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TESIS DOCTORAL

Metabolomic-driven evaluation of processed onion in the prevention of cardiovascular and liver disease in a model of diet-induced hypercholesterolemia

Evaluación metabolómica del efecto de cebolla procesada en la prevención de enfermedad cardiovascular y hepática en un modelo de hispercolesterolemia inducida por la dieta

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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UNIVERSIDAD COMPLUTENSE DE MADRID
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EVALUACIÓN METABOLÓMICA DEL EFECTO DE CEBOLLA PROCESADA EN LA PREVENCIÓN DE ENFERMEDAD CARDIOVASCULAR Y HEPÁTICA EN UN MODELO DE HIPERCOLESTEROLEMIA INDUCIDA POR LA DIETA

European Doctoral dissertation presented by:

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Las Dras. Concepción Sánchez-Moreno González y Begoña de Ancos Siguero, ambas Investigadores Científicos del Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), perteneciente al Consejo Superior de Investigaciones Científicas (CSIC).

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Madrid, 15 de Marzo de 2017

UNIVERSIDAD COMPLUTENSE DE MADRID
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EUROPEAN DOCTORAL THESIS

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CSIC Consejo Superior de Investigaciones Científicas
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INSTITUTO DE CIENCIA Y TECNOLOGÍA DE ALIMENTOS Y NUTRICIÓN

To my family and friends,

unconditional support and peers of experiences

To my nephews,

my brigthest light and passion in life

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Y llegó el momento de escribir ésta, la última página de un proyecto, pero la primera de este libro y de la nueva etapa que comienza con él. No hay manera de saber con certeza qué camino será el que nos conduzca a alcanzar nuestros objetivos, pero hay algo que tengo claro: no importa lo duro que sea el viaje si aprendes a compartirlo. Sobre todo, si consigues disfrutar de la gente que te acompaña en él. No hay esfuerzo que no tenga recompensa, y es por ello, que escribo orgullosa cuál es hasta hoy la mía: <vosotros>.

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Call me crazy… but
I love to see people happy and succeeding.
Life is a journey, not a competition
Unknown Author

Madrid, 29 de Marzo de 2017
The G.P.–D?.

PREFACE

This PhD Thesis has been conducted at the Department of Characterization, Quality and Safety at the Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), Madrid (Spain), as part of the team "Quality and Functionality of Plant Foods" (BIOACTIVEG) under the supervision of Dr. Concepción Sánchez-Moreno González and Dr. Begoña de Ancos Siguero.

During the PhD Thesis period a close collaboration was established with three outstanding research groups, where several parts of the investigation where undertaken:

- The Vascular Pharmacology and Metabolism (FARMAVASM) research group at the Department of Pharmacology, Faculty of Medicine, Autonomous University of Madrid (Madrid, Spain), lead by Dr. Concepción Peiró and Dr. Carlos F. Sánchez-Ferrer.
- The Center for Metabolomics and Bioanalysis (CEMBIO) at the Faculty of Pharmacy, San Pablo CEU University (Madrid, Spain), where I spent one valuable year and several work-visits under the supervision of Dr. Coral Barbas and the collaboration of her research team, with a special attention from Dr. Antonia García and Dr. Danuta Duzkit.
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Diana González Peña

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SUMMARY

The consumption of functional foods offers potential in the prevention and management of prevalent and worldwide diseases of concern. The known health-related properties of onion have revealed its promising value in the prevention of risk factors and reduction of symptoms associated to several diseases, including cardiovascular disease (CVD) and liver disease. This motivates the design of commercially stable onion products with a high content of phytochemicals, which includes those processed by non-thermal technologies, such as high-pressure processing. However, the examination of those effects *in vivo* requires both the understanding of individual pathologies and the use of new high-throughput analytical tools able to provide solid scientific evidence. In this sense, metabolomics allows for phenotype-characterization of live systems or organisms under specific conditions, where the effect of the diet can also be linked to specific metabolic patterns associated to either health status or disease evolution. The metabolome, understood as the metabolic result of a complex network of dynamic interconnected pathways, reflects the end point of gen-transcription-protein-metabolite interactions and allows the traceability of the changes induced by both epigenetic stimuli and adaptive responses to disease development. These responses can be modulated to a big extent by medical treatments and/or diet interventions.

In this context, the main purpose of this PhD Thesis was to study the potential preventive effect of high-pressure processed onion in cardiovascular and liver diseases from an innovative perspective. The assessment of biological functions and mapping of the metabolome in a model of diet-induced hypercholesterolemia has driven the research and motivated an interest to explore new approaches involving the mechanism of action and elucidate the metabolic pathways impaired by nutritional imbalance. This would contribute to generate novel hypothesis about the mechanisms of action behind the consumption of onion at the core of prevalent diseases and raise awareness about the importance of including complementary approaches that allow data integration in the experimental design. Moreover, the need to address different analytical platforms to improve the understanding of diet and supplementation in the differential phases of disease progression is discussed throughout this thesis as a sound basis to redirect future research of nutrition within integrative system biology.

Therefore, to achieve this objective, the PhD Thesis has been divided into three sections organised according to the sequence of research, the analytical methodology and the metabolomic strategy followed:

i) **Biochemical, vascular and microbiological analyses** to determine vascular and hepatic impairment and the preventive role of onion on well-known biomarkers.

ii) **Non-targeted metabolomics studies (fingerprinting)** describing metabolic impairments associated to hypercholesterolemia and potential modulating effects of onion.

iii) **Targeted metabolomics studies (profiling)** addressing specific pathways of interest.

Whereas the first section has mainly addressed oxidative stress, vascular reactivity and specific markers of hepatic and CVD functions, the second has focused on the metabolic modifications induced by a high-cholesterol diet and the prevention obtained by the onion ingredient included in the diet. This work was undertaken by a multiplatform of metabolic fingerprinting accomplished by LC–MS, CE–MS and GC–MS.

In the third section relevant pathways potentially involved in inflammation and assimilation of nutrients, also indicated by the non-targeted approaches (lipid mediators and bile acids) were selected and analysed by LC–MS targeted approaches in different biological samples (plasma, tissues–liver, spleen, heart– and faecal material). Each section is embodied by the research papers compiling these studies, classified in chapters with one additional chapter, which integrates the results from all the experiments into a comprehensive discussion.

In conclusion, the current PhD Thesis highlights the benefits of the consumption of functional ingredients such as high-pressurized onion in the two prevalent pathologies studied (CVD and liver disease). It makes a strong emphasis on the use of metabolomics, which is pivotal to the research and evidences the advantages and promising aspects of the application of metabolomics to the understanding of the close relationship between diet and disease. It opens new perspectives to manage health status by tackling risk factors such as inflammation and other impairments associated to disease through the inclusion of functional ingredients in the diet. Furthermore, it reinforces the path toward the use of new tools of diagnosis and prognosis of disease with predictive analysis towards an individual nutritional assessment designed to prevent risk and achieve an optimal personalised nutrition.

RESUMEN

El consumo de alimentos funcionales ofrece nuevas posibilidades de estudio dirigidas a la prevención y el tratamiento de enfermedades altamente prevalentes en la población a nivel mundial. Las propiedades beneficiosas de la cebolla en la salud han mostrado potencial en la prevención de factores de riesgo y la reducción de los síntomas asociados a múltiples enfermedades, entre las que se incluyen las enfermedades cardiovasculares y hepáticas. Estas circunstancias motivan el diseño de productos de cebolla que sean comercialmente estables y con un elevado contenido en sustancias fitoquímicas, destacando para ello, la aplicación de tecnologías de procesado no térmico y más concretamente el procesado por alta presión hidrostática. No obstante, el análisis de los efectos de la cebolla *in vivo* requiere entender cada patología a nivel individual y el uso de tecnologías analíticas de alto rendimiento, capaces de proporcionar sólidas evidencias científicas. En este sentido, las técnicas metabolómicas permiten la caracterización fenotípica de sistemas vivos u organismos, bajo condiciones específicas, permitiendo así la asociación entre el efecto de la dieta con perfiles metabólicos específicos, asociados a un estado concreto de salud o cierta fase en la evolución de una enfermedad.

El metaboloma, entendido como el resultado metabólico de un complejo entramado de rutas metabólicas interconectadas, refleja el punto final de las interacciones ocurridas entre los genes, la transcripción, la formación de proteínas y metabolitos. Este conocimiento facilita la trazabilidad de los cambios inducidos por los estímulos epigenéticos y las respuestas adaptativas en el desarrollo de cualquier enfermedad, permitiendo además considerar el grado de modulación de las respuestas fisiológicas ante tratamientos médicos combinados o no con intervenciones dietéticas.

En este contexto, el objetivo principal de la presente Tesis Doctoral fue el estudio del posible efecto preventivo de un ingrediente de cebolla procesada por alta presión hidrostática en enfermedades cardiovasculares y hepáticas desde un punto de vista innovador. La evaluación de las funciones biológicas y el mapeado de los cambios inducidos en el metaboloma de un modelo animal de hipercolesterolemia inducida por la dieta han sido los temas principales de la investigación realizada en esta Tesis, explorarando nuevos enfoques sobre los cambios y alteraciones inducidas en rutas metabólicas a través de la dieta, así como en los posibles mecanismos de acción involucrados. Este enfoque facilitará la formulación de nuevas hipótesis sobre los mecanismos de acción involucrados en los efectos derivados del consumo de cebolla

en la prevención y evolución de enfermedades altamente prevalentes. Además, los resultados obtenidos demuestran la necesidad e importancia de integrar simultáneamente distintos enfoques experimentales, desde el diseño experimental hasta la posterior integración de los datos y su interpretación. A lo largo de esta Tesis se discute la necesidad de utilizar diferentes plataformas analíticas para mejorar el conocimiento sobre el efecto de una dieta y la suplementación en diferentes fases de la intervención y de la progresión de una enfermedad, como las bases fundamentales para redirigir futuras investigaciones en nutrición hacia el estudio integral de sistemas biológicos.

Por lo tanto, con la finalidad de lograr dichos objetivos, la presente Tesis Doctoral ha sido estructurada en tres secciones, organizadas de acuerdo a la secuencia seguida para su realización, teniendo en cuenta la metodología analítica empleada y las estrategias analíticas aplicadas de acuerdo a las posibilidades que ofrece la metabolómica:

i) Análisis de carácter biológico/bioquímico, vascular y microbiológico para determinar disfunciones vasculares y hepáticas, así como el rol preventivo del ingrediente de cebolla en biomarcadores de función conocida.

ii) Estudios metabolómicos no dirigidos (fingerprinting) que describen alteraciones metabólicas asociadas a hipercolesterolemia y el potencial efecto modulador de la cebolla.

iii) Estudios metabolómicos dirigidos (profiling) enfocados a rutas metábolicas de interés específico.

Mientras que en la primera sección se evalúan estrés oxidativo, reactividad vascular y marcadores de función hepática y cardiovascular específicos, en la segunda sección el estudio se centra en las modificaciones metabólicas inducidas por una dieta rica en colesterol y el efecto preventivo ejercido por la incorporación simultánea del ingrediente de cebolla en la misma dieta. Para la realización de este trabajo se utilizó una multiplataforma de fingerprinting metabólico compuesta por tres técnicas diferentes de separación y análisis: cromatografía líquida (LC), electroforesis capilar (CE) y gases (GC), acopladas a espectrometría de masas (MS).

Así mismo, la tercera sección recoge los estudios realizados en rutas metabólicas involucradas en los procesos de inflamación y asimilación de nutrientes (mediadores lipídicos y ácidos biliares) que fueron seleccionadas con anterioridad en base a los resultados obtenidos en los análisis no dirigidos. Estos estudios fueron realizados en plataformas seleccionadas de

análisis dirigidos por cromatografía líquida acoplada en tándem a espectrometría de masas (LC–MS/MS), en diferentes muestras biológicas (plasma, tejidos –hígado, bazo, corazón– y contenido fecal).

Así, cada sección está constituida por una serie de artículos científicos que recogen dichos estudios, clasificados en capítulos y un capítulo final adicional que integra los resultados de todos los experimentos en una discusión global.

En conclusión, la presente Tesis Doctoral subraya los beneficios del consumo de ingredientes funcionales como es el ingrediente de cebolla procesada por alta presión descrito en esta memoria, en dos patologías prevalentes, enfermedad cardiovascular y hepática. Haciendo especial énfasis en el empleo de técnicas de metabolómica como pilar fundamental de la investigación realizada, evidenciando las ventajas y aspectos prometedores de la aplicación de técnicas metabolómicas para entender la estrecha relación entre la dieta y los diferentes estadios de una enfermedad. Dicha estrategia abre nuevas perspectivas para el mantenimiento de un estado de salud óptimo, abordando factores de riesgo como inflamación y otros desequilibrios asociados a la progresión de las enfermedades, a través de la incorporación de ingredientes funcionales en la dieta. Además, los resultados obtenidos en esta Tesis refuerzan el camino hacia el uso de nuevas herramientas de diagnóstico y pronóstico de enfermedades mediante la creación de modelos de análisis predictivo que se dirigen a posibilitar una evaluación nutricional individualizada para prevenir riesgos y alcanzar un estado nutricional personalizado óptimo.

Chapter 1 – General Introduction

1. NUTRACEUTICALS/FUNCTIONAL FOODS IN HEALTH AND DISEASE PREVENTION. THE POTENTIAL OF ONION

The interest in nutraceuticals and functional foods has become fashionable and is growing in popularity. The concept of "functional foods and nutraceuticals" has gained support and worldwide acceptance reinforcing the link between nutrition and health. These supplementary foods show promise in both the prevention and clinical therapy of diseases, as they have the potential to significantly reduce either the risk and symptoms of diseases or the side effects associated with treatments. An example of this is the etiology of cardiovascular or liver disease, which reveals many risk factors that may be alleviated by nutraceutical or functional food intervention. The benefits of these functional products have an impact not only on individual health, but also enhance the overall health of the general population offering possible reductions in the global cost of the health care system. Therefore, the ability of nutraceuticals/functional foods to have a positive influence on cardiovascular, hepatic and other risk factors needs to be recognized as an enormous opportunity in the health promotion and the disease prevention.

The progress in this field has been developed by research focusing on the identification of the chemical and biological properties that characterize functional substances. As a result, this will permit the development of potential applications to promote health and well-being in response to the interest and concerns of the general public as well as satisfying the demand of consumers.

In this area, many foods may attract the attention of scientists due to their potential properties on health. However, given the favourable conditions for the growth of some crops and the unique features of the Mediterranean diet in Spain, the functional properties of onion have been chosen, since it is cultivated on a large scale and used extensively as an ingredient in traditional cuisine.

In this sense, there are positive human health effects linked to the consumption of onion which are attributed to its bioactive composition. Both the individual bioactive components found in onion and the complex composition of the whole onion matrix with potential synergistic effects have a proven influence in multiple physiological mechanisms. Therefore, antioxidant, antiinflammatory, anticancer, antibacterial and a long etc. of other onion biological properties remain under exploration as potential regulators of the health status and the onset and progression of disease at both the *in vitro* and *in vivo* system levels of research.

1.1. Definition of terms: nutraceuticals or functional foods? – *Regulations for health claims related to functional foods in the EU Framework*

The concept of functional foods and nutraceuticals allows for different interpretations. The definitions have been transformed with time, depending on the point of view of the authors and the purpose and design of the new products. As a result, there is as yet, no universally accepted definition.

In general, a **functional food** can be defined as a food recognized to have one or more compounds with biochemical and physiological functions beneficial to human health. Whereas, a **nutraceutical** is considered a food, or part of a food, that provides medical or health benefits, including the prevention and/or treatment of a disease (Brower, 1998). Indeed, the latter term was coined from "nutrition" and "pharmaceutical" to encourage clinical research and trials aimed at examining the true health effects of these substances. However, this term which has also been commonly used in marketing, still lacks a firm regulatory definition; whereas the term "functional foods" may not be the ideal descriptor for this emerging food category, it has been recognized as the term more readily preferred by consumers in comparison with other terms such as "nutraceutical" or "designer foods" (IFIC, 2002).

Therefore, this widespread use and general acceptance of the term "functional foods" by the media, scientists, and consumers has led the major organizations to work within this framework rather than introduce a new, more descriptive term. However, several organizations have attempted to define this emerging food category. The position statements and definitions from the most relevant worldwide organizations have briefly been compiled (Figure 1.1).

According to all these declarations, it is necessary clarify that unmodified whole foods such as fruit and vegetables, including bulbs, represent the simplest form of functional food. For example, broccoli, carrots, tomatoes or onions would be considered functional foods because they are rich in physiologically active components such as sulforaphane, β-carotene, lycopene and flavonols, respectively. Modified foods, including those that have been fortified with nutrients or enhanced with phytochemicals also fall within the realm of functional foods. In the future, food biotechnology will continue to provide new venues for functional food development.

The European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) reflected on its consensus document *"Scientific Concepts of Functional Foods in Europe"* the following working definition: *"A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease."* Then, *"Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern."* (Diplock, 1999; Stein & Rodríguez-Cerezo, 2008).

The Academy of Nutrition and Dietetics presented its position by saying: "*although all foods provide some level of physiological function, the term functional foods is defined as whole foods along with fortified, enriched, or enhanced foods that have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis at effective levels based on significant standards of evidence."* Thus, *"The Academy supports Food and Drug Administration approved health claims on food labels when based on rigorous scientific substantiation.*" (Crowe & Francis, 2013).

The American Dietetic Association (ADA) stated: *"functional foods, including whole foods and fortified, enriched, or enhanced foods, have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis, at effective levels."* In this regard, *"The Association supports research to define further the health benefits and risks of individual functional foods and their physiologically active components. Dietetics professionals will continue to work with the food industry, the government, the scientific community, and the media to ensure that the public has accurate information regarding this emerging area of food and nutrition science."* (Hasler *et al*., 2004).

Information concerning the position adopted by *The International Food Information Council (IFIC), The International Life Sciences Institute of North America (ILSI), Health Canada, The Institute of Medicine of the National Academy of Sciences, Institute of Food Technologists, International Life Sciences Institute* and *The Japanese Ministry of Health, Labour, and Welfare,* can be found gathered in the documents cited above.

Figure 1.1. Position statement and "functional food" definitions from relevant worldwide organizations

Nowadays, the market offers an extensive range of functional foodstuff with multiple purposes in which health claims, along with other allegations, must be scientifically demonstrated. In Europe, the organization responsible for the regulation of nutritional claims and their conditions of use is the "European Food Safety Authority" (EFSA), which also acts as a feedback to motivate research. Thus, the EFSA is the organism that evaluates the safety of regulated food ingredients before they can be authorised for use on the European market.

The legal framework, which was founded on the basis established by FUFOSE and PASSCLAIM initiatives to underpin the criteria for the scientific substantiation of nutrition and health claims (Aggett *et al*., 2005; Diplock, 1999), has been established since December 2006, when the EU adopted the Regulation (EC) Nº 1924/2006 on nutrition and health claims made on foods. This regulation lays down EU-wide rules for the use of health or nutritional claims on foodstuffs based on their nutrient profiles. The regulation stated by the Commission Regulation (EC) N^{o} 353/2008 implements rules for the application for authorisation of health claims and the Commission Regulation N^{o} 1169/2009 amends the previous (EC) Nº 353/2008. Moreover, the list of permitted health claims established by the Commission Regulation (EU) N^{o} 432/2012 is regularly updated with newly authorised health claims subject to individual application submitted in pursuant of Article 13(5) of Regulation (EC) N^{o} 1924/2006, ensuring that any claim made on a food label in the EU is clear and substantiated by scientific evidence.

In this context, according to the European Commission, a **health claim** is any statement about a relationship between food and health. In more detail, it refers to any statement on labels, advertising or other forms of marketing claiming that health benefits may result from consuming a given food, such as claiming that a food product may help reinforce the body's natural defences or enhance learning abilities. It should be born in mind that health claims differ from **nutrition claims** which state or suggest that a food has beneficial nutritional properties (*e.g.* "low fat", "no added sugar" and "high in fibre").

The Health Claims are classified into the following types:

– 'Function Health Claims' (*Article 13*): *Health claims other than those referring to the reduction of disease risk a) relating to the growth, development and functions of the body; b) referring to psychological and behavioural functions and c) on slimming or weight-control.*

– 'Risk Reduction Claims' (*Article 14*) *on reducing a risk factor in the development of a disease. For example: "Plant stanol esters have been shown to reduce blood cholesterol. Blood cholesterol is a risk factor in the development of coronary heart disease".*

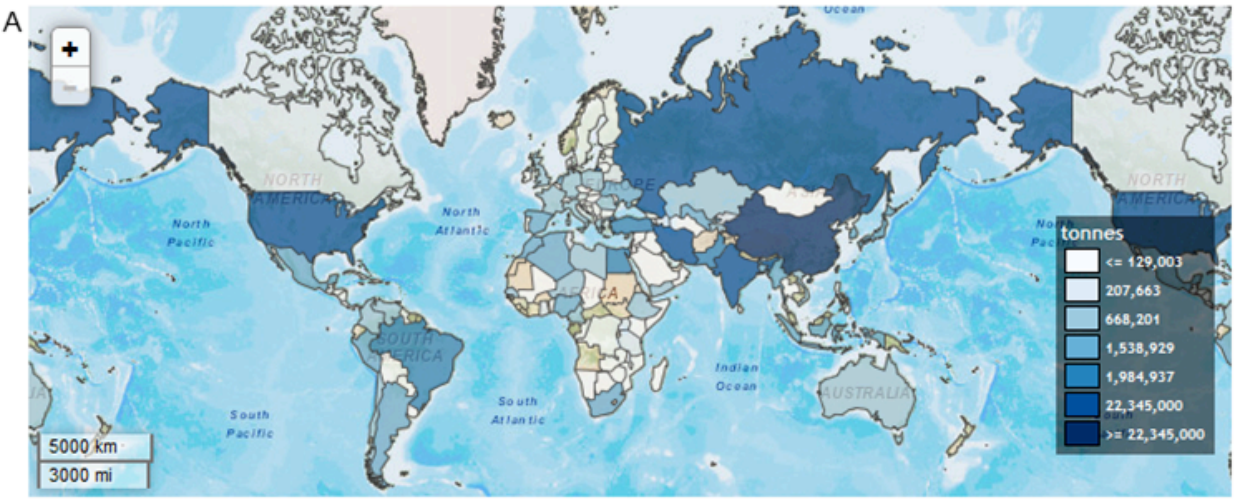
– Health 'Claims referring to children's development' (*Article 14). For example: "Vitamin D is needed for the normal growth and development of bone in children".*

1.2. Perspective of onion and derived products as functional ingredients in the market – *From the beginnings to our table today*

Although it is unable to determine the exact time and place for the first cultivation of onions, written records locate the origin in central Asia, from where it spreads across the entire world.

The tolerance of onions to grow in a wide range of soil types and climates together with the ease of drying, preservation and storage during winter has made this species of the *Allium* genus a useful source of food since the earliest civilizations. Numerous citations reveal their use in both traditional and religious ceremonies of many cultures, with references to their prophylactic and therapeutic properties (Chutani & Bordia, 1981; Fenwick & Hanley, 1985). In fact, from the empirical observation of popular use, the transformation and consumption of onion has been transformed into a rigorous field of scientific research.

A



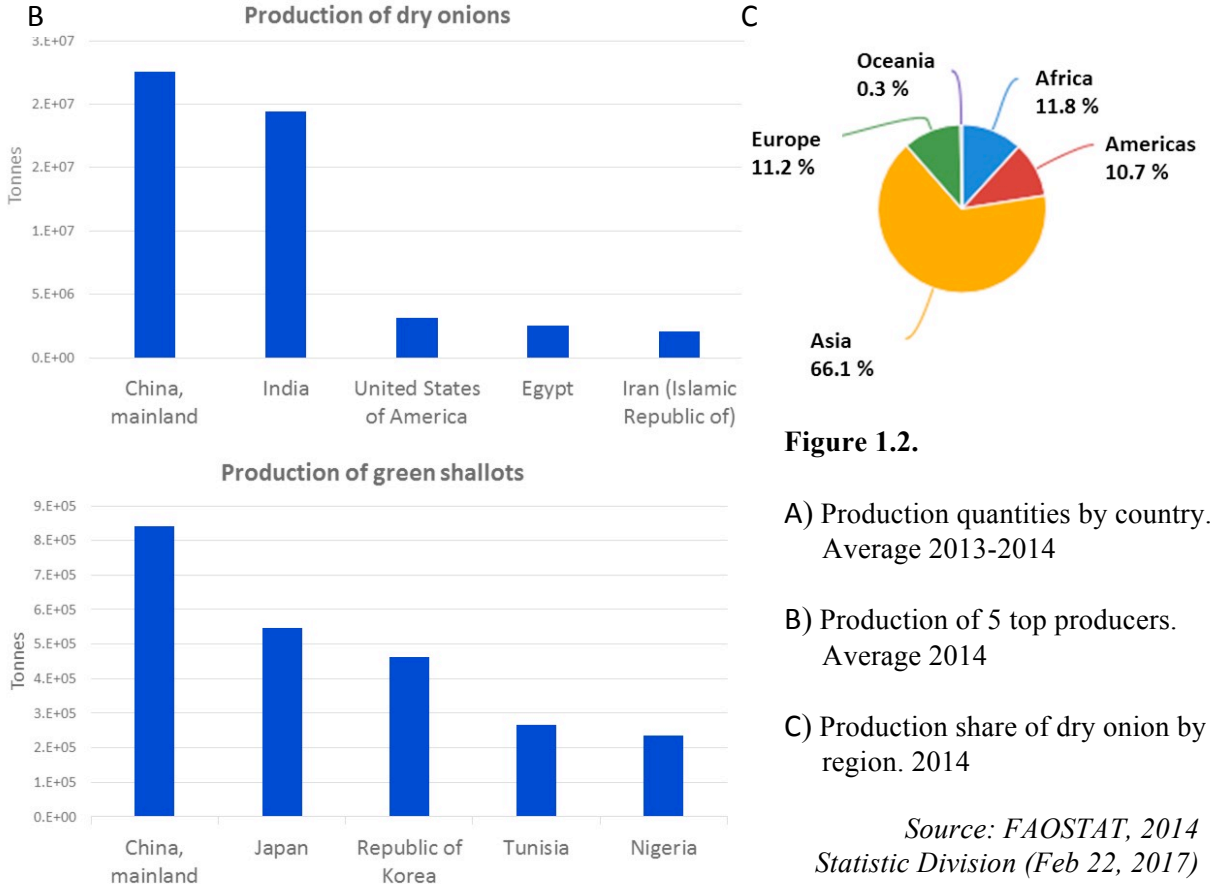


Figure 1.2.

A) Production quantities by country. Average 2013-2014

B) Production of 5 top producers. Average 2014

C) Production share of dry onion by region. 2014

Source: FAOSTAT, 2014
Statistic Division (Feb 22, 2017)

To date, the Food and Agriculture Organization (FAO) of the United Nations recognizes the growth of onions in at least 175 countries, although average production varies greatly (Figure 1.2A-C, FAOSTAT, 2014). China is the first producer of onion in the world, followed by India and the USA (Figure 1.2B, FAOSTAT, 2014). These three countries produce about 50% of the total world production, which was recorded in 2014 at 88 million tonnes (FAOSTAT, 2014).

In a EU-28 context (2015), the onion production ranges at around 6.1 million tonnes which represents 7% of the world volume, with Spain being positioned as the second largest producer after the Netherlands. Spain holds an important potential for production, exporting both within the EU (Germany, UK, Portugal, France) as well as outside (Brazil and UAE).

In Spain, the harvest of onion is mainly from the regions of Castilla-La Mancha, Andalucía, Comunidad Valenciana and Aragón, with a cultivated surface extension of onions which has remained more or less constant of 22-24,000 hectares (with the exception of 2008). The onion consumed in Spain has also remained stable with the cultivar 'Grano de oro or Valenciana' positioned at the top of the onion ranking for crop production (MAPAMA,

2013). According to the Ministry of Agriculture, Food and Environment (©Statista 2016), the volume of fresh onions consumed in Spain from 2008 to 2015 ranged from 328 to about 369 million kilograms.

In terms of human consumption, the domestic uses are widely extended, without overlooking a growing application in commercial chains and institutional caterings. Onion, which can be found in yellow, red or white coloured bulbs (FAO, 2003), adds value to many dishes due to its flavour and nutritional value used either raw, cooked or in transformed forms. It is usually consumed in tender state, raw, ripe, pickled or in the form of powder and is added in soups, stews, gazpachos, sauces, dips and fries among other cooked products. The wide application of onion in commercial channels has extended the ways of presentations in easy-to-use format, which maintain active the processing and manufacture of onion in the food industry.

Already in 2002, plant extracts (including herbal extracts, oleoresins, essential oils and fruit and vegetable extracts) had a market value of over €1 billion in Western Europe. The market share for fruit and vegetable extracts and powders was around €410 million (Stein Alexander Jivraj, 2008). More recently, a European Market report gave a general overview of the European market for phytochemicals and plant extracts and estimated a growth at a CAGR of 8.4% from 2014 to 2019. Until 2014, this European market was dominated by Germany, followed by France and Italy. However, Cargill Inc. (U.S.), Chr. Hansen (Denmark), Arboris LLC (U.S.), and BASF SE (Germany), among others, are some of the new key companies investing heavily in this high-growth market (Micro Market Monitor, 2015).

Following this tendency, the producers have also developed modern methods for the transformation of onions using both bulb onions and derived by-products to satisfy the demands of the market. Product innovation is continuously involved in a competitive environment of research and development.

Thus, innovation in cultivation and harvesting (Tocmo, Lin, & Huang, 2014), new emerging technologies of processing (*e.g.* high-pressure processing, microwave-assisted extraction (Kumar *et al*., 2014; Li *et al.*, 2015; Vazquez-Gutierrez *et al*., 2013), and storage under different atmospheric, temperature and light conditions (Islek, Nilufer-Erdil, & Knuthsen, 2015) allow to reach new onion products as dry products, oils or extracts (Antunes, *et al*., 2014; Jung *et al*., 2015; Mnayer *et al*., 2015). In consequence, the onion

commercialization continues to expand as the fields of investigation, development and innovation offer new insights into the possibilities for use and applications of onion.

The perspectives of a growing market for onion utilised as a functional ingredient let to conclude that the field of onion transformation focus its efforts on the addition of value over both regular products and by-products from manufacturing. With this aim, food processing is developed in order to optimize and improve the quality, nutritional composition and content in phytochemicals of the new processed products (Perez-Gregorio *et.al*., 2014).

1.3. Onion nutritional and bioactive composition

Allium cepa L. var. *cepa*, is the most widely cultivated specie of the genus *Allium*, it belongs to the Liliaceae family within the order of Liliales (Figure 1.3).

Levels – Classification
- Kingdom –Plantae (Plants)
- Subkingdom – Tracheobionta (Vascular plants)
- Superdivision – Spermatophyta (Seed plants)
- Division – Magnoliophyta (Flowering plants)
- Class – Liliopsida (Monocotyledons)
- Subclass – Liliidae
- Order – Liliales
- Family – Liliaceae (Lily family)
- Genus – *Allium* L. (onion)
- Species – *Allium cepa* L. (garden onion)

Allium cepa L. var. *cepa*

Figure 1.3. Classification from Kingdom Plantae to the Specie *Allium cepa* L. *USDA Natural Resources Conservation Service- United State Department of Agriculture*

Onion (*Allium cepa* L.) has a high content of water (~ 90%) and a low content in calories (~ 40 kcal/100 g). This is mainly due to the amount of dietary fibre and sugars and the low content of lipids and amino acids. Arginine and glutamic acid are exceptions in the composition which are found at higher levels. In terms of vitamins and minerals, the sodium content is low, while vitamins B_6 and C, folic acid, calcium, magnesium, phosphorus and potassium content are relatively high. The nutritional content of raw onion is shown in Table 1.1. However, the composition differs between varieties and is affected by several elements, such as the agronomic factors. Moreover, the application of processing technologies also affects the nutritional composition. While some thermal processing technologies may degrade the nutritional composition, others such as high-pressure (HP), freeze-drying and

pulverization may affect cell wall structure favouring the extraction, bioaccessibility and absorption of certain nutrients.

Table 1.1 Raw onion (*Allium cepa* L.) composition in 100 grams of edible portion

Proximates	**Units**	**Value ± S.E.**
Water	g	89.11 ± 0.248
Energy	Kcal	40 ± 0
Energy	KJ	166 ± 0
Protein	g	1.10 ± 0.036
Total lipid (fat)	g	0.10 ± 0.005
Ash	g	0.35 ± 0.003
Carbohydrate	g	9.34 ± 0
Fibre, total dietary	g	1.7 ± 0.048
Sugars, total	g	4.24 ± 0
Sucrose	g	0.99 ± 0.050
Glucose (dextrose)	g	1.97 ± 0.054
Fructose	g	1.29 ± 0.052
Minerals	**Units**	**Value ± S.E.**
Calcium, Ca	mg	23 ± 0.568
Iron, Fe	mg	0.21 ± 0.08
Magnesium, Mg	mg	10 ± 0.152
Phosphorus, P	mg	29 ± 0.584
Potassium, K	mg	146 ± 2.951
Sodium, Na	mg	4 ± 0.158
Zinc, Zn	mg	0.17 ± 0.004
Copper, Cu	mg	0.039 ± 0.002
Manganese, Mn	mg	0.129 ± 0.004
Selenium, Se	µg	0.5 ± 0.143
Fluoride, F	µg	1.1 ± 0.100
Amino Acids	**Units**	**Value ± S.E.**
Tryptophan	g	0.014 ± –
Threonine	g	0.021 ± –
Isoleucine	g	0.014 ± –
Leucine	g	0.025 ± –
Lysine	g	0.039 ± –
Methionine	g	0.002 ± –
Cystine	g	0.004 ± –
Phenylalanine	g	0.025 ± –
Tyrosine	g	0.014 ± –
Valine	g	0.021 ± –
Arginine	g	0.104 ± –
Histidine	g	0.014 ± –
Alanine	g	0.021 ± –
Aspartic acid	g	0.091 ± –
Glutamic acid	g	0.258 ± –
Glycine	g	0.025 ± –
Proline	g	0.012 ± –
Serine	g	0.021 ± –

Vitamins	**Units**	**Value ± S.E.**
Vitamin C, total ascorbic acid	mg	7.4 ± 0.053
Thiamin	mg	0.046 ± 0.001
Riboflavin	mg	0.027 ± 0.002
Niacin	mg	0.116 ± 0.003
Pantothenic acid	mg	0.123 ± 0.002
Vitamin B-6	mg	0.120 ± 0.004
Folate, total	µg	19 ± 0.059
Choline, total	mg	6.1 ± 0
Betaine	mg	0.1 ± 1
Carotene, beta	µg	1 ± 0
Vitamin A, IU	IU	2 ± 0
Lutein + zeaxanthin	µg	4 ± 0
Vitamin E (α-tocopherol)	mg	0.02 ± 0
Vitamin K (phylloquinone)	µg	0.4 ± 0.011
Lipids	**Units**	**Value ± S.E.**
Fatty acids, total saturated	g	0.042 ± –
14:0	g	0.004 ± 0.00
16:0	g	0.034 ± 0.003
18:0	g	0.004 ± 0.00
Fatty acids, total Monounsaturated	g	0.013 ± –
18:1 undifferentiated	g	0.013 ± 0.002
Fatty acids, total Polyunsaturated	g	0.017 ± –
18:2 undifferentiated	g	0.013 ± 0.002
18:3 undifferentiated	g	0.004 ± 0.00
Phytosterols	mg	15 ± –
Flavonoids (Flavonols)	**Units**	**Value ± S.E.**
Isorhamnetin	mg	5.0 ± 690
Kaempferol	mg	0.7 ± 100
Quercetin	mg	20.3 ±780

USDA National Nutrient Database for Standard Reference, Release 28 (2017). Food Group: Vegetables and Vegetable Products. Raw onion (*Allium cepa* L.) Refuse: 10% (Stem ends, sprouts and defects).

Although the content of phytochemical found in onion may cover a wide spectrum of chemical groups and functions, those which are found in higher concentrations and which have properties related to beneficial health effects have received the most attention and been subjected to greater study in depth. The potential effects of onion consumption have been primary attributed to three groups of bioactive compounds: flavonoids, organosulfur compounds (OSCs) and dietary fibre.

1.3.1. Flavonoids. Quercetin and quercetin glucosides

Flavonoids are a large family of polyphenolic secondary plant metabolites contributing substantially to the non-caloric part of the human diet (Noteborn *et al.,* 1997). The flavonoids of dietary significance are usually divided into six principal classes, termed flavones, flavonols, flavanones, isoflavones, flavanols (including catechins and tannins), and anthocyanins (Harborne & Williams, 2000). Flavonoids are generally found in high concentrations in the *Allium* genus; onion (*Allium cepa*) is characterized by a higher concentrations compared with others cultivated plant species such as garlic (*Allium sativum*) and leek (*Allium porrum*) (Fattorusso *et al*., 2002). However, only two flavonoid subgroups are usually found in onion, anthocyanins which are characterised by the red/purple colour of some varieties and flavonols such as quercetin and its derivatives which are responsible for the yellow and brown skin colours of many other varieties (Griffiths, *et al*., 2002).

In general, the non-edible skin part of the onion bulb is richer in total flavonoids compared to the edible flesh. The flavonoids present in the skin are mainly aglycones due to flavonol glucoside hydrolysis during the formation of the outer skin layers (Price & Rhodes, 1997; Takahama & Hirota, 2000). The onion bulb contains a wide range of quercetin, isorhamnetin, and kaempferol derivatives in varying proportions (Bilyk, Cooper & Sapers, 1984) with a tend to increasing contents of quercetin glucosides from the inner to outer scales (Nemeth & Piskula, 2007; Patil & Pike, 1995; Tsushida & Svzuki, 1996; Wiczkowski *et al*., 2003).

Of at least 25 flavonols that have been characterized, quercetin and quercetin derivatives are the most important forms in all onion cultivars, with their glycosyl moieties (β-*O*-glycosides, usually D-glucose), mainly attached to the 4', 3, and/or 7-positions of the aglycones (Figure 1.4). Analogous derivatives of kaempferol and isorhamnetin have also been identified as minor onion pigments (Slimestad, Fossen & Vagen, 2007).

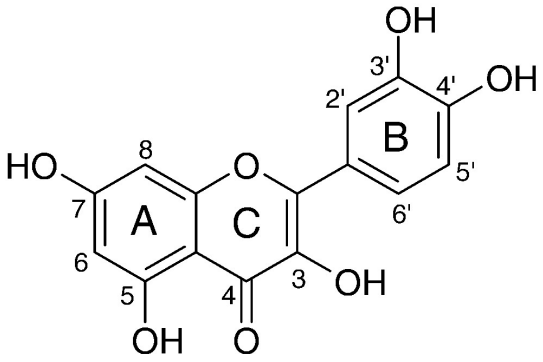


Figure 1.4. Chemical structure of quercetin

Quercetin 3,4'-diglucoside and quercetin 4'-glucoside represent about the 90% of the overall contents in different types of *Allium* species (including *Allium cepa* L. and *Allium ascalonicum* Hort.) (Bonaccorsi *et al*., 2008). However, the ratios and distribution of quercetin and its glycosides within the onion bulbs differs among cultivars and are modified by factors such as onion processing, cooking and exposure to light (Ewald *et al*., 1999; Mogren *et al*., 2008; Mogren, Olsson & Gertsson, 2007; Nemeth & Piskula, 2007).

The metabolism of quercetin and its glycosides undergoes several and complex processes on consumption beginning in the oral cavity and continuing through the stomach, small intestine, colon, and liver until the partial elimination by the urinary pathway, all of which together affects the final bioavailability of flavonoid metabolites (Nemeth & Piskula, 2007). The mechanism proposed for the absorption of quercetin glucosides from the gastrointestinal tract involves deglycosylation by luminal lactase phloridzin hydrolase and/or cleavage within the enterocyte by cytosolic β-glucosidase (Day, *et al.,* 2003). Then, this is followed by metabolism of the aglycone which leads to the appearance of quercetin sulphate and glucuronide conjugates in the circulatory system (Day & Williamson, 2003).

Moreover, it has been demonstrated in several studies that quercetin glycosides are absorbed more efficiently than quercetin aglycone (Hollman *et al*., 1995; Moon *et al*., 2000) irrespective of the position of the glucose moiety (Olthof *et al*., 2000). The efficiency of intestinal absorption of flavonoids is strongly affected by the food compositions and compound's solubility in the vectors (Piskula & Terao, 1998). A more recent study by Mullen *et al*., (2006) also demonstrates that extensive modification of quercetin glucosides occurs after ingestion of onion which involves a complex combination of deglycosylation, glucuronidation, sulfation, methylation and possibly deglucuronidation steps, with the subsequent appearance of metabolites in the blood stream and urine. However, the sequence

and location where these modifications occur after the initial deglycosylation is a matter of speculation and a topic that still requires further investigation.

1.3.2. Organosulfur compounds (OSCs)

The *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs), also referred to as flavour precursors, are produced by cellular disruption by the enzyme alliinase (EC 4.4.1.4) in the cytoplasm (Block, 1992). When the tissue is disrupted, the ACSOs are enzymatically degraded to iminopropionic acid, which spontaneously hydrolyses to form ammonia and pyruvic (non-flavour compounds) and unstable alk(en)yl cysteine sulfenic acids (flavour compounds). These sulfenic acids (methyl-, propyl- and prop-1-enyl sulfenic acid) decompose spontaneously (non-enzymatically) to form thiosulfinates which evolve to thiosulfonates, zwiebelanos and cepaenes; while prop-1-enyl sulfenic acid is responsible for thiopropanal S-oxide formation, the onion lachrymatory factor (Block *et al*., 1993) (Figure 1.5). These sulfur compounds are responsible for the 'onion pungency', well manifested by the strong smell, burning sensation in the back of the mouth and throat, or in milder cases, the pleasant flavour typical to onion.

Three ACSO compounds are naturally occurring and commonly found in onion: trans-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide (PRENCSO), (+)-*S*-methyl-L-cysteine sulfoxide (MCSO) and (+)-*S*-propyl-L-cysteine sulfoxide (PCSO) (Lancaster and Boland, 1990). Thiosulfinates themselves are unstable and decompose to form a complex mixture of compounds, in which mono-, di-, tri- and tetra-sulfides predominate (Rose, Whiteman, Moore & Zhu, 2005). Dipropyl disulfide, dipropyl trisulfide, and propenil disulfides are the major constituents of onion volatiles, although many others compounds have been identified, including dipropyl sulfide and dipropenyl sulfide (Munday & Munday, 2001).

The biological effects of organosulfur compounds of the *Allium* genus of vegetable (in particular garlic and onion) have been associated with the prevention of multiple pathologies, including cancer, cardiovascular disease and inflammatory disorders (Rose *et al*., 2005). Among the anticarcinogenetic properties described in the literature in association with the consumption of onion, the capacity to modify the enzymatic activity (such as that of the phase II detoxification enzymes), the inhibition of tumour cell proliferation and induction of apoptosis in cancer cells and the modulation of inflammatory cascades are a subject of keen interest (Rose *et al*., 2005). Moreover, the antioxidant properties of individual sulfur

compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS), *S*-methylcysteine (SMC) and *S*-propylcysteine as inhibitors of the damage caused by free-radical agents, together with the antimicrobial, antifungal and anti-parasitic properties which are derived from the use and consumption of onion are constantly under study (Corzo-Martínez & Villamiel, 2012; Kumari & Augusti, 2002; Llana-Ruiz-Cabello *et al*., 2015; Senthilkumar *et al*., 2015).

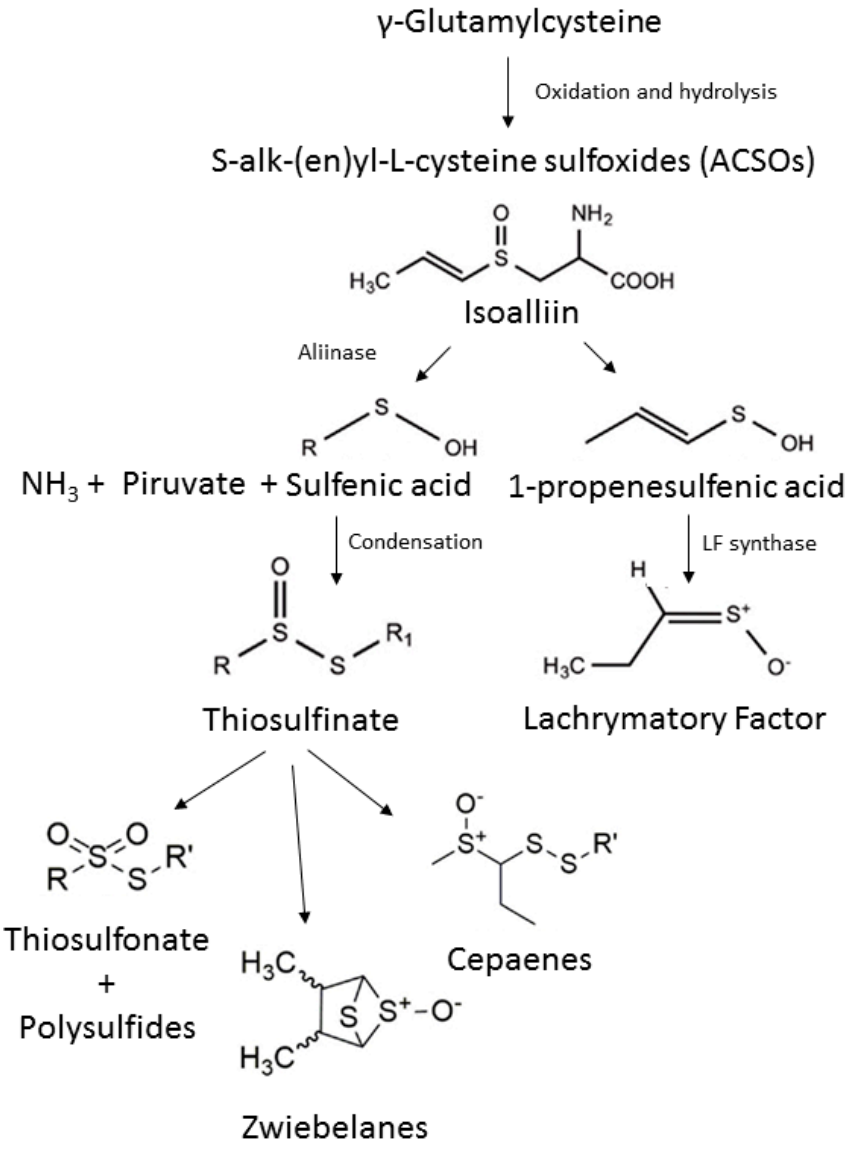


Figure 1.5. Scheme of organosulfur formation in processed onion (*Allium cepa* L.)

1.3.3. Fructans and fructooligosaccharides (FOS)

Onion is a perennial herbaceous plant rich in fructooligosaccharides (FOS) inulin (IN) type structure. FOS/IN are generally referred to as fructans belonging to the category of non-digestible carbohydrates (Roberfroid, 2005). It is generally accepted that FOS is a common term used only to refer to fructose oligomers that are mainly composed of kestose (GF_2), nystose (GF_3) and fructofuranosylnystose (GF_4), in which fructosyl units (F) are bound by β-linkage at the position of the sucrose (glucose+fructose-GF) (Jaime *et al*., 2001).

In onion, the predominant non-structural carbohydrates are glucose, fructose, sucrose and low-molecular-weight fructans, while starch and raffinose are absent. FOS appear as the main carbohydrate reserve of onion comprising polyfructosyl sucroses of varying molecular size. These FOS accumulate during bulb formation and are then catabolized during regrowth and sprout development of the bulbs (Benkeblia, 2005).

It is particular characteristic of onion tissue to have a predominance of kestose (GF_2) and an absence of highly polymerized fructans. The degree of fructan polymerization in a typical onion bulb ranges mainly between 3 and 15 (Kahane *et al*., 2001). The tissues richest in fructans are the fleshy part of the onion bulbs, which explains why the outer two fleshy layers constitute a useful by-product of onion and is a source of fructans and FOS (Jaime *et al*., 2001; Jaime *et al*., 2002).

The content, distribution and structure of FOS in onion bulb were initially investigated by Bacon (1959) and Darbyshire and Henry (Darbyshire & Henry, 1981; Darbyshire & Henry, 1978). Nowadays, short chain fructans (with a degree of polymerization less than 5) are potentially used as natural low-calorie sweeteners, while onion bulbs with of a high degree of fructan polymerization may be used for lipid replacement with consequential health benefits (Kahane *et al*., 2001). The quantification of fructans and FOS is undertaken in the study and control of prebiotics, while their properties are tested in relation to several physiological situations. FOS are fermented to a high degree by *Bifidobacterium* into short-chain carboxylic acids in the large intestine. This produces a decrease in pH and changes in gastric emptying and intestinal motility, which are associated with important physiological properties, as for example a decrease in circulating levels of cholesterol, triglycerides and phospholipids (Jaime *et al*., 2001).

Moreover, the presence of fructans in the tract stimulates the growth of the beneficial microbiota usually constituted by *Bifidobacteria* and *Lactobacilli* and also produces positive

results in the colon, including effects on colonic inflammation and increased intestinal absorption of calcium and magnesium leading to improved bone density (Lara-Villoslada *et al*., 2006; Ernst & Feldheim, 2000; Roberfroid, 2007). In more recent studies, the immunomodulatory properties of onion have also been attributed to the presence of FOS. For example, structural and *in vitro* results have indicated that onion FOS comprising tri- to hexasaccharide units promote immunostimulatory activity towards murine lymphocytes and macrophages (Kumar, Prashanth & Venkatesh, 2015).

1.3.4. Other bioactive onion compounds

Although classical phenolic and organosulfur compounds have accumulated most of the attention, still new compounds are being isolated from onion with new functions and characteristics which are being described in the scientific publications.

Saponins have gained much attention due to their antispasmodic activity, which helps explain the traditional use of onion and garlic in the treatment of intestinal spasms, instigating the study of *in vivo* effects of the isolated metabolites in models of intestinal hyperactivity (Lanzotti, 2012).

In 2008, four **furostanol saponins**, two of which were new compounds, termed ceparoside A and ceparoside B were isolated from the seeds of *Allium cepa* L. (Yuan *et al*., 2008; Yuan *et al*., 2009). Some years later, three new furostanol saponins metabolites, ceposides A-C, were also isolated and structurally determined, and were shown to have significant antifungal activity related to the resistance of *A. cepa* to pathogen attack (Lanzotti *et.al*., 2012).

Xiao and Parking (2007) were able to isolate and identify five potentially active cancer chemopreventive agents, related to the induction phase II enzymes, from methanolic extracts of green onion: 5-hydroxy-3-methyl-4-propylsulfanyl-5H-furan-2-one was reported as a new compound, in addition to four others known as 5-(hydroxymethyl) furfural, acetovanillone, methyl 4-hydroxyl cinnamate and ferulic acid methyl ester (Xiao & Parkin, 2007).

A **gamma-glutamyl peptide** from onion was mentioned as a possible inhibitor of the resorption activity of osteoclasts *in vitro* (Langos *et al*., 2007; Wetli *et al*., 2005).

More recently, **onionin A** (1) has been identified as a new stable, sulfur-containing compound isolated from acetone extracts of onion bulbs (*Allium cepa*). The structure is characterized as 3,4-dimethyl-5-(1E-propenyl)-tetrithydrothiophen-2-sulfoxide-S-oxide (El-

Aasr *et al*., 2010). In addition, two more stable sulfur-containing compounds identified as the stereoisomers of onionin A(1), classified as onionins A(2) and A(3) were also isolated, showing the potential to suppress tumour-cell proliferation by inhibiting the polarization of M2 alternatively activated macrophages (Nohara *et al*., 2014). Onionin A has been shown to inhibit epithelial ovarian cancer proliferation by the suppression of STAT3 activation in tumour cells and macrophages (Nakao *et al*., 2014) and is currently considered as a useful additional treatment for patients with ovarian cancer owing to its suppression of the protumour activation of TAMs and direct cytotoxicity against cancer cells (Tsuboki *et al*., 2016).

1.4. Effects of onion consumption in health and disease

As mentioned above, the particular composition of onion *Allium cepa* L. var. *cepa* makes this vegetable an important source of elements for the organisms with direct repercussion on human health. The health promoting benefits of consuming onion have been supported by *in vitro*, *in vivo*, and *ex-vivo* experimentation. Antioxidant, antiinflammatory, anticarcinogenic, hypolipidemic, hypoglycaemic, or antiaggregatory properties of onion have been demonstrated by a large number of investigations (Ali, Thomson & Afzal, 2000; Griffiths *et al*., 2002; Suleria *et al*., 2015; Wilson & Demmig-Adams, 2007). Antimicrobial effects of onion and substances isolated from these plants have also been described, showing their effectiveness against pathogenic microbes (Lanzotti, Bonanomi & Scala, 2013) and modulating properties on the gut microflora. Moreover, the consumption of onions has been related to a reduction of the risk of several illness, including cardiovascular disease, hepatic impairments, diabetes mellitus, metabolic syndrome, asthma and different types of cancer (Akash, Rehman & Chen, 2014; Nicastro, Ross & Milner, 2015; Suleria *et al*., 2015). Enhancement of the immune system has been associated with components of onion such as FOS (Kumar, Prashanth & Venkatesh, 2015) and research into the effects on immunological cells in rats is being undertaken (Mirabeau & Samson, 2012) as well on experimental infections such as in Schistosoma mansoni (Mantawy, Ali & Rizk, 2011).

Less well-understood but not for that less interesting properties of onion are those shown by Matheson *et al*. (2009) and Law *et al*. (2016), reporting that onion and onion juice consumption seems to have a beneficial effect on bone density and inhibits osteoclastogenesis and its differentiation in premenopausal and postmenopausal women and in healthy middle-aged subjects. Likewise, studies by Sakakibara *et al*. (2008) suggest that onion exert

antidepressant-like activity in a behavioural model of depression (Law *et al*., 2016; Matheson, Mainous & Carnemolla, 2009; Sakakibara *et al.,* 2008).

All these findings make the consumption of onions not only recommendable as a healthy food product to be included in our diet for the health promotion and disease prevention, but also for use as a complementary approach for tackling some of the side-effects resulting from the treatment of several pathologies. Thus, onion supplementation has promise as a functional ingredient contributing to future nutritional and dietary interventions.

1.4.1. The role of onion in hypercholesterolemia, inflammation and oxidative stress

Hypercholesterolemia, inflammation and oxidative stress are highly prevalent disorders associated with the initiation and onset of disease. The occurrence of inflammation and oxidative stress also appears as response to hypercholesterolemia, and as hypercholesterolemia acts as a feedback it leads to permanent damage and the progression of disease.

In this regard, atherosclerosis is a well-defined pathology that can be used as a clear illustration of the implication and close link between these impairments. Although the exact cause of atherosclerosis is not well known, its aetiology point it to be classified as a systemic inflammatory disease where hypercholesterolemia is one of the major risk factors for its promotion (Tall & Yvan-Charvet, 2015). At the same time, the inflammatory processes triggered are characterized by induced oxidative stress and a reduced cellular antioxidant capacity (Khansari, Shakiba & Mahmoudi, 2009).

Dietary habits have been largely related with these three factors as well as with the prevention and prevalence of diseases. The dietary habit of eating onion has attracted a lot of attention in this field and there are many publications describing this relationship:

- The hypolipidemic, hypocholesterolemic or more generally lipid-lowering effects of onion have been widely documented in a diversity of preparations: fresh or treated onion, onion powder, onion juice, essential oils, aqueous extracts or skin by-products. Decreased blood cholesterol concentration has generally been observed in the majority of these studies, taking into account either genetically modified models or dietary interventions under conditions of a normal, high-fat, high-cholesterol or high-sucrose diet. However, findings are heterogeneous as they depend on several factors such as the model of study, experimental design, time of intervention, subject and variety of onion tested in the experiments. By way of

example, the hypolipidemic effect of onion skin was studied in ob/ob mice concluding that it could be beneficial on hyperlipidemia and hepatic lipid accumulation (Kang *et al*., 2010). A dehydrated onion product was evaluated in experimental Wistar rats, showing significant reductions of cholesterol (LDL) and triglycerides and an increase in HDL cholesterol (Vidyavati *et al*., 2010). Anti-obesity effects of an onion extract were determined in obesity and diabetes-prone Zucker diabetic fatty rats by measuring diverse markers, which revealed reduced triglyceride and free fatty acid serum levels in the onion-fed groups, despite not finding significant differences in the levels of cholesterol (Yoshinari, Shiojima & Igarashi, 2012). The effect of ultra-high pressure processed onion extracts was also tested on serum cholesterol levels in high cholesterol-fed rats showing its capacity to prevent hyperlipidemia by improving lipid metabolism via decreased liver lipid contents and increased fecal lipid excretion (Jung *et al*., 2015). Likewise, a quercetin rich onion juice was able to markedly suppress cholesterol levels and elevate total antioxidant status in mildly hypercholesterolemic subjects after eight weeks of intervention (Lu *et al*., 2015).

- Onion has also been established as having antiinflammatory effects, intervening in different mechanisms of action. It is briefly exemplified by various studies (Suleria *et al*., 2015; Wilson & Demmig-Adams, 2007):

The antiinflammatory effect of some of its bioactive components such as quercetin was initially investigated by Wagner *et al*. (1990), on prostaglandins, leukotrienes, histamine release, and subsequent antiasthmatic activity. The organosulfur compounds of onions such as thiosulfinates and capaenes have been related to antiinflammatory activities by means of modification of enzyme activities (COX, LOX), blood platelet aggregation and inhibition of the arachidonic acid metabolic pathway. The discoveries about the inhibitory effect of onion on the conversion of arachidonic acid into eicosanoids metabolism were summarized by Ali *et al*. (2000). Furthermore, diverse components of onion such as quercetin derivatives have evidenced its protective effect by modulating the release of proinflammatory mediators and vasoactive biomarkers *e.g.* cytokine, nitric oxide, endothelin-1, etc. (Brüll *et al*., 2015; Ford *et al*., 2016; Sekhon-Loodu *et al*., 2015).

- Generation of reactive oxygen species (ROS) and other free radicals during metabolism is a normal process which is ideally compensated by the antioxidant defence system. Nevertheless, an imbalance in these mechanisms leads to an overproduction of free radicals that cause oxidative stress and damage at different levels. ROS induce lipid peroxidation in cellular membranes by generation of lipid peroxides, whereas endothelial cell

injury is induced by hydrogen peroxide, superoxide anion, and hydroxyl radicals and is often observed in the early stages of many pathological disorders (Pham-Huy, He & Pham-Huy, 2008; Zorov, Juhaszova & Sollott, 2014).

Oxidative stress is now believed to be the primary cause for not only many degenerative diseases, such as cardiovascular diseases, inflammatory diseases, cancer, cataracts, type-2 diabetes, neurodegenerative diseases, etc., but also the inevitable process of aging. The relationship between diet and chronic diseases has been increasingly understood in recent decades and dietary onion seems to have important applications as a natural antioxidant agent by delaying or suppressing oxidative stress, in both animals and humans (Srinivasan, 2014). To cite a few recent examples, the antioxidant and antigenotoxic properties of onion peel extracts were demonstrated in both non-cellular and cellular systems (Kim, Kim & Park, 2013). Hur *et al*. (2013) studied the effect of an onion extract in mouse brain lipids and its structural change during *in vitro* human digestion, finding that the antioxidative effect of onion extract was greater in high-fat-fed mouse brain lipids than that in low-fat-fed mouse brain lipids. Another study showed that onion extract therapy reduced Cd-induced oxidative stress and apoptosis (Alpsoy *et al*., 2014). Onion juice consumption showed a significant positive change in the levels of alkaline phosphatase (ALP), free radicals, total antioxidant capacity (TEAC) and various antioxidants in middle-aged and post-menopausal healthy subjects (Law *et al*., 2016). However, as previously mentioned, results present great variability. In this sense, Jeon *et al*. (2013) assayed the effect of onion juice supplementation on antioxidant status in participants with mild hypercholesterolemia, failing to find any antioxidant effect of onion supplementation in subjects with mild hypercholesterolemia.

In this context, we have to cite previous studies undertaken by our research group that have also shown the lipid lowering, antiinflammatory and antioxidant properties of diverse onion products submitted in this case to diverse treatments of high-pressure processing (González-Peña *et al*., 2013; Roldán-Marín *et al*., 2010; Roldán-Marín *et al*., 2009a; Roldán-Marín *et al*., 2009b, Roldán *et al*., 2008; Vázquez-Gutiérrez *et al*., 2013).

1.4.2. Onion in atherosclerosis and cardiovascular disease (CVD) prevention

Cardiovascular disease (CVD) groups together a series of disorders of the heart and blood vessels, including coronary and rheumatic heart disease, cerebrovascular disease, congenital heart disease, deep vein thrombosis and pulmonary embolism (WHO, 2016). This set of

pathologies is recognized as the main cause of death in the world, while atherosclerosis is the major and prevalent cause of CVD initiation and progression. Multiple risks such as cholesterol, hypertension, Angiotensin II, smoking or diabetes are pointed as inducers for its development and cholesterol is investigated as a key risk factor in cardiovascular prevention strategies.

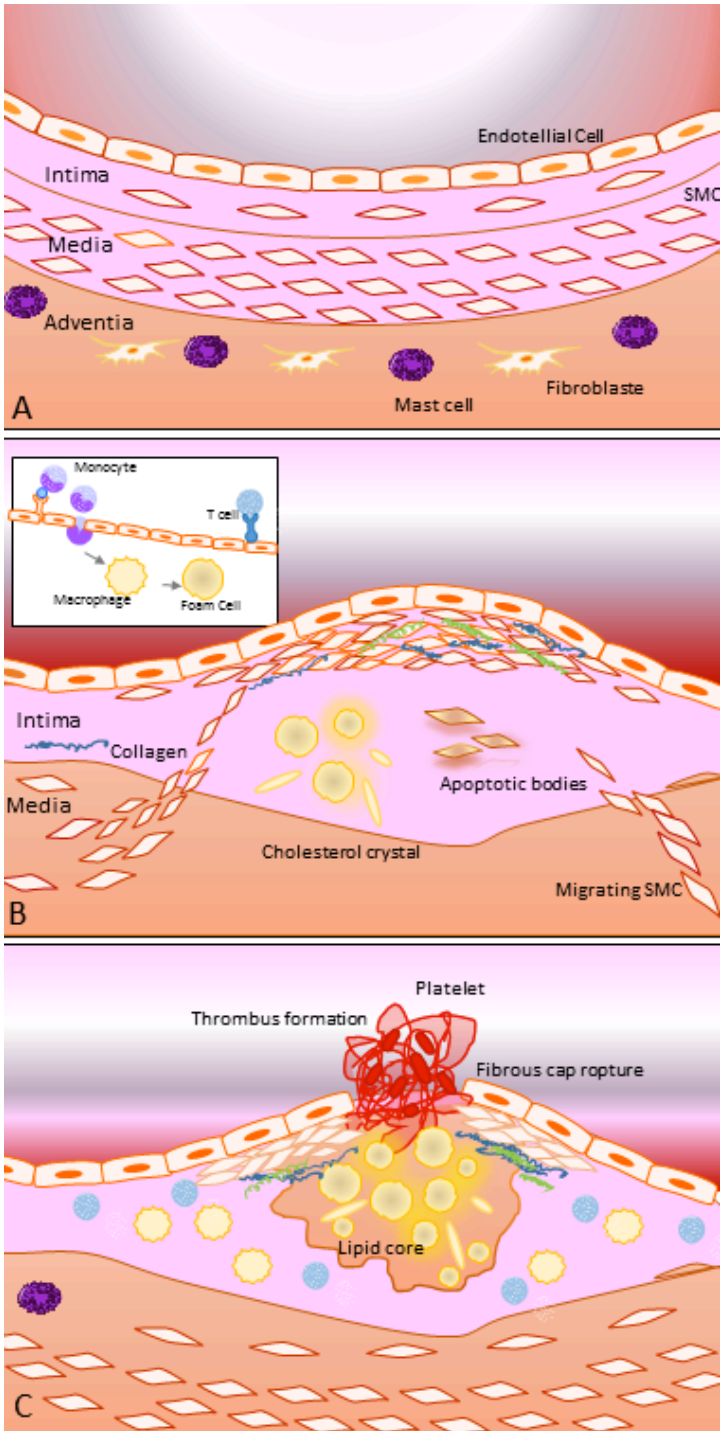


Figure 1.6. Schematized phases in the development of atherosclerosis.
Adapted from Libby et al. (2011)

Atherosclerosis involves the development of atheromatous plaques on the inner lining of the arteries (Figure 1.6). This process, which has been recently represented by Libby *et al*. in *Nature* (Libby, Ridker & Hansson, 2011), starts with an initial qualitative change in the monolayer of endothelial cells that lines the inner arterial surface, when subjected to an irritative stimuli (*e.g.* dyslipidaemia, hypertension or proinflammatory mediators). Arterial endothelial cells express adhesion molecules that capture leukocytes on their surfaces in parallel to changes in endothelial permeability and the composition of the extracellular matrix, which beneath the endothelium promote the entry and retention of cholesterol-containing low-density lipoprotein (LDL) particles in the artery wall (Figure 1.6B).

Biochemical components of these particles induce leukocyte adhesion, and monocyte-derived

macrophages undergo endocytosis, which leads to the accumulation of intracellular cholesterol. The migration of the bound leukocyte is directed by chemoattractant mediators within the tunica intima while the monocytes in the artery wall differentiate into tissue macrophages. These mononuclear phagocytes engulf lipoprotein particles and become foam cells (Figure 1.6B). Macrophages in the atheroma may also have proinflammatory functions, which produce high levels of effectors such as the cytokines interleukin-1β (IL-1β) and tumour-necrosis factor (TNF-α). Some mononuclear phagocytes in plaques act with the antigen-presenting functions of dendritic cells, while other leukocyte classes (such as lymphocytes) and mast cells also accumulate in atheromata.

The atheroma formation also involves the recruitment of smooth muscle cells (SMCs) from the tunica media into the tunica intima and some proliferate in response to mediators such as platelet-derived growth factor. SMCs produce extracellular matrix molecules in the intima, including interstitial collagen and elastin, which form a fibrous cap that covers the plaque with massive macrophage-derived foam cells, some of which die (for example, by apoptosis) and release lipids that accumulate extracellularly (Figure 1.6C).

The inefficient clearance of dead cells (efferocytosis) can promote the accumulation of cellular debris and extracellular lipids, forming a lipid-rich pool called the necrotic core of the plaque. Plaques generally cause clinical manifestations by producing flow-limiting stenosis that leads to tissue ischaemia, or by provoking thrombi that can interrupt blood flow locally or embolize and lodge in distal arteries. Complications arise with the physical disruption of the plaque triggering thrombosis. Infiltrated inflammatory cells interact with the intrinsic arterial cells promoting lesion formation and complications (*text summarized from Libby et al., 2011).*

It seems to be clear that some foods and nutraceuticals are able to intercede at different stages in the development of atherosclerosis and CVD. In this sense, onion and some of its bioactive compounds such as flavonols and organosulfur compounds have been linked with the amelioration of these pathologies. The cardioprotective effects of onion also rely on its antioxidant, antiinflammatory, hypolipidemic and antitrombotic properties (Suleria *et al*., 2015). Additionally, the anti-apoptotic effects of onion have been explored; administering a daily dose of 1 mL onion (*Allium cepa*) extract for 14 proved to be a suitable cardioprotector against the toxic effects of doxorubicin (DOX) (Alpsoy *et al*., 2011).

The first study in Mediterranean countries on the relationship between onion and garlic intake and acute myocardial infarction was recently reported by Galeone *et al*. (2009),

suggesting that a diet rich in onions may have a favourable effect on this risk. Nevertheless, investigation into the effects of onions and its compounds on several biomarkers leading to a comprehension of cardiovascular events began at a much earlier date.

The description of quercetin properties can be used as a good example since it is one the best documented and predominant flavonol constituents of onions, which may be considered a potential therapeutic agent against atherosclerosis (Gormaz, Quintremil & Rodrigo, 2015). Dietary polyphenols such as quercetin act as regulators of endothelial function (Yamagata, Tagami & Yamori, 2015). Studies of quercetin in a variety of models have shown that it can exert an antioxidant activity through different mechanism of action, such as the enhancement of antioxidant-defence mechanisms, direct radical scavenging, modulation of nitric oxide production or inhibition of xanthine oxidase activity (Nijveldt *et al*., 2001). Quercetin also favours a diminishing formation of inflammatory metabolites involved with the cyclooxygenase (COX) and lipoxygenase (LOX) activities as well as showing a capacity to inhibit platelet aggregation *in vitro* and to reduce thromboxane synthesis *in vivo* (Hubbard *et al*., 2004; Nijveldt *et al*., 2001). The lipid lowering properties of onion, although not always evidenced, have been demonstrated in several studies, where either whole onion, extracts or some of its fractions were administered under differential pathological conditions, leading to improvements at a vascular level. As examples, red onion consumption had a positive result in women with polycystic ovary syndrome (Ebrahimi-Mamaghani *et al*., 2014); a dehydrated onion product proved significant cholesterol lowering in experimental hypercholesterolemic rats (Vidyavati *et al*., 2010) and a daily supplementation with onion peel extract, improved blood lipid profiles, glucose, and blood pressure in healthy male smokers, suggesting a beneficial role against cardiovascular risk (Lee *et al*., 2011).

However, taking into consideration the possible interactions and synergistic effects of onion components, it is interesting to mention that the consumption of whole onion can offer benefits over the consumption of its individual components. This fact should direct the use of whole onion products and complex mixed with components to design new products useful as a supplement to prevent or treat vascular dysfunction linked to initial stages of atherosclerosis.

1.4.3. Effects of onion on liver disease

Non-alcoholic fatty liver disease (NAFLD) has increased significantly over the last decades and is the most prevalent chronic liver disease worldwide. NAFLD is caused by an excessive accumulation of fat in liver hepatocytes (>5% of liver weight or volume) which is prone to progression to non-alcoholic steatohepatitis (NASH), and in severe cases to cirrhosis and hepatocellular carcinoma (Katsiki, Mikhailidis & Mantzoros, 2016).

The intrahepatic lipid accumulation occurs in the form of droplets as a result of lipid metabolism abnormalities such as an increased lipolysis, free fatty acid (FFA) liver uptake, very low density lipoprotein (VLDL) synthesis, reduced FFA oxidation and triglyceride (TG) export (Katsiki *et al*., 2016). All these alterations in lipid metabolism are linked to an induction of inflammation and oxidative stress as well as to abnormal adipokine and cytokine production that affect signaling pathways and involve endothelial dysfunction. Although the mechanisms by which NAFLD contributes to CVD are not fully understood, the association with an increased mortality due to cardiovascular disease make NAFLD a priority for consideration as an independent cardiovascular risk factor (Francque, van der Graaff & Kwanten, 2016). However, it must be clear that NAFLD is usually part of a complex multisystem disease with multiple relationships (Figure 1.7).

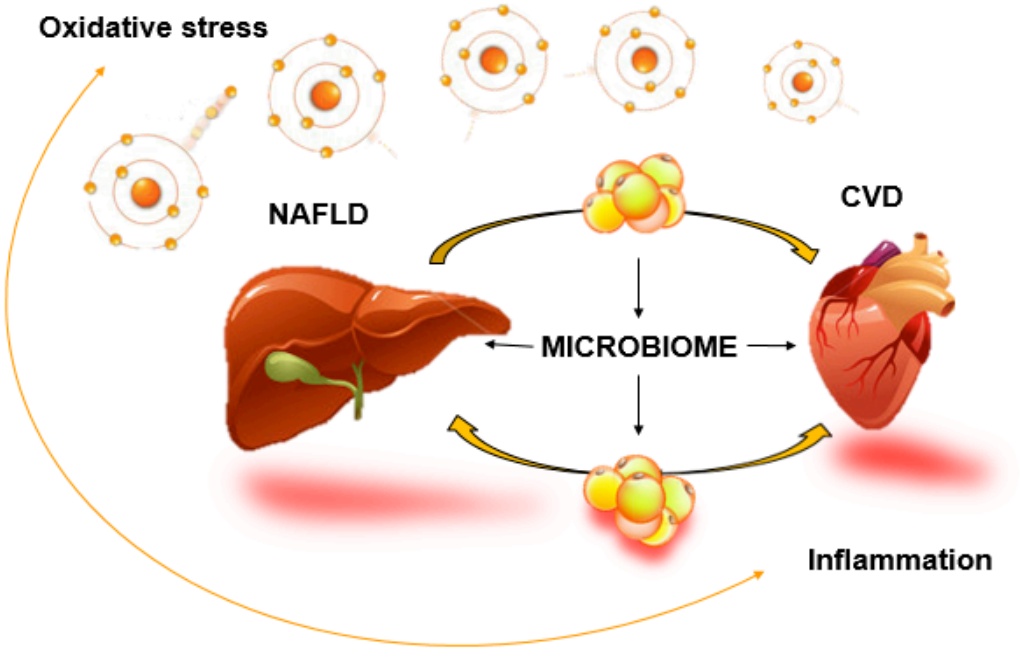


Figure 1.7. Schematic representation of some interlinked factors influencing NAFLD

Furthermore, dietary habits are the largest environmental factor that has a substantial influence on several risks leading to hepatic dysfunction. The diet alters the microbiota composition in the gut, which simultaneously is linked to multiple functions that involve the regulation of cardio-metabolic disorders such as diabetes, obesity, NAFLD/NASH, and atherosclerosis among others. Recognition and understanding of these relationships will led to new discoveries about the complex human host physiology and disease, motivating and driving the search for new biomarkers in the microbiome and testing of innovative therapeutic approaches (Zamparelli *et al*., 2016).

The particular effects of onion consumption have been explored in the treatment of NAFLD in experimental models of disease (El-Din *et al*., 2014; Emamat *et al*., 2016). Moreover, several hepatic markers have shown to be ameliorated by the consumption of onion products. For example, it has been demonstrated that the consumption of an onion peel extract containing high quercetin improves the hypoglycemic and insulin-sensitizing capacity by alleviating metabolic dysregulation of FFA, suppressing oxidative stress, and down-regulating inflammatory gene expression in liver in high fat diet/streptozotocin-induced diabetic rats (Jung *et al*., 2011). Red onion and onion powder (*Allium cepa* L.) improved the protective barriers against oxidative stress in Sprague-Dawley rats and suppressed oxidative stress and the formation of preneoplastic foci in diethylnitrosamine-induced rat hepatocellular carcinogenesis, respectively (Bang & Kim, 2010; Lee, Jung & Kim, 2012). The hepatoprotective effects of onion peel extracts have been indicated in CCl_4-induced liver injuries by reducing lipid peroxidation, improving blood cholesterol profiles and enhancing antioxidant properties (Chyun, Park & Yim, 2013).

In this sense, the beneficial effects of flavonoid consumption in NAFLD have been prominent in both animal and human studies. In general, the underlying protective mechanisms against hepatosteatosis have pointed an increased fatty acid oxidation in the liver and a decrease in fat deposition in visceral tissues and body weight. The antioxidant properties of flavonoids are also related to the inhibition of nuclear factor kappa B (NF-κB), attenuating the release of inflammatory cytokines and the increase in adiponectin, which improve insulin sensitivity, glucose tolerance, ameliorate dyslipidemia and blood pressure in patients with NAFLD (Akhlaghi, 2016). More specifically, the role of quercetin – as important flavonol present onion–, has reported favorable effects against hepatosteatosis in terms of decreased lipid accumulation and peroxidation in liver, plasma glucose, insulin

resistance, inflammatory cytokines, and hepatic enzyme levels in animal models of NAFLD and NASH (Kim *et al*., 2015; Vidyashankar, Sandeep Varma & Patki, 2013).

2. METABOLOMICS, NUTRIMETABOLOMICS AND FOODOMICS

Nutritional and food research is undergoing a remarkable transformation driven by new technologies. The complexity of the metabolome requires the application of advanced and comprehensive high-throughput approaches, which provide large amounts of data which need to be analysed and interpreted based on their dynamic biochemical networks. The interaction of food and its components in living organisms also modifies the metabolic responses, which can be measured in the metabolome by identifying and quantifying the metabolites (small molecule <1500 Da). These metabolites are considered the end products synthetized by the cellular regulatory processes (Bagchi, Lau & Bagchi, 2010; Lindon, Nicholson & Holmes, 2006).

Omics technologies have attracted much attention since they adopt a holistic view and have a broad range of applications. These differ from traditional studies, which are largely hypothesis-driven or reductionist.

The evolution of the omics cascade (Figure 1.8) reflects why metabolomics has gained such a considerable position in biomedical research. The reasoning was clearly exposed by Patty *et al*. 2012 in *Nature Reviews Molecular Cell Biology* with the following statement *"Whereas genes and proteins are subject to regulatory epigenetic processes and post-translational modifications, respectively, metabolites represent downstream biochemical end products that are closer to the phenotype. Accordingly, it is easier to correlate metabolomic profiles with the phenotype compared to genomic, transcriptomic and proteomic profiles"* (Patti, Yanes & Siuzdak, 2012). Therefore, the finality of studying the metabolome includes the discovery of biomarkers, but also the comprehension of the mechanisms of biological processes and from a nutritional perspective, an understanding of the role of food in health and disease.

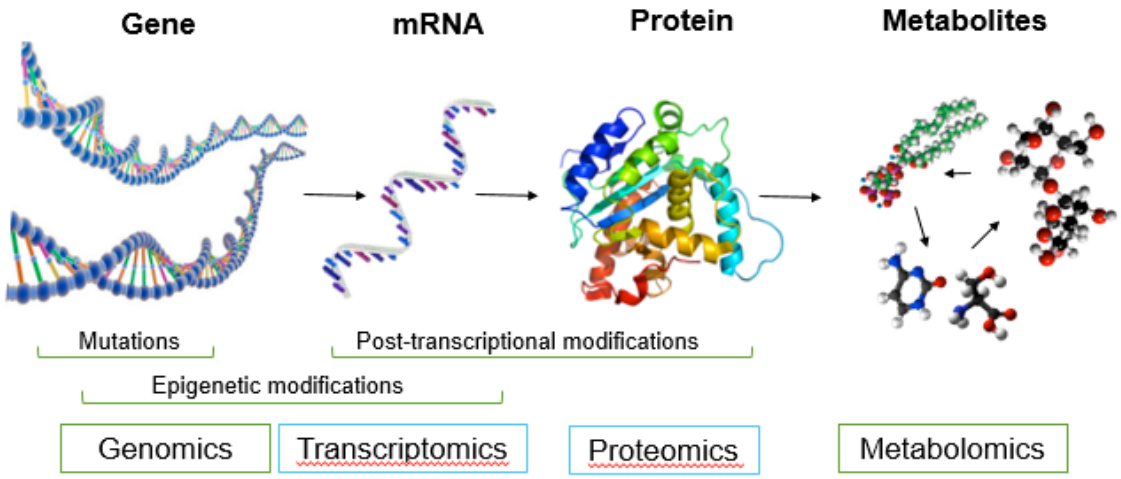


Figure 1.8. Flow of omics technologies in biomedical research

In this regard, the impressive development of omics technologies in combination with the interest in food science and nutrition have led to new and complementary disciplines, which coin new terms such as nutrimetabolomics and foodomics. Metabolomics, considered as the baseline of these metabolite studies, frequently overlaps in both objectives and methodologies with these current nutritional or food –omics categories, which can lead to a certain degree of confusion with the use of all these interchangeable terms. To clarify, a quick revision of the situation follows:

The term **"metabolomics"** was coined in the late 1990s, following the use of the term "metabolome" which was used for the first time in 1998 by Oliver *et al.,* (1998). Metabolomics aims to obtain the complete quantitative and qualitative analysis and identification of all metabolites present in an organism or a biological system, under a given set of conditions (Fiehn, 2001). Since its apparition, it has been the focus of multiple authors and has received increasing attention by the scientific community in nutritional studies [*e.g.* (Astarita & Langridge, 2013; Bagchi *et al*., 2010; Gibbons, O'Gorman & Brennan, 2015; Jones, Park & Ziegler, 2012; McGhie & Rowan, 2012; Rezzi *et al*., 2007; Scalbert *et al*., 2009; Wishart, 2008)].

In the years 2007-2009 the term **"nutrimetabolomics"** started to be used in some publications as an alternative term for metabolomics studies referring to nutritional interventions, while the use of the term **"foodomics"** also began to appear in different webpages and scientific meetings. However, a formal definition for foodomics was not published in an SCI journal until 2009, when Cifuentes (2009) outlined foodomics as a "*new*

discipline that studies the food and nutrition domains through the application of advanced omics technologies to improve consumer´s well-being, health, and confidence". Thus, the scope of nutrimetabolomics was limited to the discovery of new biomarkers in human nutritional research, which lead to a further classification into three main categories: I) *the assessment of nutritional and dietary interventions,* II) *the monitoring of diet exposure and food consumption* and III) *the phenotype and metabolic impact of diet on health* (Llorach *et al*., 2012). Whereas in comparison, the term foodomics was presented as a wider global discipline that brings together all working areas involving food, nutrition and advanced omics tools (Table 1.2) (Cifuentes, 2013).

However, a point in common to all these metabolomics-type studies is that they rely primarily on mass spectrometry (MS) coupled with chromatography or the use of nuclear magnetic resonance (NMR) generating huge amount of data.

Table 1.2. Areas covered by foodomics applying omics tools (genomics, epigenomics, transcriptomics, proteomics and metabolomics)

Nutrition	Gene-based differences among individuals in response to a specific dietary pattern
	Biochemical, molecular, and cellular mechanisms that underlie the beneficial or adverse effects of certain bioactive food components
	Effect of bioactive food constituents on crucial molecular pathways
	Identity of genes involved in the stage previous to the onset of the disease, and therefore possible molecular biomarkers
	Global role and functions of gut microbiome
Safety **Quality** **Traceability**	Effects in GM crops
	Stress adaptation responses of food-borne pathogens to ensure food hygiene, processing and preservation
	Use of food microorganisms as delivery systems including the impact of gene inactivation and deletion systems
	Comprehensive assessment of food safety, quality, and traceability ideally as a whole
Agronomy **Environmental factors**	Molecular basis of biological processes with agronomic interest and economic relevance such as the interaction between crops and pathogens, as well as physicochemical changes that take place during the ripening of fruit
	Postharvest phenomena through a global approach than links genetic and environmental response and identifies the underlying biological networks

Adapted text from Cifuentes (2013)

At the moment, in order to understand the biological meaning of the complex information obtained from metabolomics platforms and all complementary omics disciplines, the information is being encompassed into a bigger category called **"Systems Biology"**. Thus, Systems Biology has born as an integrative approach that aims to unify all information concerning molecules, cells, organs and individuals generated by genomics, proteomics and metabolomics, resulting in the most complex level of comprehension available today. Moreover, it is also considered pivotal for the development of rational intervention strategies for the prevention of disease in a nutritional context (Panagiotou & Nielsen, 2009).

2.1. Strategies for metabolomics screening

Different strategies are designed to obtain and handle the amount of data generated from metabolomics studies. These are divided into non-targeted and targeted studies. Non-targeted approaches are applied to hypothesis-generating studies, while targeted assays are usually applied to validations and to the confirmation of the novel discoveries after an initial hypothesis-generating study.

2.1.1. Non-Targeted studies

A non-targeted approach attempts to detect as many metabolites as possible in a given set of samples. The signal of each possible entity detected is assigned as a feature, which is noted as a peak area used to perform statistical analysis. The complete set of features constitutes a characteristic pattern of the studied biological system or situation. When the signal obtained for each pattern is compared among groups, unexpected changes may be observed and further interrogated until the structure can be identified. The biological interpretation is performed at the end of the experimental procedure and allows for the creation of new hypothesis, thus justifying the stage as "hypothesis-generating". However, one single analytical method cannot detect all the metabolites in a biological system. It is therefore desirable to combine multiple analytical approaches to maximise the number of metabolites detected and increase the coverage of the metabolome.

In this modality, there is no comparison with calibration curves constructed with chemical standards, so the annotations are referred to as *relative quantification*. However, the screening can be done against a comprehensive library of compounds whether or not, where unknown compounds may be new chemical structures in need of structural elucidation. Metabolite

identification is currently one of the major limitations and highly debated topics in metabolomics.

Currently, the platforms widely used in non-targeted metabolomics include proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, direct infusion mass spectrometry (DIMS), and mass spectrometry (MS) combined with a separation technique, for example GC–MS, LC–MS and CE–MS, which allow for the detection of metabolites of different chemical classes. However, there are multiple analytical techniques that can be applied in metabolomics studies (Table 1.3).

– *Metabolic Fingerprinting* is the most commonly used strategy in non-targeted studies. It is an unbiased global analysis, based on obtaining characteristic patterns considered as "fingerprints" of metabolites that change in response to disease or stress exposure, and also reflect the effects in the phenotype from multiple epigenetic factors such as environment, microbiome, diet, drugs, etc. The intention is not to identify each compound observed but to compare patterns, often NMR, mass spectra or chromatograms. This is performed using statistical tools such as hierarchical cluster analysis or multivariate analysis (Kosmides *et al*., 2013; Fiehn, 2002).

– *Metabolic footprinting* differs from metabolic fingerprinting because it does not rely on the measurement of intracellular metabolites. The strategy focuses on analysing and monitoring the changes in metabolites produced by the cells or tissues in the surrounding medium either by absorption or excretion. This approach is also called exo–metabolome or secretome and the growth medium produced in batch cultures is usually analysed by using DIMS (Hollywood, Brison & Goodacre, 2006; Kell *et al*., 2005).

Table 1.3. Summary of analytical techniques applied in metabolomics

Abbreviated name	Technique	References
^{1}H NMR	Proton nuclear magnetic resonance	Beckonert *et al*., 2007
(HR–MAS) NMR	High resolution–magic angle spinning NMR	Beckonert *et al*., 2010
LC–MS	Liquid chromatography–mass spectrometry	Zhou *et al*., 2012
UPLC–MS	Ultra–performance liquid chromatography–MS	Want *et al*., 2010
LC–EC	LC–electrochemical array	Gamache *et al*., 2004
LC–NMR	LC–nuclear magnetic resonance	Wolfender *et al*., 2003
HILIC–UPLC–MS	Hydrophilic interaction–UPLC–MS	Spagou *et al*., 2011

LC x LC–MS	Two-dimensional LC–MS	Guo *et al*., 2001; Donato *et al*., 2012
GC–MS	Gas chromatography–mass spectrometry	García *et al*., 2011
GC x GC–MS	Two-dimensional GC–MS	Mal *et al*., 2012
FTIR	Fourier transform infrared spectroscopy	Ellis *et al*., 2006
DIMS	Direct infusion–MS	Han *et al*., 2003
MALDI–MS	Matrix assisted laser desorption–MS	Milne *et al*., 2013; Vaidyanathan *et al*., 2006
FT–ICR–MS	Fourier transform ion cyclotron resonance–MS	Aharoni *et al*., 2002
CE–MS	Capillary electrophoresis–MS	Ramautar *et al*., 2009
CE–UV	CE–ultraviolet detector	Barbas *et al*., 2011
CE-LIF	Capillary electrophoresis-laser-induced fluorescence	Barbas *et al*., 2011

Table adapted from Hollywood et al. (2006) and *Villaseñor (2014)*

2.1.2. Targeted studies

A targeted approach analyses a relatively small and specific number of metabolites. These metabolites are chemically characterised and biochemically annotated with known biological importance at the start of the study, before data acquisition is performed. The methods used in a targeted mode have a greater selectivity and sensitivity than non-targeted methods. Quantification of the metabolites is performed through the use of internal standards and authentic chemical standards to construct calibration curves for each of the metabolites. In this case, sample preparation can be optimised to retain the metabolites of interest and to remove other biological species and analytical artefacts which are excluded from the downstream analysis. This simplifies the analysis of the data and interpretation of the biological significance.

The strategy is especially useful to answer well defined biological questions. However, a targeted study cannot be performed unless an authentic chemical standard of the target metabolite is available.

– *Metabolic Profiling* is the term utilised when target metabolites are selected previously and they are assessed using specific analytical methods. The metabolite search always focuses on the analysis of a group of metabolites related to a specific metabolic pathway or analyses metabolites with similarities in their chemistry (*e.g.* amino acids, FFA, etc.) (Dunn & Ellis, 2005; Hollyw*ood et al*., 2006).

2.2. Metabolomics in cardiovascular and liver disease: hypercholesterolemia, inflammation and oxidative stress

Among its multiple potential uses, the application of metabolomics in the area of prevention, diagnostic and prognostic of cardiovascular and liver diseases (and associated metabolic impairments) has occupied an outstanding position during recent decades.

Cardiovascular disease (CVD) is the first cause of death worldwide, representing the 31% of all global deaths (WHO, 2016). Although CVD may be partly prevented by addressing behavioural risk factors (*e.g.* tobacco, diet, physical inactivity, alcohol, etc.), it is expected that the prevalence of CVD will rise as the population ages. Therefore, the need for early detection and management in which more than one risk factor (like hypertension, diabetes or hyperlipidaemia) becomes a priority. In addition, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) have now been identified as the first cause of liver disease in numerous western countries. This disease confers additional risk to vascular dysfunction, the development of atherosclerosis and in final instance, cardiovascular-related mortality (Long *et al*., 2015).

As a result, the search for new cardiovascular biomarkers is an active area of investigation, in which metabolomics and proteomics offer useful approaches for potential biomarkers screening. This is due to their expected capability to improve the knowledge of disease and atherosclerosis-associated mechanisms, in diagnosis and the possibility to predict future cardiovascular events (Gerszten & Wang, 2008).

Metabolomics has been already applied in cardiology for the analysis of gene modifications, substrate selection or toxicity monitoring. Moreover, and given the metabolic basis behind cardiovascular events, changes in metabolic patterns have been used to detect blood plasma and urine metabolites highly correlated to CVD under specific conditions (atherosclerosis, myocardial ischemia and infarction or diabetic cardiomyopathy) (Griffin, Atherton, Shockcor, & Atzori, 2011). A recent review by Kordalewska & Markuszewski (2015) illustrates the potential of metabolomics to study pathomechanisms of cardiovascular-related disorders. In this work, the authors show a collection of studies related to the use of metabolomics in the analysis of atherosclerosis, prevention and diagnosis of coronary heart disease, stroke and heart failure, hypertension, atrial fibrillation, pulmonary heart disease, peripheral arterial disease, kidney and metabolic disorders. They also include a list of altered metabolites detected by untargeted and targeted analyses. The impact of diet in the onset of

disease also launches the applications of metabolomics in CVD. Overall, it is known that a given common dietary component not only influence the health status, but can also be converted by the gut microbiota into harmful metabolites linked to heart disease (Rak & Rader, 2011).

In parallel, a major effort is being made to use high-throughput metabolomics techniques to identify potential biomarkers for hepatic disease, which should be used when applied to clinical practice. Although no simple biomarkers have yet been validated, it has been stated that *"a metabolomic window has been opened into the changing biochemistry occurring in the transitional phases between a healthy liver and hepatocellular carcinoma or cholangiocarcinoma"* (Beyoğlu & Idle, 2013). Figure 1.9 represents the metabolic remodelling produced in the transition from a healthy liver (Phase 0) to liver carcinoma (Phase 3).

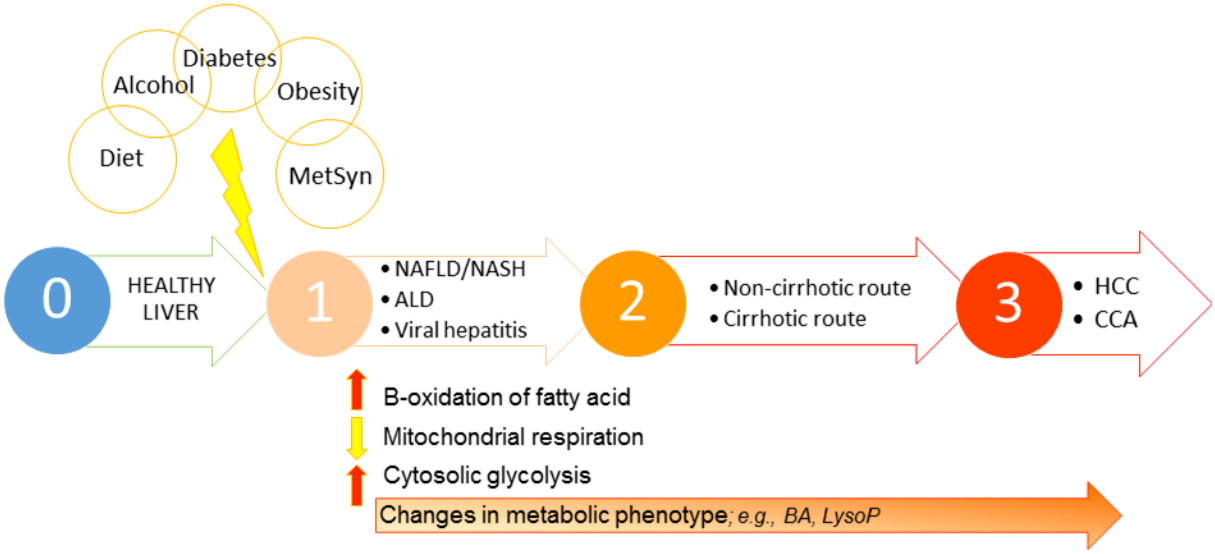


Figure 1.9. Metabolic remodelling induced as response to different stimuli in the progression of liver disease. *Adapted from Beyoğlu & Idle (2013)*

Different reviews have summarized the work done in metabolomics analysing the development of non-alcoholic fatty liver disease (NAFLD), including a broad spectrum of liver abnormalities such as non-alcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), alcoholic liver disease (ALD), viral hepatitis, cholecystitis, cholestasis, liver transplantation, and acute hepatotoxicity, in both human and animal models (Beyoğlu & Idle, 2013; Crişan *et al*., 2015; Holmes *et al*., 2015; Nie *et al*., 2016; Verhelst *et al*., 2013; Wang, Zhang & Sun, 2013).

Of the tools available for liver diagnosis, a liver biopsy gives definitive results, along with some approaches that combine several parameters to develop a predictive test. Nevertheless, these tools have a negative consideration due to the invasive nature, costs and risk of complications, as well as an incapacity to identify those patients at risk of NASH and advanced fibrosis (Ariz *et al*., 2010). These limitations open the way for new opportunities in the use of metabolomics-mediated phenotyping strategies taking advantage of the possibility to accurately identify and stratify early stages of liver disease (Holmes *et al*., 2015), as well as studying the underlying mechanisms by which nutrients and diet influence on NAFLD and progression into NASH (De Wit *et al*., 2012).

Common to these prevalent pathologies and for countless other health disorders is the impact of **hypercholesterolemia**, **oxidative stress** and **inflammation** which are either evaluated as individual risk factors or joined together in a complex network of related pathways. These three features, as previously presented, play a key role in the initial stages of atherosclerosis and hepatic impairments, and they have been highlighted as accelerators in the progression and deterioration of CVD and NAFLD.

Diet remains an important metabolic contributor to these pathogenesis, with a remarkable direct implication in: (I) the development of hypercholesterolemia (JAMA, 2001; Mannu *et al*., 2013); (II) the influence of oxidative stress, which is a key mechanism in vascular functions and liver damage (Ariz e*t al*., 2010; Santilli, D'Ardes & Davi, 2015); and (III) the modulation of inflammatory regulators and proinflammatory signaling cascades involved in atherosclerosis, liver inflammation and disease progression (Galland, 2010; Kritchevsky, 1976; Oliveira *et al*., 2016; Saita, Kondo & Momiyama, 2015; Sullivan, 2010). In addition, hypercholesterolemia and inflammation are closely linked processes, which are clearly implicated in non-alcoholic fatty liver disease and atherosclerosis (Kleemann *et al*., 2007). This explains why these multiple interactions are key objectives in the application of metabolomics studies. Therefore, metabolomics shows promise, monitoring relative risk and tracking the effect of treatments by associating the changes caused by hypercholesterolemia, oxidative stress and inflammation and both cardiovascular and liver diseases. These new possibilities will drive the basic understanding of the metabolic cascades which lead to pathophysiology.

2.3. Metabolomics for the evaluation of nutraceuticals/functional foods

Functional foods and nutraceuticals are natural or modified foods that claim to improve health, quality of life, or wellbeing. However, the evidence supporting a health claim must meet certain standards and these foods need to be fully evaluated to make sure they meet current scientific and regulatory standards. Thus, the objective of any evaluation is to obtain a body of sound and relevant scientific data and evidence. This is achieved firstly by discovering or verifying the relevant properties or functions and secondly, by being able to support the validity of different substance/health relationships. Metabolomics may be of help in supporting all the stages of research such as for example, the demonstration of safety (an essential requirement that must be satisfied before any further research into uses and applications of a functional ingredient) and/or the discovery of biomarkers.

2.3.1. Food safety and discovery of biomarkers

The studies conducted to support the safety of functional ingredients are product-specific and can include multiple analytical choices: compositional analysis, structure/toxicity analysis, evaluation of historical and intended exposure, animal studies, clinical/epidemiologic studies and evaluation of special considerations of potential adverse food or drug interactions (Kruger & Mann, 2003). Despite the complexity and regardless of the regulatory framework, nutraceuticals and functional ingredients may be substantiated by few basic concepts (Table 1.4).

Table 1.4. Basic concepts in the use of nutraceuticals/functional foods

I	A range of outcomes may be produced in the body at different levels of intake. Different dose levels may provoke responses ranging from insufficient physiological action passing through desirable therapeutic effects but then extend on to acute toxicity. It is important to be able to predict the result by understanding the pharmacologic activity and toxicological potential
II	The composition may vary from single component ingredients to complex extracts of either derived or processed products; it is essential to undertake analysis of the composition to determine the safety on a case-by-case basis
III	The intended use and potential exposure must be compared to a safe level of ingestion in accordance with the compound in question, historical exposure and/or scientific studies (animal toxicology, absorption, distribution, metabolism and excretion (ADME), clinical trials)
IV	The potential for contraindicated drug and food interactions should be determined when possible

Text adapted from Kruger & Mann (2003)

To support all these aspects with the purpose of ensuring the safety of the functional ingredients multiples approaches have been assayed. However, it is of relevant importance to highlight the role and possibilities played and offered by the application of metabolomics.

The metabolic analysis is a potentially powerful tool that can be used to address both applied and basic research questions. Likewise, metabolomics studies can participate in the demonstration of safety as well as being applied to obtain further information such as that concerning mechanisms of action, effectivity, or the discovery of new interesting biomarkers and compounds.

As early examples of these potential applications, the scientific publications describe the successful effects of the nutraceutical rosemary extract plus ω-3 PUFAs in diabetic children (Balderas *et al*., 2010); the analytical profiling of bioactive constituents from herbal products (Satheeshkumar *et al*., 2012); the metabolomics driven analysis of artichoke leaf and its commercial products (Farag *et al*., 2013); the determination of polyphenols in grape-based nutraceutical (Lopez-Gutierrez *et al*., 2016); or the metabolic fingerprint after acute and under sustained consumption of a functional beverage based on grape skin extract in healthy human subjects (Khymenets *et al*., 2015).

A special emphasis must be made on the applications of metabolomics for the discovery of dietary biomarkers (O'Gorman, Gibbons & Brennan, 2013). Two different approaches taken to explore for potential candidates are illustrated in Figure 1.10.

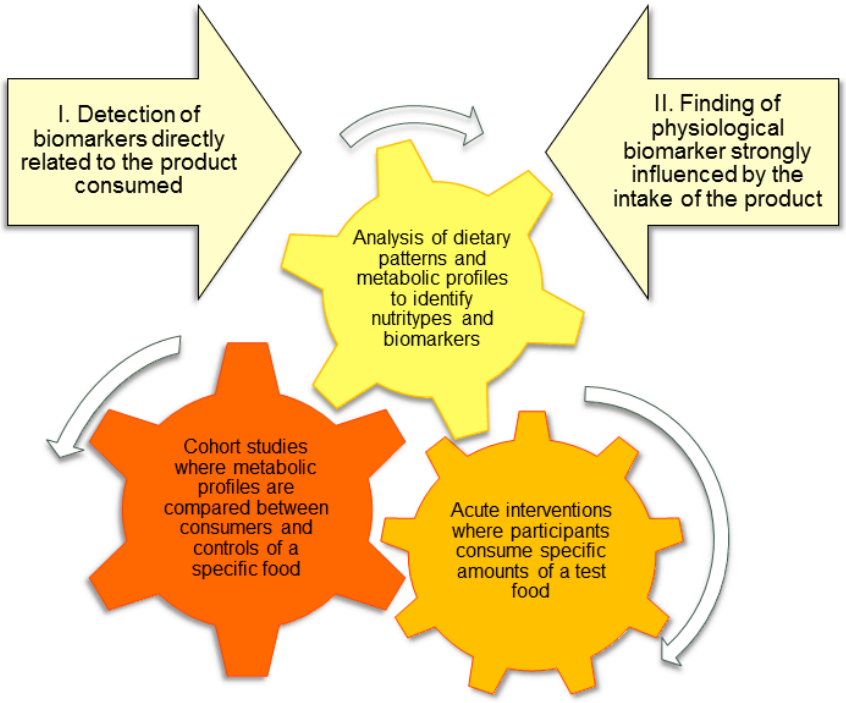


Figure 10. Metabolomics strategies followed in dietary biomarker discovery.
Adapted from O'Gorman, Gibbons & Brennan, 2013

It has to be mentioned that among the possible sources of functional foods, plants have been demonstrated to be of outstanding importance. In this area, metabolomics has been applied to demonstrate the general principle that a plant-based diet, based on cereals and rich in fruit and vegetables can reduce the risk of chronic disease (Andres-Lacueva *et al*., 2015; Rangel-Huerta & Gil, 2016). Likewise, metabolomics is used to examine the composition of foodstuff in specific phytochemicals and to demonstrate the properties of crops such as oats, soy, flaxseed, *Cruciferae* and *Solanaceae* family vegetables (such as onion, garlic, tomatoes), citrus fruits, berries (*e.g.* grapes, cranberries) as well as derived products including oils, juices and wine (Arbona, Iglesias & Gómez-Cadenas, 2015; D'Urso *et al*., 2016; O'Gorman, Gibbons & Brennan, 2013; Schmidtke *et al*., 2013; Simó *et al*., 2014; Song *et al*., 2014).

Other aspects facilitates by metabolomics are, for example, the possibility to predict sensory attributes of food and its applications in food authentication.

2.3.2. Challenges faced in nutritional metabolomics

First of all, it must remain clear that any metabolomic application has to face realistic challenges such as the fact that no unique technology will measure all metabolites present in a given biological sample. Each of the different technology platforms provides differential but also complementary information and each has its own advantages and disadvantages (Brennan, 2008). Likewise, the validation of the results in an independent study to confirm the discovery of a specific biomarker is a crucial step to substantiate the finding and confirm reliability and support further usage. Several factors such as lifestyle and physiological factors could distort the measure of biomarkers, taking into account that even the sampling procedure or the analytic methodology could be the cause of a trend and influence the results (Gibbons & Brennan, 2016).

More specific issues are the difficulties to interpret the information from different models and in particular to translate results from an animal model to humans. Equally variations within the human population also complicate any conclusions that may be drawn from results. All of which demonstrates how much there is still to be understood about the influence of genetic background, host–microbial interactions and other phenotypic characteristics such as body composition on metabolic profiles.

Other issues are the consideration of surrogate end points that do not always correlate with clinically important health benefits; the size of clinical studies and the lack of involvement of more than one centre will also considerably reduce the success of a study (Heyland, 2001).

Furthermore, the response to food is multifactorial, given that food delivers thousands of nutrients, phytochemicals and elements that will elicit multiple organ responses. Thus, the influence of diet and food ingredients with all the constituents on metabolic profiles should not be underestimated in the effects on many diseases. Finally, topping all the challenges facing the advancement of metabolomics in human nutritional research there is the ability to make a correct interpretation of the metabolites identified in a biologically relevant and meaningful manner (Gibbons & Brennan, 2016).

2.3.3. Nutraceuticals and functional foods to prevent or ameliorate cardiovascular and liver disease: opportunities offered by the application of metabolomics

Nutraceuticals and functional foods may be considered new complementary tools in the management of cardiovascular and liver disease. These bioactive products help handle and prevent serious risk factors such as hyperlipidemia or hypercholesterolemia using targeted strategies, especially those addressing lipid lowering or lipid profile improvement, *e.g.* reducing total cholesterol, triglyceride, and low-density lipoprotein cholesterol or elevating high-density lipoprotein cholesterol (Chen *et al*., 2014; Hunter & Hegele, 2017; Mannarino, Ministrini & Pirro, 2014). Likewise, the improvement of redox status (Serafini & Peluso, 2016; Xu *et al*., 2017), prevention of metabolic syndrome (Peluso, Romanelli & Palmery, 2014), reduction of obesity or management of insulin resistance syndrome, type 2 diabetes or NAFLD (Saez-Lara *et al*., 2016) are also areas where the use of bioactive products offer benefits.

Sharma and Singh (2010) showed examples of nutraceuticals with their corresponding benefits in different cardiovascular diseases and mechanisms of cardioprotective action. A recent review has evidenced, with a collection of *in vitro* and clinical studies, the potential cardiovascular benefits of nutraceuticals such as ω-3 and ω-6 PUFA, hydroxytyrosol, allicin, phytosterols, flavanols, vitamins C and E and dietary fibre, extolling the advantageous effects at different stages. Other less-consumed nutraceuticals including carnosine, coenzyme Q10, curcumin, lycopene, resveratrol, and berberine were also mentioned as possible therapies for atherosclerosis (Moss & Ramji, 2016).

Likewise, natural antioxidants for NAFLD such as phenolic compounds, *e.g.* flavonols (quercetin, rutin and troxerutin), flavanols (flavan-3-ols from green and black tea), polyphenols, anthocyanins, flavones/isoflavones (luteolin, baicalein, genistein, daidzein), flavanones (naringenin, hesperitin), etc. have been tested in *in vitro* and *in vivo* models of NAFLD and in patients with NAFLD (Salomone, Godos, & Zelber-Sagi, 2016).

All these applications and growing perspectives for the use of bioactive foods and new ingredients/nutraceuticals in the prevention of cardiovascular and liver disease, serve as the guide to conduct some of the new applications of metabolomics.

Therefore, metabolomics applied to the study of nutraceutical and/or functional foods shows potential in the following directions:

- Associating products or phytochemical consumption with biomarkers for intake and/or biomarkers of physiological response, allowing not only for the study of prevention but also regression in the state of disease.
- Identifying whether the cardio- or hepato-protective effects of an individual's diet are attributable to a specific compound or the result of a combination of elements.
- Elucidating the biochemical mechanism of action underlying the cardio- and hepato-protective effects.

In addition and most importantly, the use of metabolomics could guide research towards the answers to major issues that remain as yet unresolved and will be critical for the continuity in the use of nutraceuticals and functional foods, such as the analysis of the side effects, the dosage and associated mechanism of action, and the establishment of guide lines for efficient and effective usage.

3. METABOLOMICS APPLIED TO THE STUDY OF ONION

The evidence linking the functional properties of onion with positive human health effects have stimulated exploration of its uses as a potential regulator of health status and limiter of the onset and progression of disease. However, to advance into the possible applications, the investigation of both intrinsic and altered metabolites by consumption in both, *in vitro* and *in vivo* systems, are needed to elucidate the complex nutritional mechanisms underlying the simple action of adding onion to our diet with the objective of improving our dishes with some extra flavor.

Metabolomics may pave the way towards understanding the effects of onions by shedding new light on metabolite composition and its association with metabolite pathways altered by its consumption. For this reason, the possibilities of metabolomics in this field are divided into two separated blocks, firstly concerning the screening of the raw or processed product, and secondly for the final detection of changes in metabolites related to onion consumption and, when possible, in association with improved health status.

3.1. Metabolomics applied to the study of raw or processed onion

In recent years, the study of metabolomics has expanded into food analysis. The application of metabolomics in the study of onion has reported advantages at multiple levels, from the characterization of composition among cultivars to the discovery of substances and the prediction of sensory attributes.

In this sense, the increasing evidence that flavonols exert beneficial properties for human health has promoted the analyses of content and characterization of phytochemicals present in onion. Profiling and quantification of flavonols such as quercetin glucosides in onion varieties (*Allium cepa* L.) and treated or processed onion have been performed using capillary zone electrophoresis (CE) and high performance liquid chromatography procedures (HPLC-DAD and HPLC-ESI-MS) (Caridi *et al*., 2007; González-Peña *et al*., 2013; Pérez-Gregorio *et al*., 2011; Pérez-Gregorio *et al*., 2010; Rodrigues *et al*., 2009; Rodrigues *et al*., 2010; Sharma *et al*., 2014). Analysis of the distribution of flavonoids in the different parts of the onion bulb (inner, middle and outer layers) has also been determined using HPLC–DAD in 16 onion cultivars (Beesk *et al*., 2010).

On the other hand, the determination of volatile compounds in onion has been mainly performed by gas chromatography-mass spectrometry (GC–MS) (*e.g.* Colina-Coca, *et al*., 2013). Combination of liquid chromatography−Fourier transform ion cyclotron resonance-mass spectrometry (LC−FTICR-MS) with ^{13}C–Labeling has been also used for the chemical profiling of sulfur-containing metabolites in onion bulbs (Nakabayashi *et al*., 2013). Liquid chromatography electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) has been applied to study the changes in organosulfur compounds in onion and the quality of heat processing by analyzing the *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs) methiin, isoalliin, propiin, and cycloalliin after different heat treatments (boiling, frying, steaming, and microwaving) (Kim *et al.,* 2016). Table 1.5 summarizes a list of methods developed for the

analysis of volatile compounds from onion. Moreover, the metabolite profiling of volatiles in onion bulbs, analysed by GC–MS, has been proposed for the development of rule based models to distinguish infections produced by pathogens, such as *Erwinia carotovora* ssp. *carotovora*, *Fusarium oxysporum* and *Botrytis allii* (Prithiviraj *et al.*, 2004).

Table 5. Methods for the analysis of volatile compounds from onion

Technique	Full name	Reference
E/RSD	Extraction / Rapid spectrophotometric determination	Freeman & Whenham, 1975
E/HPLC CHIRAL	E / High performance liquid chromatography with chiral column	Bauer *et al.,* 1991
E/HPLC/1H-NMR	E / HPLC / Proton nuclear magnetic resonance	Block *et al*., 1992,1993
E/HPLC–UV (DAD)	E / HPLC–UV with a diode–array detection	Mondy *et al*., 2001
E/GC	E / Gas chromatography	Schmidt *et al*., 1996 Hong *et al*., 2000
SC-CO_2/GC–MS	Supercritical extraction with CO_2 / Gas chromatography– mass spectrometry	Sinha *et al*., 1992
SD/GC-MS	Steam distillation / GC–MS	Sinha *et al*., 1992
E/CRYOGENIC GC–MS	E / Cryogenic GC–MS	Block *et al.*, 1992,1993
SFE-MS	Supercritical fluid extraction–Mass spectrometry	Calvey *et al*., 1994
SFE/GC–MS	Supercritical fluid extraction / GC–MS	Calvey *et al*., 1994b
SFE/LC–MS	Supercritical fluid extraction / Liquid chromatography–MS	Calvey *et al*., 1997
E/GC–MS	E / Gas chromatography–mass spectrometry	Mondy *et al*., 2001 Zhang *et al*., 2014 Li *et al*., 2011
SPME/GC–MS	Solid phase micro extraction / GC–MS	Mondy *et al*., 2002 Soto *et al*., 2015
SPME/LC–MS	Solid phase micro extraction / LC–MS	Mondy *et al*., 2002
E/PTV–GC–AED	E / programmed temperature vaporization injection- gas chromatography– atomic emission spectrometry	Junyapoon *et al*., 1999
PTV–GC–ITD/MS	Programmed temperature vaporization injection– Gas chromatography with ion trap mass spectrometer	Junyapoon *et al*., 1999
E/HS GC–MS	E / Head space GC–MS	Colina-Coca *et al*., 2013
GSA	Gas sensor array	Li *et al*., 2009
E/DART	E / Direct analysis in real time	Li, 2012

Table updated from Lanzotti, 2012

At other levels of complexity, more comprehensive and integrative approaches have contemplated the analysis of large number of bioactive metabolites simultaneously. For example, the combined use of constrained total-line-shape ^{1}H NMR and LC–MS/MS in yellow onion and other different edible *Allium* species has permitted the identification and

quantification of amino acids, organic acids, sugars and flavonols (Soininen *et al*., 2014; Soininen *et al.,* 2012). Gas chromatography–mass spectrometry (GC–MS) has been used for the unbiased profiling of primary metabolites in plants and the characterization of cultivar and regional differences in the metabolite composition of onion bulbs (Kimura *et al*., 2014). GC–MS based metabolic profiling of onion has been used to demonstrate the existence of biochemical differences among 11 different onion varieties in India (Das *et al.,* 2015).

The integration of multiple omics approaches also allows for the interpretation of associations between metabolites and genes, improving the understanding of some metabolic response. Focusing on the study of onion, profiling of non-digestible carbohydrate products has been applied to a complete set of alien monosomic addition lines to explain the genetic controls over the metabolism, suggesting the possibility of controlling non-digestible carbohydrate content in the bulbs via assisted selections of the key candidate genes (Yaguchi *et al.,* 2009). Transcriptome and target metabolome obtained by liquid chromatography–tandem mass spectrometry (LC–MS/MS) provides insight into the adaptability to abiotic stress in doubled haploids of *Allium cepa* (Abdelrahman *et al*., 2015).

Furthermore, these technologies may be applied to the tracking of substances affecting the quality and safety of the onion products including issues related to manipulation. LC–MS/MS methods have been recently developed for the simultaneous determination of pesticides such as spinosad, thiacloprid and pyridalyl in spring onions and the estimation of their degradation time (Dasenaki *et al*., 2016). Likewise, with regard to the prevention of contamination in the consumption and manipulation of onions from elevated mycotoxin concentrations on the onion skins and the risk of airborne endotoxin exposure, the detection and quantification of mycotoxins has been streamlined with the use of LC–MS/MS in risk assessment (Mayer *et al*., 2016).

3.2. Detection of metabolites and effects related to onion exposure

The advance in the knowledge in food composition has also attracted the interest of further studies focused on the detection of specific substances in models *in vivo* and humans.

Initially, the studies addressing the consumption of onion in the identification of metabolites were directed at establishing the bioavailability of certain phytochemicals in bio-fluids such as urine and blood. Subsequent, studies were then carried out to detect and quantify the biomarkers of onion exposure. Finally, studies have been designed to detect both

outstanding and subtle changes in dietary patterns which leads to the suggestion of biomarkers affected and which are modulated in a positive manner by the consumption of onion.

Furthermore, the presence and inconvenience of chelating agents and the beneficial potential of synergistic effects can be considered present and future challenges to be addressed.

3.2.1. Onion bioactive compounds bioavailability and onion intake biomarkers

The interest in the metabolites derived from food such as onion or garlic has attracted attention for many years. This section summarises chronologically a set of relevant studies focused on metabolites derived from onion consumption.

In 1987, Jandke and Spiteller reported the presence in urine of *"unusual" peaks due to nutritional status*. GC–MS allowed for the detection of urinary organic acids after the consumption of onion and garlic, with the detection and identification of N-acetyl-*S*-(2-carboxypropyl)cysteine and N-acetyl-*S*-allylcysteine, which were described as "*metabolites of peptides introduced with onion or garlic*".

However, it was not until the late 90s when the advantage of omics allowed for the study in greater depth of the metabolites derived from onion metabolism. Flavonols such as quercetin and its derivatives along with sulfur compounds have been the main families of metabolites screened by a wide range of metabolic approaches.

Gross *et al*. (1996) developed one of the first high performance liquid chromatography (HPLC) based assay for quantification of quercetin metabolites in human urine. The platform included the metabolic products 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid), metahydroxyphenylacetic acid, and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid). Nevertheless, the production of these metabolites was not only derived from the diet but mainly produced by the metabolism of catecholamines and certain amino acids. In addition, the fact that multiple flavonols from an identical parent compound, quercetin, may yield several identical phenolic metabolites add complexity in the interpretation of human dietary studies.

The absorption and excretion of conjugated flavonols was tested in a study where five healthy volunteers were given 300 g of lightly fried yellow onions. Urine and plasma were

collected over a 24 h period and were analysed by HPLC and post-column derivatization. This study showed that there was a proportionally higher accumulation of isorhamnetin-4'-glucoside than quercetin conjugates, including quercetin-3,4'-diglucoside and quercetin-4'-glucoside, in the plasma and urine samples (Aziz *et al*., 1998).

The potential use of plasma concentrations and urinary excretion of quercetin and kaempferol as biomarkers of dietary intake was studied in humans fed with either black tea (1600 mL/d) or fried onions (129 g/d) for 3 d. Flavonols were determined by HPLC with detection by fluorescence. The authors concluded that "*flavonols in plasma and urine reflected short-term flavonol intake and that quercetin could distinguish between high and low flavonol consumption in epidemiologic studies"* (de Vries *et al*., 1998).

Noroozi *et al*. (2000) also concluded that the presence of flavonols in plasma and urine could possibly be used for the prediction of dietary flavonol consumption. The trial quantified the flavonols by reversed-phase HPLC in the diets and biological samples from ten patients with type 2 diabetes administered with either a low flavonoid diet or a diet supplemented with tea and 400 g fried white onion in olive oil with or without ketchup and herbs.

In 2001, Wittig *et al*. published a method for the identification of the quercetin glucuronides in plasma samples after the consumption of fried onion by means of HPLC–UV–MS/MS.

In 2004, Hong & Mitchell were able to identify twenty-one phase II metabolites of quercetin as conjugates, or mixed conjugates, of glucuronic acid, sulfate or a methyl group by LC/ESI-MS/MS in human urine after the consumption of 200 g of cooked onion. This study reinforced the potential of monitoring quercetin metabolites to obtain biomarkers to probe the individual response to dietary manipulation.

In a similar study, also using LC/ESI–MS/MS, the glutathione-related conjugates (mercapturic acids) were also observed. These mercapturic acid derivatives (dihydroxyacetic acid, dihydroxypropionic acid, hydroxycinnamic acid, dihydroxytoluene, and dihydroxybenzaldehyde) were degradation products of quercetin as a result of colonic microbial activity. Quercetin metabolites in human urine revealed evidence for the glutathionylation of quercetin. This was supported by the observation that mercapturic acids of common hydroxyphenylacetic acids generated by the microbial degradation of quercetin exist after the consumption of 200 g of cooked onion (~ 74 mg of quercetin) (Hong & Mitchell, 2006).

Plasma and urine from six volunteers after recent ingestion of lightly fried red onions (270 g) were analysed by reversed-phase HPLC with photodiode array and tandem mass spectrometric detection HPLC–DAD UV–MS^2. This was the first study using full scan MS^2 rather than HPLC using either MS^2 with selected reaction monitoring or single stage MS in the selected ion monitoring mode to analyse flavonol. This enabled the detection of twenty-three flavonols including sulphate, methyl, glucuronide and glucoside derivatives of quercetin (Mullen *et al*., 2004).

Not long afterwards, Mullen *et al*. (2006) designed a new experiment collecting plasma and urine over a 24 h period after consumption of lightly fried onions containing 275 mmol flavonol (principally quercetin-4'-glucoside and quercetin-3,4'-diglucoside). Samples were also analysed by HPLC-DAD–MS/MS detection, providing detailed quantitative concentrations of twenty-three metabolites of methyl-, glucuronyl- and sulpho-conjugates of quercetin in the plasma and urine of human subjects after ingestion of onions, thus revealing data of great importance in understanding the role of dietary flavonols in the prevention of chronic disease (Mullen *et al*., 2006).

In 2009, Winning *et al*. evaluated the metabolome of thirty-two rats following ingestion of onion by-products. Urine samples collected over 24 h were analysed using 1H NMR spectroscopy identifying dimethyl sulfone and 3-hydroxyphenylacetic acid as two dietary biomarkers of onion intake, possibly beneficial as a control in diet intervention studies.

An ultrahigh pressure liquid chromatography accurate mass quadrupole time-of-flight mass spectrometry with electrospray ionization (UHPLC-(ESI)QTOF MS/MS) method was also developed for measuring individual quercetin metabolites in human plasma. In this case, volunteers were either fed apple peel powder-enriched applesauce (AP) or onion powder-enriched applesauce (OP) for breakfast (Lee *et al*., 2012).

The profile of quercetin metabolites in pigs has also been studied. In this case, urine from seventeen pigs was collected and analysed by HPLC-MS/MS with similar results as those from humans after ingestion of dry onion skin (Wiczkowski *et al*., 2014).

More recently, a two-period, two-sequence cross over study in humans was conducted to compare the bioavailability of quercetin in the form of a fresh red onion meal (onion soup made from 100 g fresh red onion containing naturally glycosylated quercetin) compared with a dietary supplement (aglycone quercetin). Urine samples were collected over 24 h and quantified by LC-MS providing both a guideline for the design of future human studies when

using supplements and foods and facilitating a comparison of studies in existing publications (Shi & Williamson, 2015).

3.2.2. Detection of biomarkers influenced and regulated by onion consumption

The consumption of onion has been linked with the prevention and improvement of multiple disorders. In consequence, raw, cooked, onion juice, powdered onion flesh, onion skin or other onion extracts and by-products have been extensively studied in the search for associations between their consumption and the ameliorations of health status related markers.

The effects of onion consumption and some of its components have been tested as biomarkers of antioxidant, antihypertensive, hypolipidemic, hypercholesteraemic, antiinflammatory, antithrombotic, antipathogenic, antidepressant, antidiabetic, hepatoprotective and cardioprotective activities. However, the number of *in vivo* studies is limited. In this sense, it is remarkable how the study of metabolites by high-throughput technologies and statistical tools has facilitated the work detecting modifications and quantifying the magnitude of the effect provoked.

To sum up the evolution of this field, Table 1.6 summarizes in chronological order some of the findings obtained in *in vivo* studies through the use of diverse technologies, highlighting with an asterisk those studies where the detection of metabolites by the use of chromatography and mass spectrometry were indispensable.

The goal for most of these studies was to confirm a hypothesis by targeted driven approaches. Equally, most studies were directed at markers of risk with some influence or directly related to the development of cardiovascular disease and/or its associated impairments.

However, from a different perspective, studies of the modifications caused by the consumption of onion have scarcely been undertaken by non-targeted approaches. In this sense, the multi-metabolomics approaches presented in this PhD Thesis have been one of the first studies directed to reveal the influence of onion consumption at different levels of the metabolome, indicating new potential affected markers that may help create new hypothesis to focus further research.

Therefore, this PhD Thesis strengthens the use of metabolomics, which is pivotal to the research and evidences the advantages and possible applications of these techniques to

understand the effects of onion at a multi-organic level. This knowledge would help to establish new links with the modifications reflected in plasma, facilitating the generation of hypothesis to explain the close relationship between supplementation with onion and the benefits promoted in health, and at a more general level, the understanding of the relationship between diet and the progression of disease. Thus, the present work contributes to establish the basis to redirect further research assessing the effects of functional ingredients. It also shows the usefulness of considering metabolomics as a tool for early diagnosis and the management of health status by tackling risk factors, such as inflammation and other impairments associated to disease, by including functional ingredients at the core of personalized diets. These diets are expected not too far into the future to be designed to attend individual nutritional requirements and thus to achieve an optimal personalised nutrition.

Table 1.6. *In vivo* studies related to effects of onion consumption

Product	Model / Type of sample	Effect or marker studied	Authors
Raw / boiled Welsh onion juice	Male Sprague-Dawley rats / Blood-plasma	Antithrombotic effects	Chen *et al*., 2000
Cooked onion slices	Human (women, 20-21 years) / Blood-plasma	Accumulation of quercetin conjugates	Moon *et al*., 2000
Onion powder	Male Wistar rats / Blood-plasma	Effect of lipids and emulsifiers on the accumulation of quercetin metabolites	*Azuma et al*., 2003
Yellow and white chopped, dry-fried onions	Healthy human (men-women; 24-35 years) / Blood-plasma, lymphocytes	Effect of quercetin metabolites in COX-2 mRNA expression	de Pascual-Teresa *et al*., 2004
Green-leafy and white-sheath types of Welsh onion (*Allium fistulosum* L.)	Old male Sprague-Dawley rats / Blood-plasma, thoracic aorta	Antioxidative and antihypertensive effects	Yamamoto *et al.,* 2005
Yellow-type freeze-dried pulverized onion	Streptozotocin-Induced Diabetic Rats– Old male Wistar rats / Blood-plasma, urine, liver	Effect on oxidative stress biomarkers	Azuma *et al*., 2007*
Onion peel, flesh freeze-dried powder and ethanol extract	Male 16-months-old Sprague Dawley rats / Blood-plasma, kidney, liver	Effect on antioxidant status in aged	Park *et al.,* 2007*
Yellow-type onion 'Doctor Quercy' freeze-dried and pulverized	9-week-old male Wistar rats / Blood-plasma/serum, liver, kidney-urine	Antioxidative effect in high cholesterol-fed rats	Azuma *et al.,* 2010*
Powdered freeze-dried green or white type of Welsh onion	7-week-old male Sprague-Dawley rats / Blood-plasma, liver	Effect on hyperlipidaemia induced by high-fat high-sucrose diet	Yamamoto & Yasuoka, 2010*
Onion peels freeze-dried concentrated	Male healthy smokers (30-60 years) /	Effect on cardiometabolic risks	Lee *et al*., 2011b

powder (capsules)	Blood-plasma/serum		
Cold-water, hot-water, ethanolic extract (A. fistulosum)	Male BALB/c mice (CT-26 mouse tumor model) / Tumor tissue samples	Inhibition of colorectal tumor growth	Arulselvan *et al*., 2012*
Onion juice	Male Wistar rats / Blood-plasma, liver, kidney	Cadmium-induced organ toxicity and dyslipidaemia	Ige & Akhigbe, 2013
Onion peel extracts, freeze-dried powder in capsules	Healthy normal-weight women (20-25 year) / Blood-plasma/erythrocyte	Lipid profile and antioxidative status	Kim *et al*., 2013*
Onion peel extract, freeze-dried powder	Male Sprague–Dawley rats (high fat diet) / Blood-plasma/serum, adipose tissues	Antiobesity effects (3T3-L1 preadipocytes and adipogenesis)	Moon *et al*., 2013
Onion ingredient HPP-freeze-dried powder	Male Wistar rats (high-cholesterol diet) / Blood-plasma/erythrocyte, liver, adipose tissues	Fatty acid metabolism	Colina-Coca *et al*., 2014*
Onion skin powder	Male Sprague-Dawley rats / Blood-serum, cells	Modulation on BCRP and MRP 4	Lin *et al*., 2014*
Quercetin-rich onion skin extract in powder	Overweight-to-obese patients with (pre-) hypertension (25–65 year) / Blood-plasma/serum, urine	Blood pressure and endothelial function	Brüll *et al*., 2015*
Yellow onion juice	Healthy subjects (aged between 40 and 80) / Blood-plasma/serum/ erythrocytes	Modulation of oxidative stress in bone disorders	Law *et al.,* 2016
Quercetin-rich onion peel extract (capsules)	Overweight and obese subjects (males and females) / Blood, anthropometric measurement	Effects on the body composition	Lee *et al.*, 2016
Onion peel extract	Male C57BL/6J mice (high cholesterol diet) / Blood-serum, livers, faecal samples	Hypocholesterolemic effect	Kang *et al.,* 2016
Onion juice + cinnamon	Female Wistar rats exposed to power frequency electric and magnetic fields / Blood-serum	Sex hormones and total antioxidant capacity	Mansouri *et al.,* 2016
Onion powder	Sprague–Dawley rats (high-fat diet) / Blood-plasma, livers	Metabolic, histologic, and inflammatory features of NAFLD	Emamat *et al.,* 2016
Onion skin extract powder	Overweight-to-obese patients with (pre)hypertension / Blood-plasma/serum	**S**erum leptin and adiponectin concentrations	Brüll et al., 2016*
Onion peel derived extracts	4-week-old male C57BL/6 mice / Blood-plasma, WATs	Mechanisms of adipocyte browning using 3T3-L1 cells	Lee *et al.,* 2017
Onion powder	Male Sprague–Dawley rats (high-fat, high sugar diet) / Blood-plasma, livers	Prevention of NAFLD. Biochemical parameter, Hepatic TNF-α Gene Expression and histopathological features	Emamat *et al.,* 2017

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Chapter 2 – Research Questions and Objectives

RESEARCH QUESTIONS

1. *What would be the impact of an onion ingredient in the prevention of risk factors associated to the development of atherosclerosis and liver dysfunction?*

Objective 1 addresses the question. Alteration of cardiovascular and hepatic parameters, inflammation and oxidative stress biomarkers and the modifications of the vascular response in mesenteric microvessels are primary indicators of the onset of atherosclerosis and liver disease – **Research Papers I** and **II** revealed the positive effect of onion used as a functional ingredient in these biomarkers and the vasculature of hypercholesterolemic Wistar rats.

2. *Would metabolomics offers new insights for the study of nutraceuticals and the effects on health? More precisely, is a Wistar rat diet-induced model of hypercholesterolemia suitable to evaluate the preventive effect of processed onion intake on metabolic impairments?*

Objective 2 and **3** address these questions. The application of metabolomics in diet-related disease provides new insights for the elucidation of mechanism of action implicated in the pathologies and the potential detection of biomarkers – **Research Papers III** and **IV** show that a multi-metabolomic platform applied to describe the metabolic impairments caused by a high-cholesterol diet, evidenced the model's capability and described the impact caused by onion supplementation.

3. *The use of non-targeted approaches generate new hypothesis for the study of onion properties in vivo. How does processed onion affect plasma and liver profiles in hypercholesterolemia and associated metabolic impairments?*

Objective 3 and **4** address the question. The value of applying metabolomics has been demonstrated by indicating metabolites and showing pathways of interest modified by the supplementation with functional ingredients – **Research Papers IV** and **V** show the changes induced by the consumption of the onion ingredient in the plasma and liver metabolic profile of hypercholesterolemic Wistar rats.

4. *Are changes in lipid mediators honest indicators in the inflammatory process associated to hypercholesterolemia and could these changes be modulated by onion intake? If so, would the changes in lipid mediators be directly associated with the positive effect found in the vasculature and biomarkers of hypercholesterolemic rats fed with onion?*

Objective 5 and **6** address these questions. Lipid mediators are chemical messengers released in response to biological stress or injury, and they are also critical in resolving inflammation – **Research Paper VI** shows the changes in oxylipins and sphingolipids of hypercholesterolemic Wistar rats in different biological matrices and evaluates the effects of onion as a functional ingredient on those changes. The positive effects found in the vasculature and other biomarkers of hypercholesterolemic Wistar rats after onion consumption have not been associated to changes in oxylipins and sphingolipids in plasma. However, the modulation of some oxylipins in liver and sphingolipids in various tissues pointed to interesting changes.

5. *Does the measurement of faecal bile acids inform about the changes induced by the onion ingredient?*

Objective 7 addresses the question. Faecal bile patterns offers a snap shot at the endpoint of the metabolism that can contribute to the detection of important imbalances not only related to the hepatic and intestinal functions but also to other metabolic disorders – **Research Paper VI** shows the role of dietary onion on faecal bile acid content in hypercholesterolemic rats.

OBJECTIVES

Cardiovascular disease (CVD) and liver pathologies are prevalent diseases influenced by several factors, which can be conditioned and modified by nutritional interventions. The nutritional and bioactive properties of onion have a promising value in the prevention of some risk factors and the reduction of imbalances associated to these pathologies. However, the underlying mechanisms are complex and require novel high throughput analytical screening to reveal unsubstantiated alterations amenable to dietary intervention.

The **main objective** of this work is to explore the potential preventive effect of a high-pressure processed onion ingredient in cardiovascular and liver impairments associated to diet-induced hypercholesterolemia. To achieve this, the application of metabolic phenotyping initiates the exploration of both the mechanism of action underlying the hypercholesterolemic model and the search of altered metabolites and metabolic routes modified in response to onion consumption.

To achieve this goal the following **specific objectives** were established:

1. Assess inflammation, oxidative stress and vascular reactivity associated to hypercholesterolemia and evaluate the changes induced by onion supplementation.
2. Describe the metabolic changes induced in hypercholesterolemia by plasma non-targeteted fingeprinting using LC–MS, CE–MS, GC–MS analytical platforms.
3. Evaluate the changes induced by the onion ingredient in the metabolomic profile of plasma in hypercholesteromic Wistar rats.
4. Develop a protocol suitable for liver in non-targeted analysis to evaluate the changes induced by the onion ingredient in the metabolomic profile of liver in hypercholesteromic Wistar rats.
5. Analyse the oxylipin profiles and their potential implication in liver inflammation associated to hypercholesterolemia and describe the changes induced by onion supplementation.
6. Describe the changes in sphingolipids of different biological matrices in hypercholesterolemic Wistar rats and study the effects of onion as a functional ingredient on sphingolipid alterations.
7. Develop a targeted LC–MS method for the analysis of bile acids in faeces and evaluate the impact of onion consumption on bile acid excretion in hypercholesterolemic Wistar rats.

Capítulo 2 – Preguntas Científicas y Objetivos

PREGUNTAS CIENTÍFICAS

1. *¿Qué efecto produce el consumo de un ingrediente de cebolla en relación al desarrollo de aterosclerosis y la disfunción hepática?*

El **Objectivo 1** aborda esta cuestión. Alteraciones en parámetros cardiovasculares y hepáticos, así como en los biomarcadores de inflamación y estrés oxidativo y la modificación de la respuesta vascular en microvenas mesentéricas, actúan como marcadores del inicio de aterosclerosis y de las enfermedades hepáticas – Los **Artículos I** y **II** muestran el efecto positivo de la ingesta de cebolla, en forma de ingrediente funcional, en múltiples biomarcadores y en el sistema vascular de ratas Wistar hipercolesterolémicas.

2. *¿Contribuyen los estudios metabolómicos a generar conocimiento sobre los nutracéuticos y sus efectos en la salud? Concretamente, un modelo de hipercolesterolemia inducido por la dieta ¿puede resultar adecuado para evaluar los efectos derivados de la ingesta de cebolla procesada sobre el desarrollo de desequilibrios metabólicos?*

Los **Objectivos 2** y **3** abordan estas cuestiones. El empleo de técnicas metabolómicas en enfermedades relacionadas con la dieta permite generar conocimiento dirigido a la elucidación de los mecanismos implicados en las patologías y en la detección de sus correspondientes biomarcadores – Los **Artículos III** y **IV** muestran que una multiplataforma de análisis metabolómico ha permitido describir los cambios causados por una dieta rica en colesterol a nivel metabólico, evidenciado la capacidad del modelo para describir el impacto causado por la suplementación con cebolla.

3. *El empleo de técnicas de análisis no dirigidas contribuye a la generación de nuevas hipótesis en el estudio de las propiedades de la cebolla in vivo. ¿Cómo afecta el consumo de cebolla procesada en los perfiles plasmáticos y hepáticos de ratas hipercolesterolémicas y en los desequilibrios metabólicos asociados?*

Los **Objectivos 3** y **4** abordan esta cuestión. El potencial de la aplicación de técnicas metabolómica ha sido demostrado indicando metabolitos y rutas metabólicas de interés que han sido modificados por el consumo del ingrediente funcional – Los **Artículos IV** y **V** muestran los cambios inducidos por el consumo del ingrediente de cebolla en el plasma y el hígado de ratas Wistar hipercolesterolémicas.

4. *Cambios en mediadores lipídicos podrían actuar como indicadores clave en los procesos de inflamación asociados a hipercolesterolemia ¿Pueden dichos cambios ser modulados por la ingesta de cebolla? De ser así, ¿puede encontrarse una asociación directa entre los cambios en mediadores lipídicos y los efectos positivos descritos en el sistema vascular y ciertos biomarcadores de las mismas ratas hipercolesterolémicas alimentadas con cebolla?*

Los **Objectivos 5 y 6** abordan estas cuestiones. Los mediadores lipídicos actúan como mensajeros químicos liberados en respuesta al estrés y/o daño, además de jugar un papel crítico en la resolución de los procesos de inflamación – El **Artículo VI** muestra los cambios en oxilipinas y esfingolípidos en diferentes muestra biológicas de ratas hipercolesterolémicas, evaluando el efecto del consumo de cebolla en forma de ingrediente funcional en estos cambios. Los efectos positivos encontrados en el sistema vascular y en ciertos biomarcadores evaluados en las ratas hipercolesterolémicas, no han sido directamente asociados con cambios en oxilipinas y esfingolípidos determinados en plasma. No obstante, ciertas oxilipinas cuantificadas en el hígado así como esfingolípidos determinados en distintos tejidos, mostraron cambios interesantes.

5. La determinación de ácidos biliares en heces ¿podría proporcionar información sobre los cambios inducidos por el ingrediente de cebolla?

El **Objectivo 7** aborda esta cuestión. El perfil de ácidos biliares en heces ofrece información sobre la fase final de las reacciones del metabolismo, pudiendo contribuir a la detección de importantes desequilibrios relacionados con la función hepática, intestinal y otros desórdenes de carácter metabólico – El **Artículo VI** muestra el efecto del consumo de cebolla, como parte habitual de la dieta, en el contenido de ácidos biliares en heces de ratas hipercolesterolémicas.

OBJETIVOS

Las enfermedades cardiovasculares y hepáticas son patologías prevalentes, afectadas por múltiples factores, que pueden ser influenciados y modificados mediante intervención nutricional. Las propiedades nutricionales y bioactivas de la cebolla, resultan prometedoras en la prevención de factores de riesgo y la disminución de alteraciones asociadas a dichas patologías. No obstante, los mecanismos de acción involucrados son complejos y requieren del empleo de técnicas de screening analítico, que permitan descubrir las alteraciones potencialmente regulables a través de una modificación en la dieta.

El **objetivo principal** de esta Tesis es explorar el potencial efecto preventivo y la repercusión de un ingrediente de cebolla, procesado por altas presiones, en los desequilibrios cardiovasculares y hepáticos asociados al desarrollo de hipercolesterolemia, inducida por la dieta. Para alcanzar dicho objetivo se han empleado técnicas de fenotipado metabólico que han permitido iniciar la investigación de los mecanismos de acción involucrados en el modelo de hipercolesterolemia, y en la búsqueda y detección de metabolitos y de las rutas metabólicas alterados y modificables en respuesta al consumo de cebolla.

Con esta finalidad, se establecieron los siguientes **objetivos específicos**:

1. Evaluar marcadores de inflamación, estrés oxidativo y la reactividad vascular, asociados a hipercolesterolemia, evaluando los cambios inducidos por la suplementación con cebolla.
2. Describir los cambios metabólicos inducidos en plasma, con la aplicación de técnicas de análisis no dirigidos (fingerprinting) y con el empleo de distintas plataformas analíticas LC–MS, CE–MS, GC–MS.
3. Evaluar los cambios inducidos por el ingrediente de cebolla en el perfil metabólico del plasma en ratas Wistar hipercolesterolémicas.
4. Desarrollar un protocolo para efectuar el análisis no dirigido de tejido hepático, y posteriormente evaluar los cambios inducidos por el ingrediente de cebolla en el perfil metabólico del hígado en ratas Wistar hipercolesterolemicas.
5. Analizar el perfil de oxilipinas y su potencial implicación en la inflamación hepática asociada a hipercolesterolemia, describiendo así los cambios inducidos por el consumo de cebolla.

6. Describir los cambios en esfingolípidos en diferentes muestras biológica en ratas Wistar hipercolesterolemicas, estudiando los efectos del consumo de cebolla en las modificaciones del perfil de esfingolípidos.
7. Desarrollar un método de análisis dirigido a la cuantificación de ácidos biliares en heces, evaluando así el impacto del consumo de cebolla en la excreción de ácidos biliares en ratas Wistar hipercolesterolemicas.

Chapter 3 – Overview of the Experimental Work

OVERVIEW OF THE EXPERIMENTAL WORK

OVERVIEW OF THE EXPERIMENTAL WORK

This PhD Thesis is based on the evaluation of the metabolic-driven impact of processed onion in the prevention of cardiovascular and liver diseases. The research has been conducted at the following research centres:

(i) the research group Quality and Functionality of Plant Foods (BIOACTIVEG) at the Department of Food Characterization, Quality and Safety at Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), Madrid, Spain.

(ii) the group of research in Vascular Pharmacology and Metabolism at the Department of Pharmacology, Faculty of Medicine, Autonomous University of Madrid (UAM), Madrid, Spain.

(iii) the Center for Metabolomics and Bioanalysis (CEMBIO) at the Faculty of Pharmacy, San Pablo CEU University, Madrid, Spain.

(iv) the Integrative Molecular Phenotyping (IMP) group at the Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden.

A brief overview of the experimental work undertaken for the realization of this PhD Thesis is presented below, showing the process to obtain the onion product and summarizing the techniques and analysis performed. Detailed information and descriptions about the analytical platforms and the methodologies, together with the interpretation of the results, are presented in the corresponding "research paper" within each chapter.

Firstly, the production of the functional onion ingredient used during the animal experiment and its characterization was carried out at the ICTAN-CSIC. The selection of this onion product was based on a previous work addressing the effects of high-pressure processing (HPP) and stabilization procedures in *Allium cepa* L. var. *cepa* 'Recas'. These findings have already been published and form part of the PhD Thesis *"Evaluación de las propiedades antiiflamatorias, antioxidantes e hipolipidémicas de cebolla procesada como ingrediente funcional in vitro y en un modelo animal"*. Thus, the present PhD Thesis continues the study initiated to obtain and characterize a stable functional onion ingredient, rich in bioactive compounds, by testing its properties and functionality *in vivo*.

1. Production of the onion functional ingredient and diets

Briefly, onion (*Allium cepa* L. var. *cepa*, 'Recas') was processed by HPP and stabilized by freeze-drying and pulverization. Figure 3.1 of the workflow summarizes the steps followed to obtain the functional ingredient.

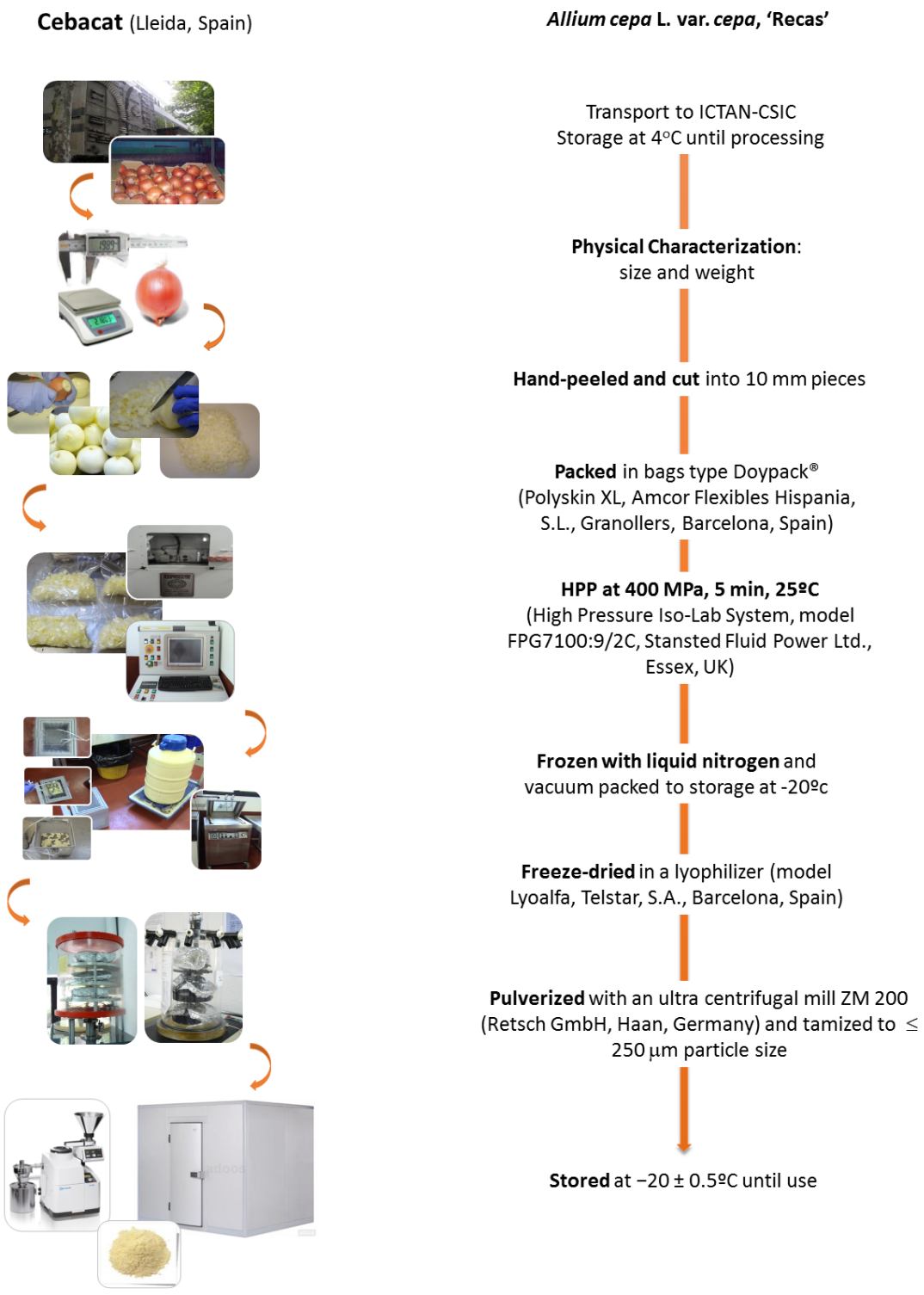


Figure 3.1. Workflow to obtain a stable high-pressure processed onion ingredient

An approximate amount of 2000 g of onion powder was prepared for the *in vivo* study and kept at -20ºC until use. This onion powder was characterized by determining its nutritional composition (protein, lipids, carbohydrates, total fructans and dietary fibre–soluble/insoluble), phytochemical content (total phenols, flavonoids, total ACSOs, sulphur-compounds, ascorbic acid and total vitamin C) and *in vitro* antiinflammatory and antioxidant activities (hyaluronidase inhibiting activity, $NO^{\bullet}$, $ABTS^{\bullet+}$, and $DPPH^{\bullet}$ scavenging capacity, ferric reducing antioxidant power, and antioxidant capacity by photochemiluminescence). The detailed composition of this ingredient, and the methodologies utilized for the analysis, can be found in the corresponding research papers.

Commercial rat pellets and three semi-synthetic diets based on the AIN-93M semi-purified rodent diet were ordered from Panlab, SLU (Barcelona, Spain). The final experimental diets were prepared weekly by adding the corresponding amount of cholesterol, cholic acid and the onion ingredient and kept at 4ºC. The prepared diets were portioned into individual daily bags to record the consumption during the experiment. It is important to mention that the amount of maize starch in the diets was adjusted in each diet to compensate for the addition of cholesterol, cholic acid and the onion powder.

Table 3.1. Experimental diets *(detailed composition described on the research papers)*

I	Control (**C**) diet	composed of a homogeneous mixture of 100 % rodent diet
II	High-cholesterol (**HC**) diet	control diet with 2 % cholesterol and 0.5 % cholic acid
III	High-cholesterol enriched with onion (**HCO**) diet	HC diet with 10 % onion powder, balancing the dietary fibre with cellulose powder

2. Animal model and experimental design

The present study was approved by the Spanish Ministry of Science and Innovation Advisory Committee [project AGL2010-15910 (subprogram ALI)] and by the Ethics Committee of the Complutense University of Madrid (Spain). All experiments were performed in compliance with the Directive 2010/63/UE regarding the protection of animals used for scientific purposes. Twenty four male Wistar rats with a body weight of approximately of 250 g were obtained from Harlan Laboratories Models (Harlan, SL, Barcelona, Spain).

The animals were fed for 3 days with the commercial rat pellets for adaptation to environmental conditions and then distributed into three groups of eight animals each and each group with approximately the same average body weight. The rats were housed

individually in metabolic cages, in a temperature-light controlled room and fed on the control diet for 4 days. After this time, the experimental feeding period began and lasted for seven weeks, during which water and food were provided daily *ad libitum* (Figure 3.2).

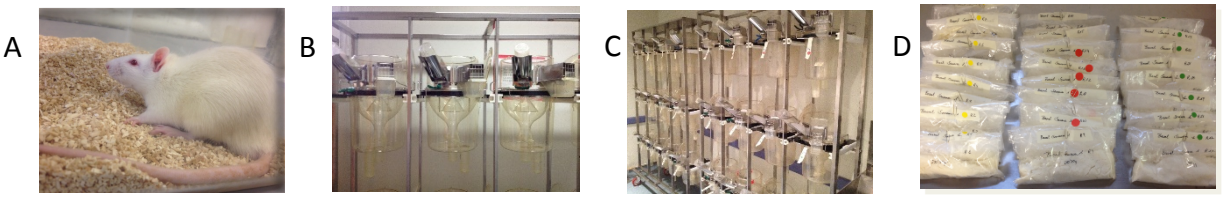


Figure 3.2. Experimental design A) 8 week-old Wistar rat in a regular cage B) Metabolic cages C) Rats in metabolic cages in a temperature/light controlled room D) Bags of the daily portions of the three different experimental diets

In order to evaluate food digestibility, food intake and faecal weight were recorded daily and body weight was recorded weekly. Individual volume of urine was also checked daily. Both faeces and urine (with the addition of NaN_3) were kept at –20 ºC (Figure 3.3).

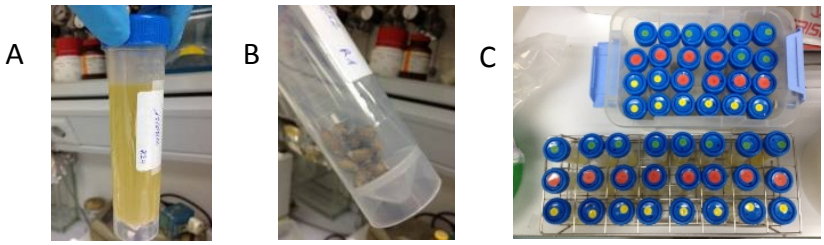


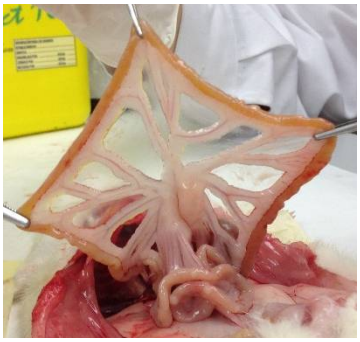
Figure 3.3. Sample collection. A) Urine collected from one rat over a 24 h period B) Faeces from one rat after 24 h C) Complete set of samples of urine and faeces as collected each day

At the end of the experimental feeding, in order to avoid inter-assay variations that could affect the comparison of data from the different groups, animals in fasting conditions were anaesthetized and euthanized by extracting blood by cardiac puncture, taking one animal at a time randomly, from each of the three groups.

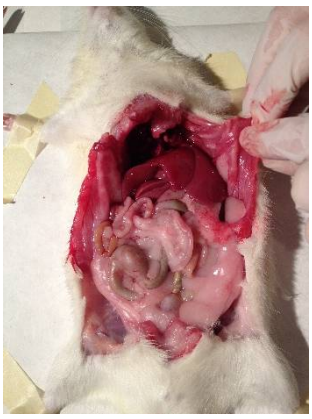
Blood was collected from the heart with a syringe and injected into tubes with EDTA as anticoagulant.

Plasma was recovered after centrifugation (1500 *g*, 15 min) at 4ºC and immediately stored in aliquots at −80ºC until analysis.

Erythrocytes fraction was also kept at −80ºC.



Mesentery and omentum specimens were obtained and placed into ice-cold Krebs-Henseleit solution (KHS; composition in mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 24.9, glucose 11, KH_2PO_4 1.2 and EDTA 0.027). Samples were transported to the laboratory at the Faculty of Medicine (UAM) for dissection of mesenteric arteries within 2 h of collection.



The other organs and tissues (liver, spleen, heart, kidneys, lungs, caecum, intestine, testicles, brain and adipose tissue) were collected, the fat removed and weighed before being frozen in liquid nitrogen and stored at −80ºC in sterile material until analysis.

A representation of the rat anatomy is presented in Figure 3.4, indicating the organs collected and used in the metabolomics studies.

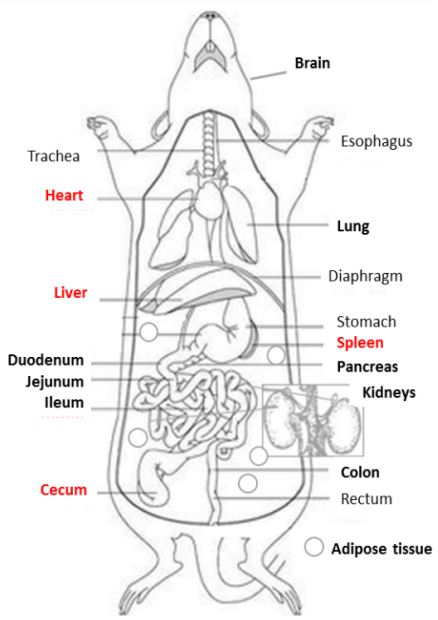


Figure 3.4. Scheme of the rat physiology, showing in bold all organs collected and in red the organs utilized for either targeted or non-targeted studies presented in this PhD Thesis

3. Analysis and techniques applied to address the *in vivo* functionality of the processed functional ingredient of onion

The work has been divided into three sections organized according to the sequence of research and the analytical methodology followed. A general overview of the research papers generated from these studies can be found in Table 3.3.

3.1. SECTION I: Biochemical, vascular and microbiological analyses

Biochemical parameters, enzyme activities, vascular reactivity and microbiological modifications were evaluated. More detail descriptions about the methodology followed for each analysis is detailed in the corresponding chapters.

3.1.1. Biochemical Analysis

3.1.1.1. Lipid profile: Total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C) and triacylglycerols (TG) in plasma were measured in a COBAS INTEGRA 400 plus system (Roche Diagnostics Ltd., Rotkreuz, Switzerland). The HDL-C and LDL-C fractions were obtained using precipitating reagent (SPINREACT, SA/SAU, Girona, Spain).

3.1.1.2. Other biochemical parameters: Glucose, urea, uric acid, creatinine, albumin, alanine aminotransferase (ALT/GPT, EC 2.6.1.2), aspartate aminotransferase (AST/GOT, EC 2.6.1.1), gamma-glutamyl transpeptidase (GGT, EC 2.3.2.2) and total bilirubin were measured in rat plasma samples using a COBAS INTEGRA 400 plus system (Roche Diagnostics Ltd., Rotkreuz, Switzerland).

3.1.2. Oxidative stress parameters

3.1.2.1. Antioxidant enzyme activities

Superoxide dismutase (SOD, EC 1.15.1.1), Catalase (CAT, EC 1.11.1.6), Glutathione peroxidase (GPx, EC 1.11.1.9) activities in erythrocytes and liver were determined using Assay Kits. Total protein content in the liver homogenates was also measured using a commercial protein assay kit (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories).

3.1.2.2. Protein carbonyl content

Protein carbonyls were measured in liver homogenates (Cayman Protein Carbonyl Colorimetric Assay Kit, 10005020).

3.1.2.3. NADPH oxidase activity

The activity of NADPH oxidase (NOX) was measured in mesenteric microvessels by lucigenin-derived chemiluminiscence.

3.1.2.4. Other measurements of antioxidant activity

2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) scavenging capacity and ferric reducing antioxidant power (FRAP) in plasma and caecum were measured by methods adapted to 96-well microplate format in the laboratory.

3.1.3. Inflammation and cardiovascular risk biomarkers

Interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon-gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), leptin, monocyte chemoattractant protein-1 (MCP-1) and vascular endothelial growth factor (VEGF) were determined in plasma using a Rat Cytokine/Chemokine magnetic bead panel kit (RECYTMAG-65K; EMD, Millipore Corp, Missouri, USA) and measured using a Luminex xMAP® bead-based technology.

The cardiovascular biomarkers plasminogen activator inhibitor 1 (PAI-1), tissue inhibitor of matrix metalloproteinases type 1 (TIMP-1) and von Willebrand Factor (vWF) were determined with a Milliplex® Map Rat Cardiovascular Panel 1 (RCVD1-89K).

The adhesion molecules, soluble intercellular adhesion molecule-1 (sICAM-1) and soluble E-selectin (sE-selectin), were also quantified in rat plasma with Milliplex® Map Rat Cardiovascular Panel 2 (RCVD2-89K), (EMD, Millipore Corp, Missouri, USA).

3.1.4. Vascular reactivity

Arterial ring segments (2 mm long) from the third branch mesenteric arteries were mounted on microvascular wire myographs (J.P. Trading, Aarhus, Denmark) for isometric tension recordings. Contractile and relaxation responses to K^+ and noradrenaline were determined as well as the response to cumulative additions of acetylcholine. The effects of superoxide scavenging and inhibition of NOX on endothelium-dependent and -independent

responses were determined with TEMPOL or apocynin, and sodium nitroprusside, respectively.

3.1.5. pH and microbiological analyses

pH of the caecal content was measured using a microelectrode (Crison micropH2000).

The bacterial charge and the microbiological culture used for their growth are specified in Table 3.2. Colony-forming units were defined as colonies measuring at least 1 mm in diameter.

Table 3.2. Determinations of bacterial charge estimated to determine the prebiotic effect

Bacteria type	**Agar type / T[a]**	**Agar type Specifications**
Total aerobic bacteria count	PCA / 30ºC	Plate count agar; Cultimed-Panreac, Spain
Enterobacter spp.	VRBG / 30ºC	Violet Red Bile Glucose agar; Cultimed-Panreac, Spain
Lactobacillus spp.	MRS / 30ºC	Man, Rogosa and Sharpe agar; Merck, Germany
Bifidobacterium	MRS / 37ºC	Man, Rogosa and Sharpe agar; Merck, Germany
β−glucuronidase (+) *Escherichia coli*	Coli ID / 47 ºC	ChromID Coli; Biomérieux, España S.A.
Total coliform bacteria count	Coli ID /47 ºC	ChromID Coli; Biomérieux, España S.A.
Sulphite-reducing *Clostridium* spp.	SPS / 46ºC	Sulphite Polymixin Sulfadiazine agar; Cultimed-Panreac, Spain

3.2. SECTION II: Non-targeted metabolomics studies: Fingerprinting

In order to describe the metabolic impairments associated to hypercholesterolemia and the potential modulating effects of the onion ingredient, the metabolic profiles of plasma and liver of the C, HC and HCO-fed rats were analysed by a multiplatform approach for metabolic fingerprinting using three different techniques: LC−, CE− and GC− separation techniques coupled to mass spectrometry. The general sequence followed in non-targeted metabolomics is summarized in this section.

As no single analytical approach can capture all metabolites in one sample, the following metabolomics platforms were applied to obtain a greater coverage of the metabolome in the Wistar rats.

3.2.1. LC-MS Analysis

LC–QTOF–MS system, composed of a UHPLC system (Agilent 1290 Infinity LC System) coupled with a Q-TOF LC/MS (6550 iFunnel) system (Agilent Technologies), was used to analyse plasma samples.

Agilent 6550 iFunnel Q-TOF LC/MS System

LC–QTOF–MS system, composed of a UHPLC system (Agilent 1200 LC System) coupled with a Q-TOF LC/MS 6520 system (Agilent Technologies), was used to analyse liver tissue samples.

Agilent 6520 Accurate-Mass Q-TOF LC/MS System

These LC-MS platforms were run in negative and positive ionization mode, allowing for the analysis of a wide range of metabolites, in particular non-polar and semi-polar compounds, as well as compounds that are either non-volatile or difficult to volatize and therefore undetectable by GC-MS.

3.2.2. CE-MS Analysis

CE–TOF–MS accomplished by an Agilent 7100 Capillary Electrophoresis system coupled to a TOF mass spectrometer (6224 Agilent), was used to analyse plasma and liver tissue samples.

Agilent 7100 CE/6224 TOF LC/MS system

This platform targets the separation of small, water-soluble ions, mainly amino acids.

3.2.3. GC-MS Analysis

GC–EI–Q–MS composed of an Agilent GC system–7890A interfaced with a 5975C inert mass spectrometer with a triple-Axis detector (Agilent Technologies), was used to analyse plasma and liver tissue samples.

Agilent 7890A GC–5975C Q-MS System

The analytical approach includes low-polarity volatile metabolites of lipids and esters, and high-polarity metabolites of amino acids and organic acids converted into volatile derivatives.

General workflow for non-targeted metabolomics studies

The general workflow followed in non-targeted metabolomics studies can be schematized in five principal stages (Figure 3.5). Each of these stages can be split in several steps according to the material used, analytical platform and objective of the study. As a step previous to the analyses, the preparation and use of quality control (QC) samples must be considered. These QCs are required for the extraction procedure and to follow and monitor the analytical procedure.

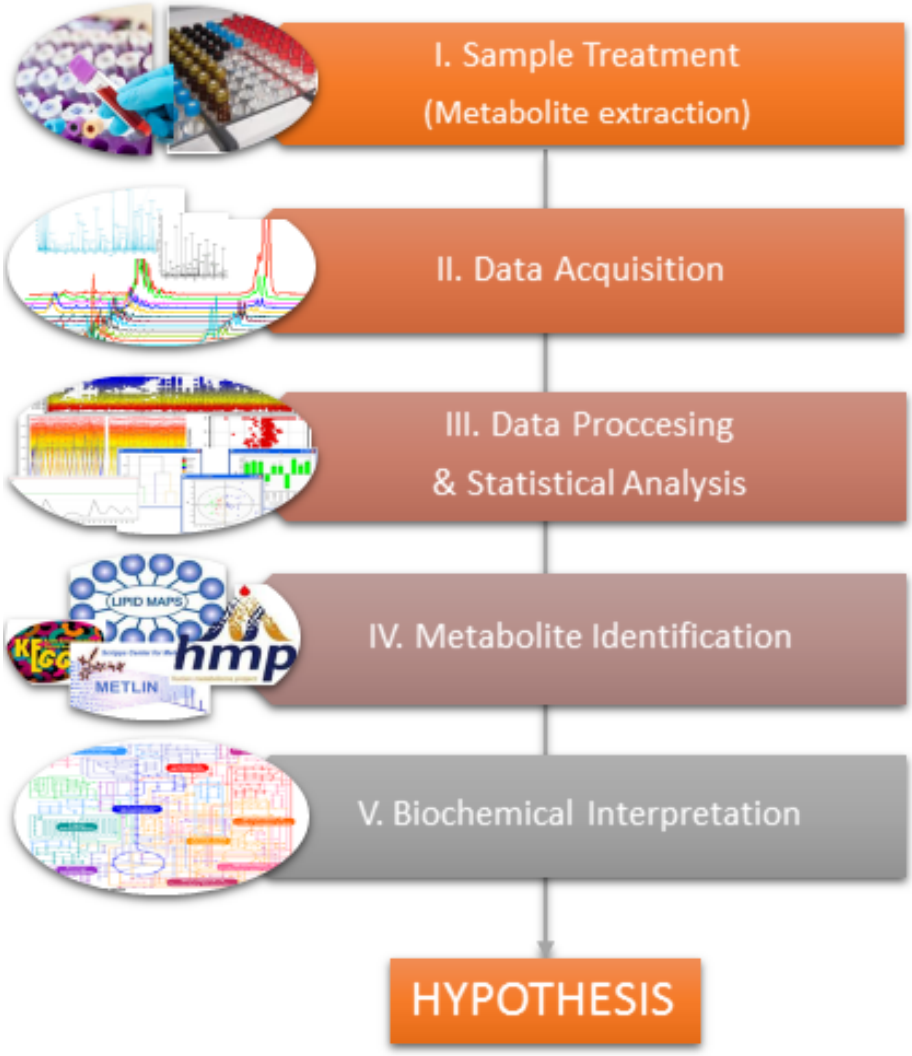


Figure 3.5. Workflow applied in non-targeted metabolomics studies

(I) Sample extraction follows standard protocols in order to obtain the greatest response to a maximum number of metabolites, which can be measured by the selected analytical platform in the next step.

(II) According to the objective, the treated samples are run (simultaneously or not) in optimized platforms (method, column, conditions, etc.) to acquire data for screening.

(III) A molecular feature extraction is performed by the use of software, creating raw data for further processing. This processing combines several steps (normalization, scaling, filtering, cleaning, etc.) in order to obtain reliable and manageable data. Once cleaned, data are subjected to multivariate analysis and several chemometric tools.

(IV) Metabolites statistically indicated as potential markers in the study allow for the creation and discrimination of models. The distinct metabolites are further investigated to be identified chemically or structurally through the exploration and comparison of databases, libraries, MS/MS and/or injection of standards.

(V) After metabolite identification, the compounds are related to their corresponding biochemical pathway and their regulation is interpreted in a biological context.

The interpretation of these results leads to the next stage of hypothesis generation, which relies on a further validation of these results.

3.3. SECTION III: Targeted metabolomics studies: Profiling

The process of inflammation in the course of hypercholesterolemia and the assimilation of nutrients are outstanding sources of information to elucidate the mechanism of action involved in the onset and progression of atherosclerosis and liver impairments. Lipid mediators, including oxylipins and sphingolipids, as well as bile acids, some of which were indicated by the non-targeted approaches, were selected for analysis and determination through the application of targeted approaches.

Thus, three different LC–MS approaches were optimized to address the changes in these specific pathways from different biological samples. The general workflow utilized in target studies can also be found in this section.

3.3.1. LC–MS Quantitative Analysis of oxylipins

ACQUITY UPLC System/Xevo® TQ-S

UPLC-MS/MS composed of an ACQUITY UPLC System and Xevo® TQ-S (Waters) in negative ESI mode, was used for the analysis of ω-6 (AA-, LA-, DHGLA-derived) oxylipins and ω-3 (α-LA-, EPA- and DHA-derived) oxylipins in plasma and tissues (heart, liver, spleen and brain).

3.3.2. LC–MS Analysis of sphingolipids

ACQUITY UPLC System/Xevo® TQ MS

UPLC-MS/MS composed of an ACQUITY UPLC System and Xevo® TQ (Waters) in negative ESI mode, was used for the analysis of sphingolipids (sphingoid bases, ceramides, sphingomyelins, hexosylceramides, lactoylceramides) in plasma and tissues (heart, liver, spleen and brain).

3.2.3. LC–MS Analysis of bile acid

Agilent 1200 LC MSD 6530A-QTOF MS

HPLC-MS/MS, composed of an Agilent 1200 LC MSD System and 6530A-QTOF accurate mass (Agilent) in negative ESI mode, was used for the analysis of bile acids in faeces.

General workflow followed in targeted metabolomics studies

The general workflow utilized in target studies can be outlined as follows (Figure 3.6). Identified compounds or a family of chemically or pathway related metabolites are defined as targets of the study.

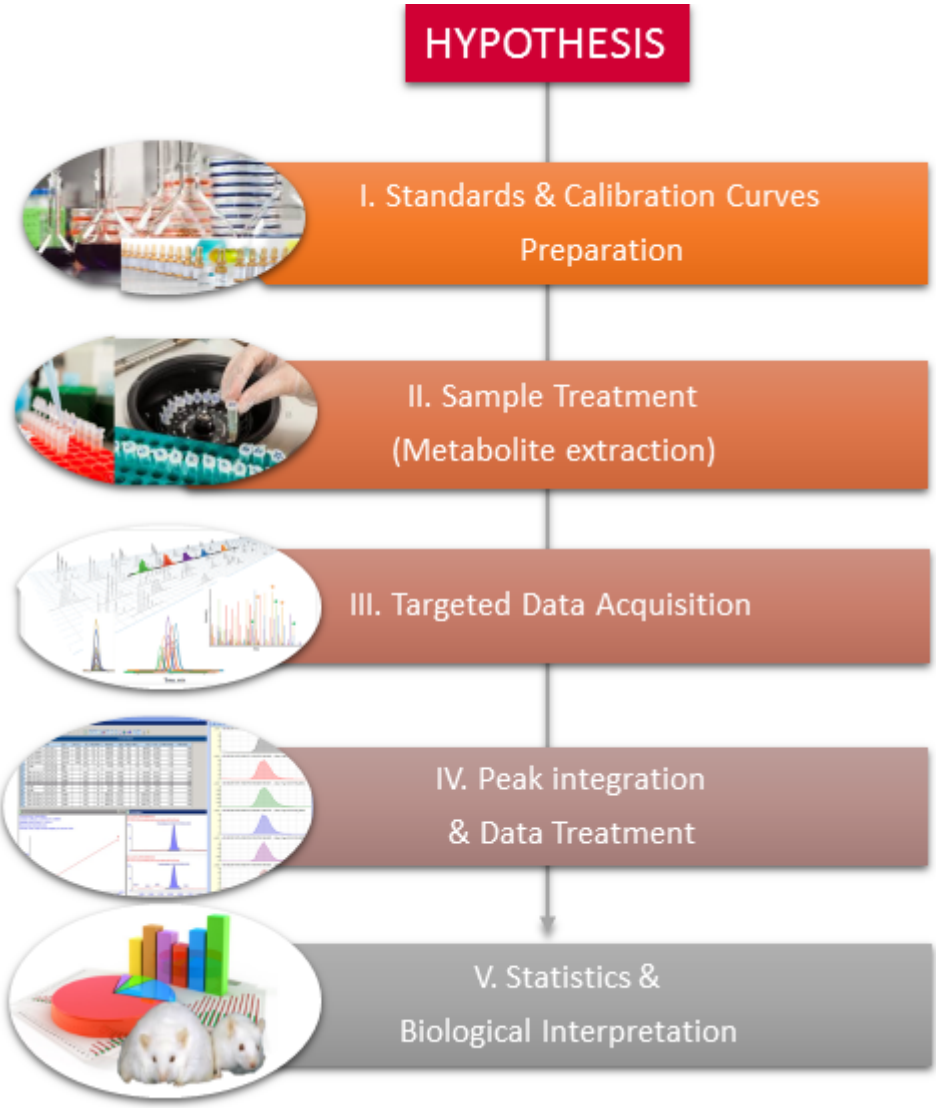


Figure 3.6. Workflow applied in targeted metabolomics studies

(I) Preparation of known standards (used as internal, external and surrogated standards) and calibrations curves are prepared prior the analysis.

(II) Sample treatment, which aims to clean samples, remove interferences and includes the addition of IS to both trace the procedure and quantify the compounds.

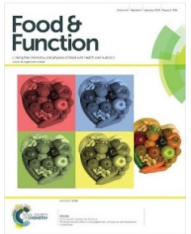
(III) Data acquisition is targeted and MS calibrated to detect the metabolites of interest.

(IV) Data treatment includes the creation of methods for extraction of metabolites of interest, peak integration, concentration calculations and dismissal of the metabolites outside the analytical criteria established (LOD, LOQ, reproducibility, etc.).

(V) Statistic analysis reveals the direction of the regulation and the magnitude of changes, providing solid results for further interpretation.

The preparation and use of QC samples is required for the extraction procedure and to trace the procedure throughout the instrumental analysis.

Table 3.3. Overview of the research papers included in the PhD Thesis

SECTION	BIOCHEMICAL, VASCULAR & MICROBIOLOGICAL ANALYSES		NON-TARGETED METABOLOMICS STUDIES: FINGERPRINTING			TARGETED METABOLOMICS STUDIES: PROFILING	
	Paper I	Paper II	Paper III	Paper IV	Paper V	Paper VI	Paper VII
Title	High-cholesterol diet enriched with onion affects endothelium-dependent relaxation and NADPH oxidase activity in mesenteric microvessels from Wistar rats	Dietary onion ameliorates antioxidant defence, inflammatory response, and cardiovascular risk biomarkers in hypercholesterolemic Wistar rats	Multiplatform metabolomic fingerprinting as a tool for understanding hypercholesterolemia in Wistar rats	Evaluation of onion as a functional ingredient in the prevention of metabolic impairments associated to diet-induced hypercholesterolaemia using a multiplatform approach based on LC-MS, CE-MS and GC-MS	Metabolomic fingerprinting in the comprehensive study of liver changes associated with onion supplementation in hypercholesterolemic Wistar rats	New insights about the effects of onion consumption on lipid mediators using a diet-induced model of hypercholesterolemia	Role of dietary onion on faecal bile acids content in rats fed a high-cholesterol diet
Journal	Metabolism & Nutrition 	Free Radical Biology & Medicine (Under Review) 	European Journal of Nutrition 	Journal of Functional Foods 	International Journal of Molecular Sciences 	Redox Biology 	Food & Function (Under Review) 
Type of sample	Plasma Messenteric microvessels	Plasma Erythrocytes Faeces	Plasma	Plasma	Liver	Plasma Liver, Spleen, Heart	Faeces
Analytical platform	Antioxidant assays, Vascular reactivity	Multiple biochemical assays, Microbiological Analyses	LC–MS, CE–MS, GC–MS	LC–MS, CE–MS, GC–MS	LC–MS, CE–MS, GC–MS	UPLC–MS/MS	LC–MS

SECTION I –

BIOCHEMICAL, VASCULAR AND MICROBIOLOGICAL ANALYSES

This section contains two chapters corresponding to a primary evaluation of the vascular and hepatic status induced by the high-cholesterol diet and the preventive role of onion on well-known biomarkers of oxidative stress, vascular reactivity and specific markers of hepatic and CVD functions.

Chapter 4. High-cholesterol diet enriched with onion affects endothelium-dependent relaxation and NADPH oxidase activity in mesenteric microvessels from Wistar rats

(Research Paper I)

Chapter 5. Dietary onion ameliorates antioxidant defence, inflammatory response, and cardiovascular risk biomarkers in hypercholesterolemic Wistar rats

(Research Paper II)

SECTION I – Chapter 4

High-cholesterol diet enriched with onion affects endothelium-dependent relaxation and NADPH oxidase activity in mesenteric microvessels from Wistar rats

D. González-Peña, J. Angulo, S. Vallejo, C. Colina-Coca, B. de Ancos, C.F. Sánchez-Ferrer, C. Peiró, C. Sánchez-Moreno

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Nutrition&Metabolism

RESEARCH Open Access

High-cholesterol diet enriched with onion affects endothelium-dependent relaxation and NADPH oxidase activity in mesenteric microvessels from Wistar rats

Diana González-Peña[1], Javier Angulo[2], Susana Vallejo[3], Clara Colina-Coca[1], Begoña de Ancos[1], Carlos F Sánchez-Ferrer[3], Concepción Peiró[3] and Concepción Sánchez-Moreno[1*]

Abstract

Background: The aim of the present study was to examine the effects of onion as functional ingredient on the oxidative status, lipoprotein levels (total cholesterol-TC, HDL-C, LDL-C), triacylglycerides (TAG) and vascular reactivity of mesenteric arteries in hypercholesterolemic Wistar rats.

Methods: Twenty-four animals were fed with three different diets [control, high-cholesterol diet (HC) and high-cholesterol enriched with onion diet (HCO)]. After seven weeks of experimental feeding the rats were euthanized for blood and tissues collection. TC, HDL-C, LDL-C and TAG were measured, and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) scavenging capacity and ferric reducing antioxidant power (FRAP) were determined in plasma. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activities were assayed in erythrocyte lysates. Endothelium-dependent vasodilation to acetylcholine was evaluated in mesenteric arterial segments. NADPH oxidase (NOX) was also measured by lucigenin-derived chemiluminiscence.

Results: The dietary cholesterol content significantly affected plasma lipoprotein levels, increased superoxide generation from NOX, and caused impaired endothelium-dependent vasodilation in the rat mesenteric arteries. Onion ingredient improved antioxidant status in HCO group, as it was evidenced by $ABTS^{\bullet+}$ and FRAP values and SOD and GPx enzyme activities compared to the HC-fed group, reduced the increment in NOX activity and reversed endothelial dysfunction promoted by the HC diet. Scavenging of superoxide with TEMPOL or inhibition of NOX with apocynin improved endothelium-dependent vasodilation only in HC-fed rats.

Conclusions: Enrichment of diet with onion as functional ingredient could be proposed as a complementary approach to prevent or partially modulate vascular dysfunction, reducing some of the risk indexes linked to initial development of atherosclerosis.

Keywords: Onion, Functional foods, Dietary cholesterol, Mesenteric microvessels, NADPH oxidase

SECTION I – Chapter 5

Dietary onion ameliorates antioxidant defence, inflammatory response, and cardiovascular risk biomarkers in hypercholesterolemic Wistar rats

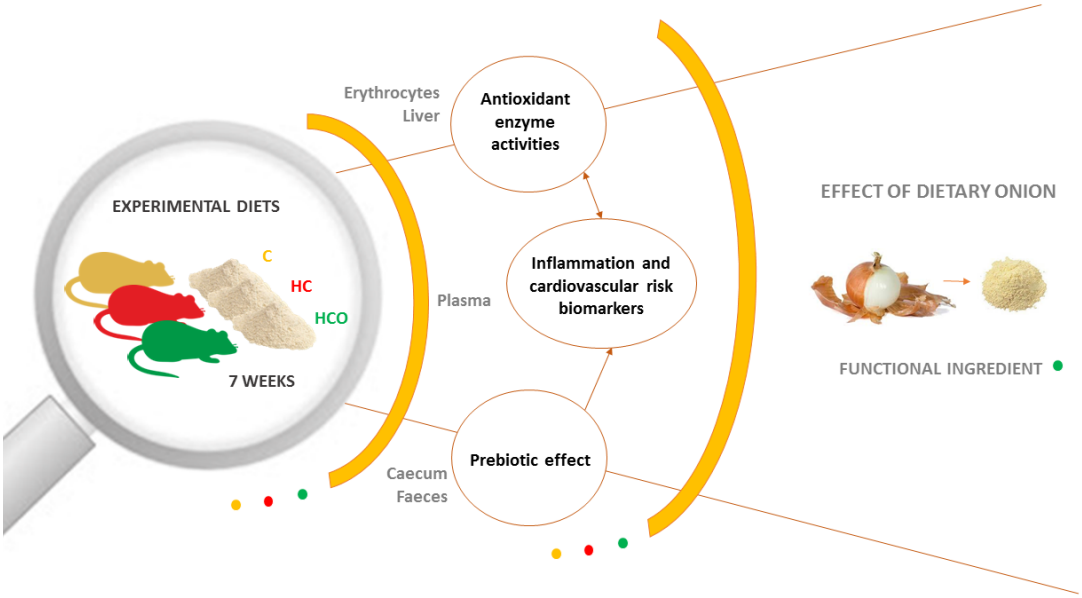
C. Colina-Coca, D. González-Peña, B. de Ancos, C. Sánchez-Moreno

Under Review in:

Free Radical Biology & Medicine

REF: FRBM-D-17-00282

Graphical Abstract



Highlights

- Onion ingredient enhanced antioxidant defence mechanisms in high-cholesterol-fed rats
- Hepatotoxicity evidenced by plasma ALT and AST levels was prevented by onion
- Onion supplementation ameliorated inflammatory response associated with atherogenesis
- Inflammation and cardiovascular risk biomarkers were positively modulated by onion
- Beneficial and pathogenic gut microflora was modified by onion ingredient

Dietary onion ameliorates antioxidant defence, inflammatory response, and cardiovascular risk biomarkers in hypercholesterolemic Wistar rats

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Abstract

Hypercholesterolemia is a major risk factor for the development of atherosclerosis and endothelial dysfunction; onion supplementation has been proposed as nutritional intervention to prevent or improve some of its associated biological impairments. The objective of this study was to investigate the effects of onion as a functional ingredient on antioxidant defence, inflammatory response, and cardiovascular risk biomarkers in hypercholesterolemic male Wistar rats.

Rodents (n = 24) were fed three different diets: control (C), high-cholesterol (HC), and high-cholesterol enriched with onion (HCO). After 7 weeks of experimental feeding, blood and organs were collected. Antioxidant enzymes were measured in erythrocytes and liver, whereas plasma was used for the analysis of cytokines, chemokines, and adhesion molecules, among other cardiovascular parameters. Furthermore, caecum and faeces were analysed to evaluate the changes produced on the microflora. Rats fed the HC diet significantly decreased erythrocyte SOD, CAT and GPx activities compared with the C group. Interestingly, the HCO group showed a significant increase in SOD, CAT and GPx activities compared with the HC group. Plasma inflammatory parameters, namely IL-10, MCP-1, VEGF, s-ICAM-1, sE-selectin, PAI-1, v-WF, and TIMP-1 were significantly increased in the HC group and onion enrichment modulated this increment. Additionally, the HCO diet reduced the presence of sulphite-reducing *Clostridium* spp. *versus* the HC diet. Taken together, these results suggest an improvement on the antioxidant and antiinflammatory response as well as on the cardiovascular risk biomarkers promoted by the onion ingredient.

Keywords: Functional ingredient; Antioxidant enzymes; Protein carbonyls; Oxidative stress; Cytokines; Adhesion molecules

SECTION II –

NON-TARGETED METABOLOMICS STUDIES (FINGERPRINTING)

This section comprises three chapters based on corresponding research papers.
These studies describe metabolic impairments associated to hypercholesterolemia followed by the potential modulating effects of onion by analysing plasma and liver samples by non-targeted approaches. The work was undertaken by a multiplatform of metabolic fingerprinting comprising LC–MS, CE–MS and GC–MS.

Chapter 6. Multiplatform metabolomic fingerprinting as a tool for understanding hypercholesterolemia in Wistar rats

(Research Paper III)

Chapter 7. Evaluation of onion as functional ingredient in the prevention of metabolic impairments associated to diet-induced hypercholesterolemia using a multiplatform approach based on LC-MS, CE-MS and GC-MS

(Research Paper IV)

Chapter 8. Metabolomic fingerprinting in the comprehensive study of liver changes associated with onion supplementation in hypercholesterolemic Wistar rats

(Research Paper V)

SECTION II – Chapter 6

Multiplatform metabolomic fingerprinting as a tool for understanding hypercholesterolemia in Wistar rats

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ORIGINAL CONTRIBUTION

Multiplatform metabolomic fingerprinting as a tool for understanding hypercholesterolemia in Wistar rats

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Abstract
Purpose The aim was to investigate the impact of hypercholesterolemic diet on the metabolome of male Wistar rats by a multiplatform metabolomic fingerprinting.
Methods Male Wistar rats were fed with two different diets [control (C) and high-cholesterol diet (HC)—containing 2 % cholesterol and 0.5 % cholic acid]. After 7 weeks of experimental feeding, the rats were euthanized for blood collection and plasma recovery. The metabolite fingerprint was then achieved by applying a multiplatform comprising LC–MS, GC–MS and CE–MS.
Results Multivariate statistical analysis showed a clear separation between the C and HC groups. Individual differences in metabolites were evaluated using univariate statistical analysis, and multiple metabolites were identified and confirmed in the plasma. A global profiling integrates for the first time pathways affected by high-cholesterol diet intake and allowed us to elucidate some of the associated alterations underlying the hypercholesterolemia event in Wistar rats.
Conclusions HC feeding stimulated the alteration of multiple pathways in Wistar rats, warning of the risk of developing important diseases, which can be modulated by the diet. Further studies are required to investigate the possibilities to revert or ameliorate the negative effects triggered by HC intake.

SECTION II – Chapter 7

Evaluation of onion as a functional ingredient in the prevention of metabolic impairments associated to diet-induced hypercholesterolaemia using a multiplatform approach based on LC-MS, CE-MS and GC-MS

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Evaluation of onion as a functional ingredient in the prevention of metabolic impairments associated to diet-induced hypercholesterolaemia using a multiplatform approach based on LC-MS, CE-MS and GC-MS

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ABSTRACT

The preventive and modulating effects of onion in metabolites affected by a high-cholesterol diet and related to molecular pathway dysfunction were examined. Plasma obtained from 24 male Wistar rats after seven weeks of experimental feeding was analysed through a non-targeted multiplatform metabolomics approach based on LC-MS, CE-MS and GC-MS methods. A cross-comparison of the three metabolic profiles [I) control (C) group, II) high-cholesterol (HC) group and III) high-cholesterol enriched with onion (HCO) group] pointed out two sulphur metabolites, *S*-methyl-L-cysteine and *S*-methylcysteine sulphoxide, as markers of onion intake and evidenced the possibilities to regulate key metabolites. Thus, the methylation cycle, arginine and tryptophan pathways, the modulation of 3-methylhistidine, as well as the routes involved in the glycerophospholipids transformation have been highlighted. This study suggests that onion's biologically active compounds take a prominent role in ameliorating hypercholesterolaemia and related complications through the regulation of altered metabolic pathways.

SECTION II – Chapter 8

Metabolomic fingerprinting in the comprehensive study of liver changes associated with onion supplementation in hypercholesterolemic Wistar rats

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Article

Metabolomic Fingerprinting in the Comprehensive Study of Liver Changes Associated with Onion Supplementation in Hypercholesterolemic Wistar Rats

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Abstract: The consumption of functional ingredients has been suggested to be a complementary tool for the prevention and management of liver disease. In this light, processed onion can be considered as a source of multiple bioactive compounds with hepatoprotective properties. The liver fingerprint of male Wistar rats (n = 24) fed with three experimental diets (control (C), high-cholesterol (HC), and high-cholesterol enriched with onion (HCO) diets) was obtained through a non-targeted, multiplatform metabolomics approach to produce broad metabolite coverage. LC-MS, CE-MS and GC-MS results were subjected to univariate and multivariate analyses, providing a list of significant metabolites. All data were merged in order to figure out the most relevant metabolites that were modified by the onion ingredient. Several relevant metabolic changes and related metabolic pathways were found to be impacted by both HC and HCO diet. The model highlighted several metabolites (such as hydroxybutyryl carnitine and palmitoyl carnitine) modified by the HCO diet. These findings could suggest potential impairments in the energy−lipid metabolism, perturbations in the tricarboxylic acid cycle (TCA) cycle and β-oxidation modulated by the onion supplementation in the core of hepatic dysfunction. Metabolomics shows to be a valuable tool to evaluate the effects of complementary dietetic approaches directed to hepatic damage amelioration or non-alcoholic fatty liver disease (NAFLD) prevention.

Keywords: functional food; hypercholesterolemia; mass spectrometry; LC-MS; CE-MS; GC-MS; metabolome; non-targeted metabolomics; non-alcoholic fatty liver disease (NAFLD)

SECTION III –

TARGETED METABOLOMICS STUDIES (PROFILING)

SECTION III – Chapter 9

New insights into the effects of onion consumption on lipid mediators using a diet-induced model of hypercholesterolemia

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Research Paper

New insights into the effects of onion consumption on lipid mediators using a diet-induced model of hypercholesterolemia

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ABSTRACT

The levels and roles of lipid mediators can be modified in response to nutritional stimuli. The aim of this study was to investigate shifts in oxylipin and sphingolipid profiles stimulated by a hypercholesterolemic (HC) diet along with the modulating effects of onion introduced as an antioxidant functional ingredient characterized in the diet (HCO). Oxylipin and sphingolipid profiles were determined in plasma and tissues from Wistar rats using LC-MS/MS. Plasma ω-3 and ω-6 PUFA-derived oxylipins decreased in rats after 7 weeks of HC feeding, but did not evidence a further shift with HCO diet. Onion ingredient supplementation modulated the hepatic concentrations of prostaglandins and enhanced ω-3 oxylipins in the liver of HCO-fed rats relative to the HC group. The HC diet induced shifts in plasma sphingolipids, increasing sphingoid bases, dihydroceramides and ceramides, whilst the sphingomyelin, hexosylceramide and lactosylceramide families decreased. The HCO diet modified some HC diet-induced changes in sphingolipids in liver and spleen tissue. Onion supplementation effected changes in lipid mediator levels in diet-induced hypercholesterolemic Wistar rats. The potential of onion as regulator of pro-inflammatory mediators, and possible enhancer of pro-resolution pathways, warrants further study of the interaction of functional ingredients with bioactive lipid mediators and their potential impact on inflammation, oxidative stress and organ dysfunction.

SECTION III – Chapter 10

Role of dietary onion on faecal bile acids content in rats fed a high-cholesterol diet

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Abstract (50–250 words)

The determination of faecal bile patterns offers new opportunities in the search for non-invasive biomarkers of disease status. The objective of this study was to describe the shifts in the faecal bile acid (BA) composition induced by feeding a high-cholesterol/cholic acid diet (HC), over 7 weeks of experimental feeding in Wistar rats, evaluating the effects of onion included as a functional ingredient (HCO). A HPLC–MS/MS allowed for the detection of 29 bile acids, 10 of which were tentatively identified and 12 confirmed and quantified by means of IS-calibration curves. The excretion of bile acids revealed a discriminating bile acid profile between the HC and HCO groups compared with the C group. HCO feeding indicated significant changes in specific primary and secondary BA in both unconjugated and conjugated forms caused by the addition of the onion ingredient to the diet. The results suggest the induction of microbiome modifications by the HC and HCO diets acts as a critical modifier of the faecal bile acid composition. These modifications might reflect and be linked to changes in the reabsorption of BA at an intestinal level and the processes of BA deconjugation in the course of hypercholesterolemia.

Keywords: Faeces; Hypercholesterolemia; LC-MS; Profiling; NAFLD

1. Introduction

The secretion from the liver and absorption from the intestine of bile acids (BA) are the two major processes involved in the enterohepatic circulation, which is simultaneously coordinated by the action of lipid activated nuclear hormone receptors regulating the cholesterol metabolism and bile acid synthesis [1]. However, BA are also involved in the regulation of a complex network of metabolic pathways including lipid, glucose and energy metabolism [2], where they also act as important and versatile signaling molecules and they become the specific ligands for activation of nuclear and membrane receptors [2].

The balance in the content of the BA pool prevents both functional impairments and cytotoxic accumulation, thus as the bile acid pool size increases, a feedback mechanism is activated to inhibit *de novo* BA synthesis. Nevertheless, the equilibrium of this system is critically affected by the composition of the diet. The intake of dietary cholesterol and its supplementation with cholic acid (CA), has been assayed in several studies [3-5]. Charach *et al.* [6] demonstrated an inverse correlation between the degree of hypercholesterolemia and the rate of bile acid elimination, which was suggested to depend on the animal's ability to convert cholesterol and associated with the subsequent development of atherosclerosis. Dietary supplementation of a specific bile acid has shown to increase the hepatic and serum concentrations of that bile acid administered in a dose-dependent manner, as well as their known metabolites [7, 8]. Moreover, the influence of different dietary supplements on cholesterol and bile acids can promote a differential response in faecal bile acid excretion and composition. For example, the use *of S*-methyl cysteine sulfoxide from *Allium cepa* Linn. in high cholesterol diet fed rats evidenced increases in the excretion of bile acids and sterols [9].

On the other hand, the consumption of onion and onion powder have been shown to exert a direct impact in the modulation of biliary cholesterol, which influence: the cholesterol nucleation and contribute to their anti-lithogenic potential [10, 11]; the lipid profile in conditions of metabolic disorders and diseases [12] and the hepatic conversion of cholesterol to bile acids exerting hepatoprotection [13]. In this regard, the hepatoprotective effect of both dietary onion and some of its principal bioactive components has consistently been shown through the enhancement of the antioxidant capability and the activities of the enzyme systems [14]. In addition, a recent study by our group evidenced the preventive effect of an onion ingredient in both hepatic markers

and inflammatory responses in hypercholesterolemic Wistar rats. This could suggest a possible removal of fats in the liver, which would be directed by different mechanisms, such as the excretion of bile acids. However, multiple mechanisms and metabolic responses may be behind the development of hypercholesterolemia that remain unexplored. Overall, when disease and physiopathology are the result of a specific diet such as HC, this situation is amenable to change with the aim of improving the health status (*e.g*. by supplementation with onion).

Therefore, the aim of the present study had a double purpose of describing the changes induced by feeding a high-cholesterol (HC) diet in the faecal BA composition during a 7-weeks experiment and the assessment of the effect of a supplementation with an onion ingredient on the excretion of BA, thus evaluating its concentration in faeces with a chronological perspective.

2. Material and Methods

2.1 Experimental design

Twenty four male Wistar rats with a body weight of approximately of 250 g at the outset were obtained from Harlan Laboratories Models (Harlan, SL, Barcelona, Spain). The animals were housed individually in metabolic cages in a temperature-controlled room (22.5 ± 0.5ºC) with a 12 h light–12 h dark cycle. The rats were fed commercial rat pellets (Panlab, SLU, Barcelona, Spain) for the adaptation to environmental conditions and then distributed into three groups of eight animals each, according to average body weight. For the acclimatisation to metabolic cages, animals were nourish a control diet based on the AIN-93M semi-purified rodent diet [15], whose composition is specified in ESI Table 1.

The three experimental semi-synthetic diets were prepared and given according to: (1) the control (C) diet, composed of a homogeneous mixture of 100 % rodent diet; (2) the high-cholesterol (HC) diet, composed by the control diet with 2 % cholesterol and 0.5 % cholic acid; and (3) the high-cholesterol enriched with onion (HCO) diet, identical to the HC diet but with 10% onion powder, balancing the dietary fibre with cellulose powder. The amount of maize starch in the HC and HCO diets was adjusted to compensate for the addition of cholesterol and cholic acid in the HC diet, and onion powder in the HCO diet (ESI Table 2). The experimental period last for 7-weeks for

which water and food were provided *ad libitum*. Then, food consumption and faeces deposits were registered daily, while body weight was recorded weekly.

The present study was approved by the Spanish Ministry of Science and Innovation Advisory Committee [project AGL2010-15910 (subprogram ALI)] and by an Ethics Committee of the Complutense University of Madrid (Spain). All experiments were performed in compliance with the Directive 2010/63/UE regarding the protection of animals used for scientific purposes.

2.2 Chemicals and reagents

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), hyodeoxycholic acid (HDCA), ursodeoxycholic acid (UDCA), taurocholic acid (T-CA), taurochenodeoxycholic acid (T-CDCA), taurodeoxycholic acid (T-DCA), taurolithocholic acid (T-LCA), glycocholic acid (G-CA), glycochenodeoxycholic acid (G-CDCA), and glycodeoxycholic acid (G-DCA) were purchased from Sigma–Aldrich (St. Louis, MO). HPLC-grade methanol was provided by Lab-Scan (Gliwice, Poland). Ethanol and formic acid were purchased from Panreac (Barcelona, Spain). Acetic acid was obtained from Merck (Darmstadt, Germany). Sep-Pak® C18 cartridges were purchased from Waters (Milford, MA, USA).

2.3 Faecal sample collection and extraction of faecal bile acids (BA)

Faecal samples were collected daily from individual metabolic cages, weighed and kept at −20 ± 0.5 ºC until use. Sample from selected days were individually freeze-dried using a lyophilizer (model Lyoalfa, Telstar, S.A., Barcelona, Spain). The dried samples were pulverized using a small grinder and kept in tubes at 4ºC. 250 mg of dried faecal material from the 8 rats of each group (C, HC and HCO groups) were weighed and pooled together each week obtaining a final pool of faecal powder for each group and week (3 pools per week during 7 weeks, starting at week 0).

5 mg of each pool sample was weighed in a pirex tube and 0.5 mL of 50 mM sodium acetate buffer (pH 5.6) was added. The tube was top up to 2 mL with methanol and vortexed. Samples were incubated at 80ºC for 1 hour and centrifuged at 1600 *g* for 5 min at 4ºC. Supernatants were recovered and ¼ diluted with milliQ water. SPE extraction was performed using Sep-Pak® C18 cartridges (360 mg, 55-105 µm, Waters, Milford, Massachusetts, USA) according to Kakiyama *et al.* [16], with minor

modifications and dried using a Speed-Vac (Savant SPD131DDA-RVT4104TRAP, Thermo). Dried samples were re-suspended in 500 µL of methanol (50%) and filtered with 0.45 µm nylon syringe filters before injection. All extractions were performed in duplicate. A general overview of the experimental workflow is represented in Fig. 1.

2.4 Identification and quantification of bile acids

HPLC-DAD and HPLC-ESI-QTOF-MS analysis was performed on an Agilent 1200 series HPLC (Agilent Technologies, Waldbroon, Germany), comprised of a quaternary pump (G1311A) with integrated degasser (G1322A), an autosampler (G1367B), a thermo stated column compartment (G1316A) a diode array detector (DAD) (G1315B) and a hybrid mass spectrometer quadrupole-time of flight via an electrospray ionization source (ESI) with JetStream technology (Agilent Accurate Mass QTOF LC-MS, Waldbronn, Germany) in series in the same chromatographic line. Two extracts from each pool were taken and duplicate samples of 1 and 10 µL each were injected into a C18 Hypersil ODS stainless steel column (250 mm × 4.6 mm, 5 µm; Teknokroma, Barcelona, Spain). Chromatographic and operating conditions were based on Kakiyama *et al.* [16] and Bobeldijk *et al.* [17] protocols, and optimized as followed: mobile phase (A) used 5mM of ammonium acetate in water and mobile phase (B) used methanol. The gradient started after 8 min at 65% B, increasing to 75 % B (8-15 min) and 95% B (15-20 min) and afterwards restored to 65% B (20-25 min). An isocratic mode was kept for the last 5 min until total run time of 30 min. The flow rate was fixed at 0.8 mL/min. HPLC-ESI-MS/MS operated in negative ion mode, with voltage set at −3500 V, fragmentor 200 V, nebulizing gas flow rate 12 L/min, nebulizer pressure 45 psig, and drying temperature 300 °C. Mass spectrometry data was acquired in the scan mode (mass range 100−1500 m/z). MS/MS collision energy was set at 35 V. Data acquisition and MS and MS/MS data were processed through MasshunterWorkstation software (version B.04.00, Agilent Technologies, Waldbronn, Germany).

Identification of bile acids was carried out by comparing the mass, retention time and fragmentation pattern with those of the authentic commercial standards, and data reported in the literature for biological samples obtained from rodents. Quantification was achieved by employing calibration curves as an external standard. To optimize the method, stock solutions with 2 mg/mL were prepared for each standard. Then, 1 mL from each stock was diluted into one of the two multi-standard curves prepared to reach

a concentration range of ~35 µg/mL to 2 µg/mL and then combined into a new unique multi-standard, including all quantifiable compounds.

Final determination of external curves was performed in duplicate and injected twice along the sequence. The analysis also included curves with the concentration range needed of each standard for sample quantification (20-0.3 µg/mL for HDCA and DCA; 5-0.078 µg/mL for T-CA, UDCA, CA and CDCA; 500-142.85 µg/mL for CA; 5-0.156 µg/mL for G-CA, G-CDCA and G-DCA; 2-0.05 µg/mL for T-CDCA, T-DCA). Samples were randomized in a sequence and injected along with the combined multi standard as QC every 12 samples to ensure the system's performance and stability.

2.5 Statistical analysis

Results are reported as mean values with their standard deviation (SD). Data were analysed using two-way ANOVA or *t*-test for multiple or pair comparisons, as appropriate. In order to verify the homogeneity of the variances a Levene's test was applied. Tamhane's T2 (equal variances not assumed) or Bonferroni (equal variances assumed) *post hoc* tests were used to determine differences within groups ($p < 0.05$). Analyses were performed using the IBM SPSS Statistics 23 (SPSS Inc., an IBM Company).

3. Results & Discussion

3.1 Qualitative and quantitative analysis of faecal bile acids

A simple HPLC–MS/MS method for the simultaneous quantification of 12 BA in samples of faeces has been developed (Fig. 2), allowing to quantify their shifts and the detection of other non-targeted BA among the three groups of experiment. A detailed list of detected compounds classified by unconjugated (primary / secondary), conjugated bile acids and unidentified compounds is shown in Table 1, specifying the sample groups where they were found and indicating the possibilities of detection with time (T*i*–T*f*). Quantified peaks in the three groups over 7 weeks of experiment is summarized in Table 2, evidencing whether the influence of time or group have an impact on the final results. The putative identification of BA or the confirmation of their identities was possible through searches on databases, data reported in the literature[18, 19] and the use of commercial standards. Fig. 3 shows a merged extracted-ion

chromatogram (EIC) of BA for the C, HC, and HCO groups, verifying the differential profile on faecal extracts among the HC and HCO groups compared with the C group.

3.2 Effect of high-cholesterol diet in faecal bile acid profile

The regulation of bile acid metabolism is directly involved with the cholesterol homeostasis. Dietary cholesterol and BA affect cholesterol metabolism, modifying the composition of the major and minor BA found in different compartments, including the final excretion of faecal BA. Although most BA (95%) delivered to the intestine are re-absorbed into the enterohepatic circulation, the analyses of faecal BA can offer some advantages in the search of biomarkers.

In addition to the non-invasive nature of the sampling procedure, the screening of faecal BA reflects changes caused in the small intestine, and thus takes into account the changes produced by the colonic microbiota.

A high-cholesterol (HC) diet containing cholic acid (CA) has been classically considered as potentially atherogenic [20] and diverse findings also established a clear relationship with hepatic damage which is characterized by steatosis, inflammation and fibrosis [20, 21]. Feeding the animals with a HC diet modified the faecal BA by increasing its subsequent excretion (Table 2). Muricholic acids (MCA), hyodeoxicholic acids (HDCA) and deoxycholic acid (DCA) were the main BA found at the beginning of the experiment in all groups and remained dominant throughout the experiment in the C group. The primary BA, CA and chenodeoxycholic acid (CDCA) underwent a strong increase in HC and HCO groups, due to the dietary cholesterol/CA overload. The exceedingly high levels of CA attained were also responsible for the higher concentrations of DCA and ursodeoxycholic acid (UDCA) found in hypercholesterolemic rats. Conversely, litocholic acid (LCA) and hyodeoxycholic acid (HDCA) showed a marked and significant decrease in HC-fed group compared with the C group.

The synthesis of MCA in rodents is known to be one of the most important differences with humans and a reason why part of the metabolism and physiological functions of MCA remains unclear. The supplementation with CA in mice is known to increase the absorption of cholesterol in the liver by around 60%, while MCA-BA are simultaneously reduced by 80-90% [22]. In this study, α, β and ω– MCA were putatively identified in the faeces of Wistar rats. Nevertheless, HC-feeding caused a remarkable

and progressive increase in the excreted concentrations of MCA compared to the C group until the week-5, point from which the trend was modified and showed a partial decrease relative to the HC group.

Stimulation of BA production was observed by an increased faecal BA excretion of conjugated forms. Glycine and taurine conjugated forms were only detected by feeding HC and HCO diets, thus remaining at very low levels and precluding its detection in C group during the 8 points analysed in the experiment. The concentration of BA conjugated with glycine was higher than the taurine forms in HC-fed rats. Whereas most non-conjugated BA showed a more regular trend, conjugated BA such as taurocholic acid (T-CA) or glycocholic acid (G-CA) presented marked oscillating cycles (of increasing and decreasing concentrations) when rats were fed the high cholesterol (HC- and HCO-) diets. Moreover, taurolitocholic acid (T-LCA) was not detected at any point of the experiment, whereas its unconjugated form decreased in HC group from week 1 to week 4, showing a partial recovery after that point. The concentration of most BA varied markedly between weeks in the different groups during the experiment.

The rates of biosynthesis of different bile acids are known to be altered by the overload of cholesterol/cholic acid and regulated by the joint activity of CYP7A1, CYP8B1 and CYP27 enzymes which differ widely according to the animal model [3]. The interest in the action of nuclear Farnesoid X receptor (FXR) and Short Heterodimer Partner (SHP) is also a priority since they are both involved in the control of bile acid synthesis [23] and describe interactions with the lipid metabolism, which is also modified by the composition of the diet [24]. However, it is important to highlight that bile acids found in rat faeces are extensively modified by the intestinal microflora, which is simultaneously modified in response to the diet and conditioned by the presence and type of BA [25]. There is a complex modification of the gut microbiome when rats are fed bile acids. As an example, Islam et al., 2011, demonstrated that CA intake produced drastic increases in Clostridia [26]. A study of our group also corroborated these finding by showing a 7-fold increase in sulfite-reducing *Clostridium* spp. seen in faeces from the same diet-induced hypercholesterolemic model. Thus, evidences suggest that the changes in faecal bile acids observed in this study should be interpreted in an integrated way, where faeces reflects the changes in BA produced in response to HC-feeding, and acts as a determinant of enzymes, nuclear receptors, the gut microbiota and its metabolites, and subsequently modifies the quantity of BA excreted.

3.2 Effect of the onion ingredient in bile acid profile of high-cholesterol fed-rats

The effects of onion supplementation on lipid metabolism and its role in preventing hepatic impairments have been the objective of several studies. Nevertheless, the particular consequences of a HC diet evaluated in parallel with the effect of onion on BA excretion have not been stablished until this study.

The supplementation of HC diet with the onion ingredient caused significant changes in the composition of specific BA found in the faeces (Table 2). The general trend found in the HCO group was similar to that of HC group. However, CA, MCA, CDCA and UDCA concentration increases were partial but significantly prevented in the HCO group. The trend showed by litocholic acid also revealed a partial prevention induced by the onion ingredient compared with the HC group. Likewise, the oscillating trend found in glyco- and tauro-conjugated forms seemed to be greater in HCO-fed rats compared with those rats fed the HC diet. An overview of the shifts in the pattern of faecal BA between week 0 and week 7, as a consequence of HC- and HCO-feeding is represented in Fig. 4.

The consumption of garlic, onion powder and some of their component such *S*-methyl cysteine sulphoxide has been reported to induce the enhancement of faecal sterol excretion and up-regulation of LXRα and CYP7A1 in hypercholesterolemic conditions [9, 27, 28]. Amelioration of cholesterol levels in response to onion consumption usually provokes an increase in its synthesis that is compensated by an increase in excretion of cholesterol and BA. Nevertheless, the addition of an onion ingredient to the HC diet in this study did not evidence an increase in the excretion of BA, except for DCA and some conjugated forms. These results support previous findings where body weight and cholesterol levels of this animal model were not improved by adding onion to the diet, whereas cardiovascular, hepatic and inflammatory benefits suggested a more complex differential mechanism active behind this model [29].

According to Hagey and Krasowski [30], excreted BA are the result of the summed products of commensal bacterial modification in the small intestine; non-absorbed (passively or actively) BA along the length of the entire intestine and those bile acids trapped within or adsorbed by dietary components such as fibre. In this sense, despite the amount of dietary fibre in HCO diet compensated with cellulose powder, onion contributed to the diet with a more complex composition of dietary fibre, where fructans or fructooligosacarides with prebiotic effect were also present. Thus, the differential faecal excretion of BA might suggest an increased in the retention of BA produced by

298 the components of onion, which are able to stimulate the fermentative processes and the
299 changes in microflora that promote modifications in the permeability of membranes. All
300 these circumstances together may have driven the changes in metabolism toward an
301 increased reabsorption of BA at an intestinal level. Fig 5 outlines the modifications
302 found in faecal BA within each group after 7 weeks of HC and HCO-feeding.
303 In addition, the gut microbiota is responsible for converting primary BA to secondary
304 BA and affects the ratio of conjugated forms [31]. At the end of the experiment, the HCO
305 group showed a tendency to augment the concentration of conjugated forms compared
306 with the HC group, which could indicate an amelioration over the negative impact of
307 HC-feeding. Some studies have suggested that taurine-conjugated BA may help prevent
308 the development of NAFLD [32-34]. Recently, a new study suggested that development of
309 NAFLD was partly associated with a decreased in the levels of taurine-conjugated BA
310 and butyrate-producing microbiota [35]. In this sense, the fermentation of onion products
311 have also demonstrated that the presence of fructans increase the production of total
312 short fatty acids, including propionate, acetate and butyrate in the caecum of rats
313 [36].Therefore, supplementation with onion might contribute to reduce BA deconjugation,
314 which usually counts as a risk factor for worsening NAFLD.
315
316 **4. Conclusions**
317 A HPLC–MS/MS method for the measurement of BA in faecal samples has allowed
318 for the description of the shifts produced over 7 weeks of HC feeding and the
319 supplementation with an onion ingredient. Faeces samples displayed discriminating BA
320 profiles between the HC and HCO groups relative to the C group, whereas HCO pointed
321 to significant changes in specific primary and secondary BA in both unconjugated and
322 conjugated forms. The results indicate that supplementation with onion might have
323 contributed to changes in metabolism that suggest an increase in the reabsorption of BA
324 at an intestinal level and the decrease in BA deconjugation. Furthermore, the importance
325 and influence of diet in the microbiome is highlighted as a possible crucial modifier of
326 the metabolic responses found in the course of hypercholesterolemia.
327 It is remarkable that the comparison of faecal bile patterns offers a snap shot at the
328 end point of the metabolism. This can contribute to the detection of important
329 imbalances not only related to the hepatic and intestinal functions but also to other
330 metabolic disorders, giving new insights about the understanding of the relationship
331 between diseases and the search of non-invasive biomarkers. However, further studies

are also needed to facilitate the understanding about how the diet and functional ingredients such as onion may influence the enterohepatic circulation regulation and the homeostatic feedback mechanisms with the luminal microbiota that affects the BA signalling in the progression of hypercholesterolemia.

Conflicts of interest statement

The authors declare that there are no conflict of interest.

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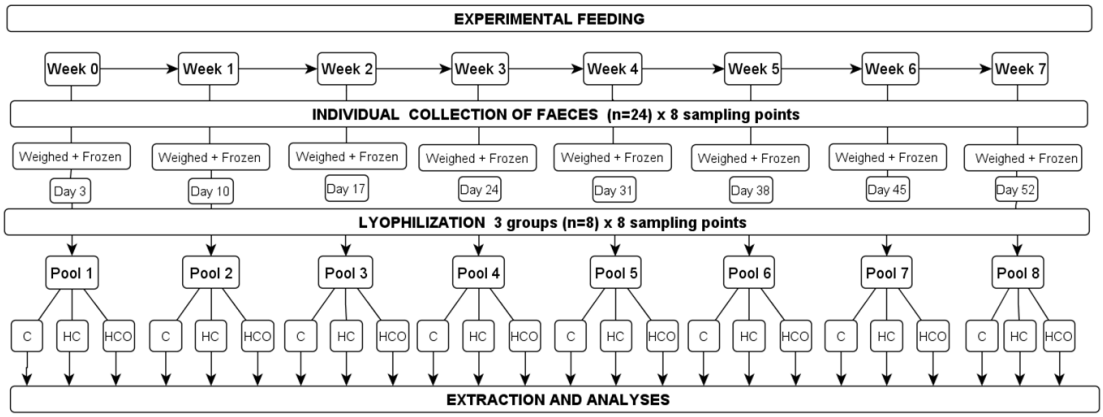


458

459 **Fig. 1.** Scheme of the fecal sampling procedure in the experimental design.

460 C: control group; HC: high-cholesterol group; HCO: high-cholesterol enriched with

461 onion group

462

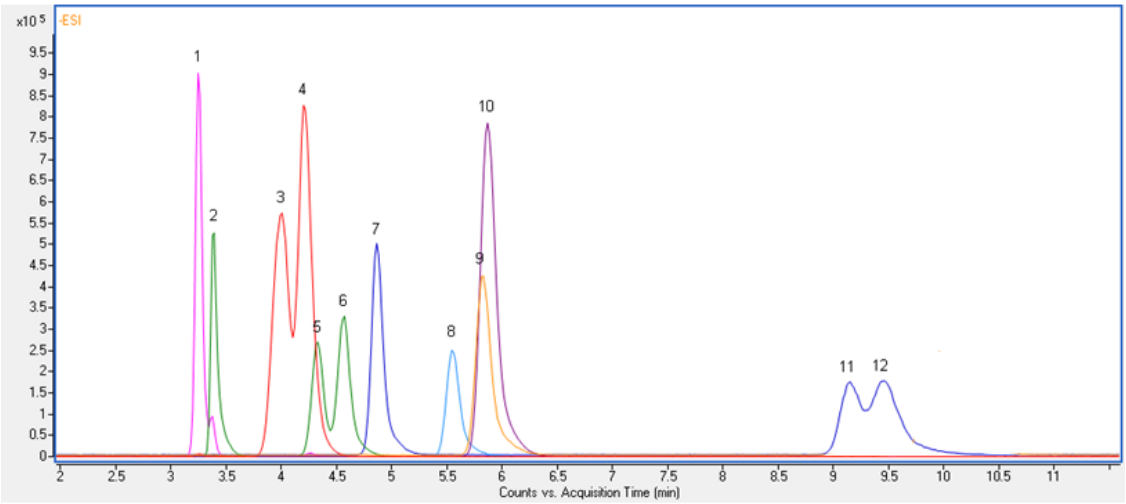
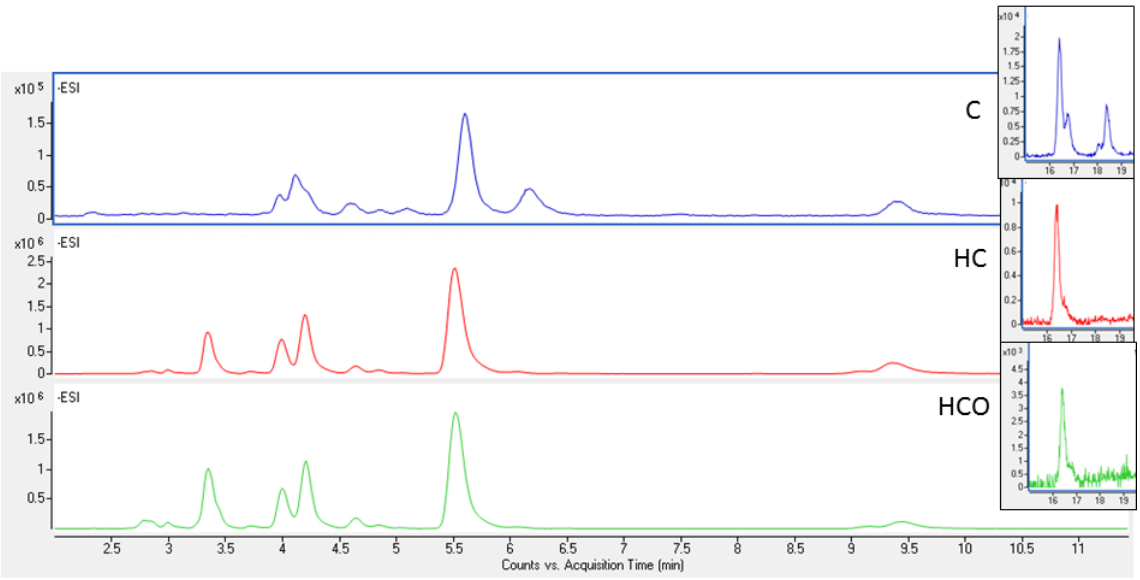


Fig. 2. EIC of a standard mixture containing the 12 bile acids quantified. Upper numbers correspond with the bile acids identities indicated in Table 1.



466

467 **Fig. 3.** Masses merged into one chromatogram for comparison of the bile acids profile
468 of the three experimental groups.
469 C: control group; HC: high-cholesterol group; HCO: high-cholesterol enriched with
470 onion group
471

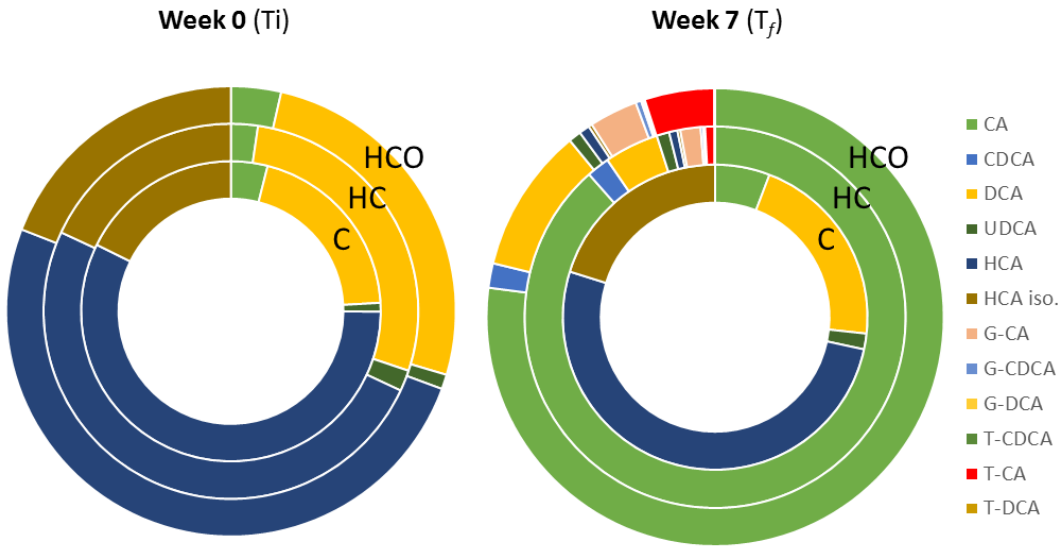


Fig.4. Representation of the changes induced by HC and HCO diets in the pattern of faecal bile acids between week 0 and week 7.

C: control group; HC: high-cholesterol group; HCO: high-cholesterol enriched with onion group

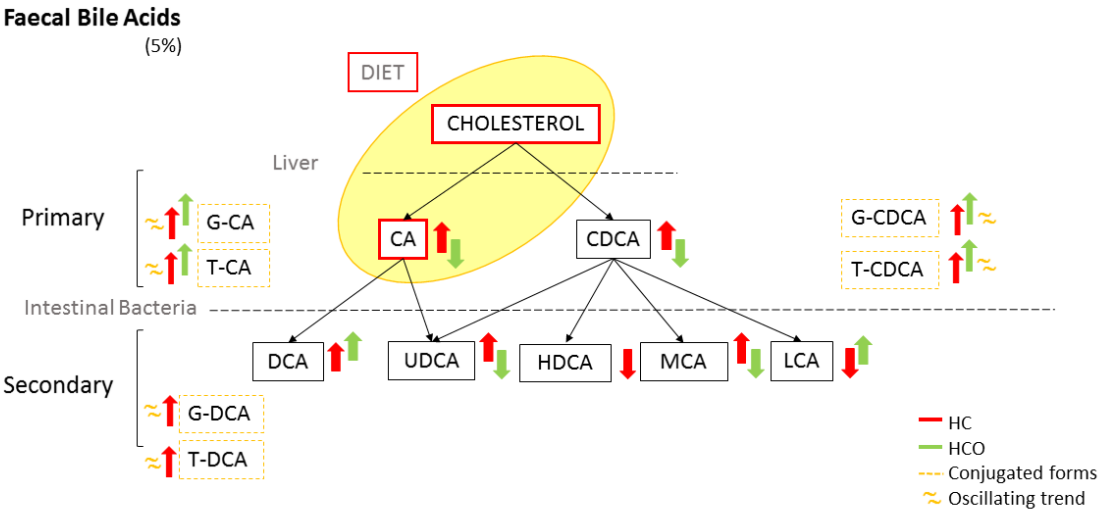


Fig. 5. Schematic representation of trends in primary and secondary bile acids in both unconjugated and conjugated forms, found in faecal bile acids within each group after 7 weeks of experimental feeding.

Red and green arrows show the comparison of HC *vs.* C and HCO *vs.* HC groups, respectively. C: control group; HC: high-cholesterol group; HCO: high-cholesterol enriched with onion group

485 **Table 1.** Bile acids detected and identified in faeces from control (C), high-cholesterol
486 (HC) and high-cholesterol with onion (HCO) groups at T_i and T_f of the experimental
487 feeding.

Classification	Compound name	Formule	MW (M)	$[M-H]^-$	t_R (min)	Groups	T_i - T_f
Primary	Cholic acid [8]	$C_{24}H_{40}O_5$	408.57	407.2803	5.54	C, HC, HCO	√- √
	Chenodeoxycholic acid [11]	$C_{24}H_{40}O_4$	392.57	391.2854	9.15	HC, HCO	x- √
Secondary	Deoxycholic acid [12]	$C_{24}H_{40}O_4$	392.57	391.2854	9.46	C, HC, HCO	√- √
	Ursodeoxycholic acid [7]	$C_{24}H_{40}O_4$	392.57	391.2854	4.86	C, HC, HCO	√- √
	Hyodeoxycholic acid [9]	$C_{24}H_{40}O_4$	392.57	391.2854	5.82	C, HC, HCO	√- √
	Hyodeoxycholic acid isom.*	$C_{24}H_{40}O_4$	392.57	391.2854	6.17	C, HC, HCO	√- √
	ω-Muricholic acid [DB]*	$C_{24}H_{40}O_5$	408.57	407.2803	3.96	C, HC, HCO	√- √
	α-Muricholic acid [DB]*	$C_{24}H_{40}O_5$	408.57	407.2803	4.21	C, HC, HCO	√- √
	β-Muricholic acid [DB]*	$C_{24}H_{40}O_5$	408.57	407.2803	4.64	C, HC, HCO	√- √
	Lithocholic acid [DB]	$C_{24}H_{40}O_3$	376.57	375.2905	16.4	C, HC, HCO	√- √
Conjugates	Glycocholic acid [2]	$C_{26}H_{43}NO_6$	465.62	464.3018	3.39	HC, HCO	x- √
	Glycochenodeoxycholic acid [5]	$C_{26}H_{43}NO_5$	449.62	448.3068	4.33	HC, HCO	x- √
	Glycodeoxycholic acid [6]	$C_{26}H_{43}NO_5$	449.62	448.3068	4.57	HC, HCO	x- √
	Taurocholic acid [1]	$C_{26}H_{45}NO_7S$	515.70	514.2844	3.24	HC, HCO	x- √
	Taurochenodeoxycholic acid [3]	$C_{26}H_{45}NO_6S$	499.70	498.2895	4.01	HC, HCO	x- √
	Taurolithocholic acid [10]	$C_{26}H_{45}NO_5S$	483.70	482.2946	5.87	C, HC, HCO	x- x
	Taurodeoxycholic acid [4]	$C_{26}H_{45}NO_6S$	499.70	498.2895	4.20	HC, HCO	x- √
Unknown	Unknown			407.2803	2.99	HC, HCO	x- √
	Unknown			407.2803	3.35	HC, HCO	x- √
	Tauroursodeoxycholic acid *			498.2895	2.83	HC, HCO	x- √
	α-Tauromurocholic acid *			514.2844	2.77	C, HC, HCO	√- √
	β- Tauromurocholic acid *			514.2844	3.07	HC, HCO	x- √
	Taurohycholic acid*			514.2844	4.10	C, HCO	√-√
				514.2844	4.27	C, HC, HCO	√- √
	Unknown			464.3018	2.85	HC, HCO	x- √
	Unknown			498.2895	2.99	HC, HCO	x- √
	Taurohyodeoxycholic acid*			498.2895	3.38	HC, HCO	x- √
	Unknown			375.2905	16.75	C	√- √
						HC, HCO	√- x
	Unknown			375.2905	18.41	C	√-√
						HC, HCO	√- x

488 MW–molecular weight; T_i– initial time; T_f–final time; t_R– retention time;

489 [Numbered]Compounds were identified by comparison with commercial standards. Numbers
490 correspond with standard bile acids peaks shown in Fig. 2.

491 *Compounds tentatively identified by literature review

492 [DB] Compounds tentatively identified by using databases

493

494

495

Table 2. Quantified bile acids in faeces from control (C), high-cholesterol (HC) and high-cholesterol with onion (HCO) groups over 7 weeks of experimental feeding

Compound name / *Group*	***Week 0***	***Week 1***	***Week 2***	***Week 3***	***Week 4***	***Week 5***	***Week 6***	***Week 7***
Cholic acid [8]								
C	0.480 ± 0.26[a]	n.d.	0.279 ± 0.05[a]	3.375 ± 0.71[a]*	0.286 ± 0.02[a]*	0.414 ± 0.12[a]	0.385 ± 0.03[a]	0.715 ± 0.05[a]*
HC	0.284 ± 0.02[a]	245.744 ± 21.35[a]*	192.136 ± 43.37[b]	234.352 ± 37.20[b]	243.029 ± 0.74[c]	230.271 ± 41.85[b]	246.662 ± 7.20[c]	200.351 ± 4.34[c]*
HCO	0.479 ± 0.20[a]	184.453 ± 37.84[a]*	208.449 ± 24.41[b]	175.184 ± 28.03[b]	203.440 ± 12.77[b]	200.965 ± 12.69[b]	140.794 ± 30.59[b]	152.392 ± 2.21[b]
Chenodeoxycholic acid [11]								
C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HC	n.d.	3.242 ± 0.11[a]	3.018 ± 0.42[a]	4.668 ± 0.36[b]*	5.647 ± 0.20[b]	4.906 ± 0.73[a]	5.181 ± 0.30[b]	4.409 ± 0.15[b]
HCO	n.d.	3.140 ± 0.10[a]	3.094 ± 0.46[a]	3.506 ± 0.48[a]	3.773 ± 0.01[a]	4.178 ± 0.13[a]	3.397 ± 0.54[a]	3.415 ± 0.001[a]
Deoxycholic acid [12]								
C	2.452 ± 0.50[a]	2.579 ± 0.18[a]	2.908 ± 0.04[a]	2.664 ± 0.15[a]	2.808 ± 0.117[a]	2.739 ± 0.18[a]	2.192 ± 0.007[a]	2.574 ± 0.11[a]
HC	3.409 ± 0.21[b]	17.469 ± 2.13[bc]*	6.271 ± 0.95[b]*	7.144 ± 1.18[b]	4.021 ± 0.102[b]	8.340 ± 0.78[b]*	10.181 ± 0.72[b]	10.437 ± 0.70[b]
HCO	3.455 ± 0.25[b]	13.112 ± 2.49[b]*	12.545 ± 2.18[bc]	16.251 ± 0.45[c]	13.741 ± 0.179[c]	16.383 ± 0.96[c]	15.091 ± 2.68[c]	20.262 ± 0.21[c]*
Ursodeoxycholic acid [7]								
C	0.114 ± 0.06[a]	0.114 ± 0.01[a]	0.150 ± 0.01[a]	0.196 ± 0.02[a]	0.265 ± 0.060[a]	0.218 ± 0.11[a]	0.335 ± 0.07[a]	0.205 ± 0.02[a]
HC	0.222 ± 0.00[b]	0.737 ± 0.03[c]*	0.844 ± 0.15[b]	1.614 ± 0.17[c]*	1.927 ± 0.144[c]	2.476 ± 0.21[b]*	2.543 ± 0.07[c]	2.424 ± 0.002[c]
HCO	0.140 ± 0.003[ab]	0.411 ± 0.19[b]	0.865 ± 0.13[b]	1.096 ± 0.28[b]	1.470 ± 0.099[b]	2.288 ± 0.07[b]*	1.764 ± 0.24[b]*	1.760 ± 0.11[b]
Hyodeoxycholic acid [9]								

	C	6.969 ± 1.55[a]	4.920 ± 0.56[b]	8.608 ± .007*	7.476 ± 1.24[b]	6.639 ± 0.04[c]	6.072 ± 0.89[b]	6.946 ± 0.63[b]	6.332 ± 0.30[b]
	HC	6.124 ± 0.02[a]	0.282 ± 0.002[a]*	n.d.	1.699 ± 0.13[a]	1.044 ± 0.00[a]*	1.867 ± 0.20[a]*	2.077 ± 0.30[a]	1.605 ± 0.41[a]
	HCO	6.698 ± 1.31[a]	n.d.	n.d.	2.142 ± 0.22[a]	1.889 ± 0.01[b]	2.164 ± 0.25[a]	1.865 ± 0.24[a]	1.481 ± 0.17[a]
Hyodeoxycholic acid									
(Iso.)	C	2.152 ± 0.98[a]	2.033 ± 0.15[b]	3.432 ± 0.006[b]	3.140 ± 0.65[b]	2.875 ± 0.037[c]	2.562 ± 0.29[b]	3.415 ± 0.45[b]	2.482 ± 0.17[b]
	HC	2.216 ± 0.21[a]	2.160 ± 0.21[b]	1.457 ± 0.25[a]	0.669 ± 0.07[a]	0.619 ± 0.003[b]	0.536 ± 0.06[a]	0.760 ± 0.14[a]	0.552 ± 0.11[a]
	HCO	2.539 ± 0.22[a]	1.430 ± 0.26[a]*	1.656 ± 0.28[a]	0.486 ± 0.10[a]*	0.452 ± 0.021[a]	0.466 ± 0.12[a]	0.389 ± 0.006[a]	0.500 ± 0.15[a]
Glycocholic acid [2]									
	C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	HC	n.d.	4.411 ± 0.22[a]	2.515 ± 0.60[a]*	5.034 ± 0.53[a]*	4.905 ± 0.30[a]	3.319 ± 0.51[a]*	3.067 ± 0.08[a]	3.887 ± 0.09[a]
	HCO	n.d.	7.077 ± 1.13[a]	5.727 ± 0.49[b]	3.959 ± 0.48[a]*	5.756 ± 0.13[b]*	5.329 ± 0.16[b]	2.436 ± 0.41[a]*	6.770 ± 0.64[b]*
Glycochenodeoxycholic acid [5]									
	C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	HC	n.d.	0.307 ± 0.057[a]	0.099 ± 0.01[a]*	0.559 ± 0.01[b]*	0.610 ± 0.04[b]	0.511 ± 0.08	0.457 ± 0.03[b]	0.502 ± 0.03[a]
	HCO	n.d.	0.304 ± 0.08[a]	0.373 ± 0.01[b]	0.219 ± 0.09[a]	0.428 ± 0.003[a]	n.d.	0.213 ± 0.04[a]	0.777 ± 0.17[a]*
Glycodeoxycholic acid [6]									
	C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	HC	n.d.	0.872 ± 0.08[b]	0.127 ± 0.01[a]*	0.357 ± 0.31[b]*	0.318 ± 0.01[a]	0.191 ± 0.04*	0.218 ± 0.03[b]	0.273 ± 0.001[a]
	HCO	n.d.	0.258 ± 0.05[a]	0.292 ± 0.002[b]	0.124 ± 0.19[a]*	0.355 ± 0.01[b]*	n.d.	0.073 ± 0.01[a]	0.254 ± 0.04[a]*
Taurocholic acid [1]									

C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HC	n.d.	2.206 ± 0.11[a]	0.535 ± 0.10[a]*	0.943 ± 0.18[a]	1.590 ± 0.08[a]*	0.756 ± 0.13[a]*	0.773 ± 0.16[a]	1.807 ± 0.39[a]*
HCO	n.d.	3.287 ± 0.75[a]	1.706 ± 0.31[b]*	0.710 ± 0.14[a]	2.975 ± 0.12[b]*	0.898 ± 0.01[a]*	0.704 ± 0.17[a]	9.738 ± 0.97[b]*
Taurochenodeoxycholic acid [3]								
C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HC	n.d.	0.085 ± 0.01[a]	n.d.	0.072 ± 0.01[a]	0.095 ± 0.02[a]	n.d.	n.d.	0.079 ± 0.001[a]
HCO	n.d.	0.113 ± 0.02[a]	0.113 ± 0.02	0.134 ± 0.02[b]	0.129 ± 0.01[b]	n.d.	n.d.	0.278 ± 0.03[b]
Taurolitocholic acid [10]								
	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Taurodeoxycholic acid [4]								
C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HC	n.d.	0.235 ± 0.01[b]	n.d.	0.038 ± 0.01[a]	0.043 ± 0.01[a]	n.d.	n.d.	0.105 ± 0.01[a]
HCO	n.d.	0.101 ± 0.02[a]	0.085 ± 0.01	0.162 ± 0.03[b]*	0.204 ± 0.01[b]	n.d.	n.d.	0.122 ± 0.02[a]

Values are expressed as the mean ± SD; n.d.–non detected; * indicates significant difference between consecutive weeks; Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

Electronic supplementary information (ESI)

Table 1. Composition of the experimental diets [control (C), high-cholesterol (HC) and high-cholesterol enriched with onion (HCO)]

Ingredient (g/kg)	C	HC	HCO
Onion powder	–	–	100
Casein	200	200	200
Sucrose	100	100	100
Maize starch	470.49	445.49	368.69
Soya oil	50	50	50
Maize oil	80	80	80
Mineral mixture*	35	35	35
Vitamin mixture†	10	10	10
Cellulose powder	50	50	26.8
Choline bitartrate	2.5	2.5	2.5
tert-butylhydroquinone	0.010	0.010	0.010
L-cystine	2	2	2
Cholesterol	–	20	20
Cholic acid	–	5	5

* Mineral mix for the AIN-93M diet, g/kg (AIN-93M-MX): calcium carbonate anhydrous, 357.00; potassium phosphate monobasic, 250.00; potassium citrate, tripotassium monohydrate, 28.00; sodium chloride, 74.00; potassium sulphate, 46.00; magnesium oxide, 24.00; ferric citrate, 6.06; zinc carbonate, 1.65; sodium meta-silicate $9H_2O$, 1.45; manganous carbonate, 0.63; cupric carbonate, 0.30; chromium potassium sulfate $12H_2O$, 0.275; boric acid, 0.0815; sodium fluoride, 0.0635; nickel carbonate, 0.0318; lithium chloride, 0.0174; sodium selenate anhydrous, 0.01025; potassium iodate, 0.0100; ammonium paramolybdate $4H_2O$, 0.00795; ammonium vanadate, 0.0066; powdered sucrose, 209.806.

† Vitamin mix for the AIN-93M diet, g/kg (AIN-93-VX): niacin, 3.000; calcium pantothenate, 1.600; pyridoxine-HCl, 0.700; thiamin-HCl, 0.600; riboflavin, 0.600; folic acid, 0.200; biotin, 0.200; vitamin B12 (0.1%), 2.500; vitamin E (all-*rac*-α-tocopheryl acetate, 500 IU/g), 15.000; vitamin A (all-*trans*-retinyl palmitate, 500,000 IU/g), 0.800; vitamin D3 (400,000 IU/g), 0.250; vitamin K1, 0.075; powdered sucrose, 974.655.

‡ Diet energy content was calculated using the factors 16.73 kJ/g (4 kcal/g) for protein, 15.69 kJ/g (3.75 kcal/g) for monosaccharides, 16.53 kJ/g (3.95 kcal/g) for disaccharides, 17.49 kJ/g (4.18 kcal/g) for starch, 8.37 kJ/g (2 kcal/g) for dietary fibre, and 37.65 kJ/g for fat. Control diet, 18540.9 kJ/kg (4431.4 kcal/kg); HC diet, 18856.6 kJ/kg (4506.8 kcal/kg); HCO diet, 18642.4 kJ/kg (4455.6 kcal/kg).

Table 2. Nutritional composition, phytochemical compounds, and antioxidant activity of onion powder

	Onion powder
Protein (g/100 g)	9.75 ± 0.08
Lipids (g/100 g)	1.30 ± 0.06
Carbohydrates (g/100 g)	80.10 ± 2.89
Glucose (g/100 g)	27.7 ± 1.73
Fructose (g/100 g)	20.7 ± 0.46
Sucrose (g/100 g)	4.3 ± 0.11
Total fructans (g/100 g)	4.2 ± 0.10
Total dietary fibre (g/100 g)	23.2 ± 1.15
Soluble fibre (g/100 g)	3.2 ± 0.09
Insoluble fibre (g/100 g)	20.0 ± 0.17
Ash (g/100 g)	4.63 ± 0.07
Total phenols (mg GAE/100 g)	1629.6 ± 60.0
Quercetin 3-glucoside (mg/100 g)	32.22 ± 0.90
Quercetin 4′-glucoside (mg/100 g)	950.00 ± 2.99
Quercetin 3,4′-diglucoside (mg/100 g)	1368.89 ± 8.77
Quercetin 7,4′-diglucoside (mg/100 g)	31.56 ± 0.33
Quercetin 3,7,4′-triglucoside (mg/100 g)	9.16 ± 0.32
Isorhamnetin 4′-glucoside (mg/100 g)	45.16 ± 1.54
Isorhamnetin 3,4′-diglucoside (mg/100 g)	32.00 ± 0.19
Total ACSOs (mg BCSOE/100 g)	4120.89 ± 89.43
Propionaldehyde (mg/100 g)	245.04 ± 39.61
1-Propanethiol (mg/100 g)	23.54 ± 0.90
Hexanal (mg/100 g)	0.04 ± 0.001
2-Methyl 2-pentenal (mg/100 g)	10.80 ± 0.67
Propyl thioacetate (mg/100 g)	0.45 ± 0.03
Dimethyl trisulfide (mg/100 g)	66.41 ± 5.02
Dipropyl disulfide (mg/100 g)	89.45 ± 3.29
Methyl propyl trisulfide (mg/100 g)	42.28 ± 2.14
Dipropyl trisulfide (mg/100 g)	25.50 ± 2.45
Ascorbic acid (mg/100 g)	62.31 ± 0.77
Total vitamin C (mg/100 g)	104.26 ± 4.07
Scavenging of $NO^{\bullet}$ (μmol TE/100 g)	1706.00 ± 49.61
$ABTS^{\bullet+}$ (μmol TE/100 g)	4936.67 ± 72.65
$DPPH^{\bullet}$ (μmol TE/100 g)	1135.00 ± 82.21
FRAP (μmol TE/100 g)	12245.14 ± 60.45

Values are expressed as the mean ± SD (n = 3).
GAE, gallic acid equivalents; ACSOs, *S*-alk(en)yl-L-cysteine sulfoxide; BCSOE, *S*-Butyl-L-cysteine sulfoxide equivalents; $NO^{\bullet}$, nitric oxide radical $ABTS^{\bullet+}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; FRAP, ferric reducing antioxidant power; TE, trolox equivalents

Chapter 11 – General Discussion Future Prospects

GENERAL DISCUSSION

The transformation of onion products has been developed in order to attend the consumer demand in the modern day market. The latest demands rely on the addition of value to regular products and by-products of manufacturing procedures, by both optimizing the processing and improving the quality, nutritional composition and content of phytochemicals. This stimulates the application of high-pressure (HP) processing which combined with other preserving technologies creates a stable and easy-to-use product. The transformation of fresh onion into a HP-processed onion ingredient offers advantages over the fresh products such as the conservation of its composition (diminishing the loss of phytochemicals produced by other technologies or processing methods) as well as providing a greater bioaccessibility of the active components. Its final presentation as a dry powder form also facilitates its conservation, storage and offers great versatility of use due to its facility to be added daily in cuisine not only as a flavouring agent but also as a functional ingredient. In contrast with the market of fresh products, the demonstration of added value in the functional ingredients must be endorsed by solid scientific evidence, which in the final instance will justify the corresponding health claim for commercialization.

The study of the biological responses caused by the consumption of this HP-processed onion *in vivo* has verified certain health benefits directly related to the prevention of risk factors associated to prevalent diseases such as liver and CVD. Due to the association between NAFLD and atherosclerosis it is interesting to study in conjunction both pathologies since they share common risk factors such as oxidative stress (Polimeni *et al.,* 2015). The hypercholesterolemic and potentially pro-atherosclerotic diet (HC) incorporated in the experimental design has been directly linked to imbalances in oxidative stress defence mechanisms and inflammatory reactions that lead to abnormal metabolic responses. The changes induced in organ morphology were outstanding showing an evident hepatomegaly and spleen dystrophy in comparison to the control group (Figure 11.1). The plasma and liver fingerprinting also distinguished clear differential patterns reflecting the multiple changes produced in the metabolome. According to previous research by our group, the characterization of this ingredient and its evaluation *in vitro* showed antioxidant and antiinflammatory capacities likely related to the prevention of certain disorders *in vivo* (González-Peña *et al*., 2013). This has been supported by other onion products previously described in the scientific literature (Roldán *et al*., 2008; Roldán-Marín *et al*., 2009; Roldán-Marín *et al.*, 2009b; Suleria *et al*., 2015). The addition of the onion ingredient in a HC diet for seven weeks improved the antioxidant defence mechanisms, ameliorated hepatotoxicity

and positively modulated the inflammatory response of certain markers associated with atherogenesis in the diet-induced hypercholesterolemic rats.

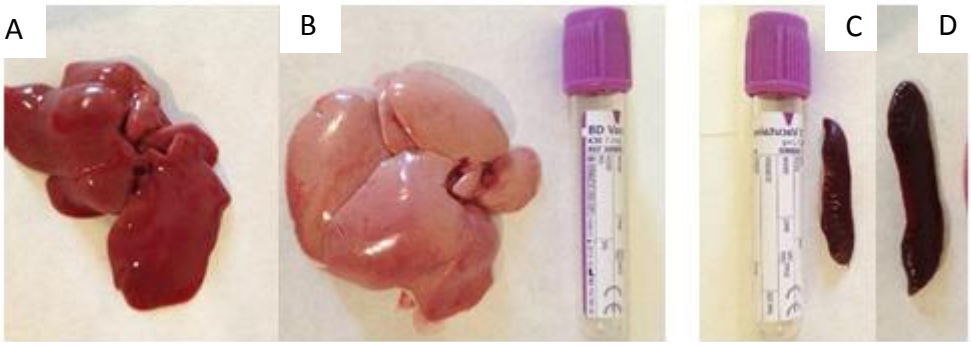


Figure 11.1. Livers (A-B) and spleens (C-D) collected from Wistar rats after 7 weeeks of experimental feeding. A-C) Control rats B-D) Hypercholesterolemic rats

It is interesting to note that most of the benefits derived from the intake of onion in pathological circumstances have been related to the amelioration of hypercholesterolemia, which is clearly recorded by a decrease in cholesterol or lipid levels, and the enhancement of the antioxidant defence system. These ameliorative effects have generally been linked to the presence of flavonoids, FOS and organosulfur compounds (Griffiths *et al.*, 2002; Roldán-Marín *et al*., 2012). It is probable that the functions of the onion component go beyond these effects in complex synergistic and interacting mechanisms modulating the outcome. This singularity has been demonstrated in our experimental model, where the vascular and hepatic improvements produced by onion supplementation were revealed by certain plasma markers, but could not be directly correlated to a decrease in total cholesterol levels found in plasma and liver.

Even though it may result obvious that additional investigation is required to understand the complexity of the mechanism of action involved in this pathological model, it is worth remarking that while the study of isolated functions is useful to a certain extent, to ascertain what occurs in the whole living system requires a complex projection in both the design and workflow applied. Studying the properties of onion products, it was observed that although the benefits of onion are related to several pathologies, research in this field has made little progress, focusing on the systematic repetition of analyses of usual parameters related to the hypolipidemic and antioxidant capacities (*e.g.* Corzo-Martínez & Villamiel, 2012; Kang e*t al*., 2016; Kim & Noh, 2016; Lu *et al*., 2015; Roldán-Marín *et al*., 2012). Advancement in the knowledge of the mechanisms involved in a living system does not necessarily follow a straight line and from time to time requires a change of perspective, taking a step aside, to analyse what

other changes are simultaneously occurring and then to focus the research on those pathways or functions most affected by the particular conditions. This will be the approach of the new research.

In light of this projection, the present PhD Thesis has been divided into three different phases. The first phase is based on the confirmation of the forced pathological circumstances and the effect of the onion supplementation on known biomarkers (section I). The second phase focuses on the description of the general metabolic alterations promoted by a hypercholesterolemic diet and the subsequent effect of the onion ingredient on the metabolic responses associated to vascular and hepatic impairments (section II) and the third phase analyses some of the specific altered pathways to obtain more detailed information on downstream metabolites (section III). Thus, this research concentrates on obtaining specific information that confirms an initial basic hypothesis and moves from the particular to the general (applying non-targeted metabolomics) with the aim to generate information to help create new hypothesis. It then moves back again from the general to the particular to initiate new research lines to elucidate some of the possible underlying mechanisms.

Another noteworthy aspect in this study is the statistical analysis applied and the mode of interpreting the results. In this sense, the effect of HC feeding was unbiased by comparing the HC-fed group with the C group. Nevertheless, the extent to which the onion ingredient may affect the regulation induced by HC-feeding is analysed from two different angles. On the one hand, the differences found between the HCO-fed group and the HC-fed group indicate big changes in magnitude and significance in the metabolic pattern and thus, changes in the possible underlying activities that lead to those metabolites. However, on the other hand, small changes should not to be overlooked since they could reflect a big difference in the system function, being easily and unfortunately under evaluated by comparing HCO-fed group *vs.* HC-fed group. Thus, the relative comparison of the HCO and HC groups in relation to the C group reveals more subtle changes that may have biological significance. In this regard, the statistics applied to the global metabolomics analysis support a direct multiple comparison of the three groups, highlighting relevant changes between the HCO and HC groups. The analysis of lipid mediators, which are found in very low concentrations (10^{-6} to 10^{-9}) but which may act as very important signalling mediators, focuses on the significance of the fold changes generated by comparison of the HC and HCO groups relative to the C group.

Additionally, it is interesting to highlight that the objective of this experimental design was to show a preventive effect following regular onion consumption. Although blood and organs

were examined at the end of the experimental feeding, the results obtained differ slightly with those which pursue a direct measure of effect reversion. The present design cannot ensure that feeding this onion ingredient (though likely) would revert the stages of established pathological situations but it does provide evidence about the positive influence on a delay in onset and deceleration in the progression of the pathology.

Therefore, the originality of this PhD Thesis relies on several aspects that gave rise to seven research papers. Each of these research papers presents new work that provides insight regarding the effects of the onion ingredient. Due to the novelty of these findings some of the results are presented as large sets of descriptive data which cannot be compared with former studies. From all the results, four aspects need to be emphasised and stand out in particular due to either the approach utilized or to the effect exerted by the onion ingredient:

- The onion diet enrichment preserved endothelium dependent relaxation functions in the mesenteric arteries of Wistar rats fed a daily high-cholesterol diet, and modulated positively cardiovascular risk biomarkers such as MCP-1, s-ICAM, sE-selectin, IL-10, VEGF, PAI-1, vWF, TIMPs related to endothelial dysfunction and the coagulation system.
- Metabolomics was used as a research engine to explore unknown effects produced by onion consumption in relation to vascular and hepatic dysfunction. It has provided a wide description of the changes stimulated in the plasma and liver metabolome of hypercholesterolomic rats, facilitating lists of metabolites and indicating pathways that need further exploration and may lead to new lines of research.
- The role of onion as a regulator of proinflammatory mediators and possible enhancer of pro-resolution pathways is underlined. The analysis of lipid mediators produces relevant information to help elucidate the interactive effect of functional ingredients within the diet and the potential impact on mechanisms that may lead to organ dysfunction.
- Changes in the gut microflora were explored indirectly, firstly, by analysing microbial composition of faeces at the beginning and at the end of the experimental feeding and secondly, by exploring changes in the excretion of bile acids. These studies attract attention to the influence of onion modifying the microbiome, and how diet induces changes in a microbiome and is able to interact and modulate the progression of disease.

In addition, to distinguish between the two pathologies under study, the most relevant findings from different parts of the investigation have been integrated allowing us to obtain the following pictures on the mechanism whereby the onion supplementation has had an effect:

- *Onset and progression of hypercholesterolemia toward CVD.*

The induced endothelial dysfunction found in this hypercholesterolemic model fitted with the pathological status indicated by multiple markers. Likewise, the effects observed in the group of rats fed the onion ingredient reveals the counteracting effects of onion in the progression of the pathophysiological framework toward atherosclerosis.

The results obtained suggest a possible decrease in nitric oxide NO production and bioavailability promoted by ROS such as $O_2^{\bullet -}$ in the hypercholesterolemic group, the greater generation levels of which were confirmed by the activity of NADPH oxidase. These negative outcomes were also reflected by the activities of SOD, CAT and GPx enzymes. Nevertheless, these activities were shown to be partially stabilized by the enhancement of the antioxidant defence system and a significant drop of NOX activity produced by onion supplementation. The described oxidative mechanisms may be also connected to the dyslipidemic status, which in turn is linked to endothelial dysfunction.

The endothelial impairments are additionally characterized by an increase in the plasma ADMA levels. ADMA is known to compete with L-arginine for the binding site on eNOS and its uncoupling results in O_2^- production and a decrease in NO (Park & Parck, 2015). Interestingly, the increase of ADMA in the blood stream of the hypercholesterolemic rats was controlled significantly by the addition of onion, at the same time that arginine plasma concentration was found to increase. Additionally, the dysregulation of the kyrunenine levels was ameliorated in the group fed the onion ingredient, thus improving the efficacy of onion by keeping the altered concentrations of relevant markers similar to the control group. These facts reinforce the possible contribution of the onion ingredient *via* regulation of cytokines that help suppress the impact of high cholesterol in the pathogenesis of atherogenesis. In this regard, proinflammatory cytokines such as TNF-α, IL-1β and IFNγ tended to be increased by the high-cholesterol diet and ameliorated by the onion ingredient.

In addition, the present work suggests that the regulation of NF-kB (which is involved in arterial inflammation) may have been affected by several factors that were positively modulated by the onion ingredient supplementation. Among these factors, the increased production of ROS and lipids that induced proinflammatory changes in the endothelial phenotype and the inflammation mediated by cytokines and adhesion molecules are strongly indicated as potential modifiers.

The significant decrease of MCP-1, sICAM-1 and E-selectin produced by onion indicated a decline in the progression of atherosclerosis, suggesting improvements in the endothelial function (as was confirmed by the study of vascular reactivity). In addition to MCP-1 levels, the response of VEGF and the decrease in PAI-1 and vWF concentrations in the HCO group may have contributed to the prevention of microvascular complications and an improvement of the anticoagulation system.

Therefore, taken as a whole, this suggests that the onion ingredient may not only contribute to improving the microcirculation by acting as a potential exogenous NO pathway activator and ROS scavenger, but also by maintaining ADMA, kyrunenine, some cytokines, chemokines and adhesion molecules at normal levels in the course of hypercholesterolemia. In addition, it acts as a modifier of the methylation cycle, the arginine and tryptophan pathways as well as the routes involved in the transformation of glycerophospholipids.

- *Hepatic dysfunction induced by hypercholesterolemia*

As previously mentioned, but now focusing on the induced liver dysfunction, the diet enrichment with the onion ingredient (HCO) produced significant improvements by ameliorating hepatotoxicity, decreasing oxidative stress and modulating inflammation in the liver. The elevated circulating levels of ALT and AST and the activity of enzymes such as CAT, SOD and GPx measured in liver, reflect the development of liver injury caused by the HC feeding and the simultaneous amelioration produced by the addition of the onion ingredient. Furthermore, liver protein carbonyls, which are identified as biomarkers for protein damage caused by oxidized amino acid residues in stress conditions, revealed that the HCO feeding produced a significant decrease compared with the HC diet, which was significantly correlated with liver SOD activity and liver GPx activity.

Additionally, most acute and chronic liver diseases are characterized by inflammation which shows enhanced expression of various pro- and antiinflammatory cytokines in the liver (Tilg, Kaser & Moschen, 2006). However, it should be born in mind that the interpretation of cytokine levels is controversial since the same mediators intervene in both hepatic inflammation and damage, as well as possible regeneration (Tilg, 2001). Among these cytokines, special attention must be drawn to TNFα and IL-10, which play important roles in inflammation and both of which were found with modified levels in the group supplemented with the onion ingredient of this study. However, these changes might reflect the positive modulation of other mediators, which simultaneously induced a decline in TNFα and IL-10 levels. Although some of the

benefits of the consumption of onion were linked to liver function instead of vascular dysfunction, it is very important to remember that amelioration of liver impairment will also have a positive impact on prevention of cardiovascular disease.

Furthermore, the non-targeted metabolomics approach in liver tissues indicated that specific metabolites such as hydroxybutyrylcarnitine and palmitoylcarnitine along with some glucosyl forms, methylated and free fatty acids, all tentatively identified, were significantly affected by the HC diet, and reflected an impact in response to onion consumption at the core of hepatic dysfunction.

Among the disturbances induced by the HC diet in the lipids profile, the modification of free fatty acids in plasma and liver attracted an interest to study the changes in the AA pathway. In this respect, although the hepatoprotective effect triggered by the intake and metabolism of the onion ingredient could not be directly linked with improvements in the hepatic lipid profile with direct removal of lipids (total cholesterol, LDL, TAG), interesting findings were found in some lipid mediators such as oxylipins and sphingolipids. The changes observed in oxylipins as oxidation products of fatty acids [arachidonic acid (AA), linoleic acid (LA), α-linolenic acid (α-LA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] have newly attracted our interest concerning the potential protective effect for health of onion.

Whereas the onion ingredient did not revert the decline in circulating oxylipins in the hypercholesterolemic rats, it did ameliorate HC diet-related changes in the hepatic concentrations of PGs (PGD_2 and PGE_2 as well as PGD_1 and PGE_1, both COX metabolites derived from AA and DHGLA, respectively) and modified the concentration of some CYP450/LOX derived oxylipins in the liver of HCO-fed rats. In this case, a common link might also be established between the inhibition of NF-κB, the subsequent COX-2 activity and the final PGs concentration. Additionally, the levels of ω-3-derived oxylipins were increased, which encourages further investigation of the effect of onion on pro-resolution pathways.

Likewise, the levels of Cer and other sphingolipids such as HexCer and LacCer in the HCO-fed rats suggest a significant amelioration in the liver associated to the intake of the onion ingredient. However, certain modifications could be interpreted as a metabolic response to offset the diet-induced hepatic steatosis and inflammation, rather than a plausible direct impact on the metabolite synthesis or transformation.

Therefore, whereas the changes found in the eicosanoid and other PUFA-derived oxylipins and the sphingolipid metabolism are not suggested to be the principal modifiers of the

vasculature of this model, the role played by these metabolites in both the progress of inflammation and the potential resolution in organs may be crucial. All these findings help to conclude that the consumption of onion as a functional ingredient may be useful to ameliorate the impact of hypercholesterolemia in liver inflammation. However, as the chemistry behind these metabolites is very complex, a focused study of the synthetic pathways of both oxylipins and sphingolipids is still necessary for a comprehensive interpretation of the data. Accordingly, this warrants further study of the interaction of functional ingredients with bioactive lipid mediators and their potential impact on inflammation and organ dysfunction.

Finally, the analysis of faeces also indicated changes in the hypercholesterolemic rats that compromised the liver functions. In parallel, the onion ingredient contributed to changes in metabolism that suggest an increase in the reabsorption of bile acids (BA) at an intestinal level and a decrease in BA deconjugation, which highlights the importance and influence of diet in the microbiome as a potentially crucial modifier of the metabolic responses found in the course of hypercholesterolemia. However, it is clear that to facilitate the understanding about how onion may influence the enterohepatic circulation regulation and the homeostatic feedback mechanisms with the luminal microbiota that affects the bile acid signalling in the progression of hypercholesterolaemia requires extensive and more exhaustive investigation.

Apart from the interest relative to these pathologies, during the search for biomarkers of onion consumption throughout this PhD Thesis, it has become evident that the analysis of metabolites directly derived from onion metabolism should be addressed by direct targeted approaches. However, the detection of some of these compounds by non-targeted approaches may serve to emphasize the importance of key metabolites. This was the case for *S*-methyl-L-cysteine and *S*-methylcysteine sulphoxide, the detection of which was possible in plasma samples applying non-targeted CE–MS.

In general terms, it is worth remarking that elucidating the effects of processed onion, as for any other functional ingredient, in the metabolic responses triggered at the core of hypercholesterolemia and vascular and/or hepatic dysfunctions is exceedingly complex and requires an extensive view of all the modifications and adaptations of functions that are provoked by the imbalance. In addition, as it has been stressed in the research papers, the chemical diversity and large differences in the concentration range detected in the analysis of biological samples evidences the fact that no single technique is capable of detecting all the metabolites involved. This supports the need to utilize complementary approaches for metabolic screening to increase the metabolome coverage. At the same time, it must remain

clear that each analytical technique has different capabilities and is only suitable for the analysis of a specific range of compounds. Therefore, in order to consider the strengths and weaknesses of each platform is essential to choose the right analytical technique in accordance with the specific groups or families of compounds which are to be detected and quantified.

Even so, despite the complexity, with the technology currently available, investigation of the response of entire living systems to a specific diet is not only a realistic objective but also is within the budget of scientific investigation. With this availability, the techniques may help comprehend both the aetiology of disease as well as the modulatory influence of the diet in the progression of disease, the amelioration or reduction of symptoms and the speed of progression of the impairments produced by disease or an unbalanced diet.

Therefore, the present PhD Thesis supports the use of metabolomics and the movement towards the application of systems biology in the field of nutrition. This movement will help identify the association between dietary patterns and risk factors of disease. In addition, it will allow for the development of nutritional strategies to help enhance general public health as well as manage pathologies or fulfil specific health requirements. As a result, not too far into the future, the integration of data into the formulation of predictive models and model-based personalized diagnostics could be achieved.

FUTURE PROSPECTS

To fully understand the complex mechanisms of action behind this functional onion ingredient in hypercholesterolemia and its repercussion in CVD and liver dysfunction further research in greater depth is required at both the *in vitro* and *in vivo* level. However, the present project has established some basis to redirect the focus of new work. Of the multiple lines of investigation to continue the research, the options offered by metabolomics are outstanding.

In general terms, the identities putatively assigned to metabolites need to be confirmed as well as the downstream metabolites in the pathways of interest by means of targeted analysis. This validation of the models is an essential primary step that needs to be taken before going any further. When confirmed, this would guide future planning of new experiments *in vitro* to verify the influence of the onion ingredient on metabolic reactions and enzyme systems, starting at a simple and progressing towards more complex levels of organization (cell-tissue-organs-organ systems-organisms), to finally upgrade the research to the highest level of experimentation in humans. In addition, the exploration and identification of unknown significant compounds by both LC–MS and CE–MS, although a laborious task, would provide novel and valuable information. An additional step would be to follow the specific pathways in different organs, tissues and fluids such as plasma/serum or urine, permitting a correlation between the metabolites considered as potential metabolic markers and then to correlate these with other biochemical markers of interest for the pathology under analysis such as inflammation, vascular and hepatic dysfunction, etc. This practice will facilitate the extrapolation of the modifications occurring at an organ level to modifications quantifiable in bio-fluids, thus avoiding the need to apply invasive practices.

In another category, the research on biomarkers of onion intake by targeted approaches orientated to identify the different families of metabolites will facilitate the assessment of individual diets and the effects of supplementation with complementary dietary ingredients. This could lead to the design of dietary interventions aimed to diminish hepatic damage and subsequent vascular deterioration, both associated to NAFLD and cardiometabolic dysfunctions.

The progress offered by the use of the new technologies such as metabolomics opens the door to multiple new lines of investigation, and the results presented in this PhD Thesis is a contribution to the interest in the application of global approaches to characterize the changes

at multiple functional levels which efficiently permits the assessment of the effects of functional ingredients, such as the onion ingredient presented in this research project.

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Chapter 12 – Conclusions

CONCLUSIONS

This PhD Thesis comprises seven complementary research papers interpreting the effects of an onion ingredient at the core of diet-induced hypercholesterolemia. These studies have been presented in three sections, from which the following conclusions can be extracted:

SECTION I – Biochemical, vascular, and microbiological analyses

- Feeding the animals with a diet composed of cholesterol (2%) and cholic acid (0.5%) for seven weeks leads to hypercholesterolemia, oxidative stress, inflammation, vascular impairments and liver dysfunction.
- The onion ingredient enhanced the antioxidant defence mechanisms, ameliorated hepatotoxicity and improved the inflammatory response associated with atherogenesis in the hypercholesterolemic rats. This was reflected by an increase in the activity of CAT, SOD and GPx enzymes in both erythrocytes and liver, a reduction of ALT and AST in plasma, and a modulation of cardiovascular risk biomarkers such as MCP-1, s-ICAM, sE-selectin, IL-10, VEGF, PAI-1, vWF, TIMPs related to endothelial dysfunction and the coagulation system.
- Onion ingredient enrichment of the hypercholesterolemic diet modulated vascular dysfunction, reducing some of the risk indexes linked to initial stages of atherosclerosis. Preservation of the endothelium dependent relaxation functions in the mesenteric arteries and in the activity of NOX was observed.
- Diet enrichment with onion promoted beneficial modification in gut microflora. Initial phases of the onion supplementation showed increases in *Lactobacillus* spp. and *Bifidobacterium* spp., while the pathogenic *Clostridium* spp. decreased after seven weeks of experimental feeding.

SECTION II – Non-targeted metabolomics studies: Fingerprinting

- Plasma fingerprinting of hypercholesterolemic rats permitted the description of circulating metabolic changes induced by a high-cholesterol diet. A series of changes occurred in the metabolome of Wistar rats which indicated key metabolites and metabolic routes, the monitoring of which could contribute to the prevention of vascular and hepatic impairments.

- The intake of the onion ingredient was associated with modulations of the metabolic profile of hypercholesterolemia. The study underlines the need to study in depth the methylation cycle, arginine and tryptophan pathways, the modulation of 3-methylhistidine, as well as the routes involved in the glycerophospholipids transformation. *S*-methyl-L-cysteine and *S*-methylcysteine sulphoxide were indicated as potential metabolites to be targeted in the evaluation of biomarkers of onion intake.
- The hepatic metabolic signature of hypercholesterolemic Wistar rats and the changes induced by the onion ingredient indicated metabolite candidates for group discrimination (C, HC, HCO). The energy and lipid metabolism including perturbations in the TCA cycle and β-oxidation, as well as the bile acid synthesis and possibly the methionine metabolism, reflected a significant impact in response to onion consumption. Hydroxybutyrylcarnitine, palmitoylcarnitine, some glucosyl forms, and methylated and free fatty acids were the main tentatively identified metabolites affected by the consumption of the onion ingredient.

SECTION III – Targeted analysis metabolomics studies: Profiling

- The shifts in plasma ω-3 and ω-6 PUFA-derived oxylipins induced by the hypercholestolemic diet and the onion ingredient are described. The onion ingredient enrichment modulated the hepatic concentrations of prostaglandins and enhanced ω-3 oxylipins in the liver of the hypercholesterolemic rats.
- The high-cholesterol diet induced a specific plasma sphingolipid pattern with increased sphingoid bases, dihydroceramides and ceramides, while the sphingomyelin, hexosylceramide and lactosylceramide families decreased. Onion supplementation modified some changes in sphingolipids induced by the hypercholesterolemic diet in liver and spleen tissue.
- Onion is proposed for study as a regulator of proinflammatory mediators, and possible enhancer of pro-resolution pathways.
- Specific primary and secondary bile acids (BA) in both unconjugated and conjugated forms indicated that onion supplementation might increase the BA reabsorption at intestinal level and decrease the BA deconjugation in the course of hypercholesterolaemia. The microbiome modification induced by the onion ingredient as a critical modifier of the faecal bile acid composition is proposed.

Capítulo 12 – Conclusiones

CONCLUSIONES

La presente Tesis Doctoral se compone de siete artículos científicos cuyo contenido es complementario, permitiendo así lograr una interpretación integrada sobre los efectos de un ingrediente de cebolla en hipercolesterolemia inducida por la dieta. Estos estudios han sido estructurados en tres secciones, de las cuales, se pueden obtener las siguientes conclusiones:

SECCION I – Análisis bioquímicos, vasculares y microbiológicos

- La administración de una dieta compuesta por colesterol (2%) y ácido cólico (0.5%) durante siete semanas indujo el desarrollo de hipercolesterolemia, estrés oxidativo, inflamación, alteraciones vasculares y disfunción hepática en ratas Wistar.
- La ingesta del ingrediente de cebolla mejoró los mecanismos de defensa antioxidante, redujo el nivel de hepatotoxicidad y mejoró la respuesta inflamatoria asociada con aterogénesis en las ratas hipercolesterolémicas. Dichos cambios fueron reflejados por un aumento en la actividad de las enzimas catalasa (CAT), superóxido dismutasa (SOD) y glutation peroxidasa (GPx) en eritrocitos e hígado, una disminución de los niveles de alanina transaminasa (ALT) y aspartato transaminasa (AST) en plasma, y la modulación de biomarcadores de riesgo cardiovascular, como la proteína quimiotáctica de monocitos-1 (MCP-1), molécula de adhesión intercelular (s-ICAM), selectina E (sE-selectin), interleucina 10 (IL-10), el inhibidor del activador de plasminógeno-1 (PAI-1), el factor de Von Willebrand (vWF) y los inhibidores tisulares de metaloproteinasas (TIMPs), todos relacionados con la disfunción endotelial y el sistema de coagulación.
- La suplementación de la dieta hipercolesterolémica con el ingrediente de cebolla moduló el grado de disfunción vascular inducido, reduciendo algunos índices de riesgo relacionados con estadios iniciales de aterosclerosis en las ratas hipercolesterolémicas. También se observó el mantenimiento de la función de relajación dependiente de endotelio en las arterias mesentéricas y en la actividad de NOX.
- La suplementación con el ingrediente de cebolla indujo modificaciones beneficiosas en la microflora del tracto intestinal. Fases iniciales de la suplementación con cebolla mostraron aumentos en *Lactobacillus* spp. y *Bifidobacterium* spp., mientras que los patógenos *Clostridium* spp. disminuyeron tras siete semanas de alimentación experimental.

SECCION II – Análisis metabolómicos no dirigidos: Fingerprinting

- El análisis por fingerprinting del plasma de ratas hipercolesterolémicas permitió describir cambios metabólicos inducidos por la dieta rica en colesterol. Los cambios ocurridos en el metaboloma de ratas Wistar indicaron metabolitos clave en rutas metabólicas, cuya monitorización puede contribuir en la prevención de alteraciones vasculares y hepáticas.
- La ingesta del ingrediente de cebolla fue asociada con la modulación del perfil metabólico inducido en hipercolesterolemia. El estudio evidencia la necesidad de estudiar en profundidad el ciclo de la metilación, las rutas metabólicas de la arginina y del triptófano, la modulación de 3-metil histidina, así como las rutas involucradas en la transformación de glicerofosfolípidos. *S*-metil-L-cisteina y *S*-metilcisteina sulfóxido son indicados como metabolitos candidatos de evaluación, mediante análisis dirigidos en la búsqueda y confirmación de biomarcadores de la ingesta de cebolla.
- El perfil metabólico del hígado en ratas Wistar hipercolesterolémicas y los cambios inducidos por el ingrediente de cebolla mostraron metabolitos candidatos para la discriminación entre los grupos en estudio (C, HC y HCO). El metabolismo energético y lipídico, incluyendo la alteración en el ciclo de los ácidos tricarboxílicos y la β-oxidación, así como la síntesis de ácidos biliares y el metabolismo de la metionina reflejaron un impacto significativo, en respuesta al consumo de cebolla. Hidroxibutirilcarnitina, palmitoilcarnitina, así como algunas formas glucosiladas, además de ácidos grasos libres y metilados, fueron los principales metabolitos tentativamente identificados que se afectaron por el consumo del ingrediente de cebolla.

SECCION III – Análisis metabólicos dirigidos: Profiling

- Se han descrito diferencias en oxilipinas derivadas de ácidos grasos poliinsaturados ω-3 y ω-6 en plasma, estimuladas por la dieta hipercolesterolémica y el ingrediente de cebolla. El enriquecimiento de la dieta con el ingrediente de cebolla moduló las concentraciones de prostaglandinas hepáticas y mejoró los niveles de oxilipinas ω-3 en el hígado de ratas hipercolesterolémicas.
- La dieta rica en colesterol indujo un perfil plásmatico de esfingolípidos, caracterizado por un aumento en la concentración de bases esfingoides y dihidroceramidas, mientras

que las esfingomielinas, hexosilceramidas y lactosilceramidas disminuyeron. La suplementación con cebolla causó algunos cambios en los niveles de esfingolípidos inducidos por la dieta hipercolesterolémica en hígado y bazo. Se propone el estudio de la ingesta de cebolla como posible regulador de mediadores proinflamatorios y estimulador de las rutas de proresolución en procesos de inflamación.

- Los ácidos biliares primarios y secundarios en forma conjugada y no conjugada indicaron que la suplementación con cebolla podría aumentar la reabsorción de ácidos biliares a nivel intestinal, modificando el nivel de conjugación en el curso de hipercolesterolemia. Se propone que las modificaciones en el microbioma inducidas por el ingrediente de cebolla pueden ser un modificador crucial de la composición de ácidos biliares fecales.

Annex I – Abbreviations

A

AA arachidonic acid
ABTS $^{\bullet+}$ 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
ACh acetylcholine
ACN acetonitrile
ACSOs *S*-alk(en)yl-L-cysteine sulfoxides
ADA American Dietetic Association
ADMA asymmetric dimethylarginine
AED atomic emission detector
AI atherogenic index
ALD alcoholic liver disease
ALP alkaline phosphatase
ALT alanine aminotransferase
AMDIS Automated mass spectrometry deconvolution and identification system
ANOVA analysis of variance test
AST aspartate aminotransferase

B

BA bile acids
BCRP breast cancer resistance protein
BGE background electrolyte
BSTFA *N*,*O*-Bis(trimethylsilyl) trifluoroacetamide

C

C control
CA cholic acid
CAT catalase
CCA cholangiocarcinoma
CDCA chenodeoxycholic acid
CE capillary electrophoresis
CE–LIF capillary electrophoresis – laser induced fluorescence
CE–MS capillary electrophoresis coupled to mass spectrometry
Cer ceramides
CE–TOF–MS capillary electrophoresis–time of flight– mass spectrometry.
mass spectrometry.
CE–UV capillary electrophoresis –ultraviolet detector
CI chemical ionization
CID collision induced dissociation
CoA coenzyme A
COX cyclooxygenase
CYP cytochrome P450
CYP7A1 cholesterol 7-alfa-hydroxylase
CV coefficient of variation
CVD cardiovascular disease
CZE capillary zone electrophoresis
cGMP cyclic guanosine monophosphate

D

DA discriminant analysis
DAD diode array detector
DADS diallyl disulphide
DAG diacylglycerides
DART direct analysis in real time
DAS diallyl sulphide
DB database
DHA docosahexaenoic acid
DhCer dihydroceramides
DHGLA dihomo-gamma-linolenic acid
DiHETE dihydroxy-eicosatetraenoic acid
DiHETrE dihydroxy-eicosatrienoic acid
DiHOME dihydroxy-octadecenoic acid
DIMS direct infusion to mass spectrometry
DMSO dimethyl sulfoxide
DOX doxorubicin
DPPH$^{\bullet}$ 2, 2-diphenyl-1-picrylhydrazyl radical
dw dry weight

E

E extraction
EDHF endothelium-derived hyperpolarizing factor
EDTA ethylenediaminetetraacetic acid
EFSA European Food Safety Authority
EGTA ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EI electron impact ionization
EIC extracted ion chromatogram
EKODE epoxy-keto-octadecenoic acid
EOF electroosmotic flow
EPA eicosapentaenoic acid

EpDPE epoxy-docosapentaenoic acid
EpETE epoxy-eicosatetraenoic acid
EpODE epoxy-octadecadienoic acid
EpOME epoxy-octadecenoic acid
EpETrE epoxy-eicosatrienoic acid
ERK1/2 Extracellular signal-regulated protein kinases 1 and 2
ESI electrospray ionization
EU European Union

F

FAME fatty acid methyl esters
FAO Food and Agriculture Organization
FAS fatty acid synthase
FbF find by formula
FDR false discovery rate
FER food efficiency ratio
FFA free fatty acids
FOS fructooligosaccharides
FRAP ferric reducing antioxidant power
FTIR Fourier transform infrared spectroscopy
FT–ICR–MS Fourier transform ion cyclotron resonance–mass spectrometry
FUFOSE Functional Food Science in Europe
FXR-α farnesoid X receptor α

G

G-CA glycocholic acid
G-CDCA glycochenodeoxycholic acid
G-DCA glycodeoxycholic acid
GalCer galactosylceramide
GC gas chromatography
GC–EI–Q–MS gas chromatography–electron impact–single quadrupole–mass spectrometry
GC–MS gas chromatography coupled to mass spectrometry
GGT gamma glutamyl transferase
GL glycolipids
GM genetically modified
GPx glutathione peroxidase
GR glutathione reductase
GSA gas sensor array

H

1H NMR proton nuclear magnetic resonance
HC high-cholesterol
HCC hepatocellular carcinoma
HCO high-cholesterol enriched with onion
HDCA hyodeoxycholic acid
HDL high density lipoprotein
HDoHE hydroxy-docosahexaenoic acid
HEPE hydroxy-eicosapentaenoic acid
HETE hydroxy-eicosatetraenoic acid
HETrE hydroxyl-eicosatrienoic acid
HexCer hexosylceramide
HHP high hydrostatic pressure
HHTrE hydroxy-heptadecatrienoic acid
HMDB Human Metabolome Database
HO-1 heme oxygenase-1
HODE hydroxy-octadecadienoic acid
HOTE hydroxy-octadecatrienoic acid
HP high-pressure
HPLC high performance liquid chromatography
HPLC–DAD high performance liquid chromatography – diodo array
HPP high-pressure processing
HPLC–UV–MS/MS
(HR-MAS) NMR High resolution-magic angle spinning NMR spectroscopy
HS GC–MS headspace gas chromatography-mass Spectrometry

I

ICAM-1 Intercellular adhesion molecule-1
ID identification
IEM incremental elastic modulus
IFIC The International Food Information Council
IFN-γ interferon-gamma
IL-x interleukin-x
ILSI The International Life Sciences Institute of North America
IS internal standard

J

JK Jack–Knife

K

KEGG Kyoto Encyclopedia of Genes and Genomes
KETE oxo-eicosatetraenoic acid
KODE oxo-octadecadienoic acid
KOTE oxo-octadecatrienoic acid
KYN kynurenine

L

LA linoleic acid
LacCer lactosylceramides
LC liquid chromatography
LCAT lecithin-cholesterol acyltransferase
LC–EC liquid chromatography–electrochemical array
LC/ESI-MS/MS liquid chromatography electrospray ionization–tandem mass spectrometry
LC−FTICR-MS liquid chromatography−Fourier transform ion cyclotron resonance-mass spectrometry
LC–MS liquid chromatography coupled to mass spectrometry
LC–MS/MS liquid chromatography – tandem mass spectrometry
LC–NMR liquid chromatography coupled to nuclear magnetic resonance
LC–QTOF–MS liquid chromatography–quadrupole time of flight–mass spectrometry
LDL low density lipoprotein
LOD limit of detection
LOQ limit of quantification
LOX lipoxygenase
LPA lysophosphatidic acid
LPC lysophosphatidylcholine
LPE lysophosphatidylethanolamine
LPG lysophosphatidylglycerol
LPI lysophosphatidylinositol
LPS lysophosphatidylserine
LTB_4 leukotriene B_4
LXA_4 lipoxin A_4
α-LA alpha-linolenic acid

M

m/z mass/charge ratio
MALDI–MS matrix assisted laser desorption−mass spectrometry
MAPK mitogen-activated protein kinase
MCA muricholic acids
MCSO *S*-methyl-L-cysteine sulfoxide
METLIN Scripps Center for Metabolomics and Mass Spectrometry
MFE molecular feature extractor
MMPs matrix metalloproteinases
MPO myeloperoxidase
MPP mass profiler professional
MRM multiple reaction monitoring
MRP 4 multidrug resistance-associated protein 4
MS mass spectrometry
MS/MS tandem mass spectrometry
MVA multivariate analysis
mRNA messenger ribonucleic acid
3-MH 3-methylhistidine

N

NA noradrenaline
NADPH nicotinamide adenine dinucleotide phosphate
NAFLD non-alcoholic fatty liver disease
NASH non-alcoholic steatohepatitis
NF-κB nuclear factor kappa B
NIH National Institutes of Health
NIST National Institute of Standards and Technology
NMR nuclear magnetic resonance
NO nitric oxide
NOX NADPH oxidase

O

ob/ob
OCT RF Vpp octopole radio frequency voltage
OPLS ortogonal partial least squares
OPLS–DA ortogonal partial least squares – discriminant analysis

OSCs organosulfur compounds

P

PA phosphatidic acid
PAI-1 plasminogen activator inhibitor 1
PASSCLAIM The process for the assessment of scientific support for claims of foods
PBM probability-based matching
PC phosphatidylcholine
PCA principal components analysis
PCSO *S*-propyl-L-cysteine sulfoxide
PE phosphatidylethanolamine
PG phosphatidylglycerol
PGs prostaglandins
PGE_x prostaglandin E
PGD_x prostaglandin D
PGF_x prostanglandin F
PI phosphatidylinositol
PLS–DA partial least squares – discriminant analysis
ppm parts per million
PRENCSO trans-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide
PS phosphatidylserine
PUFA polyunsaturated fatty acid

Q

Q quadrupole
Q^2 prediction power
QC quality control
QTOF quadrupole time of flight

R

R^2 goodness of sample classification
RI retention index
ROS reactive oxygen species
RP reversed phase
RT retention time
RTL retention time locking

S

S1P sphingosine-1-phosphate
SCFA short chain fatty acids
SD standard deviation
SFA supercritical fluid extraction
SHP short heterodimer partner
SM sphingomyelins
SMC *S*-methylcysteine
SMCs smooth muscle cells
SNP sodium nitroprusside
SOD superoxide dismutase
Spa sphinganine
Spa1P sphinganine-1-phosphate
SPE solid phase extraction
Sph sphingosine
SPME solid phase micro extraction
SREBP sterol response element-binding protein
SRM selected reaction monitoring
STAT3 signal transducer and activator of transcription 3
sE-selectin soluble E selectin
sICAM-1 soluble intercellular adhesion molecule-1

T

T-CA taurocholic acid
T-CDCA taurochenodeoxycholic acid
T-DCA taurodeoxycholic acid
T-LCA taurolithocholic acid
TAG triacylglycerides
TAMs tumour-associated macrophages
TC total cholesterol
TCA tricarboxylic acid cycle
TEAC total antioxidant capacity
TG triglyceride
TIC total ion chromatogram
TIMP-1 matrix metalloproteinases type 1
TNF tumour-necrosis factor
TP total phenols
TQ total quercetin
TXA_2 thromboxane A2
TXB_2 thromboxane B2
3T3-L1 3-day transfer, inoculum 3 x 10^5 cell line

U

UDCA ursodeoxycholic acid

UHPLC ultra–high performance liquid chromatography
UPLC ultra–performance liquid chromatography
UPLC–MS ultra–performance liquid chromatography coupled to mass spectrometry
UV–scaling unit variance scaling
UV/Vis ultravioleta / visible

V

Var variable
VCAM-1 vascular cell adhesion molecule 1
VEGF vascular endothelial growth factor
VIP variable variable influence on the projection
VLDL very low density lipoprotein
vWF von Willebrand Factor