Leccino) fruit were harvested from an orchard located in Perugia (Italy), in two different seasons (2016 and 2017) in correspondence of three different ripening stages: a) SEMI-RIPE (SR), Jaén ripening index (0-7 scale) of 2.69 ± 0.08 and 2.50 ± 0.07 ; b) RIPE (RP), Jaén ripening index of 3.82 ± 0.04 and 4.58 ± 0.18 ; for 2016 and 2017, respectively c) ADVANCED RIPENING (AR), Jaén ripening index 5.10 ± 0.15 , only for the 2017 season. Within 24 hrs after harvest, fruit were divided in three groups of equal weights (7kg) and washed using water at three different temperatures (4, 12 and 19 °C). When the inner (mesocarp) temperature reached the values of 10, 15 and 19 °C for the three water conditions, respectively, fruit were removed from the water and immediately processed using a lab-scale olive mill. After crushing, malaxation duration was set at 20 min for all samples with no control of temperature. At the end of malaxation the temperature of the paste was of 14, 17 and 21 °C, for the three temperature conditions, respectively. Olive oil was extracted via centrifugation and then re-centrifuged for 2min at 14,000 rpm at 4 °C in order to remove any remaining water residues. Oil samples have been stored in dark glass vials at 4 °C until further analysis.

Olive oil analyses

NMR. For NMR sample preparation, ≈ 60 mg of olive oil was dissolved in 600 μ L of deuterated chloroform (CDCl_3). 600 μ L of the prepared mixture, after 20 s of vortexing, was transferred into a 5 mm NMR tube. NMR experiments were performed using an AVANCE III 400 MHz Bruker spectrometer working at 300K. Two different ^1H -NMR experiment were performed for each sample⁹⁰: A) ZG1H: a standard single pulse experiment, i.e. RD-P(90°)-acquisition of the free induction decay (FID). The nonselective 90° hard pulse P(90°) was adjusted to 10 μ s. The relaxation delay (RD), and acquisition time (AQ) were set to 4s, and ~ 2.66 s, respectively, resulting in a total recycle time of ~ 6.66 s. FIDs were collected into time domain (TD) = 65536 (64K) complex data point by setting: dummy scans (DS) = 4, number of scans (NS) = 64, spectral width (SW) = 20.5155 ppm and receiver gain (RG) = 1. FIDs were processed by applying an exponential line broadening (LB) of 0.3 Hz before Fourier transformation; B) NOESYGPPS: a one-dimensional ^1H -NMR pulse sequence with suppression of the strong saturating signals. The setting for parameters RD, P (90°), AQ, TD, NS, DS, and LB were the same as those of ZG1H experiment. An amplitude and phase modulation shaped pulse were applied during RD with a frequency spectrum of 16 highly selective bands with 3.7 Hz RF-field each to achieve highly selective suppression of multiple dominating signals leaving the rest of the spectrum undistorted (figure of acquired spectra in supplementary material, Fig. S1). Resulting NMR spectra were aligned to β -sitosterol (0.7 ppm). Input variables for statistical analysis were generated via bucketing (binning). Bucketing of ZG1H spectra was obtained considering whole spectra with the exception of chloroform residual signal (7.24 ppm). Bucketing of NOESYGPPS spectra was obtained removing all suppressed saturating signals (Fig. S1, supplementary material). Then, binned spectra were scaled using oil weight measured

for samples preparation. Finally, total area normalization was applied concluding the 1D spectral processing phase.

GC/MS. For volatile organic compound (VOC) analysis, oil (2g) samples were transferred into a 20mL vial and thermostated at 50 °C for 90min. A SPME fiber 50/30um DVB/CAR/PDMS (Supelco, Bellefonte, PA, USA) was used. Prior to analysis, the fiber was conditioned by introducing the fiber into the injector which was set at 270 °C for 30' in a stream of helium. The fiber was inserted into the GC injector and set in splitless mode using a splitless inlet liner of 0.75 mm i.d. for thermal desorption where it was left for 5 min. A Clarus® 680 Gas Chromatograph equipped with a split/splitless injector (PerkinElmer®, Waltham, Massachusetts) was used. A fused-silica capillary column was employed (DB-Wax-ETR, 60 m, 0.32 mm ID, 0.25 µm film thickness; Restek, Bellefonte, PA). The operating gas was helium with a flow rate of 2mL/min for 5min during fiber desorption and then a constant flow of 1ml/min. The GC oven heating was started at 40 °C. This temperature was maintained for 6 min, increased to 120 °C at a rate of 5°C/min, increased to 160 °C at a rate of 3 °C/min, increased to 240°C at a rate of 15°C/min, and finally increased to 210 °C at a rate of 3.6 °C/min, where it was held for 5min; the total time of analysis was 46 min. The injector temperature was maintained at 250°C. Each sample was analysed in 10 replicates. The identification of the compounds was performed by a PerkinElmer® Clarus® 600 MS. Component assignment was based on computer matching of EI spectra with the NIST 08 spectral library (National Institute of Standards and Technology, Gaithersburgh, MD), taking into account the coherence of the retention indices (Kovat's indices) of the analysed compounds with those reported in the literature when available.

Spectrophotometric and HPLC analyses. In order to assess the total polyphenol content of the oil samples, the Folin-Ciocalteu (FC) assay was employed as described by Alessandri et al. (2014) with some modifications. Briefly, a total of 2 g of oil was diluted in 4 mL of n-hexane, vortexed for 1min and extracted with 10min of centrifugation at 6000 rpm with 4mL of CH₃OH–H₂O (80:20 v/v). 1 mL of the extract was added to 0.25 mL of FC reagent, 1.5 mL of Na₂CO₃ (20% w/v), in a 10mL volumetric flask reaching the final volume with purified water. Each sample was incubated for 2 hrs at 25 °C. Spectrophotometric analysis was performed at $\lambda = 750$ nm. The final result, expressed in mg/kg of gallic acid, obtained via a calibration curve with range from 1 to 15 µg mL⁻¹ ($R^2 = 0.998$). The quantification of specific phenol compounds was carried out by HPLC according to the International Olive Oil Council method with some modifications. More specifically, the eluents used were acidified H₂O (1 % Acetic Acid) (A), methanol (B) and acetonitrile (C). The analysis was performed via a multistep linear solvent gradient as follows: 0–40 min, gradient 96 % A, 2 % B, 2 % C; 40–45min, gradient 50 % A, 25 % B, 25 % C; 45 – 60 min, gradient 40 % A, 30 % B, 30 % C; 60 – 72 min, gradient 50 % B, 50 % C; 72 – 82 min, gradient 96 % A, 2 % B, 2 % C. Total time of analysis 82 min, oven temperature 27 °C; flow rate 0.8 mL min⁻¹. Four different wavelengths (260, 280, 320, 370 nm) were selected for the quantification of the different phenols. The single compounds were quantified via their calibration curves constructed with the following standards: gallic acid, hydroxytyrosol, tyrosol, cinnamic acid, vanillic acid, oleuropein, apigenin, all obtained by Sigma-Aldrich. All calibration curves had an $R^2 \geq 0.998$.

Statistical Analysis. Multivariate analysis on metabolomics data was conducted on processed NMR spectra. Principal component analysis (PCA) was used as first exploratory analysis. Orthogonal projections to latent structure discriminant analysis (OPLS/DA) was applied to obtain the supervised separation of the analysed groups²¹⁵. The confusion matrices and the accuracies reported for NMR data were assessed by means of Leave-one-out cross validation scheme (LOOCV). Twenty metabolites, corresponding to well defined and resolved peaks in the spectra, were assigned using ZGH1 and NOESYGPPS experiments. Signal identification was performed using a library of NMR spectra of pure organic compounds, public databases (e.g., FooDB, PhytoHub, PhenolExplorer) storing reference and literature data^{37,111,308–310}. The concentrations of the various metabolites in the different spectra were calculated by integration of the signal area, and the relative concentrations were analysed to determine the discriminating metabolites among the group of olive oil.

A PLSDA model was built to analyse the influence of the washing temperature on the aroma and metabolic profiles of the olive oil samples considering all the analysed variables (GCMS, HPLC, ¹H-NMR). JMP 13 Software (© SAS Institute) was used to perform the multivariate analysis and variable importance in projection (VIP) scores have been employed to filter variables that contributed the most to clusters separation.

Results

Based on free fatty acid, peroxide number, K232, K270 and Delta K values (data not shown), all oil samples were classified as extra-virgin.

A total of 45 spectra (15 of SR, 15 of RP and 15 of AR samples) were acquired via ¹H-NMR analysis. PCA statistical analysis was applied as preliminary unsupervised approach on the entire buckets of all 45 acquired spectra to obtain a simplified view of the variation in the data and to check the sample quality excluding the presence of outliers. PC1, accounting for 82% of variation in ZG1H and 85% in NOESYGPPS models, separates oil samples according to the year of collection (2016 and 2017), instead the general distribution of samples shows the maturation stage of olives at the time of the harvest (**Fig. S2a and b, supplementary material**). Information regarding the direct effect of temperature cannot be derived by an unsupervised approach. Consequently, supervised OPLS/DA statistical analysis was applied on the same dataset demonstrating a high predictive accuracy (98% on both models) in identifying oil samples treated under different temperatures (**Fig. 1a and 1b**).

Moreover, 20 metabolites corresponding to well defined signals in all oil ¹H-NMR spectra have been assigned (**supplementary material, table S1**). A heatmap (**Figure 2**) reporting the fold-change (FC) of the identified compounds in the 2016 and 2017 samples revealed that the most marked changes concerning the NMR-identified compounds are present considering two terpenes (terpene2 resonating at 4.68 ppm and terpene3 resonating at 4.73 ppm) and a secoiridoid derivative (hemiacetal, 9.26 ppm), that increases in oils obtained from ripe olives, indicating a strong association of these metabolites with the evolution of ripening. An opposite trend has been observed for trans-2-hexenal.

The low temperatures (10°C and 15°C) applied to the olives before entering the crushing step were effective in decreasing the content in the oil samples of the ripening-associated secoiridoids hemiacetal, with a more marked effect when SR olives were processed after temperature conditioning at 10°C. No significant changes induced by temperature conditioning were

detected for the two ripening-associated terpenes. Higher trans-2-hexenal levels were in general detected in oil samples obtained from RP and AR olives kept at both 10° and 15°C. Thus, decreasing olive temperatures at the post-harvest level induces significant changes in oil metabolic profiles as indicated also by PCA results obtained by considering the buckets of the aldehydic region (10-7.6 ppm) where 19°C samples of SR group were shifted along the positive PC1 (**supplementary data, Fig. S3**).

The results of the TPC quantification indicated that when lower temperature was applied in SR fruit, it resulted in significantly higher TPC in oils for both low temperature treatments when compared to 19°C. The same trend was observed for the oil deriving from AR fruit in respect to 15°C but not 10°C. On the contrary, both low temperatures significantly decreased the polyphenol content in oil in comparison to 19°C (**Figure 3**).

Further analyses were performed in order to assess the content of seven single phenolics, recognized as important components of the oil. Different trends have been observed in relation to the decrease of temperature, with higher levels of gallic acid and apigenin but lower amounts of cinnamic acid and oleuropein detected in the oil samples (**Supplementary data, Fig. S4**). Tyrosol, hydroxytyrosol and vannilic acid were higher in the oils obtained from SR fruit maintained at lower temperatures from ripe fruit the pattern was the opposite. Lastly, tyrosol, hydroxytyrosol and vannilic acid were reported slightly decreased, stable and decreased respectively, when compared to 19°C, regarding the olive oils of the latest harvest.

A total of 20 volatile organic compounds (VOCs) were identified (**Supplementary data table S2**). The results indicated that the predominant (quantitatively) volatile compound of all the olive oils, regardless the treatment and the ripening stage in respect to their relative intensity was 2-Hexenal.

Hexanal and 2-Hexenal displayed the same trend for all ripening stages and all treatments (**Figure 4**). More specifically, both C6 aldehydes were reported significantly higher when fruit were kept at lower temperatures regardless the ripening stage. This effect was more pronounced in oils obtained from RP and AD ripe fruit. Hexanal and 2-Hexenal are considered to be two of the most significant volatile organic compounds present in the olive oil, due to their association with its leafy and bitter/almond sensory notes. Hence, the results point out that even in later harvests when the fruit are mature beyond the point of regular harvest, once the fruit are conditioned in cold temperature, the resulting olive oil preserves the aforementioned sensory traits. Among the 20 VOC identified, some are associated with sensory defects. Ethyl alcohol, acetic acid, 1-penten-3-ol, 2-penten-1-ol (*Z*)- and 1-penten-3-one were reported significantly lower when the fruit were conditioned in cold temperature for the first two harvests, with ethyl alcohol to display the most profound decrease (**Figure 4**).

Regarding the aforementioned compounds in respect to the latest harvest, 1-penten-3-ol, 2-penten-1-ol, and 1-penten-3-one were found to be increased and stable, respectively, in the oils deriving from the fruit incubated at 15 °C when compared to 19°C. Acetic acid was reported to be significantly lower and higher for 15 and 10 °C respectively when compared with 19°C. Lastly, ethyl alcohol followed the same trend as mentioned before, and was found to be profoundly decreased in both oils obtained from low temperature conditioned fruit. The presence of ethyl alcohol, acetic acid, 1-penten-3-ol, 2-penten-1-ol (*Z*)- and 1-penten-3-one in olive oil has been associated with the perception of winey, vinegary, mold, ripe and metallic perception, respectively. Therefore, the results suggest that by lowering the temperature of the

pulp before the fruit undergo crushing, results in the reduction of aroma compounds responsible for specific defects (**Figure. 4**).

The results of the panel test (**Figure 5**) showed that the different pulp temperatures affect the sensory characteristics of the resulting oil. More specifically, the oil samples obtained from semi-ripe fruit which were conditioned in low temperature were characterized by higher scores concerning the “green”, “fruity” and “almond”, attributes, whereas semi-ripe olives kept 19°C produced oil with higher scores regarding spicy and bitter attributes. Regarding the oil samples from the ripe olives, the oil extracted from fruit incubated at 15°C was evaluated as more bitter, spice and green, than oils from the two other fruit samples (10 and 19°C). The panel test of the oil samples extracted from the ripe+ olives indicated that the highest scores in terms of spiciness and green attributes were obtained by the oil of the olives incubated at 10 °C. On the other hand, oil of 19°C samples reported higher scores in terms of almond and artichoke attributes when compared to the lower temperature samples. No defects were detected in all samples analysed

A heatmap has been created in order to associate groups or single metabolites identified by NMR and GC/MS with the attributes detected by the panelists. As shown in **figure 6**, different clusters can be identified. In particular, a first cluster includes compounds such as hydroxytyrosol, saturated fatty acids and linolenic acid, hexanal and hexenal and hydroperoxides which show positive correlations with bitter, spicy and green attributes, but are negatively correlated with fruity and artichoke traits. A second cluster includes a larger number of metabolites: alcohols, 2,4- hexadienal, wax, squalene, 1-penten-3-one, two terpenes, vanillic and acetic acids, gallic and cinnamic acids, propanoic and pentanoic acids, unsaturated acids are negatively correlated with bitter, spicy and green and positively correlated with fruity and artichoke. The almond trait appears to be positively correlated with saturated acids and negatively with unsaturated acids, propanoic and pentanoic acids.

Partial least squares (PLS) analysis was employed to better assess the whole dataset obtained from the different oils in respect to both ripening and cold temperature treatment. To do so, the ripening index of the fruit and the pre-crushing temperature were assigned as response variables while the identified compounds were set as predictor variables. The PLS (**Figure 7**) revealed a marked effect of the pre-crushing temperature on oil composition, with a clear separation among the different ripening stages. More specifically, the ripening evolution follows a left-to-right direction, whereas lowering the mesocarp temperature an up-to-bottom direction. As a general trend, lowering olive fruit temperature before crushing, allows to produce oils more associated with aldehydes (hexenal, 2-hexenal), with a marked effect on sensorial parameters, and in particular on traits such as bitter, spicy and green that resulted increased (**Figs. 5 and 6**). In general, the effects of lowering fruit temperature have a more pronounced effects on oils extracted from ripe and ripe+ olives. Compared to the oil obtained from semi-ripe olives, which are associated with aldehydes, linolenic acid, hydroxytyrosol, oils extracted from ripe and ripe + olives show association with alcohols and most of the compounds included in the second cluster of fig. 6. In these oils the effect of lowering the temperature of olives before crushing seems to have a more pronounced effect than that observed in semi-ripe olives, as demonstrated by the shift from the upper to the low quadrants in the PLS. In addition, the oil samples obtained from ripe and ripe+ olives cooled down to 10 and 15°C lose the association with the group of

compounds sitting in the upper right quadrant (alcohols, 1-penten-3-one) negatively correlated with bitter, spicy and green and positively associated with ripe fruit trait (**figure. 6**).

Discussion

Olive oil quality can be affected by several parameters, one of which is the temperature during the olive oil extraction. This particular parameter can be considered as a limiting step regarding the final quality of the olive oil but at the same time it is a parameter that can be controlled either right after harvest and prior to crushing or during the malaxation step. Due to the sensitivity of the involved metabolic pathways responsible for the production of both VOCs and phenolics against temperature alterations, it is imperative to identify the optimum range of temperature in which simultaneously high quality and quantity of olive oil can be obtained.

Fruit metabolism do not stop after harvest. On the contrary, fruit remain highly active on the post-harvest level, and there is a strict correlation between temperature and metabolism. Temperature can accelerate several metabolic processes which indeed can have a profound impact on the quality of the final product. As the climate changes rapidly, and the fluctuations of temperature become more and more severe, olive growers tend to harvest earlier in order to achieve better olive oil quality. This on the other hand, frequently results in poor harvest yields. Our study focused on the reduction of the inner fruit temperature, without further control of temperature of the olive paste during malaxation in order not to complicate the experimental design which can lead us to false hypothesis and uncomprehensive interpretation of our data. Our results indicate that controlling the temperature of the fruit right before the latter undergo crushing, can certainly induce compositional changes in the resulting olive oil. This reduction, may affect the enzymatic activities of the enzymes of the LOX pathway in such way that the ratio of oxidative processes which characterize and are initiated in both ripening and senescence, is lower when compared with our control temperature. Furthermore, there is a positive correlation between oxidation and production of volatiles associated with off-flavors and lower sensorial quality parameters. Our data pointed out that reduction of the aforementioned volatiles is proportional to the temperature; lower inner temperature of the fruit resulted in lower abundance of off-flavor related VOCs. What is worth mentioning is the higher reduction of the aforementioned VOCs particularly in the olive oils obtained from ripe and advanced ripe olive fruit. This can allow olive growers to harvest later and thus having higher yield without taking the risk of compromising their olive oil quality.

On the contrary, VOCs which are correlated to high sensorial quality olive oil, such as C6 aldehydes (characteristic green aroma), were found in higher abundance when the inner temperature of the fruit was reduced. Another interesting observation was that of the preservation of green and fresh attributes in olive oil produced by ripe and advanced ripe fruit that have undergone pre-cooling; when these oils were compared with their controls, were found to resemble, in terms of aroma composition, oils of earlier harvests.

However, utilizing very low temperatures such as 10°C, appeared to have a negative effect on the phenolic composition of the resulting olive oil regardless the ripening stage of the fruit. Also, this temperature gave the lowest oil yield when compared with the other two (15 and 19°C). A slightly higher temperature (15°C) appeared to be the most suitable since it resulted in olive oils which preserved their positive sensorial attributes, such as their green and fresh aroma traits. This trend became even more profound when ripe olives were utilized.

As mentioned above, the olive oil VOCs are generated by the LOX pathway. The activity of all the enzymes is strictly temperature specific and highly temperature sensitive, and the enzymes involved in the LOX pathway do not differ. Their range of optimal temperature activity is estimated from 15°C to 35°C. More specifically, the HPL's optimal activity temperature is 15°C (Anthon and Barrett, 2003), and thus it might be more active in the fruit incubated at 15°C. This can be the reason higher levels of C6 aldehydes were observed in the oils deriving from such fruit. On the other hand, ADH activity declines with ripening (Salas and Sanchez, 2000), and hence a reduction in the ratio of decrease in alcohols due to its lower activity was observed as ripening progressed (second and third harvest). Last but not least, the AAT enzyme has an optimal temperature activity at 35°C and thus lower temperatures can probably partly inhibit its activity. However, the absence of esters in our samples can be also attributed to the fact that Italian cultivars have been categorized as poor in esters but rich in C6 aldehydes (Perez et al. 1993).

Overall, our results indicate that modifications of the aroma profile of olive oil can be achieved at the pre-processing level, while the fruit are still intact. The suggested protocol can be followed and can be applicable on the industrial level and not only in an experimental olive mill, and thus we believe that with our study we provide a new insight regarding the crucial issue of balancing profitable yield without compromising olive oil quality that many olive growers are facing.

Tables and Figures

Table 1: SPME GC-MS identified volatile compounds, along with their odour threshold and sensory description

Volatile Compounds	Odour threshold	Sensory description	Reference
<i>Aldehydes</i>			
Hexanal	80	Green apple, grassy	Morales et al. (2005)
2-Hexenal	420	Bitter almonds, Green	Morales et al. (2005)
Nonanal	150	Fatty, waxy, pungent	Morales et al. (2005)
3-Hexanal (Z)		Walnut husk	Angerosa et al. (2002)
2,4-Hexadienal (E,E)		Fruity, aromatic, soft	
<i>Alcohols</i>			
Ethyl alcohol	30000	Alcohol	Morales et al. (2005)
1-penten-3-ol		Wet earth, Undesirable	
2-penten-1-ol (Z)		Fruity, banana	Morales et al. (1997)
1-Hexanol	400	Fruit, banana, soft	Aparicio and Morales (1998)
3-Hexen-1-ol (Z)	1500	Green	Aparicio and Morales (1998)
2-Hexen-1-ol (E)	5000	Green grass, leaves	Morales et al. (2005)
<i>Ketones</i>			
1-penten-3-one	50	Green	Aparicio and Luna (2002)
Acetone		Acetone	
<i>Carboxylic acids</i>			

Acetic acid	500	Sour, vinegary, Undesirable	Morales et al. (2005)
Propanoic acid			
Pentanoic acid			

Other Compounds

3-Ethyl-1,5-octadiene			
Butyrolactone	50		Burdock, G.A. (2005)
Benzyl Alcohol			
Phenylethyl Alcohol			

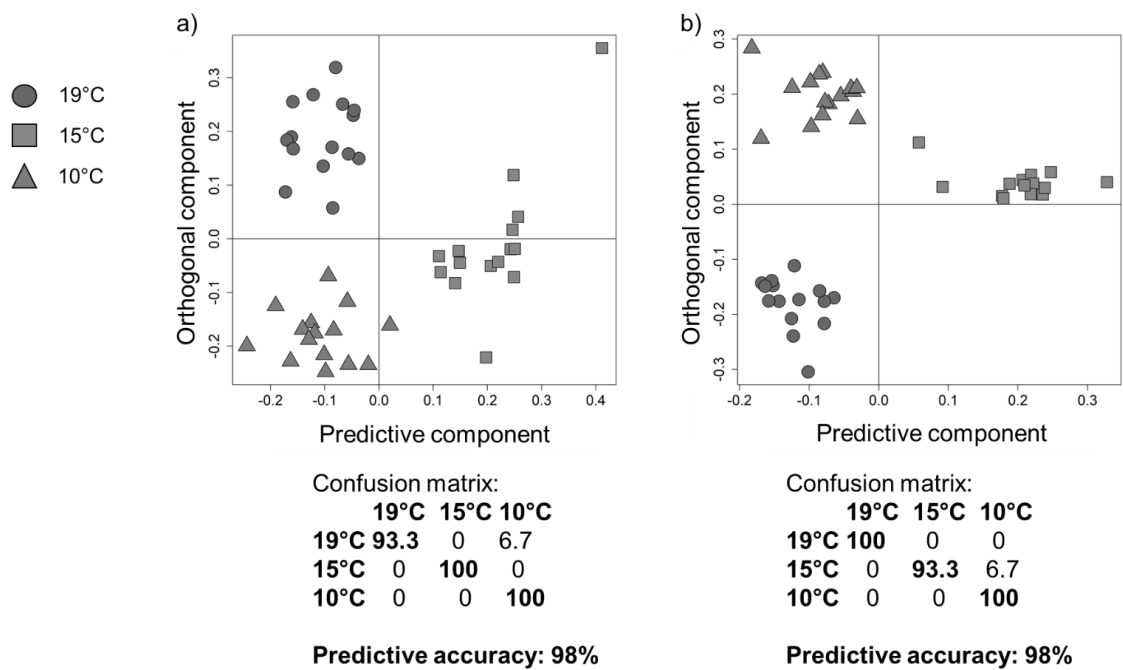


Figure 1. OPLS/DA analysis of ¹H-NMR olive oil spectra. (from 2016 and 2017) Score plots discriminating 19°C (dots, n=15), 15°C (rectangles, n=15) and 10°C (triangles, n=15). a) ZG1H experiment; b) NOESYGPPS experiment. Confusion matrix and prediction accuracy of leave-one-out cross validation are reported for both models

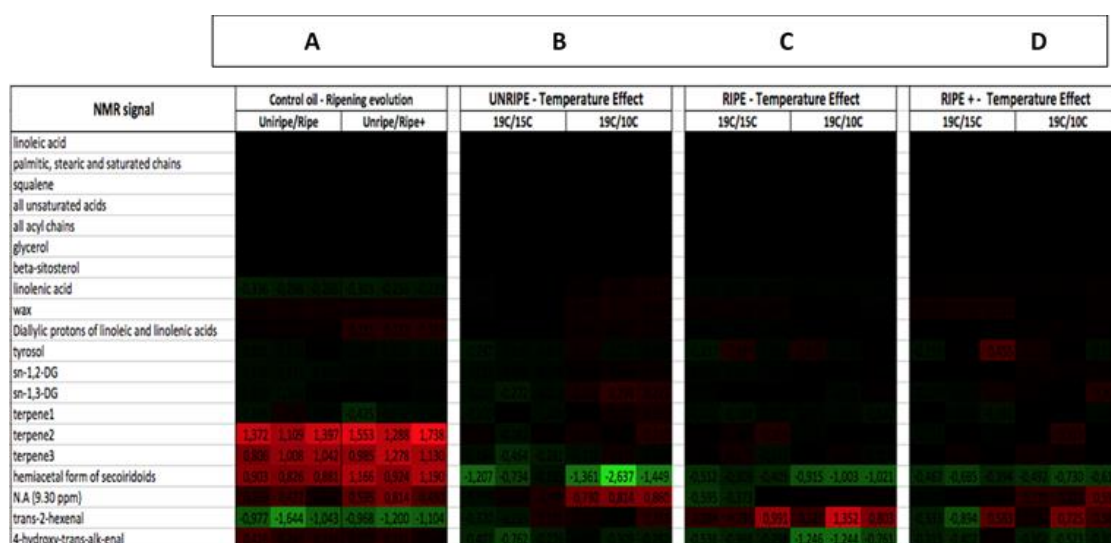


Figure 2. Heatmap reporting the fold change of the identified metabolites via ^1H -NMR in oil samples. Relations of the olive ripening stage (SR vs RP and SR vs AR, panel A) and the effects of different temperatures (19°C vs 15°C and 19°C vs 10°C) applied before processing to olives harvested in correspondence of different ripening stages (SR, panel B; RP, panel C; AR, panel D).

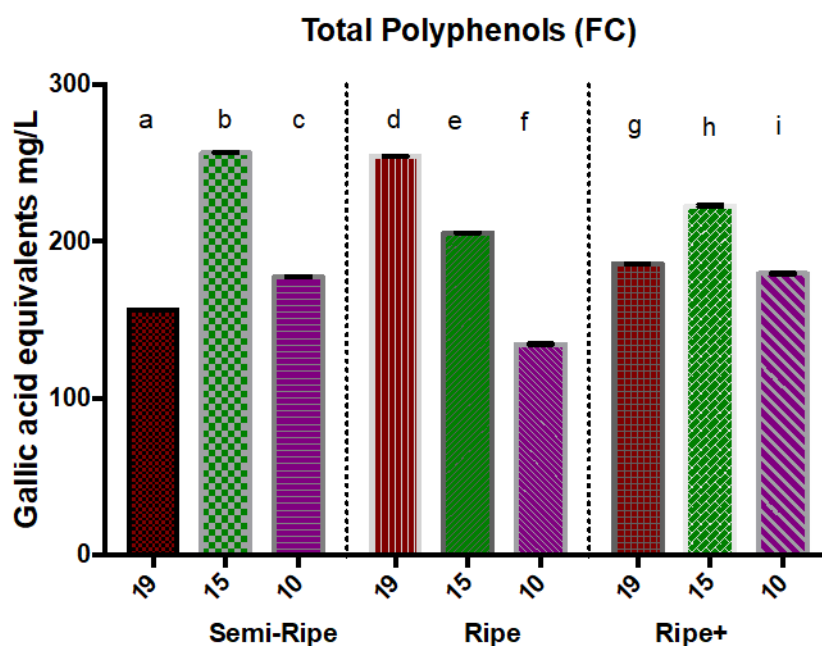


Figure 3: Total Polyphenol Quantification in oil samples obtained from olives harvested at SR, RP, and AR stages. Red colour accounts for control (19°C), green for 15°C and purple for 10°C .

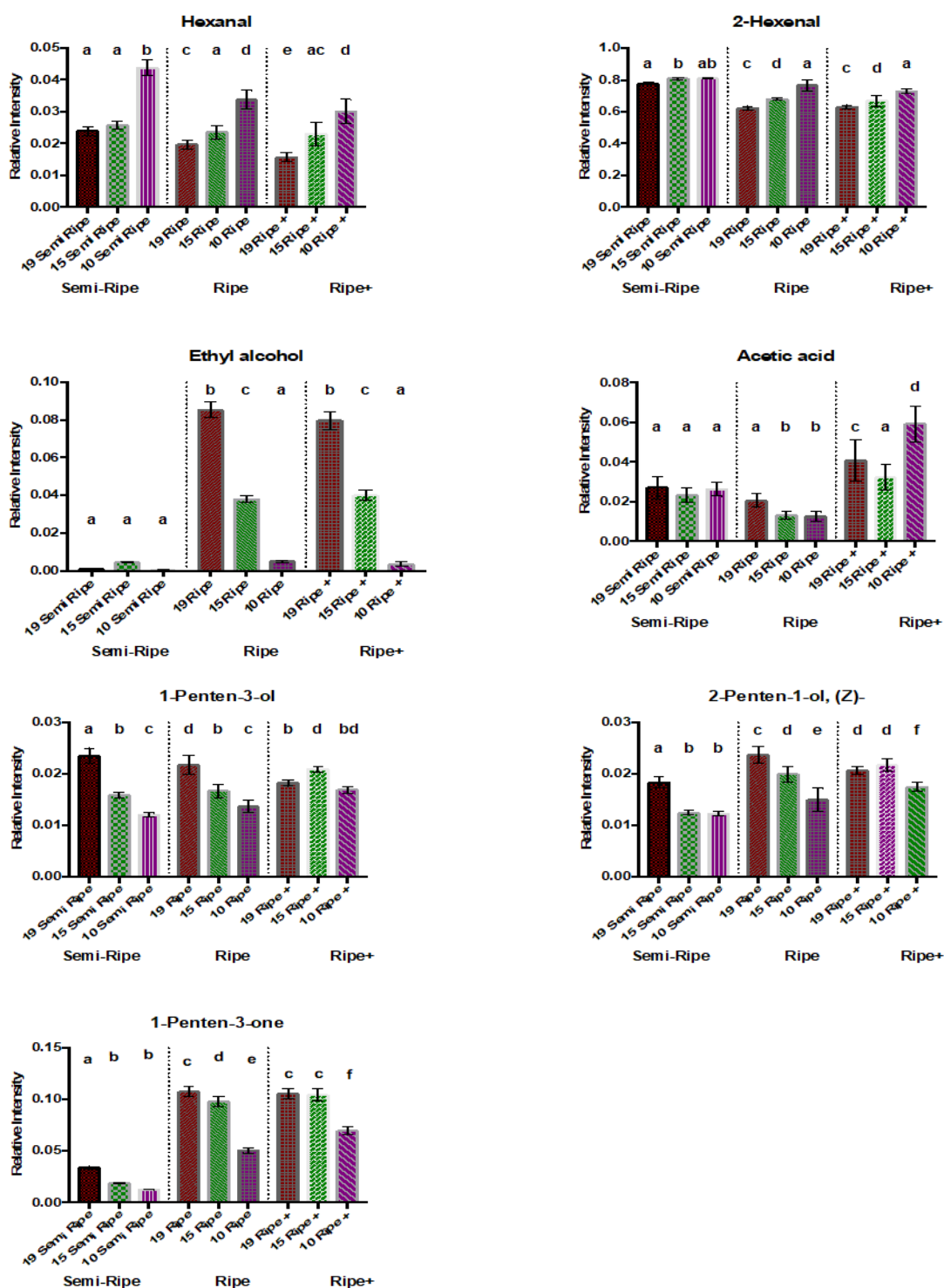


Figure 4: Specific volatile organic compounds in oils identified by the SPME-GC/MS analysis. Red colour accounts for control (19°C), green for 15°C and plump for 10°C.

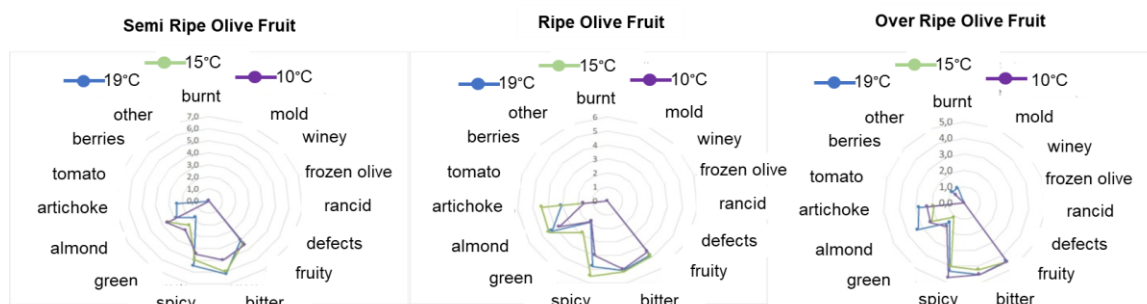


Figure 5. Panel test of oils obtained from olives harvested at different ripening stages and processed with pulp temperature of 19 (control), 15 or 10°C.

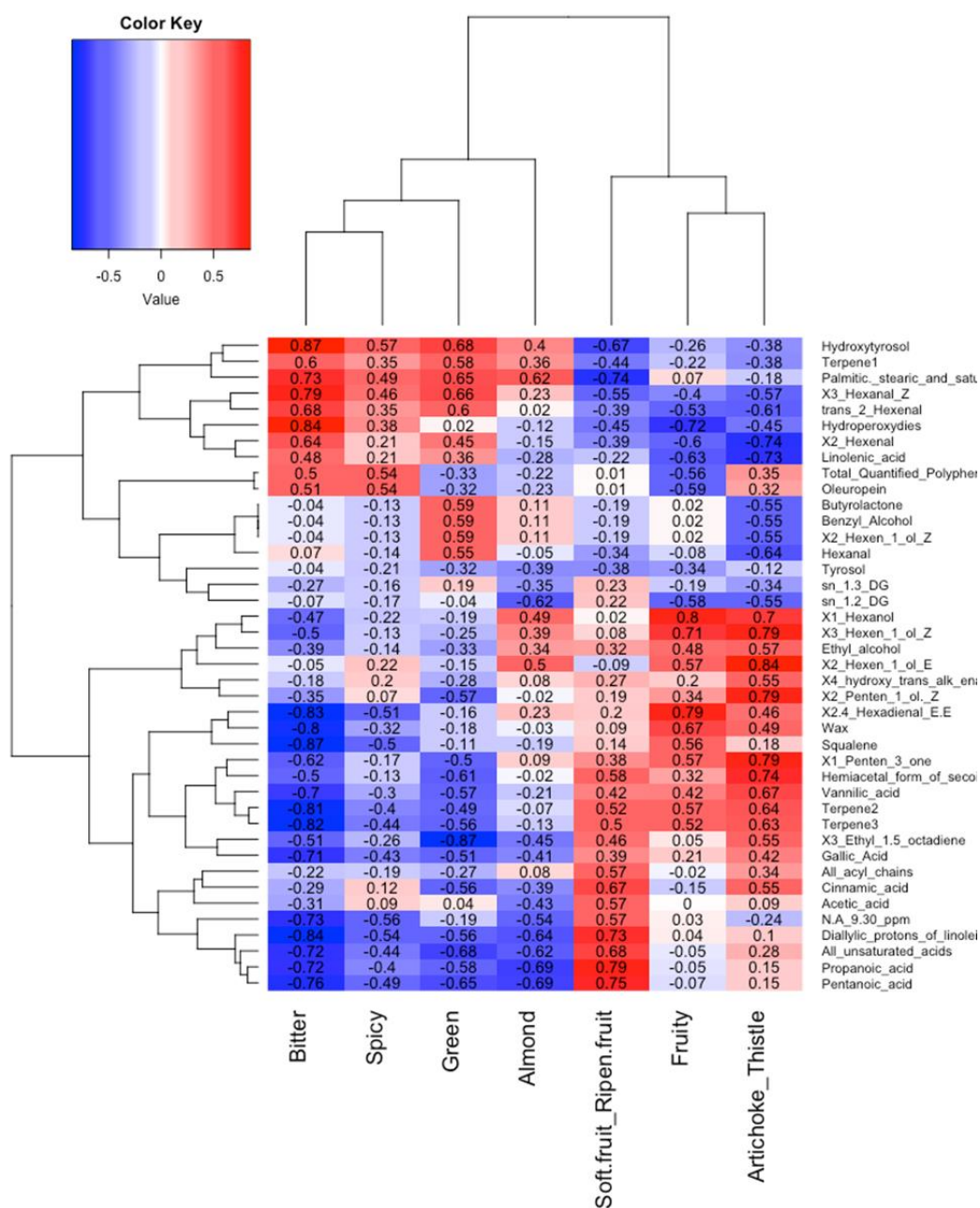


Figure 6. Heat-map correlating sensory scores (panel test) with identified metabolites

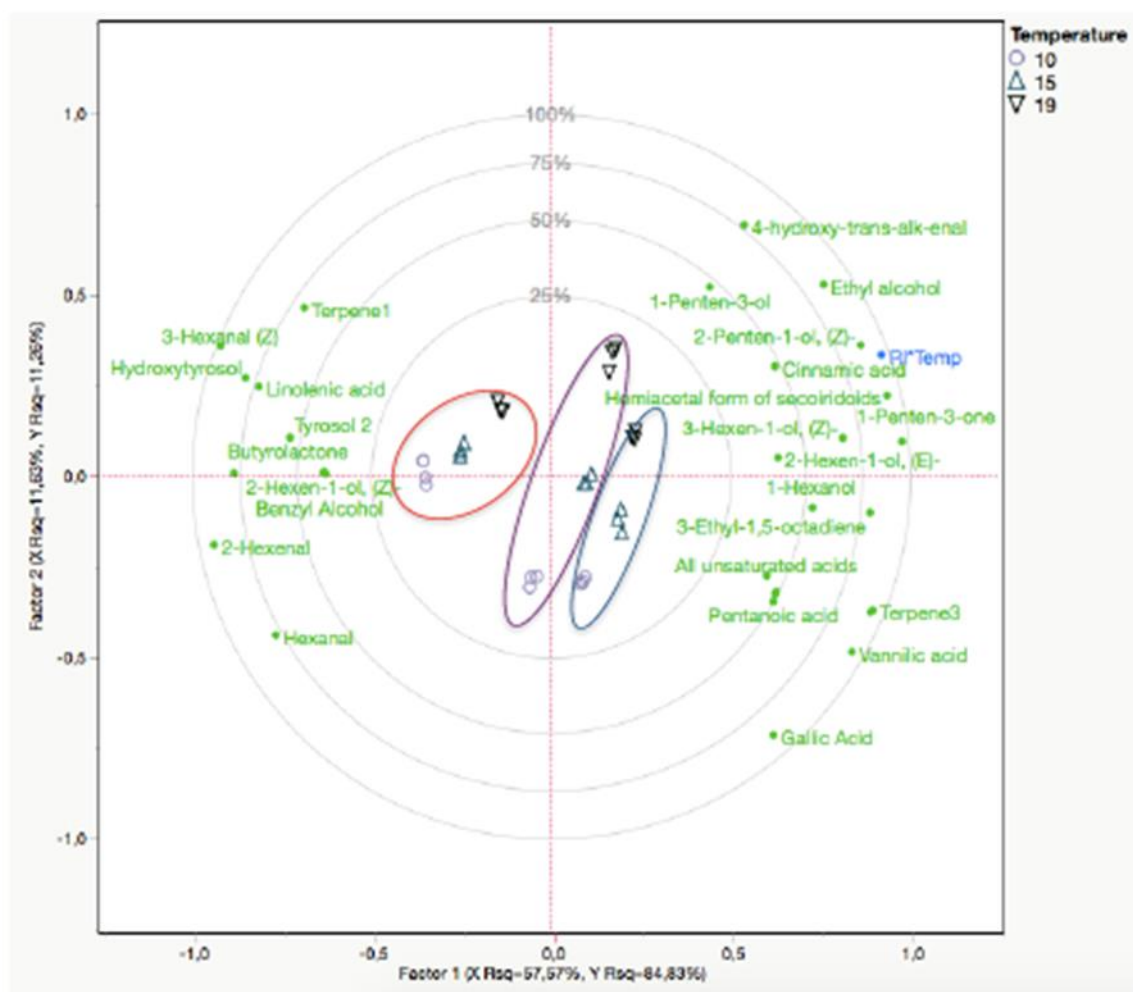


Figure 7: Partial least squares analysis (PLS) of all datasets. Ripening index and temperature are assigned as response variables whereas identified VOCs, metabolites, and phenols as predictor variables. Light purple circle indicated the samples at 10°C, blue triangle samples at 15°C and reversed black triangle samples of control (19°C). Red = oil samples from semi-ripe olives; purple= oil samples from ripe olives; blue= oil samples from ripe+ olives

Supplementary Material

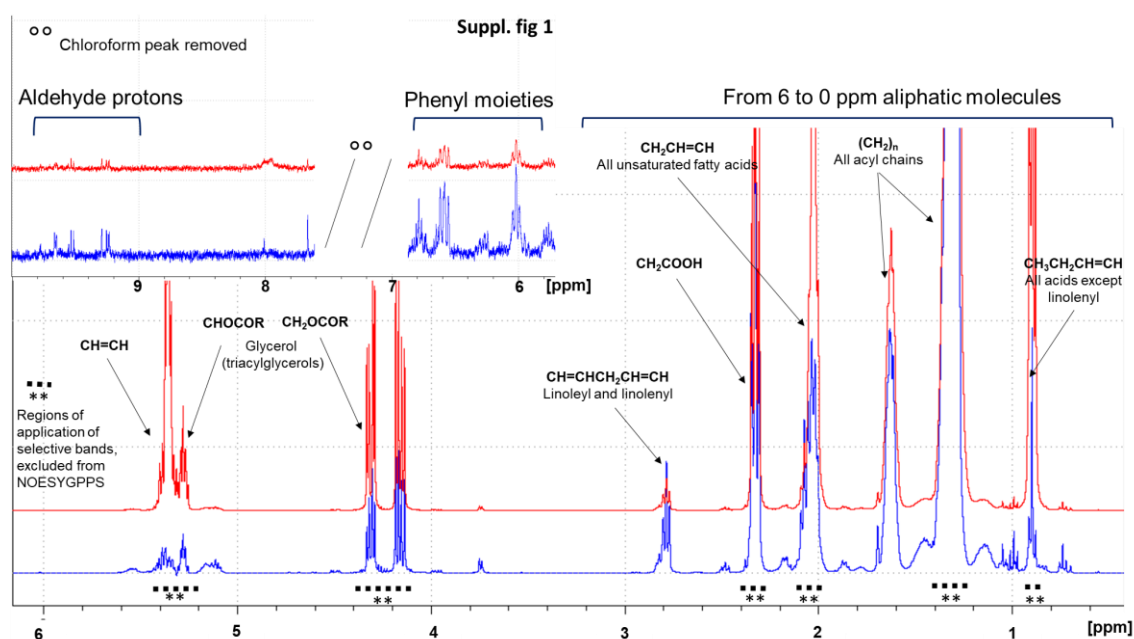
Supplementary table 1. Concentrations in arbitrary units (mean $\pm$ SD) of the metabolites assigned in Leccino olive oil samples, ppm (δ_H) of the integrated signals are also reported.

<i>metabolites</i>	<i>ppm</i> (δ_H)	CTR (19°C)		MED (15°C)		LOW (10°)		SEMI-RIPE		RIPE		RIPE+	
		<i>mean</i>	$\pm SD$	<i>mean</i>	$\pm SD$	<i>mean</i>	$\pm SD$	<i>mean</i>	$\pm SD$	<i>mean</i>	$\pm SD$	<i>mean</i>	$\pm SD$
linoleic acid	0.9	8.8	$\pm 5 \cdot 10^{-2}$	8.7	$\pm 3.5 \cdot 10^{-2}$	8.7	$\pm 4.1 \cdot 10^{-2}$	8.72	$\pm 4.4 \cdot 10^{-2}$	8.7	$\pm 4.7 \cdot 10^{-2}$	8.7	$\pm 6 \cdot 10^{-2}$
palmitic, stearic and saturated chains	1.3	63.6	$\pm 3 \cdot 10^{-1}$	63.8	$\pm 2.45 \cdot 10^{-1}$	63.6	$\pm 3 \cdot 10^{-1}$	63.8	$\pm 1.7 \cdot 10^{-1}$	63.8	$\pm 2 \cdot 10^{-1}$	63.2	$\pm 1.04 \cdot 10^{-1}$
squalene	1.64	66.3	$\pm 3.4 \cdot 10^{-2}$	6.6	$\pm 3.2 \cdot 10^{-2}$	6.6	$\pm 4.4 \cdot 10^{-2}$	6.6	$\pm 5.1 \cdot 10^{-2}$	6.6	$\pm 2.5 \cdot 10^{-2}$	6.63	$\pm 1.1 \cdot 10^{-2}$
all unsaturated acids	2.04	10.6	$\pm 1.1 \cdot 10^{-1}$	10.5	$\pm 1.2 \cdot 10^{-1}$	10.6	$\pm 1.3 \cdot 10^{-1}$	10.5	$\pm 8.6 \cdot 10^{-2}$	10.6	$\pm 4.1 \cdot 10^{-2}$	10.8	$\pm 4.3 \cdot 10^{-2}$
all acyl chains	2.34	6.1	$\pm 9 \cdot 10^{-3}$	6.0	$\pm 6.5 \cdot 10^{-3}$	6.1	$\pm 8.4 \cdot 10^{-3}$	6.05	$\pm 8.4 \cdot 10^{-3}$	6.05	$\pm 9 \cdot 10^{-3}$	6.06	$\pm 5.8 \cdot 10^{-3}$
glycerol	4.16	1.97	$\pm 4.2 \cdot 10^{-3}$	1.96	$\pm 2.3 \cdot 10^{-3}$	1.97	$\pm 2.9 \cdot 10^{-3}$	1.97	$\pm 4 \cdot 10^{-3}$	1.97	$\pm 4.1 \cdot 10^{-3}$	1.97	$\pm 3.7 \cdot 10^{-3}$
beta-sitosterol	0.74	$1.4 \cdot 10^{-1}$	$\pm 1.1 \cdot 10^{-3}$	$1.4 \cdot 10^{-1}$	$\pm 1.2 \cdot 10^{-3}$	$1.4 \cdot 10^{-1}$	$\pm 9.8 \cdot 10^{-4}$	$1.4 \cdot 10^{-1}$	$\pm 9.7 \cdot 10^{-4}$	$1.4 \cdot 10^{-1}$	$\pm 1.3 \cdot 10^{-3}$	$1.4 \cdot 10^{-1}$	$\pm 1.1 \cdot 10^{-3}$
linolenic acid	0.97	$3 \cdot 10^{-2}$	$\pm 4.2 \cdot 10^{-3}$	$3 \cdot 10^{-2}$	$\pm 3.7 \cdot 10^{-3}$	$3.1 \cdot 10^{-2}$	$\pm 4.8 \cdot 10^{-3}$	$3.4 \cdot 10^{-2}$	$\pm 2.4 \cdot 10^{-3}$	$2.6 \cdot 10^{-2}$	$\pm 2.1 \cdot 10^{-3}$	$3 \cdot 10^{-2}$	$\pm 4.6 \cdot 10^{-4}$
wax	1.05	$1.4 \cdot 10^{-1}$	$\pm 6 \cdot 10^{-3}$	$1.5 \cdot 10^{-1}$	$\pm 1 \cdot 10^{-2}$	$1.4 \cdot 10^{-1}$	$\pm 4.1 \cdot 10^{-3}$	$1.4 \cdot 10^{-1}$	$\pm 4.7 \cdot 10^{-3}$	$1.5 \cdot 10^{-1}$	$\pm 5 \cdot 10^{-3}$	$1.5 \cdot 10^{-1}$	$\pm 5.6 \cdot 10^{-3}$
Diallylic protons of linoleic and linolenic acids	2.78	1.2	$\pm 1.6 \cdot 10^{-1}$	1.14	$\pm 1.6 \cdot 10^{-1}$	1.2	$\pm 1.8 \cdot 10^{-1}$	1.12	$\pm 8.8 \cdot 10^{-2}$	1.1	$\pm 1.5 \cdot 10^{-1}$	1.4	$\pm 1.5 \cdot 10^{-2}$
tyrosol	3.53	$9.6 \cdot 10^{-4}$	$\pm 1 \cdot 10^{-4}$	$9.4 \cdot 10^{-4}$	$\pm 1.2 \cdot 10^{-4}$	$9.8 \cdot 10^{-4}$	$\pm 9.3 \cdot 10^{-5}$	$9.5 \cdot 10^{-4}$	$\pm 1.1 \cdot 10^{-4}$	$9.5 \cdot 10^{-4}$	$\pm 9.7 \cdot 10^{-5}$	$9.8 \cdot 10^{-4}$	$\pm 1.3 \cdot 10^{-4}$
sn-1,2-DG	3.74	$8.6 \cdot 10^{-2}$	$\pm 5.8 \cdot 10^{-3}$	$8.5 \cdot 10^{-2}$	$\pm 4.6 \cdot 10^{-3}$	$8.9 \cdot 10^{-2}$	$\pm 5.8 \cdot 10^{-3}$	$8.5 \cdot 10^{-2}$	$\pm 7.5 \cdot 10^{-3}$	$8.6 \cdot 10^{-2}$	$\pm 3.7 \cdot 10^{-3}$	$9 \cdot 10^{-2}$	$\pm 1.9 \cdot 10^{-3}$
sn-1,3-DG	4.23	$1.8 \cdot 10^{-3}$	$\pm 8.3 \cdot 10^{-4}$	$1.9 \cdot 10^{-3}$	$\pm 5 \cdot 10^{-4}$	$2.2 \cdot 10^{-3}$	$\pm 5 \cdot 10^{-4}$	$1.7 \cdot 10^{-3}$	$\pm 8.8 \cdot 10^{-4}$	$2 \cdot 10^{-3}$	$\pm 2.6 \cdot 10^{-4}$	$2.4 \cdot 10^{-3}$	$\pm 1.2 \cdot 10^{-4}$
terpene1	4.6	$5.5 \cdot 10^{-3}$	$\pm 7.9 \cdot 10^{-4}$	$5.3 \cdot 10^{-3}$	$\pm 6.4 \cdot 10^{-4}$	$5.4 \cdot 10^{-3}$	$\pm 8.6 \cdot 10^{-4}$	$6 \cdot 10^{-3}$	$\pm 4.1 \cdot 10^{-4}$	$5 \cdot 10^{-3}$	$\pm 7.8 \cdot 10^{-4}$	$5 \cdot 10^{-3}$	$\pm 2.5 \cdot 10^{-4}$
terpene2	4.68	$2.6 \cdot 10^{-3}$	$\pm 1.1 \cdot 10^{-3}$	$2.7 \cdot 10^{-3}$	$\pm 1.2 \cdot 10^{-3}$	$2.7 \cdot 10^{-3}$	$\pm 1.1 \cdot 10^{-3}$	$1.4 \cdot 10^{-3}$	$\pm 1.5 \cdot 10^{-4}$	$3.3 \cdot 10^{-3}$	$\pm 2 \cdot 10^{-4}$	$4 \cdot 10^{-3}$	$\pm 2.4 \cdot 10^{-4}$
terpene3	4.73	$2.8 \cdot 10^{-3}$	$\pm 9.8 \cdot 10^{-4}$	$2.7 \cdot 10^{-3}$	$\pm 1.1 \cdot 10^{-3}$	$3 \cdot 10^{-3}$	$\pm 1.1 \cdot 10^{-3}$	$1.6 \cdot 10^{-3}$	$\pm 1.8 \cdot 10^{-4}$	$3.4 \cdot 10^{-3}$	$\pm 1.6 \cdot 10^{-4}$	$4 \cdot 10^{-3}$	$\pm 1.8 \cdot 10^{-4}$
hemiacetal form of secoiridoids	9.26	$6.8 \cdot 10^{-4}$	$\pm 2.4 \cdot 10^{-4}$	$4.7 \cdot 10^{-4}$	$\pm 2.1 \cdot 10^{-4}$	$3.6 \cdot 10^{-4}$	$\pm 2 \cdot 10^{-4}$	$2.8 \cdot 10^{-4}$	$\pm 1.3 \cdot 10^{-4}$	$6 \cdot 10^{-4}$	$\pm 1.7 \cdot 10^{-4}$	$7.8 \cdot 10^{-4}$	$\pm 1.7 \cdot 10^{-4}$
N.A	9.3	$6.1 \cdot 10^{-4}$	$\pm 3.5 \cdot 10^{-4}$	$7.3 \cdot 10^{-4}$	$\pm 2 \cdot 10^{-4}$	$1 \cdot 10^{-3}$	$\pm 3 \cdot 10^{-4}$	$7.2 \cdot 10^{-4}$	$\pm 3.7 \cdot 10^{-4}$	$6.6 \cdot 10^{-4}$	$\pm 2 \cdot 10^{-4}$	$1.2 \cdot 10^{-3}$	$\pm 1.7 \cdot 10^{-4}$

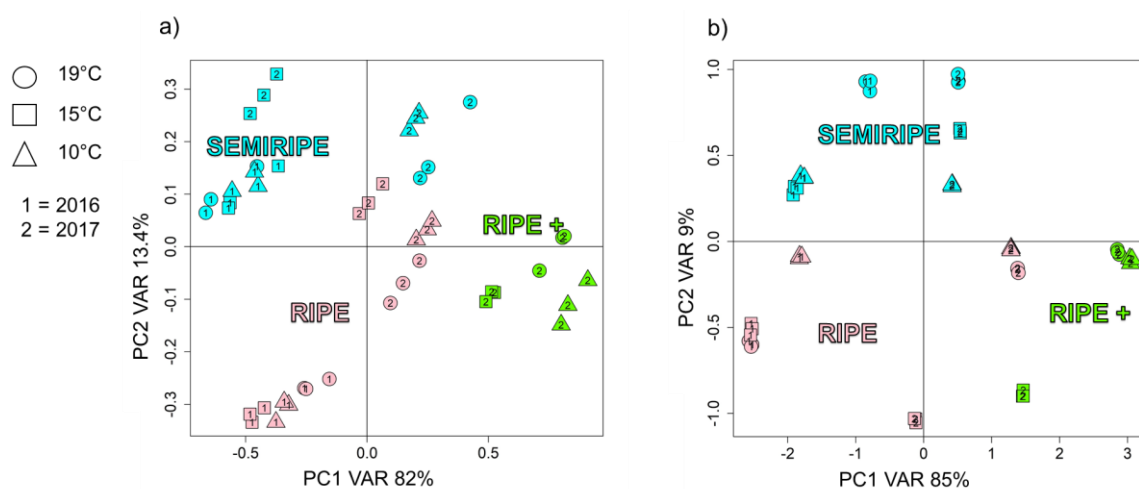
trans-2-hexenal	9.51	2·10 ⁻⁴	±9·10 ⁻⁵	2.6·10 ⁻⁴	±8.7·10 ⁻⁵	3.2·10 ⁻⁴	±8.8·10 ⁻⁵	3.4·10 ⁻⁴	±9.2·10 ⁻⁵	2.2·10 ⁻⁴	±6.2·10 ⁻⁵	2·10 ⁻⁴	±5.4·10 ⁻⁵
4-hydroxy-trans-alk-enal	9.55	2·10 ⁻³	±2.1·10 ⁻⁴	1.4·10 ⁻³	±4.3·10 ⁻⁴	1.3·10 ⁻³	±2.8·10 ⁻⁴	1.4·10 ⁻³	±4.1·10 ⁻⁴	1.6·10 ⁻³	±5.3·10 ⁻⁴	1.8·10 ⁻³	±2.4·10 ⁻⁴

Supplementary table 2. SPME GC-MS identified volatile compounds, along with their odour threshold and sensory description

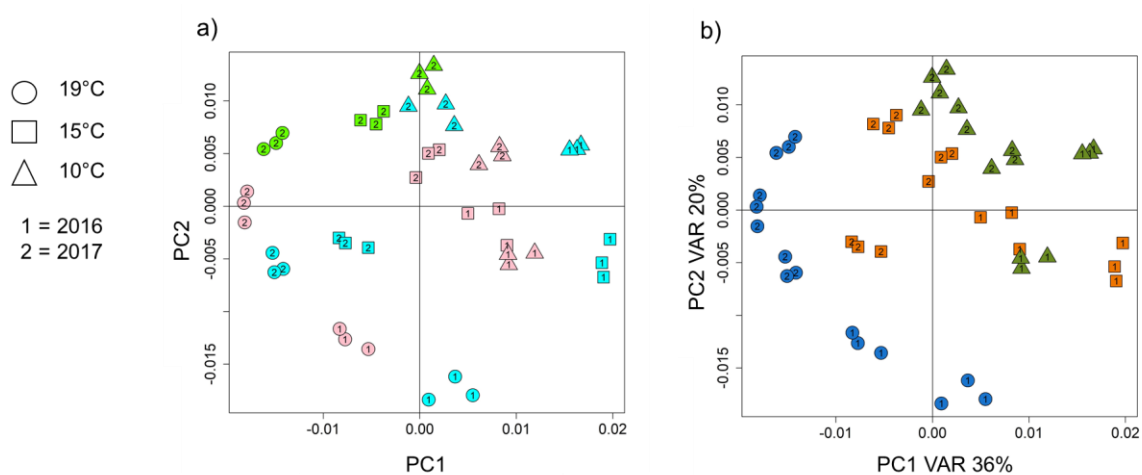
Volatile Compounds	Odour threshold	Sensory description	Reference
<i>Aldehydes</i>			
Hexanal	80	Green apple, grassy	Morales et al. (2005)
2-Hexenal	420	Bitter almonds, Green	Morales et al. (2005)
Nonanal	150	Fatty, waxy, pungent	Morales et al. (2005)
3-Hexanal (Z)		Walnut husk	Angerosa et al. (2002)
2,4-Hexadienal (E,E)		Fruity, aromatic, soft	
<i>Alcohols</i>			
Ethyl alcohol	30000	Alcohol	Morales et al. (2005)
1-penten-3-ol		Wet earth, Undesirable	
2-penten-1-ol (Z)		Fruity, banana	Morales et al. (1997)
1-Hexanol	400	Fruit, banana, soft	Aparicio and Morales (1998)
3-Hexen-1-ol (Z)	1500	Green	Aparicio and Morales (1998)
2-Hexen-1-ol (E)	5000	Green grass, leaves	Morales et al. (2005)
<i>Ketones</i>			
1-penten-3-one	50	Green	Aparicio and Luna (2002)
Acetone		Acetone	
<i>Carboxylic acids</i>			
Acetic acid	500	Sour, vinegary, Undesirable	Morales et al. (2005)
Propanoic acid			
Pentanoic acid			
<i>Other Compounds</i>			
3-Ethyl-1,5-octadiene			
Butyrolactone	50		Burdock, G.A. (2005)
Benzyl Alcohol			
Phenylethyl Alcohol			



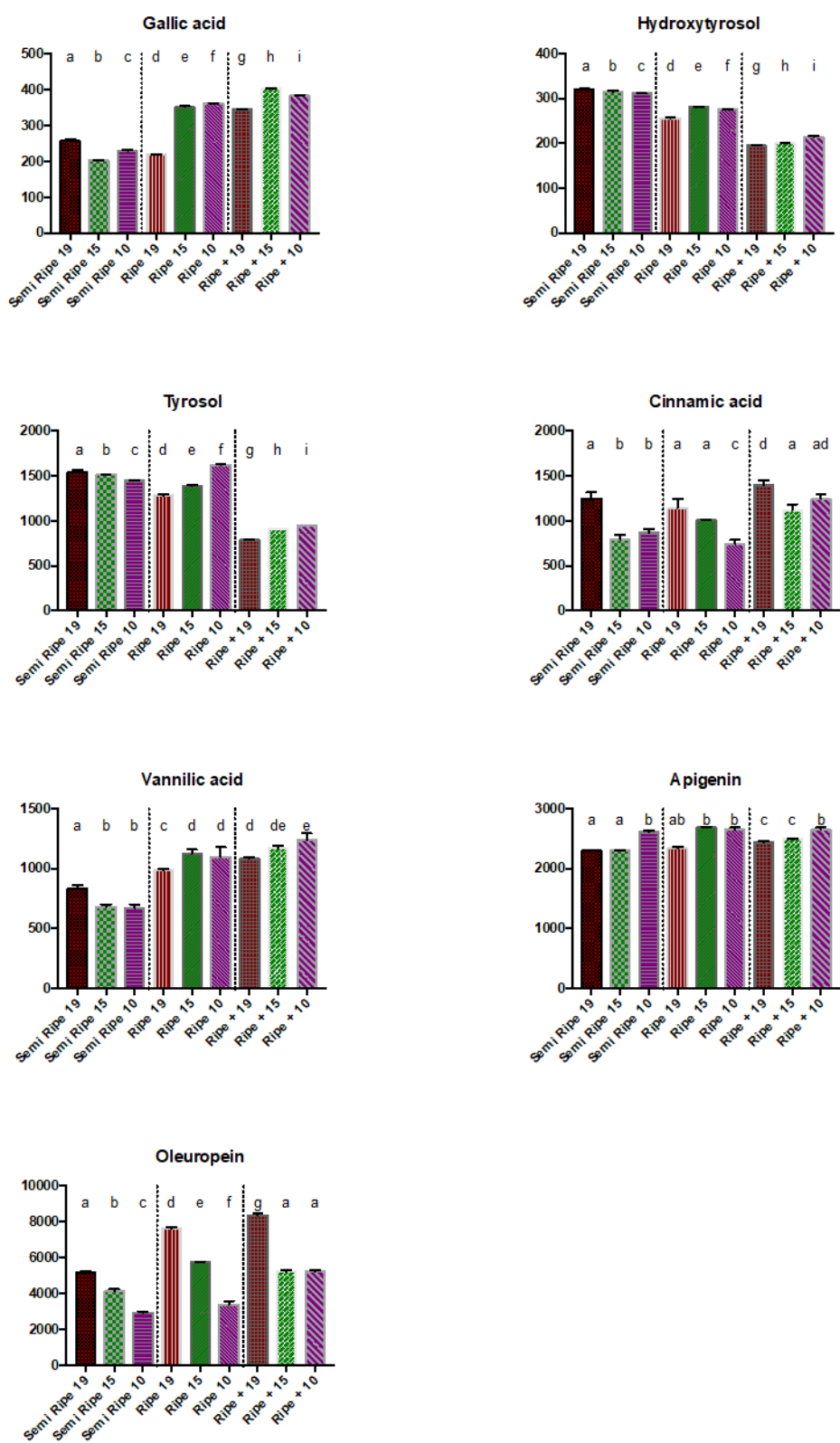
Suppl. Fig. S1. ¹H-NMR 1D Leccino oil spectra. ZG1H (red) and NOESYGPPS (blue) ¹H-NMR spectra to illustrate the effect of selective suppression of saturating multiple signals and the assignment of the main resonances in the ¹H-NMR spectrum of Leccino olive oil. Dotted lines indicate where the selective bands were applied, those regions, where consequently excluded from the buckets of NOESYGPPS for statistical analysis.



Suppl. Fig. S2. PCA score plot of whole ¹H-NMR spectra collected in 2016 and 2017 seasons. Symbols are as follows: Circles = 19°C (n=15), squares = 15°C (n=15), triangles = 10°C (n=15), cyanide = SR (n=18), pink = RP (n=18), green = AR (n=9). Symbols labeled with “1” indicate oil samples obtained from olives harvested in 2016 (n=21), symbols labeled with “2” indicate oil samples obtained from olives harvested in 2017 (n=24). a) ZG1H experiment; b) NOESYGPPS experiment. PC1 and PC2 variance are also reported for both models.



Suppl. Fig. S3. PCA score plot of aldehydic region of ^1H -NMR spectra. Model obtained by considering only the bucket region between 10 and 7.6 ppm of NMR spectra. Circles = 19°C (n=15), squares = 15°C (n=15), triangles = 10°C (n=15), cyanide = SR (n=18), pink = RP (n=18), green = AR (n=9). Symbols labeled with "1" indicate oil samples obtained from olives harvested in 2016 (n=21), symbols labeled with "2" indicate oil samples obtained from olives harvested in 2017 (n=24). a) PCA score plot colored according to the evolution of olive ripening; b) PCA score plot colored according to different temperatures.



Suppl. Fig. S4. HPLC quantification analysis of seven phenol compounds in oil samples

5.2 NMR on the side of small farms: metabolomics in food traceability

In the last years metabolomics has demonstrated to be a fast and reliable tool to provide consumers with true information about the origin and traceability of agro-food products. Each NMR spectrum could be regarded as the individual “fingerprint” of a food sample, which includes information (in just one measurement) about variety, origin, vintage, physiological state, technological treatments, and others and can be used to extract some chemical features that can be related to potential health benefits and/or organoleptic properties.

NMR was first used to investigate the properties of milk in the 1950s and since then has been employed in a wide range of studies, most of them focusing on linking milk metabolites content with nutritional aspects, discovery of biomarkers of cow’s diseases^{40,311–313} and analysis of milk bioactive compounds^{314,315}.

More recently, milk and cheese products origin has been studied using metabolite fingerprinting techniques by means of NMR^{316,317}. To date there are not fingerprinting based studies assessing the traceability of milk samples from different farms of the same restricted geographical area.

In this thesis (§5.2.1) we employed a novel pre-analytical procedure for NMR-based metabolomics analysis of milk samples; the use of dichloromethane for the extraction of soluble metabolites offers good spectra as chloroform do but has several advantages over chloroform: it is less toxic and β -hydroxybutyrate and creatine signals are better visible.

The detection of these two compounds can provide important information: β -hydroxybutyrate is known to be a diagnostic biomarker for cow ketosis³¹⁸ and it also an indicator of mastitis infection⁴⁰. Creatine is found in milk and dairy products but in a relatively small amount. Estimating the amount of creatine can be useful to assess the nutritional value of milk.

Further, the metabolomic approach has proven to be a valid method in assessing the origin of authentic products of nine different farms located in a Tuscan small geographic valley (Mugello) by revealing 97% of the overall predictive accuracy by considering both 2013 and 2014 milk collection. Indeed, seasonal variations need to be considered using repeated collections, due to the changes over time of the composition of milk caused mainly by modification of caws nutrition. This method provides important information about the nutrition of the cows, opening scenarios for the monitoring of the production of dairy products (*e.g.* Parmesan cheese), although its potential in fraud detection needs to be further investigated.

Seasonal variation, climate and soil are important variables that can contribute to particular signs (“*terroir*”) useful to detect the origin many plants or animals derived food. Indeed, if combined with other factors such as agronomical practices and post-harvest manipulation they can be very important to assess the precise production origin. Coffee is one of the most important internationally traded products, and precise authentication represents a very important opportunity especially for small coffee producers and growers (producing high quality coffee, *special coffees*), severely beaten by multinationals market.

We think that the identification of the origin of coffee beans coming from small, geographically condensed Colombian plantations is the ideal case to proof the power of the NMR fingerprint approach in recognizing Colombian local producers (§5.2.2). 51 batches coming from different coffee growers have been collected and screened for a total of 357 green coffee beans samples (*Arabica*) analysed (7 replicates for each batch). Using a cross-validated OPLS-DA on the

spectral data, the 51 cultivars have been well-recognised showing an overall predictive accuracy of 90.7 %.

In parallel, 16 of those batches, were sent for roasting to a professional coffee trader. The statistical model built on roasted coffee beans shows an overall predictive accuracy of 95% in identifying the farms (origin). We correlated metabolites with batch characteristic (*e.g.* altitude of the plantation, time of fermentation *etc.*) and organoleptic scores finding weak correlations (alanine/altitude = $R\ 0.56$; $p < 0.001$; sucrose/altitude = $R\ 0.53$; $p < 0.001$; maleate/bitterness = $R\ 0.60$; $p < 0.05$), however using green coffee fingerprints, the model built shows to be quite effective in predicting coffees with a quality score above 70 (*i.e.* of remarkable quality).

The utility of these results is twofold: rapidity of the approach in recognizing producers from a restricted geographical area, and promising preliminary evidences suggesting the possibility to correlate remarkable quality coffees by means of its NMR fingerprint providing to coffee traders the possibility to control coffee parameters in a “scientifically supervised” way, in order to produce high quality and specially flavoured coffees, ready to collect high scores from classical panels.

5.2.1 NMR metabolomic fingerprinting distinguishes milk from different farms

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Candidate's contributions: acquisition of NMR data, statistical analysis and interpretation of data and review of the manuscript.



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NMR metabolomic fingerprinting distinguishes milk from different farms

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ABSTRACT

A fast and reproducible protocol for milk Nuclear Magnetic Resonance (NMR) metabolomic fingerprinting was developed, allowing for an accurate discrimination among milk samples from large-scale distribution, as well as among milk sample from different farms located in the same restricted geographical area. Seasonal variations in milk composition and correlations with cows' nutritional patterns are also assessed, underlining relationships between feeding and metabolites. The most important difference was related to the use of silage feeding. This finding is relevant to assess the suitability of milk for different dairy products. A prominent example is parmesan cheese, the preparation protocol of which excludes milk from silage-fed cows.

1. Introduction

Bovine milk is an important diet constituent. Lipids and lactose are the two main nutrients, but milk also contains a wide range of bioactive compounds such as immunoglobulins and other immune proteins, peptides and nucleotides (Ulrik K. Sundekilde, Larsen, & Bertram, 2013). Being a biological fluid, the composition of milk is influenced by several factors such as breed, individual metabolism of the animals, season, health status, nutrition and milking protocols (Lamanna, Braca, Di Paolo, & Imperato, 2011; Tian et al., 2016). All these factors contribute to the variability of milk metabolite profiles; in fact, milk metabolites can originate from different metabolic pathways in the animal organism. The chemical composition of milk is important to understand its nutritional value, including bioactive compounds, its technological properties, and the potential use of biomarkers in milk as diagnostic tools for cows health (Sundekilde, Poulsen, Larsen, & Bertram, 2013). Metabolomics allows for the simultaneous characterization of large amounts of compounds in biological matrices and can provide a more detailed molecular picture of food composition, food consumption or about the consequences of diets (Wishart, 2008). Milk metabolome has been studied using different metabolomics approaches in order to extrapolate information on fat composition (Kalo, Kemppinen, & Kilpeläinen, 1996) or structural changes in caseins and other proteins (Leslie, Irons, & Chapman, 1969). Through metabolomics it was

possible to find biomarkers that are correlated with the health status of bovines: for example β -hydroxybutyrate, phosphocholine and glycerophosphocholine could be used as biomarkers for ketosis (M. S. Klein et al., 2010). The composition of milk is also important for the production of dairy products because many factors are known to influence the coagulation properties of milk (Bittante, Penasa, & Cecchinato, 2012). Two additional aspects are also related to consumers' expectation: the first concerns the nutritional value of milk, and ^1H NMR spectroscopy has also been used to validate the labelling in relation to particular nutritional features (Monakhova, Kuballa, Leitz, Andlauer, & Lachenmeier, 2011); the second is related to the authentication and the control of geographical origin. In fact, the commercial value of many dairy products is closely associated with the origin and composition of milk. A few cases have been reported about the use of NMR spectroscopy for traceability of geographical origin of cow and buffalo milk (Brescia, Monfreda, Buccolieri, & Carrino, 2005)(Sacco et al., 2009). However, they were focused on the identification of provenance from a large territorial basis (e.g. different provinces or countries), relied on a limited number of samples, and did not address the relation between milk composition and cows' feedings.

In the present study, to provide a proof of concept for the ability of NMR-based metabolomics to distinguish different milk samples, we first determined the metabolic profiles of three different milk brands obtained from large-scale distribution. In doing so we developed an

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original protocol for metabolites extraction from milk to allow for a fast and reproducible acquisition of NMR spectra and compared our procedure with the most common methods described in the literature. Then we applied the same analytical protocol to monitor the origin of milk samples collected in ten farms located in a relatively small valley in the north of Tuscany (Italy), called Mugello. The rationale for this design was to prove that NMR-metabolomics is sensitive enough to accurately assess the provenance of milk even at the scale of each individual farm in a small territory. We analysed also cow feeding data in order to understand the contribution of different nutritional profiles on the changes of the milk metabolic profile.

2. Materials and methods

2.1. Sample collection

Whole pasteurized milk was collected from the large-scale distribution in three different periods: Autumn 2013 (September/October), Spring 2014 (April/May) and Autumn 2014 (September/October). Packaged milk of three major Italian brands ("Latte Coop", "Granarolo" and "Mukki Mugello") was collected (twenty milk samples for each brand in each period).

Samples coming from ten different farms located in the Mugello valley were collected in two different periods, Autumn 2013 and Spring 2014, in parallel with large-scale distribution (supermarket) milk. For each period, twenty raw milk samples were collected from each farm in twenty different days. Each sample consisted of a mixture of the milk produced in the same day by all bovines in a single farm. A total of 40 samples per farm were thus collected in the two collection periods (200 samples in Autumn 2013 and 200 samples in Spring 2014) and analysed.

The ten Mugello farms belongs to two categories, "organic" and "non-organic". The organic farms meet the requirements stated in the European Regulations (N. 1804/1999, 834/2007, 889/2008), about organic productions and labelling of organic products. The organic group is composed by three farms, whereas the remaining seven farms compose the non-organic group. Once the milk coming from these farms arrives at the Milk Factory of Florence, Italy, it is pooled in two distinct tanks, one for the organic and one for non-organic milk. This bulk raw milk is then pasteurized and packaged. In addition to the collections at the individual farms, bulk raw milk samples (before pasteurization and packaging) coming from non-organic and organic farms were collected in Autumn 2013 and Spring 2014 directly from the Milk Factory; in both collections, 20 samples from each of the two kinds of bulk milk were analysed. Finally, eight samples coming from eight randomly selected Mugello farms were collected in 2015 (2nd March) as a blind dataset to be used to test the predictive ability of the milk fingerprints obtained in the two previous collections. A summary diagram of collected samples is reported in Supplementary Fig. 1.

2.2. Cow feeding data

Regarding raw milk samples from farms, for each day of collection we recorded also the cow feeding rations, reported as kilograms of food per bovine. In particular, we divided the nutritional rations in eight categories: silages, wrapped hays, dry hays, legumes flours and proteic foods, cereal flours and energetic foods, protein supplements, energy supplements, mineral salts and vitamin supplements.

2.3. Sample preparation for NMR analysis

All milk samples were processed in the same way in order to minimize errors due to the pre-analytical phase. All samples were stored at 4 °C for at most 12 h before sample preparation. Freezing was avoided to prevent breaking of the somatic cells and the leaking of cell metabolites in the milk.

In order to evaluate the best preparation procedure, we compared a method developed in our laboratory with respect to other methods described in literature. The metabolite profiling following our own method consists of mixing 700 µl of milk with 700 µl of dichloromethane (CH₂Cl₂). The mixture is homogenized by vortexing and then is incubated for 10 min at room temperature. The mixture is then centrifuged at 14000 rpm for 30 min and the supernatant is recovered.

For comparison, we acquired also NMR spectra after using other published extraction procedures. We followed four different protocols described in literature, in particular: chloroform and deuterated chloroform instead of dichloromethane was used in 1:1 ratio with the sample (Lamanna et al., 2011); filtration of milk using a 10 kDa cut-off was performed in order to remove high-molecular weight molecules (Matthias S. Klein et al., 2012; U. K. Sundekilde, Poulsen, et al., 2013); a 75 min ultracentrifugation followed by a centrifugation of the obtained supernatant was also tested (Lu et al., 2013); the analysis of the whole milk without any manipulation was also finally performed (Ulrik Kræmer Sundekilde, Frederiksen, Clausen, Larsen, & Bertram, 2011).

In all cases 300 µl of the samples obtained by the various methods were mixed with 300 µl of a sodium phosphate buffer (70 mM Na₂HPO₄; 20% (v/v) ²H₂O; 0.025% (w/v) NaN₃; 0.8% (w/v) sodium trimethylsilyl [2,2,3,3-²H₄]propionate (TSP); pH 7.4). A total of 450 µl of this mixture was transferred into a 4.25 mm NMR tube (Bruker BioSpin srl) for analysis.

2.4. NMR analysis

One-dimensional ¹H NMR spectra of milk extracts were measured on a Bruker spectrometer operating at 600.13 MHz proton Larmor frequency and equipped with a 5 mm CPTCI 1H-13C/31P-2H cryo-probe including z-axis gradient coil, an automatic tuning-matching (ATM) and an automatic sample changer. A PT 100 thermocouple provided temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 3 min inside the NMR probe for temperature equilibration (310K). One-dimensional NMR spectra were acquired with water peak suppression adopting a standard pulse sequence (NOESYpresat, Bruker), using 64 free induction decays (FIDs), 64 k data point, a spectral width of 12,019 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s and a mixing time of 100 ms. 2D ¹H–¹H COSY with presaturation during relaxation delay using gradient pulses for selection (cosygpqrqf, Bruker) was acquired using 2 k to 512 data points, 8 scans with a relaxation delay of 4 s, acquisition time of direct and inverse dimensions of 0.15 s and 0.038 s respectively. 2D ¹H–¹³C HSQC via double incept transfer using sensitivity improvement, phase sensitive using Echo/Antiecho-TPPI gradient selection with decoupling during acquisition using trim pulses in incept transfer with gradients in back-incept (hsqcetgpsi2, Bruker) was acquired using 1 k to 256 data points, 32 scans with a relaxation delay of 2 s, acquisition time of direct and inverse dimensions are 0.77 s and 0.005 s respectively.

2.5. Spectral processing

Free induction decays were multiplied by an exponential function equivalent to a 1.0 Hz line-broadening factor before applying Fourier transform. Transformed spectra were automatically corrected for phase and baseline distortions and calibrated (TSP peak at 0.00 ppm) using TopSpin (Bruker). For multivariate analysis, each 1D spectrum in the range between 0.02 and 10.00 ppm was segmented into 0.02-ppm chemical shifts bins, and the corresponding areas were integrated using AMIX software (Bruker BioSpin). The dichloromethane and water regions (between 4.5 and 5.65 ppm) were removed. After having excluded that signals of lactose, that account for a major part of the total area, vary too much between farms/seasons etc. (Supplementary Fig. 2), the total spectral area was calculated on the remaining bins and was used for the normalization of the data prior to pattern recognition.

Normalization to the external reference (TSP) has been avoided in this study since TSP levels are not stable, suggesting the interaction with some proteins or peptides in the samples (Supplementary Fig. 3). Normalization using probabilistic quotient normalization (Dieterle, Ross, Schlotterbeck, & Senn, 2006) (PQN) was also used for comparison (see section 3.3).

2.6. Statistical analysis

Data reduction was carried out by means of projection into a Partial Least Square (PLS) subspace, and the canonical analysis (CA), as a method to post-process the PLS results (Ergon, n.d.; Ghadiri, Rezaei, Tabatabaei, Shahsavari, & Shahsavari, 2016), was applied to enhance the supervised separation of the analysed groups (Yu & MacGregor, 2004). Accuracy, sensitivity and specificity for the different classifications were assessed by means of 100 cycles of a Monte Carlo cross-validation scheme (MCCV, R script in-house developed). Briefly, 90% of the data were randomly chosen at each iteration as a training set to build the PLS-CA model. Then, the PLS-CA scores for the remaining 10% (test set) were obtained by projecting the test spectra into the training PLS-CA space. For classification, k-Nearest Neighbours (k-NN) method ($k = 5$) was applied (Cover & Hart, 1967) using the PLS-CA scores of the training set to drive the classification of the calculated PLS-CA scores of the test set. Test samples were predicted according to the position of the respective PLS-CA scores in the PLS-CA space calculated using the training set. Sensitivity, specificity and accuracy for the classification were assessed. The procedure was randomly repeated 100 times to derive an average discrimination accuracy for each group of samples.

Principal Component Analysis (PCA) was used to analyse cow feedings data obtained for each farm in order to highlight common nutritional profiles.

For the analysis of metabolite content, all resonances of interest were manually checked, and signals were assigned on template one-dimensional NMR profiles by using matching routines of AMIX 7.3.2 (Bruker BioSpin) in combination with the BBIORFCODE (Version 2-0-0; Bruker BioSpin), reference database and published literature when available (Sundekilde, Larsen, & Bertram, 2013). The relative concentrations of each metabolite were calculated by integrating the corresponding signals in the spectra. Variations were reported as $\log_2$ (Fold Change) and significant differences were assessed using the Wilcoxon test. *P*-values were adjusted for multiple comparisons using Bonferroni correction (Bonferroni, 1935) in which the *P*-values are multiplied by the number of comparisons.

All data analysis were performed using the R statistical environment ("R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria," n.d.).

3. Results

3.1. A novel preanalytical protocol for NMR analysis of milk

All the procedures described in Materials and Methods section can be considered equally effective for the analysis of milk samples. In Fig. 1, a comparison among spectra obtained from the same milk sample using different extraction methods is reported. In the case of the use of whole milk without any pre-processing (Ulrik Kræmer Sundekilde et al., 2011) (spectrum 6), signals derived from high-molecular weight molecules and lipids are clearly visible and they tend to cover signals derived from metabolites. To remove macromolecules, filtration with 10 kDa cut-off (Matthias S. Klein et al., 2012; Sundekilde, Larsen, & Bertram, 2013) (spectrum 4) or ultracentrifugation (Lu et al., 2013) (spectrum 5) permit the obtainment of good quality spectra, but they are time consuming procedures and, in the case of filtration, they are also expensive when a large number of samples need to be

processed. Using a 10 kDa cut-off it is possible to eliminate the macromolecules and the obtained spectrum shows well resolved signals and no baseline distortion: the effectiveness of this method was also demonstrated in (M. S. Klein et al., 2010), where the authors were able to assign a total of 44 metabolites in filtered milk using both GC-MS and NMR (25 and 23 metabolites, respectively). With ultracentrifugation, followed by another centrifugation of the supernatant, the quality of the spectrum is lower with respect to filtration because some broader signals due to lipids and proteins are still present.

The use of an organic solvent (Lamanna et al., 2011) is the fastest way for the preparation of milk samples. Chloroform stabilized with 1% of ethanol has been used in several cases, therefore ethanol peaks are present in the NMR spectrum and cover important spectral regions (spectrum 1). Conversely dichloromethane has only a singlet peak near the water peak, which can be easily removed before pattern recognition analysis. Significantly, the comparison of spectra obtained after extraction with deuterated chloroform (spectrum 2) and dichloromethane (spectrum 3) demonstrates that β -hydroxybutyrate and creatine signals can be better detected when dichloromethane is used. Moreover, considering also that non-deuterated solvents are cheaper, we eventually decided to proceed using dichloromethane extraction. Last but not least, dichloromethane is the least toxic of the chlorohydrocarbons (Brown-Woodman et al., 1998; Honma & Suda, 1997).

3.2. Metabolomic fingerprinting of large-scale distribution milk

According to the experimental scheme reported in Supplementary Figs. 1, 60 samples from three Italian milk brands (20 samples each) were collected in a supermarket during three periods: Autumn 2013, Spring 2014, and Autumn 2014, for a total of 180 milk samples retrieved from the large-scale distribution. Our first aim was to test our system and to evaluate whether it was possible to discriminate each single brand.

The preanalytical and analytical approaches chosen proved to be particularly efficient for the NMR analysis of milk samples. The acquired spectra resulted of high quality, well resolved and with many visible peaks. Supplementary Fig. 4 shows a superimposition of the 60 spectra from the Autumn 2013 collection. From the figure, the high reproducibility of the spectra can be inferred. Supplementary Fig. 5 and Supplementary Table 1 show in detail the assigned peaks. Supplementary Figs. 6 and Supplementary Fig. 7 show the 2D spectra, ^1H - ^1H COSY and ^1H - ^{13}C HSQC respectively, used to confirm 1D assignments; Supplementary Fig. 8 shows a zoomed region of the HSQC spectra.

The analysis of the metabolic fingerprints of milk samples was performed using PLS-CA and Monte Carlo cross-validation: for all the three collection periods, it was possible to clearly distinguish the three brands (Fig. 2A, B and C), with a mean cross-validated accuracy over 95% in all the three cases (Supplementary Table 2), demonstrating the efficacy of both the chosen extraction protocol and the statistical strategy.

3.3. Metabolomic fingerprinting of milk from specific farms

To assess whether NMR-metabolomics, coupled with suitable pre-analytical and statistical approaches, is sensitive enough to accurately identify the origin of milk samples even from individual farms in a limited geographic area, twenty samples from ten different farms located in the Mugello valley (about 1100 Km²) were collected in Autumn 2013 and Spring 2014, for a total of 400 milk samples (Supplementary Fig. 1).

Using PLS-CA on the spectral data (Autumn 2013 collection), the ten farms were well-discriminated, except two of them (3 and 4) that were not distinguishable (Fig. 3A). In this case, the mean cross-validated accuracy was 82%. It later turned out that farms 3 and 4 indeed were a single farm, represented by two different company names used

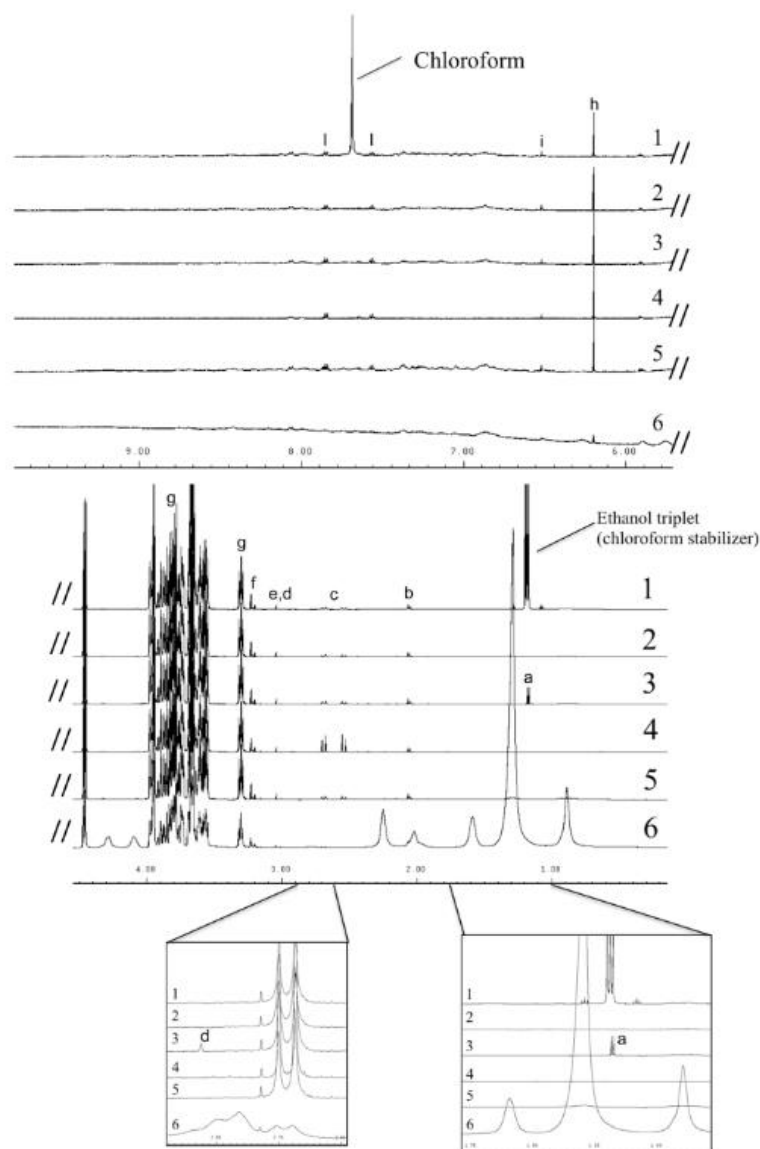


Fig. 1. Comparison among ¹H NMR spectra obtained from the same milk sample using different extraction methods. Chloroform (1), deuterated chloroform (2), dichloromethane (3), filtration with 10 kDa cut-off (4), ultracentrifugation (5), raw milk (6). Some metabolites are highlighted: β-hydroxybutyrate (a), *N*-acetyl group (b), citrate (c), creatine (d), creatinine (e), choline (f), lactose (g), orotate (h), fumarate (i), hippurate (l).

to market different dairy products: the cows were actually the same, living in the same environment and receiving the same kind of feedings. Correctly considering these two group of samples as coming from a single farm, the cross-validated accuracy for the recognition (of nine farms) became very high (97%, see Supplementary Table 3), demonstrating that each farm is characterized by a specific metabolic fingerprint of its milk. The same situation (Fig. 3B) and similar average accuracy (97%) was found also for the 2014 collection. To test the effect of a different kind of normalization on the recognition accuracy, PQN (Dieterle et al., 2006) was also used, showing comparable results (97.5% and 96.8% of accuracy for the 2013 and 2014 collections,

respectively). Total area normalization was then used in all the subsequent analyses.

The comparison between bulk raw milk samples coming from organic and non-organic farms, resulted in a very high cross-validated accuracy for the discrimination (about 99%, Supplementary Fig. 9).

3.4. Metabolomic fingerprinting of seasonal variations

In order to evaluate whether the pattern recognition was preserved independently of the season, all the data collected for milk from the nine farms (the two different collections) were merged together. The

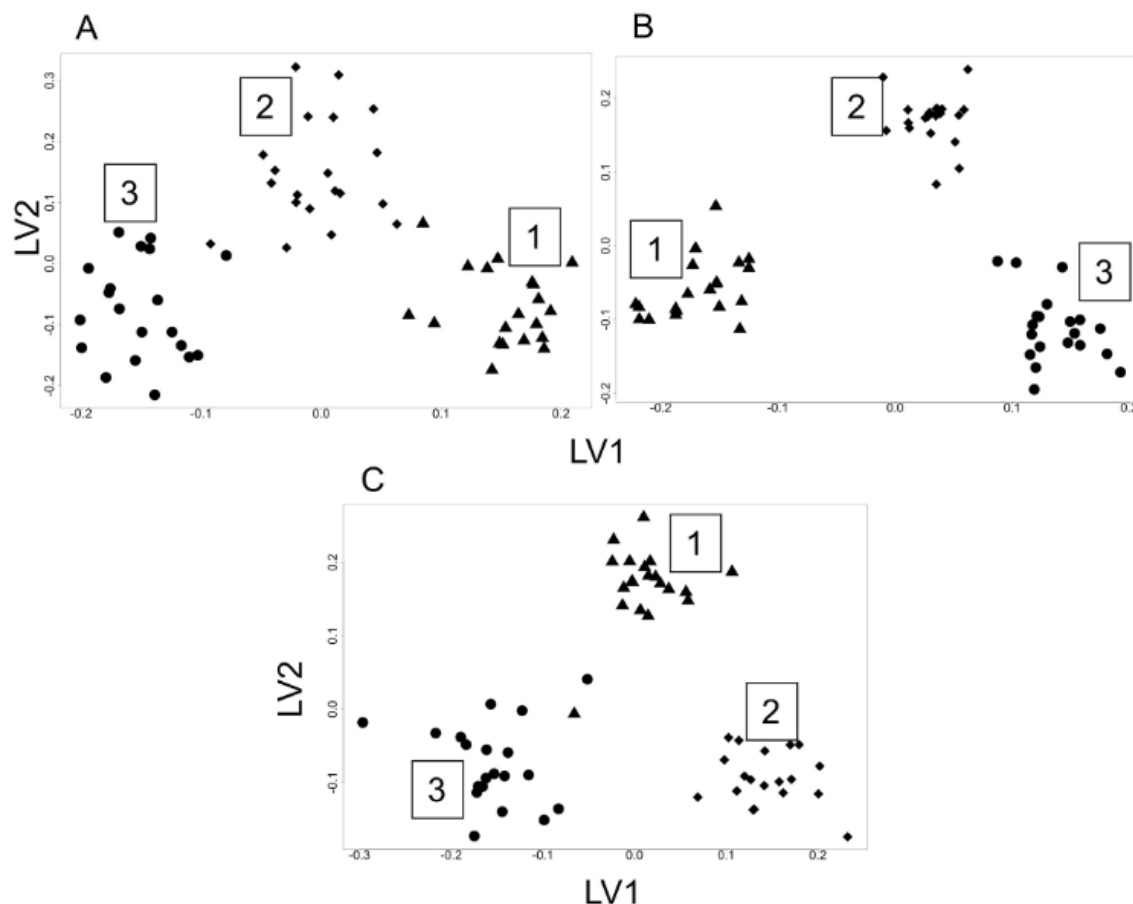


Fig. 2. PLS-CA plot of large scale distribution milk samples. Brand 1, triangles; brand 2, rhombus; brand 3, circles. Collection 2013, A; collection 2014, B; collection 2015, C.

cross-validated accuracy obtained for farms identification was 96% (Fig. 4A). Merging together the data for large-scale distribution milk (three different collections) the accuracy for brand identification was still 95% (Fig. 4C). These findings demonstrate that the characteristic metabolic fingerprint of milk samples exists independently of the season.

To compare the two collections of farm milk and the three collections of large-scale distribution milk, the statistical analysis described above for discrimination was repeated distinguishing samples from the same farm (or brand) originated in different collections as they were different farms (*pseudofarms*) or different brands (*pseudobrands*). In this case we obtained a total of 18 *pseudofarms* and 9 *pseudobrands*. The results of this analysis demonstrate that farm milk samples collected in the two seasons are indeed different, and the overall cross-validated accuracy is 96% (Fig. 4B). Considering the plot of the PLS-CA for *pseudofarms*, it is possible to observe that samples of 2014 are “shifted” to the bottom of the plot and mostly separated along the second latent variable. Instead, the first latent variable (LV1) of the same PLS-CA model tends to separate the samples according to farms. The loadings of this PLS-CA model are reported in Supplementary Fig. 10 to show which parts of the spectra contribute to the discrimination of the two different year of collection (LV2, Supplementary Fig. 10B), and which parts of the spectra mostly contribute to the farms characterization

(LV1, Supplementary Fig. 10A). The two sets of discriminating variables are not completely orthogonal, meaning that at least some features are affected by both seasonality and provenance, thus making more difficult to recognise farms without considering also the season of collection. Consequently, using the samples of the first collection (Autumn 2013) as a training set to blindly predict the farms of the samples of the second collection (Spring 2014), the accuracy drops to 78.5%. Analogously, the accuracy is 87.3% for the reverse analysis (i.e. predicting the farms of the 2013 samples using the 2014 samples as training set). For large-scale distribution milk, we collected samples in three periods to have a clearer scenario of the seasonal variability. The first collection (Autumn 2013) and the second collection (Spring 2014) are well discriminated and the cross-validated accuracy is 90%. The third collection (Autumn 2014) is still different from both the collections mentioned before and this means that although the period of the year for the third collection is the same as the first, the composition of milk is different (Fig. 4D). In practice, although the recognition accuracy inside each collection is very high, seasonal variations need to be considered if the aim is to build a metabolomic method for origin assessment and traceability.

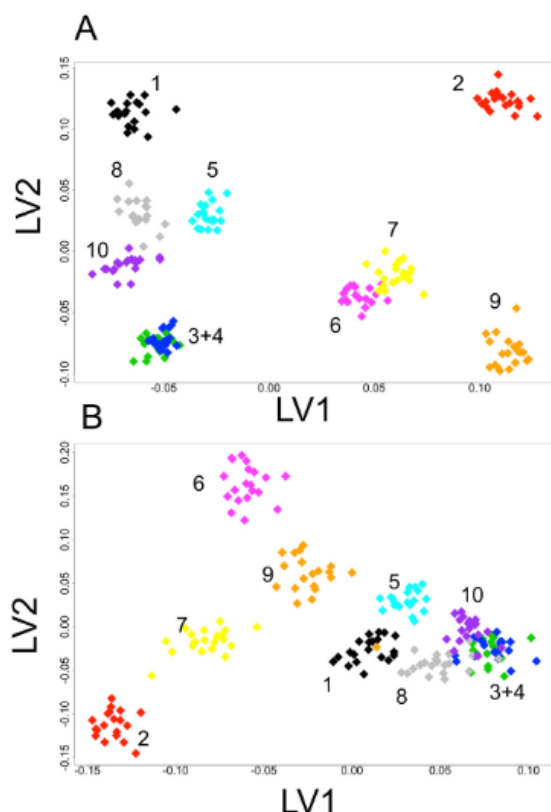


Fig. 3. PLS-CA plot of Mugello farms, each colour represents a distinct farm. Collection 2013, A; collection 2014, B.

3.5. Blind prediction of the origin of unknown samples

To evaluate the power of the metabolomic approach in the traceability of milk, we were provided with additional eight raw milk samples from the Mugello farms, collected in March 2015. We used these samples as a blind test set for assessing the predictive ability of the samples collected in 2013 and/or 2014 used as a training set. Three and four test samples were correctly assigned using, respectively, the 2013 and 2014 collections for training. Using both collection together in the training set, six out of eight test samples were unambiguously assigned to the correct farm. This latter result is quite promising, considering both the complexity of the task (identifying the correct group among ten possible choices) and the different period of the year in which these new samples were collected (late Winter) with respect to the samples included in the training set.

3.6. Analysis of cow feeding data

For each day of collection, raw milk samples coming from the Mugello's farms were accompanied by data about cow feeding, reported as kilograms of foods per bovine. These data are divided in eight different nutritional groups described in Materials and Methods section. By means of PCA analysis of the cow feedings data, we selected the farms presenting common nutritional profiles. Both for the 2013 and for the 2014 collection, we found three different nutritional groups (Supplementary Figs. 11A and 11B). For the 2013 collection, group 1 is composed by two farms that use silage and hays as cow feedings; group

2 is composed by four farms mostly using silage as cow feeding; group 3 is composed by four farms, and in this case hays and cereal flour constituted the major components of the feeding ration. In the 2014 collection, the feeding composition of the three groups did not change from those of 2013 and the only difference regarded group 1 that is composed by two more farms previously belonging to the group 3 of 2013 (farm nr 2 and 7, Fig. 5B). The features of the three groups with their composition in terms of feedings are reported in Supplementary Table 4 and bar-plots of the composition of the three groups in terms of foods are reported in Supplementary Figs. 12 and 13.

In order to evaluate the influence of cow feedings on the metabolic fingerprint of milk, we considered the discriminant plot obtained through PLS-CA and we checked the distribution of the three nutritional groups into this plot. It appears that the metabolic fingerprint of milk reflects cow feeding both in the 2013 and the 2014 collections. The collection of 2013, as shown in Fig. 5A, is characterized by three distinct groups corresponding to the kind of nutrients given: in particular is clearly visible the separation from farms that use silage (groups 1 and 2) and farms not using silage (groups 3). The same finding is true for the 2014 collection (Fig. 5B), where quite good separation of the three groups is maintained and the separation of farms that use silage (groups 1 and 2) from farms not using silage is mainly visible along the second latent variable (LV2). Farms 2 and 7 are more shifted towards group 3 along the LV1. These are the two farms belonged to group 3 in the 2013 collection, moving to silage feed, are now belonging to group 1 (collection 2014, Fig. 5B). Finally, the PLS-CA model built to distinguish farms using silage from farms not using silage revealed 98.8% predictive accuracy in discriminating the two kinds of feed (Supplementary Figs. 14 and 15).

3.7. Effects of silages on the metabolic profile of milk

A total of 19 metabolites were assigned in milk spectra. The variations in metabolites content have been analysed in order to point out differences between farms using or not using silages. Both collections show some metabolites that are statistically different between the two categories: for example the farms that did not use silages in the collection of 2013 present a weak but significant increase (positive $\log_2$ FC values in Fig. 6A) of 2-oxoglutarate, choline, methionine, hippurate, acetone, alanine and glutamate, and statistically significant lower levels of acetate, citrate, *N*-acetyl carbohydrates, creatinine, creatine, lactate and very low levels of an unknown metabolite (< 2.5 -fold) if compared with the farms that use silages (negative $\log_2$ FC values in Fig. 6A). The unknown metabolite presents the same trend also in the 2014 collection: in this case, samples from the two farms that did not use silages present a 3-fold reduction of this metabolite. This unknown peak could belong to a trimethylamine group. However, this signal cannot be unambiguously assigned to any corresponding molecule. A good candidate could be lecithin, considering the intense signal resonating at 1.28 ppm (δ H), 30 ppm (δ C) that could be attributed to the methylene groups of the acyl chains (Supplementary Fig. 6). Less marked but statistically relevant common variations are also present for other metabolites in both collection (Fig. 6A and B). Six metabolites present a statistically relevant common trend: three metabolites are lower (choline, methionine, hippurate) and three are higher (the unknown, creatinine and lactate) in milk samples coming from farms where silages are used.

4. Discussion

The peculiar features of several dairy products are related to the quality and the origin of the milk used (Lamanna et al., 2011). In fact, milk of bovine origin is both consumed fresh and processed into a variety of dairy products (cheese, fermented milk products) and the nutritional quality and the processing capabilities are closely associated to composition and origin. In this sense, metabolomics is an ideal

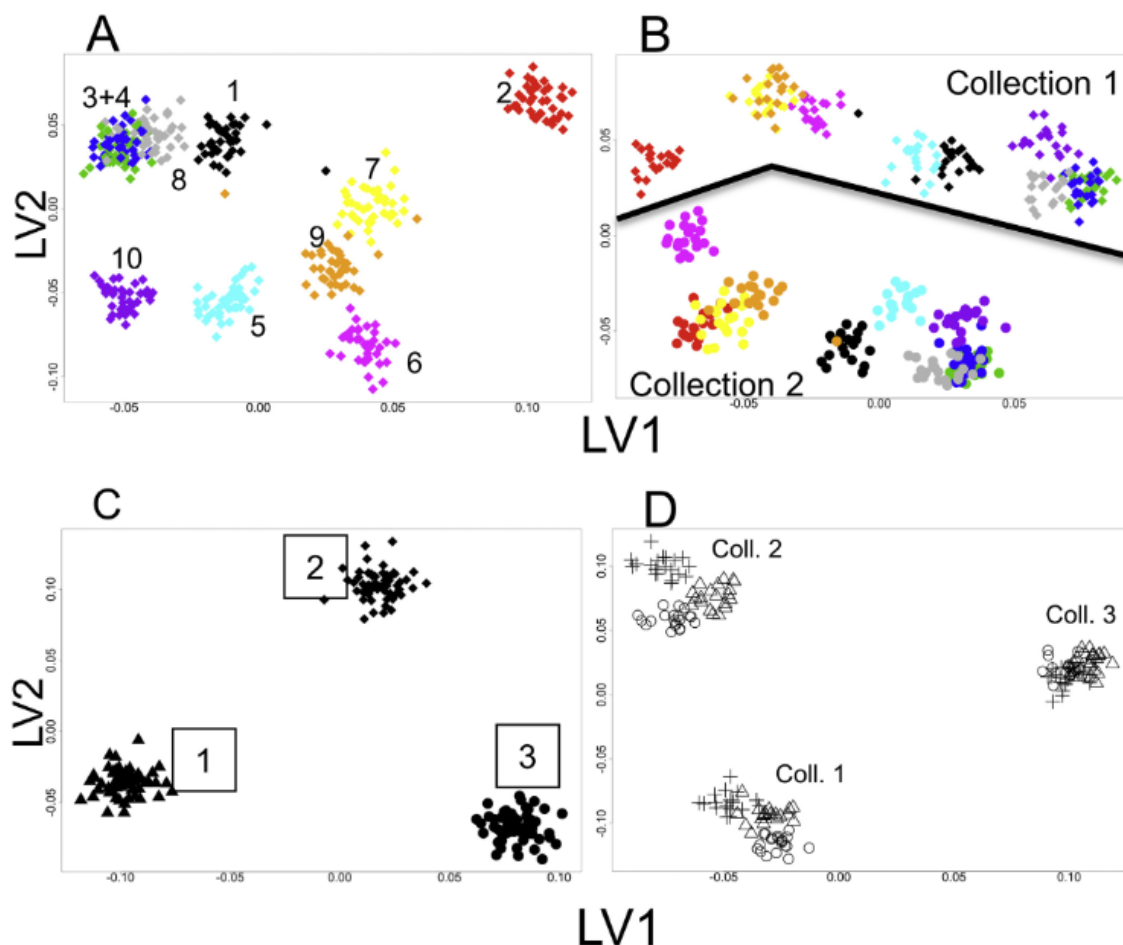


Fig. 4. PLS-CA plot of milk samples considering all the collections together. Discrimination among different farms, aggregating samples from the same farm originated in different collections, A; discrimination among different farms, distinguishing samples from the same farm originated in different collections as they were different farms (pseudofarms), B (diamonds: samples from Collection 1, circles: samples from Collection 2); discrimination among different brands, aggregating samples from the same brand originated in different collections, C; discrimination among different brands, distinguishing samples from the same brand originated in different collections as they were different brands (pseudobrands), D (the same symbol identify the same brand in the three collections). The separation line in panel B is manually drawn only for visualization purposes.

approach because it provides a good profiling of low-molecular-weight compounds in milk (Sundekilde, Poulsen, et al., 2013). Many applications related to the analysis of milk through NMR have been proposed, most of them focusing on linking milk metabolites content with nutritional aspects, discovery of biomarkers of cow's diseases (Enjalbert, Nicot, Bayourthe, & Moncoulon, 2001; M. S. Klein et al., 2010; Matthias S. Klein et al., 2012; Sundekilde, Poulsen, et al., 2013) and analysis of bioactive compounds (Garcia et al., 2012; Holmes, Snodgrass, & Iles, 2000).

In the present study, before considering metabolic fingerprints of different kinds of milk samples, a novel procedure for NMR-based metabolomics analysis of milk samples has been developed. Comparing different pre-analytical procedures for obtaining soluble metabolites from a heterogeneous fluid such as milk, the use of dichloromethane has given good results in terms of quality and reproducibility of the spectra. Spectra of milk samples obtained using ultracentrifugation and filtration with 10 kDa cut-off are good, especially in the case of filtration, in which no broader signals are present. Our experience confirms

that sample preparation based on organic solvent such as chloroform or dichloromethane is a viable alternative. The quality of the spectra obtained is comparably good: the signals of metabolites are clearly visible, and no broader signals are present. Although chloroform and dichloromethane provide similar spectra, the preparation with dichloromethane appears preferable. First, dichloromethane is less toxic than chloroform, second the signals of β -hydroxybutyrate, and creatine are better visible after the extraction with CH_2Cl_2 . The detection of these two compounds can provide important information: β -hydroxybutyrate is known to be a diagnostic biomarker for cow ketosis (Matthias S. Klein et al., 2012) and it also increases in relation to somatic cells count that is normally used as an indicator of mastitis infection (Sundekilde, Poulsen, et al., 2013). Creatine plays a key role in cellular energetics and its deficiency is associated with several neurological manifestations. Creatine is also found in milk and dairy products but in a relatively small amount. Estimating the amount of creatine can be useful to assess the nutritional value of milk.

Metabolic profiles of milk samples reflect with high accuracy the

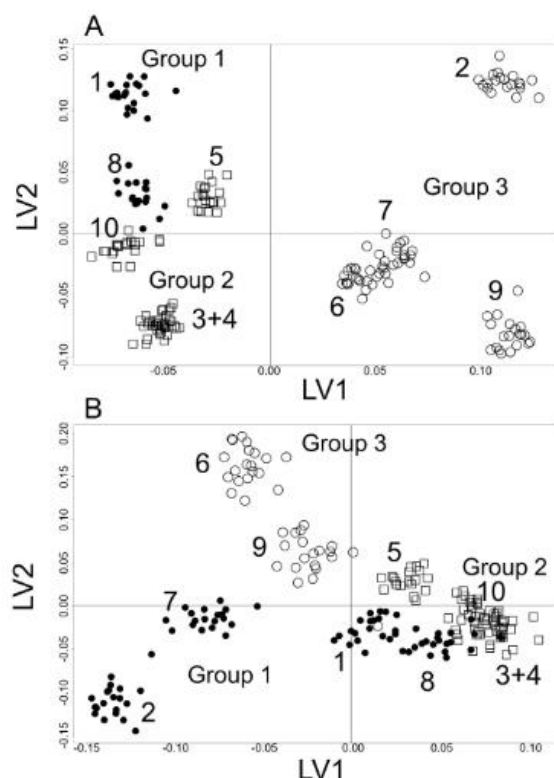


Fig. 5. PLS-CA plot for the discrimination among farms, highlighting the nutritional groups obtained by means of PCA on nutritional data. Black dots, group 1; white squares, group 2; white dots, group 3. Collection 2013, A; collection 2014, B.

farm of origin. Also considering two distinct milk collections (summer/autumn 2013 and spring 2014), the results did not change and the accuracy for the discrimination among farms is very high. Milk coming from organic and non-organic farm can also be identified, and this is likely due to the different cow feeding. Extending our approach to large-scale distribution milk it is possible to observe a clear separation between the three brands that we have considered, although in this case each brand of milk originates from a more extensive geographical area.

Milk samples collected in different periods are indeed different. Identifying the reasons of this seasonal variability is not an easy task: in different seasons several factors can change, e.g. living environment, nutrition, metabolism, etc. Our results show that considering each collection period separately, milk presents some features that allow us to correctly identify each farm (for the Mugello samples) or each brand (for the supermarket samples). For raw milk coming from Mugello farms, the distribution of the two collections in the PLS-CA plot is almost the same; however, the 2014 samples are shifted to the bottom part of the plot, demonstrating a seasonal metabolic shift. Due to this variability, the accuracy for predicting the farm of provenience in one collection, using the other as training, is suboptimal (78.5% and 87.3%). However, pooling together the two collections permits the correct prediction of 6 out of 8 “unknown” samples, demonstrating that repeated collections can average these variations, and confirming the possible role of metabolomics for the traceability of products. In any case, further analyses with a larger number of samples in different seasons are needed to fully validate this approach.

Analysis of nutritional profiles during the collection periods has

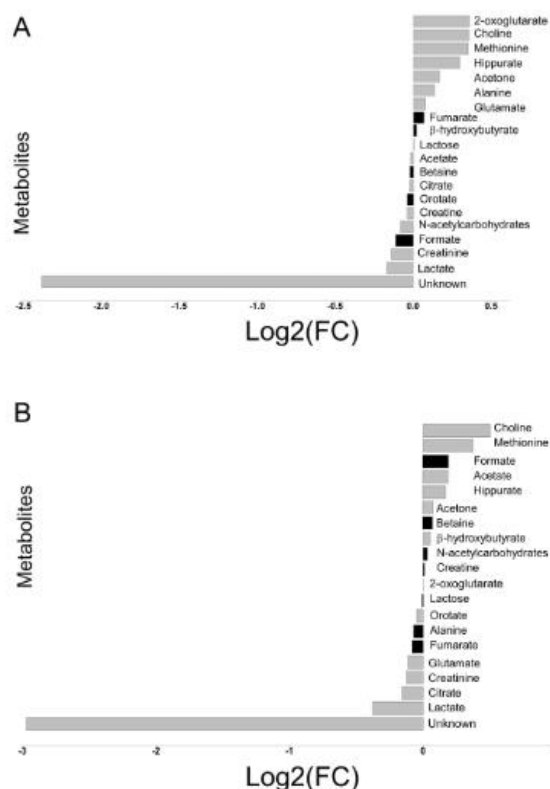


Fig. 6. $\text{Log}_2(\text{FC})$ analysis between farms using feeding without silages ($\text{Log}_2 \text{FC}$ positive values) vs. farms using feeding with silages ($\text{Log}_2 \text{FC}$ negative values). Grey bars are related to metabolites statistically different between the two groups ($p < 0.05$). Collection 2013, A; collection 2014, B.

been performed in order to evaluate the influence of cow nutrition on the metabolic profile of milk. Both for the 2013 and for the 2014 collections, we obtained three different groups: the composition of the three groups in terms of foods did not change drastically, except that two farms that in 2013 did not use silages started to use silages in 2014. The three nutritional groups are perfectly discriminated in the PLS-CA plot of the metabolic profiles, confirming the great influence of cow feeding in the composition of milk. In particular, the three groups are well-discriminated in both collections, even if in the 2014 PLS-CA plot two farms belonging to nutritional group 1 are shifted near group 3. We have noticed also that the most important difference between the three groups is the evident separation between farms using and not using silages. In fact, in both collections, groups 1 and 2 are composed by farms that use high quantity of silages, at variance with farms belonging to group 3 that mainly use hays. This finding is very important for the production and authentication of dairy products: for example, the production protocol of the Italian cheese “Parmigiano Reggiano” (Parmesan cheese), that is strictly regulated by the European Union, forbids the use of milk coming from bovines fed with silages (Council Regulation 510/2006) to avoid contamination of the product by clostridium bacteria that cause malformations in the wheels of cheese during maturation. Thus, analysis of metabolic profiles could be a fast and reliable tool to screen milk for the presence of silage in the animal feedings, and possibly to prevent fraud in the preparation of original cheeses. Although in the two collections it is possible to identify several common metabolites that are statistically different between farms

where silages are used or not, the most important difference is related to an unknown signal resonating at 3.11 ppm (δ H), that is strongly reduced in farms where silages are not used, both in 2013 (2.5-fold reduction) and 2014 (3-fold reduction) collections.

5. Conclusion

The present work supports NMR-based metabolomics as a useful tool for the analysis of milk. Sample preparation is very important in order to obtain reliable results, and it should be fast and accurate in order to analyse several samples together and to obtain good quality and reproducible spectra. For this purpose, after testing protocols from the literature and comparing them with the one developed in our laboratory, we propose extraction using dichloromethane: it proved to be a fast and cheap method, and provides information about two important metabolites for the metabolic status of the cow (β -hydroxybutyrate) and nutritive value of the milk (creatine). Further, the metabolomic approach has proven to be a valid method in assessing the origin of authentic products, both considering a small geographic valley (Mugello) and a larger area (large-scale distribution). However, seasonal variations need to be considered using repeated collections, due to the changes over time of the composition of milk. This method is also able to provide information about the nutrition of the cows, opening scenarios for the monitoring of the production of dairy products (e.g. Parmesan cheese), although its potential in fraud detection needs to be further investigated. In this respect, the NMR analysis performed directly on cheese samples can be the natural continuation of the present work.

Acknowledgments

We acknowledge the Cooperlatte s.c.a. associated farmers for providing us with the milk samples.

Conflict of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2018.06.066>.

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5.2.2 From beans to brew: NMR based metabolomic approach to assess traceability of coffee producers within a restricted geographical area of Colombia

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In Preparation

Candidate's contributions: acquisition of NMR data, statistical analysis and interpretation of data and writing the draft

ABSTRACT

The precise identification of the origin of the coffee plantation is a really challenging task. Colombia is the world's third coffee-producing country, after Brazil and Vietnam. We think that the identification of the origin of coffee beans coming from small, geographically localized Columbian plantations is the ideal case to proof the power of the NMR fingerprint approach. Our preliminary results are based on the collection of 51 batches of green coffee beans (*arabica*) coming from different Columbian producers. After setting the most suitable protocol for the analysis, from each batch we prepared 7 different samples undergoing NMR analysis. OPLS-DA model was built on 357 green coffee bean spectra acquired using NOESYGPPS pulse sequence. The model, validated using leave-one-out cross-validation scheme, shows an overall of 90.7% in predictive accuracy. In parallel, 18 of those batch, were sent for roasting to a professional coffee trader. The statistical model built on roasted coffee beans shows an overall predictive accuracy of 95% in identifying the farms (origin). We correlated metabolites with batch characteristic (e.g. altitude of the plantation, time of fermentation etc.) and organoleptic properties finding weak correlations (alanine/altitude = R 0.56; $p < 0.001$; sucrose/altitude = R 0.53; $p < 0.001$; maleate/bitterness = R 0.60; $p < 0.05$), using green coffee fingerprints we correctly predict the coffees with a quality score above 70 (i.e. of remarkable quality).

Introduction

The two major species of coffee, *Coffea arabica* and *C. canephora* (Robusta), differ considerably in quality and therefore price. *C. arabica* (commonly known as Arabica coffee) is most appreciated by consumers due to its intense aroma, low bitterness and lower caffeine content and this is reflected in the selling price which is usually 20–25% higher than the one in Robusta. Another important aspect on coffee authenticity refers to geographical origin declaration. In recent years there has been an increase on falsification of the product declaration. Therefore, it is easy to understand the relevance of the availability of suitable analytical methods to differentiate between these two coffee species. Agronomic practices and environmental conditions such as altitude, climate and soil type can affect the chemical composition of the plant. Most commercially available coffees are varietal blends which makes the detection of adulteration very difficult. In addition, the genetic variability associated with these conditions can affect the acidity of the coffee and the content of sugars, chlorogenic acids, lipids and caffeine (Garrett et al., 2013). Different methods mainly based on the quantification of chemical markers such as caffeine, fatty acids or sugars are described in the literature to distinguish Arabica from Robusta coffee (Garrett et al., 2012). However they are based on elaborated methodologies that are time consuming and limited in terms of fraud screening because of the small number of components monitored. Therefore a metabolomic approach could be a powerful way to investigate differences in the profile of secondary metabolites in coffees. NMR metabolomics has been demonstrated to be a useful tool for distinguishing the species and origins of green coffee bean samples of Arabica and Robusta from six different geographic regions (Wei F. et al., 2012). By the same study significantly different levels of 14 metabolites of green coffee beans were identified as significant for the classification. Besides geographical origin, metabolomics can be used to assess the relationship between organoleptic properties and chemical composition of food matrices. NMR spectroscopy can be considered a kind of "magnetic tongue" for the characterization and prediction of the tastes of foods, since it provides a wealth of information in a non-destructive and untargeted manner. The taste of commercial coffee beans, identified by human sensory tests, has been successfully predicted based on their NMR metabolite profiles using multivariate statistics on the whole spectral profile (Wei F. et al., 2014).

These preliminary results provide a proof of concept for the ability of NMR-based metabolomics to distinguish different coffee of the same specie (Arabica) collected in fifty-one cultivars located in Columbia. Colombia is the world's third coffee-producing country, after Brazil and Vienna. From "Cafè de Colombia" has been registered as Protection of Geographical Indications granted Originally the production of coffee in Colombia was in the hands of wealthy landowners. Due to both a sharp fall in coffee price around 1900 and the civil war, "Guerra de los Mil Dias", these large estates fell into decline. They were divided into very small plantations, measuring on average 1.4ha with minuscule lots (50kg). 94% of the coffee producers plant coffee on less than 5ha of their land. Therefore the precise identification of the plantation origin is a really challenging task.

The rationale for this study design was to prove that NMR-metabolomics is sensitive enough to accurately assess the provenance of green and roasted coffee beans even at the scale of each individual Colombian cultivars. We analyzed also individual coffees characteristics provided by each cultivar (e.g. altitude of the cultivar, time of fermentation etc.) and panel test values in

order to understand the contribution of different environmental conditions or agronomic practices on metabolites levels of coffee samples.

Materials and methods

Coffee Beans. Green coffee beans were collected from 51 different coffee-growers localised in Huila district of Colombia (**Figure 1, Supplementary Table 1**), among them 18 samples, corresponding to different coffee-growers, were roasted by a professional coffee trader (Sandalj Trading company Spa, Trieste, Italy).

NMR Samples. 7 beans for each sample were grounded using a Caso 1830 coffee grinder which was thoroughly cleaned between the grinding of each sample. A total of ~0.2 g of crushed beans was dissolved in H₂O (1 mL) and centrifuged 5 min at 14000 RCF (room temperature), then samples were incubated at 95°C in a closed Eppendorf for 1h. The extracts were centrifuged for 5 min at 14000 RCF at 4°C.

300 uL of the supernatant were removed to a new tube and mixed with 300 uL of phosphate buffer (1.5 M K₂HPO₄, 100% (v/v) ²H₂O, 2 mM NaN₃, 5.8 mM TMSP; pH 7.4) and vortexed for 20 s. A total of 450 uL of this mixture was transferred in a 4.25 mm NMR tube.

NMR Spectroscopic Analysis and Data Processing. One-dimensional (1D) ¹H-NMR spectra were measured at 400 MHz using an AVANCE III Bruker spectrometer equipped with a 5mm BBI 400S1 H-BB-D-05Z probe. The probe temperature was regulated at 300K and for each spectrum 64 scans were collected using the *Noesygpps1d* sequence with P1 = 10.63 us (9.7 W), a spectral width of 12.47 ppm, a relaxation delay of 4 s and a total acquisition time of 8 min. The receiver gain was adjusted at 203. FIDs were zero-filled and transformed using exponential line broadening (0.6 Hz) resulting in spectra of 16,384 points.

NMR Data Analysis. Resulting NMR spectra were aligned to TMSP resonating signal (0 ppm) and input variables for statistical analyses were generated via bucketing (binning) by segmenting each spectrum in buckets of 0.02 ppm in the range between 0.4 and 10 ppm after excluding the region of residual water (4.5 ppm-5.24 ppm). Buckets were then normalized on measured weight of crushed beans, and finally, Probabilistic Quotient Normalization (PQN) was applied. The resulting dataset was used to perform multivariate statistical analysis.

Statistical Analysis. Multivariate analysis on metabolomics data was conducted on processed NMR spectra. Principal component analysis (PCA) was used as first exploratory analysis. Orthogonal projections to latent structure discriminant analysis (OPLS/DA) was applied to obtain the supervised separation of the analysed groups (Westerhuis, van Velzen, Hoefsloot, & Smilde, 2010). The confusion matrices and the accuracies reported for NMR data were assessed by means of Leave-one-out cross validation scheme (LOOCV). Metabolites, corresponding to well defined and resolved peaks in the spectra, were assigned 1D Noesygpps experiments. Signal identification was performed using a library of NMR spectra of pure organic compounds, public databases (e.g., FooDB, PhytoHub) storing reference and literature data (Kwon et al. 2015, Wei et al. 2012). Multivariate logistic regression models were applied to the buckets of roasted coffee batches and organoleptic data resulted from the panel test activity (**Supplementary Table 3**),

P-values were calculated. The concentrations of the various metabolites in the different spectra were calculated by integration of the signal area, and the relative concentrations were analysed to determine the discriminating metabolites among the groups under study. Kruskal-Wallis test was chosen to infer differences among the groups on the biological assumption that metabolite concentrations are not normally distributed. Pearson correlation coefficients have been also calculated to assess the correlations among metabolites' levels and local Colombian coffee's cultivars data.

Results

Metabolic fingerprint of different Colombian local coffee-growers. To assess whether NMR-metabolomics, coupled with suitable pre-analytical and statistical approaches is sensitive enough to accurately identify the origin of green coffee beans of the same species cultivated in the same geographical area (Huila department, Colombia), 51 batches coming from different coffee growers have been collected and analysed for a total of 357 samples analysed (7 replicates for each batch). Using a cross-validated OPLS-DA on the spectral data, the 51 cultivars have been well-recognised showing an overall predictive accuracy of 90.7 % (**Figure 2**). Among them only two batches (n° 51, and 4) have been recognized with an individual predictive accuracy of 57 %.

The same approach has been applied on roasted coffee beans. From all the 51 batches of green coffee beans, 18 have been roasted by a professional coffee trader. A total of 126 samples (7 replicates for each batch) have been used to build a cross-validate OPLS-DA model (**Figure 3**) showing a mean of predictive accuracy in recognizing each grower over 95%. The analyses of the metabolic fingerprint of Colombian coffee demonstrate the efficacy of both the chosen extraction protocol and the statistical strategy in distinguishing different close coffee producer of same geographical area. This approach is effective both with green and roasted coffee beans.

Multivariate analysis on the metabolic profile of coffee beans: altitude and variety. Each batch of coffee beans was provided by producer information regarding the altitude of the plantation and the variety of coffees.

Since has been already noticed in other edible plant species that altitude (Ohno et al. 2011) and variety (Son et al. 2009) are important parameters determining the quality of the product, we also decided to see if effects of these factors are detectable in green and roasted coffee beans by ¹H-NMR base metabolomics approach. PCA analysis was firstly attempted to visualize sample distribution (**Supplementary Figure 1**) dividing all the batches in three different groups according to calculated tertiles: group 1 (n = 133, blue) ≤1600 m; 1600 m < group 2 < 1750 m (n = 84, yellow); group 3 (n = 140, green) ≥1750 m. PC1 seems to conceal a sort of separation especially between group 1 and group 3, however considering the first five principal component of the PCA is not possible to ensure the visibility of altitude effect on those samples using this unsupervised approach. Therefore we applied OPLS-DA modelling to the 1H NMR spectra datasets from the three calculated groups of cultivar altitude. The model built seems to be quite effective in discriminating samples according to the altitude of the corresponding cultivar as shown in **Figure 4** and the related cross-validation (75% mean of predictive accuracy). The same approach has been applied on the 18 batches of black coffees to check if such information is still present in the metabolic fingerprint undergoing roasting process (**Figure 5a**).

In this case tertiles have been calculated again to obtain a comparable number of samples per groups: group 1 < 1721.3 m; 1721.3 m < group 2 < 1800 m; group 3 ≥ 1800 m. The resulted overall predictive accuracy of black coffee beans is 78%, therefore to see how much of cultivar altitude information is lost during the roasting process, a new model has been created using the 18 respective green batches (**Figure 5b**). The very high resulting accuracy (90%) obtained by this model suggests that roasting process attenuates somehow the altitude information contained in the metabolic profile of the coffee.

Moreover we checked if the same approach could be effective in defining the fingerprint of the different varieties present in the 18 different batches: 2 producers (samples nr 2 and 13) cultivating bi-varietal coffees Caturra-Castillo, 14 producers (nr 4, 6, 9, 12, 15, 18, 24, 28, 34, 41, 44, 47, 50 and 51) cultivating mono-varietal coffees Caturra, 1 producer (nr 38) cultivating bi-varietal coffees Caturra-Colombia and another producer (nr 21) cultivating bi-varietal Castillo-Caturra. Therefore, we tried to classify each coffee variety building two models, both for green and roasted coffee beans (**Figure 6 a and b**).

The cross-validated model explains that it is possible classifying samples according to different varieties and different combination of them. Unfortunately for multi-varietal batches the percentage of the variety is not known. Therefore, the little differences in the prediction accuracies of the two models can be explained by the fact the beans were randomly taken from the batches for sample preparation. However, by selecting available mono-varietal coffee batches (Castillo, Caturra and Colombia) from the 51 cultivars we were not able to see an improvement in the recognition accuracies of groups (84%, **Supplementary Figure 2**). This should mean that other environmental factors or/and agronomic practice mask somehow the information of the variety in green coffee beans.

Univariate analysis on metabolites. A total of 16 metabolites have been assigned on green coffee beans spectra and a total of 22 in roasted coffee beans spectra (**Figure 7a and 7b**). Metabolites levels have been compared for all batches analysed (**Supplementary Figure 3 a, b**). Moreover, correlations with data available (producers' characteristics and organoleptic tests) have been performed. Relative concentrations of green coffee beans metabolites have been correlated with altitude (meters), fermentation (hours) and drying (days). Results show significant ($P < 0.001$) positive weak correlations ($r \sim 50$) between metabolites (alanine, caffeine, sucrose, quinic acid, valine isoleucine and GABA) and altitude (**Figure 8a, Supplementary Table 2**), confirming results obtained by multivariate analysis on green coffee fingerprint.

Roasted coffee beans metabolites have been correlated to organoleptic result (**Supplementary Table 3**) gained from panel test (**Figure 8b, Supplementary Table 2**), resulting again in weak correlations ($r < 70$).

Another attempt was performed by trying to predict for each batch of the 18 producers the goodness score assigned by the panel test. Results reported in **Figure 9**, shows that the PLS-DA model, using the entire spectra of green coffee beans we are able to correctly identify the coffees with a score above 70 (*i.e.* of interesting quality).

Conclusions and future perspectives

This pilot project has been performed in 2018. We collected 51 batches of green beans of coffee, each from 51 different farms within a restricted region of the Huila department in Colombia. From each batch we prepared 7 different samples for NMR analysis, using green beans. An accuracy of about 90% was obtained in correctly identify the farms.

In parallel, 16 of those batch was also sent for roasting to a professional coffee trader.

We then prepared samples for NMR analysis using roasted beans and again we were able to obtain ~90% accuracy in identifying the farms (origin). Then we compared roasted and green beans and we found interesting molecular changes after the roasting process.

Consequently, we correlated NMR spectra with organoleptic score of the tests and we were able to find (weak) correlations between some metabolites and organoleptic properties and correctly identify the coffees with a score above 70 (i.e. of interesting quality).

It is the first large scale pilot proof of concept of NMR analysis for coffee production origin of a restricted area and quality assessment.

However it need to be validated in several directions such as including more farms in the database of NMR spectra, including more samples for each farm (more than 7 samples) to strength the statistical power of the method, repeating the collection in different periods to check for the seasonal/annual variations in the metabolic fingerprint (in principle this is a crucial point, but there are evidence in the literature that the coffee fingerprint is quite stable), strength the quality assessment by including more organoleptic tests (beyond the 16 already performed)

Figures and Tables

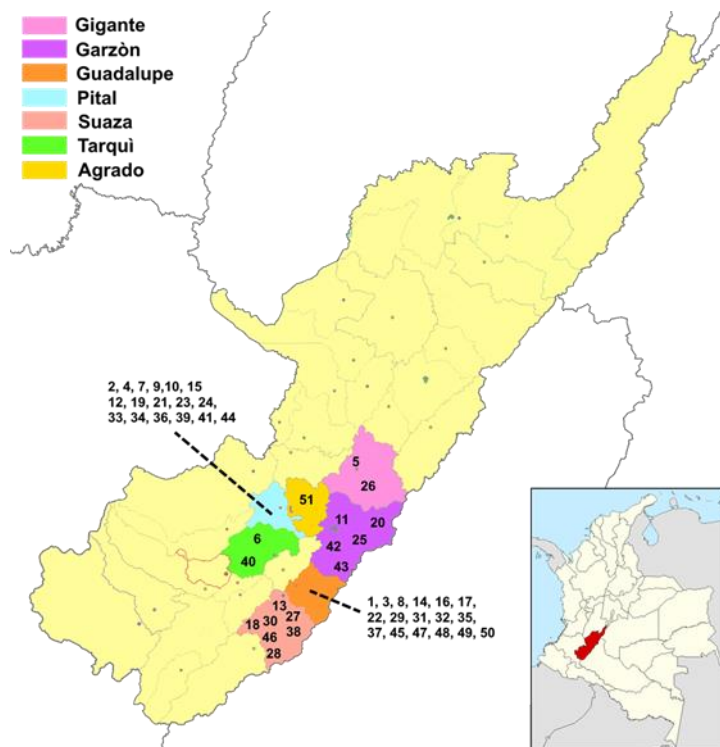


Fig 1. City halls (Gigante, Garzòn, Guadalupe, Pital, Suaza, Tarqui, Agrado) of the Huila department of Colombia, corresponding to the provenance of 51 different producers of green coffee. Number on the maps refers to codes corresponding to different coffee cultivars (see **ST1**).

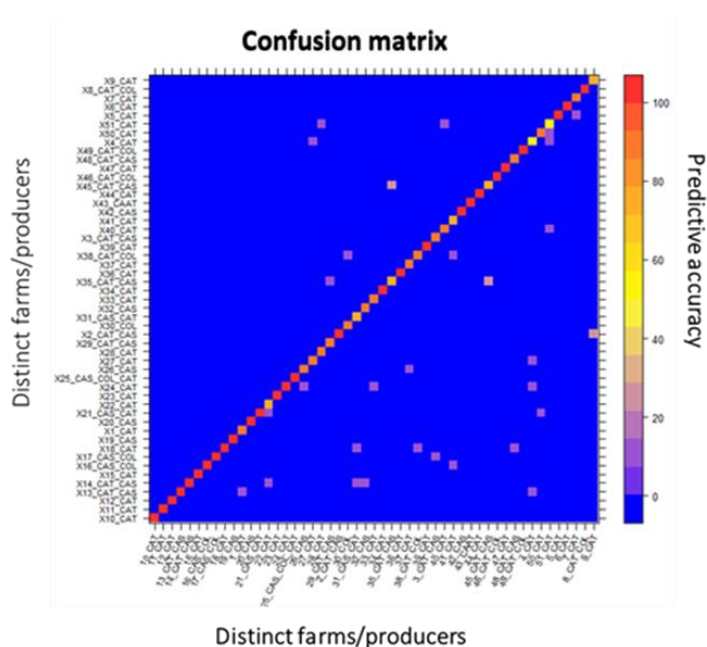


Fig 2. Heatmap of the confusion matrix for the recognition of each Colombian coffee farm (51) using an OPLS-DA model built on green coffee beans water soluble extracts.

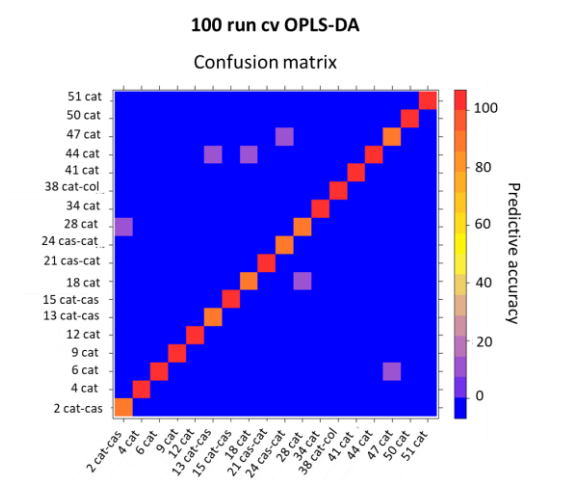


Fig 3. Heatmap of the confusion matrix for the recognition of each Colombbian coffee farm (18) using an OPLS-DA model built on roasted coffee beans water soluble extracts.

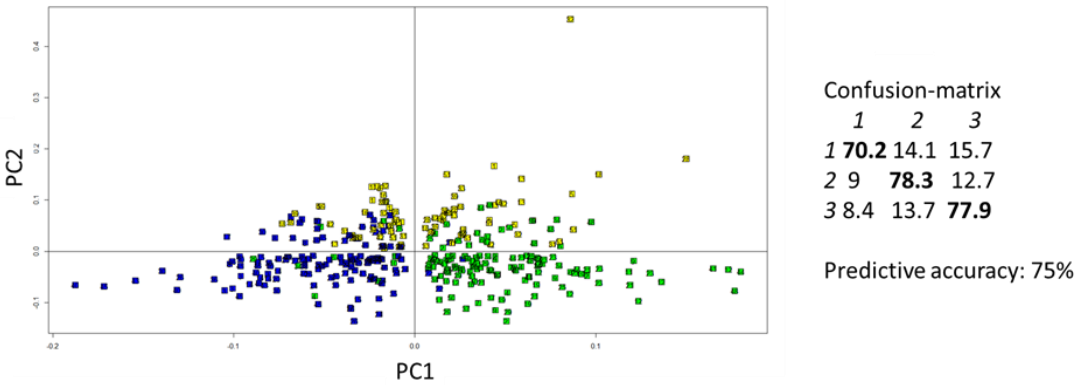


Fig 4. OPLS-DA on green coffee batches, three groups have been created as following by dividing samples in tertiles according to the altitude (m) of each cultivar: HCV, HBV and HC 1H-NMR serum spectra. Score plots of OPLS-D group 1 (n = 133, blue) ≤ 1600 m; $1600 \text{ m} < \text{group 2} < 1750$ m (n = 84, yellow); group 3 (n = 140, green) ≥ 1750 m. Confusion matrix and prediction accuracy of Monte Carlo cross-validation are also reported.

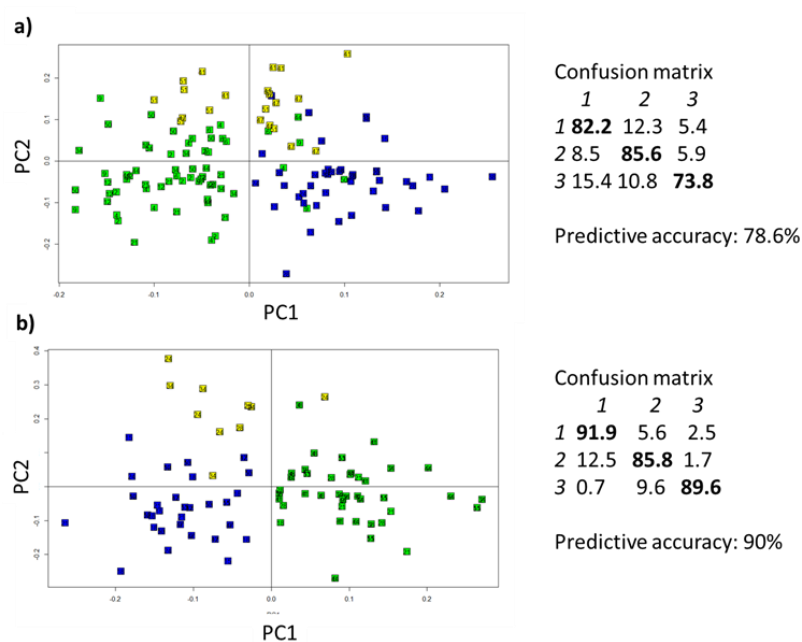


Fig 5. OPLS-DA on roasted coffee beans (a) and green coffee beans (b) 18 batches. The two score plots shown 3 groups divided as following on the basis of cultivar altitude (m): group 1(blue) < 1721.3 m; 1721.3 m < group 2 (yellow) < 1800 m; group 3 (green) $\geq$ 1800 m. On the right are reported the confusion matrix and prediction accuracy of both models

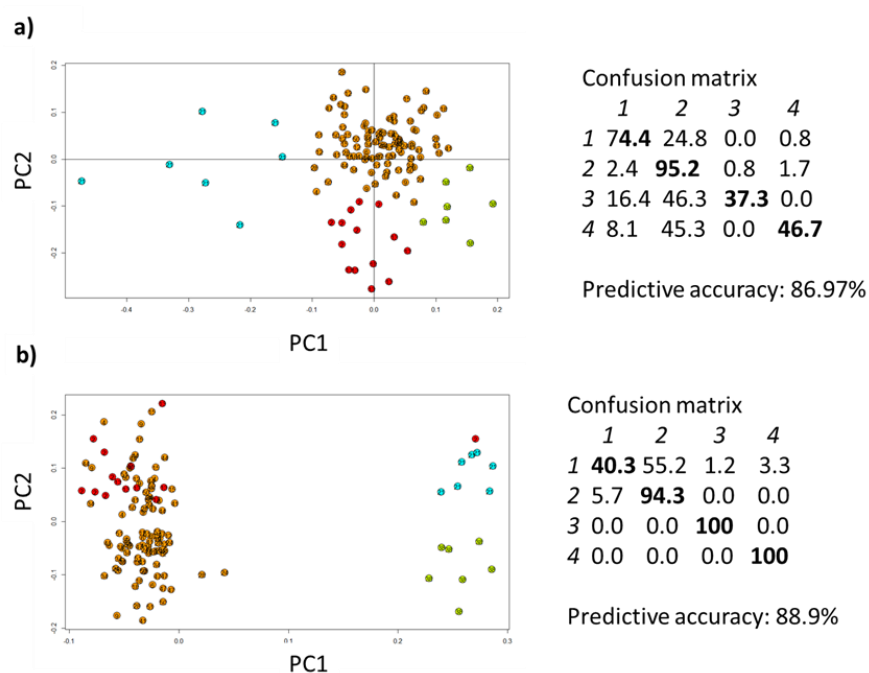


Fig 6. OPLS-DA on green coffee beans (a) and roasted coffee beans (b) 18 batches. The two score plots shown 3 groups divided on the basis of the four bi- and mono- varietal batches present. *Caturra-Castillo* bi-varietal producers n°1 in the confusion-matrices (red dots); *Caturra* mono-varietal producers n°2 (orange dots); *Caturra-Colombia* producer n°3 (green dots); *Castillo-Caturra* producer n°4 in the confusion matrices (cyan dots)

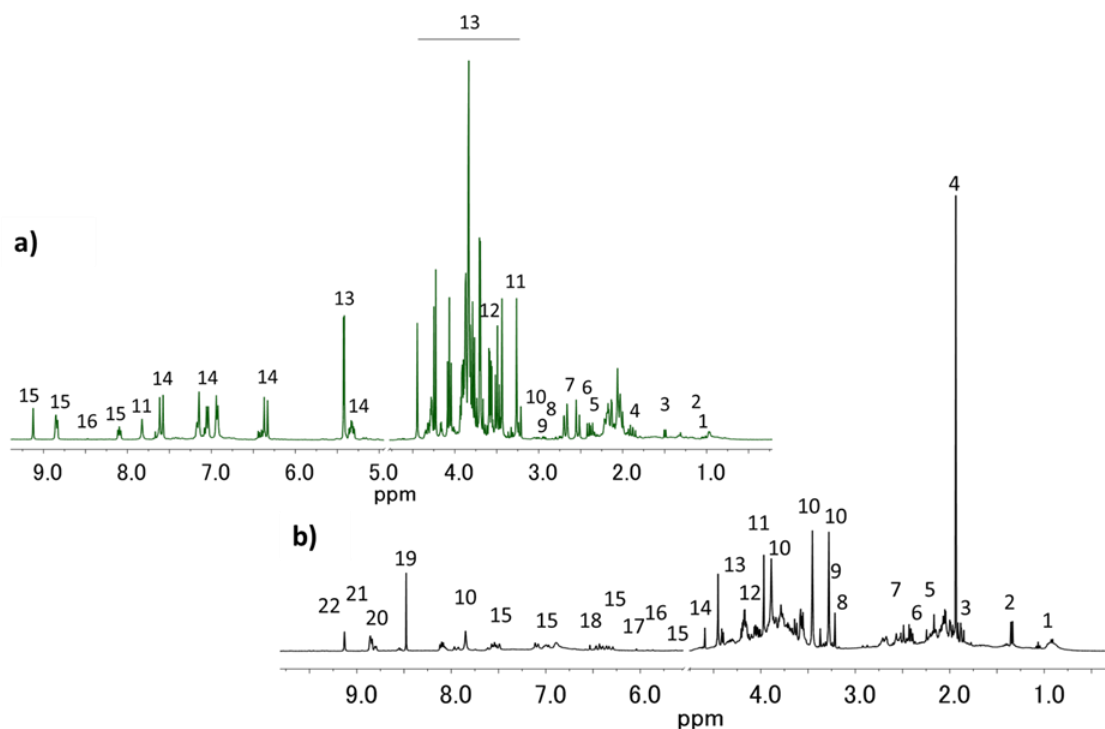


Fig 7. ^1H -NMR Colombian coffee water soluble extracts. A) 1D Noesygpps spectrum of green coffee beans: 1) isoleucine; 2) valine; 3) alanine; 4) quinic acid; 5) succinate; 6) malic acids; 7) citric acid; 8) sarcosine; 9) asparagine; 10) gaba; 11) caffeine; 12) 1,3-dimethylurate; 13) sucrose; 14) chlorogenic acids; 15) trigonelline; 16) formic acid; B) 1D Noesygpps spectrum of roasted coffee beans: 1) propionate; 2) lactate; 3) quinic acid; 4) acetic acid; 5) acetone; 6) gamma-butyrolactone; 7) pyroglutamate; 8) choline; 9) 16-methylcafestol; 10) caffeine; 11) glycolate; 12) malic acid; 13) myo-inositol; 14) 2-furfuryl methanol; 15) chlorogenic acids; 16) citraconate; 17) maleate; 18) fumarate; 19) formic acid; 20) N-methylpyridinium; 21) nicotinic acid; 22) trigonelline. Water region has been removed from both spectra

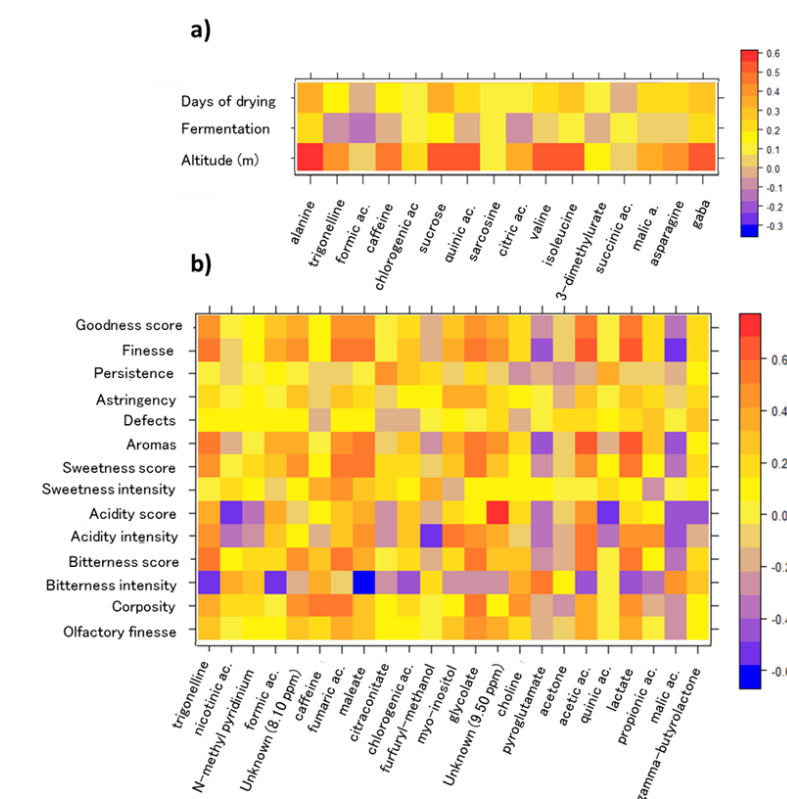


Fig 8. Heatmap reporting the correlations between identified metabolites in green coffee beans and coffee data relatives to each producer (a), and in roasted coffee beans and organoleptic test Coloured bars on the left show r values.

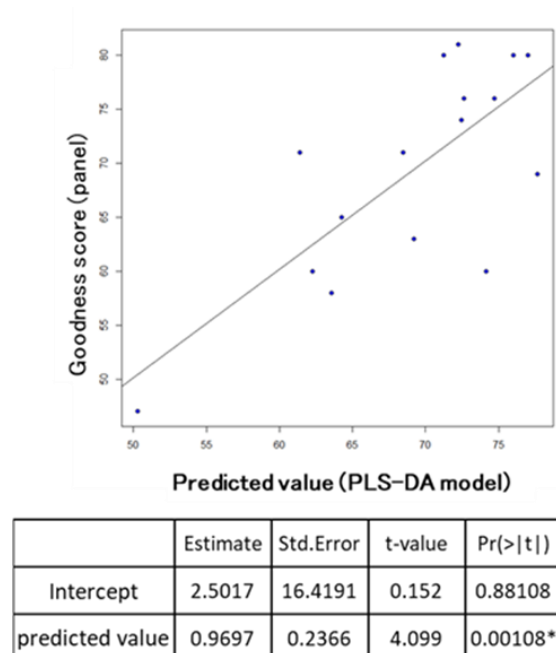


Fig 9. Correlation ($r = 0.74$) of predicted goodness score (x axis) based on entire NMR spectra of roasted coffee beans and goodness score expressed by the panel test. Each point in the plots represents a certain cultivar calculated by the mean of the 7 spectra analyzed for each distinct producer.

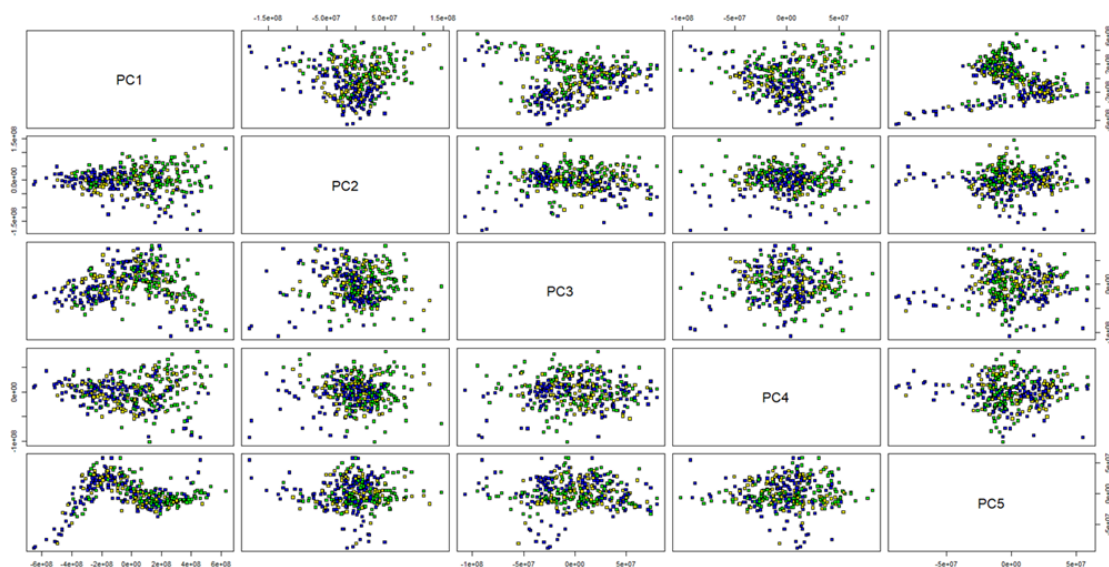
Supplementary Information

Supplementary Table 1. Characteristics of the 51 coffee batches.

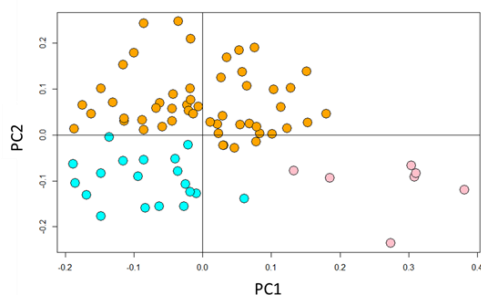
	City hall	Borough	Estate	Altitude (meters)	Variety	Fermentation (hours)	Drying (days)	Roasted
1	Guadalupe	Filo Salazar	La Argentina	1628	Caturra-Castillo	18	15	
2	Pital	Santa Rosa	La Batalla	1800	Caturra-Castillo	22	22	yes
3	Guadalupe	La Australia	La Esperanza	1489	Caturra-Castillo	16	12	
4	Pital	El Recreo	El Guayabo	1800	Caturra	22	20	yes
5	Gigante	La Pradera	La Esmeralda	1629	Caturra	18	15	
6	Tarqui	Quebraditas	Laureles	1800	Caturra	20	18	yes
7	Pital	Las Minas	Las Brisas	1750	Caturra	20	18	
8	Guadalupe	La Australia	Bella Vista	1411	Caturra-Colombia	18	15	
9	Pital	La Florida	Bella Vista	1900	Caturra	22	20	yes
10	Pital	El Recreo	Villa Milena	1760	Caturra	20	15	
11	Garzòn	El Socorro	La Esperanza	1835	Caturra	20	20	
12	Pital	Las Minas	El Progreso	1900	Caturra	22	20	yes
13	Suaza	Bajo Horizonte	El Tesoro	NA	Caturra-Castillo	20	18	yes
14	Guadalupe	Alto Meson	Primavera	1598	Caturra-Castillo	17	14	
15	Pital	El Vegòn	Tamanà	1600	Caturra	22	20	yes
16	Guadalupe	La Bernarda	El Higueron	1700	Castillo-Colombia	18	15	

17	Guadalupe	La Danta	El Rubi	1270	Castillo-Colombia	13	12	
18	Suaza	Campo Hermoso	La Esperanza	1700	Caturra	20	18	yes
19	Pital	El Recreo	La Mireya	1800	Castillo	22	20	
20	Garzòn	La Pita	La Esperanza	1500	Castillo	36	30	
21	Pital	El Recreo	La Esperanza	1900	Castillo-Caturra	22	20	yes
22	Guadalupe	La Miguela	Aguasa Claras	1600	Caturra	20	18	
23	Pital	El Recreo	La Victoria	1850	Caturra	22	20	
24	Pital	Santa Rosa	La Mirada	1600	Caturra	18	15	yes
25	Garzòn	Santa Marta	Villa Adriana	1750	Castillo-Colombia-Caturra	24	18	
26	Gigante	El Salado	Villa Daniela	1478	Castillo	16	15	
27	Suaza	Fatima	Los Pinos	1600	Caturra	18	18	
28	Suaza	Alto Horizonte	Primavera	1700	Caturra	20	18	yes
29	Guadalupe	Alto Meson	El Cachingo	1973	Caturra-Castillo	22	20	
30	Suaza	Divino Niño	La Esmeralda	1573	Colombia	20	18	
31	Guadalupe	Potrerillos	El Morro	1705	Castillo-Caturra	18	15	
32	Guadalupe	Filo de Salazar	El Descanso	1630	Castillo	18	15	
33	Pital	El Socorro	La Montaña	1700	Caturra	20	18	
34	Pital	Capoalegre	La Unión	1800	Caturra	22	20	yes
35	Guadalupe	Las Palmeras	Canova	1347	Caturra-Castillo	18	15	
36	Pital	El Amparo	La Esperanza	1600	Caturra	18	15	

37	Guadalupe	La Australia	La Trinidad	1793	Caturra	20	18	
38	Suaza	Bajo Horizonte	El Triunfo	1573	Caturra-Colombia	20	18	yes
39	Pital	Minas	El Higuero	1700	Caturra	18	15	
40	Tarqui	Tablon de Bèlgica	Los Altares	1600	Caturra	18	15	
41	Pital	La indipendencia	Porvenir	1750	Caturra	18	15	yes
42	Garzòn	Panorama	La Meseta	1700	Castillo	22	20	
43	Garzòn	Panorama	Buena Vista	1600	Caturra	18	15	
44	Pital	El Recreo	Santa Clara	1800	Caturra	20	18	yes
45	Guadalupe	El Carmen	El Caimo	1554	Caturra-Castillo	17	14	
46	Suaza	Fatima	El Mirador	1600	Caturra-Colombia	18	15	
47	Guadalupe	El Triunfo	La Villa	1789	Caturra	20	18	yes
48	Guadalupe	Alto Meson	El Meson	1895	Caturra-Castillo	22	20	
49	Guadalupe	La Australia	La Esperanza	1471	Caturra-Colombia	18	15	
50	Guadalupe	Las Palmeras	Palmeras	1982	Caturra	24	20	yes
51	Agrado	El Carmen	La Esperanza	1735	Caturra	20	18	yes



Supplementary Figure 1. PCA analysis on entire green coffee beans spectra: in the score plots are shown the first 5 principal components. Each plot is seen as a transformation in a two-dimensional space where the two components are the new coordinate system. Each dot represents a single NMR spectrum of 7 grinded green coffee beans of a batch/farm. Three groups have been created as following by dividing samples in tertiles according to the altitude (m) of each cultivar: group 1 ($n = 133$, blue) ≤ 1600 m; $1600 \text{ m} < \text{group 2} < 1750$ m ($n = 84$, yellow); group 3 ($n = 140$, green) ≥ 1750 m.

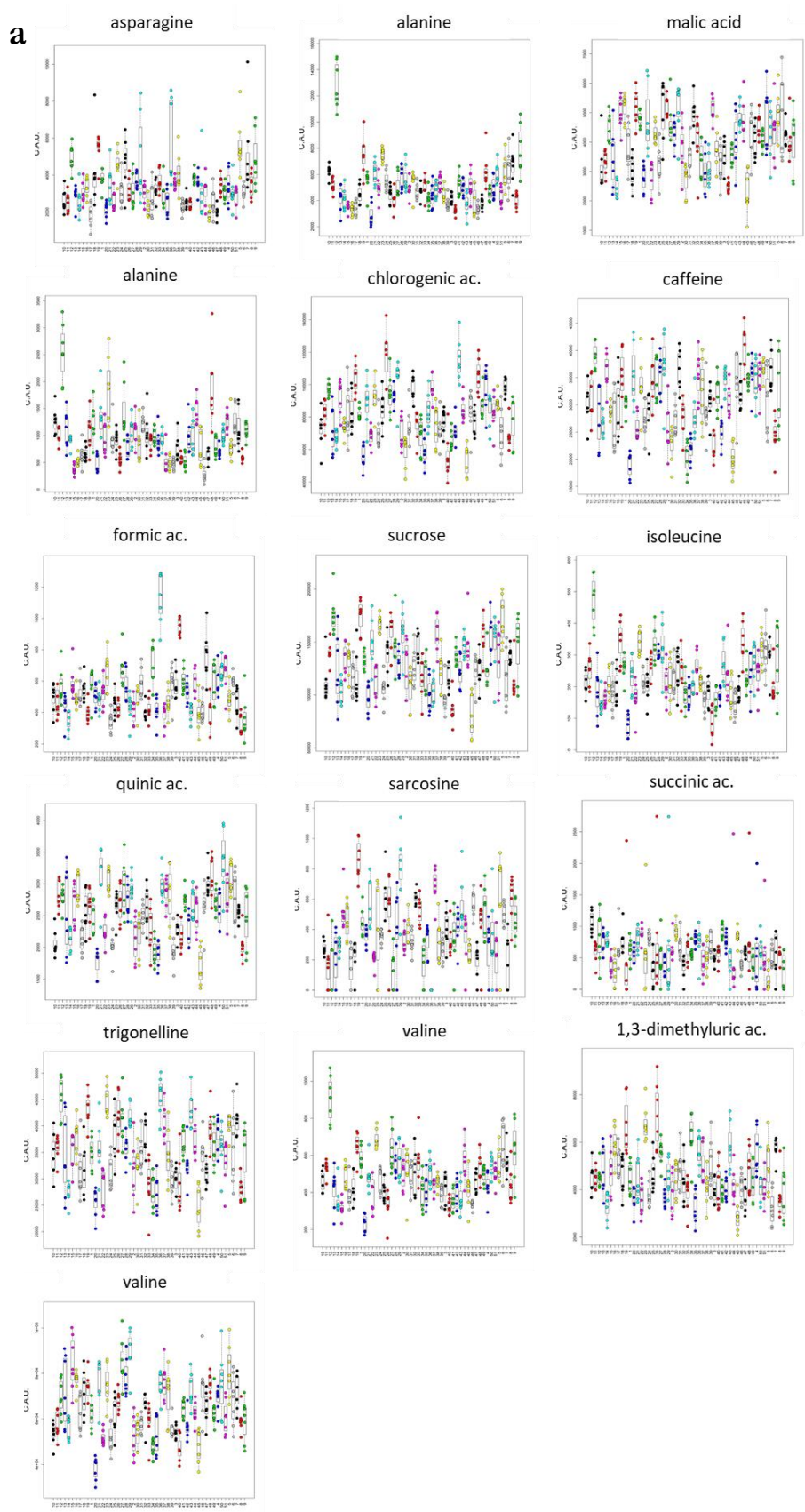


Confusion matrix

	1	2	3
1	85.4	12.0	2.7
2	21.2	78.8	0.0
3	12.5	0.0	87.5

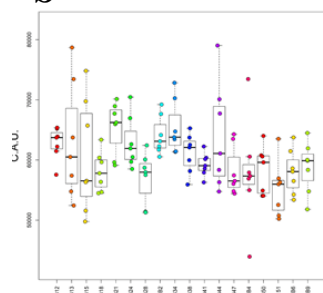
Predictive accuracy: 84%

Supplementary Figure 2. OPLS-DA on randomly selected green coffee beans showing three groups divided on the basis of the three monovarietal batches present: group 1 (orange), Caturra; group 2 (cyan), Castillo; group 3 (pink), Colombia. The cross-validated confusion matrix and prediction accuracy are reported.

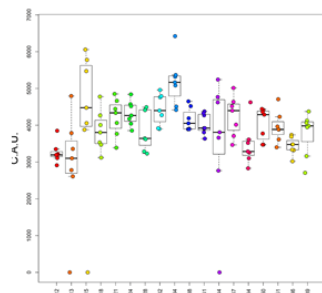


b

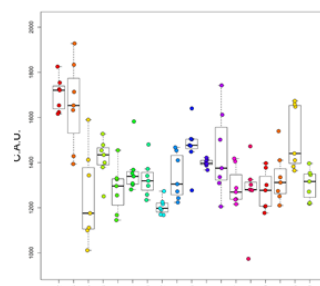
glycolate



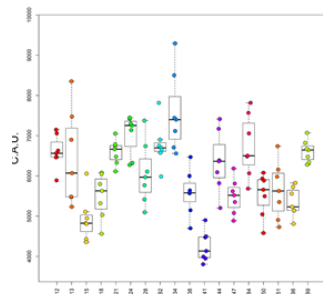
chlorogenic ac.



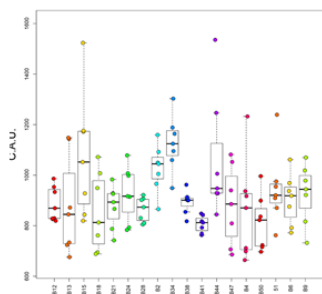
Gamma-butyrolactone



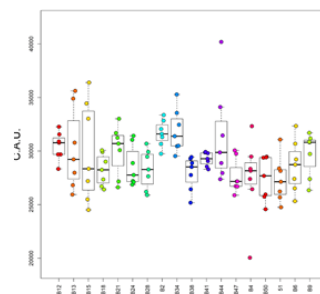
myo-inositol



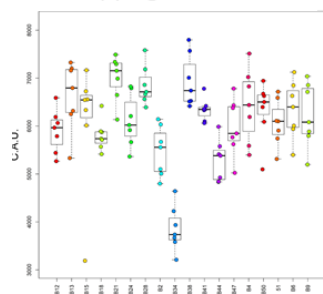
malate



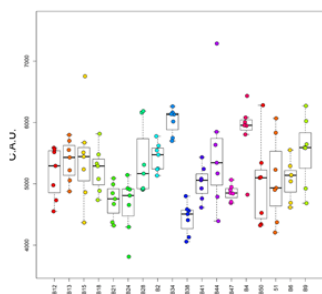
lactate



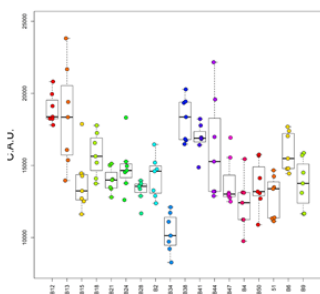
pyroglutamate



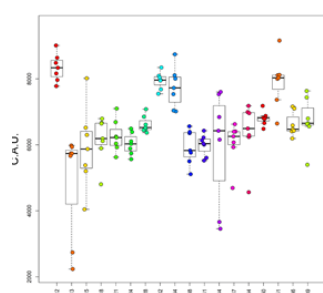
propionate



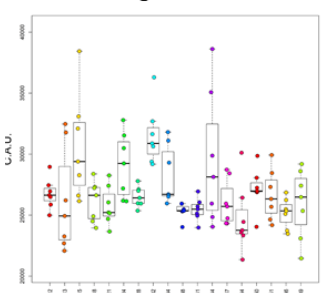
N-methyl pyridinium



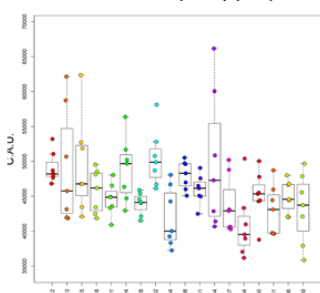
Unknown (9.5 ppm)



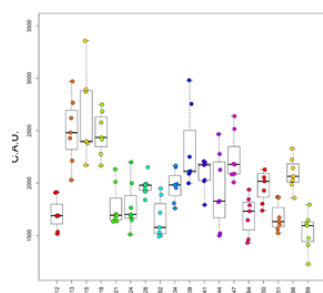
trigonelline



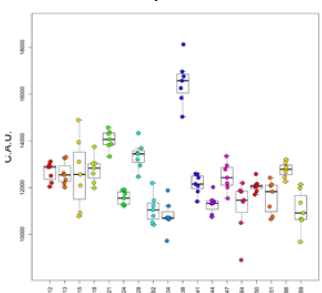
Unknown (8.1 ppm)



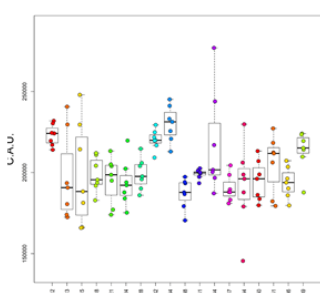
16-O-methylcafestol

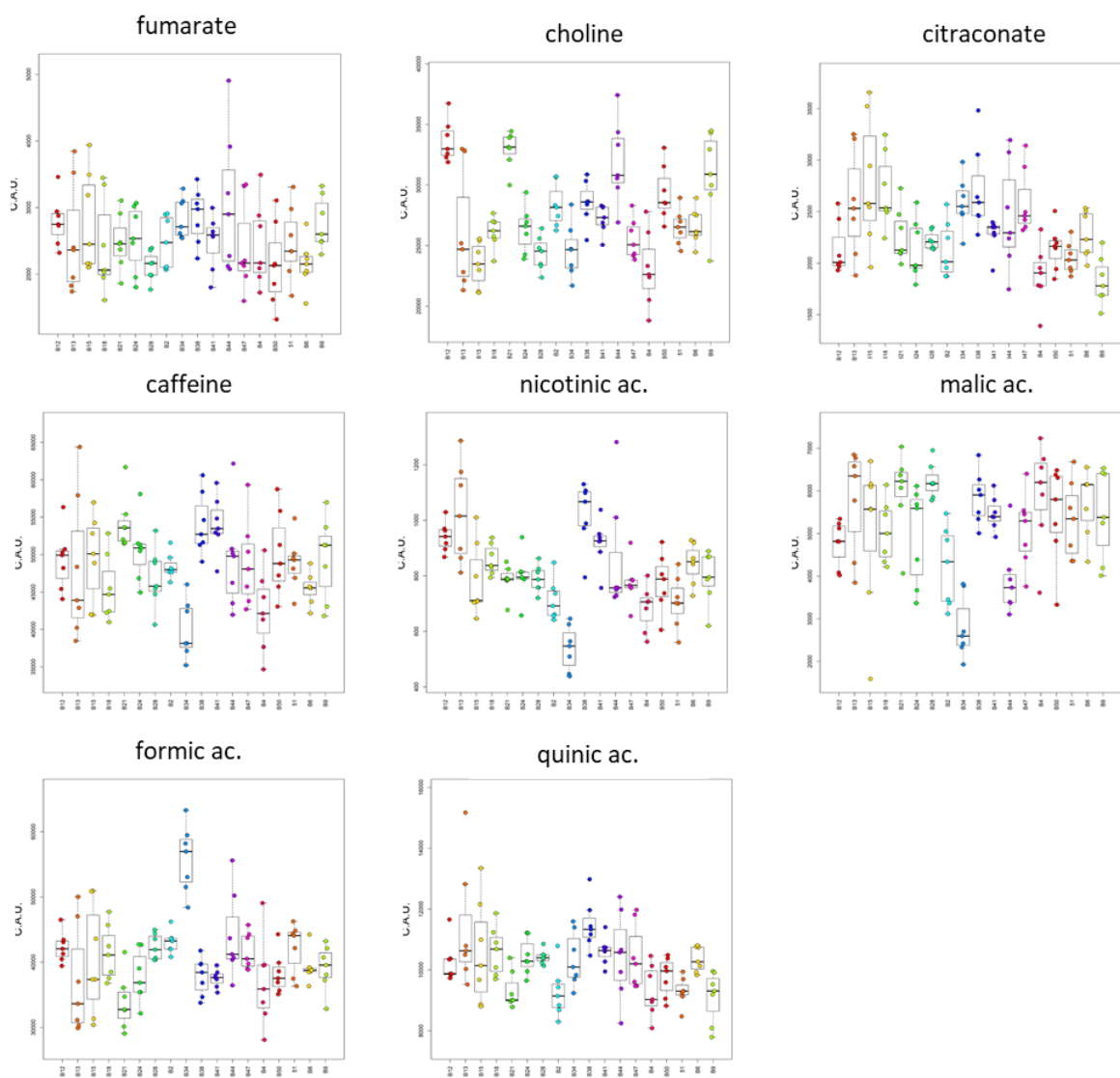


Furfuryl-methanol



acetic ac.





Supplementary Figure 3. Concentration levels (C.A.U = Concentration reported in arbitrary units, refers to metabolite concentration calculated by peak/NMR signal integration) of metabolites in green (a) and black (b) Colombian coffee among different producers; a) boxplots from left to right :producer n°10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 2, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 3, 40, 41, 41, 43, 44, 45, 46, 47, 48, 49, 4, 50, 51, 5, 6, 7, 8, 9; b) boxplots from left to right :producer n°12, 13, 15, 18, 21, 24, 28, 2, 34, 38, 41, 44, 47, 4, 50, 51, 6, 9.

Supplementary Table 2. Statistically significant correlations ($p < 0.001$ in green batches; $p < 0.05$ in black batches) between metabolites' concentrations and coffee information.

<i>P</i>	<0.001		<i>P</i>	<0.05	
metabolites	Var2	r	metabolites	Var2	r
alanine	Altitude (m)	0.55	trigonelline	bitterness intensity	-0.5631964

caffeine	Altitude (m)	0.45	formic ac.	bitterness intensity	-0.5506525
sucrose	Altitude (m)	0.53	maleate	bitterness intensity	-0.5835488
quinic acid	Altitude (m)	0.51	furfuryl-methanol	acidity intensity	-0.5150787
valine	Altitude (m)	0.51	nicotinic ac.	acidity score	-0.5153189
isoleucine	Altitude (m)	0.50	malic ac.	Finesse	-0.5146894
gaba	Altitude (m)	0.50	caffeine	Corposity	0.5045952
			fumarate	Corposity	0.554102
			glycolate	Corposity	0.5353483
			pyroglutamate	bitterness intensity	0.5049162
			trigonelline	bitterness score	0.5145393
			fumarate	bitterness score	0.5268139
			glycolate	bitterness score	0.5062552
			acetic ac.	bitterness score	0.5365232
			lactate	bitterness score	0.5697828
			myo-inositol	acidity intensity	0.5614976
			acetic ac.	acidity intensity	0.5209357
			unknown (9.30)	acidity score	0.6845426
			fumarate	sweetness score	0.5721654
			maleate	sweetness score	0.561329
			glycolate	sweetness score	0.5032284
			lactate	sweetness score	0.5446557
			trigonelline	Aromas	0.5411903
			maleate	Aromas	0.5502545
			glycolate	Aromas	0.5629667

acetic ac.	Aromas	0.6596523
Lactate	Aromas	0.6821937
Trigonelline	Finesse	0.5920886
Fumarate	Finesse	0.5030731
Maleate	Finesse	0.59021
Glycolate	Finesse	0.5467755
acetic ac.	Finesse	0.6680801
Lactate	Finesse	0.6546978
Fumarate	goodness score	0.5014215
acetic ac.	goodness score	0.5446621
Lactate	goodness score	0.5655648

Supplementary Table 3. Olfactory and gustative scores given by panel test (Sandaly Trading Company) to the 18 distinct batches of Colombian roasted coffees. Negative values represent a high score of astringency and defects.

Prod ucer	Olfactory finesse	Corp osity	Bitterness intensity	Bitterness score	Acidity intensity	Acidity score	Sweetness intensity	Sweetnes s score	Aro mas	Defe cts	Astring ency	Persist ence	Fine sse	Goodness score
12	80	75	4	85	4	80	3	80	78	0	0	4	82	80
13	77	78	4	79	4	3.5	2	78	72	0	0	4.5	73	76
15	76	75	3	85	4	79	4	82	76	0	0	4.5	79	80
18	75	66	3	71	1.5	55	2	60	60	0	0	3	60	65
21	76	76	4	74	3	70	3	65	69	-20	0	4	66	69
24	76	75	3.5	78	4	74	3	75	72	0	0	3.5	73	76
28	58	55	3	56	2	55	2	53	55	0	-10	3	56	58
2	79	76	2	83	5	84	2	80	80	0	0	3	81	80
34	80	75	2.5	80	5	85	3	81	80	0	0	5	83	81
38	75	76	4	70	2	66	4	75	60	0	0	4	65	71
41	72	76	4	80	3	66	3	64	66	0	-10	4	66	71
44	60	73	4	70	2	55	2	60	60	-20	-10	3	63	63
47	59	67	4	51	2	50	1.5	55	53	-50	-40	4	50	47
4	62	60	4	60	4	61	2	55	55	0	0	4	50	60

6	60	60	4	56	1.5	52	3	61	59	0	-10	3	58	60
9	75	78	4	75	4	75	3	74	75	0	-10	0	71	74
50	78	70	5	69	2.5	63	2	55	60	-20	-10	4.5	62	63
51	78	81	4	81	3.5	76	3	75	71	0	0	4	76	77

Chapter 6

Conclusions

The results achieved during these three years of Ph.D. activity and reported in this thesis have allowed me to further examine the potential of the untargeted NMR-based metabolomic approach in different fields, ranging from biomedicine to food research.

Metabolomics has proven itself an optimal tool for biological systems characterization, enables a deep comprehension of environmental effects, physiological status, and is helpful in monitoring medical treatments for *in vivo* and *in vitro* models. Our results show how metabolomics can lead to design new non-invasive, rapid, economic, and sensitive diagnostic tests, useful also for prognostic purposes.

In particular, a metabolomic approach has proved to be an effective tool to predict variations of response to treatment. Collecting multiple samples for each individual at different time points represents an important potential for such studies, enabling to eliminate inter-individual variations and making a more accurate picture of the treatment effect on the population studied. Significant variations have been detected for the first time within the serum metabolic profile of HCV infected patients successfully undergoing DAAs therapy, as well as in the saliva of patients with periodontitis undergoing non-surgical therapy.

Concentrated metabolomics efforts at specific time points, focusing on immediate molecular changes induced by the causative factor, are indeed expected to produce sufficient insight to promote understanding of therapeutic responses, but could also provide pivotal information for patient management. Monitoring patient profile is more and more advocated for omics studies. From this point of view, saliva, being an easily accessible, non-invasive biofluid and more stable if compared to blood and urine, could represent a great potential for such approach.

Indeed, we have seen that using repeated follow-up samples not only provides a less noisy diagnosis model by eliminating inter-individual variation, but also enables dynamic evolution in time of patients, allowing us to trace the path of each individual from healthiness to pathology or *vice versa*.

This dynamic evolution could be even better corroborated by collecting data also before the onset of the disease, *i.e.* under epidemiological studies.

The individual metabolic fingerprint contains information that can be regarded as informative of the individual biological age, since genetics as well as environment are essential in defining it. There is a growing awareness within the scientific community that age-related diseases could be considered manifestations of the acceleration of ageing. We think that untargeted metabolomics could nowadays provide one of the best choices to identify specific metabolic perturbations that are responsible for the deviation from the trajectories of healthy ageing. These signatures could also contribute to better understanding of Parkinson's disease *vs.* a healthy ageing status and could help in defining new potential targets for PD. The preliminary results obtained could be considered really promising, since they corroborate the presence of oxidative stress in PD patients for the first time by analyzing a large cohort of *de novo* PD patients by means of an NMR based metabolomic approach. Moreover, we have also obtained the first evidence of PD signature in urine through NMR based metabolomics.

However, these results will be soon validated with other matched cohorts of patients.

In general, omics approaches to diseases are mostly comparative. Indeed, data from healthy and diseased individuals are compared and the difference is assumed to be directly related to disease.

However, in complex phenotypes both “healthy” and “disease” groups are heterogeneous with respect to many confounding factors such as population structure, batch effects and many other unknown factors. Thus, a crucial aspect for the success of metabolomics, as well as of all the other omic studies, is the collection of large population and datasets that accurately capture sources of variance in the background community.

One of the most important challenges of these studies will be also to integrate metabolomics data collected with those from other -omics (proteomics, transcriptomics, and genomics); surely this would be the best way to provide a holistic perspective on complex interactions occurring in biological systems and will improve the understanding of the implied metabolic pathways.

In this thesis, NMR based fingerprinting has proven an efficient diagnostic tool to assess hepatitis A and B, Parkinson’s disease, periodontitis in human patients and sepsis in newborn calves. Especially in this last case, NMR based metabolomics has an advantage also over the standard laboratory techniques for sepsis diagnosis, that instead are time consuming and seem not to be sufficiently specific.

It is increasingly difficult to find a specific single biomarker of a pathological condition, in general because pathological conditions can be variable according to different immunological responses or to different degrees of aggressiveness of the disease. As it appears in the case of lack of molecular diversification of GCP (Generalized Chronic Periodontitis) and AgP (Aggressive Periodontitis), it could be plausible that maybe it is not always correct to assume that patients can be subdivided into only two diagnosis groups when actually the disease diversification could be much more heterogeneous. In this regard it could be interesting to evaluate the metabolomic profile of patients with their immunological profile.

This thesis also corroborates the growing evidence that the entire metabolomic profile, the fingerprint, better describes, in all the mentioned cases, the pathological status, if compared to the analyses based on the identified metabolites. This fact supports the growing idea that the most reliable biomarker is actually to be found in a modulation of several alterations that are identifiable considering the NMR spectra in its entirety.

For this kind of studies, the individual fingerprint could represent the best choice to describe the global response resulting from the sum genetics, infection, individual immune response, *etc.* Integration of metabolomic-based diagnostic principles will for example likely become an essential tool for the development of personalized medicine for decision-making diseases management.

Metabolomics has proven also a useful tool for the characterization of *in vitro* models. Cultured cell metabolomics, indeed, has offered an important opportunity to characterize for the first time the metabolic profile of a heterogeneous group of breast cancer cells resistant to palbociclib by identifying the existence of a common metabolic drift driven by the development of the resistance. Moreover, this untargeted approach has suggested the need of deeper investigations on choline metabolism regarding its role on palbociclib response and tumour progression. Understanding how genes and proteins regulate the levels of GPC (glycerophosphocholine) during treatment and at the time of resistance might further clarify our observations and elucidate the role of GPC in palbociclib resistance. Certainly, NMR based metabolomics is not the best technique to perform targeted studies, however, since it offers the possibility to visualize a broad panel of molecules, it could provide the first view on the group of metabolites to be interrogated by a targeted approach. However, one of the main limits of cultured cell metabolomics remain translating the findings into patient’s samples.

Despite the importance of the collected data, the majority of these studies, as most metabolomic researches in the literature, are small scale studies, in which the number of samples ranges from

tens to hundreds. Overcoming this limitation by much larger studies, covering the analysis of tens of thousands of specimens, represents the next challenge for metabolomics and is expected to provide solid bases for the use of metabolomics for large population screening.

Metabolomics and the increasing popularity of lifestyle tracking devices and social media has created an unprecedented opportunity for studying environmental factors that contribute to disease development and progression on a large scale, and further integration with other omics data may provide additional guidance for personalization of treatment of the future.

Another important chapter of this thesis is dedicated to NMR metabolomics in food research. One of the goals has been the definition of some peculiar metabolic profiles present in olive fruitlets and olive oil able to provide us with some useful information to improve the quantity and quality of the final product. Hence also the advantage to use NMR to assess the relationship between organoleptic properties, resulting from panel test analysis, and chemical composition of food matrices. Indeed, NMR spectroscopy is to be considered a kind of “magnetic tongue” for correlation and prediction of the metabolic profile with food taste.

Another important topic of this thesis, which I believe helps to highlight the potential of the untargeted NMR approach, is food traceability.

This thesis contributes for the first time to define and characterize milk and coffee samples (green and roasted beans) from small farm localized in the same geographic area, resulting in the definition of their precise provenance given by their fingerprint occupying a precise “metabolic space”, where samples from each distinct farm can be discriminated from the others with optimal accuracies. The NMR fingerprinting technique coupled with advanced statistics has demonstrated to be an excellent tool especially in characterizing food samples of different producers even when located in the same geographical area. Farms are defined by particular environmental condition, soil, feeding, pre- and post-harvest manipulation that influence and control livestock as well as plantation (*i.e.* cows and coffee plants) development and their derivatives. These factors, although not easy to be singularly isolated, are present in their NMR spectra and significantly contribute to their fingerprint. Preliminary results have provided a successful correlation between the goodness score evaluated by the panel test and green coffee fingerprint. Taken together, these results corroborate the idea that considering a well-structured samples population, NMR metabolomics provides a really unique tool, particularly useful for building traceability and quality food models. These potentials are in line with the growing request to develop new methods for the detection of food adulterants and the integration of new approaches as routine methods in official authorities. This is necessary because such scientific questions usually require complex and demanding laboratory studies and leads to the identification of food fraud at the center of the new control strategy.

Even if the published material that constitute this Ph.D thesis may look very heterogenous, as it tackles very diverse research areas ranging from biomedicine to food research, I hope it contributes significantly to the demonstration that NMR-based metabolomics, coupled with complex statistical analyses, can be considered as a whole as a “universal” quantitative analytical technique.

Offering an unbiased and untargeted view of samples composition and the ability to simply quantify multiple compounds simultaneously, could be considered a very powerful choice for diagnosis studies as well as for the characterization and quality control of complex biological/natural samples such as biofluids, foods, plants and cellular extracts.

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Acknowledgments

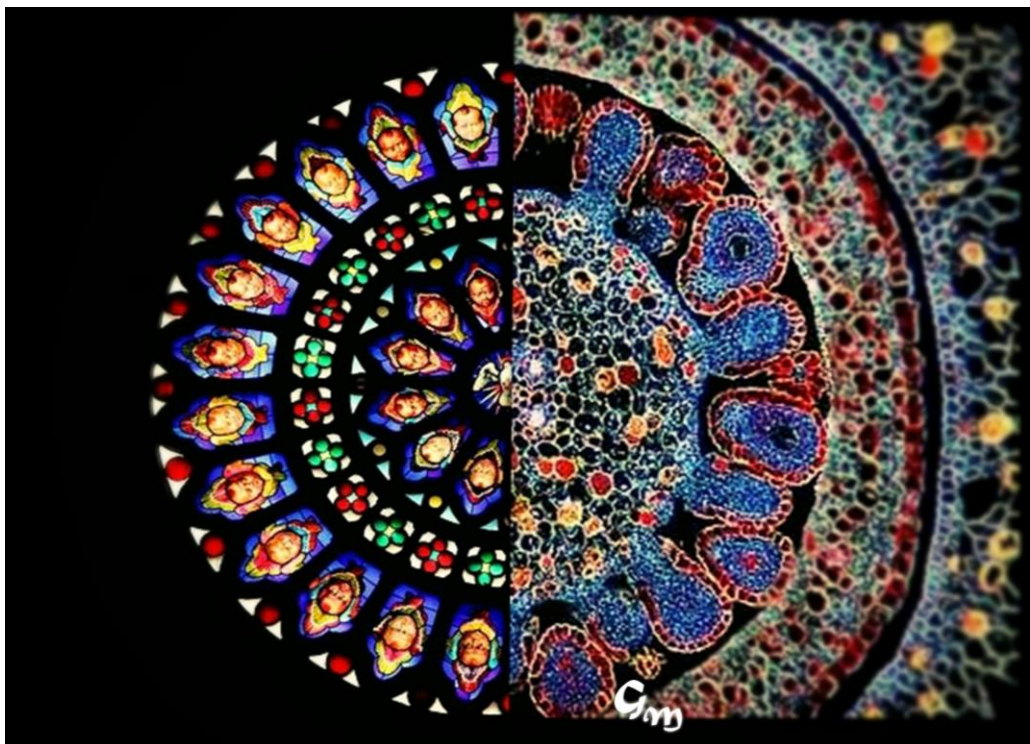
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Art & Science: rosette of the Todi Cathedral / section of flower ovary by Ray Nelson; picture of Gaia Meoni.